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Potent inhibition of spontaneous rhythmic contraction by a novel β_2-adrenoceptor agonist, HSR-81, in pregnant rat uterus

Tetsuo Ohashi [a], Shigeki Hashimoto [a,*], Kouji Morikawa [a], Hideo Kato [a], Yasuo Ito [a], Masahisa Asano [b], Hiroshi Azuma [c]

[a] *Research and Development Division, Hokuriku Seiyaku Co., Ltd., Katsuyama, Fukui 911, Japan*

[b] *Department of Pharmacology, Nagoya City University Medical School, Mizuho-ku, Nagoya, Aichi 467, Japan*

[c] *Department of Medicinal Chemistry, Institute for Medical and Dental Engineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101, Japan*

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Abstract

We examined the effect of HSR-81 ((−)-(*R*)-α-[(*tert*-butylamino)methyl]-2-chloro-4-hydroxybenzyl alcohol L-tartrate), a newly developed, potent and selective β_2-adrenoceptor agonist, as well as ritodrine and isoproterenol, on the spontaneous rhythmic contraction in uteri isolated from late pregnant, middle pregnant and non-pregnant (dioestrous and oestrous) rats. The three agonists inhibited the spontaneous rhythmic contraction at all the stages in a concentration-dependent manner. The pD_2 value for HSR-81 was greater in late pregnancy than in dioestrus and oestrus. In the uterine preparations of late pregnancy and dioestrus, ICI-118,551 (1-(7-methylindan-4-yloxy)-3-isopropyl-aminobutan-2-ol, a selective β_2-adrenoceptor antagonist) and atenolol (a selective β_1-adrenoceptor antagonist) produced a parallel rightward shift of the concentration-response curves for HSR-81. The pK_B values for ICI-118,551 and atenolol suggest that the inhibitory effect of HSR-81 was mediated through β_2-adrenoceptors in the two stages. In the membranes prepared from rat uteri in late pregnancy and dioestrus, the equilibrium dissociation constant for [^{125}I]iodocyanopindolol binding was not significantly different between the two stages. The three β-adrenoceptor agonists and the two antagonists competed for the specific [^{125}I]iodocyanopindolol binding and the pK_i values were not significantly different between the two stages. However, the maximum number of binding sites was significantly greater in late pregnancy than in dioestrus. The configuration of the competition curves and the pK_i values for the two antagonists confirmed the fact that these membranes contain predominantly β_2-adrenoceptor subtype. These results indicate that the potent inhibition of the spontaneous rhythmic contraction by HSR-81 in the pregnant uterus may be due to the increased number of β_2-adrenoceptors.

Keywords: Uterus, rat; β_2-Adrenoceptor, subtype; β_2-Adrenoceptor agonist; Pregnancy

1. Introduction

Stimulation of β-adrenoceptors in the myometrium leads to an increase of cellular cyclic AMP through the activation of adenylate cyclase, and the subsequent activation of cyclic AMP-dependent protein kinase, resulting in the inhibition of myometrial contraction (Kroeger and Marshall, 1974; Andersson et al., 1980; Krall et al., 1981; Hatjis, 1985; Riemer et al., 1988; Roberts et al., 1989). Functional and receptor binding studies have shown that the myometrium predominantly contains the β_2-adrenoceptor subtype in various species including human (Kenakin, 1982; Hayashida et al., 1982; Mattsson et al., 1982; Hatjis, 1985; McPherson et al., 1985; Dattel et al., 1986; Maltier and Legrand, 1988). The selective β_2-adrenoceptor agonists have been successfully used in clinical practice for the prevention of premature labor (Johnson, 1993; Travis and McCullough, 1993).

Previous studies of the number of myometrial β-adrenoceptor binding sites and of responsiveness to β-adrenoceptor agonists during pregnancy and the oestrous cycle have provided conflicting results. In tissues from pregnant rats, the number of β_2-adrenoceptors, measured by an agonist ligand, [^{3}H]hydroxybenzylisoproterenol, was found to be decreased at a period immediately before parturition (Legrand et al., 1987; Maltier and Legrand, 1988; Maltier et al., 1989), but was not changed when an antagonist ligand, [^{125}I]iodocyanopindolol, was used (El Alj et al., 1989). Salbutamol, a selective β_2-adrenoceptor agonist, was reported to inhibit the spontaneous contraction in the

* Corresponding author.

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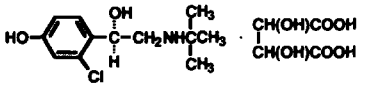

Fig. 1. Chemical structure of HSR-81.

rat uterus and was more potent at the end of pregnancy than at the middle of pregnancy (Chernaeva, 1984). However, the inhibitory effect of isoproterenol, a non-selective β-adrenoceptor agonist, on the spontaneous contraction was not changed during pregnancy (Chow and Marshall, 1981). In non-pregnant rats, [^{3}H]dihydroalprenolol binding sites in the myometrial membranes were reported to be significantly elevated during proestrus and oestrus (Krall et al., 1978) and treatment with estradiol or progesterone increases the [^{3}H]dihydroalprenolol binding sites in ovariectomized rats (Krall et al., 1978; Kano, 1982). However, the inhibitory effect of isoproterenol on the spontaneous contraction was not to be changed during the oestrous cycle (Abdel-Aziz and Bakry, 1973; Hartley and Pennefather, 1981) but was more potent in progesterone-treated uterus than in estrogen-treated one (Chow and Marshall, 1981).

We developed a potent and selective β_2-adrenoceptor agonist, HSR-81 ((−)-(*R*)-α-[(*tert*-butylamino)methyl]-2-chloro-4-hydroxybenzyl alcohol L-tartrate) (Fig. 1), for preventing premature labor (Hashimoto et al., 1994). The β_2 (uterus)/β_1 (atrium) selectivity of HSR-81 was higher than that of ritodrine (Hashimoto et al., 1994). To clarify the action of HSR-81 on the late pregnant uterus, we compared an inhibitory effect of HSR-81 on the spontaneous rhythmic contraction in uteri isolated from late pregnant, middle pregnant and non-pregnant rats. In addition, the radioligand binding studies were performed in uteri from late pregnant and non-pregnant rats, and the effect of HSR-81 to compete for the specific binding was examined. The effects of a selective β_2-adrenoceptor agonist, ritodrine, which is a useful drug to prevent premature labor (Johnson, 1993; Travis and McCullough, 1993), and a non-selective β-adrenoceptor agonist, isoproterenol were also examined. Our results suggest the possibility that the potent inhibition of the spontaneous rhythmic contraction by HSR-81 in the late pregnant uterus is due to the increased number of β_2-adrenoceptors. A preliminary account of these findings was presented to the 67th Annual Meeting of the Japanese Pharmacological Society (Ohashi et al., 1994).

2. Materials and methods

2.1. Functional experiments

Non-pregnant (virgin, 205–270 g) and pregnant (270–480 g) Wistar rats were used. In the non-pregnant rats, the oestrous cycle was determined by microscopic observation of vaginal smears. The female rats were housed with male rats overnight and successful mating was determined by the presence of the plug in the vagina (day 0 of pregnancy). The rats in dioestrus, oestrus, 13–15th (middle pregnancy) and 19–21st days of gestation (late pregnancy) were stunned and exsanguinated, and the uterine horns were isolated. The uterine horns except for the 19–21st days of gestation were cut approximately 10 mm in length and the adhering tissues were cleared away. The uterine horns in the 19–21st days of gestation were opened and strips (approximately 3 mm in width and 10 mm in length of longitudinal direction) were prepared except for the placental region. The preparations were mounted vertically to permit recording of contractions of the longitudinally arranged muscle layer in an organ bath containing 10 ml of Krebs-Henseleit solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 24.9 mM $NaHCO_3$ and 11.1 mM glucose. The bath solution was maintained at 37°C; pH 7.4 and was equilibrated with 95% O_2 and 5% CO_2. A resting tension of 0.5 *g* was applied and the spontaneous rhythmic contraction was recorded isometrically with a force-displacement transducer (T7-30-240; Orientec, Tokyo, Japan). All preparations were equilibrated for about 60 min before starting the experiments.

Effects of HSR-81, ritodrine (selective β_2-adrenoceptor agonists), isoproterenol (a non-selective β-adrenoceptor agonist) and forskolin (a direct activator of adenylate cyclase) on the spontaneous rhythmic contraction were examined. These drugs were added to the bath solution. Cumulative concentration-response curves for the drugs were determined with a stepwise three-fold increase in the concentration of an drug at 20 min (non-pregnant preparations) or 10 min (pregnant preparations) intervals because of the different contractile frequencies between non-pregnancy and pregnancy (see Results). Spontaneous rhythmic contraction activity was measured as the sum of contractile force for 10 or 20 min. For example, if the force of consecutive contraction was 3 *g*, 4 *g* and 3 *g*, the sum was 10 *g*. Then, % response of the each period was calculated as 100% was the sum of contractile force for 10 or 20 min before the application of the drug. EC_{50} value, the concentration of drug that elicited 50% of the maximal inhibition, was calculated from a plot of % response vs. log concentration of drug and expressed as a negative log (pD_2 value). In other experiments, the effects of β-adrenoceptor antagonists on the inhibitory responses to HSR-81 were studied in the uterine preparations from animals in dioestrus and late pregnancy. The cumulative concentration-response curve for HSR-81 in the presence of β-adrenoceptor antagonist (ICI-118,551 (1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol), β_2 selective; atenolol, β_1 selective) was obtained after 30 min incubation with the antagonist. The apparent antagonist potency was calculated as follows (Furchgott, 1972): apparent pK_B = negative log $[B/DR - 1]$, where B is the concentration of

antagonist and DR is ratio of the EC_{50} value in the presence of the antagonist/EC_{50} value in the absence of the antagonist.

2.2. Binding experiments

The radioligand binding studies were performed using uteri from animals in dioestrus and the 19–21st days of gestation. The uterine horns were quickly dissected and adhering tissues were cleared away. The uteri were then frozen and stored at −80°C. Thawed tissues were cut into small pieces by a surgical blade and homogenized with a glass-Teflon homogenizer in 50 volumes of ice-cold buffer: 150 mM NaCl, 20 mM Tris-HCl and 1 mM EDTA; pH 7.5 at 25°C. The homogenates were centrifuged at $1500 \times g$ for 10 min at 4°C and the resulting supernatant was then recentrifuged at $100000 \times g$ for 30 min at 4°C. The supernatant was discarded and the remaining membrane pellet was suspended in a reaction mixture (50 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM EDTA; pH 7.5 at 25°C) to give a protein concentration of 75–130 μg ml^{-1}. The membrane suspension was used immediately in the radioligand binding assay.

Saturation binding assay was performed as described previously (Asano et al., 1991). Briefly, 200 μl membrane suspension was incubated with seven concentrations (1.25–120 pM) of [^{125}I]iodocyanopindolol in a final volume of 300 μl at 37°C for 60 min. The reaction was terminated by rapid vacuum filtration, and radioactivity retained on the Whatman GF/F glass fiber filters was measured in an Aloka autowell gamma counter. Non-specific binding was defined as the amount of [^{125}I]iodocyanopindolol binding measured in the presence of 100 μM isoproterenol and was subtracted from the total to obtain specific binding. Saturation radioligand binding isotherms were analyzed by Scatchard transformation and Hill plot (Bennett and Yamamura, 1985) and the maximum number of binding sites (B_{max}), the equilibrium dissociation constant (K_d) and the Hill coefficient (n_H) were calculated.

Studies of competition between β-adrenoceptor agonists and antagonists and [^{125}I]iodocyanopindolol for binding to uterine membranes were performed with a constant radioligand concentration (18 pM) and different concentrations of competitors (0.1–3000 nM for HSR-81, 0.003–100 μM for ritodrine, 0.1–10000 nM for isoproterenol, 0.03–300 nM for ICI-118,551 and 0.03–100 μM for atenolol). Competition radioligand binding data were subjected to computer analysis using programs of Equilibrium Binding Data Analysis (EBDA; McPherson, 1985) and a negative log of equilibrium dissociation constant (pK_i) and the slope factor (pseudo Hill coefficient) were calculated. When the slope factors were significantly < 1 and the Runs test indicated that a one-site model was not optimal, two-site curve-fitting analysis was carried out using programs of EBDA/LIGAND (McPherson, 1985).

Protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

2.3. Statistical analysis

Unless specified, the results are expressed as means ± S.E.M. (n = number of preparations each from a separate animal with an exception of forskolin experiments where 12 preparations from 3 late pregnant rats and 12 preparations from 7 dioestrous rats were studied). Statistical analysis of the data was done by Student's t-test for unpaired data, or by Duncan's multiple range test, or by test for parallelism (Pharmacologic Calculation System Version 4.0; Tallarida and Murray, 1986), depending on which test was statistically appropriate. Two groups of data were considered to be significantly different when $P < 0.05$.

2.4. Drugs and chemicals

The following drugs were used: HSR-81 ((−)-(*R*)-α-[(*tert*-butylamino)methyl]-2-chloro-4-hydroxybenzyl alcohol L-tartrate; Hokuriku Seiyaku, Katsuyama, Fukui, Japan), atenolol, (±)-isoproterenol hydrochloride, prazosin

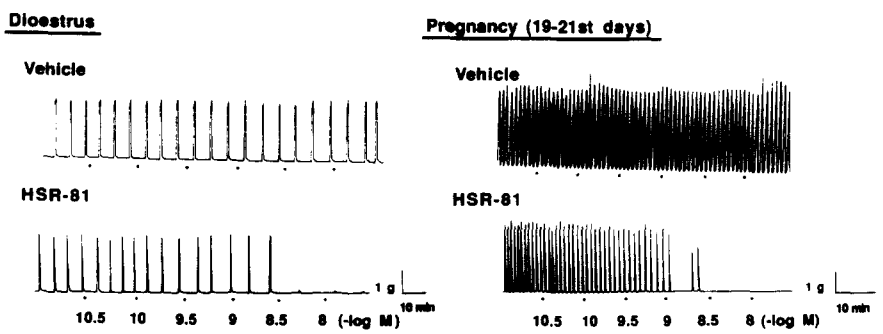

Fig. 2. Typical traces of the effects of HSR-81 on the spontaneous rhythmic contraction in the uterine preparations isolated from dioestrous and late pregnant (19–21st days of gestation) stages of rats. HSR-81 in concentrations ranging from 0.03 to 10 nM (expressed as a negative log of the concentration) was cumulatively added to the bath solution at 20 min (dioestrus) or 10 min (pregnancy) interval (see text).

hydrochloride, ritodrine hydrochloride, tetrodotoxin (Sigma Chemical, St Louis, MO, USA), forskolin (Research Biochemicals, Natick, MA, USA), ICI-118,551 (erythro-*dl*-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol hydrochloride; Cambridge Research Biochemicals, Cheshire, UK), atropine sulfate (Wako Pure Chemical Industries, Osaka, Japan) and [^{125}I](−)-iodocyanopindolol (specific activity 2200 Ci/mmol) (DuPont/NEN Research Products, Boston, MA, USA). Forskolin (10 mM) was prepared using ethanol, with further dilution in distilled water.

3. Results

3.1. Functional experiments

Uterine preparations isolated from non-pregnant and pregnant rats exhibited spontaneous rhythmic contraction which was well maintained during the experimental period (Fig. 2). However, the frequency of the contraction varied: the frequency was low in dioestrus (1.8 ± 0.1 10 min^{-1}, $n = 36$) and oestrus (1.5 ± 0.1 10 min^{-1}, $n = 24$), and was high in middle (9.6 ± 0.5 10 min^{-1}, $n = 36$) and late (12.5 ± 1.1 10 min^{-1}, $n = 35$) pregnancy. Thus, the frequency was gradually increased with the gestational stage. The spontaneous rhythmic contractions in tissues from dioestrous and late pregnant animals were not affected by atropine (1 μM), prazosin (0.1 μM) or tetrodotoxin (0.3 μM ($n = 4$ for each drug: data not shown), confirming the fact that the contraction is myogenic in origin.

The addition of HSR-81 (0.03–30 nM) inhibited the spontaneous rhythmic contraction in all uterine preparations tested. The inhibition was concentration-dependent and the higher concentrations (> 10 nM) of HSR-81 abolished the contractions (Figs. 2 and 3). The pD_2 values for HSR-81 in non-pregnant and pregnant stages are shown in Table 1. The pD_2 value for HSR-81 was significantly greater in late pregnancy than in dioestrus and oestrus. The pD_2 values for HSR-81 in late pregnancy were not significantly different between 20 min of contact time (9.57 ± 0.10, $n = 6$) and 10 min of contact time (9.47 ± 0.12, $n = 12$). Then, the contact times for each concentration of agonist were 10 min (pregnant preparations) and 20 min (non-pregnant preparations) because of the different contractile frequencies between pregnancy and non-pregnancy. There was no significant difference in the pD_2 value for HSR-81 between dioestrus and oestrus, and between middle and late pregnancy. Ritodrine (0.1–1000 nM) and isoproterenol (0.01–30 nM) also inhibited the spontaneous rhythmic contraction in these preparations (Fig. 3, Table 1). The pD_2 value for ritodrine was significantly greater in late pregnancy than in oestrus. However, in each stage, the pD_2 value for HSR-81 was much greater than that for ritodrine. On the other hand, the pD_2 value for isoproterenol was not significantly different between non-pregnancy and pregnancy. However, these pD_2 values for isoproterenol were slightly greater than those for HSR-81.

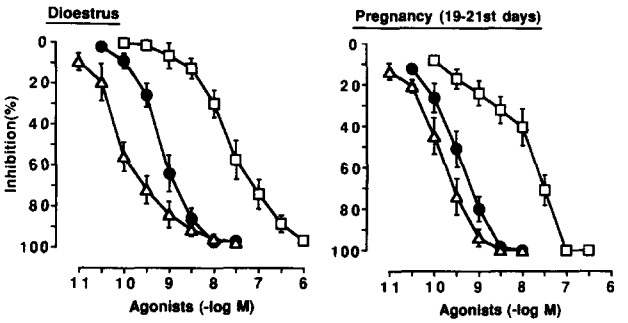

Fig. 3. Inhibitory effects of HSR-81 (●), ritodrine (□) and isoproterenol (△) on the spontaneous rhythmic contraction in the uterine preparations isolated from dioestrous and late pregnant (19–21st days of gestation) stages of rats. Experimental conditions were the same as in Fig. 2. Data points are means of 11–12 preparations, and S.E.M. is shown by vertical bars.

Table 1
Inhibitory effects of HSR-81, ritodrine, isoproterenol and forskolin on the spontaneous rhythmic contraction in the uterine preparations isolated from non-pregnant and pregnant stages of rats [a]

Stages	pD_2 [b]			
	HSR-81	Ritodrine	Isoproterenol	Forskolin
Non-pregnancy				
Dioestrus	9.14 ± 0.10 [c]	7.66 ± 0.19 [d]	10.03 ± 0.14 [d]	6.85 ± 0.10 [d]
Oestrus	9.03 ± 0.16 [c]	7.50 ± 0.19 [c,d]	9.63 ± 0.16 [d]	NT [e]
Pregnancy				
13–15th days	9.27 ± 0.07	7.84 ± 0.15 [d]	9.84 ± 0.10 [d]	NT [e]
19–21st days	9.47 ± 0.12	8.10 ± 0.19 [d]	9.85 ± 0.13	6.80 ± 0.12 [d]

[a] Experimental conditions were the same as in Figs. 2 and 3.
[b] Negative log of EC_{50} value. Values are expressed as the means ± S.E.M. ($n = 7$–12).
[c] Significantly different from the value in the 19–21st days of gestation ($P < 0.05$, Duncan's multiple range test).
[d] Significantly different from the value of HSR-81 ($P < 0.05$, Duncan's multiple range test).
[e] NT, not tested.

Since HSR-81 showed higher potency in inhibiting the spontaneous rhythmic contraction in tissues from late pregnant than from non-pregnant animals, we examined which component of the β-adrenoceptor-adenylate cyclase-relaxation system is responsible for the increased responsiveness. Therefore, the effect of forskolin, a direct activator of adenylate cyclase (Daly, 1984), was examined in dioestrus and late pregnancy. Although forskolin (10–3000 nM) concentration dependently inhibited the spontaneous rhythmic contraction, there was no difference in the inhibitory potency of this drug between dioestrus and late pregnancy (Table 1).

The effects of ICI-118,551 and atenolol on the inhibitory responses to HSR-81 were studied in dioestrus and late pregnancy. ICI-118,551 (10 nM) and atenolol (30 μM) did not affect the spontaneous rhythmic contraction, but produced a parallel rightward shift of the concentra-

tion-response curve for HSR-81. The calculated pK_B value for ICI-118,551 was not significantly different between dioestrus (9.05 ± 0.21, $n = 5$) and late pregnancy (9.35 ± 0.13, $n = 5$). The calculated pK_B value for atenolol was not significantly different between dioestrus (5.52 ± 0.14, $n = 10$) and late pregnancy (5.76 ± 0.23, $n = 7$).

3.2. Binding experiments

Saturation experiments were examined with [^{125}I]-iodocyanopindolol at concentrations ranging from 1.25 to 120 pM to label β-adrenoceptors of rat uterine membranes. The characteristics of [^{125}I]iodocyanopindolol binding to membranes prepared from the uteri in dioestrus and late pregnancy were shown in Fig. 4. The non-specific binding was linear with increasing concentrations of the radioligand and represented < 4% of the total binding at [^{125}I]iodocyanopindolol concentrations around the K_d values. The specific binding of [^{125}I]iodocyanopindolol was saturable and of high affinity. Scatchard analysis of the saturation [^{125}I]iodocyanopindolol binding isotherms resulted in a straight line, indicating a single class of binding sites in the two stages. The K_d value was not significantly different between the two stages (Table 2). However, the B_{max} was significantly greater in late pregnancy than in dioestrus (Table 2). The n_H of these data showed no evidence of cooperativity or of multiple receptors of different affinity (Table 2).

The ability of the β-adrenoceptor antagonists and agonists to compete for the specific [^{125}I]iodocyanopindolol binding to membranes was determined in dioestrus and late pregnancy. To label the binding sites 18 pM [^{125}I]iodocyanopindolol was used. ICI-118,551 (0.03–300 nM) competed for the binding in a monophasic manner with a high pK_i value in dioestrus and late pregnancy (Fig. 5, Table 3). On the other hand, atenolol (0.03–100 μM) competed for the binding with a low pK_i value. Although the competition curves for atenolol showed a biphasic pattern (Fig. 5), the slope factors were not significantly different from unity (Table 3). The pK_i values for the two β-adrenoceptor antagonists were not significantly different between dioestrus and late pregnancy (Table 3). HSR-81 (0.1–3000 nM), ritodrine (0.003–100 μM) and isoproterenol (0.1–10000 nM) also competed for the binding in dioestrus and late pregnancy (Fig. 6). The rank order of the pK_i value was HSR-81 > isoproterenol > ritodrine. There was no difference in the pK_i value for these β-adrenoceptor agonists between dioestrus and late pregnancy (Table 3). Among the three β-adrenoceptor agonists, isoproterenol had the lowest slope factors (Table 3). Two-site curve-fitting analysis was carried out for isoproterenol. The relative proportions of the high- (43 ± 1 vs. 41 ± 2%,

Table 2
Binding characteristics of [^{125}I]iodocyanopindolol to membranes prepared from uteri in dioestrous and late pregnant (19–21st days of gestation) stages of rats [a]

Stages	n [b]	K_d [c] (pM)	B_{max} [d] (fmol mg^{-1} protein)	Hill coefficient (n_H)
Dioestrus	12	15.3 ± 1.0	149 ± 6	1.10 ± 0.05
19–21st days	12	13.8 ± 0.6	233 ± 20 [e]	1.08 ± 0.04

[a] Experimental conditions were the same as in Fig. 4. Values are expressed as means ± S.E.M.
[b] Number of determinations each performed in duplicate.
[c] Equilibrium dissociation constant of [^{125}I]iodocyanopindolol.
[d] Maximum number of binding sites.
[e] Significantly different from the value in the dioestrus ($P < 0.001$, Student's t-test).

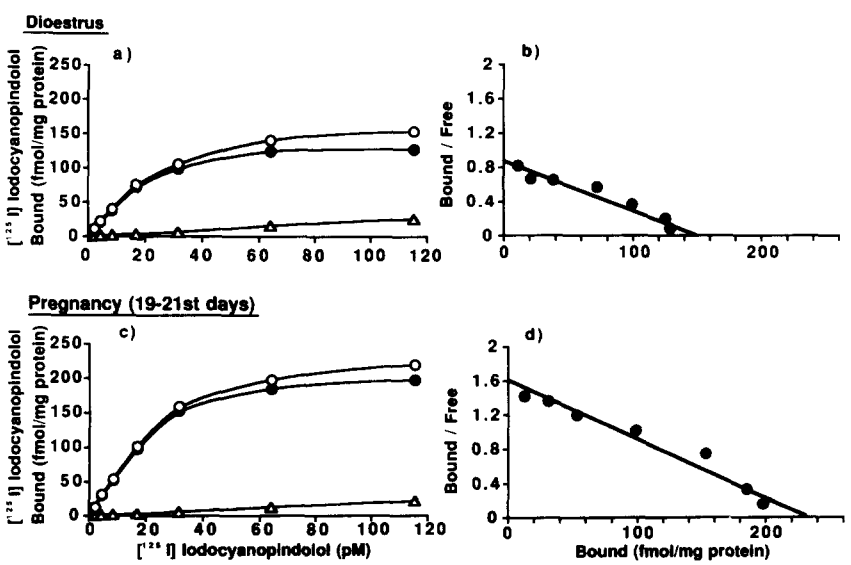

Fig. 4. Saturation isotherms (**a** and **c**) and Scatchard plots (**b** and **d**) of [^{125}I]iodocyanopindolol binding to membranes prepared from uteri in dioestrous (**a** and **b**) and late pregnant (19–21st days of gestation; **c** and **d**) stages of rats. **a** and **c**: The binding of the indicated free concentrations of [^{125}I]iodocyanopindolol was evaluated in the absence (total binding, ○) and presence (non-specific binding, △) of 100 μM isoproterenol. The specific binding (●) was obtained by subtracting the non-specific binding from the total binding. **b** and **d**: Scatchard analysis of the specific binding data in **a** and **c**.

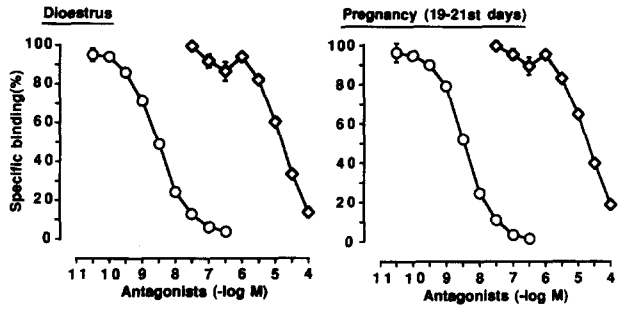

Fig. 5. Competition by ICI-118,551 (○) and atenolol (◇) of [^{125}I]iodocyanopindolol binding to membranes prepared from uteri in dioestrous and late pregnant (19–21st days of gestation) stages of rats. [^{125}I]iodocyanopindolol (18 pM) was incubated with various concentrations of ICI-118,551 or atenolol under assay conditions described in Materials and methods. Data points are means of 5 determinations each performed in duplicate, and S.E.M. is shown by vertical bars.

Table 3
Competition by β-adrenoceptor antagonists and agonists of [^{125}I]-iodocyanopindolol binding to membranes prepared from uteri in dioestrous and late pregnant (19–21st days of gestation) stages of rats [a]

Compounds	Dioestrus		19–21st days	
	pK_i	Slope factor	pK_i	Slope factor
Antagonists				
ICI-118,551	8.94±0.07	0.881±0.020	8.85±0.07	1.008±0.015
Atenolol	5.16±0.03	1.136±0.099	5.07±0.03	0.985±0.077
Agonists				
HSR-81	7.34±0.05	0.814±0.045	7.26±0.06	0.884±0.026
Ritodrine	5.91±0.02	0.850±0.057	5.88±0.04	0.877±0.040
Isoproterenol	6.92±0.06	0.718±0.056	6.82±0.04	0.743±0.029

[a] Experimental conditions were the same as in Figs. 5 and 6. The concentration of [^{125}I]iodocyanopindolol used was 18 pM. Values are expressed as means±S.E.M. of 4–5 determinations each performed in duplicate.

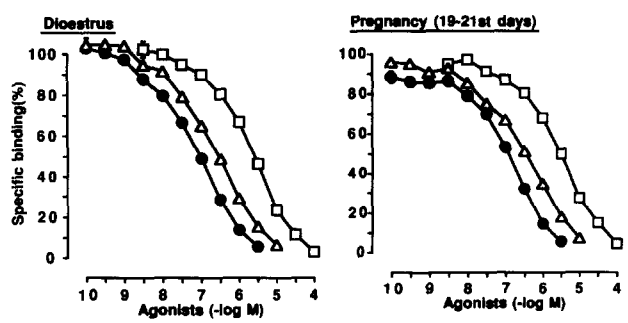

Fig. 6. Competition by HSR-81 (●), ritodrine (□) and isoproterenol (△) of [^{125}I]iodocyanopindolol binding to membranes prepared from uteri in dioestrous and late pregnant (19–21st days of gestation) stages of rats. Experimental conditions were the same as in Fig. 5. Data points are means of 4 determinations each performed in duplicate, and S.E.M. is shown by vertical bars.

$n = 3$) and low-affinity sites (57 ± 1 vs. 59 ± 2%, $n = 3$) for isoproterenol were not significantly different between dioestrus and late pregnancy. The pK_i values for the high- (8.01 ± 0.11 vs. 8.20 ± 0.20, $n = 3$) and low-affinity sites (6.47 ± 0.07 vs. 6.49 ± 0.003, $n = 3$) were not significantly different between dioestrus and late pregnancy.

4. Discussion

The present study clearly demonstrated that HSR-81 inhibited the spontaneous rhythmic contraction via the activation of β_2-adrenoceptors in isolated rat uteri, and the inhibition was more potent in the pregnant stage than in the non-pregnant stage. The potent inhibition by HSR-81 in pregnant uteri may be due to the increased number of β_2-adrenoceptors. This conclusion arises from the following observations: (1) the inhibition by HSR-81 of the spontaneous rhythmic contraction was selectively antagonized by ICI-118,551, a selective β_2-adrenoceptor antagonist; (2) [^{125}I]iodocyanopindolol binding experiments showed the existence of β-adrenoceptors in the rat uteri and this binding was selectively competed for by ICI-118,551; (3) the number of β_2-adrenoceptors was greater in the pregnant stage than in the non-pregnant stage; (4) the inhibition by HSR-81 and ritodrine of the spontaneous rhythmic contraction was also greater in the pregnant stage than in the non-pregnant stage; and (5) the inhibition of the spontaneous rhythmic contraction by forskolin, a direct activator of adenylate cyclase, was the same in the pregnant and non-pregnant stages.

ICI-118,551, reportedly a selective β_2-adrenoceptor antagonist (Bilski et al., 1983), antagonized the inhibitory effect of HSR-81 in the uterine preparations of dioestrus and late pregnancy with a similar pK_B value. These pK_B values for ICI-118,551 were in good agreement with the pK_B values for this antagonist reported for β_2-adrenoceptors (Hartley and Pennefather, 1985; Abrahamsson, 1986; Rimele et al., 1988). On the other hand, atenolol, reportedly a selective β_1-adrenoceptor antagonist (Rimele et al., 1988), also antagonized the inhibitory effect of HSR-81 in the two stages with a similar pK_B value. These pK_B values were in good agreement with the values for this antagonist reported for β_2-adrenoceptors rather than the values for β_1-adrenoceptors (Hartley and Pennefather, 1985; Rimele et al., 1988). Therefore, our results suggest that the inhibitory effects of these β-adrenoceptor agonists on the spontaneous rhythmic contraction in both non-pregnant and pregnant stages are mainly mediated through β_2-adrenoceptor subtype. This is in consistent with previous reports showing that the muscle relaxation induced by β-adrenoceptor agonists in rat myometrium is due to the stimulation of β_2-adrenoceptor subtype (Kenakin, 1982;

Mattsson et al., 1982; Abrahamsson, 1986; El Alj et al., 1989).

In the membranes prepared from rat uteri in dioestrus and late pregnancy, Scatchard analysis of the saturation [^{125}I]iodocyanopindolol binding isotherms indicates a single class of binding sites. ICI-118,551 and atenolol competed for the specific [^{125}I]iodocyanopindolol binding. The pK_i values for ICI-118,551 and atenolol in the two stages were in good agreement with the pK_i values for this antagonist reported for β_2-adrenoceptor subtype (Hedberg et al., 1985; El Alj et al., 1989; May et al., 1985; Tsuchihashi et al., 1989). In addition to this, the pK_i values were comparable to the pK_B values in the present functional studies. These results suggest that uterine membranes from the two stages predominantly contain β_2-adrenoceptor subtype. This is in consistent with previous reports showing that rat myometrial membranes contain predominantly β_2-adrenoceptor subtype (Legrand et al., 1987; Maltier and Legrand, 1988; Tolszczuk and Pelletier, 1988; El Alj et al., 1989). From the above functional and binding studies, it is concluded that the potent inhibition of the spontaneous rhythmic contraction by HSR-81 and ritodrine in late pregnancy is not due to the altered affinity of β_2-adrenoceptors.

The present study clearly showed the increased number of β_2-adrenoceptors in late pregnant rats. It has been shown that β-adrenoceptors exist in the states of high or low affinity for agonists. The high-affinity agonist binding indicates the interaction of the receptor with a G_S-protein (Kent et al., 1980). In the present study, the competition curves for these β-adrenoceptor agonists were shallow, suggesting the existence of the high- and low-affinity states. Since the configuration of these competition curves was similar between the dioestrus and the late pregnancy, it is unlikely that the relative proportion of the high- and low-affinity state is changed during the pregnancy, as shown by the two-site curve-fitting analysis. Therefore, the increased responsiveness to selective β_2-adrenoceptor agonists in late pregnancy may be due to the increase of absolute number of high-affinity state of β_2-adrenoceptors.

It has been reported that the rat myometrium has a large β-adrenoceptor reserve and only $< 10\%$ occupation of β-adrenoceptors results in a half-maximum relaxing effect of isoproterenol (Krall et al., 1981; Kenakin, 1982). The present study clearly demonstrated that although HSR-81 had the highest affinity for β_2-adrenoceptor binding among the three agonists, the inhibitory potency of HSR-81 was less than that of isoproterenol in functional experiments, therefore suggesting a small β-adrenoceptor reserve for HSR-81 compared with isoproterenol. More receptors may be necessary for HSR-81 than isoproterenol to produce the same functional responses. Thus, the increase in the number of β_2-adrenoceptors in late pregnancy may occur judging from the increased responsiveness to HSR-81 in this stage. Because we used racemic form of isoproterenol in the present study, *d*-isomer may have some antagonistic action and bind with different affinity for adrenoceptors at high concentrations.

β-Adrenoceptor-mediated relaxation in the myometrium, as well as in other smooth muscles, has been proposed to involve increased cellular cyclic AMP through the activation of adenylate cyclase, and the subsequent activation of cyclic AMP-dependent protein kinase (Kroeger and Marshall, 1974; Andersson et al., 1980; Krall et al., 1981; Hatjis, 1985; Riemer et al., 1988; Roberts et al., 1989). The functional and binding experiments of the present study showed that the increased responsiveness to selective β_2-adrenoceptor agonists in late pregnancy occurs at a level of the β_2-adrenoceptors. Since the inhibitory effect of forskolin, a direct activator of the catalytic subunit of adenylate cyclase (Daly, 1984), on the spontaneous rhythmic contraction were the same in dioestrus and late pregnancy, it may be concluded that the components of the β_2-adrenoceptor-adenylate cyclase system distal to and including adenylate cyclase are probably not responsible for the increased responsiveness.

In the guinea-pig myometrium, the stimulation of G_S-protein by Gpp(NH)p, a non-hydrolyzable analog of GTP, and NaF resulted in a selective enhancement of the adenylate cyclase activity during pregnancy, indicating an increased function of G_S-protein (Arkinstall and Jones, 1990). In the rat myometrium, however, the stimulation of G_S-protein by GTP, NaF and cholera toxin resulted in a sharp fall of the adenylate cyclase activity during the first half of gestation, followed by a full restoration of this activity before parturition, indicating an altered function of G_S-protein (Tanfin and Harbon, 1987). Thus, it is less likely that the altered function of G_S-protein may contribute to the increased actions of β_2-adrenoceptor agonists in the present study.

In conclusion, the present study suggests the possibility that the potent inhibition of the spontaneous rhythmic contraction by HSR-81 in late pregnant rat uterus is due to the increased number of β_2-adrenoceptors. The potent inhibition by HSR-81 in pregnant rat can maintain the uterine quiescent during pregnancy. From the present study and our previous report (Hashimoto et al., 1994), it may be considered that HSR-81 is a useful drug for the treatment of uterine contraction-associated diseases, such as premature labor.

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