Heme oxygenase (HO)-1, which is a rate-limiting enzyme involved in the catabolism of heme, is upregulated by a variety of stimuli including oxidative stresses and inflammatory cytokines, in many cell types. Recent studies have suggested that upregulation of HO-1 might provide cytoprotection and immunomodulatory functions in addition to its obvious role in heme metabolism. In this study, we examined whether HO-1 was upregulated following degranulation in mast cells that initiate vigorous immunity reactions. To trigger degranulation, rat basophilic leukemia (RBL)-2H3 cells were passively sensitized using an antiserum collected from ovalbumin (OA) immunized-Brown Norway rats, and the cells were stimulated by treatment with OA. Degranulation was confirmed by measuring the release of β-hexosaminidase. HO-1 mRNA and presence of HO-1 protein were detected using Northern blot and Western blot analyses, respectively. The effect of the antioxidant N-acetyl-l-cysteine (NAC) on HO-1 expression was also tested. HO-1 mRNA transiently increased at 1—2 h after RBL-2H3 cells were stimulated to degranulate. Its mRNA increases were dependent on the extent of degranulation. Following the upregulation of HO-1 mRNA, HO-1 protein was also increased. We also detected intracellular production of reactive oxygen species following degranulation in RBL-2H3 cells. NAC attenuated the HO-1 expression in a dose-dependent manner. This is the first report to reveal induction of both HO-1 mRNA and protein by degranulation in RBL-2H3 cells. We showed that NAC inhibited HO-1 upregulation. These results suggest that oxidative stress in activated RBL-2H3 cells results in the upregulation of HO-1.

Key words degranulation; heme oxygenase; oxidative stress; RBL-2H3

Heme oxygenase-1 (HO-1) is a stress protein induced by a variety of stimuli, including oxidative stresses and inflammatory cytokines, in many cell types. HO-1 is the rate-limiting enzyme in the conversion of heme to biliverdin, carbon monoxide (CO) and free iron. In mammals, biliverdin is rapidly reduced to bilirubin by biliverdin reductase. Several studies have suggested that the induction of HO-1 contributes to cytoprotection through these products, which possess anti-inflammatory, antioxidant and anti-apoptotic properties. Moreover, recent reports using a variety of immunocytes, including mast cells, basophils, macrophages and T cells, have suggested that HO-1 and its products can modulate cell activation and function, thus regulating immune responses.

Mast cells are important in allergic and other immune responses by releasing mediators such as histamine, prostaglandins, and inflammatory cytokines following activation. Mast cells generate intracellular reactive oxygen species (ROS) following the aggregation of IgE receptors by specific antigens. Although mast cells were also exposed to these stresses, mast cells can recover after degranulation, and play important roles in allergy and immunity. These facts raise the possibility that HO-1 is induced following mast cell activation. In this study, we examined changes in HO-1 expression following degranulation using rat basophilic leukemia (RBL)-2H3 cells, which are a mucosal mast cell type and a model for high affinity IgE receptor (Fc epsilon RI)-mediated cell degranulation. We also investigated whether an antioxidant drug, N-acetyl-l-cysteine (NAC), could inhibit HO-1 expression. Our data demonstrate that HO-1 expression is induced by degranulation in RBL-2H3 cells and that NAC inhibited this HO-1 induction.

MATERIALS AND METHODS

Cell Culture RBL-2H3 cells were maintained in RPMI 1640 medium (Sigma-aldrich, Inc., St. Louis, MO, U.S.A.) supplemented with 10% heat-inactivated fetal bovine serum (ICN, Aurora, Ohio), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA, U.S.A.). Cells were treated with 0.25% Trypsin-0.02% EDTA-2Na (JRH BIOSCIENCES, Lenexa, KS, U.S.A.), and were seeded at 10^5 cells/ml in culture medium for 48 h before use in experiments.

Stimulation of RBL-2H3 Cells Anti-ovalbumin (OA) serum was prepared according to a modification of the method of Levine and Vaz. Briefly, Brown Norway rats (Japan SLC, Inc., Hamamatsu, Japan) were actively sensitized by subcutaneous injection of 1 mg OA in 0.5 ml (2X 10^10 bacilli/ml) Bordetella pertussis vaccine. To demonstrate that the rats are in fact sensitized to OA, the passive cutaneous anaphylaxis (PCA) titer of the anti-OA serum was measured in Wistar rats (Japan SLC, Inc.). Anti-OA serum with a PCA titer of 1/256 was diluted 64-fold with medium, and RBL-2H3 cells were passively sensitized for 2 h. Next, the RBL-2H3 cells were washed twice with phosphate-buffered saline (PBS), and then stimulated with 1—100 µg/ml OA for various times. Cells were costimulated with 0—10 mNAC and 10 µg/ml OA to study the effect of NAC on HO-1 expression. We tested the effect of NAC on cell viability under our experimental conditions by the trypan blue exclusion test.

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Sn (IV) Mesoporphyrin IX Dichloride (SnMP) (Frontier Scientific, Inc.) was dissolved in an arginine containing solution (153 mM arginine, 40% 1,2-propanediol, 10% ethanol, pH 8.7), and diluted in medium just before use. Sensitized RBL-2H3 cells were stimulated in the absence or presence of SnMP (10 μg/ml). The cell viability after 24 h of degranulation was measured with estimating trypsin blue exclusion.

**β-Hexosaminidase Release Assay** Degranulation of RBL-2H3 cells was confirmed by measuring β-hexosaminidase release. The activities of the released and residual β-hexosaminidase were measured 30 min after stimulating degranulation with OA. Aliquots (20 μl) of medium and cell lysate (in 2 ml of 0.1% Triton X-100) were incubated with 20 μl of 1 mM p-nitrophenyl-β-D-glucosaminide (Sigma) in 0.1 M sodium citrate buffer (pH 4.5) at 37 °C for 1 h. Then, 250 μl of a buffer containing 0.1 M Na2CO3 and 0.1 M NaHCO3 buffer (pH 10) was added. Absorbance was measured at 405 nm. The percentage of β-hexosaminidase release was calculated as follows:

$$\% \text{ of released } \beta\text{-hexosaminidase} = \left( \frac{\text{released } \beta\text{-hexosaminidase}}{\text{released } \beta\text{-hexosaminidase} + \text{residual } \beta\text{-hexosaminidase}} \right) \times 100$$

**Detection of HO-1 mRNA and Protein Following Degranulation** Total RNA was isolated using the TRIZOL reagent (Invitrogen), according to the manufacturer's instructions, at 0.5—24 h post stimulation by 10 μg/ml OA, and used to quantify expression of HO-1 mRNA. HO-1 mRNA was detected by Northern blot analysis using 10 μg total RNA.

Protein samples were collected 1—6 h after degranulation was stimulated by 10 μg/ml OA. Samples were used to detect HO-1 protein and were analyzed using the ECL Western blotting analysis system (Amersham Biosciences, U.K.) and a rabbit anti-HO-1 polyclonal antibody (Stressgen Biotechnologies Corp., Victoria, B.C., Canada) as a first antibody. Total protein bands were visualized by Ponceau S (Sigma) staining of the membrane.

**Measurement of Intracellular ROS Production by Fluorometry** A fluorometric assay was used to determine intracellular levels of ROS. The production of ROS following RBL-2H3 cells activation was measured using the cell-permeable oxidation-sensitive dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Invitrogen). For analyses, RBL-2H3 cells (2×10^5 cells/ml) plated on a 96-well plate were incubated with a 64-fold dilution of anti-OA serum for 2 h at 37 °C. The cells were then washed with physiological salt solution (145 mM NaCl, 2.7 mM KCl, 1 mM CaCl2 and 5 mM HEPES, 5.6 mM glucose, pH 7.4), and incubated with 5 μM H2DCFDA for 15 min at 37 °C before stimulating them with 1—100 μg/ml OA. H2DCFDA is reduced by cellular esterase activity after incorporation into the cells, and is rapidly oxidized to florescent 2',7'-dichlorofluorescein (DCF) by intracellular ROS. DCF was analyzed at 1 min intervals for 10 min using a LS-PLATE manager 2000 (excitation and emission at 485 and 535 nm, respectively).

**Statistics** Results are expressed as mean±S.E.M., n equals the sample size. Statistical comparisons were performed by unpaired t-test or one-way analysis of variance (ANOVA) with Bonferroni correction. p values less than 0.05 were considered statistically significant.

**RESULTS**

**Time Course of HO-1 mRNA Upregulation Following Degranulation in RBL-2H3 Cells** The passively sensitized RBL-2H3 cells were exposed to 10 μg/ml OA for 0.5—24 h, and HO-1 mRNA was analyzed by Northern blot analysis. HO-1 mRNA was induced significantly at 1—2 h after application of the degranulation stimulus (Fig. 1). The maximum induction of HO-1 mRNA was reached 2 h after stimulation with 10 μg/ml OA and was approximately 2.4 fold greater than stimulation with medium (0 μg/ml OA).

**OA Concentration-Dependent Induction of HO-1 mRNA Expression** OA stimulated degranulation was induced in a dose dependent manner over the range of concentrations tested (1—100 μg/ml) (Fig. 2A). Furthermore, we detected intracellular ROS following OA stimulation of cells sensitized with anti-OA serum (Fig. 2B). After adding OA to the sensitized cells, DCF fluorescence was significantly increased above the basal level in a time-dependent and OA dose dependent manner. DCF fluorescence reached a plateau at 10 μg/ml OA.

In addition, the dose-dependent increase in fluorescence was also consistent with the induction of HO-1 mRNA (Fig. 2C).

**Synthesis of HO-1 Protein by RBL-2H3 Cell Degranulation** Western blot analysis confirmed HO-1 upregulation at the protein level after RBL-2H3 cells were stimulated to degranulate (Fig. 3A). HO-1 protein was increased 2—4 h after stimulation. HO-1 protein was not upregulated in sensitized RBL-2H3 cells without OA stimulation or in non-sensitized RBL-2H3 cells stimulated with OA (Fig. 3B). Ponceau
S staining confirmed that total protein levels were nearly constant in these experiments (data not shown). Moreover, the passive sensitization of RBL-2H3 cells with serum from naïve rats didn’t induce the expression of HO-1 protein (data not shown).

**The Effect of NAC on the Upregulation of HO-1**

We first tested the effect of NAC on RBL-2H3 cell viability. Cell viability at 2 h after degranulation stimulation was 95.5 ± 1.3% without NAC and 98.3 ± 0.1% with NAC (10 μM). NAC (10 μM) did not affect cell viability as assessed by trypan blue exclusion. Furthermore, 10 mM NAC alone did not influence HO-1 mRNA expression in non-sensitized RBL-2H3 cells (Fig. 4A). We then analyzed HO-1 mRNA expression with Northern blot analysis, and showed that HO-1 expression at 2 h after application of the degranulation stimulus was inhibited in a dose-dependent manner by NAC (Fig. 4A). NAC did not have a significant effect on degranulation (Fig. 4B).

**The Effect of SnMP on Survival of RBL-2H3 Cells after Degranulation**

RBL-2H3 cells were passively sensitized with a 64-fold dilution of anti-OA serum and exposed to 10 μg/ml OA in the presence of 0—10 mM NAC. Data are means ± S.E.M. of five independent experiments.
DISCUSSION

We report here that HO-1 mRNA and protein were upregulated in RBL-2H3 cells after degranulation. Additionally, the antioxidant drug NAC inhibited the induction of HO-1 mRNA. Previous studies had also reported that mast cell activation by Fc epsilon RI cross-linking induced ROS production.\(^1\)\(^2\) Moreover, it is known that oxidative stress induces HO-1 in various cell types, including RBL-2H3 cells.\(^3\) Therefore, our current studies suggest that HO-1 induction in activated RBL-2H3 cells is mediated by ROS. To verify intracellular production of ROS in our experiment, we measured the increase in DCF fluorescence after adding OA to cells loaded with H\(_2\)DCFDA. Once incorporated into cells, H\(_2\)DCFDA is reduced by cellular esterase activity and rapidly oxidized to fluorescent 2',7'-dichlorofluorescein (DCF) by intracellular ROS, such as hydrogen peroxide, peroxynitrite, and hydroxyl radicals. Therefore, it is indicated that OA stimulation increased intracellular production of ROS. One of previous reports suggested that the IgE-mediated production of ROS is most likely due to activation of an NAD(P)H oxidase.\(^7\) Thus, it is likely that activation of an NAD(P)H oxidase may be involved in the mechanism of ROS production by OA stimulation in the present study.

Biliverdin, CO and iron are produced from heme by the action of HO-1 on heme containing proteins such as P450 and cyclooxygenase. Each of these end products ameliorates the oxidative susceptibility of cells through distinct mechanisms.\(^1\)\(^2\)\(^9\) Mast cells can survive after degranulation and be reactivated,\(^10\) and the anti-oxidant production by HO-1 may contribute to the survival of these long-lived mast cells. To study these possibilities, we investigated whether SnMP, an inhibitor of HO activity, could modulate cell recovery or cell viability after degranulation; however, no differences were detected in the presence or absence of the inhibitor. The H25A mutant HO-1, which has no HO activity, protects cells from hydrogen peroxide-induced cytotoxicity in the human monoblastic lymphoma cell line U937.\(^1\)\(^3\) Moreover, mast cells have additional mechanisms for protection from ROS, such as intracellular superoxide dismutase and glutathione,\(^4\)\(^15\) and the levels of these components are also enhanced following mast cell activation. Therefore, HO-1 may partially contribute to cell protection after degranulation in mast cells.

Recent reports have suggested that, in a variety of immunocytes including T cells and macrophages, the products of HO activity modulate cell activation and functions which regulate immune responses.\(^3\)\(^6\) Mast cell functions may also be regulated by these HO-1 products. Further research will be needed in order to characterize the specific roles of HO-1 in mast cells.

The present study is the first to provide evidence that HO-1 is upregulated in activated mast cells that were stimulated to degranulate. Previously, HO-1 or its product CO has been detected in several cell types, including macrophages, eosinophils and endothelial cells,\(^16\)\(^17\) but not mast cells. Moreover, Bachelet et al. reported that degranulation in RBL-2H3 cells does not represent a physiological stress, and HO-1 was not directly linked to the degranulation process.\(^12\) These results differ from our current results, and this difference may be due to the conditions under which HO-1 expression was measured. Because we investigated a detailed time course, we were able to detect the transient induction of HO-1 protein at approximately 2—4 h after degranulation.

In summary, we first revealed that HO-1 was induced in RBL-2H3 cells by stimulating degranulation, and its expression may be implicated in mast cell self-protection, or, alternatively, mast cells may play a role in regulation of allergic or immune responses through the products of HO-1 activity.

REFERENCES