INFLAMMATION AND STRESS INCREASE VARIOUS PROTEINS

Three different nitric oxide (NO) synthases generate NO from L-arginine. Of these, two are constitutive isoforms, while the other is Ca\(^{2+}\)-independent iNOS, which is expressed only following transcriptional activation of its gene (Xie et al., 1992; Lorsbach et al., 1993) in acute and chronic inflammatory disease (Grisham et al., 1999). Physiological levels of NO production following iNOS up-regulation are associated with increased healing and repair in tissue injury (Yamasaki et al., 1998). However, marked production of NO has a number of detrimental effects, including neurological disorders (Koprowski et al., 1993), septic shock (Xu et al., 2002), atherosclerosis (Chen et al., 2003), and apoptotic cell death (Kim et al., 1999).

HO-1 and constitutively expressed heme oxygenases (HO-2 and HO-3) catalyze the rate-limiting step of heme oxidation to biliverdin, carbon monoxide (CO), and iron (Abramson et al., 1996). Biliverdin is rapidly converted to bilirubin, a potent endogenous antioxidant. All products of the HO reaction (biliverdin/bilirubin, CO, and iron/ferritin) participate in cellular defense (Stocker et al., 1987). HO-1 functions as a stress marker because of its rapid inducible response to various types of oxidative stress (Oguro et al., 2002).

\(\text{iNOS (Hamid et al., 1993) and HO-1 (Horváth et al., 1998) are known to be expressed in the bronchial epithelium of asthma patients. Over expression of iNOS has been demonstrated in the alveolar wall of patients with COPD (Ricciardol et al., 2005). It is suspected that these} \)}
enzymes play roles in the pathogenesis of and may therefore be useful as targets in the treatment of asthma and COPD. β₂-adrenoceptor (AR) agonists such as salbutamol are mainstay bronchodilators in the treatment of asthma and COPD. Malfait et al. (1999) demonstrated that salbutamol exhibited therapeutic effects against rheumatoid arthritis model rats with collagen-induced arthritis. In a clinical study, inhalation of a long-acting β₂-AR agonist by patients with mild asthma yielded a novel antineutrophilic effect (Jeffery et al., 2002).

Based on these findings we examined whether β₂-AR agonists inhibit proinflammatory cytokine and stress-inducible protein production by in vitro cultured abdominal macrophages, in an attempt to determine novel targets in the treatment of inflammatory diseases.

**MATERIALS AND METHODS**

**Materials**

Salbutamol (hemisulfate, minimum purity 98%) and PD98059, an extracellular stimulus-responsive kinase (ERK) 1/2 inhibitor, were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). LPS from *Escherichia coli* and ICI118551, a selective β₂-adrenoceptor antagonist, were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). The rat IL-6 Immunoassay KIT and rat TNF-α ELISA KIT were purchased from BioSource International Inc. (Camarillo, CA, USA). Primer sets, including those for iNOS oligo (RA008296), HO-1 oligo (RA008256), and β-actin oligo (RA006466), were used for complementary DNA synthesis and purchased from Takara Bio Inc. (Shiga, Japan). The primary antibodies for immunoblotting of iNOS, HO-1, ERK1/2, phosphorylated ERK1/2, and β-actin, were anti-NOS2 (sc-7271, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Heme-Oxygenase-1 (OSA-111, Stressgen Bioreagents, Victoria, BC, Canada), anti-ERK1&2 (MAPK) (SA-278, BIOMOL, Plymouth Meeting, PA, USA), anti-ERK1&2 (pTpY 185/187) (SA-275, BIOMOL), and anti-β-actin (AC-15, GenTex, San Antonio, TX, USA) as an internal standard for iNOS and HO-1 proteins, respectively.

**Animals**

Male Sprague-Dawley rats (7 weeks of age) were purchased from SLC (Shizuoka, Japan). The rats were fed a commercial diet and water *ad libitum*. After a quarantine and acclimation period of 7 days, rats (8 weeks of age, body weight 260-290 g) were treated as described below. The experiments were conducted according to the Guide to the Care and Use of Experimental Animals of the Toxicology Research Laboratory of Kissei Pharmaceutical Co., Ltd. and the regulations of the Animal Care and Use Committee of the same laboratory.

**Cell preparation**

Macrophages were collected according to the method of Rocha et al. (1997). Briefly, rats were injected intraperitoneally with sterile 3% thioglycolate, and then peritoneal exudate corpuscles were collected 4 days later by lavage with phosphate-buffered saline containing penicillin (100 IU/ml) and streptomycin (100 µg/ml). Cell suspensions were seeded in 24-well cell culture plates containing RPMI-1640 medium American Type Culture Collection (ATCC), Manassas, VA, USA supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), 10% (v/v) heat-inactivated fetal bovine serum (ATCC), and 2 mM of L-glutamine. After 2 hr incubation at 37°C in a humidified atmosphere of 5% CO₂ in air, each culture plate was washed with fresh RPMI-1640 medium to remove non-adherent cells. It was confirmed by microscopic examination following May-Giemsa staining that most of the adherent cells were macrophages. The cells were used after 48 hr incubation, with exchange of fresh RPMI-1640 medium every 24 hr.

**Experimental conditions**

To demonstrate suppression by salbutamol of LPS-induced responses, the cells were cultured for 3 or 24 hr with LPS (0.1 to 100 ng/ml) in combination with salbutamol (1 µM), ICI118551, or PD98059. Times of incubation and concentrations of LPS were determined in a preliminary study (data not shown).

**Cell viability**

For assessment of cell viability, lactate dehydrogenase (LDH) released by cells into medium after treatment was assayed using a 7180 autoanalyzer (Hitachi, Tokyo, Japan).

**Determination of proinflammatory cytokines released from macrophages**

Culture medium was collected after incubation to determine the concentrations of TNF-α and IL-6 released by cells. Enzyme-linked immunosorbent assay (ELISA) kits were used according to the protocols provided by the manufacturers.

**Quantitative real-time polymerase chain reaction**

After removal of the culture medium, the cells were recovered in lysis buffer and used for the measurement of iNOS and HO-1 mRNA by quantitative real-
time polymerase chain reaction (qPCR). Total RNA was extracted using an RNA isolation kit (QIAamp® RNA Blood Mini, QIAGEN, Valencia, CA, USA). Complementary DNA (equivalent to 10 ng of total RNA) synthesized using the ExScript™ RT reagent Kit (Takara Bio Inc.) was mixed with SYBR® Green PCR Master mix (Applied Biosystems, Foster City, CA, USA) and 100 nM primer sets. PCR was performed using the GeneAmp® 5700 Sequence Detection System (Applied Biosystems) as follows: 50°C for 2 min and 95°C for 2 min as pretreatment, 40 cycles of 95°C for 15 sec for denaturation, and 60°C for 60 sec for annealing/extension. Relative differences among samples in the various treatment groups were determined using the ΔΔCt (Ct: cycle threshold) method as outlined in the Applied Biosystems protocol. Briefly, a ΔCt value was calculated for each sample using the Ct values for β-actin as internal standard and target genes. ΔΔCt values were then calculated by subtracting the ΔCt of the control from the ΔCt of a treatment group. ΔΔCt values were converted to fold differences versus the control by raising 2 to the power ΔΔCt (2^ΔΔCt).

Western blotting

After removal of the culture medium, the cells were recovered in lysis buffer 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, protease inhibitor cocktail including (p-amidinophenyl) methanesulfonyl fluoride, aprotinin, E-64, leupeptin, vestatin, and pepstatin (Nacalai Tesque Inc., Kyoto, Japan), and phosphatase inhibitor cocktail (sodium fluoride, sodium orthovanadate, sodium pyrophosphate, and β-glycerophosphate), Pierce Biotechnology, IL, USA and frozen at -80°C until analysis. The protein content of lysate was determined using a Protein Assay Reagent Kit (Bio-Rad Laboratories, Hercules, CA, USA) and 100 nM primers. PCR was performed using the ExScript™ RT-PCR reagent Kit (Takara Bio Inc.) and 100 nM primers. PCR was performed using the GeneAmp® 5700 Sequence Detection System (Applied Biosystems) as follows: 50°C for 2 min and 95°C for 2 min as pretreatment, 40 cycles of 95°C for 15 sec for denaturation, and 60°C for 60 sec for annealing/extension. Relative differences among samples in the various treatment groups were determined using the ΔΔCt (Ct: cycle threshold) method as outlined in the Applied Biosystems protocol. Briefly, a ΔCt value was calculated for each sample using the Ct values for β-actin as internal standard and target genes. ΔΔCt values were then calculated by subtracting the ΔCt of the control from the ΔCt of a treatment group. ΔΔCt values were converted to fold differences versus the control by raising 2 to the power ΔΔCt (2^ΔΔCt).

Statistical analysis

All values are the mean ± S.E. Differences between the control or LPS-alone group and each treatment group were examined by Dunnett’s test for multiple comparisons.

RESULTS

The amount of LDH released into medium did not differ between all treatment groups (92 ± 8.6% to 122 ± 15.8%) and the control group (100 ± 2.5%).

The effects of co-culture of LPS and salbutamol for 3 or 24 hr on the release of TNF-α and IL-6 into medium are shown in Figs. 1 and 2. Rat macrophages responded strongly to the addition of LPS with release of TNF-α and IL-6 into medium. Induction by LPS of TNF-α and IL-6 was significantly reduced (P < 0.05) by salbutamol in concentration-dependent fashion. IC118551 inhibited the reduction in levels of cytokines by salbutamol. LPS released TNF-α and IL-6 into medium in concentration-dependent fashion. Induction of TNF-α was higher with incubation for 3 hr than for 24 hr with either concentration of LPS, whereas induction of IL-6 was higher with incubation for 24 hr than for 3 hr. Salbutamol (1 µM) significantly inhibited (P < 0.05) levels of both cytokines after either length of incubation.

Cells strongly expressed both iNOS mRNA and protein in concentration-dependent fashion following LPS treatment (Figs. 3A and 4B). When the cells were co-incubated with LPS and salbutamol for 24 hr, induction by LPS of iNOS mRNA and protein expression was significantly inhibited (P < 0.05). On the other hand, salbutamol did not inhibit LPS-induced HO-1 mRNA and protein expression (Figs. 3B and 4C).

Table 1 shows the effects of the ERK1/2 inhibitor PD98059 on induction by LPS of proinflammatory cytokines and iNOS mRNA in rat macrophages. PD98059 significantly suppressed (P < 0.05) LPS induction of IL-6 but not TNF-α after incubation for 3 and 24 hr. LPS induction of iNOS mRNA was significantly suppressed (P < 0.05) by incubation with the inhibitor for 24 hr. Fig. 5 shows the effects of salbutamol on LPS-induced phosphorylation of ERK1/2. The cells responded to the addition of LPS, and phosphorylation was maximal after incubation for 60 min. The LPS-induced phosphorylation was markedly inhibited by salbutamol.

DISCUSSION

LPS is the main outer membrane component of Gram-negative bacteria and induces proinflammatory cytokines such as TNF-α and IL-6 and stress-inducible proteins...
Fig. 1. Effects of various concentrations of salbutamol on induction by LPS of proinflammatory cytokines. Release of TNF-α (A) and IL-6 (B) into medium following LPS with or without salbutamol (SB) and ICI118551 (ICI) was assayed by ELISA after 3 hr incubation. Concentration of agents are indicated in the figure. Values are the mean ± S.E. of four experiments. * P < 0.05 and ** P < 0.01 versus LPS alone.

Fig. 2. Changes in LPS-induced proinflammatory cytokines in cell culture medium with or without salbutamol. Release of TNF-α (A, C) and IL-6 (B, D) into medium by LPS (0 to 100 ng/ml) stimulation with (open columns) or without (closed columns) salbutamol (1 µM) was assayed by ELISA after 3 hr (A, B) and 24 hr (C, D) of incubation. Values are the mean ± S.E. of four experiments. * P < 0.05 and ** P < 0.01 versus LPS alone.
Fig. 3. Effects of salbutamol on LPS-induced mRNA expression of iNOS and HO-1 in rat macrophages. mRNA of iNOS (A) and HO-1 (B) in cells following LPS (0 to 100 ng/ml) stimulation with (open columns) or without (closed columns) salbutamol (1 µM) was assayed by qPCR after 24 hr of incubation. Values are the mean ± S.E. of four experiments. *P < 0.05 and **P < 0.01 versus LPS alone.

Fig. 4. Effects of salbutamol on LPS-induced protein expression of iNOS and HO-1 in rat macrophages. Rat macrophages were cultured with LPS alone (0 to 100 ng/ml) or LPS plus salbutamol (1 µM) for 24 hr, followed by immunoblotting with antibodies. A representative immunoblot is shown in (A). Induction of expression of iNOS (B) and HO-1 (C) proteins in cells by LPS (0 to 100 ng/ml) with (open columns) or without (closed columns) salbutamol (1 µM) was assayed by western blotting after 24 hr of incubation. Values are the mean ± S.E. of four experiments. *P < 0.05 and **P < 0.01 versus LPS alone.

Anti-inflammatory effects of salbutamol
such as iNOS and HO-1 (Ulmer et al., 2002; Ohta et al., 2003). LPS stimulated release by rat macrophages of the proinflammatory cytokines TNF-α and IL-6 into medium and induced the expression of iNOS and HO-1 mRNA and protein in the cells. These findings indicate that the rat macrophages obtained by thioglycolate stimulation were appropriate for assessment of LPS-triggered inflammatory responses.

Decreases in proinflammatory cytokine release and of iNOS and HO-1 expression in macrophages are of great importance in the treatment of inflammatory diseases. In the present study, we demonstrated that salbutamol inhibited LPS-induced TNF-α and IL-6 release from rat macrophages, and that these inhibitory effects of salbutamol were eliminated by pretreatment with β₂-AR antagonist. The inhibitory effect of salbutamol was thus due to its β₂-AR agonistic effect. In addition, salbutamol suppressed the expression of LPS-inducible iNOS mRNA and protein. These findings are expected that β₂-AR agonists are a novel, potent suppressor of inflammatory responses and might be useful for the treatment of sepsis, endotoxemia, rheumatoid arthritis, asthma, COPD, and other related conditions. In fact, as noted above, Malfait et al. (1999) found that salbutamol exhibited therapeutic effects against rheumatoid arthritis model rats with collagen-induced arthritis, while in a clinical study inhalation of a long-acting β₂-AR agonist by patients with mild asthma yielded a novel antineutrophilic effect (Jeffery et al., 2002).

β₂-ARs couple to G stimulatory (Gs) proteins and activate adenyl cyclase (AC), resulting in elevation of cyclic adenosine 3’, 5’-monophosphate (cAMP) levels and subsequent activation of protein kinase A (PKA) (Brode et al., 1995). β₂-ARs couple to G inhibitory (Gi) proteins in myocytes, as well (Abramson et al., 1988; Xiao et al., 1995, 1999; Kilts et al., 2000; Communal et al., 1999), and Gi not only inhibits AC activity but also initiates signaling of mitogen-activated protein kinase (MAPK) by the Gβγ subunit of Gi (Daaka et al., 1997). These findings suggest that salbutamol may exert effects on inflammation through macrophage Gi. Our findings showed that PD98059, which inhibits ERK1/2 in the classical MAPK

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Agents</th>
<th>TNF-α (pg/ml)</th>
<th>IL-6 (pg/ml)</th>
<th>iNOS mRNA (Ratio to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 hr</td>
<td>LPS alone</td>
<td>1515.42 ± 104.19</td>
<td>215.24 ± 10.42</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LPS+PD98059</td>
<td>1372.61 ± 156.08</td>
<td>BLQ**</td>
<td>-</td>
</tr>
<tr>
<td>24 hr</td>
<td>LPS alone</td>
<td>699.83 ± 89.69</td>
<td>1482.59 ± 102.30</td>
<td>41.34 ± 8.20</td>
</tr>
<tr>
<td></td>
<td>LPS+PD98059</td>
<td>958.64 ± 129.02</td>
<td>20.65 ± 16.17**</td>
<td>5.84 ± 0.14**</td>
</tr>
</tbody>
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Release of TNF-α and IL-6 into medium following LPS (1 ng/ml) stimulation was assayed by ELISA after 3 and 24 hr of incubation. mRNA expression of iNOS in cells following LPS (1 ng/ml) stimulation was assayed by qPCR after 24 hr incubation. The effects of the ERK1/2 inhibitor PD98059 (10 µM) on cytokine and mRNA expression were assessed using rat macrophages co-incubated with LPS. Values are the mean ± SD of four experiments. BLQ: Values below the lower limit of quantification (8 pg/ml) were considered zero in statistical analyses. ** P < 0.01 versus LPS alone.
pathway, inhibited the LPS-induced IL-6 in rat macrophages, whereas PD98059 had no effect on the expression of TNF-α. Kim et al. (2004) have also reported that PD98059 inhibits the induction by LPS in IL-6 in macrophages, but not TNF-α. Moreover, in the present study, the ERK1/2 inhibitor decreased in LPS-induced iNOS mRNA and protein expressions in cells. Suh et al. (2006) have also shown that U0126, an inhibitor of ERK, significantly down-regulates LPS-induced iNOS expression. These findings suggest that salbutamol inhibits LPS-induced iNOS expression at least in part through the MAPK signal-transduction pathway and subsequent increase in IL-6. Thus, although it is unclear whether TNF-α is involved in iNOS expression in macrophages, salbutamol clearly inhibits the release of TNF-α from macrophages following LPS stimulation. The reactivity of iNOS observed in inflammatory cells including macrophages is significantly higher in patients with sepsis, endotoxemia (Parratt, 1998), asthma and COPD (Ichinose et al., 2000) than in healthy subjects. Diseases with inflammatory cells which express high levels of proinflammatory cytokines and iNOS might be targets of treatment with salbutamol.

We predicted that salbutamol treatment would decrease induction by LPS of HO-1 mRNA and protein in rat macrophages, since expression of HO-1 mRNA was induced by cell culture with a NO donor (Chen and Maines, 2000). However, this induction was not affected by salbutamol treatment. Ulmer et al. (2002) found that a wide array of signal-transduction pathways is activated by LPS, and that elucidation of such pathways is complicated by the fact that various types of cross-talk exist among them. It thus appeared that there were no effects of salbutamol on LPS-induced HO-1 in rat macrophages. HO-1 inducers such as heme, heavy metals, and NO donor were found to be potent inhibitors of cytokine and iNOS expression in a human intestinal epithelial cell line (Cavicchi et al., 2000) and in a rat model of endotoxin-induced uveitis (Ohta et al., 2003). The potential of HO-1 for the cell protective effects was suggested by the experimental finding that HO-1-deficient cells are more sensitive to cytotoxic injury following exposure to the strong HO-1 inducers hemin and cadmium (Pess and Tonogawa, 1997). These reports suggest that HO-1 protein and its enzymatic products ameliorate inflammatory disease via anti-inflammatory effects.

Izeboud et al. (1999) and Verhoeckx et al. (2005) have demonstrated that β2-AR agonists had the inhibition effects on the LPS-induced production of proinflammatory cytokines both in human U-937 cell line (monocyte-derived macrophages) and in rats. Liaw et al. (2003) have revealed that β2-AR agonists suppressed TNF-α and NO production via iNOS in LPS treated rats. This study also demonstrated that salbutamol inhibited the induction by LPS of these proinflammatory cytokines, and suppressed the expression of LPS-induced iNOS mRNA and protein Therefore, it was considered that β2-AR agonists had the capability to suppress the over expressions of proinflammatory cytokines and iNOS in vivo as well as in vitro. Moreover, in the present study, it was clarified that the anti-inflammatory effects of salbutamol were exerted via ERK pathway and were keeping the expression of HO-1, which had an antioxidant effect. Based on the findings, it can be reasonable expected that β2-AR agonists treatment to the patients with inflammatory disorders such as asthma and COPD produces not only smooth muscle relaxation but also yields anti-inflammatory effects in the trachea or bronchi without the involvement of HO-1 protein, which has been shown to play important roles in cellular defenses against oxidative stress, among its other effects.

REFERENCES


S. Tanaka et al.