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ORIGINAL ARTICLE

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Terbutaline increases the cervical resistance of the pregnant rat in vitro

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Abstract Cervical ripening is a crucial process leading to delivery. Early dilation of the pregnant cervix can contribute to premature labour. The maturity of the cervix can be characterized by its resistance to mechanical stretching. Although a number of compounds are considered to increase cervical resistance (e.g., progesterone, nitric oxide synthase inhibitors and nonsteroidal anti-inflammatory drugs), none of them seem to be safe for clinical application. Other compounds, such as β_2-adrenergic receptor (β_2-AR) agonists, have been used for several decades to stop premature myometrium contractions, but their cervical action has never been investigated. The aim of this study was to detect the effects of the β_2-AR agonist terbutaline on nonpregnant and late-pregnant (day 18, 20, 21 or 22) cervices isolated from Sprague–Dawley rats. Cervical resistance was measured by means of a mechanical stretching test in vitro, the β_2-AR density was determined by Western blot analysis, the β_2-AR mRNA was determined by RT-PCR, while the G-protein activation following cervical β_2-AR stimulation with terbutaline was evaluated via a [^{35}S]GTPγS binding assay.

Terbutaline at 10^{-6} M increased the cervical resistance of the late-pregnant samples in vitro from day18 to day 22, but did not alter the resistance of the nonpregnant samples. This cervical resistance-increasing effect was concentration dependent and antagonized with propranolol on day 21. Terbutaline was ineffective on cervical samples when gradual stretching was omitted. RT-PCR and Western blot studies revealed increased β_2-AR mRNA and β_2-AR levels respectively on day 18 of pregnancy compared with the nonpregnant cervix, but no further changes were detected up to the end of pregnancy. The [^{35}S]GTPγS binding assay demonstrated a decreased G-protein activation on the days of pregnancy investigated, but no activation was found in the nonpregnant samples. The degree of decrease in G-protein activation by terbutaline was in harmony with its cervical resistance-increasing action. On day 21, the G-protein activation-decreasing effect of terbutaline was antagonized with propranolol.

We presume that the cervical resistance-increasing effect of terbutaline is a consequence of its G-protein activation-decreasing property via β_2-ARs, which finally leads to an increased muscle resistance against mechanical stretching. This action of terbutaline seems unique among the smooth muscles, and may open up a new perspective in the prevention of premature labour. Clinical experience indicates that β_2-AR agonists will not be sufficient to stop the overall process, but their combination with more potent inhibitors of uterine contractions may be of clinical benefit.

Keywords β_2-adrenergic receptors · Cervical ripening · Premature labour · Cervical resistance · [^{35}S]GTPγS binding · Pregnancy · Rat · Terbutaline

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Introduction

Cervical ripening is one of the most important initial steps leading to delivery. This process includes the dilation, softening and finally effacement of the cervix (Hayashi 1993). The cervical maturation is controlled by several factors, e.g., progesterone (Bienkiewicz 1995), oestrogens (Wang et al. 2001), prostaglandins (Hertelendy and Zakar 2004), cytokines (Sennstrom et al. 2000), oxytocin (Umscheid et al. 1998), nitric oxide (Vaisanen-Tommiska et al. 2003) and connexins (Lenhart et al. 1999). This process is also crucial in premature labour, when the early uterine contractions are accompanied by softening of the cervical tissue, as a result of the decreased cervical collagen content

(Uldbjerg et al. 1983). As a consequence, premature babies have a lower chance of life and a higher risk of complications such as respiratory distress syndrome (Hulsmann and van den Anker 1997). In spite of tremendous efforts, the prevention of premature birth has not yet been resolved. Clinical practice has some medical tools with which to elongate the time of delivery, but these possibilities are quite limited (Sanchez-Ramos and Huddleston 2003). Theoretically, enhancement of the cervical resistance could be one aim of tocolytic therapy, besides the inhibition of premature contractions of the myometrium. Among the drugs most frequently used for tocolysis (the prevention of premature labour) are the β_2-adrenergic receptor (β_2-AR) agonists or betamimetics (Gyetvai et al. 1999). These compounds can decrease the uterine activity in the third trimester of pregnancy (Souney et al. 1983), but their cervical action is not well known. Some human data suggest that the betamimetics have only slight or insignificant action on cervical ripening (Goeschen et al. 1985; Lewis et al. 1997), while others indicate that the betamimetics inhibit the cervical contractions elicited by noradrenaline in vitro (Bryman et al. 1986). However, no studies have been carried out to investigate the effects of betamimetics on the resistance of the pregnant cervical smooth muscle.

The aim of our study was to investigate the in vitro effects of terbutaline on cervices isolated from nonpregnant and late-pregnant (days 18–22) rats. Cervical resistance was characterized by the tissue extensibility in response to mechanical stretching. The amount of β_2-ARs was determined by Western blot analysis, and the β_2-AR mRNA by means of a reverse transcriptase polymerase chain reaction (RT-PCR). The G-protein activation of the β_2-ARs was investigated via the [^{35}S] guanosine-5′-*O*-(3-thiotriphosphate) ([^{35}S]GTPγS) binding assay.

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 were mated in a special mating cage. A metal door, which was movable by a small electric engine, separated the rooms for the male and female animals. A timer controlled the function of the engine. 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 1,200 times. If the search 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.

Measurement of the cervical resistance

Cervical tissues were removed from nonpregnant and late-pregnant (gestational days 18, 20, 21 or 22) rats. The cervix was defined as the least vascular tissue with two parallel lumina between the uterine horns and the vagina. The two cervical rings were separated and mounted with their longitudinal axis vertically by hooks in an organ bath containing 10 ml de Jongh buffer (in mM: 137 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 12 $NaHCO_3$, 4 NaH_2PO_4, 6 glucose; pH 7.4). The organ bath was maintained at 37°C and carbogen (95% O_2+5% CO_2) was bubbled through it. The lower sides of the cervices were fixed to the bottom of the tissue holders in the organ chambers, while the upper parts were hooked to gauge transducers (SG-02, Experimetria, Budapest, Hungary). After mounting, the rings were equilibrated for about 1 h before experiments were undertaken, with a buffer change every 15 min. The initial tension was set to about 1.00 g.

Cervical resistance was investigated by gradual increasing by the tension in the tissues. The cervices were stretched in incremental steps and allowed to relax for 5 min. After every 5 min the next initial tension was set in the following sequence (in g): 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. The tension was increased manually via the control screw of a gauge transducer. The precise initial tension and the relaxation of the cervices were followed with an online computer, using the SPEL Advanced Isosys Data Acquisition System (Experimetria). The resultant stress–strain curves had saw-tooth shapes (Fig. 1). In the evaluation of the cervical resistance, the initial tension of the cervix was plotted versus the stretch after 5 min. Straight lines were fitted by linear regression and the slopes of the lines were used to express the degree of resistance. A steeper slope reflected higher resistance. Fig. 2 depicts representative straight lines derived from resistance studies on nonpregnant and day 22 pregnant cervices (Fig. 2). When the effects of terbutaline were investigated, 10^{-6} M (10^{-9}–10^{-4} M with or without 10^{-7} M propranolol on day 21) of the drug was added to the organ bath and the cervices were incubated for 5 min. The data were analysed with the Prism 2.01 (GraphPad Software, San Diego, CA, USA) computer program, and the slopes of the fitted straight lines were compared. The ANOVA Neuman–Keuls test was used for statistical evaluations.

When the effect of terbutaline was investigated on basal (2 g precontraction at the beginning of incubation period) and precontracted (5 g precontraction at the beginning of incubation period) cervical tension, cumulative concentration–response curves of terbutaline were constructed in the concentration range of 3×10^{-9}–3×10^{-5} M on 21-day pregnant cervices.

RT-PCR studies

Tissue isolation Cervical tissues from nonpregnant and late-pregnant animals were removed and frozen in liq-

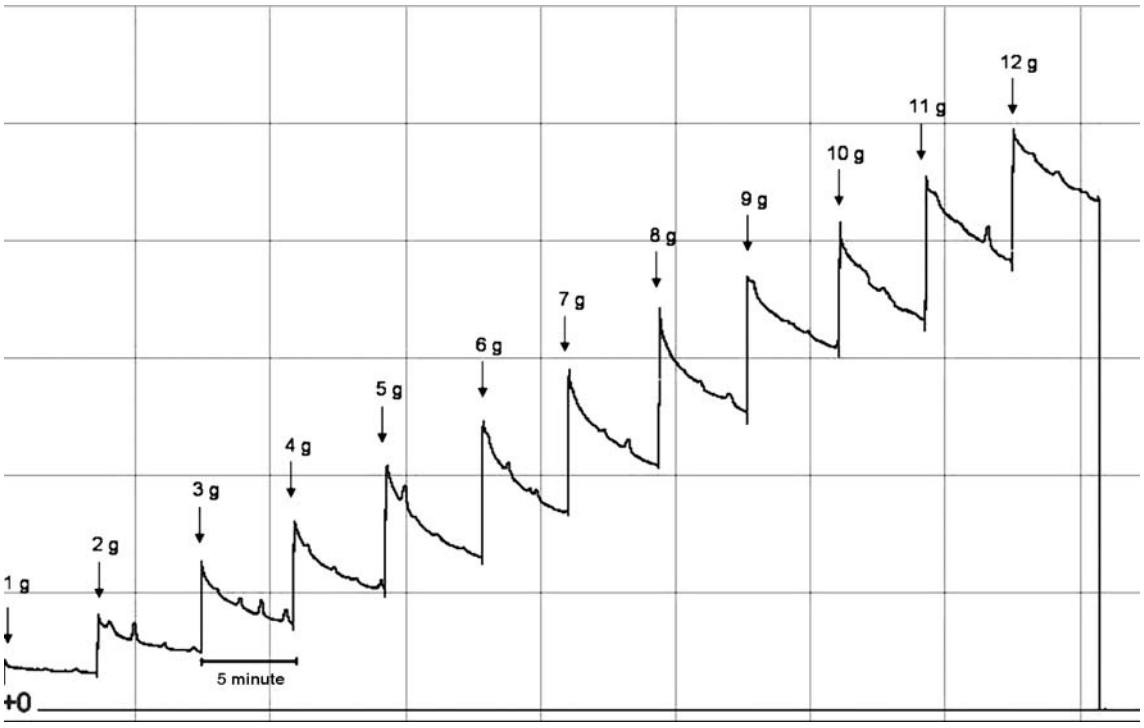

Fig. 1 Representative stress–strain curve of a 22-day pregnant rat cervix in vitro. The cervices were stretched in incremental steps and allowed to relax for 5 min. After every 5 min, the next initial tension value was adjusted. The series of stretching and relaxation resulted in a saw-tooth shape. The initial tensions were plotted against the tensions recorded after 5 min to create regression lines (see Fig. 2).

uid 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 and Sacchi (1987). After precipitation with isopropanol, the RNA was washed three times 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 absorbance 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, Paisley, UK), 20 U RNase inhibitor (Invitrogen) and 200 µM dNTP (Sigma-Aldrich, Budapest, Hungary) in First Strand Buffer (Invitrogen), in a final reaction volume of 20 µl. After the mixture had been cooled to 4°C, 20 U M-MLV reverse transcriptase (Gibco, Paisley, UK) and RNase H Minus (Invitrogen) were added, and the mixture was incubated at 37°C for 60 min.

The PCR was carried out with 5 µl cDNA, 25 µl Ready-Mix REDTaq PCR reaction mix (Sigma-Aldrich), 2 µl 50 pM sense and antisense primer of the β_2-AR subtype and 16 µl DNase and RNase-free distilled water. For the rat β_2-AR cDNA, a 343-bp PCR product resulted with the forward primer 5′-TCT TCG AAA ACC TAT GGG AAC GGC-3′ and the reverse primer 5′-GGA TGT GCC CCT TCT GCA AAA TCT-3′ (Engstrom et al. 2001). A rat GAPDH probe was used as internal control in all samples (Tso et al. 1985). The PCR was performed with a PCR Sprint thermal cycler (Hybaid, Ashford, UK) with the following cycle parameters: after initial denaturation at 95°C for 2 min, the reactions were taken through 27 cycles of 45 s at 95°C, 45 s at 54°C and 1 min at 72°C, followed by lowering of the temperature to 4°C. The RT-PCR products were separated on 2% agarose gels, stained with ethidium bromide and photographed under a UV

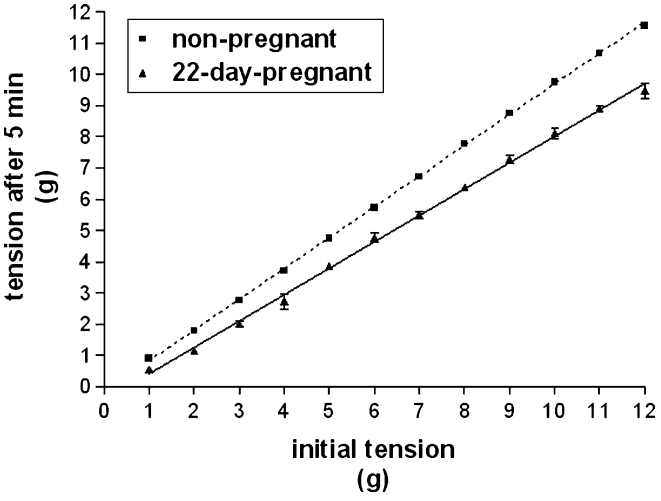

Fig. 2 Representative regression lines fitted to nonpregnant and 22-day pregnant rat stress–strain curves in vitro. *Intermittent* and *continuous lines* are the regression lines from the nonpregnant and the 22-day pregnant cervix respectively. The slopes of the *straight lines* denote the cervical resistance. A steeper slope means a higher resistance. The lower resistance of the 22-day pregnant cervix is a consequence of the ripening process leading to delivery.

transilluminator. Semiquantitative analysis was performed by densitometric scanning of the gel with Kodak EDAS290 (Csertex, Budapest, Hungary). For statistical evaluations, data were analysed by ANOVA with the Neuman–Keuls test.

Western blot analysis

Ten micrograms of protein per well was subjected to electrophoresis on 10% sodium dodecylsulfate polyacrylamide gels in Series Standard Dual Cooled Units (BioRad, Budapest, Hungary). Proteins were transferred from gels to nitrocellulose membranes (Scheicher and Schuell, Dassel, Germany), using a semidry blotting technique (BioRad). The membranes were blocked with 5% nonfat dry milk in Tris saline buffer (in mM: 50 Tris, 200 NaCl; pH 7.4) containing 0.1% Tween, overnight at 4°C. After washing, the blots were incubated for 1 h, at room temperature, on a shaker with β_2-AR polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-570, 1:200) in the blocking buffer. The blots were washed and incubated with 1:5,000 peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology; sc-2004). The antibody binding was detected with an enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK) and exposed on Kodak X-Omat film (Sigma-Aldrich).

[^{35}S]GTPγS binding assay

Rat cervix membrane fractions (≈10 μg of protein/sample) were incubated at 30°C for 60 min in Tris–EGTA buffer (in mM: 50 Tris–HCl, 1 EGTA, 3 $MgCl_2$, 100 NaCl; pH 7.4) containing 20 MBq/0.05 cm^3 [^{35}S]GTPγS (0.05 nM) and increasing concentrations (10^{-10}–10^{-6} M) of terbutaline in the presence of excess GDP (30 pM) in a final volume of 1 ml according to Sim et al. (1995), and Traynor and Nahorski (1995) with slight modifications. On day 21, the experiment was also carried out in the presence of propranolol (10^{-7} M). Nonspecific binding was determined with 10 μM GTPγS and subtracted. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/B filters with a Millipore manifold. Filters were washed with 3×5 ml ice-cold buffer, and the radioactivity of the dried filters was determined in a toluene-based scintillation cocktail in a Wallac 1,409 scintillation counter (Turku, Finland). The percentage stimulation caused by terbutaline was plotted against the concentration of the drug. Dose–response curves were fitted, and the concentrations eliciting half of the maximum effect (EC_{50}) and the maximum effect (E_{max}) were calculated and statistically analysed by means of the ANOVA Neuman–Keuls test.

Results

In the isolated organ bath studies, we found a very limited extensibility of the nonpregnant cervices. In essence, the initial tension remained unchanged (slope~1.00) after 5 min. From day 18, the cervical resistance continuously decreased towards term, reaching a trough value on days 21 and 22 (Fig. 3, grated bars). Terbutaline had no effect on the nonpregnant cervices but elicited cervical resistance-increasing action from day 18 to day 22. This effect was most marked on days 21 and 22 (Fig. 3, black bars). The cervical resistance-increasing effect of terbutaline was concentration dependent within a range of 10^{-9} to 10^{-5} M (Fig. 4a, control) on day 21. This concentration–response curve was shifted to the right in the presence of 10^{-7} M propranolol (Fig. 4a, propranolol; Table 1). Terbutaline had no effect on the basal (Fig. 5a1) and precontracted (Fig. 5b1) cervical muscle tone on day 21 within a concentration range of 3×10^{-9} to 3×10^{-5} M, while the spontaneous activity of the nontreated cervices decreased after 15 min (Fig. 5a2, b2).

The RT-PCR studies revealed an increase in cervical β_2-AR mRNA level on day 18. No further change was detected up to the end of pregnancy (Fig. 6).

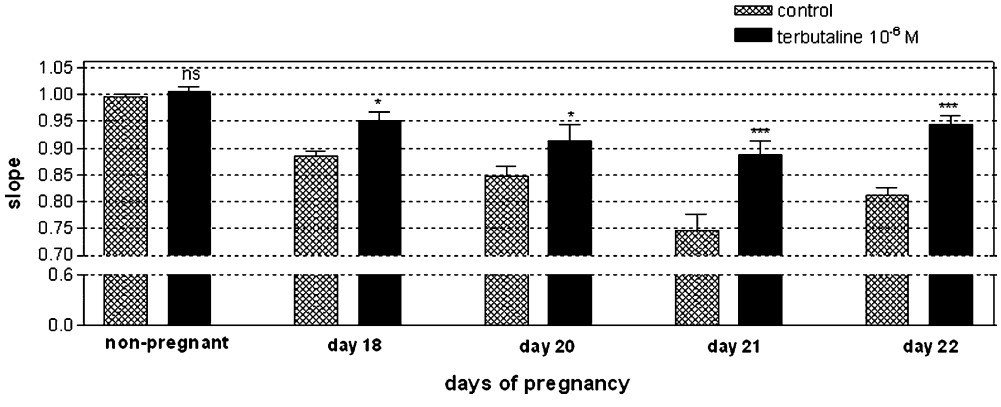

Fig. 3 Effects of terbutaline on the cervical resistance of nonpregnant and late-pregnant rat cervices in vitro (n=8). The resistance is expressed as the slope of the regression lines fitted to the stress–strain curves (see Figs. 1, 2). The Y axis is segmented into two in order to present a higher magnification of the changes in slopes. *Grated bars* show the slopes from nontreated, and *black bars* those from terbutaline-treated cervices in vitro. One each day, the level of significance relates to the comparison with the nontreated (control) sample. *ns* not significant, *p<0.05, ***p<0.001

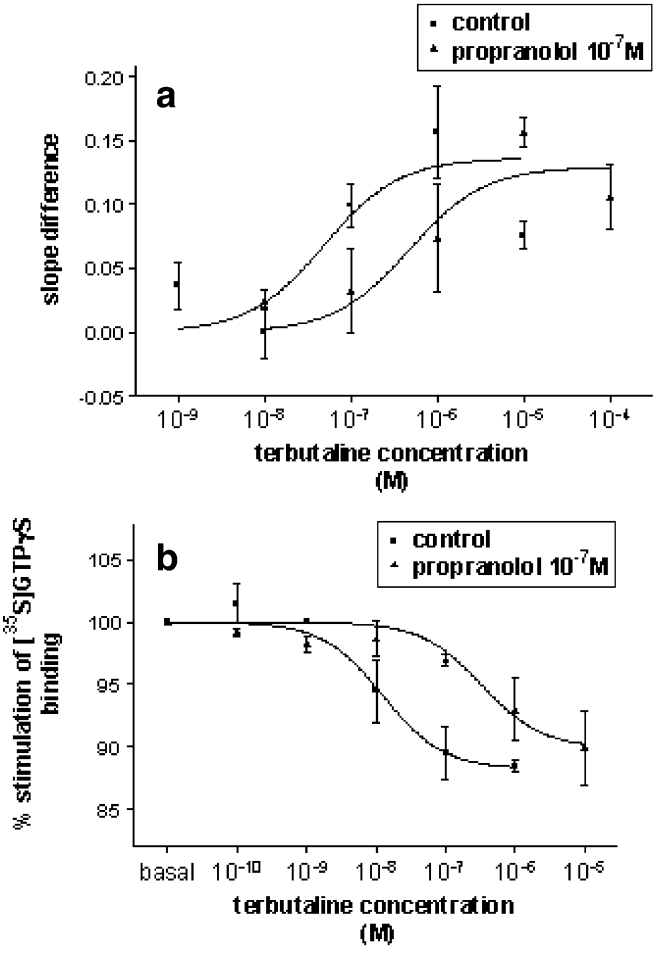

Fig. 4 The effect of propranolol **a** on the cervical resistance-increasing and **b** G-protein-activating action of terbutaline in 21-day pregnant cervices (number of independent experiments = 6; number of animals used for each curve = 12). **a** The effect of terbutaline on cervical resistance was expressed as slope difference, which was calculated by the subtraction of the average slope (0.7473) of nontreated samples from the slope of the treated ones. The drug elicited a concentration-dependent increase in slope difference (control curve). This curve was shifted to the right without significant change in its maximum value in the presence of 10^{-7} M propranolol (propranolol curve; see also Table 1), suggesting the β-adrenergic receptor-mediated character of terbutaline action on cervical resistance. **b** Terbutaline decreased the [^{35}S]GTPγS binding of 21-day pregnant cervix membranes in a concentration-dependent manner (control curve). Basal refers to the value of [^{35}S]GTPγS binding without terbutaline. Nonspecific binding (13% of specific binding) was determined by subtracting the binding in the presence of 10 μM unlabelled GTPγS from the total (nonstimulated) value. Data are given as percent stimulation/inhibition over the basal (nonstimulated, taken as 100%) level. Typical specific binding values representing constitutive G-protein activity (basal level) was 2,809±206 dpm, while nonspecific binding was found to be 358±102. The terbutaline curve was shifted to the right without significant change in its maximum value in the presence of 10^{-7} M propranolol (propranolol curve; see also Table 2). This suggests that the activated G-protein-decreasing effect of terbutaline is mediated via β-adrenergic receptors.

Western blot analysis pointed to an approximate doubling of the optical density of the β_2-ARs on day 18 compared with the nonpregnant samples. No subsequent change in this elevated optical density was observed up to the day of delivery (Fig. 7).

Terbutaline did not alter the [^{35}S]GTPγS binding in the nonpregnant preparations compared with the basal value. From day 18 to day 22, terbutaline caused not stimulation, but a decline in [^{35}S]GTPγS binding. The curve on day 22 was shifted to the left compared with the earlier days (Fig. 8). The degrees of inhibition of [^{35}S]GTPγS binding were similar on days 18 and 20; higher inhibitions were detected on days 21 and 22 (Table 2). The curve of day 21 was shifted to the right in the presence of 10^{-7} M propranolol (Fig. 4b; Table 2).

Table 1 EC_{50} and E_{max} values of terbutaline cervical resistance-increasing effect on 21-day pregnant rat in vitro (n=6). *EC_{50}* concentration of terbutaline eliciting half of E_{max}, *E_{max}* maximum slope difference increasing effect of terbutaline (slope difference is calculated by the subtraction of the average slope [0.7473] of nontreated samples from the treated ones), *SEM* standard error of mean, *ns* not significant

	EC_{50}±SEM (M)	E_{max}±SEM
Terbutaline	$4.9\times10^{-8}\pm1.3\times10^{-8}$	0.14±0.02
Terbutaline + propranolol 10^{-7} M	$4.8\times10^{-7}\pm1.1\times10^{-7}$**	0.13±0.03 (ns)

**p<0.01

Discussion

The cervical resistance plays a determining role in pregnancy. During pregnancy, the cervix is normally firm and closed. At the end of gestation, however, the cervical tissue becomes softer and dilates as the myometrial contractions increase. The harmonised cooperation of these two parts of the uterus results in safe delivery. In some cases, premature cervical dilation contributes to premature delivery of the foetus (Bouyer et al. 1986), which can impair the chances of the neonate for life. Compounds that increase resistance can be beneficial in the prevention of premature complications, but the number of such compounds is quite limited. Progesterone is the main sex hormone responsible for high cervical resistance; antigestagens accelerate cervical ripening (Chwalisz and Garfield 1994). However, the use of progesterone is not widely accepted for the inhibition of premature labour; little information is available in this respect (Fuchs and Stakemann 1959; Noblot et al. 1991). It is very probable that the diversified actions of progesterone also contribute to its rare use in late pregnancy, although new efforts have been made to take advantage of its clinical benefit (Brancazio et al. 2003). Drugs acting against prostaglandins (nonsteroidal anti-inflammatory drugs—NSAIDs) and nitric oxide (a nitric oxide synthase inhibitor—NOSI) may also have beneficial effects on the cervical resistance in early ripening (Garfield et al. 1998; Shi et al. 2000). NSAIDs have been used in pregnancy, but their adverse effects on the foetus limit their clinical importance (Loudon et al. 2003). NOSIs exert paradox action on the pregnant uterus: they can enhance cervical resistance, but

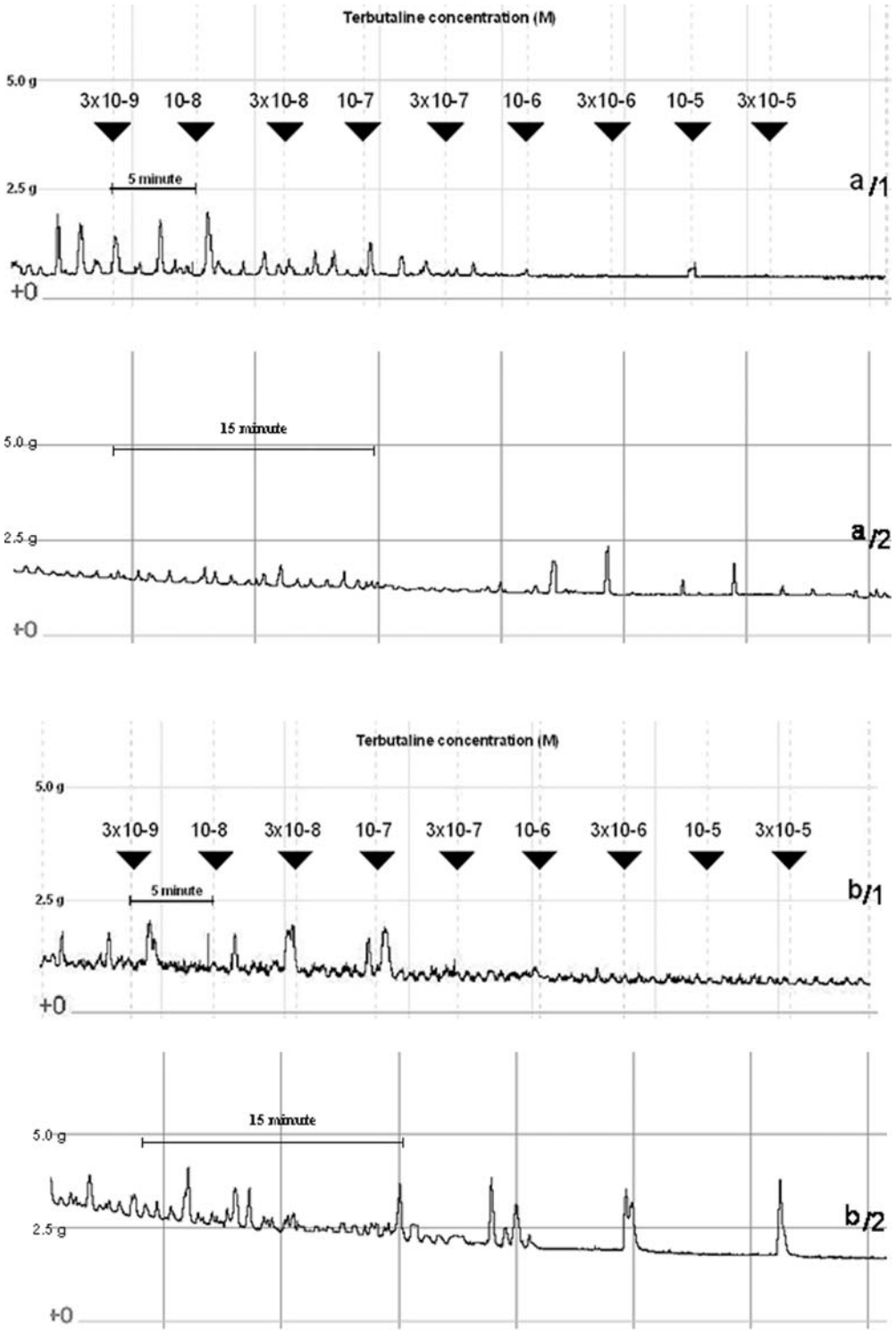

Fig. 5 Effects of terbutaline on the smooth muscle tone in **a** the basal and **b** precontracted cervices on the 21st day of pregnancy in vitro. **a***1* In the basal experiments, the pretension of the cervices was 2 g at the beginning of the incubation period. This tension was decreased to about 0.6–0.8 g after 1 h. The doses of terbutaline were added in every 5 min. The cumulative administration of terbutaline did not alter the cervical tension. Although the cervical sample contractile activity was decreased after the dose of 10^{-7} M, this phenomenon is a natural weakening in cervical contractility after 15 min, as was shown on **a***2* the nontreated sample. **b***1* The pretension of precontracted samples was 5 g and decreased to about 1.2–1.5 g at the end of the incubation period. Terbutaline was not able to increase the cervical tone; however, a further decrease was detected in the first 15 min of the experiment. The contractile response of cervix was also diminished after 15 min, as was also proved on **b***2* the nontreated sample.

they also enhance the myometrial contractions (Maul et al. 2003), diminishing their own potency in tocolysis. On the basis of these facts, it can be claimed that the drugs known to increase the cervical resistance have serious therapeutic disadvantages and/or have not been tested in human pregnancy.

β_2-AR agonists have been used in tocolytic therapy for several decades (Ingegarsson 1976). In spite of the doubts, they are still among the drugs of first choice for this aim, although their advantages over others (calcium channel blockers, NSAIDs, magnesium and ethanol) are continuously questioned (Berkman et al. 2003). While the uterine contractility-decreasing effects of β_2-AR agonists have been studied extensively, no reliable information is available concerning their cervical action. To know more, how-

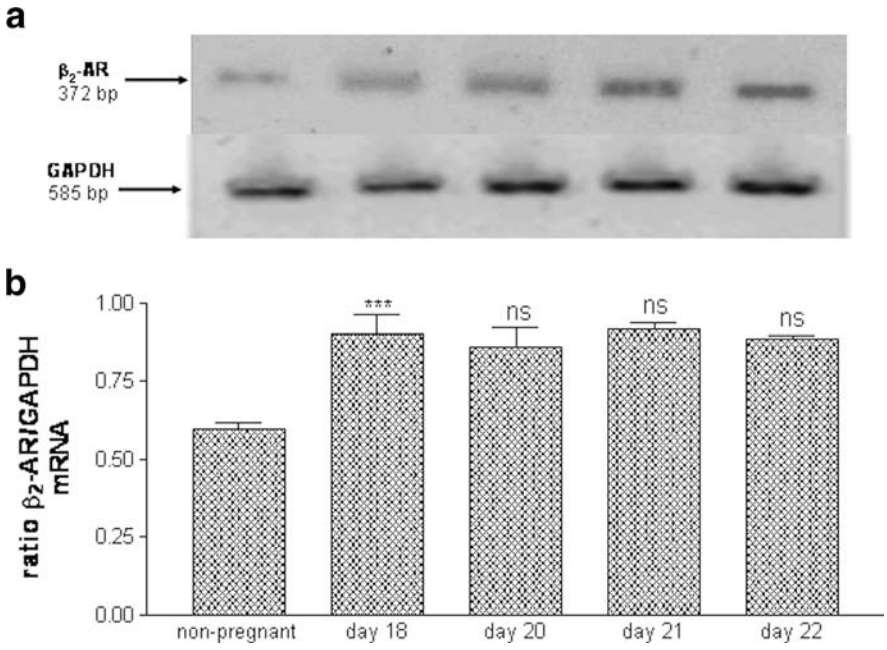

Fig. 6 Change in mRNA level of the β_2-adrenergic receptor in nonpregnant and late-pregnant rat cervices (n=6). **a** The β_2-adrenergic receptor (β_2-*AR*) reverse transcriptase-polymerase chain reaction and GAPDH products from the total cervical RNA of a nonpregnant animal and a pregnant animal on days 18, 20, 21 and 22 of pregnancy. The 372- and 585-base pair (*bp*) PCR products are the mRNA of β_2-ARs and GAPDH respectively. Each band contains RNA from the cervix of one animal. The RT-PCR products were stained with ethidium bromide and photographed under a UV transilluminator. Semiquantitative analysis was performed by densitometric scanning of the gel, and **b** the result was expressed in the ratio of the optical densities of β_2-AR/GAPDH. The level of significance relates to the comparison with the previous investigated day. ***p<0.001

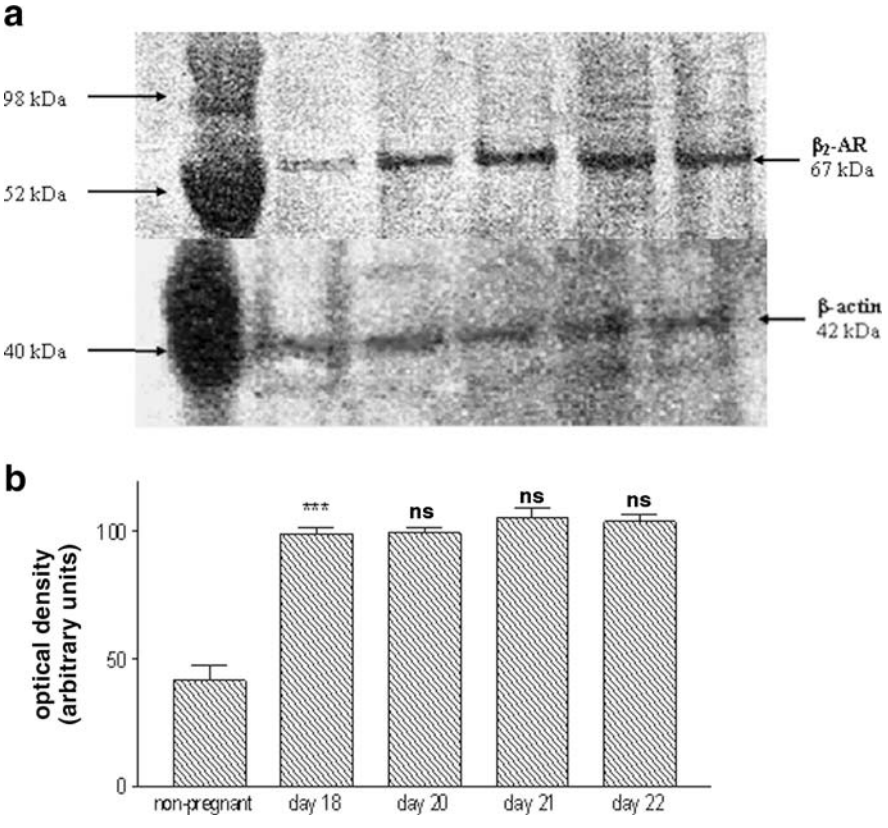

Fig. 7 Change in the β_2-adrenergic receptor (β_2-*AR*) level in nonpregnant and late-pregnant rat cervices (n=6). **a** The β_2-AR and β-actin Western blot products from nonpregnant and 18-, 20-, 21- and 22-day-pregnant rat cervices. The 67- and 42-kDa Western blot products are β_2-ARs and β-actin respectively. Samples were subjected to gel electrophoresis on 10% polyacrylamide gel. The antibody binding was detected with an enhanced chemiluminescence detection system, and **b** expressed as optical density (semiquantitative) data. The level of significance relates to the comparison with the previous investigated day. ***p<0.001

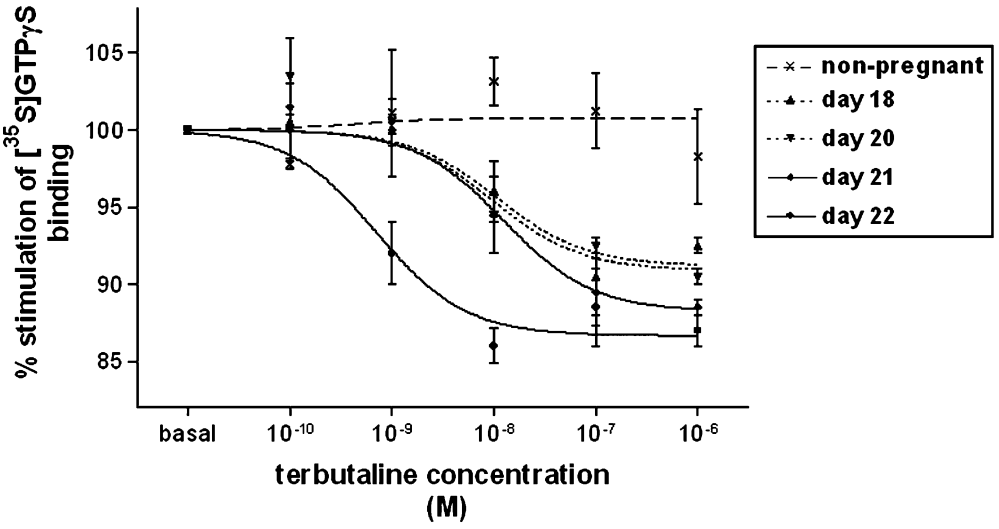

Fig. 8 Change in [^{35}S]GTPγS binding following terbutaline treatment in nonpregnant and late-pregnant rat cervical membranes (number of independent experiment = 6; number of animal used for each curve = 12). The percentage stimulation caused by terbutaline was plotted against the concentration of the drug. Basal refers to the value of [^{35}S]GTPγS binding without terbutaline. Nonspecific binding (10–13% of specific binding) was determined by subtracting the binding in the presence of 10 μM unlabelled GTPγS from the total (non-stimulated) value. Data are given as percentage stimulation over the basal (nonstimulated, taken as 100%) level. Typical specific binding values representing constitutive G-protein activity (basal level) were 2,809±206 dpm on each day. At the highest terbutaline concentration (10^{-6} M) the specific binding values were 2,760±154; 2,598±141; 2,542±151; 2,485±137 and 2,443±129 dpm in nonpregnant, 18-, 20-, 21- and 22-day pregnant cervices respectively. The *declining curves* indicate the decreased G-protein activation following the addition of terbutaline to the cervical membrane preparations.

ever, we first had to investigate the changes in cervical resistance during pregnancy.

In the isolated organ studies, we found that the rat cervical resistance declines towards delivery, a finding in harmony with the results of others (Shi et al. 1999; Vedernikov et al. 2000). Our stretching method was more robust (a 1-g increase in the initial tension every 5 min) than in the previous studies, where the degree of incremental stretching was 0.2 mm/min (Shi et al. 1999). (Although we have no means of determining the exact stretching in millimetres corresponding to a 1-g increase in cervical tension, it seems very probable that a 200-μm change in cervical diameter generates less than a 1-g increase in tension.) A discrepancy may be observed in the slopes of the fitted curves characterizing the cervical resistance. In our experience, the slopes were steeper, reflecting the different method of stretching. Nevertheless, our findings in respect of cervical ripening have the same outcome as that reported earlier, and our method is therefore also considered appropriate to investigate the changes in cervical resistance during pregnancy.

Terbutaline administered in vitro to the organ bath increased the cervical resistance in the late-pregnant rats, but was not active on the nonpregnant samples. On days 18 and 20, this resistance-increasing effect was moderate, but on days 21 and 22 it was pronounced. On these latter days, the measured increases in slope exceeded 0.1, i.e., very high values in our system, the difference in slope between the nonpregnant and the 22-day pregnant non-treated cervices, for example, being 0.18. These results indicate that terbutaline is a very potent agent, eliciting an acute increase in cervical resistance against mechanical stretching.

It is known that the smooth muscle content of the cervix is quite low, and is reduced further by pregnancy-induced apoptosis (Leppert and Yu 1994). Therefore, the possibility that this effect of terbutaline is not a β_2-ARs-mediated action has been considered too. In order to create a

Table 2 EC_{50} and E_{max} values of terbutaline [^{35}S]GTPγS binding-inhibitory effect on late-pregnant rat cervix membrane preparations (n=6)

Day of pregnancy	Terbutaline		Terbutaline + propranolol 10^{-7} M	
	EC_{50}±SEM (M)	E_{max}±SEM (%)	EC_{50}±SEM (M)	E_{max}±SEM (%)
18	$1.1\times10^{-8}\pm0.4\times10^{-8}$	8.8±0.8	–	–
20	$9.6\times10^{-9}\pm1.8\times10^{-9}$ (ns)	9.1±1.0 (ns)	–	–
21	$1.3\times10^{-8}\pm0.5\times10^{-8}$ (ns)	11.8±0.9*	$3.3\times10^{-7}\pm0.8\times10^{-7}$**	10.1±1.9 (ns)
22	$7.1\times10^{-10}\pm0.9\times10^{-10}$***	13.3±1.1 (ns)	–	–

The level of significance in the terbutaline column relates to the comparison with the previous investigated day. The level of significance of E_{max} values on days 21 and 22 was <0.05 and <0.01, compared with nonpregnant values, respectively. The level of significance in the terbutaline + propranolol column relates to the comparison with values of 21-day cervices in the terbutaline column
*p<0.05; **p<0.01; ***p<0.001

concentration–response curve, 21-day pregnant cervical samples were used, because the difference between the nontreated and 10^{-6} M terbutaline-treated resistance was highest on this day. In organ bath studies we proved that the cervical resistance-increasing action of terbutaline is concentration dependent, and this effect can be antagonised with the nonselective β-AR blocker propranolol. We know that 90% of the β-ARs are β_2-ARs in the pregnant rat uterus (Principe et al. 1997); accordingly, we can conclude that the cervical resistance-increasing property of terbutaline is dominantly related to β_2-ARs, although we cannot exclude the roles of β_1-ARs. However, terbutaline was inefficient on the basal cervical muscle tone, when gradual stretching was omitted. The contractile response of the cervix samples was eliminated after 15 min, but—as was proved on nontreated cervical samples—it should rather be considered as a natural consequence of tissue fatigue to contract than as a result of pharmacological action. These results suggest that terbutaline increases the resistance of the pregnant cervix against incremental stretching, but apparently does not influence the smooth muscle basal tone.

We next attempted to find an explanation for this action because this smooth muscle effect of terbutaline was very unusual. First, we determined the change in β_2-AR mRNA by RT-PCR. The receptor synthesis was found to be elevated in the late-pregnant rat cervices compared with the nonpregnant samples, but no differences were detected among the investigated pregnant tissues. The same held true for the β_2-AR protein density determined by Western blot analysis. Although these methods were only semiquantitative (we followed the changes in optical density in both methods), we concluded that the amount of β_2-ARs is much higher in the late-pregnant cervix than in the nonpregnant tissues, but this difference itself cannot fully explain the inefficiency of terbutaline in the nonpregnant cervix. For further explanation, we carried out [^{35}S]GTPγS binding studies.

The [^{35}S]GTPγS binding assay measures the level of G-protein activation following agonist occupation of the G-protein-coupled receptor. This method detects the functional consequence of receptor occupancy in one of the earliest receptor-mediated events. In the assay, the [^{35}S]GTPγS 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 and Traynor 2003). Surprisingly, in our studies β_2-AR occupancy by terbutaline resulted in a moderate decrease in [^{35}S]GTPγS binding compared with the basal value in the pregnant samples, while no effect was found in the nonpregnant cervices, indicating the lack of further G-protein activation. This latter phenomenon is quite strange, but not unique, because uncoupled β_2-ARs were already reported in 24-month-old rat aorta (Gurdal et al. 1995). It seems very probable that the relaxing action mediated through β_2-AR is not necessary in the nonpregnant myometrium. The coupling of this receptor to G-protein may therefore be a result of a later process during pregnancy.

On the other hand, only a few studies have investigated the changes in [^{35}S]GTPγS binding caused by β_2-AR agonists on different preparations, but none of them reported a fall in [^{35}S]GTPγS level (Cerione et al. 1985; Garnier et al. 1999). This decrease means that β_2-AR stimulation reduces the level of activated G-protein in the pregnant rat cervix. The degrees of inhibition in [^{35}S]GTPγS binding (E_{max} values) were in harmony with the increases in cervical resistance elicited by terbutaline. On day 21 the terbutaline action was antagonized with propranolol, which provides further evidence for the β-AR-mediated drug effect. The shift to the left in the terbutaline dose–response curve and the decrease in EC_{50} on day 22 indicates the higher sensitivity of the β-ARs to this special action of the drug on the day of delivery.

We assume that the effect of terbutaline is connected with stimulatory G-proteins (G_s), although an interaction with inhibitory G-protein (G_i) cannot be excluded, because protein kinase A-regulated G_i-coupled β_2-ARs have already been reported (Zamah et al. 2002). Additionally, the assessment of G_s activation seems more difficult than G_i detection in the [^{35}S]GTPγS binding because G_s subunit can dissociate from the plasma membrane into the cytosol (Lee et al. 1999). Nevertheless, G_s exists, at least in part, in a palmitoylated form closely associated with the membrane providing the possibility of [^{35}S]GTPγS measurements (Mumby 1997). We presume that the changes in level of activated G_s and/or G_i mediate an intracellular process that is not sufficient to alter the smooth muscle basal tone, but can provide a stronger resistance against stretching forces.

These facts lead us to conclude that terbutaline has a unique smooth muscle resistance-increasing effect against mechanical stretching on the isolated pregnant rat cervix, which can be explained by the decreased level of activated G-protein. To the best of our knowledge, this is the first example of a G-protein-inhibiting effect of terbutaline. It was shown that nonselective β-AR agonist isoprenaline inhibits the spontaneous porcine cervical contractions in vitro (Kitazawa et al. 2001), while other data prove the opposite on isolated human cervix, although terbutaline behaved as an inhibitor in this experiment (Bryman et al. 1986). These facts, including our results, suggest some genus-specific reaction of cervical smooth muscle to β_2-AR agonists. Nevertheless, none of the earlier studies investigated the β_2-AR agonist effect against mechanical stretching. This action of terbutaline may be promising for enhancing cervical resistance and sustaining pregnancy in cases of threatened premature labour. In the light of the clinical experience it seems very probable that β_2-AR agonists will not be sufficient to stop the whole process, but their combination with more potent inhibitors of uterine contractions may have clinical benefits. Certain clinical data support this possibility (e.g., the successful combination of terbutaline with magnesium sulfate for tocolysis), though without any relation to cervical ripening (Kosasa et al. 1994). The cervical resistance-increasing effect of terbutaline may open up new perspectives for the clinical

use of β_2-AR agonists in obstetrics. Further studies are needed to determine the actions of other β_2-AR agonists (e.g., fenoterol, ritodrine or hexoprenaline) in order to compare the cervical resistance-increasing effects of clinically used β_2-mimetics.

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