



Heme oxygenase-1 inhibits cytokine production by activated mast cells

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Abstract

Heme oxygenase-1 (HO-1) is thought to contribute to host defense reactions against various stresses. In addition, recent reports have suggested that HO-1 modulates immunocyte activation and functions. HO-1 suppresses mast cell degranulation, but whether HO-1 suppresses cytokine synthesis as well is not yet known. We examined whether rat HO-1 cDNA transfected rat basophilic leukemia (RBL)-2H3 cells have altered cytokine production in response to stimulation with anti-ovalbumin (OA) serum/OA compared to Mock transfected RBL-2H3 cells. HO-1 inhibited anti-OA serum/OA-induced IL-3 and TNF- α production. Inhibition of HO-1 activity by Zn (II) protoporphyrin IX, a specific HO-1 inhibitor, prevented the suppression of TNF- α production. The cytokine inhibition by HO-1 was associated with selective suppression of the DNA-binding activity of AP-1 transcription factors. The suppression of mast cell cytokine production by HO-1 may be an important aspect of the processes that lead to resolution of allergic inflammation.

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Heme oxygenase-1 (HO-1) is a stress responsive protein whose expression is upregulated by a variety of stimuli, including heme, various oxidative stresses, LPS, and inflammatory cytokines. HO-1 is the rate-limiting enzyme in heme catabolism and converts heme to biliverdin, carbon monoxide (CO), and free iron. In mammals, biliverdin is rapidly converted to bilirubin by biliverdin reductase. Increases in HO-1 have been detected in inflammatory diseases such as allergic asthma [1–3] and rhinitis [4,5], and mast cells are widely recognized as the effector cells.

The induction of HO-1 has been suggested to contribute to host defense reactions against various stresses. Moreover, recent reports have suggested that HO-1 induced in immunocytes, including T cells and macrophages, modulates cell activation and functions, resulting in modulation

of the immune response. For example, HO-1 inhibits T cell proliferation stimulated by exposure to anti-CD3 and anti-CD28 antibodies [6,7], pro-inflammatory cytokine production in LPS-exposed macrophages [8,9], and cytokine production in hypoxia exposed-macrophages [9].

HO-1 suppresses compound 48/80-, calcium ionophore A23187-, or IgE-induced mast cell degranulation *in vitro* [10,11]. *In vivo*, HO-1 is induced in mast cells in rats exposed to hemin, an inducer of HO-1, and inflammation was attenuated through suppression of mast cell activity [10]. However, whether HO-1 suppresses cytokine synthesis in mast cells is unknown. There is little information available with regard to the mechanism of action of HO-1 in mast cells, other than the activation of guanylate cyclase by CO [11]. In particular, the means by which HO-1 is able to modify mast cell-dependent inflammatory responses and the specific molecular mechanisms involved are not understood.

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To address these questions, we used rat HO-1 cDNA transfected rat basophilic leukemia (RBL)-2H3 cells, which are a mucosal mast cell type, and examined whether HO-1 overexpressing RBL-2H3 cells display altered cytokine production in response to anti-ovalbumin (OA) serum/OA treatments compared to mock transfected RBL-2H3 cells. Additionally, we have shown that HO-1 inhibits anti-OA serum/OA-induced mast cell synthesis of inflammatory cytokines such as IL-3 and TNF- α . This effect is correlated with selective suppression of the anti-OA serum/OA-induced DNA-binding activity of AP-1, which is a hetero- or homo-dimeric complex composed of members of the Fos and/or Jun families of transcription factors [12]. The suppression of mast cell cytokine production by HO-1 may be an important mechanism in the resolution of allergic inflammation.

Methods

Cell culture. The RBL-2H3 cells were obtained from NIIH (JCRB; cell number JCRB0023). Parental RBL-2H3 cells, Mock transfected RBL-2H3 cells (Mock) [10] and rat HO-1 cDNA transfected RBL-2H3 cells (rHO-1) [10] were maintained in Eagle's minimal essential medium (Sigma–Aldrich, Inc., St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (ICN, Aurora, Ohio), 100 U/mL penicillin and 100 μ g/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C, 5% CO₂. For experimental procedures, cells were detached with trypsin (JRH Biosciences, Lenexa, KS, USA), washed, resuspended in fresh medium, seeded at a density of 2×10^5 cells in 2 mL of culture medium in 35-mm diameter wells, and cultured for 48 h before use.

Stimulation of RBL-2H3 cells. Anti-ovalbumin (OA) serum was prepared according to a modification of the method of Levine and Vaz [13]. Briefly, Brown Norway rats (Japan SLC, Inc., Hamamatsu, Japan) were actively sensitized by subcutaneous injection of 1 mg OA in 0.5 mL (2×10^{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 Mock and rHO-1 cells were passively sensitized for 2 h. Next, the cells were washed twice with phosphate-buffered saline (PBS), and then stimulated with 10 μ g/mL OA for various times.

The calcium ionophore ionomycin was used to stimulate calcium mobilization. Ionomycin (Sigma) was dissolved in DMSO. Zn (II) Protoporphyrin IX (ZnPP) (Calbiochem, San Diego, CA) was dissolved in 0.01 N NaOH and diluted in medium just before use. rHO-1 cells were pretreated with ZnPP (1–20 μ M) for 8 h before anti-OA serum/OA stimulation. Parental RBL-2H3 cells were treated with lipopolysaccharide isolated from *Escherichia coli* 0111:B4 (LPS) (Sigma).

Flow cytometry. For analysis of IgE binding to the surface of cells sensitized with anti-OA serum for 2 h, sensitized cells were detached with trypsin, washed with PBS, and incubated with FITC labeled mouse anti-rat IgE antibodies (Serotec, Ltd) for 15 min at room temperature in the dark. The cells were resuspended in PBS containing 0.5% paraformaldehyde. An FITC conjugated mouse IgG₁ isotype antibody (eBioscience) was used for the negative control.

Ribonuclease protection assay and RT-PCR. Total RNA was isolated using the TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. The production of the cytokine mRNA was quantitatively measured using the Multi-Probe RNase Protection Assay System (incorporating probes for IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, TNF- α , TNF- β , IFN- γ , GAPDH, and L32) (BD Biosciences). Twenty micrograms of total RNA was used in an RNase protection assay, which was performed according to the protocol provided by the manufacturer.

TNF- α mRNA expression in response to calcium ionophore or anti-OA serum/OA stimulation was also measured by RT-PCR. Total RNA (2 μ g) was used for first strand cDNA synthesis using SuperScript II Reverse Transcriptase (Invitrogen) and oligo-dT primers (Invitrogen). Primers for amplification of TNF- α and β -actin were obtained from Hokkaido System Sciences. The primer sequences are: TNF- α , forward primer 5'-T GAGCACAGAAAGCATGATCCGAG-3', reverse primer 5'-T GAAATGGCAAATCGGCTGACG-3'; β -actin, forward primer 5'-TGG AATCCTGTGGCATCCATGAAAC-3', reverse primer 5'-TAAAACG CAGCTCAGTAACAGTCCG-3'. PCR conditions were 1 cycle of 95 °C for 1 min, 15 cycles (β -actin) or 22 cycles (TNF- α) of 94 °C for 0.5 min, 58 °C for 1 min, 72 °C for 1.5 min, and final extension at 72 °C for 7 min.

TNF- α detection by ELISA. The TNF- α content of cell culture supernatants obtained after stimulation with OA or calcium ionophore was determined using a rat TNF- α ultrasensitive ELISA Kit (BioSource International, Inc. Flynn Road Camarillo, California, USA) according to the manufacturer's instructions.

β -Hexosaminidase release assay. Degranulation of Mock and rHO-1 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.

Each aliquot (20 μ L) of medium and cell lysate (in 2 mL of 0.1% Triton X-100) was incubated with 20 μ L of 1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide in 0.1 M sodium citrate buffer (pH 4.5) at 37 °C for 1 h. At the end of the incubation, 250 μ L of a 0.1 M Na₂CO₃, 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:

$$\beta\text{-hexosaminidase release (\%)} = \frac{\text{released } \beta\text{-hexosaminidase}}{\text{released } \beta\text{-hexosaminidase} + \text{residual } \beta\text{-hexosaminidase}} \times 100.$$

Immunoblotting. The cells were lysed in Lysis buffer (20 mM Hepes, pH 7.4, 0.1 M NaCl, 1% Triton X-100, 20 mM EGTA, 50 mM NaF, 2 mM Na₃VO₄), then separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (ADVANTEC Toyo Roshi Kaisha, Ltd., Japan). After blocking with 10% skimmed milk, the membranes were then incubated with a rabbit anti-HO-1 polyclonal antibody (Stressgen Biotechnologies Corp., Victoria, BC, Canada). Detection was performed by the ECL Western blotting analysis system (Amersham Biosciences, UK) according to the manufacturer's instructions.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared using a Nuclear Extraction Kit (Panomics, Redwood City, CA, USA). A biotin-labeled probe containing the consensus transcription factor binding site for AP-1 (5'-CGCTTGATGACTCAGCCGAA-3') was purchased from Panomics. For binding reactions, nuclear extracts (8 μ g) were incubated with labeled probe in the presence of poly-d(I-C) at room temperature for 30 min. DNA-protein complexes were separated on a non-denaturing 6% polyacrylamide gel and shifted bands corresponding to protein-DNA complexes were visualized using a chemiluminescence-imaging system according to the manufacturer's protocols. For competition experiments, excess unlabeled AP-1 probe was added to the reaction mixtures.

Statistics. Results are expressed as means $\pm$ SEM, *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 and discussion

In this study, we used rHO-1 cDNA transfected-RBL-2H3 cells (rHO-1) to reveal the effect of HO-1 on mast cell cytokine synthesis. First, we investigated the characteristics of mock transfected RBL-2H3 cells (Mock) and rHO-1 cells. Cells were plated at equal densities and cultured for 48 h before use in experiments. Initial cell numbers did not differ significantly between Mock and rHO-1 cells (data not shown). We showed that resting rHO-1 cells expressed

significant amounts of HO-1 protein (Fig. 1A). Mock and rHO-1 cells sensitized with rat anti-OA serum for 2 h showed no differences in the binding of IgE to the FcεRI on the cell surface (Fig. 1B). Degranulation of Mock and rHO-1 cells was confirmed by measuring β-hexosaminidase release. Degranulation by Mock and rHO-1 cells was $43.7 \pm 3.5\%$ and $27.5 \pm 1.8\%$, respectively (Fig. 1C). These data indicate that degranulation was suppressed in rHO-1 cells. These results were consistent with those of previous studies [10].

Next, we examined the effects of HO-1 on cytokine expression in activated mast cells by analyzing levels of transcription of cytokine genes using an RNase protection assay. Antigen induced cytokine transcription was substantially suppressed in sensitized rHO-1 cells compared to levels in sensitized Mock cells (Fig. 1D). IL-3 and TNF-α mRNAs in sensitized Mock cells were upregulated for at least 1 h after stimulation with OA. However, in rHO-1 cells IL-3 mRNA was not upregulated, and TNF-α mRNA was only slightly upregulated. In contrast, no differences were detected in production of the control housekeeping genes, L32 and GAPDH, in Mock or rHO-1 cells.

AP-1 is essential for transcription of the IL-3 and TNF-α genes in FcεRI-IgE stimulated mast cells [14]. Therefore, the DNA-binding activity of AP-1 after anti-OA serum/OA stimulation was compared between Mock and rHO-1 cells (Fig. 2A). As demonstrated by EMSA, a biotin

labeled AP-1 consensus binding site DNA probe bound to nuclear proteins extracted 10 or 30 min after OA-stimulation of sensitized Mock cells, and protein–DNA complexes were disrupted by addition of excess cold competitor. In contrast, AP-1 protein–DNA complexes in rHO-1 cells were almost undetectable. These data suggest that overexpression of HO-1 in mast cells may suppress antigen induced transcription of IL-3 and TNF-α genes by inhibiting AP-1 binding to target sites in the DNA.

Although OA stimulated DNA-binding by AP-1 increased in Mock cells, only minimal binding was detected in rHO-1 cells. AP-1 is essential for transcription of both IL-3 and TNF-α in response to FcεRI-IgE stimulation [14], but our data indicate that while IL-3 transcription was largely suppressed in rHO-1 cells, TNF-α transcription was only partially suppressed. This contradiction between IL-3 and TNF-α expression could indicate that another pathway for TNF-α expression exists, for example, a pathway dependent on calcium influx [15–17]. Ionomycin was used to stimulate calcium mobilization. We examined the effect of HO-1 on anti-OA serum/OA-induced (Fig. 2B) and calcium ionophore-induced (Fig. 2C) TNF-α expression, at both the transcriptional and translational levels. RT-PCR and ELISA showed that anti-OA serum/OA-induced TNF-α mRNA was partially inhibited in rHO-1 cells, and TNF-α release by rHO-1 cells was approximately 65% lower than in Mock cells (Fig. 2B). In contrast, iono-

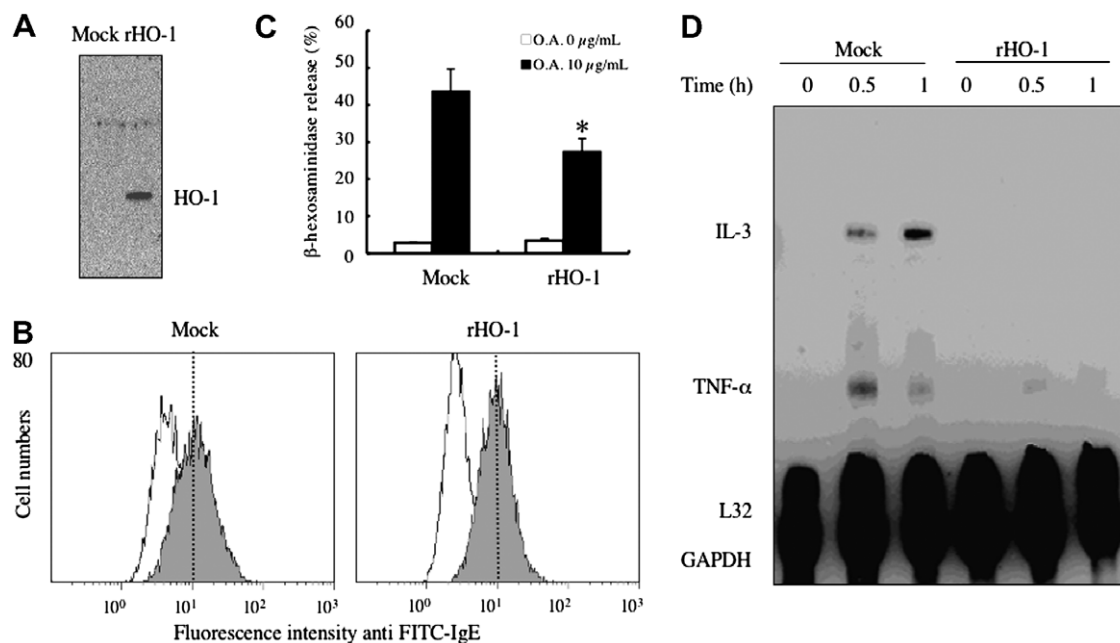


Fig. 1. (A) Western blot analysis of HO-1. HO-1 protein in resting Mock cells and rHO-1 cells was detected by Western blot analysis. The results are representative of three experiments. (B) Flow cytometry analysis of IgE binding to the cell surface. IgE binding to cells after sensitization was analyzed by flow cytometry using FITC-labeled anti-IgE antibodies. Cells labeled by the FITC-labeled isotype control antibodies are shown in white; cells labeled by FITC-labeled anti-IgE antibodies are shown in gray. The results are representative of three experiments. (C) Effects of HO-1 overexpression on OA-induced degranulation in RBL-2H3 cells. Mock and rHO-1 cells were treated with anti-OA serum for 2 h, and then incubated in the presence or absence of 10 μg/mL OA for 30 min. Degranulation was measured as percent release of β-hexosaminidase. Data are means $\pm$ SEM for four separate experiments. * $p < 0.05$ followed by two-tailed unpaired t -test compared to Mock cells. (D) Effects of HO-1 overexpression on OA-induced cytokine gene expression. Total RNA was extracted from cells challenged with 10 μg/mL OA for the indicated times, and cytokine mRNA expression was detected using an RNase protection assay. The results are representative of three experiments.

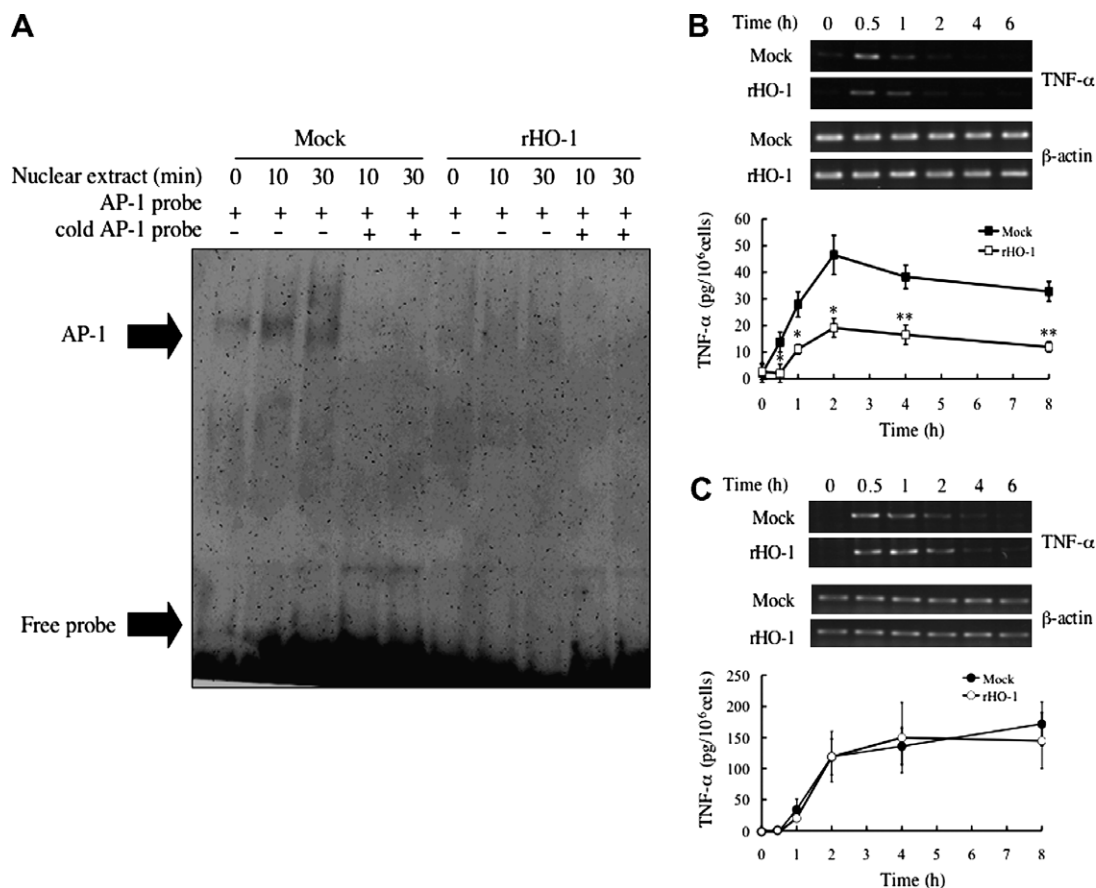


Fig. 2. (A) Effects of HO-1 overexpression on OA-induced AP-1 activation. Nuclear extracts were prepared from cells challenged with 10 $\mu\text{g/mL}$ OA for the indicated times, and the DNA-binding activity of AP-1 was detected by EMSA. (B) Effects of HO-1 overexpression on OA-induced TNF- α expression in RBL-2H3 cells. Mock and rHO-1 cells were treated with anti-OA serum for 2 h, and challenged with 10 $\mu\text{g/mL}$ OA for the indicated times. Upper panels show the results of RT-PCR for TNF- α and β -actin. The lower panel quantifies TNF- α release from Mock and rHO-1 cells in response to anti-OA serum/OA stimulation. Data are means $\pm$ SEM of four separate experiments. * $p < 0.05$, ** $p < 0.01$, by two-tailed unpaired t -test compared to mock transfected cells. (C) Effects of HO-1 overexpression on ionomycin-induced TNF- α expression in RBL-2H3 cells. Mock and rHO-1 cells were treated with ionomycin for the indicated times. Upper panels show the results of RT-PCR for TNF- α and β -actin. The lower panel quantifies TNF- α release in response to ionomycin. Mock and rHO-1 cells were treated with 1 μM ionomycin for the indicated times. Data are means $\pm$ SEM for three separate experiments.

mycin induced equivalently levels of TNF- α expression and release in Mock and rHO-1 cells (Fig. 2C). Moreover, TNF- α expression is generated *de novo* in antigen stimulated RBL-2H3 cells [18]. Taken together, these data suggest that HO-1 overexpression partially suppresses TNF- α synthesis, primarily by inhibiting an AP-1 dependent pathway.

We investigated whether ZnPP, an inhibitor of HO activity, could reverse the inhibition of TNF- α release from rHO-1 cells (Fig. 3). Although 20 μM ZnPP alone did not markedly increase TNF- α release from sensitized rHO-1 cells, ZnPP dose-dependently increased TNF- α release from rHO-1 cells by anti-OA serum/OA stimulation. These data suggest that HO-1 overexpression in mast cells inhibited TNF- α release through HO reaction products, such as CO, free iron, biliverdin, and bilirubin.

Furthermore, to confer a pathophysiological role on HO-1 in mast cells, we investigated whether HO-1 is induced after treatment of this parental cell line with physiological stimuli, such as LPS. Parental RBL-2H3 cells

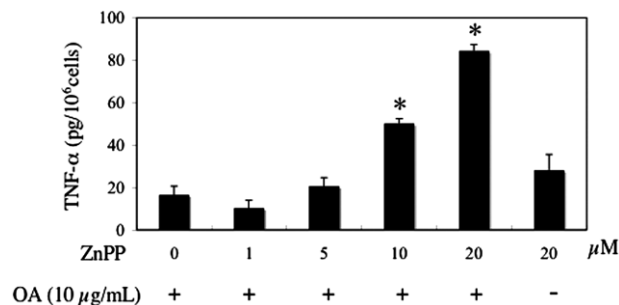


Fig. 3. The effect of ZnPP on TNF- α release from sensitized rHO-1 cells. rHO-1 cells were pretreated with ZnPP (1–20 μM) for 8 h before anti-OA serum/OA stimulation. The culture supernatants of cells incubated in the presence or absence of 10 $\mu\text{g/mL}$ OA for 2 h were collected, and released TNF- α was measured using an ultrasensitive ELISA kit. Data are means $\pm$ SEM for three separate experiments. * $p < 0.01$ followed by one-way ANOVA with Bonferroni correction compared with 0 μM .

were exposed to 1 $\mu\text{g/mL}$ LPS for 8–30 h, and HO-1 induction was analyzed by Western blot analysis (Fig. 4A). HO-1 protein was induced at 24 h after stimulation with LPS.

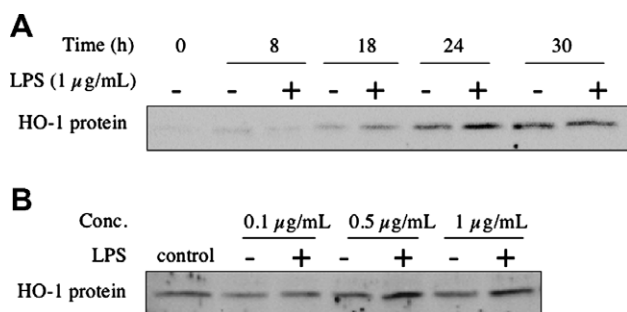


Fig. 4. (A) Time course of HO-1 expression after LPS stimulation of parental RBL-2H3 cells. RBL-2H3 cells were exposed to 1 µg/mL LPS for the indicated times and extracts were prepared. The expression of HO-1 was detected by Western blot analysis. The results are representative of five experiments. (B) Increases of HO-1 expression are dependent on LPS concentration. RBL-2H3 cells were exposed to the indicated concentration of LPS for 24 h. The expression of HO-1 was detected by Western blot analysis. The results are representative of four experiments.

The cells were then exposed to a range of 0.1–1 µg/mL LPS concentrations for 24 h. LPS increased HO-1 protein in a dose-dependent manner in RBL-2H3 cells (Fig. 4B). It has been shown that LPS can reduce asthma and allergic inflammation in mice and guinea pigs [19–21]. Our current findings suggest that HO-1 may be part of a mechanism that LPS protects against asthma and allergic inflammation. In addition, we showed in previous study that HO-1 was also upregulated by degranulation in RBL-2H3 cells [22].

The crucial role of mast cell produced TNF- α in immediate hypersensitivity reactions and immune responses, has been demonstrated in studies using TNF- α -deficient mice [23–25]. In these studies, TNF- α supported the persistence of inflammation by stimulating eosinophil infiltration, enhancing T cell activity, and increasing ICAM-1 expression by endothelial cells. TNF- α is also an important mast cell survival factor [26]. IL-3 is important for mast cell survival [26] and eosinophil proliferation [27], while stimulation of human endothelium with IL-3 induces selective basophil accumulation [28]. Taken together, it is suggested that HO-1 confers anti-inflammatory effect by decreasing cytokine expression in mast cells through HO reaction products.

In conclusion, the present study suggests that HO-1 regulates mast cell cytokine production via AP-1 inhibition. HO-1 might be an important protein in the resolution or self-limitation of allergic inflammation. Therefore, we propose that HO-1 is an appropriate target for development of novel anti-allergy drugs and gene therapy for immune diseases.

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