Original Article

Salbutamol inhibits lipopolysaccharide-induced inflammatory responses in rat peritoneal macrophages

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ABSTRACT — Acute and chronic inflammatory diseases are associated with the induction of inducible nitric oxide synthase (iNOS) and inducible heme oxygenase (HO-1). These inducible enzymes are up-regulated in macrophages subjected to inflammatory stimuli and oxidative stress. β_2 -Adrenoceptor (AR) agonists, which function as bronchial dilators, are widely used for the treatment of asthma and chronic obstructive pulmonary disease (COPD). We examined whether salbutamol, a classical β_2 -AR agonist, inhibits the induction of proinflammatory cytokines and stress inducible proteins. Rat macrophages obtained from the abdominal cavity were incubated with lipopolysaccharide (LPS) with or without salbutamol. Induction by LPS of tumor necrosis factor (TNF)- α and interleukin (IL)-6 was significantly inhibited ($P < 0.05$) by salbutamol treatment. Induction by LPS of iNOS mRNA and protein was also significantly inhibited ($P < 0.05$) by salbutamol. LPS-mediated increases in HO-1 mRNA and protein were not appreciably affected by salbutamol. One of the anti-inflammatory mechanisms of salbutamol was thus found to be inhibition of induction by LPS of extracellular stimulus-responsive kinase (ERK) 1/2 in macrophages. These findings suggest that salbutamol has the potential for use as an anti-inflammatory agent due to its suppression of LPS-induced TNF- α , and IL-6 and iNOS via ERK pathway without affecting HO-1 expression.

Key words: Salbutamol, TNF- α , IL-6, iNOS, LPS

INTRODUCTION

Inflammation and stress increase various proteins that act as defense-related substances, such as inducible nitric oxide synthase (iNOS) and inducible heme oxygenase (HO-1). 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; Lorschbach *et al.*, 1993) in acute and chronic inflammation (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), atheroscle-

rosis (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).

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

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enzymes play roles in the pathogenesis of and may therefore be useful as targets in the treatment of asthma and COPD. β_2 -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 β_2 -AR agonist by patients with mild asthma yielded a novel antineutrophilic effect (Jeffery *et al.*, 2002).

Based on these findings we examined whether β_2 -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 β_2 -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, GeneTex, 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 Tox-

icology 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 μ g/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 μ g/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 μ M), 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 $\Delta\Delta C_t$ (C_t : cycle threshold) method as outlined in the Applied Biosystems protocol. Briefly, a ΔC_t value was calculated for each sample using the C_t values for β -actin as internal standard and target genes. $\Delta\Delta C_t$ values were then calculated by subtracting the ΔC_t of the control from the ΔC_t of a treatment group. $\Delta\Delta C_t$ values were converted to fold differences versus the control by raising 2 to the power $\Delta\Delta C_t$ ($2^{-\Delta\Delta C_t}$).

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). Cell lysate containing 20 μ g of protein was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories) and immunoblotted with the primary antibodies, and then with biotin-conjugated IgG antibody (Pierce Biotechnology) as a secondary antibody and avidin-alkaline phosphatase (Bio-Rad Laboratories). Bound antibodies were detected by adding a 1-Step™ NBT/BCIP (Pierce Biotechnology) to blots. Bands were analyzed using Image J free software (NIH, <http://rsb.info.nih.gov/ij/>).

Statistical analysis

All values are the mean $\pm$ S.E. Differences between the control or LPS-alone group and each treatment group were examined by Dunnett's test for multiple compari-

son. Differences between the LPS-alone and co-culture of LPS and SB or PD98059 were examined by Student's and Welch's t-tests. For each test, differences were considered significant at $P < 0.05$.

RESULTS

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

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. ICI118551 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 μ M) 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 $\pm$ 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 μ M) was assayed by ELISA after 3 hr (A, B) and 24 hr (C, D) of incubation. Values are the mean $\pm$ S.E. of four experiments. * $P < 0.05$ and ** $P < 0.01$ versus LPS alone.

Anti-inflammatory effects of salbutamol

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 μ M) was assayed by qPCR after 24 hr of incubation. Values are the mean $\pm$ 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 μ M) 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 μ M) was assayed by western blotting after 24 hr of incubation. Values are the mean $\pm$ S.E. of four experiments. * $P < 0.05$ and ** $P < 0.01$ versus LPS alone.

Table 1. Effects of ERK1/2 inhibitor on induction by LPS of proinflammatory cytokines and iNOS mRNA in rat macrophages

Incubation time	Agents	TNF- α (pg/ml)	IL-6 (pg/ml)	iNOS mRNA (Ratio to control)
3 hr	LPS alone	1515.42 $\pm$ 104.19	215.24 $\pm$ 10.42	-
	LPS+PD98059	1372.61 $\pm$ 156.08	BLQ**	-
24 hr	LPS alone	699.83 $\pm$ 89.69	1482.59 $\pm$ 102.30	41.34 $\pm$ 8.20
	LPS+PD98059	958.64 $\pm$ 129.02	20.65 $\pm$ 16.17**	5.84 $\pm$ 0.14**

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 μ M) on cytokine and mRNA expression were assessed using rat macrophages co-incubated with LPS. Values are the mean $\pm$ 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.

Fig. 5. Effects of salbutamol on LPS-activated ERK1/2 in rat macrophages. Rat macrophages were cultured with LPS alone (1 ng/ml) or LPS plus salbutamol (1 μ M) for 2 hr, followed by immunoblotting with antibodies. P-ERK1/2 and ERK1/2 show bands of phosphorylated and non-phosphorylated ERK1/2 protein, respectively.

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 β_2 -AR antagonist. The inhibitory effect of salbutamol was thus due to its β_2 -AR agonistic effect. In addition, salbutamol suppressed the expression of LPS-inducible iNOS mRNA and protein. These findings are expected that β_2 -AR agonists are a novel, potent suppressor of inflammatory responses and might be useful for the treatment of sepsis, endo-

toxemia, 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 β_2 -AR agonist by patients with mild asthma yielded a novel antineutrophilic effect (Jeffery *et al.*, 2002).

β_2 -ARs couple to G stimulatory (Gs) proteins and activate adenylyl cyclase (AC), resulting in elevation of cyclic adenosine 3', 5'-monophosphate (cAMP) levels and subsequent activation of protein kinase A (PKA) (Brodde *et al.*, 1995). β_2 -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 G $\beta\gamma$ 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

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 (Poss and Tonegawa, 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 β_2 -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 β_2 -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 β_2 -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 β_2 -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.

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