Up-regulation of heme oxygenase-1 in alveolar macrophages of newly diagnosed asthmatics

T. Harju*, Y. Soini†, P. Pääkkö‡ and V. L Kinnula*

*Department of Internal Medicine and †Department of Pathology, University of Oulu and Oulu University Hospital, Oulu, Finland

Abstract Exhaled carbon monoxide (CO), which has been found to be elevated in asthma, is generated primarily by heme oxygenase 1 (HO-1), an enzyme induced by oxidant stress and cytokines. The aim of this study was to assess the distribution and expression of HO-1 in various human lung cells in acute and stable asthma.

Normal lung tissue biopsies (from 6 non-smoking subjects operated on for a lung tumour) and macrophages from induced sputum (from 5 healthy controls, 5 untreated asthmatics, 7 stable treated asthmatics and 5 asthmatics recovering from exacerbation and being on systemic steroids) were investigated for HO-1 by immunohistochemistry. The time response of HO-1 induction was examined in cultured monocytes, which are known to mature into monocyte-derived macrophages in culture. Lung biopsies showed prominent HO-1 immunoreactivity only in alveolar macrophages. Macrophages in the induced sputum of healthy controls showed no HO-1 immunoreactivity, with the exception of one case. Moderate or intense HO-1 immunoreactivity could be observed in alveolar macrophages in 4/5 cases with recent asthma, and 2/7 with stable asthma, but in none of the patients treated with systemic corticosteroids for acute exacerbation. Experiments with cultured cells revealed that HO-1 was induced by oxidants within the first 24 h, but the induction was reversed during the next 48 h. HO-1 is mainly expressed in alveolar macrophages of human lung. Macrophages of induced sputum show prominent but transient HO-1 immunoreactivity, in untreated asthmatics, but not in asthmatics treated with corticosteroids. © 2002 Elsevier Science Ltd

Keywords heme oxygenase-1; asthma; induced sputum; macrophage; cell culture.

INTRODUCTION
Carbon monoxide (CO) is elevated in the exhaled air of asthma patients (1–4). This finding is probably associated with the up-regulation of heme oxygenase-1 (HO-1), since previous studies have shown that total cellular CO is generated primarily via heme degradation by HO-1. (5–7). Immunoreactivity of HO-1 has also been detected in the bronchial biopsies (8) and bronchoalveolar lavage fluid of asthma patients (1).

The HO system consists of three proteins, an oxidative stress-induced form of the enzyme, called HO-1, and constitutive forms of the enzyme, all of which are products of distinct genes (7,9). Due to its molecular weight and rapid induction by heat and stress, HO-1 has been called heat shock protein 32 (5,7,10). Heme oxygenase catalyses the rate-limiting step in the oxidative degradation of heme to biliverdin, a reaction which also releases CO and iron.

No systematic studies have been conducted on HO-1 expression or distribution in human lung tissue. A recent study on bronchial biopsies of segmental airways obtained by bronchoscopy showed a similar expression of HO-1 in healthy controls and asthmatics (8). In our recent study on patients with sarcoidosis and interstitial pneumonia, however, HO-1 was expressed only in alveolar macrophages (10). Studies on rat lung have revealed HO-1 immunoreactivity in bronchial epithelium and inflammatory cells of the airways after hyperoxia and in alveolar macrophages after hypoxia exposures (11,12). HO-1 expression has also been detected in human monocytes, where it is induced during erythropagocytosis and cell activation (13,14). It is also known that HO-1 is stimulated not only by oxidants but also by endotoxin and inflammatory cytokines (5,11,15–18).

In this study, we first compared HO-1 expression in various cell types of human lung and in small and large
airways using both paraffin-embedded and frozen tissue samples. These studies confirmed our recent findings on HO-1 expression mainly in alveolar macrophages. To better understand the regulation of HO-1 in asthma, we therefore assessed the expression of HO-1 in alveolar macrophages obtained by sputum induction from healthy subjects and asthma patients. The material included samples from healthy non-smoking individuals, recently diagnosed and untreated asthma patients, stable treated asthmatics and patients in the acute phase of asthma exacerbation under treatment. Furthermore, the time response of HO-1 was assessed in human monocyte-derived macrophages in culture.

METHODS

Lung tissue biopsies

The biopsy material of paraffin-embedded tissues consisted of lung tissue samples from 6 subjects (5 non-smokers, one ex-smoker), all of whom had been operated on for a bronchial carcinoid tumour or malignancy (lung carcinoma, sarcoma or metastasis). The control subjects had no other diseases and no medication, their lung function was normal and the lung biopsies had been taken from areas with normal-looking histology. The biopsies were collected from the files of the Department of Pathology, Oulu University Hospital.

Frozen tissue samples

Frozen tissue samples were obtained from additional 5 patients operated on for a lung tumour. The samples included central cartilage containing bronchus and, from one patient, peripheral lung tissue.

Induced sputum

Patients and processing of samples

Induced sputum was obtained from 5 patients with recently diagnosed untreated asthma, 7 patients with stable asthma on inhaled corticosteroids and 5 asthmatics treated in hospital for asthma exacerbation with systemic steroids for at least 3 days before sputum sampling. The new and stable asthmatics were recruited from the outpatient clinic of Oulu University Hospital and the asthmatics with acute exacerbation from the pulmonary ward of the same hospital. All the patients were non-smokers. Forced expiratory flow in 1 sec (FEVI) varied within 63–124% of the reference value in the patients with asthma. The asthma diagnosis was based on reversible airway disease, as shown by a positive histamine or methacholine challenge test, a ≥ 15% increase in FEVI or PEF after the administration of an inhaled bronchodilator, or daily PEF variability ≥ 20%. The controls for the induced sputum group consisted of 6 healthy non-smoking individuals with normal spirometry findings and no history of asthma or other lung diseases (Table I).

Two patients with asthma exacerbation produced adequate sputum after inhalation of nebulised bronchodilators (salbutamol and ipratropium). In all other cases, sputum was induced by inhalation of 5 ml of 3% saline solution using an ultrasonic nebuliser (Omron UI, Omron, Germany). Both the healthy controls and the asthmatics were given 0.2 mg of salbutamol as dry powder inhalation 15 min before the sputum induction. Peak expiratory flow (PEF) values were measured before and after the induction to ensure the safety of the procedure. The subjects were asked to cough during and after the inhalation. Sputum was collected in a clean plastic container. If PEF decreased by more than 20% or subjective symptoms appeared, the patients were given 0.2 mg of inhaled salbutamol.

The sputum was processed by the method of Pizzichini and colleagues (19). Sputum which was macroscopically free of salivary contamination was selected and treated with dithiotreitol (Sputolyisin 10%, Calbiochem Corp., San Diego, CA, U.S.A.) and phosphate-buffered saline. Five hundred micro litres of the filtered suspension was used for the cell count and cytospin preparations. Only adequate samples with less than 80% squamous epithelial cell contamination from saliva (20) were accepted. In order to study the expression of HO-1 in macrophages, additional cytospin preparations were fixed in acetone for 10 min.

Immunohistochemistry

The histological slides were immunostained as follows. The sections (4 μm) were deparaffinised in xylene and dehydrated in graded ethanol. They were then incubated with 30% hydrogen peroxide in absolute methanol to consume the endogenous peroxidase. Background staining was minimised by treatment with 2% non-fat dry milk. The primary HO-1 antibody (Transduction Laboratories, Lexington, KY, U.S.A.) was used in a dilution of 1:100, and the colour was developed with aminoethyl carbazole substrate solution (Zymed Laboratories). They were then incubated with the secondary antibody followed by Histostain TM-PLUS kit (Zymed Laboratories, South San Francisco, CA, U.S.A.). The colour was developed with aminoethyl carbazole (AEC) substrate solution (Zymed Laboratories). The sections were counterstained with a light haematoxylin stain. The negative controls consisted of PBS at pH 7.2 and the mouse serum isotype recommended by the manufacturer (Zymed Laboratories).

Cytospin slides were fixed in acetone for 10 min and incubated with 8% hydrogen peroxide in water to consume
the endogenous peroxidase. The background staining was minimised by treatment with 2% non-fat dry milk. The same primary HO-1 antibody as for histological slides was used in a dilution of 1:100.

The frozen sections were cut into 4μm sections, fixed for 10 min in cold acetone and air-dried overnight. In order to consume endogenous peroxidase, the slides were incubated in 30% hydrogen peroxide in absolute methanol for 10 min. Non-specific staining was blocked by incubating the slides in fetal calf serum. Primary antibody for HO-1 was then applied (dilution 1:100) to the slides for 60 min, followed by secondary anti-mouse antibody for 30 min, and the peroxidase–antiperoxidase complex (Dako) for 30 min. The chromogen used was diaminobenzidine and the slides were then lightly counterstained with haematoxylin.

HO-1 immunoreactivity was assessed semiquantitatively by two experienced lung pathologists independently and the interobserver repeatability was excellent (k = 0.714). Immunoreactivity was graded as follows: — negative, + weak, ++ moderate, +++ strong and ++++ very strong. All analyses were conducted blindly without information about the clinical characteristics of the subjects.

**Cell cultures and exposures**

Monocytes were isolated from buffy coats (Finnish Red Cross Helsinki, Finland) using a modified method described by Böym (21). The buffy coats were diluted with phosphate-buffered saline (PBS), and the cell suspension was layered over Ficoll-Paque (Pharmacia, Biotech AB, Uppsala, Sweden) and centrifuged. Mononuclear cells were collected at the interface, washed with PBS and suspended in RPMI-1640 medium (Gibco Europe, Paisley, U.K.) supplemented with L-glutamine (Gibco), penicillin–streptomycin (Gibco) and 20% human serum (Finnish Red Cross). After isolation, the cells were incubated in collagen-coated (Vitrogen, Collagen-Corporation, Palo Alto, CA, U.S.A.) Petri dishes for 1h at 37°C. After incubation, non-adherent cells were removed by washing with PBS. Adherent monocytes were cultured under 5% CO₂ at 37°C for up to 72 h which has been shown to induce maturation of monocytes into monocyte-derived macrophages. The monocytes were exposed to formylated peptide fMLP (10⁻⁷M) (Sigma) or 100 and 500μM H₂O₂ for 24 or 72 h. FMLP is known to cause a respiratory burst of monocytes and macrophages, thereby mimicking oxidative stress in vivo.

**Western blotting**

The cell pellets were mixed with the electrophoresis sample buffer and boiled for 5 min at 95°C. The protein concentration of the samples was measured using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA), and 75 μg of the cell protein was applied to 12% sodium dodecyl sulphate-polyacrylamide gels. The gels were electrophoresed for 1-5 h (90 V) at room temperature, and the protein was transferred (45 min, 100 V) onto Hybond ECL nitrocellulose membranes (Amersham, Arlington Hights, IL, U.S.A.) in a Mini-PROTEAN II Cell (Bio-Rad). The blotted membranes were incubated with mouse antibody to HO-1 (diluted 1:250) followed by treatment with anti-mouse secondary antibody (diluted 1:2000) conjugated to horseradish peroxidase (Amersham). The reactivity was detected by an enhanced chemiluminescence system (ECL, Amersham), and the luminol excitation was imaged on X-ray film.

The study protocol was accepted by the ethical committee of the University of Oulu and Oulu University Hospital.

**Statistical analysis**

The statistical analyses were performed with SPSS for Windows software (SPSS, Chicago, IL, USA). The significance of the associations was determined using Fisher’s exact probability test designed for small sample groups. P-values of less than 0.05 were considered statistically significant.

**RESULTS**

**Distribution and expression of HO-1 in the lung**

The immunohistochemical reactivity of lung tissue was investigated in 6 non-smoking individuals with normal lung histology. With the exception of one case, all control lung biopsies showed strong HO-1 immunoreactivity in alveolar macrophages [Fig. 1(c), Table 2]. One biopsy from a subject with a carcinoid tumour showed no HO-1 immunoreactivity in any cells of the biopsy. Given the fact that HO-1 may show different expression patterns in the large and small airways, we also assessed HO-1 immunoreactivity in the large, cartilage-containing airways. However, even these samples were negative for HO-1 immunoreactivity.

Frozen lung samples from additional 5 patients were assessed for HO-1 immunoreactivity. These results showed no HO-1 reactivity in bronchial epithelium, but alveolar macrophages showed positive immunoreactivity.

**Induced sputum**

Macrophages in the induced sputum of non-smoking controls, with the exception of one case, showed no HO-1...
immunoreactivity [Table 1 and Fig. 1(a)]. In induced sputum, HO-1 immunoreactivity appeared to be present more often in the macrophages of new asthmatics without inhaled anti-inflammatory therapy (4/5) than in cases with stable asthma and inhaled anti-inflammatory therapy (2/7) ($P=0.027$, Fisher’s exact probability test) [Table 1 and Fig. 1(b)]. No HO-1 immunoreactivity could be detected in the sputum of asthmatics recovering from acute exacerbation who had been on systemic corticosteroid therapy for at least 3 days ($P=0.023$, Fisher’s exact probability test).

**Cell cultures**

The time response of HO-1 induction was investigated in cultured monocytes, which are known to mature into macrophage-like cells in culture (22). Monocytes showed scant expression of HO-1, which was enhanced by 500 μM H$_2$O$_2$ and /MLP within the first 24 h of exposure. The induction was, however, only transient, since no elevated HO-1 expression could be detected after 72 h of exposure any more (Fig. 2).

**DISCUSSION**

The present study shows that HO-1 is expressed in alveolar macrophages of human lung. It also appears that oxidant stress causes a rapid but transient induction of this enzyme in inflammatory cells *in vitro*. These results are in line with the expression levels of HO-1 *in vivo*, since the present patient samples also showed a tendency towards higher HO-1 reactivity in the alveolar macrophages of untreated asthmatics but not in subjects with stable disease or after 72 h of treatment.

Tissue specimens obtained from surgical operations of non-smokers showed that HO-1 is expressed mainly in alveolar macrophages. This result does not exclude the possibility that small amounts of this enzyme could also be expressed in other cells of human lung since immunohistochemistry may not be sensitive enough to detect minor amounts of the enzyme protein. Our present findings are in agreement with our recent study on paraffin-embedded biopsies of sarcoidosis and interstitial pneumonias (10), which showed that HO-1 is expressed in macrophages. There is one recent study on bronchial biopsies obtained by bronchoscopy that investigated HO-1 immunoreactivity from frozen samples of segmental bronchial epithelium. In that study, similar positive HO-1 immunoreactivity was detected in the epithelium of healthy controls and asthmatic patients (8). In view of inducibility of HO-1, however, bronchoscopy may cause induction of this enzyme both in healthy subjects and in asthmatics, and induction may also occur in healthy lung during surgery. In our study, additional stainings were also made on frozen lung biopsies, and even these results revealed immunoreactivity only in macrophages. Different levels of the bronchial tree may also show variable HO-1 immunoreactivity, but based on our findings, the large airways did not express HO-1, either. Thus, these additional studies did not change the overall conclusion that alveolar macrophages represent the cell population mainly responsible for HO-1 expression in human lung.

Degradation of heme by HO-1 generates CO, and exhaled CO has been suggested to be a sensitive marker of inflammation in asthma (1, 9, 23). The exhaled CO concentration has, however, been shown to be higher in...
atopic subjects than in non-atopic individuals (24) and similarly elevated in allergic rhinitis without asthma (25). Exhaled CO is also elevated in respiratory infections (23,26) and smokers (27), which makes the interpretations of exhaled CO in asthma even more difficult. The purpose of this study was not to investigate CO, but in agreement with the results on exhaled
CO, our study showed HO-1 to be up-regulated in the alveolar macrophages of non-treated asthmatics, the immunoreactivity of HO-1 also being higher in untreated than treated subjects with stable asthma. This is also consistent with the rapid inducibility of this enzyme and with the results on cultured monocytes showing transient up-regulation of HO-1 in oxidant-exposed cells.

In conclusion, our study shows that HO-1 is induced especially in the alveolar macrophages of human lung and that alveolar macrophages are probably the most important cell type to explain high CO in the exhaled air of asthma patients. Heme oxygenase may play a role in the acute inflammation of human lung, but due to its rapid induction by oxidants, cytokines, infections and cigarette smoke, the specificity of HO-1 in the differential diagnosis of asthma as well as in the clinical follow up of asthmatics appears to be modest.

### Table 1.
HO-1 reactivity (0—++++) in induced sputum of healthy controls, new untreated asthmatics, stable asthmatics on anti-inflammatory treatment and asthmatics after the treatment of acute asthma exacerbation

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>% of HO-1—positive macrophages</th>
<th>Intensity of HO-1 positivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls (group 1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>M</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>28</td>
<td>M</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>56</td>
<td>M</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>51</td>
<td>M</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>22</td>
<td>F</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>38</td>
<td>M</td>
<td>10</td>
<td>++</td>
</tr>
<tr>
<td>New asthma, no anti-inflammatory treatment (group 2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>F</td>
<td>40</td>
<td>+++</td>
</tr>
<tr>
<td>39</td>
<td>F</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>21</td>
<td>F</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>44</td>
<td>F</td>
<td>10</td>
<td>++</td>
</tr>
<tr>
<td>36</td>
<td>M</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>Stable asthma, inhaled corticosteroid therapy (group 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>F</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>49</td>
<td>M</td>
<td>5</td>
<td>++</td>
</tr>
<tr>
<td>25</td>
<td>F</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>48</td>
<td>F</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>53</td>
<td>M</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>46</td>
<td>F</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>56</td>
<td>M</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>Asthma exacerbation, systemic corticosteroid therapy (group 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>M</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>45</td>
<td>F</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>69</td>
<td>F</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>55</td>
<td>F</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>53</td>
<td>F</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

*Heme oxygenase-1 immunoreactivity was graded as follows: — negative, + weak, ++ moderate, +++ strong and ++++ very strong.

According to Fisher’s exact probability test the following statistically significant associations were found: group 2 vs groups 3–4: \( P = 0.027 \); group 2 vs group 4: \( P = 0.023 \).

![Western blotting showing transiently elevated expression of HO-1 in monocytes exposed to 100 and 500 μM H$_2$O$_2$ and to fMLP (10$^{-7}$ M) for 24 and 72 h.](image)
Acknowledgements

This study was supported by the Finnish Anti-Tuberculosis Association Foundation and Ju selius Foundation. The technical assistance of Ms Raija Sirviö and Mr Manu Tuovinen is kindly acknowledged.

REFERENCES