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Clinical Record

Immunolocalisation of heme oxygenase isoforms in human nasal polyps

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Abstract

Background: Carbon monoxide is an endogenous vasodilator gas produced by the enzyme heme oxygenase (HO). HO is expressed in human nasal mucosa, but its pathophysiological role in nasal inflammatory diseases is not fully understood. The aim of this study was to detect and compare the expression of HO-1 and -2 isoforms in nasal polyps with normal nasal mucosa.

Methods: Immunohistochemical analysis using antibodies specific for HO-1 and -2 was conducted on nasal polyps from nine patients with allergic nasal polyposis, and on normal nasal mucosa from six controls.

Results: Intense HO-1 immunoreactivity was observed in nasal polyp epithelium but was absent in normal nasal mucosa. HO-2 staining was observed in respiratory epithelium, vascular endothelium and seromucous glands, with no difference observed between nasal polyps and normal nasal mucosa.

Conclusions: HO-1 expression is up-regulated in nasal polyp epithelium, supporting the theory that respiratory epithelium plays a role in the pathogenesis of nasal polyposis.

Key words: Heme Oxygenase; Carbon Monoxide; Nasal Polyps; Immunohistochemistry

Introduction

In Western countries, nasal polyposis has a prevalence of around 2 per cent.[1–2] The aetiology of nasal polyp diseases is poorly understood. The characteristic histological features of nasal polyps comprise eosinophilic inflammation and destruction of connective tissue. Nasal respiratory epithelium has been suggested to play an important role in inflammatory diseases of the nose. In particular, various pro-inflammatory mediators released by the epithelium have been identified. These include cysteinyl leukotrienes, cationic proteins, eosinophil peroxidase, interleukins, matrix metalloproteinases and neuropeptides.[3–7] Recently, much attention has focused on the role of epithelial-derived nitric oxide (NO) in inflammatory diseases of the upper airway, including nasal polyposis.[8–10]

Like NO, carbon monoxide (CO) is also implicated in the pathophysiology of inflammatory airway diseases. CO is an endogenous gas mediator produced by the enzyme heme oxygenase (HO).[11] HO enzymes are members of the stress/heat shock protein 30 family, which catalyses the first rate-limiting step of heme degradation to CO, iron and biliverdin. There are three forms of HO. HO-1 (heat shock protein 32), the inducible form, is a major stress protein. It is induced by hypoxia, heme, cytokines and oxidants, including NO, – indeed, any stimuli that induce cellular stress.[12] HO-2 is constitutively expressed and is virtually uninducible, with the exception of glucocorticoids.[13] Very little is known about HO-3. It lacks the catalytic activity shared by the other two HO isoforms, and has been suggested to act as a binding protein of the heme molecule.[14] CO is thought to play a cytoprotective role against airway oxidative stress, and it has been put forward as a useful marker for inflammatory conditions in the lower respiratory tract. Increased levels of exhaled CO have been found in patients with bronchiectasis, cystic fibrosis and asthma.[15–17]

Nasal respiratory epithelium has been postulated to serve as the major site for CO production within the nose and paranasal sinuses. Nasal CO is found in both the maxillary sinus and the nasal cavity in healthy individuals, and HO-1 and -2 immunoreactivity have been identified in normal human nasal respiratory epithelium.[18,19] Increased nasal CO has been observed in patients with upper respiratory tract infection and allergic rhinitis,[20] and up-regulation of HO-1 has been reported in the inferior turbinate submucosa of patients with allergic rhinitis,[19] suggesting that CO may also play a potential role in regulating inflammatory disease processes within the upper respiratory tract.

Since the activity of HO isoforms in nasal polyposis had not been previously investigated, the aim of the current study was to detect and compare the expression of HO in human nasal polyp tissue and in normal nasal mucosa.

Materials and methods

Immunohistochemical staining for type one and two HO isoforms was carried out in nine nasal polyp specimens from patients who had undergone endoscopic sinus surgery for allergic nasal polyposis. The same staining was also conducted in six inferior turbinate control

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specimens obtained from patients who had undergone septoplasty for mechanical nasal obstruction in the absence of a history or clinical signs of rhinitis.

Universal exclusion criteria included smokers, subjects who had taken systemic or topical steroids or antihistamine therapy during the four weeks prior to surgery, children under 16 years of age, and pregnant women. Patients who presented with nasal polyposis as a result of cystic fibrosis, aspirin sensitivity or due to infective causes such as fungal sinusitis were also excluded.

The study was approved by the local research ethics committee. Informed consent was obtained from all participants.

All specimens were fixed in 10 per cent formalin, serially sectioned to 6-μm thickness and embedded in paraffin.

Immunohistochemical analysis was then performed on the sections, using the streptavidin–biotin technique. HO-1 expression was detected using a mouse monoclonal antibody (OSA110, Stressgen Biotech, Victoria, British Columbia, Canada) at a dilution of 1:1000 for 25 minutes. HO-2 expression was detected using a rabbit polyclonal antibody (OSA200, Stressgen Biotech) at 1:1500 for 25 minutes. The signal was visualised using diaminobenzidine and sections were counterstained with haematoxylin. Negative control sections were incubated in the absence of primary anti-HO antibodies. HO immunoreactivity was analysed blindly by two independent assessors (SL and SDP) using the method described by Brennan *et al.*[21]

Each case was given a score that was the sum of the scale of the observed area of staining (viewed at a magnification of ×40) and the intensity of staining (viewed at ×200). The area of staining was scored on a scale of zero to four, where: zero = no cell staining in any microscopic field; one = less than 25 per cent of tissue showing immunopositivity; two = 25 to 50 per cent immunopositivity; three = 50 to 75 per cent immunopositivity; and four = greater than 75 per cent immunopositivity. The intensity of heme oxygenase expression was scored on a scale of zero to three, where: zero = no staining; one = mild staining; two = moderate staining; and three = intense staining. The total score therefore ranged from zero to seven.

When the two assessors' scores for a single slide differed, the slide was reassessed blindly and independently until an identical score was reached.

The staining results were analysed statistically using the Statistical Package for the Social Sciences software (SPSS Inc, Chicago, Illinois, USA). The Mann–Whitney U test was used to compare the staining in the nasal polyp epithelial cells with that in the normal nasal respiratory epithelium.

Results and analysis

Within all six inferior turbinate control specimens, there was no evidence of HO-1 protein expression in respiratory epithelial cells or in the underlying stroma (containing vessels, seromucous glands and scattered inflammatory cells) (Figure 1a).

Cytoplasmic immunoreactivity to HO-1 was observed in the epithelial layer of seven out of the nine nasal polyp specimens, with distinct staining in the apical region of the cells (Figure 1b).

For epithelial HO-1 immunoreactivity, the difference between nasal polyps and normal mucosa was statistically significant ($p = 0.012$; Table I).

Macrophages in both nasal polyps and normal nasal mucosa exhibited positive HO-1 immunostaining, with no difference in the intensity of staining. HO-2 immunostaining was observed in respiratory epithelium, vascular endothelium and seromucous glands in both the control inferior turbinate mucosa (average stain score seven; Figure 2a) and the nasal polyps (nine of nine)

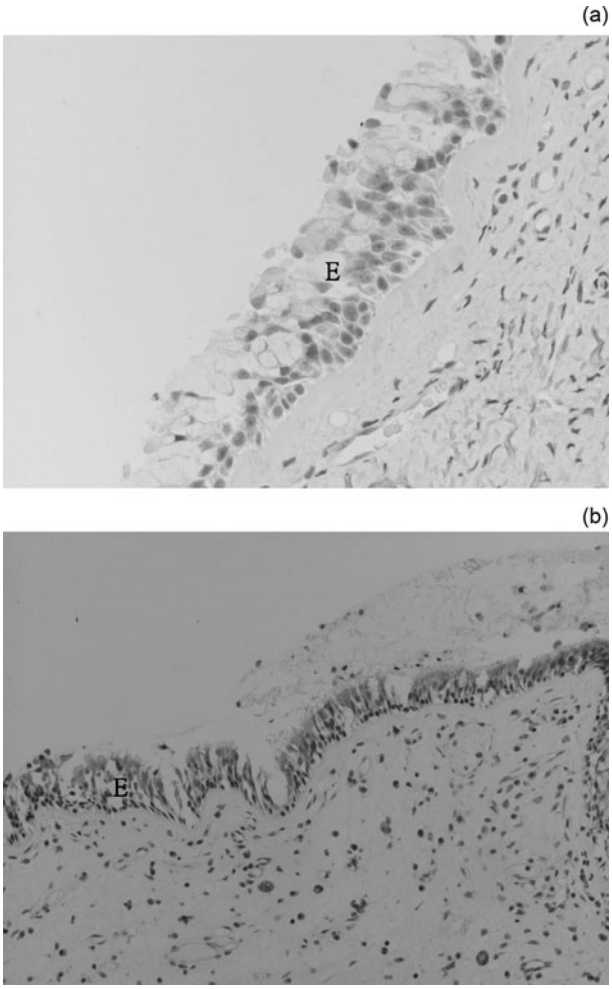

FIG. 1

(a) HO-1 immunostaining of normal inferior turbinate. No positive staining is seen (×40). (b) HO-1 immunostaining of nasal polyp. Positive cytoplasmic staining is strongest in the apical region of the epithelial cells overlying the polyp (×40). E = respiratory epithelium.

TABLE I

HO-1 EXPRESSION: NORMAL NASAL RESPIRATORY EPITHELIUM *VS* NASAL POLYP EPITHELIUM*

Normal nasal respiratory epithelium†	Nasal polyp epithelium‡
0	7
0	7
0	7
0	5
0	4
0	4
	4
	0
	0

Data shown are visual analogue scale scores. *$p = 0.012$. †$n = 6$; ‡$n = 9$

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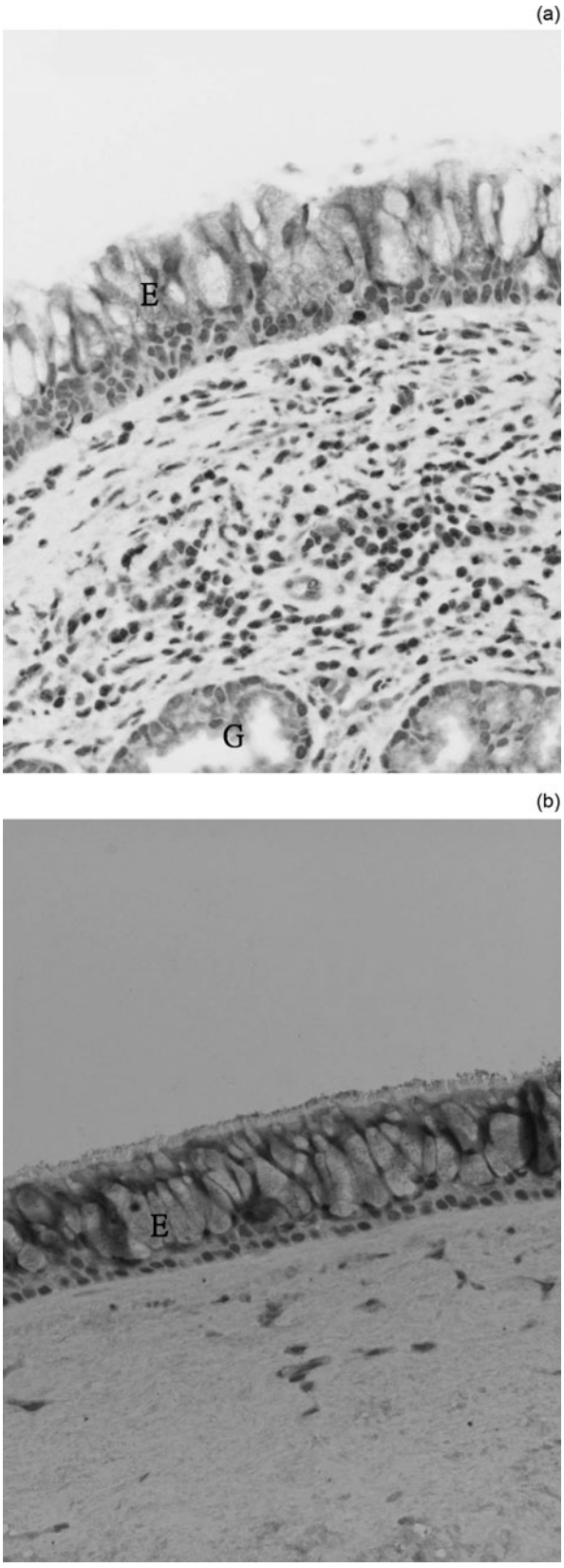

FIG. 2

(a) HO-2 immunostaining of normal inferior turbinate. Positive staining is seen in the epithelial cells of the respiratory mucosa, seromucous glands and endothelial cells lining vessels (×40). (b) HO-2 immunostaining of nasal polyp. Positive staining is shown in epithelial cells covering the polyp (×40). E = respiratory epithelium; G = seromucous glands.

(average stain score seven; Figure 2b), with equal intensity ($p = 1.00$).

Discussion

Recent research has examined the role of the endogenous gas CO in the respiratory tract. In particular, much work has focused on the ability of respiratory epithelium to express HO and release CO. The expression of HO-1 and -2 has been demonstrated in both lower and upper airway respiratory epithelium.[18,19,22–24] However, the role of CO in airway regulation remains poorly understood.

Lim *et al.* reported extensive distribution of HO-1 and -2 immunoreactivity in airway epithelium and submucosal macrophages in normal subjects.[23] When compared with asthmatic subjects, these authors found no difference in intensity and distribution of immunostaining. Following corticosteroid inhalation, in subjects with asthma the expression and distribution of HO-1 and -2 also remained unchanged. Although the levels of exhaled NO were significantly reduced, exhaled CO levels were not altered. Based on these findings, Lim *et al.* suggested that HO may be an important endogenous antioxidant enzyme which serves to protect cells against environmental agents that induce oxidative stress.

Subsequently, Lakari *et al.* reported a differential distribution of HO-1 in healthy human lung.[24] HO-1 immunoreactivity was mainly localised to alveolar macrophages, with staining varying from moderate to intense. In contrast, in bronchial epithelium, alveolar epithelium, endothelium and interstitium, the immunoreactivity varied from very low to undetectable.

Biochemical and immunohistochemical studies using bronchioalveolar lavage fluid and lung tissue performed in patients with established acute respiratory distress syndrome showed HO-1 elevation. Concentrations of HO-1 in bronchioalveolar lavage fluid correlated positively with changes in the concentrations of ferritin and the iron saturation of transferrin. Elevated levels of HO-1 were postulated to contribute to the changes in iron mobilisation, signalling and regulation seen in acute respiratory distress syndrome.[25]

However, nasal HO expression and CO in inflammatory diseases of the upper airway is less well characterised. Andersson *et al.* demonstrated that CO is endogenously produced in the human nose and paranasal sinuses by the HO isoenzymes.[18] A different study, on the other hand, reported no evidence of basal CO production within the nasal airway.[26] Increased CO levels were reported in both the nasal and exhaled air of patients with seasonal allergic rhinitis and those with upper respiratory tract infection.[20,27,28]

We subsequently reported that, in the inferior turbinate tissue of patients with allergic rhinitis, HO-1 immunoreactivity is up-regulated in vascular endothelium within the submucosal layer, compared with normal inferior turbinate tissue; however, no HO-1 staining was observed in the nasal respiratory epithelium in either group.[19] We postulated that, in patients with allergic rhinitis, HO-1 may play a role at the vascular interface within the submucosa, rather than within nasal respiratory epithelium.

In the current study, inducible HO-1 immunoreactivity was found in nasal polyp epithelium but not in normal nasal respiratory epithelium, suggesting up-regulation of HO-1 in nasal polyposis. Based on these findings, we hypothesise that the expression of nasal polyp HO-1 may be induced by the inflammatory pathways that are associated with the formation of nasal polyps. HO-1 can be induced by any stimulus that causes cellular stress.[12] The high level of endogenous expression of HO-1 present

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within the nasal polyp epithelium may directly result from upper airway exposure to normal daily environmental oxidative insults, such as pollutants, which contain ozone, nitrogen dioxide and particulates.[29,30]

- **The aetiology of nasal polyp diseases is poorly understood**
- **Much attention has focused on the role of epithelial-derived NO in the pathophysiological process of nasal polyposis**
- **Heme oxygenase-derived CO is thought to play a cytoprotective role against airway oxidative stress, and has been put forward as a useful marker for inflammatory conditions in the lower respiratory tract**
- **This is the first study to demonstrate up-regulation of epithelial HO-1 in nasal polyposis, compared with normal nasal mucosa**
- **The current results support the theory that locally released CO may play a role in the pathogenesis of nasal polyposis**

On the other hand, HO-1 expression may be intrinsically induced by the inflammatory mediators released from the epithelium and from the infiltrating inflammatory cells that have been implicated in the pathogenesis of nasal polyposis.[31] Induced NO synthase expression is up-regulated in nasal polyp epithelium.[8] NO is known to induce HO-1, and the subsequent production of CO has the ability to inhibit induced NO synthase activity by binding the heme moiety of this enzyme.[32] It is therefore possible that locally released CO serves a regulatory role in limiting NO production in nasal polyp epithelium, and protects cells against toxic levels of NO.

Harju *et al.* reported that HO-1 immunoreactivity could be readily observed in alveolar macrophages in newly diagnosed asthmatic patients not using inhaled anti-inflammatory therapy, but not in those treated with systemic corticosteroids for acute exacerbation.[33] These authors also observed a rapid but transient induction of HO-1 by oxidants in cultured monocytes within the first 24 hours, but no HO-1 could be detected during the next 48 hours. We observed only weak HO-1 immunostaining of macrophages in both nasal polyps and normal nasal mucosa, with no difference in intensity. None of the patients in our study had received nasal or systemic steroid therapy for at least one month prior to their nasal surgery. Immunohistochemistry was probably not sensitive enough to detect minor differences of expression of this enzyme protein. Further, detailed studies on nasal polyp macrophages, using more sensitive techniques involving nasal lavage fluid and Western blot analysis, are warranted.[23]

Different levels of the respiratory tract have been shown to have variable HO-1 immunoreactivity. In the bronchial tree, HO-1 is mainly expressed in alveolar macrophages, and expression is increased in asthmatic patients.[33] Within the nose, HO-1 is up-regulated within the vascular endothelium in patients with allergic rhinitis.[19] The difference in the distribution of HO-1 immunoreactivity between patients with nasal polyps and those with atopic conditions of both the upper and lower respiratory tract implies that the underlying pathophysiological processes of these conditions may be different. Whilst reduced nasal NO and increased exhaled NO have been observed in patients with nasal polyposis,[9] nasal and exhaled CO concentrations have not been investigated in these patients. Hence, it would be interesting to determine whether the concentration of nasal and exhaled CO is altered in patients with nasal polyp disease.

HO-2, the constitutively expressed form of heme oxygenase, is found to be expressed with the same intensity in both nasal polyp epithelium and normal nasal respiratory epithelium. We did not expect up-regulation of HO-2 in nasal polyp tissue, since the expression of this enzyme is not known to be altered by any inflammatory processes.[13] Although nasal polyp tissue typically consists of an outer lining of epithelium surrounding an underlying stroma of oedema, glandular hyperplasia, fibrosis and cellular infiltrate, the histological features of nasal polyp epithelium remain similar to those of normal nasal respiratory epithelium.[34] Our study results show that nasal polyp epithelium retains the ability to express HO-2, suggesting that nasal polyp lining has the ability to continually produce CO. Further investigations are required to determine whether this is related to the process of nasal polyp formation.

Conclusion

The findings of the current study further support the role of respiratory epithelium in the pathogenesis of nasal polyposis, and locally released CO may play a part in this process.

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References

1 Klossek JM, Neukirch F, Pribil C, Jankowski R, Serrano E, Chanal I *et al.* Prevalence of nasal polyposis in France: a cross-sectional, case-control study. *Allergy* 2005;**60**:233–7
2 Johansson L, Akerlund A, Holmberg K, Melen I, Bende M. Prevalence of nasal polyps in adults: the Skovde population-based study. *Ann Otol Rhinol Laryngol* 2003;**112**:625–9
3 Peters-Golden M, Henderson WR Jr. The role of leukotrienes in allergic rhinitis. *Ann Allergy Asthma Immunol* 2005;**94**:609–18, 618–20, 669
4 Gelfand EW. Inflammatory mediators in allergic rhinitis. *J Allergy Clin Immunol* 2004;**114**(suppl 5):S135–8
5 Bradley DT, Kountakis SE. Role of interleukins and transforming growth factor-beta in chronic rhinosinusitis and nasal polyposis. *Laryngoscope* 2005;**115**:684–6
6 Bhandari A, Takeuchi K, Suzuki S, Harada T, Hayashi S, Imanaka-Yoshida K *et al.* Increased expression of matrix metalloproteinase-2 in nasal polyps. *Acta Otolaryngol* 2004;**124**:1165–70
7 Fang SY, Shen CL, Ohyama M. Presence of neuropeptides in human nasal polyps. *Acta Otolaryngol* 1994;**114**:324–8
8 Watkins DN, Lewis RH, Basclain KA, Fisher PH, Peroni DJ, Garlepp MJ *et al.* Expression and localization of the inducible isoform of nitric oxide synthase in nasal polyp epithelium. *Clin Exp Allergy* 1998;**28**:211–19
9 Gilain L, Bedu M, Jouaville L, Guichard C, Advenier D, Mom T *et al.* Analysis of nasal and exhaled nitric oxide concentration in nasal polyposis. *Ann Otolaryngol Chir Cervicofac* 2002;**119**:234–42
10 Kang BH, Huang NC, Wang HW. Possible involvement of nitric oxide and peroxynitrite in nasal polyposis. *Am J Rhinol* 2004;**18**:191–6
11 Rodgers PA, Vreman HJ, Dennery PA, Stevenson DK. Sources of carbon monoxide (CO) in biological systems and applications of CO detection technologies. *Semin Perinatol* 1994;**18**:2–10

12 Elbirt KK, Bonkovsky HL. Heme oxygenase: recent advances in understanding its regulation and role. *Proc Assoc Am Physicians* 1999;**111**:438–47
13 Maines MD. The heme oxygenase system and its functions in the brain. *Cell Mol Biol* 2000;**46**:573–85
14 McCoubrey WK Jr, Huang TJ, Maines MD. Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. *Eur J Biochem* 1997;**247**: 725–32
15 Horvath I, Loukides S, Wodehouse T, Kharitonov SA, Cole PJ, Barnes PJ. Increased levels of exhaled carbon monoxide in bronchiectasis: a new marker of oxidative stress. *Thorax* 1998;**53**:867–70
16 Horvath I, Borka P, Apor P, Kollai M. Exhaled carbon monoxide concentration increases after exercise in children with cystic fibrosis. *Acta Physiol Hung* 1999;**86**:237–44
17 Paredi P, Leckie MJ, Horvath I, Allegra L, Kharitonov SA, Barnes PJ. Exhaled carbon monoxide is elevated during allergen challenge in asthmatic patients. *Eur Respir J* 1998;**28**(suppl):127–8
18 Andersson JA, Uddman R, Cardell LO. Carbon monoxide is endogenously produced in the human nose and paranasal sinuses. *J Allergy Clin Immunol* 2000;**105**:269–73
19 Lo S, Di Palma S, Pitkin L, McCombe AW. Localisation of heme oxygenase isoforms in allergic human nasal mucosa. *Eur Arch Otorhinolaryngol* 2005;**262**:595–8
20 Andersson JA, Uddman R, Cardell LO. Increased carbon monoxide levels in the nasal airways of subjects with a history of seasonal allergic rhinitis and in patients with upper respiratory tract infection. *Clin Exp Allergy* 2002; **32**:224–7
21 Brennan PA, Umar T, Buckley J, Zaki GA, Langdon JD, Spedding A *et al*. Expression of nitric oxide synthase in pleomorphic adenomas of the parotid. *Br J Oral Maxillofac Surg* 2000;**38**:338–42
22 Donnelly LE, Barnes PJ. Expression of heme oxygenase in human airway epithelial cells. *Am J Respir Cell Mol Biol* 2001;**24**:295–303
23 Lim S, Groneberg D, Fischer A, Oates T, Caramori G, Mattos W *et al*. Expression of heme oxygenase isoenzymes 1 and 2 in normal and asthmatic airways: effect of inhaled corticosteroids. *Am J Respir Crit Care Med* 2000;**162**: 1912–18
24 Lakari E, Pylkas P, Pietarinen-Runtti P, Paakko P, Soini Y, Kinnula VL. Expression and regulation of hemoxygenase 1 in healthy human lung and interstitial lung disorders. *Hum Pathol* 2001;**32**:1257–63
25 Mumby S, Upton RL, Chen Y, Stanford SJ, Quinlan GJ, Nicholson AG *et al*. Lung heme oxygenase-1 is elevated in acute respiratory distress syndrome. *Crit Care Med* 2004;**32**:1130–5
26 Lundberg JO, Palm J, Alving K. Nitric oxide but not carbon monoxide is continuously released in the human nasal airways. *Eur Respir J* 2002;**20**:100–3
27 Monma M, Yamaya M, Sekizawa K, Ikeda K, Suzuki N, Kikuchi T *et al*. Increased carbon monoxide in exhaled air of patients with seasonal allergic rhinitis. *Clin Exp Allergy* 1999;**29**:1537–41
28 Yamaya M, Sekizawa K, Ishizuka S, Monma M, Mizuta K, Sasaki H. Increased carbon monoxide in exhaled air of subjects with upper respiratory tract infections. *Am J Respir Crit Care Med* 1998;**158**:311–14
29 Seaton A, MacNee W, Donaldson K, Godden D. Particulate air pollution and acute health effects. *Lancet* 1995; **345**:176–8
30 Li XY, Gilmour PS, Donaldson K, MacNee W. Free radical activity and pro-inflammatory effects of particulate air pollution (PM10) in vivo and in vitro. *Thorax* 1996;**51**:1216–22
31 Djukanovic R. Nasal polyps – a model of chronic respiratory mucosal inflammation. *Clin Exp Allergy* 1995;**25**:582–5
32 Durante W, Kroll MH, Christodoulides N, Peyton KJ, Schafer AI. Nitric oxide induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle cells. *Circ Res* 1997;**80**:557–64
33 Harju T, Soini Y, Paakko R, Kinnula VL. Up-regulation of heme oxygenase-I in alveolar macrophages of newly diagnosed asthmatics. *Respir Med* 2002;**96**:418–23
34 Slavin RG. Relationship of nasal disease and sinusitis to bronchial asthma. *Ann Allergy* 1982;**49**:76–9

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