

Heme oxygenase-1 (HO-1) protein induction in a mouse model of asthma

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Summary

Background and objective Carbon monoxide (CO) is known to be present in measurable quantities in the exhalation of asthmatic patients. Corticosteroid treatment resulted in a decrease in exhaled CO levels in asthmatic patients, raising the possibility that an increase in exhaled CO concentration reflects inflammation of the asthmatic airway. Heme oxygenase-1 (HO-1) protein, also called HSP32, is the rate-limiting enzyme in the catabolism of heme to biliverdin, free iron and CO. However, it is unknown whether an expression of HO-1 within the lung tissue is related to allergic airway inflammation. We studied the expression of HO-1 in lung tissue and bronchoalveolar lavage cells in a mouse model of asthma.

Methods Ovalbumin (OVA)-sensitized C57BL/6 mice were challenged with aerosolized OVA. HO-1 positive cells were identified by immunostaining in lung tissue and bronchoalveolar lavage fluid (BALF) after the challenge.

Results HO-1 positive cell numbers increased in the subepithelium of the bronchi after OVA challenge. In cytospin preparations from BALF after OVA challenge, HO-1 was localized to alveolar macrophages. Inside the macrophages, HO-1 reactivity was expressed in the cytoplasm, and the perinuclear region in particular.

Conclusion The expression of HO-1 is increased within the lung tissue in allergic airway inflammation. Measurement of HO-1 activity may be clinically useful in the management of asthma.

Keywords: HO-1, macrophage, asthma, mice

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Introduction

Increased expression levels of antioxidant enzymes and heat shock proteins (HSPs) are indicators of oxidative stress. Though some types of inflammation have been associated with the expression of HSPs, the inflammatory process is considered a paradigm for cellular environmental stress [1]. The response of HSPs has been implicated in the pathogenesis of many pulmonary diseases, including adult respiratory distress syndrome (ARDS), interstitial pulmonary

fibrosis, and chronic inflammatory disorders such as chronic obstructive pulmonary disease (COPD) and asthma [2–4].

To date, three isoforms of heme oxygenase have been characterized, HO-1, 2 and 3. The three isoforms are different gene products [5–7] and differ in their molecular biochemical characteristics. HO-1 protein, also called HSP32, is the rate-limiting enzyme in the catabolism of heme to biliverdin, free iron, hormones and CO, as well as agents causing oxidative stress [8,9]. Its expression has been associated with protection against oxidative stress and ischaemia, vascular regulation via the heme oxygenase/CO pathway, and potential use as a biomarker for exposure to

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environmental arsenic [10,11]. In contrast, HO-2 is present chiefly in the brain and testes and is virtually uninducible [5,6]. HO-3 has very low activity and a physiological function in the binding of heme [7]. Previous studies have shown that increased expression of HO-1 provides cellular protection against oxidant injury such as kidney failure or endotoxemia [12,13]. Conversely, deficient HO-1 expression in mammalian cells weakens stress defences [14]. Poss and Tonegawa [15] reported that HO-1-deficient mice were highly susceptible to hepatic necrosis or death when subsequently challenged with endotoxin. These results suggest that the induction of HO-1 in response to injurious stimuli is an important mechanism for moderating the severity of cell damage.

Among the many classes of stress proteins, HO-1 is characterized with respect to lung biology, which is predictable given the highly conserved nature of stress response [16]. Chronic airway inflammation is a characteristic feature of asthma and increased levels of reactive oxygen species (ROS), nitric oxide (NO) and cytokines including interleukin (IL)-6 and tumour necrosis factor (TNF)- α have been demonstrated to induce the expression of HO-1 [17,18]. Zayasu *et al.* [19] reported that exhaled CO concentrations were higher in asthmatic patients than in control subjects. Therefore, HO-1 and its metabolized product CO may be important factors for allergic response and regulate help to allergic asthma; however, little is known about the expression and activity of HO-1 within lung tissue under such conditions.

In this report, we investigated the involvement of HO-1 in the pathogenesis of asthma in mice exposed to allergen. We also attempted to determine which cells express HO-1 in allergic airway inflammation.

Materials and methods

Animal preparations

Specific pathogen-free male C57BL/6 mice, 6–8 weeks old, were purchased from Charles River Laboratories (Atsugi, Japan). Each experimental group consisted of six animals. Immunization and challenge were carried out as described previously [20]. Mice were immunized by intraperitoneal (i.p.) injection of 50 mg OVA (Sigma Chemical, St Louis, MO, USA) adsorbed to 1 mg of alum on days 0 and 12. Control mice were injected i.p. with 0.5 mL of sterile saline. On day 24 mice were challenged three times with an aerosol of OVA (10 mg/mL) in 0.9% saline for 30 min with an ultrasonic nebulizer. The challenge was repeated every second day for 8 days. Non-sensitized saline-injected mice were exposed to nebulized sterile saline using the same protocol. On day 31, airway inflammation and HO-1 expression were

compared between the mice challenged with saline and with OVA.

Lung histology

Lung sections (4 μ m) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and were subjected to Giemsa staining. For immunohistochemical analysis of neutrophils, CD4⁺ and CD8⁺ T cells, macrophages and HO-1 positive cells in the infiltrates in lung sections, the tissue samples were fixed with acetone at room temperature (23–24°C) for 5 min. All sections were treated with 0.3% H₂O₂ in methanol overnight at room temperature to quench the endogenous peroxidase. Immunostaining was performed overnight at 4 °C with the following antibodies: rat anti-mouse neutrophil (7/4) monoclonal antibody (Biosource, Camarillo, CA, USA), rat anti-mouse CD4 (L3T4), rat anti-mouse CD8 (Ly-2), rat anti-mouse macrophage (clone F4/80) monoclonal antibodies (Pharmingen, San Diego, CA, USA) and rabbit anti-HO-1 polyclonal antibody (StressGen Biotechnologies, Victoria, British Columbia, Canada). These were diluted 1 : 200 with 1% normal goat serum (NGS) in PBS.

After incubation with biotinylated secondary antibody (goat anti-rat IgG; Tago, Camarillo, CA, USA) diluted 1 : 500 with 1% NGS in PBS, immune reaction product was detected by the avidin-biotin peroxidase complex technique (Vectastain ABC Kit; Vector, Burlingame, CA, USA). The specificity of each antibody was confirmed by replacement of first antibodies with non-immunized rat IgG 2 κ (clone G155–178). The eosinophils, neutrophils, CD4⁺ and CD8⁺ T cells, macrophages and HO-1 positive cells present in the medium were enumerated in each section using a light microscope. Morphometric analysis was performed using the Interaktive Bild-Analyse System (IBAS; Kontron-Bildanalyse, Oberkochen, Germany). These counts reflect the number of cells per unit length of bronchial basement membrane in the airway.

Bronchoalveolar lavage

After airway responsiveness had been measured, the lungs were lavaged six times with one 0.5-mL aliquot of PBS supplemented with 0.05 mM sodium EDTA and 0.1% BSA (both from Sigma Chemical). The bronchoalveolar lavage fluid (BALF) was centrifuged at 400 g for 10 min at 4 °C, the supernatant removed for cytokine analysis, and the cell pellet resuspended in normal saline after a brief hypotonic exposure to lyse red blood cells. Total cell count was determined in a fresh fluid specimen using a haemocytometer. Differential cell counts were assessed in cytologic preparations as described [20]. The slides were prepared using a cytospin apparatus (Cytospin 2; Cytospin, Shandon,

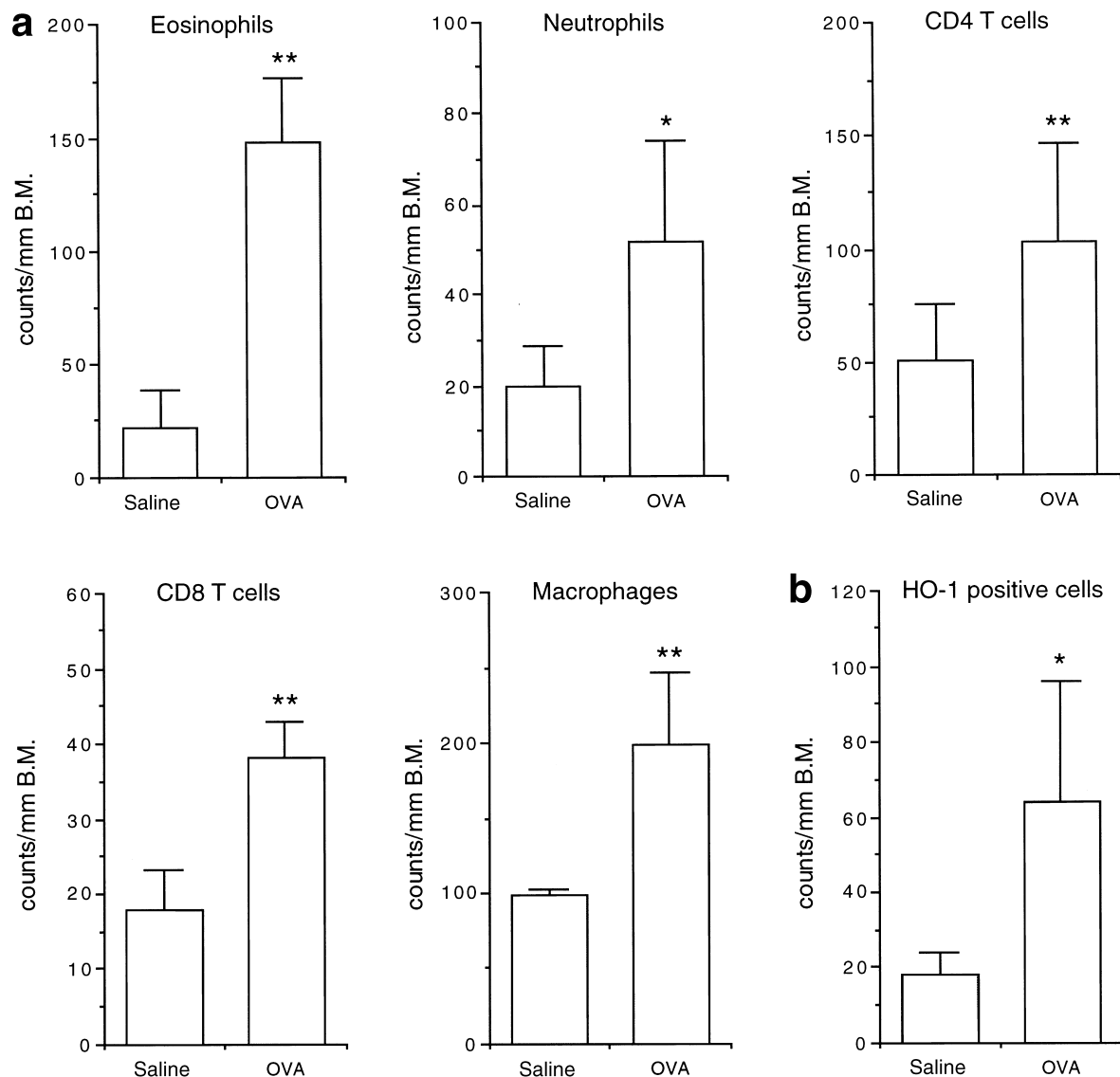


Fig. 1. Quantitative analysis of lung HO-1-positive and inflammatory cells in C57BL/6 mice sensitized and treated with aeroallergen. Data represent the mean $\pm$ SD for groups of mice and are representative of two separate experiments. * $P < 0.05$ and ** $P < 0.01$ compared with the control saline-treated mice.

UK) and stained with Diff-Quik (Baxter, McGraw Park, IL, USA). Cell types were identified and scored as percentages based on the light microscopic evaluation of > 500 cells/slide.

Morphological analysis

To identify the source of HO-1 positive cells, double-immunofluorescence staining was performed. Antibodies were diluted 1 : 200 with 1% NGS in PBS. Lung sections were sequentially incubated overnight at 4 °C in the dark with rabbit anti-HO-1 polyclonal antibody, followed by the rhodamine-labelled antibody to rabbit IgG, rat anti-mouse

CD4 (L3T4), rat anti-mouse macrophage monoclonal antibodies (F4/80) and finally FITC-conjugated antibody to rat IgG1. BAL cells samples were subjected to immunocytochemistry for HO-1. No immunostaining was observed in controls. Furthermore, confocal fluorescence microscopic images of HO-1-positive cells were taken under a confocal scanning fluorescence microscope system (MRC-600; Bio Rad, Cambridge, MA, USA) with inverted epifluorescence microscope (Nikon TMD-EFQ; Tokyo, Japan)[21]. The temperature of the observation chamber was controlled at 37 °C. The fluorescence images were analysed with a digital image processor (Image Sigma II

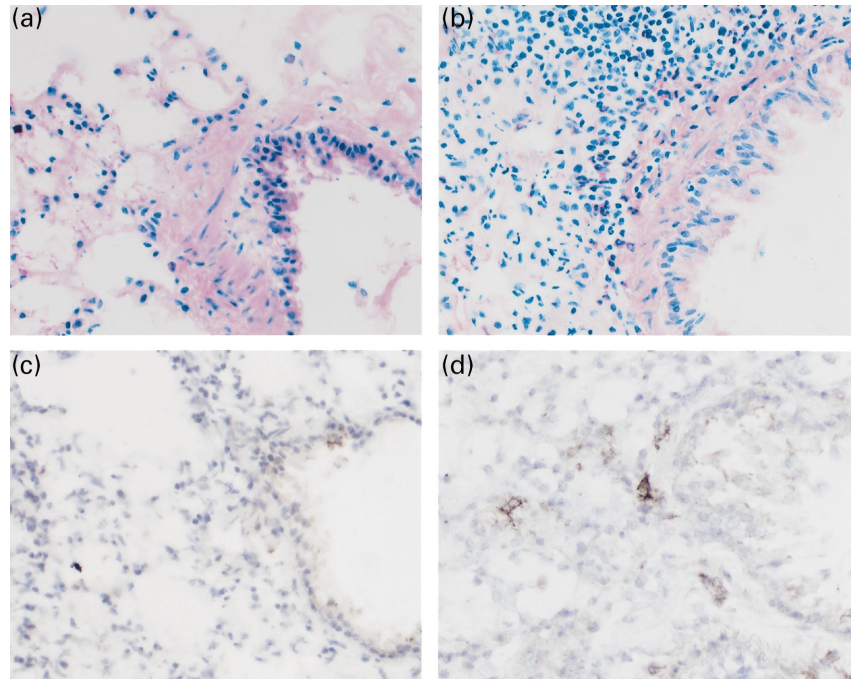


Fig. 2. Histological staining of inflammatory cells and HO-1-positive cells in lung sections from C57BL/6 mice. Light micrographs of formalin-fixed tissue from sensitized C57BL/6 mice exposed to an aerosol of saline (a and c) or to OVA (b and d). The lung sections were stained with Giemsa (a and b) or for immunohistochemistry for HO-1 (c and d). Dark brown-stained HO-1 positive cells were present in the subepithelium of the bronchus after OVA challenge (d).

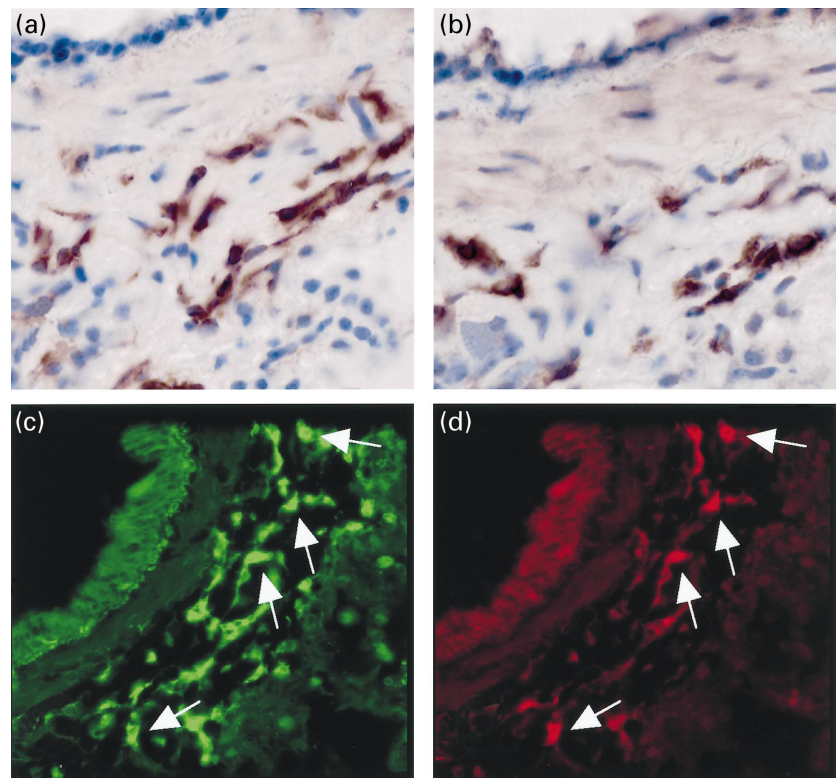


Fig. 3. Sequential 4- μ m sections of bronchial mucosa taken after OVA challenge, stained with rabbit anti-HO-1 ab (a) and rat anti-mouse F4/80 ab to macrophages (b). Immunofluorescent double-stained cells in the subepithelium of the bronchi after OVA challenge. Lung sections were incubated with the following Abs: rabbit anti-HO-1 (c), rat anti-mouse F4/80 (d). Rhodamine-labelled anti-rabbit Ig and FITC-conjugated anti-rat Ig were used as revelation systems. The same microscopic field was photographed twice; first with a filter specific for rhodamine (c) and next with one for FITC (d). Double-positive cells are indicated by arrows.

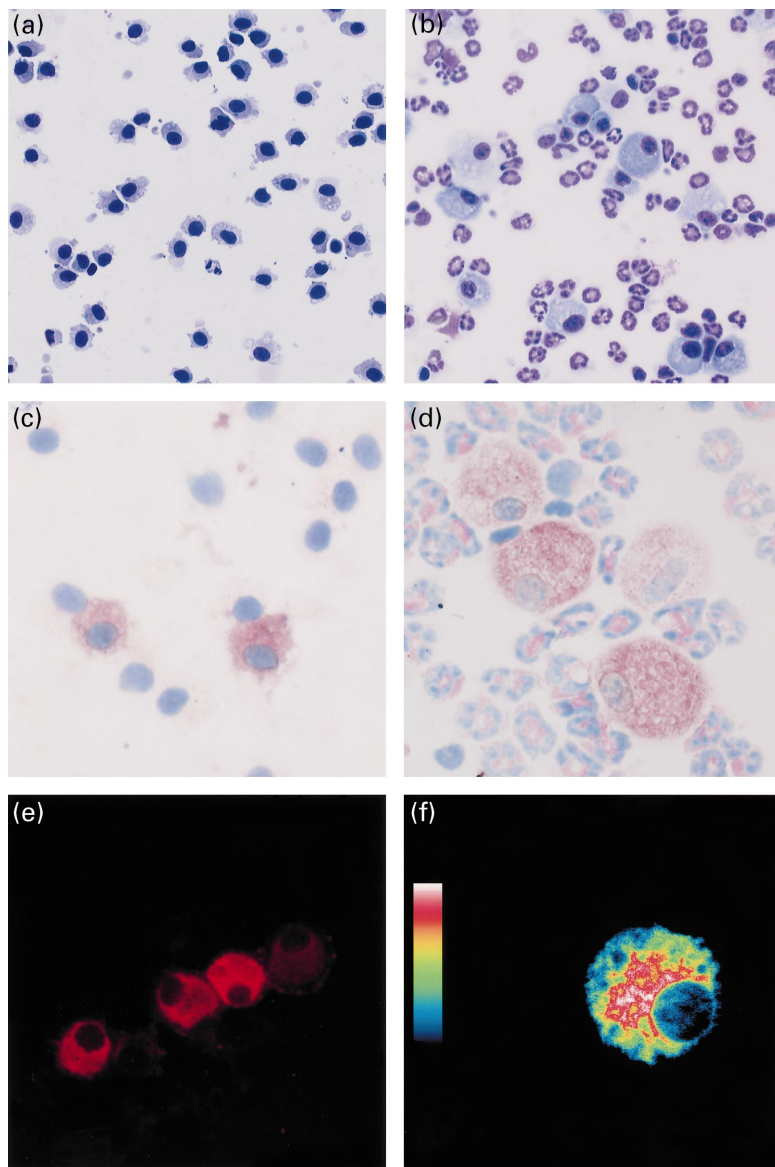


Fig. 4. Bronchoalveolar lavage was performed after saline or OVA challenge and cells in the BALF were enumerated on cytospin smears stained with Diff-Quik (a and b) and immunocytochemical staining for HO-1 with Giemsa's solution (c and d). Confocal fluorescence micrographs showing HO-1 immunoreactivity; note heterogeneous distribution of label the cytoplasm (e). Confocal fluorescence pseudo-images of rhodamine-labelled HO-1 (f); note distribution in the perinuclear region in particular. (a and c) control; (b, d, e and f) OVA inhalation.

and TVIP-2000; Avionics, Tokyo, Japan), which was controlled by a microcomputer [22].

Statistical analysis

Results are expressed as the mean $\pm$ SD. The significance of differences between experimental groups was analysed with unpaired Student's *t*-tests using Excel 5 for Apple Macintosh (Microsoft Corporation, Redmond, WA, USA). Significance was assumed at *P*-values of < 0.05 .

Results

The results of infiltration of inflammatory cells in the lung are summarized in Fig. 1(a). There were no significant

histological changes in the lungs of the age-matched control mice, but marked allergic pulmonary inflammation and inflammatory cell infiltration were observed in the lungs of the OVA-challenged mice. In actively immunized C57BL/6 mice, the number of inflammatory cells infiltrating the lung tissue after exposure to OVA was increased in comparison with the number in mice exposed only to saline. Infiltration of eosinophils, neutrophils, CD4⁺ T cells, CD8⁺ T cells and macrophages was significantly increased in the subepithelial area of the bronchus after antigen challenge (Fig. 1a). Analysis of BAL cells also revealed a marked expression of the eosinophil population (37.8 ± 25.0 vs. $\leq 0.1 \times 10^5/\text{mL}$) and slight increases in the numbers of neutrophils (2.3 ± 0.8 vs. $\leq 0.1 \times 10^5/\text{mL}$), lymphocytes (6.5 ± 1.6 vs. $0.2 \pm 0.1 \times 10^5/\text{mL}$)

and macrophages (9.3 ± 4.8 vs. $2.7 \pm 0.8 \times 10^5/\text{mL}$) (Figs 4 a and b). In addition, airway responsiveness to intraventricular acetylcholine was measured. In C57BL/6 mice, the OVA challenge significantly increased the airway response to acetylcholine compared with the saline-challenged mice (data not shown).

The number of cells expressing HO-1 within the lung sections is summarized in Fig. 1(b). Immunostaining of the lung revealed a low level of HO-1 in the saline-treated mice. HO-1-positive cells were rarely detected in the subepithelial area in sensitized mice given aerosolized saline. After the OVA challenge, the number of HO-1-positive cells was increased, along with that of inflammatory cells in the subepithelium of the bronchi (Fig. 2).

To identify the cells expressing HO-1 in allergic airway inflammation, double-immunofluorescence techniques for HO-1 and macrophage were performed in the lung sections. A similar pattern of immunostaining in adjacent thin sections was observed in the OVA challenged lungs (Fig. 3). Coexpression of HO-1 and F4/80-positive macrophages was detected in the OVA-challenged mouse lung by double-immunofluorescence staining (Fig. 3), suggesting that HO-1-expressing cells are mostly F4/80-positive macrophages. In cytospin preparations from BALF, immunocytochemistry for HO-1 was observed in macrophages (Figs 4 c and d). From the morphological patterns of HO-1 images in Fig. 4(e), the parts of the bright fluorescence seemed to the Golgi apparatus in macrophages. The fluorescence intensity of HO-1 was located in the perinuclear region in particular (Fig. 4f).

Discussion

A previous study suggested that HSPs play a role in inflammatory and allergic diseases, including eosinophilic inflammation [2]. The possible involvement of stress response in the pathogenesis of asthma has only begun to attract attention. Allergic airway inflammatory diseases are associated with 'stress proteins', so termed because of their broad cytoprotective role in heat shock response, as well as the variety of inflammatory inducers [23]. Data obtained from animal models suggest that stress proteins have an important cytoprotective role during pulmonary inflammation and injury [13,24]. Therefore, an index of oxidative stress would provide considerable insight into the importance of oxidative injury and be of potential therapeutic value in allergic inflammation.

In this study, we investigated the expression of HO-1 enzyme, also known as HSP32, in a mouse model of allergic airway inflammation. The production of HO-1 in inflamed lung tissue and BAL cells was examined by immunostaining. We found that expression of HO-1 in the airway lumen and bronchial submucosa increased after

antigen exposure. In the saline-challenged mice, HO-1-positive cells were rarely detected in the lung tissue and BALF. These results suggest that the induction of HO-1 expression is regulated by the allergic asthmatic response in allergic inflammation.

A previous study found that HO-1 was expressed at high levels in sputum in asthmatic subjects [25], which is consistent with our finding in the bronchial submucosa. Recently, Mosley et al. [26] reported the induction of HO-1 expression in both glomeruli and tubules in nephrotoxic nephritis rats, and they concluded that the source of glomerular HO-1 is infiltrating macrophages. On the other hand, another report [27] suggested that human CD4 positive clone expressed HO-1 protein *in vitro*. We found evidence of HO-1 expression in the airway and lung macrophages, but not in CD4 T cells, after antigen challenge (data not shown). The reason for differences is not clear. Species may vary in the expression of HO-1 enzyme. The distribution of HO-1 during allergic inflammation of the lung suggests that there is a gradient of infiltrating macrophages in terms of stress response. In addition, from the confocal fluorescence images of rhodamine-labelled HO-1 antibody in Fig. 4, HO-1 reactivity was expressed to the cytoplasm, and the perinuclear region in particular. This suggested the possibility that the HO-1 enzyme may work on the axis of Golgi network [28].

Severe oxidative stress is thought to be a major mediator of apoptosis in several cellular systems. Soares et al. [29] reported that HO-1 had potent anti-apoptotic activity in a model of inflammation. In addition, recent reports demonstrated that CO is an anti-apoptotic, anti-inflammatory molecule [9,30], suggesting that up-regulation of HO-1 prolongs survival of macrophages in allergic airway inflammation. The functional significance of heme oxygenase induction in the allergic response remains to be elucidated.

There is evidence to suggest that HO-1 is induced not only by the substrate heme but also by a variety of non-heme inducers such as heavy metals, endotoxin, heat shock, prostaglandins and inflammatory cytokines [8,10,31]. Many cytokines secreted by macrophages are involved in asthmatic inflammation, including IL-1, IL-6 and TNF- α . These cytokines activate the transcription of the HO-1 gene *in vitro*, suggesting that the induction of HO-1 in asthmatic patients enhances allergen-induced pulmonary inflammation [17,32]. One of the products of HO-1 metabolism, CO, commonly classified as a toxic gas and biological waste, is considered a heme of gaseous cellular messenger. Horvath et al. [25] found that induction of HO-1 led to high exhaled CO concentrations in asthmatic subjects. Furthermore, an increase in HO-1 expression reflects induction of HO-1 which may be inhibited by corticosteroids such as anti-inflammatory drugs.

Increased production of HO-1 and CO may have pro-inflammatory effects because CO is a potent vasodilator, which is thought to play a key role in the modulation of vascular tone and increase plasma exudation from airway vessels [33,34]. Although the functional significance of the induction of HO-1 by oxidative stress is not well understood, many studies are supportive of the hypothesis that increased HO-1 and CO production provide cellular protection against both heme- and non-heme-mediated oxidant injury [12,13,24,35,36]. HO-1 induction provided protection against H₂O₂-induced injury of the cultured human airway epithelial cells in part via the HO-bilirubin pathway as reported in an *in vitro* study by Yamada and coworkers [37]. Similar to nitric oxide, CO increases cellular cyclic guanosine monophosphate (cGMP) via the activation of guanylcyclase [34,38], and a role for cGMP in the protection against cellular oxidant injury has been suggested [39,40]. Among other physiological responses, increased cellular cGMP causes smooth-muscle relaxation. These results suggest that induction of HO-1 plays a crucial role in the cellular protective system. Indeed, the induction of CO via HO-1 may participate in the resolution of inflammation and play a cytoprotective role in heme- and oxidant-induced injury. Further study of HO-1 functions should provide additional insight into allergic airway inflammation.

In conclusion, we demonstrated that expression of HO-1 was up-regulated within the lung tissue in allergic airway inflammation. Immunostaining provided evidence for HO-1 induction in macrophages in allergic inflammation.

Understanding the induction of HO-1 expression may provide for better management of asthma that involves oxidative stress.

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