Inhibitory effects of formoterol on lipopolysaccharide-induced premature delivery through modulation of proinflammatory cytokine production in mice

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Previous studies have demonstrated that formoterol, a β_2 -adrenoceptor agonist, has potent tocolytic effects in rats. The aim of the present study was to determine whether formoterol treatment affects proinflammatory cytokine production in a lipopolysaccharide (LPS)-treated premature delivery mouse model. Formoterol was continuously administered by osmotic pump and the number of fetuses in the uteri were counted. Samples of amniotic fluid and plasma were collected 8 and 16 h after

systemic administration of LPS. LPS induced premature delivery and an increase in prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), interleukin 1α (IL- 1α), IL-6 and IL-10 in the amniotic fluid, and an increase in IL-6 in plasma. Formoterol blocked all changes except the increase in IL-10. These data indicate that formoterol exerts inhibitory effects on proinflammatory cytokine production, and these effects may play an important role in the prevention of premature delivery.

Introduction

Formoterol fumarate (formoterol) is a highly potent long-lasting β_2 -adrenoceptor agonist used for asthma therapy. Other drugs, such as ritodrine, are used extensively for the prevention of premature delivery in the clinical field. Widespread use of β -adrenoceptor agonists is not associated with any significant improvement in neonatal outcome (Johnson, 1993). However, Shinkai and Takayama (2000) demonstrated that formoterol suppresses rat uterine activity at 100–1000-fold lower doses than ritodrine.

Premature delivery is associated with increased concentration of proinflammatory cytokines in the amniotic fluid (Gomez *et al.*, 1997). Romero *et al.* (1992, 1993) proposed that the proinflammatory cytokines interleukin 1 (IL-1) and IL-6 are predictors of microbial colonization of the amniotic cavity and premature delivery in the setting of infection. An increase in IL-1 and IL-6 stimulates prostaglandin production, causing uterine contraction (Mitchell *et al.*, 1990, 1991). IL-6 and IL-8 are known to increase during human cervical ripening, and presumably play an important role in uterine contraction (Sennstrom *et al.*, 2000). IL-6 causes an increase in the activity of the gelatinases or type IV collagenases matrix metalloproteinase 2 (MMP-2) and MMP-9 in human cytotrophoblastic cells (Meisser *et al.*, 1999).

IL-10 generally inhibits the transcription of proinflammatory cytokines such as IL-1, tumour necrosis factor α (TNF- α), IL-6 and IL-8 (Moore *et al.*, 1993; Wang *et al.*, 1995). IL-10 is produced in monocytes and macrophages as well as in gestational tissues, including amnion, chorion and decidual cells (Trautman *et al.*, 1997). IL-10 concentrations in amniotic fluid of women who experience preterm labour are lower than those seen in that of women who undergo normal term delivery in the absence of infection. However, an increase in IL-10 has been reported for preterm labour associated with intrauterine infection, indicating an immunoregulatory role for IL-10 (Greig *et al.*, 1995).

Studies have also indicated that β -adrenergic agonists are involved in immunoregulatory processes. For example, adrenaline induces IL-6 in endothelial cells and enhances lipopolysaccharide (LPS)-induced IL-6 production (Gornikiewicz *et al.*, 2000). In contrast, the β_2 -adrenoceptor agonist, clenbuterol, suppresses the LPS-induced release of IL-6 and TNF- α from macrophages (Izeboud *et al.*, 1999), and isoproterenol and formoterol decrease IL-6 and TNF- α gene transcription in rat renal macrophage cells (Nakamura *et al.*, 1999). Furthermore, IL-10 secretion induced by LPS is enhanced by the β_2 -adrenoceptor agonist TA2005 in macrophages (Izeboud *et al.*, 2000), whereas terbutaline does not affect LPS-induced IL-10 production in rat mesangial cells (Nakamura *et al.*, 2000). Thus, the effects of β -adrenoceptor agonists on cytokine production may depend on the experimental conditions, such as the cell

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Table 1. The influence of formoterol administration on the number of fetuses in uteri 8 h after lipopolysaccharide (LPS) treatment in mice

Treatment	Number of fetuses in uteri	
	Initial	8 h after LPS treatment
Control	7.0 ± 0.5	7.0 ± 0.5 (6.8)
LPS (30 µg per mouse)	8.8 ± 0.8	8.8 ± 0.8 (4.4)
+ formoterol (0.006 mg kg ⁻¹ h ⁻¹)	7.4 ± 0.2	7.4 ± 0.2 (5.8)
+ formoterol (0.06 mg kg ⁻¹ h ⁻¹)	6.8 ± 0.8	6.8 ± 0.8 (4.2)

LPS was administered intraperitoneally 4 h after implanting osmotic pumps containing formoterol solution. Values represent the mean ± SEM ($n = 5$ or 6 mice). The numbers of live fetuses in uteri are shown in parentheses.

strain used or the $\beta 2$ -adrenoceptor ligand. In particular, it remains unclear whether $\beta 2$ -adrenoceptor agonists influence cytokine secretion by amnion tissues. If $\beta 2$ -adrenoceptor agonists such as formoterol cause not only relaxation of the uterus but also the secretion of cytokines, they could be used for women at high risk of premature delivery.

Therefore, the present study investigated the effects of formoterol on LPS-induced IL-6, IL-1 α , IL-10 and prostaglandin F_{2 α} (PGF_{2 α}) production *in vivo*.

Materials and Methods

Animals

C3H/HeN strain pregnant mice (Charles River, Yokohama) at day 15 of gestation were used. They were housed at 23 ± 2°C, relative humidity of 55 ± 15% and with a 12 h light: 12 h dark cycle (light on at 8:00 h), and allowed access to mouse or rat diet (F-2, pelleted form; Funabashi Farm, Funabashi) and tap water *ad libitum* throughout the experiment. All experimental procedures were carried out in accordance with the in-house guidelines of the Institutional Animal Care and Use Committee of Saitama Daiichi Pharmaceutical Co Ltd.

Drugs

Formoterol fumarate was obtained from Yamanouchi Pharmaceutical Co. Ltd (Tokyo). LPS was purchased from Sigma Chemical Co. (St Louis, MO). Both drugs were dissolved in 0.9% (w/v) physiological saline (Otsuka Pharmaceutical Co. Ltd, Tokyo).

Procedure

Osmotic pumps (Model 2001D, Alza Corporation, Mountain View, CA) were filled with 200 µl formoterol (Yamanouchi) or saline and implanted subcutaneously into the backs of mice on day 15 of gestation, under ether anaesthesia. The osmotic pumps injected drug solution with a pumping rate of 7.9 µl h⁻¹ for 24 h. A

dose of LPS (30 µg ml⁻¹) that gave consistent premature delivery without maternal death was administered intraperitoneally 4 h after implantation and mice were killed 8 or 16 h thereafter. Saline solution was used as a control and comparisons were made of the numbers of total fetuses and live fetuses in uteri. Samples of amniotic fluid and plasma were collected by injection syringe (1 ml) and pooled from each individual mouse, and stored at -80°C until assayed for PGF_{2 α} (Cayman Chemical, Ann Arbor, MI) and IL-1 α , IL-6 and IL-10 (Amersham Pharmacia Biotech, Little Chalfont) using ELISA kits.

Statistical analysis

The results, expressed as mean ± SEM, were analysed by Kruskal–Wallis non-parametric one-way ANOVA. Further statistical analysis for *post hoc* comparisons was accomplished with a Steel's non-parametric multiple comparison test. Student's *t* test was used for pairs of sample means. The criterion for significance was $P < 0.05$ in all statistical evaluations.

Results

The number of fetuses in uteri before LPS treatment (30 µg ml⁻¹) did not vary among the groups (Tables 1 and 2). Treatment of pregnant mice with LPS caused a decrease in the number of total fetuses and live fetuses in uteri 16 h after administration, and this was used as an index of premature delivery (Table 2). This decrease was significantly inhibited by the injection of formoterol (0.006 or 0.06 mg kg⁻¹ h⁻¹).

LPS treatment increased the IL-6 concentration in plasma 16 h after administration, but did not change PGF_{2 α} or IL-10 concentration (Table 3). The concentration of IL-1 α in plasma was lower than the limit of detection in all samples. Formoterol treatment significantly reduced the increase in IL-6 induced by LPS.

LPS significantly increased PGF_{2 α} and IL-6 concentrations in the amniotic fluid 8 h after administration and to a greater extent 16 h after administration (Figs 1 and 2).

Table 2. The influence of formoterol administration on the number of fetuses in uteri 16 h after lipopolysaccharide (LPS) treatment in mice

Treatment	Number of fetuses in uteri	
	Initial	8 h after LPS treatment
Control	8.3 ± 0.5	8.3 ± 0.5 (8.3)
LPS (30 µg per mouse)	7.2 ± 0.4	2.2 ± 1.0* (0.0*)
+ formoterol (0.006 mg kg ⁻¹ h ⁻¹)	6.3 ± 1.0	6.3 ± 1.0* (5.3 ^{##})
+ formoterol (0.06 mg kg ⁻¹ h ⁻¹)	7.3 ± 0.5	7.3 ± 0.5* (4.3 [#])

LPS was administered intraperitoneally 4 h after implanting osmotic pumps containing formoterol solution. Values represent the mean ± SEM (*n* = 5 or 6 mice). The numbers of live fetuses in uteri are shown in parentheses. **P* < 0.01 versus control; **P* < 0.05; ^{##}*P* < 0.01 versus LPS alone.

Table 3. Changes in cytokine and prostaglandin F_{2α} (PGF_{2α}) plasma concentration in mice 16 h after administration of lipopolysaccharide (LPS) alone or with formoterol

Treatment	Concentration (pg ml ⁻¹)			
	PGF _{2α}	IL-1α	IL-6	IL-10
Control	1688.5 ± 190	ND	33.4 ± 13	3598.9 ± 323
LPS (30 µg per mouse)	1425.0 ± 216	ND	313.4 ± 57*	3922.2 ± 333
– formoterol (0.006 mg kg ⁻¹ h ⁻¹)	1308.0 ± 213	ND	132.4 ± 10 [#]	4357.0 ± 324
+ formoterol (0.06 mg kg ⁻¹ h ⁻¹)	1203.0 ± 165	ND	108.1 ± 11 [#]	4304.5 ± 283

Values represent the mean ± SEM (*n* = 9 or 10 mice). Interleukin 1α (IL-1α) concentrations were lower than the limit of detection (ND). **P* < 0.01 versus control; [#]*P* < 0.01 versus LPS alone.

Fig. 1. Effects of formoterol on lipopolysaccharide (LPS)-induced increase of prostaglandin F_{2α} (PGF_{2α}) in amniotic fluid in mice (□) 8 h and (■) 16 h after administration of LPS. Values are mean ± SEM (*n* = 8–11 mice). **P* < 0.05; ***P* < 0.01 versus control; [#]*P* < 0.01 versus LPS alone. **Fig. 2.** Effects of formoterol on lipopolysaccharide (LPS)-induced increase of interleukin 6 (IL-6) in amniotic fluid in mice (□) 8 h and (■) 16 h after administration of LPS. Values are mean ± SEM (*n* = 8–11 mice). **P* < 0.05; ***P* < 0.01 versus control; [#]*P* < 0.05; ^{##}*P* < 0.01 versus LPS alone.

Discussion

IL-1α and IL-10 concentrations were also increased after 16 h (Figs 3 and 4).

Combined treatment of LPS with formoterol resulted in a significant suppression of the change in PGF_{2α}, IL-6 and IL-1α at 0.006 or 0.06 mg formoterol kg⁻¹ h⁻¹. The concentration of IL-10 was not affected by the formoterol treatment.

Formoterol administration inhibited premature delivery induced by LPS in mice and increased the number of live fetuses in uteri 16 h after treatment with LPS. However, formoterol administered intraperitoneally twice a day did not influence LPS-induced premature delivery (data not shown). Osmotic pumps can maintain the blood concentration of formoterol for over 20 h. Thus, it may

Fig. 3. Effects of formoterol on lipopolysaccharide (LPS)-induced increase of interleukin 1α (IL-1α) in amniotic fluid in mice (□) 8 h and (■) 16 h after administration of LPS. Values are mean ± SEM ($n = 9-10$ mice). * $P < 0.01$ versus control; # $P < 0.05$ versus LPS alone.

Fig. 4. Effects of formoterol on lipopolysaccharide (LPS)-induced increase of interleukin 10 (IL-10) in amniotic fluid in mice (□) 8 h and (■) 16 h after administration of LPS. Values are mean ± SEM ($n = 9-10$ mice). * $P < 0.01$ versus control.

be of importance that formoterol was continuously and uniformly administered.

In the present study, LPS (30 µg per mouse) caused a marked increase in IL-1α, IL-6, IL-10 and PGF_{2α} concentrations in amniotic fluid 16 h after administration. Furthermore, an increase in plasma IL-6 concentration was observed. In general, TNF-α is the first cytokine produced after treatment with LPS (Chensue *et al.*, 1991), and this can drive the synthesis of other proinflammatory cytokines such as IL-1α or IL-6 (Shalaby *et al.*, 1989), which are in turn capable of stimulating prostaglandin production by intrauterine tissues (Romero *et al.*, 1989; Mitchell *et al.*, 1990, 1991). The present study attempted to measure TNF-α concentrations, but they proved to be lower than the detection limit in all samples (data not shown). The concentration of TNF-α

in amniotic fluid is known to be very low, with transient increases observed only within 4 h after systemic administration of LPS (Fidel *et al.*, 1994). In the present study IL-6 and PGF_{2α} concentrations were increased at 8 h, whereas IL-1α was changed at 16 h. Similarly, IL-10 was increased 16 h after systematic administration of LPS, as reported by Barsig *et al.* (1995). An increase in IL-10 production in LPS-treated mice may maintain pregnancy by inhibition of over-expression of proinflammatory cytokines such as IL-1, IL-6 and TNF-α, mainly at the level of gene transcription (Wang *et al.*, 1994).

In the present study, the β₂-adrenoceptor agonist formoterol ameliorated the LPS-induced decrease in the percentage of fetuses in uteri (an index of premature delivery) and this appears to be related to the suppression of the increase in concentrations of IL-1α, IL-6 and PGF_{2α} in the amniotic fluid. Moreover, formoterol inhibits an increase in IL-6 in plasma. There is much evidence that β₂-adrenoceptor agonists may be involved in immunoregulation, although little is known about the signal pathways involved. However, Nakamura *et al.* (2001) demonstrated that downregulation of Shiga toxin 2-induced TNF-α production by terbutaline is mediated by an inhibitory effect of β₂-adrenoceptor activation on the nuclear factor κB (NF-κB), exerted through a cAMP-protein kinase pathway and a cAMP-independent mechanism. Moreover, an increase in intracellular cAMP concentration may decrease NF-κB-mediated TNF-α gene transcription (Oliver *et al.*, 1996). The present study did not examine whether formoterol influences the production of TNF-α. Future investigations will concentrate on early TNF-α production in order to clarify the inhibitory mechanisms of formoterol. However, the present study has shown that formoterol inhibits IL-1α and IL-6 production in amnion tissues after the synthesis of PGF_{2α}. This effect may also be associated with the suppression of activation or transcription of NF-κB or TNF-α.

Formoterol administration did not affect the LPS-induced increment in the anti-inflammatory cytokine inhibitor IL-10 in amniotic fluid in the present study. IL-10 may cause inhibition of NF-κB activation in LPS-stimulated human monocytes (Wang *et al.*, 1995), and thus suppress cytokine production. It remains unclear whether an increase in the IL-10 concentration in amniotic fluid may contribute to negative feedback in the cytokine network, but it should be noted that the plasma concentration of IL-10 is approximately 160-fold higher than that in amniotic fluid in normal pregnant mice. Thus, the effect of IL-10 on IL-1α or IL-6 might be expected to be much less in amniotic fluid than in plasma. In the present study, LPS treatment increased the IL-10 concentration in amniotic fluid, albeit only approximately threefold compared with the control. The fact that IL-10 was not affected by formoterol, despite the significant reduction in other cytokines, indicates a beneficial effect with regard to the suppression of premature delivery.

In conclusion, the present study has shown that formoterol can protect against premature delivery induced by systemic administration of LPS in pregnant mice, and the associated reduction in the cytokines IL-1 α and IL-6. Whether this activity is mediated through β 2-adrenoceptors remains to be clarified, but the results of this study indicate that formoterol may have potential for the treatment of women at high risk of premature delivery.

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