Eur Respir J 2001; 18: 420–430
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European Respiratory Journal
ISSN 0903-1936

MEETING REPORT

"Haemoxygenase-1 induction and exhaled markers of oxidative stress in lung diseases", summary of the ERS Research Seminar in Budapest, Hungary, September, 1999

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ABSTRACT: In recent years, there has been increasing interest in noninvasive monitoring of airway inflammation and oxidative stress. Several volatile and nonvolatile substances can be measured in exhaled breath and have been suggested as potential biomarkers of these events.

Exhaled gases, including carbon monoxide (CO), alkanes (ethane, pentane), and substances measured in breath condensate, such as hydrogen peroxide (H_2O_2) and isoprostanes were all suggested as potential markers of oxidative stress in the lung.

A European Respiratory Society (ERS) International Research Seminar entitled "Haemoxygenase-1 induction and exhaled markers of oxidative stress in lung diseases" was organized by the Airway Regulation and Provocation Group of the Clinical Allergy and Immunology Assembly in Budapest, Hungary in September, 1999 to integrate the latest knowledge on these issues and accelerate further improvement in this area. During this 2-day event several issues were raised about: the use and standardization of measurements in exhaled breath; problems of measuring expired H_2O_2 and other mediators in breath condensate; role and regulation of haemoxygenase (HO)-1 in the lung; and conditions and factors influencing exhaled CO.

This report is a summary of the main presentations at the seminar, together with the current areas of research in this rapidly expanding field.
Eur Respir J 2001; 18: 420–430.

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Keywords: Breath condensate
exhaled air
haemoxygenase
hydrogen peroxide
inflammation
oxidative stress

Received: March 27 2001
Accepted: March 27 2001

Inflammation and oxidative stress are involved in the pathogenesis of a variety of pulmonary diseases. These pathological processes are present in several inflammatory lung conditions including asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF). The main objectives of this international research seminar were to discuss recent results on exhaled markers of oxidative stress and the role of haemoxygenase (HO)-1 induction in different lung diseases. There were four major sections concentrating on different aspects of the mechanism and role of oxidative stress in different lung diseases and its noninvasive monitoring by the use of exhaled breath samples: The first section entitled "Oxidative stress in inflammatory lung diseases" contained three topics concentrating on the mechanism of oxidative stress in the lung, the antioxidant defences on the airway surface and the link between clinical symptoms, inflammation and oxidative stress in chronic inflammatory lung diseases. The second section (Markers of oxidative stress in exhaled breath condensate) included talks on methods and problems of exhaled H_2O_2 measurement, exhaled markers of oxidative stress in lung diseases and on the questions of oxygen radical-mediated tissue damage in CF. In the third section (Carbon monoxide (CO) in exhaled breath and in the lung), the introductory talks dealt with the localization of HO enzymes in the airways, the increase of exhaled CO in different lung conditions, factors influencing the level of exhaled CO and the protective effect of CO against oxidative stress. Finally, in the fourth section (Haemoxygenase-1 induction in the lung) the topics concentrated on HO-1 induction from different aspects: as a vascular protective response, its role in the inflammatory response, and its regulation by nitric oxide (NO) and cytokines in the airway epithelium. All sections were closed by a discussion when several issues were raised about these topics. In this report the introductory talks of the seminar and the main areas discussed are presented.

Oxidative stress response in inflammatory lung diseases

Mechanisms of oxidative stress in the lung

The major source of increased oxidant burden in inflammatory lung diseases is the release of reactive oxygen species and nitrogen species (ROS and RNS, respectively) from inflammatory leukocytes and also from airway epithelial cells. In addition, inhaled oxidants such as those in cigarette smoke and air pollutants also contribute to oxidative stress, and endogenous intracellular generation of ROS and RNS also occurs.

Oxidative stress is an imbalance between oxidants and antioxidants which occurs either from an increase in oxidants and/or decrease in antioxidants. A number of antioxidant systems, both enzyme systems and sacrificial antioxidants, are present in the lungs and these vary in their importance in different sites in the respiratory tract. Oxidative stress can have a number of consequences both locally and systemically such as inactivation of antiproteineases, epithelial injury, neutrophil sequestration and migration in the lungs, and signal transduction and gene expression for pro-inflammatory mediators. Considerable research has focused on the regulation of redox sensitive transcription factors such as nuclear factor-κB (NF-κB) and activation protein-1 (AP-1) which have fundamental roles in the inflammatory response. Both ROS and RNS can activate NF-κB and increase the nuclear binding of NF-κB and AP-1. Synergistic activation of NF-κB can be demonstrated between ROS/RNS and inflammatory mediators such as tumour necrosis factor-α (TNF-α) which itself creates oxidative stress. Oxidative stress also produces a response involving the up-regulation of AP-1, c-Jun N-terminal kinase (JNK) and p38. One aspect of this stress response is the upregulation of protective antioxidants such as γ-glutamylcysteine synthetase, and consequently, increased glutathione production. A critical factor may therefore be the balance between these pro- and anti-inflammatory effects of oxidative stress. A further effect of oxidative stress in relation to the inflammatory response is the induction of apoptosis for which oxidants are an important signal. Although there is a considerable amount of circumstantial evidence implicating oxidative stress in the pathogenesis of many inflammatory lung diseases, this hypothesis remains to be proven. One of the problems with proving this hypothesis is the lack of effective antioxidant therapy. The development of molecules with potent antioxidant properties should allow studies on proof of the concept that redressing the oxidant/antioxidant balance using effective antioxidants may be a novel treatment in inflammatory lung diseases [1–5].

Oxidative stress and antioxidant defences on the surface of the lung

Under normal circumstances, oxidative injury of the respiratory epithelium is minimized as the extracellular surface of the lung is buffered by a thin layer of respiratory tract lining fluid (RTLF), rich in antioxidant defences. Nonenzymatic antioxidants include reduced glutathione, uric acid, vitamin C (ascorbic acid), and vitamin E (α-tocopherol). Enzymatic antioxidants include superoxide dismutase, glutathione peroxidase and catalase. It is likely that the quantity and quality of this airway antioxidant network is an important determinant of the susceptibility of the underlying respiratory epithelium to resist oxidative stress.

There are marked differences in antioxidant defences between the upper and lower respiratory tract. For example, bronchoalveolar lavage (BAL) fluid obtained from the lower respiratory tract contains the proteins caeruloplasmin and transferrin, abundant amounts of the low molecular weight antioxidants reduced glutathione and vitamin C, and low concentrations of uric acid and α-tocopherol. In contrast, lavage of the nasal cavity contain uric acid in large quantities, with much smaller amounts of tripeptide glutathione (GSH) and vitamin C. As the lavage procedure leads to variable dilution of RTLF from different regions of the respiratory tract, it is difficult to fully quantify these differences. For this reason, the ERS working group on "Acellular Components of BAL Fluid" have recently suggested that standardized techniques are used and all data are reported per mL of recovered fluid.

RTLF antioxidant status can differ markedly even between normal individuals and these differences appear to be consistent with time. Even so, there is still relatively little information available regarding the normal range of antioxidant defences in RTLF. Moreover, the sources of RTLF antioxidant defences are still unclear. For example, there is a steep concentration gradient between the RTLF and plasma glutathione pools, suggesting local synthesis or secretion of glutathione into RTLF. On the other hand, ascorbic acid concentrations appear to be similar in RTLF and plasma suggesting free movement between the circulating and airspace pools of this antioxidants. Uric acid concentrations are about half the circulating level in the lower respiratory tract, but in the nasal cavity they are at least equivalent suggesting free movement of uric acid in the upper airways. These findings suggest that different mechanisms exist for regulating the RTLF pool sizes of ascorbic acid, GSH and uric acid. At this time, the status (and response) of the RTLF antioxidant defence network in different lung pathologies is unclear. For example, in two conditions which involve inflammation of the respiratory tree, namely cigarette smoking and patients with idiopathic pulmonary fibrosis, glutathione status is quite different. In cigarette smokers, RTLF glutathione concentration is elevated with respect to controls while in patients with idiopathic pulmonary fibrosis (IPF) is decreased. Recently, it has been reported that subjects with mild asthma have low RTLF vitamin C and E concentrations. The relative importance of each antioxidant *in vivo*, *i.e.* the relative contribution of each antioxidant to the total antioxidant capacity of BALF, is not presently understood. It is likely that in certain

circumstances, many antioxidants will act cooperatively to provide protection against oxidative damage. This question, and how RTLF antioxidant status relates individual sensitivity to oxidative stress, are both important considerations for future study design [6–8].

The link between clinical symptoms, inflammation and oxidative stress in the "puzzle" of chronic inflammatory lung diseases

Many chronic inflammatory pulmonary diseases are associated with inflammation and oxidative stress. A complex interplay between the specific cause of the disease, host characteristics and the type and intensity of inflammation and oxidative stress results in the clinical picture and likelihood of progression of that specific disease. The presence of inflammation and oxidative stress has well been established in obstructive lung diseases (asthma and COPD), interstitial lung disease, cystic fibrosis and bronchiectasis, and adult respiratory distress syndrome (ARDS). Non-invasively obtained markers of inflammation and oxidative stress have been identified, and, ideally, should represent the intensity of the ongoing pathological processes within the lungs. From a clinical point of view, physicians may expect that exhaled markers reveal insight into the pathophysiology, have a role in the assessment of the severity of inflammation/oxidative stress, predict lung function deterioration, and provide individual guidance during (pharmaco)therapy.

H_2O_2 is formed by inflammatory cells in the upper and lower airways. Increased levels have been demonstrated in patients with asthma, COPD, bronchiectasis, and ARDS. In general, exhalation of H_2O_2 appears to increase during unstable disease, and is (in asthma) related to the total number of eosinophils in sputum, and (in COPD) with the total number of polymorphonucleates (PMN)s in induced sputum. Inhaled corticosteroids (ICS) reduce the production and exhalation of H_2O_2. CO is produced by HO, and has been shown to be increased in smokers, in patients with CO poisoning, in asthma, and in upper respiratory tract infections. In asthma, a correlation has been noted between exhaled CO and eosinophil cell counts in sputum. Exhaled H_2O_2 and CO have provided an insight into the pathophysiology of chronic inflammatory lung diseases, are to some extent related with the severity of inflammation/oxidative stress, and are influenced by interventions. Their predictive value and their role in assessing individual patients remains to be established [9–19].

During the discussion part of this section the role of oxidative stress in the clinical picture of inflammatory lung diseases, the relationship between symptoms and oxidant-antioxidant imbalance and factors which are capable of influencing oxidative stress response were discussed.

Markers of oxidative stress in exhaled breath condensate

Methods and problems of measuring hydrogen peroxide in breath condensate

Two methods have been described to measure H_2O_2 in breath condensate: the spectrophotometric method and the fluorometric method (table 1).

Spectrophotometric method. The interaction of H_2O_2, 3,3′,5,5′-tetramethylbenzidine (TMB) and horse radish peroxide (HRP) forms 3,3′,5,5′-tetramethyl-1,1′-diphenoquinon-4,4′-diamine which can be measured with an absorbance of 450 nm.

Fluorometric method. The interaction of H_2O_2, p-hydroxyphenylacetic acid (pHPA) and HRP forms 2,2′-dihydroxybiphenyl-5,5′diacetate ($p(HPA)_2$) and the fluorescence of the dimer reflects the concentration of H_2O_2.

Several factors may influence the concentration of exhaled H_2O_2: smoking status, upper respiratory tract infections, breathing pattern, contamination with saliva and body temperature [9–19].

Exhaled markers of oxidative stress in lung diseases

Traditionally, oxidative stress has been monitored by measuring increased production of reactive oxygen

Table 1. – Methods of exhaled hydrogen peroxide measurement

Reference	Pretreatment	Stability/storage	Detection limit
Spectrophotometric method:			
[10]	No	?	?
[11]	No	0 days	?
[12]	No	6 h	0.1 μM
[18]	No	2 days	0.1 μM
[19]	No	2 days	0.1 μM
Fluorometric method:			
[14]	HPA and HRP	1 month	?
[13]	HPA and HRP	?	0.1 μM
[15]	No	14 days	0.1 nM
[16]	No	14 days	0.1 nM
[17]	No	14 days	0.083 μM

HPA: hydroxyphenylacetic acid; HRP: horse radish peroxide; ?: not stated.

species or end-products of oxidation in circulating cells and plasma. Recently, several techniques have been developed to detect oxidative stress using breath samples, which would more directly sample local production in the lungs. The major advantage of breath analysis is that it can easily be repeated, can be used in children and can be applied in patients with very severe disease where more invasive measurements are inappropriate. Several studies have reported an increase in H_2O_2 in expired condensates in lung diseases. Other molecules including nitrite and nitrate, nitrosothiols, peroxynitrite, lipid mediators, 8-isoprostane and cytokines can also be detected in breath condensate [20–21].

Isoprostanes are stable prostaglandins formed by oxidation of arachidonic acid *via* a nonenzymatic pathway [22] and have been advocated as a novel index of oxidative stress. The most prevalent isoprostane species in humans is 8-isoprostane (8-epiprostaglanding $F_{2\alpha}$). Concentrations of 8-isoprostane are increased in expired condensates of patients with asthma and its level correlates with disease severity and exhaled CO level [23]. Levels of 8-isoprostane are also increased in cigarette smokers, in patients with COPD and in CF [24].

The volatile gases, ethane and pentane, can also be detected in the breath and have been used to measure lipid peroxidation to reflect oxidative stress. Exhaled pentane is increased during asthma exacerbations and decreases during recovery [25]. Exhaled ethane level is increased in cigarette smokers [26] and is reduced by high doses of antioxidant vitamins C and E, but not by vitamin E alone [27, 28]. Exhaled ethane levels are increased in CF and COPD, where there is a correlation with disease severity and with other markers of oxidative stress, such as exhaled CO. Exhaled ethane is also increased in asthma, and the levels are lower in patients treated with inhaled corticosteroids [29]. Its level is also increased in interstitial lung diseases, suggesting that it may also be a marker of lung parenchymal inflammation [30]. A disadvantage of this measurement is that it is expensive and time consuming, and avoidance of contamination with environmental ethane is important. However, it is useful as way of validating other and easier measurements of oxidative stress, such as exhaled CO [20–30].

New markers of oxidative stress in the breath

The major problems in human breath testing are:

Breath volatile organic compound collection and assay. Most volatile organic compounds (VOCs) in breath are present in picomolar concentrations, and require specialized instruments for their detection. A portable breath collection apparatus (BCA) has been developed, which collects breath VOCs onto sorbent traps for subsequent analysis by gas chromatography and mass spectroscopy. This has made it possible to perform clinical studies of breath VOCs at sites remote from a research laboratory.

Compensation for volatile organic compounds present in room air and in breath. Most VOCs in breath are also present in room air. It is therefore necessary to distinguish the breath signal from an artefact of room air contamination. Samples of breath and air VOCs are collected in order to determine the alveolar gradient of each VOC (concentration in breath-concentration in air). The alveolar gradient varies with the rate of synthesis-the rate of clearance of a VOC.

Characterization of breath volatile organic compounds in normals subjects. In a study of 50 normal subjects, 3,481 different VOCs were observed (alveolar gradient positive in 1,753, negative in 1,728). However, a central core common to subjects comprised only 27 VOCs. These were principally isoprene, alkanes, methylalkanes, and benzene derivatives.

Determination of breath markers of oxidative stress. Alkanes are products of oxidative stress produced by lipid peroxidation of polyunsaturated fatty acids by ROS. A new breath marker of oxidative stress has been identified, the breath alkane profile. This comprised the alveolar gradient of C4–C20 alkanes plotted as a function of carbon chain length. In a study of 50 normal subjects, the mean breath alkane profile was negative from C4–C11 and positive from C12–C20. The mean age of the older half of the group was significantly greater than the younger half (47.56 yrs *versus* 29.88 yrs, $p<0.0001$), and the mean alveolar gradients of four alkanes (C5–C8) were significantly more positive in the older subjects ($p<0.05$). There were no significant differences between males and females. It was concluded that the spectrum of alkanes in normal human breath contains apparently new markers of oxidative stress. The mean rate of clearance (*via* cytochrome P450 enzymes) exceeded the mean rate of synthesis (by ROS-mediated oxidative stress) for C4–C11 alkanes, while synthesis was greater than clearance for C12–C20 alkanes. The elevated alkane profile in older subjects was consistent with an age-related increase in oxidative stress.

Detection of disease with breath markers of oxidative stress. The sensitivity and specificity of the breath alkane profile as a screening test for a number of diseases, including lung cancer, breast cancer, heart transplant rejection, and ischaemic heart disease are currently being investigated [31–32].

Does oxygen radical-mediated tissue damage occur in patients with cystic fibrosis and chronic lung infection? Patients with CF often suffer from chronic bacterial lung infections [33]. The exact mechanisms for bacterial airways colonization with several opportunistic pathogens, particularly *Pseudomonas aeruginosa*, in CF are still speculative but are most probably related to CF specific host factors [34]. Despite a vigorous and rapid influx of functional peripheral blood polymorphonuclear leukocytes (neutrophils) into the airways lumen, and the production of high titres of specific antibodies against the infecting pathogens, bacterial eradication is not generally achieved [34]. It was hypothesized that neutrophils cannot produce oxygen radicals in CF

airways. Therefore, H_2O_2 levels were determined in breath condensates of 63 CF patients and 51 normal subjects. Furthermore, in CF sputum samples, activities and concentrations of myeloperoxidase (MPO) and catalase (CAT) were determined as well as MPO/H_2O_2-mediated cytotoxicity of CF sputum in cell culture assays. It was found that H_2O_2 levels were similar in CF patients and normals [35]. This may be due to the presence of scavengers of H_2O_2 in CF sputum specimens. Alternatively, H_2O_2 may not be produced at all in CF airways. High concentrations and activities of human CAT and MPO were detectable in 38 CF sputa. Further experiments revealed that CF sputum, in the presence of H_2O_2, did not induce cytotoxicity *in vitro*, even when CAT was removed from sputum. This surprising result was due the immobilization of the highly positively charged MPO to negatively charged macromolecules present abundantly in CF sputum. In order to investigate whether H_2O_2 may not be produced at all in CF airways, it was investigated whether the oxygen partial pressure (P_{O_2}) is reduced in the CF sputum. The P_{O_2} in sputum plugs *in vivo* in six CF patients and 14 sputum specimens *in vitro* were assessed. The high P_{O_2} in sputum-free CF bronchi dropped rapidly and significantly when the oxygen probe entered the plugs, suggesting that CF plugs are anaerobic. Lack of oxygen may markedly reduce bacterial killing by neutrophils due to the inability to produce ROS and thus helps to explain the chronicity of the lung infection in CF patients and possibly chronic lung infections in other patient groups [33–35].

The handling, storage of breath condensate, reproducibility of measurements, significance of elevated levels of mediators in condensate and the relationship between disease activity and marker levels were discussed in more detail.

Carbon monoxide in exhaled breath and in the lung

Localization of haemoxygenases in the airway wall of normal and asthmatic subjects: the effect of corticosteroid therapy

HO is an anti-oxidant enzyme which catabolizes haem to produce CO and biliverdin. Induction of HO activity in the lung has a protective effect against oxidant-induced inflammation and bronchial hyperresponsiveness, such as that induced by exposure to ozone. The expression and distribution of HO-1 and HO-2, two isoenzymes of HO was determined, in the airways of patients with asthma, and determined the effect of inhaled corticosteroid therapy. Immunostaining for both enzymes was widely distributed in the airways submucosa, particularly in airway epithelium and submucosal macrophages (CD68+) as determined by double-immunostaining. There was no difference in intensity and extent of staining in biopsies from normal (n=10) and mild asthmatic (n=10) subjects. Following 1 month of treatment with inhaled corticosteroids (budesonide 1,600 $\mu g \cdot day^{-1}$), there was no significant change in the expression and distribution of either HO-1 or HO-2 in the airways submucosa in eight mild asthmatics, despite a significant reduction in airway eosinophils and a reduction in bronchial responsiveness to methacholine. Levels of exhaled NO were significantly reduced, but exhaled CO levels remained unchanged by the treatment. Treatment with a placebo inhaler (n=8) had no effect on these parameters. Thus, both HO-1 and HO-2 are extensively distributed equally in normal and asthmatics, and are not modulated by inhaled corticosteroid therapy in asthmatics. HO may be an important endogenous antioxidant enzyme [36, 37].

Increased carbon monoxide in acute asthma, upper respiratory infections and allergic rhinitis

To examine the role of endogenous CO in airway inflammatory diseases, exhaled CO concentrations were measured on a CO monitor (EC50 analyser, Bedfort Technical Instruments Ltd, Sittingbourne, UK). CO was reproducibly detectable in the exhaled air of all subjects. The exhaled CO concentrations were higher in asthmatic patients not receiving inhaled corticosteroids and similar in asthmatic patients receiving inhaled corticosteroids, compared with those in nonsmoking healthy control subjects. All of 12 patients with symptomatic asthma had reductions in exhaled CO concentration 4 weeks after the initiation of inhaled corticosteroid treatment. Changes in exhaled CO level were significantly related to those in the eosinophil cell counts in sputum. These findings suggest an elevation of exhaled CO in asthmatic patients, which decreases with corticosteroid therapy. Increases in the exhaled CO levels may, therefore, reflect inflammation in the asthmatic airways [38]. The time course of changes in exhaled CO concentrations after treatment of acute exacerbation of asthma was also examined. Exacerbations of asthma caused a fall in peak expiratory flow rate (PEFR) and a rise in exhaled CO in all patients, and treatment with oral glucocorticoids reversed these changes in both parameters. An improvement of PEFR was closely associated with a reduction of exhaled CO after treatment [39]. Whether upper respiratory tract infections increase the concentration of CO in exhaled air of normal persons was studied. At the time of symptoms, exhaled CO concentrations were elevated, and were decreased during recovery. Recovery values of exhaled CO were similar to those in age-matched nonsmoking control subjects [40]. To determine whether levels of CO are increased in patients with seasonal allergic rhinitis, measurements of exhaled CO were made in 86 patients with allergic rhinitis during and out of the cedar pollen season [38–40]. During the season, exhaled CO concentrations were elevated, and decreased to normal out of the season.

Exhaled carbon monoxide in lung diseases

Exhaled CO levels are increased in various inflammatory lung diseases and may be a useful marker of inflammation/oxidative stress. The source of exhaled CO is likely to be the HO enzyme family. Evidence for

this is that HO-1 expression is increased in airway macrophages and bilirubin levels are elevated in induced sputum of asthmatic patients, who present elevated exhaled CO level. Furthermore, exhaled CO increases after inhalation of the HO substrate haemin in normal subjects [41].

The levels of exhaled CO are increased in patients with asthma who are not treated with inhaled corticosteroids [38, 41]. The levels are usually within the normal range in patients who are treated with inhaled corticosteroids, but patients with severe asthma who may be on high doses of inhaled steroid or maintenance oral steroids have higher levels, suggesting that exhaled CO may reflect disease severity [42]. CO level is also increased in childhood asthma, but only in children who are symptomatic [43]. Exhaled CO is increased in some atopic adults who are asymptomatic, although to a lesser extent than exhaled NO [44]. Exhaled CO levels are increased after allergen exposure during the late response, and also during the early response within minutes of allergen challenge [45]. Patients with COPD have a neutrophil inflammation in their airways and the levels of exhaled CO are increased in COPD. This is due, in part, to cigarette smoking which causes a marked elevation in exhaled CO, but the levels of exhaled CO are also increased in exsmokers. Cigarette smoking causes a similar increase in exhaled CO in normal subjects and asthmatic patients and this effect lasts several hours. The levels of exhaled CO are increased in patients with bronchiectasis and it is not reduced in patients treated with inhaled corticosteroids [46]. In cystic fibrosis (CF) there is an increase in the level of exhaled CO and this is further increased during infective exacerbations [47, 48]. While in patients with stable CF inhaled steroids do not appear to affect the level of CO, oral corticosteroids are associated with lower values. The implication of these studies is that exhaled CO may be useful to detect deterioration in cystic fibrosis and may be a reflection of increased oxidative stress, which also causes exhaled NO to be low by the rapid reaction of different oxidants with NO [49]. Nasal and exhaled NO are diagnostically low in patients with primary ciliary dyskinesia (PCD) and Kartagener's syndrome. By contrast, exhaled CO is elevated in these patients and may reflect the chronic inflammation in the lower airways of these patients [50]. The levels of exhaled CO are elevated in normal subjects after upper respiratory tract infections [40], in a similar manner to the increase in exhaled NO. Viral infections might directly activate HO-1 or inducible nitric oxide synthase (iNOS) *via* transcription factor activation, or may induce these enzymes by increasing pro-inflammatory cytokines. Exhaled CO is also elevated in patients with lower respiratory tract infections and is reduced by antibiotic treatment.

Since HO is so widely distributed and is activated by many stimuli, it is likely that CO production is increased in many systemic diseases, and this could be reflected by an increase in exhaled CO. Little work has been done is this area, but in the future it will be necessary to determine how different conditions might affect exhaled CO. An increase in exhaled CO has been found in patients with diabetes and is related to blood glucose level and may be a reflection of the increased oxidative stress in this condition and activation of HO by glucose [51].

In summary, exhaled CO measurements are easy to make and are reproducible. CO levels are increased in several diseases of the airways, which are associated with inflammation and oxidative stress. The levels are more closely related to disease severity and symptoms than exhaled NO, and are of potential value in diseases such as COPD, CF and primary ciliary dyskinesia (PCD) where exhaled NO levels are of little value. The CO analysers are relatively simple and cheap so this measurement may be widely available. The measurement of exhaled CO is not complicated by nasal contamination, which is a major issue with exhaled NO measurements. On the other hand, exhaled CO levels are markedly affected by environmental CO, which may fluctuate considerably during the course of the day, particularly in cities. Previous exposure to high environmental CO levels may also result in subsequent increases in exhaled CO, as CO dissociated from carboxyhaemoglobin. Active and passive smoking markedly affects exhaled CO levels and it may be important to check smoking status by measuring urinary cotinine or Nikcheck reagent strips (DynaGen Inc., Cambridge, Ma, USA). Less is known about the origin of exhaled CO than of NO. While it is likely that some exhaled CO is derived from airways, a large proportion of exhaled CO may be derived from the alveoli and may diffuse from the circulation [38–51].

Factors influencing exhaled carbon monoxide level

Exhaled CO is produced endogenously in healthy nonsmokers and increases in different inflammatory lung conditions. It has been suggested to be useful in monitoring inflammatory lung diseases. Before using this measurement as a noninvasive marker of disease activity, however, more needs to be known about factors that may influence exhaled CO level.

Concerning the production of CO, the effect of exercise on the concentration and total output of exhaled CO in healthy subjects has been studied. During symptom-limited exercise, exhaled CO concentration showed a gradual decrease. However, CO output was increased approximately three-fold by the end of exercise test. CO output returned to normal 10 min after the test [52]. This increase in the output was similar in healthy children and adults, in asthmatic patients with or without steroid treatment, and also in patients with CF. This increase may be attributed to increased production of CO in the lung, probably because of sudden activation of HO-2; however, further studies should be performed to clarify the effect of changes in CO gradient between the airway wall and the ambient air.

From experimental studies, hypoxia is known to be a factor modulating CO production. In patients with pronounced arterial hypoxia as a result of non-pulmonary diseases, for example cardiac failure, increased levels of exhaled CO have been found, as

compared to nonhypoxic patients with the same condition. Improvement of their clinical condition was associated with the decrease of exhaled CO. In patients on steroid treatment, there was no elevation in exhaled CO level, regardless of the presence of great severity of hypoxia, suggesting that the source for increased CO in exhaled breath is HO-1 [53]. Not only chronically pre-existing hypoxia is able to cause an increase in exhaled CO, but acute hypoxia may also be able to. This was shown in a study investigating children with CF in a stable condition. Some subjects developed hypoxia during symptom-limited exercise testing, which was followed by an increase in exhaled CO level after the test [52].

As the increase in CO production is assumed to be regulated by factors leading to oxidative stress, it seemed reasonable to investigate the effect of antioxidants on exhaled CO in asthma. Oral supplementation with high doses of vitamins C and E for two weeks, however, did not modulate the increased CO concentration in a double-blind, cross-over study in 14 mild asthmatic patients. More potent antioxidants and local application of antioxidants into the airways may influence exhaled CO level. Finally, while atopy by itself is known to modulate the level of exhaled NO in asthmatic patients, this does not seem to be the case for exhaled CO. There is no difference in exhaled CO level between atopic and nonatopic patients with the same severity of the disease [53].

In summary, several factors are able to influence exhaled CO level and their possible effects should be taken into account before interpreting data obtained by this measurement [52–54].

Protective effects of haemoxygenase-1 and carbon monoxide against oxidative stress

HO catalyses the first and rate limiting step in the degradation of haem, to yield equimolar quantities of biliverdin IXa, CO and iron [55]. Three isoforms of HO exist: HO-1 is highly inducible while HO-2 and HO-3 are constitutively expressed. Although haem is the major substrate of HO-1, a variety of nonhaem agents including heavy metals, cytokines, hormones, endotoxin and heat shock are also strong inducers of HO-1 expression [56]. This diversity of HO-1 inducers has provided further support for the speculation that HO-1, besides its role in haem degradation, may also play a vital function in maintaining cellular homeostasis. Furthermore, HO-1 is highly induced by a variety of agents causing oxidative stress, including H_2O_2, GSH depletors, UV irradiation, endotoxin and hyperoxia [56–58]. One interpretation of this finding is that HO-1 can serve as a key biological molecule in the adaptation and/or defense against oxidative stress, which has been strengthened by recent findings in HO-1-deficient mice and humans [59, 60]. It has been shown that induction of HO-1 provides protection both *in vivo* and *in vitro* in various models of oxidative stress [56–58, 61]. Furthermore, the present authors will review additional observations that suggest exogenous administration of HO-1 by gene transfer also provides protection against oxidant stress [61], and that CO may mediate this protection [62]. CO may play an important protective role in other inflammatory disease states and thus has potential therapeutic implications.

A.M.K. Choi′s laboratory have examined whether exogenous administration of HO-1 *via* transgene delivery can induce HO-1 expression in the rat lung and can confer protection against oxidant induced lung injury using a rat model of hyperoxia. HO-1 messenger ribonucleic acid (mRNA) and HO-1 protein increased just hours after Ad5-HO-1 administration. The control rats exhibited marked lung haemorrhage, oedema, alveolar septal thickening, influx of inflammatory cells and fibrin deposition after 56 h of hyperoxia, but lung architecture was preserved in rats receiving AD5-HO-1. The present authors have also observed that rats exposed to hyperoxia alone exhibit a highly significant induction in the lung apoptotic index when compared to control rats in normoxia. In contrast, rats exposed to hyperoxia after pretreatment with Ad5-HO-1 demonstrated a significant reduction in the lung apoptotic index when compared to animals exposed to hyperoxia alone. Finally, animals receiving vehicle control all died by 66 h of hyperoxia exposure while a significant level of tolerance against hyperoxia was observed in rats receiving Ad5-HO-1.

Rats exposed to hyperoxia alone all died within 72 h, while rats exposed to hyperoxia in the presence of a low concentration of CO exhibited highly significant tolerance to hyperoxia: all these animals were alive at the 72 h time point. This protective effect of CO is concentration dependent, with effects seen in the range 50–500 ppm. Carboxyhaemoglobin levels, a standard measurement of CO levels in the blood, correlated with increasing concentrations of CO exposure and survival of animals to lethal hyperoxia. Rats exposed to low concentrations of CO (50–500 parts per million (ppm)) alone did not exhibit any untoward effects [62].

In rats exposed to hyperoxia in the presence of a low concentration of CO, a marked inhibition in the amount of pleural effusion was observed, as were significantly lower levels of protein accumulation as compared to rats exposed to hyperoxia alone. Marked lung haemorrhage, oedema, alveolar septal thickening, influx of inflammatory cells, and fibrin deposition were observed in rats exposed to hyperoxia alone. In contrast, the lungs of rats exposed to hyperoxia in the presence of CO were both macroscopically and microscopically normal. Rats exposed to hyperoxia in the presence of CO exhibited significant reductions in neutrophil influx and in the lung apoptotic index when compared to animals exposed to hyperoxia alone.

In summary, the potent anti-inflammatory and anti-apoptotic effects described in this report and recent accumulating data demonstrating the anti-inflammatory effects of HO-1, highlight the major mechanisms by which HO-1 serves to protect cells, organs and host organisms against oxidative stress. The potential role of CO in mediating the protective effects of HO-1 has also been highlighted. Future studies need to address the mechanism by which CO mediates these protective

effects, either independently or in conjunction with ferritin and bilirubin [9, 10], other by-products of HO-1 [55–62].

Haemoxygenase-1 induction in the lung

Haemoxygenase-1 a vascular protective response

This section attempts to summarize the protective effects of HO-1 induction and exogenous CO administration relating to a model of xenotransplanting mouse hearts to rats. If the rats receive treatment which inhibits complement activation (cobra venom factor (CVF)) and blocks T-cell responses (CyA), the mouse hearts survive indefinitely in them. If the rats are not treated, the mouse hearts are rejected in 3–5 days. Examination of the long-term surviving hearts reveals that HO-1 is up-regulated in endothelial cells (EC) and smooth muscle cells (SMC) of the heart, whereas there is much less or no up-regulation in hearts that are rejected. In order to test whether the HO-1 plays a role in survival of the hearts, HO-1-deficient hearts were transplanted to rats receiving the same immunosuppression given above (CVF+CyA). Despite this treatment, the HO-1-deficient hearts were rejected in 3–7 days, clearly showing that HO-1 plays a crucial role in assuring long-term survival of the transplanted hearts in this model. To test whether carbon monoxide (CO), one of the three products of HO-1 action on haem, is in part or wholly responsible for the action of HO-1 in allowing long-term survival, tin protoporphyrin (SnPP) was used to inhibit HO-1 action. Normal (wild-type) mouse hearts transplanted to rats receiving CVF+CyA+SnPP were rapidly rejected, in a similar fashion to the HO-1-deficient hearts. The rats receiving the mouse hearts were then exposed to CO at 400 ppm. Under these conditions, *i.e.* in the absence of HO-1 action but in the presence of exogenously administered CO, the mouse hearts survived long-term. The experiments showed that, in this model, CO can fully replace the action of HO-1. Production of bilirubin was measured to ensure that HO-1 action was inhibited in the presence or absence of CO. Mouse hearts that are rejected by rats show severe evidence of inflammation, whereas those hearts that are not rejected, do not show such signs. CO fully suppressed the inflammatory response associated with rejection. It has been shown that CO, in some situations, can substitute for the role of HO-1, and that either agent is highly anti-inflammatory [63]. These findings could lead to clinical application of HO-1 and/or CO [63].

Role of haemoxygenase-1 induction in inflammatory conditions

WILLIS *et al.* [64] have analysed the activity of HO in inflammatory cells isolated from a model of carrageenin-induced acute inflammation in the rat. HO activity was significantly higher 24 h after induction of inflammation. This increase in activity coincided with the appearance of the highly inducible isoform of HO, as determined by Western Blot analysis. Pretreatment of animals with an HO-inducer, ferriprotoporphyrin, decreased inflammatory cell number by 50% and cell exudate by 73% at 24 h, compared to controls. These results suggest that HO may represent an endogenous protective mechanism against free radicals in acute inflammation, and may be involved in the resolution of acute inflammation. HO-1 may therefore represent a novel therapeutic target for the modulation of the inflammatory response [64].

Nitric oxide and cytokine regulation of haemoxygenase-1 induction in epithelial cells

HO-1 can be induced by a variety of cellular stresses and inflammatory mediators [56, 65, 66]. CO, a product of HO activity, is elevated in the exhaled breath of subjects with various airway diseases. The cellular source of CO is unclear, although increased levels of HO-1 protein have been detected in sputum macrophages from asthmatic patients, when compared to normal subjects [41]. This section aims to examine whether the airway epithelium could contribute to the increased exhaled CO seen in many airway diseases.

Human primary airway epithelial cells were treated with interleukin (IL)-1β, interferon (IFN)-γ and tumour necrosis factor (TNF)-α (cytomix) for up to 24 h and expression of HO-1, HO-2, iNOS and reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) measured by reverse transcription polymerase chain reaction. After exposure to cytomix, HO-1 expression increased at 4 h, but decreased at 12 h, to return to basal levels by 24 h, but HO-2 expression did not change. The expression of iNOS followed a different time course with maximum expression at 24 h. HO-1 expression and iNOS activity (nitrite accumulation in culture media) were not inhibited by dexamethasone. The specific iNOS inhibitor, 1400W, inhibited cytomix-stimulated induction of HO-1, indicating that iNOS derived NO may stimulate HO-1 induction in human primary epithelial cells. The NO donor, NOC-18 (detanonoate), induced HO-1, but had no effect on HO-2 induction. HO-1 induction was not inhibited by dexamethasone. Addition of the guanylyl cyclase inhibitor, [1-H-[1,2,4]oxadiaole[4,3-a]quinoxalin-1-one (ODQ), and the protein kinase G inhibitor, KT5823, failed to inhibit NOC-18 induction of HO-1, implying that the NO induction of HO-1 in human primary epithelial cells is *via* a cyclic guanosine monophosphate (GMP) independent pathway. HO-1 protein was not expressed under basal conditions; however, 24-h exposure to cytomix induced HO-1 protein, which was not inhibited by dexamethasone. HO-2 protein was expressed constitutively in these cells and was upregulated in the presence of dexamethasone. Cytomix treatment induced HO activity 13-fold; however, NOC-18 treatment increased HO activity in a dose-dependent manner, with maximum activity at 500 μM (23-fold). This was quite different to the effect of NOC-18 on mRNA induction. This would imply that NO may activate HO directly. There are currently no

Table 2. – Publications relating to the use of exhaled markers in different lung conditions

	Asthma	COPD	Bronciectasis	Cystic fibrosis	Other conditions
Hydrogen peroxide	[13, 14, 16, 18]	[12, 17]	[19]	[35, 69]	[9–11, 15]
Nitrite/nitrate	[70]			[21, 67]	
Isoprostanes	[23]	[71]		[24]	[71]
Adenosine	[68]				
Ethane/pentane	[25, 29]				[26–28, 30]
VOCs					[31, 32]
Carbon monoxide	[38, 39, 41–43, 45, 54]		[46]	[47, 48, 52]	[40, 44, 50, 51, 53]

VOCs: volatile organic compounds; COPD: chronic obstructive pulmonary disease.

specific enzyme inhibitors and, therefore, it is not possible to determine whether this increase in activity is due to the activation of HO-1 or HO-2.

In summary, human airway epithelial cells can be induced with cytomix to express HO-1 mRNA and protein and this is not inhibited by glucocorticosteroids. NO can also induce HO-1 and again is not steroid-sensitive. Therefore, the airway epithelium may be a source of exhaled CO seen in airway diseases [65–66].

Conclusion

Carbon monoxide production in the body, factors and conditions influencing exhaled carbon monoxide, and, possible sources of exhaled carbon monoxide were discussed. The regulation of haemoxygenase-1 and haemoxygenase-2 activity, mechanisms of haemoxygenase-1 induction by inflammatory mediators and reactive oxygen species, and the multiple effects and role of the end-products of haemoxygenase activity under physiological and pathophysiological conditions were discussed in detail. Table 2 summarizes the references on the most studied exhaled biomarkers in different lung conditions (exhaled nitric oxide is not included).

Acknowledgements. The seminar was sponsored by the European Respiratory Society with additional help from the Hungarian Respiratory Society and the Science and Technology Programme of the Hungarian Ministry of Education.

Seminar chair: P.J. Barnes. **Initiator and coordinator**: I. Horváth. **Participants**: I. Adcock, UK; I. Rahman, UK; K. Thethi, UK; L. Otterbein, USA; D. Dyck, USA; M. Soares, USA; S. Loukides, Greece; P.J. Barnes, UK; S. Petruzzelli, Italy; R. Foresti, UK; G. Döring, Germany; G. Joos, Belgium; A. Antczak, Poland; G. Becher, Germany; F. Kelly, UK; A.M.K. Choi, USA; P.N.R. Dekhuijzen, the Netherlands; W. MacNee, UK; M. Phillips, USA; F. Bach, USA; M. Yamaya, Japan; Z. Csoma, Hungary; G. Losonczy, Hungary; P. Magyar, Hungary; G. Papp, Hungary; É. Huszár, Hungary; E. Barát, Hungary; G. Böszörményi-Nagy, Hungary; R. Horváth, Hungary; I. Herjavecz, Hungary; J. Strausz, Hungary; Z. Lohinai, Hungary; L. Donnelly, UK; J. Homolka, Czech Republic; E. Hídvégi, Hungary; K.F. Chung, UK; M. Leckie, UK; N. Sterfors, Sweden; D. Willis, UK; T. Lawrence, UK; P. Paredi, UK; B. Bálint, Hungary; C. Dunster, UK; J.T. Chapman, USA; K. Ganas, Greece; Z. Novák, Hungary; R. Motterlini, UK; D. Worlitzsch, Germany; M. Krol, Poland; K. Nakayama, Japan; S. Culpitt, UK; K. Torén, Sweden; A-C. Olin, Sweden; I. Horváth, Hungary.

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