TISSUE- AND DOSE-DEPENDENT ALTERATION OF STRESS-INDUCIBLE PROTEINS BY $\beta_2$-ADRENOCEPTOR AGONIST, SALBUTAMOL, IN RATS

Satoru TANAKA, Ryoichi YAMAGISHI, Masaru TSUTSUI, Tomoyuki KISHIDA, Makoto MURAKAMI, Junji KURODA and Takemi YOSHIDA

Division of Toxicological Research, Kissei Pharmaceutical Co. Ltd., 2320-1 Maki, Hotaka, Azumino, Nagano 399-8305, Japan

Department of Biochemical Toxicology, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa, Tokyo 142-8555, Japan

(Received July 6, 2005; Accepted August 30, 2005)

ABSTRACT — The effects of selective $\beta_2$-adrenoceptor agonists on proinflammatory cytokines and the expression of stress-inducible proteins have not yet been clarified. We investigated the effect of a higher dose (60 mg/kg intravenously) of salbutamol, a selective $\beta_2$-adrenoceptor agonist, on the induction of interleukin (IL)-1$\beta$, IL-6 and tumor necrosis factor (TNF)-$\alpha$ in plasma and the expression of protein and mRNA of metallothioein-1 (MT-1), heme oxygenase-1 (HO-1) and inducible nitric oxide synthase (iNOS) in heart, lung, liver and spleen in rats. The plasma IL-6 concentration was significantly increased after administration with a maximum increase at 3 hr in a dose-dependent manner, but IL-1$\beta$ and TNF-$\alpha$ concentrations were not changed. MT-1 mRNA increased in heart, lung and liver, but not in spleen, and MT-1 protein increased in endocardium, fibroblasts of lung and periporal regions in liver. HO-1 mRNA was not changed in lung, decreased at 3 hr in liver and spleen, and increased at 6 hr in liver. Contrary to liver, HO-1 mRNA in the heart increased at 3 hr and decreased at 6 hr. HO-1 protein increased in cardiomyocytes and centrilobular regions in the liver. iNOS mRNA increased in lung, liver and spleen, but decreased in the heart, and iNOS protein increased in alveolar type II cells and hepatocytes, and decreased in necrotic cardiomyocytes. In contrast, a lower dose (6 mg/kg intravenously) of salbutamol suppressed lipopolysaccharide-induced HO-1 and iNOS mRNA. We conclude that salbutamol tissue- and dose-dependently alters the expression of stress-inducible proteins.

KEY WORDS: Salbutamol, MT-1, HO-1, iNOS, IL-6, $\beta$-adrenoceptor

INTRODUCTION

$\beta_2$-Adrenoceptor is involved in the relaxation of bronchial smooth muscle, the gastrointestinal tract, the blood vessels and the uterus. Therefore the selective $\beta_2$-adrenoceptor agonists are widely used for the treatment of asthma and premature birth. Salbutamol was developed as a first $\beta_2$-adrenoceptor selective agonist (Waldeck, 2002). The major toxic effects of salbutamol when rats are treated with high dose levels have been reported to be myocardial necrosis and fibrosis (Magnusson and Hansson, 1973; Libretto, 1994; Tanaka et al., 2004). High doses of salbutamol cause various biological alterations related to tissues expressing $\beta_1$- and $\beta_2$-adrenoceptors. However, the relationship between the inducible proteins and $\beta_2$-adrenoceptor stimulation is not fully understood.

Metallothioein-1 (MT-1), heme oxygenase-1 (HO-1) and inducible nitric oxide synthase (iNOS), known as stress-inducible proteins, are induced by various agents in vivo in order to protect the organs from endogenous stress. MT-1 forms chelate complexes with metals in many organs and is induced by metal ions, certain hormones, cytokines, growth factors, tumor promoters and various oxidants (Naganuma, 1997; Nath et al., 2000). HO-1 localizes in the endoplasmic...
reticulum and is induced by metal ions, cytokines, various drugs and chemicals, heat shock and hormones. HO-1 breaks down heme into biliverdin, carbon monoxide and Fe^{2+}, and these forms are rapidly reduced into bilirubin (Yoshida, 1997; Agarwal and Nick, 2000). iNOS is induced by inflammatory cytokines and lipopolysaccharide (LPS) and produces nitric monoxide (Guzik et al., 2003). These stress-inducible proteins are also induced by proinflammatory cytokines such as interleukin (IL)-1β, IL-6 or tumor necrosis factor (TNF)-α. Recent reports have reviewed the gene regulation of MT-1 (Sato and Kondoh, 2002), HO-1 (Shibahara, 2003) and iNOS (Morris et al., 2003), and the relationship between the protein expression and various types of biological damage (Kondoh and Sato, 2002; Sikorski et al., 2004; Tuteja et al., 2004; Giordano, 2005).

In the present study, we investigated the effect of salbutamol on plasma proinflammatory cytokine production and higher doses of salbutamol on tissue-specific induction of these three stress-inducible proteins in heart, lung, liver and spleen in rats. We also demonstrated that the lower dose of salbutamol suppressed the LPS-induced expression of HO-1 and iNOS mRNA as an anti-inflammatory effect of salbutamol.

**MATERIALS AND METHODS**

**Materials**

Salbutamol was purchased from Wako Pure Chemical Industries, Ltd. (hemi-sulfate; minimum 98.0%, Osaka, Japan). LPS and IC111851, selective β2-adrenoceptor antagonists, were purchased from SIGMA-ALDRICH Inc. (MO, USA). Rat IL-1β ELISA Kit, Rat IL-6 Immunoassay Kit and Rat TNF-α ELISA KIT were purchased from Pierce Biotechnology, Inc. (IL, USA), BioSource International (CA, USA) and Diaclone Research (Besançon, France), respectively. The primers were synthesized by QIAGEN (CA, USA) (Table 1). Monoclonal mouse MT-1, monoclonal mouse HO-1 and polyclonal rabbit iNOS were purchased from DAKO JAPAN Co., Ltd. (Kyoto, Japan), Stressgen (BC, Canada) and Santa Cruz Biotechnology, Inc. (CA, USA) and each antibody was diluted 1:50, 1:1000 and 1:200 in 1% BSA solution, respectively. All other reagents were of the highest available grade.

**Animals and treatments**

All animal experiments were carried out in accordance with the Guide to the Care and Use of Experimental Animals of the Toxicological Research Lab. of Kissei Pharmaceutical Co. Ltd. and were approved by the Animal Care and Use Committee of the Toxicological Research Lab. of Kissei Pharmaceutical Co. Ltd. Male Sprague-Dawley rats 6 weeks old were purchased from SLC (Hamamatsu, Japan). The rats were given a commercial diet and water ad libitum. After 7 days of a quarantine and acclimation period, rats (7 weeks-old and 230 – 260 g in body weight) were treated with the drugs as described in the following sections. Drugs for intravenous and subcutaneous administration were dissolved in saline. Control rats were treated with saline.

**Determination of plasma proinflammatory cytokine**

Rats were treated intravenously with salbutamol (60 mg/kg) and their blood collected at 0 (pre-dosing), 1, 3, 6 and 24 hr after dosing, using heparin as anticoagulant, for determination of IL-1β, IL-6 and TNF-α. Blood of rats treated intravenously with salbutamol was collected at 3 hr after dosing for measurement of IL-6. Plasma was obtained by centrifugation of the blood samples and stored at −80°C until use. The

**Table 1.** MT-1, HO-1, iNOS and GAPDH primer sequences.

<table>
<thead>
<tr>
<th></th>
<th>MT-1</th>
<th>HO-1</th>
<th>iNOS</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5'-gaatgcaatgatacctgac-3'</td>
<td>5'-ggacagattgcgctc-3'</td>
<td>5'-gagactaagcagctgtg-3'</td>
<td>5'-gctactcctggttttgcag-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-gaatgcaatgatacctgac-3'</td>
<td>5'-ggacagattgcgctc-3'</td>
<td>5'-gagactaagcagctgtg-3'</td>
<td>5'-gctactcctggttttgcag-3'</td>
</tr>
<tr>
<td></td>
<td>189 bp</td>
<td>615 bp</td>
<td>217 bp</td>
<td>306 bp</td>
</tr>
</tbody>
</table>

MT-1, methallothionein-1; HO-1, inducible heme oxygenase; iNOS, inducible nitric oxide synthase; GAPDH, glyceraldehydes 3-phosphate dehydrogenase.

Vol. 30 No. 4
Alteration of stress-inducible proteins by salbutamol.

plasma concentrations of IL-1β, IL-6 and TNF-α were assayed by the respective ELISA kits.

Treatment of animals for determination of stress-inducible proteins and microscopic observation

Rats treated intravenously with salbutamol at 60 mg/kg were exsanguinated under anesthesia with diethyl ether. Heart, lung, liver and spleen were isolated at 0 (pre-dosing), 3, 6 and 24 hr after dosing for mRNA assay and at 24 hr for microscopic observation and immunohistochemistry.

LPS treatment

To examine the β2-adrenoceptor-mediated suppression of LPS-inducible mRNA rats were subcutaneously administered 2 mg/kg LPS at 15 min after the intravenous injection of 6 mg/kg salbutamol and the tissues isolated at the times described above for the mRNA assay. To inhibit the action of the β2-adrenoceptor, IC118551 at a dose of 1 mg/kg was administered intravenously 30 min before dosing LPS.

Reverse transcription-polymerase chain reaction (RT-PCR)

The apex of the heart, right lung lobe, left liver lobe and a spleen central part were separated from the tissues, immediately frozen in liquid nitrogen, and stored at −80°C until use for extraction of total RNA. Total RNA was extracted from homogenized tissue using an RNA isolation kit (QIAamp® RNA Blood Mini, QIAGEN). Reverse transcription and PCR (RT-PCR) were performed using an RT-PCR Kit (QIAGEN® OneStep RT-PCR Kit, QIAGEN) according to the standard protocol of the kit. Briefly, RT-PCR was done in QIAGEN® OneStep RT-PCR buffer reacted with both sense and antisense primers, omniscript and sensiscript reverse transcriptases, HotStarTaq DNA polymerase, and 100 ng of template RNA from each tissue, using a thermal cycler (Bio-Rad Laboratories, Inc., CA). After reverse transcription at 50°C for 30 min, HotStarTaq DNA polymerase was activated, omniscript and sensiscript reverse transcriptases were inactivated, and the cDNA template was denatured at 95°C for 15 min simultaneously. PCR was performed at 26 cycles for MT-1, HO-1, iNOS and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) with each cycle consisting of denaturation at 94°C, annealing at 55°C, and extension at 72°C for 1 min. GAPDH of the housekeeping protein was used as an internal standard for evaluation of the changes in mRNA. PCR products were separated by electrophoresis on 4 – 20% polyacrylamide gradient gel (Daiichi Chemical, Tokyo, Japan) with a Tris-glycine buffer (pH 8.4). Bands were dyed with SYBR® Green I (Molecular Probes, Inc., OR) in Tris-EDTA buffer (pH 8.0) and visualized by ultraviolet-induced fluorescence. Each band taken from a photograph was analyzed using an image J free software (NIH, http://rsb.info.nih.gov/ij/).

Microscopic observation and immunohistochemistry

Parts of the tissues used for the RT-PCR were fixed with 4% paraformaldehyde. Three μm thin slices of the histopathological specimens were stained with hematoxylin and eosiin (HE) and observed microscopically. Continuous slices of 5 μm for immunohistochemistry were placed in a microwave for antigen retrieval of MT-1 and HO-1 and irradiated for 5 min in an antigen retrieval technique using a Buffer for Anti-gen Retrieval (DAKO JAPAN Co., Ltd.). To inhibit the endogenous peroxidase activity of the tissue, the slices were treated with 3% H2O2 solution for 10 min. Thereafter, the sections were rinsed thoroughly with TBS and then pre-incubated in Non-specific Staining Blocking Reagent (Protein Block Serum-Free, DAKO JAPAN Co., Ltd.) for 10 min. After the blocking step, the sections were incubated with the primary antibody for 24 hr at 4°C in a humidified chamber. At each of the following steps, the sections were rinsed three times with TBS buffer. An ENVISION kit (DAKO JAPAN Co., Ltd.) was used to stain the sections, followed by 3,3-diaminobenzidine (DAB) staining. Sections for the negative control were treated with the same protocol as described above in the absence of primary antibody.

Statistical analysis

All data are presented as the mean ± SE. Statistical comparisons were made by using the Student’s and Welch’s t test (Yoshimura, 1987). For each test differences were considered statistically significant at p<0.05.

RESULTS

Plasma proinflammatory cytokine concentrations after intravenous administration of salbutamol

Plasma concentrations of the proinflammatory cytokines, IL-1β, IL-6 and TNF-α, were measured after a single intravenous administration of salbutamol at the dose of 60 mg/kg. As shown in Fig. 1, IL-6 was significantly increased with a maximum level seen at 3 hr after dosing with salbutamol, but IL-1β and TNF-α were not changed appreciably. The plasma IL-6 level
was increased at 3 hr in a dose-dependent manner with salbutamol doses of 0, 6, 20 and 60 mg/kg (Fig. 2).

**Expression of mRNAs of MT-1, HO-1 and iNOS after salbutamol treatment**

Fig. 3 shows the time course of the changes in MT-1, HO-1 and iNOS mRNA expression, as determined by RT-PCR, in the apex of the heart, the right caudal lobe of the right lung, the posterior segment of the right lobe of the liver and a central part of the spleen after the intravenous administration of salbutamol at the dose of 60 mg/kg. MT-1 mRNA was increased in the heart, lung and liver; however, it was constant in the spleen. HO-1 mRNA was not changed in the lung and decreased in the spleen, and was decreased at 3 hr and increased at 6 hr after the administration in liver, and contrary to liver, HO-1 mRNA in heart was increased at 3 and decreased at 6 hr after the administration. iNOS mRNA was increased in the lung, liver and spleen; however, it was reduced in the heart and then returned to the pre-dose level at 24 hr after the drug treatment.

**Histological pathology and immunohistochemistry**

Photo 1 shows the representative results of HE staining and the changes in the inducible proteins, MT-1, HO-1 and iNOS, stained by immunohistochemistry using continuous sections of the heart (A), lung (B) and liver (C) at 24 hr after the intravenous administration with a higher dose of salbutamol. The specimens were the same parts of the tissues used for the mRNA measurements. The experimental results obtained from each tissue were as follows.

1. **Heart**

Necrosis was observed in the submyocardial cells, and infiltration of neutrophils was also observed. MT-1 was induced in the endocardium. HO-1 was uniformly induced in the cardiomyocytes. iNOS expression was reduced in the region of the necrotic cells by drug treatment.

2. **Lung**

Hemorrhage with neutrophils and macrophage chemotaxis was observed. MT-1 was induced in some fibroblasts. The macrophages expressing HO-1 and iNOS were increased in treated animals in comparison with control animals. The higher dose of salbutamol...
Alteration of stress-inducible proteins by salbutamol.

Fig. 3. Effect of overdosage of salbutamol on mRNA of stress-induced protein. MT-1, HO-1 and iNOS mRNAs in heart, lung, liver and spleen were measured by RT-PCR after overdosage of salbutamol at the indicated time (The method is shown in the Materials and Methods section). The increases in mRNA are indicated as level of increase over control value. The mRNA expression-time profiles are shown in heart, lung, liver and spleen. Each column represents the ratio of the treated group to control at each time (0, 3, 6 and 24 hr after dosing) with the mean ± SE for four or five animals. Statistical significance: *p<0.05 and **p<0.01 versus pre-dosing groups (0 hr).
induced the iNOS expression in alveolar type II cells.

3. Liver
No pathological findings by the higher dose of salbutamol were observed. HO-1 was induced in the centrilobular regions and MT-1 induced in the periporal regions that did not express HO-1. iNOS was uniformly expressed in hepatocytes in treated animals as well as control animals.

4. Spleen
No pathological findings or changes in protein expression were observed following treatment with salbutamol (data not shown).

Suppressive effect of salbutamol on LPS-induced mRNA
LPS-treated rats were used to examine the mechanism of the reduction of iNOS mRNA in the heart and HO-1 mRNA in the liver and spleen after treatment with salbutamol. Fig. 4 shows the effect of salbutamol on LPS-induced iNOS mRNA expression and that of ICI1118551, selective β2-adrenoceptor antagonist. In the heart, although LPS did not significantly enhance iNOS mRNA, similar effects with salbutamol and ICI1118551 were observed. These results indicate that the dose of salbutamol used effectively inhibits LPS-induced inflammation. In the lung, liver and spleen, iNOS mRNA levels were significantly increased by LPS treatment and this increase also suppressed by salbutamol. The suppressive effects of salbutamol were cancelled by pretreatment with ICI1118551 before injecting salbutamol. Likewise, expression of HO-1 mRNA was seen in the LPS-injected rats’ liver and spleen (Fig. 5).

DISCUSSION
In the present study, we chose to give a high dose of salbutamol to rats to evaluate β2-adrenoceptor agonist activity on the plasma levels of inflammatory cytokines and levels of stress-inducible proteins in various tissues. The dose of salbutamol of 60 mg/kg was chosen as a toxic dose, because this dose produces inflammation and myocardial fibrosis in rats at 7 days after intravenous administration (Tanaka et al., 2004).

The administration of salbutamol caused a significant increase in plasma IL-6 levels, but not IL-1β or TNF-α, in a dose-dependent manner, indicating that this increase in IL-6 is mediated by β-adrenoceptor stimulation. IL-6 production is also stimulated by high doses of terbutaline, a selective β2-adrenoceptor agonist (Nakamura et al., 1999). These results suggest that toxic doses of β2-adrenoceptor agonists mainly induce

![Graph](image-url)
IL-6 rather than IL-1β and TNF-α. IL-6 is known to induce tissue MT-1 (Nath et al., 2000), HO-1 (Yoshida, 1997) and iNOS (Kanai, 1997). Therefore, we further investigated the effect of toxic doses of salbutamol on the expression of stress-inducible proteins in the major tissues of rats under the same experimental conditions.

Salbutamol produced histological changes in the heart at 24 hr after its administration, leading to myocardial fibrosis 7-days later (Tanaka et al., 2004). Unlike other tissues, treatment with salbutamol resulted in a significant decrease in iNOS mRNA in the heart. Salbutamol caused inflammation with macrophages which expressed HO-1 and iNOS but not MT-1 in the lung. This could be one of the mechanisms by which β2-adrenoceptor agonists induce HO-1 and iNOS proteins in the lung.

Salbutamol significantly induced MT-1 protein expression in the liver compared to the other organs.

Therefore, MT-1 mRNA significantly increased at 3 hr and tended to increase 6 and 24 hr in liver by a higher dose of salbutamol. IL-6 is the main cytokine that enhances MTs (Sato et al., 1994), suggesting that the observed increase in MT-1 with the salbutamol treatment is via the increase in this cytokine. Salbutamol also enhanced HO-1 protein expression in the liver but not iNOS. Evidence has been accumulating that induction of HO-1 as well as MT-1 is a compensatory phenomenon for protecting tissue damage from endogenous and exogenous insults. Therefore, it is reasonable to point out that centrilobular induction of HO-1 after salbutamol treatment will be related to cytochrome P450 regulation which is highly expressed in the same region. It consumes most of the newly synthesizing heme and also produces reactive oxygen species through its enzymatic reaction. Treatment of rats with salbutamol did not change the expression of the stress-inducible proteins including HO-1 in the spleen, despite the increased mRNAs. This could be partly due to the highly constant induction of HO-1 protein in the spleen leading to the increased heme breakdown, which is one of the major physiological roles occurring in this tissue. No pathological findings were observed in the spleen even after toxic doses of salbutamol to other tissues. These findings suggest that sensitivity to β2-adrenoceptor agonists in the spleen is lower than in the organs, in addition to the presence of a high concentration of antioxidant bilirubin, a product of HO-1 mediated heme breakdown, in this tissue.

We also investigated the suppressive effect of salbutamol on LPS-induced expression of mRNAs of iNOS and HO-1, because toxic doses of salbutamol reduced mRNA of iNOS in the heart and HO-1 in the liver and spleen. LPS enhanced iNOS mRNA expression in the lung, liver and spleen in a dose-dependent manner. This increase was suppressed by pretreatment with a lower dose (6 mg/kg) of salbutamol. ICI118551, a β2-adrenoceptor antagonist that restored the inhibitory effect of salbutamol on LPS-mediated mRNA expression, suggesting that the suppressive effect of salbutamol is related to β2-adrenoceptor stimulation. The heart has a specific function for regulation of iNOS. Essentially identical results were obtained for HO-1 in both liver and spleen.

It has been reported that the clinically effective doses of β2-adrenoceptor agonists suppress any adverse effects through the β2-adrenoceptors (Kanail, 1997; Jayachandran et al., 2001; Looms et al., 2002). Nakamura et al. (1999) have shown that IL-6 production in LPS-stimulated renal macrophage cells was
suppressed by low doses of \( \beta_2 \)-adrenoceptor agonists but increased by high doses. They have also shown that two pathways of signal transduction exist for IL-6 production: induction through protein kinase A and reduction through mitogen-activated protein kinase (MAPK). In particular, MAPK plays an important role in the signal transduction in response to cytokine stimulation for the expression of iNOS (Guzik et al., 2003) and HO-1 (Numazawa and Yoshida, 2004). Recent reports have shown that these opposing regulation of \( \beta_2 \)-adrenoceptor agonists are attributed to the activation of different G proteins, \( \mathrm{G}_q \) and \( \mathrm{G}_\beta \gamma \) (Chesley et al., 2000; Foerster et al., 2003). We found that the low dose of salbutamol effectively inhibited LPS-induced HO-1 and iNOS. The results suggest that \( \beta_2 \)-adrenoceptor agonists produce tissue-dependent effects on LPS-mediated biological actions.

In summary, salbutamol has been shown to increase plasma IL-6 levels but not IL-1\( \beta \) and TNF-\( \alpha \) levels. The expression of MT-1, HO-1 and iNOS were altered tissue-dependently by a higher dose of salbutamol. The suppressive effect of a lower dose of salbutamol in the LPS-treated inflammatory model was produced through \( \beta_2 \)-adrenoceptors by changes in iNOS and HO-1. The present study has demonstrated that salbutamol has biphasic actions with a tissue-protective effect and inflammatory effects.

**ACKNOWLEDGMENT**

We thank Ms. R. Nakano and Mr. T. Takahashi for pathological and animal treatment in the Division of Toxicological Research, Kissei Pharmaceutical Co. Ltd., Nagano 399-8305, Japan.

**REFERENCES**


