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Selective activation of adrenergic β_1 receptors induces heme oxygenase 1 production in RAW264.7 cells

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Abstract **We hypothesized that catecholamines through β-adrenoceptor might modulate macrophage function. We showed that isoproterenol concentration-dependently induced HO-1 production through β_1-but not β_2-adrenoceptor. Production was increased by forskolin and inhibited by pretreatment with the PKA inhibitor, H-89. Furthermore, induction of HO-1 by isoproterenol effectively protected RAW264.7 cells from effects of glucose oxidase treatment, which was abrogated either by HO-1 inhibitor, ZnPP IX and β-adenoceptor antagonist, propranolol. Thus, stimulation of HO-1 production through β_1-adenoceptors, and via the PKA pathways by isoproterenol, can enable RAW264.7 cells to resist oxidant stress, suggesting that catecholamine hormones may be necessary, at least, to maximize defending role of macrophages.**

Keywords: β_1 Adrenoceptor; Catecholamines; HO-1; Isoproterenol; Macrophage; Oxidative stress

1. Introduction

Macrophages are among the first cells of the host to confront microbes and are important effector cells in the body's innate resistance to intracellular microbial pathogens. The outcome of this initial encounter with an intracellular pathogen is that either the macrophage resists the growth of the microorganism or the microorganism adapts and replicates within the macrophage. The ability of the macrophage to resist the growth of the microorganism is dependent on the activation state of the macrophage. Cytokines, such as interferon gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor and tumor necrosis factor alpha, activate macrophages to resist the growth of intracellular pathogens by enhancing the production of the major antimicrobial effector molecules, including reactive oxygen species (ROS) and nitric oxide [1–3].

On the other hand, the sympathetic nervous system acts to maintain homeostasis during periods of stress by releasing norepinephrine at sympathetic nerve endings, and epinephrine from the adrenal medulla [4,5]. These catecholamine hormones modulate the activities of cells, including those of the immune system [6]. Macrophage function can be either activated [7–9] or suppressed [10–13] by catecholamines. Boomershine et al. [14] showed that the addition of epinephrine to IFN-γ-activated mouse peritoneal macrophages inhibited the ability of the macrophages to resist the growth of *Mycobacterium avium*. This effect was mediated by the β_2-adrenergic receptor and was correlated with a decrease in nitric oxide production. In contrast, when resting peritoneal macrophages were treated with epinephrine, the inhibition of mycobacterial growth increased, suggesting that in this situation the catecholamine activated the macrophages [7]. Macrophage activation was mediated by α_2-adrenergic stimulation; thus, the α_2-adrenergic agonist (α_2-agonist) clonidine increased the ability of the macrophages to inhibit mycobacterial growth, and the effect of epinephrine was blocked by the α-adrenergic antagonist phentolamine. However, it is unclear how activated macrophages defend themselves from the reactive ROS they produce.

Heme oxygenase (HO)-1 is now recognized as a beneficial molecule in protecting against the oxidative stresses induced by many stimuli [15,16]. However, regulation of the activity of the gene for *ho-1* in macrophages under stress conditions is not fully understood. While HO-1 production is induced by stress [17,18], recent evidence suggests that increased intracellular cAMP levels induce HO-1 production via a protein kinase A (PKA)-dependent pathway [19,20]. Intracellular cAMP levels are increased by several receptor-dependent pathways including β-adrenoceptor-agonists.

We hypothesized that HO-1 may protect macrophages from oxidant stresses as in other cells, in which catecholamines through β-adrenoceptors play an important role for the induction of this enzyme. Therefore, the purpose of this study was to explore the effect of isoproterenol, one of the synthetic catecholamines having β-adrenergic activity, on the production of HO-1 in macrophages, the RAW264.7 cell line. In addition, we investigated the mode of HO-1 induction and which subtype of β-adrenergic receptor (AR) might be responsible for it.

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Abbreviations: AR, adrenergic receptor; GOX, glucose oxidase; HO, heme oxygenase; IFN-γ, interferon gamma; PKA, protein kinase A; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; TBS-T, tris–buffered saline/Tween 20; ZnPP, zinc protoporphyrin

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2. Materials and methods

2.1. Materials

DMEM medium, fetal bovine serum, penicillin, streptomycin, and glutamine were supplied by Gibco BRL (Rockville, MD). PRO-PREP protein extract solution and ECL western blotting detection reagents were supplied by iNtRON Biotechnology (Sungnam, Korea). MTT, isoproterenol, propranolol, butoxamine, metoprolol, forskolin, H-89, and RuCO were supplied by Sigma–Aldrich (St. Louis, MO). Glucose oxidase (GOX) from *Aspergillus niger* and an anti-HO-1 antibody were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). Zinc protoporphyrin (ZnPP) IX was supplied by Calbiochem (La Jolla, CA).

2.2. Cell viability assay (MTT assay)

Cell viability was analyzed using the MTT assay. RAW264.7 cells at the exponential growth phase were seeded at 10^4 cells/well in 24-well plates. After different treatments, 20 μL aliquots of 5 mg/mL MTT solution was added to each well (0.1 mg/well) and incubated for 4 h. Supernatants were aspirated and the formazan crystals in each well were dissolved in 200 μL dimethyl sulfoxide (DMSO) for 30 min at 37 °C and light absorbance at 570 nm was read on a Microplate Reader (Bio-Rad).

2.3. Western blotting

Cells were lysed in PRO-PREP protein extract solution. The sample was centrifuged at $10\,000 \times g$ for 20 min at 4 °C. Protein concentration was determined by the Bradford method. An equal volume of 2× SDS sample buffer (0.1 M Tris–CI, 20% glycerol, 4% SDS and 0.01% bromophenol blue) was added to an aliquot of the supernatant fraction from the lysates and the mix was boiled for 5 min. Aliquots of 30 μg of protein were subjected to 10% SDS–polyacrylamide gel electrophoresis for 1 h 30 min at 110 V. The separated proteins were transferred to PVDF membranes for 2 h at 20 mA with SD Semi-dry Transfer Cell (Bio-Rad). The membranes were blocked with 5% nonfat milk in Tris–buffed saline containing 0.05% Tween 20 (TBS-T) for 2 h at room temperature. The membranes were then incubated with goat polyclonal anti-mouse HO-1 at 1:500 concentration in 5% skim milk in TBS-T overnight at 4 °C, and bound antibody was detected by horseradish peroxidase conjugated anti-goat IgG. The membranes were washed and then developed using a Western Blotting Luminol Reagent system and autoradiography.

2.4. HO activity

After incubation for various times, confluent macrophages were washed twice with phosphate-buffered saline, gently scraped off the dish, and centrifuged ($10\,000 \times g$, 5 min, 4 °C). The cell pellet was suspended in 2 mM $MgCl_2$ phosphate buffer (100 mM, pH 7.4), frozen at −70 °C, thawed three times and finally sonicated on ice before centrifugation at 13 000 rpm for 15 min at 4 °C. The supernatant (400 μL) was added to a NADPH-generating system. This contained 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate-1-dehydrogenase, and 2 mg protein of rat liver cytosol prepared from the $105\,000 \times g$ supernatant fraction as a source of biliverdin reductase, potassium phosphate buffer (100 mM, pH 7.4), and hemin (10 μM) in a final volume of 200 μL. The reaction was incubated for 1 h at 37 °C in the dark and terminated by the addition of 600 μL chloroform. The extracted bilirubin was calculated from the difference in light absorption between 464 and 530 nm wavelengths using a quartz cuvette. HO activity was measured as picomoles of bilirubin formed per milligram of macrophage cell protein per hour.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) for β-AR subtypes

Total RNA was extracted from the RAW264.7 cells by a single-step guanidine thiocyanate–phenol–chloroform extraction procedure, using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA (2 μg) was reverse transcribed at 37 °C for 90 min with 100 U of recombinant M-MLV reverse transcriptase in the presence of 0.1 μg DNA random hexamers, 40 U RNase inhibitor, and 1.4 mM dNTPs in a final volume of 50 μL. The reaction was stopped by heating at 95 °C for 5 min, then held at 4 °C for 5 min. A negative control without reverse transcriptase was done to verify that amplification did not ensue from residual genomic DNA. PCR amplification was carried out on cDNA equivalent to 100 ng of starting mRNA, using specific oligonucleotide primers for β_1-AR (forward, 5′-CATCGTAGTGGGCAACG-TGTTG-3′ and reverse, 5′-AAATCGCAGCACTTGGGGTC-3′), for β_2-AR (forward, 5′-ACCTCCTTCTTGCCTATCCA-3′ and reverse, 5′-TAGGTTTTCGAAGAAGACCG-3′), and GAPDH (forward, 5′-ATCACCATCTTCCAGGAGCGAGA-3′ and reverse, 5′-CAAAGTTGTCATGGATGACCTT-3′). The cDNA was heated for 5 min at 95 °C, then amplified using 35 cycles for β_1-AR (94 °C for 1 min, 61 °C for 1 min, 72 °C for 2 min) and β_2-AR (94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min), and 25 cycles for GAPDH (94 °C for 30 s, 58 °C for 30 s, 72 °C for 45 s), followed by 5 min of extension at 72 °C. The PCR products were electrophoresed on 2% ethidium bromide stained agarose gels.

2.6. DNA extraction and electrophoresis

The characteristic ladder pattern of DNA breakage caused by apoptosis was analyzed by agarose gel electrophoresis. Briefly, DNA from the RAW264.7 cells (3×10^6 cells per group) was isolated by a Genomic DNA extraction kit (iNtRON Biotechnology, Sungnam, Korea). Isolated genomic DNA was subjected to 1.5% agarose electrophoresis at 100 V for 1 h. DNA was visualized by staining with ethidium bromide under UV light.

2.7. Statistical evaluation

Values are expressed as means ± S.E.Ms. Differences from the respective control for each experimental test condition were assessed using paired Student's *t*-tests, and $P < 0.05$ was assumed significant.

3. Results

3.1. Effect of isoproterenol on HO-1 gene expression in RAW264.7cells

There is abundant evidence that HO-1 is a stress-response enzyme with cytoprotective effects, including pulmonary defense against LPS and hypoxia. To assess the role of isoproterenol for induction of HO-1 gene expression, murine macrophage cell line RAW264.7 cells were treated for 6 h with isoproterenol at doses of 1–200 μM. Western blot analysis showed that there was no measurable induction of HO-1 protein production by isoproterenol at doses between 1 and 10 μM, but it was evident at doses of 50 μM or higher (Fig. 1A). Treatment with 100 μM of isoproterenol increased HO-1 levels in a time-dependent manner (Fig. 1B).

3.2. Induction of HO-1 production through PKA-stimulating agents

Because intracellular cAMP levels are increased by β-AR agonists, and increased intracellular cAMP levels induce HO-1 via a PKA-dependent pathway (19), we tested whether an activator of adenylate cyclase forskolin could induce HO-1 gene expression in RAW264.7 cells. Indeed, forskolin induced HO-1 levels in a dose-dependent manner (1–30 μM) after 6 h treatment (Fig. 2A). Following pretreatment with the PKA inhibitor, H-89, HO-1 production was inhibited by forskolin at 30 μM (Fig. 2B). H-89 also decreased isoproterenol-induced HO-1 production, suggesting a PKA-dependent induction of HO-1 gene expression in these cells (Fig. 2C).

3.3. Stimulation of HO-1 production via β_1-adrenoceptors

To examine the role of β-adrenoceptors for the production of HO-1, we used the specific antagonist propranolol. As shown in Fig. 3A and B, isoproterenol effectively induced HO-1 levels and activity at 100 μM, and this was inhibited by pretreatment with propranolol in a dose-dependent

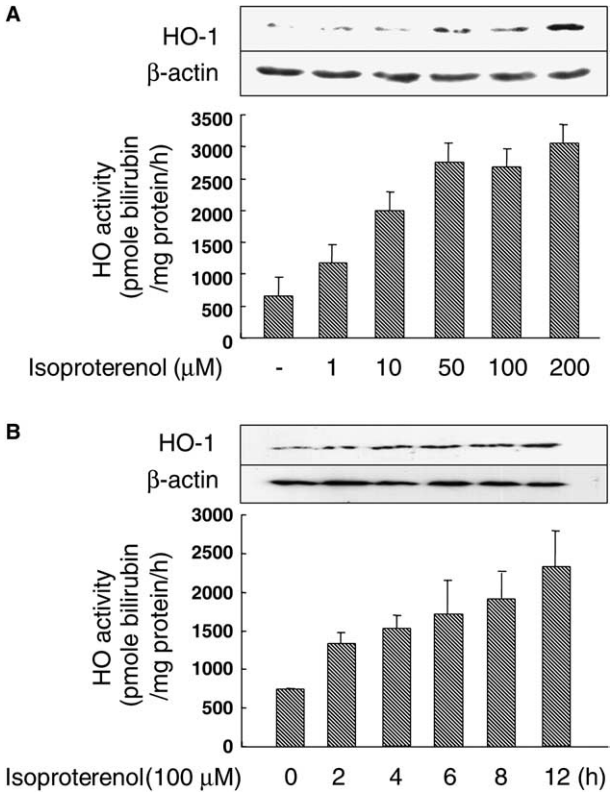

Fig. 1. Effect of isoproterenol on HO-1 production and activity in RAW264.7 cells. Cells were treated with isoproterenol for 6 h at doses from 1 to 200 μM (A), and at the dose of 100 μM for 2, 4, 6, 8 and 12 h (B). HO-1 protein levels and activity were measured from isoproterenol-treated cells as described in Section 2.

manner. We then aimed to specify the subtype of β-adrenoceptor involved in this. Isoproterenol-induced HO-1 production was significantly blocked by the specific β_1-AR antagonist metoprolol, but not by the β_2-AR-specific antagonist butoxamine (Fig. 3C and D). Fig. 3E shows that isoproterenol significantly increased the level of expression of β_1-AR mRNA even though the level expressed in resting cells was weak. On the other hand, β_2-AR mRNA levels were very low in control or isoproterenol-treated cells. These results suggest that isoproterenol induced HO-1 gene expression through the β_1-specific adrenoceptor.

3.4. Protection of RAW264.7 from oxidative stress via HO-1 expression

As shown in Fig. 4A, isoproterenol protected RAW264.7 cells from GOX-induced cytotoxicity. When the cells were treated with GOX for 6 h, the cells showed about 38% viability, however the presence of isoproterenol significantly increased this to 60%. Pretreatment with propranolol, a β-AR antagonist, abrogated this protective effect. To verify that this was mediated by HO-1, cells were preincubated with the HO-1 inhibitor ZnPP IX for 30 min before addition of isoproterenol. Fig. 4B clearly shows that the effect of isoproterenol was significantly inhibited by the presence of ZnPP IX, indicating that HO-1 plays a role in protecting cells against GOX-induced injury. Because it is well known that CO produced via HO-1 activity mainly contributes to its protective effect, we investigated whether RuCO was able to protect the cells from

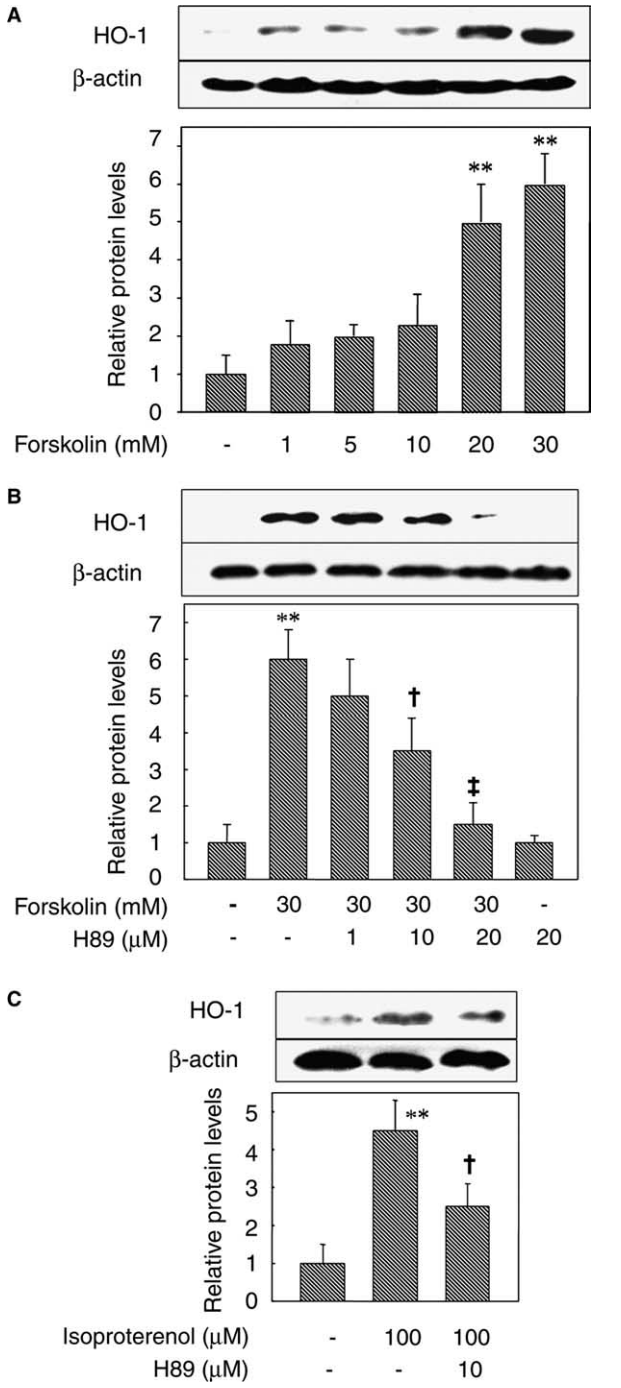

Fig. 2. Induction of HO-1 production by isoproterenol via the phosphokinase A (PKA) pathway. (A) Dose-dependent induction of HO-1 production by the adenylate cyclase activator forskolin (1, 5, 10, 20, 30 μM) in RAW264.7 cells. Cells were treated with forskolin for 6 h at the doses indicated and western blot analysis was performed as described in Section 2. (B) Prevention of forskolin-induced HO-1 production by pretreatment with the PKA inhibitor H-89. Cells were pretreated with H-89 (1, 10, 20 μM) for 1 h and then treated with forskolin (30 μM) for 6 h. After treatment, protein was extracted from the cells and HO-1 protein level was determined by western blot analysis. (C) Prevention of isoproterenol-induced HO-1 production by pretreatment with the PKA inhibitor H-89. Cells were pretreated with PKA inhibitors H-89 (10 μM) for 1 h and then treated with isoproterenol (100 μM) for 6 h. Values in the graphs represent the densitometric data from three independent experiments (means ± S.E.M.). ($^{**}P < 0.01$ compared with control; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ compared with forskolin or isoproterenol).

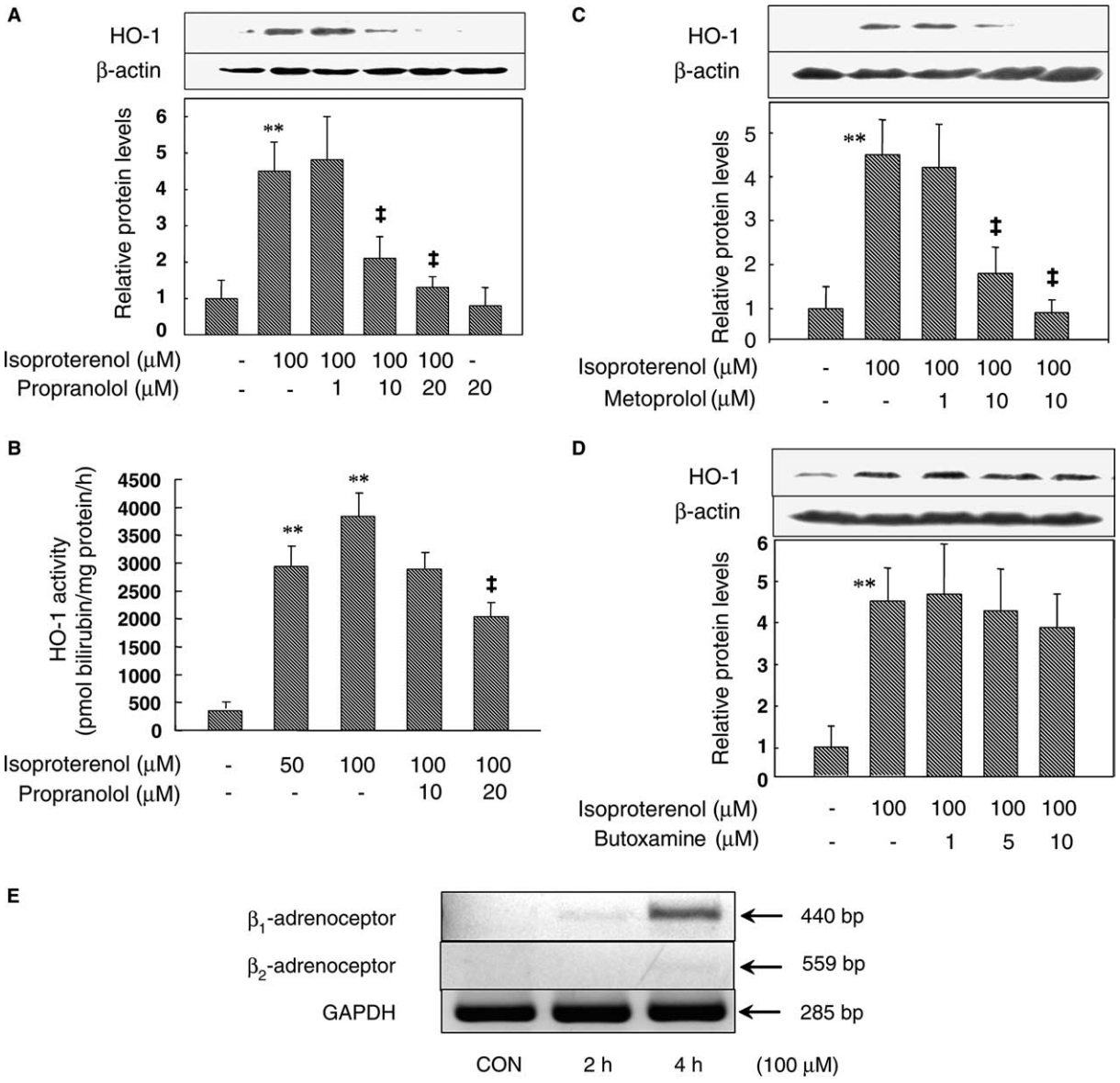

Fig. 3. Induction of HO-1 protein production and activity by isoproterenol via the β-adrenoceptor (AR). Propranolol, a β-AR antagonist, was administered (1, 10, 20 μM) 1 h before treatment with 100 μM of isoproterenol. After 6 h (A) HO-1 protein and (B) enzyme activity levels were detected as described in the Materials and Methods. (C) Involvement of β_1- AR in isoproterenol-induced HO-1 gene expression. Cells were pretreated with (C) the β_1-AR antagonist metoprolol (1, 10 μM) or (D) the β_2-AR antagonist butoxamine (1, 5, 10 μM) for 1 h and then treated with isoproterenol for 6 h. Cells were then harvested for western blot analysis. Each bar represents the means ± S.E.M. of experiments ($^{**}P < 0.01$ compared with control, $^{‡}P < 0.01$ compared with isoproterenol). (E) Detection of β_1- or β_2-AR mRNA in isoproterenol-treated RAW264.7 cells by RT–PCR. Total RNA was extracted from the cells treated with isoproterenol (100 μM for 2 or 4 h). RT-PCR was performed as described in Section 2 and GAPDH was used as an internal standard. The data represent three separate experiments.

GOX. RuCO effectively protected the cells from GOX in a dose-dependent manner, suggesting that isoproterenol-induced HO-1 expression may exert a protective effect through CO activity. To further determine the protective effect of isoproterenol on RAW264.7 cells from GOX, which is caused by isoproterenol-induced HO-1 gene expression, we performed DNA fragmentation analysis for apoptosis. As shown in Fig. 4C, GOX at 15 mU/mL effectively induced DNA strand breakage after 6 h. Isoproterenol blocked this GOX-induced DNA laddering, however in the pretreatment of ZnPP IX, isoproterenol failed to prevent the cells from apoptosis. Propranolol, a β-AR antagonist, also inhibited the protective effect of isoproterenol (Fig. 4D). Thus, we suggest that isoproterenol induces HO-1 gene expression through β adrenoceptors, especially the β_1-adrenoceptor, and that isoproterenol-induced HO-1 gene expression protects macrophage cells from oxidative stress.

4. Discussion

The aim of this study was to examine whether the induction of HO-1 gene expression in macrophages by catecholamine is associated with self-defense against oxidant stress. The adrenergic β_1-receptor was clearly responsible for increased levels of the stress-related enzyme HO-1, which is known to protect

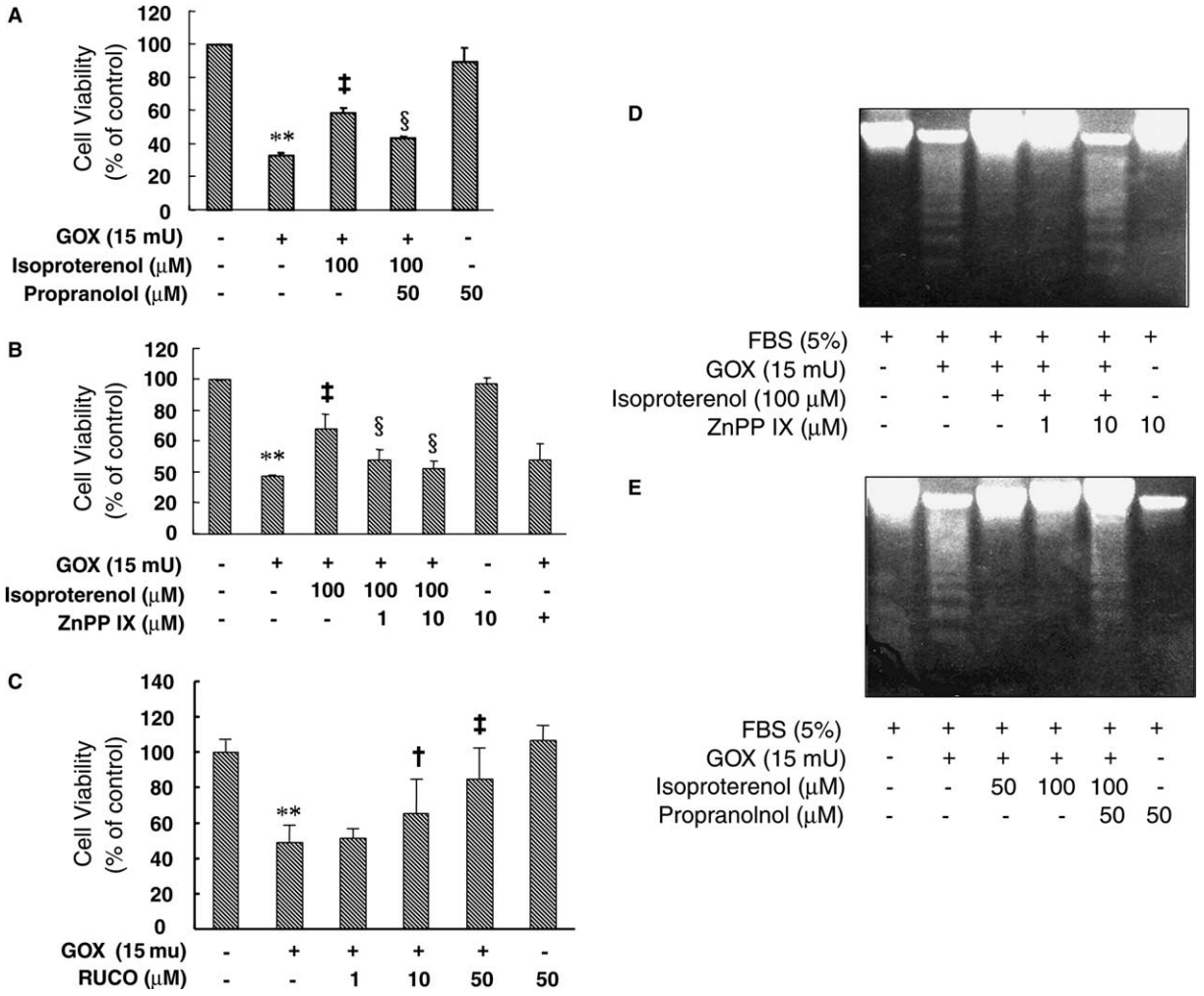

Fig. 4. Protection of RAW264.7 from oxidative stress via isoproterenol-induced HO-1 gene expression. (A) Propranolol was administered at 50 μM, 1 h before treatment with 100 μM of isoproterenol and 15 mU of GOX. Six hours later, cell viability was determined by MTT assay. (B) ZnPP IX (1, 10 μM) was administered for 1 h, and then cells were treated with isoproterenol and GOX for 6 h. (C) Cells were treated with GOX with or without RuCO for 6 h. Each bar represents the means ± S.E.M. of experiments. ($^{**}P < 0.01$ compared with control; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ compared with GOX, $^{\S}P < 0.05$ compared with isoproterenol). (D,E) Protection of RAW264.7 cells from GOX-induced apoptosis by isoproterenol. RAW264.7 cells were incubated with GOX (15 mU/ml) for 6 h with or without isoproterenol (100 μM). To determine the role of HO-1 or the involvement of β-adrenoceptors in isoproterenol-mediated cell protection, cells were pretreated with ZnPP IX (D) or propranolol (E) 1 h before GOX or isoproterenol administration, respectively. Treatment with 15 mU/mL GOX increased apoptosis 6 h after treatment. DNA laddering was visualized using ethidium bromide staining under UV light.

cells against oxidant injury. We used the nonselective β-AR agonist, isoproterenol, as a source of catecholamines, because among the natural catecholamines, norepinephrine is classified as an α-agonist, epinephrine as an α- and β-agonist, and dopamine as a weak α- and β-agonist. We expected that β_2-AR stimulation by isoproterenol might be involved in HO-1 gene expression in the activation of macrophages, because β_2-AR is known to regulate immunological responses. However, the results showed that β_1-AR rather than β_2-AR was involved in the induction of HO-1 gene expression by isoproterenol. β_1-AR is coupled via G-proteins to the intracellular second messenger system, adenylate cyclase. Thus, cAMP is the major second messenger of β-AR activation. In line with this, the adenylate cyclase activator forskolin increased HO-1 gene expression. However, HO-1 gene induction by isoproterenol, but not by forskolin, was inhibited by the nonselective β-blocker, propranolol (data not shown), indicating that β-AR activation was necessary. We speculate that increases in intracellular levels of cAMP may lead to activation of the cAMP-dependent PKA. This was supported by our finding that the PKA inhibitor H-89 inhibited the observed induction of HO-1 after both isoproterenol and forskolin treatments, suggesting that the PKA pathway plays a role in the induction of HO-1 gene expression in macrophages. However, assuming an increase of intracellular cAMP, it was reasonable to think that activation of β_2-adrenergic receptor could also induce HO-1 gene expression. Unfortunately, this idea proved to be wrong, because butoxamine, a selective β_2-AR antagonist, did not inhibit the induction of HO-1 gene expression by isoproterenol. To address this apparently puzzling question – why activation of β_2-adrenoceptor in RAW264.7 cells failed to induce HO-1 gene expression even though it presumably increased cAMP levels – we examined the mRNA expression of β-adrenoceptor subtypes. Surprisingly, we found that the subtype activated by isoproterenol in RAW264.7 cells was predominantly β_1, although both β_1- and β_2-adrenoceptors are

weakly expressed in resting cells. This finding is quite different from that for peritoneal macrophages, in which the β_2-AR subtype is mainly involved in the immune response [14].

On the other hand, HO-1 plays a fundamental role in cell protection against oxidative stress [21]. HO-1 gene induction appears to be part of a generalized cellular response that protects cells against oxidative stress and other toxic insults [22,23]. We were interested in how macrophages can protect themselves against oxidative stress, because macrophages are widely distributed in varying numbers throughout most normal tissues and organs [24] and play an important role in the regulation of the immune system [24–27]. Based on reports that the introduction of oxidative stress leads to macrophage activation or to cytotoxic effects such as apoptosis and necrosis [27–29], we hypothesized that macrophages also have antioxidant defense pathways involving catecholamines. The term "macrophage" actually encompasses different cell types of monocytic origin that acquire particular properties as a function of their environment. All macrophages express the multicomponent enzyme NADPH oxidase and generate superoxide to various extents. As macrophages are clearly involved in both propagating inflammation through the phagocytosis of apoptotic and necrotic cells, a better understanding of the possible mechanisms by which ROS or RNS may affect their response could have implications on the development of anti-inflammatory therapeutics. Furthermore, macrophages produce ROS such as O_2^-, H_2O_2 and $^{\cdot}OH$ that contribute not only to the pathogenesis of diseases such as inflammation and atherosclerosis but also damage to themselves. Cells have multiple defense systems against those ROS, and upon exposure to ROS and electrophilic agents, murine peritoneal macrophages induce stress proteins to protect themselves [30]. Induction of HO-1 gene expression and the related production of CO in macrophages play essential roles in protecting the cells against inflammation or oxidative stress [31–34]. Therefore, we focused on the role of catecholamines in macrophages, because catecholamines are released not only from sympathetic nerve terminals when the body is under stress, but macrophages themselves produce catecholamines in response to various stimulants that may affect physiological functions, especially antioxidant protection. Indeed, induction of HO-1 gene expression by isoproterenol protected the cells from glucose oxidase (GOX) treatment. GOX treatment in the presence of glucose generates H_2O_2 [35,36], which causes apoptotic cell death in many cells including the RAW264.7 cells studied here. RuCO, a CO donor, also protected the cells from GOX treatment. It remains to be determined whether CO is responsible for the isoproterenol-induced HO-1-mediated protection from GOX. Although both α_2 and β_2-adrenoceptors are involved in immune regulatory functions, we suggest that macrophages may have a novel role for self defense mechanism by catecholamines via HO-1 expression through β_1-AR. Thus, it is possible that macrophages can protect themselves from those amounts of nitric oxide they produce to kill other microorganisms, because NO is a strong inducer of HO-1 gene expression in many cells including macrophages.

In conclusion, in this study we showed an upregulation of HO-1 gene expression by the β adrenoceptor agonist, isoproterenol, via β_1-AR activation and the PKA signaling pathway, and demonstrated a protective effect against oxidative stress produced by GOX in RAW264.7 cells.

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