Antioxidants in chronic upper airway inflammation

Gerrit Jan Westerveld

Antioxidants in chronic upper airway inflammation

The studies described in this thesis were financially supported by the Netherlands Asthma Foundation, grant number 93.72, and were performed at the Department of Otorhinolaryngology – Head and Neck Surgery (Head: Prof. Dr. G.B. Snow), University Hospital Vrije Universiteit Amsterdam, the Department of Pharmacochemistry (Head: Prof. Dr. A. Bast), Vrije Universiteit Amsterdam and the Department of Pharmacology and Toxicology (Head: Prof. Dr. A. Bast), University of Maastricht.

Sponsors: Abbott BV, ALK-Abello BV, Almirall Prodesfarma, Alphatron Medical & Microwave Systems BV, Artu Biologicals BV, Aventis Pharma BV producent van Telfast®, Beltone Netherlands B.V., Byk Nederland bv, Emid Elektro Medical Instruments BV, Entermed B.V., Enthoven-de Vries, Friesland Bank Witmarsum, Glaxo Wellcome, GN ReSound bv, Halmon Laboratoria Beheer B.V. producent van Depothal®, Interim Care BV, Makker Hoortoestellen Laren, Mediprof Medical Products BV, Medtronic BV, Mundipharma Pharmaceuticals B.V., Nutricia Nederland B.V., Ortho Biotech, Pfizer bv, Schoonenberg Hoortoestellen, Schering-Plough BV makers van Aerius® en Nasonex®, Stichting tot Bijstand van Ziekenhuis Hilversum, UCB Pharma B.V., Veenhuis Medical Audio B.V., Zambon Nederland BV

Cover: "Big Deal", Ruud de Rode

Printed by: Ponsen & Looijen bv, Wageningen

VRIJE UNIVERSITEIT

Antioxidants in chronic upper airway inflammation

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Vrije Universiteit Amsterdam,
op gezag van rector magnificus
prof.dr. T. Sminia,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Geneeskunde
op woensdag 25 september 2002 om 13.45 uur
in het auditorium van de universiteit,
De Boelelaan 1105

door

Gerrit Jan Westerveld

geboren te Harlingen

promotoren: prof.dr. G.B. Snow

prof.dr. A. Bast

copromotor: dr. R.A. Scheeren

Het leven is wat je gebeurt terwijl je andere plannen maakt (Acda & de Munnik)

Ter nagedachtenis aan mijn moeder

Aan mijn vader en Gré

Voor Sandra en Lisalotte

Contents

Preface

Preface

The upper airways function as an "airconditioner" of the inspired air as they warm, humidify and filter it before it enters the lungs.[1] Disorders of the upper airways may often result in an impaired lung function which implicates a close pathophysiological relationship between the upper- and the lower airways. For example, the relationship between sinusitis and asthma is well known.[2] During the last decade, numerous investigations have been reported over the oxidative stress-induced cellular damage underlying the pathogenesis of several human pulmonary diseases.[3,4,5] Although considerably less has been written on the role of oxidative stress in upper airway disorders, there are increasing scientific data that oxidative cell injury may result in pathologic conditions.

During inflammation, activated granulocytes show enhanced oxidative metabolism which may result in cell damage, ciliary dysfunction and hyperresponsiveness of the upper airways.[6,7] In addition, increased levels of markers of oxidative stress induced tissue damage have been found in chronic airway inflammation.[8] The inhaled air may also cause damage to the upper airway mucosa. Direct exposure to ozone, nitrogenoxide, nitrogendioxide, sulfur dioxide and even pure oxygen may impair upper airway function.[9] But also the presence of a wide array of oxidative enzymes which play a role in the bioactivation or detoxification of xenobiotics may be important in cellular injury.[10] All these mechanisms share the involvement of free radical mediated cell damage, which may open perspectives for antioxidant pharmacotherapy. The purpose of this thesis is to investigate and discuss of the role of oxidative stress in chronic upper airway inflammation, focussing on antioxidant defenses.

References

1. Procter DF. The upper airway. In The nose: upper airway physiology and the atmospheric environment. Procter DF, Andersen I. eds. Amsterdam/New York/Oxford: Elsevier Biomedical Press 1982:23-43.
2. Slavin RG. Asthma and sinusitis. *J Allergy Clin Immunol* 1992; 90:534-537.
3. Doelman CJA, Bast A. Oxygen radicals in lung pathology. *Free Radical Biol Med* 1990; 9:381-399.
4. Heffner JE, Repine JE. Pulmonary strategies of antioxidant defense. *Am Rev Respir Dis* 1989; 140:531-554.
5. Barnes PJ. Reactive oxygen species and airway inflammation. *Free Radical Biol Med* 1990; 9:235-243.
6. Feldman C, Anderson R, Kanthakumar K, Vargas A, Cole PJ, Wilson R. Oxidant mediated ciliary dysfunction in human respiratory epithelium. *Free Radic Biol Med* 1994; 17:1-10.
7. Turner PJ, Foreman JC. Hyperresponsiveness in the human nasal airway: new targets for the treatment of allergic airway disease. *Med Inflamm* 1999; 8:133-146.

8. Dogru H, Delibas N, Döner F, Tüz M, Uygur K. Free radical damage in nasal polyp tissue. *Otolaryngol Head Neck Surg* 2001; 124:570-572.

9. Effects of air pollution on the upper respiratory tract of children. Koltai PJ. *Otolaryngol Head Neck Surg* 1994; 111:9-11.

10. Xenobiotic-metabolizing enzymes in human respiratory nasal mucosa. Gervasi PG, Longo V, Naldi F, Panattoni G, Ursino F. *Biochem Pharmacol* 1991; 41:177-184.

Chapter I-1

Anatomy and physiology of the upper respiratory tract

Introduction

The upper respiratory tract consists of the nasal cavity, the paranasal sinuses, the middle ear cavity, and the pharynx and is separated from the trachea and the lungs by the larynx. The nasal cavity and the paranasal sinuses form the first line of defense against invading microorganisms, dust particles and allergens present in inhaled air before they enter the lungs. Except for this filtering function the upper airways also humidify and heat the inspired air.[1] In this thesis when we speak of the upper airways the nose and paranasal sinuses are meant. Inflammatory diseases of the upper airways predominantly occur in the nose and the paranasal sinuses and are frequently present in man. They form a major problem in health care. For instance in the United States of America, in 1995, 37 million cases of chronic sinusitis were reported, which means 141.3 cases per 1000 inhabitants.[2] Also in the USA in 1996 and 1997, 787.4 millions visits to a general practitioner and 67.1 million outdoor patient clinic visits were registered for upper respiratory tract inflammation.[3] Recently it was reported that the overall health care expenditures attributable to sinusitis in the United States were $5.8 billion in 1996.[4] In the Netherlands, in 1998, 8.9 % of the Dutch population, approximately 1.4 million patients, suffered from chronic upper airway inflammation.[5] In 1990 this figure was 6.4 %, indicating that the prevalence of these disorders is rising.[6] Compared to the prevalence of other major chronic health problems like asthma, chronic bronchitis or chronic obstructive pulmonary disease (8.3 %), chronic back problems (8.0 %), hypertension (7.6 %), joint arthrosis (7.2 %), migraine (6.2 %) and, diabetes mellitus (2.0 %) chronic upper airway inflammation is the most frequent chronic disease in the Netherlands. Furthermore, in 1998, 3.4 % of all operative procedures performed were carried out for chronic inflammation of the nose and the paranasal sinuses with a mean hospital stay of 3.4 days.[5] These latter figures indicate the enormous impact of these disorders for Dutch health care. As the exact cause of chronic upper respiratory tract inflammation, i.e. chronic rhinosinusitis and nasal polyps is unknown, many research has been performed upon their pathogenesis.[7,8,9] For sinusitis it is widely accepted that ostial obstruction, either due to anatomic variations or mucosal inflammation, is a primary pathophysiologic mechanism.[10] However, modifications of nasal secretions, deficiency of mucociliary clearance and immunodeficiencies may also play a role in the pathogenesis.[11] For nasal polyposis, chronic inflammation of upper airway mucosa seems to provoke its occurence.[12] Chronic inflammation of the upper respiratory tract often coincides with inflammation in the lower airways. Chronic sinusitis has been mentioned as an important trigger for asthma.[13] An

increased prevalence of asthma is reported in patients with nasal polyps.[14] Furthermore, it has been shown that treatment of sinusitis or nasal polyps is beneficial regarding lung hyperactivity and asthma symptoms.[15,16] Currently, the treatment of chronic sinus disease predominantly consists of functional endoscopic sinus surgery, with or without adjuvant treatment with decongestants, nasal- or oral corticosteroids or antibiotics.

The anatomy

The nose

The external nose is a pyramid shaped midfacial structure which in its upper third is composed of bone and in its lower two-thirds of cartilage. The internal nose extends about 7.5 cm from external nares or nostrils to the choanal opening, which connects the nose with the nasopharynx.

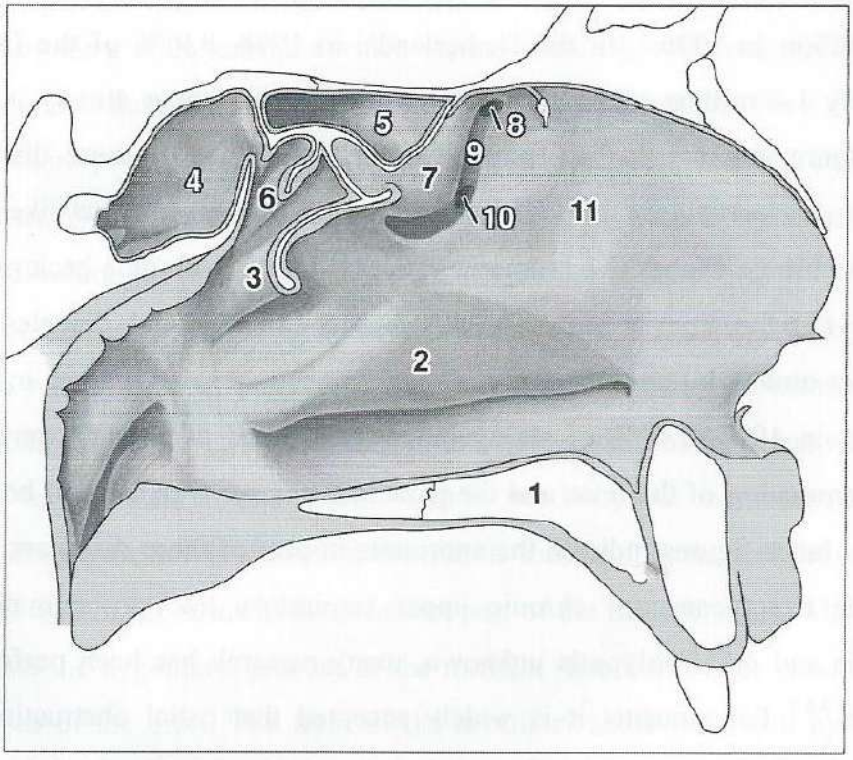


Figure 1. Saggital section of the lateral nasal wall. The middle turbinate has been partially removed. 1 hard palate; 2 inferior turbinate; 3 middle turbinate; 4 sphenoid sinus; 5 ethmoidal cell; 6 superior turbinate; 7 ethmoid bulla; 8 frontal recess; 9 hiatus semilunaris; 10 ostium of the maxillary sinus; 11 agger nasi

Over its entire length the internal nose is bisected into two nasal cavities by the nasal septum. Cranially the roof of the internal nose is formed by the frontal sinus floor anteriorly and the cribiform plate posteriorly. The nasal floor, which lies 6 cm below its roof, is formed by the hard palate. The part of the internal nose just proximal to the nostrils is called the nasal vestibule. It contains hairs and is lined with stratified squamous epithelium. Its function is protection from gross particles in the inspired air which are filtered out by the present hairs or vibrissae.[17]

Just behind this area the nasal valve, or internal ostium is located. This is the narrowest part of the nose and separates the nasal vestibule from the nasal cavity. From front to back the nasal cavity in divided into three meati, which are formed by the presence of three nasal turbinates which project from the lateral nasal wall into the internal nose. The largest is the inferior turbinate which lies just above the nasal floor and extends throughout the most of the length of the nose. More cranially the smaller middle and superior turbinates are located. Sometimes even a fourth, supreme, turbinate is present. The meati contain the orifices of the paranasal sinuses and the nasolacrimal duct (Figure 1).

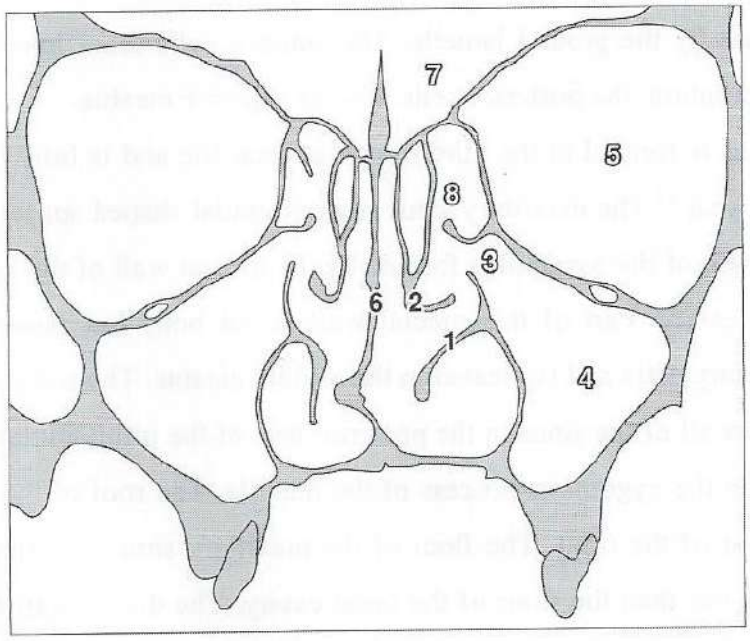


Figure 2. Coronal section of the nose and paranasal sinuses. 1 inferior turbinate; 2 middle turbinate; 3 infundibulum; 4 maxillary sinus; 5 orbit; 6 septum; 7 frontal sinus; 8 ethmoid sinus

The paranasal sinuses

The paranasal sinuses are air-filled, mucosa-lined spaces within the facial and cranial bones which communicate with the internal nose. They consist of the frontal, the ethmoid, the maxillary and the sphenoid sinus (Figure 2).

The *frontal sinus* is developed from the age of 3.5 years and is radiological visible at the age of 6 years.[18] There is a wide variety in its size and shape between people. In 3 % the frontal sinus is absent.[19] The septum between both sides is not always in the midline and sometimes there are more septa. The ostium of the frontal sinus is located in the medial aspect of the sinus floor, where it usually drains through the frontal recess in the middle meatus. The frontal recess may or may not be continuous with the infundibulum.[20]

The *ethmoidal sinus* starts to develop during fetal life and reaches its final form at adolescent age. It is an unpaired skull bone which is divided by its perpendicular plate in a left and right labyrinth. Each labyrinth is composed of numerous cells which are laterally bordered by the lamina papyracea which separates the ethmoid from the orbit. The medial wall of the ethmoid is largely formed by the middle turbinate which separates the ethmoid from the internal nose. Cranially the ethmoid is bordered by the cribiform plate which forms the floor of the anterior cranial fossa. In antero-posterior direction the ethmoid labyrinth is divided in an anterior part and a posterior part by the ground lamella. The anterior cells drain into the middle meatus through the infundibulum, the posterior cells into the superior meatus.

The *maxillary sinus* is formed in the third month of fetal life and is fully developed between the 14th and 18th year.[21] The maxillary sinus is a pyramidal shaped space within the body of the maxilla. The base of the pyramid is formed by the medial wall of the sinus which borders it from the nasal cavity. Part of this medial wall is not bony but membranous. This part contains the accessory ostia and is located in the middle meatus. The natural ostium is located high in the medial wall of the sinus in the posterior part of the infundibulum. The apex of the pyramid points into the zygomatic process of the maxilla. The roof of the maxillary sinus is formed by the floor of the orbit. The floor of the maxillary sinus is formed by the alveolar process and lies lower than the floor of the nasal cavity. The dorsal wall separates the sinus from the pterygopalatine fossa, a space which amongst others contains the maxillary artery and vein.

The *sphenoid sinus* is located within the body of the unpaired sphenoid bone. Pneumatisation of the sphenoid bone starts at the age of three years. Cranially the sinus is bordered by the pituitary fossa. The anterior wall separates the sinus from the spheno-ethmoidal recess, a part

of the cavum nasi, in which the sphenoid sinus drains through its ostium. The sphenoid sinus is in 76 % divided by one or more septa.[22]

The *osteomeatal complex* is the area of the final common drainage pathways of the frontal, maxillary and anterior ethmoid sinuses. It consists of the maxillary sinus ostia, the ethmoid infundibulum, the semilunar hiatus, the middle meatus, the frontal recess, the ethmoid bulla and the uncinate process.[23] Blockage of this region has been implicated in the pathogenesis of paranasal inflammation.[24]

Histology of the nose and paranasal sinuses

The nose and paranasal sinuses are, except from the nasal vestibule and olfactory region, mainly lined with pseudostratified ciliated columnar epithelium.[25] On each columnar cell approximately 200 cilia project into the free mucosal surface. Other cell types such as globet cells, basal cells and non-ciliated cells with microvilli are also present.[26] Furthermore, migrating inflammatory cells such as lymphocytes, macrophages, eosinophils, neutrophils and mast cells may be present. In the submucosal area known as the lamina propria, which is separated from the mucosa by the basement membrane, both serous and mucoid glands constantly excrete their secretions onto the mucosal surface. Moreover, in this layer the cavernous sinusoids are found, which have the capability of marked variation in luminal capacity. The submucosa in the paranasal sinuses is thin, dense and relatively avascular compared to the submucosa of the nose which is, especially at the lower septum and the inferior and middle turbinate, loose, rich of glands and very vascular. This region represents the nasal surface most prominently exposed to inspired air. The respiratory epithelium is innervated by both sympathetic and parasympathetic fibers which regulate the type of mucus secretion and the tone of the contractile sinusoids.

The physiology of the nose and the paranasal sinuses

The nose

The most important functions of the nose are to warm, humidify and filter the inhaled air before it enters the lungs.[27] In addition, the nose also provides the sense of smell. The "air conditioning" function of the nose is very efficient as was shown by the experiments of Ingelstedt in 1956 and Proctor in 1977 who both showed that the temperature and humidity of inspired air remains constant regardless of the starting point.[28,29]

The filtering function of the nose is effectuated by the mucociliary clearing system which consists of a mechanical and immunological barrier. Trapped particles are removed from the nose by the epithelial cilia which stick through a deeper layer (sol) of the mucous blanket into the superficial layer (gel) and move it in the direction of the nasopharynx at a speed of 6 mm/min, the sol layer itself is not transported.[30] The mucous blanket on the ciliated cells is 10 to 15 μm thick and traps the inhaled particles in its outer (gel) layer which contains mastcells, leukocytes, eosinophils, lysozym, and secretor immunoglobuline A, which form an immunological barrier against invading microorganisms.[31] Disturbances in the mucociliary clearing system may give rise to infectious disorders of the nose and paranasal sinuses.[32]

The paranasal sinuses

The exact function of the paranasal sinuses is unknown. Several theories have been proposed such as, protecting the skull from trauma, contributing to olfaction, lightening the weight of the skull, adding voice resonance, contributing to air heating and air humidification, holding air residues, protection of the brain and craniofacial development. However, none of these theories has been proven in controlled research.[33] The paranasal sinuses have ostia through which they drain their mucus to the nose. To prevent retention of sinus secretions which would lead to paranasal infection the paranasal sinuses have the same mechanical and immunological barrier as the nose. Furthermore, patency of the ostia is important to achieve adequate drainage. The mechanisms of transport of secretions in the various sinuses have been thoroughly described.[34,35,36,37] In the ethmoid the secretions are directly guided to the ostium. In the maxillary sinus, where the ostium is located rather high in the medial wall, the mucous layer is transported in a star-like pattern from the floor of the sinus in the direction of the ostium. In the sphenoid sinus the transport is conducted in a spiral fashion. In the frontal sinus a part of the mucous layer is directly transported to the ostium, whereas the other part is recycled in the sinus before leaving through the ostium.

Pathophysiology of chronic sinusitis and nasal polyps

Chronic sinusitis

Chronic sinusitis was primarily defined as sinus disease which lasts for more than 3 months.[38] However, more recently chronic sinusitis has been defined as a persistent inflammation documented with imaging techniques at least 4 weeks after initiating appropriate medical therapy in the absence of an intervening acute episode.[39] Although the exact pathophysiology

is unknown, the general idea is that persistent inflammation of the mucosa of the upper respiratory tract leads to irreversible damage to the mucociliary clearance system which results in the loss of its function and aggravation of the disease. Therefore, chronic sinusitis is in principle considered to be a non infectious disease. Although the opinions differ on the etiology of chronic sinusitis, a similar set of predisposing factors that contribute to acute sinusitis account for chronic sinusitis. Some of these factors will be discussed below in more detail.

1. Ostial blockage

Blockage of the osteomeatal region is thought to be a key factor in the development of chronic sinusitis.[40,41] Anatomical variations in this area are reported to influence aeration and mucociliary clearance, leading to mucostasis and subsequent inflammation and eventually to further blockage.[42] The sinuses draining to the osteomeatal complex, i.e., the maxillary sinus and the anterior ethmoid sinus, are the most commonly affected sinuses in both acute and chronic sinusitis.[9]

2. Abnormal recovery of mucociliary function

Diminished mucociliary clearance is a feature which has been consistently shown in patients with chronic sinusitis.[43,44] A factor which seems to influence mucociliary function negatively is hypoxia. It has been shown that sinus hypoxia, which may be due to a diminished ostial size, leads to a decrease in ciliary beating frequency and to failure of the mucociliary transport.[45,46,47] A decreased oxygen content and even an absence of oxygen has been demonstrated in acute sinusitis and chronic sinusitis respectively.[48,49,50] The reduction of ciliary beat frequency in chronic sinusitis is accompanied by morphological changes in the mucosal epithelium also leading to diminished mucociliary clearance.[51] At this point it should be noted that chronically damaged sinus mucosa, always has the potency to regain its function after adequate aeration has been restored, regardless of the duration of the disease. It has been shown that mucustransport in the maxillary sinus improves after endoscopic sinus surgery. The rate of restoration may however be slow and incomplete leading to residual impairment in mucociliary clearance and recurrence of disease.[43,52] Other factors that predispose for impairment of mucociliary function such as, immotile cilia disorder (Kartagener syndrome), diminished quality of sinus mucus present in cystic fibrosis, IgA deficiency and hypogammaglobulinemia are also important but fall outside the scope of this review.[53,54]

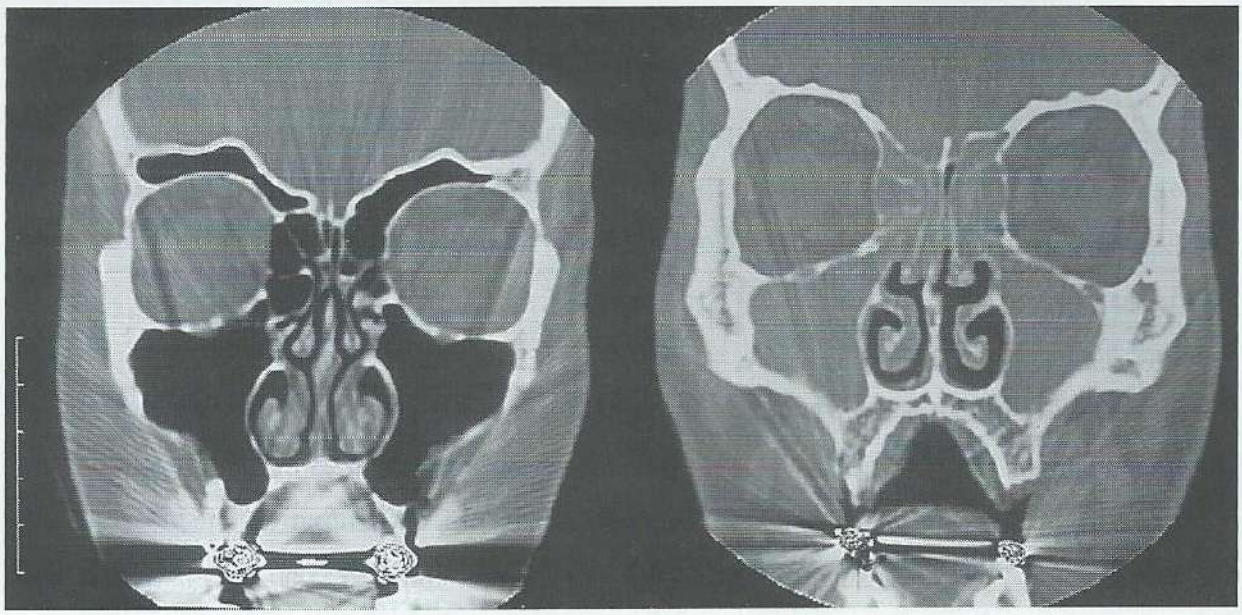


Figure 3. Coronal CT-scan of the nose and paranasal sinuses. Left: normal scan, all paranasal sinuses are air filled. The infundibulum on both sides is patent. Right: pansinusitis, maxillary sinus and ethmoid sinus are totally opacified.

3. Microbial factors

The nasal cavity and the paranasal sinuses are, under normal circumstances, colonized with several similar types of aerobic and anaerobic bacteria.[55] The normal flora can cause infection as the environmental changes, in case of ostial closure, that occur as a result of blockage of the ostium provide excellent growth conditions for bacteria. This makes it difficult to distinguish the pathogenic bacteria from the non-pathogenic bacteria. Bacterial infection in chronic sinusitis is, in contrast to acute sinusitis, probably secondary. Moreover, bacterial cultures from patients with chronic sinusitis show a great interindividual diversity and often several types of bacteria are cultured simultaneously.[56] It has been suggested that bacteria present in sinusitis may change over time. This possibly may also explain the diversity of bacteria found in cultures of patients with chronic sinusitis.[57] The most common identified microorganisms in chronic sinusitis are staphylococcus aureus, coagulase negative staphylococcus and anaerobic bacteria.[9] Except from their direct inflammatory action, some bacteria have been found to release factors that either decrease or disorganize ciliary activity or even destroy cilia.[58,59] Relative to bacteria, much less is known about the role of viruses in the pathogenesis of chronic sinusitis.[60]

Nasal polyps

Nasal polyps are benign mucosal swellings which present as glistering, pale gray, smooth, soft, freely movable tumors attached by a pedicle to predominantly the nasal mucosa of the middle turbinate or the ostia of the ethmoid and maxillary sinus. They occur in the general population with a prevalence of 1-4 %. In patient with cystic fibrosis, acetylsalicic acid sensitivity or asthma much higher frequencies are found.[12] Histopathologically, polyps are mainly composed of edema and a few mucous glands. The surface epithelium generally reveals squamous metaplasia. In persons without cystic fibrosis, eosinophils are the predominant cell type to occur in nasal polyps.[9] As chronic sinusitis, the pathogenesis of nasal polyps is far from clear. However, chronic inflammation of the mucosa either caused by infection or allergy seems to be essential. Local factors, such as aerodynamic conditions, hemodynamic factors and changes in local immunity may contribute to the formation of nasal polyps.[61]

Diagnosis and therapy

Chronic sinusitis and nasal polyps are disorders which are clinically characterized by nasal congestion, facial pressure (headache), (post)-nasal discharge, and hyposmia. However, the signs may be subtle.[62] Next to the clinical presentation the diagnosis is made on rhinologic and radiological examination. Although on anterior rhinoscopia only a small portion of the nasal cavity is visualized, it gives a good overview of the status of the nasal mucosa and possible abnormalities of the septum. To obtain a more detailed overview of the middle meatus, nasal endoscopy is essential.[63] To date, CT-scanning of the nose and paranasal sinuses is the imaging modality of choice to visualize the surgical anatomy and extension of the disease (Figure 3).[64] Medical management of chronic rhinosinusitis is diverse. The choice of therapy is made on patient and disease characteristics. Beneficial effects of decongestants, antihistamines, topical and systemic corticosteroids, antibiotics and immunotherapy have been described.[65] Nowadays endoscopic surgery is the preferred surgical procedure although more traditional forms of surgery are still acceptable. The outcome of the surgical procedure may differ considerably and is mainly determined by its indication.[66]

References

1. Proctor DF. The upper airway. In: The nose. Upper airway physiology and the atmospheric environment. Proctor DF, Andersen I, eds. Amsterdam/New York/Oxford: Elsevier Biomedical Press 1982:22-43.

2. Vital and health statistics. Series 10, No. 199. National center for health statistic/Center for disease control and prevention.
3. Vital and health statistics. Series 13, No 134. National center for health statistic/Center for disease control and prevention.
4. Ray NF, Baraniuk JN, Thamer M, Rinehart CS, Gergen, PJ, Kaliner M, Josephs S, Pung YH. Healthcare expenditures for sinusitis in 1996: contributions of asthma, rhinitis and other airway disorders. *J Allergy Clin Immunol* 1999; 103:408-414.
5. Vademecum gezondheidsstatistiek nederland 1999. Centraal bureau voor de statistiek/ Ministerie van volksgezondheid, welzijn en sport.
6. Vademecum gezondheidsstatistiek nederland 1992. Centraal bureau voor de statistiek/ Ministerie van volksgezondheid, welzijn en sport.
7. Kaliner MA, Osguthorpe JD, Fireman P, Anon J, Georgitis J, Davis ML, Naclerio R, Kennedy D. Sinusitis: bench to bedside. Current findings, future directions. *J All Clin Immunol* 1997; 99:S829-S848.
8. Hamilos DL. Chronic sinusitis. *J All Clin Immunol* 2000; 106:213-227.
9. Salvin RG. Nasal polyps and sinusitis. *JAMA* 1997; 278:1849-1854.
10. Wageman M, Naclerio RM. Anatomic and physiologic considerations in sinusitis. *J Allergy Clin Immunol* 1992; 90:419-423.
11. Scheeren RA. Chronic upper airway infections. The role of p15E-like proteins and defects in cell-mediated immunity. Academical thesis. Amsterdam 1992.
12. Baan S van der. Epidemiology and natural history. In: Nasal polyposis. An inflammatory disease and its treatment. Mygind N, Lildholdt T, eds. Munksgaard Copenhagen 1997:13-15.
13. Slavin RG. Asthma and sinusitis. *J Allergy Clin Immunol* 1992; 90:534-537.
14. Reichelman H. Operatieve sanierung der Nasennebenhöhlen und Asthma. *Allergologie* 1994; 2:78-79.
15. Hosemann W. Einfluss der endonasalen Nasennebenhöhlenchirurgie auf die Lungenfunction des Patienten mit Asthma bronchiale. *Laryngol Rhino Otol* 1990; 69:521-526.
16. Rachelefski GS, Katz RM, Siegel SC. Chronic sinus disease with associated reactive airway disease in children. *Pediatrics* 1984; 73:526-529.
17. Cottle MH. The structure and function of the nasal vestibule. *Arch Otolaryngol* 1955; 62:173-181.
18. Ritter FN. The surgical anatomy of the nasal sinuses. *Otolaryngol Clin North Am* 1971; 4:3-11.
19. Simpson WL. The ethmosphenofrontal operation. *Arch Otolaryngol* 1937; 26:270-282.
20. Messenklinger W. Uber den Recessus frontalis und seine Klinik. *Laryngol Rhinol Otol* 1982; 61:217-223.
21. Schaeffer JP. The sinus maxillaris and its relations in the embryo, child and adult man. *Am J Anat* 1910; 10:313-367.
22. Renn WH, Rhoton AL. Microsurgical anatomy of the sellar region. *J Neurosurg* 1975; 43:288-298.
23. Kennedy DW, Zinreich SJ, Rosenbaum A, Johns ME. Functional endoscopic sinus surgery: Theory and diagnostic evaluation. *Arch Otolaryngol Head Neck Surg* 1985; 111:576-582.
24. Stammberger H. Nasal and paranasal sinus endoscopy. A diagnostic and surgical approach to recurrent sinusitis. *Endoscopy* 1986; 18:213-218.

25. Boysen N. The surface structure of the human nasal mucosa I. Ciliated and metaplastic scanning/transmission electron and light microscopy. *Virchows Arch B (Cell Pathol)* 1982; 40;279-294.
26. Irvani J, As v A. Mucus transport in the tracheobronchial tree of normal and bronchitic rats. *J Pathol* 1972; 106:81-93.
27. Proctor DF, Andersen I. The nose. Upper airway physiology and the atmospheric environment. Elsevier biomedical press. Amsterdam, 1982.
28. Ingelstedt S. Studies on the conditioning of air in the respiratory tract. *Acta Otolaryngol* 1956; 131 (suppl):1-80.
29. Proctor DF. The upper airways I. Nasal physiology and defense of the lungs. *Am Rev Resp Dis* 1977; 115:97-129.
30. Wilson WR, Allansmith MR. Rapid, atraumatic method for obtaining nasal mucus samples. *Ann Rev Otol Rhinol Laryngol* 1976; 85:391-395.
31. Richmore JT, Marshall ML. Cytology of nasal secretions: Furthet diagnostic help. *Laryngoscope* 1976; 86:516.
32. Baan van der S. Primaire ciliare dyskinesie. Academical thesis. Amsterdam1985.
33. Rys Evans PH. The paranasal sinuses and other enigmas: an aquatic evolutionary theory. *J Laryngol Otol* 1992; 106:214-225.
34. Hilding AC. The physiology of drainage of the nasal mucus I. The flow of the mucus currents through the drainage system of the nasal mucosa and its relation to cilliary activity. *Arch Otolaryngol* 1932; 15:92-100.
35. Hilding AC. The physiology of drainage of the nasal mucus IV. Drainage of the accessory sinuses in man. *Ann Otol Rhinol* 1944; 53:35-41.
36. Messenklinger W. Uber die Drainage der menschlichen Nasennebenhöhlen unter normale und pathologische Bedingungen I. *Mitteilung. Mschr Ohr Hk* 1966; 100:56-68.
37. Messenklinger W. Uber die Drainage der menschlichen Nasennebenhöhlen unter normale und pathologische Bedingungen II. Mitteilung: Die Stirnhöhle und ihr Ausfiihrungssystem. *Mschr Ohr Hk* 1966; 101:313-326.
38. Melen I, Lindahl L, Andreasson L, Rundcranz H. Chronic maxillary sinusitis. Definition, diagnosis and relation to dental infections and nasal polyposis. *Acta Otolaryngol (Stockh)* 1986; 43:110-113.
39. Stammberger HR, Kennedy DW. Paranasal sinuses: anatomic terminology and nomenclature. The anatomic terminology group. *Ann Otol Rhinol Laryngol Suppl* 1995; 167:7-16.
40. Messenklinger W. Nasendoskopie: der mittlere nasengang und seine unspezifischen Entzündungen. *HNO* 1972 (b); 20:212-215.
41. Senior BA, Kennedy DW. Management of sinusitis in the asthmatic patient. *Ann Allergy Asthma Immunol* 1996; 77:6-19.
42. Stammberger H. Endoscopic endonasal surgery concepts in treatment of recurring rhinosinusitis Part I. Anatomic and pathophysiologic considerations. *Otolaryngol Head Neck Surg* 1986 (a); 94:143-147.
43. Elwany S Hisham M, Gamaee R. The effect of endoscopic sinus surgery on mucociliary clearance in patients with chronic sinusitis. *Eur Arch Otorhinolaryngol* 1998; 255:511-514.

44. Passali D, Ferri R, Becchini G, Passali GC, Bellussi L. Alterations of nasal mucociliary transport in patients with hypertrophy of the inferior turbinates, deviations of the nasal septum and chronic sinusitis. *Eur Arch Otorhinolaryngol* 199; 256:335-337.

45. Aust R, Drettner B. The functional size of the human maxillary ostium in vivo. *Acta Otolaryngol (Stock)* 1974 (a); 78:432-435.

46. Drettner B. Pathophysiology of the paranasal sinuses with clinical implications. *Clin Otolaryngol* 1980; 5:277-284.

47. Reimer A, Huberman D, Klementsson K, Toremalm NG. The mucociliary activity of the respiratory tract. II. Experiments on the effect of changing pO_2 and pCO_2 in ambient atmosphere and arterial blood. *Acta Otolaryngol (Stockh)* 1980 (b); 91:139-148.

48. Braverman I, Wright ED, Wang CG, Eidelman D, Frenkiel S. Human nasal ciliary beat frequency in normal and chronic sinusitis subjects. *J Otolaryngol* 1998; 27:145-152.

49. Nuutinen J, Rauch-Toskala E, Saano V, Joki S. Ciliary beating in chronic sinusitis. *Arch Otolaryngol Head Neck Surg* 1993; 119:645-647.

50. Carenfelt C, Lundberg C.The role of local gas composition in pathogenesis of maxillary sinus empyema. *Acta Otolaryngo (Stockh)* 1978; 85:116-121.

51. Ohashi Y, Nakai Y. Reduced ciliary action in chronic sinusitis. *Acta Otolaryngol* 1983; S397:3-9.

52. Dal T, Onerci M, Caglar M. Mucociliary function of the maxillary sinuses after restoring ventilation. A radioisotopic study of the maxillary sinus. *Eur Arch Otorhinolaryngol* 1997; 254:205-207.

53. Baan van der S, Veerman AJP, Wulffraat N, Feenstra L. Primary ciliary dyskinesia and ciliary activity. *Acta Otolaryngol* 1986; 102:274-281.

54. Cuyler JP, Monaghan AJ. Cystic fibrosis and sinusitis. *Otolaryngology* 1989; 18:173-175.

55. Cauwenberge van P, Mijnsbrugge van der AM, Ingels KJO. The microbiology of acute and chronic sinusitis and otitis media. A review. *Eur Arch Otorhinolaryngol* 1993; 250:S3-S6.

56. Hartog B. Diagnosis and treatment of chronic maxillary sinusitis. Results of a randomized clinical trial, supplemented with epidemiological and methodological research. Academical thesis. Utrecht 1997.

57. Ramadan HH. The role of anaerobes in chronic sinusitis. *Arch Otolaryngol Head Neck Surg* 1995; 121:591.

58. Wilson R, Roberts D, Cole P. Effects of bacterial products on human ciliary function in vivo. *Thorax* 1985; 40:125-131.

59. Lundberg C, Engquist S. Localisation of bacteria and the cause of tissue destruction in maxillary sinusitis. *Acta Otolaryngol (Stockh)* 1984; S407:30-32.

60. Subauste MC, Jacoby DB, Richards SM, Proud D. Infection of a human respiratory epithelial cell line with rhinovirus: induction of cytokine release and modulation of susceptibility to infection by cytokine exposure. *J Clin Invest* 1995; 96:549-557.

61. Mygind N, Lildholdt T. Nasal polyposis; an inflammatory disease and its treatment. Munksgaard Copenhagen 1997.

62. Clinical evaluation of rhinosinusitis. History and physical examination. Hadley JA, Schaefer SD. *Otolaryngol Head Neck Surg* 1997; 117:S8-S11.

63. Vleming M. Endoscopische neusbijholten chirurgie. Academical thesis. Amsterdam 1991.

64. Zinreich SJ. Rhinosinusitis: radiologic diagnosis. *Otolaryngol Head Neck Surg* 1997; 117:S27-S34.

65. Benninger MS, Anon J, Mabry RL. The medical management of rhinosinusitis. *Otolaryngol Head Neck Surg* 1997; 117:S41-S49.

66. Anand VK, Osguthorpe JD, Rice D. Surgical management of adult rhinosinusitis. *Otolaryngol Head Neck Surg* 1997; 117:S50-S52.

Chapter I-2

Biochemical aspects of oxidative stress

Introduction

Free radicals or oxidants play an important role in a broad spectrum of physiological biological processes. Despite their physiological importance, there is increasing evidence that free radicals can be harmful and may be responsible for tissue injury in several human diseases. The main evidence for this hypothesis comes from *in vitro* models and experimental animal studies. However, during the last two decades evidence has also been obtained in human studies. Various diseases of the lungs and the heart are related to oxidative cell damage, although given a sufficient burden of oxidants, any tissue can be damaged. For instance, in the liver, drug metabolism can result in excessive free radical production causing direct tissue damage. Except from organ dysfunction due to direct tissue damage, it has been shown that free radicals can cause DNA damage resulting in malignant potential. Furthermore, oxidative stress may lead to immune dysfunction by damaging components of the cellular immune system.[1] To prevent against free radical damage the most obvious strategy is to reduce the oxidative burden. However, for most oxidant-related human disorders, limiting the oxidant burden is not possible as the disease is ongoing. In these circumstances, the most obvious approach is to develop a therapeutic strategy of augmentation of the antioxidant defense mechanisms to re-establish the oxidant-antioxidant balance in favor of the antioxidant defense. [2]

Oxidants responsible for human disease originate from three sources:

1) oxidants generated through normal biological reactions, in excessive amounts or in an environment in which the normal antioxidant defense is insufficient.
2) oxidants released through inflammatory cells
3) oxidants entering the human system from exogenous sources, either direct (i.e. cigarette smoke or ozone) or indirect (i.e. metabolism of xenobiotics).

In this chapter the basal concepts of the chemistry of free oxygen radicals, the antioxidant defenses and their involvement in human disease are discussed.

Chemical background

Electrons in an atom or molecule occupy spatial volume elements defined by energy and probability functions of quantum mechanics, called orbitals. each of which can contain two electrons under certain restrictions. The orbitals contain the electrons of a compound, usually as pairs, each electron spinning in an opposite direction. A normal chemical covalent bond

frequently consists of a pair of electrons also spinning in opposite directions, each component of the bond donating one of the electrons. In contrast, a free radical is a molecule or molecular fragment that contains one or more unpaired electrons in its outer orbital.[3,4]

A molecule becomes a free radical either by gaining an additional electron, i.e. the molecule is reduced, or by donating one, the molecule is oxidized. The presence of an unpaired single electron in the outer orbital of a free radical is mostly represented by a superscript dot ($R^{\cdot}$).

Reduction: $R + e^- \rightarrow R^{\cdot -}$

Oxidation: $R - e^- \rightarrow R^{\cdot +}$

Although most free radicals are reactive due to the unpaired electrons, some are relatively stable. These stable radicals often have an unpaired electron that is making it less reactive.

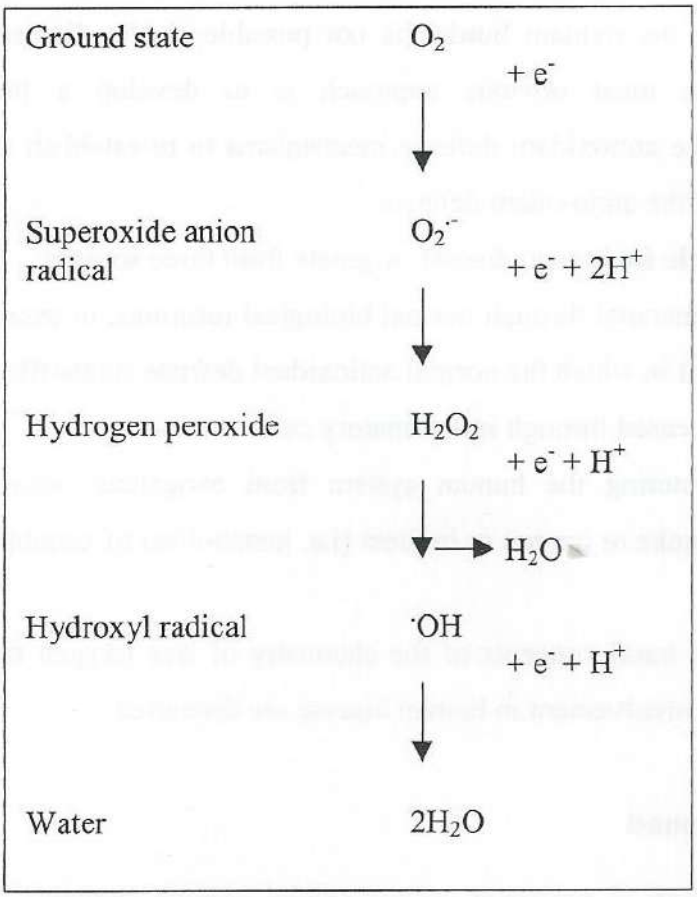


Figure 1. The sequential univalent reduction of molecular oxygen

When two radicals meet, they share their unpaired electrons and form a stable covalent bond. When a radical reacts with a non-radical is either donates an electron to the non-radical or it scavenges an electron from the non-radical. Through both reactions the non-radical becomes a free radical which might further induce a chain reaction of free radical formation.

Oxygen derived free radicals

In biological systems molecular oxygen (O_2) is considered to be the most important source of radicals and oxidants. Molecular oxygen itself has to be regarded as a biradical as it contains two unpaired electrons in each of its two outer orbitals. The expected reactivity is reduced by the quantum mechanical restrictions caused by the parallel directions of spin of these electrons.[5]

During normal cell metabolism adenosine triphosphate (ATP) is formed from adenosine diphosphate (ADP) by mitochondrial oxidative phosphorylation through cytochrome oxidase. Parallel to this process tetravalent reduction of O_2 into water (H_2O) occurs. The oxygen free radicals formed during these reactions are tightly bound to the active sites of the cytochrome oxidase enzyme preventing them to cause cell damage.

Approximately 5% of the O_2 which enters the respiratory cycle in the mitochondria leaves this cycle as sequential univalent reduction occurs. Through this mechanism several nonbound free oxygen radicals are produced that can disrupt normal functioning of the cell (Figure 1).

In figure 1 the protonated an unprotonated intermediate forms of molecular oxygen during its reduction to water are shown. Not all of these intermediate forms are actually free radicals and therefore it is more appropriate to speak of reactive oxygen species (ROS) instead of oxygen free radicals.[6] Beside the reactive oxygen species shown in figure 1, other biologically important reactive species are known. Singlet oxygen ($^1O_2$) is formed by the impute of energy causing the inversion of the spin of one of the two electrons in the outer orbitals. Although singlet oxygen is not a free radical by definition it is capable of rapidly oxidizing molecules in biologic systems. Ozone (O_3) is produced by industry and contributes to air pollution, in contrast it forms a protective shield in the atmosphere against solar radiation. Ozone is regarded as a strong oxidant.[7] Nitric oxide ($NO^{\cdot}$), although not a classical reactive oxygen metabolite, is also a potent oxidant which has been implicated in inflammatory cell response.

Reactive oxygen species formation in biological systems

The free radical burden to the human body depends on the external exposure to oxidants, such as, cigarette smoke and ozone and to the exposure to free radical formation inducing systems such as ionizing radiation and certain types of light.[7,8] However, the most important source of free radicals are the constant oxidation-reduction reactions that occur diffusely in the body. A variety of enzyme systems, such as oxidases, dehydrogenases, and several peroxidases catalyze the univalent reduction of O_2 to superoxide anion radicals ($O_2^{\cdot-}$) .[9,10] However, univalent reduction of O_2 can also occur during non enzymatic oxidation-reduction reactions and during auto-oxidation reactions. Moreover, free oxygen radicals are also generated during the respiratory mitochondrial electron transport system and during the respiratory burst present in inflammatory cells.[6]

Superoxide anion radicals are converted under the catalytic influence of superoxide dysmutase (SOD) into hydrogen peroxide (H_2O_2) (reaction 1).[11]

$$2O_2^{\cdot-} + 2H^+ \rightarrow (SOD) \rightarrow H_2O_2 + O_2 \qquad (1)$$

In the presence of transition metals, such as iron (Fe) or copper (Cu), superoxide anion radicals ($O_2^{\cdot-}$) give rise to the formation of hydroxyl radicals ($^{\cdot}OH$) in the so-called Fenton reaction (reaction 2).[12]

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^- \qquad (2)$$

Another important reaction that produces hydroxyl radicals in the presence of transition metals is the Haber-Weiss reaction (reaction 3).[12]

$$O_2^{\cdot-} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$
$$\underline{H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+}}$$
$$O_2^{\cdot-} + H_2O_2 \rightarrow {}^{\cdot}OH + OH^- + O_2 \qquad (3)$$

In biological systems, hydroxyl radicals are the most reactive radicals known as they are known to initiate and propagate several free radical (chain) reactions, such as DNA damage and lipid peroxidation, leading to cell dysfunction and cell death.[13,14] During lipid peroxidation the poly unsaturated fatty acids located in the cell membrane become

peroxidized. In this process primarily a hydrogen atom is abstracted from a poly unsaturated fatty acid (LH) through a radical, e.g. ·OH, forming a lipid radical (L·). Subsequently the lipid radical can react with molecular oxygen forming an lipid peroxyl radical (LOO·), which in turn can react with another membrane lipid molecule forming a lipidhydroperoxide (LOOH) and a new lipid radical (reaction 4, 5, 6).[14]

$$LH + {}^{\cdot}OH \rightarrow L^{\cdot} + H_2O \quad (4)$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (5)$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad (6)$$

Myeloperoxidase (MPO), an enzyme expressed in activated leukocytes, catalyses the oxidation of chloride ions (Cl^-) by H_2O_2 into hypochlorous acid (HOCl), which is also a potent oxidant (reaction 7).

$$H_2O_2 + Cl^- \rightarrow (MPO) \rightarrow HOCl + OH^- \quad (7)$$

From HOCl itself hydroxyl radicals can be generated through either a reaction with superoxide anion radicals (reaction 8) or iron ions (reaction 9).

$$HOCl + O_2^{\cdot -} \rightarrow {}^{\cdot}OH + Cl^- + O_2 \quad (8)$$

$$HOCl + Fe^{2+} \rightarrow {}^{\cdot}OH + Cl^- + Fe^{3+} \quad (9)$$

Although it is not a classical reactive oxygen metabolite, nitric oxide (NO·) is a biologically important free radical and is produced by various mammalian cells.[15] It is synthesized through the enzymatic action of nitric oxide synthase (NOS) which coverts the amino acid L-arginine into L-citrulline (Figure 2).[16,17,18] Under the influence of NO·, guanosine triphosphate (GTP) is converted to cyclic guanosine monophosphate (c-GMP) which leads to relaxation of smooth muscle cells. Therefore, NO· was initially characterized as endothelium derived relaxing factor (EDRF). The isolation of NOS was first reported by Bredt and Snyder in 1990.[19] At this moment three isoforms of NOS have been described.[20] There are two constitutive types (cNOS), one first described in neural tissue (nNOS, NOS type I) and the other in endothelial cells (eNOS, NOS type III).

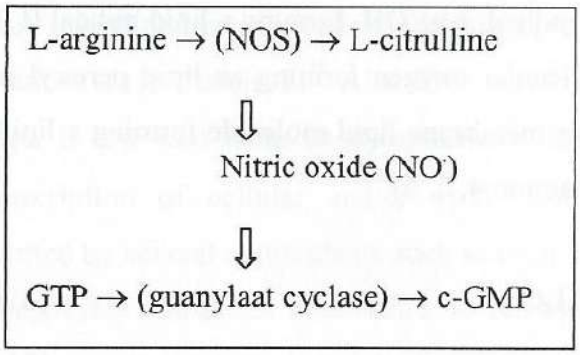


Figure 2. Schematic representation of the formation of nitric oxide.

It has now become apparent that both enzymes are also expressed in other cells, such as epithelial cells. Both enzymes are activated by a rise in intracellular calcium ions (Ca^{2+}) and produce small amounts of NO·. The third isoform, inducible NOS (iNOS, NOS type II) is not normally expressed but is induced by inflammatory mediators such as, endotoxins and cytokines.[17] This form is less Ca^{2+} dependent as calmodulin is integral to the enzyme. Once induced iNOS produces large amounts of NO· over many hours.[21] NO· derived from iNOS is involved in inflammatory diseases of the airways and in host defense against infection.[22] NO· can rapidly react with $O_2^{\cdot-}$ To form peroxynitrite ($ONOO^-$) (reaction 10).

$$O_2^{\cdot-} + NO^{\cdot} \rightarrow ONOO^- \qquad (10)$$

Peroxynitrite itself is a more potent oxidant than NO· and $O_2^{\cdot-}$ And can attack a wide array of biological targets. Moreover, from $ONOO^-$ the very reactive ·OH can be formed (reaction 11).[23,24]

$$ONOO^- + H^+ \rightarrow ONOOH \rightarrow NO_2^{\cdot} + {}^{\cdot}OH \qquad (11)$$

Nitric oxide can also react with other oxidants such as, such as H_2O_2, releasing large amounts of chemiluminescence with the characteristics of singlet oxygen.[25] In addition, when combined with HOCl and $O_2^{\cdot-}$, nitric oxide forms nitrosylchloride, also a very strong oxidant.[26]

Antioxidants

An antioxidant is defined as any substance that, when present in low concentrations compared to an oxidizable substrate, significantly delays or inhibits oxidation of that substrate.[27] The assault of endogenous and exogenous oxidants mandates that all metabolically active cells assemble an antioxidant strategy that utilizes a broad array of resources to prevent or limit oxidant injury (table 1). These antioxidant mechanisms are provided by several cell processes[28,29]:

1. prevention of formation of free radicals
2. compartmentalization of reactive species away from vital cellular structures
3. elimination of oxidants or conversion of oxidants into less toxic species
4. reparation of molecular damage by free radicals

At this point it should be noted that an antioxidant can not distinguish between radicals that play a physiologic role and those that cause damage. Moreover, antioxidants can function alone or in combination with others.

Prevention of formation of free radicals

Free radical metabolites are continuously formed during normal cellular respiration. The tetravalent reduction of oxygen into water, catalyzed by the cytochrome oxidases is in fact a powerful antioxidant mechanism as the majority of the free radicals formed during this process are bound to the active sites of the enzyme keeping it apart from other cellular constituents. For instance, in lungs the mitochondrial cytochrome oxidase is capable of binding and metabolizing 90 % of the lung cell oxygen in a way that free radical formation is prevented.[30] When cytochrome oxidase activity is reduced, as is the fact in hypoxic circumstances increased amounts of radicals are formed from the mitochondrial electron transport chain.[31]

Compartmentalization of reactive species away from vital cellular structures

The primary antioxidant strategy of cells is limitation of hydroxyl radical formation through diminishing transition metal availability. Safe storage of transition metals such as iron and copper ions is therefore crucial in preventing hydroxyl radical production.[32] In humans approximately 70 % of the iron is stored in hemoglobin which in turn is predominantly stored in erythrocytes. Free hemoglobin is rapidly bound to haptoglobin and hemopexin as it is known to release its iron easily under the influence of peroxides.[33,34,35]

Table 1. Antioxidant systems in biological systems[28]

System	Type	Tissue site	Actions
Enzymatic			
SOD	CuZn-SOD	Primarily cytosol, also nucleus	Catalyzes dysmutation of $O_2^{\cdot-}$ into H_2O_2
	Mn-SOD	Primarily mitochondria	
	Cu-SOD	Primarily plasma	
Catalase	Tetrameric hemoprotein	Intracellular; peroxisomes	Catalyzes dysmutation of H_2O_2 and other peroxides
GSH-peroxidase	Selenium or non-selenium dependent protein	Primarily cytosol, also mitochondria	Catalyzes reduction of H_2O_2 and other hydroperoxides
GSH-transferase	Non-selenium dependent protein	Primarily cytosol, also mitochondria	Catalyzes reduction of organic hydroperoxides
GSH-reductase	Dimeric-protein	Primarily cytosol, also mitochondria	Catalyzes reduction of low molecular weight disulfides
G-6-PD and 6-PGD	NADP dependent enzymes	Extramitochondrial cytosol	Supply NADPH for GSH reductase
Non-enzymatic			
Vitamin E	α-tocopherol, fat soluble vitamin	Lipid membranes, extracellular fluids	Converts $O_2^{\cdot-}$, $^{\cdot}OH$ and lipid peroxyl radicals into less reactive species. Breaks lipid peroxidation chain reactions
Vitamin C	Ascorbic acid, water soluble vitamin	Wide distribution in intra- and extracellular fluids	Scavenges $O_2^{\cdot-}$ and $^{\cdot}OH$. Neutralizes oxidants from stimulated neutrophils. Contributes to the regeneration of vitamin E
Uric acid	Oxidized purine base. Water soluble	Wide distribution	Scavenges $^1O_2$, $^{\cdot}OH$, oxoheme oxidants, and peroxyl radicals. Prevents oxidation of vitamin C. Binds transition metals
GSH	Tripeptide. Water soluble	Largely intracellular	Substrate in GSH redox cycle. Cofactor of GSH peroxidase and GSH transferase. Scavenging of $O_2^{\cdot-}$, $^{\cdot}OH$, and organic free radicals
β-carotene	Metabolic precursor of vitamin A. Fat soluble	Cell membranes	Scavenging of $O_2^{\cdot-}$, peroxyl radicals, and $^1O_2$
Aminoacids	Cysteine Tryptophane Methionine residues	Wide distribution	Scavenges oxidants as sacrificial antioxidant. Binds transition metals
	Taurine	Intracellular in cells with high rates of radical generation	Conjugates xenobiotics, reacts with HOCl
	Tyrosine	High concentration in semen plasma	Scavenges $^{\cdot}OH$ and peroxynitrite
Glucose	Carbohydrate	Wide distribution	Scavenging of $^{\cdot}OH$
Billirubin	Degradation product of heme	Blood and tissues	Chainbreaking antioxidant
Proteins	Albumin	Wide distribution	Binds transition metals. Scavenges oxidants as sacrificial antioxidant
	Ferritin	Intracellular	Binds and stores transition metals
	Hemosiderine	Intracellular	Binds and stores transition metals
Glycoproteins	Lactoferrin	Present in blood	Binds and transports transition metals
	Transferrin	Present in blood	Binds and transports transition metals
	Ceruloplasmin	Present in blood	Binds transition metals. Promotes iron binding to transferrin

Abbreviations: GSH = glutathione; Cu = copper; Zn = zinc; Mn = manganese; $O_2^{\cdot-}$ = superoxide anion; H_2O_2 = hydrogen peroxide; G-6-PD = glucose 6 phosphate dehyrogenase; 6-PGD = 6 phosphogluconate dehydrogenase; NADPH = nicotinamide adenine dinucleotide phosphate; $^{\cdot}OH$ = hydroxyl radical; $^1O_2$ = singlet oxygen; HOCl = hypochlorous acid.

Ten percent of the iron is stored in myoglobin, and a small portion in iron containing enzymes, such as lactoferrin and transferrin, that serve as transport proteins and prevent iron to participate in free radical forming reaction through the high affinity of their metal binding sites.[12] Transferrin, is under normal conditions only 30 % occupied with iron. At pH levels below 5.6, which may occur under ischemic conditions, iron may dissociate from transferrin. However, lactoferrin has a greater affinity for iron than transferrin at low pH. The remainder of the iron is stored in intracellular storage proteins, such as ferritin and hemosiderin, to prevent it to participate in the Haber-Weiss and Fenton reactions. [6] Under certain circumstances, however, such as acidosis or a reductive environment iron dissociates from ferritin and becomes available for catalyzing ·OH formation. Next to the ferritin bound iron, cells contain a transition pool of iron from which iron is withdrawn to form iron containing proteins. Although iron from these stores is available for free radical formation it seems that these stores are compartmentalized away from free radical reaction sites.[36]
The main binder of copper ions in plasma is albumin.[37] Also the glycoprotein ceruloplasmin binds copper. Moreover, ceruloplasmin, by means of the enzyme ceruloplasmin ferroxidase, promotes the iron binding to transferrin as it catalyses the oxidation of Fe^{2+} to Fe^{3+}.[38]

Eliminating oxidants or conversion of oxidants into less toxic species

The most studied group of antioxidants in the intracellular and extracellular spaces are those compounds that eliminate oxidants or converse them into less reactive species. Roughly these agents can be divided in enzymatic and non enzymatic antioxidants.

1. Enzymatic antioxidants

Superoxide dysmutase catalyses the dysmutation of $O_2^{\cdot-}$ into H_2O_2 through primarily reducing and subsequent oxidation by two superoxide anions of the metal ion (Cu, Zn, Mn) of the enzyme (reaction 12).

$$\text{SOD-Cu}^{2+} + O_2^{\cdot-} \rightarrow \text{SOD-Cu}^{+} + O_2$$

$$\underline{\text{SOD-Cu}^{+} + O_2^{\cdot-} + 2H^{+} \rightarrow \text{SOD-Cu}^{2+} + H_2O_2}$$

$$\text{Overall:} \qquad 2O_2^{\cdot-} + 2H^{+} \rightarrow O_2 + H_2O_2 \qquad (12)$$

The rate constant of this reaction is 10^4 times the rate constant of spontaneous dysmutation of $O_2^{\cdot-}$ at normal pH.[39] This means that normally no superoxide anion is available for reaction

with hydrogen peroxide to form the very reactive hydroxyl radicals. There are several forms of SOD. The copper and zinc containing enzymes are predominantly found in the cytoplasm. The manganese containing form is found in the mitochondrial matrix. In the extracellular fluid the high molecular weight copper containing form is found.[40] In circumstances of oxidative stress cellular synthesis of SOD has been shown to increase.[39] It should be noted at this point that H_2O_2, a reaction product of dysmutation of $O_2^{\cdot-}$ is also capable of inactivation of SOD by the formation of $^{\cdot}OH$.[41] However, as H_2O_2 has a low affinity for SOD compared to catalase and peroxidases, interplay between them offers adequate antioxidant protection.

Catalase catalyzes the conversion of H_2O_2 into water and oxygen (reaction 13).

$$2H_2O_2 \rightarrow (\text{catalase}) \rightarrow 2H_2O + O_2 \qquad (13)$$

It has appreciable reductive activity for only small peroxide molecules such as, H_2O_2, methyl- and ethyl hydroperoxides and does not metabolize larger molecules such as lipid hydroperoxides formed during lipid peroxidation. Catalase is primarily located in intracellular peroxisomes, which contain many enzymes that generate H_2O_2 in aerobic cells, and is therefore highly compartmentalized in mammalian cells.[28] Catalase activity is highest in erythrocytes.

Glutathione peroxidase is the key enzyme in the so-called glutathione redox cycle which is considered the central mechanism of intracellular reduction of hydroperoxides (Figure 3). The redox cycle is complementary to catalase in the elimination of H_2O_2 but exceeds catalase in its capacity to reduce other toxic hydroperoxides.[42] A biologically important source of hydroperoxides are the lipid hydroperoxides formed by free radical attack on the polyunsaturated lipid cell membranes through lipid peroxidation. These lipid hydroperoxides are reduced by the selenium containing GSH peroxidase into their corresponding, less reactive alcohols at the expense of GSH that is oxidized to GSSG (reaction 14).

$$ROOH + 2GSH \rightarrow GSSG + ROH + H_2O \qquad (14)$$

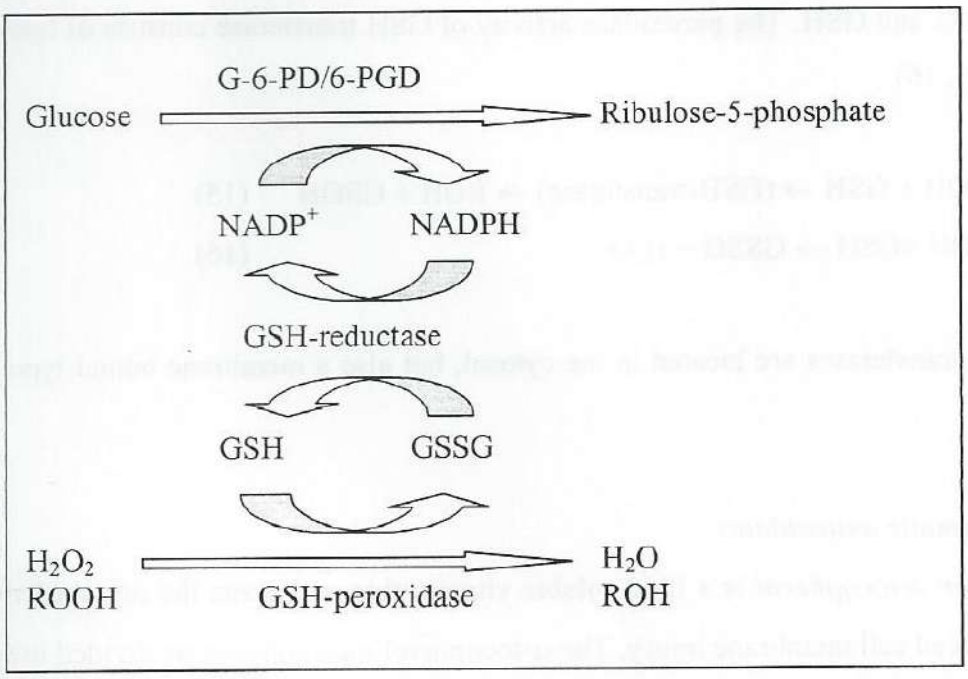


Figure 3. Schematic representation of the GSH redox cycle. Glucose 6 phosphate dehydrogenase (G-6-PD) and 6 phosphogluconate dehydrogenase (6-PGD) enzymatically metabolize glucose to generate NADPH.

Under normal conditions cells keep a high intracellular ratio GSH/GSSG. On one hand this high ratio ensures the availability of reduced glutathione (GSH) to deal with radical stress. On the other hand a excess of GSSG is avoided as GSSG is known to be cytotoxic.[43] The cell possesses two mechanisms by which high levels of GSSG are avoided. First, GSSG can actively be transported out of the cell by a transmembrane transport mechanism.[43] Second, the enzyme GSSG-reductase is capable of reducing GSSG back into GSH. The necessary NADPH is provided by the hexose monophosphate shunt. The antioxidant effect is indirect through the capacity to generate NADPH which is used in the redox cycle. NADPH does not directly scavenge oxidants.[44] Like GSH-peroxidase, GSH-reductase is present in the cytosol however, it also exists in mitochondria and plasma. Cells that are deficient of G-6-PD activity are more sensitive to oxidant mediated damage. SOD, catalase, and GSH-peroxidase work together in limiting oxidant stress on cells. Moreover, they protect each other against oxidant inactivation. As stated before H_2O_2 can inactivate SOD but also $O_2^{\cdot-}$ can inhibit catalase and peroxidase function.[45]

Most cells have a different class of GSH-peroxidases which are the non selenium dependent GSH transferases. GSH-transferases catalyze the reaction between organic hydroperoxides, but not H_2O_2, and GSH. The peroxidase activity of GSH transferase consists of two reactions (reaction 15, 16).

$$ROOH + GSH \rightarrow (GSH\text{-transferase}) \rightarrow ROH + GSOH \quad (15)$$

$$GSOH + GSH \rightarrow GSSG + H_2O \quad (16)$$

Most GSH-transferases are located in the cytosol, but also a membrane bound type has been described.[14]

2. Non-enzymatic antioxidants

Vitamin E or α-tocopherol is a lipid soluble vitamin that represents the principal defense in radical induced cell membrane injury. The α-tocopherol molecule can be divided in two parts, a chroman head and a phytyl chain (Figure 4). The phytyl chain is believed to anchor between the fatty acid residues of the phospohlipids. The chroman group, which is responsible for the antioxidant effect, faces the cytosol, although the chroman ring is still located in the hydrophobic zone of the lipid bilayer.[14]

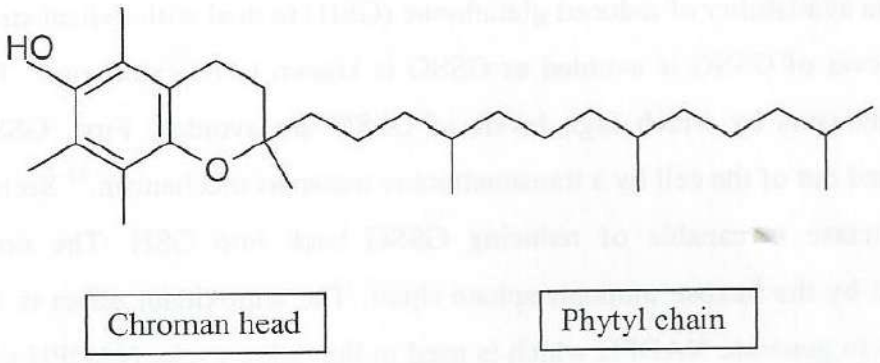


Figure 4. Chemical structure of α-tocopherol

In the antioxidant activity of vitamin E, a radical (LOO·) abstracts a hydrogen atom from the aromatic hydroxyl group of the chroman head (vitE-OH) and forms a chromanoxyl radical (vitE-O·) (reaction 17) which is fairly stable due to delocalisation of the unpaired electron.

$$LOO^{\cdot} + \text{vitE-OH} \rightarrow LH + \text{vitE-O}^{\cdot} \qquad (17)$$

Vitamin E is a particularly effective antioxidant and converts $O_2^{\cdot-}$, ·OH and lipid peroxyl radicals to less reactive forms.[46] Moreover, tocopherols are scavengers of singlet oxygen.[47] In order to give efficient protection, vitamin E has to be regenerated from the vitamin E radical. Both vitamin C and GSH are able to mediate the regeneration of vitamin E (vide infra). Although vitamin E is present in extracellular fluids and blood plasma, its main antioxidant activity is expressed in the cell membrane where it inhibits lipidperoxidation by interrupting the chain reaction through scavenging the lipid peroxyl radicals (LOO·). Inhibition of lipid peroxidation in blood by vitamin E is marginal, other yet undefined compounds may possibly play a more significant role in defending polyunsaturated lipids in serum from peroxidation.[48] It should be noted that a relatively high concentration of vitamin E may cause radical formation and therefore can function as a pro-oxidant.[2]

Vitamin C is a hydrophilic vitamin with well known antioxidant properties. Its water solubility allows it to be widely available in both the extracellular and intracellular spaces. In relatively high concentrations vitamin C can directly scavenge $O_2^{\cdot-}$, and ·OH under the formation of the vitamin C radical (dehydroascorbate radical, vit C·) (reaction 18).[49,50]

$$\text{Vit C} + {}^{\cdot}OH \rightarrow \text{Vit C}^{\cdot} + H_2O + H^+ \qquad (18)$$

Although not very efficient, due to its hydrophilic character, vitamin C is also known to scavenge lipidperoxyl radicals and by this means break the chain reaction of lipid peroxidation. Furthermore, vitamin C functions as an antioxidant by regeneration vitamin E.[51] In lower concentrations vitamin C is also known to have distinct pro-oxidant properties.[14,52] In the presence of iron or copper also the vitamin C radical can be formed by the reduction of Fe^{3+} to Fe^{2+} (reaction 19).

$$Fe^{3+} + \text{Vit C} \rightarrow Fe^{2+} + \text{Vit C}^{\cdot} + 2H^+ \qquad (19)$$

Although the vitamin C radical itself is a relatively, non reactive species, it may either terminate the propagation via dysmutation, resulting in the production of vitamin C and dehydroascorbate (DHA) (reaction 20), or reduce another Fe^{3+} ion (reaction 21).[49]

$$2\text{Vit C}^{\cdot} + 2H^{+} \rightarrow \text{VitC} + \text{DHA} \qquad (20)$$

$$\text{Vit C}^{\cdot} + Fe^{3+} \rightarrow \text{DHA} + Fe^{2+} \qquad (21)$$

During the oxidation of vitamin C, H_2O_2 is formed (reaction 22).

$$\text{Vit C} + O_2 \rightarrow \text{DHA} + H_2O_2 \qquad (22)$$

All these reactions provide the necessary ingredients for the Fenton reaction (reaction 3) to occur and form ·OH radicals, which may induce tissue damage. It has become clear that the degree of iron reduction determines the prevalence of vitamin C to act as a pro- or antioxidant.[2]

Glutathione (GSH) is a tripeptide containing a thiol (SH) group which plays a pivotal function in several antioxidant protective systems. The GSH-peroxidases and transferases participating in the GSH redox cycle have already been described. In addition, the water soluble GSH can function as an antioxidant unrelated to the GSH-redox cycle as direct scavengers of radicals like $O_2^{\cdot-}$, ·OH, and organic free radicals.[53] Furthermore, GSH is shown to act as an antioxidant by the regeneration of vitamin E through a GSH selective free radical reductase containing an essential and vulnerable thiol moiety itself (Figure 5).[54,55,56,57]

There is also an interplay between GSH and vitamin C. First, GSH can react with dehydroascorbate (DHA) forming GSSG and vitamin C (reaction 23). Second, The GSH radical, called the thiyl radical (GS·), can react with vitamin C forming the vitamin C radical and GSH (reaction 24).[58,59]

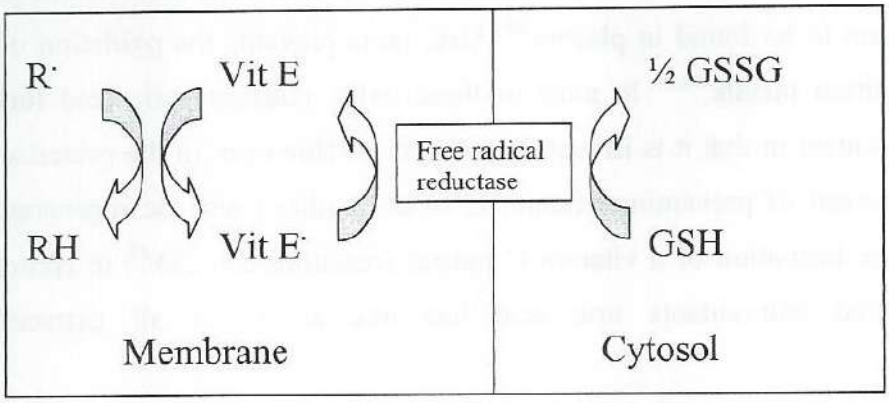


Figure 5. Schematic representation of regeneration of vitamin E by glutathione through catalytic activity of free radical reductase.

$$2\ GSH + DHA \rightarrow GSSG + Vit\ C \qquad (23)$$

$$GS^{\cdot} + Vit\ C \rightarrow GSH + Vit\ C^{\cdot} \qquad (24)$$

GSH has no direct pro-oxidant activity, since it is not able to reduce free iron.[54] However, GSH can regenerate vitamin C (reaction 23), providing indirect a reducing equivalent for the reduction of Fe^{3+} to Fe^{2+} and by this means GSH can indirectly act as a pro-oxidant. On the other hand due to delocalisation the unpaired electron in the vitamin C radical is more stable than in the thiyl radical leading to an indirect antioxidant effect.[49] The physiological relevance of the interplay between GSH and vitamin C is not fully clear yet.[60]

Uric acid is formed from the catabolism of purines by the enzyme xanthine dehydrogenase (XDH) (reaction 25). The enzyme uses NAD^+ as the electron acceptor, but may be converted from this XDH form into the xanthine oxidase (XO) form, which transfers the reducing equivalents directly to molecular oxygen (reaction 26).[61] During for instance ischemia adenosine triphosphate forms hypoxanthine providing a substrate for xanthine oxidase.

$$xanthine + H_2O + NAD^+ \rightarrow (XDH) \rightarrow urate + NADH + H^+ \qquad (25)$$

$$xanthine + H_2O + 2O_2 \rightarrow (XO) \rightarrow urate + 2O_2^{\cdot-} + 2H^+ \qquad (26)$$

Uric acid has powerful antioxidant properties.[62] At physiological pH, 99% of the uric acid is present in the form of its monovalent anion urate which means that only reactions involving urate are important.[61] Urate has been proven to directly scavenge hydroxyl radicals, singlet

oxygen and peroxyl radicals.[63,64] Furthermore, urate represents the major inhibitor of ozone induced oxidation to be found in plasma.[65] Also, urate prevents the oxidation of vitamin C and binds transition metals.[66,67] In most of these redox reactions uric acid functions as a sacrificial antioxidant in that it is irreversibly degraded. However, in the presence of vitamin C, urate can instead of preventing vitamin C to be oxidized also be regenerated from its radical under the formation of a vitamin C radical (reactions 27, 28).[68] In contrast to other compartmentalized antioxidants uric acid has free access to all extracellular fluid compartments.

$$^{\cdot}OH + \text{urate} \rightarrow OH^{-} + \text{urate}^{\cdot} \quad (27)$$

$$\text{urate}^{\cdot} + \text{Vitamin C} \rightarrow \text{urate} + \text{Vit C}^{\cdot} \quad (28)$$

In addition to the aforementioned antioxidants there are several other fat- and water soluble antioxidants, such as β-carotene, glucose, and the amino acids cysteine, tyrosine and taurine which have their own specific antioxidant properties but will not be discussed here in detail.[4]

Reparation of molecular damage by free radicals

The repair of oxidant injury depends on the initiation of both the repair or removal of injured cellular components and on the increased cellular proliferation to replace damaged structures.[28] The molecular repair of oxidant mediated injury to nucleic acids, leading to DNA strand breakage, is well known.[69] Furthermore, repair of oxidant induced cell membrane damage by phospholipase A_2 and selenium dependent phospholipid hydroperoxide glutathione peroxidase has been reported.[70,71] Although the exact stimulus for cell proliferation after oxidant damage is uncertain, cell proliferation has been shown for lung cells and endothelial cells.[72] Cell proliferation may not only allow replacement of injured cells during hyperoxia but also cells with greater tolerance to oxidative stress may be formed.

Free oxygen radicals in human disease

Oxidative stress has been involved in the pathogenesis of various human diseases such as rheumatoid arthritis, adult respiratory distress syndrome, chronic obstructive pulmonary disease, reperfusion injury after myocardial ischemia, atherosclerosis, AIDS, cystic fibrosis and carcinogenesis.[4,73] This wide range of disorders implies that reactive oxygen species accompany tissue injury, as tissue injury leads to oxidative stress. Some times reactive

oxygen species make a significant contribution to the disease pathology however, at other times they may not (Figure 6).[4]

Disease which is associated with oxidative stress either results from:[74]

1. a diminished antioxidant defense through mutations affecting antioxidant defense enzymes or depletion of antioxidant defense.
2. an increased production of reactive oxygen species through exposure to elevated external levels of oxidants or excessive activation of endogenous oxidant generating systems.

Cells deal with oxidative stress through adaptive mechanisms. As a result of mild oxidative stress usually the antioxidative defense systems are up-regulated which may offer partial protection, complete protection or even permanent resistance against oxidative stress. Otherwise, oxidative stress may cause cell damage. Important cellular targets for oxidative stress are DNA, proteins, carbohydrates, and membrane lipids.[6] DNA is an important early target for oxidative stress. Strand breaks may occur, a feature which has been related to carcinogenesis.[75] Furthermore, oxidative stress can activate transcription factors such as NF-κB promoting the expression of pro-oxidant proteins and enzymes. NF-κB activation has been related to severe diseases including atherosclerosis.[76] Also oxidation of membrane phospholipid molecules, better known as lipid peroxidation, and leading to irreversible membrane damage has been implicated in the pathogenesis of several diseases such as rheumatoid arthritis.[73]

It should be noted that in most human diseases oxidative stress is a consequence and not a cause of the primary disease process. The primary tissue injury, i.e. caused by infection, trauma, radiation, ischemia or toxins leads to the formation of all kinds of mediators such as prostaglandins, interleukins, cytokines, and reactive oxygen species.[74] Nevertheless, free radical mediated damage has a substantial contribution to the final tissue injury. In order to relate reactive oxygen species to disease the ROS should be present at the site of injury. Moreover, its time course of formation should be coincide with the time course of the tissue injury and direct application of the ROS to the tissue at concentrations within the range found in vivo should reproduce most of all damage observed. Finally, removing the agent or inhibiting its formation should diminish the injury to an extent related to the degree of removal of the agent or inhibition of its formation.[4]

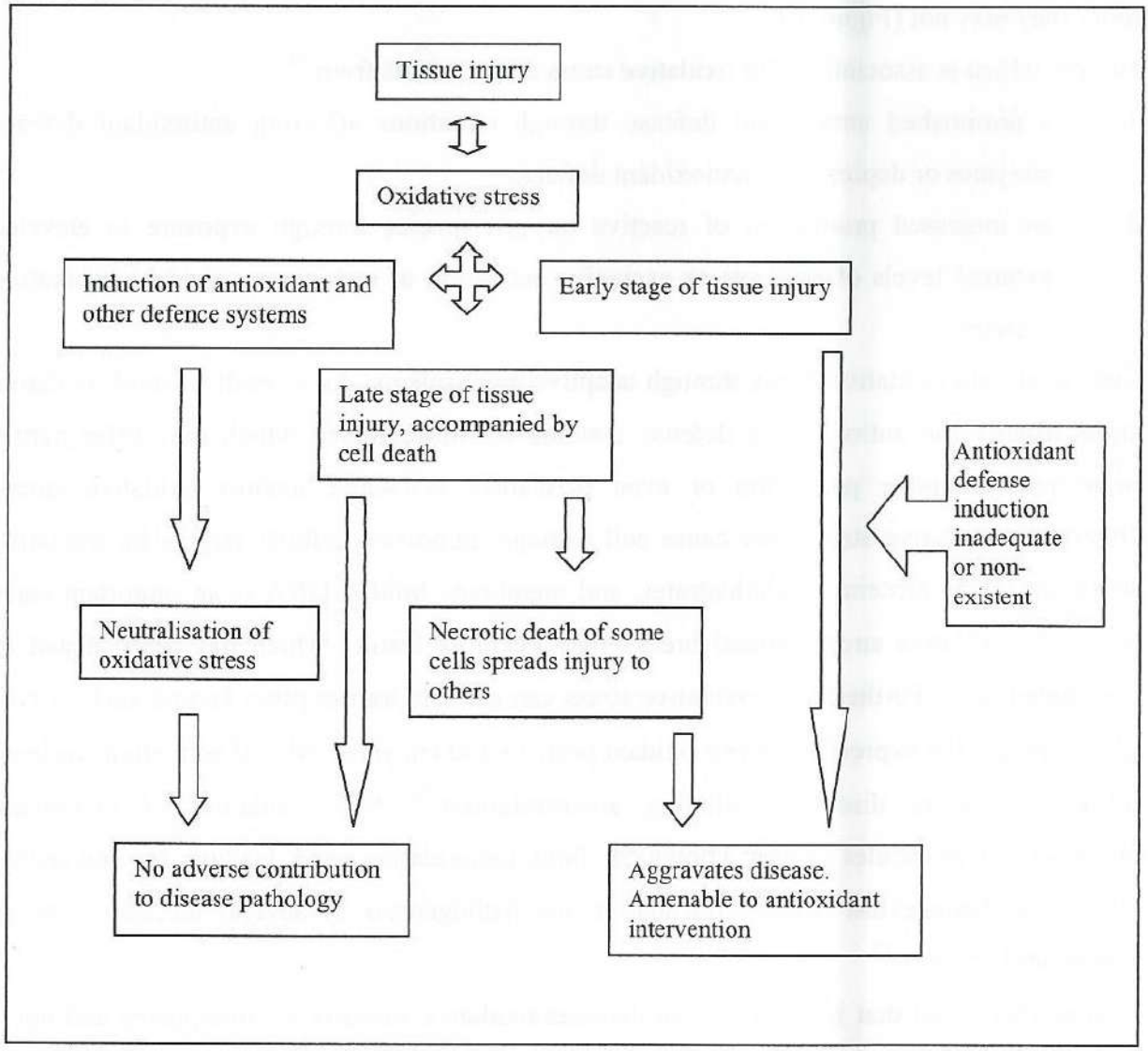


Figure 6. The significance of oxidative stress in human disease. Flowchart according to Halliwell and Gutteridge.[4]

Antioxidant therapy consists of antioxidants that prevent disease and antioxidant pharmacotherapy. Preventive antioxidants are predominantly diet derived. For instance vitamin E has been shown to have a protective role in cardiovascular disease. Also vitamin C, β-carotene, and selenium are implicated in preventing cardiovascular disease.[77] Furthermore, it has been shown that a dietary combination of vitamin E, selenium and β-carotene, but not vitamin C, gives a drop in gastric cancer deaths. However, for lung cancer this was not proven.[4]

Pharmacotherapeutic antioxidants ideally encompasses drugs that can be targeted to cellular locations where undesirable excessive radical formation occurs. Moreover, they should not interfere with physiologically important radical processes. Pharmacotherapy may consist of

naturally occurring antioxidants or of synthetic molecules. Naturally occurring antioxidants such as flavonoids and lipoic acid have been shown to have beneficial effects in respectively protection against doxorubicin induced cardiotoxicity and diabetic poly neuropathy.[78,79] The synthetic antioxidant N-acetylcysteine has been implicated in the treatment of various inflammatory pulmonary disorders.[80,81] Furthermore, a wide range of other endogenous and exogenous antioxidants have been described to have distinct therapeutic use in free oxygen radical mediated diseases.[82]

References

1. Crystal RG. Summary. *Am J Med* 1991; 91(suppl 3C):145S.
2. Bast A, Crystal RG, eds. GSH system, glutathione in antioxidant defense. Excerpta Medica, Amsterdam, Hong Kong, Princeton, Sydneay, Tokio 1992.
3. Southorn PA, Powis G. Free radicals in medicine. I. Chemical nature and biologic reactions. *Mayo Clin Proc* 1988; 63:381-389.
4. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 3th edition. Oxford Science Publications 1999.
5. Taube H. Mechanisms of oxydation with oxygen. *J Gen Physiol* 1965; (Part 2):2950.
6. Bast A, Haenen GRMM, Doelman CJA. Oxidants and antioxidants: state of the art. *Am J Med 1991*; 91 (suppl 3C):2S-13S.
7. Crystal RG. Oxidants and respiratory tract epithelial injury: pathogenesis and strategies for therapeutic intervention. *Am J Med* 1991; suppl 3C;39S-44S.
8. Pryor WA. Free radical biology: xenobiotics, cancer, and aging. *Ann NY Acad Sci* 1982; 393:1-22.
9. McCord JM, Fridovich I. The reduction of cytochrome c by milk xantine oxidase. *J Biol Chem* 1968; 243:5753-5760.
10. Bast A. Is formation of reactive oxygen by cytochrome P-450 perilous and predictable ? *Pharmacol Sci* 1986; 7:266-270.
11. Fridovich I. Superoxide dysmutases. *Adv Enzymol* 1974; 41:35-48.
12. Halliwell B, Gutteridge JMC. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* 1984; 219:1-14.
13. Aruoma OI, Halliwell B, Dizdaroglu M. Iron ion-dependent modification of bases in DNA by the superoxide radical-generating system hupoxantine/xantine oxidase. *J Biol Chem* 1989; 264:13024-13028.
14. Haenen GRMM. Thiols in oxidative stress. Some implications for catecholamine toxicity.Academical thesis. Amsterdam 1989.
15. Snyder SH, Bredt DS. Biological roles of nitric oxide. *Sci Am* 1992; 266:68-77.
16. Moncada S, Palmer RMJ, Higgs EA. Biosynthesis of nitric oxide from l-arginine. A pathway for the regulation of cell functions and communication. *Biochem Pharmacol* 1989; 38:1709-1715.

17. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* 1991; 43:109-142.

18. Moncada S. The l-arginine nitric oxide pathway. *Acta Physiol Scand* 1992; 145:201-222.

19. Bredt DS, Snyder SH. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci* 1990; 87:682-685.

20. Nathan C, Xie Q. Regulation of biosynthesis of nitric oxide. *J Biol Chem* 1994; 269:13725-13728.

21. Hibbs JB, Taintor RR, Vavrin Z. Macrophage cytotoxicity: role for L-arginine deiminase activity and imino nitrogen oxidation to nitrite. *Science* 1987; 235:473-476.

22. Barnes PJ. Nitric oxide. In: Asthma; basic mechanisms and clinical management, 3th ed. Barnes PJ, Rodger IW, Thomson NC eds. Academic Press. 1998.

23. Pryor WA, Squadito GL. The chemistry of peroxynitrite: a product from the reaction of nitric oxide and superoxide. *Am J Physiol* 1995; 268:L699-L722.

24. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990; 87:1620-1624.

25. Noronha-Dutra AA, Epperlein MM, Woolf N. Reaction of nitric oxide with hydrogen peroxide to produce potentially cytotoxic singlet oxygen as a model for nitric oxide-mediated killing. *FEBS Lett* 1993; 321:59-62.

26. Koppenol WH. Thermodynamic considerations on the formation of reactive species from hypochlorite, superoxide and nitrogen monoxide. *FEBS Lett* 1994; 347:5-8.

27. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. 2nd ed. Oxford, Claridon Press 1989.

28. Heffner JE, Repine JE. Pulmonary startegies of antioxidant defense. *Am Rev Respir Dis* 1989; 140:531-554.

29. Sies H. Antixoidant activity in cells and organs. *Am Rev Respir Dis* 1987; 136:478-480.

30. Wharton DC, Gibson QH. Studies of the oxygen generated compound of cytochrome oxidase. *J Biol Chem* 1968; 243:702-706.

31. Sugano T, Shino NO, Chance B. Mitochondrial function under hypoxic conditions. The steady state of cytochrom reduction and of energy metabolism. *Biochim Biophys Acta* 1974; 347:340-358.

32. Halliwell B. Oxygen radicals and metal ions potential antioxidant intervention strategies. *Ann Intern Med* 1987; 107:526-545.

33. Gutteridge JNC. The antioxidant activity of haptoglobin toward hemoglobin-stimulated lipid peroxidation. *Biochim Biophys Acta* 1987; 917:219-223.

34. Gutteridge JMC, Smith A. Antioxidant protection by heamopexin of heam stimulated lipid peroxidation. *Biochem J* 1988; 256:861-865.

35. Gutteridge JNC. Iron promotors of the Fenton reatcion and lipid peroxidation can be released from haemoglobin by peroxides. *FEBS Lett* 1986; 201:291-295

36. Halliwell B, Gutteridge JNC. Oxygen free radicals and iron in relation to biology and medicine: some problems and some concepts. *Arch Biochem Biophys* 1986; 246:501-514

37. Halliwell B. Albumin- an important extracellular anti-oxidant? *Biochem Pharmacol* 1988; 37:569-571.

38. Chidambaram MV, Barnes G, Frieden E. Ceruloplasmin and the reactions forming diferric transferrin. *FEBS Lett* 1983; 159:137-140.

39. Fridovich I. Superoxide radical: an enogenous toxicant. *Annu Rev Pharmacol Toxicol* 1983; 23:239-257.

40. Marklund SL, Holme E, Hellner L. Superoxide dismutase in extracellular fluids. *Clin Chim Acta* 1982; 126:41-51.

41. Yim MB, Chock PB, Stadtman ER. Copper zinc superoxide dismutase catalyzes hydroxyl radical production from hydrogen peroxide. *Proc Natl Acad Sci USA* 1990; 87:5006-5010.

42. Chance B, Sies H, Boveris A. Hydroperoxidase metabolism in mammalian organs. *Physiol Rev* 1979; 59:527-605.

43. Sies H. Akerboom TPM. Glutathione disulfide (GSSG) efflux from cells and tissues. *Methods Enzymol* 1984; 105:445-51.

44. Koufos A, Sagone AL. Effects of oxidant stress on the hexose monophosphate shunt pathway of platelets. *Blood* 1980; 165:195-199.

45. Blum J, Fridovich I. Inactivation of glutathione peroxidase by superoxide radical. *Arch Biochem Biophys* 1985; 240:500-508.

46. Tappel AL. Vitamin E as biological lipid antioxidant. *Vitam Horm* 1962; 20:493-510.

47. Kaiser S, Dimascio P, Murphy ME, Sies H. Physical and chemical scavenging of singlet molecular oxygen by tocopherol. *Arch Biochem Biophys* 1990; 227:101-108.

48. Wayner DDM, Burton GW, Inghold KU, Locke S. Quantitative measurement of total peroxyl radical trapping antioxidant capability of human blood plasma. *FEBS Lett* 1985; 187:33-37.

49. Bielski BHJ, Richter HW. Some properties of the ascorbate free radical. *Ann NY Acad Sci* 1975; 258:231-237.

50. Patel Jm, Edwards DA. Vitamin E, membrane order and antioxidant behaviour in lung mocrosomes and reconstituted vesicles. *Toxicol Appl Pharmacol* 1988; 96:101-114.

51. Doba T, Burton GW Inghold KU. Antioxidant and co-oxidant activity of vitamin C. *Biochem Biophys Acta* 1985; 835:298-303.

52. Lee SH, Oe T, Blair IA. Vitamin C induced decomposition of lipid hydroperoxides to endogenous genotoxins. *Science* 2001; 292:2083-2086.

53. Ketterer B. Detoxification reactions of glutathione and glutathione transferases. *Xenobiotica* 1986; 16:957-973.

54. Haenen GRMM, Bast A. protection against lipidperoxidation by a microsomal glutathione dependent heat labile factor. *FEBS Lett* 1983; 159:24-28.

55. Haenen GRMM, Tan Tin Tsoi JNL, Vermeulen NPE, Timmerman H, Bast A. 4-Hydroxy-2,3-trans-nonenal stimulates microsomal lipid peroxidation by reducing the glutathione dependent protection. *Arch Biochem Biophys* 1987; 259:449-456.

56. Haenen GRMM, Vermeulen NPE, Timmerman H, Bast A. Effect of several thiols on lipid peroxidation in rat liver microsomes. *Chem Biol Interact* 1989; 71:201-212.

57. Haenen GRMM, Vermeulen NPE, Tjai Tin Tsoi JNL, Ragetli HMN, Timmerman H, Bast A. Actvation of microsomal glutathione-S-transferase and reduction of the glutathione dependent protection by acrolein. *Biochem Pharmacol* 1988; 37:1933-1938.

58. Bast A, Haenen GRMM Savenije-Chapel EM. Inhibition of rat hepatic microsomal lipid peroxidation by mesna via glutathione. *Arzneim. Forsch* 1987; 37:1043-1045.

59. Forni LC, Wilson RL. Vitamin C and consecutive hydrogen atom and electron transfer in free radical reactions. A novel catalytic role for glutathione. In: McBrein DC, Slater TF, eds. Protective agents in cancer. Academic press, New York 1983; 161-173.

60. Leung HW, Morrow PE. Interactions of glutathione and ascorbic acid in guinea pig lungs exposed to nitrogen dioxide. *Res Comm Chem Pathol Pharmacol* 1981 ;31:111-118.

61. Becker BF. Towards the physiologocal function of uric acid. *Free Rad Biol Med* 1993; 14:615-631.

62. Ames BN, Cathcart R Schwiers E, Hochstein P. Uric acid provides an antioxidant defense in humans against oxidant and radical caused aging and cancer. A hypothesis. *Proc Natl Acad Sci USA* 1981; 78:6858-6862.

63. Wayner DD, Burton GW, Ingold KU, Barclay LRC, Locke SJ. The relative contributions of vitamin E urate ascorbate and proteins to the total peroxyl trapping antioxidant activity of human blood plasma. *Biochim Biophys Acta* 1987; 924:408-419.

64. Thomas MJ. Urate causes the human polymorphonuclear leukocyte to secrete superoxide. *Free Radic Biol Med* 1992; 12:89-91.

65. Cross CE, Motchnik PA, Bruener BA, Jones DA, Kaur H, Ames BN, Halliwell B. Oxidative damage to plasma constituents by ozone. *FEBS Lett* 1992; 298:269-272.

66. Sevenian A, Davies KJA, Hochstein P. Conservation of vitamin C by uric acid in blood. *Free Rad Biol Med* 1985; 1:117-124.

67. Davies KJA, Sevenian A Muakkassah-Kelly SF, Hochstein P. Uric acid iron ion complexes: a new aspect of the antioxidant functions of uric acid. *Biochem J* 1986; 235:747-754.

68. Simic MG, Jovanovic SV. Antioxidation mechanisms of uric acid. *J Am Chem Soc* 1989; 111:5778-5782.

69. Sancar A. DNA excision repair. *Annu Rev Biochem* 1996; 65:43-81.

70. Sevenian A, Muakkassah-Kelly SF, Montestruqe S. The influence of phospholipase A_2 and glutathione peroxidase on the elimination of membrane lipid peroxides. *Arch Biochem Biophys* 1983; 223:441-452.

71. Kuyk FJGM , Sevenian A, Handelman GJ, Dratz EA. A new role for phospholipase A_2: protection of membranes from lipid preoxidation damage. *Trends Biochem Sci* 1987; 12:31-34.

72. Thet LA, Parra SC, Shelburne JD. Repair of oxygen induced lung injury in adult rats. The role of ornithne decarboxylase and polyamines. *Am Rev Resp Dis* 1984; 129:174-181.

73. Southorn PA, Powis G. Free radicals in medicine II. Involvement in human disease. *Mayo Clin Proc* 1988; 63:390-408.

74. Halliwell B, Gutteridge JM, Cross CE. Free radicals, antioxidants and human disease. Where are we now ? *J Lab Clin Med* 1992; 119:598-620.

75. Cerutti PA. Oxyradicals and cancer. *Lancet* 1994; 344:862-863.

76. Collins T. Endothelial nuclear factor κB and the initiation of the atherosclerotic lesion. *Lab Invest* 1993; 68:499-508.

77. Brussaard JH, van Dis SJ, van Poppel G. Antioxidantia en de preventie van hart en vaartziekten. *Ned Tijdschr Geneesk* 1992; 43:2110-2114.

78. van Acker SABE. Flavonoids as protective agents against doxorubicin-induced cardiotoxicity. Academical thesis. Amsterdam 1996.

79. Biewenga G. Lipoic acid. A pharmacochemical study. Academical thesis. Amsterdam 1997.

80. van Herwaarden CLA, Bast A, Dekhuijzen PNR. The role of N-acetylcysteine in the treatment of chronic obstructive pulmonary disease. *Neth J Med* 1995; 47:45-48.

81. Doelman CJA. Reactive oxygen species and airway hypereactivity. Academical thesis. 1991.

82. Halliwell B. Drug antioxidant effects. A basis for drug selection ? *Drugs* 1991: 42:569-605.

Chapter I-3

Oxidative stress in upper respiratory tract inflammation

Gerrit-Jan Westerveld[1], Robert A Scheeren[1], and Aalt Bast[2]

[1] Department of Otorhinolaryngology and Head and Neck Surgery, Medical Center Vrije Universiteit, Amsterdam, The Netherlands.

[2] Department of Pharmacology and Toxicology, University of Maastricht, The Netherlands.

Submitted

Abstract

The mucosa of the nose and the paranasal sinuses can be damaged by oxidants. Inflammation, exposure to air pollution, such as ozone, and even exposure to high levels of pure oxygen can be harmful to the mucosa and may aggravate inflammatory disease through oxidative damage. Also the metabolism of inhaled xenobiotics may produce potential dangerous products leading to oxidant stress. The reactive oxygen en nitrogen species responsible for the mucosal damage and the endogenous antioxidant defense mechanisms dealing with them are discussed in this review. Possible pharmacotherapeutic antioxidant strategies in the treatment of several upper respiratory tract pathologies are mentioned.

Introduction

For efficient function of the upper respiratory tract the mucosa of the nose and the paranasal sinuses needs to be in optimal condition. Damage to the mucosa may lead to upper respiratory tract infection or secondary pulmonary disorders, due to impaired air-conditioning of the inhaled air. The upper airways can be damaged in different ways. During inflammation, leukocytes, macrophages and mast cells may release mediators, among others, leading to diminished function of the mucociliary system. Inhaled air may also damage the upper respiratory tract mucosa. Exposure to cigarette smoke, ozone and other airborne pollutants and even high doses of pure oxygen may impair the mucosal function. Furthermore, metabolizing inhaled xenobiotics may destruct nasal mucosa. All these mechanisms have one thing in common i.e. the damage is at least partially mediated via reactive oxygen species (ROS). Important ROS are, the superoxide anion radical ($O_2^{\cdot-}$), the hydroxyl radical ($^{\cdot}OH$), and hydrogen peroxide (H_2O_2). Other, especially biologically important ROS are the lipid peroxyl radical ($LOO^{\cdot}$), singlet oxygen ($^1O_2$) and peroxynitrite ($ONOO^-$). The latter is formed from the reaction between $O_2^{\cdot-}$ and the nitric oxide radical ($NO^{\cdot}$), a reactive nitrogen species (RNS). To cope with the assault of endogenous and exogenous oxidants tissues contain a broad spectrum of antioxidant defense mechanisms. Except from preventing reactive oxygen species to be formed by diminishing transition metal availability by compounds such as hemoglobin and albumin, free radicals can be inactivated by several enzymatic and non-enzymatic substances. The most important antioxidative enzymes are superoxide dysmutase (SOD), catalase, and glutathione peroxidase (GSH-px). The main non-enzymatic antioxidant compounds are vitamin E, vitamin C, GSH, and uric acid.[1]

Numerous reports have pointed out that oxidant induced cellular injury may underlie the pathogenesis of various inflammatory human respiratory tract diseases. For the lungs extensive reviews have been published indicating that an imbalance between the presence of free oxygen radicals and antioxidative defense mechanisms is at least partially responsible for several pulmonary disorders. [1,2,3] Although the upper respiratory tract and the lungs form a pathophysiologic continuum, considerably less is known about a possible pathogenetic relationship between oxidative stress and inflammatory diseases like rhinosinusitis and nasal polyps.

In this review we describe mechanisms of oxidative stress leading to damage of upper airways and the development of subsequent disease. Moreover, possible antioxidative strategies are discussed.

Reactive oxygen species produced by inflammatory cells

During inflammation, inflammatory cells such as neutrophils, eosinophils, macrophages and monocytes become activated. Under resting conditions these cells consume little oxygen (O_2), since they rely mainly on glycolysis for ATP production. However, activation of these cells leads to a marked increase in O_2 uptake also known as the respiratory or oxidative burst. In normal phagocytosis, the cytotoxic reactive oxygen species are used to destroy invading microorganisms. [4] The respiratory burst is triggered by various stimulants such as opsonized bacteria and viruses, immune complexes, complement fragments, phorbol esters, platelet activating factor (PAF), leukotriene B_4 (LTB_4) and interleukin-8 (IL-8).[5] All stimulating substances interact with specific receptors and elicit the specific responses that are required for the defense function of the cell, i.e. the respiratory burst. In contrast to activation of inflammatory cells, 'priming' of these cells does not stimulate the respiratory burst itself. However, priming leads to a much more vigorous respiratory burst on subsequent stimulation.[6] Priming agents include several cytokines, bacterial lipopolysaccharide and complement peptides.[7] During the respiratory burst, superoxide anions are produced in a reaction which is catalyzed by the enzyme reduced nicotinamide adenine dinucleotide phosphate oxidase (NADPH-oxidase). This enzyme is located on the exterior surface of the cell membrane including that lining the phagocytic vacuole (Figure 1).

NADPH-oxidase reduces oxygen to superoxide anion radicals by utilizing an electron derived from the oxidation of NADPH into $NADP^+$. NADPH is provided by the cytosolic hexose monophosphate shunt. Although the mechanism of activation of the NADPH-oxidase is not totally elucidated it has been shown that occupancy of a cell membrane receptor by a stimulating agent leads to a phospholipase C (PLC) catalyzed cleavage of membrane phospholipids generating two separate second messengers i.e. diacylglycerol and polyphosphoinositol. The link between the receptor and the PLC is a guanosine triphosphate (GTP) consuming G-protein. Diacylglycerol activates protein kinase C (PKC) which activates the NADPH-oxidase system via phosphorylation of a number of proteins. The polyphosphoinositol groups generate increased cytosolar calcium (Ca^{2+}) levels from intracellular stores and from the extracellular environment which greatly facilitates the ability of diacylglycerol to activate PKC.[8] The produced superoxide anion radicals dysmutate into hydrogen peroxide by one superoxide anion radical acting on another. Dysmutation may

occur either spontaneously (rate constant 1 x 10^{-5} M^{-1} s^{-1}), or catalyzed by the intracellular enzyme superoxide dysmutase (SOD) (rate constant 1 x 10^{-9} M^{-1} s^{-1}).[9]

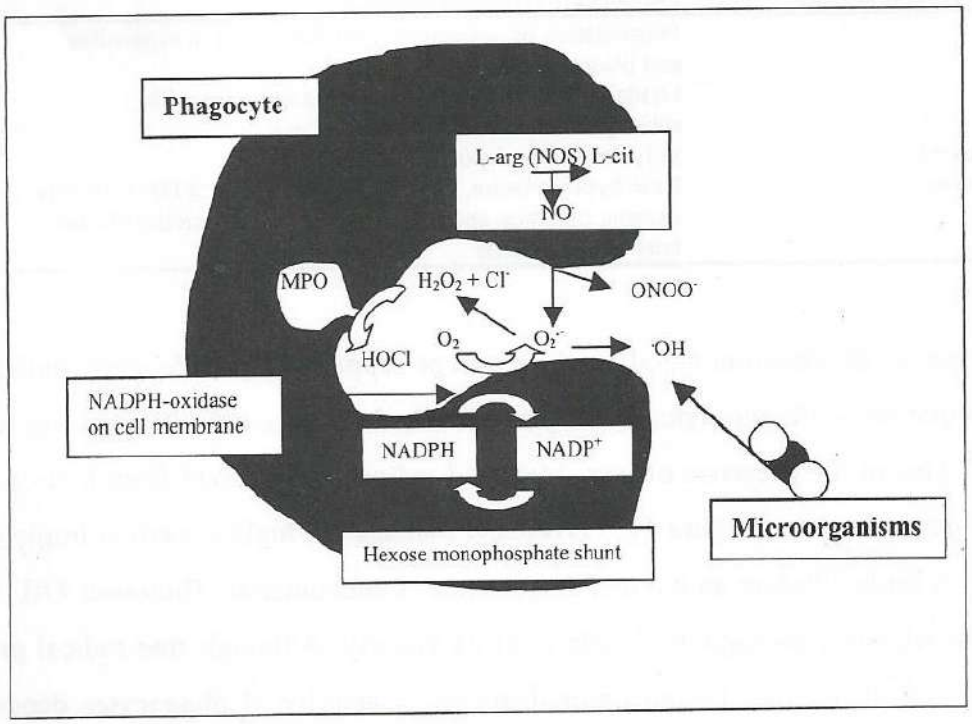


Figure 1. The respiratory burst by phagocytes. The membrane bound NADPH-oxidase generates $O_2^{\cdot-}$ from O_2 during phagocytosis. The enzyme myeloperoxidase (MPO) oxidizes the halide chloride (Cl^-) to form the potent bactericidal hypochlorous acid (HOCl). The extremely reactive ·OH are formed from $O_2^{\cdot-}$ in the presence of transition metals. NO· is formed from the conversion of L-arginine to L-citruline by nitric oxide synthase (NOS). The reaction of $O_2^{\cdot-}$ with NO· leads to the formation of peroxynitrite ($ONOO^-$).

Superoxide anion radicals are moderately reactive compounds capable of acting as an oxidant or reductant in biologic systems. This relative inactivity allows $O_2^{\cdot-}$ to diffuse for considerable distances before it exerts its toxic effects. extracellular generated $O_2^{\cdot-}$ can gain access to intracellular targets via cellular anion channels.[10]

Hydrogen peroxide is a more reactive oxidant than $O_2^{\cdot-}$, and readily diffuses across cell membranes. In addition, H_2O_2 may serve as the substrate for the enzymes myeloperoxidase (MPO) in neutrophils and eosinophil peroxidase (EPO) in eosinophils which can oxidize various halides such as chlorine and iodine to produce hypohalous acids, such as, hypochlorous acid (HOCl), which has potent antimicrobial action.[11] Moreover, MPO and EPO are cationic enzymes, allowing them to stick to the cell surfaces and perhaps enhancing

their potential for cell injury by increasing the local concentration of hypohalous acid at the cell membrane.

Table 1. Cellular damage by ROS

Cellular compartment	Damage by free radicals
Lipids	Peroxidation of polyunsaturated fatty acids in organelles and plasma membranes
Proteins	Oxidation of sulfhydryl-containing enzymes with subsequent inactivation of the enzyme
Carbohydrates	Polysaccharide depolymerization
Nucleic acids	Base hydroxylation, cross-linking, scission of DNA strands causing mutation and inhibition of protein, nucleotide and fatty acid synthesis

In the presence of transition metals such as iron or copper, superoxide anion radicals give rise to the formation of the biologically very reactive hydroxyl radicals through the Haber-Weiss reaction. Also in the presence of iron, hydroxyl radicals are formed from hydrogen peroxide via the Fenton reaction (Figure 1).[12] Hydroxyl radicals are highly reactive implying that $^{\cdot}OH$ diffusion is limited before an oxidizable substrate is encountered. Therefore $^{\cdot}OH$ is known to react with whatever biologic molecule is in its vicinity. Although free radical production is essential to host defense, i.e. an optimal microbial activity of phagocytes depends on free radical production, it exhibits a broad spectrum of biotoxicity.[13] Free radicals in general and hydroxyl radicals in particular can damage proteins, cause breakage of DNA strands and initiate lipid peroxidation (table 1).[14,15] Next to oxygen derived oxidant species, nitrogen derived compounds are important in regulating inflammation. Nitric oxide ($NO^{\cdot}$), in mammalian cells is formed by the enzyme nitric oxide synthase (NOS).[16] There are three isoforms of NOS of which two are of the constitutive type (cNOS) and one is of the inducible type (iNOS). All types oxidize L-arginine to L-citrulline under the formation of $NO^{\cdot}$.[17] The constitutive isoforms are found in neuronal and endothelial cells. Constitutive NOS responds to changes in intracellular calcium concentration resulting in an intermittent production of $NO^{\cdot}$ necessary for physiologic processes such as neurotransmission and blood pressure regulation. Inducible NOS is expressed in many celltypes such as hepatocytes, respiratory epithelial cells and macrophages. Its activity is independent of intracellular calcium concentrations but is modulated by cytokines, microorganisms and microbial products indicating its function in host defense and inflammation. Once activated iNOS produces large amounts of $NO^{\cdot}$ over prolonged periods of time.[18] Once formed $NO^{\cdot}$ has the ability to act as an oxidizing agent alone or interact with $O_2^{\cdot-}$ forming peroxynitrite ($ONOO^{-}$) and ultimately

·OH via decomposition.[19,20] Peroxynitrite itself is a more potent oxidant than NO· and $O_2^{\cdot-}$ (Figure 1).

Inflammatory diseases of upper respiratory tract

During inflammation of the upper respiratory tract first a non-specific immune response occurs through influx of inflammatory cells via the post capillary venules. The influx of inflammatory cells is triggered by the production of vasoactive and chemotactic factors due to tissue injury elicited by the causative agent. After approximately 24-hours granulocytes invade the site of inflammation followed by macrophages and lymphocytes. In case of acute inflammation the main infiltrating cell type is the neutrophilic granulocyte. In case of chronic inflammation the main infiltrating cell types are eosinophils, macrophages, mast cells and lymphocytes. Phagocytic cells contain and excrete various hydrolytic and proteolytic enzymes and generate ROS and RNS all designed to eliminate the causative agent and prevent further tissue injury. However, releasing these toxic substances in the extracellular milieu may also contribute to tissue damage.[21] In addition, accumulating evidence implies that ROS and RNS may contribute to the complex pathogenesis of diseases associated with inflammation.[22]

Acute upper airway inflammation

Viral infection

Acute upper airway infection is predominantly viral of origin. The rhinoviruses are the most common pathogens however, other viruses such as influenza virus and adenovirus have also been described to cause rhinitis.[23] The level of ROS and RNS production of mammalian cells after viral infection exceeds that required for the physiologic process of cell activation which is mediated through the activation of redox-sensitive transcription factors.[24] This phenomenon nicely illustrates the dual role of ROS and RNS: metabolic regulation of cells and a phagocytic defense mechanism.[25] Although it is difficult to distinguish between association and causation of a given virus and ROS or RNS mediated cellular injury the existing evidence for the role of oxidative stress in the pathogenesis of viral upper airway infection is reviewed here.

Virus induced activation of phagocytes is associated with oxidative stress, not only because ROS are released but also because activated phagocytes may release pro-oxidant cytokines, such as tumor necrosis factor (TNF) and interleukin-1 (IL-1).[26] Influenza viruses have been shown to prime and activate monocytes and polymorphonuclear leukocytes to produce ROS

in vitro.[6,27] TNF may be produced by activated phagocytes or infected host cells. In either case it may act on host cell mitochondrial respiration inducing a pro oxidant effect. This effect can be inhibited by the antioxidant vitamin E.[28] Also TNF may cause the release of nuclear transcription factor kappa B (NF-κB) from its cytoplasmatic inhibitor protein leading to induction of the transcription of cellular and/or viral genes. NF-κB induced gene transcription can be inhibited by several antioxidants such as thiol donors.[29,30] The production of IL-1 by activated phagocytes stimulates neutrophils to release lactoferrin.[31] Lactoferrin binds, stores and transports iron to the reticuloendothelial system.[32] Furthermore, interaction of lactoferrin with phagocytes prevents iron catalyzed oxidant formation directly through trapping of iron and indirectly by binding lipopolysaccharide preventing priming of phagocytes for O_2^- production, thereby limiting tissue injury.[33,34] On the other hand when the accumulated iron exceeds the cellular iron binding capacity unbound iron may react with superoxide anions and hydrogen peroxide to produce hydroxyl radicals via the Haber Weiss and Fenton reaction respectively.[11]

In addition to activated phagocytes, respiratory epithelial cells themselves are also capable of producing ROS after viral challenge with, for instance, rhinovirus.[35] Production of oxidative species after rhinovirus stimulation, mediates the elaboration of the proinflammatory cytokine interleukin-8 (IL-8) through subsequent activation of NF-κB. A direct correlation has been reported between the severity of symptoms associated with rhinovirus infection and the concentration of IL-8 in nasal secretions.[36] N-acetylcysteine (NAC) inhibits NF-κB activation and IL-8 elaboration.[35,37] However, rhinovirus stimulated increases in H_2O_2 release are not altered by NAC, suggesting that rhinovirus stimulation of IL-8 in respiratory epithelium was mediated through the production of ROS and the subsequent activation of NF-κB.[37]

An alternative pathway of oxygen radical formation is via the enzymatic production of superoxide anion radicals by xanthine oxidase (XO), which catalyzes the oxidation of (hypo)-xanthine to uric acid. In fact is has been shown that, in influenza infected animals, both the level of XO and the generation of superoxide anion radicals was increased in broncho-alveolar lavage fluid.[25,38]

Another aspect of the role of ROS in the pathogenesis of viral infections is the modulating role of ROS in initiating the immune response. In the defense against viral infection the cellular immune response, mediated through T-lymphocytes, plays an important role. It has been shown in vitro that rhinoviruses, although they do not directly induce superoxide anion radical production from peripheral blood eosinophils, do cause T-cell activation and

proliferation enhancing the airway inflammatory respons.[39] Several antioxidants inhibit the activation and proliferation of T-lymphocytes, suggesting an oxidant mediated mechanism.[40,41] In addition, virus induced apoptosis, i.e. by influenza viruses, can be counteracted with antioxidants such as NAC and glutathione peroxidase. In addition, some proteins of adenovirus may inhibit apoptosis via an antioxidant pathway.[42]

Finally, viruses can also affect the host cell pro-antioxidant balance by increasing cellular pro-oxidants such as iron or by inhibiting the synthesis of antioxidant enzymes such as superoxide dysmutase.[25,26] In contrast, infection of human airway epithelial cells with human influenza A virus resulted in increased mRNA expression of the antioxidant enzymes superoxide dysmutase and indoleamine 2,3-dioxygenase.[43]

The efficacy of antioxidant therapy in viral infections is unclear. It may be particularly useful for those viruses which thus far have not responded well to classical antiviral therapies.[26]

Until now, a few reports have been published in which antioxidant pharmacotherapy for instance, superoxide dysmutase has been successfully used in mice for the treatment of influenza virus.[44,45] Furthermore, NAC showed to have beneficial effects on local and systemic symptoms in patients with influenza compared to a placebo.[46] In addition, antioxidant effects of the nasal decongestants, oxymetazoline and xylometazoline, frequently used in the topical treatment of acute viral upper airway infection, have been shown *in vitro* experiments.[47]

Next to ROS, formation of RNS may play a role in the pathogenesis of several viral diseases. As a cytotoxic or cytostatic molecule, $NO^{\cdot}$ has been thought to have an physiologic antimicrobial action against various pathogens.[48] On the other hand pathologic overproduction of $NO^{\cdot}$ by iNOS, leading to aggravation of the inflammation, has been implicated in the development of several viral infections. For instance, infection with influenza virus A leads to expression of iNOS in macrophages and epithelial cells mediated by proinflammatory cytokines, i.e. interferon γ (INF-γ) and TNF-α.[49,50] In addition, the well known antiviral effector molecule in host defense, IFN-$\alpha\beta$, downregulates the $NO^{\cdot}$ production of virus infected macrophages. Similar down regulation of iNOS expression was reported for the cytokines IL-4 and IL-10 during influenza virus infection in mice preventing excessive $NO^{\cdot}$ production.[50] Although $NO^{\cdot}$ suppresses virus replication in situ, overproduction of $NO^{\cdot}$ together with the production of $O_2^{\cdot-}$, leading to the formation of $ONOO^{-}$, powerfully impairs the physiologic functions of the host cells. In addition, some viruses express or induce selfprotective molecules having antioxidant and antiapoptotic activity. All these

characteristics contradict an important role of NO· in the antiviral host defense. In fact it suggests that NO· is actually a proinflammatory mediator in viral disease. A presumption which is supported by the fact that treatment of influenza virus infected animals, with the NOS inhibitor N-monomethyl-L-arginine (L-NMMA), show significant improvement of survival after (sub)-lethal infection.[49] Recently it was shown that the nasal decongestants oxymetazoline and xylometazoline frequently used in the treatment of viral rhinitis, are potent inhibitors of iNOS expression, a feature possibly beneficial to their therapeutic effect.[51]

Bacterial infection

Viral respiratory pathogens may significantly alter local and/or systemic physical and immune defense mechanisms, paving the way for secondary bacterial invasion. In acute rhinosinusitis the Streptococcus pneumoniae, the Haemophilus influenzae, and the Moraxella catharrhalis are considered to be the most important pathogens. But also other Streptococcus species, Staphylococcus aureus and anaerobes are found. [52,53]

The major source of oxidant production during bacterial infection is the excretion of free radicals by phagocyting inflammatory cells. Free radicals are very effective in killing microorganisms. Anaerobic microorganisms are especially susceptible to oxidative damage as they often lack antioxidant mechanisms observed in aerobic microorganisms. Although free radicals are physiologically important for host defense, these toxic compounds may also be responsible for tissue damage as they leak to the extracellular environment when the phagosome is closing.

The impossibility to generate free radicals, i.e. in patients with chronic granulomatous disease who have a defect in the NADPH-oxidase complex, results in recurrent infections by microorganisms normally rapidly killed by ROS. Also patients that lack the enzyme MPO show delayed killing of bacteria and fungi .[54] In addition, neutrophils can only kill Staphylococcus aureus under aerobic circumstances.[55] Physiologically the most important mechanism of phagocyte mediated oxidant generation and microbial toxicity seems to be the iron catalyzed ·OH production via $O_2^{\cdot-}$ and H_2O_2. Indeed in vitro studies have shown that ·OH mediated killing of Staphylococcus aureus by phagocytes is enhanced by increased bacterial iron levels.[56] In contrast to ROS, the contribution of NO· phagocytic defense against bacterial infection has not been clearly established yet.[13] Except from free radical production by inflammatory cells a number of microorganisms generate oxidants which may exert both beneficial and toxic effects on the host and other microrganisms.[57] For instance, H_2O_2 produced by Streptococcus pneumoniae may play a role in host cellular injury as it has been

shown to damage rat alveolar epithelial cells.[58] Also, as a result of inflammation, endothelial cells and epithelial cells produce ROS contributing to the highly oxidative environment during bacterial infection.[59]

One of the main targets of oxidative damage is the cellular membrane. The polyunsaturated fatty acids are susceptible for lipid peroxidation. This process is initiated when a free radical abstracts a hydrogen atom from the methylene carbon of an unsaturated fatty acid. This results in a carbon centered radical which reacts with molecular oxygen to from a peroxy radical. The peroxy radical is able to propagate the reaction by abstracting a hydrogen atom from another unsaturated fatty acid. By this means a chain reaction of lipid peroxidation is activated.[15] Lipid peroxidation decreases the fluidity and barrier function of membranes eventually leading to cell death. In healthy tissues lipid peroxidation occurs at a very low level. Therefore, lipid peroxidation products such as malondialdehyde (MDA) and lipid hydroperoxide (LOOH) can be used as a marker of free radical induced tissue damage. Recently, it has been shown that in experimental maxillary sinusitis caused by Staphylococcus aureus in rabbits increased ROS production occurred as serum MDA levels and erythrocyte SOD levels were elevated compared to control blood samples. Moreover, mucosal MDA levels were significantly higher in the experimental group than in the control group.[60] Although not a common pathogen in acute sinusitis Pseudomonas aeruginosa derived products pyocyanin and 1-hydroxyphenazine impair respiratory epithelial ciliary activity via a free oxygen radical mechanism.[61] Ciliary dysfunction promotes mucosal inflammation. In an experimental model of acute otitis media, induced by Streptococcus pneumoniae, elevated levels of the hypochlorous acid generating enzyme MPO have been found in middle ear effusions.[62] Also in pneumococcal otitis media in guinea pigs, mucosal levels of both LOOH and MDA were significantly elevated compared to control measurements. In this study, a strong correlation was found between these biochemical findings and histopathologic signs of infection in the mucosa, such as inflammatory cell infiltration, submucosal edema, hyperemia and effusion formation.[63] More recently, it was found that compared to controls increased levels of LOOH were found in middle ear mucosa up to 30 days after infection with Streptococcus pneumoniae. This indicates that ongoing radical injury continues after acute infection contributing to chronicity of the inflammation.[64] Hydrogen peroxide is presumed to be an important oxidant in the pathogenesis of otitis media. Apart from the fact that H_2O_2 is generated by a number of bacteria which are known to cause acute upper airway infection, H_2O_2 has indeed been identified in the middle ear fluid of a guinea pig model of pneumococcal otitis media and was correlated with the inflammatory changes of the middle

ear mucosa.[65] Also the H_2O_2 producing enzyme SOD has been found in middle ear fluid of children with chronic serous otitis media.[66] Furthermore, elevated levels of catalase, an antioxidant which catalyses the transformation of H_2O_2 into water and oxygen, have been found in middle ear mucosa of animals with otitis media.[67] Another study showed diminished overall superoxide dysmutase levels in infected middle ear mucosa of guinea pigs indicating a possible impaired mucosal defense for $O_2^{\cdot-}$.[68] Recently, reduced lipid peroxidation in infected middle ear mucosa of guinea pigs was observed after intraperitoneal injection with the lazaroid U-74389G, a known antioxidant.[69] This latter finding indicates that antioxidative pharmacotherapy may be beneficial in upper airway infection. In contrast to antioxidant pharmacotherapy, microorganisms have specific defense strategies for oxidative damage contributing to their pathogenicity. Avoidance of encounters with phagocyte derived oxidants has been described for streptococcus species and staphylococcus aureus as they generate a toxin which kills the phagocyte before they can be killed by the phagocyte. In addition, pneumococci resist phagocytic uptake by covering their surface with a hydrophobic capsule.[13] Also pneumococci are shown to generate a specific respiratory burst inhibitor of polymorphonuclear leucocytes.[70,71] Nearly all bacteria, except from the obligate anaerobes, have developed effective enzymatic pathways for preventing oxidant inactivation. In particular catalase and/or peroxidases are important.[72] Accordingly, it has been shown that neutrophils easily kill low-, but not high-catalase producing Staphylococcus aureus strains.[55] Next to catalase and peroxidase enzymatic oxidant inactivation by bacteria is effectuated by SOD. Several species of bacteria, amongst which Haemophilus species, have been found to contain copper-zinc SOD.[73]

De role of ROS in host-microbial interaction is complex. Oxidants can have beneficial and detrimental properties in both the host and the microorganism. Tissue injury at site of infection may be the result of the host inflammatory response to the pathogen rather than cytotoxic components of the microorganism. Although a role of oxidants in the pathogenesis of upper respiratory tract microbial pathophysiology is anticipated the exact role needs to be further established.

Chronic upper airway inflammation

The mucociliary clearing system

In contrast to acute rhinosinusitis chronic inflammation of the upper respiratory tract is in principle non-infectious of origin. Chronic sinusitis is characterized by ongoing inflammation

for which the causative agent is often unknown. Inflammation results in mucosal damage which by itself contributes to the chronicity of the mucosal disease. A key factor in the development of chronic sinusitis is dysfunction of the mucociliary clearing system. This system consists of a two layered mucous blanket which rests on the ciliated epithelial cells and is transported in the direction of the natural ostia of the paranasal sinuses. Except from this mechanical cleaning mechanism the mucous blanket contains inflammatory cells and secretory cell products which form a immunological defense against invading pathogens.[74,75] Ciliated epithelial cells are an important target for oxidant mediated tissue injury in the human airways. In experiments using human nasal epithelial cells (HNECs) it has been shown that exposure of HNECs to reactive oxidants such as $O_2^{\cdot-}$, generated by a enzymatic xanthine-xanthine oxidase system, or H_2O_2, generated by glucose-glucose oxidase system, or reagent H_2O_2 or HOCl results in significant alterations in ciliary beating.[76] The earliest change noted was the presence of ciliary slowing and dyskinesia, eventually progressing to complete ciliary stasis. These negative effects on ciliary movement could be prevented by incubating the cells with enzymatic antioxidants such as SOD and catalase acting alone or in combination. Also the free radical scavenger 3-aminobenzamide, a DNA repair enzyme inhibitor, significantly prevented ciliary slowing caused by superoxide anions and H_2O_2 but not by HOCl. This may indicate that free radical mediated damage to DNA may be at least partially responsible of the detrimental effects on ciliary movement of HNECs. In addition, the effects of HOCl could be neutralized by adding the HOCl scavenger methionine. [76] Although it has also been reported that low doses of superoxide anion radical give a temporally stimulatory effect on ciliary beat frequency in HNECs higher doses of superoxide anion radicals, generated by a xanthine-xanthine oxidase enzymatic system, consistently give impaired movement of the epithelial cilia.[77,78] This finding indicates a dose-dependent effect of free radicals on ciliary beat frequency in HNECs.[76]

The role of the eosinophil

In chronic sinusitis and nasal polyposis the main infiltrating celltype is the eosinophil.[79,80] The eosinophil is considered to be an important effector molecule in chronic inflammatory disease of the paranasal sinus epithelium as there is evidence that it mediates injury to respiratory tissue. For instance major basic protein (MBP), a granule stored protein of the eosinophil, has been proven to be cytotoxic to the respiratory epithelium.[81] In human paranasal sinus mucosa it has been shown that in addition to infiltration with eosinophils, epithelial desquamation, thickening of the basement membrane, hyperplasia of mucus producing cells and edema of

the stroma occurs. In this study a striking association was found between the deposition of eosinophil granule MBP indicating extensive eosinophil degranulation and destruction of sinus epithelium. In addition, in areas were sinus epithelium was intact a scarcity of eosinophils was observed with minimal or no signs of eosinophil degranulation.[82] Furthermore, MBP has been proven to cause ciliostasis in human nasal sinus mucosa.[83] During eosinophil granulation except from MBP other proteins such as eosinophil peroxidase (EPO) are released. It has been shown that human nasal epithelial cells are severely injured when they are exposed to EPO-H_2O_2-halide system. A process which could be significantly inhibited by catalase and azide.[84] It these experiments it was observed that damage to human nasal epithelial cells by MBP requires a longer incubation period than cell damage by the oxidant forming EPO-H_2O_2-halide system.[84] Extrapolating this to the situation in vivo higher eosinophil to target cell ratios are necessary for cell injury via MBP release than via the EPO-H_2O_2-halide system, indicating that the latter is an important biological mechanism of oxidative tissue damage in inflammatory nasal disease. Moreover, MBP is thought to be responsible for tissue edema based on increased vascular permeability. As a result of increased vascular permeability outpouring of plasma proteins, albumin in particular, into the nasal mucosa and into the nasal secretions occurs.[85,86,87] Albumin is an important extracellular antioxidant as it binds copper ions preventing them to serve as transition metals in radical generating reactions.[88]

Another granular eosinophilic cell product which is excreted under inflammatory conditions is eosinophilic cationic protein (ECP). It has been shown that in nasal secretions from patients with nasal inflammation ECP levels were significantly higher than in nasal secretions from healthy controls. In serum samples of the same patients no differences in ECP levels were detected. In patients with chronic sinusitis a significant correlation between the subjective symptoms score and the nasal ECP level was found.[89] This may be an interesting finding as ECP is known to stimulate lactoferrin release from airway submucosal glands and lactoferrin may serve as an important antioxidant by strongly binding free iron at low pH, a situation which frequently occurs during inflammation.[12,90]

Although eosinophils are the main infiltrating cells in chronic upper respiratory tract inflammation, in nasal lavage fluid (NAL) also increased levels of neutrophils have been described.[91] Moreover, elevated concentrations of soluble intercellular adhesion molecule-1, an important adhesion molecule for neutrophil recruitment, were found in chronic sinusitis indicating that also neutrophils may be important in the pathogenesis of chronic upper respiratory tract inflammation.[92] In fact, the levels of the neutrophilic oxidant producing

enzyme MPO were found to be significantly higher in NAL of patients with chronic sinusitis compared to NAL of healthy controls. Moreover, in these experiments a strong correlation between MPO and the pro-inflammatory cytokine IL-8 levels was found.[93] The chemotactic and activating properties of IL-8 for inflammatory cells may be responsible for mucosal damage or thickening and thus chronicity of the disease.[94] In addition, another important cytokine in chronic upper airway inflammation, eotaxin, has been shown to be a key factor in eosinophilic accumulation and activation in nasal polyps.[95,96] Human eotaxin is a potent activator of the respiratory burst, releasing ROS, and therefore contributes to eosinophilic tissue damage.[97] It may be by this mechanism that recently significantly higher levels of MDA were found in nasal polyp tissue compared to healthy nasal mucosa indicating a pathophysiological relationship between free radical damage and nasal polyp development.[98]

Allergic inflammation

A separate entity in chronic eosinophilic nasal inflammation is the allergic rhinitis. Except from being one of the most common chronic upper airway diseases in itself, nasal allergic inflammation may be a causative factor in the development of chronic sinusitis as it may lead to sinus ostial narrowing. Allergic rhinitis is characterized by nasal inflammation and nasal airway hyperresponsiveness (AHR) to allergens and a number of other stimuli. The contribution of the eosinophil to epithelial damage, tissue edema, and mucus hypersecretion leading to nasal inflammation has been discussed above (vide supra). The exact mechanism by which AHR occurs remains unclear however, a multifactorial pathway can be anticipated. Antigen challenge may cause activation of mast cells and macrophages causing the release of several mediators such as platelet activating factor (PAF), leukotriens and cytokines. These chemoattractants lead to infiltration of inflammatory cells, i.e. eosinophils, which in turn release mediators, amongst which ROS and RNS, which in collaboration may lead to AHR.[99] Eosinophils of patients with allergic rhinitis have been shown to have increased oxidative metabolic function compared to normal controls.[100] Moreover, the role of superoxide anions in the development of AHR has been shown in cats.[101] PAF, which is a naturally occurring phospholipid, seems to play a pivotal role in AHR in man. Except from inducing inflammatory cells to release cytotoxic mediators, such as ROS, which contribute to AHR, it also induces eosinophil independent ROS formation by epithelial cells leading to AHR. This latter feature can be prevented by catalase and SOD.[102] In addition, PAF induced hyperresponsiveness in the human nasal airway can be almost abolished by pretreatment with vitamin E.[103] Next to ROS, RNS are thought to play an major role in nasal allergy.[104,105,106]

The role of RNS in upper airway inflammation is discussed in a separate paragraph (vide infra).

Nitric oxide

Site of formation

In healthy subjects exhaled NO· mainly originates from the upper respiratory tract with a relatively minor contribution from the lower airways.[107,108] The predominant location for NO· production is thought to be the paranasal sinusses.[109] Via immunohistochemical and mRNA in situ hybridizations studies it was found that a NOS, most closely resembling the inducible isoform, is constitutively expressed apically in sinus epithelium. In contrast only weak NOS activity was found in the epithelium of the nasal cavity.[110] These findings are confirmed by the fact that exhaled and nasal NO· concentrations are markedly lower in animals without paranasal sinuses than in mammals with paranasal sinuses.[111] Although the paranasal sinuses seem to be the most important source of upper airway derived NO·, NO· is also produced at other sites in the upper respiratory tract such as the nose itself and the nasopharynx.[112,113,114] In fact, strong positive iNOS immunoreactivity, but no significant cNOS positivity has been found in ciliated cells of human nasal turbinates.[115] In contrast, Furukawa et al, showed strong immunostaining for cNOS in vascular endothelium, surface epithelium and submucosal glands in human nasal turbinate tissue. Moderate iNOS immunostaining was found in surface epithelium, glandular cells, inflammatory cells, vascular endothelial cells and smooth muscle cells in specimens from patients with rhinitis only.[116]

Pathophysiology

In the nose and the paranasal sinuses NO· is thought to have an important role in several physiological mechanisms and host defense against infections.[117] NO· has been shown to stimulate mucociliary clearance both in vitro and in vivo. The mucociliary activity of rabbit sinus mucosa was increased dose dependently through the NO-substrate L-arginine, an effect which could be fully blocked by the NOS inhibitor N-nitro-L-arginine (L-NNA).[117] Furthermore, addition of the NO-donor sodium nitroprusside (SNP) to the nasal cavity also resulted in significant increase in mucociliary activity. In these same experiments strong to moderate NOS immunoreactivity and NADPH-diaphorase activity, which is a marker for NOS activity, was observed in the cell bodies of the sphenopalatine ganglion and the peripheral mucosal nerves. This correlation between the results of measurements of two

independent methods supports the evidence that cNOS, probably of the neuronal subtype is present in these nerves structures. [118] Also strong iNOS activity has been shown in human nasal ciliated cells.[115] These findings may support the biological significance of NO· in mucociliary clearance. Clinically, the absence of nasal NO· in Kartagener's syndrome and significant reduction of nasal NO· in children with cystic fibrosis has been reported.[119,120] This may at least partially explain the recurrent infections occurring in these patients.

Nitric oxide has been shown to mediate nasal fluid production by increasing vascular permeability through the effector arm of the nasonasal reflex, but not the sensory nerve afferent pathway, in a rat model.[121,122] In these experiments vascular albumin leakage could be totally blocked by N-nitro-L-arginine methylester (L-NAME), an inhibitor of NO· synthesis. Mucin secretion however was not inhibited by L-NAME, indicating that glandular secretion is not primarily under NO· control. In contrast, other investigations have shown that in the dog nasal mucosa NO· could act as a non–cholinergic parasympathetic neurotransmitter in both vascular and secretory control as NADPH-diaphorase activity was histochemically revealed in parasympathetic nerve fibers in the nasal muscosa.[123]

In pig nasal mucosa it was observed that NO· is involved in the basal vascular regulation. Inhibition of NOS by L-NNA induced a reduction in nasal vascular conductance with a concomitant increase in nasal cavity volume.[124,125] In these same experiments L-arginine mainly reversed the L-NNA mediated reduction of nasal vascular conductance whereas the effects on nasal cavity volume were small. This finding indicates that NO· may be of greater importance for resistance vessel (arteries and arterioles) than for capacitance vessel (veins and venous erectile tissue) function. However, this observation was contradicted by Ruffoli et al, who found strong NADPH diaphorase activity in the cavernous sinuses of human inferior turbinates which function as capacitance vessels. Furthermore, occasional activity was found in the arterioles and no activity was found in the capillaries which function as resistance vessels.[126] In addition, Ruffoli et al., found strong enzymatic activity of NOS in the smooth muscle cells of patients with vasomotor rhinitis suggesting a role for NO· in the vascular disorder of vasomotor rhinitis.[127] A positive relation between nasal NO· and nasal resistance was also found by Imada et al. [128] Moreover, NOS immunoreactivity has been detected in mast cells of human nasal mucosa indicating that mast cells contain a particulate isoform of cNOS which is rapidly removed from granules upon exocytosis and thus playing a role in regulating the blood supply to the nasal mucosa.[129] In contrast, more recent investigations failed to show such a relationship.[130,131,132] The difference in these results may be methodological of origin. The latter studies use high flow rate insufflation techniques which

may mimic the physiological transnasal airflow better than the low flow techniques which may underestimate the measured NO· output.[133] Another explanation for the discrepancy may be the fact that the NO· levels measured in the nasal air are predominantly produced by the calcium independent form of NOS found in the sinus mucosa which is not responsible for vascular tone regulation as this is controlled by nasal vascular cNOS.[110,126,134,135]

At this point it should be noted that there is no standardized technique for measuring nasal NO·. *In vivo*, indirect methods, in which nitrate and nitrite as stable end products of NO· metabolism are measured or immunohistochemical techniques, which detect NOS in tissue, are used. The direct methods measure airborne NO· via chemiluminescence analysis.[117] Recently measurement techniques have been compared by Silkoff et al.[136] Nasal NO· concentrations were found to be inversely correlated with transnasal airflow which makes it vital to use a constant flow rate during measurements. Therefore, the fixed flow exhalation method is considered to be the preferred method. In addition, NO· contribution from the lower airways should be either excluded or subtracted.

Another important physiological function of nasal NO· is that it may function as an aerocrine messenger for the lungs as it has beneficial effects in regulating pulmonary blood flow optimizing the ventilation/perfusion ratio.[137,138,139]

Inflammation

Nitric oxide is detectable in the nasally exhaled air of normal subjects and its level is not related to age, gender or diurnal variation. NO· may therefore be a useful measure for monitoring upper airway disease.[140] Within the respiratory tract pathogenic microorganisms have been shown to induce NOS.[141] NO· has strong antiviral and moderate antibacterial effects.[142,143] The airborne NO· may even represent the very first line of defense in the airways, possibly acting on pathogens even before they reach the mucosa.[137] During viral upper respiratory tract infection (URTI) it has been shown that during the acute phase the exhaled NO· concentration increases significantly. During recovery exhaled NO· returned to normal.[144] These findings indicate a role for NO· in regulating URTI. In contrast, Ferguson et al., did not find any difference in exhaled NO· levels between the acute stage of a common cold and the values obtained when the subjects were healthy.[145] Also, Lindberg et al, showed no difference in NO· between healthy volunteers and patients suffering from a common cold.[146] Furthermore, only low concentrations of nitrite were found in nasal washings of patients with a rhinovirus infection, levels which were not different from sham-challenged subjects. Nitrite concentrations did not change over the course of rhinovirus infection.[147]

These latter findings may contradict a role of NO· in the pathogenesis of URTI.

In patients with allergic rhinitis it has been shown that nasal NO· is increased compared to non allergic subjects.[105] Treatment with topical steroids has been shown to significantly lower the nasal NO· production, a feature which is explained by inhibition of iNOS.[106,148] In contrast, although NO· metabolites, nitrite and nitrate, are detectable in nasal washings of patients with house dust mite allergy, intranasal steroids did not affect the NO· levels measured.[104] Increased expression of iNOS, but not of cNOS, was shown in nasal turbinate tissue of patients with house dust mite allergy compared to healthy controls.[149] This was confirmed by Kang et al, who showed strong immunostaining of iNOS especially in the submucosal glands of patients with allergic rhinitis. Furthermore, they found increased production of peroxynitrite ($ONOO^-$), as represented by the accumulation of 3-nitrotyrosine, suggesting that this toxic NO· metabolite may be at least partially responsible for the symptoms of allergic rhinitis.[150] Sato et al, also found increased levels of NO· and nitrotyrosine in nasal mucosa of patients with allergic rhinitis compared to healthy controls.[151] They proposed a vicious cycle of perennial nasal allergy. As a response to the allergic reaction, mast cells and inflammatory cells release cytokines which increase vascular permeability and cause edema. At the same time, cytokines induce NOS with subsequent formation of NO·. Superoxide anions are also produced through inflammatory cells and through the ischemia reperfusion phenomenon of the nasal cycle. In this latter process xanthine oxidase produces $O_2^{\cdot-}$ during reperfusion. From NO· and $O_2^{\cdot-}$ rapidly $ONOO^-$ is formed which aggravates the edema. In turn the edema produces ischemia.[151] The key cells in the formation of $ONOO^-$ may be the eosinophil as it has been recently shown that intranasal administered eotaxin, a potent chemoattractant for eosinophils, causes clinical symptoms of nasal allergy with elevated levels of NO·, and nitrotyrosine.[152]

In the pathophysiology of chronic upper respiratory tract inflammation NO· also seems to be involved. The majority of NO· is produced in the paranasal sinuses which suggest a role in host defense in these areas. Indeed in patients with chronic sinusitis nasal concentrations of NO· have been proven to be significantly lower than in healthy controls.[146] Decreased nasal NO· may be theoretically caused by two factors. First, through diminished production of NO· in ciliated cells of the paranasal and nasal mucosa. Second, through narrowing of the ostia of the paranasal sinuses. Diminished production of NO· in ciliated cells might be due to metaplasia of ciliated epithelium into cuboidal and squamous epithelium a feature occurring in chronic sinusitis and in smoking. Indeed, nasal NO· has been reported significantly lower in smokers than in healthy subjects.[153,154] Narrowing of the sinus ostia occurs frequently in

chronic sinusitis due to edema of the mucosa. Blockage of flow of NO· from the paranasal sinuses into the nose occurs leading to decreased nasal NO· but also increased NO· levels within the paranasal sinuses. Recently Schlosser et al, demonstrated significantly increased levels of NO· metabolites in sinus lavage fluid of rabbits with chronic sinusitis, which resolved after restoring sinus ventilation. These authors speculate that NO· autotoxicity plays a role in the pathogenesis of chronic sinusitis.[155] However, in this respect it should be noted that hypoxia, a situation frequently occurring during chronic sinusitis, depresses nasal nitric oxide output and may serve as a controlling mechanism that prevents further rising of NO·.[156] Finally, in patients with non allergic nasal polyps, nasal NO· has been reported to be low compared to healthy controls and patients with polyposis and allergy.[157] As allergic rhinopathy is characterized by high nasal NO· levels this may very well compensate the low nasal NO· levels caused by sinus ostium obstruction in patients with only nasal polyps. Although nasal NO· may be low in nasal polyposis it should be noted that upregulation of iNOS activity has been shown in nasal polyps suggesting a role in their development.[158]

Airpollution and hyperoxia

Aipollution

Ozone (O_3) is the major irritant gas in photochemical smog. Due to its extreme oxidative reactivity it has been suggested that about 90 % of the O_3 which enters the airways reacts with constituents from the epithelial lining fluid, before it reaches the epithelial membrane.[159,160]

The epithelial lining fluid covers the surface of the airways. It contains various molecules, such as unsaturated fatty acids, proteins, nucleic acids, thiols, vitamin C, and vitamin E, which may function as a reactant for O_3. The thickness of the epithelial lining fluid in the upper respiratory tract (5-10 μM) is larger than in the alveoli (0.2-0.5 μM) and therefore direct cellular damage is expected in the lower respiratory tract , whereas secondary products, such as aldehydes and hydrogen peroxide are responsible for the deleterious effects in upper airways.[161] The capacity of O_3 to oxidize unsaturated lipids and protein sulfhydryl groups is presumed to be the mechanism by which O_3 exerts its cellular toxicity.[162] Moreover, the antioxidants GSH, uric acid, and vitamin C directly react with O_3 and serve as sacrificial antioxidants.

Within the nasal cavity O_3 exposure leads to an inflammatory response due to the influx of neutrophils, which may be indicative for cellular injury.[163] Also significant increases of neutrophils in nasal lavages in healthy subjects exposed to ozone have been reported.[164]

Moreover, a transient but marked increase in DNA replication, indicating cell proliferation, was seen in nasal epithelial cells of rats after exposure to ozone.[165] Cell proliferation of cells lining the respiratory tract is usually very low. However, after cell injury epithelial cell proliferation increases dramatically and therefore increase in cell proliferation is frequently used as an indicator of toxicant-induced cell damage.[166] Next to cell proliferation, repeated ozone exposure of rat nasal mucosa leads to hyperplastic and secretory cell metaplastic changes which may protect it from subsequent exposures.[167,168] In a cell culture of human upper respiratory tract epithelial cells, ozone has been shown to induce subacute damaging effects. After one week of continuous exposure to intermediate and high concentrations of ozone the levels of cytotoxic cells increased and the ciliary beat frequency and IL-8 production decreased in vitro.[169] Although is this study the proinflammatory cytokine IL-8 decreased after exposure to ozone, other studies have shown increased release of proinflammatory cytokines and other mediators of inflammation.[170,171] For instance, Steerenberg et al, showed the presence of leukocytes, the proteins myeloperoxidase and eosinophil cationic protein and, IL-8 in nasal lavage fluid after ozone exposure. Moreover, the exudation markers albumin, urea and uric acid were also detectable.[172] These factors may contribute to the development of chronic inflammation.

Ozone is also known to contribute to airway hyperreactivity. In rats, it was found that O_3 induces increased airway responsiveness to acetylcholine and bradykinin. This effect was inhibited by the antioxidants apocynin, allopurinol and deferoxamine indicating involvement of superoxide anions released by inflammatory cells.[173]

Next to ozone, formaldehyde is a major air contaminant which alone or in combination with ozone may cause airway epithelial damage.[174] Significant reduced ciliary beat frequency has been shown after exposure to high levels of formaldehyde.[175] Furthermore, when healthy volunteers were exposed to formaldehyde *in vivo*, they showed strong nasal eosinophilia, chronic inflammation and metaplasia.[176,177,178] Although formaldehyde is not a true oxidant in itself these findings may very well be mediated via an oxidative mechanism. Glutathione conjugation is involved in the detoxification of both ozone and formaldehyde. A role of glutathione or glutathione dependent enzymes in the pathogenesis of nasal adverse effects induced by ozone, formaldehyde, or a combination of both can therefore be anticipated, however, such a role has not been proven yet.[179] Other oxidants found in airpollution such as nitrogen dioxide and sulfur dioxide, are also implicated in nasal disease. Nitrogen dioxide exposure results in epithelial eosinophilia leading to mucosal injury and decreased ciliary activity possibly contributing to airway hyperresponsiveness.[180,181] These effects may very

well be caused by lipid peroxidation as this has been shown to occur in rat nasal tissue after exposure to nitrogen dioxide.[182] As vitamin E levels in rat nasal tissue were relatively high, the peroxidation in this site seemed not attributable to a high sensitivity to oxidants. However, the minimal capacity of the mucous lining layer to scavenge oxidants was suggested to be responsible for the peroxidative effects of nitrogen dioxide.[182] Inhalation of the oxidant sulfur dioxide in higher concentrations is known to result in suppression of mucociliary clearance, mucosal edema, and increased nasal resistance.[170,183] In mice early lesions were observed in the nasomaxillary turbinates consisting of edema, necrosis and desquamation of the respiratory epithelium.[184] Furthermore, sulfur dioxide exposure leads to influx of mast cells and lymphocytes into nasal lavage fluid. These factors may contribute to upper respiratory tract inflammation.[185] In fact sulfur dioxide containing outdoor pollution has been shown to have adverse effects on the symptoms of allergic rhinitis.[186]

Hyperoxia

In addition to airborne pollutants, oxygen itself is in higher concentrations capable of injuring the nasal mucosa. The hyperoxic damage seems to be partially mediated via reactive oxygen species.[187] Hyperoxia has been shown to induce hypertrophy but not hyperplasia of rat nasal mucosa. In these same experiments the mucosal activity of the antioxidants superoxide dysmutase, catalase, glutathione peroxidase, and glucose-6-phosphate dehyrogenase were markedly increased after hyperoxic exposure. Moreover, an increased cell replication was seen reflecting replacement of susceptible cells by more resistant cells.[188,189] The former morphologic and biochemical alterations suggest an protective mechanism of nasal epithelia to subsequent hyperoxic exposure.

Xenobiotic metabolism

An important source of free radical formation are the constant oxidation-reduction reactions that occur diffusely in the body. The nose represents a major port of entry into the body for volatile xenobiotics and therefore the respiratory mucosa contains various oxidative enzymes to metabolize potentially hazardous compounds.[190] In some cases this nasal biotransformation process of xenobiotics may lead to a cytotoxic bioactivation of the metabolized substances. The superfamily of cytochrome P450 enzymes are involved in the oxidation of a wide array of substances at the expense of O_2, and has been described to occur in nasal mucosa of animals and humans.[190,191] In addition, although in low concentrations, all other components of the mono-oxygenase system such as cytochrome b_5, NADH and NADPH-cytochrome c

reductase, and the NADH-cytochrome b_5 reductase are present in human respiratory nasal tissue.[191] Cytochrome P450 may function as a mono-oxygenase, using NADPH as a co-factor, in which one atom of the oxygen enters the substrate and the other forms water. Except from this mono-oxygenase activity cytochrome P450 may also function as a peroxidase using peroxides as electron donors.[15] Whether the oxidation of a substrate is catalyzed by cytochrome P450 with either NADPH/O_2 or peroxide as cofactor ROS play a central role in the reaction cycle and may cause adjuvant cellular damage.[192] For instance, cytochrome P450 may, under certain xenobiotic induced circumstances, generate excessive amounts of superoxide anion radicals which may dysmutate to H_2O_2 and other ROS which have been implicated in the initiation of lipid peroxidation.[193] This phenomenon has not been shown specifically for human nasal mucosa. However, hyperoxic exposure to rat nasal epithelia has been shown to give a significant decrease in cytochrome P450- dependent monooxygenase activity which may indicates a protective response of nasal epithelium against potential oxidative stress.[188]

Next to cytochrome P450, GSH-S-transferases have been repeatedly reported to occur in rat and human nasal mucosa.[194,195] GSH-S-transferase is an enzyme which is well known as a catalyst of the reaction of GSH with endogenous compounds and xenobiotics. For instance, cytotoxic aldehydes which are produced during lipid peroxidation are detoxified by conjugation to GSH. Furthermore, organic hydroperoxides are metabolized to oxidized glutathione (GSSG) and water.[196,197]

Although GSH-S-transferase is predominantly an antioxidant enzyme, strongly increased activity may cause an decrease in GSH concentration, thereby impairing the antioxidant capacity and indirectly function as a pro oxidant.[7] In addition, combined exposure of rat nasal epithelium to formaldehyde and ozone results in decreased activity of GSH-S-transferase without a decrease in GSH, indicating also an indirect prooxidant effect.[179]

Antioxidants in general

Naturally occurring antioxidants

Airway mucosal surfaces are potentially subjected to a variety of oxidant stresses. Except from oxidant attacks from inflammatory cells, exogenous oxidants such as ozone (O_3), sulfurdioxide (SO_2), nitrogen dioxide (NO_2), tobacco smoke and even pure oxygen may damage the epithelium. To cope with this assault, respiratory epithelium uses several antioxidant strategies. Cells contain many antioxidants, some of which are non-enzymatic

radical scavengers, others that prevent radical formation, and others that enzymatically detoxify ROS.[198]

Antioxidant enzymes

The most important antioxidant enzymes are SOD, catalase, and GSH dependent enzymes such as GSH peroxidase (GSH-px). SOD catalyses the dysmutation of superoxide anions to oxygen and H_2O_2 at a rate constant 10^4 times the rate constant of spontaneous dysmutation.[9,199] There are several isoforms of SOD. The copper and zinc containing enzymes are predominantly found in the cytoplasm. The manganese isoform is found in the mitochondrial matrix. Catalase and GSH-px predominantly detoxify the common oxidant H_2O_2 but also other peroxide molecules such as lipid hydroperoxides may be reduced. Catalase converts H_2O_2 into water and oxygen and is mainly located in intracellular peroxisomes.[1] GSH-px, a cytosolic selenium dependent enzyme, catalyses the reaction in which H_2O_2 is converted to water or, another toxic hydroperoxide is converted into its corresponding alcohol. During this reaction GSH is oxidized into glutathione disulfide (GSSG).[200] Another GSH dependent enzyme GSH reductase reduces GSSG back into GSH by this means providing sufficient levels of GSH to deal with radical stress. To do so GSH reductase oxidates NADPH, which is provided through the hexose monophosphate shunt, into $NADP^+$.[201] Airway epithelium also contains a non-selenium dependent class of GSH-px. These so-called GSH-transferases catalyze the reaction between GSH and organic hydroperoxides, but not H_2O_2, to form GSSG and water.[202]

Non-enzymatic antioxidants

The non-enzymatic antioxidants found in respiratory epithelium include low and high molecular weight compounds. The most important low molecular weight antioxidants are vitamin E, vitamin C, uric acid, thiols, β-carotene, and taurine. Vitamin E is lipophilic and serves as a chain breaking antioxidant in the process of lipid peroxidation.[202] Vitamin C is a more hydrophilic antioxidant and may quench radicals in the nonlipid cellular compartments. Furthermore vitamin C regenerates vitamin E.[203] Uric acid, present as urate under physiological pH, is also hydrophilic and has powerful antioxidant properties as it directly scavenges ROS, prevents the oxidation of vitamin C and binds transition metals.[204,205] Uric acid has free access to all extracellular fluid compartments. From the thiols the hydrophilic tripeptide GSH plays a pivotal role in several antioxidant systems. Except from its function in the GSH-dependent enzymes, GSH can directly scavenge ROS.[206] By this means the

important sulfhydryl groups on proteins are protected. Furthermore, GSH may regenerate vitamin E.[207] The fat-soluble β-carotene and the hydrophilic amino acid taurine serve as free radical scavengers.[1]

Larger molecular weight antioxidants include transferrin, lactoferrin, albumin, and ceruloplasmin. Lactoferrin and transferrin serve as transport proteins of iron preventing it from participating in free radical generating reactions, such as the Haber-Weis-, and the Fenton reaction, through their specificity of their binding sites.[12] Albumin and ceruloplasmin bind another important transition metal, copper.[88,208]

Antioxidants in the upper respiratory tract

Epithelial antioxidants

In the nasal mucosa several antioxidants have been shown to occur. For instance, in rat nasal mucosa the different forms of SOD have been detected via electron spin resonance spin trapping and immunohistochemical techniques.[209] In these experiments it was found that manganese and copper-zinc SOD were immunohistochemically clearly expressed in the epithelial cells and the subepithelial glands, implying an important role of the epithelial cells in protecting the nasal mucosa against cytotoxic superoxide anions. In the epithelial globet cells and the connective tissue of the lamina propria no positive immunostaining was found which make these latter two more vulnerable to damage caused by superoxide anions. The overall activity of SOD was found to be highest in the nasal olfactory areas suggesting a site specific different requirement of the nasal cavity in detoxifying superoxide anions. In middle ear mucosa of guinea pigs with otitis media decreased levels of SOD were found compared to healthy animals, Indicating an impaired antioxidant defense against $O_2^{\cdot-}$.[68] In contrast, elevated levels of catalase were found in infected guinea pig middle ear mucosa suggesting a increased antioxidant potential in the defense against H_2O_2.[67]

GSH, has also been detected in rat nasal epithelium.[210] Although the exact role of GSH within the nasal epithelia remains unclear it was found that the concentration in respiratory epithelium is rather high (2.67 ± 0.42 μmol/g tissue) compared to other tissues. The liver (6.1 μmol/g tissue) and the kidney (3.96 μmol/g tissue), where the importance of GSH has well been established have similar or higher concentrations.[211] Furthermore, a high GSH turnover rate was observed in the nasal respiratory epithelium after intravenous cysteine administration, which is indicative for a high metabolic activity. As was mentioned earlier GSH is used by the antioxidant GSH-S-transferase to detoxify xenobiotics and organic

hydroperoxides. GSH-S-transferase has been shown in animal and human nasal mucosa.[194,195] Although in considerable lower concentrations compared to rat nasal tissue (0.6 ± 0.2 μmol/g wet weight tissue vs. 2.67 ± 0.42 μmol/g tissue), GSH has also been found to be present in human nasal mucosa of healthy controls. Furthermore, in patients with chronic sinusitis a significant lower level of GSH was found suggesting a diminished antioxidant defense through direct scavenging of ROS produced during inflammation and conversion of GSH by GSH peroxidase. The expected increase in mucosal GSSG was not observed. [212] This might be explained by the efflux of GSSG out of the cells into the blood stream and reduction of GSSG by the enzyme GSSG reductase.[213]

Uric acid has been reported to occur in human nasal mucosa.[212,214] However, in these same experiments the presence of xanthine oxidase, the enzyme responsible for uric acid synthesis, could not be detected. It is speculated therefore that uric acid is taken up by nasal glands from plasma.[214] In addition it was found that the mucosal level of uric acid was decreased in patients with chronic sinusitis, again suggesting diminished antioxidant defense.[212]

Vitamin E levels have been shown not to differ significantly between patients with chronic sinusitis and healthy controls.[212] Although vitamin E is the principal defense against oxidant induced lipid peroxidation, the consumption of vitamin E begins when other antioxidants have been consumed. Furthermore, vitamin E can be regenerated from its radical by GSH and vitamin C.[215] This latter may suggest mucosal consumption of vitamin C a feature which was already hypothesized in 1968 for patients with allergic rhinitis who had considerably lower blood levels of vitamin C than normal subjects, indicating a unsaturated state of the tissue.[216]

Epithelial associated antioxidants

Antioxidants contained in the respiratory tract lining fluids (RTLFs) can be expected to provide the initial defense against inhaled environmental oxidants. The major antioxidants in the RTLFs are mucin, uric acid, albumin, ascorbic acid and GSH. Antioxidants in RTLFs can be augmented by processes such as transudation/exudation of plasma constituents, secretory processes from glandular and epithelial cells, and cellular antioxidants derived from lysis of epithelial and inflammatory cells.[161] Accurate measurement of RTLFs constituents is difficult as substantial dilution and influx of solutes from non-RTLF sources into the instilled saline may represent a significant source of errors in calculating RTLF constituent concentrations. The single-cycle lavage procedure described by Peterson et al., seems to lessen these problems and provides accurate and reliable results.[217]

1. Uric acid

Uric acid has been shown to be a major low molecular weight antioxidant in upper respiratory tract lining fluid.[214,218,219] The approximate level of uric acid in nasal lining fluid has been reported to be 160 μM.[161] However, more accurate determinations show a level of 225 ± 105 μM.[220] Uric acid functions as a sacrificial antioxidant as it protects not only upper respiratory tract epithelial cells but also other constituents of nasal RTLF from oxidant injury. For instance, uric acid is one of the most potent scavengers of ozone.[221] During normal breathing it has been shown that the nasal airways take up approximately 40 % of the inspired ozone independent from the O_3 concentration.[222] Urate has indeed been proven to be depleted in human nasal lavage fluid after in vitro O_3 exposure confirming its protecting effect on the upper respiratory tract from the oxidant effects of ozone.[223] Although the levels of uric acid in nasal lavage fluids is lower in women than in men, and age may effect lavage uric acid, it may be used as a marker for O_3 sensitivity.[224] Furthermore, as the urate concentration in nasal secretions is similar to the concentration in plasma, it provides a useful marker for nasal epithelial lining fluid volume to which other lining fluid constituents, such as antioxidants, can be related.[225]

2. Glutathione, vitamin A, and vitamin E

Glutathione, ascorbic acid and vitamin E have been shown to occur in RTLFs. Approximate estimated levels of glutathione and ascorbic acid, both extracellular antioxidants, were reported to be 40 μM for both compounds.[161] The level of vitamin E in nasal epithelial lining fluids is not known. It should be noted that of the total glutathione amount measured only a small amount is present as GSH. Testa et al., found GSH levels of 1.27 ± 1.72 μM.[226] Blomberg et al., found GSH levels in nasal lavage fluid between 0.6-2.5 μM.[227] Housely et al., found GSH levels between 0.02 and 0.16 μM.[223] All these authors used spectrophotometric techniques to measure GSH. More recently van der Vliet et al., could not detect any GSH at all in nasal lavage fluid using a HPLC technique.[220] These low levels of GSH might be explained by the fact that, like the situation in plasma, glutathione is largely present as GSSG, although Housley et al., could not detect GSSG in nasal lavage fluid.[223] Furthermore, the low GSH concentration may be due to the formation of mixed disulfides. These latter may be formed with mucin thiols which are abundantly present in upper respiratory tract lining fluids.[86]

In addition to earlier reports ascorbic acid levels in nasal epithelial lining fluids have been refined and estimated at 28 ± 19μM.[161,220,223,227] It should be noted that ascorbate is almost exclusively present in its reduced form indicating its function as a oxidizable substrate. This redox state is maintained by reducing mechanisms at the epithelial surface or by active uptake of oxidized ascorbate, intracellular reduction and subsequent secretion in extracellular fluid.[220]

In bronchoalveolar lavage fluid the levels of GSH, vitamin A, and vitamin E, are influenced by the level of oxidative stress, i.e. smoking modulates antioxidant concentrations either through direct oxidant effect or through an indirect oxidant effect caused by the inflammatory respons.[228] This suggests that this may also be true for nasal epithelial lining fluid. Although diesel exhaust exposure has shown to increase the level of vitamin C in the nasal lavage fluid other investigations have not shown such effects.[227] In fact no measurable change in vitamin C and GSH levels in nasal lavage fluid was found after exposure of normal human volunteers to O_3 compared to exposure to air.[223,226] Therefore the exact role of GSH and vitamin C as antioxidants in nasal epithelial lining fluid remains unclear.

3. Proteins

In addition to the afore mentioned antioxidants, nasal epithelial lining fluid contains a spectrum of metal binding proteins which serve as antioxidant by either preventing transition metals to participate in ROS generating reactions or by other antioxidant properties.[229] The most important metal binding proteins are albumin, lactoferrin, ceruloplasmin and transferrin.[161] Albumin is a copper binding plasma protein which is secreted in the epithelial lining fluid under circumstances of increased vascular permeability. Increased vascular permeability has been observed in nasal and sinus inflammation indicating a role of albumin in the host defense in these disorders.[85,87] Also increased oxidant burden caused by ozone has been shown to increase albumin levels in nasal lavage fluids suggesting increased antioxidant potential.[230,231,232]

Lactoferrin, a nasal glandular protein, is an important scavenger of iron and presumably kills bacteria by this action as it has been shown that lactoferrin is both bacteriostatic and bactericidal to susceptible bacteria.[233] During cholinergic stimulation the lactoferrin concentration rises significantly in nasal lavage fluid.[214,218] Furthermore, the antioxidant activity of nasal lavage fluid is significantly related to this cholinergic rise in lactoferrin concentration.[218] Although patients with recurrent sinusitis have high baseline secretions of glandular products, such as lactoferrin, they have an abnormal cholinergic responses leading

to diminished mucus and thus lactoferrin secretion. This glandular abnormality may represent a diminished antioxidant defense contributing to the disease.[87,234] In addition, activated neutrophils, frequently found in sinusitis, produce lactoferrin.[31] Also, MPO, an oxidant producing enzyme expressed by inflammatory cells, has been shown to be significantly increased in nasal lavage fluid of sinusitis patients.[31] This may also suggest an additional antioxidant effect of lactoferrin. Indeed in patients with sinusitis an increased neutrophil related titer of lactoferrin has been found compared to healthy controls.[235]

Antioxidant pharmacotherapy

Reports on antioxidant pharmacotherapy in upper respiratory tract inflammation are sparse. However, several papers indicate that antioxidant pharmacotherapy may have beneficial effects on ROS related upper respiratory tract mucosal injury. These papers are discussed here. In principle there are three ways to cope with oxidant stress. First, by inhibiting the ROS production. Second, by supply of the endogenous antioxidant systems present in the upper airways, and third by the use of exogenous antioxidants.

Inhibition of ROS formation

During inflammation superoxide anion radicals are formed by phagocytes in a reaction catalyzed by the enzyme NADPH-oxidase.[4] Although the direct inhibition of this free radical generating system has been extensively described no data are available on the clinical effects of this inhibition in upper respiratory tract inflammation.[236] Indirect inhibition of the phagocytic free radical generating system by corticosteroids has been described for inflammatory lung disease.[237,238] It might be speculated that this mechanism may also account for chronic upper respiratory tract inflammation as steroid treatment is effective in chronic sinusitis and nasal polyps.[53] Other indirect inhibitory effects, i.e. inhibition of pro-oxidant cytokines such as TNF and IL-8, on superoxide production have been described for vitamin E and NAC. Furthermore, NAC inhibits the pro inflammatory transcription factor NF-κB.[28,35,37] Recently, the antimicrobial ketolides HMR 3004 and HMR 3647, have been shown to antagonize the injurious effects of phagocyte induced bioactive phospholipids in human nasal epithelial cells.[239] Deferoxamine is an iron chelating compound which has been shown to protect against ROS damage in lung injury.[1] In experiments in which rats were exposed to ozone upper respiratory tract hyperreactivity was decreased by treatment with deferoxamine

suggesting prevention of hydroxyl formation from superoxide anions by transition metal availability.[172]

Also treatment with allopurinol, a substrate for the alternative superoxide generating system xanthine oxidase which prevents superoxide anions to be formed, showed diminished nasal airway hyperreactivity in rats.[172]

Supplementation of endogenous antioxidant systems

Superoxide dysmutase and catalase are not suitable for oral administration as there is no uptake from the gastrointestinal tract.[240] In contrast, aerosolisation of SOD in sheep has been shown effective in increasing both the amount of SOD and anti-superoxide anion activity in epithelial lining fluid.[241] This finding might be of importance as in upper respiratory tract infections levels of mucosal SOD seems to be impaired. [65,67,68,242] In fact, ciliary slowing of human respiratory epithelial cells, caused by ROS, could be prevented by incubation of these cells with SOD and catalase.[76] Moreover, catalase and SOD have been shown to prevent ROS induced hyperreactivity in animal tracheal epithelial cells.[102]

Glutathione is thought to be the major cellular antioxidant. To increase GSH levels within the cell, the compound needed for GSH synthesis, i.e. cysteine, has to be provided.[1] NAC provides, after deacetylation, cysteine the precursor for glutathione. Except from contributing to GSH synthesis, NAC has also direct antioxidant properties by scavenging hydroxyl radicals and hypochlorous acid.[243] Both species have been implicated in upper respiratory tract inflammation.[76,84] Intramuscular NAC has been proven efficacious in the treatment of maxillary sinusitis in children.[244] Furthermore, GSH given by aerosol to healthy controls and patients with chronic rhinitis increased the GSH levels in the nasal mucosa significantly in both groups and improved symptoms dramatically for longer periods of time.[226] In addition, nebulization with S-carboxymethylcysteine, a derivative of N-acetylcysteine, showed significant improvement in rabbit sinus mucosa after exposure to oxidative cell damage by sulfur dioxide.[245]

Intake of ascorbic acid has been reported to improve hyperreactivity in patients with upper airway infection.[246] In contrast, Fortner et al., found no beneficial effect of relatively high doses of ascorbic acid on the nasal response to allergen in patients with seasonal allergic rhinitis.[247] Although the opinions about the treatment with ascorbic acid of patients with nasal allergy differs, clinical studies have shown beneficial effects.[248,249] This latter is also true for the treatment of patients with a common cold, of which improvement of symptoms has been shown.[250,251] No effect of vitamin C supplementation was observed on nasal mucosal

dysplasia after exposure of human subjects to ozone containing photochemical smog in a placebo controlled study.[252]

The lipophilic antioxidant vitamin E is the main protector of cell membranes against the process of lipid peroxidation. In principle vitamin E should be considered as a compound suitable for antioxidant pharmacotherapy. However, elevation of tissue vitamin E levels is very slow when supplemented dietary. This makes vitamin E unsuitable for the treatment of acute diseases.[1] The role of supplementary vitamin E in the treatment of upper respiratory tract inflammation is unclear. It has been suggested that dietary antioxidants, amongst which vitamin E, have antioxidant effects in nasal mucosa exposed to air pollution, thereby preventing allergic rhinosinusitis to occur.[253] This suggestion may be supported by the fact that PAF induced hyperresposiveness in the human nasal airway can almost be completely prevented by pretreatment with vitamin E.[103] However, in a double-blind randomized, placebo controlled clinical trial using 100 international units of vitamin E in patients exposed to oxidant containing photochemical smog no beneficial effects were seen on the development of mucosal damage.[252]

Exogenous antioxidants

The topical or systemic administration of exogenous antioxidants for upper respiratory tract inflammation in humans has to our best knowledge not been reported in Anglo-Saxon literature. However, there is some circumstantial evidence that some compounds may be used in this respect. Diminished lipid peroxidation was observed in a rat model after intraperitoneal injection of a lazaroid U-74389G which is known as a antioxidant.[69] Furthermore, the nasal decongestants oxymetazoline and xylometazoline have been shown to have specific antioxidant effects *in vitro*. A dose dependent inhibition of oxidative burst by leukocytes has been reported by oxymetazoline and xylometazoline.[254] In addition, both compounds proved to be potent scavengers of hydroxyl radicals, and oxymetazoline but not xylometazoline showed dose dependent inhibition of lipid peroxidation.[47] Finally nasal decongestants have been shown to cause reduced nitric oxide levels in the nasal airways. A feature occurring mainly as result of inhibition of the induction of iNOS.[51]

Although the role of antioxidant pharmacotherapy in upper respiratory tract pathology is far from clear, a beneficial role may be anticipated. Further research need to be performed using selective pharmacotherapeutics alone or in combination with others.

References

1. Hefner JE, Repine JE. Pulmonary strategies of antioxidant defense. *Am Rev Respir Dis* 1989; 140:531-554.
2. Doelman CJA, Bast A. Oxygen radicals in lung pathology. *Free Rad Biol Med* 1990; 9:381-400.
3. Barnes PJ. Reactive oxygen species and airway inflammation. *Free Rad Biol Med* 1990; 9:235-243.
4. Barbior BM. Oxygen dependent microbial killing by phagocytes (in two parts). *N Engl J Med* 1978; 298:659-668;721-725.
5. Thelen M, Dewald B, Baggiolini M. Neutrophil signal transduction and activation of the respiratory burst. *Physiol Rev* 1993; 73:797-821.
6. Busse WW, Vrtis RF, Steiner R, Dick EC. In vitro incubation with influenza virus primes human polymorphonuclear leukocyte generation of superoxide. Am J Respir Cell Mol Biol 1991; 4:347-354.
7. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. (3ed) Oxford University Press 1999.
8. Sadler KL, Badwey JA. Second messengers involved in superoxide production by neutrophils. *Hematol Oncol Clin North Am* 1988; 2:185-200.
9. Southorn PA, Powis G. Free radicals in medicine. I. Chemical nature and biologic reactions. *Mayo Clin Proc* 1988; 63:381-389.
10. Roos D, Eckmann CM, Yazdanbakhsh M, Hamers N, Boer de M. Excretion of superoxide by phagocytes measured with cytochrome c entrapped in resealed erythrocyte ghosts. *J Biol Chem* 1984; 259:1770-1775.
11. Weiss SS. Tissue destruction by neutrophils. *N Engl J Med* 1989; 320:365-376.
12. Halliwell B, Gutteridge JMC. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* 1984; 219:1-14.
13. Miller RA, Britigan BE. Role of oxidants in microbial pathophysiology. *Clin Microbiol Rev* 1997; 10:1-18.
14. Southorn PA, Powis G. Free radicals in medicine. II. Involvement in human disease. *Mayo Clin Proc* 1988; 63:390-408.
15. Bast A, Haenen GRMM, Doelman CJA. Oxidants and antioxidants: state of the art. *Am J Med* 1991: 91 (suppl 3C):2S-13S.
16. Snyder SH, Bredt DS. Biological roles of nitric oxide. *Sci Am* 1992; 266:68-77.
17. Nathan C, Xie Q. Regulation of biosynthesis of nitric oxide. *J Biol Chem* 1994; 269:13725-13728.
18. Hibbs JB, Taintor RR, Vavrin Z. Macrophage cytotoxicity: role for L-arginine deiminase activity and imino nitrogen oxidation to nitrite. *Science* 1987; 235:473-476.
19. Pryor WA, Squadito GL. The chemistry of peroxynitrite: a product from the reaction of nitric oxide and superoxide. *Am J Physiol* 1995; 268:L699-L722.
20. Beckman JS, Beckman TW, Chen J, Marshall PA Freeman BA. Apparent hyroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990; 87:1620-1624.
21. Henson PM, Johnston RB. Tissue injury in inflammation: oxidants, proteinases and cationic proteins. *J Clin Invest* 1987; 79:669-674.

22. Fantone JC, Ward PA. Role of oxygen derived free radicals and metabolites in leucocyte dependent inflammatory reactions. *Am J Pathol* 1982; 107:397-418.

23. Krimpen JLL. Infecties van de bovenste luchtwegen. In: Microbiologie en infectieziekten. Hoepelman IM, Noordaa J van der, Sauerwein R, Verburgh HA, eds. Bohn Stafleu Van Loghum, Houten/Diegem 1999.

24. Burdon RH. Superoxide and hydrogenperoxide in relation to mammalian cell proliferation. *Free Rad Biol Med* 1995; 18:775-794.

25. Peterhans E. Oxidants and antioxidants in viral diseases: disease mechanisms and metabolic regulation. *J Nutr* 1997; 127:962S-965S.

26. Schwarz KB. Oxidative stress during viral infection: a review. *Free Radic Biol Med* 1996; 5:641-649.

27. Peterhans E, Grob M, Bürge T, Zanoni R. Virus induced formation of reactive oxygen intermediates in phagocytic cells. *Free Radic Res Commun* 1987; 3:39-46.

28. Schultze-Oshoff K, Baker AC, Vanhaesbroeck B, Beyaer R, Jacob WA, Fiers W. Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. *J Biol Chem* 1992; 167:5317-5323.

29. Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of NF-κB transcription factor and HIV-1. *EMBO J* 1991; 10:2247-2258.

30. Schreck R, Meier B, Männel DN, Dröge W, Baeuerle PA. Dithiocarbamates as potent inhibitors of nuclear factor B activation in intact cells. *J Exp Med* 1992; 175:1181-1194.

31. Klempner MS, Dinarello CA, Gallis JI. Human leukocyte pyrogen induces release of specific granule contents from human neutrophils. *J Clin Invest* 1978; 61:1330-111336.

32. Halliwell B, Gutteridge JMC. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* 1984; 219:1-14.

33. Molloy AL, Winterbourn CC. Release of iron from phagocytosed Escherichia coli and uptake of neutrophil lactoferrin. *Blood* 1990; 75:984-989.

34. Cohen MS, Mao J, Rasmussen GT, Serodi JS, Britigan BE. Interaction of lactoferrin and lipopolysaccharide (LPS): effects on the antioxidant property of lactoferrin and the ability of LPS to prime human neutrophils for enhanced superoxide formation. *J Infect Dis 1992*; 166:1375-1378.

35. Biagioli MC, Kaul P, Singh I, Turner RB. The role of oxidative stress in rhinovirus induced elaboration of IL-8 by respiratory epithelial cells. *Free Rad Biol Med* 1999; 26:454-462.

36. Turner RB, Weingand KW, Yeh CH, Leedy DW. Association between interleukin-8 concentration in nasal secretion and symptoms of experimental rhinovirus colds. *Clin Infect Dis* 1998; 28:840-846.

37. Kaul P, Biagioli MC, Singh I, Turner RB. Rhinovirus induced oxidative stress and interleukin-8 elaboration involves p47-phox but is independent of attachment to intercellular adhesion molecule-1 and viral replication. *J Infect Dis* 2000; 181:1885-1890.

38. Akaike T, Ando M, Oda T, Ijiri S, Araki S, Maeda H. Dependence on O_2^- generation by xantine oxidase of pathogenesis of influenza virus infection in mice. *J Clin Invest* 1990; 85:739-745.

39. Handzel ZT, Busse WW, Sedgewick JB, Vrtis R, Lee WM, Kelly EA, Gern JE. Eosinophils bind rhinovirus and activate virus specific T-cells. *J Immunol* 1998; 160:1279-1284.

40. Chandhri G, Clark IA, Hunt NH, Cowden WB Ceredig R. Effect of antioxidants on primary alloantigen-induced T-cell activation and proliferation. *J Immunol* 1986; 137:2646-2652.

41. Hunt NH, Fragonas JC. Effects of antioxidants on ornithine decarboxylase in mitogenically activated T-Lymphocytes. *Biochim Biophys* 1992; 113:261-267.

42. White E, Sabbatini P Debbas M, Wold WS, Kusher DI, Gooding LR. The 19-kilodalton adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by tumor necrosis factor alpha. *Mol Cell Biol* 1992; 12:2570-2780.

43. Jacoby DB, Choi AMK. Influenza virus induces expression of antioxidant genes in human epithelial cells. *Free Rad Biol Med* 1994; 16:821-824.

44. Oda T, Akaike T, Hamamoto T, Suzuki F, Hirano T, maeda H. Oxygen radicals in influenza induced pathogenesis and treatment with pyran polymer-conjugated SOS. *Science* 1989; 244:974-976.

45. Sidwell RW, Huffman JH, Bailey KW, Wong MH, Nimrod A, Panet A. Inhibitory effects of recombinant manganese superoxide dismutase on influenza virus infections in mice. *Antimicrob Agents Chemother* 1996; 40:2626-2631.

46. De Flora S, Grassi C, Carati L. Attenuation of influenza like symptomatology and improvement of cell immunity with long term N-acetylcysteine treatment. *Eur Respir J* 1997; 10:1535-1541.

47. Westerveld GJ, Scheeren RA, Dekker I, Griffioen DH, Voss HP, Bast A. Antioxidant actions of oxymetazoline and xylometazoline. *Eur J Pharmacol* 1995; 291:27-31.

48. Nathan CF, Hibbs JB. Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr Opin Immunol* 1991; 3:65-70.

49. Akaike T, Noguchi Y, Ijiri S, Setoguchi K Suga M, Zheng YM, Doetzschoold B, Maeda H. Pathogenesis of influenza virus induced pneumonia: involvement of both nitric oxide and oxygen radicals. *Proc Natl Acad Sci USA* 1996; 93;2448-2453.

50. Akaike T, Suga M, Maedea H. Free radicals in viral pathogenesis: molecular mechanisms involving superoxide and NO. *PSEMB* 1998; 217:64-73.

51. Westerveld GJ, Voss HP, Hee van der RM, Haan-Koelewijn de GJN, hartog den GJ, Scheeren RA, Bast A. Inhibition of nitric oxide synthase expression and activity by nasal decongestants oxymetazoline and xylometazoline. *Eur Resp J* 2000; 16:437-444.

52. Gwaltney JM, Scheld WM, Sande MA, Sydnor A. The microbial etiology and antimicrobial therapy of adults with acute community-acquired sinusitis: a fifteen year experience at the university of Virginia and review of other selected studies. *J Allergy Clin Immunol* 1992; 90:457-462.

53. Benninger MS, Anon J, Mabry RL. The medical management of rhinosinusitis. *Otolaryngol Head Neck Surg* 1997; 117:S41-S49.

54. Parry MF, Root RK, Metcalf JA, Delaney KK, Kaplow LS, Richar WJ. Myeloperoxidase deficiency: prevalence and clinical significance. *Ann Intern Med* 1981; 95:293-301.

55. Mandell GL. Catalase, superoxide dismutase, and virulence of staphylococcus aureus: in vitro and in vivo studies with emphasis on staphyloccocal leukocyte interaction. *Infect Immun* 1975; 9:337-341.

56. Hoepelman IM, Bezemer WA, Vandenbroucke-Grauls CMJE, Marx JJM, verhoef J. Bacterial iron enhances oxygen radical mediated killing of staphylococcus aureus by phagocytes. *Infect Immun* 1990; 58:26-31.

57. Whittenbury R. Hydrogen peroxide formation and catalase activity in the lactic acid bacteria. *J Gen microbiol* 1964; 35:13-26.
58. Duane PG, Rubins JB, Weisel HR, Janoff JN. Identification of hydrogen peroxide as a streptococcus pneumoniae toxin for rat alveolar epithelial cells. *Infect Immun* 1993; 61; 4392-4397.
59. Moncada S, Higgs A. Mechanisms of disease: the L-arginine-nitric oxide pathway. *N Eng J Med* 1993; 329:2002-2012.
60. Dönner F, Delibas N, Dogru H, Sari I, Yorgancigil B. Malondyaldehyde levels and superoxide dysmutase activity in experimental sinusitis. *Auris Nasus Larynx* 1999; 26:287-291.
61. Jackowski JT, Szepfalusi Z, Wanner DA, Seybold Z, Sielczak MW, Lauredo IT, Adams T, Abraham WM, Wanner A. Effects of P. aeruginosa derived bacterial products on tracheal ciliary function: role of O_2 radicals. *Am J Physiol* 1991; 260:61-67.
62. Kawana M, Kawana C, Yokoo T, Quie PG, Giebink GS. Oxidative metabolic products released from polymorphonuclear leukocytes in middle ear fluid during experimental pneumococcal otitis media. *Infect Immun* 1991; 59:4084-4088.
63. Parks RR, Huang CC, Haddad J. Evidence of oxygen radical injury in experimental otitis media. *Laryngoscope* 1994; 104:1389-1392.
64. Haddad J. Lipoperoxidation as a measure of free radical injury in otits media. *Laryngoscope* 1998; 108:524-530.
65. Takoudes TG, Haddad J. Hydrogen peroxide in acute otitis media in guinea pigs. *Laryngoscope* 1997; 107:206-210.
66. Ovensen T, Borglum JD. Superoxide dysmutase in middle ear fluid from children with secretory otits media. *Acta Otolaryngol (Stockh)* 1992; 112:1017-1024.
67. Parks RR, Huang CC, Haddad J. Middle ear catalase distribution in an animal model of otitis media. *Eur Arch Otorhinolaryngol* 1996; 253:445-449.
68. Parks RR, Huang CC, Haddad J. Superoxide dysmutase in an animal model of otitis media. *Eur Arch Otorhinolaryngol* 1995; 252:153-158.
69. Haddad J, Egusa K, Takoudes TG. Effects of 21-aminosteroid U-74389G on acute otitis media in a guinea pig model. *Otolaryngol Head Neck Surg* 1998; 118:44-48.
70. Perry FE, Elson CJ, Greenham LW, Catterall JR. Interference with the oxidative response of neutrophils by streptococus pneumoniae. *Thorax* 1993; 48:364-369.
71. Perry FE, Elson CJ, Mitchell TJ, Andrew PW, Catterall JR. Characterisation of an oxidative response inhibitor produced by streptococus pneumoniae. *Thorax* 1994; 49:676-683.
72. McCord JM, Keele BB, Fridovich I. An enzyme based theory of obligate anaerobiosis: the physiological function of superoxide dysmutase. *Proc Natl Acad Sci USA* 1971; 68:499-506.
73. Langford PR, Loynds BM, Kroll JS. Copper-zinc superoxide dysmutase in haemophilus species. *J Gen Microbiol* 1992; 138:517-522.
74. Baan van der S. Primaire ciliaire dyskinesie. Academical thesis. Amsterdam 1985.
75. Passali D, ferri R, Becchini G, Passali GC, Bellussi L. Alterations of nasal mucociliary transport in patients with hypertrophy of the inferior turbinates, deviations of the nasal septum and chronic sinusitis. *Eur Arch Otorhinolaryngol* 1999; 256:335-337.

76. Feldman C, Anderson R, Kanthakumar K, Vargas A, Cole PJ, Wilson R. Oxidant mediated ciliary dysfunction in human respiratory epithelium. *Free Radic Biol Med* 1994; 17:1-10.

77. Yoshitsugu M, Matsunaga S, Hanamure Y, Rautiainen M, ueno K, Miyanohara T, Furuta S, Fukuda K, Ohyama. Effects of oxygen radicals on ciliary motility in cultured human respiratory epithelial cells. *Auris Nasus Larynx* 1995; 22:178-185.

78. Min YG, Ohyama M, Lee KS, Rhee CS, Oh SH, Sung MW, Yun JB, Jung IH. Effects of free radicals on ciliary movement in the human nasal epithelial cells. *Auris Nasus Larynx* 1999; 26:159-163.

79. Demoly P, Crampette L, Mondain M, Campbell AM, Lequeux N, Enander I, Schwartz LB, Guerrier B, Michel FB, Bousquet J. Assessment of inflammation in noninfectious chronic maxillary sinusitis. *J All Clin Immunol* 1994; 94:95-108.

80. Jankowski R. Eosiniphils in the pathophysiology of nasal polyposis. *Acta Otolaryngol (Stockh)* 1996; 116:160-163.

81. Gleich GJ, Frigas E, Loegering DA, Wassom DL, Steinmuller D. Cytotoxic properties of the eosinophil major basic protein. *J Immunol* 1979; 123:2925-2927.

82. Harlin SL, Ansel DG, Lane SR, Myers J, Kephart GM, Gleich GJ. A clinical and pathologic study of chronic sinusitis: the role of the eosinophil. *J All Clin Immunol* 1988; 81:867-875.

83. Hitsamatsu K, Tetsuya G, Nakazawa T, Murakami Y, Gleich GJ, Makiyama K, Koyama H. Cytotoxicity of human eosinophil granule major basic protein to human nasal sinus mucosa in vitro. *J All Clin Immunol* 1990; 86:52-63.

84. Ayars GH, Altman LC, McManus MM, Agosti JM, Baker C, Luchtel DL, Loegering DA, Gleich GJ. Injurous effect of eosinophil peroxide-hydrogen peroxide-halide system and major basic protein on human nasal epithelium in vitro. *Am Rev Respir Dis* 1989; 140:125-131.

85. Raphael GD, Druce HM, Baraniuk JN, Kaliner MA. Pathophysiology of rhinitis. I. Assessment of the sources of protein in metacholine induced nasal secretions. *Am Rev Respir Dis* 1988; 138:413-420.

86. Kaliner MA. Human nasal respiratory secretions and host defense. *Am Rev Resp Dis* 1991; 144:S52-S56.

87. Kaliner MA. Human nasal host defense and sinusitis. J All Clin Immunol 1992; 90:424-430.

88. Halliwell B. Albumin an important extracellular antioxidant ? *Biochem Pharmacol* 1988; 37:569-571.

89. Rasp G, Thomas PA, Bujia J. Eosinophil inflammation of the nasal mucosa in allergic and non-allergic rhinitis measured by eosinophil cationic protein levels in native nasal fluid and serum. *Clin Exp All* 1994; 24:1151-1156.

90. Lundgren JD, Davey RT, Lundgren B, Mullol J, Marom Z, Logun C, Baraniuk J, Kaliner MA, Shelhamer JH. Eosinophil cationic protein stimulates and major basic protein inhibits airway mucus secretion. *J All Clin Immunol* 1991; 87:689-698.

91. Müns G, Rubinstein I, Singer P. Phagocytosis and oxidative burst of granulocytes in the upper respiratory tract in chronic and acute inflammation. *J Otolaryngol* 1995; 24:105-110.

92. Kramer MF, Ostertag P, Pfrogner E, Rasp G. Nasal interleukin-5, immunoglobulin E, eosinophilic cationic protein, and soluble intercellular adhesion molecule-1 in chronic sinusitis, allergic rhinitis, and nasal polyposis. *Laryngoscope* 2000; 110:1056-1062.

93. Demoly P, Crampette L, Mondain M, Enander I, Jones I, Bousquet J. Myeloperoxidase and interleukin-8 levels in chronic sinusitis. *Clin Exp All* 1997; 27:672-675.

94. Min YG, Lee CH, Rhee CS, Hong SK, Kwon SH. Increased expression of IL-4, IL-5, INF-γ, IL-6, IL-8 and TGF-γ mRNAs in patients with chronic sinusitis. *Am J Rhinol* 1999; 13:339-343.

95. Shin SH, Park JY, Jeon CH, Choi JK, Lee SH. Quantitative analysis of eotaxin and rantes messenger RNA in nasal polyps: assosiation of tissue and nasal eosinophils. *Laryngoscope* 2000; 110:1353-1357.

96. Bachert C, Gevaert P, Holtappels G, Cuvelier C, Cauwenberge van P. Nasal polyposis: from cytokines to growth. *Am J Rhinol* 2000; 14:279-290.

97. Elsner J, Höchstetter R, Kimming D, Kapp A. Human eotaxin represents a potent activator of the respiratory burst of human eosinophils. *Eur J Immunol* 1996; 26:1919-1925.

98. Dogru H, Delibas N, Döner F, Tüz M, Uygur K. Free radical damage in nasal polyp tissue. Otolaryngol Head Neck Surg 2001; 124:570-572.

99. Turner PJ, Foreman JC. Hyperresponsiveness in the human nasal airway: new targets for the treatment of allergic airway disease. *Med Inflamm* 1999; 8:133-146.

100. Shult PA, Graziano FM, Busse WW. Enhanced eosinophil luminol dependent chmiluminescence in allergic rhinitis. J All Clin Immunol 1986; 77:702-708.

101. Katsumata U, Miura M, Ichinose M, kimura K, Takahashi Y, Inoue H, Takishima T. Oxygen radicals produce airway constriction and hyperresponsiveness in anaethetised cats. *Am Rev Resp Dis* 1990; 141:1158-1161.

102. Webber SE, Morikawa T, Widdicombe JG. PAF-induced muscarinic cholinoceptor hyperresponsiveness of ferret tracheal smooth muscle and gland secretion in vitro. *Br J Pharmacol* 1992; 105:230-237.

103. Austin CE, Foreman JC. The effect of platelet activating factor on the responsiveness of the human nasal airway. *Br J Pharmacol* 1993; 110:113-118.

104. Garrrelds IM, Amsterdam van JGC, Graaf de-in 't Veld C, Gerth van Wijk R. Nitric oxide metabolites in nasal lavage fluid of patients with house dust mite allergy. *Thorax* 1995; 50:275-279.

105. Martin U, Bryden K, devoy M, Howarth P. Increased levels of exhaled nitric oxide during nasal and oral breathing in subjects with seasonal rhinitis. *J All Clin Immunol* 1996; 97:768-772.

106. Kharitonov SA, Rajakulasingam K, O'Connor B, Durham SR, Barnes PJ. Nasal nitric oxide is increased in patients with asthma and allergic rhinitis and may be modulated by nasal glucocorticoids. *J All Clin Immunol* 1997; 99:58-64.

107. Lundberg JO, Weitzberg E, Lundberg JM, Alving K. Nitric oxide in exhaled air. *Eur Resp J* 1996; 9:2671-2680.

108. Schedin U, Frostell C, Persson MG, Jakobsson J, Andersson G, Gustafsson LE. Contribution from upper and lower airways to exhaled endogenous nitric oxide in humans. *Acta Anaesthesiol Scand* 1995; 39:327-332.

109. Lundberg JON, Rinder J, Weitzberg E, Lundberg JM, Alving K. Nasally exhaled nitric oxide in humans originates mainly in the paranasal sinuses. *Acta Physiol Scand* 1994; 152:431-432.

110. Lundberg JON, Farkas-Szallasi T, Weitzberg E, Rinder J, Lindholm J, Änggård A, Hökfelt T, Lundberg JM, Alving K. High nitric oxide production in human paranasal sinuses. *Nat Med* 1995; 1:370-373.

111. Lewandowski K, Busch T, Lohbrunner H, Rensing S, Keske U, Gerlag H, Falke KJ. Low nitric oxide concentrations in exhaled gas and nasal airways of mammals without paranasal sinuses. *J Appl Physiol* 1998; 85:405-410.

112. Haight JS, Djupesland PG, Qjan W, Chatkin JM, Furlott H, Irish J, Witterick I, McClean P, Fenton RS, Hoffstein V, Zamel N. Does nasal nitric oxide come from the sinuses ? *J Otolaryngol* 1999; 28:197-204.

113. Chatkin JM, Qjan W, McClean P, Zamel N, Haight JS, Silkoff P. Nitric oxide accumulation in the nonventilated nasal cavity. *Arch Otolaryngol Head Neck Surg* 1999; 125:682-685.

114. Kimberly B, Nejadnik B, Giraud GD, Holden WE. Nasal contribution to exhaled nitric oxide at rest and during breathholding in humans. *Am J Respir Crit Care Med* 1996; 153:829-836.

115. Rosbe KW, Mims JW, Prazma J, Petrusz P, Rose A, Drake AF. Immunohistochemical localization of nitric oxide synthase activity in upper respiratory epithelium. *Laryngoscope* 1996; 106:1075-1079.

116. Furukawa K, Harrison DG, Saleh D, Shennib H, Chagnon FP, Giaid A. Expression of nitric oxide synthase in the human nasal mucosa. *Am J Respir Crit Care Med* 1996; 153:847-850.

117. Conway PJ, Jones NS. The nose and nitric oxide: a review. *Clin Otolaryngol* 2000; 25:337-341.

118. Runer T, Cervin A, Lindberg S, Uddman R. Nitric oxide is a regulator of mucociliary activity in the upper respiratory tract. Otolaryngol Head Neck Surg 1998; 119:278-287.

119. Lundberg JON, Weitzberg E, Nordvall SL, Kuylenstierna R, Lundberg JM, Alving K. Primarily nasal origin of exhaled nitric oxide and absence in Kartagener's syndrome. *Eur Respir J* 1994; 7:1501-1504.

120. Lundberg JON, Nordvall SL, Weitzberg E, Kollberg H, Alving K. Exhaled nitric oxide in paediatric asthma and cystic fibrosis. *Arch Dis Child* 1996; 75:323-326.

121. Lane AP, Prazma J, Baggett HC, Rose AS, Pillsbury HC. Nitric oxide is a mediator of neurogenic exudation in the nose. *Otolaryngol Head Neck Surg* 1997; 116:294-300.

122. Lane AP, Prazma J, Gibbons PJ, Rose AS, Pillsbury HC. The role of nitric oxide in the neural control of nasal fluid production. *Am J Rhinol* 1997; 11:303-311.

123. Lacroix JS, Potter EK, McLachlan E. Nitric oxide and parasympathetic vascular and secretory control of the dog nasal mucosa. *Acta Otolaryngol (Stockh)* 1998; 118:257-263.

124. Rinder J. Sensory neuropeptides and nitric oxide in vascular regulation. *Acta Pysiol Scand Suppl* 1996; 157:7-45.

125. Rinder J, Lundberg JM. Nasal vasoconstriction and decongestant effects of nitric oxide synthase inhibition in the pig. *Acta Physiol Scand* 1996; 157:233-244.

126. Ruffoli R, Fattori B, Soldani P, Giambelluca MA, Giannessi F. Ultrastructural localisation of NADPH-diaphorase activity in the endothelial cells of human nasal respiratory mucosa. *Am J Rhinol* 1999; 13:49-53.

127. Ruffoli R, Fattori B, Giambelluca MA, Soldani P, Giannessi F. Ultrastructural localisation of the NADPH-diaphorase activity in the human nasal respiratory mucosa in vasomotor rhinitis. *Laryngoscope* 2000; 110:1361-1365.

128. Imada M, Iwamoto J, Nonaka S, Kobayashi Y, Unno T. Measurement of nitric oxide in human nasal airway. *Eur Resp J* 1996; 9:556-559.

129. Bacci S, Arbi-Riccardi, Mayer B, Rumio C, Borghi-Cirri MB. Localization of niyric oxide synthase immunoreactivity in mast cells of human nasal mucosa. *Histochemistry* 1994; 102:89-92.

130. Ferguson EA, Eccles R. Relationship between nasal nitric oxide concentration and nasal airway resistance. *Rhinology* 1997; 35:120-123.

131. Chatkin JM, Djupesland PG, Qian W, McClean P, Furlott H, Gutierrez C, Zamel N, Haight JS. Nasal nitric oxide is independent of nasal cavity volume. Am J Rhinol 1999; 13:179-184.

132. Silkoff PE, Roth Y, McClean P, Cole P, Chapnik J, Zamel N. Nasal nitric oxide does not control basal nasal patency or acute congestion following allergen challenge in allergic rhinitis. *Ann Oto Rhinol Laryngol* 1999; 108:368-372.

133. Djupesland PG, Chatkin JM, Qian W, Cole O, Zamel N, McClean P, Furlot H, Haight JS. Aerodynamic influences on nasal nitric oxide output measurements. *Acta Otolaryngol* 1999; 119:479-485.

134. Lundberg JON, Weitzberg E, Rinder J, Rudehill A, Jansson O, Wiklund NP, Lundberg JM, Alving K. Calcium-independent and steroid resistent nitric oxide synthase activity in human paranasal sinus mucosa. *Eur Resp J* 1996; 9;1344-1347.

135. Rinder J, Lundberg JON, Änggård A, Alving K, Lundberg JM. Effects of topical nasal decongestants, l-arginine and nitric oxide synthase inhibition, on nasal cavity nitric oxide levels and nasal cavity volume in man. *Am J Rhinol* 1996; 10:399-408.

136. Silkoff PE, Chatkin J, Qian W, Chakravorty S, Gutierrez C, Furlott H, McClean P, Rai S, Zamel N, Haight J. Nasal nitric oxide: a comparison of measurement techniques. Am J Rhinol 1999; 13:169-178.

137. Lundberg JO. Airborne nitric oxide: inflammatory marker and aerocrine messenger in man. *Acta Physiol Scand Suppl* 1996; 633:1-27.

138. Lundberg JO, Settergen G, Gelinder S, Lundberg JM, Alving K, Weitzberg E. Inhalation of nasally derived nitric oxide modulates pulmonary function in humans. *Acta Physiol Scand* 1996; 158:343-347.

139. Jenkins IR, Langlois D. Quantitative measurement of nasal production of nitric oxide in awake humans. *Anaesth Intens Care* 1995; 23;702-705.

140. Bartley J, Fergusson W, Moody A, Wells AU, Kolbe J. Normal adult values , diurnal variation, and repeatability of nasal nitric oxide measurement. *Am J Rhinol* 1999; 13:401-405.

141. Flak TA, Goldman WE. Autotoxicity of nitric oxide in airway disease. *Am J Respir Crit Care Med* 1996; 154:S202-S206.

142. Croen KD. Evidence for an antiviral effect of nitric oxide:inhibition of herpes simplex virus type 1 replication. *J Clin Invest* 1993; 91:2446-2452.

143. Mancinelli RL, McKay CP. Effects of nitric oxide and nitrogen dioxide on bacterial growth. *Appl Environ Microbiol* 1983; 46:198-202.

144. Kharitonov SA, Yates D, Barnes PJ. Increased nitic oxide in exhaled air of normal human subjects with upper respiratory tract infections. *Eur Respir J* 1995;6:295-297.

145. Ferguson EA, Eccles R. Changes in nasal nitric oxide concentration associated with symptoms of common cold and treatment with a topical nasal decongestant. *Acta Otolaryngol (Stockh)* 1997; 117:614-617.

146. Linberg S, Cervin A, Runer T. Nitric oxide production in the upper airways is decrease in chronic sinusitis. *Acta Otolaryngol (Stockh)* 1997; 117:113-117.

147. Kaul P, Singh I, Turner RB. Effect of nitric oxide on rhinovirus replicatin and virus induced interleukin-8 elaboration. *Am J Respir Crit Care Med* 1999; 159:1193-1198.

148. Baraldi E, Azzolin NM, Carra S, Dario C, Marchesini L, Zacchello F. Effcet of topical steroids on nasl nitric oxide production in children with perennial allergic rhinitis: a pilot study. *Respir Med* 1998; 92:558-561.

149. Kawamoto H, Takeno S, Yain K. Increased expression of inducible nitric oxide synthase in nasal epithelial cells in patients with allergic rhinitis. *Laryngoscope* 1999; 109:2015-2020.

150. Kang BH, Chen SS, Jou LS, Weng PK, Wang HW. Immunolocalization of inducible nitric oxide synthase and 3-nitrotyrosine in the nasal mucosa of patients with rhinitis. *Eur Arch Otorhinolaryngol* 2000; 257:242-246.

151. Sato M, Fukuyama N, Sakai M, Nakazawa H. Increased nitric oxide in nasal lavage fluid and nitrotyrosine formation in nasal mucosa. Indices for severe perennial nasal allergy. *Clin Exp All* 1998; 28:597-605.

152. Hanazawa T, Antuni JD, Kharitonov SA, Barnes PJ. Intranasal administration of eotaxin increases nasal eosinophils and nitric oxide in patients with allergic rhinitis. J All Clin Immunol 1999; 105:58-64.

153. Gerlach H, Rossaint R, Pappert D, Knorr M, Falke KJ. Autoinhalation of nitric oxide after endogenous synthesis in nasopharynx. *Lancet* 1994; 343:518-519.

154. Olin AC, Hellgren J, Karlsson G, Ljungkvist G, Nolkrantz K, Torén K. Nasal nitric oxide and its relationship to nasal symptoms, smoking and nasal nitrate. *Rhinology* 1998; 36:117-121.

155. Schlosser RJ, Spotnitz WD, Peters EJ, Fang K, Gaston B, Gross CW. Elevated nitric oxide metabolite levels in chronic sinusitis. Otolaryngol Head Neck Surg 2000; 123:357-362.

156. Haight JSJ, Qian W, Daya H, Chalmers P, Zamel N. Hypoxia depresses nitric oxide output in the human nasal airways. *Laryngoscope* 2000; 110:429-433.

157. Arnal JF, Flores P, Rami J, Murris-Espin M, Bremont F, Pasto I Aguilla M, Serrano E, Didier A. Nasal nitric oxide concentration in paranasal sinus inflammatory diseases. Eur Respir J 1999; 13:307-312.

158. Ramis I, Lorente J, Roselló-Catafau J, Quesada P, Gelpi E, Bulbena O. Differential activity of nitric oxide synthase in human nasal mucosa and polyps. Eur Respir J 1996; 9:202-206.

159. Pryor WA. How far does ozone penetrate into the pulmonary air/tissue boundary before it reacts ? *Free Radic Biol Med* 1992; 12:83-88.

160. Pryor WA. Mechanisms of free radical formation from reactions of ozone with target molecules in the lung. *Free Radic Biol Med.* 1994; 17:451-465.

161. Cross CE. Vliet van der A, O'Neill CA, Louie S, Halliwell B. Oxidants, antioxidants, and respiratory tract lining fluids. *Environ Health Perspect* 1994; 102 (Suppl 10):185-191.

162. Mustafa MG. Biochemical basis of ozone toxicity. *Free Radic Biol Med* 1990; 9:245-265.

163. Hotchkiss JA, Harkema JR, SUN JD, Henderson RF. Comparison of acute ozon-induced nasal and pulmonary responses in rats. *Toxicol Appl Pharmacol* 1989; 98:289-302.

164. Graham D, House D. Neutrophil influx in nasal lavages of humans exposed to ozone. *Arch Environ Health* 1988; 43:228-233.

165. Johnson NF, Hotchkiss JA, Harkema JR, Henderson RF. Proliferative responses of rat nasal epithelia to ozone. *Toxicol Appl Pharmacol* 1990; 103:143-155.

166. Chang JCF, Gross EA, Swenberg JA, Barrow CS. Nasal cavity deposition, histopathology, and cell proliferation after single or repeated formaldehyde exposures in B6C3F$_1$ mice and F344 rats. *Toxicol Appl Pharmacol* 1983; 68:161-176.

167. Harkema JR, Hotchkiss JA, Henderson RF. Effects of 0.12 and 0.80 ppm ozone on rat nasal and nasopharyngeal epithelial mucosubstances: quantitative histochemistry. *Toxicol Pathol* 1989; 17:525-535.

168. Harkema JR, Hotchkiss JA, Henderson RF. Effect of cumulative ozone exosure on ozone-induced nasal epithelial hyperplasia and secretory metaplasia in rats. *Exp Lung Res* 1991; 17:589-600.

169. Gosepath J, Schaefer D, Brommer C, Klimek L, Amedee RG, Mann WJ. Subacute effects of ozone exposure on cultivated human respiratory mucosa. *Am J Rhinol* 2000; 14:411-418.

170. Riechelmann H. The nose versus the environment: 1982 and today. *Am J Rhinol* 2000; 14:291-297.

171. Kopp MV, Ulmer C, Ihorst G, Seydewitz HH, frischer T, Forster J, Kuehr J. Upper airway inflammation in children exposed to ambient ozone and potential signs of adaptation. *Eur Respir J* 1999; 14:854-861.

172. Steerenberg PA, Fischer PH, Gmelig Meyling F, Willighagen J, Geerse E, Vliet van de H, Ameling C, Boink ABTJ, Dormans JAMA, Bree Van L, Van Loveren H. Nasal Lavage as tool for health effect assessment of photochemical air pollution. *Hum Exp Tox* 1996; 15:111-119.

173. Tsukagoshi H, Haddad EB, Sun J, Barnes PJ, Chung KF. Ozone induced airway hyperresponsiveness: role of superoxide anions, NEP, and BK receptors. *J Appl Physiol* 1995;78:1015-1022.

174. Reuzel PGJ, Wilmer JWGM, Woutersen RA, Zwart A, Rombout PJA, Feron VJ. Interactive effects of ozone and formaldehyde on nasal respiratory lining epithelium in rats. *J Toxicol Environ Health* 1990; 29:279-292.

175. Schäfer D, Brommer C, Riechelmann H, Mann JW. In vivo and in vitro effect ozone and formaldehyde on human nasal mucociliary transport system. Rhinology 1999; 37:56-60.

176. Pazdrak K, Gorsik P, Krakowiak A, Ruta U. Changes in nasal lavage fluid due to formaldehyde inhalation. *Int Arch Occupl Environ Health* 1993; 64:515-519.

177. Edling C, Hellquist H, Odkvist L. Occupational exposure to formaldehyde and histopathological changes in nasal mucosa. *Br J Ind Med* 1988; 45:761-765.

178. Falk J, Juto J, Stridh G, Bylin G. Dose response study of formaldehyde on nasal mucosal swelling – a study on residents with nasal distress at home. *Am J Rhinol* 1994; 8:143-146.

179. Cassee FR, Feron VJ. Biochemical and histopathological changes in nasal epithelium of rats after 3-day intermittent exposure to formaldehyde and ozone alone or in combination. *Toxicol Lett* 1994; 72:257-268.

180. Ohashi Y, Nakai Y, Sugiura Y, Ohno Y, Okamoto H. Nitrogen dioxide induced eosinophilia and mucosal injury in the trachea of the guinea pig. *ORL* 1993; 55:36-40.

181. Carson JL, Collier Am, Hu SC, Delvin RB. Effect of nitrogen dioxide on human nasal epithelium. *Am J Respir Cell Mol Biol* 1993; 9:264-270.

182. Cavanagh D, Morris JB. Mucus protection and airway peroxidation following nitrogen dioxide exposure in the rat. *J Toxicol Environ Health* 1987; 22:313-328.

183. Riechelman H, Maurer J, Kienast K, Hafner B, ann WJ. Respiratory epithelium exposed to sulfur dioxide. Functional and ultrastructural alterations. Laryngoscope 1995; 105:295-299.

184. Giddens WE, Fairchild GA. Effects of sulfur dioxide on nasal mucosa of mice. Arch Environ Health 1972; 25:166-121.

185. Koltai PJ. Effects of air pollution on the upper respiratory tract in children. *Otolaryngol Head Neck Surg* 1994; 111:9-11.

186. Keles N, Ilicali C, Deger K. The effects of different levels of air pollution on atopy and symptoms of allergic rhinitis. *Am J Rhinol* 1999; 13:185-190.

187. Freeman BA, Crapo JD. Biology of disease. Free radicals and tissue injury. *Lab Invest* 1982; 47:412-426.

188. Nikula KJ, Sabourin PJ, Frietag BC, Birdwhistell AJ, Hotchkiss JA, Harkema JR. Biochemical and morphologic responses of rat nasal epithelia to hyperoxia. *Fund Appl Toxicol* 1991; 17:675-683.

189. Sabourin PJ, Nikula KJ, Birdwhistell AJ, Freitag BC, Harkema JR. Biochemical and morphological responses of nasal epithelia to hyperoxia. In: Biological Reactive Intermediates IV. Eds. Witmer et al. Plenum Press New York, 1990.

190. Dahl AR, Hadley WM. Nasal cavity enzymes involved in xenobiotic metabolism: efects on the toxicity of inhalants.*Toxicology* 1991; 21:345-372.

191. Gervasi PG, Longo V, Naldi F, Panattoni G, Ursino F. Xenobiotic metabolizing enzymes in human respiratory nasal mucosa. *Biochem Pharmacol* 1991; 41:177-184.

192. Bast A. Is formation of reactive oxygen species by cytochrome P450 perilous and predictable ? *Trends Pharmacol Sci* 1986; 7:266-270.

193. Bast A, Haenen GRMM. Cytochrome P450 and glutathione what is the significance of their interrelationship in lipid peroxidation ? *Trends Biochem Sci* 1984; 9:510-513.

194. Banger KK, Foster JR, Lock EA, Reed CJ. Immunohistochemical localisation of six glutathione S-tarnsferases within the nasal cavity of the rat. *Arch Toxicol* 1994; 69:91-98.

195. Aceto A, Di Ilio C, Angeluccin S, Longo V, Gervasi PG, federici G. Glutathione transferases in human nasal mucosa. Toxicology 1989; 63:427-431.

196. Ålin P, Danielson UH, Mannervik B. 4-Hydroxyalk-2-enals are substrates for glutathione transferase. *FEBS Lett* 1985; 179:267-270.

197. Prohaska JR. The glutathione peroxidase activity of the glutathione-S-transferases. *Biochim Biophys Acta* 1980; 611:87-98.

198. Wright DT, Cohn LA, Li H, Fischer B, Li CM, Adler KB. Interactions of oxygen radicals with airway epithelium. *Environ Health Perspect 1994*; 102(Suppl 10):85-90.

199. Fridovich I. Superoxide radical: an enogenous toxicant. *Annu Rev Pharmacol Toxicol* 1983; 23:239-257.

200. Chance B, sies H, Boveris A. Hydroperoxidase metabolism in mammalian organs. *Physiol Rev* 1979; 59:527-605.

201. Koufos A, sagone AL. Effects of oxidants stress on the hexose monophosphate shunt pathway of platelets. *Blood* 1980; 165:195-199.

202. Haenen GRMM. Thiols in oxidative stress. Some implications for catecholamine toxicity. Academical thesis. Amsterdam 1989.

203. Doba T, Burton GW, Inghold KU. Antioxidant and co-antioxidant activity of vitamin C. *Biochem Biophys Acta* 1985; 835:298-303.

204. Sevenian A, Davies KJA, Hochstein P. Conservation of vitamin C by uric acid in blood. *Free Radic Biol Med* 1985; 1:117-124.

205. Davies KJA, sevenian A, Muakkassah-Kelly SF, Hochstein P. uric acid iron ion complexes: a new aspect of the antioxidant functions of uric acid. *Biochem J* 1986; 235:747-754.

206. Ketterer B. Detoxification reactions of glutathione transferases. Xenobiotica 1986; 16:957-973.

207. Haenen GRMM, Vermeulen NPE, Timmerman H, Bast A. Effect of several thiols on lipid peroxidation in rat liver microsomes. *Chem Biol interact* 1989; 71:201-212.

208. Chidambaram MV, Barnes G, Frieden E. Ceruloplasmin and the reactions forming diferric transferrin. *FEBS Lett* 1983; 159:137-140.

209. Lai MT, Ohmichi T, Ogawa T, Kazunori N, masuda Y. Electron spin resonance spin trapping assay and immunohitochemical localization of superoxide dismutases in rat nasal mucosa. *Acta Otolaryngol (Stockh)* 1997; 117:437-446.

210. Potter DW, Finch L, Udinski. Glutathione content and turnover in rat nasal epithelia. *Toxicol Appl Pharmacol* 1995; 135:185-191.

211. Potter DW, Tran TB. Apparent rates of glutathione turnover in rat tissues. *Toxicol Appl Pharmacol* 1993; 120:186-192.

212. Westerveld GJ, Dekker I, Voss HP, bast A, Scheeren RA. Antioxidant levels in the nasal mucosa of patients with chronic sinusitis and healthy controls. *Arch Otolaryngol Head Neck Surg* 1997; 123:201-204.

213. Sies H. Oxidative stress: from basic research to clinical application. *Am J Med (Suppl 3C)* 1991;31S-38S

214. Peden DB, Swiersz M, Ohkubo K, Hahn B, Emery B, Kaliner MA. Nasal secretion of the ozone scavenger uric acid. *Am Rev Respir Dis* 1993; 148:455-461.

215. Acker van SABE, Koymans LNH, Bast A. Molecular pharmacology of vitamin E: structural aspects of antioxidant activity. *Free Radic Biol Med* 1993; 20:311-328.

216. Authors unknown. Ascorbic acid levels of blood and urine in patients with allergic rhinitis. *Eye Ear Nose Throat Monthly* 1968; 47:63-64.

217. Peterson BT, Griffith DE, Tate RW, Clancy SJ. Single-cycle bronchoalveolar lavage to determine the solute concentrations in epithelial lining fluid. *Am J Respir Dis* 1993; 147:1216-1222.

218. Peden DB, Brown ME, Wade Y, Raphael GD, Berkebile C, Kaliner MA. Human nasal glandular secretion of novel antioxidant activity: cholinergic control. *Am Rev Respir Dis* 1991; 143:545-552.

219. Peden DB, Hohman R, Brown ME, mason RT, Berkebile C, Fales HM, Kaliner MA. Uric acid is a major antioxidant in human nasal airway secretions. *Proc Natl Acad Sci USA* 1990; 87:7638-7642.

220. Vliet van der A, O'Neill CA, Cross CE, Koostra JM, Volz WG, Halliwell B, Louie S. Determination of low-molecular-mass antioxidant concentrations in human respiratory tract lining fluids. *Am J Physiol* 1999; 276:L289-L296.

221. Cross CE, Motchik PA, Bruener BA, Jones DA, Kaur H, Ames BN, Halliwell B. Oxidative damage to plasma constituents by ozone. *FEBS Lett* 1992; 298:269-272.

222. Gerrity TR, Weaver RA, Bentsen J, House DE, O'Neil JJ. Extrathoracic and intrathoracic removal of O_3 in tidal breathing humans. *J Appl Physiol* 1988; 65:393-400.

223. Housley DG, Mudway I, Kelly FJ, Eccles R, Richards RJ. Depletion of urate in human nasal lavage following in vitro ozone exposure. *Int J Biochem Cell Biol* 1995; 27:1153-1159.

224. Housely DG, Eccles R, Richards RJ. Gender difference in the concentration of the antioxidant uric acid in human nasal lavage. *Acta Otolaryngol (Stockh)* 1996; 116;751-754.

225. Kaulbach HC, White MV, Igarashi Y, Hahn BK, Kaliner MA. Estimation of nasal epithelial lining fluid using urea as a marker. *J All Clin Immunol* 1993; 92:457-465.

226. Testa B, Mesolela M, Testa D, Giuliano A, Costa F, Maione F, Iaccarino F. Glutathione in the upper respiratory tract. *Ann Otol Rhinol Laryngol* 1995; 104:117-119.

227. Blomberg A, sainsbury C, Rudell B, Frew AJ, Holgate ST, Sandstrom T, Kelly FJ. Nasal cavity lining fluid ascorbic acid concentration increases in healthy human volunteers following short term exposure to diesel exhaust. *Free Radic Res* 1998; 28:59-67.

228. Crissman KM, Slade R, Norwood J, Koren H, Hatch GE. Differences between cigarette smokers and non-smokers in bronchoalveolar lavage fluid fatty acids, protein and antioxidants. *Am Rev Respir Dis* 1990; 141:A781.

229. Wasil M, Halliwell B, Hutchison CS, Baum H. The antioxidant action of human extracellular fluids. *Biochem J* 1987; 243:219-223.

230. Koren HS, Hatch GE, Graham DE. Nasal lavage as a tool in assessing acute inflammation in response to inhaled pollutants. *Toxicology* 1990; 60:15-25.

231. Graham DE, Koren HS. Biomarkers of inflammation in ozone-exposed humans. Comparison of nasal and bronchoalveolar lavage. *Am Rev Respir Dis* 1990; 142:152-156.

232. Bascom R, naclerio RM, Fitzgerald TK, Kagey-Sobotka A, Proud D. Effect of ozoen inhalation on the response to nasal challenge with antigen of allergic subjects. *Am Rev Respir Dis* 1990; 14:594-601.

233. Masson PI, Heremans JF, ive CH. An iron binding protein common to may external secretions. *Clin Chim Acta* 1966; 14:735-739.

234. Jeney EV, Raphael GD, Meredith SD, Kaliner MA. Abnormal nasal glandular secretion in recurrent sinusitis. *J Allergy Clin Immunol* 1990; 86:10-18.

235. Niehaus MD, Gwaltney JM, Hendley JO, Newman MJ, Heymann PW, Rakes GP, Platts-Mills TAE, Guerrant RL. Lactoferrin and eosinophillic cationic protein in nasal secretions of patients with experimental rhinovirus colds, natural colds,and presumed acute community-acquired bacterial sinusitis. *J Clin Microbiol* 2000; 38:3100-3102.

236. Cross AR. Inhibitors of the leukocyte superoxide generating oxidase: mechanisms of action and methods for their elucidation. *Free Rad Biol Med* 1990; 8:71-93.

237. Schleimer RP. Effects of glucocorticosteroids on inflammatory sells relevant to their therapeutic application in asthma. *Am Rev Respir Dis* 1990; 141:S59-S69.

238. Majori M, Vachier I, Godard P, Farce M, Bousquet J, Chanez P. Superoxide anion production by monocytes of corticosteroid-treated asthmatic patients. *Eur Respir J* 1998; 11:133-138.

239. Feldman C, Anderson R, Theron A, Mokgobu I, Cole PJ, Wilson R. the effects of ketolides on bioactive phospholipid-induced injury to muman respiratory epithelium in vitro. *Eur Res J* 1999; 13: 1022-1028.

240. Greenwald RA. Superoxide dismutase and catalase as therapeutic agents in human diseases. A critical review. *Free Rad Biol Med* 1990; 8:201-209.

241. Gillissen A, Roum JH, Hoyt RF, Crystal RG. Aerosolization of superoxide dismutase. Augmentation of respiratory epithelial lining fluid antioxidant screen by aerosolization of recombinant human Cu^{++}/Zn^{++} superoxide dysmutase. *Chest* 1993; 104:811-815.

242. Lee ES, Woo JS, Hwang SJ, Lim HH, Suh HK. Protective role of superoxide dismutase in rat eustachian tube mucosa against acute otitis media induced by upper respiratory tract infection. *J Laryngol Otol 2000*; 114:832-826.

243. Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide and hypochlorous acid. *Free Radic Biol Med* 1989; 6:593-597.

244. Boner AL, Valleta EA, Andreoli A, Vallone G, Baronio L. A combination of cefuroxime and N-acetylcysteine for the treatment of maxillary sinusitis in children with respiratory allergy. *Int J Clin Pharmacol Ther Toxicol* 1984; 22:511-514.

245. Sugiura Y, Ohashi Y, Nakai Y. Improvement of mucosal pathology of the sinuses after exposure to sulfur dioxide by nebulization of S-carboxymethylcysteine. *Acta Otolaryngol (Stockh) Suppl* 1997; 531:10-16.

246. Bucca C, Rolla G, Arossa W, Caria E, Elia C, Nebiolo F, Baldi S. Effect of ascorbic acid on increased responsiveness during upper airway infection. *Respiration* 1989; 55:214-219.

247. Fortner BR, Danziger RE, Rabinowitz PS, Nelson HS. The effect of ascorbic acid on cutaneous and nasal response to histamin and allergen. *J All Clin Immunol* 1982; 69:484-488.

248. Podoshin L, Gertner R, Fradis M. Treatment of perennial allergic rhinitic with ascorbic acid solution. *Ear Nose Throat J* 1991; 70:54-55.

249. Thornhill SM, Kelly AM. Natural treatment of perennial allergic rhinitis. *Altern Med Rev* 2000; 5:448-454.

250. Spannhake EWM, Menkes HA. Vitamin C: new tricks for an old dog. *Am Rev Respir Dis* 1983; 127:139-140.

251. Wilson CWM, Loh HS. Common cold and vitamin C. *Lancet* 1973; 1:638-641.

252. Calderon-Garciduenas L, Roy-Ocotla G. Nasal cytology in southwest metropolitan mexico city inhabitants: a pilot intervention study. *Environ Health Perspect* 1993; 101:138-144.

253. Trevino RJ. Air pollution and its effects on the upper respiratory tract and on allergic rhinosinusitis. *Otolaryngol Head Neck Surg* 1996; 114:239-241.

254. Bjerkness R, Steinsvåg SK. Inhibition of human neutrophil actin polymerization, phagocytosis and oxidative burst by components of decongestive nosedrops. *Pharmacol Toxicol* 1993; 73:41-45.

Chapter II-1

Antioxidant levels in the nasal mucosa of patients with chronic sinusitis and healthy controls

Gerrit-Jan Westerveld[1], Robert A Scheeren[1], Ingrid Dekker[2], Hans-Peter Voss[2], and Aalt Bast[2]

[1] Department of Otorhinolaryngology and Head and Neck Surgery, University Hospital Vrije Universiteit, Amsterdam, The Netherlands.

[2] Leiden/Amsterdam Center for Drug Research, Department of Pharmacochemistry, Division of Molecular Pharmacology, Vrije Universiteit, Amsterdam, The Netherlands.

Arch Otolaryngol Head Neck Surg 1997; 123:201-204

Abstract

Although chronic upper airway infections occur frequently in man, the pathogenesis is not clear. As imbalances between oxidant formation and antioxidative defense are associated with the pathogenesis of several chronic inflammatory disorders of the respiratory tract, a role of oxidative stress in chronic upper airway infections can be anticipated. It was hypothesized that in chronic sinusitis a reduced antioxidative tissue status may be present.

The levels of three biologically important antioxidants, i.e., glutathione (reduced (GSH) and oxidized (GSSG)), uric acid and vitamin E, were determined biochemically in mucosal biopsies from the uncinate process of patients with chronic sinusitis and healthy controls.

Inflamed mucosa was obtained from 9 patients with chronic sinusitis during functional endoscopic sinus surgery. Normal mucosa was collected from 10 healthy controls during surgery for nasal obstruction. The data show a significant reduction ($p \leq 0.05$) of GSH levels (0.3 $\pm$ 0.1 μmol/g wet weight) and uric acid levels (2.7 $\pm$ 0.4 μmol/ g wet weight) in mucosa obtained from patients with chronic sinusitis compared to healthy controls (0.6 $\pm$ 0.2 and 3.4 $\pm$ 0.6 μmol/ g wet weight respectively). No difference was found in GSSG (24 $\pm$ 8 vs 25 $\pm$ 15 nmol/ g wet weight) and vitamin E (20.5 $\pm$ 7.9 vs 22.5 $\pm$ 6.9 nmol/ g wet weight) levels between both groups. It was concluded that reduced levels of both GSH and uric acid in patients with chronic sinusitis lead to a diminished antioxidant defense, which may be associated with the pathogenesis. Vitamin E seems less important. This concept may offer perspectives for pharmacotherapeutic intervention with antioxidants.

Introduction

Chronic upper airway infections are inflammatory disorders of the (para-)nasal respiratory mucosa, clinically characterized by nasal obstruction, (purulent) nasal discharge, headache and anosmia. Although several factors such as modifications of nasal secretions, deficiency of mucociliary clearance, immunodeficiencies and anatomic abnormalities are thought to be involved, the exact pathogenesis is unclear.

Reactive oxygen species (ROS), like the superoxide anion radical ($O_2^{\cdot-}$) and the hydroxyl radical ($^{\cdot}OH$), which are produced by activated granulocytes during the respiratory burst, play an important role in mucosal defense against invading microorganisms.[1] However, relative overproduction of ROS, which occurs in inflammation, can also cause severe oxidative tissue damage.[2] To prevent this oxidative damage, the mucosa contains a broad spectrum of naturally occurring protective antioxidants. A disturbance in the delicate balance between oxidant production and local antioxidant defense, also known as oxidative stress, may result in pathological conditions. For chronic inflammatory disorders of the lungs, like asthma and chronic obstructive pulmonary disease, these imbalances between oxidant formation and antioxidative capacity have been implicated in the pathogenesis.[3,4] Moreover, the importance of sufficient mucosal antioxidant defense in pulmonary inflammation has been stressed repeatedly.[5,6] As inflammatory disorders of the upper- and lower respiratory tract are pathophysiologically related, i.e., the relationship between chronic sinusitis and bronchial asthma is well-known [7], a role of oxidative stress in the pathogenesis of chronic upper airway inflammations can be anticipated.

In the present study it was hypothesized that reduced antioxidant levels, which can lead to oxidative stress, may be present in chronically inflamed nasal mucosa. Therefore, we investigated the antioxidant defense systems, viz., glutathione, uric acid and vitamin E in the nasal mucosa of patients with chronic sinusitis and healthy controls. In addition, the activity of xanthine oxidase, the enzyme responsible for uric acid synthesis, was examined.

Materials and Methods

Collection of human nasal mucosa

Subjects

The subjects studied in these experiments were either patients suffering from chronic sinusitis or healthy volunteers. The study protocol was approved by the institutional medical ethical committee and informed consent was obtained from all subjects of which nasal mucosa was collected. Patients (n = 9, 6 males, 3 females, age ranging from 23 to 65 years, mean 47 years) all suffered from nasal obstruction, headache, and nasal

discharge for more than 3 months. All patients were diagnosed to have a chronic rhinosinusitis which was objectified by a computerized tomogram (CT) of the paranasal sinuses, which showed opacification of the paranasal sinuses including the anterior ethmoid. All patients were tested for allergy using a skin-prick test (a panel of graspollen, treepollen, animal dander and house dust mite was tested), a radio-allergosorbent (RAST) test and a total IgE test. None of the patients showed any signs of an atopic constitution. Moreover, no patient suffered from any other known disease. All patients were non-smokers and none of them had used any medication or dietary vitamin supplementation for at least 3 months prior to the investigation. All patients underwent functional endoscopic sinus surgery, and mucosal specimens of the osteomeatal area, i.e. the uncinate process, were obtained during surgery. On all specimens histopathologic examination was performed, showing chronic inflammation and eosinophilia in all cases. The controls (n = 10, 9 males, 1 female, age ranging from 26 to 51 years, mean 37 years) were healthy volunteers who were operated on for complaints of nasal obstruction caused by either a nasal septum deviation (n = 5) or hypertrophy of the inferior turbinate
(n = 5). Of the controls 6 were smokers and 4 were non-smokers. None of the controls had suffered from sinusitis or rhinitis during the last year and none of them used medication or dietary vitamin supplementation during the last 3 months before entering the study. Mucosal specimens from the controls were obtained from the osteomeatal unit, i.e. the uncinate process, during surgery.

Sample preparation

Immediately after surgery all mucosal specimens were homogenized (10 mg/ml) in ice cold phosphate buffered saline. Than the samples were split for the various antioxidant determinations, snap frozen, and stored in an acid environment at -80°C until further use. The enzymatic catabolism by the enzyme γ-glutamyltranspeptidase as well as the spontaneous degradation of glutathione were prevented by storing these samples in an acid environment.[8]

Antioxidant assays

Glutathione assay

Reduced (GSH) and oxidized (GSSG) glutathione levels were measured with minor modifications according to the microtiter plate assay described by Baker and co-workers.[9] The method is based on the enzymatic recycling reaction of GSH and GSSG combined with a chromogenic reaction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to the thiolate anion (TNB). In short, 191.4 µl sample homogenate was mixed with 8.6 µl perchloric acid (70 %; DBH Laboratories Supplies, Poole, UK). For the determination of GSSG the thioalkylating agent N-ethylmaleimide (NEM) (stock solution: 110 mM; Sigma Chemical Co., St. Louis, Missouri, USA) was added to the sample. For the determination of total glutathione 20 µl water was added. To adjust the pH for alkaline hydrolysis all samples were treated with 66 µl potassium phosphate (3 M, pH = 13; Merck, Darmstadt, Germany). After 15 mins all samples were neutralized with 99 µl of 10 % (v/v) perchloric acid. After centrifugation (10 mins, 11.000 x g at 4° C) 50 µl supernatant of each sample was mixed with 100 µl reagent containing 0.2 mM NADPH (Boehringer Mannheim, Germany), 0.15 mM DTNB (Jansen Chimica, Geel, Belgium) and 1 unit/ml GSSG reductase (Sigma) in a microtiter plate. Glutathione measurements were done by using an Argus 400 microplate reader (Canberra Packard, Tilburg, The Netherlands) The settings were, single

wavelength kinetics with a 405 nm filter and a read out after 10 mins. The formation rate of TNB per minute indicates either the total glutathione or the GSSG concentration of the sample. Standard curve data, for GSH (Sigma) and GSSG (Sigma), were obtained in the same fashion as the sample data. Levels of GSH and GSSG were expressed as µmol per gram wet weight and nmol per wet weight respectively. To determine the GSH concentration the numerical difference between the total glutathione concentration and the GSSG concentration was calculated.

Uric acid assay

This spectrophotometric method is based on the ability of the enzyme uricase to irreversibly metabolize uric acid to allantoin.[10] The homogenates were centrifuged at 11.000 x g (10 min at 4°). Subsequently 50 µl of the supernatant was placed into 450 µl of 0.7 M glycine buffer (pH = 9.4; Sigma) after which the absorption of uric acid at 292 nm was determined using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, UK). After adding 10 µl of uricase (stock solution: 1 unit/ml; Sigma) and allowing the reaction to proceed for 10 mins at 25° C the absorption was again determined at 292 nm. A uricase blank was subtracted from determinations containing this enzyme and all values were corrected for non-uric acid absorption. The difference in absorbance before and after addition of the excess of uricase was measured which indicated the uric acid concentration. The standard curve data for uric acid (Janssen) were obtained in the same fashion as the sample data. Uric acid levels were expressed as µmol per gram wet weight.

Vitamin E assay

This method is based on the extraction of vitamin E from the cell membrane into the apolar solvent heptane [11], and determined by means of a high performance liquid chromatograph (HPLC) (Gilson 305 pump, Gilson 232 sample injector, Gilson dilutor 401; Gilson, Villiers, France) with fluorometric detection (excitation 295 nm, emission 340 nm; Perkin Elmer 3000 Fluorescence Spectrometer, The Netherlands). In short, 200 µl sample homogenate was mixed (1 min) with 2 ml of phosphate buffer (50mM, pH = 7.4) containing 0.1 mM EDTA and 1 ml solubilization solvent containing 100 mM sodium dodecyl sulphate (USB, Cleveland, Ohio, USA), 5mM EDTA and 5mM ascorbic acid (J.T. Baker). Then 3 ml of ethanol (J.T. Baker) and 4ml of n-heptane (HPLC-grade; J.T. Baker) were added after which the samples were thoroughly mixed for 20 mins (Griffin Flash Shaker, London, UK). Centrifugation (5mins,1500 x g at 4° C) was carried out to obtain phase separation. Three milliliters of the heptane (supernatant) were transferred into an incubation tube after which the heptane was evaporated at 60° C under a continuous nitrogen flow. Finally the residue was dissolved in 200 µl methanol (HPLC-grade; J.T. Baker) of which 30 µl was injected onto a reversed phase C18 nucleosil column (Chrompac, Middelburg, The Netherlands) and eluted with 98 % methanol at a flow rate 0f 0.6 ml/min. Levels of vitamin E were calculated from the height of the vitamin E peak in the chromatogram and expressed as nmol per gram wet weight. Data for a standard curve of vitamin E (Merck) were obtained in the same fashion as the sample data.

Xanthine oxidase assay

This spectrophotometric method is based on the reduction of cytochrome C by superoxide anion radicals which are formed during the conversion of xanthine to uric acid catalyzed by xanthine oxidase.[12] All sample homogenates were centrifuged at 11.000 x g (10 mins at 4° C). Before each measurement 2.5 ml substrate

containing 60 µM xanthine (Sigma) and 11.8 µM Fe^{3+} -cytochrome C (from horse heart, Sigma) was incubated for 10 mins at 25° C. The reaction was started by adding either 500 µl sample supernatant or 500 µl xanthine oxidase (stock solution: 0.0214 units/ml; Sigma, to obtain the standard curve). The formation rate per minute of reduced Fe^{2+} cytochrome C, which can be measured spectrophotometrically at 550 nm (PU8720 UV/VIS scanning Spectrophotometer, Philips, Eindhoven, The Netherlands), indicates the activity of the enzyme xanthine oxidase.

Statistics

For each determination all tissue samples were measured in duplicate, the standards were measured in triplicate. Intergroup comparisons were made by using an unpaired Student's t test . The criterion for statistical significance was set at $p \leq 0.05$.

Results

Determination of reduced and oxidized glutathione

The mean GSH value ± S.D. in the patient group was 0.3 ± 0.1 µmol/g wet weight.This was significantly lower ($p \leq 0.05$) than the mean GSH value ± S.D. in the control group which was 0.6 ± 0.2 µmol/g wet weight (Figure 1). The mean GSSG value ± S.D., 24 ± 8 nmol/g wet weight or the patient group and 25 ± 15 nmol/g wet weight for the control group, did not differ significantly between the two groups.

Determination of uric acid

The allowed reaction time (10 mins) for uricase to irreversibly convert uric acid to allantoin gave a total disappearance of the absorption peak at 292 nm for all samples and standard curve concentrations. This proved that in this time-period all uric acid was converted and the absorbance at 292 nm reflects the uric acid concentration.

The mean value ± S.D. of uric acid was 2.7 ± 0.4 µmol/g wet weight for the patient group and differed significantly ($p \leq 0.05$) from this value for the control group, which was 3.4 ± 0.6 µmol/g wet weight. (Figure 2).

Determination of vitamin E

The mean value ± S.D. of vitamin E in the samples of patients with chronic upper airway infections (20.5 ± 7.9 nmol/ g wet weight) and in the samples of the healthy controls (22.5 ± 6.9 nmol/g wet weight) did not differ significantly.

Determination of xanthine oxidase (XO)

None of the samples demonstrated any measurable xanthine oxidase activity (lower limit of detection, 4.2×10^{-2} U XO/g wet weight).

For all determinations (GSH,GSSG, uric acid, vitamin E) no significant difference was found between smokers and none-smokers in the control group. Therefore, despite the small sample size of smokers and non-smokers in the control group, smoking did not seem to confound the results of any of the determinations.

Discussion

Oxidative stress is an important causal factor in a number of diseases. For example, in atherosclerosis, carcinogenesis, rheumatoid arthritis and several pulmonary disorders oxidants have a significant contribution in the pathogenesis.[13] From asthmatic patients, for instance, it was shown that alveolar macrophages and hypodense eosinophils produce increased amounts of superoxide anion radicals.[14,15] The role of oxidants in the pathogenesis of asthma is interesting because the pathophysiology of asthma and of chronic upper airway infections are related.[7] Important in the relationship between chronic inflammatory diseases of the upper airways and asthma is the eosinophil which acts as an effector cell in these disorders.[16] It is known that the production of potentially toxic secretory products of eosinophils, like major basic protein and eosinophil cationic protein, is associated with chronic inflammatory diseases of the (para-) nasal respiratory mucosa.[17-19] Moreover, oxidant mediated ciliary dysfunction of human nasal ciliated epithelium and toxic effects of human eosinophil peroxidase to cultured nasal epithelial cells have been described.[19,20] In order to deal with the deleterious effects of an increased oxidant generation, the mucosal antioxidant defense has to be intact. Pulmonary tissue and pulmonary epithelial lining fluid have been shown to contain a broad spectrum of antioxidant activity.[3]

In nasal secretions glutathione, ascorbic acid and uric acid are known to occur and especially uric acid is thought to be a major antioxidant in human nasal airway secretions.[10,21,22] Except for uric acid, which has been demonstrated to occur in nasal mucosa [23], considerably less is known about the antioxidative state of nasal mucosa, in particular during inflammatory conditions.

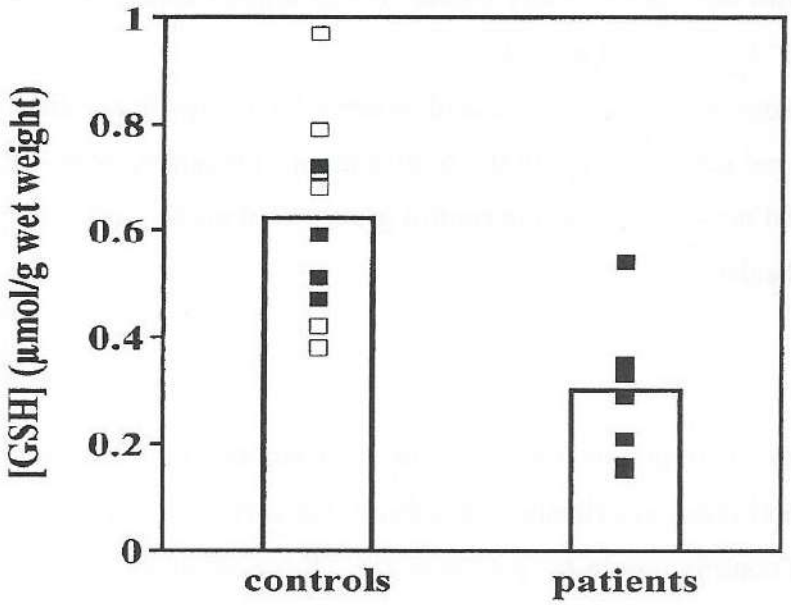


Figure 1. The levels of GSH in nasal mucosa of healthy controls (n = 10) and patients with chronic sinusitis (n = 9) expressed as µmol/g wet weight. The individual data points (smokers (□), non-smokers (■)) are shown. The mean GSH value (bar) in the patient group is significantly lower ($p \leq .05$) as compared to the mean value (bar) found for the control group.

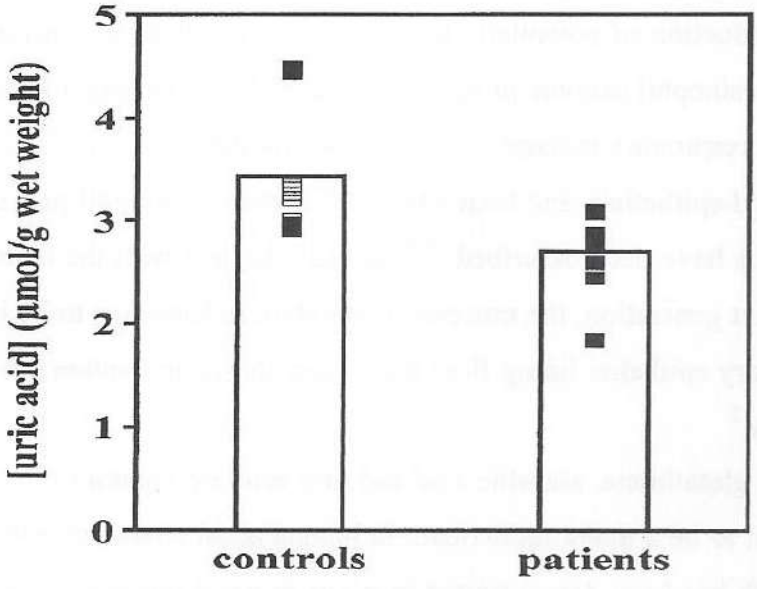


Figure 2. The levels of uric acid in nasal mucosa of healthy controls (n = 10) and patients with chronic sinusitis (n = 9) expressed as µmol/g wet weight. The individual data points (smokers (□), non-smokers (■)) are shown. In the patient group the mean uric acid level (bar) is significantly lower ($p \leq .05$) as compared to the mean value (bar) found for the control group.

In the present study we assessed the concentrations of the antioxidants: glutathione, uric acid and vitamin E, in nasal mucosa of patients with chronic sinusitis and compared the results with the results found in nasal mucosa of healthy controls. It was expected to find a reduced antioxidative defense in the inflamed mucosa of patients with chronic sinusitis.

GSH was indeed significantly lower ($p \leq 0.05$) in the nasal mucosa of patients with chronic sinusitis compared to controls. This might be explained by the fact that during inflammation an increased oxidant production leads to consumption of GSH through direct scavenging of ROS. Moreover, conversion of GSH to GSSG in the GSH redox cycle as a result of the GSH-peroxidase activity occurs. The expected increase in GSSG, however, could not be shown, a feature which might be due to the cellular efflux of GSSG to the circulation and the reduction of GSSG by the enzyme GSSG reductase.[24]

Uric acid was also found to be significantly lower ($p \leq 0.05$) in the patient group compared to the control group. The mean uric acid concentration we found was 3.4 µmol/g wet weight for the healthy control group, which is approximately 7 times higher than found by Peden and co-workers, which is a difference we cannot explain.[23] Uric acid is a very powerful radical scavenger. In addition, uric acid prevents the oxidation of vitamin C and binds transition metals in forms that will not stimulate free radical reaction.[25] This 'sacrificial' behavior, which means that uric acid is irreversibly degraded, may explain the fact that uric acid is lowered under inflammatory conditions by increased oxidant generation. Additionally, we investigated the levels of the uric acid producing enzyme xanthine oxidase. However, the nasal tissue did not show any presence of this enzyme, which is in accordance with the findings of Peden and co-workers.[23] This suggests that uric acid is not locally formed but taken up by the nasal glands from the peripheral blood.

Vitamin E levels did not differ significantly between the patient group and the control one. Although vitamin E represents the principal defense against oxidant induced lipid peroxidation in the lipophilic compartment of cellular membranes, the consumption of vitamin E begins when the other antioxidants have been consumed. In addition, vitamin C and GSH seem to be able to regenerate vitamin E from its radical.[26] This may explain that our results do not show a relationship between nasal inflammation, and vitamin E tissue level.

In conclusion, the reduced levels of GSH and uric acid in the nasal mucosa of patients with chronic sinusitis, compared to healthy controls, reflect an impaired antioxidant defense which may be associated with the pathogenesis. This concept may offer perspectives for antioxidative pharmacotherapeutic intervention.

References

1. Southern PA, Powis G. Free radicals in medicine. I. Chemical nature and biologic reactions. *Mayo Clin Proc* 1988; 63:381-389.
2. Henson PM, Johnson RB. Tissue injury in inflammation. Oxidants, proteinases and cationic proteins. *J Clin Invest* 1987; 79:669-674.
3. Doelman CJA, Bast A. Oxygen radicals in lung pathology. *Free Radic Biol Med* 1990; 9:381-400.
4. Riley DJ, Kerr JS. Oxidant injury of the extracellular matrix: Potential role in the pathogenesis of pulmonary emphysema. *Lung* 1985; 163:1-13.
5. Heffner JE, Repine JE. Pulmonary strategies of antioxidant defense. *Am Rev Respir Dis* 1989; 140:531-554.
6. White CW, Repine JE. Pulmonary antioxidant defense mechanisms. *Exp Lung Res* 1985; 8:81-96.
7. Slavin RG. Relationship of nasal disease and sinusitis to bronchial asthma. *Ann Allergy* 1982; 49:76-80.
8. Roberts JC, Francetic DJ. The importance of sample preparation and storage in glutathione analysis. *Anal Biochem* 1992; 211:183-187.
9. Baker MA, Cerniglia CJ, Zaman A. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Anal Biochem* 1990; 360:360-365.
10. Peden DB, Hohman R, Brown ME, Mason RT, Berkebile C, Fales HM, Kaliner MA. Uric acid is a major antioxidant in human nasal airway secretions. *Proc Natl Acad Sci* 1990; 87:7638-7642.
11. Burton GW, Webb A, Ingold KU. A mild, rapid and efficient method of lipid extraction for use in determing vitamin E/lipid ratios. *Lipids* 1986; 20:29-33.
12. McCord JM, Fridovich I. The reduction of cytochrome C by milk xanthine oxidase. *J Biol Chem* 1968; 10:5753-5760.
13. Southern PA, Powis G. Free radicals in medicine. II. Involvement in human disease. *Mayo Clin Proc* 1988; 63:90-408.
14. Calhoun WJ, Salisbury SM, Bush RK, Busse WW. Increased superoxide release from alveolar macrophages in symptomatic asthma. *Am Rev Respir Dis* 1987; 135:A224.
15. Sedgwick JB, Geiger KM, Busse WW. Superoxide generation by hypodense eosinophils from patients with asthma. *Am Rev Respir Dis* 1990; 142:120-125.
16. Slavin GS. Asthma and sinusitis. *J Allergy Clin Immunol* 1992; 90:534-537.
17. Harlin SL, Ansel DG, Lane SR, Meyers J, Kephart GM, Gleich GJ. A clinical and pathologic study of chronic sinusitis: The role of the eosinophil. *J Allergy Clin Immunol* 1988; 81:867-875.
18. Hisamatsu K, Ganbo T, Nakazawa T, Murakami Y, Gleich GJ, Makiyama K, Koyama H. Cytotoxicity of human eosinophil granule major basic protein to human nasal sinus mucosa in vitro. *J Allergy Clin Immunol* 1990; 86:52-63.
19. Ayars GH, Altman LC, McManus MM, Agosti JM, Baker C, Luchtel DL, Loegering DA, Gleich GJ. Injurous effect of the eosinophil peroxide-hydrogen peroxide-halide system and major basic protein on human nasal epithelium in vitro. *Am Rev Resp Dis* 1989; 140:125-131.

20. Feldman C, Anderson R, Kanthakumar K, Vargas A, Cole PJ, Wilson R. Oxidant-mediated ciliary dysfunction in human respiratory epithelium. *Free Rad Biol Med* 1994; 17:1-10.

21. Peden DB, Brown ME, Wade Y, Raphael GD, Berkebile C, Kaliner MA. Human nasal glandular secretion of novel antioxidant activity: cholinergic control. *Am Rev Resp Dis* 1991; 143:545-552.

22. Cross CE, Van der Vliet A, O'Neill CA, Louie S, Halliwell B. Oxidants, antioxidants, and respiratory tract lining fluids. *Environ Health Perspect* 1994; 102(Suppl 10):185- 191.

23. Peden DB, Swiersz M, Ohkubo K, Hahn B, Emery B, Kaliner MA. Nasal secretion of the ozone scavenger uric acid. *Am Rev Resp Dis* 1993; 148:455-461.

24. Bernard GR. N-acetylcysteine in experimental and clinical acute lung injury. *Am J Med* 1991; 91(suppl 3C):54S-9S.

25. Davies KJA, Sevenian A, Muakkassah-Kelly SF, Hochstein P. Uric acid-iron ion complexes: a new aspect of the antioxidant functions of uric acid. *Biochem J* 1986; 235:747-754.

26. Van Acker SABE, Koymans LMH, Bast A. Molecular pharmacology of vitamin E: structural aspects of antioxidant activity. *Free Rad Biol Med* 1993; 20:311-328.

Chapter II-2

Blood antioxidant levels in patients with chronic upper respiratory tract inflammation and healthy controls

Gerrit-Jan Westerveld[1], Robert A Scheeren[1], Marc Fischer[2], Guido RMM Haenen[2], and Aalt Bast[2]

[1] Department of Otorhinolaryngology and Head and Neck Surgery, Medical Center Vrije Universiteit, Amsterdam, The Netherlands.

[2] Department of Pharmacology and Toxicology, University of Maastricht, The Netherlands.

Submitted

Abstract

The exact pathogenesis of chronic upper respiratory tract inflammation is, although multifactorial, still unclear. Oxidative stress, which represents an imbalance between oxidant generation and antioxidant defense, has been associated with the pathogenesis of chronic inflammatory disorders such as chronic sinusitis and nasal polyps. Alterations in the blood concentrations of the biologically important antioxidants, glutathione, vitamin E and uric acid, were determined in erythrocytes and blood plasma of patients with chronic sinusitis, patients with nasal polyps and healthy controls. In addition in the same subject groups the blood levels of the total antioxidant capacity were investigated and expressed as TEAC. The data show (presented as mean ± SD) a significant reduction ($p \leq .05$) in total glutathione level in blood samples obtained from patients with chronic sinusitis (1977 ± 480 μM) and patients with nasal polyps (2088 ± 417 μM) compared to the control group (2335 ± 343 μM). Also a significant reduction ($p \leq .05$) in vitamin E level was found in patients with chronic sinusitis (16 ± 12 g/mmol triglyceride) when compared to healthy controls (24 ± 14 g/mmol triglyceride). This could not been shown for patients with nasal polyps (19 ± 10 g/mmol triglyceride). Uric acid levels were found to be significantly higher ($p \leq .05$) in blood samples of patients with either chronic sinusitis (291 ± 71 μM) or nasal polyps (282 ± 72 μM) compared to the values of healthy controls (214 ± 58 μM). Total TEAC levels did not differ between the blood samples of patients chronic sinusitis (411 ± 106 μM), patients with nasal polyps (384 ± 80 μM) and healthy controls (429 ± 81 μM). However, the residual antioxidant capacity, obtained after subtraction of the contribution of uric acid from the total TEAC, showed a significant reduced ($p \leq .05$) level in patients with chronic sinusitis (128 ± 87 μM) and patients with nasal polyps (102 ± 74 μM) compared to the healthy controls (216 ± 56 μM). In conclusion, the altered levels of glutathione, vitamin E, and residual TEAC may reflect an impaired antioxidant defense in patients with a chronic inflammatory disorder of the upper respiratory tract which may be implicated in the complex multifactorial pathogenesis. Increased uric acid levels might be due to oxidative stress induced xanthine oxidase activity. These findings offer perspectives for pharmacotherapeutic intervention with antioxidants.

Introduction

Chronic upper respiratory tract infections, such as chronic sinusitis and nasal polyps, are frequently occurring disorders which are clinically characterized by nasal congestion, (purulent) nasal discharge, headache, and hyposmia. In the Netherlands, in 1998, 8.9 % of the population, approximately 1.4 million patients, suffered from chronic upper airway inflammation.[1] In 1990 the prevalence was 6.4 %, indicating that the prevalence is rising.[2] Although several factors, alone or in combination, such as, anatomic variations, disturbances in mucociliary clearance, modifications in nasal secretions, and immunodeficiencies, are thought to be involved, the exact pathogenesis of chronic upper respiratory tract infections has not been elucidated.[3,4]

During mucosal inflammation, activated granulocytes generate reactive oxygen species (ROS) such as superoxide anion radicals and hydroxyl radicals via the respiratory burst, which defend the mucosa against invading microorganisms.[5] In contrast, ROS have been shown to cause tissue injury by damaging, cellular proteins, membrane poly-unsaturated fatty acids, nucleic acids in DNA, and cytosolar carbohydrates, leading to cellular dysfunction and eventually cell death.[6,7] To prevent this oxidative cell damage to occur, the respiratory mucosa contains a broad spectrum of antioxidant defense mechanisms.[8] Under physiologic conditions the ROS production is balanced by the antioxidant defense. Disturbance of this delicate balance, also known as oxidative stress, may result in pathologic conditions. For chronic inflammatory diseases in the lungs, such as asthma or chronic obstructive pulmonary disease, a role of oxidative stress in their pathogenesis has been established.[9,10,11] Inflammatory disorders of the upper respiratory tract and the lungs are pathophysiologically related. For instance, the relationship between chronic sinusitis and bronchial asthma is well known.[12] Therefore a role of oxidative stress in the pathophysiology of chronic upper airway inflammation may be anticipated.

Indeed, in patients with chronic sinusitis increased levels of myeloperoxidase (MPO) a neutrophil-derived ROS producing enzyme, are found in nasal lavage fluid (NAL) compared to healthy controls.[13] This indicates an increased ROS production. Moreover, in patients with chronic sinusitis significant diminished mucosal levels of reduced glutathione (GSH) and uric acid were found indicating impaired mucosal defense against ROS attack.[14] Furthermore, significantly higher levels of malondialdehyde (MDA), a marker of ROS induced lipid peroxidation, has been observed in human nasal polyp tissue.[15] Under these circumstances in which ROS threat to overwhelm the antioxidant capacity of upper respiratory tract mucosa it can be expected that the mucosal cells recruit additional antioxidant resources from the

circulating blood. In fact, uric acid is been thought to be taken up by nasal glands from plasma, as xanthine oxidase, the enzyme responsible for uric acid synthesis, cannot be detected in the nasal mucosa itself.[16] In the present study it was hypothesized that alterations of blood antioxidants may occur due to consumption of these antioxidants by the chronically inflammed mucosa of the upper respiratory tract. By this means these antioxidants can be used as markers of oxidative stress. Therefore, we investigated the antioxidants levels of total glutathione, uric acid, and vitamin E, in the blood of patients with chronic sinusitis and patients with nasal polyps and compared them to the levels of healthy controls. In addition the total plasma antioxidant capacity, expressed as TEAC, in these patient groups was determined.

Subjects, materials, and methods

Subjects

The subjects studied in these experiments were either patients with chronic sinusitis, patients with nasal polyps or healthy controls. The study protocol was approved by the institutional medical ethical committee of the Vrije Universiteit medical center Amsterdam, The Netherlands, and informed consent was obtained from all participating subjects.

The controls (n = 18, 8 males and 10 females; age range 18-42 years, mean age, 28.8 years) were healthy volunteers who underwent corrective surgical procedures of the upper and lower jaw performed by the maxillofacial surgeons. None of the controls had suffered from upper airway inflammation during the past year and none of them was known with an atopic constitution. On rhinologic examination in none of the controls abnormalities were seen. All of the controls were non-smokers. Patients with chronic sinusitis (n = 42, 25 males and 21 females; age range 22-59 years, mean age, 39.5 years) and patients with nasal polyps (n = 46, 30 males and 16 females; age range 25-69 years, mean age, 45 years) all had complaints of nasal obstruction, headache, and recurrent nasal discharge for more than 3 months. In all patients the diagnosis was confirmed on nasal endoscopy and by a computerized tomogram (CT) of the paranasal sinuses, which showed opacification of the paranasal sinuses including the anterior ethmoid. All patients were tested for allergies using a radioallergosorbent test and a total IgE test. Patients were considered positive when the total blood IgE level was higher than 100 U/ml and a positive reaction was seen in one or more allergens from the radioallergosorbent test (a panel of grass pollen, tree pollen, animal dander, and house dust mite was used). Of the patients with chronic sinusitis 12 patients showed positive reactions and 30 were negative. Of the patients with nasal polyps 24 patients showed positive reactions and 22 were negative. Ten of the patients with chronic sinusitis smoked and 32 were non-smokers. Of the patients with nasal polyps 7 smoked and 39 did not. None of the subjects, controls and patients, used medication or any dietary vitamin or other antioxidant supplements during the 3 months prior to the study. None of the subjects suffered from any other known disease.

Sample preparation

Venous blood was drawn from the cephalic vein using a venoject system®. Samples for vitamin E and triglyceride determinations were collected in non coated reagent tubes. Uric acid samples were collected in heparin gel tubes and gluthatione samples in heparin coated tubes. All tubes were centrifuged at 1500 rpm for 10 min. Plasma for vitamin E, triglyceride, and uric acid determination was snap frozen and stored at –80°C until further use. In the gluthatione samples erythrocytes were separated from the plasma. The plasma was snap frozen at –80°C and used for total antioxidant capacity assay. The erythrocytes were lyzed by Symex™ quicklyzer (Toa Medical Electronics Co., LTD, Kobe, Japan) after which the lyzate was centrifuged at 3000 rpm for 10 min. The supernatant was snap frozen and stored in an acid environment at –80°C until further use.

Antioxidant assays

Chemicals

NADPH, glutathione reductase, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), RRR-α-tocopherol (vitamin E), (±)-α-tocopherol nicotinate (vitamin E nicotinate) and uric acid were obtained from Sigma Chemical Co., St. Louis, USA. 5'5'dithiobis 2-nitrobenzoic acid (DTNB) and reduced glutathione (GSH) were obtained from ICN Biomedicals Inc. Costa Mesa, USA. 2,2'-azino-bis(2-amidinopropane) dihydrochloride (ABAP) was obtained from Brunschwig Chemicals, Amsterdam, The Netherlands. All other chemicals were from analytical purity.

Glutathione assay

Total glutathione was determined using the recycling method.[17] To 50 µl of a lysed erythrocyte sample (stored in presence of sulfosalicylic acid in a final concentration of 1.3 %) 100 µl of a 0.4 mM NADPH/ 0.3 mM DTNB solution in 143 mM phosphate buffer pH 7.4, containing 6.3 mM EDTA, is added in a 96- well plate. Then the reaction is started by adding 50 µl of a solution containing 4 U/ml glutathione reductase. The increase of the absorption (ΔA/min) is followed for 2 minutes at 405 nm. The ΔA/min of the samples is compared to that of the calibrators and the concentration of total glutathione is calculated.

Uric acid assay

Uric acid was determined in plasma ultra filtrates using a HPLC method described by Lux *et al.*[18] with minor modifications. Two hundred µl plasma was filtered over a 3 kD filter unit (Centrex UF 0.5, Schleicher & Schuell, Dassel, Germany) by centrifugation for 45 minutes at 14.000 x g and 4 °C. 25 µl of the filtrates was analyzed on the HPLC. The system (Agilent, Palo Alto, CA, USA) consisted of an Hypersil BDS C-18 end-capped column, 125 x 4 mm, particle size 5 µm (Agilent, Palo Alto, CA, USA) with a mobile phase of 5 mM sodium phosphate buffer pH = 3.3. Detection was performed using a variable wavelength detector set on a wavelength of 292 nm. Plasma concentrations uric acid were calculated using a calibration curve of uric acid.

Vitamin E assay

Vitamin E concentrations in plasma were determined using a HPLC method. Vitamin E was extracted from the plasma by adding to 100 µl plasma, 20 µl internal standard (vitamin E nicotinate, 200 µg/ml), 1 ml water and 2

ml ethanol. After shaking for 5 minutes, 3 ml hexane was added and the mixture was shaken again (10 min.). The hexane layer was evaporated and the residue was dissolved in 100 µl isopropylalcohol. The analysis was performed using a HPLC system equipped with a variable wavelength detector, set on 295 nm, and a Nucleosil C18 column, 125 x 4 mm, particle size 5 µm (Agilent, Palo Alto, CA, USA). Samples were eluted with 99 % methanol. Plasma concentrations were calculated using a calibration curve of vitamin E. Vitamin E levels were expressed per mmol triglyceride. The triglyceride content was determined using a commercially available kit (Sigma Diagnostics, Sigma Chemical Co., St Louis, USA). Triglycerides are first hydrolyzed by lipoprotein lipase to glycerol and free fatty acids. Glycerol is than phosphorylated by adenosine triphosphate forming glycerol-1- phosphate and adenosine-5-diphosphate in the reaction catalyzed by glycerol kinase. Glycerol-1-phosphate is than oxidized by glycerol phosphate oxidase to dihydroacetone phosphate and hydrogen peroxide. The amount of hydrogen peroxide formed is quantified by the formation of a quinoneimine dye from 4-aminoantipyrine and sodium N-ethyl-N-(3-sulfopropyl)-m-aniside, catalyzed by a peroxidase. The increase in absorbance at 540 nm due to the formation of the quinoneimine dye is directly proportional to the triglyceride concentration in the sample. Glycerol is used as standard.

Total antioxidant capacity assay

The total antioxidant capacity assay was carried out as described by van den Berg *et al.* with some modifications.[19] The $ABTS^{\bullet+}$ radical was produced by incubating a solution of 0.23 mM ABTS and 2.3 mM ABAP in 100 mM sodium phosphate buffer pH 7.4 at 70°C until the absorption of the solution at 734 nm was between 0.680 and 0.720. Plasma samples were deproteinated by adding to 100 µl plasma 100 µl of a solution of 10 % (w/v) trichloro acetic acid. After centrifugation (14.000 x g 5 minutes) the supernatant was used for the analysis. 950 of the $ABTS^{\bullet+}$ radical solution was preheated for 1 minute at 37°C. Then 50 µ of the sample was added and after incubating for 5 minutes the absorption (A) at 734 nm was measured. The decrease in absorption after 5 minutes of the samples, subtracted from that of the blank (buffer), was related to the decrease in absorption of trolox standards. The TEAC value gives the concentration of trolox needed to get a similar decrease in absorption as found for the sample.

Statistics

For each determination each sample was measured in duplicate and the standards were measured in triplicate. Intergroup comparisons were made using a two tailed Student *t* test. The criterion for statistical significance was set at $p \leq .05$.

Results

Blood total glutathione levels.

The mean (± SD) total glutathione value in the sinusitis group was 1977 ± 480 μM. The mean (± SD) total glutathione value in the nasal polyps group was 2088 ± 417 μM. Both levels were significantly lower ($p \leq .05$) than the mean (± SD) total glutathione value in the control group, which was 2335 ± 343 μM (Figure 1).

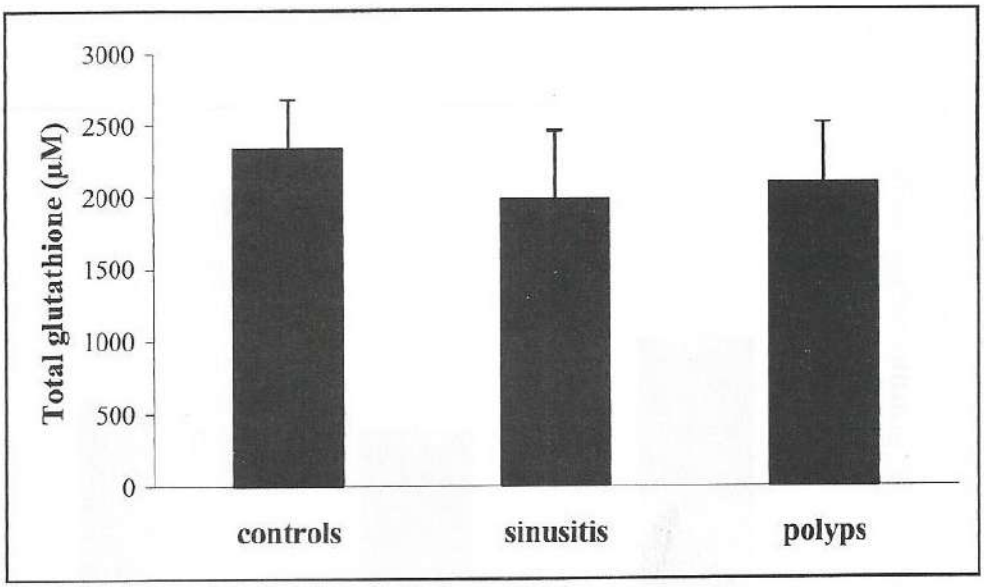


Figure 1. The levels of total glutathione in blood erythrocytes of healthy controls (n = 18), patients with chronic sinusitis (n = 42) and patients with nasal polyps (n = 46). The mean glutathione values of the patient groups were significantly lower ($p \leq .05$) than the mean value found for the control group.

Blood plasma vitamin E levels

The mean (± SD) vitamin E level in the sinusitis group, 16 ± 12 g/mmol triglyceride, was significantly lower ($p \leq .05$) than the vitamin E level in the control group, 24 ± 14 g/mmol triglyceride. The vitamin E level in the nasal polyps group, 19 ± 10 g/mmol triglyceride, was not significantly different from that of the control group (Figure 2).

Blood plasma uric acid levels.

The mean (± SD) uric acid level in the sinusitis group was 291 ± 71 μM. The mean (± SD) uric acid level in the nasal polyp group was 282 ± 72 μM. Both levels were significantly

higher ($p \leq .05$) than the mean (± SD) uric acid level in the control group, which was 214 ± 58 μM (Figure 3).

Blood plasma TEAC levels

The mean (± SD) TEAC levels in the sinusitis group, 411 ± 106 μM, and in the nasal polyp group, 384 ± 80 μM were not significantly different from the TEAC levels in the control group, 429 ± 81 μM.

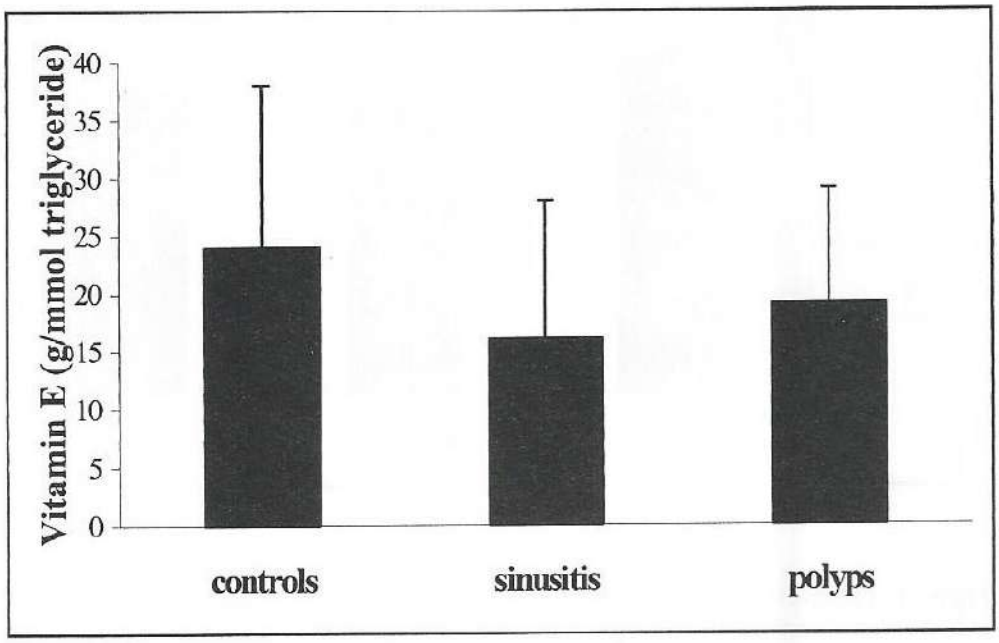


Figure 2. The levels of vitamin E in blood plasma of healthy controls (n = 18), patients with chronic sinusitis (n = 42) and patients with nasal polyps (n = 46). The mean vitamin E value of the chronic sinusitis group was significantly lower ($p \leq .05$) than the mean value found for the control group. For the nasal polyps group no significant difference could be found.

Non urate antioxidant capacity

Although the total TEAC levels between the groups does not differ significantly, the contribution of uric acid to the TEAC value rises in the sinusitis-, and nasal polyps groups (Figure 3). Furthermore, there is a simultaneous, significant decrease ($p \leq .05$) in residual plasma antioxidant capacity (mean ± SD) in the sinusitis (128 ± 87), and the nasal polyps group (102 ± 74) compared to the control group (216 ± 56) (Figure 4).

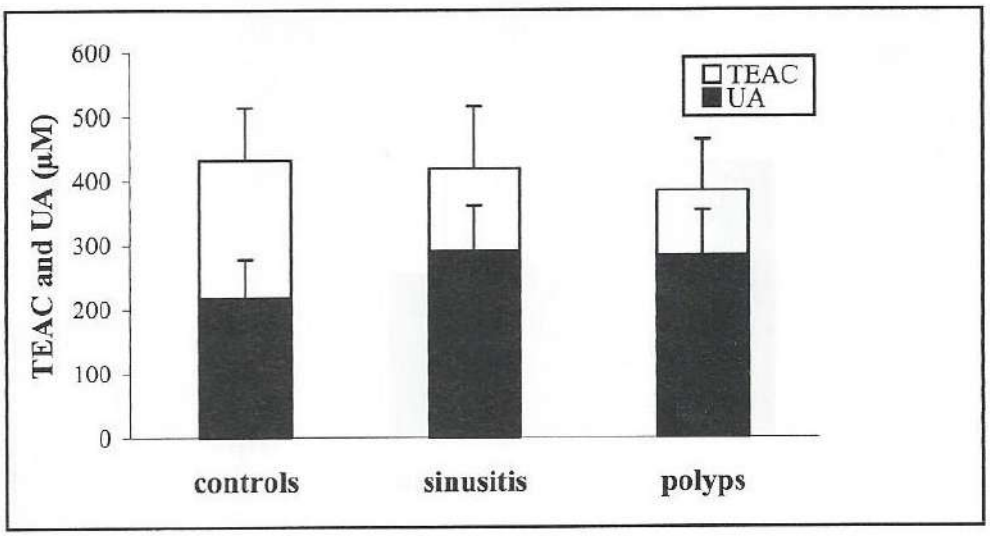


Figure 3. The levels of total TEAC and uric acid (UA) in blood plasma of healthy controls (n = 18), patients with chronic sinusitis (n = 42) and patients with nasal polyps (n = 46). The mean total TEAC levels of the patient did not differ from the mean value found for the control group. However, mean uric acid levels were significantly higher ($p \leq .05$) in the chronic sinusitis and nasal polyps group compared to the control group.

The influence of smoking and atopic constitution on glutathione, uric acid, vitamin E and TEAC levels in patients with chronic sinusitis and nasal polyps.

For all determinations (total glutathione, vitamin E, uric acid, and TEAC) no significant difference was found between smokers and nonsmokers and atopics and nonatopics within the sinusitis-, and nasal polyps groups. Therefore, neither smoking nor atopic constitution did seem to confound the results of any of the determinations.

Discussion

Oxidative stress is an important causal factor in a number of diseases. For example, in atherosclerosis, carcinogenesis, rheumatoid arthritis and several pulmonary disorders, oxidants significantly contribute to the pathogenesis.[5] Especially in patients with chronic inflammatory pulmonary diseases, the pathogenetic role of oxidants is interesting because the upper and lower respiratory tract form a pathophysiologic continuum.[12] Upper airway mucosal surfaces are subjected to various sources of oxidant stress.

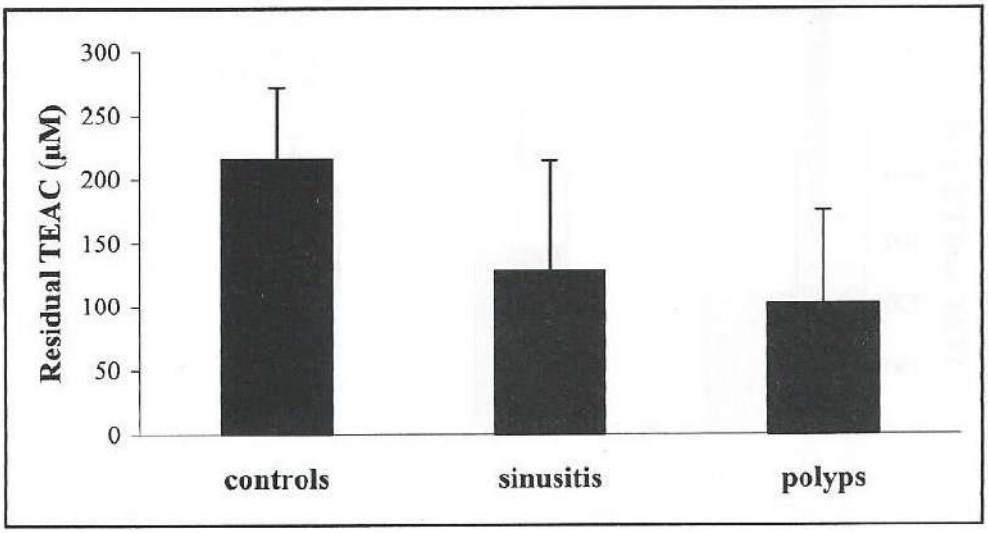


Figure 4. The levels of residual TEAC in blood plasma of healthy controls (n = 18), patients with chronic sinusitis (n = 42) and patients with nasal polyps (n = 46). The residual TEAC value is the TEAC of plasma that does not originate from uric acid. The mean residual TEAC values of both patient groups were significantly lower ($p \leq .05$) than the mean value found for the control group.

Beside external sources, such as air pollution, tobacco smoke and even pure oxygen, endogenously produced oxidants, mainly generated by inflammatory cells, may damage the epithelium. To defend the mucosa against oxidative damage, cells and extracellular fluids contain a variety of antioxidants, some of which are non-enzymatic free radical scavengers, others that prevent radical formation, and others that enzymatically detoxify ROS.[20] In the upper respiratory tract, antioxidants have been shown to occur in the mucosa and in epithelial lining fluid.[14,21] Although the exact role of antioxidants in upper respiratory tract inflammation is not clear, it has been shown that antioxidant concentrations in either epithelial lining fluid and mucosa may change under inflammatory conditions indicating a pathophysiological role.[14,22,23,24]

As the determination of antioxidant levels in upper respiratory tract mucosa and epithelial lining fluid is often technically difficult, antioxidant determinations in plasma are more convenient and may accordingly be used as markers of upper airway inflammation. In healthy volunteers blood levels of urate, glutathione, and vitamin E have been determined.[21] Moreover plasma TEAC levels have also been established.[25,26] However, to our best knowledge these levels have not been determined under inflammatory conditions of the upper respiratory tract. In this report we assessed the concentrations of the low molecular mass antioxidants glutathione, urate and vitamin E in erythrocytes and plasma of patients with chronic sinusitis, and patients with nasal polyps and compared the results with the levels

found in blood of healthy controls. Moreover, the TEAC values were determined. Although individual antioxidants play a specific role in the antioxidative defense system, these antioxidants may act co-operatively *in vivo* to provide synergistic protection to the organs against oxidative damage. Therefore, it may be more meaningful to assess antioxidant status by measuring both the individual and the overall antioxidant capacity.

Glutathione

Glutathione is a hydrophilic tripeptide serving as one of the most important intracellular antioxidants. According to literature human blood contains approximately 1 mM glutathione of which only 1 – 3 μM is present in plasma. The major pool of reduced glutathione (GSH) in blood are the erythrocytes containing 240 μg GSH/ml blood. The reduced glutathione versus the oxidized glutathione (GSH/GSSG) ratio in blood is higher than 10/1.[27] We measured a mean total glutathione level of approximately 1.6 mM in lyzed erythrocytes of healthy controls, which is in the same order of magnitude as is reported in other studies.[27] Total blood glutathione levels were significantly lower ($p \leq .05$) in patients with chronic sinusitis and patients with nasal polyps than in healthy controls. This difference might be explained by the fact that during mucosal inflammation an increased oxidant production leads to consumption of GSH through direct scavenging of reactive oxygen species at the inflammatory site. This utilization of GSH may be supplemented by systemic GSH transported by erythrocytes form the blood into the inflamed mucosa. In addition, mucosal conversion of GSH into GSSG e.g. as a result of GSH-peroxidase activity, occurs. GSSG leaks away to the bloodstream by cellular efflux and by this means does not increase total glutathione levels measured in lyzed erythrocytes. [28] Although the contribution of a diminished blood glutathione level to the disease pathology is uncertain, several other inflammatory diseases, such as AIDS, hepatitis, and pulmonary fibrosis, have been associated with systemic glutathione reduction.[29]

Vitamin E

Vitamin E is a lipophilic antioxidant which represents the principal defense against oxidant induced lipid peroxidation in the lipophilic compartment of cellular membranes. In experimental maxillary sinusitis in rabbits, elevated tissue and serum malondialdehyde (MDA), a product of ROS attack on polyunsaturated free fatty acids of the cell membrane (lipid peroxidation), levels were found.[30] Elevated levels of lipid hydroperoxides (LOOH), also a product of lipid peroxidation, and MDA were found in middle ear mucosa during

experimental otitis media.[31,32] Recently, high levels of MDA were found in tissue of human nasal polyps compared to mucosal samples of controls.[15] These data support a role of oxidant damage in the pathogenesis of upper airway inflammation. Vitamin E may play a protective role in this process. Indeed we found significant lower plasma concentrations of vitamin E in patients with chronic sinusitis suggesting a ROS-mediated vitamin E consumption. In patients with nasal polyps however such a reduction in plasma vitamin E level could not been shown. It has been reported that there is an efficient recycling of vitamin E by other antioxidants such as GSH and vitamin C.[33] It can therefore be speculated that the higher glutathione level in the nasal polyps group compared to the sinusitis group indicates that in the nasal polyps group more glutathione needs to be consumed before vitamin E levels starts to drop.

TEAC and uric acid

The total antioxidant capacity of plasma was measured by using the ABTS assay. We found in the plasma of healthy controls a TEAC value of approximately 0.5 mM which is in the same order of magnitude of values found in literature.[25,26] Major contributors to the total antioxidant capacity are urate (35-65 %) and vitamin C (24 %). The known antioxidants do not account for the whole of the measured TEAC value, i.e. there is an unidentified component.[27] In our measurements we did not find a significant difference in plasma TEAC values between healthy controls and either patients with sinusitis or nasal polyps. However, a significant lower uric acid level was found in the plasma of the controls compared to both the patient groups. At this point it should be noted that the plasma levels of uric acid of healthy controls found in our experiments equal those found in literature.[34] The finding that controls have lower plasma uric acid levels might be explained by the fact that during inflammation increased xanthine oxidase activity is present. Xanthine oxidase is the enzyme responsible for uric acid synthesis and has been reported to increased in plasma in patients suffering from chronic inflammatory disorders such as atherosclerosis and rheumatic diseases.[35,36] Furthermore, increased plasma uric acid levels are also found in other chronic inflammatory diseases such as gout and rheumatoid arthritis.[27] In contrast, mucosal uric acid levels are diminished in patients with chronic upper respiratory tract inflammation compared to healthy controls.[14] This is probably due to the sacrificial behavior of uric acid, meaning that uric acid is irreversibly degraded at the site of inflammation by increased oxidant production. Xanthine oxidase cannot be measured in nasal mucosa.[14,37] This suggests that uric acid is not formed locally but taken up from the peripheral bloodstream. It might therefore be speculated that under inflammatory conditions increased plasma levels are necessary to supplement the uric

acid utilization at the inflammatory site which may explain the elevated uric acid plasma levels in both the patient groups.

When the contribution of uric acid to TEAC was subtracted from the TEAC value, the residual antioxidant capacity containing factors such as, vitamin C, proved to be significantly lower in all patients suffering from chronic upper respiratory tract inflammation than in the control group. As vitamin C regenerates vitamin E, which is consumed during inflammation, this may very well partly explain the diminished residual TEAC value in the patient groups. This mucosal utilization of vitamin C was already hypothesized in 1968 for patients with allergic rhinitis who had considerably lower blood levels of vitamin C than normal subjects, indicating an unsaturated state of the tissue.[38] In addition, increased xanthine oxidase activity does not only lead to increased levels of uric acid which contribute to the antioxidant defense, but also produces ROS which may consume antioxidants, i.e. vitamin C or unidentified components, which contribute to the residual TEAC.

In conclusion, the antioxidant levels of glutathione, vitamin E and uric acid and the total antioxidant parameter TEAC in peripheral blood of patients with chronic sinusitis and nasal polyps were measured and compared to the levels in healthy controls. The reduced levels of glutathione, vitamin E and residual (non-urate) TEAC, may reflect the consumption of antioxidants and point to a role of oxidative stress in the pathogenesis of chronic upper respiratory tract inflammation. Increased uric acid levels might be due to oxidative stress induced xanthine oxidase activity. Although further research needs to be done to establish the exact role of oxidative stress in the multifactorial pathogenesis of upper respiratory tract inflammation, these findings may render perspectives for pharmacotherapeutic intervention.

References

1. Vademecum gezondheidsstatistiek nederland 1999. Centraal bureau voor de statistiek/ Ministerie van volksgezondheid, welzijn en sport.
2. Vademecum gezondheidsstatistiek nederland 1992. Centraal bureau voor de statistiek/ Ministerie van volksgezondheid, welzijn en sport.
3. Kaliner MA, Osguthorpe JD, Fireman P, Anon J, Georgitis J, Davis ML, Naclerio R, Kennedy D. Sinusitis: bench to bedside. Current findings, future directions. *J All Clin Immunol* 1997; 99:S829-S848.
4. Hamilos DL. Chronic sinusitis. *J All Clin Immunol* 2000; 106:213-227.
5. Southorn PA, Powis G. Free radicals in medicine II. Involvement in human disease. *Mayo Clin Proc* 1988; 63:390-408.
6. Henson PM, Johnston RB. Tissue injury in inflammation: oxidants, proteinases and cationic proteins. *J Clin Invest* 1987; 79:669-674.

7. Freeman BA, Crapo JD. Biology of disease. Free radicals and tissue injury. *Lab Invest* 1982; 47:412-426.

8. Wright DT, Cohn LA, Li H, Fischer B, Li CM, Adler KB. Interactions of oxygen radicals with airway epithelium. *Environ Health Perspect 1994*; 102(Suppl 10):85-90.

9. Hefner JE, Repine JE. Pulmonary strategies of antioxidant defense. *Am Rev Respir Dis* 1989; 140:531-554.

10. Doelman CJA, Bast A. Oxygen radicals in lung pathology. *Free Rad Biol Med* 1990; 9:381-400.

11. Barnes PJ. Reactive oxygen species and airway inflammation. *Free Rad Biol Med* 1990; 9:235-243.

12. Slavin RG. Asthma and sinusitis. *J All Clin Im*munol 1992; 90:534-537.

13. Demoly P, Crampette L, Mondain M, Enander I, Jones I, Bousquet J. Myeloperoxidase and interleukin-8 levels in chronic sinusitis. *Clin Exp All* 1997; 27:672-675.

14. Westerveld GJ, Dekker I, Voss HP, bast A, Scheeren RA. Antioxidant levels in the nasal mucosa of patients with chronic sinusitis and healthy controls. *Arch Otolaryngol Head Neck Surg* 1997; 123:201-204.

15. Dogru H, Delibas N, Döner F, Tüz M, Uygur K. Free radical damage in nasal polyp tissue. *Otolaryngol Head Neck Surg* 2001; 124:570-572.

16. Peden DB, Swiersz M, Ohkubo K, Hahn B, Emery B, Kaliner MA. Nasal secretion of the ozone scavenger uric acid. *Am Rev Respir Dis* 1993; 148:455-461.

17. Vandeputte C, Guizon I, Genestie-Dennis I, Vannier B, Lorenzon G. A microtiter plate assay for total glutathionee and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. *Cell Biology and Toxicology* 1994; 10: 415-421.

18. Lux O, Naidoo D, Salonikas C. Improved HPLC method for the simultaneous measurement of allantoin and uric acid in plasma. *Ann. Clin. Biochem* 1992; 29: 674-675.

19. Berg van den R, Haenen G.R.M.M, Berg van den H, Bast A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for the evaluation of the anitoxidant capacity measurements of mixtures. *Food Chemistry* 1999; 66: 511-517.

20. Wright DT, Cohn LA, Li H, Fischer B, Li CM, Adler KB. Interactions of oxygen radicals with airway epithelium. *Environ Health Perspect* 1994; 102(Suppl 10):85-90.

21. Vliet van der A, O'Neill CA, Cross CE, Koostra JM, Volz WG, Halliwell B, Louie S. Determination of low-molecular-mass antioxidant concentrations in human respiratory tract lining fluids. *Am J Physiol* 1999; 276:L289-L296.

22. Parks RR, Huang CC, Haddad J. Middle ear catalase distribution in an animal model of otitis media. *Eur Arch Otorhinolaryngol* 1996; 253:445-449.

23. Parks RR, Huang CC, Haddad J. Superoxide dysmutase in an animal model of otitis media. *Eur Arch Otorhinolaryngol* 1995; 252:153-158.

24. Peden DB, Hohman R, Brown ME, mason RT, Berkebile C, Fales HM, Kaliner MA. Uric acid is a major antioxidant in human nasal airway secretions. *Proc Natl Acad Sci USA* 1990; 87:7638-7642.

25. Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin Sci (Lond)* 1993; 84:407-412.

26. Wayner DDM, Burton GW, Inghold KU, Barclay LR, Locke SJ. The relative contributions of vitamin E, urate, ascorbic acid and proteins to the total peroxyl radical trapping antioxidant activity of human blood plasma. *Biochim Biophys* 1987; 924:408-419.

27. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. (3ed) Oxford University Press 1999.

28. Sies H. Akerboom TPM. Glutathione disulfide (GSSG) efflux from cells and tissues. *Methods Enzymol* 1984; 105:445-51.

29. Uhlig S, Wendel A. The physiological consequences of glutathione variations. *Life Sci* 1992; 51:1083-1094.

30. Dönner F, Delibas N, Dogru H, Sari I, Yorgancigil B. Malondyaldehyde levels and superoxide dysmutase activity in experimental sinusitis. *Auris Nasus Larynx* 1999; 26:287-291.

31. Parks RR, Huang CC, Haddad J. Evidence of oxygen radical injury in experimental otitis media. *Laryngoscope* 1994; 104:1389-1392.

32. Haddad J. Lipoperoxidation as a measure of free radical injury in otits media. *Laryngoscope* 1998; 108:524-530.

33. Van Acker SABE, Koymans LMH, Bast A. Molecular pharmacology of vitamin E: structural aspects of antioxidant activity. *Free Radic Biol Med* 1993; 20:311-328.

34. Cross CE. Vliet van der A, O'Neill CA, Louie S, Halliwell B. Oxidants, antioxidants, and respiratory tract lining fluids. *Environ Health Perspect* 1994; 102 (Suppl 10):185-191.

35. Mohacsi A, Kozlovszky B, Kiss I, Seres I, Fulop T. Neutrophils obtained from obliterative atherosclerotic patients exhibit enhanced respiratory burst and increased degranulation in response to various stimuli. *Biochim Biophys Acta* 1996; 1316:210-216.

36. Miesel R, Zuber M. Elevated levels of xanthine oxidase in serum of patients with inflammatory and autoimmune rheumatic diseases. *Inflammation* 1993; 17:551-561.

37. Peden DB, Swiersz M, Ohkubo K, Hahn B, Emery B, Kaliner MA. Nasal secretion of ozone scavenger uric acid. *Am Rev Respir Dis* 1993; 148:455-461.

38. Authors unknown. Ascorbic acid levels of blood and urine in patients with allergic rhinitis. *Eye Ear Nose Throat Monthly* 1968; 47:63-64.

Chapter II-3

Effects of N-acetylcysteine on blood antioxidant levels in patients with chronic sinusitis and nasal polyps

Gerrit-Jan Westerveld[1], Robert A Scheeren[1], Marc Fischer[2], Guido RMM Haenen[2], and Aalt Bast[2]

[1] Department of Otorhinolaryngology and Head and Neck Surgery, Medical Center Vrije Universiteit, Amsterdam, The Netherlands.

[2] Department of Pharmacology and Toxicology, University of Maastricht, The Netherlands.

Submitted

Abstract

Antioxidant pharmacotherapy has been proven successful in various oxidant mediated diseases. In recent years it has become clear that the pathophysiology of chronic upper respiratory tract inflammation is at least partially mediated by oxidative stress. As it was shown that antioxidant levels of nasal mucosa and peripheral blood of patients with nasal polyposis and patients with chronic sinusitis are decreased beneficial effects of N-acetylcysteine (NAC) in the management of these disorders, which are otherwise difficult to treat, can be anticipated. The blood levels of total glutathione, uric acid, TEAC, and the residual antioxidant capacity were measured in patients with either chronic sinusitis or nasal polyps who were additionally treated with 600 mg of oral NAC twice a day for 6 months in a placebo controlled randomized double blind fashion after functional endoscopic sinus surgery. Furthermore, the clinical status was scored subjectively by the patients and objectively by nasal endoscopy. In both the patient groups (nasal polyps and chronic sinusitis) which were treated with NAC the total glutathione levels, the uric acid levels, the TEAC levels, and the residual antioxidant capacity levels increased significantly after 6 months of oral NAC. This was not observed for the patient groups treated with placebo. Furthermore, in the NAC treated patients no nasal complaints were reported. Also nasal endoscopy of middle meatus showed an open osteomeatal unit in all cases. In contrast, in the placebo treated patients no effect was observed on either subjective well-being or nasal endoscopy.

It was concluded that oral NAC improves the antioxidant defense mechanisms and by this means contributes to the difficult therapeutic management of nasal polyposis and chronic sinusitis.

Introduction

Reactive oxygen species such as superoxide anions ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), are produced by several types of inflammatory cells and are known to contribute to tissue damage during chronic inflammation.[1] To cope with oxidative damage, tissues contain a broad spectrum of antioxidant defense mechanisms. Enzymatic systems such as superoxide dysmutase, catalase and glutathione dependent enzymes and non-enzymatic antioxidants such as glutathione, uric acid, vitamin E and vitamin C are present in respiratory tract mucosa. Disturbance of the delicate balance between free radical production and antioxidant defense may lead to disease. For instance, several chronic inflammatory disorders of the lungs are at least partially mediated by oxidative stress.[2,3,4] Also the pathogenesis of chronic upper respiratory tract inflammation has been related to oxidative stress.[5,6,7] It has been shown that mucosal and blood antioxidant levels are diminished in chronic sinusitis and nasal polyposis.[6,8] For this reason possible beneficial effects of antioxidant pharmacotherapy can be anticipated.

N-acetylcysteine (NAC) is a known antioxidant with specific direct and indirect antioxidant properties. On one hand NAC is a powerful scavenger of $^{\cdot}OH$ and HOCl.[9] On the other hand NAC serves as precursor of glutathione which is known to be one of the major antioxidants in human tissues. Being a precursor of glutathione, NAC has been shown to supplement consumed intracellular glutathione stores as a result of increased ROS formation. For instance, hart muscle damage after ischemic – reperfusion injury can be largely prevented by oral NAC pharmacotherapy due to a dose dependent increase of myocardial glutathione content.[10] In addition, oral NAC administration as antidote treatment for acetaminophen overdose has been proven successful based on supplementation of hepatic cell glutathione content.[11]

In inflammatory airway diseases in which oxidative stress plays a role in the pathophysiology, oral NAC treatment also has been proven to be beneficial. Peroral NAC increases glutathione levels in plasma and bronchoalveolar lavage fluid in normal subjects and in patients with chronic obstructive pulmonary disease and suggesting an increase in the antioxidant defense against ROS attack.[12] In addition, it has been postulated that NAC may protect lung cells from oxidative stress by increasing intra and extracellular glutathione.[13] All these findings may be extrapolated to chronic inflammation of the upper respiratory tract. In fact, oral N-acetylcysteine has been proven to be beneficial in the treatment of chronic sinusitis and serous otitis media.[14,15] However, in these studies treatment was short and only clinical parameters were monitored. In this paper we investigated the effects of additional oral

NAC treatment in patients with chronic sinusitis an nasal polyps on plasma antioxidant defense and clinical presentation.

Subjects, materials and methods

Subjects

The subjects studied in these experiments were either patients with chronic sinusitis (n = 13, 8 males and 5 females; age range 31-61 years, mean age 46.5 years) or patients with nasal polyps (n = 22, 11 males and 11 females; age range 29-64 years, mean age 47.4 years). All patients underwent functional endoscopic sinus surgery at time of inclusion of the study and were treated with an effervescent tablet twice a day containing 600 mg of N-acetylcysteine or a placebo during 6 months in a double blind randomized fashion. The study protocol was approved by the institutional medical ethical committee of the Vrije Universiteit medical center Amsterdam, The Netherlands, and informed consent was obtained from all participating subjects.

Patients with chronic sinusitis and patients with nasal polyps all had complaints of nasal obstruction, headache, and recurrent nasal discharge for more than 3 months. In all patients the diagnosis was confirmed on nasal endoscopy, which showed either hyperplastic mucosa or evident polyposis in the middle meatus, and by a computerized tomogram (CT) of the paranasal sinuses, which showed opacification of the paranasal sinuses including the anterior ethmoid. Histopathologic examination of the resection specimen at time of inclusion showed chronic inflammation with moderate to severe signs of eosinophilia in all subjects. All patients were tested for allergies using a radioallergosorbent test and a total IgE test. Patients were considered positive when the total blood IgE level was higher than 100 U/ml and a positive reaction was seen in one or more allergens from the radioallergosorbent test (a panel of grass pollen, tree pollen, animal dander, and house dust mite was used). None of the patients with chronic sinusitis or nasal polyps showed positive reactions. None of the subjects smoked. None of the subjects, controls and patients, used medication or any dietary vitamin or other antioxidant supplements during the 3 months prior to the study. None of the subjects suffered from any other known disease. At time of inclusion blood samples were drawn to obtain pretreatment levels of total glutathione, TEAC and uric acid. These measurements were repeated after 6 month of pharmacotherapy. Furthermore, at time of inclusion as well as at 6 months the patient complaints were scored subjectively as no complaints, moderate complaints and severe complaints. Also after 6 months of pharmacotherapy nasal endoscopy was performed scoring the mucosal picture at middle meatus level as open osteomeatal complex, swollen mucosa, or recurrence of disease.

Sample preparation

Venous blood was drawn from the cephalic vein using a venoject system®. Uric acid samples were collected in heparin gel tubes and gluthatione samples in heparin coated tubes. All tubes were centrifuged at 1500 rpm for 10 min. Plasma for uric acid determination was snap frozen and stored at –80°C until further use. In the gluthatione samples erythrocytes were separated from the plasma. The plasma was snap frozen at –80°C and used for total antioxidant capacity assay (TEAC). The erythrocytes were lyzed by Symex™ quicklyzer (Toa Medical Electronics Co., LTD, Kobe, Japan) after which the lyzate was centrifuged at 3000 rpm for 10 min. The supernatant was snap frozen and stored in an acid environment at –80°C until further use.

Antioxidant assays

Chemicals

NADPH, glutathione reductase, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and uric acid were obtained from Sigma Chemical Co., St. Louis, USA. 5'5'dithiobis 2-nitrobenzoic acid (DTNB) and reduced glutathione (GSH) were obtained from ICN Biomedicals Inc. Costa Mesa, USA. 2,2'-azino-bis(2-amidinopropane) dihydrochloride (ABAP) was obtained from Brunschwig Chemicals, Amsterdam, The Netherlands. All other chemicals were from analytical purity.

Glutathione assay

Total glutathione was determined using the recycling method.[16] To 50 µl of a lyzed erythrocyte sample (stored in presence of sulfosalicylic acid in a final concentration of 1.3 %) 100 µl of a 0.4 mM NADPH/ 0.3 mM DTNB solution in 143 mM phosphate buffer pH 7.4, containing 6.3 mM EDTA, is added in a 96- well plate. Then the reaction is started by adding 50 µl of a solution containing 4 U/ml glutathione reductase. The increase of the absorption (Δ A/min) is followed for 2 minutes at 405 nm. The Δ A/min of the samples is compared to that of the calibrators and the concentration of total glutathione is calculated.

Uric acid assay

Uric acid was determined in plasma ultra filtrates using a HPLC method described by Lux *et al.*[17] with minor modifications. Two hundred µl plasma was filtered over a 3 kD filter unit (Centrex UF 0.5, Schleicher & Schuell, Dassel, Germany) by centrifugation for 45 minutes at 14.000 x g and 4 °C. 25 µl of the filtrates was analyzed on the HPLC. The system (Agilent, Palo Alto, CA, USA) consisted of an Hypersil BDS C-18 end-capped column, 125 x 4 mm, particle size 5 µm (Agilent, Palo Alto, CA, USA) with a mobile phase of 5 mM sodium phosphate buffer pH = 3.3. Detection was performed using a variable wavelength detector set on a wavelength of 292 nm. Plasma concentrations uric acid were calculated using a calibration curve of uric acid.

Total antioxidant capacity assay

The total antioxidant capacity assay was carried out as described by van den Berg *et al.* with some modifications.[18] The $ABTS^{\bullet+}$ radical was produced by incubating a solution of 0.23 mM ABTS and 2.3 mM ABAP in 100 mM sodium phosphate buffer pH 7.4 at 70°C until the absorption of the solution at 734 nm was between 0.680 and 0.720. Plasma samples were deproteinated by adding to 100 µl plasma 100 µl of a solution of 10 % (w/v) trichloro acetic acid. After centrifugation (14.000 x g 5 minutes) the supernatant was used for the analysis. 950 of the $ABTS^{\bullet+}$ radical solution was preheated for 1 minute at 37°C. Then 50 µl of the sample was added and after incubating for 5 minutes the absorption (A) at 734 nm was measured. The decrease in absorption after 5 minutes of the samples, subtracted from that of the blank (buffer), was related to the decrease in absorption of trolox standards. The TEAC value gives the concentration of trolox needed to get a similar decrease in absorption as found for the sample.

Statistics

For each determination each sample was measured in duplicate and the standards were measured in triplicate. Intergroup comparisons, at time of inclusion between the groups treated with N-acetylcysteine and placebo were

made using a two tailed Student *t* test. Comparison of the blood antioxidant levels of patients before and after 6 months of pharmacotherapy were made using paired *t* tests. The criterion for statistical significance was set at $p \leq .05$.

Results

Total blood glutathione levels and the effect of N-acetylcysteine

At time of inclusion there was no significant difference ($p > .05$) in total glutathione value between the patient groups (chronic sinusitis and nasal polyps) which were treated with NAC compared to the groups which received placebo. After NAC treatment the total glutathione levels in both the nasal polyp group ($p = .0013$) and the chronic sinusitis group ($p = .0043$) increased significantly. In the placebo treated groups no significant alteration in total glutathione level was found (Figure 1,2).

Blood plasma uric acid levels.

At time of inclusion there was no significant difference ($p > .05$) in uric acid level between the patient groups (chronic sinusitis and nasal polyps) which were treated with NAC compared to the groups which received placebo.

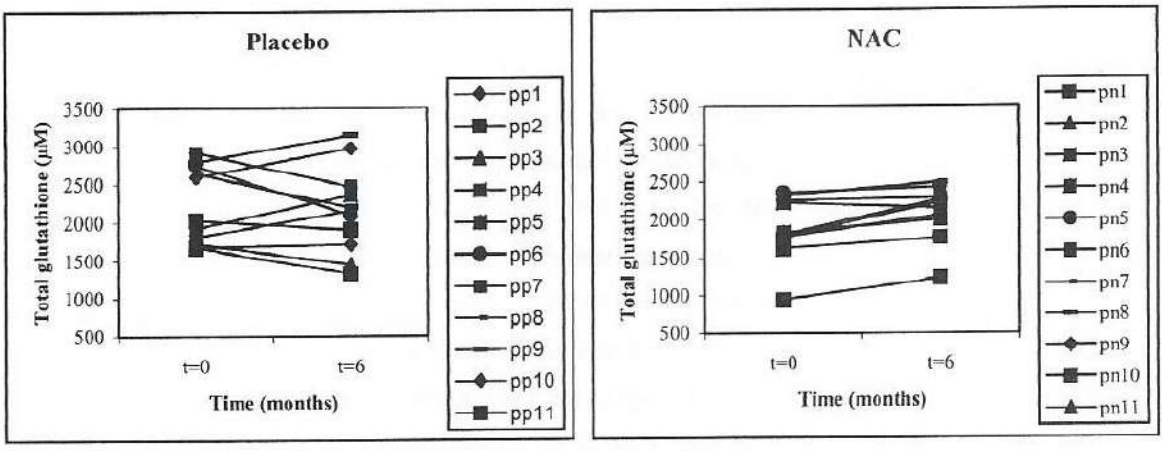


Figure 1. The effects of oral NAC versus placebo on blood total glutathione levels in patients with nasal polyps.

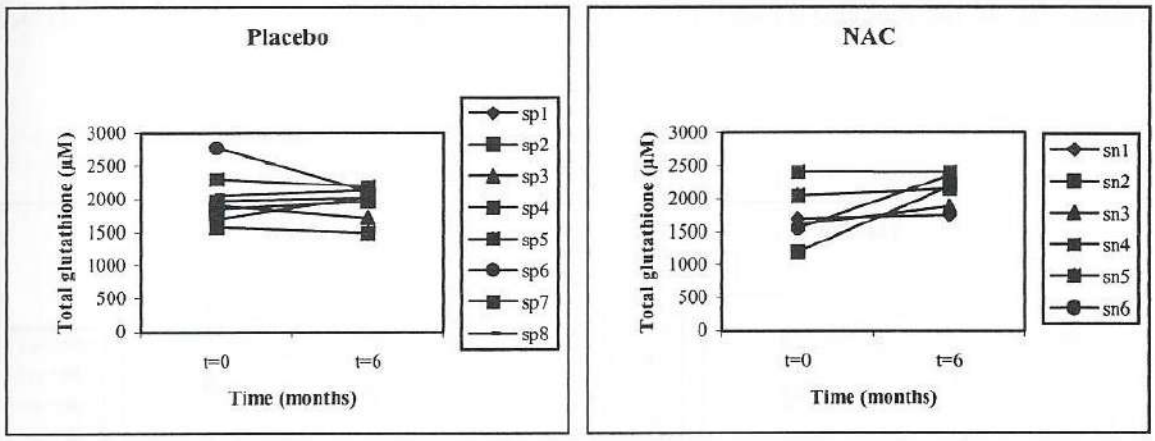


Figure 2. The effects of oral NAC versus placebo on blood total glutathione levels in patients with chronic sinusitis.

After NAC treatment the uric acid levels in both the nasal polyps group ($p = .0096$) and the chronic sinusitis group ($p = .033$) increased significantly. In the placebo treated groups no such significant effect was found (Figure 3,4).

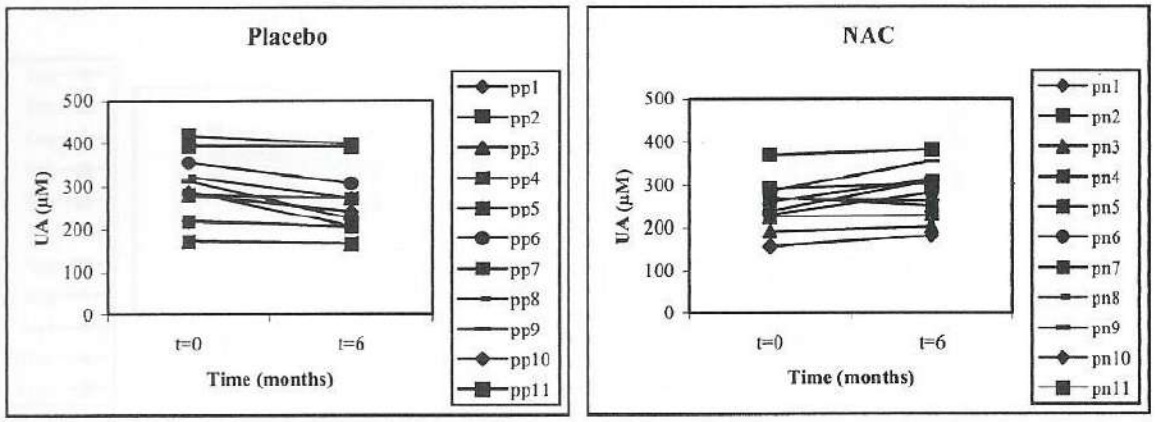


Figure 3. The effects of oral NAC versus placebo on blood uric acid levels in patients with nasal polyps.

Blood plasma TEAC levels

At time of inclusion there was no significant difference ($p > .05$) in TEAC level between the patient groups (chronic sinusitis and nasal polyps) which were treated with NAC compared to the groups which received placebo. After NAC treatment the TEAC levels in both the nasal

polyps groups ($p = 5.1 \times 10^{-5}$) and the chronic sinusitis group ($p = .0019$) increased significantly. In the placebo treated groups no such significant effect was found (Figure 5,6).

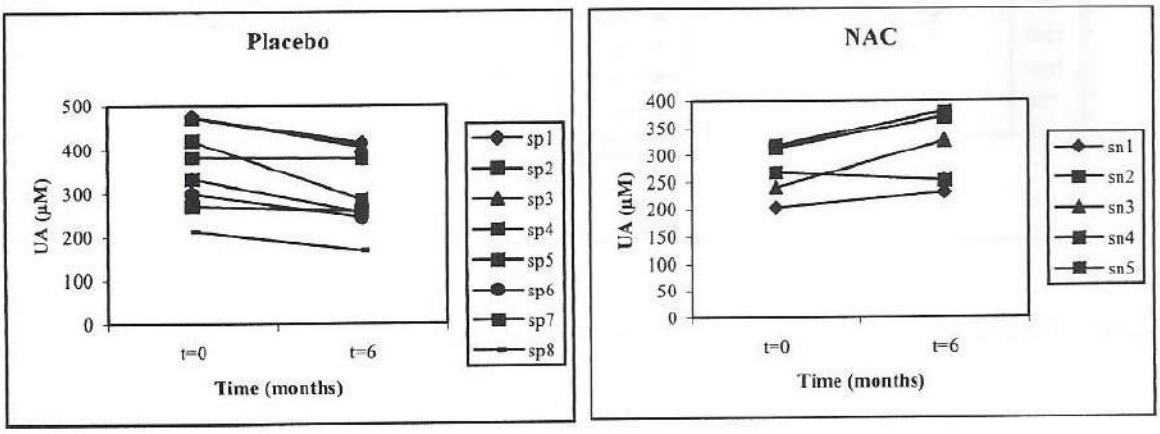


Figure 4. The effects of oral NAC versus placebo on blood uric acid levels in patients with chronic sinusitis.

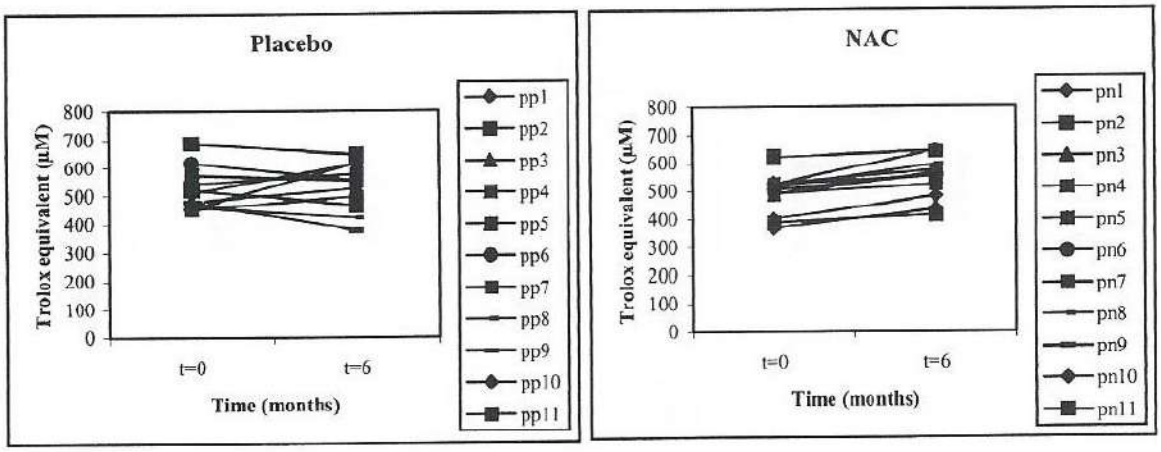


Figure 5. The effects of oral NAC versus placebo on blood TEAC levels in patients with nasal polyps.

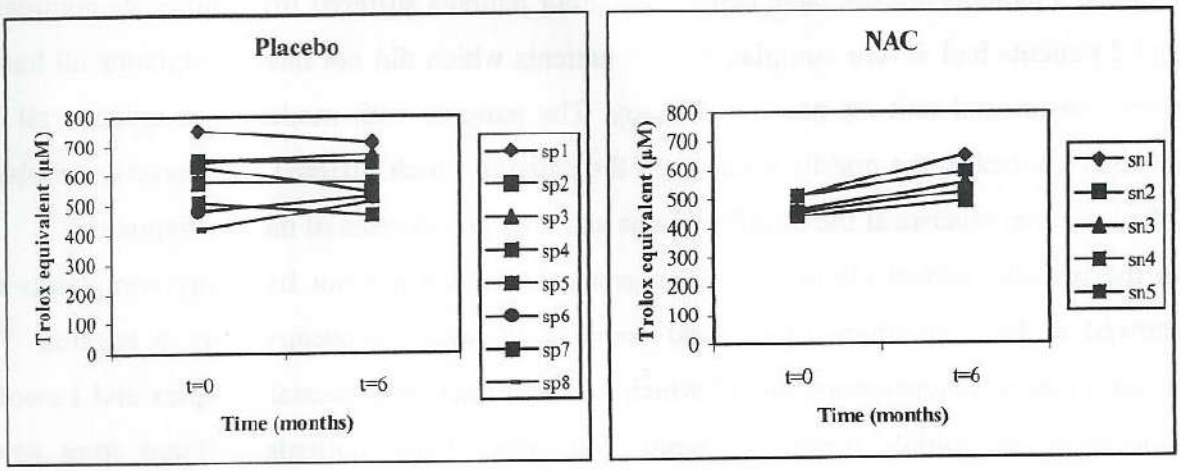


Figure 6. The effects of oral NAC versus placebo on blood TEAC levels in patients with chronic sinusitis.

Contribution of other antioxidants to blood plasma TEAC

The residual antioxidant capacity consisting of TEAC minus uric acid did not differ significantly ($p > .05$) between the patient groups (chronic sinusitis and nasal polyps) which were treated with NAC compared to the groups which received placebo. After NAC treatment the residual antioxidant capacity levels in both the nasal polyps group ($p = .0088$) and the chronic sinusitis group ($p = .048$) increased significantly. In the placebo treated groups no such significant effect was found. (Figure 7,8)

In the patient groups treated with NAC the separate contributions to the increase in total TEAC by the increase in uric acid and the increase in residual antioxidant capacity levels are shown in the figures 9 and 10. In the nasal polyps group 7 patients had a main increase in residual antioxidant capacity and a minor increase or decrease in uric acid level. In 4 patients with nasal polyposis the increase in uric acid was the most important contribution with a minor increase or decrease in residual antioxidant capacity. (Figure 9) In the chronic sinusitis group it was shown that in 2 patients the increase in residual antioxidant capacity was most important. However, in 3 patients the major contribution to the increase in total TEAC was caused by the increase in uric acid. (Figure 10)

Clinical effects of NAC treatment

In the patient groups (nasal polyps and chronic sinusitis) which were treated with NAC none of the subjects had complaints after 6 months of pharmacotherapy. On nasal endoscopy in all subjects an open osteomeatal unit was seen. In the placebo treated nasal polyps group at 6

months, 5 patients did not have complaints. Four patients suffered from moderate complaints and 2 patients had severe complaints. The patients which did not have complaints all had an open osteomeatal unit on nasal endoscopy. The patients with moderate complaints all had swollen musoca in the middle meatus. Of the patients which suffered from severe complaints 1 had swollen mucosa at the middle meatus and 1 had recurrence of nasal polyposis.
In the placebo treated chronic sinusitis group 3 patients did not have any complaints and showed to have an open osteomeatal complex at nasal endoscopy after 6 months. Two patients had moderate complaints of which 1 had an open osteomeatal complex and 1 swollen mucosa at the middle meatus at nasal endoscopy. Three patients suffered from severe complaints of those 1 had an open osteomeatal complex and 2 had recurrence of disease.
In both the patient groups (nasal polyps and chronic sinusitis) treated with placebo no relation could be detected between the patients symptoms and nasal endoscopic picture on one hand and the change in the levels of either GSH, UA, TEAC and residual antioxidant capacity on the other hand. (data not shown)

Discussion

Chronic inflammatory diseases of the upper respiratory tract, such as chronic sinusitis and nasal polyposis, form a major problem in health care. For instance, in the USA in 1995, 37 million cases of chronic sinusitis were reported.[19] In the Netherlands, in 1998, 8.9 % of the Dutch population, approximately 1.4 million patients, suffered from chronic upper respiratory tract inflammation and the prevalence is rising.[20] Although of lesser importance for chronic sinusitis than for nasal polyposis, the recurrence rate for these disorders after surgery is rather high despite adjuvant pharmacotherapy. Although the different studies on the outcome of functional endoscopic sinus surgery for chronic sinusitis and nasal polyps are hard to compare due to the lack of uniform criteria, recurrence percentages up to 50 % within a year follow-up have been reported.[21] The pathogenesis of chronic upper respiratory tract inflammation seems to be influenced by oxidative stress. In previous research we have shown that mucosal as well as blood antioxidant levels were diminished in chronic upper respiratory tract infections such as chronic sinusitis and nasal polyps.[6,8] Beneficial effects of adjuvant antioxidant pharmacotherapy can therefore be anticipated and may lead to a better therapeutic management. These considerations offer perspectives for pharmacotherapeutic intervention with oral NAC.

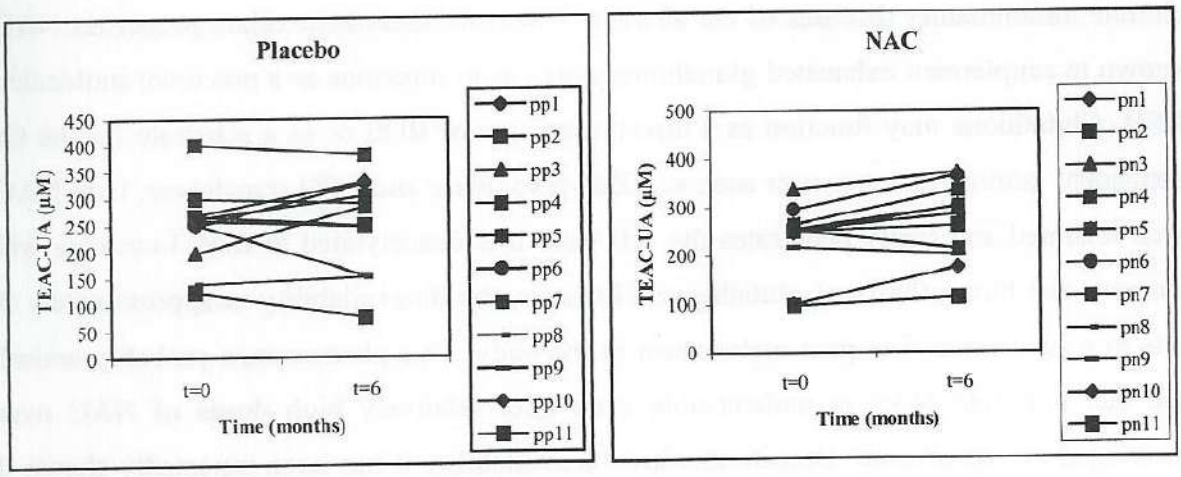


Figure 7. The effects of oral NAC versus placebo on blood residual (TEAC-UA) antioxidant levels in patients with nasal polyps.

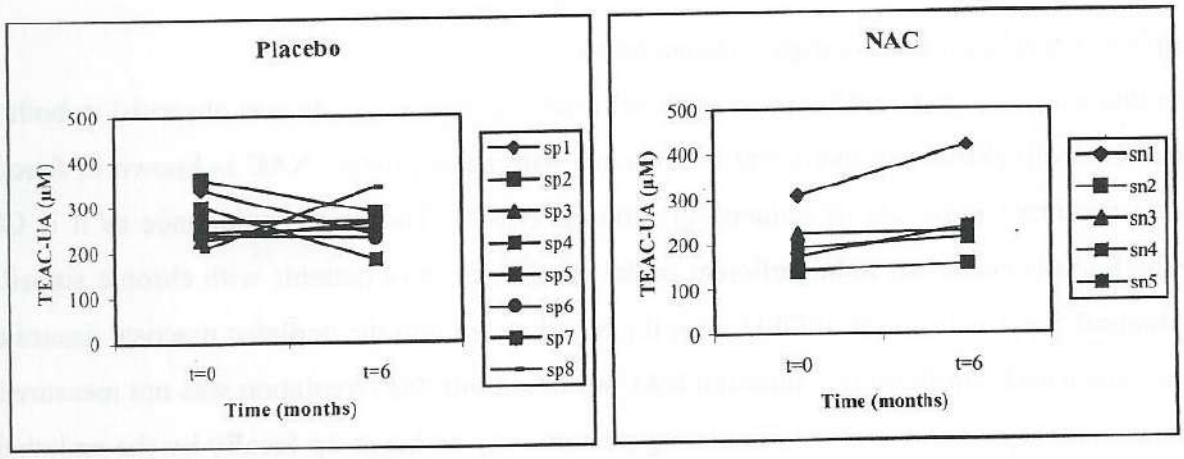


Figure 8. The effects of oral NAC versus placebo on blood residual (TEAC-UA) antioxidant levels in patients with chronic sinusitis.

Oral NAC pharmacotherapy has been proven beneficial in several oxidative stress related chronic inflammatory diseases of the airways.[22] Next to direct antioxidant properties NAC is known to supplement exhausted glutathione stores as in functions as a precursor molecule for GSH. Glutathione may function as a direct scavenger of ROS or as a substrate for the GSH dependent antioxidant enzymes such as, GSH-peroxidase and GSH transferase. Oral NAC is well tolerated and easily penetrates the cell were it is deacetylated to form l-cysteine which supports the biosynthesis of glutathione. However, the bioavailability is approximately 5%, due to a substantial first pass metabolism in the body. This phenomenon probably underlies the fact that free NAC is undetectable even after relatively high doses of NAC over a prolonged period of time. Despite this low bioavailability it has been repeatedly shown that circulatory levels of GSH are elevated in humans after oral NAC administration.[23] We used a dosage of 600 mg twice, for a period of 6 months a day which is in accordance with other clinical studies.[12,24,25] Chronic inflammation of the upper respiratory tract does not only lead to diminished blood and mucosal levels of glutathione, but it also effects blood and mucosal levels of uric acid and TEAC. Therefore, not only glutathione but also these other parameters were examined after pharmacotherapy with NAC.

Effect of NAC on total blood glutathione levels

In this study a significant increase in blood total glutathione levels was observed in both the patients with chronic sinusitis and the patients with nasal polyps. NAC is known to function as a precursor molecule of reduced glutathione (GSH). This is of importance as it is GSH which has been shown to be deficient in the nasal mucosa of patients with chronic sinusitis.[6] Mucosal supplementation of GSH from the bloodstream into the depleted mucosal tissues can be anticipated. Furthermore, although NAC absorbed into the circulation was not measured as bioavailability is low, a small circulating portion may be taken up locally by the endothelial cells of the nasal mucosa and deacylated, forming L-cysteine leading to local GSH formation. By these mechanisms tissue antioxidant defense may be increased leading to less inflammatory cell damage and improvement of the disease state.

TEAC and uric acid

TEAC represents the total antioxidant capacity of plasma. The most important contributor to TEAC is uric acid. Other contributors are vitamin C and vitamin E. Furthermore, the known antioxidant contributors do not account for the whole measured TEAC value, so there is an unidentified component.[26] Although, we did not find a significant difference in plasma TEAC

levels between patients with either chronic sinusitis or nasal polyps and healthy controls in former research, in these same experiments a significant decreased level of uric acid was found in the patient groups (chronic sinusitis and nasal polyps) compared to the healthy controls.[8] Furthermore, the residual antioxidant capacity, resulting from the subtraction of the contribution of uric acid from the TEAC value, was significantly lower in the patient groups suffering from chronic sinusitis and nasal polyps compared to healthy controls. These findings were explained by the fact that during chronic inflammation the activity of the plasma enzyme xanthine oxidase, which is responsible for uric acid synthesis, is increased as is also found in other chronic inflammatory disorders such as atherosclerosis and rheumatic diseases.[27,28] Based on this assumption lower uric acid levels could be expected after antioxidant pharmacotherapy due to a lower xanthine oxidase activity as a result of reduced inflammation. However, a significant rise in uric acid levels was shown in the chronic sinusitis and nasal polyps groups compared to the placebo treated groups. The reason for this finding is still unclear. A higher uric acid level might be due to a lower uric acid consumption as a result of lower oxidative stress after antioxidant therapy. Plasma TEAC levels were shown to increase significantly in both patients groups treated with NAC compared to the placebo treated group. Furthermore, the residual antioxidant capacity (non-uric acid TEAC) increased significantly in the patient groups treated with NAC. As uric acid was shown to increase significantly and uric acid is the main contributor to TEAC, the increase in TEAC was expected to be mainly due to the increase in uric acid level. However, this proved to be only partially true. In the nasal polyps group 7 of the 11 patients had a significant higher rise in residual antioxidant capacity than in uric acid levels. In only 4 of these patients the increase in uric acid seemed to be primarily responsible for the increase in TEAC. In the patients group with chronic sinusitis, in only 3 subjects the increase in TEAC was due to an elevated uric acid level. Apparently, NAC treatment results in an improved antioxidant network, as illustrated by increased TEAC. However, the effect of NAC treatment on the individual antioxidants is divers since it varies between subjects. This might be the result of the interaction of the antioxidants in the antioxidant network, the interaction of the antioxidant supplement in the antioxidant network, and the difference between subjects in this antioxidant network.

Clinical effects of NAC treatment

Although the beneficial effects of oral NAC in the treatment of chronic bronchopulmonary inflammation are well documented the results of NAC in the treatment of chronic upper

respiratory tract inflammation are sparse.[25] In 1980 Panosetti reported positive effects of NAC treatment in chronic maxillary sinusitis.[14] In addition, in the same year Balli showed enhancement of the functional effects of middle ear drainage for serous otitis media by oral NAC.[15] Furthermore, intramuscular injection of NAC has been shown to be effective in the treatment of maxillary sinusitis in children.[29]

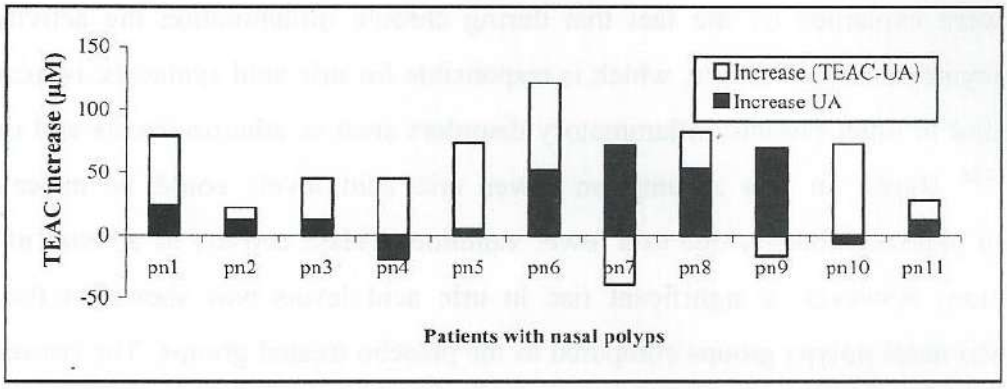


Figure 9. Contributions of the increase in uric acid (UA) and the increase in residual antioxidant capacity (TEAC-UA) to the total increase in blood TEAC value in patients with nasal polyps recieving NAC.

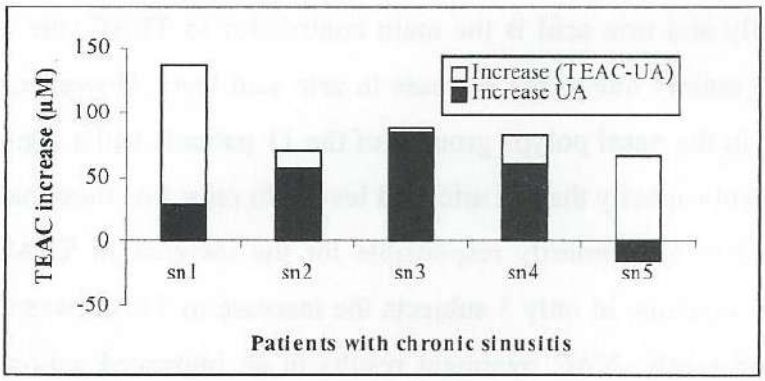


Figure 10. Contributions of the increase in uric acid (UA) and the increase in residual antioxidant capacity (TEAC-UA) to the total increase in blood TEAC value in patients with chronic sinusitis recieving NAC.

Also GSH given by an aerosol to patients with chronic rhinitis did not only increase GSH levels in the nasal mucosa significantly but also improved symptoms dramatically for longer periods of time.[30] Despite these encouraging results no additional studies on the effects of oral NAC in chronic upper respirator tract inflammation are available. We found that after 6 months of oral NAC in combination with functional endoscopic sinus surgery both the

patients with nasal polyps and the patients with chronic sinusitis were free of complaints and had an open osteomeatal area on nasal endoscopy. This suggests an additional beneficial effect from NAC as in the placebo treated control groups, 6 from the 11 patients in the nasal polyp group and 5 from the 8 patients in the chronic sinusitis group, had both moderate to severe complaints and mucosal swelling to recurrence of disease on nasal endoscopy.

In conclusion, the blood levels of the antioxidant systems total glutathione, uric acid, TEAC and residual antioxidant capacity are measured in peripheral blood of patients with chronic sinusitis and nasal polyps before and after treatment with 600 mg oral NAC twice a day during 6 months. The results were compared to control groups who received in a double blind randomized fashion a placebo. It was found that the blood levels of all antioxidant parameters increased significantly after treatment an effect which could not be found in the control groups. Furthermore, the clinical features improved in all NAC treated patients in contrast to the placebo treated patients in which only the minority of both groups showed clinical improvement 6 months after surgery. These findings strongly suggest that adjuvant antioxidant therapy, i.e. oral NAC supplementation, improves antioxidant defense systems and via this mechanism leads to a better therapeutic management of chronic inflammatory disorders of the upper respiratory tract.

References

1. Henson PM, Johnston RB. Tissue injury in inflammation: oxidants, proteinases and cationic proteins. *J Clin Invest* 1987; 79:669-674.
2. Hefner JE, Repine JE. Pulmonary strategies of antioxidant defense. *Am Rev Respir Dis* 1989; 140:531-554.
3. Doelman CJA, Bast A. Oxygen radicals in lung pathology. *Free Rad Biol Med* 1990; 9:381-400.
4. Barnes PJ. Reactive oxygen species and airway inflammation. *Free Rad Biol Med* 1990; 9:235-243.
5. Demoly P, Crampette L, Mondain M, Enander I, Jones I, Bousquet J. Myeloperoxidase and interleukin-8 levels in chronic sinusitis. *Clin Exp All* 1997; 27:672-675.
6. Westerveld GJ, Dekker I, Voss HP, bast A, Scheeren RA. Antioxidant levels in the nasal mucosa of patients with chronic sinusitis and healthy controls. *Arch Otolaryngol Head Neck Surg* 1997; 123:201-204.
7. Dogru H, Delibas N, Döner F, Tüz M, Uygur K. Free radical damage in nasal polyp tissue. *Otolaryngol Head Neck Surg* 2001; 124:570-572.
8. Westerveld GJ, Scheeren RA, Fischer M, Haenen GRMM, Bast A. Blood antioxidant levels in patients with chronic upper respiratory tract inflammation and healthy controls. *Submitted for publication.*

9. Aruoma OI, Halliwell B, Hoey BM, Butler J. The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic Biol Med* 1989; 6:593-597.

10. Ferrari R, Ceconi C, Curello S, Cargnoni A, Alfieri O, Pardini A, Marzollo P, Visioli O. Oxygen free radicals and myocardial damage: protective role of thiol-containing agents. *Am J Med* 1991; 91(suppl 3C):95S-105S.

11. Smilkstein MJ, Knapp GL, Kulig KW, Rumack BH. Efficacy of oral N-acetylcysteine in the treatment of acetaminophen overdose. *N Engl J Med* 1988; 319:1557-1562.

12. MacNee W, Bridgeman MME, Marsden M, Drost E, Lannan S, Selby C, Donaldson K. The effects of N-acetylcysteine and glutathione on smoke-induced changes in lung phagocytes and epithelial cells. *Am J Med* 1991; 91(suppl 3C):60S-66S.

13. Moldeus P, Cotgreave I, Bergren M. Lung protection by thiol-containing antioxidant, N-acetylcyteine. *Respiration* 1986; 50(suppl 1):31-42.

14. Panosetti E. Clinical trial with oral acetylcysteine in chronic sinusitis. *Eur J Respir Dis* 1980; 61(suppl 111):159.

15. Balli R. Controlled trial on the use of oral acetylcysteine in the treatment of glue-ear following drainage. *Eur J Respir Dis* 1980; 61(suppl 111):158.

16. Vandeputte C, Guizon I, Genestie-Dennis I, Vannier B, Lorenzon G. A microtiter plate assay for total glutathionee and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. *Cell Biology and Toxicology* 1994; 10: 415-421.

17. Lux O, Naidoo D, Salonikas C. Improved HPLC method for the simultaneous measurement of allantoin and uric acid in plasma. *Ann. Clin. Biochem* 1992; 29: 674-675.

18. Berg van den R, Haenen G.R.M.M, Berg van den H, Bast A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for the evaluation of the anitoxidant capacity measurements of mixtures. *Food Chemistry* 1999; 66: 511-517.

19. Vital and health statistics. Series 10, No. 199. National center for health statistic/Center for disease control and prevention.

20. Vademecum gezondheidsstatistiek nederland 1999. Centraal bureau voor de statistiek/ Ministerie van volksgezondheid, welzijn en sport.

21. Vleming M. Endoscopische neusbijholten chirurgie. Academical thesis. Amsterdam 1991.

22. Ziment I. Acetylcycteine: a drug with an interesting past and a fascinating future. *Respiration* 1986; 50(suppl 1):26-30.

23. Cotgreave IA. N-acetylcysteine: pharmacological considerations and experimental and clinical applications. *Adv Pharmacol* 1997; 38:205-227.

24. Behr J, Maier K, Degenkolb B, Krombach F, Vogelmeier C. Antioxidative and clinical effects of high dose N-acetyl cysteine in fibrosing alveolitis. *Am J Respir Crit Care Med* 1997; 156:1897-1901.

25. Grandjean EM, Berthet P, Ruffmann R, Leuenberger P. Efficacy of oral long term N-acetylcysteine in chronic bronchopulmonary disease: a meta-analysis of published double-blind, placebo-controlled clinical trials. *Clin Therapeut* 2000; 22:209-221.

26. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. (3ed) Oxford University Press 1999.

27. Mohacsi A, Kozlovszky B, Kiss I, Seres I, Fulop T. Neutrophils obtained from obliterative atherosclerotic patients exhibit enhanced respiratory burst and increased degranulation in response to various stimuli. *Biochim Biophys Acta* 1996; 1316:210-216.

28. Miesel R, Zuber M. Elevated levels of xanthine oxidase in serum of patients with inflammatory and autoimmune rheumatic diseases. *Inflammation* 1993; 17:551-561.

29. Boner AL, Valleta EA, Andreoli A, Vallone G, Baronio L. A combination of cefuroxime and N-acetylcysteine for the treatment of maxillary sinusitis in children with respiratory allergy. *Int J Clin Pharmacol Ther Toxicol* 1984; 22:511-514.

30. Testa B, Mesolela M, Testa D, Giuliano A, Costa F, Maione F, Iaccarino F. Glutathione in the upper respiratory tract. *Ann Otol Rhinol Laryngol* 1995; 104:117-119.

Chapter III-1

Anti-oxidant actions of oxymetazoline and xylometazoline

Gerrit-Jan Westerveld[1,2], Robert A Scheeren[2], Ingrid Dekker[1], Désirée H Griffioen[1], Hans-Peter Voss[1] and Aalt Bast[1]

[1]Leiden/Amsterdam Center for Drug Research, Department of Pharmacochemistry, Division of Molecular Pharmacology, Vrije Universiteit, Amsterdam, The Netherlands

[2]Department of Otorhinolaryngology and Head and Neck Surgery, University Hospital Vrije Universiteit, Amsterdam, The Netherlands.

Eur J Pharmacol 1995; 291:27-31.

Abstract

Antioxidant actions of oxymetazoline and xylometazoline were investigated by measuring inhibition of microsomal lipid peroxidation and hydroxyl radical scavenging activity. Oxymetazoline was shown to be a potent inhibitor of lipid peroxidation (IC_{50} = 4.9 μM at t = 15 min, IC_{50} = 8.1 μM at t = 30 min), in contrast to xylometazoline. Both compounds were excellent hydroxyl radical scavengers. Their rate constants (k_s = 1.1 x 10^{12} $M^{-1}s^{-1}$ for oxymetazoline and k_s = 4.7 x 10^{10} $M^{-1}s^{-1}$ for xylometazoline) exceeded the rate constant of a powerful known scavenger cimetidine (k_s = 1.8 x 10^{10} $M^{-1}s^{-1}$). The difference in inhibiting lipid peroxidation might be explained by the fact that only oxymetazoline has a hydroxyl group which can donate a hydrogen atom and terminate the chain reaction of lipid peroxidation. The mechanism of hydroxyl radical scavenging activity is still unclear. Moreover, oxymetazoline seems to have a different mode of action in scavenging hydroxyl radicals than xylometazoline and cimetidine resulting in a extremely high rate constant. Because oxidants play a role in tissue damage in inflammation, it was hypothesized that especially oxymetazoline and to a lesser extent xylometazoline may have additional beneficial effect due to their antioxidant properties, in the topical treatment of nasal inflammation.

Introduction

In inflammation reactive oxygen species (ROS), produced by activated granulocytes, have an important function by destroying invading microorganisms.[1] However, these ROS can also cause severe oxidative tissue injury.[2] Oxidant damage is prevented by antioxidants in tissue.[3] It is known that ROS contribute to respiratory epithelial damage in inflammatory diseases of the lungs.[4,5] Since there is a relationship between nasal inflammation and asthma, a role of oxidants in upper respiratory inflammations can be anticipated.[6,7] Hydroxyl radicals, which are among the most reactive radicals known, are frequently associated with biological damage as they can injure cellular constituents such as nucleic acids, proteins, carbohydrates and lipids.[8,9] An important manifestation of tissue damage is lipid peroxidation, in which the polyunsaturated free fatty acids of the biomembrane are transformed to lipid hydroperoxides.[10] This process occurs as a result of free radical attack on the cell membrane leading to impairment of membrane functions and eventually to cell death. In upper airway infections, oxymetazoline and xylometazoline are frequently used for mucosal decongestion. They are known to cause vasoconstriction due to their direct α-adrenoceptor stimulation on the vascular smooth muscle in the nasal mucosa.[11] Both drugs are commonly used in the symptomatic treatment of rhinitis and sinusitis. It has been suggested that antioxidant therapy may have beneficial effect in the treatment of oxidant-mediated diseases.[3] Possible antioxidant properties of oxymetazoline and xylometazoline may further explain the therapeutic effect of these compounds and were therefore characterized.

Material and methods

Chemicals

Oxymetazoline, xylometazoline, cimetidine and 2-deoxy-D-ribosec were obtained from Sigma Chemical (St Louis, USA). All other chemicals were of reagent grade.

Preparation of microsomes

Male Wistar rats (Harlan Olec, Zeist, The Netherlands), 200-220 g, were killed by decapitation. Livers were removed and washed with ice-cold Na-K-phosphate buffer (50 mM, pH 7.4) containing 0.1 mM EDTA; non-liver tissue was removed. The livers were weighed and homogenized in phosphate buffer (1:2, w/v) using a Polytron (PT 3000 Kinematica; 25000 rpm, 2 x 60 s at 4°C) and potter homogenizer (Heidolph-Electro; Rührkopf II; 250 rpm, 2 x 60 s at 4°C). The homogenate was centrifuged at 10000 x g (20 min at 4°C). Subsequently the supernatant was centrifuged at 100000 x g (60 min at 4°C). The pellet was resuspended in phosphate buffer and centrifuged again at 100000 x g (60 min at 4°C). The microsomal pellet was resuspended in the phosphate buffer (2 g liver/ml). During continuous stirring, the resuspended pellet was divided in 1 ml parts

and directly frozen in liquid nitrogen and stored at –80°C until use.

Determination of inhibition of microsomal lipid peroxidation

Before use the protein concentration of the microsomes was determined by the method described by Bradford (1976) sing the commercially available Bio-Rad reagent, with bovine serum albumin as a standard.[12] The protein concentration was approximately 24 mg/ml. Microsomes were thawed and diluted with approximately 30 volumes of ice-cold Tris-HCl buffer (50 mM, pH 7.4) containing 0.9% NaCl. The buffer was removed by centrifugation at 115000 x g (45 min) and the pellet was heated for 90 s at 100°C. The microsomes were diluted to 1 mg protein per ml, in ice0cold Tris-HCl buffer. Microsomes (1 ml) were incubated in the Tris-HCl buffer (830 µl) while shaking at 37°C in open plastic incubation tubes. Different concentrations of oxymetazoline and xylometazoline were added to the incubation tubes. Ascorbic acid (100 µl, 0.2mM final concentration) and $FeSO_4$ (20 µl, 10 µM final concentration) were dissolved in N_2 de-aerated water. Reactions were started by adding the freshly prepared $FeSO_4$ solution. Lipid peroxidation was assayed by measuring thiobarbituric acid reactive substances. At different times an aliquot of the incubation (0.3 ml) was taken and the reaction was stopped by mixing it with 2 ml of an ice-cold solution containing thiobarbituric acid (4.16 mg thiobarbituric acid in 1 ml trichloroacetic acid (168 g/l, 0.125 M HCl)) and HCl butylated hydroxytoluene (1.5 mg HCl butylated hydroxytoluene in 1 ml ethanol). The thiobarbituric acid-HCl butylated hydroxytoluene solution consisted of 1volume of HCl butylated hydroxytoluene solution per 10 volumes of thiobarbituric acid solution. After heating (15 min at 80°C) and centrifugation (15 min, 3000 rpm) the absorbance at 535 nm vs 600 nm in the supernatant was determined (LKB Novospec II spectrophotometer). Corrections were made for the absorbance of the thiobarbituric acid-HCl butylated hydroxytoluene solution, the microsomes and the Tris-HCl buffer using a sample containing 2ml thiobarbituric acid-HCl butylated hydroxytoluene solution, 150 µl Tris-HCl and 150 µl microsomes as a blank. Three independent experiments were carried out to obtain IC_{50} values.

Determination of hydroxyl radical scavenging activity

Hydroxyl radicals are generated via the Fenton reaction and determined by the detector molecule deoxyribose (DR). The hydroxyl radicals cause a degradation of this sugar molecule and the fragments after degradation react with thiobarbituric acid. [13] This is shown by the following equations:

Fe^{3+}-EDTA + ascorbic acid → Fe^{2+}-EDTA +oxidized ascorbic acid

Fe^{2+}-EDTA + H_2O_2 → OH^- + $^{\cdot}OH$ + Fe^{3+}-EDTA **(Fenton reaction)**

OH^- + deoxyribose → fragments (heat with thiobarbituric acid/HCl butylated hydroxytoluene) → malondialdehyde

A hydroxyl radical scavenger added to the reaction will compete with the deoxyribose molecule for the hydroxyl radical. The rate constant for reaction of the scavenger with the hydroxyl radical can be calculated using the following equation:

$1/A = 1/A^0 \{1 + k_s[S]/k_{DR}[DR]$

A is the absorbance in the presence of a scavenger S at concentration [S] and A^0 is the absorbance in the absence of a scavenger. When a linear plot is made of 1/A against [S] under the condition that the rate constant (k_{DR}) for the reaction of deoxyribose with the hydroxyl radical is 3.1×10^9 $M^{-1}s^{-1}$ and the concentration of deoxyribose [DR] ≥ 2.8 mM, the rate constant (k_s) for the reaction of S with the hydroxyl radical can be obtained from the slope ($= k_s/k_{DR}[DR]A^0$) of the line.[13] The reaction mixtures contained, in a final volume of 1 ml, the following reagents at these final concentrations: KH_2PO_4-KOH buffer (20 mM, pH 7.4), $FeCl_3$ (100 μM), ascorbic acid (100 μM), EDTA (100 μM), H_2O_2 (1mM) and deoxyribose (2.8 mM). The ascorbic acid and the $FeCl_3$ were freshly made before use and the Fe Cl_3 was the last compound added. Oxymetazoline and xylometazoline, dissolved in water, were added at different concentrations. The incubations were carried out for 1 h at 37°C and stopped by adding 2 ml of a thiobarbituric acid-HCl butylated hydroxytoluene solution. Then the incubations were heated for 15 min at 90°C and centrifuged at 3000 rpm for 15 min. The thiobarbituric acid reactive fragments were determined by measuring the absorbance at 535 nm. The thiobarbituric acid-HCl butylated hydroxytoluene solution was made the same way as described in the section: *"Determination of inhibition of lipid peroxidation"*. EDTA was present in the incubations as iron chelator since it is known that iron easily binds to deoxyribose. As reference compound the known hydroxyl radical scavenger cimetidine was also tested in the assay.[14]

Results

Xylometazoline used in concentrations between 100 μM and 1 mM gave no inhibition of lipid peroxidation in a time dependent manner (Figure 1). In contrast oxymetazoline showed clear inhibition of lipid peroxidation at a concentration of 5 μM and higher (Figure 2). This was shown by the occurrence of a lag-time which lasted until 10 min of incubation for a 5 μM concentration of oxymetazoline and until 30 min of incubation for 10 μM of oxymetazoline. Higher concentrations completely inhibited lipid peroxidation during an incubation period of 60 min. Concentrations lower than 5 μM of oxymetazoline gave no significant inhibition of lipid peroxidation (data not shown). The $IC_{50} \pm$ SD value, a relative measure of the capacity to inhibit lipid peroxidation, of oxymetazoline was 4.9 ± 1.2 μM at t = 15 min and 8.1 ± 4.1 μM at t = 30 min. Both oxymetazoline and xylometazoline are powerful hydroxyl radical scavengers as shown by their rate constants ($k_s \pm$ SD) $1.1 \times 10^{12} \pm 5.0 \times 10^{11}$ $M^{-1}s^{-1}$ (n = 9) and $4.7 \times 10^{10} \pm 1.3 \times 10^{10}$ $M^{-1}s^{-1}$ (n = 11) respectively. As reference compound the rate constant of cimetidine $k_s = 1.8 \times 10^{10} \pm 5.4 \times 10^9$ $M^{-1}s^{-1}$ (n = 5) was determined. The concentrations used for the compounds ranged from 10 μM to 100 μM. The concentration range used for

cimetidine was 10 μM to 1 mM, although figure 3 only shows 10 μM to 100 μM.[14] However, as the measured absorbance (A^0) of both xylometazoline and cimetidine equaled the A^0 which was calculated by extrapolating the linear curve fit to the y-axis, this was not found for oxymetazoline. In fact the measured A^0 of oxymetazoline was repeatedly found to be higher than the calculated A^0.To investigate this phenomenon we performed a number of experiments (n = 9) in which we used a concentration range of 0.25 μM to 10 μM, assuming that oxymetazoline was very effective as a hydroxyl scavenger in a dose dependent manner even at low concentrations (Figure 3). These experiments showed that the scavenging activity of oxymetazoline was biphasic. The k_s for this compound was calculated by using the steepest slope of the biphasic curve. To investigate possible iron chelating properties of the compounds the experiment was also carried out in the absence of EDTA. Compounds with good ion chelating properties will show a higher k_s value in the absence of EDTA, because hydroxyl radicals are then formed in the direct environment of the compound and site-specific scavenging occurs. This means that the formed hydroxyl radicals are immediately available for scavenging by the compound. However, this was not found, i.e. in the absence of EDTA no scavenging was seen for both compounds. The plots of 1/A against [S] showed horizontal lines (data not shown).

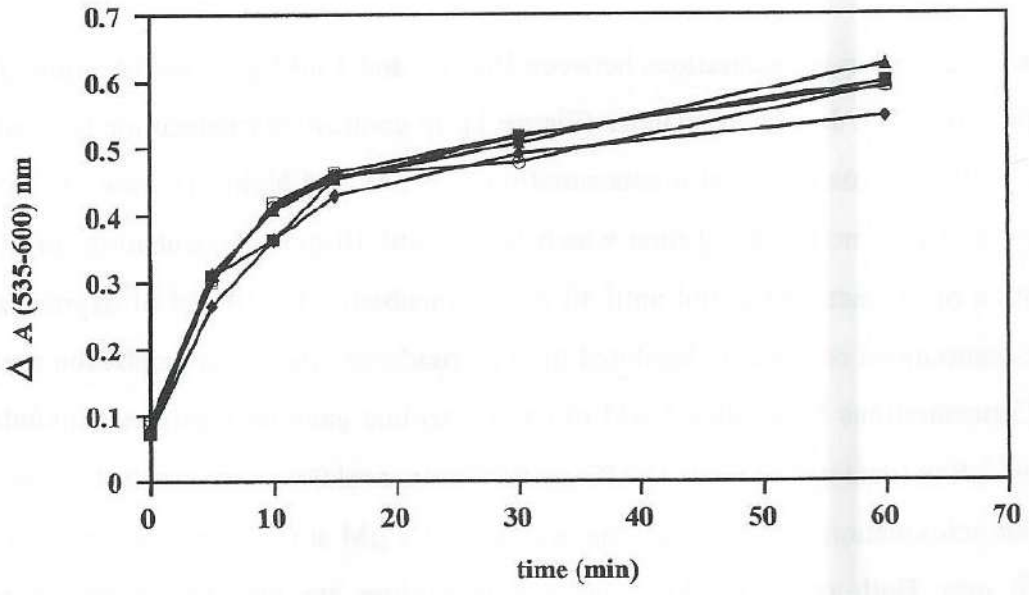


Figure 1. The influence of xylometazoline ((■) 100 μM; (□) 250 μM; (○) 500 μM; (♦) 1 mM) on a time dependent Fe^{2+}/ascorbic acid induced liver microsomal lipid peroxidation ((▲) control). No inhibition is observed. A typical example out of three independent experiments is shown.

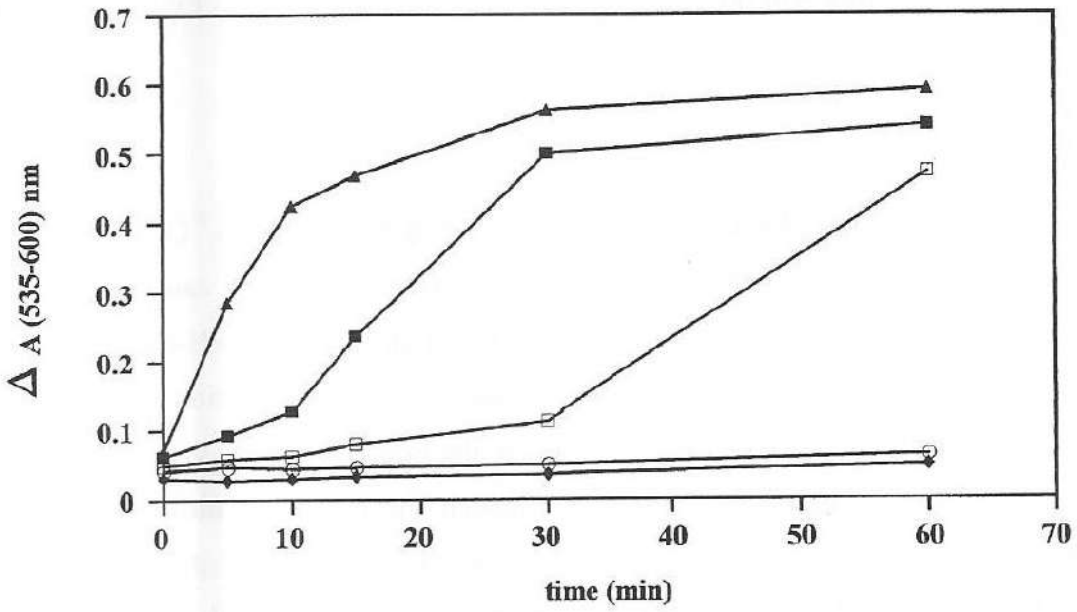


Figure 2. The influence of oxymetazoline ((■) 5 μM; (□) 10 μM; (○) 15 μM; (◆) 20 μM) on a time dependent Fe^{2+}/ascorbic acid induced liver microsomal lipid peroxidation ((▲) control). The different lag-times are evident. A typical example out of three independent experiments is shown.

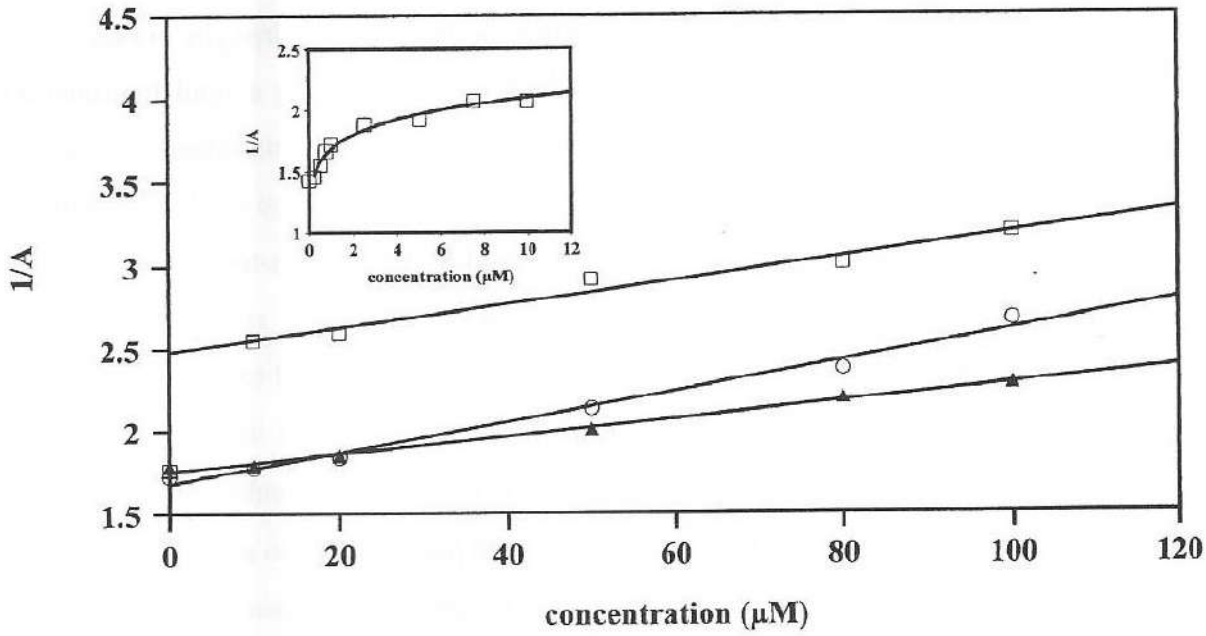
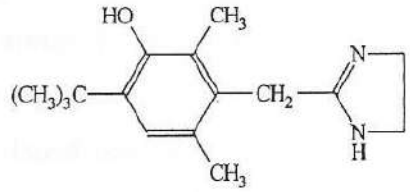


Figure 3. A typical example of the determination of the rate constant (k_s) for oxymetazoline (□), xylometazoline (○) and cimetidine (▲). Various concentrations of the compounds are plotted against the reciprocal value of absorption of thiobarbituric acid reactive fragments of deoxyribose after degradation. The k_s of xylometazoline and cimetidine were calculated using the slope of the linear curve fit according to the equation: k_s = slope x k_{DR} x[DR] x A^0. The k_s of oxymetazoline was calculated using the slope of the steepest part of the biphasic curve (inset). This experiment was conducted at least 5 times for each compound.

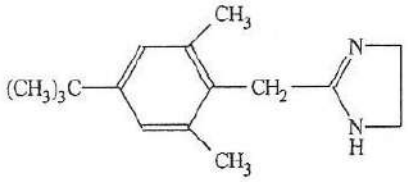
iron chelation therefore will not play a role in the antioxidant action of either oxymetazoline and xylometazoline.

Discussion

Oxidants can play a pivotal role in tissue damage during inflammation.[9] Oxidants are released by activated inflammatory cells as a result of their respiratory burst caused by a variety of stimuli.[15] Due to these stimuli the membrane bound enzyme NADPH-oxidase is activated leading to an increased production of ROS such as superoxide anion radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) eventually resulting in the formation of the very reactive hydroxyl radical ($^{\cdot}OH$) In the present study we investigated the possible antioxidant properties of oxymetazoline and xylometazoline because such actions may contribute to explain their therapeutic effect. Oxymetazoline and xylometazoline are frequently used in the topical treatment of inflammatory diseases of the upper airways. Through α-adrenoceptor stimulation and subsequent vasoconstrictive action both drugs lead to decreased nasal congestion followed by diminished nasal discharge and better ventilation of the paranasal sinuses.[16] The antioxidant action of oxymetazoline in lipid peroxidation might be explained by the possibility of donating a hydrogen atom, originating from the hydroxylic moiety on the phenyl ring (Figure 4), to the lipid peroxyl radical ($LOO^{\cdot}$) forming a lipid hydroperoxide (LOOH) instead of oxidizing another polyunsaturated acid and by this means terminate the chain reaction of lipid peroxidation. The oxymetazoline radical will be relatively stable, due to delocalisation of the unpaired electron, similar to the α-tocopherol radical.[17] Xylometazoline does not have such a hydroxy group on the phenyl ring (Figure 4) which explains the lack of antioxidant activity in lipid peroxidation compared to oxymetazoline. The IC_{50} value of α-tocopherol is approximately 2 μM for an incubation time of 20 min.[18] We found a IC_{50} value for oxymetazoline of approximately 5 μM (incubation time 15 min) indicating that this compound inhibits microsomal lipid peroxidation to a similar extent as α-tocopherol. Moreover, in comparison to especially designed antioxidant compounds such as 21-amino steroids (U74006 and U74500A) which have IC_{50} values ranging from 2 to 60 μM oxymetazoline has also a comparable potency in inhibiting microsomal lipid peroxidation.[18] As the biomembrane is an important target of free radical attack following inflammation, protection of this membrane, by inhibiting its peroxidation, may contribute in anti-inflammatory therapy.



Oxymethazoline



Xylomethazoline

Figure 4. The chemical structures of oxymetazoline and xylometazoline are shown. Note the hydroxy group on the phenyl ring of oxymetazoline, which is absent in xylomethazoline.

Hydroxyl radicals are very reactive and often related to biological damage in inflammation.[19] Furthermore, it has been suggested that some anti-inflammatory drugs might exert their effect by hydroxyl radical scavenging.[20] Both oxymetazoline and xylometazoline were more effective hydroxyl radical scavengers than the known scavenger cimetidine, as judged from their respective k_s values.[14] The rate of hydroxyl radical scavenging of oxymetazoline is extremely high when compared to the rate of scavenging of xylometazoline and cimetidine. Since for oxymetazoline a biphasic character of scavenging was observed, more than one mode of action in hydroxyl radical scavenging is suggested. Such an atypical behavior was not found before and is not yet fully comprehended. However, this finding is not caused by possible iron-chelating properties of the compound. The exact mechanism by which ascorbic acid and iron ions cause microsomal lipid peroxidation is not clear. It is thought that the pro-

oxidant activity of ascorbic acid is found in combination with a specific ratio of Fe^{3+}/Fe^{2+}, giving oxidation of polyunsaturated free fatty acids.[21] Hydroxyl radicals do not play a role in iron-induced microsomal lipid peroxidation.[22] We therefore established the antioxidative properties of both compounds by either inhibition of lipid peroxidation and hydroxyl radical scavenging. Our findings suggest a possible additional beneficial role of oxymetazoline and xylometazoline in the treatment of nasal inflammation by their antioxidative properties. Moreover, the commercially available decongestive nosedrops will probably give even higher local concentrations of both compounds than those used in our assays. Decongestive nosedrops, containing xylometazoline or oxymetazoline, decrease nasal mucociliary clearance.[23,24] This effect, however, seems to be mainly caused by the added preservatives. Another study showed that both oxymetazoline and xylometazoline had an inhibitory effect on neutrophil oxidative burst.[25] These two findings indicate that both compounds can also give rise to a diminished mucosal defense, acting as pro-inflammatory instead of anti-inflammatory compounds.

We conclude that both oxymetazoline and xylometazoline have specific antioxidant capacities, which have not been described before. Until now these compounds were used in the treatment of upper airway inflammation based only on their decongestive effect on the mucosa. Presuming that oxidants play a role in upper airway inflammation, these drugs may also act as anti-inflammatory compounds by their antioxidant properties in hydroxyl radical scavenging and lipid peroxidation (only oxymetazoline). It should be noted, however, that these compounds, at least in their pharmaceutical matrix, also have unfavorable effects on the nasal mucosa.

References

1. Southern PA, Powis G. Free radicals in medicine. I. Chemical nature and biologic reactions. *Mayo Clin Proc* 1988; 63:381-389.
2. Henson PM, Johnston RB. Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins. *J Clin Invest* 1987; 79:669-674.
3. Bast A, Haenen GRMM, Doelman CJA. Oxidants and antioxidants: state of the art. *Am J Med* 1991; 91:2S-12S.
4. Barnes PJ. Reactive oxygen species and airway inflammation. *Free Rad Biol Med* 1990; 9:235-243.
5. Doelman CJA, Bast A. Oxygen radicals in lung pathology. *Free Rad Biol Med* 1990; 9:381-399.
6. Slavin RG. Relationship of nasal disease and sinusitis to bronchial asthma. *Ann Allergy* 1982; 49:76-80.

7. Rachelefsky GS, Katz RM, Siegel SC. Chronic sinus disease with associated reactive airway disease in children. *Pediatrics* 1984; 73:526-529.
8. Southern PA, Powis G. Free radicals in medicine. II. Involvement in human disease. *Mayo Clin Proc* 1988; 63:390-408.
9. Cochrane CG. Cellular injury by oxidants. *Am J Med* 1991; 91:23S-30S.
10. Freeman BA, Crapo JD. Biology of disease. Free radicals and tissue injury. *Lab Invest* 1982; 47:412-426.
11. Bowman WC Rand MJ. Textbook of pharmacology, sec. edition (Blackwell scientific publications Oxford) 1980;chp. 11:32.
12. Bradford MM. A rapid and sensitive methods for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72:248-254.
13. Halliwell B, Gutteridge JMC, Aruoma OI. The deoxyribose method: a simple "test-tube" assay for determination of rate constants for reactions of hydroxylradicals. *Anal. Biochem* 1987; 165:215-219.
14. Ching T-L, Haenen GRMM, Bast A. Cimetidine and other H_2 receptor antagonists as powerful hydroxyl radical scavengers. *Chem Biol Int*eract 1993; 86:119-127.
15. Thelen M, Dewald B, Baggiolini M. Neutrophil signal transduction and activation of the respiratory burst. *Physiol Rev* 1993; 73:797-821.
16. Johnson DA, Hricik JG. The pharmacology of α-adrenergic decongestants. *Pharmacotherapy* 1993; 13:110S-115S.
17. van Acker SABE, Koymans LMH, Bast A. Molecular pharmacology of vitamin E: structural aspects of antioxidant activity. *Free Rad Biol Med* 1993; 15:311-328.
18. Braughler JM, Pregenzer JF, Chase RL, Duncan LA, Jacobsen EJ, McCall JM. Novel 21-amino steroids as potent inhibitors of iron-dependent lipid peroxidation. *J Biol Chem* 1987; 262:10438-10440.
19. Samuni A, Black CDV, Krishna CM, Maleeh HL, Bernstein EF, Russo A. Hydroxyl radical production by stimulated neutrophils reappaised *J Biol Chem* 1988; 263:13797-13801.
20. Halliwell B, Hoult JB, Blake DR. Oxidants, inflammation, and anti-inflammatory drugs. *FASEB* J 1988; 2:2867-2873.
21. Braughler JM, Duncan LA, Chase RL. The involvement of iron in lipid peroxidation. *J Biol Chem* 1986; 261:10282-10289.
22. Bast A, Steeghs MHM., Hydroxyl radicals are not involved in NADPH dependent microsomal lipid peroxydation. *Experientia* 1986; 42:555-556.
23. van de Donk HJM, van den Heuvel AGM, Zuidema J, Merkus FWHM. The effect of nasal drops and their additives on human nasal mucociliary clearance. *Rhinology* 1982; 20:127-137.
24. Deitmer T, Scheffler R., The effect of different preparations of nasal decongestants on ciliary beat frequency in vitro. *Rhinology* 1993; 31:151-153.
25. Bjerknes R, Steinsvag SK. Inhibition of human neutrophil actin polymerization, phagocytosis and oxydative burst by components of decongestive nosedrops. *Pharm Toxicol* 1993; 73:41-45.

Chapter III-2

Inhibition of nitric oxide synthase by nasal decongestants

Gerrit-Jan Westerveld[1], Hans-Peter Voss[2], Regine M van der Hee[2],
Gabriëlle JN de Haan-Koelewijn[2], Gert-Jan M den Hartog[1,3], Robert A Scheeren[1],
and Aalt Bast[3]

[1] Department of Otorhinolaryngology and Head and Neck Surgery, University Hospital Vrije Universiteit, Amsterdam, The Netherlands.

[2] Leiden/Amsterdam Center for Drug Research, Department of Pharmacochemistry, Division of Molecular Pharmacology, Vrije Universiteit, Amsterdam, The Netherlands.

[3] Department of Pharmacology and Toxicology, University of Maastricht, The Netherlands.

Eur Resp J 2000; 16:437-444

Abstract

The nasal decongestants oxymetazoline and xylometazoline are frequently used in the topical treatment of rhinitis and sinusitis. As NO is thought to play a role in inflammation of the upper respiratory tract, the aim of this study was to examine the in vitro effects of these compounds on the activity and the expression of NO producing enzymes, iNOS and cNOS.

Experiments concerning the effects on enzymatic activity and enzyme induction of iNOS by both compounds were performed in a LPS induced rat alveolar macrophage cell line (NR8383) using the Griess assay and the ^{3}H-citrulline assay respectively. The effects on cNOS were examined in fresh rat synaptosomes using the ^{3}H-citrulline assay. The direct scavenging properties of both compounds were investigated using a amperometric NO sensor.

Oxymetazoline and xylometazoline were shown to have a dose dependent inhibitory effect on total iNOS activity indicated by nitrite/nitrate formation in the Griess assay. This effect was found to be due to an inhibition of induction of the enzyme rather than inhibition of the enzyme activity, as was investigated in two separate experiments using the ^{3}H-citrulline assay. Inhibition of cNOS was moderate and in the same order of magnitude as the inhibition of enzymatic iNOS activity. Direct scavenging of NO could not be detected. As cNOS activity is thought to serve beneficial physiological functions and exaggerated iNOS activity may cause exacerbation of the inflammatory process, pharmacological treatment influencing the NO generating system should focus on inhibition of iNOS alone. The specific characteristics of these decongestants *in vitro* suggests suitability for this application and may indicate an additional beneficial effect in the treatment of upper respiratory tract inflammation.

Introduction

Nitric oxide (NO) is a free radical which is known to have an important function as mediator in several biological processes amongst which vasorelaxation, cell-mediated immune responses, inhibition of platelet aggregation and neurotransmission are extensively investigated.[1-6] The formation of NO has been described in many mammalian cell types and can be addressed to three isoforms of nitric oxide synthase (NOS), which all oxidize L-arginine to L-citrulline. The two constitutive forms of NOS (cNOS), which are Ca^{2+}/calmodulin dependent, are typically found in neural and vascular endothelial cells. Endothelial NOS is located on the cell membrane and releases NO for short periods of time in response to receptor or physical stimulation. Neuronal NOS has been identified in the cytosol of central and peripheral neurons.[7] The inducible form of NOS (iNOS), which is Ca^{2+}-independent, can be found in inflammatory cells, such as macrophages, after they are activated by proinflammatory mediators like oxidants, cytokines and endotoxins. Once this enzyme is expressed it produces significantly larger amounts of NO for longer periods than the constitutive isoform does.[8,9]

Nitric oxide is known to play an important role in airway function and seems to be implicated in the pathophysiology of several lower airway diseases. Since there is an evident relationship between nasal inflammation and inflammatory pulmonary disease a role of NO in upper respiratory inflammations can be anticipated.

It has been reported that the levels of NO in exhaled air of patients with upper respiratory tract infections are increased.[10,11] Also it has been shown that considerable levels of NO metabolites were present in nasal lavage fluid of patients with house dust mite allergy and that nasal polyps contain higher levels of NOS than normal nasal mucosa.[12,13] In addition, the exact localization of NOS in nasal mucosa has been identified.[14,15] Furthermore, NOS activity seems to be involved in nasal vascular conductance and in nasal cavity volume.[16,17] Also NO, upon reaction with the superoxide anion radical and forming peroxynitrite, has been shown to act as potent cytotoxic effector molecule.[18] Both features may contribute to nasal inflammation and indicate the involvement of NO in the pathogenesis of upper respiratory tract inflammation.

In upper airway inflammation, nasal decongestants such as oxymetazoline and xylometazoline, are frequently used in the topical treatment. Their effectivity is thought to be due to direct α-adrenergic receptor stimulation on the vascular smooth muscle in the nasal mucosa, leading to vasoconstriction. However, it is justified to presume that, in the clinically used concentrations, which are in the millimolar range, other molecular mechanisms play a

role in the therapeutic effect. Recently we have reported on the distinct anti-oxidant properties of oxymetazoline and xylometazoline i.e., hydroxyl radical scavenging and lipid peroxidation inhibition.[19] Presuming that the NO generating system is involved in the pathogenesis of upper respiratory tract inflammation, the effects of oxymetazoline and xylometazoline on activity and expression of cNOS and iNOS were determined in an in vitro model. In addition, possible NO scavenging properties were investigated.

Materials and Methods

Chemicals

RPMI 1640 medium, HAM's F-12 medium, glutamine and Fungibact were obtained from BioWhittaker (Walkersville, MD, USA). Foetal calf serum (FCS), HEPES, Tris and EDTA were purchased from Life Technologies (Paisley, UK). Dowex was obtained from ACROS (Geel, Belgium). Leupeptin, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol, aprotin, lipopolysacharide (LPS), NADPH, L-arginine, N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), L-nitroarginine methyl ester (l-NAME), oxymetazoline, xylometazoline, N-(1-naphtyl)ethylenediamine, sulfanylamide and 8-anilino-1-naphtalene-sulfonic acid were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Trypsin inhibitor from soybean was obtained from Difco Laboratories Inc. (Detroit, Michigan, USA). Sucrose and EGTA were obtained from Merck (Darmstadt, Germany). Hydrogen peroxide was purchased from J.T. Baker (Deventer, Holland). [2,3,4,5]-^{3}H-L- arginine was obtained from Amersham International plc. (Little Chalfont, Buckinghamshire, UK). All other used chemicals were of reagent grade.

The macrophage cell line, NR8383 was kindly provided by Dr. R.J. Helmke, Department of Pediatrics, University of Texas Health Science Center, San Antonio, Texas, USA.

Klebsiella Pneumoniae were a kind gift from Dr. J. van Amsterdam, RIVM, Bilthoven, The Netherlands.

Study design

To determine the effects of oxymetazoline and xylometazoline on iNOS and cNOS experiments according to the diagram shown in figure 1 were conducted (Fig 1.). Inducible NOS was expressed in rat alveolar macrophages by adding LPS in the presence and absence of the test compounds. Primarily the effects on total iNOS activity were measured by the formation of nitrate and nitrite which was determined in the Griess-assay.[20] To elucidate the findings, a second set of experiments was performed using the ^{3}H-citrulline assay, in which the conversion of tritiated L-arginine to L-citrulline is measured.[21] By adding the test compounds only during LPS induction or only after LPS induction, effects on respectively enzyme induction and enzymatic activity could be separated. In addition the effects of the test compounds on cNOS activity were examined, using the same ^{3}H-citrulline assay. NO scavenging was investigated using a amperometric NO sensor.

All experiments were carried out in triplicate. Results are presented as mean ± SD. The different parts of the experiments are described in more detail here below.

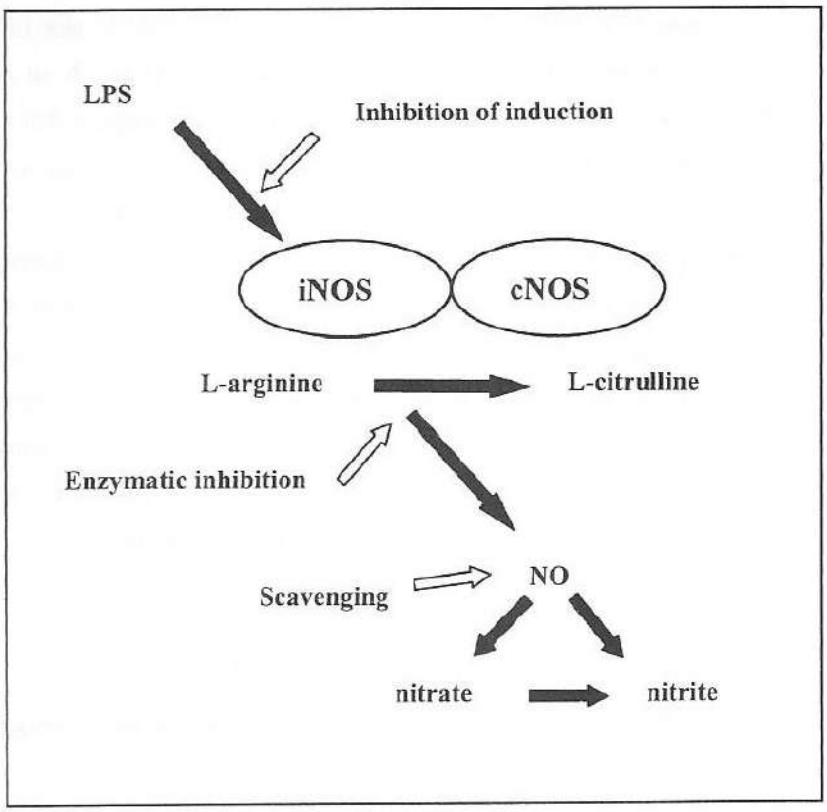


Figure 1. Study design. In order to establish the overall effect of oxymetazoline and xylometazoline and reference compound L-nitroarginine methyl ester (L-NAME) on the activity of lipopolysaccharide (LPS) induced nitric oxide synthase (iNOS), total nitrite production was measured first (Griess-assay). To separate the effects of the test compounds on induction inhibition and enzymatic activity inhibition, the formed amount of radioactive L-citrulline was measured in a second experiment (^{3}H-citrulline assay). In these experiments test compound was only added during LPS induction or during conversion of L-arginine to L-citrulline. In addition, the effects of test compounds on constitutive nitric oxide synthase (cNOS) activity (^{3}H-citrulline assay) and nitric oxide scavenging (amperometric NO sensor method) were investigated.

Cell culture and induction of NO-synthase

A rat pulmonary alveolar macrophage cell line, NR8383, was used as the source of inducible NO-synthase.[22,23] Cells were maintained in culture at a floating cell concentration of 10^5 cells/ml in RPMI 1640 medium containing 2 mM glutamine, 0.5% Fungibact (50 U/ml penicillin, 50µg/ml streptomycin) and 10% heat inactivated FCS. Cells were grown on 162-cm^2 flasks (Costar Cambridge, MA) in a humidified, 37 °C, 5% CO_2 incubator. Cells were collected (112 x g, 1000 rpm, Heraeus Labofuge 400R, 5 min, 25 °C) and resuspended at a cell concentration of 10^6 cells/ml in HAM's F-12 medium containing 2 mM glutamine and 0.5% Fungibact (50 U/ml penicillin, 50µg/ml streptomycin). NO-synthase was induced by addition of 10 µg bacterial LPS /10^6 cells with or without the test compounds oxymetazoline and xylometazoline in different concentrations. The samples were incubated during 24 h

(37 °C, 5% CO_2). As reference compound the known NOS inhibitor L-NAME was used.

Reduction of nitrate to nitrite and determination of nitrite: the Griess-assay

In order to measure the effect of LPS on total nitrite/nitrate formation as well as the inhibition by L-NAME, oxymetazoline and xylometazoline on the LPS effect, cells were incubated for 24 h with LPS and in the absence

and presence of the test compounds in final concentrations varying between 10^{-5} M and 10^{-3} M. An aliquot of 150 µl of the cell suspension was deproteinated by adding 20 µl NaOH (1 M) and 20 µl $ZnSO_4$ (1 M). After shaking firmly the incubations were left on ice for 5 minutes and centrifuged (5 min, 1,500 x g at 4 °C, Heraeus Christ, Biofuge A). For the conversion of nitrate to nitrite Klebsiella Pneumoniae (1.4 mg/ml in 50mM PBS, pH = 7.4) was mixed with 2 vol. of 0.2 M TES (pH = 7.0) and 2 vol. of 0.5 M sodium formate. Of this mixture 50 µl was added to 100 µl of the deproteinated sample. Nitrate reduction was done at room temperature in a vacuum desiccator containing a dish of water to reduce evaporisation of the reaction mixtures. After 30 minutes the tubes were removed and 0.5 ml of nitrogen flushed water was added. After centrifugation (5 min, 15.000 x g at 4° C) 0.5 ml of the incubate mixture was added to 50 µl of sulfanyl amide (1% in 5% H_3PO_4) and the tubes were placed at 4 °C for 10 minutes. After this incubation 50 µl of 0.1% ($^{w}/_{v}$) N-(1-naphtyl) ethylenediamine was added and within 30 minutes the absorption of nitrite was determined at 540 nm in a Packard Argus 400 microplate reader. Standards containing 0 - 150 µM $NaNO_2$ and $NaNO_3$ were used for quantification.

Determination inhibition of NO-synthase activity: ^{3}H-citrulline assay

In order to separate the effects of the test compounds on induction of iNOS and on the enzymatic iNOS activity per se, two different sets of experiments were performed.

To establish the effect of the test compounds on iNOS induction, a 24 h incubation of the N8383 cells with LPS was performed in the presence of test compounds. After the 24 h incubation period the test compounds were removed by washing. To establish the effect of the test compounds on iNOS enzymatic activity cells were preincubated for 24 h with LPS in the absence of test compounds. These were only added during the conversion of L-arginine to L-citrulline in the ^{3}H-citrulline assay.

After incubation with LPS, with or without test compounds (10^{-4} to 10^{-3} M), N8383 cells were collected and resuspended at a cell concentration of 10^7 cell/ml in Tris/HCl-buffer (50 mM, pH = 7.4 at 4 °C) containing 2µM leupeptin, 1 mM PMSF, 1 mM dithiothreitol, 10 µg/ml trypsin inhibitor from soybean, 2 µg/ml aprotinin, 0.1 mM EDTA and 230 mM sucrose. The cell suspension was subsequently sonificated for 5 minutes at 4° C. Of the obtained cell suspension 40 µl was added to 60 µl of a Tris/HCl-buffer (50 mM, pH = 7.4 at 37 °C) containing 1 mM NADPH, 10 µM L-arginine and 20 nCi [^{3}H]-L-arginine (specific activity; 63 Ci/mmol). For measuring the enzymatic inhibition test compounds were added in a concentration of 10^{-4} to 10^{-3} M. Activity of iNOS was measured in the presence of 1mM EGTA whereas cNOS activity was determined using 2 mM $CaCl_2$. Non-enzymatic conversion was determined by heating the cell suspension (100 °C for 2 min). Both cNOS activity and non-enzymatic conversion were not found. The reaction was carried out for 45 minutes at 37 °C and terminated by adding 1 ml of ice-cold Hepes-buffer (20 mM, pH = 5.5 at 4 °C) and putting the vials on ice. L-arginine was removed from the reaction mixture by adding 1 ml of the ion-exchanger Dowex (H_2O/Dowex-50W, 1:1, Na^+-form, 200-400 mesh, 8% cross-links, pH = 7.0) and shaking firmly. After centrifugation (15 min, 2,100 x g at 4 °C) the reaction product [^{3}H]-L-citrulline was measured by transferring 1 ml of the supernatant to scintillation vials containing 4 ml Packard Ultima Gold scintillation fluid. Radioactivity was counted in a Packard Tri-carb (1900 CA) liquid scintillation analyzer. All samples were corrected for background radioactivity.

The same ^{3} H-citrulline assay was used to determine the possible inhibitory effects of oxymetazoline and xylometazoline (concentrations between 10^{-4} M and $3x10^{-3}$ M) on cNOS activity. In these experiments enzymatic activity was measured in the presence of
2mM $CaCl_2$. L-NAME was used as reference compound. However these measurements were performed in synaptosomes obtained from the cerebella of male Wistar rats as these cells are known to contain high levels of cNOS. Activity of iNOS could not be detected in this tissue as was shown by using a calcium free buffer.

Determination of cell viability

In the experiments in which the alveolar cell line, NR 8383, was used, the viabilty of the cells was tested at different stages of the experiments using thrypan blue. When in a sample more than 5 % of the cells were stained with thrypan blue and therefore not viable the sample was not further used.

Determination of NO-scavenging

Possible NO-scavenging properties of both compounds were investigated by using a amperometric NO-sensor.[24] As reference compound the well known NO scavenger hemoglobin was used. Two µl of NO-spiked deoxygenated water was added to 20 ml 50 mM phosphate buffer (pH = 7.4) in a thermostated vessel (37° C). The vessel was kept under a N_2 atmosphere. The decrease in NO concentration, which was measured with an Iso-NO meter (World Precision instruments, Sarasota FL, USA) coupled to both a MacLab™ interface (ML020 MacLab/8, AdInstruments, London, UK) on an Apple Macintosh computer with "Chart" software an a chart recorder (Kipp, Delft, The Netherlands), was followed in time in the presence and absence of the test compounds oxymetazoline and xylometazoline (final concentrations up to 10^{-3} M) and reference compound (final concentrations used were
3.9×10^{-7} and 7.8×10^{-7}) in solution. During this procedure the reaction mixture was mixed using a magnetic stirrer.

Results

Determination of inhibition of iNOS activity

1. Nitrite formation after LPS stimulation.

The effects of L-NAME, oxymetazoline and xylometazoline on the production of nitrite (NO_2^-) and nitrate (NO_3^-), which are formed after oxidation of the unstable NO, were determined in the Griess-assay. The amount of nitrite measured after nitrate reduction, reflects the iNOS activity. Nitrite formation could only be measured in LPS induced cells. When no test compound was added the LPS induced cells gave a mean total Nitto concentration of 117 ± 12 µM. All data concerning iNOS are presented as the fraction of this value. All compounds showed a dose dependent inhibitory effect on the nitrite formation (Figure 2). In contrast to L-NAME, oxymetazoline and xylometazoline showed no inhibition of nitrite formation upto a concentration of 10^{-4} M whereas at this concentration L-NAME already gave 25 % inhibition.

However, oxymetazoline and xylometazoline inhibited the nitrite formation completely at a concentration of 1 mM whereas L-NAME only inhibited approximately 60%.
To exclude possible inhibition of cell growth or reaction with components of the Griess-assay by the compounds, control experiments were performed. None of these confounding reactions occurred.

2. Citrulline formation after LPS stimulation in the presence of test compounds.

To elucidate the mode of action of this inhibitory effect, the compounds were investigated in the ^{3}H-citrulline assay in which the effect of the compounds on iNOS induction and enzymatic iNOS activity could be separated. L-citrulline is formed from L-arginine by NOS under the production of NO. The amount of L-citrulline production reflects the enzymatic activity of NOS. When cells were not stimulated with LPS, no L-citrulline could be detected. The baseline radioactivity value of the formed ^{3}H-citrulline was determined in LPS induced cells to which no test compound was added. The inhibition by the test compounds is presented as a fraction of the LPS induced value. Different concentrations of L-NAME, oxymetazoline and xylometazoline were present during LPS induced iNOS induction for 24 hours and then removed by washing. Oxymetazoline and xylometazoline showed a prominent dose dependent reduction of L-citrulline formation (Figure 3). More than 50 % inhibition of L-citrulline formation was obtained for both compounds at a concentration of 0.3 mM, whereas complete inhibition for both compounds was obtained at 1 mM. L-NAME also showed some reduction however, at a concentration of 1 mM only 50 % reduction was found.

3. Citrulline formation after LPS stimulation in the absence of test compounds: inhibition of enzymatic iNOS activity

When cells were not stimulated with LPS, no L-citrulline could be detected. The baseline radioactivity value of the formed ^{3}H-citrulline was determined in LPS induced cells to which no test compound was added. The inhibition by the test compounds is presented as a fraction of the LPS induced value.
Oxymetazoline and xylometazoline showed a moderate concentration dependent inhibitory effect on the LPS stimulated iNOS activity (Figure 4). At a concentration of 1 mM, added after 24 hours of LPS stimulation, both compounds inhibited iNOS activity for approximately 50 %, whereas L-NAME inhibited almost completely (85 %) even at a 10 times lower concentration. The test compounds did not influence the separation of ^{3}H-citrulline and ^{3}H-arginine.

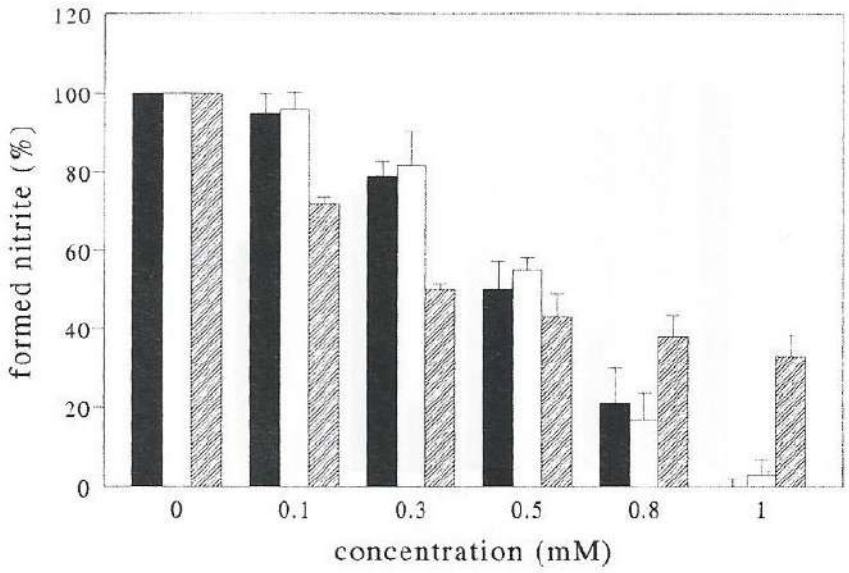


Figure 2. The overall inhibitory effect of oxymetazoline (■) and xylometazoline (□) on inducible nitric oxide synthase (iNOS) was investigated by measuring nitrite production in the Griess-assay. L-nitroarginine methyl ester (L-NAME) (▨) was used as a reference compound. The inhibition of nitrite formation was concentration dependent for all compounds. At a concentration of 1 mM total inhibition of NO production was shown for oxymetazoline and xylometazoline. L-NAME, on the other hand, only inhibited nitrite production for approximately 60 %.

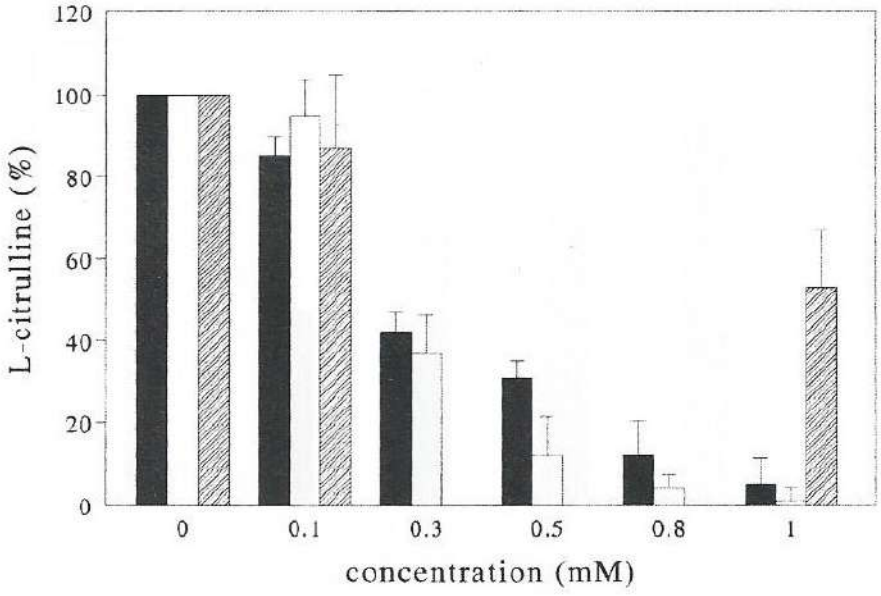


Figure 3. The inhibitory effect of oxymetazoline (■) and xylometazoline (□) on the lipopolysaccharide (LPS) induced inducible nitric oxide synthase (iNOS) induction measured by the formation of L-citrulline using the ^{3}H-citrulline assay. L-nitroarginine methyl ester (L-NAME) (▨)was used as a reference compound. In contrast to L-NAME a strong concentration dependent reduction of L-citrulline formation was seen for oxymetazoline and xylometazoline, which completely inhibited iNOS induction at a concentration of 1 mM. At this concentration L-NAME only gave 50 % inhibition.

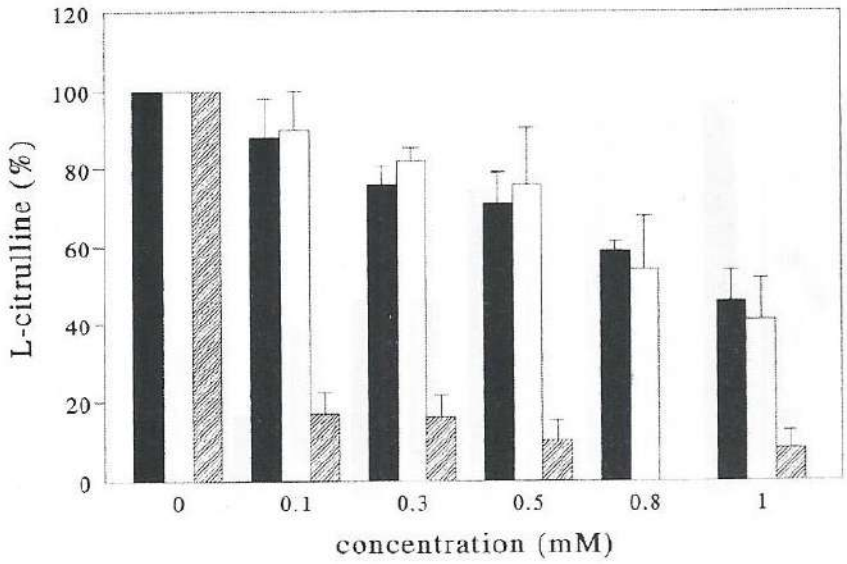


Figure 4. The inhibitory effect of oxymetazoline (■) and xylometazoline (□) on the enzymatic inducible nitric oxide synthase (iNOS) activity measured by formation of L-citrulline using by the ^{3}H-citrulline assay. L-nitroarginine (L-NAME) (▨) was used as reference compound. Compounds were only added to the reaction mixture during incubation with L-arginine. A moderate concentration dependent reduction in L-citrulline formation was seen for oxymetazoline and xylometazoline. In contrast to L-NAME, which gave prominent inhibition of enzymatic iNOS activity at a concentration of 10^{-4} M, oxymetazoline and xylometazoline inhibited L-citrulline formation for approximately 50 % at a concentration of 10^{-3} M.

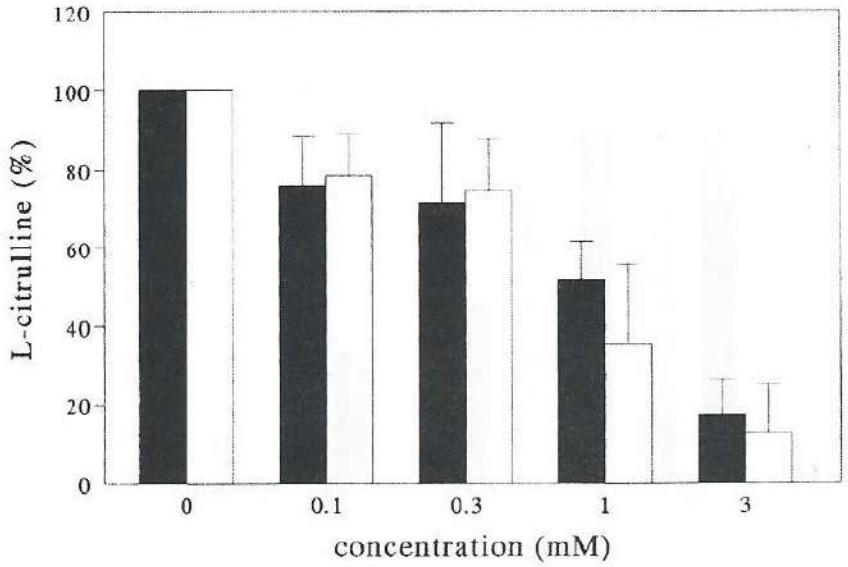


Figure 5. The inhibitory effect of oxymetazoline (■) and xylometazoline (□) on the enzymatic acivity of constitutive nitric oxide synthase (cNOS) measuring L-citrulline formation using the ^{3}H-citrulline assay. The L-citrulline production was concentration dependent diminished by both compounds. At a concentration of 10^{-3} M oxymetazoline inhibited cNOS by 50 % and xylometazoline by 65 %. The effects of both compounds on the enzymatic activity of cNOS are comparable to their effects on the enzymatic activity of inducible nitric oxide synthase (iNOS).

Determination of the inhibition of cNOS

The baseline radioactivity value of the formed ^{3}H-citrulline was determined in samples to which no test compound was added. The data are presented as fractions of the absolute baseline values.

The inhibition of cNOS activity in rat brain tissue by oxymetazoline and xylometazoline was concentration dependent and in the same order of magnitude as inhibition of enzymatic iNOS activity measured by the ^{3}H-citrulline assay. At a concentration of 1 mM oxymetazoline and xylometazoline inhibited L-citrulline formation for 50 % and 65 % respectively (Figure 5). The inhibition activity of L-NAME, as was expected, was much more prominent. At a concentration of 0.3 μM already 50% of cNOS activity was inhibited (data not shown).

Determination of NO radical scavenging

In contrast to the reference hemoglobin which showed potent scavenging, no scavenging of NO radicals occurred by either oxymetazoline or xylometazoline added to the reaction mixture at final concentrations up to 10^{-3} M (Figure 6).

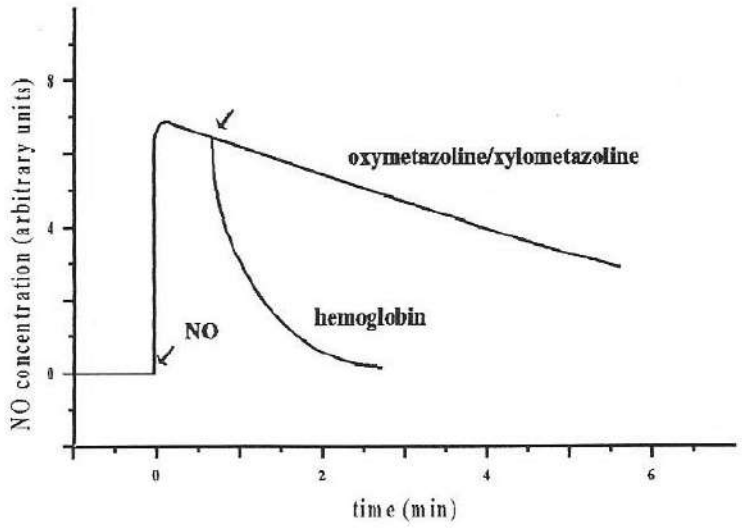


Figure 6. Characteristic example of scavenging experiment. Time dependent degradation of nitric oxide (NO) in buffer in the presence of a possible scavenger (arrow). A concentration of 1mM of both test compounds did not enhance the degradation of measured NO in time, indicating that no scavenging of NO occured. In contrast to the decongestive compounds (oxymetazoline and xylometazoline), the reference compound hemoglobin (7.8×10^{-7}) showed potent time dependent NO degradation.

Discussion

We investigated the effects of the nasal decongestants oxymetazoline and xylometazoline on the induction and activity of iNOS and the activity of cNOS. NOS activity is of importance in inflammatory disorders of the upper respiratory tract via at least two mechanisms. First the regulation of nasal vascular tone is thought to be under cNOS control and secondly iNOS is important in the modulation of the inflammatory and immunological response.[14,16,18,25] As high doses of oxymetazoline and xylometazoline are frequently used in the topical treatment of upper respiratory tract inflammation, the effect of these compounds on NO regulation is of interest.

LPS was used to induce iNOS in a macrophage cell line. As was measured by the nitrite/nitrate formation during 24 h the false substrate for NOS, L-NAME, was able to decrease the amount of NO metabolites dose dependently, although not completely. Since oxymetazoline and xylometazoline in the same concentration were capable of complete inhibition of iNOS, this finding suggests at least an additional mode of pharmacological action of these compounds. It should be noted that in the nitrite/nitrate experiments NO was formed as an intermediate and that the inhibitory effects on NOS could be due to scavenging of NO by the used test compounds. To exclude this, the test compounds were investigated for possible NO scavenging properties. No nitric oxide scavenging occurred.

Because decreased NO formation could be explained by either, the inhibition of enzymatic activity or the inhibition of induction, these were investigated separately. Oxymetazoline and xylometazoline were not full inhibitors of both iNOS and cNOS activity, measured by ^{3}H-citrulline formation. This in contrast to L-NAME which showed complete enzymatic inhibition of NOS, which is in accordance with literature.[26] We suggest that the incomplete enzymatic inhibition of both iNOS and cNOS by oxymetazoline and xylometazoline may be due to binding of the imidazoline part of these compounds to the heme domain of NOS. This mode of action is comparable but less efficient than for arginine derivatives such as L-NAME.[27]

In this way it was proven that the inhibitory effect of oxymetazoline and xylometazoline could not be fully explained by enzyme inhibition. Inhibition of induction of the iNOS enzyme might therefore be involved as well. This was investigated in an experiment in which iNOS enzyme activity was measured after washing away the test compounds. I.e. the test compounds were present during LPS induction of iNOS. Both compounds, in contrast to L-NAME, completely inhibited spontaneous iNOS activity and thus expression of iNOS.

It is known that the induction of iNOS is regulated by transcription factors, of which the most important is nuclear factor-κB (NF-κB).[28] As NF-κB in cells is activated by many factors such as microorganisms, oxidants, antigens and LPS a possible site of action of oxymetazoline and xylometazoline may be the inhibition of the activation of NF-κB.[29]

The nasal mucosa has an important function in preparing the inspired air before entering the lungs through filtering particles and regulating humidity and temperature. NO seems to play a pivotal role in these physiological functions as amongst others it has been shown that NO is involved in the regulation of the vascular tone in the nasal mucosa.[25,30] By this means NO is thought to modulate nasal vascular conductance and nasal cavity volume, which are correlated to the nasal airway resistance.[16,17] It should be noted however, that recent investigations, using high flow rate insufflation, contradict the existence of such a relationship.[31,32] The difference in these results is thought to be due to underestimation of measured NO outputs as low flows were used instead of higher and probably more physiologic transnasal airflows.[33] Another possibility is that this discrepancy might be explained by the fact that NO levels measured in nasal air, which are predominantly produced by a Ca^{2+} independent form of NOS in epithelial cells in the paranasal sinuses are not responsible for vascular tone regulation which may be predominantly under control of nasal vascular epithelial cNOS.[34,35] This speculation might be supported by the finding that lidocain showed strong inhibition of sinus NO production in contrast to nasal NO production.[36]

Furthermore, it has been shown that human nasal mast cells, play, next to alpha adrenoceptor effects, a major role in the regulation of the blood supply to the nasal mucosa. They contain a particulate isoform of NOS which besides other vasoactive substances may effect the congestive state of the nasal mucosa.[37] In addition, nasal exudation, of importance in nasal inflammation, also seems to be mediated by NO via increase of the vascular permeability.[38] NO, generated via iNOS, which is induced in inflammatory cells and epithelium of the upper respiratory tract, is an important mediator in modulating the inflammatory and immunological response.[18,39] Involvement of NO was shown for rhinitis based on house dust mite allergy, seasonal rhinitis, chronic rhinitis and nasal polyps.[11,13,14,15] NO levels are high in exhaled air, which was shown to be originating mainly from the nasal and paranasal airways.[34,40,41,42] These high concentrations of the reactive NO may contribute to aggravate airway inflammation, which has been shown after e.g. viral airway infection.[43] The involvement of iNOS may be supported by the finding that glucocorticoids, which amongst others inhibit the induction of iNOS and have beneficial effects in treating airway inflammation, inhibit the increase in exhaled NO in asthmatic patients.[44]

All formerly mentioned studies indicate that NO has a role in upper respiratory tract inflammation. This role may be influenced by nasal decongestants like oxymetazoline and xylometazoline. Although, the role of nasal NO in nasal vascular regulation is not clearly established most studies support the observation that administration of nasal decongestants significantly decreases nasal NO concentration.[30,31] The mechanism by which decongestants reduce NO concentration is not known. It was speculated to be due to vasoconstriction of the nasal venous sinusoids which may lead to a decreased epithelial surface area affecting the NO diffusion into the lumen of the nasal cavity.[45] It may also be related to the reduction of bloodflow leading to a diminished availability of substrates for NOS or by a toxic effect of the decongestant in the chain of reactions that produces NO.[31] Until now a specific site of action could not be ascertained.

In our study we tried to elucidate this mode of action as a direct inhibitory effect on enzymatic activity of cNOS and iNOS, but especially induction of iNOS was shown. In addition, no direct scavenging effect of both compounds could be found.

The results were obtained in rat alveolar macrophages as these cell lines are a reliable source of iNOS.[22,23] As it is known that differences in the NOS systems have been found between cell types of animals of different species and humans, our results can not be directly extrapolated to human upper airways.[9] However, although this is an in vitro model the results may contribute to the understanding of NO in upper respiratory tract pathophysiology and the influence of nasal decongestants herein. Presuming that the NO generating system plays a role in the pathogenesis of upper respiratory tract inflammations, our findings may suggest an additional beneficial effect of the nasal decongestants oxymetazoline and xylometazoline in the topical treatment of these disorders. It should be noted, however, that these compounds, at least in their pharmaceutical matrix, also have unfavorable effects on nasal mucosa like decreasing mucociliary clearance, and inducing rhinitis medicamentosa.[46,47,48]

In conclusion, studies mentioned above imply that cNOS activity serves beneficial physiological functions in the nose, whereas an exaggerated iNOS activity, which is induced by overexpression, causes an exacerbation of the inflammatory process. Possible treatment via NO mechanisms should therefore primarily focus on inhibition of iNOS alone, leaving the necessary physiological control via cNOS intact. In an in vitro model oxymetazoline and xylometazoline showed only moderate effects as direct inhibitors of the NO-producing enzymes. However, these compounds, used in their clinically applied concentration, are able to inhibit the induction of iNOS completely, which renders them selective tools for attenuation of iNOS activity, a mode of action which has not been described before.

References

1. Furchgott RF, Zawadski JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980; 288:373-376.
2. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhury G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc Natl Acad Sci USA 1987*; 84:9265-9269.
3. Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishnor JS. Macrophage oxidation of L-arginine to nitrite and nitrate: Nitric oxide is an intermediate. *Biochemistry* 1988; 27:8706-8711.
4. Hibbs JB Jr, Taintor RR, Vavrin Z, Rachlin EM. Nitric oxide: A cytotoxic activated macrophage effector molecule. *Biochem Biophys Res Commun* 1988;157:87-94.
5. Radomski MW, Palmer RMJ, Moncada S. Endogenous nitric oxide inhibits human platelet adhesion in vascular endothelium. *Lancet* 1987; 2:1037-1038.
6. Knowles RG, Palacios M, Palmer RMJ, Moncada S. Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. *Proc Natl Acad Sci USA* 1989; 86:5159-5162.
7. Bredt DS, Glatt CE, Fotuhi M, Dawson TM, Snyder SH. Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. *Neuron* 1991; 7:615-624.
8. Moncada S, Palmer RMJ, Higgs EH. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharm Rev* 1991; 43:109-142.
9. Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J* 1994; 298:249-258.
10. Kharitonov SA, Yates D, Barnes PJ. Increased nitric oxide in exhaled air of normal human subjects with upper airway respiratory tract infections. *Eur Resp J* 1995; 8:295-297.
11. Martin U, Bryden K, Devoy M, Howarth P. Increased levels of exhaled nitric oxide during nasal and oral breading in subjects with seasonal rhinitis. *J Allergy Clin Immunol* 1996; 97:768-772.
12. Garrelds IM, Amsterdam van JCG, De Graaf-in't Veld C, Gerth van Wijk R, Zijlstra FJ. Nitric oxide metabolites in nasal lavage fluid of patients with house dust mite allergy. *Thorax* 1995; 50:275-279.
13. Ramis I, Lorente J, Rosello-Catafau J, Quesada P, Gelpi E, Bulbena O. Differential activity of nitric oxide synthase in human nasal mucosa and polyps. *Eur Resp J* 1996; 9:202-206.
14. Furakawa K, Harrison DG, Saleh D, Shennib H, Chagnon FP, Glaid A. Expression of nitric oxide synthase in the human nasal mucosa. *Am J Respir Crit Care Med* 1996; 153:847-850.
15. Rosbe KW, Mims JW, Prazma J, Petrusz P, Rose A, Drake AF. Immunohistochemical localisation of nitric oxide synthase activity in upper respiratory epithelium. *Laryngoscope* 1996; 106:1075-1079.
16. Rinder J, Lundberg JM. Nasal vasoconstriction and decongestant effects of nitric oxide synthase in the pig. *Acta Physiol Scand* 1996; 157:233-244.
17. Imada I, Iwamoto J, Nonaka S, Kobayashi Y, Unno T. Measurement of nitric oxide in human nasal airway. Eur Resp J 1996; 9:556-559.
18. Albina JE, Reichner JS. Nitric oxide in inflammation and immunity. *New Horizons* 1995; 3:46-64.

19. Westerveld GJ, Scheeren RA, Dekker I, Griffioen DH, Voss H-P, Bast A. Antioxidant levels of oxymetazoline and xylometazoline. *Eur J Pharmacol* 1995; 291:27-31.

20. Phizackerly PJR, Al-Dabbagh SA. The estimation of nitrate and nitrite in saliva and urine. *Anal Biochem* 1983;131:242-245.

21. Bredt DS, Snyder SH. Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *Proc Natl Acad Sci USA* 1990; 87:682-685.

22. Helmke RJ, Boyd RL, German VF, Mangos JA. From growth factor dependence to growth factor responsiveness: the genesis of an alveolar macrophage cell line. *In Vitro Cell Development Biol* 1987; 23:567-574.

23. Helmke RJ, German VF, Mamgos JA. A continuous alveolar macrophage cell line: comparisons with freshly derived alveolar macrophages. *In Vitro Cell Development. Biol* 1989; 25:44-48.

24. Vriesman MF, Haenen GRMM, Westerveld GJ, Paquay JBG, Voss H-P, Bast A. A method for measuring nitric oxide radical scavenging activity. Scavenging properties of sulfur containing compounds. *Pharmacy World & Sci* 1997; 19:283-286.

25. Rinder J. Sensory neuropeptides and nitric oxide in nasal vascular regulation. *Acta Physiol Scand* 1996; 157:7-45.

26. Mitchell JA, Kohlhaas KL, Sorrentino R, Warner TD, Murad F, Vane JR. Induction by endotoxin of nitric oxide synthase in the rat mesentery: lack of effect on action of vasoconstrictors. *Br J Pharmacol* 1993; 109:265-270

27. Peterson DA, Peterson DC, Archer S, Weir EK. The non specific nitric oxide inhibitors. *Biochem Biophys Res* 1992; 187:797-801.

28. Xie Q-W, Kashiwabara Y, Nathan C. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J Biol Chem* 1994; 269:4705-4708.

29. Barnes PJ, Karin M. Nuclear factor κB - A pivotal transcription factor in chronic inflammatory disease. *New Engl J Med* 1997; 336:1066-1071.

30. Rinder J, Lundberg JON, Änggård A, Alving K, Lundberg JM. Effects of topical nasal decongestants, L-arginine and nitric oxide synthase inhibition, on nasal cavity nitric oxide levels and nasal cavity volume in man. *Am J Rhinol* 1996; 10:399-408.

31. Chatkin JM, Djupesland PG, Qian W, McClean P, Furlott, Gutierrez C, Zamel N, Haight JS. Nasal nitric oxide is independent of nasal cavity volume. *Am J Rhinol* 1999; 13:179-184.

32. Silkoff PE, Chatkin J, Qian W, Chakravorty S, Gutierrez C, Furlott H, McClean P, Rai S, Zamel N, Haight J. Nasal nitric oxide: a comparison of measurement techniques. *Am J Rhinol* 1999; 13:169-178.

33. Djuppesland PG, Chatkin JM, Qian W, Cole P, Zamel N, McClean P, Furlott H, Haight JS. Aerodynamic influences on nasal nitric oxide output measurements. *Acta Otolaryngol* 1999; 119:479-485.

34. Lundberg JON, Farkas-Szallasi T, Weitzberg E, Rinder J, Lidholm J, Änggård A, Hökfelt T, Lundberg JM, Alving K. High nitric oxide production in human paranasal sinuses. *Nature Med* 1995; 1:370-373

35. Lundberg JON, Weitzberg E, Rinder J, Rudehill A, Jansson O, Wiklund NP, Lundberg JM, Alving K. Calcium-independent and steroid-resistant nitric oxide synthase activity in human paranasal sinus mucosa. *Eur Respir J* 1996; 9;1344-1347.

36. Haight JS, Djupesland PG, Qian W, Chatkin JM, Furlott H, Irish J, Witterick I, McClean P, Fenton RS, Hoffstein V, Zamel N. Does nasal nitric oxide come from the sinuses ? *J Otolaryngol* 1999; 28:197-204.

37. Bacci S, Arbi-Riccardi R, Mayer B, Rumino C, Borghi-Cirri MB. Localisation of nitric oxide synthase immunoreactivity in mast cells of human nasal mucosa. *Histochemistry* 1994; 102:89-92.

38. Lane AP, Prazma J, Baggett HC, Rose AS, Pillsbury HC. Nitric oxide is a mediator of neurogenic vascular exudation in the nose. *Otolaryngol Head Neck Surg* 1997; 116:294-300.

39. Croen KD. Evidence for an antiviral effect of nitric oxide. *J Clin Invest* 1993; 91:2446-2452.

40. Schedin U, Frostell C, Persson MG, Jakobsson J, Andersson G, Gustafsson LE. Contribution from upper and lower airways to exhaled endogenous nitric oxide in humans. *Acta Anaesthesiol Scand* 1995; 39:327-332.

41. Lundberg JON, Weitzberg E, Nordvall SL, Kuylenstierna R, Lundberg JM, Alving K. Primarily nasal origin of exhaled nitric oxide and absence in Kartagener's syndrome. *Eur Resp J* 1994; 7:1501-1504.

42. Kimberly, Nejadnik B, Giraud GD, Holden WE. Nasal contribution to exhaled nitric oxide at rest and during breathholding in humans. *Am J Respir Crit Care Med* 1996; 153:829-836.

43. Barnes, PJ, Liew FY. Nitric oxide and asthmatic inflammation. *Immunol Today* 1995; 1:128-130.

44. Kharitonov SA, Yates D, Robbins RA, Thomas PS, Barnes PJ. Corticosteroids decrease exhaled nitric oxide. *Am J Respir Crit Care Med* 1994; 149:A201.

45. Ferguson EA, Eccles R. Changes in nasal nitric oxide concentration associated with symptoms of common cold and treatment with a topical nasal decongestant. *Acta Otolaryngol (Stockh)* 1997; 117:614-617.

46. Van de Donk HJM, Van den Heuvel AGM, Zuidema J, Merkus FWHM. The effect of nasal drops and their additives on human mucociliary clearance. *Rhinology* 1982; 20:127-137.

47. Deitmer T, Scheffler R. The effects of different preparations of nasal decongestants on ciliary beat frequency in vitro. *Rhinology* 1993; 31:151-153.

48. Graf PM, Hallen H. One year follow-up of patients with rhinitis medicamentosa after vasoconstrictor withdrawal. *Am J Rhinol* 1997; 11:67-72.

Chapter IV

General discussion and summary

General discussion

In the Netherlands chronic upper respiratory tract inflammations are the most frequent occurring chronic diseases (8.9 %) with a rising prevalence.[1] Although extensive research has been done, the exact multifactorial pathogenesis of disorders such as, chronic sinusitis and nasal polyps remains unclear. In addition, therapeutic management of these disorders by either surgery, medication or a combination of both, is characterized by a generally moderate outcome with a relatively high number of recurrences.[2,3] It has become clear that oxidative stress is associated with the pathophysiology of chronic inflammatory disorders of the lungs.[4] As it has also been reported that there is a pathophysiologic relationship between chronic inflammation of the lower- and upper respiratory tract, a role of oxidative stress in the pathogenesis of chronic upper airway inflammation can be anticipated. In fact, recently the involvement of oxidative stress has been reported.[5,6] To elucidate this subject further we have elaborated on the possible role of oxidative stress in the pathogenesis of chronic upper respiratory tract inflammation focussing on antioxidant mechanisms.

Oxidative stress and disease

Oxidants such as, reactive oxygen species (ROS) and reactive nitrogen species (RNS), play an important role in a broad spectrum of physiological processes. Despite their physiological importance there is increasing evidence that these species are also potentially harmful and responsible for tissue damage.[7] During inflammation, activated inflammatory cells infiltrate the upper respiratory tract mucosa and release large amounts of oxidants, such as the superoxide anion radical ($O_2^{\cdot-}$), and the nitric oxide radical ($NO^{\cdot}$). From these oxidants other reactive products i.e., the hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), hypochlorous (HOCl), and peroxynitrite ($ONOO^-$) can be formed. To protect the mucosa against the assault of these reactive species, it contains an extensive network of enzymatic and non enzymatic antioxidant defense mechanisms.[8] When there is an imbalance between local oxidant production and antioxidant defense, a situation known as oxidative stress, tissue injury contributing to the disease may occur. It is thought that in most human diseases oxidative stress is a consequence and not a cause of the primary disease as the release of ROS and RNS is secondary to the primary tissue injury caused by for instance, the infection.[9] However, oxidative stress induced tissue injury may very well contribute to the chronicity of the disease, especially when the causative agent has disappeared. This feature may be of importance in the pathogenesis of chronic upper respiratory tract inflammation as this is considered to be a non infectious disease. In fact this phenomenon has been shown for chronic

middle ear inflammation, in which ongoing oxidative mucosal damage was shown in absence of microorganisms.[10] Direct measurements on ROS and RNS production in human tissue and blood are technically difficult. One of the main reasons for this is the extremely short half lifetimes of most of these species due to their high reactivity. It is therefore more common to measure more stable products, so-called biomarkers, that reflect ROS induced tissue injury.[6,10,11,12] Another reliable method to get more insight in oxidative stress related diseases is to measure the antioxidant status. In fact, measurements have been done on antioxidant mechanisms in the upper respiratory tract.[13,14]

Antioxidants in chronic upper airway inflammation

In chapter I-3 we summarized the available literature on oxidants and antioxidants occurring in the upper respiratory tract both under normal as under inflammatory circumstances. Although some reports are contradictionary we feel that it is justified to say that a dysbalance between oxidant formation and antioxidant defense mechanisms mediates various types of upper respiratory tract inflammations. The majority of reports concerning oxidative stress in upper respiratory tract inflammation consist of measurements of antioxidants in upper respiratory tract lining fluids. Surprisingly, much less information is available about the antioxidant levels in upper respiratory tract mucosa and blood of humans, especially under inflammatory conditions of the upper respiratory tract. In addition, most reports show results of measurements performed on animals which may not directly be extrapolated to the human situation. To get more insight into this subject we measured, the levels of three biologically important antioxidants in nasal mucosa of patients with chronic sinusitis and healthy controls (chapter II-1). The levels of the intracellular antioxidant glutathione (GSH) and the extracellular antioxidant uric acid (UA) proved to be significantly lower in patients suffering from chronic sinusitis than in healthy control material. Vitamin E levels did not differ significantly between both subject groups. Although the number of subjects in both groups were small the measured differences were statistically significant indicating an impaired mucosal antioxidant defense under chronic inflammatory conditions. This lowered mucosal antioxidant defense can be explained by either an increased oxidant production leading to increased antioxidant consumption or diminished antioxidant supply from the bloodstream to the mucosa. We did not find directly increased oxidant production in nasal mucosa. However, there is circumstantial evidence in literature that this phenomenon in fact occurs in chronic upper respiratory tract inflammation.[5,6] To investigate the antioxidant supply from the bloodstream to the mucosa we measured the antioxidant levels of the same antioxidants in

peripheral blood of patients with chronic upper respiratory tract inflammation and compared them to healthy controls (chapter II-2). It was found that the total glutathione levels and vitamin E levels were significantly lower in the subjects suffering from chronic inflammation than in the controls. In contrast uric acid levels were significantly higher in the inflammatory groups than in the control group. It can be speculated that the lowered mucosal antioxidant levels under chronic inflammatory conditions, due to either mucosal scavenging of oxidants or enzymatic conversion, can not be supplemented sufficiently by systemic antioxidants from the blood stream and therefore contribute to the chronicity of the inflammation. This finding offers perspectives for specific antioxidant pharmacotherapy with N-acetylcysteine (NAC) as this compound has distinctive direct and indirect antioxidant properties.[15] Furthermore, oral NAC has been proven to be beneficial in the treatment of chronic sinusitis and serous otitis media.[16,17] We investigated, in a placebo controlled randomized double blind fashion, the effects of oral NAC in patients with chronic sinusitis and nasal polyps on the blood antioxidant defense and clinical outcome (chapter II-3). It was shown that NAC treatment increased the total glutathione levels, the uric acid levels, the TEAC levels and the residual antioxidant capacity levels in the peripheral blood of all patients. Furthermore, the clinical presentation after 6 months of NAC pharmacotherapy was much better in all patients treated with NAC compared to the placebo treated group. It was already known that oral NAC increases blood levels of glutathione.[15] Therefore, we expected the blood levels of glutathione to increase after NAC pharmacotherapy in the patients suffering from chronic inflammation. As both mucosal and systemic glutathione levels are low in patients suffering from chronic sinusitis or nasal polyps inappropriate local synthesis or mucosal supplementation of glutathione from the bloodstream into the mucosa can be anticipated. Uric acid levels were low in mucosa of chronic sinusitis patients however, in peripheral blood they were higher than in controls. This difference can be explained by the fact that although uric acid is locally consumed through its sacrificial behavior it not locally formed, as we were not able to detect any xanthine oxidase in the mucosal specimens, a finding which is in accordance with literature.[18] However, in circulating blood increased activity of xanthine oxidase has been found under chronic inflammatory conditions.[19] This might explain the increased blood levels of uric acid in chronic upper respiratory tract inflammation. It can be hypothesized that increased blood levels of uric acid are needed to supplement the uric acid utilization at the inflammatory site. Additional increase of blood uric acid levels by NAC pharmacotherapy can be beneficial. Although the mechanism by which this rise occurs is unclear. In addition, it should be noted that increased activity of xanthine oxidase not only increases uric acid levels

but also leads to increased superoxide anion radical formation. It can be expected that these superoxide anion radicals are neutralized by various systems in the peripheral blood and therefore do not contribute to mucosal damage in the upper respiratory tract. Although the exact mechanism of this feature has to be elucidated, the components contributing to TEAC might play a role in this phenomenon as the residual antioxidant capacity (TEAC – uric acid) is indeed lowered in patients with chronic respiratory tract inflammation. The increase of TEAC and residual antioxidant capacity in chronic upper respiratory tract infection after NAC supplementation can not be explained from the experiments we performed in this thesis and have to be investigated further by studying the effects of NAC on the separate contributors of residual antioxidant capacity. However, a synergistic interplay between the various antioxidant systems can be anticipated. The fact that the residual antioxidant capacity rises after NAC pharmacotherapy may be of particular importance in dealing with the increased superoxide anion radical production by xanthine oxidase under chronic inflammatory conditions.

The positive clinical effect of NAC pharmacotherapy can be explained by increased antioxidant defense however, other factors, such as the mucolytic activity of NAC, may also play a role. Furthermore, a follow-up of 6 months in these type of disorders may be rather short. Therefore, longer trials with possibly larger patient groups have to be performed.

Antioxidant effects of local pharmacotherapy

Beside the investigations made on biologic material of patients with chronic upper respiratory tract inflammations and healthy controls we performed measurements on the possible antioxidant properties of the decongestive compounds, oxymetazoline and xylometazoline (chapter III-1 and chapter III-2). These compounds are frequently used as topical medication in these disorders based on their α-adrenergic receptor stimulating, decongestive effects. However, as oxidative stress may play a role in the pathogenesis of chronic upper respiratory tract inflammation, possible antioxidant actions may further explain the therapeutic effect. Oxymetazoline, but not xylometazoline was shown to be a potent inhibitor of lipid peroxidation. Lipid peroxidation is an important result of oxidative stress and results by its chain reaction in cell membrane damage which contributes to ongoing inflammation. Oxymetazoline contains a hydroxyl moiety on its phenylring which can donate a hydrogen atom and by this means terminate the chain reaction of lipid peroxidation. Increased lipid peroxidation has indeed been found in upper respiratory tract inflammation.[6,10,11,12] Xylometazoline lacks such a hydrophyl moiety and is not capable of inhibiting lipid

peroxidation. In addition, both compounds showed to be powerful hydroxyl radical scavengers. Especially oxymetazoline proved to have an extremely high rate constant for scavenging hydroxyl radicals, a characteristic which we were not able to explain. As hydroxyl radicals are strongly related to various types of biological damage during inflammation, scavenging them may, limit the inflammatory response. It would in this respect be interesting to investigate the hydyroxyl radical production in the inflamed upper respiratory tract mucosa and the effects both compounds on this hydroxyl radical formation.

Although the investigations were *in vitro* experiments and extrapolation to clinical relevant situation should be done cautiously, we strongly believe that the distinct antioxidant properties of oxymetazoline and xylometazoline contribute to their therapeutic effect.

It has been reported that $NO^{\cdot}$ and $NO^{\cdot}$ metabolites, such as peroxynitrite, seem to play a role in chronic upper airway inflammation.[20,21,22,23,24] We therefore investigated the *in vitro* effects of oxymetazoline and xylometazoline on the $NO^{\cdot}$ producing system NOS. NOS consists of several isoforms of which iNOS is thought to be related to inflammation. cNOS plays a role in physiological processes such as, vascular tone regulation and neurotransmission. In contrast, iNOS is considered to be an important mediator in inflammation. We found a dose dependent inhibitory effect of oxymetazoline and xylometazoline on iNOS activity based on inhibition of induction of the enzyme rather than on inhibition of the enzyme activity. Furthermore, a moderate inhibition of cNOS activity for both compounds was found. Direct scavenging of $NO^{\cdot}$ could not be found. Presuming that cNOS serves beneficial physiological functions and exaggerated iNOS activity causes exacerbation of the inflammation the specific inhibitory effects of oxymetazoline and xylometazoline may contribute to an inhibitory effect in inflammation. This speculation might be strengthened by the fact that the iNOS inhibitors glucocorticosteroids have beneficial effects in the treatment of chronic upper respiratory tract inflammation.[25] Again it should be noted that these were *in vitro* experiments and extrapolation to the in vivo situation should be done with care. For instance in the experiments no human nasal cells but instead rat cells (rat cerebellar synaptosomes and lung alveolar macrophages) were used. This was done to prevent methodological errors in the measurements as the assays used were designed to use these types of rat cells. Experiments using human nasal mucosal cells and inflammatory cells can be performed to gain more knowledge on the in vivo situation. However, we think that we made the first step in elucidating these unknown features of these "old" compounds.

Conclusions

Based on the research described in this thesis the following can be concluded:

The levels of several biologically important antioxidants are altered in nasal mucosa and peripheral blood of patients suffering from chronic upper respiratory tract inflammation. These findings suggest that there is an diminished antioxidant defense which may be associated with the multifactorial pathogenesis of chronic sinusitis and nasal polyps. Antioxidant pharmacotherapy using NAC does not only supplement the deficient systemic levels of the antioxidants but also proves to be clinically effective. In addition, nasal decongestants, oxymetazoline and xylometazoline, used as local pharmacotherapeutics showed unexpected but distinct antioxidative properties which may contribute to their beneficial effect in the treatment of chronic upper respiratory tract infection.

Although, the research described herein does not pretend to fully explain the pathogenesis of chronic upper respiratory tract inflammation, it suggests a role for oxidative stress. Furthermore, it offers perspectives for further elucidation of this interesting field.

References

1. Vademecum gezondheidsstatistiek nederland 1999. Centraal bureau voor de statistiek/ Ministerie van volksgezondheid, welzijn en sport.
2. Kaliner MA, Osguthorpe JD, Fireman P, Anon J, Georgitis J, Davis ML, Naclerio R, Kennedy D. Sinusitis: bench to bedside. Current findings, future directions. *J All Clin Immunol* 1997; 99:S829-S848.
3. Hamilos DL. Chronic sinusitis. *J All Clin Immunol* 2000; 106:213-227.
4. Doelman CJA, Bast A. Oxygen radicals in lung pathology. *Free Rad Biol Med* 1990; 9:381-400.
5. Demoly P, Crampette L, Mondain M, Enander I, Jones I, Bousquet J. Myeloperoxidase and interleukin-8 levels in chronic sinusitis. *Clin Exp All* 1997; 27:672-675.
6. Dogru H, Delibas N, Döner F, Tüz M, Uygur K. Free radical damage in nasal polyp tissue. *Otolaryngol Head Neck Surg* 2001; 124:570-572.
7. Henson PM, Johnston RB. Tissue injury in inflammation: oxidants, proteinases and cationic proteins. *J Clin Invest* 1987; 79:669-674.
8. Bast A, Haenen GRMM, Doelman CJA. Oxidants and antioxidants: state of the art. *Am J Med* 1991: 91 (suppl 3C):2S-13S.
9. Halliwell B, Gutteridge JM, Cross CE. Free radicals, antioxidants and human disease. Where are we now ? *J Lab Clin Med* 1992; 119:598-620.
10. Haddad J. Lipoperoxidation as a measure of free radical injury in otits media. *Laryngoscope* 1998; 108:524-530.
11. Dönner F, Delibas N, Dogru H, Sari I, Yorgancigil B. Malondyaldehyde levels and superoxide dysmutase activity in experimental sinusitis. *Auris Nasus Larynx* 1999; 26:287-291.

12. Parks RR, Huang CC, Haddad J. Evidence of oxygen radical injury in experimental otitis media. *Laryngoscope* 1994; 104:1389-1392.
13. Cross CE. Vliet van der A, O'Neill CA, Louie S, Halliwell B. Oxidants, antioxidants, and respiratory tract lining fluids. *Environ Health Perspect* 1994; 102 (Suppl 10):185-191.
14. Peden DB, Swiersz M, Ohkubo K, Hahn B, Emery B, Kaliner MA. Nasal secretion of the ozone scavenger uric acid. *Am Rev Respir Dis* 1993; 148:455-461.
15. Cotgreave IA. N-acetylcysteine: pharmacological considerations and experimental and clinical applications. *Adv Pharmacol* 1997; 38:205-227.
16. Panosetti E. Clinical trial with oral acetylcysteine in chronic sinusitis. *Eur J Respir Dis* 1980; 61(suppl 111):159.
17. Balli R. Controlled trial on the use of oral acetylcysteine in the treatment of glue-ear following drainage. *Eur J Respir Dis* 1980; 61(suppl 111):158.
18. Peden DB, Swiersz M, Ohkubo K, Hahn B, Emery B, Kaliner MA. Nasal secretion of the ozone scavenger uric acid. *Am Rev Resp Dis* 1993; 148:455-461.
19. Miesel R, Zuber M. Elevated levels of xanthine oxidase in serum of patients with inflammatory and autoimmune rheumatic diseases. *Inflammation* 1993; 17:551-561.
20. Sato M, Fukuyama N, Sakai M, Nakazawa H. Increased nitric oxide in nasal lavage fluid and nitrotyrosine formation in nasal mucosa. Indices for severe perennial nasal allergy. *Clin Exp All* 1998; 28:597-605.
21. Hanazawa T, Antuni JD, Kharitonov SA, Barnes PJ. Intranasal administration of eotaxin increases nasal eosinophils and nitric oxide in patients with allergic rhinitis. J All Clin Immunol 1999; 105:58-64.
22. Schlosser RJ, Spotnitz WD, Peters EJ, Fang K, Gaston B, Gross CW. Elevated nitric oxide metabolite levels in chronic sinusitis. Otolaryngol Head Neck Surg 2000; 123:357-362.
23. Arnal JF, Flores P, Rami J, Murris-Espin M, Bremont F, Pasto I Aguilla M, Serrano E, Didier A. Nasal nitric oxide concentration in paranasal sinus inflammatory diseases. Eur Respir J 1999; 13:307-312.
24. Ramis I, Lorente J, Roselló-Catafau J, Quesada P, Gelpi E, Bulbena O. Differential activity of nitric oxide synthase in human nasal mucosa and polyps. Eur Respir J 1996; 9:202-206.
25. Kharitonov SA, Yates D, Robbins RA, Thomas PS, Barnes PJ. Corticosteroids decrease exhaled nitric oxide. *Am J Respir Crit Care Med* 1994; 149:A201.

Summary

Chronic upper respiratory tract infections form a major problem in health care. In the Netherlands in 1998, approximately 9 % of the population suffered from these type of disorders. As the exact pathophysiology underlying these disorders is unknown definite treatment remains difficult. Accumulating scientific data suggest a role for oxidative stress in chronic airway pathology. This thesis describes both clinical and experimental investigations on the role of oxidative stress in the pathogenesis of chronic upper respiratory tract inflammation, focussing on the antioxidant defense mechanisms.

In **chapter I-1** the anatomy and physiology of the nose and paranasal sinuses is described. Furthermore, general aspects of chronic inflammatory disorders of the upper respiratory tract are discussed.

In **chapter I-2** the basic concepts of the chemical nature of oxidants and antioxidants are outlined. In addition, the role of oxidative stress in human disease is discussed.

In **chapter 1-3** a review of literature is given on the role of oxidative stress in upper respiratory tract inflammation. Oxidant damage to nasal –, and paranasal mucosa caused by inflammation, airpollution, xenobiotic inhalation, and pure oxygen is discussed. Furthermore, endogenous and pharmacotherapeutic antioxidant mechanisms and strategies are elaborated.

In **chapter II-1** antioxidant levels in the nasal mucosa of patients with chronic sinusitis and of healthy controls are described. Beforehand it was hypothesized that in chronic sinusitis a reduced antioxidant tissue status may be present. The levels of three biologically important antioxidants, i.e., glutathione (reduced (GSH) and oxidized (GSSG)), uric acid and vitamin E, were determined biochemically in mucosal biopsies from the uncinate process of patients with chronic sinusitis and healthy controls.

Inflamed mucosa was obtained from 9 patients with chronic sinusitis during functional endoscopic sinus surgery. Normal mucosa was collected from 10 healthy controls during surgery for nasal obstruction. The data show a significant reduction of GSH levels (0.3 ± 0.1 µmol/g wet weight) and uric acid levels (2.7 ± 0.4 µmol/g wet weight) in mucosa obtained from patients with chronic sinusitis compared to healthy controls (0.6 ± 0.2 and 3.4 ± 0.6 µmol/g wet weight respectively). No difference was found in GSSG (24 ± 8 vs 25 ± 15

nmol/g wet weight) and vitamin E (20.5 ± 7.9 vs 22.5 ± 6.9 nmol/g wet weight) levels between both groups. It was concluded that reduced levels of both GSH and uric acid in patients with chronic sinusitis lead to a diminished antioxidant defense, which may be associated with the pathogenesis. Vitamin E seems less important. This concept may offer perspectives for pharmacotherapeutic intervention with antioxidants.

In **chapter II-2** measurements on blood antioxidant levels in patients with either chronic upper respiratory tract inflammation and healthy controls are described. Alterations in the blood concentrations of the biologically important antioxidants, glutathione, vitamin E and uric acid, were determined in erythrocytes and blood plasma of patients with chronic sinusitis, patients with nasal polyps and healthy controls. The data (presented as mean ± SD) show a significant reduction in total glutathione level in blood samples obtained from patients with chronic sinusitis (1977 ± 480 μM) and patients with nasal polyps (2088 ± 417 μM) compared to the control group (2335 ± 343 μM). Also a significant reduction in vitamin E level was found in patients with chronic sinusitis (16 ± 12 g/mmol triglyceride) when compared to healthy controls (24 ± 14 g/mmol triglyceride). This reduction could not been shown for patients with nasal polyps (19 ± 10 g/mmol triglyceride). Uric acid levels were found to be significantly higher in blood samples of patients with either chronic sinusitis (291 ± 71 μM) or nasal polyps (282 ± 72 μM) compared to the values of healthy controls (214 ± 58 μM). In addition, in the same subject groups the blood levels of the total antioxidant capacity were investigated and expressed as TEAC. TEAC levels did not differ between the blood samples of patients chronic sinusitis (411 ± 106 μM), patients with nasal polyps (384 ± 80 μM) and healthy controls (429 ± 81 μM). However, the residual antioxidant capacity, obtained after subtraction of the contribution of uric acid from the TEAC, showed a significant reduced level in patients with chronic sinusitis (128 ± 87 μM) and patients with nasal polyps (102 ± 74 μM) compared to the healthy controls (216 ± 56 μM). In conclusion, the altered levels of glutathione, vitamin E, uric acid and residual TEAC may reflect an impaired antioxidant defense in patients with a chronic inflammatory disorder of the upper respiratory tract which may be implicated in the complex multifactorial pathogenesis. These findings offer perspectives for pharmacotherapeutic intervention with antioxidants.

In **chapter II-3** the effects of oral pharmacotherapy with N-acetylcysteine (NAC) on blood antioxidant levels of patients with chronic sinusitis and nasal polyps are reported.

Furthermore, the clinical effects of this treatment were scored. Antioxidant pharmacotherapy has been proven successful in various oxidant mediated diseases. In recent years it has become clear that the pathophysiology of chronic upper respiratory tract inflammation is at least partially mediated by oxidative stress. As it was shown that antioxidant levels of nasal mucosa and peripheral blood of patients with nasal polyposis and patients with chronic sinusitis are decreased, beneficial effects of NAC in the management of these disorders, which are otherwise difficult to treat, can be anticipated. The blood levels of total glutathione, uric acid, TEAC, and the residual antioxidant capacity were measured in patients with either chronic sinusitis or nasal polyps who were additionally treated with 600 mg of oral NAC twice a day for 6 months in a placebo controlled, randomized double blind fashion after functional endoscopic sinus surgery. Furthermore, the clinical status was scored subjectively by the patients and objectively by nasal endoscopy. In both the patient groups (nasal polyps and chronic sinusitis) which were treated with NAC the total glutathione levels, the uric acid levels, the TEAC levels, and the residual antioxidant capacity levels increased significantly after 6 months of oral NAC. This was not observed for the patient groups treated with placebo. Furthermore, in the NAC treated patients, after 6 months, no nasal complaints were reported. Also nasal endoscopy after 6 months of middle meatus showed an open osteomeatal unit in all cases. In contrast, in the placebo treated patients no significant effect was observed on either subjective well-being or nasal endoscopy.
It was concluded that oral NAC improves the antioxidant defense mechanisms and in this way contributes to the difficult therapeutic management of nasal polyposis and chronic sinusitis.

In **chapter III-1** the antioxidant actions of the nasal decongestants oxymetazoline and xylometazoline were investigated by measuring inhibition of microsomal lipid peroxidation and hydroxyl radical scavenging activity. Oxymetazoline was shown to be a potent inhibitor of lipid peroxidation (IC_{50} = 4.9 μM at t = 15 min, IC_{50} = 8.1 μM at t = 30 min), in contrast to xylometazoline. Both compounds were excellent hydroxyl radical scavengers. Their rate constants (k_s = 1.1 x 10^{12} $M^{-1}s^{-1}$ for oxymetazoline and k_s = 4.7 x 10^{10} $M^{-1}s^{-1}$ for xylometazoline) exceeded the rate constant of a powerful known hydroxyl radical scavenger cimetidine (k_s = 1.8 x 10^{10} $M^{-1}s^{-1}$). The difference in inhibiting lipid peroxidation might be explained by the fact that only oxymetazoline contains a hydroxyl moiety which can donate a hydrogen atom and terminate the chain reaction of lipid peroxidation. The mechanism of hydroxyl radical scavenging activity is still unclear. Moreover, oxymetazoline seems to have

a different mode of action in scavenging hydroxyl radicals then xylometazoline and cimetidine resulting in a extremely high rate constant. Because oxidants play a role in tissue damage in inflammation, it was hypothesized that especially for oxymetazoline and to a lesser extent for xylometazoline their antioxidant properties may contribute to the therapeutic efficacy, in the topical treatment of nasal inflammation.

In **chapter III-2** the effects of the nasal decongestants oxymetazoline and xylometazoline on the enzymatic action of nitric oxide synthase are described. As NO· is thought to play a role in inflammation of the upper respiratory tract, the aim of this study was to examine the *in vitro* effects of these compounds on the activity and the expression of the NO· producing enzymes, iNOS and cNOS.

Experiments concerning the effects on enzymatic activity and enzyme induction of iNOS by both compounds were performed in lipopolysacharide (LPS) induced rat alveolar macrophages (cell line NR8383) using the Griess assay and the ^{3}H-citrulline assay respectively. The effects on cNOS were examined in fresh rat cerebellum synaptosomes using the ^{3}H-citrulline assay. The direct scavenging properties of both compounds were investigated using a amperometric NO· sensor. Oxymetazoline and xylometazoline were shown to have a dose dependent inhibitory effect on total iNOS activity indicated by nitrite/nitrate formation in the Griess assay. This effect was found to be due to an inhibition of induction of the enzyme rather than inhibition of the enzyme activity, as was investigated in two separate experiments using the ^{3}H-citrulline assay. Inhibition of cNOS was moderate and in the same order of magnitude as the inhibition of enzymatic iNOS activity. Direct scavenging of NO· could not be detected. As cNOS activity is thought to serve beneficial physiological functions and exaggerated iNOS activity may cause exacerbation of the inflammatory process, pharmacological treatment influencing the NO generating system should focus on inhibition of iNOS alone. The specific characteristics of these decongestants *in vitro* suggests suitability for this application and may indicate an additional beneficial effect in the treatment of upper respiratory tract inflammation.

Samenvatting

Antioxidanten en chronische onstekingen van de bovenste luchtwegen

Samenvatting

Chronische bovenste luchtweginfecties vormen een groot probleem in de gezondheidszorg. In Nederland was de prevalentie van deze aandoeningen in 1998 ongeveer 9 %. Daar de exacte pathofysiologie van deze aandoeningen onbekend is, is curatieve behandeling vaak moeilijk. Er ontstaat steeds meer wetenschappelijk bewijs dat oxidatieve stress een rol speelt in chronische aandoeningen van de luchtwegen. Dit proefschrift beschrijft zowel klinisch als experimenteel onderzoek naar de rol van oxidatieve stress in de pathogenese van chronische bovenste luchtweg infecties, met name gericht of anti-oxidatieve verdedigingsmechanismen.

In **hoofdstuk I-1** wordt de anatomie en fysiologie van de neus en de neusbijholten beschreven. Daarnaast worden algemene aspecten van chronische ontstekingsprocessen in de bovenste luchtwegen besproken.

In **hoofdstuk I-2** wordt de chemie van oxidanten en antioxidanten besproken. Tevens wordt de rol van oxidatieve stress bij humane aandoeningen aangegeven.

In **hoofdstuk I-3** wordt een overzicht over de literatuur gegeven met betrekking tot de rol van oxidatieve stress bij onstekingsprocessen van de bovenste luchtwegen. Oxidatieve schade aan het slijmvlies van de neus- en de neusbijholten veroorzaakt door ontstekingsprocessen, luchtvervuiling, biotransformatie van xenobiotica en zuivere zuurstof worden besproken. Aansluitend worden endogene en farmacotherapeutische antioxidantmechanismen bediscussieerd.

In **hoofdstuk II-1** worden de antioxidant niveaus in neusslijmvlies van patiënten met chronische sinusitis en gezonde controles beschreven. Vooraf was de hypothese gesteld dat er bij patiënten met chronische sinusitis een verlaagde antioxidant weefselstatus aanwezig is. De niveaus van drie biologisch belangrijke antioxidanten, te weten, glutathion (gereduceerd (GSH) en geoxideerd (GSSG)), urinezuur en vitamine E werden bepaald in slijmvlies biopten uit de processus uncinatus van patiënten met chronische sinusitis en gezonde controles. Ontstoken slijmvlies werd verkregen tijdens endoscopische neusbijholten chirurgie bij 9 patiënten met chronische sinusitis. Normale mucosa werd verkregen bij gezonde controles die een operatie ondergingen in verband met neusobstructie. De resultaten lieten een significante verlaging zien ($p \leq .05$) van GSH (0.3 ± 0.1 µmol/g nat gewicht) en urinezuur niveaus (2.7 ± 0.4 µmol/g nat gewicht) in de mucosa verkregen bij de patiënten met chronische sinusitis

vergeleken met de gezonde controles (0.6 ± 0.2 en 3.4 ± 0.6 μmol/g nat gewicht respectievelijk). Er werd geen verschil gevonden in GSSG (24 ± 8 vs 25 ± 15 nmol/g nat gewicht) en vitamine E (20.5 ± 7.9 vs 22.5 ± 6.9 nmol/g nat gewicht) niveaus tussen beide groepen. Er werd geconcludeerd dat de verlaagde niveaus van zowel GSH als urinezuur bij patiënten met chronische sinusitis leiden tot een verminderde anti-oxidatieve verdediging hetgeen mogelijk een rol speelt in de pathogenese van deze aandoening. Vitamine E lijkt hierin van minder belang. Dit concept biedt mogelijkheden tot farmacotherapeutische interventie met antioxidanten.

In **hoofdstuk II-2** worden de metingen naar antioxidant niveaus in bloed beschreven bij patiënten met chronische bovenste luchtweginfecties en gezonde controles. Mogelijke veranderingen in de concentraties van de biologisch belangrijke antioxidanten, glutathion, vitamine E en urinezuur werden bepaald in respectievelijk erytrocyten en bloedplasma van patiënten met chronische sinusitis, patiënten met neuspoliepen en gezonde controles. De resultaten (uitgedrukt als gemiddelde ±SD) tonen een significante verlaging ($p \leq .05$) in totale glutathion concentratie in bloedmonsters van patiënten met chronische sinusitis (1977 ± 480 μM) en patiënten met neuspoliepen (2088 ± 417 μM) ten opzichte van de controles (2335 ± 343 μM). Ook werd er een significante verlaging ($p \leq .05$) in vitamine E concentratie gevonden bij patiënten met chronische sinusitis (16 ± 12 g/mmol triglyceride) ten opzichte van gezonde controles (24 ± 14 g/mmol triglyceride). Dit kon niet worden aangetoond voor patiënten met neuspoliepen (19 ± 10 g/mmol triglyceride). De urinezuur concentraties in de plasmamonsters bleken significant hoger ($p \leq .05$) bij zowel de patiënten met chronische sinusitis (291 ± 71 μM) als de patiënten met neuspoliepen (282 ± 72 μM) vergeleken met de metingen bij de gezonde controles (214 ± 58 μM). Tevens werd bij dezelfde groepen de antioxidantcapaciteit in bloed, uitgedrukt als TEAC, onderzocht. De TEAC waarden verschilden niet tussen patiënten met chronische sinusitis (411 ± 106 μM) en neuspoliepen (384 ± 80 μM) enerzijds en gezonde controles anderzijds (429 ± 81 μM). Echter, de rest antioxidantcapaciteit van plasma, verkregen door aftrek van de bijdrage van urinezuur van de TEAC waarde, was significant verlaagd ($p \leq .05$) bij patiënten met chronische sinusitis (128 ± 87 μM) en neuspoliepen (102 ± 74 μM) vergeleken met gezonde controles (216 ± 56 μM). Concluderend, suggereren de verlaagde concentraties glutathion, vitamine E, urinezuur en rest antioxidantcapaciteit, een verlaagde anti-oxidatieve verdediging bij patiënten met chronische onstekingsprocessen van de bovenste luchtwegen. Dit zou een rol kunnen spelen in de

complexe multifactoriële pathogenese van deze aandoeningen. Tevens bieden deze bevindingen perspectief voor de farmacotherapeutische interventie met antioxidanten.

In **hoofdstuk II-3** worden de effecten van orale farmacotherapie met N-acetylcysteine (NAC) op de bloed antioxidantconcentraties van patiënten met chronische sinusitis en neuspoliepen beschreven. Tevens werd gekeken naar de klinische effecten van deze behandeling. Antioxidant farmacotherapie is succesvol gebleken in diverse oxidant gemedieerde ziekten. De laatste jaren is het duidelijk geworden dat chronische ontstekingen van de bovenste luchtwegen op zijn minst gedeeltelijk worden gemedieerd door oxidatieve stress. Daar het is aangetoond dat de antioxidantniveaus in de mucosa en het perifere bloed van patiënten met chronische sinusitis en patiënten met neuspoliepen verlaagd zijn, kunnen positieve effecten van NAC in de, overigens moeilijke, behandeling van deze aandoeningen worden verwacht. De concentraties in perifeer bloed van totaal glutathione, urinezuur, TEAC en rest antioxidant capaciteit werden bepaald bij patiënten met chronische sinusitis of neuspoliepen die additioneel met NAC (2 maal daags 600 mg gedurende 6 maanden) werden behandeld na endoscopische neusbijholten chirurgie te hebben ondergaan. De behandeling met NAC was dubbelblind gerandomiseerd. Tevens werd het effect van de behandeling subjectief door de patiënt en objectief middels neusendoscopie beoordeeld. In beide patiënten groepen (chronische sinusitis en neuspoliepen) trad een significante stijging op in totale glutathion-, urinezuur-, TEAC- en rest antioxidant capaciteit niveau op na 6 maanden orale NAC. Dit werd niet waargenomen bij die patiënten die met placebo werden behandeld. In de met NAC behandelde patiënt groepen had geen van de patiënten klachten na 6 maanden. Tevens werd bij al deze patiënten op 6 maanden een open osteomeatale unit bij neusendoscopie waargenomen. Dit in tegenstelling tot de met placebo behandelde patiënten waarbij nog op het subjectieve welbevinden nog op het neus endoscopisch beeld enig positief effect werd gezien. Er werd geconcludeerd dat NAC de antioxidant verdedigingsmechanismen verbetert en hierdoor bijdraagt aan de moeilijke behandeling van neuspoliepen en chronische sinusitis.

In **hoofdstuk III-1** wordt de antioxidant werking van de decongestieve neusdruppels oxymetazoline en xylometazoline gekarakteriseerd middels remming van microsomale lipide peroxidatie en hydroxyl radicaal scavenging. Oxymetazoline bleek een potente remmer van lipide peroxidatie (IC_{50} = 4.9 µM op t = 15 min, IC_{50} = 8.1 µM op t = 30 min), dit in tegenstelling tot xylometazoline. Beide stoffen waren uitmuntende hydroxyl radicaal scavengers. De reactieconstanten ($k_s = 1.1 \times 10^{12}$ $M^{-1}s^{-1}$ voor oxymetazoline en $k_s = 4.7 \times 10^{10}$

$M^{-1}s^{-1}$ voor xylometazoline waren hoger dan de reactieconstante van een bekende scavenger cimetidine (k_s = 1.8 x 10^{10} $M^{-1}s^{-1}$). Het verschil in remmingsactiviteit van lipide peroxidatie kan worden verklaard door het feit dat alleen oxymetazoline een hydroxyl groep heeft die en waterstof atoom kan doneren waardoor de kettingreactie van lipide peroxidatie kan beëindigen. Het mechanisme van hydroxyl radicaal scavenging blijft onduidelijk. Omdat oxidanten een rol spelen in weefselschade tijdens ontsteking kan er worden gespeculeerd dat met name oxymetazoline en in mindere mate xylometazoline een additioneel gunstig effect hebben bij de topische behandeling van ontstekingsprocessen in de neus.

In **hoofdstuk III-2** worden de effecten van de decongestieve neusdruppels oxymetazoline en xylometazoline op de enzymatische activiteit van stikstof oxide synthase (NOS) beschreven. Daar NO· een rol wordt toegeschreven in ontstekingsprocessen van de bovenste luchtwegen werden de *in vitro* effecten van deze stoffen op de activiteit en expressie van NO· producerende enzymen, iNOS en cNOS onderzocht. Experimenten aangaande de effecten op de enzymatisch activiteit en enzym inductie van iNOS door beide stoffen werden uitgevoerd in een LPS geïnduceerde ratten alveolaire macrofagen cel lijn (NR8383). Hierbij werd respectievelijk gebruik gemakt van het Griess assay en het ^{3}H-citrulline assay. De effecten op cNOS werden onderzocht in verse ratten synaptosomen middels het ^{3}H-citrulline assay. De directe scavenging eigenschappen van beide stoffen werden onderzocht door middel van een amperometrische NO· sensor. Oxymetazoline en xylometazoline lieten een dosis afhankelijk remmend effect op de totale iNOS activiteit zien. Zoals aangetoond door nitriet/nitraat vorming in het Griess assay. Dit effect bleek met name te worden veroorzaakt door de remming van inductie van het enzym en in mindere mate door remming van de enzym activiteit, zoals aangetoond middels twee onafhankelijke experimenten waarbij gebruik gemaakt werd van het ^{3}H-citrulline assay. De remming van cNOS bleek matig en in dezelfde orde van grootte als de remming van enzymatische iNOS activiteit. Directe scavenging van NO kon niet worden aangetoond. Omdat cNOS activiteit wordt gerelateerd aan gunstige fysiologische effecten en omdat verhoogde iNOS activiteit een inflammatoire exacerbatie kan geven, moet farmacologische behandeling met name gericht zijn op beïnvloeding van alleen iNOS. De specifieke *in vitro* eigenschappen van deze decongestieve stoffen suggereren geschiktheid voor een dergelijke toepassing. Als gevolg hiervan kan een additioneel gunstig effect bij de behandeling van bovenste luchtweg infecties worden verwacht.

Abbreviations

A	absorbance
ADP	adenosine diphosphate
AHR	airway hyperresponsiveness
ATP	adenosine triphosphate
c-GMP	cyclic guanosine monophosphate
CT	computerized tomogram
DNA	deoxyribonucleic acid
DR	deoxyribose
ECP	eosinphil cationic protein
EDRF	endothelium derived relaxing factor
EPO	eosinophil peroxidase
G-6-PD	glucose 6 phosphate dehydrogenase
GSH	glutathione reduced
GSH-px	glutathione peroxidase
GSSG	glutathione oxidized
GS·	thiyl radical
GTP	guanosine triphosphate
HNECs	human nasal epithelial cells
HOCl	hypochlorous acid
H_2O_2	hydrogen peroxide
H_2O	water
HPLC	high performance liquid chromatograph
Ig	immunoglobulin
IL	interleukin
INF	interferon
k_s	rate constant
LH	poly unsaturated fatty acid
L-NAME	N-nitro-L-arginine methylester
L-NMMA	N-monomethyl-L-arginine
L-NNA	N-nitro-L-arginine
LOO·	lipid peroxyl radical
LOOH	lipidhydroperoxide
LPS	lipopolysaccharide
LT	leukotriene
L·	lipid radical
MBP	major basic protein
MDA	malondialdehyde
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
NAC	N-acetylcysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NAL	nasal lavage fluid
NF-κB	nuclear transcription factor kappa B
NO·	nitric oxide
NOS	nitric oxide synthase
NO_2	nitrogen dioxide
OH·	hydroxyl radical
$ONOO^-$	peroxynitrite
$^1O_2$	singlet oxygen
O_2	oxygen
$O_2^{\cdot}$	superoxide anion radical
O_3	ozone
PAF	platelet activating factor
PLC	phospholipase C
PKC	protein kinase C
6-PGD	6 phosphogluconate dehydrogenase
px	peroxidase
RAST	radio-allergosorbent test
RNS	reactive nitrogen species
ROS	reactive oxygen species
S	scavenger
SNP	sodium nitroprusside
SOD	superoxide dysmutase
TEAC	trolox equivalent antioxidant capacity
TNB	thiolate anion
TNF	tumor necrosis factor
UA	uric acid
URTI	upper respiratory tract infection
XDH	xanthine dehydrogenase
XO	xanthine oxidase

List of Publications

1. Westerveld GJ, Verkeyn JMA Klasen HJ. Acute stridor na een stomp larynxtrauma. Ned Tijdschr Traumatol 1994; 1:48-49
2. Westerveld GJ, Scheeren RA, Dekker I, Grifioen DH, Voss HP, Bast A. Anti-oxidant actions of oxymetazoline and xylometazoline. Eur J Pharmacol 1995; 291:27-31.
3. Rosman C, Westerveld GJ, Vanoeveren W, Kooi K, Bleichrodt RP. Effect of intraperitoneal antimicrobials on the concentration of bacteria, endotoxin, and tumor necrosis factor in abdominal fluid and plasma in rats. Eur Surg Research 1996; 28:351-360.
4. Westerveld GJ, Dekker I, Voss HP, Bast A, Scheeren RA. Antioxidant levels in nasal mucosa of patients with chronic sinusitis and healthy controls. Arch Otolaryngol Head Neck Surg 1997; 123:201-204.
5. Vriesman MF, Haenen GRMM, Westerveld GJ, Paquay JBG, Voss HP, Bast A. A method for measuring nitric oxide radical scavenging activity. Scavenging properties of sulfur containing compounds. Pharm World Sci 1997; 19:283-286.
6. Westerveld GJ, Nieuwkerk van EBJ, Mahieu HF. Laryngoschisis: een zeldzame oorzaak van congenitale pneumonieën. Ned Tijdschr KNO-Heelk 1998; 4:103-107.
7. Westerveld GJ, Nieuwkerk van EBJ, Mahieu HF. Imaging quiz case. Arch Otolaryngol Head Neck Surg 1999; 125:812-815.
8. Mahieu HF, Bree de R, Westerveld GJ, Leemans CR. Laryngeal suspension and upper esophageal sphincter myotomy as a surgical option for treatment of severe aspiration. Oper Techniq Otolaryngol Head Neck Surg 1999; 10:305-310.
9. Rosman C, Westerveld GJ, Kooi K, Bleichrodt RP. Local treatment of generalised peritonitis in rats; effect of bacteria, endotoxin and mortality. Eur J Surg 1999; 165:1072-1079.
10. Bree de R, Westerveld GJ, Smeele LE. Submandibular approach for excision of a large schwannoma in the base of the tongue. Eur Arch Otorhinolaryngol 2000; 257:283-286.
11. Westerveld GJ, Voss HP, Hee van der RM, Haan-Koelewijn de GJN, Hartog den GJ, Scheeren RA, Bast A. Inhibition of nitric oxide synthase expression and activity by nasal decongestants oxymetazoline and xylometazoline. Eur Resp J 2000; 16:437-444.
12. Westerveld GJ, Leemans CR, Middelweerd MJ. Reconstruction of large 3-layer nasal vestibular defects with the composite septal hinge flap. In: Facial reconstructive surgery. Eds. Leemans CR, Middelweerd MJ, Vuyk HD. 2000; p87-92.
13. Westerveld GJ, Quak JJ, Bresters D, Zwaan CM, Valk van der P, Leemans CR. Endodermal sinus tumor of the maxillary sinus. Otolaryngol Head Neck, 2001; 124:691-692.
14. Westerveld GJ, Diest van PJ, Nieuwkerk van EBJ.Neuroendocrine tumor of the sphenoid sinus. Rhinology, 2001; 39:52-54.
15. Westerveld GJ, Middelweerd MJ, Leemans CR. The hinged-door composite septal flap as structural support and lining of nasal reconstruction by a forehead flap. Rhinology 2001; 39:5-8.
16. Westerveld GJ, Nieuwkerk van EBJ, Middelweerd MJ. Traumata van de oorschelp. Ned Tijdschr KNO-Heelk (suppl) 2001; 2:S1-S16.

Dankwoord

Het belangrijkste doel in het VU ziekenhuis was altijd om Keel-, Neus- en Oorarts te worden. Voor, tijdens en na mijn opleiding heeft dit proefschrift een belangrijke rol in mijn leven gespeeld. Bij het volbrengen van het onderzoek en de totstandkoming van dit proefschrift hebben velen een onmisbare rol gespeeld. Mijn speciale dank gaat uit naar:

Mijn promotor en opleider prof. dr. G.B. Snow, beste Gordon, toen jij mij in december 1993 vroeg of ik als noorderling wel zou kunnen aarden in het westen heb ik volmondig ja geantwoord. Ik hoop dat ik je vertrouwen nooit heb beschaamd. Dank voor de genoten opleiding en mogelijkheden voor het voltooien van dit proefschrift.

Mijn promotor prof. dr. A. Bast, beste Aalt, als absolute expert op het gebied van radicaal pathologie ben ik je veel dank verschuldigd voor de inhoudelijke begeleiding van dit onderzoek. Je nooit aflatende enthousiasme was onontbeerlijk.

Mijn copromotor dr. R.A. Scheeren, beste Robert, als geestelijk vader van dit onderzoek ben ik jou de afgelopen 8 jaren in wisselende mate dankbaar geweest dat je ooit dit project hebt geschreven. In moeilijke tijden echter wist jij mij altijd weer te motiveren. Dank voor je interesse, steun en advies gedurende de afgelopen jaren. Op otologisch gebied hoop ik in de toekomst nog veel van je te mogen leren.

Dr. G.R.M.M Haenen, beste Guido, met name in de afrondende fase van het onderzoek heb jij een onmisbare rol gespeeld. Jouw gave de voor mij veelal moeilijke chemische achtergronden en overwegingen begrijpelijk te maken alsmede je pragmatische aanpak van onderzoek doen hebben dit onderzoek net dat duwtje gegeven dat nodig was voor een goede afronding. Bedankt.

Dr. H-P Voss, beste Hans-Peter, bij welk proefschrift op de afdeling farmacochemie ben je niet betrokken geweest. Naast je grote inhoudelijke kennis, is je praktische kennis alsook je kennis van apparatuur en computers ongeëvenaard. Dank voor je hulp.

Dhr. M. Fischer, beste Marc, zonder jou 'monnikenwerk' achter de labtafel was dit proefschrift er niet geweest. Bedankt voor je hulp.

Alle collega AIO's en medewerkers van de afdeling farmacochemie. Als vreemde eend in de "farmacochemische" bijt hebben jullie mij geholpen waar het kon. Bedankt voor jullie samenwerking.

Alle collega's en medewerkers van de afdeling Keel-, Neus- en Oorheelkunde dank ik hartelijk voor hun hulp bij en belangstelling voor het onderzoek.

Mijn ouders, lieve mamma, dit proefschrift is ter nagedachtenis aan jou. Jammer genoeg kun je er niet bij zijn. Beste Pa, vele discussies hebben wij gevoerd over de zin en onzin van het promoveren. We zijn er nog niet uit. Nu het klaar is denk ik dat jij en mamma trots op me zijn. Bedankt voor alle mogelijkheden die jullie mij hebben geboden alsmede het veilige thuisfront waar ik altijd op kon terugvallen.

Mevrouw Westerveld-Leegwater, beste Gré, je bent het beste wat ons en natuurlijk met name Pa na het overlijden van mamma is overkomen. Je onvoorwaardelijke liefde heeft Pa weer op de rails gezet. Bedankt!

Last but not least, lieve Sandra, woelige tijden heb jij moeten doorstaan waarbij mijn humeur mij wel eens in de steek liet. Bedankt voor je geduld, begrip en liefde de afgelopen jaren, maar vooral bedankt voor het mooiste geschenk wat ik me maar kon wensen, ons prinsesje Lisalotte.

Curriculum Vitae

Gerrit Jan Westerveld werd op 31 augustus 1967 geboren te Harlingen. Na het behalen van het Atheneum diploma aan de scholengemeenschap Simon Vestdijk te Harlingen in 1985, werd begonnen met de studie geneeskunde aan de Rijksuniversiteit Groningen. Het artsexamen werd behaald in 1992, waarna hij ruim anderhalf jaar werkzaam was als assistent geneeskundige niet in opleiding (AGNIO) bij de afdeling Heelkunde van het Academisch Ziekenhuis Groningen. Van 1 februari 1994 tot 1 juli 1996 was hij werkzaam als assistent in opleiding (AIO) op de afdeling Keel-, Neus- en Oorheelkunde van het Academisch Ziekenhuis der Vrije Universiteit Amsterdam en de afdeling Farmacochemie van de Vrije Universiteit Amsterdam. Van 1 juli 1996 tot 31 december 2000 werd hij opgeleid tot Keel-, Neus- en Oorarts in het Academisch Ziekenhuis der Vrije Universiteit te Amsterdam (opleider prof. dr. G.B. Snow). Een deel van deze opleiding werd respectievelijk in het Ziekenhuis Hilversum (opleider dr. M.J. Middelweerd) en in het Diakonessenhuis Utrecht (opleider dr. J.J. Quak) gevolgd. Vanaf 1 januari 2001 is hij werkzaam als staflid bij de afdeling Keel-, Neus- en Oorheelkunde in het Academisch Ziekenhuis der Vrije Universiteit Amsterdam. Vanaf 1 juli 2001 is hij tevens werkzaam als Keel-, Neus- en Oorarts in het Ziekenhuis Hilversum alwaar hij per 1 juli 2002 zal toetreden tot de maatschap. Gerrit Jan Westerveld is getrouwd met Sandra Aning. Zij hebben een dochter, Lisalotte.

ISBN 90-6464-419-5