

Role of Heme Oxygenase in Pulmonary Response to Antigen Challenge in Sensitized Rats in vivo

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Key Words

Reactive oxygen species · Nitric oxide · Stress enzyme · Heme degradation · Mast cells · Airway inflammation

Abstract

Heme oxygenase (HO) protects against oxidant-mediated lung injury. However, it is uncertain whether changes in HO activity modulate antigen-induced airway inflammation. We studied the effects of pretreatment with either hemoglobin, a HO inducer, or tin protoporphyrin (SnPP)-9, a specific HO inhibitor, on increases in pulmonary insufflation pressure (PIP) and plasma extravasation induced by intravenously injected ovalbumin (OA) antigen in rats sensitized to OA in vivo with Evans blue dye as a marker. Pretreatment with hemoglobin (300 mg/kg) significantly increased ($p < 0.01$) and that with SnPP-9 (50 $\mu\text{mol/kg}$) significantly decreased ($p < 0.01$) HO activity of the lung, but they failed to alter OA antigen (300 $\mu\text{g/kg}$)-induced increases in PIP. In contrast, hemoglobin pretreatment significantly decreased ($p < 0.01$) and SnPP-9 pretreatment significantly increased ($p < 0.05$) the leakage of dye induced by OA antigen in the trachea, main bronchi, and segmental bronchi. OA antigen-induced increases in plasma extravasation were also inhibited by superoxide dismutase (12,000 U/kg). These findings suggest the oxygen radicals are involved in increases in plasma extravasation induced by antigen challenge and HO protects against antigen-induced airway inflammation.

Introduction

Asthma is a chronic inflammatory disorder of the airways characterized by airway hyperresponsiveness and acute airflow limitation. Several inflammatory cells are found in asthmatic airways, including mast cells, eosinophils and neutrophils. These cells release reactive oxygen species (ROS) which are thought to participate in airway inflammation [1].

Heme oxygenase (HO), a stress enzyme, is responsible for catabolizing the heme molecule, resulting in the production of equimolar amounts of carbon monoxide and bilirubin [2]. Two distinct isoforms of HO have been identified in various tissues. One of the isozymes is inducible (HO-1), whereas the second form (HO-2) is constitutive. The inducible form of HO is affected by several factors, including oxidized hemoglobin [3, 4], heavy metal ions [5], hydrogen peroxide, ultraviolet A radiation [6], and oxygen-free radicals [7]. HO-1 is upregulated by oxidative stress and is shown to protect against oxidant-mediated lung injury [8, 9].

Since ROS contribute to the inflammatory response in asthma and HO has a protective effect against cytotoxicity induced by ROS, HO may play a role in protecting allergic inflammation of the airways. We, therefore, investigated whether changes in HO-1 activity modify bronchoconstriction and plasma extravasation induced by antigen challenge in sensitized rats.

Materials and Methods

Animal Preparation

Pathogen-free male Sprague-Dawley rats (Funabashi Farm, Shizuoka, Japan) weighing 300 g were anesthetized intraperitoneally with urethane (1 g/kg) and pentobarbital sodium (30 mg/kg). A tracheal cannula was inserted via a tracheostomy into the lumen of the trachea and connected to a constant-volume respirator (Model 607; Harvard Apparatus, South Natick, Mass.). A tidal volume of 5 ml/kg and a frequency of 90 breaths/min were used to ventilate the animals. Blood gas analysis revealed PaO_2 88 ± 2 mmHg, PaCO_2 39 ± 3 mmHg and pH, 7.40 ± 0.03 mmHg in this condition. Pulmonary insufflation pressure (PIP) was measured by means of a differential pressure transducer (MP45; Validyne Engineering, Northridge, Calif.) connected to a side arm in the expiratory limb of the ventilation tubing [10], and was recorded on a pen recorder (RECTI-HORIZ-8K; San-ei, Tokyo, Japan). To evaluate validation of the methods of measuring PIP, known resistances (from 0.1 to 1.0 cm $\text{H}_2\text{O}/\text{ml}/\text{s}$) were connected to the tracheal cannula. Linear increases in PIP were recorded with increases in resistance. The left carotid artery was cannulated with a polyethylene catheter to monitor systemic blood pressure with a pressure transducer (DTX; Viggo-Spectramed Japan, Tokyo, Japan). All drugs were administered via the jugular vein.

Measurement of Airway Plasma Extravasation

The magnitude of plasma extravasation was quantified using a modification of the method of Saria and Lundberg [11], the results of which correlate well with extravasation of radiolabeled albumin [12]. To determine the tissue content of Evans blue dye after the administration of the drug, the systemic circulation was perfused with saline to remove intravascular dye. The left ventricle was incised, a blunt-ended needle was inserted into the aorta, and the ventricles were cross-clamped. Blood was expelled from the incised right atrium at 100 mmHg pressure until the perfusate was clear (about 200 ml infused). The lung was then removed. The connective tissues, vasculature, and parenchyma were gently scraped, and airways were divided into three components: lower part of the trachea, main bronchi, and segmental bronchi. The tissues were blotted dry and weighed. To quantify the amount of extravasated Evans blue dye, each tissue was immersed in 2 ml of 100% formamide for 24 h at 50°C to extract the dye; the concentration of dye in the solution was determined spectrophotometrically by measuring the absorbance at 620 nm (220A Spectrophotometer; Hitachi, Tokyo, Japan). Tissue content (ng dye/mg wet tissue) was calculated from a standard curve of dye concentrations in the range from 0.5 to 10 $\mu\text{g}/\text{ml}$.

Experimental Protocol

Male Sprague-Dawley rats weighing 200–250 g were sensitized to ovalbumin (OA), as described previously [13]. Rats were simultaneously injected subcutaneously with 1 ml of a suspension containing 1 mg of OA and 200 mg of $\text{Al}(\text{OH})_3$ in saline and intraperitoneally with 0.5 ml of a *Bordetella pertussis* antigen solution containing 1×10^9 organisms/ml. These sensitized animals were challenged and subsequently studied at 2 weeks when they weighed 300 g. To determine the role of HO in allergic reaction, animals were divided into three groups ($n = 10$ for each group). Animals received the subcutaneous administration of either the vehicle of tin protoporphyrin (SnPP)-9 (control) or SnPP-9 (50 $\mu\text{mol}/\text{kg}$) 21 h before the administration of Evans blue dye [8]. The solutions of SnPP-9 were covered from light since light can activate protoporphyrin to produce radicals

which are toxic to tissues [14]. For hemoglobin studies, rats were intravenously administered rat hemoglobin (300 mg/kg) 16 h before the administration of Evans blue dye [8]. Evans blue dye (30 mg/kg) was injected into a jugular vein followed 1 min later by intravenously administered OA (300 $\mu\text{g}/\text{kg}$, 1 mg/ml in saline). Five minutes later, perfusion of the circulation was started. We also studied the effects of the vehicle of OA alone on PIP and Evans blue dye extravasation ($n = 10$). In preliminary studies, we found that the administration of a 400- μg OA caused decreases in mean arterial pressure from a baseline value of 71.5 ± 7.0 to 48.6 ± 4.9 mmHg ($n = 4$) after challenge, which made an interpretation of the results difficult. In contrast, a percent increase in PIP from baseline by the administration of a 200- μg OA was small ($23.6 \pm 5.2\%$, $n = 4$), so that an evaluation of treatment with either hemoglobin or SnPP-9 was difficult. Therefore, we employed a concentration of 300 μg OA following the experiments. We also found that OA antigen-induced increases in PIP appeared within 1.5 min and reached a maximum within 5 min after the cessation of OA administration. Therefore, the response to OA antigen was evaluated at the maximal PIP values.

We also studied the effects of the intravenous injection of OA antigen on PIP and Evans blue dye extravasation in unsensitized animals ($n = 10$). To determine whether ROS involved in increased plasma extravasation induced by OA antigen challenge, superoxide dismutase (SOD; 12,000 U/kg) dissolved in saline ($n = 7$) or saline ($n = 7$) was injected intravenously 15 min before OA antigen administration [15] in animals sensitized to OA.

HO Enzyme Activity

HO activity was measured by bilirubin generation [16] in rat lung microsomes. After the sensitized animals were killed, the pulmonary circulation was flushed with 20 ml of ice-cold phosphate-buffered saline via the pulmonary artery. The whole lungs were homogenized in 2 vol of MgCl_2 (2 mM)-potassium phosphate (100 mM) buffer (pH 7.4) on ice, sonicated, and centrifuged at 20,000 g for 15 min at 4°C . The supernatant obtained was successively centrifuged at 105,000 g for 60 min. The resulting pellets (microsomes) were suspended in 50 mM potassium phosphate buffer (pH 7.4) by the sonication, and assayed for HO activity. Reaction mixtures, in a final volume of 200 μl , had the following composition: 0.1 M of potassium phosphate buffer, 500 $\mu\text{g}/\text{ml}$ of microsomes, 15 μM of hemin, 100 $\mu\text{g}/\text{ml}$ of bovine serum albumin, 100 $\mu\text{g}/\text{ml}$ of a partially purified biliverdin reductase [17], and 420 $\mu\text{g}/\text{ml}$ of nicotinamide-adenine dinucleotide phosphate. The mixtures were aerobically incubated for 20 min at 37°C . The reaction was then stopped by placement on ice, and the amount of bilirubin formed was measured with a double-beam spectrophotometer (Hitachi, U-2000, Tokyo, Japan) as OD 464–530 nm (excitation coefficient, 43.5 mM/cm for bilirubin). HO activity was expressed as nanomoles of bilirubin formed per milligram of protein per hour. The protein content was determined by the method of Lowry et al. [18].

Drugs

OA, SOD, Evans blue dye, $\text{Al}(\text{OH})_3$, SnPP-9 and hemoglobin were from Sigma Chemical (St. Louis, Mo.). *B. pertussis* antigen was from Wako Chemicals (Osaka Japan).

Statistical Analysis

Values are reported as mean $\pm$ SEM. For statistical analysis, we used one-way analysis of variance. Multiple comparisons between means were performed by the Newman-Keuls test. Significance was accepted at $p < 0.05$.

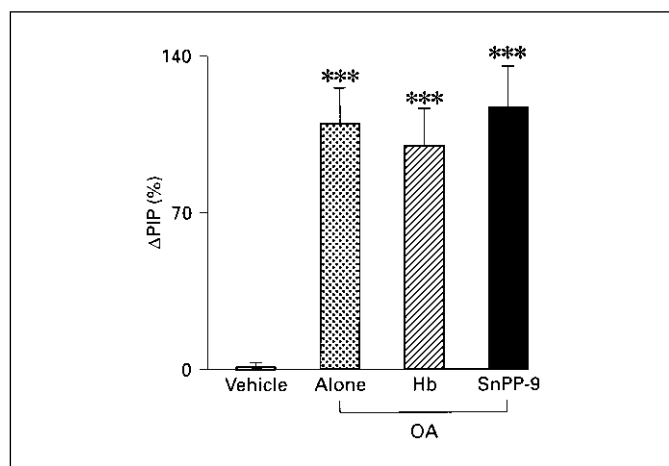


Fig. 1. Percent increase in PIP from baseline induced by OA (300 µg/kg) in the presence of hemoglobin (Hb, 300 mg/kg) or SnPP-9 (50 µmol/kg), the absence of drugs (Alone), and by the vehicle of OA. Results are reported as mean $\pm$ SEM from 10 animals. Significant differences from the vehicle alone are indicated by *** p <0.001.

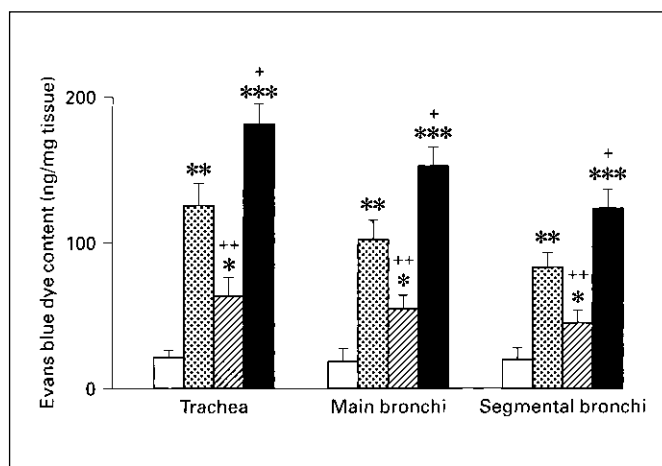


Fig. 2. Leakage of Evans blue dye induced by OA (300 µg/kg) in the trachea, main bronchi, and segmental bronchi in the presence of hemoglobin (300 mg/kg; ▨) or SnPP-9 (50 µmol/kg; ■), the absence of drugs (□), and by the vehicle of OA (□). Results are reported as mean $\pm$ SEM from 10 animals. Significant differences from the vehicle alone are indicated by * p <0.05, ** p <0.01 and *** p <0.001. Significant differences from OA alone are indicated by + p <0.05 and ++ p <0.01.

Results

The baseline values of PIP were 5.2 ± 0.3 cm H₂O in the vehicle of OA antigen, 5.3 ± 0.3 cm H₂O in OA antigen alone, 5.0 ± 0.2 cm H₂O in animals pretreated with hemoglobin and 5.0 ± 0.3 cm H₂O in animals pretreated with SnPP-9, and there were no significant differences among them (p >0.20). OA antigen failed to alter PIP (5.1 ± 0.3 cm H₂O in OA antigen vs. 5.2 ± 0.3 cm H₂O in saline control; p >0.50, n = 10) or the amount of extravasated Evans blue dye in the trachea (18.6 ± 6.9 ng/mg tissue in OA antigen vs. 20.0 ± 7.6 ng/mg tissue in saline control; p >0.20, n = 10) in unsensitized animals. In sensitized animals, OA antigen challenge alone significantly increased PIP, but pretreatment with hemoglobin or SnPP-9 failed to alter OA antigen-induced increases in PIP (p >0.20) (fig. 1).

OA antigen challenge significantly increased the leakage of Evans blue dye compared to the vehicle of OA antigen in the trachea, main bronchi, and segmental bronchi (fig. 2). Pretreatment with hemoglobin reduced and SnPP-9 potentiated OA antigen-induced increases in the leakage of Evans blue dye in all tissues (fig. 2).

SOD significantly reduced OA antigen-induced increases in the leakage of Evans blue dye in trachea (56.2 ± 10.3 ng/mg tissue in SOD vs. 131.5 ± 14.7 ng/mg tissue in saline control; p <0.01, n = 7), main bronchi (49.0

± 9.6 ng/mg tissue in SOD vs. 112.8 ± 15.6 ng/mg tissue in saline control; p <0.01, n = 7) and segmental bronchi (41.0 ± 10.1 ng/mg tissue vs. 88.3 ± 11.9 ng/mg tissue; p <0.01, n = 7).

Mean arterial pressure did not differ significantly before (70.3 ± 6.1 mmHg) and within 5 min after the intravenous administration of OA antigen (66.9 ± 5.7 mmHg; p >0.20). Likewise, the baseline values of mean arterial pressure did not differ among animals pretreated with hemoglobin (71.0 ± 6.6 mmHg) or SnPP-9 (69.3 ± 6.4 mmHg) and in animals without pretreatment (70.3 ± 6.1 mmHg; p >0.20).

HO activity 16 h after hemoglobin administration was 3.1-fold higher versus that observed in saline control (5.9 ± 0.3 nmol/mg/h in hemoglobin treatment vs. 1.9 ± 0.1 nmol/mg/h in control; p <0.01, n = 10). SnPP-9 significantly inhibited HO activity (0.1 ± 0.1 nmol/mg/h in SnPP-9 treatment vs. 1.9 ± 0.1 nmol/mg/h in control; p <0.01, n = 10).

Discussion

The present study shows that treatment of rats with hemoglobin caused a 3.1-fold increase in HO activity and that with SnPP-9 almost completely abolished HO activity. These results are in agreement with a previous study [8].

Thus, the present study suggests that HO protects against increases in plasma extravasation induced by antigen challenge in rats.

The mediators responsible for antigen-induced airway microvascular leakage and bronchoconstriction in rats are thought to be mainly leukotrienes and histamine [19, 20]. However, a variety of ROS are produced from inflammatory cells such as mast cells [21] and eosinophils [22]. Furthermore, mast cells have nitric oxide (NO) synthase [23] and NO reacts with superoxide to produce a potent cytotoxic oxygen radical, peroxynitrite [24]. A recent report shows that endogenous NO acts to increase airway microvascular leakage during allergic airway inflammation in guinea pigs [25]. ROS are suggested to cause epithelial damage, bronchoconstriction and increases in airway responsiveness [1]. They also increase vascular permeability probably via cytotoxic effects on vascular endothelial cells [26]. SOD, which inactivates ROS, attenuated OA antigen-induced increases in plasma leakage, suggesting that ROS could be involved in pulmonary responses to antigen challenge in the present study.

HO is present in the airway epithelial [27] and endothelial cells [16]. Upregulation of HO-1 activity has been shown to provide protection against oxidant-induced kidney failure [28], endotoxin shock [8] and hyperoxia-induced pulmonary epithelial cell death [27]. Increases in HO-1 activity also protect the endothelial cells from oxidative stress [29] and cultured embryonic fibroblasts from the targeted mice show hypersensitivity to cytotoxicity caused by oxidants [30]. Furthermore, the anti-inflammatory properties of HO-1 have been shown to protect mouse cardiac allografts against chronic injury [31], to determine cardiac xenograft survival [32], and to suppress carrageenin-induced inflammatory response in the pleural cavity of rats [33].

The initial degradation of heme by microsomal HO involves the loss of iron and the formation of biliverdin, which is subsequently reduced to bilirubin by the cytosolic biliverdin reductase. Motterlini et al. [29] showed that increased bilirubin levels by HO induction are a cellular defense mechanism against oxidative injury in the endothelial cells. Elevated HO activity by hemoglobin pretreatment, therefore, may attenuate antigen-induced increases in microvascular leakage by protecting endothelial cells from oxidative injury. This is consistent with the observation that SnPP-9, a specific inhibitor of HO, potentiated antigen-induced effects in the present study. However, pretreatment with either hemoglobin or SnPP-9 failed to alter antigen-induced bronchoconstriction. The reason for this inability is uncertain. Since OA antigen was infused intravenously in

the present study, allergic inflammation might occur near the endothelial cells, thereby the endothelial cells were exposed to high concentrations of ROS compared to the smooth muscle cells. Alternatively, the endothelial cells may be more susceptible to ROS than the smooth muscle cells.

ROS are generated by several inflammatory cells implicated in asthma, and an increased production of oxygen radicals has been demonstrated in various inflammatory cells isolated from asthmatic patients [1]. If certain oxygen species contribute to the inflammatory response in asthma, upregulation of HO activity could play a useful role in therapy.

In conclusion, the present study suggests that ROS are involved in antigen-induced increases in microvascular leakage and endogenous HO protects against allergic inflammation of the airways.

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