

Pregnancy-induced decrease in the relaxant effect of terbutaline in the late-pregnant rat myometrium: role of G-protein activation and progesterone

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Abstract

The effectiveness of β_2 -agonists in preterm delivery is reduced by several factors. The aim of this study was to determine the influence of late pregnancy in the uterus-relaxing effect of terbutaline in the rat *in vitro*.

Rat uterine tissues from late pregnancy (days 15, 18, 20 and 22) were used. *In vitro* electrical field-stimulation (EFS) was used to evoke contractions. The radioligand-binding technique, reverse transcription-polymerase chain reaction and radioimmunoassay technique were used to determine the β -adrenergic receptor density and mRNA level and the plasma sex hormone level, respectively. The activated G-protein level of the β -adrenergic receptors was investigated by a radiolabelled GTP binding assay.

EFS-induced contractions were inhibited by terbutaline. This effect decreased towards term with respect to both the EC_{50} and maximal inhibition values. A drop in plasma progesterone level was also detected. Binding studies revealed an increase in β -adrenergic receptor number on the last day of pregnancy, which correlated with the change in receptor mRNA level. The G-protein-activating effect of terbutaline decreased continuously between days 15 and 20. Surprisingly, terbutaline decreased the G-protein activation to below the basal level on day 22. However, progesterone pretreatment set back the uterine action of terbutaline, increased the density of the β_2 -adrenergic receptors and their mRNA level and increased the G-protein-activating property of terbutaline.

These data provide evidence of a pregnancy-induced decrease in activated G-protein level after β_2 -agonist stimulation.

The decrease in plasma progesterone level has a crucial role in this process. The effects of β_2 -adrenergic receptor agonists in tocolytic therapy may possibly be potentiated with progesterone.

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Introduction

Tocolysis is one of the greatest challenges in obstetrical practice. In spite of tremendous efforts, the incidence of premature labor is still around 5–7% and 12% in developed European countries and the US, respectively. The statistics indicate that preterm birth is the leading factor cause of neonatal morbidity and mortality (Haram *et al.* 2003, Tucker & McGuire 2004).

β_2 -adrenergic receptor (β_2 -AR) agonists are among the most frequently applied tocolytic agents. However, their use in therapy has some disadvantages. They may have several side-effects, such as tachycardia, pulmonary

edema, hypokalemia, sodium retention and glucose intolerance, mainly in consequence of the high doses used for uterus-relaxing action. Additionally, β_2 -agonists can affect the life perspectives of neonates by causing respiratory distress syndrome, intracranial bleeding and neonatal jaundice (Andreassi & Teso 1992, Smigaj *et al.* 1998, Gyetvai *et al.* 1999, Papatsonis *et al.* 2000). On the other hand, the effectiveness of these agents has been the subject of intensive debate in the literature. Some articles claim that most β_2 -mimetics can put off labor for 48–72 h (Katz & Farmer 1999), while others conclude that their duration of action is only 24–48 h (Higby *et al.* 1993). Nevertheless, it has been stated that β_2 -agonist treatment

does not influence the preterm delivery rate and the perinatal outcome (Sciscione *et al.* 1998).

The high doses applied and the ineffectiveness are most frequently attributed to the phenomenon of tachyphylaxis, when the receptor function is lost over time as a result of the continuous or repetitive therapeutic administration of β_2 -agonists (Caritis *et al.* 1987). It is thought that this phenomenon is mainly caused by agonist-promoted β_2 -AR desensitization, which is partially controlled by β -adrenergic receptor kinase and the estrogen/progesterone levels (Ruzycky & DeLoia 1997). Recently, the role of G-protein coupled receptor kinases in this process was also suggested (Simon *et al.* 2001, Simon *et al.* 2003).

Interestingly, some earlier findings suggest that pregnancy itself may alter the myometrial action of adrenergic drugs. It was found that adrenergic drugs had a lower capacity to inhibit contractions in the mouse uterus at the end of pregnancy (Cruz *et al.* 1990). The number of β -ARs also dropped during the last 7 h before term in rats, which may contribute to the weakening effect of β -AR agonists (Maltier & Legrand 1988). Cohen-Tannoudji *et al.* (1991) demonstrated a pregnancy-dependent uncoupling of β -ARs and a decreased responsiveness of rat myometrial adenylyl cyclase to isoproterenol. These changes were pronounced in the last few hours before delivery in the rat and controlled by progesterone.

These facts led us to plan an extensive study to clarify the role of pregnancy in the rat myometrial response to β -mimetics without any pretreatment with β -agonists. Late pregnant myometrial tissue was stimulated by an electrical field; the level of expression of β_2 -AR protein and transcripts of receptor protein in the uterus were detected by radioligand-binding assay and reverse transcription-polymerase chain reaction (RT-PCR), respectively. The change caused by terbutaline in the G-protein activation of β_2 -ARs was investigated with a [35 S] guanosine-5'-O-(3-thiotriphosphate) ([35 S] GTP γ S) binding assay. The changes in serum levels of sex hormones (17 β -estradiol and progesterone) were measured by means of radioimmunoassay (RIA).

Materials and Methods

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).

Mating of the animals

Mature female (180–200 g) and male (240–260 g) Sprague-Dawley rats (Charles-River Ltd, Budapest, Hungary) were mated in a special mating cage. A metal door, which was movable by a small electric motor, separated the rooms for the male and female animals. A timer controlled the function of the motor. Since rats are usually active at night, the separating door was opened before dawn. Within 4–5 h after the possibility of mating, vaginal

smears were taken from the female rats, and a sperm search was performed under a microscope at a magnification of $\times 1200$. If the smear proved positive, or if smear taking was impossible because of an existing vaginal sperm plug, the female rats were separated and were regarded as first-day pregnant animals.

Uterus preparation and electric field stimulation

Uteri were removed from rats (250–350 g) on day 15, 18, 20 or 22 of pregnancy. Muscle rings 0.5 cm long were sliced from the uterine horns and mounted vertically between two platinum electrodes in an organ bath containing 10 ml de Jongh solution (composition: 137 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 12 mM NaHCO₃, 4 mM NaH₂PO₄, 6 mM glucose, pH 7.4). The organ bath was maintained at 37 °C and carbogen (95% O₂ + 5% CO₂) was bubbled through it. After mounting, the rings were equilibrated for about 1 h before experiments were undertaken, with a solution change every 15 min. The initial tension of the preparation was set to about 1.25 g, which was relaxed to about 0.5 g at the end of equilibration. Maximum rhythmic contractions were elicited with a digital, programmable stimulator (ST-02; Experimetria Ltd Budapest, Hungary, and GraphPad Software Inc. San Diego, CA, USA), as described earlier (Gáspár *et al.* 2001). The tension of the myometrial rings was measured with a gauge transducer (SG-02; Experimetria UK Ltd) and recorded with an SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd). Non-cumulative concentration-response curves to the β_2 -agonist terbutaline (Sigma) were constructed in each experiment. Following the addition of a given concentration of terbutaline, recording was performed for 240 s. After this period, the electric field was switched off and the tissues were washed three times and left to rest for 5 min. Concentration-response curves were fitted and areas under curves (AUCs) were evaluated and analyzed statistically with the Prism 2.01 (GraphPad Software) computer program. Using AUC values, the following two values were calculated: maximal inhibitory effect of terbutaline on a given day of pregnancy (E_{max}) and concentration of terbutaline eliciting 50% of the maximal inhibition of uterine contraction (EC_{50}). For statistical evaluations, data were analyzed by the ANOVA Neuman–Keuls test.

Determination of plasma 17 β -estradiol and progesterone

Blood samples were collected by cardiac puncture immediately before removal of the uteri. After centrifugation (10800 $\times g$, 15 min, 25 °C), the plasma was separated and stored at –20 °C until determination. 17 β -estradiol and progesterone were determined by RIA. Reagent kits were purchased from the WHO Matched Reagent Program (Immunometrics Ltd London, UK). The lower limits of the 17 β -estradiol and progesterone determinations were 30 pM and 0.5 nM, respectively. No cross-

reaction was found in either case. The intraassay and inter-assay coefficients of variation for 17β -estradiol were 7.5% and 14.3% and those for progesterone were 5.2% and 12.7% respectively. Statistical analysis was carried out by the ANOVA Neuman–Keuls test as above.

Radioligand binding assays

Radioligand-binding experiments were carried out on pregnant rat uterus membrane preparations. The uterine tissues were cut and homogenized in buffer (0.01 M Tris–HCl, 0.25 M sucrose, pH 8.0) with an Ultra-Turrax T25 homogenizer (Janke&Kunkel, IKA-Labortechnik GmbH, Straufen Germany), and centrifuged ($20\,000 \times g$, 10 min, 4°C). The supernatants were stored at 4°C and the pellets were resuspended and recentrifuged. After mixing, the supernatants were centrifuged ($50\,000 \times g$, 60 min 4°C). The pellets were resuspended and stored at -70°C .

The reaction mixture contained $100\ \mu\text{l}$ membrane preparation ($\sim 0.5\ \text{mg/ml}$ protein), $100\ \mu\text{l}$ tritiated β_2 -AR selective ligand ($[^3\text{H}]\text{ICI 118551}$; Bilski *et al.* 1983) with a specific activity of $18.8\ \text{Ci/mmol}$ (Tocris Cookson Ltd, Avonmouth, UK), and $100\ \mu\text{l}$ unlabeled ligand (dihydroalprenolol, Sigma) for non-specific binding, or $100\ \mu\text{l}$ incubation buffer (consisting of 0.05 M Tris–HCl, 0.01 M MgCl_2 and 2.5% ethanol, pH 7.42) for total binding. Incubation was started by addition of the membrane suspension and continued in a shaking water bath until a steady state was achieved (30°C , 30 min). At the end of the incubation, the bound radioligand was separated from the residual free radioligand by rapid filtration on a Brandell cell harvester (SEMAT, UK) through Whatman GF/C filters (SEMAT Technical Ltd, St. Albans UK) and washed with $3 \times 10\ \text{ml}$ ice-cold buffer (Tris–HCl, pH 7.42). The bound radioactivity was determined in a HighSafe scintillation cocktail in a Wallac 1409 liquid scintillation counter (Wallac, Turku, Finland).

Saturation analysis of β -ARs was performed with 0.25 – $15\ \text{nM}$ $[^3\text{H}]\text{ICI 118551}$ in the presence or absence of $1\ \mu\text{M}$ unlabeled dihydroalprenolol. Specific binding was determined by subtracting the non-specific binding from the total binding values. All assays were carried out at least 3 times in duplicate and values are given as means \pm S.E.M. In the non-treated and progesterone-treated tissues, the K_d values of $[^3\text{H}]\text{ICI 118551}$ were 5.16 ± 0.51 and 1.95 ± 0.09 , respectively. The amount of β -AR protein (B_{max}) was calculated by Scatchard transformation of saturation curves. Statistical analysis was carried out by the ANOVA Neuman–Keuls test as above.

RT-PCR studies

Tissue isolation

Uterus tissues were removed and dissected in ice-cold saline (0.9% NaCl) containing 2 units/ml of recombinant ribonuclease inhibitor (RNasin; Promega, Southampton,

UK). The tissues were frozen in liquid nitrogen and then stored at -70°C until the extraction of total RNA.

Total RNA preparation

Total cellular RNA was isolated by extraction with guanidinium thiocyanate-acid-phenol-chloroform according to the procedure of Chomczynski & Sacchi (1987). After precipitation with isopropanol, the RNA was treated with RNase-free DNase I (Csertex Ltd, Budapest, Hungary) for 30 min at 37°C , re-extracted with phenol, precipitated with ethanol, washed with 75% ethanol and then resuspended in diethyl-pyrocyanate-treated water and the RNA concentration was determined by optical density measurements at 260 nm (Lightwave S2000 spectrophotometer; WPA Ltd, Cambridge, UK).

RT-PCR

The RNA ($0.5\ \mu\text{g}$) was denatured at 70°C for 5 min in a reaction mixture containing 20 units of RNase inhibitor (Hybaid, Ashford, UK), $200\ \mu\text{M}$ dNTP (Sigma), $20\ \mu\text{M}$ oligo(dT) (Hybaid) in 50 mM Tris–HCl, pH = 8.3, 75 mM KCl and 5 mM MgCl_2 in a final reaction volume of $19\ \mu\text{l}$. After the mixture had been cooled to 4°C , 20 units of M-MLV Reverse transcriptase, RNase H Minus (Promega, UK) was added, and the mixture was incubated at 37°C for 60 min and then at 72°C for 10 min.

PCR was carried out with $5\ \mu\text{l}$ cDNA, $25\ \mu\text{l}$ ReadyMix REDTaq PCR reaction mix (Sigma) and 50 pM sense and antisense primer. Gene-specific PCR primers for the β_2 -AR were synthesized as reported by Engelhard & Lohse (2000). The primer sequences used to amplify the β_2 -AR were 5'-CCT CCT TAA CTG GTT GGG-3' (for the forward primer) and 5'-AGT CTG GTT AGT GTC CTG-3' (for the reverse primer); these primers were anticipated to generate a 372 bp PCR product. A rat glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) probe was used as an internal control in all samples (Tso *et al.* 1985). Amplification took place in a PCR Sprint thermal cycler (Hybaid): after initial denaturation at 95°C for 2 min, the reactions were taken through 27 cycles at 94°C for 45 s, 54°C for 45 s and 72°C for 1 min. After the last cycle, incubation was continued for 5 min at 72°C , followed by lowering of the temperature to 4°C . PCR products were used immediately or stored at -70°C . The PCR products were electrophoresed in 2.0% agarose gels and visualized by performing the electrophoresis on an ethidium bromide-containing gel (Sigma). Densitometric scanning of the gel was performed with the Kodak EDAS290 system (Csertex Ltd). The amounts of PCR products were compared via their optical densities. Statistical analysis was carried out by the ANOVA Neuman–Keuls test as above.

$[^35\text{S}]\text{GTP}\gamma\text{S}$ binding assay

Rat uterus membrane preparations were prepared similarly as for the radioreceptor binding assays, but were more

diluted ($\sim 10 \mu\text{g}$ of protein/sample). The membrane fractions were incubated at 30°C for 60 min in Tris-EGTA buffer (pH 7.4) composed of 50 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl_2 , 100 mM NaCl, containing $20 \text{ MBq}/0.05 \text{ cm}^3$ [^{35}S]GTP γS (0.05 nM) and increasing concentrations (1×10^{-10} – 1×10^{-5} M) of terbutaline in the presence of excess GDP (30 μM) in a final volume of 1 ml, according to Sim *et al.* (1995) and Traynor & Nahorski (1995), with slight modifications. Non-specific binding was determined with 10 μM GTP γS and subtracted. Bound and free [^{35}S]GTP γS were separated by vacuum filtration through Whatman GF/B (Whatman Inc. USA filters with a Millipore manifold (Millipore Ltd, Hungary). Filters were washed with 3×5 ml ice-cold buffer, and the radioactivity of the dried filters was detected in a toluene-based scintillation cocktail in a Wallac 1409 scintillation counter (EG&G Wallac, Finland). E_{max} and EC_{50} values were calculated for G-protein activation of terbutaline; E_{max} : maximal [^{35}S]GTP γS binding – and hence stimulating effect of terbutaline – on a given day of pregnancy expressed as a percentage change in the basal [^{35}S]GTP γS -binding value (without terbutaline), EC_{50} : stimulating concentration of terbutaline eliciting half the maximal [^{35}S]GTP γS binding on day 20 (the highest value from all investigated days) of pregnancy.

Progesterone treatment of pregnant rats

The progesterone treatment of the non-ovariectomized rats was started with pregnant animals on day 15 of pregnancy. Progesterone was dissolved in corn oil and injected subcutaneously every day up to day 21 at a concentration of 0.5 mg/0.1 ml. On day 22, the uterine and blood samples were collected and the contractility and molecular pharmacological studies were carried out as described above.

Results

Results on non-treated uteri

The electrical field-stimulated contractions on days 15, 18, 20 and 22 of pregnancy were inhibited by terbutaline in a concentration-dependent manner. The concentration-response curves exhibited continuous rightward shift towards term (Fig. 1). The calculated EC_{50} values of terbutaline gradually increased from day 15 to day 22; Only on day 18 was the change not statistically significant at $P > 0.05$, as compared with the situation on the previous investigated day. In parallel, a decrease was found in the calculated maximal inhibitory effects of terbutaline, with statistically significant differences on days 20 and 22 (Table 1).

The plasma level of 17β -estradiol doubled from day 15 to day 18 and then remained unchanged until labor (Fig. 2, continuous line). The progesterone plasma level declined from day 15, with a plateau phase between days 18 and

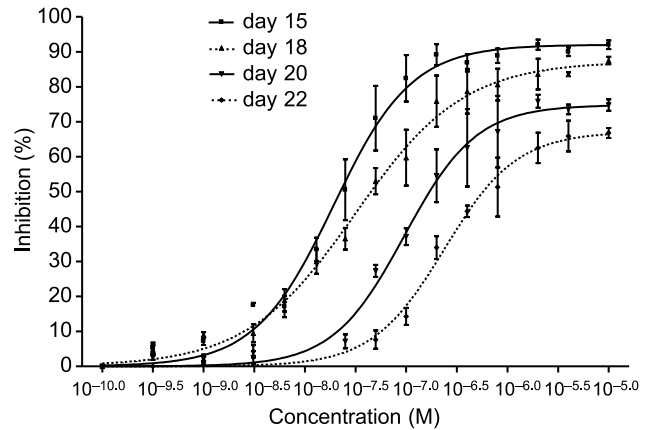


Figure 1 Changes in inhibitory effect of terbutaline on electrical field-stimulated uterine contractions in late pregnant rat *in vitro*. The 22nd day is the last day of pregnancy.

20. It dropped dramatically on day 22, resulting in an extremely low plasma concentration (Fig. 2, dashed line)

RT-PCR with β_2 -AR-specific primers resulted in the identification of β_2 -AR mRNA in the myometrial tissue. As predicted on the basis of the reported cDNA sequence for the β_2 -receptor, the size of the PCR product was 372 bp (Fig. 3a). The histograms demonstrate the changes in β_2 -AR mRNA as compared with GAPDH. There was an increase in uterine β_2 -AR mRNA between days 18 and 20, the level then remaining unchanged until day 22 (Fig. 3b). In radioligand-binding studies, the number of β -ARs was found to be unchanged until day 20, but a significant elevation was detected on day 22 (Fig. 3c).

Terbutaline was able to stimulate the [^{35}S]GTP γS binding through the β_2 -ARs on day 15, 18 and 20. The stimulatory curves were shifted to the right toward term, without significant changes in their maximal value. Although the maximal value was almost double on day 20 as compared with day 15, the difference was not significant because of the high standard error of the mean. On day 22, however, terbutaline elicited a decline in the GTP γS binding, decreasing it to below the basal level from a concentration of 1×10^{-8} M (Fig. 4; Table 2).

A strong correlation was found between the maximal inhibitory value of terbutaline on the electric-stimulated

Table 1 Changes in EC_{50} and maximal inhibitory values of terbutaline on electric field-stimulated uterine contractions in late-pregnant rat *in vitro* ($n = 6$).

Day of pregnancy	EC_{50} ($\times 10^{-8}$ M \pm S.E.M.)	E_{max} (% \pm S.E.M.)
15	2.4 ± 1.7	92.0 ± 1.4
18	3.8 ± 1.3 ns	87.3 ± 4.2 ns
20	23.5 ± 5.8 ***	74.8 ± 3.9 *
22	82.4 ± 17.9 ***	67.1 ± 1.3 **

The level of significance indicated in brackets relates to the comparison with value on the previous investigated day. S.E.M.: standard error of mean; ns: not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

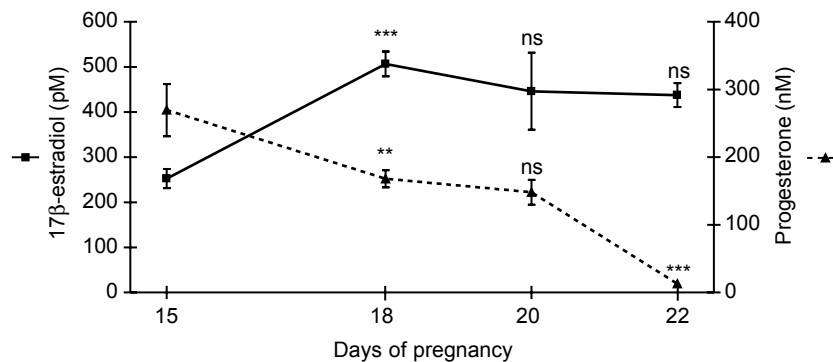


Figure 2 Changes in plasma sex hormone levels during late pregnancy in the rat. Hormone concentrations were determined by radioimmunoassay. Solid line, progesterone; Dashed line; 17β-estradiol. In the statistical analysis, the results were compared with the concentration on the previous investigated day (day 18 vs day 15; day 20 vs day 18; day 22 vs day 20). ns: not significant, ** $P < 0.01$, *** $P < 0.001$.

pregnant uterine contractions and the plasma levels of progesterone on days 15–22 of pregnancy (Fig. 5).

Results on progesterone-treated uteri

The progesterone treatment between pregnancy days 15 and 21 increased the progesterone plasma level (Fig. 6a), but did not alter the level of 17β-estradiol (Fig. 6b) in the 22-day pregnant rat.

The contraction-inhibiting effect of terbutaline was increased by progesterone treatment in the 22-day pregnant rat. The concentration-response curve was shifted to the left (EC_{50} : $3.1 \times 10^{-8} \pm 1.4 \times 10^{-8}$ M) and its maximum was also increased (E_{max} : $76.3 \pm 4.8\%$), although the slope of the curve was not so steep (Fig. 7).

The β_2 -AR mRNA level was slightly increased by progesterone treatment on day 22 (Fig. 8a and b). A similar result was found for the density of the β_2 -ARs (Fig. 8c).

The stimulation of β_2 -ARs with terbutaline followed by progesterone treatment enhanced the number of activated [35 S]GTP γ S molecules in the 22-day pregnant myometrium samples. The action of terbutaline on [35 S]GTP γ S binding was reversed (E_{max} : $15.6 \pm 3.7\%$) as compared with that for the non-treated samples (Fig. 9).

Discussion

Preterm delivery is the major determinant of infant mortality and there is a lack of treatment for this condition. The β_2 -ARs play an important role in the regulation of the contractility of the pregnant uterus. They are still one of the main targets in tocolytic therapy, although the therapeutic significance of their agonists in premature labor is constantly questioned, mainly because of the high doses required at the end of pregnancy. The high maternal risk of these compounds is also a disadvantage as compared with other tocolytics (e.g. calcium channel blockers, oxytocin antagonists, non-steroidal anti-inflammatory agents and magnesium). On the other hand, no significantly better group of agents has yet been found for tocolysis than β_2 -agonists (Berkman *et al.* 2003), although the

side effects of the oxytocin antagonist atosiban seem more favorable than that of β_2 -agonists (Moutquin *et al.* 2000, Worldwide Atosiban versus Beta-agonists Study Group 2001).

In the present study, we sought an answer as to whether the process of pregnancy itself is responsible or not for the changes in the pharmacological action of β -mimetics. In electric field stimulation studies, we demonstrated that the terbutaline potency and efficacy were decreased toward term. This means that more advanced pregnancy results in a weaker action of terbutaline on myometrial contractions.

In a search for the cause of this phenomenon, sex hormone levels were determined. We found an increasing 17β-estradiol predominance toward the end of pregnancy with the well-known dramatic drop in progesterone level at the end of the gestation period (Fuchs & Fields 1998). It has been reported that an estrogen dominance results in an increased sensitivity of the α -adrenergic receptors, while a progesterone predominance increases the β_2 -AR synthesis during pregnancy (Riemer *et al.* 1987, Roberts *et al.* 1989). Other experiments, however, proved that estrogen pretreatment enhanced the amount of β_2 -AR mRNA in the non-pregnant rat uterus. This effect of estrogen was not altered by concomitant progesterone treatment. It was concluded that the process of β_2 -AR function desensitization was independent of the sex steroids (Engstrom *et al.* 2001). Our experiment revealed increased levels of β_2 -AR mRNA and β_2 -AR on days 20 and 22, respectively. This result reaffirms the earlier findings that the estrogen predominance should not necessarily cause a decrease in the synthesis of β_2 -AR in the uterus. On the other hand, a decreased number of β_2 -ARs were found earlier in the last 7 hours of pregnancy in the rat myometrium (Maltier & Legrand 1988). Our results do not support this finding, but it should be mentioned that our experiments were carried out before the last 7 h of gestation (~10–12 h) and a β_2 -selective radioligand was used instead of the non-selective tritiated dihydroalprenolol. These differences may cause the discrepancies. Additionally, we have to emphasize that determination of

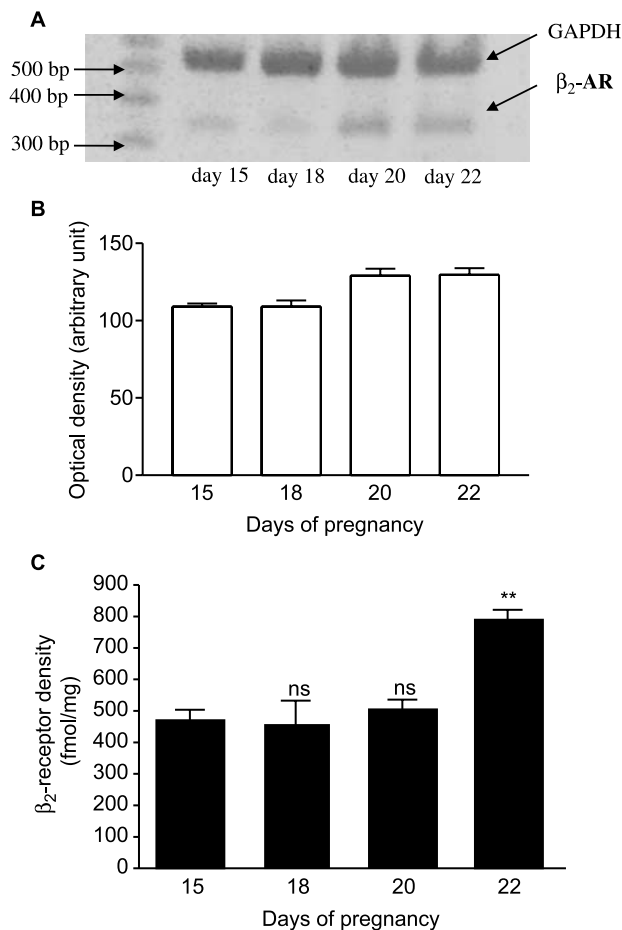


Figure 3 Changes in rat uterine β_2 -adrenergic receptor mRNA and density in late gestation. (A) GAPDH and β_2 -adrenergic receptor (β_2 -AR) reverse transcription-polymerase chain reaction (RT-PCR) products from the rat uterus total RNA on days 15, 18, 20 and 22 of pregnancy. Each band contains RNA from the uterus of one animal. The 372 base pair (bp) RT-PCR products correspond to the mRNA of β_2 -ARs. The histograms (B) demonstrate the optical density of the RT-PCR products which reflect the β_2 -AR mRNA expression level on different days of pregnancy. Values are the mean \pm S.E.M. result of 6 different preparations. (C) Densities were determined with a radioligand-binding technique using rat uterine membrane preparations at different days of pregnancy. Values are mean \pm S.E.M. result of 8 different preparations. Levels of significance were determined by comparison with the density on the previous investigated day (day 18 vs day 15; day 20 vs day 18; day 22 vs day 20). ns: not significant, ** $P < 0.01$.

last 7 h of pregnancy is quite difficult because of the individual variability of the time of delivery in the rat (Maeda *et al.* 2000). Nevertheless, it should be stated that the pregnancy-induced change in the synthesis and number of β_2 -ARs can not be responsible for the continuous decline in terbutaline action toward the end of the gestation period.

As a next step, we took terbutaline-induced G-protein activation into consideration as a possible explanation for its lower activity. The [35 S]GTP γ S binding assay measures

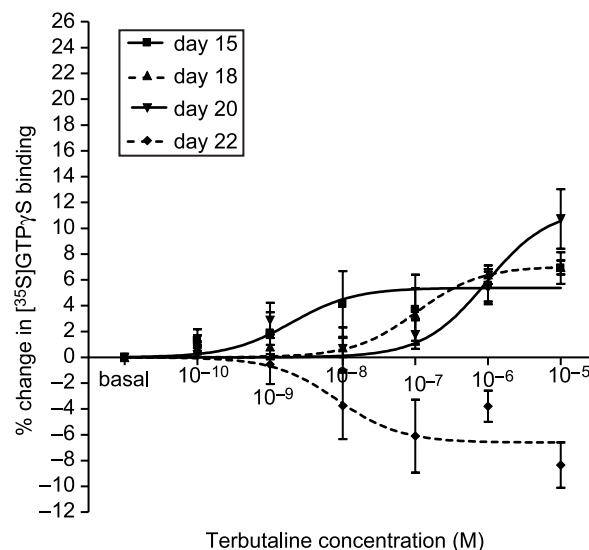


Figure 4 Change in [35 S]GTP γ S binding to rat uterine membrane from days 15, 18, 20 and 22 of pregnancy by various concentrations of terbutaline. Points are means \pm S.E.M. from 3 separate experiments carried out in triplicate. The basal value is that of [35 S]GTP γ S binding without terbutaline stimulation and was regarded as 0.

the level of G-protein activation following agonist occupation of the G-protein-coupled receptor. This method detects the functional consequences of receptor occupancy in one of the earliest receptor-mediated events. In the assay, [35 S]GTP γ S replaces endogenous guanosine triphosphate (GTP) and binds to the α subunit of G-protein (G_α). The γ -thiophosphate bond is resistant to the hydrolysis of G_α by GTPase. The labelled G_α subunits therefore accumulate and can be measured by counting the amount of 35 S incorporated (Harrison & Traynor 2003).

In the [35 S]GTP γ S binding assay, a shift to the right in the EC_{50} values was found between days 15 and 20, with a quite low maximal G-protein activation. The high NaCl content of the reaction mixture may contribute to these low values. On day 22, terbutaline was not able to enhance the basal G-protein activation; moreover, the drug decreased the amount of activated G-protein. Such a

Table 2 Changes in EC_{50} and maximal [35 S]GTP γ S-binding-stimulating effect of terbutaline in late-pregnant rats uterine membranes *in vitro* ($n = 6$).

Day of pregnancy	EC_{50} (M \pm S.E.M.)	E_{max} (% \pm S.E.M.)
15	$3.9 \times 10^{-8} \pm 1.3 \times 10^{-8}$	5.4 ± 3.6
18	$30.1 \times 10^{-8} \pm 9.6 \times 10^{-8***}$	7.0 ± 2.9 ns
20	$74.3 \times 10^{-8} \pm 12.5 \times 10^{-8*}$	10.4 ± 3.1 ns
22	$8.5 \times 10^{-9} \pm 1.3 \times 10^{-9}$	-6.6 ± 2.2

The level of significance indicated in brackets relates to the comparison with the value on the previous investigated day. The values on day 22 reveal an inhibitory action and were not compared statistically with the stimulatory values. S.E.M.: standard error of mean; ns: not significant, * $P < 0.05$, *** $P < 0.001$.

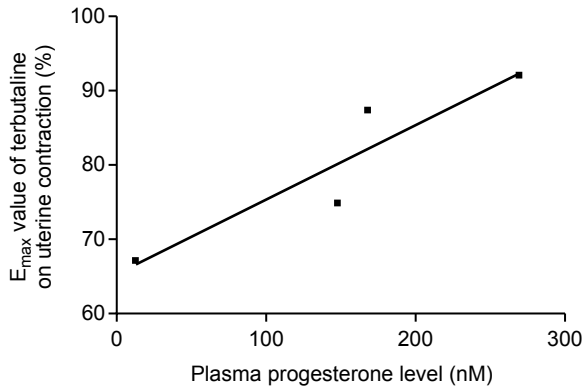


Figure 5 Correlation between the plasma progesterone level and the maximal inhibitory (E_{max}) value of terbutaline on the electric field-stimulated uterine contractions. The fitted regression line shows that these two parameters change in parallel between pregnancy days 15 and 22. The goodness of fit (r^2 value) was 0.858.

G-protein-activating property is characteristic of inverse agonists (Harrison & Traynor 2003); thus, it may be stated that terbutaline behaves as an inverse agonist toward the 22-day pregnant rat myometrium. The contraction-inhibitory action of the drug is still retained on day 22, though the maximal effect is the lowest on this day. We

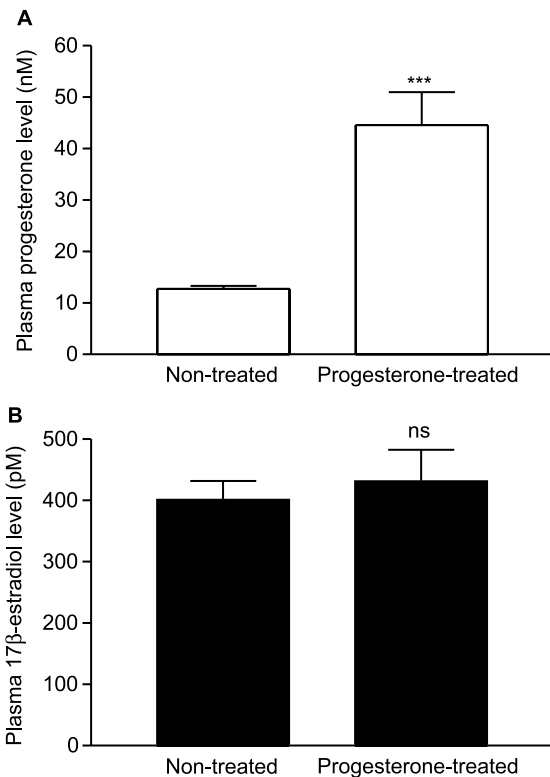


Figure 6 Effects of progesterone treatment on the plasma levels of progesterone (A) and 17 β -estradiol (B) in 22-day pregnant rat. Sex hormone levels were determined by radioimmunoassay. Values are means \pm S.E.M. of the results on 6 different animals. * $P < 0.05$, *** $P < 0.001$.

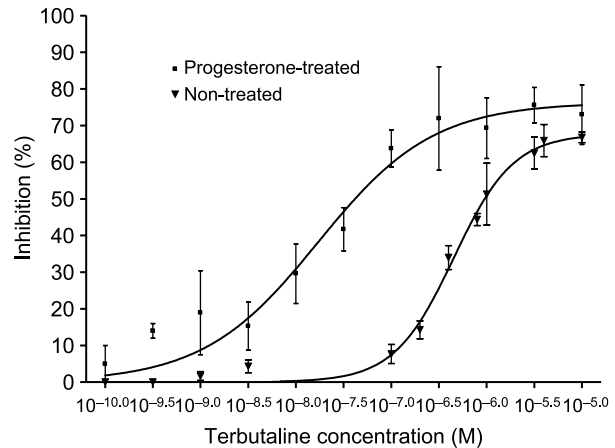


Figure 7 Effects of progesterone treatment on the contraction-inhibiting action of terbutaline in the rat myometrium *in vitro*. Values are means \pm S.E.M. of the results on 6 different animals.

presume that the decreased amounts of activated G-proteins are still sufficient to mediate the relaxant action of terbutaline. Additionally, the decreased G-protein activation may generate an up-regulation in the genetic activity of β -AR regulation in order to maintain cellular receptor homeostasis. This might explain the increase in β -AR mRNA level and protein density at term in parallel with the reduced effect of terbutaline.

In a search for the explanation of the activated G-protein-decreasing effect of terbutaline, we found that the plasma progesterone level and the inhibitory action of terbutaline changed in parallel. Earlier studies suggested that the presence or absence of progesterone can alter the effect of β_2 -AR agonists on the pregnant myometrium (Dowell *et al.* 1994, Engstrom *et al.* 2001). On the basis of the sex hormone levels at the end of pregnancy, the pregnant animals were treated with progesterone for 7 days. This treatment elevated the plasma progesterone level but did not change the level of estrogen. The progesterone supplementation restored the weakened relaxing action of terbutaline on day 22 of pregnancy, the approximate EC_{50} and E_{max} values of terbutaline being reached on days 15–18. Our results clearly demonstrate that the presence of progesterone is a determining factor for the pregnant uterine-relaxing action of terbutaline.

This correlation can be explained by the β_2 -AR density-increasing effect of progesterone. We showed that progesterone treatment caused an elevation in the number of myometrial β_2 -ARs, which was in harmony with the results of others (Hatjisi *et al.* 1988, Vivat *et al.* 1992). On the other hand, progesterone treatment inverted the dose-dependent decrease in the amount of activated G-protein of β_2 -ARs by terbutaline on day 22. Earlier findings suggested that sex hormones play a role in the regulation of G-proteins in the myometrium (Elwady-Merezak *et al.* 1994, Cohen-Tannoudji *et al.* 1995). It was also revealed that an estrogen predominance decreases the β -AR-mediated Gs-proteins

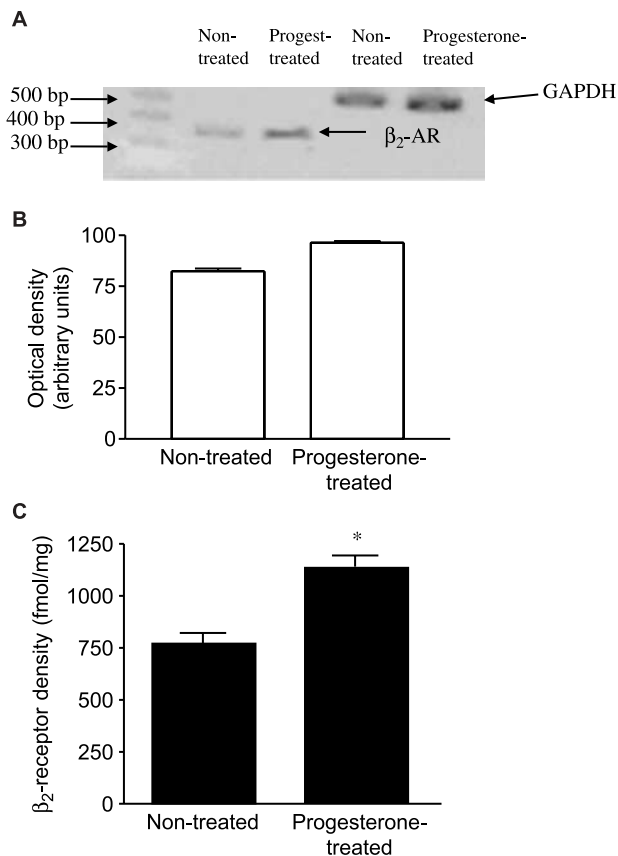


Figure 8 Changes in the rat uterine β_2 -adrenergic receptor mRNA and in the density in 22-day pregnant rat after progesterone treatment. (A) GAPDH and β_2 -AR RT-PCR products from the total RNA on day 22 of pregnancy from non-treated and progesterone-treated uteri. Each band contains RNA from the uterus of one animal. The 372 base pair (bp) RT-PCR products correspond to mRNA of β_2 -ARs. The histograms (B) demonstrate the optical densities of RT-PCR products, which reflect the β_2 -AR mRNA expressions without and with progesterone pretreatment. (C) Changes in pregnant rat uterine β_2 -receptor density on progesterone treatment. Densities were determined by a radioligand-binding technique, using rat uterine membrane preparations from non-treated and progesterone-treated rats. Values are means \pm S.E.M. of the results on 8 different animals. * $P < 0.05$.

and the cAMP level and progesterone treatment increases the number of G-protein coupled receptors (Riemer *et al.* 1988, Nimmo *et al.* 1995). Our data clearly indicate that a higher progesterone level means better G-protein activation and a stronger inhibitory action of terbutaline on late pregnant myometrial contractions.

Accordingly, we conclude that the decrease in terbutaline action in late pregnancy is caused by the drop in progesterone plasma level, which results in a significant decrease in the amount of activated G-proteins coupled to β -ARs. These findings suggest that the clinical experience of a setback in the tocolytic effect of β_2 -agonists in late pregnancy is not merely a consequence of drug-induced desensitization, but may also be a result of a pregnancy-induced decrease in the signaling mechanism of the

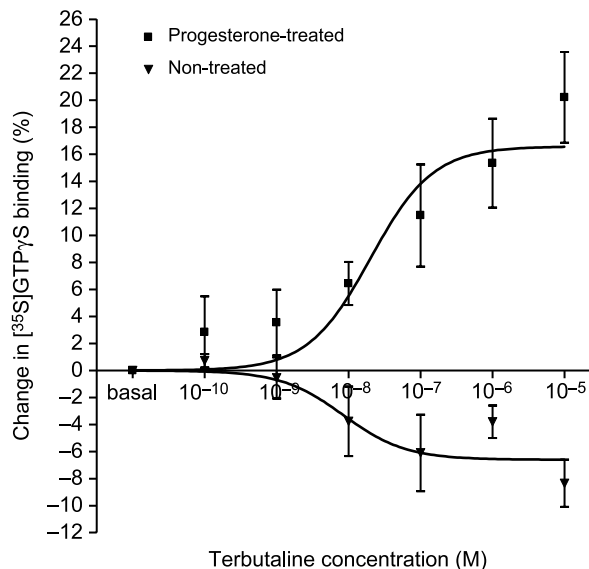


Figure 9 Effects of progesterone treatment on the [35 S]GTP γ S binding to rat uterine membrane from day 22 of pregnancy by various concentrations of terbutaline. Points are means \pm S.E.M. of the results of 3 separate experiments carried out in triplicate. The basal value is that of [35 S]GTP γ S binding without terbutaline stimulation and was regarded as 0.

β_2 -ARs. The use of progesterone and its analogs has recently been reconsidered in the prevention of preterm delivery in humans (da Fonseca *et al.* 2003, Meis *et al.* 2003, Einstein & Bracero 2004, Tita & O'Day 2004). We presume that the effects of β_2 -AR agonists in tocolytic therapy might be stronger by a combination with progesterone, although the progesterone level is not dramatically dropped at the end of human pregnancy. Such a combination may provide better pharmacological targets for β_2 -AR agonists in advanced pregnancies. The significance of these experimental findings, however, should be validated in clinical trials.

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