Effect of cigarette smoking on haem-oxygenase expression in alveolar macrophages

L. Atzoria,b, G. Caramoria, S. Lima, E. Jazrawia, L. Donnellya, I. Adcocka, P.J. Barnesa, K.F. Chunga,*

aDepartment of Thoracic Medicine, National Heart and Lung Institute, Imperial College School of Medicine, Dovehouse Street, London SW3 6LY, UK
bDepartment of Toxicology, Section of Oncology and Molecular Pathology, University of Cagliari, Cagliari, Italy

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Summary We investigated the effect of chronic cigarette smoking on the expression of haem-oxygenase (HO)-1 and HO-2. Normal subjects and asymptomatic young current smokers with normal lung function tests underwent bronchoalveolar lavage for recovery of macrophages. Reverse transcription/polymerase chain reaction (RT-PCR) analysis showed no significant difference in HO-1 and HO-2 mRNA expression between the two groups. On the other hand, Western blot analysis showed a significant (P < 0.05) reduction of HO-2 protein, but not of HO-1, in alveolar macrophages from smokers compared to normal. There was no significant differences by immunocytochemistry for HO-1 and HO-2 expression between the groups. We concluded that HO-2 expression is reduced in alveolar macrophages of smokers, possibly due to the oxidative stress of cigarette smoke. This may in turn lead to reduced protection against further oxidative insults.

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Carbon monoxide;
Smoking

Introduction

The airway epithelium is continuously exposed to oxidants inhaled from exogenous sources, such as cigarette smoke and ozone,1,2 or endogenously produced and released from neutrophils and alveolar macrophages recruited to the lungs and activated during acute and chronic airway inflammation.3 Oxidative stress has been implicated in the pathogenesis of many respiratory diseases including chronic obstructive pulmonary disease (COPD), bronchial asthma and cystic fibrosis.4,5 There is accumulating evidence that oxidative stress may play an important role in the pathogenesis of COPD. There is an increase in the concentration of hydrogen peroxide in the exhaled breath condensates of patients with COPD, particularly during exacerbations,6 and increased breath and urinary concentrations of 8-isoprostane, a marker of oxidative stress.5 Oxidative stress may exacerbate COPD through several mechanisms, including the activation of the transcription factor, nuclear factor-κB (NF-κB), which switches on the genes for TNF-α, interleukin-8, and other inflammatory proteins, and oxidative damage of anti-proteases, such as α1-antitrypsin and secretory leukoprotease inhibitor, thus enhancing inflammation and proteolytic injury.5 Chronic inhalation of tobacco smoking is the main cause of COPD around...
the world. In vitro acute exposure to tobacco smoke induces the expression of haem-oxygenase-1 through reactive oxygen intermediates. Many studies indicate that the haem-oxygenase (HO) enzyme system is involved in the lung resistance against oxidative damage.

Haem-oxygenases catalyse the rate-limiting step in the oxidative degradation of haem to biliverdin, which is then converted to the antioxidant bilirubin, carbon monoxide (CO), and free iron. Three isoforms called HO-1, HO-2, and HO-3 have been characterised. While HO-2 is constitutively expressed in most cells, HO-1 is inducible by many agents that lead to oxidative stress, including hydrogen peroxide and heavy metals such as cadmium. The HO-3 expression and function has not been fully characterised. It has been proposed that HO-1 plays an important role in lung protection against oxidant injury, but studies using knock-out mice suggest also an important role of HO-2 in the protection of the lungs against oxidative stress.

Carbon monoxide (CO) generated by catalysis of haem by HO detectable in the exhaled air and elevated levels of exhaled CO have been reported in stable asthma. The difference in exhaled CO between normal and asthmatic subjects, however, is much less than that of exhaled nitric oxide. The inducible isoenzyme HO-1 expression in the airways has been already extensively studied both in the bronchoalveolar lavage (BAL) cells and in the bronchial mucosa from normal subjects and asthmatics patients. Constitutive isoenzyme HO-2 expression has been described in the bronchial epithelium of normal subjects and asthmatics patients. However, the expression of the two isoforms of the HO system has not been examined in the alveolar macrophages from smokers. We hypothesised that the expression of inducible HO-1 would be increased in cigarette smokers. We therefore investigated the expression of HO-1 and HO-2 in bronchoalveolar macrophages collected from normal subjects and young current asymptomatic smokers.

### Methods

#### Patients

Six healthy smokers and six normal nonsmoking volunteers were studied (Table 1). The healthy smokers were current smokers (>5 pack/yr; 20–30 cigarettes per day) with a normal forced expiratory volume in one second (FEV1) and no respiratory symptoms. All study subjects were free of acute upper respiratory tract infections and none had received glucocorticoids, theophylline or antibiotics within the preceding month. All subjects had no past history of asthma or allergic rhinitis. The study was approved by the local Ethics Committee, and informed consent was obtained from each subject.

#### Fibreoptic bronchoscopy, collection and processing of bronchoalveolar lavage

Subjects attended the bronchoscopy suite at 8.30 a.m. after having fasted from midnight and were pre-treated with atropine (0.6 mg IV) and midazolam (5–10 mg IV). Oxygen (3 l/min) was administered via nasal prongs throughout the procedure and oxygen saturation was monitored with a digital oximeter. Using local anaesthesia with lidocaine (4%) to the upper airways and larynx, a fibreoptic bronchoscope (Olympus BF10 Key-Med, Southend, UK) was passed through the nasal passages into the trachea. Further lidocaine (2%) was sprayed into the lower airways. The bronchoscope was wedged in a right middle lobe bronchus and sterile saline solution at 37°C was injected in five aliquots of 50 ml each. The fluid was gently suctioned back and collected in a conical polypropylene tubes kept on ice.

#### Preparation of BAL cells

BAL fluid was strained through sterile gauze to remove mucus and the cells collected by

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**Table 1** Patient characteristics.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Atopya</th>
<th>FEV1 (% pred)</th>
<th>FEV1/FVC (%)</th>
<th>Pack-year†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smoker</td>
<td>6</td>
<td>25.7 ± 2.6</td>
<td>6M</td>
<td>1/6</td>
<td>97 ± 4.1</td>
<td>88.2 ± 1.1</td>
<td>0</td>
</tr>
<tr>
<td>Smoker</td>
<td>6</td>
<td>27.7 ± 3.9</td>
<td>4M</td>
<td>2/6</td>
<td>84 ± 4.9</td>
<td>88.3 ± 0.7</td>
<td>12.8 ± 3.8</td>
</tr>
</tbody>
</table>

Data shown as mean ± SEM.

FEV1: Forced expiratory volume in one sec; FVC: Forced expiratory volume; M: Male.

aAtopy was defined as positive response to skin prick tests to common aeroallergens.

†One pack-year of cigarette smoking is one year of smoking 20 cigarettes per day.
centrifugation. The cell pellet was resuspended in Hanks’ balanced salt solution. The total number of cells was counted in a Bürker chamber and the viability was tested by exclusion of trypan blue. 5 x 10⁶ cells/well were cultured on 6-well plates at 37°C, 5% CO₂ for 1 h in 1 ml/well of sterile Dulbecco modified Eagle’s medium (DMEM; Sigma, Poole, UK) supplemented with fetal calf serum (10%), benzylpenicillin (0.1 mg/ml), streptomycin sulphate (0.1 mg/ml) and L-glutamine. Nonadherent cells were removed by washing the monolayers with 37°C culture medium, yielding monolayers that contained at least 95% macrophages by morphologic profile. Cells for western blot analysis were immediately placed on ice and processed as described later. Cytospin slides were prepared and were dried for 30 min. Slides were wrapped in aluminum foil and stored at -70°C prior to immunostaining.

**RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)**

RNA was isolated from alveolar macrophages using the Qiagen RNeasy mini kit according to the manufacturer’s instructions. Reverse transcription (RT) was performed on 0.5 μg of RNA. RNA was heated to 70°C for 5 min and then mixed with 0.01 μg/μl random primers, 1.0 mM dNTP, 1 μg/μl RNasin, 0.25 μg/μl AMV-reverse transcriptase, in 1 x reverse transcriptase buffer and incubated at 42°C for 1 h followed by denaturation at 90°C for 4 min. The resultant complementary DNA (cDNA) was then diluted by the addition of 80 μl of water. For polymerase chain reaction (PCR), 5 μl of cDNA were incubated in a final volume of 25 μl containing 1 x KCl buffer, 2 mM dNTP, 5 ng/μl specific primers, and Taq polymerase 2U. Specific primers for HO-1 gave a specific product of 440 bp: forward primer, 5’-ATCCCCAGGATTGTGACAGC-3’; reverse primer, 5’-AGGGTTTCTGGGCAATCTTT-3’. The cycles used were 94°C for 30 s, 62°C for 30 s, 72°C for 1 min for 26 cycles followed by 72°C for 10 min. Specific primers for HO-2 gave a specific product of 523 bp: forward primer 5’-GGAAGGGACCAAGGAAGCACA-3’; reverse primer, 5’-GTTTCAGTTCCAGGGCTTCTAT-3’. The cycles used were 94°C for 30 s, 62°C for 30 s, 72°C for 1 min for 26 cycles followed by 72°C for 10 min. Specific primers for HO-2 gave a specific product of 440 bp: forward primer, 5’-ATCCCCAGGATTGTGACAGC-3’; reverse primer, 5’-AGGGTTTCTGGGCAATCTTT-3’. The cycles used were 94°C for 30 s, 62°C for 30 s, 72°C for 1 min for 26 cycles followed by 72°C for 10 min. Specific primers for HO-2 gave a specific product of 523 bp: forward primer, 5’-GGAAGGGACCAAGGAAGCACA-3’; reverse primer, 5’-GTTTCAGTTCCAGGGCTTCTAT-3’. The cycles used were 94°C for 30 s, 62°C for 30 s, 72°C for 1 min for 26 cycles followed by 72°C for 10 min. **RT-PCR of glyceraldehydes-3-phosphate dehydrogenase (GAPDH)** was performed to act as an internal control and the specific primers used gave a PCR product of 589 bp: forward primer, 5’-CCACCCTGGGCAAATTCCATGGGCA-3’; reverse primer, 5’-TCTAGACGGCAGGCTAGTGTCCCCACC-3’. The cycles used were 94°C for 30 s, 60°C for 30 s, 72°C for 1 min for 24 cycles followed by 72°C for 10 min. PCR products were identified on 2 wt/vol% agarose gels. Samples that did not contain reverse transcriptase were used as negative controls. The intensity of bands for PCR was measured densitometrically using UVP Gel works documentation system GDS800 (UVP, Cambridge, UK).

**Western blot analysis for HO-1 and HO-2 protein**

Whole-cell proteins were extracted from alveolar macrophages. At least 50 μg/lane of whole-cell proteins were subjected to a 18% SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose filters (Hybond-ECL, Amersham Pharmacia Biotech) by blotting. Filters were blocked for 45 min at room temperature in TBS, 0.05% Tween 20, 5% nonfat dry milk. The filters were then incubated with goat anti-human HO-1 (C-20, sc-7695), -2(C-20, sc-7697) antibody (Santa Cruz Biotechnology, USA) for 1 h at room temperature in TBS, 0.05% Tween 20, 5% nonfat dry milk at dilution of 1:1000. These antibodies are specific for the respective human HO proteins and do not cross-react each other. Filters were washed three times in TBS, 0.05% Tween 20 and then incubated for 45 min at room temperature with antigoat antibody conjugated to horseradish peroxidase (Dako, UK) in TBS, 0.05% Tween 20, 5% nonfat dry milk, at dilution of 1:4000. After further three washes in TBS, 0.05% Tween 20 visualisation of the immunocomplexes was performed using ECL as recommended by the manufacturer (Amersham Pharmacia Biotech, UK). As an internal control we reprobed each filter with an antihuman actin antibody (Santa Cruz Biotechnology, USA). The bands, which were visualised at approximately 43 kDa (actin), 32 kDa (HO-1) and 35 kDa (HO-2) were quantified using a densitometer with Grab-It and GelWorks software (UVP, Cambridge, UK). The individual band optical density values for each lane of HO-1 and -2 were expressed as the ratio with the corresponding actin optical density value of the same lane.

**Immunoperoxidase staining for HO-1 and HO-2 in alveolar macrophages**

Cytospins of alveolar macrophages were fixed with methanol. Cytospins were washed repeatedly with phosphate-buffered saline (PBS). The cell membranes were permeabilised by adding to the blocking serum 0.3% triton X-100. Endogenous peroxidase activity was blocked by incubating
slides in 3% hydrogen peroxide (H$_2$O$_2$) and 0.02% sodium azide in methanol for 1h, followed by washing in PBS. Nonspecific labelling was blocked by coating with blocking serum (5% normal rabbit serum) for 20 min at room temperature. After washing in PBS the cells were incubated for 1 h with goat polyclonal antihuman HO-1,-2 antibody (Santa Cruz Biotechnology) at dilutions of 1:50 of a 200 µg/ml solution. For the negative control slides, in some slides normal goat nonspecific immunoglobulins (Santa Cruz Biotechnology) were used at the same protein concentration as the primary antibody. After repeated washing steps with PBS, the sections were subsequently incubated with anti-goat biotinylated antibody (Vector Elite ABC Kit, Vector Laboratories) for 30 min at room temperature. After further washing the sections were subsequently incubated with ABC reagent (Vector Elite ABC Kit, Vector Laboratories) for 30 min at room temperature. Slides were then incubated with chromogen-fast diaminobenzidine (DAB) for 1–5 min, after which they were counterstained in hematoxylin and mounted on mounting medium (DPX).

**Data analysis**

Group data were expressed as mean±standard error of the mean (SEM). Differences between groups were analysed using unpaired t-test. Probability values of $P<0.05$ were considered as significant. Data analysis was performed using the Stat View SE Graphics program (Abacus Concepts Inc., Berkeley, CA-USA).

**Results**

**RT-PCR expression of HO-1 and HO-2 mRNA**

RT-PCR analysis of HO-1 and HO-2 mRNA expression in BAL macrophages showed no significant difference in HO-1 and HO-2 mRNA between normal and cigarette smokers subjects (Fig. 1).

**Analysis of HO-1 and HO-2 protein expression**

Western blot analysis of the alveolar macrophages showed that there was a significant reduction of the expression of HO-2 protein in alveolar macrophages from smokers compared with normal (Fig. 2). However, no significant differences were observed in HO-1 protein expression between normal subjects and smokers. Immunocytochemical staining for HO-1 and HO-2 proteins on cytopsin of alveolar macrophages revealed staining in alveolar macrophages of normal subjects and smokers, but there was no obvious difference in the intensity of the staining (Fig. 3).

**Discussion**

In this study we have examined the expression of the 2 main isoenzymes of the HO system, HO-1 and HO-2, in alveolar macrophages from normal subjects and asymptomatic current smokers with normal lung function. We found a large expression of HO-1 both in normal subjects and smokers with no significant differences between the two groups. This suggests that in the airways of the normal subjects and smokers there is an active HO system to protect against environmental oxidative stress.
In vitro studies have previously demonstrated that HO-2 is a constitutive isoenzyme, whereas HO-1 is an inducible form that is not usually expressed in unstimulated airway epithelial cells. In vitro HO-1 expression can be induced in many cells by different stimuli, particularly those that induce oxidative stress. There is evidence for increased oxidative stress in the lungs of young current smokers with normal lung function and of older patients with COPD. However in our study we found no increase in the expression of HO-1 mRNA and protein in alveolar macrophages of smokers compared with normal subjects. One possible explanation of these results is that the expression of this enzyme is already maximal due to the continuous stimulation by the environmental oxidative stresses, such as exposure to air pollutants. Basal expression of HO-1 has been described in hamster fibroblasts and this constitutive HO-1 expression was associated with resistance to hyperoxia. The variable oxidant and antioxidant effect of HO-1 in the different models may depend on the level of its expression.

By contrast, we found a significant decrease of HO-2 protein expression in alveolar macrophages of young current smokers with normal lung function compared with normal subjects using Western blot analysis, which is a more sensitive assay for detection of differences in the total amount of a protein than immunocytochemistry. The functional significance of this HO-2 protein decrease is not known. HO-2 expression in the lungs may provide cellular protection against oxidant injury. In a model of HO-2 knock-out mice, the animals were more susceptible to oxidative stress toxicity, despite an increase in HO-1. In this model, HO-1 induction may be simply a generalised response to oxidative stress, and not necessarily protective. The increased oxygen toxicity in the knockout mice also suggests that HO-2 provides an essential protective function that may be important in antioxidant defence over and above the effects of HO-1. The increased toxicity in absence of HO-2 appears to be correlated to the accumulation in the lungs of haem-induced iron.

In summary, we examined the expression of HO-1 and HO-2 isoenzymes in alveolar macrophages of normal subjects and smokers with normal lung function, and found a reduction in HO-2 protein expression. HO-2 deficiency in the alveolar macrophages of smokers may indicate that the lung of smokers may be less well protected against oxidative stress. Whether this deficiency can lead to the development of COPD deserves further study.
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