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Tocolytic activity of formoterol against premature delivery in mice

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Abstract

The tocolytic activity of formoterol (eformoterol), a long-acting potent β_2-adrenoceptor agonist, was assessed in pregnant mice, with determination of uterine effects on the 15th and 16th days of gestation. For examination in the lipopolysaccharide-induced premature delivery model, osmotic pumps filled with formoterol or saline solution were implanted subcutaneously under the back skin. The mice were sacrificed 18–20 h thereafter, and the numbers of fetuses in the uteri and the newborn were counted. The uteri, amniotic membranes and placenta were also rapidly removed for determination of IL-6 concentrations. Furthermore, the effect of formoterol on IL-6 secretion from mouse amnion cells was determined. Formoterol and ritodrine inhibited contraction responses of isolated mouse uteri and their intravenous administration resulted in lowered uterine motility. Lipopolysaccharide (30 μg mL^{-1}/mouse) induced premature delivery, attributable to increased IL-6 secretion, and formoterol suppressed this. Doses of 5–500 μg/mouse thus reduced the number of prematurely delivered newborn, and 50 μg/mouse also depressed IL-6 secretion. On histopathologic analysis, the marked oedema and slight haemorrhage in the mouse cervix induced by lipopolysaccharide were reduced by administration of the β_2-adrenoceptor agonist. Neither formoterol (10^{-7}–10^{-5} M) nor ritodrine (10^{-7}–10^{-5} M) influenced spontaneous secretion of IL-6 in amnion cells. However, at 10^{-7} and 10^{-5} M, and 10^{-6} and 10^{-5} M, respectively, they inhibited lipopolysaccharide-induced IL-6 secretion and this inhibitory effect was competitively reversed by addition of ICI-118,551 (β_2-adrenoceptor antagonist), but not atenolol (β_1-adrenoceptor antagonist). These findings strongly suggest that formoterol can suppress premature delivery mediated by its actions on IL-6 secretion.

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Introduction

Human myometrial β-adrenoceptors predominantly belong to the β_2-subtype, mediating smooth muscle relaxation (Mahon et al 1967). Thus, specific β_2-adrenoceptor agonists, such as ritodrine, have become a popular group of drugs employed for countering premature delivery in the clinical field. Ritodrine has been shown to reduce delivery within 48 h of treatment (King et al 1988), but there is little convincing evidence that any further prolongation of pregnancy can be obtained. This may be due to multiple factors. Tachyphylaxis of the β-adrenergic response system in the myometrium is known to occur in both animals and man. Also, ritodrine stimulates a relatively specific β_2-adrenoceptor and its potency is about 100–1000-fold lower than that of isoproterenol (Ikeda & Tamaoki 1984). In addition, it exerts antagonistic effects on isoproterenol-stimulated c-AMP production in human amnion cells (Collins et al 1993).

Formoterol (eformoterol) is a catecholamine analogue employed for the therapy of asthma, possessing potent β_2-adrenoceptor agonist effects with high potency and long lasting action in animal experiments (Ida 1976; Sugiyama et al 1992). It is 5–15 times more potent than salbutamol in patients with asthma, and the duration of symptom alleviation is usually at least 8–12 h (Löfdahl & Svedmyr 1989; Graff-Lonnevig & Browaldh 1990; Maesen et al 1990). We have established that formoterol inhibits uterine contraction stimulated by oxytocin in anaesthetized pregnant rats more strongly than ritodrine (Shinkai & Takayama 2000). However, it has not been hitherto examined for effects in an animal model of premature delivery.

The initiation of term is characterized by contractions of the uterus and cervical ripening. Increase in prostaglandin synthesis and secretion of cytokines, such as interleukin (IL)-1, IL-6 or IL-8 into amniotic fluid, are important as mechanisms underlying premature delivery associated with intra-uterine infection (McDuffie et al 1992; Hsu et al 1998). Especially, it has been indicated that IL-6 is a useful clinical marker for infection-mediated premature delivery (Andrews et al 1995). Increase in IL-6 synthesis stimulates prostaglandin and leukotriene production, causing dilation of cervical vessels and further promoting the extravasation of leucocytes (Winkler & Rath 1999). Also, since IL-6 and IL-8 are increased in the human cervix during ripening, they are thought to play an important role in cervical remodelling (Sennstrom et al 2000). Results from different studies indicate that the adrenergic system is involved in this process. Warrick et al (1985) reported that β-adrenergic agonists such as salbutamol, isoproterenol and adrenaline (epinephrine) stimulate prostaglandin output from dispersed cells of human amnion and decidua. Adrenaline increases release of IL-6 from cultured murine neurohypophyseal cells mediated by β_2-adrenoceptors (Christensen et al 1999). At the same time, it has been reported that the β_2-adrenoceptor agonist, clenbuterol, suppresses the lipopolysaccharide-induced release of tumour necrosis factor (TNF)-α and IL-6 from macrophages (Izeboud et al 1999). Furthermore, isoproterenol was found to decrease TNF-α and IL-6 gene transcription in rat renal resident macrophage cells (Nakamura et al 1999). If β-adrenoceptor activation influences cytokine secretion, it may also impact on premature delivery.

In this study, we therefore investigated the inhibitory effects of formoterol and ritodrine on premature delivery in pregnant mice. In addition, we assessed whether these drugs influence IL-6 secretion in the uterus, amniotic membrane and placenta induced by administration of lipopolysaccharide.

Materials and Methods

Animals

C3H/HeN strain mice (Charles River, Yokohama, Japan) at the 15th or 16th day of gestation were housed in an animal room with a room temperature of $23 \pm 2^{\circ}C$, a relative humidity of $55 \pm 15\%$, and a 12-h light–dark cycle (lights on at 0800 h). The mice were allowed free access to mouse/rat diet (F-2, pelleted form; Funabashi Farm, Funabashi, Japan) and tap water throughout the experiment.

Drugs

Formoterol (eformoterol) fumarate (Yamanouchi Pharmaceutical Co. Ltd, Tokyo, Japan), ritodrine hydrochloride (Sigma Chemical Co., St Louis, MO), atenolol (β_1-adrenoceptor antagonist; Sigma Chemical Co., St Louis, MO), ICI-118,551 (β_2-adrenoceptor antagonist; Research Biochemicals Inc., Natick, MO) and lipopolysaccharide (serotype 0.55: B5; Sigma Chemical Co., St Louis, MO) were each dissolved in 0.9% physiological saline (Otsuka Pharmaceutical Co. Ltd, Tokyo, Japan). The composition of the Ringer solution was (in mM); NaCl 150.4, KCl 5.4, $CaCl_2$ 0.36, $MgCl_2$ 0.19, $NaHCO_3$ 4.76, KH_2PO_4 0.15, Na_2HPO_4 0.56 and glucose 2.78.

Myometrial contraction

Mice were stunned and killed by decapitation. The uteri were rapidly removed and longitudinal muscle strips (2 mm wide, 10 mm long) were cut and suspended in Ringer solution at $37^{\circ}C$, bubbled with a mixture of 95% O_2 and 5% CO_2 at pH 7.4 in 10-mL organ baths. The preparations were loaded with a 0.5-g weight and contractions were recorded isometrically on an ink-writing recorder through a forced placement transducer. Muscle contraction was evaluated as a percentage of the size before the addition of drugs. IC50 values (molar concentration of agonists producing 50% of maximal response) were calculated from cumulative concentration–response curves.

Uterine motility in anaesthetized mice

Mice were anaesthetized with pentobarbital-Na (40 mg kg^{-1}, i.p.) (Dainippon Pharmaceutical Co. Ltd, Osaka, Japan) and a balloon connected to a polyethylene catheter was inserted into a uterine horn after the removal of one fetus. Uterine motility was measured through transducers with amplifiers and a recorder. Percentage inhibition of the frequency of uterine contraction was calculated as an index, with reference to the contractions occurring over a 5-min period before the administration of each drug.

Experimental design for the lipopolysaccharide-induced premature model

Osmotic pumps (Model 2001D; Alza Corporation) were filled with a solution (200 μL) of formoterol or saline and implanted subcutaneously into the backs of mice on the 15th day of gestation, under anaesthesia with pentobarbital-Na (50 mg kg^{-1}, i.p.). This osmotic pump injects drug solution with a pumping rate of 8.0 μL h^{-1} for one day. A dose of lipopolysaccharide (30 μg mL^{-1}) that gave consistent premature delivery without maternal death was administered intraperitoneally 4 h after implantation and mice were killed 18–20 h thereafter. As a control, phosphate buffered saline solution was used and comparisons were made of the numbers of fetuses in the uteri and newborn. Each cervix was fixed in 10% phosphate-buffer formalin and routinely processed for histopathological observation. Sections of 3-μm thickness were stained with haematoxylin and eosin, and examined under a light microscope. Samples of uterus, amniotic membrane and placenta were also removed, and frozen in liquid nitrogen. After being homo-

genized in ice-cold phosphate-buffered saline (5 mL (g tissue)$^{-1}$) and centrifuged at 13 000 *g* for 10 min at 4°C, they were stored at −80°C until assayed for IL-6 content with an enzyme-linked immunosorbent assay kit (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, UK). IL-6 values for tissue homogenates were standardized to the total protein content. Quantitative protein assays were performed colorimetrically with the bicinchoninic acid method (BCA Protein Reagent, Pierce, Rockford, IL).

Culture of amnion cells

C3H/HeN-strain pregnant mice on the 15th or 16th day of gestation were killed and the amnia excised. Amnion cells were isolated by enzymatic digestion following the protocol described by Glasser et al (1988). In brief, tissues were incubated in Hanks' balanced salt solution without Ca^{2+}/Mg^{2+} and phenol red (HBSS; Sigma Chemical Co., St Louis, MO), but containing 0.5% trypsin (Sigma Chemical Co., St Louis, MO) and 2.5% pancreatin (Gibco-BRL, Grand Island, NY), first for 60 min at 4°C, then for another 60 min at room temperature. After incubation, tissues were promptly diluted in HBSS with 1% fetal bovine serum (Cosmo Bio Co. Ltd, Tokyo, Japan). They were then passed in and out through a large-bore pipette and filtered through a cell dissociation sieve (mesh 70 μm) and amnion cells were collected in a 50-mL centrifuge tube. After washing twice in HBSS and re-suspension in Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (1:1) (Gibco-BRL) with 10% fetal bovine serum in 75-cm^2 culture flasks (Corning Inc., NY), they were plated in 24-well plates at a concentration of 1×10^5 to 3×10^5 cells/mL/well and incubated at 37°C in 5% CO_2 in air for determination of IL-6. After 24 h, the culture medium was changed to medium with added drugs and the cells were further incubated for 4, 8 or 24 h. Cultured supernatants were collected at each time point and stored frozen at −40°C until assayed. Determination of IL-6 concentration in supernatants was made by ELISA (Amersham Pharmacia Biotech UK Ltd).

Data analysis

The results are expressed as mean±standard deviation. Differences in the various treatments were statistically examined using the one-way analysis of variance. Individual differences between means were examined using Dunnett's test. In all cases *P* values less than 0.05 were accepted as significant.

Results

Myometrial contraction

Formoterol and ritodrine reduced the amplitude of uterine contractions in a dose-dependent manner (Figure 1), with IC50 values of 2.1×10^{-9} M and 3.2×10^{-6} M, respectively. Formoterol was approximately 1000-fold more potent than ritodrine.

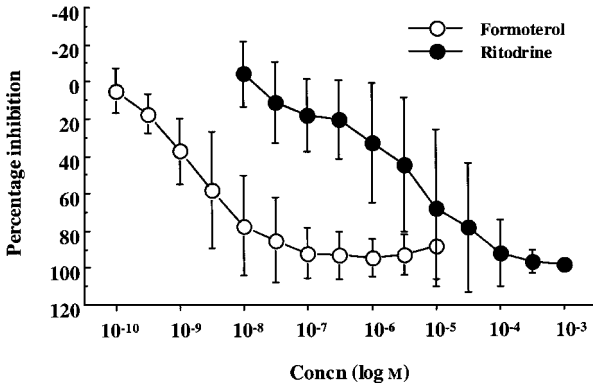

Figure 1 Effects of formoterol and ritodrine on contractile responses of isolated uteri of pregnant mice (15th–16th day of gestation). Each point and vertical bar represents the mean±s.d. of data from six experiments.

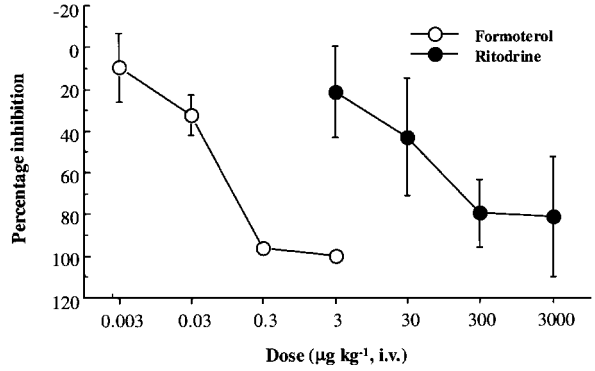

Figure 2 Effects of formoterol and ritodrine on uterine activity in pregnant mice (15th–16th day of gestation). Each point and vertical bar represents the mean±s.d. of data for four or five mice. The drugs were administered intravenously.

Uterine motility in anaesthetized mice

Figure 2 shows data for the effects of formoterol and ritodrine on uterine motility when administered intravenously to pregnant mice. In most mice, uterine motility was observed to occur spontaneously. Intravenously administered formoterol (0.003–3 μg kg^{-1}) and ritodrine (3–3000 μg kg^{-1}) inhibited the uterine motility in a dose-dependent manner, with ED50 (50% effective dose) values of 0.125 μg kg^{-1} and 120.7 μg kg^{-1}, respectively. The dose causing inhibition by formoterol was approximately 1000-fold less than that by ritodrine.

Assessment of formoterol effects on lipopolysaccharide-induced premature delivery

A marked decrease in the percentage of fetuses remaining in uteri was observed at 30 μg/mouse of lipopolysaccharide (Table 1), along with significant increment in IL-6 concentrations in the uterus, amniotic membrane and placenta. This decrease in the percentage of fetuses remaining in the uteri was reversed by administration of formoterol

Table 1 Comparison of the influence of formoterol on percentage of fetuses in mouse uteri and IL-6 concentrations in tissues after lipopolysaccharide treatment.

Treatment	Fetuses in uteri (%)	IL-6 concentration (pg (mg protein)$^{-1}$)		
		Uterus	Amniotic membrane	Placenta
No treatment with LPS	100.0±0	40.2±37	33.5±27	38.3±22
LPS 30 μg mL^{-1}/mouse	2.5±8**	171.6±137**	562.7±337**	260.1±108**
LPS+formoterol 5 μg/mouse	45.8±47#	102.5±42	190.5±182##	190.0±116
LPS+formoterol 50 μg/mouse	78.6±37##	87.3±43	101.7±67##	101.4±24#
LPS+formoterol 500 μg/mouse	51.8±49#	130.7±45	311.6±120	227.9±121

The numbers of fetuses in uteri are shown as percentages of the control value, 18–20 h after administration of lipopolysaccharide (LPS). Formoterol solution was administered by osmotic pumps. LPS was administered intraperitoneally immediately after implantation of osmotic pumps on day 15 of gestation. Values are mean±s.d. (n = 7–10). **$P < 0.01$ vs no-LPS-treatment group (t-test); #$P < 0.05$; ##$P < 0.01$ vs LPS-treated group (Dunnett's test).

(5–500 μg/mouse) (Table 1). Formoterol also reduced the marked increment in IL-6 in the uterus, amniotic membrane and placenta, significant decrease being observed for the latter two, at doses of 5 and 50 μg/mouse, and at 50 μg/mouse, respectively (Table 1). Ritodrine (40 mg/mouse) did not exert a significant effect on IL-6 secretion in the uterus, amniotic membrane or placenta.

Histologic analysis of cervical tissues

Figure 3 shows typical histopathologic features of cervical tissues from saline-treated (A), lipopolysaccharide (LPS, 30 μg/mouse)-treated (B) and LPS plus formoterol (50 μg/mouse)-treated mice (C). In the lipopolysaccharide-treated mice, marked oedema (soft structure) and haemorrhage were observed. Formoterol (50 μg/mouse) administered by osmotic pump suppressed these lipopolysaccharide-induced changes.

IL-6 secretion from mouse amnion cells

Formoterol (10^{-5} M) did not appear to influence spontaneous IL-6 secretion from cultured mouse amnion cells 4, 8 and 24 h after application (data not shown). However, lipopolysaccharide (1 μg mL^{-1}) remarkably increased IL-6 production 24 h after application (Figure 4) and treatment with formoterol (10^{-7} and 10^{-5} M) or ritodrine (10^{-6} and 10^{-5} M) significantly suppressed this effect. The inhibition by formoterol (10^{-5} M) was reversed by administration of a β_2-adrenoceptor antagonist, ICI-118,551 (10^{-5} M), but not a β_1-adrenoceptor antagonist, atenolol (10^{-5} M) (Figure 5). Atenolol (10^{-5} M) and ICI-118,551 (10^{-5} M) alone did not influence IL-6 production 4, 8 and 24 h after application (data not shown).

Discussion

β-Adrenoceptor agonists, such as ritodrine, are potent inhibitors of myometrial contractile activity and are employed extensively for the treatment of premature delivery. However, their widespread use has not been associated with any significant improvement in neonatal outcome (Johnson 1993). Ritodrine stimulates a relatively specific β_2-adrenoceptor, and only has less than one-hundredth the potency of isoproterenol (Ikeda & Tamaoki 1984), whereas formoterol's action on histamine-induced bronchoconstriction is approximately 20-fold stronger than that of isoproterenol in guinea-pigs (Ida 1976). This study showed both formoterol and ritodrine to inhibit uterine activity in pregnant mice, the potency of formoterol being approximately 1000-fold that of ritodrine. Stimulation of β_2-adrenoceptors in the myometrium results in an increase of cellular c-AMP through the activation of c-AMP-dependent protein kinases, ultimately leading to inhibition of myometrial contraction (Kroeger & Marshall 1974; Riemer et al 1988). However, it has been indicated that ritodrine has antagonistic effects on isoproterenol-stimulated c-AMP production in human amnion cells and this might limit its influence on uterine activity (Collins et al 1993).

This study confirmed that systemic administration of lipopolysaccharide to pregnant mice induces premature delivery with marked increment of IL-6 in the uterus, amniotic membrane and placenta, as reported earlier (Hirsch et al 1999). The bacterial component, released after disruption of Gram-negative bacteria, has the ability to liberate arachidonic acid from intrauterine tissues (Bennett et al 2000). Through the cyclooxygenase pathway this is metabolized to form prostaglandin E_2 or $F_{2\alpha}$, which are known to contribute to the initiation of premature delivery (Romero et al 1988). IL-6 stimulates prostaglandin production by human amnion and chorion (Kent et al 1993), and rat hypothalamic explants were found to activate vasopressin or oxytocin release, this being blocked by the cyclooxygenase inhibitor indometacin, but not the lipoxygenase inhibitor BW A4C (Yasin et al 1994). Furthermore, IL-6 elevation due to lipopolysaccharide has been implicated as a local mediator of premature delivery in pregnant mice (Fidel et al 1994). Clear suppression of lipopolysaccharide-induced increase in IL-6 concentrations in the uterus, amniotic membrane and placenta of pregnant mice was observed in this study. However, ritodrine (40 mg/

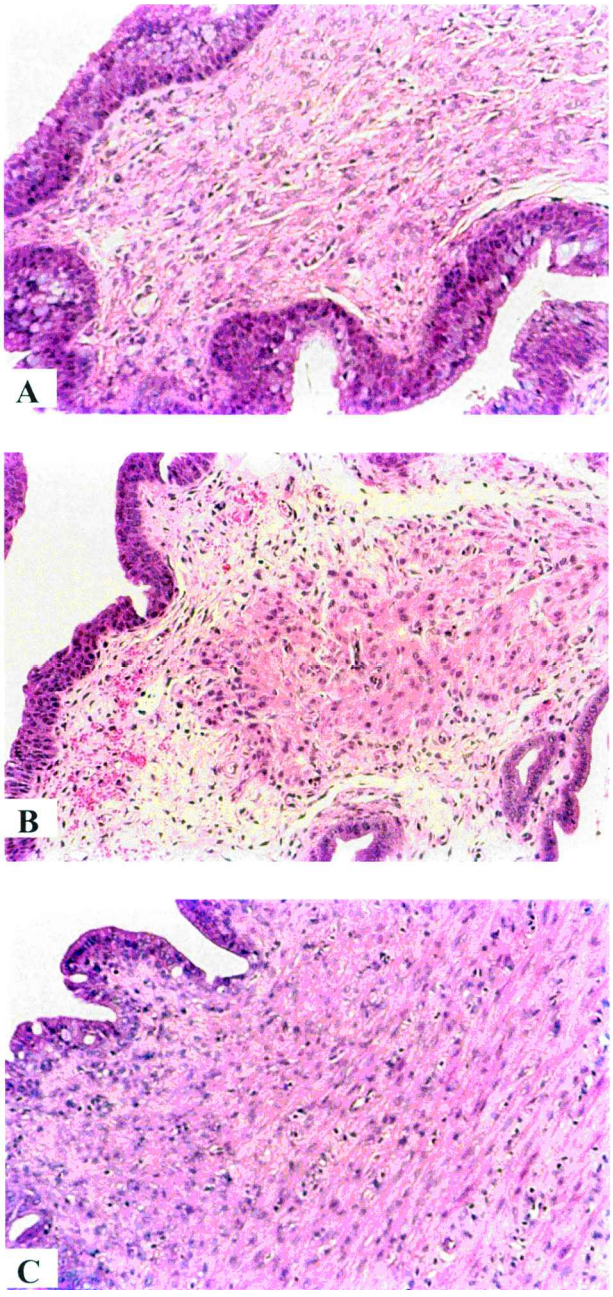

Figure 3 Representative histopathologic appearance of cervical tissue from a saline treated pregnant mouse (A), a lipopolysaccharide (LPS, 30 μg/mouse)-treated mouse at 16 h after treatment (B) and an LPS-treated mouse at 16 h after treatment with LPS and formoterol (50 μg/mouse) (C) on the 15th day of gestation. A and C show loose connective tissue. B shows marked oedema (softer structure) and slight haemorrhage. Haematoxylin and eosin staining; original magnification ×100.

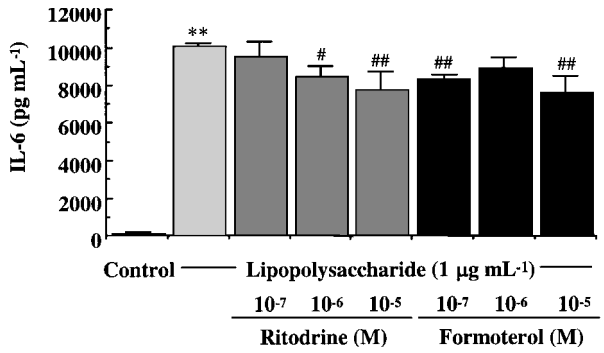

Figure 4 Effects of formoterol and ritodrine on lipopolysaccharide-induced IL-6 secretion in cultured mouse amnion cells. Each value represents the mean±s.d. of data for four determinations. $^{**}P < 0.01$ vs the control (*t*-test); $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs the lipopolysaccharide treated group (Dunnett's test).

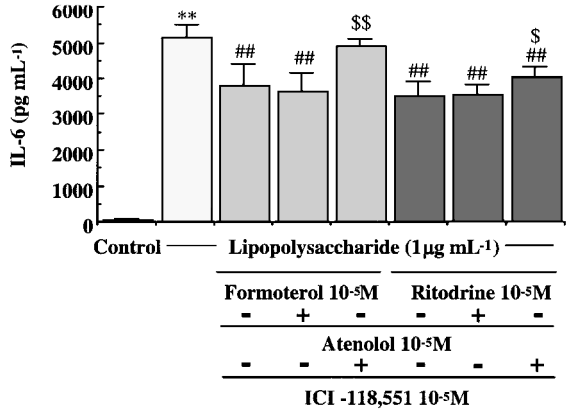

Figure 5 Effects of atenolol and ICI-118,551 on the reduction, by formoterol and ritodrine, of IL-6 secretion from cultured mouse amnion cells. Each value represents the mean±s.d. of data for four determinations. $^{**}P < 0.01$ vs the control (*t*-test); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs the lipopolysaccharide-treated group (Dunnett's test); $\$P < 0.05$, $\$\$P < 0.01$ vs the lipopolysaccharide-+-formoterol-treated group.

mouse) did not exert significant effects on IL-6 secretion in the uterus, amniotic membrane or placenta. The dose of ritodrine (40 mg/mouse) used for this study was 800-fold that of formoterol (50 μg/mouse). However, we have not yet examined whether ritodorine suppresses uterine activity with continuous administration using osmotic pumps. Formoterol and ritodrine partly inhibited IL-6 secretion induced by lipopolysaccharide in mouse amnion cells, without any appreciable effect on spontaneous secretion. This inhibition was sensitive to competition by a β_2-adrenoceptor antagonist, ICI-118,551, but not a β_1-adrenoceptor antagonist, atenolol. The potency of formoterol in suppression of uterine activity was approximately 1000-fold that of ritodrine, whereas the effects of formoterol and ritodrine on IL-6 secretion from culture cells seemed similar. Since we did not examine whether lower doses of formoterol suppress IL-6 secretion, we could not elucidate this discrepancy here.

This study showed that formoterol reduces cervical ripening (oedema) induced by lipopolysaccharide on histologic analysis. Such ripening is thought to result from accumulation of neutrophils or decrease of the collagen concentration in the cervix (Maradny et al 1995).

Accumulated neutrophils stimulate the cytokine network so that IL-6 or IL-1 are secreted. In this study, we did not observe any marked change in neutrophil accumulation in the cervix, but pro-inflammatory cytokines also are produced by the amnion or uterine tissue. In previous studies, β_2-adrenoceptor agonists were found to influence IL-6 secretion or IL-6 transcription in several cell types or tissues. For example, clenbuterol, a β_2-adrenoceptor agonist, suppressed lipopolysaccharide-induced release of TNF-β and IL-6 from macrophages (Izeboud et al 1999), and isoproterenol reduced IL-6 or TNF-α transcription in renal macrophage cells (Nakamura et al 1999), whereas adrenaline induced an increase in IL-6 from murine pituicytes and stimulated IL-6 mRNA expression in primary cultured rat hepatocytes (Christensen et al 1999; Jung et al 2000). Although the influence of β-adrenoceptor agonists on IL-6 secretion or transcription may depend on the experimental conditions, IL-6 is well established as an important cytokine in premature delivery induced by systemic infections such as pyelonephritis or pneumonia (Yoon et al 1995). It stimulates the production of prostaglandins and cytokines related to uterine contraction or cervical ripening. The available information thus suggests that stimulation of amnion cells or the uterine β_2-adrenoceptor system by formoterol could suppress premature delivery not only by direct inhibition of uterine contraction induced by an increase of c-AMP but also indirectly by reduction of uterine contraction or cervical ripening mediated by suppression of IL-6 secretion.

In summary, this study showed that formoterol and ritodrine both inhibit uterine activity in pregnant mice, the potency of formoterol being approximately 1000-fold that of ritodrine. While lipopolysaccharide induces premature delivery in pregnant mice associated with IL-6 secretion, this can be effectively suppressed by the adrenoceptor agonist formoterol. This agent may therefore be beneficial for treatment of premature delivery.

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