Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 354 (2007) 485–490
BBRC
www.elsevier.com/locate/ybbrc

Heme oxygenase-1 inhibits cytokine production by activated mast cells

Yumiko Yasui [a,*], Mao Nakamura [a], Toshihiro Onda [a], Tomohiro Uehara [a], Saori Murata [a], Nobuaki Matsui [a], Nobuyuki Fukuishi [a], Reiko Akagi [b], Makoto Suematsu [c], Masaaki Akagi [a]

[a] *Department of Pharmacology, Faculty of Pharmaceutical Sciences, Tokushima Bunri University, 180 Nishihama-bouji, Yamashiro-cho, Tokushima-shi, Tokushima 770-8514, Japan*
[b] *Faculty of Health and Welfare Science, Okayama Prefectural University, Okayama 719-1197, Japan*
[c] *Department of Biochemistry and Integrative Medical Biology, School of Medicine, Keio University, Tokyo 160-8582, Japan*

Received 28 December 2006
Available online 10 January 2007

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.

Keywords: AP-1; Heme oxygenase-1; IL-3; Mast cell; TNF-α

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.

* Corresponding author. Fax: +81 88 655 3051.
E-mail address: yumikoy@ph.bunri-u.ac.jp (Y. Yasui).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.228

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 NIHS (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 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C, 5% CO_2. 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 μg/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 μM) 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 IgG_1 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-1α, IL-1β, 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 μg) 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′-GAGCACAGAAAGCATGATCCGAG-3′, reverse primer 5′-TGAAATGGCAAATCGGCTGACG-3′; β-actin, forward primer 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, reverse primer 5′-TAAAACGCAGCTCAGTAACAGTCCG-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 μL) of medium and cell lysate (in 2 mL of 0.1% Triton X-100) was incubated with 20 μL 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 μL of a 0.1 M Na_2CO_3, 0.1 M $NaHCO_3$ buffer (pH 10) was added. Absorbance was measured at 405 nm. The percentage of β-hexosaminidase release was calculated as follows:

β-hexosaminidase release (%) = (released β-hexosaminidase/(released β-hexosaminidase + residual β-hexosaminidase)) × 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 Na_3VO_4), 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′-CGCTTGATGACTCAGCCGGAA-3′) was purchased from Panomics. For binding reactions, nuclear extracts (8 μg) 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 ± 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