Progesterone decreases the relaxing effect of the β_3 -adrenergic receptor agonist BRL 37344 in the pregnant rat myometrium

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Abstract

Although the published results regarding the function of the β_3 -adrenergic receptors (β_3 -ARs) in the regulation of smooth muscle activity are very promising, the question of the mechanism of β_3 -ARs' action in the pregnant myometrium cannot be fully answered by human investigations. To assess whether it possesses an essential role in the regulation of uterine contractility in pregnant rats, as in humans, we performed functional, western blotting and molecular biology experiments on the late-pregnant rat myometrium. The influence of progesterone on the function of the β_3 -ARs was also investigated. We demonstrated the presence and the functional activity of the β_3 -ARs in the late-pregnant rat myometrium. The maximum dose-dependent uterus-relaxing effect of the selective β_3 -agonist BRL 37344 was recorded at the end of pregnancy in rats, similarly as in humans. The extent of its relaxing action was regarded as moderate. The expression of β_3 -AR protein and mRNA remained unchanged during the investigated period. The administration of progesterone had no effect on the β_3 -AR mRNA and protein expression or the maximum relaxation effect of BRL 37344, but shifted the dose-response curve to the right and decreased the synthesis of the second messenger, cAMP. It can be concluded that the β_3 -ARs play an additional role in the regulation of the contractile activity of the pregnant rat uterus. The inhibitory effect of progesterone on the functional activity of the β_3 -ARs may have important consequences in the case of human application if this effect is also demonstrated in pregnant human myometrial tissue.

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Introduction

Two years after the identification of the third adrenergic receptor in humans, the β_3 -adrenergic receptors (β_3 -ARs) were cloned from rat tissue by Granneman *et al.* (1991). The expression of the new adrenoceptor was found to be active only in the white and brown adipose tissue. It has subsequently become known that β_3 -ARs are expressed not only in adipocytes, but in various organs containing smooth muscle, such as the small and large intestines (Anthony *et al.* 1998, Zhao *et al.* 2001), the bladder (Clouse *et al.* 2007), the lung (Martin & Advenier 1995), the vascular smooth muscle (Trochu *et al.* 1999) and additionally in the heart (Gauthier *et al.* 1996). Stimulation of the β_3 -ARs evokes smooth muscle relaxation by enhancing cAMP accumulation (Skeberdis 2004, Yuan & Bernal 2007). Bardou *et al.* (2000) presented the first evidence of the existence of β_3 -AR mRNA in the human near-term myometrium and proved its functional significance under the signal transduction pathways mediating relaxation in the pregnant uterus. The β_3 -ARs became a possible target of newly developed compounds for tocolysis, because the selective β_3 -AR agonist BRL 37344 induced relaxation of human myometrial contractions with a similar potency to that

of the most commonly used tocolytic agent, ritodrine, but with fewer adverse vascular effects (Dennedy *et al.* 2001, 2002). Various functional and biochemical experiments have demonstrated that the β_3 -ARs are the predominant β -AR subtype in the human myometrium, and during pregnancy their expression is up-regulated towards the time of parturition (Rouget *et al.* 2005). To date, only limited information is available as concerns the existence of the β_3 -AR mRNA and protein in the late-pregnant rat myometrium, or its pharmacological role in the regulation of uterine smooth muscle activity in the rat. Moreover, earlier experimental results are available on the pharmacological reactivity and receptor expression patterns of other adrenergic receptor subtypes (α_1 -, α_2 - and β_2 -ARs) in the pregnant rat myometrium (Ducza *et al.* 2002, Gáspár *et al.* 2005, 2007). If a comparison between the β_3 - and the other ARs is needed, it is necessary to have results from the same species.

The use of progesterone and its analogues in the prevention of preterm labour has recently been reassessed (Schindler 2005), because it has been demonstrated that they play a central role in the maintenance of uterine quiescence. The administration of progesterone in high doses to women with previous preterm birth reduces the myometrial activity

significantly (Mackenzie *et al.* 2006). Moreover, it has been hypothesised that the continuous substitution of progesterone at the end of pregnancy can enhance the smooth-muscle relaxant effect of the most frequently applied tocolytic agents, the β_2 -AR agonists. As verification, it has been demonstrated that a 7-day course of progesterone treatment is able to increase the potency and efficacy of the relaxing effect of terbutaline on the rat myometrium *in vitro* (Gáspár *et al.* 2005). The increased β_2 -AR density and the enhanced G-protein activation were revealed to be the explanation of this heterologous regulatory process. The improved efficacy of the gestagen- β_2 -mimetics combination was also established in hormone-induced preterm delivery in rats *in vivo* (Gálik *et al.* 2008). To date, this beneficial action of progesterone on the relaxant effect of β_2 -agonists (ritodrine in the article) has been confirmed only in an isolated organ bath study in the case of human pregnancy (Chanrachakul *et al.* 2005). However, it is not yet known how the administration of progesterone is able to modulate the physiological function of the β_3 -AR in the late-pregnant myometrium.

In the present study, our aim was to prove the existence and the function of the β_3 -ARs in the pregnant rat myometrium. We planned to investigate whether the β_3 -AR mRNA and protein expression alter towards the end of pregnancy, how the myorelaxant effect of BRL 37344 changes in the late-pregnant rat uterus, and whether a 7-day course of progesterone treatment is able to influence the β_3 -AR expression and the uterorelaxant effect of a β_3 -AR agonist compound similar to the β_2 -ARs and its agonist agents. We were also interested in the potential intracellular changes evoked in the β_3 -AR signal transduction mechanism by progesterone treatment.

Results

Results of receptor mRNA and protein studies

The presence of the β_3 -AR mRNA and protein in the late-pregnant uterine tissue was demonstrated by RT-PCR and western blotting assays. Neither the synthesis of the β_3 -AR mRNA (Fig. 1A), nor the receptor protein expression (Fig. 1B) displayed a significant increase towards term.

Results of isolated tissue studies

The selective β_3 -AR agonist BRL 37344 inhibited the KCl-induced rhythmic contractions of the late-pregnant rat myometrium on all investigated days (Fig. 2). The magnitude of the smooth muscle relaxation reached its peak on day 22 of pregnancy (Table 1). Although the efficacy of BRL 37344 was highest on day 18 of pregnancy, the maximum inhibition of the myometrial contractions was significantly weaker as compared with

that on day 22. The minimum relaxing effect of BRL 37344 ($19.8 \pm 2.3\%$) was observed on day 21. The calculated EC_{50} values of BRL 37344 did not differ significantly from each other, apart from the value measured on day 18 (Table 1).

The inhibitory effect of BRL 37344 more than doubled in the absence of the β_2 -AR antagonist (Fig. 3).

Results of progesterone treatment

After the administration of progesterone, the concentration-response curve of BRL 37344 was shifted to the right (EC_{50} : $3.1 \times 10^{-7} \pm 1.4 \times 10^{-7}$ M) (Fig. 4), though the maximum relaxing effect of BRL 37344 remained unchanged in the 22-day-pregnant rat (Table 2).

Figure 1 (A) Changes in expression of β_3 -AR mRNA in the pregnant rat uterus, measured by RT-PCR. The amount of PCR-transcripts was determined by fluorometric assay. NS denotes $P > 0.05$ as compared with the data on the previous day. Each bar represents the mean $\pm$ S.E.M., $n = 4$. The β -actin probe was used as internal control. Panels below the graphs are representative gel pictures. (B) Changes in expression of the β_3 -AR protein in the pregnant rat uterus, detected by western blotting. NS denotes $P > 0.05$ as compared with the data on the previous day. Each bar represents the mean $\pm$ S.E.M., $n = 4$. The β -actin probe was used as internal control. Panels below the graphs are representative membrane pictures.

Figure 2 Changes in inhibitory effect of BRL 37344 on KCl-evoked rhythmic uterine contractions in the late-pregnant rat *in vitro*. Each value represents the mean $\pm$ S.E.M., $n=8$.

The progesterone treatment did not evoke any significant difference in the expression of β_3 -AR mRNA in the 22-day-pregnant rat uterus relative to the normal pregnant myometrium at term (Fig. 5A). This result was demonstrated by the western blotting experiment, which revealed that there was no significant alteration in the β_3 -AR protein expression following progesterone treatment as compared with the non-treated 22-day-pregnant rat uterus (Fig. 5B).

Measurement of the amount of cAMP accumulated following progesterone administration proved that, after stimulation with forskolin, there was a significantly lower amount of cAMP in the progesterone-treated uteri than in the non-treated pregnant uteri (Fig. 6). A control experiment was conducted in the presence of a β_3 -AR antagonist, SR 59230A, which prevented the accumulation of cAMP induced in the 22-day-pregnant rat uterus by the β_3 -AR agonist BRL 37344 (Fig. 6).

Discussion

Investigation of the functional role of the β_3 -ARs in the maintenance of myometrial quiescence during pregnancy began <10 years ago. During this period, appreciable basically new information concerning their expression, their signal transduction system and their

Table 1 Changes in EC_{50} and maximum relaxation effect of BRL 37344 in the late-pregnant rat uterus *in vitro*.

Day of pregnancy	$EC_{50} \pm$ S.E.M. (M)	$E_{max} \pm$ S.E.M. (%)
18	$8.4 \times 10^{-10} \pm 5.2 \times 10^{-10}$ *	27.7 ± 3.1 NS
20	$2.0 \times 10^{-8} \pm 8.8 \times 10^{-9}$ NS	26.5 ± 2.9 NS
21	$3.4 \times 10^{-8} \pm 1.4 \times 10^{-8}$ NS	19.8 ± 2.3 †
22	$1.8 \times 10^{-8} \pm 1.0 \times 10^{-8}$	40.3 ± 2.9

The level of significance relates to the comparison with the value on day 22 of pregnancy. NS denotes $P>0.05$; * $P<0.05$; † $P<0.001$. Each value represents the mean $\pm$ S.E.M., $n=8$.

involvement in the regulation of uterine smooth muscle activity have been published. Nevertheless, their mRNA and protein expression patterns during the third part of pregnancy and the influence of progesterone treatment on the physiological function of the myometrial β_3 -ARs in the rat have not yet been fully clarified.

The present findings demonstrate the mRNA and protein expression of the β_3 -ARs in the late-pregnant rat myometrium. They also corroborate and extend the previous experimental data relating to the smooth muscle contraction-inhibiting effect of BRL 37344 reported by Yurtcu *et al.* (2006). The variance in the magnitude of BRL 37344 efficacy on myometrial contractions can be explained by the different experimental conditions. Our results reflect the contraction-inhibiting effect of the test compound acting selectively on the β_3 -ARs, since only high concentrations of BRL 37344 (10^{-6} – 10^{-5} M) were able to evoke relaxation through the β_2 -ARs in the absence of ICI 118 551. Although the β_3 -AR expression exhibited no significant changes between days 18 and 22 of pregnancy, the maximum dose-dependent uterus-relaxing effect of BRL 37344 in the rat was observed at the end of pregnancy, similarly as in humans. There was an essential difference between the results of the human and rat experiments as concerns the question of the predominance of the β_3 -ARs in the uterine tissue. The inhibition of the spontaneous contractions induced by a β_3 -AR agonist, SR 59119A, was more pronounced than for a β_2 -AR agonist, salbutamol, in the human near-term myometrium (Rouget *et al.* 2005). In contrast with what was observed in human samples, the β_2 -AR agonist terbutaline was able to relax the pregnant rat myometrium at term (Gáspár *et al.* 2005) more efficiently than did

Figure 3 Changes in inhibitory effect of BRL 37344 on KCl-evoked rhythmic uterine contractions in the 21-day-pregnant rat in the presence (metoprolol + ICI) and absence (metoprolol) of the selective β_2 -AR antagonist ICI 118 551 *in vitro*. Each value represents the mean $\pm$ S.E.M., $n=8$.

Figure 4 Effects of progesterone (P) treatment on the inhibitory action of BRL 37344 on KCl-evoked rhythmic uterine contractions in the 22-day-pregnant rat *in vitro*. Each value represents the mean $\pm$ S.E.M., $n=8$.

BRL 37344 under similar experimental conditions. Accordingly, the smooth muscle-relaxing action through the β_3 -ARs seems to be weaker in the rat than in the human.

Progesterone treatment is known to prevent myometrial contractions in the event of threatening premature delivery (da Fonseca *et al.* 2003). The inhibition of the uterine smooth muscle contractions presumably develops through the heterologous interaction of the progesterone receptor subtypes and several other, still not fully identified receptors. Among these are the β_2 -ARs. It has been reported that a progesterone predominance increases β_2 -AR synthesis (Roberts *et al.* 1989), and also enhances the amount of activated G-proteins (Gáspár *et al.* 2005), so that the smooth muscle-relaxing effect of terbutaline on the term pregnant rat uterus is increased *in vitro*. Gálik *et al.* (2008) supposed that the increased efficacy of treatment with the salmeterol and progesterone combination in hormone-induced preterm delivery is based on the previously explained mechanism in the rat *in vivo*.

On the other hand, the effects of progesterone on the uterine β_3 -AR expression and its consequences on the myometrial contractions have not been investigated to date in either human or animal studies. We have now demonstrated that progesterone administration

decreases the uterorelaxant efficacy of BRL 37344 by reducing the synthesis of cAMP, but surprisingly not altering the expression of the β_3 -AR mRNA and protein in the pregnant rat uterus. This phenomenon can be regarded as an important difference between the signal mechanisms of the β_2 - and β_3 -ARs, which should be taken into account in the design of further investigations aimed at the human use of β_3 -mimetics. The above-mentioned findings suggested the additional consequence that progesterone is able to alter the signal transduction process through the β_3 -ARs by reducing the activity of the second messenger system, while it can also modulate the extent of β_2 -AR mRNA expression and the number of binding sites (Gáspár *et al.* 2005). On the other hand, the action of progesterone treatment on the amount of β_3 -AR mRNA and/or the binding sites has not

Figure 5 (A) Effect of progesterone (P) treatment on the expression of β_3 -AR mRNA in the 22-day-pregnant rat uterus, measured by RT-PCR. The amount of PCR-transcripts was determined by fluorometric assay. NS denotes $P>0.05$ as compared with the data on the non-treated animals. Each bar represents the mean $\pm$ S.E.M., $n=4$. The β -actin probe was used as internal control. Panels below the graphs are representative gel pictures. (B) Effect of progesterone (P) treatment on the expression of the β_3 -AR protein in the 22-day-pregnant rat uterus, detected by western blotting. NS denotes $P>0.05$ as compared with the data on the non-treated animals. Each bar represents the mean $\pm$ S.E.M., $n=4$. The β -actin probe was used as internal control. Panels below the graphs are representative membrane pictures.

Table 2 Effect of progesterone treatment on EC_{50} and maximum relaxation effect of BRL 37344 in the 22-day-pregnant rat uterus *in vitro*.

	$EC_{50} \pm \text{S.E.M. (M)}$	$E_{\max} \pm \text{S.E.M. (\%)}$
Non-treated	$1.8 \times 10^{-8} \pm 1.0 \times 10^{-8}$	40.3 ± 2.9
Progesterone-treated	$3.1 \times 10^{-7} \pm 1.4 \times 10^{-7}^*$	37.6 ± 4.5 NS

The level of significance relates to the comparison with the value for non-treated animals. NS denotes $P>0.05$; $^*P<0.05$. Each value represents the mean $\pm$ S.E.M., $n=8$.

Figure 6 Effect of progesterone (P) and a β_3 -AR antagonist, SR59230A treatment on the accumulation of cAMP in the 22-day-pregnant rat uterus, measured by enzyme-immunoassay. **Denotes $P < 0.01$ as compared with the data on the non-treated animals. Each bar represents the mean $\pm$ S.E.M., $n = 6$.

yet been fully elucidated. The published experimental data relating to how the β_3 -AR mRNA expression or binding capacity is altered by progesterone administration are somewhat contradictory (Malo & Puerta 2001, Monjo *et al.* 2005).

The loss of BRL 37344 efficacy is confirmed by the alteration in activity of the second messenger system, showing a significant reduction in the accumulation of cAMP after progesterone treatment. Moreover, the results of our control experiments in the presence of a β_3 -AR antagonist proved that the decline in the cAMP accumulation is restricted only to the β_3 -ARs. Up to the present, only one investigation has been published concerning the effect of progesterone on the accumulation of cAMP after stimulation by a selective β_3 -AR agonist on smooth muscles. Yono *et al.* (2000) measured the amount of cAMP in the ovariectomised female rabbit detrusor smooth muscle after a 2-week course of progesterone treatment, but it was not changed significantly. None of the above-mentioned observations are in accordance with our experimental data relating to the effect of progesterone on β_3 -AR synthesis and the degree of cAMP accumulation. This discrepancy might be caused by the use of different animal strains and/or organs. Further investigations are therefore needed to elucidate the effect of progesterone on the signal mechanism of the β_3 -ARs in various tissues.

In summary, our present results demonstrate the continuous presence and the functional activity of the β_3 -ARs in the late-pregnant rat myometrium. The rat model will be useful in the future to determine the exact role of the β_3 -ARs in inhibiting the preterm myometrial contractions associated with intrauterine and/or systemic inflammation or diabetes mellitus. The inhibitory efficacy of BRL 37344 on smooth muscle contractions was decreased after its combination with progesterone. We conclude that progesterone is able to affect the β_3 -AR signal transduction process in the opposite way as compared with its well-known effect on the β_2 -ARs. Our results tend to suggest that the combination of progesterone and a β_3 -AR agonist might be detrimental

in putative future therapeutic use. The significance of this phenomenon should be fully clarified in further investigations of the β_3 -ARs in human pregnant myometrial samples.

Materials and Methods

Housing and handling of the animals

The animals were treated in accordance with the European communities council directives (86/609/ECC) and the Hungarian Act for the protection of animals in research (XXVIII.tv.32.§). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV/1813-1/2002). Sprague-Dawley rats (Charles-River Laboratories, Isaszeg, Hungary) were kept at $22 \pm 3^\circ\text{C}$; the relative humidity was 30–70% and the light:darkness cycle was 12 h:12 h. The animals were maintained on a standard rodent pellet diet (Charles-River Laboratories), with tap water available *ad libitum*. The animals were killed by CO_2 inhalation.

Mating of the animals

Mature female (180–200 g) and male (240–260 g) rats were mated in a special mating cage in the early morning; copulation was determined by the presence of a copulation plug or sperm in a native vaginal smear. The day of conception was considered to be the first day of pregnancy.

Unless otherwise specified, substances were purchased from Sigma-Aldrich.

RT-PCR studies

Tissue isolation

Uterine tissues from 18, 20, 21 and 22-day-pregnant animals were rapidly removed, frozen in liquid nitrogen and then stored at -70°C until total RNA extraction.

Total RNA preparation

Total cellular RNA was isolated by extraction with acid guanidinium thiocyanate-phenol-chloroform by the procedure of Chomczynski & Sacchi (1987). After precipitation with isopropanol, the RNA was washed thrice with ice-cold 75% ethanol and then dried. The pellet was resuspended in 100 μl DNase- and RNase-free distilled water. The RNA concentrations of the samples were determined from their absorbances at 260 nm.

RT-PCR

The RNA (0.5 μg) was denatured at 70°C for 5 min in a reaction mixture containing 20 μM oligo (dT) (Invitrogen), 20 U RNase inhibitor (Invitrogen), 200 μM dNTP in 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 5 mM MgCl_2 in a final reaction volume of 20 μl . After the mixture had been cooled to 4°C , 20 U MMLV reverse transcriptase (Invitrogen) was added, and the mixture was incubated at 37°C for 60 min.

The PCR was carried out with 5 µl cDNA, 25 µl ReadyMix Taq PCR reaction mix, 2 µl 50 pM sense and antisense primers of the β₃-AR (Table 3) and 16 µl DNase- and RNase-free distilled water.

Rat β-actin primers were used as internal controls in all samples (Table 3). The PCR was performed with a PCR Sprint thermal cycler (Hybaid Corp., Ashford, UK). After the initial denaturation at 95 °C for 5 min, the reactions were taken through 31 cycles for β₃-AR: 60 s at 95 °C, 60 s at a coupling temperature of 56 °C and 60 s at 72 °C, followed by lowering of the temperature to 4 °C. This PCR protocol furnished optimised conditions and linear phase amplification for the primer set employed. The optimum number of cycles for the applied primers was determined by performing kinetic analyses.

The RT-PCR products were separated on 2% agarose gels, stained with ethidium bromide and photographed under a u.v. transilluminator (Kodak EDAS290, Csrtex Ltd, Budapest, Hungary). The amount of PCR products in each sample was measured by fluorometric assay using the Qubit fluorometer (Csrtex Ltd). For statistical evaluations, data were analysed by a one-way ANOVA followed by Neuman–Keuls post test.

Western blotting studies

Uterine membrane fraction was prepared as described by Chernogubova *et al.* (2005) with some modifications. Samples were homogenised in an ice-cold homogenisation buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% Triton-X 100. After the centrifugation procedure each sample was resuspended in 100 µl homogenisation buffer and 20 µl Protease Inhibitor Cocktail, and 15 µl 200 mM phenylmethylsulphonyl fluoride was added to each.

Twenty micrograms of protein per well were subjected to electrophoresis on 10% sodium dodecylsulfate polyacrylamide gels in X-Cell Sure Lock (Invitrogen). Proteins were transferred from gels to nitrocellulose membranes, using a semidry blotting technique (Hoefer Pharmacia Biotech, San Francisco, CA, USA). The membranes were blocked with 5% (w/w) non-fat dry milk in Tris saline buffer (50 mM Tris, pH 7.4, 200 mM NaCl) containing 0.1% (w/v) Tween 20 for 3 h at 4 °C on a shaker. After washing, the blots were incubated overnight at 4 °C with β₃-AR and β-actin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:10 000) in the blocking buffer. Immunoreactive bands were visualised with the Millipore Immobilon Western Chemiluminescent Reagent (Biocenter Ltd, Szeged, Hungary) and photographed with a Kodak Image Station 2000R (Csrtex Ltd). After densitometric quantification of the bands, data were analysed by a one-way ANOVA followed by Neuman–Keuls post test.

Isolated tissue studies

Uterine rings were dissected from the horns of pregnant rats. Two muscle rings were sliced per horn of the uterus and mounted vertically in a tissue bath containing 10 ml de Jongh buffer (137 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 12 mM NaHCO₃, 4 mM Na₂HPO₄ and 6 mM glucose, at pH 7.4). The temperature of the tissue bath was set to and maintained at 37 °C, and O₂ containing 5% (v/v) CO₂ was perfused continuously through the bath. Tissue samples were equilibrated under these conditions for 90 min before the experiments were started. The tensions of the myometrial rings were measured with a strain gauge transducer and recorded with an Isosys Data Acquisition System (Experimetria Ltd, Budapest, Hungary). The initial tension of the uterus rings was set to 1.5 g, which dropped to ~0.5 g by the end of the equilibration period. The tones of the isolated uterine rings were very similar at the end of the incubation period; their average initial tension was 0.542 g±0.045. During the equilibration period, the buffer in the chambers was changed every 15 min. With this method, we measure the overall contractions generated by both the circular and the longitudinal layers of the rat myometrium.

Cumulative dose–response curves were constructed for BRL 37344 in the concentration range of 10^{−11}–10^{−5} M (a total of seven doses). The chamber contained metoprolol (10^{−6} M) to block the β₁-ARs, and ICI 118 551 (10^{−6} M) to inhibit the β₂-ARs (Yurtcu *et al.* 2006). At the beginning of the experiment, 25 mM KCl was added to the chamber and the evoked contractions were recorded for 5 min. Concentration–response curves were fitted and areas under curves (AUCs) were evaluated and analysed statistically with the Prism 4.01 (GraphPad Software, San Diego, CA, USA) computer program. From the AUCs, the following values were calculated; the maximum inhibitory effect of BRL 37344 on a given day of pregnancy (*E*_{max}), and the concentration of BRL 37344 eliciting 50% of the maximum inhibition of uterine contractions (*EC*₅₀). For statistical evaluations, data were analysed by a one-way ANOVA followed by Neuman–Keuls post test.

Detection of myometrial cAMP

Myometrial cAMP accumulation was measured with a commercial cAMP enzyme immunoassay kit (Sigma–Aldrich). Briefly, samples containing cAMP, alkaline phosphatase conjugated with cAMP and a polyclonal rabbit antibody of cAMP are simultaneously incubated at room temperature in a secondary antibody coated microwell plate. The excess reagents are then washed away and *p*-nitrophenyl phosphate,

Table 3 Primer pairs used for amplification of the β₃-adrenergic receptor (β₃-AR) and β-actin, the GenBank access numbers and the length of PCR products.

Receptor	Primer sequence	GenBank access no.	Product size (bp)
β ₃ -AR	5'-ATC ATG AGC CAG TGG TGG CGT GTA G-3' 5'-GCG ATG AAA ACT CCG CTG GGA ACT A-3'	NM_013108	473
β-Actin	5'-ATC GTG GGC CGC CCT AGG CAC-3' 5'-TGG CCT TAG GGT TCA GAG GGG C-3'	NM_031144	313

a substrate of alkaline phosphatase, is added to the reaction mixtures. The *p*-nitrophenol generated can be determined via its yellow colour at 405 nm. The more intense the colour, the lower the amount of intracellular cAMP.

Uterine tissue samples from 22-day-pregnant rats were incubated in an organ bath (10 ml) containing de Jongh buffer (37 °C, perfused with carbogen). Isobutylmethylxanthine (10^{-3} M), metoprolol (10^{-6} M) and ICI 118 551 (10^{-6} M) were incubated with the tissues for 20 min. During control experiments SR 59230A (10^{-6} M) was also added to the samples for 20 min. BRL 37344 (10^{-7} M) was then added for 10 min. At the end of the BRL 37344 incubation period, forskolin (10^{-5} M) was added for another 10 min, as described by Roberts *et al.* (1998). After this, the samples were immediately frozen and stored in liquid nitrogen until cAMP extraction. The tissue samples were next ground under liquid nitrogen, weighed, homogenised in 10 volumes of ice-cold 5% (v/v) trichloroacetic acid and centrifuged at 600 g for 10 min. The supernatant was extracted with three volumes of water-saturated diethyl ether. After drying, the extracts were stored at -70 °C until the cAMP assay. The cAMP content was expressed in pmol/mg tissue. For statistical evaluations, data were analysed by an unpaired Student's *t*-test.

Progesterone treatment of pregnant rat

The progesterone treatment of the pregnant animals started on day 15 of pregnancy. Progesterone was dissolved in corn oil and injected subcutaneously every day up to day 21 of pregnancy at an amount of 0.5 mg in 0.1 ml (Gáspár *et al.* 2005). On day 22, the studies were carried out as described above. The experimental data on the non-treated and the progesterone-treated animals were analysed by an unpaired Student's *t*-test.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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