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**Role of the adrenergic system in regulation of the  
resistance of the cervix of pregnant rat**

Ph.D. Thesis

by

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## **1. List of abbreviations**

Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature: Nomenclature and symbolism for Amino Acids and Peptides (J Biol Chem 1984; 219: 345-373)

ADP	adenosine diphosphate
AR	adrenoceptor
cAMP	cyclical adenosine monophosphate
DAG	diacyl glycerol
EC <sub>50</sub>	half of the maximum effect
EGTA	ethylenedis(oxyethylenenitrilo)tetraacetic acid
E <sub>max</sub>	maximum effect
G protein	heterotrimeric guanine nucleotide binding regulatory protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine-5'-diphosphate
GTP <sub>γ</sub> S	guanosine-5'-O-(γ-thio)triphosphate
IP <sub>3</sub>	inositol triphosphate
NAD	nicotinamide adenine dinucleotide
NOSI	nitric oxide synthase inhibitor
NSAID	nonsteroidal anti-inflammatory drug
PTX	pertussis toxin
RT-PCR	reverse transcriptase – polymerase chain reaction
SEM	Standard error of mean
Tris-HCl	tris(hydroxymethyl)aminomethane

## **2. Introduction**

### **2.1. Occurrence of preterm birth**

The normal uterus is spontaneously contractile, and the progesterone secreted from the placenta suppresses the activity of the uterus during pregnancy, keeping the fetus within the uterus. The cervix remains firm and noncompliant. At term, changes occur in the cervix that makes it softer, and the uterine contractions become more frequent and regular. The precise mechanisms of these changes remain obscure. Changes in the ratio of estrogen to progesterone, in the fetal steroid secretion, and in the tension of the uterine wall as the fetus grows all play determining roles in the induction of labor.

Most pregnancies last around 40 weeks. Babies born between 37 and 42 weeks of pregnancy are called full term. Babies born before 37 completed weeks of pregnancy are said to be premature or preterm. In 2004, 4 115 590 babies were born in the USA; the preterm birth rate that year was 12.5 percent in this year, i.e. more than 560 000 preterm babies in only one year. In Hungary in the same year, 96 804 babies were born, 8.5 percent of them (about 8300 preterm babies) with a birth weight of less than 2500 g. Of those, the majority (84 percent) were born between 32 and 36 weeks of gestation, about 10 percent between 28 and 31 weeks of gestation, and about 6 percent at less than 28 weeks of gestation.

While babies born near term often have few or no problems, babies born before 32 to 34 weeks of gestation may have a number of complications. In some cases, these complications may be fairly mild; in other cases, however, they are severe and may lead to long-term medical problems (respiratory distress syndrome, apnea, intraventricular hemorrhage, patent ductus arteriosus, necrotizing enterocolitis, retinopathy of prematurity, jaundice, anemia, chronic lung disease - also called bronchopulmonary dysplasia and infections) or even death.

First and last, tocolysis is one of the greatest challenges in obstetrical practice, because of the lack of treatment for this condition.



## **2.2. Functioning of the cervix; agents which can influence cervical ripening**

The human uterus is composed of 2 basic parts, the fundus and the cervix. The fundus is composed of the myometrium, predominantly smooth muscle cells, and the endometrium. In pregnancy, the uterine cervix serves 2 major functions. First, it maintains its firmness (i.e. physical integrity) during pregnancy as the uterus dramatically enlarges. This physical integrity is critical so that the developing fetus can remain in the uterus until the appropriate time for delivery. Second, in preparation for labor and delivery, the cervix softens and becomes more distensible, a process called cervical ripening. Chemical and physical changes are required for cervical dilatation, labor and delivery of the fetus.

The cervix in normal human pregnancy measures 3.5 cm or longer. It consists mainly of extracellular connective tissue. The predominant molecules of this extracellular matrix are type 1 and type 3 collagen. Intercalated among the collagen molecules are glycosaminoglycans and proteoglycans, predominantly dermatan sulfate, hyaluronic acid and heparin sulfate. Fibronectin and elastin also run among the collagen fibers. In contrast with the fundus, it has only 10-15% smooth muscle.

In response to uterine contractions, the ripened cervix dilates, leading to reorientation of the tissue fibers in the cervix in the direction of the stress. The myometrial contractions cause the cervix to passively dilate and it is pulled over the presenting fetal part.

In some cases, premature cervical dilation contributes to premature delivery of the fetus (Bouyer *et al.*, 1986) which can impair the chances of the neonate for life. Compounds that increase the 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). The substitutive therapy of progesterone is difficult to carry out because of the high doses; even so it is at the forefront of attention again in the US (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 utilize its clinical benefit (Brancazio *et al.*, 2003). Drugs acting against prostaglandins (nonsteroidal anti-inflammatory drugs - NSAIDs) and nitric oxide (nitric oxide synthase inhibitors - NOSIs) 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 fetus limit their clinical importance (Loudon *et al.*, 2003). NOSIs exert paradox action on the uterus in pregnancy: they can enhance the cervical resistance, but they also enhance the myometrial contractions (Maul H *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.

$\beta_2$ -Adrenoceptor ( $\beta_2$ -AR) agonists have been used in tocolytic therapy for several decades (Ingemarsson 1976). In spite of the doubts, they are still among the drugs of first choice for this aim, although their advantages over others ( $\text{Ca}^{2+}$  channel blockers, NSAIDs, magnesium and ethanol) continue to be questioned (Berkman *et al.*, 2003).

### 2.3. Adrenoceptors

$\alpha$ -ARs exist on peripheral sympathetic nerve terminals and are divided into two subtypes  $\alpha_1$ , and  $\alpha_2$ . These subtypes were at first classified by their anatomical location;  $\alpha_1$  is found mostly postsynaptically, whilst  $\alpha_2$ , although typically sited presynaptically, can also occur postsynaptically.

$\alpha_1$ -ARs are found in both the central and peripheral nervous systems. In the central nervous system they are situated mostly postsynaptically and have an excitatory function. Peripherally, they are responsible for contraction and are situated on vascular and on nonvascular smooth muscle.  $\alpha_1$ -ARs on vascular smooth muscle are located intrasynaptically and function in response to neurotransmitter release.

There are three subtypes of  $\alpha_1$ -AR:  $\alpha_{1A}$ ,  $\alpha_{1B}$ , and  $\alpha_{1D}$ . Most tissues express mixtures of the three subtypes, but the relative expression levels have been found to be different in different reports. These subtypes appear to coexist in different densities and ratios, and in most cases responses to  $\alpha_1$ -AR-selective agonists are probably due to the activation of more than one subtype.

All  $\alpha$ -ARs are members of the heterotrimeric guanine nucleotide-binding regulatory protein (G protein) coupled receptor superfamily.  $\alpha_1$ -ARs are coupled through the  $G_p/G_q$  mechanism, whereas  $\alpha_2$ -ARs are coupled through  $G_i/G_o$ . The  $\alpha_1$  class of ARs belongs among the  $G_{q/11}$  type of G-protein. An agonist acting at the  $\alpha_1$ -AR-binding site causes  $G_{q/11}$  to activate the phospholipase C-dependent hydrolysis of phosphatidyl inositol 4,5-bisphosphate. The conversion of this compound by phospholipase C results

in the generation of inositol triphosphate and diacyl glycerol. Inositol triphosphate acts to release  $\text{Ca}^{2+}$  from intracellular stores in the sarcoplasmic reticulum. Diacyl glycerol synergizes with  $\text{Ca}^{2+}$  to activate protein kinase C, which phosphorylates specific target proteins in the cell to change their function.

The  $\beta$ -ARs were initially divided into  $\beta_1$ - and  $\beta_2$ -ARs defined in terms of agonist potencies. Further experimentation with  $\beta$ -antagonists revealed another AR subtype, which appeared to be insensitive to typical  $\beta$ -AR antagonists; this was classified the  $\beta_3$ -AR (Gauthier *et al.*, 1996). Pharmacological evidence has also emerged in support of a further AR subtype: the  $\beta_4$ -ARr (Sarsero *et al.*, 1998), although as yet there are no selective compounds for this particular subtype.

$\beta_2$ -ARs are also mainly postsynaptic and are located on a number of tissues, including blood vessels, bronchi, the gastrointestinal tract, the skeletal muscle, liver and mast cells; catecholamines acting on  $\beta_2$ -ARs mediate a number of tissue responses. Bronchial smooth muscle is strongly dilated by the activation of  $\beta_2$ -ARs. Uterine smooth muscle responds in a similar way, and  $\beta_2$ -agonists are frequently used to delay premature labor. For all  $\beta$ -ARs transduction occurs via G proteins coupled to the intracellular second messenger adenylate cyclase. All  $\beta$ -ARs are positively coupled to adenylate cyclase via the activation of  $G_s$  protein; however, activation of the  $\beta_2$  and  $\beta_3$ -ARs results in the stimulation or the stimulation and inhibition of adenylate cyclase. Activation of the  $\beta_1$ - and  $\beta_4$ -ARs results in an increase in the formation of cAMP and the subsequent stimulation of cAMP-dependent protein kinase.

$\beta_2$ -ARs are positively coupled to the membrane-bound enzyme adenylate cyclase via the activation of  $G_s$  protein.  $\beta_2$ -AR activation causes relaxation of the smooth muscle through the activation of adenylate cyclase. Stimulation of adenylate cyclase produces alterations in cellular activity by increasing levels of cAMP. The resulting increased levels of cAMP activate protein kinase A, which phosphorylates and inactivates myosin light chain kinase, the contractile machinery of smooth muscle.

means that more advanced pregnancy results in a weaker action of terbutaline on myometrial contractions.

## 2.4. Pregnancy-induced decrease in the relaxant effect of terbutaline in the myometrium of late-pregnant rat

The effectiveness of  $\beta_2$ -AR agonists has been the subject of intensive debate in the literature. Some articles claim that most  $\beta_2$ -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  $\beta_2$ -agonist treatment does not influence the preterm delivery rate and the perinatal outcome (Sciscione *et al.*, 1998). 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). Experiments were carried out to clarify these changes, and the role of pregnancy in the rat myometrial response to  $\beta$ -mimetics without any pretreatment with  $\beta$ -agonists. 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 continuously shifted downward toward term (Fig. 1).

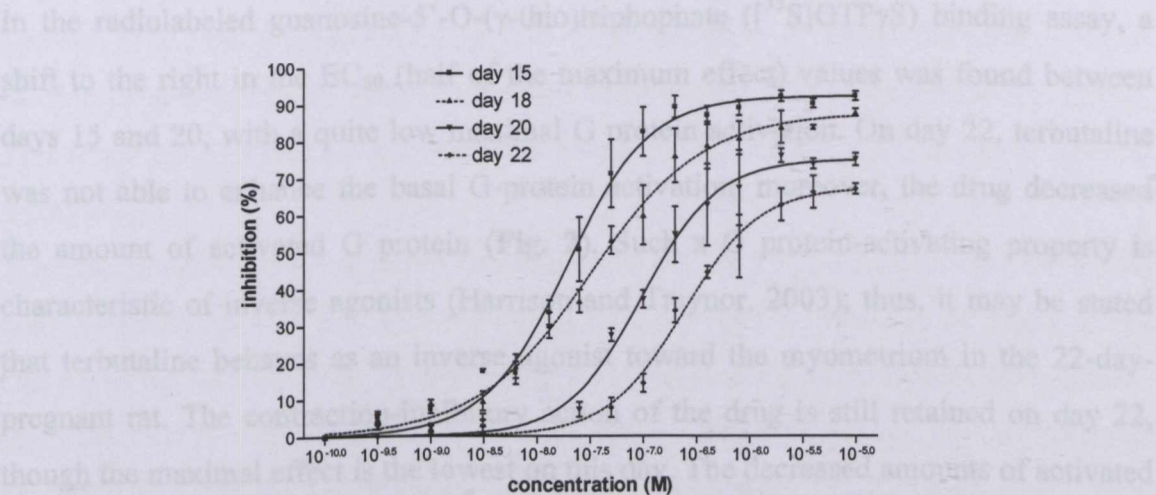


Figure 1. Changes in inhibitory effect of terbutaline on electrical field-stimulated uterine contractions in late-pregnant rat *in vitro*. Day 22 is the last day of pregnancy.

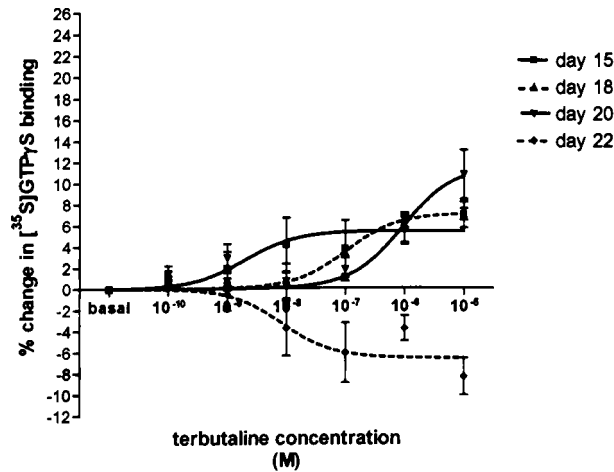
The calculated  $EC_{50}$  values of terbutaline gradually increased from day 15 to day 22; the change was nonsignificant as compared with the situation on the previous investigated day only on day 18. In parallel, a decrease was found in the calculated maximal inhibitory effects of terbutaline, with significant differences on days 20 and 22. 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. The  $17\beta$ -estradiol predominance increased toward the end of pregnancy, with the well-known dramatic drop in progesterone level at the end of the gestation period (Fuchs and Fields, 1998). It has been reported that an estrogen predominance results in an increased sensitivity of the  $\alpha$ -AR, while a progesterone predominance increases  $\beta_2$ -AR synthesis during pregnancy (Riemer *et al.*, 1987; Roberts *et al.*, 1989).

There was an increase in uterine  $\beta_2$ -AR mRNA between days 18 and 20, the level then remaining unchanged until day 22. In radioligand-binding studies, the number of  $\beta$ -ARs was found to be unchanged until day 20, but a significant elevation was detected on day 22. This result reaffirms the earlier findings that an estrogen predominance should not necessarily cause a decrease in the synthesis of  $\beta_2$ -AR in the uterus. Nevertheless, it should be stated that the pregnancy-induced change in the synthesis and number of  $\beta_2$ -ARs can not be responsible for the continuous decline in terbutaline action toward the end of the gestation period.

In the radiolabeled guanosine-5'-O-( $\gamma$ -thio)triphosphate ( $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ ) binding assay, a shift to the right in the  $\text{EC}_{50}$  (half of the maximum effect) values was found between days 15 and 20, with a quite low maximal G protein activation. On day 22, terbutaline was not able to enhance the basal G-protein activation; moreover, the drug decreased the amount of activated G protein (**Fig. 2**). Such a G protein-activating property is characteristic of inverse agonists (Harrison and Traynor, 2003); thus, it may be stated that terbutaline behaves as an inverse agonist toward the myometrium in the 22-day-pregnant rat. The contraction-inhibitory action of the drug is still retained on day 22, though the maximal effect is the lowest on this day. 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 upregulation in the genetic activity of  $\beta$ -AR regulation in order to maintain cellular receptor homeostasis. This might explain the increase in  $\beta$ -AR mRNA level and protein density at term, in parallel with the reduced effect of terbutaline.



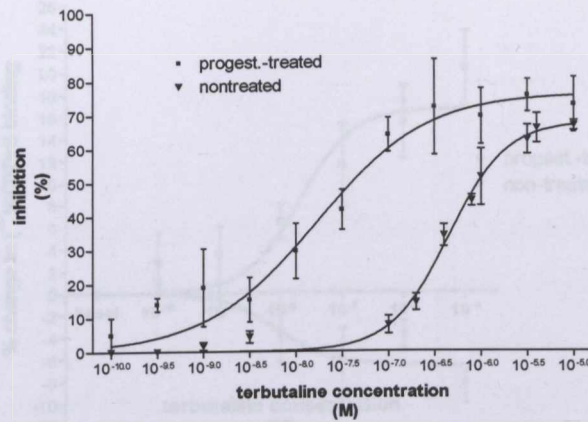
**Figure 2. Change caused in [ $^{35}$ S]GTP $\gamma$ S binding to rat uterine membrane from days 15, 18, 20 and 22 of pregnancy by various concentrations of terbutaline**

Points are means  $\pm$  SEM from 3 separate experiments carried out in triplicate. The basal value is that of [ $^{35}$ S]GTP $\gamma$ S binding without terbutaline stimulation, and was regarded as 0.

Moreover, 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  $\beta_2$ -AR agonists on the myometrium in pregnancy (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 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. 3). Accordingly, 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. These data clearly demonstrate that the presence of progesterone is a determining factor for the uterine-relaxing action of terbutaline in pregnancy.





**Figure 3. Effects of progesterone treatment on the contraction-inhibiting action of terbutaline in the rat myometrium *in vitro***

Values are means  $\pm$  SEM of the results on 6 different animals.

This correlation can be explained by the  $\beta_2$ -AR density-increasing effect of progesterone. Furthermore, progesterone treatment caused an elevation in the number of myometrial  $\beta_2$ -ARs, which was in harmony with the results of others (Hatjis *et al.*, 1988; Vivat *et al.*, 1992).

On the other hand, the stimulation of  $\beta_2$ -ARs with terbutaline followed by progesterone treatment enhanced the number of activated [<sup>35</sup>S]GTP $\gamma$ S molecules in the myometrium in 22-day-pregnant animals. The action of terbutaline on [<sup>35</sup>S]GTP $\gamma$ S binding was reversed ( $E_{\max}$ :  $15.6 \pm 3.7\%$ ) as compared with that for the nontreated samples (**Fig 4**). Earlier findings suggested that sex hormones play a role in the regulation of G proteins in the myometrium (Elwardy-Merezak *et al.*, 1994; Cohen-Tannoudji *et al.*, 1995). It was also revealed that an estrogen predominance decreases the  $\beta$ -AR-mediated  $G_s$  proteins and the cAMP level, and progesterone treatment increases the number of G protein-coupled receptors (Riemer *et al.*, 1988; Nimmo *et al.*, 1995). These data indicate that a higher progesterone level means better G protein activation and a stronger inhibitory action of terbutaline on the myometrial contractions in late pregnancy. Accordingly, 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  $\beta_2$ -ARs.

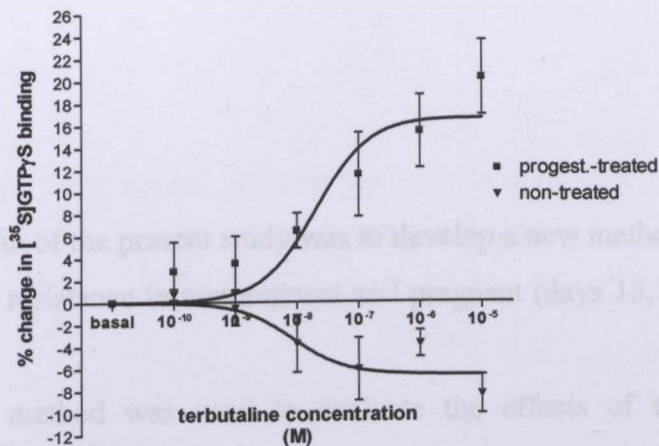
### 3. Aims

1. The primary aim of the present study was to develop a new method for investigation of the cervical myometrium in pregnant rats (days 13, 18, 20, 21 and 22) *in vitro*.

2. This *in vitro* model was used to investigate the effects of the  $\beta_2$ -AR agonist terbutaline and the  $\beta_1$ -AR antagonist metoprolol on the myometrium of pregnant rats (day 22 of pregnancy).

3. The mRNA levels and protein density of the  $\beta_1$ - and  $\beta_2$ -AR subtypes were measured by means of the reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot techniques in the cervixes of nonpregnant and pregnant rats.

4. To clarify the mechanisms of action of the investigated compounds on the  $\beta_1$ - and  $\beta_2$ -AR subtypes, experiments were carried out with the  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding technique. Experiments were also initiated in an attempt to reveal the exact functioning of these ARs, so these experiments were repeated in the presence of antagonist agents, and in a special case, with pertussis toxin (PTX).



**Figure 4.** Effects of progesterone treatment on  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding to rat uterine membrane from day 22 of pregnancy at various concentrations of terbutaline

Points are means  $\pm$  SEM of the results of 3 separate experiments carried out in triplicate. The basal value is that of  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding without terbutaline stimulation, and was regarded as 0.



### 3. Aims

1. The primary aim of the present study was to develop a new method for investigation of the cervical resistance in nonpregnant and pregnant (days 15, 18, 20, 21 and 22) rats *in vitro*.
2. This *in vitro* method was used to evaluate the effects of the  $\beta_2$ -AR agonist terbutaline and three subtype selective  $\alpha_1$ -AR inverse agonists ( $\alpha_{1A}$ -AR: WB 4101,  $\alpha_{1B}$ -AR: AH 11110A, and  $\alpha_{1D}$ -AR: BMY 7379) on the cervical resistance in nonpregnant and late-pregnant rats.
3. The mRNA level and protein density of the  $\beta_2$ - and  $\alpha_1$ -AR subtypes were measured by means of the reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot techniques in the cervixes of nonpregnant and pregnant rats.
4. To clarify the mechanisms of action of the investigated compounds on the  $\beta_2$ - and  $\alpha_1$ -AR subtypes, experiments were carried out with the [ $^{35}$ S]GTP $\gamma$ S binding technique. Experiments were also initiated in an attempt to reveal the exact functioning of these ARs, so these experiments were repeated in the presence of antagonist agents, and in a special case, with pertussis toxin (PTX).

## **4. 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), which is in harmony with the regulation of the European Union.

### **4.1. 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 1200 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.

### **4.2. Organ samples**

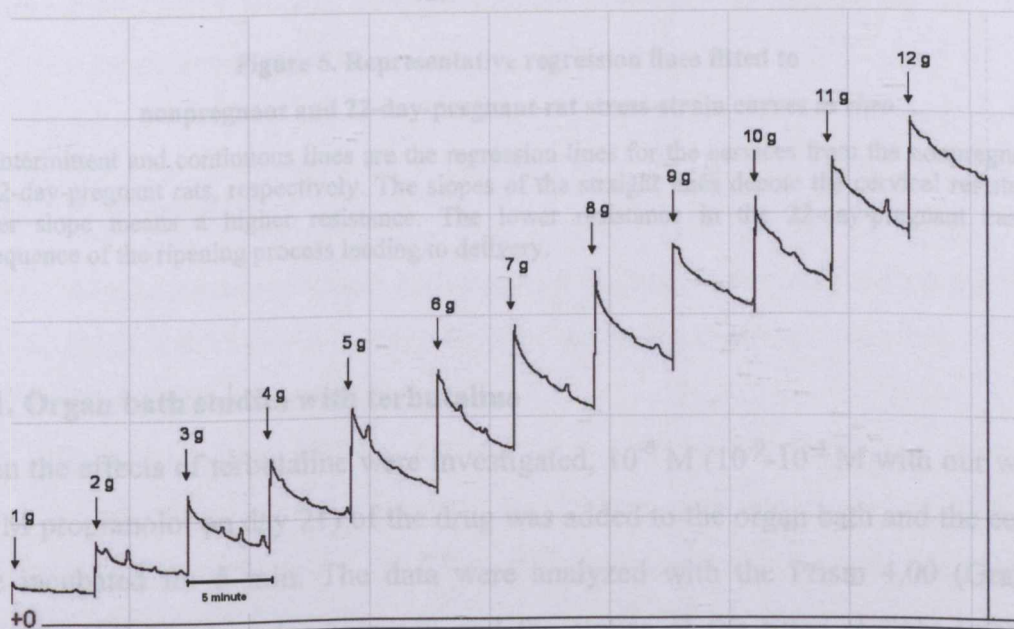
The cervical resistance was investigated *in vitro* in cervical samples from nonpregnant and pregnant rats. Organ samples from the pregnant rats were obtained on days 18, 20, 21 and 22 (term) of pregnancy.

### **4.3. Measurement of cervical resistance**

Cervical tissues were removed from nonpregnant and late-pregnant (gestational day 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<sub>2</sub>, 1 MgCl<sub>2</sub>, 12 NaHCO<sub>3</sub>, 4 NaH<sub>2</sub>PO<sub>4</sub>, 6 glucose, pH 7.4). The organ bath was maintained at 37 °C and carbogen (95% O<sub>2</sub> + 5% CO<sub>2</sub>) was bubbled through it. The lower sides of the cervixes 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 Ltd, 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 increase of the tension in the tissues. The cervixes 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 cervixes were followed with an online computer, using the S.P.E.L. Advanced Isosys Data Acquisition System (Experimetria Ltd, Hungary). The resultant stress-strain curves had saw-tooth shapes (Fig. 5).



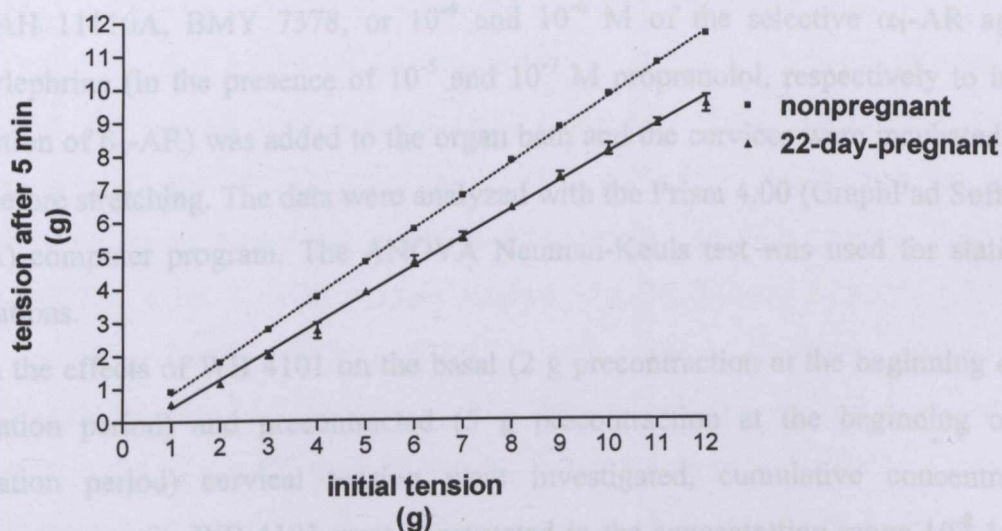
**Figure 5. Representative stress-strain curve of cervix from a 22-day-pregnant rat *in vitro***

The cervixes 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. 6).

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. **Figure 6** depicts representative straight lines derived from resistance studies on cervixes from nonpregnant and day-22-pregnant rats.



**Figure 6. Representative regression lines fitted to nonpregnant and 22-day-pregnant rat stress-strain curves *in vitro***

The intermittent and continuous lines are the regression lines for the cervixes from the nonpregnant and the 22-day-pregnant rats, respectively. The slopes of the straight lines denote the cervical resistance. A steeper slope means a higher resistance. The lower resistance in the 22-day-pregnant case is a consequence of the ripening process leading to delivery.

#### 4.3.1. Organ bath studies with terbutaline

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 cervixes were incubated for 5 min. The data were analyzed with the Prism 4.00 (GraphPad Software, U.S.A) computer program, and the slopes of the fitted straight lines were compared. The ANOVA Neuman-Keuls test was used for statistical evaluations.

When the effects of terbutaline were investigated on the basal (2 g precontraction at the beginning of the 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  $3 \times 10^{-9}$  -  $3 \times 10^{-5}$  M for cervixes from 21-day-pregnant rats.

#### 4.3.2. Investigation of the *in vitro* effects of $\alpha$ -AR inverse agonists

When the effects of these drugs were investigated,  $10^{-6}$  M of the subtype-selective  $\alpha_1$ -AR inverse agonist WB 4101 ( $10^{-8}$ - $10^{-4}$  M, with or without  $10^{-6}$  M phentolamine on day 22), AH 11110A, BMV 7378, or  $10^{-4}$  and  $10^{-6}$  M of the selective  $\alpha_1$ -AR agonist phenylephrine (in the presence of  $10^{-5}$  and  $10^{-7}$  M propranolol, respectively to inhibit the action of  $\beta_2$ -AR) was added to the organ bath and the cervixes were incubated for 5 min before stretching. The data were analyzed with the Prism 4.00 (GraphPad Software, U.S.A) computer program. The ANOVA Neuman-Keuls test was used for statistical evaluations.

When the effects of WB 4101 on the basal (2 g precontraction at the beginning of the incubation period) and precontracted (5 g precontraction at the beginning of the incubation period) cervical tension were investigated, cumulative concentration-response curves for WB 4101 were constructed in the concentration range  $10^{-8}$ - $10^{-4}$  M for 22-day-pregnant cervixes.

#### 4.4. RT-PCR studies

Cervix tissues from nonpregnant and pregnant animals were removed on gestational days 18, 20, 21 and 22 ( $n = 6$  on each day), frozen in liquid nitrogen and then stored at  $-70$  °C until total RNA extraction.

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  $\mu$ L of DNase and RNase-free distilled water. The RNA concentrations of the samples were determined from their absorbance at 260 nm.

The RNA (0.5  $\mu$ g) was denatured at 70 °C for 5 min in a reaction mixture containing 20  $\mu$ M oligo(dT) (Invitrogen, UK), 20 U of RNase inhibitor (Invitrogen, UK), 200  $\mu$ M dNTP (Sigma-Aldrich, Hungary) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 5 mM  $MgCl_2$  in a final reaction volume of 20  $\mu$ L. After the mixture had been cooled to 4 °C, 20 U of M-MLV reverse transcriptase (GIBCO, UK) and RNase H Minus (Invitrogen, UK) were added, and the mixture was incubated at 37 °C for 60 min.

PCR was carried out with 5  $\mu$ L of cDNA, 25  $\mu$ L of ReadyMix REDTaq PCR reaction mix (Sigma-Aldrich, Hungary) and 50 pm of each of the forward and reverse primers. For the rat  $\beta_2$ -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). The primer sequences used to amplify the  $\alpha_{1A}$ -AR were 5'-GTA GCC AAG AGA GAA AGC CG-3' (for the forward primer) and 5'-CAA CCC ACC ACG ATG CCC AG-3' (for the reverse primer); these primers were anticipated to generate 212 bp PCR product. For the rat  $\alpha_{1B}$ -AR cDNA, a 300 bp PCR product resulted with the forward primer 5'-GCT CTT CTA CAT CCC GCT CG-3' and the reverse primer 5'-AGGGGAGCCAACATAAGATGA-3'. The primers for the  $\alpha_{1D}$ -AR were 5'-CGT GTG CTC CTT CTA CCT ACC-3' (for the forward primer) and 5'-GCA CAG GAC GAA GAC ACC CAC-3' (for the reverse primer) (Scofield *et al.*, 1995). A rat  $\beta$ -actin probe was used as an internal control in all samples (Murata and Higuchi, 2003). The PCR was performed with a PCR Sprint thermal cycler (Hybaid Corp. U.K.), with the following cycle parameters:

- $\beta_2$ -AR: 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.
- $\alpha_1$ -ARs: after initial denaturation at 95 °C for 3 min, the reactions were taken through 35 cycles of 1 min at 94 °C, and annealing at 54 °C ( $\alpha_{1B}$ - and  $\alpha_{1D}$ -AR) or 50 °C ( $\alpha_{1A}$ -AR) for 1 min and at 72 °C for 2 min. After the last cycle, incubation was continued for 10 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 transilluminator. Quantitative analysis was performed by densitometric scanning of the gel with Kodak EDAS290 (Csertex Ltd., Hungary). For statistical evaluations, data were analyzed by ANOVA with the Neuman-Keuls post test.

#### 4.5. Western blot analysis

20 µg of protein per well was subjected to electrophoresis on 10% sodium dodecylsulfate polyacrylamide gels in Series Standard Dual Cooled Units (BioRad, Hungary). Proteins were transferred from gels to nitrocellulose membranes (Scheicher and Schuell, Germany), using a semidry blotting technique (BioRad, Hungary). The membranes were blocked with 5% non-fat dry milk in Tris saline buffer (50 mM Tris, pH 7.4, 200 mM NaCl) containing 0.1% Tween, overnight at 4 °C. After washing, the blots were incubated for 1 h on a shaker at room temperature with  $\beta_2$ -,  $\alpha_{1A}$ -,  $\alpha_{1B}$ - and  $\alpha_{1D}$ -AR and  $\beta$ -actin polyclonal antibody (Santa Cruz Biotechnology, California, 1:200) in the blocking buffer. Antibody binding was detected with a Western Breeze Chromogenic Western blot immunodetection kit (Invitrogen, Hungary). Quantitative analysis was performed by densitometric scanning of the gel with Kodak EDAS290 (Csrtex Ltd., Hungary). For statistical evaluations, data were analyzed by ANOVA with the Neuman-Keuls post test.

#### 4.6. [ $^{35}$ S]GTP $\gamma$ S binding assay

Rat cervix membrane fractions ( $\approx 10$  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<sub>2</sub>, 100 NaCl; pH 7.4) containing 20 MBq/0.05 cm<sup>3</sup> [ $^{35}$ S]GTP $\gamma$ S (0.05 nM) and increasing concentrations ( $10^{-10}$ - $10^{-6}$  M) of terbutaline, or increasing concentrations ( $10^{-10}$ - $10^{-6}$  M) of the subtype-selective  $\alpha_1$ -AR inverse agonist WB 4101, AH 11110A or BMY 7378 and the subtype-nonselective  $\alpha_1$ -AR agonist phenylephrine, 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 with terbutaline was also carried out in the presence of propranolol ( $10^{-7}$  M). The G<sub>i</sub> protein-activating effect of WB 4101 was measured in the presence of 500 ng of PTX. Nonspecific binding was determined with 10 µM GTP $\gamma$ S and subtracted. Bound and free [ $^{35}$ S]GTP $\gamma$ S were separated by vacuum filtration through Whatman GF/B filters with a Millipore manifold. Filters were washed with 3x5 ml of ice-cold buffer, and the radioactivity of the dried filters was determined in a toluene-based scintillation cocktail in a Wallac 1409 scintillation counter (Turku, Finland). The percentage stimulation caused by terbutaline or the subtype-selective  $\alpha_1$ -AR inverse agonist was plotted against the concentration of the

drug. Dose-response curves were fitted, and the concentrations  $EC_{50}$  and  $E_{max}$  were calculated and statistically analyzed by means of the ANOVA Neuman-Keuls test.



5. Results

5.1. Isolated organ bath studies

In the isolated organ bath studies, a very limited extensibility of the cervixes from the nonpregnant rats was found. In essence, the initial tension remained unchanged (slope ~1.00) after 5 min. From day 18, the cervical resistance continuously decreased toward term, reaching a trough value on days 21 and 22 (Fig. 7, grated bars). Terbutaline had no effect on the cervixes in the nonpregnant cases, but elicited cervical resistance-increasing action from day 18 to day 22 of pregnancy. This effect was most marked on days 21 and 22 (Fig. 7, black bars).

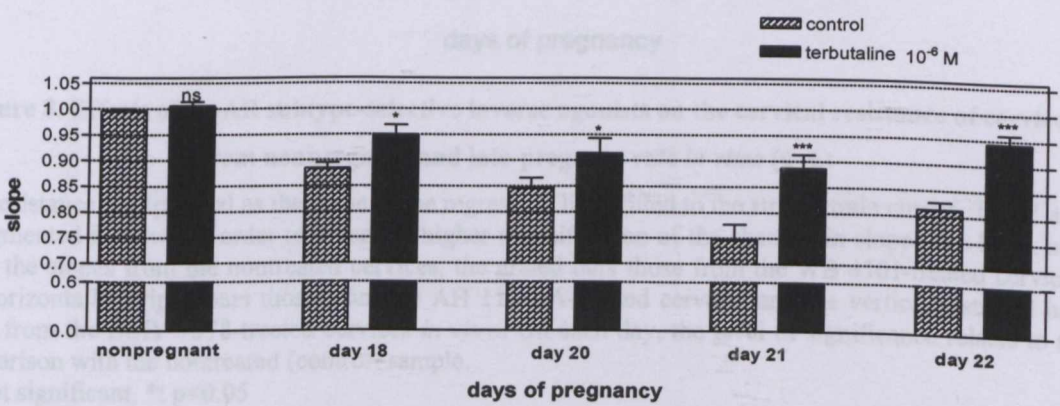


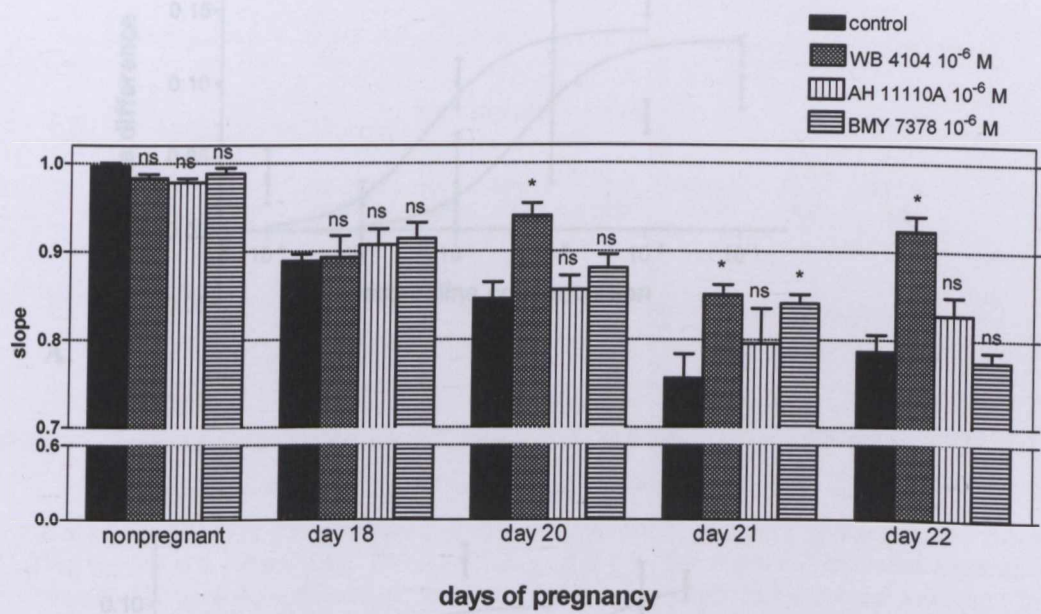
Figure 7. Effects of terbutaline on the cervical resistance of cervixes from nonpregnant and late-pregnant rats *in vitro* (n=8)

The resistance is expressed as the slope of the regression lines fitted to the stress-strain curves (see Figs 5 and 6). The Y axis is segmented into two in order to present a higher magnification of the changes in slopes. The grated bars show the slopes from the nontreated, and the black bars those from the terbutaline-treated cervixes *in vitro*. On each day, the level of significance relates to the comparison with the nontreated (control) sample.

ns: not significant, \*: p<0.05; \*\*\*: p<0.001

As compared with the nontreated cervixes, WB 4101 had no effect on the tissues in the nonpregnant and 18-day-pregnant cases, but elicited cervical resistance-increasing action from day 20 to day 22 of pregnancy. This effect was most marked on days 21 and 22 (Fig. 8, grated bars). AH 11110A had no effect on the cervical resistance *in vitro*

(Fig. 8, horizontally striped bars). BMY 7378 increased the cervical resistance only on day 21 (Fig. 8, vertically striped bars).



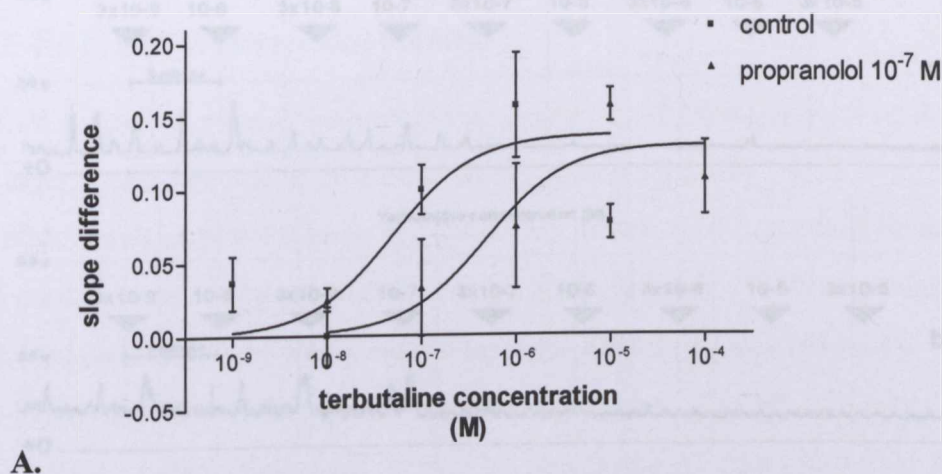
**Figure 8. Effects of  $\alpha_1$ -AR subtype-selective inverse agonists on the cervical resistance of cervixes from nonpregnant and late-pregnant rats *in vitro* (n=6)**

The resistance is expressed as the slope of the regression lines fitted to the stress-strain curves. The Y axis is segmented into two in order to present a higher magnification of the changes in slope. The black bars show the slopes from the nontreated cervixes, the grated bars those from the WB 4101-treated cervixes, the horizontally striped bars those from the AH 11110A-treated cervixes and the vertically striped bars those from the BMY 7378-treated cervixes *in vitro*. On each day, the level of significance relates to the comparison with the nontreated (control) sample.  
 ns: not significant, \*:  $p < 0.05$

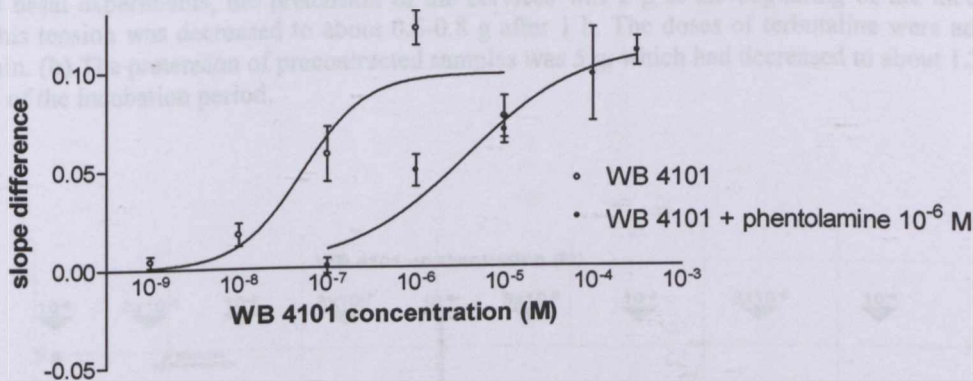
The cervical resistance-increasing effect of terbutaline was concentration-dependent in the range  $10^{-9}$ - $10^{-5}$  M (Fig. 9/A, control) on day 21, and the effect of WB 4101 was concentration-dependent in the range  $10^{-8}$ - $10^{-4}$  M (Fig. 9/B, control) on day 22. This concentration-response curve was shifted to the right in the presence of  $10^{-7}$  M propranolol (Fig. 9/A, propranolol, Table 1), and shifted to the right, without a significant change in its maximum value, in the presence of  $10^{-6}$  M phentolamine (Fig. 9/B, WB 4101 + phentolamine, Table 2), respectively. These data suggest the  $\beta$ -AR-mediated character of the action of terbutaline and the  $\alpha$ -AR-mediated character of WB 4101 on the cervical resistance.

Neither terbutaline nor WB 4101 had an effect on the basal (Fig. 10/a and Fig. 11/A) or precontracted (Fig. 10/b and Fig. 11/B) cervical muscle tone on day 21 or day 22.





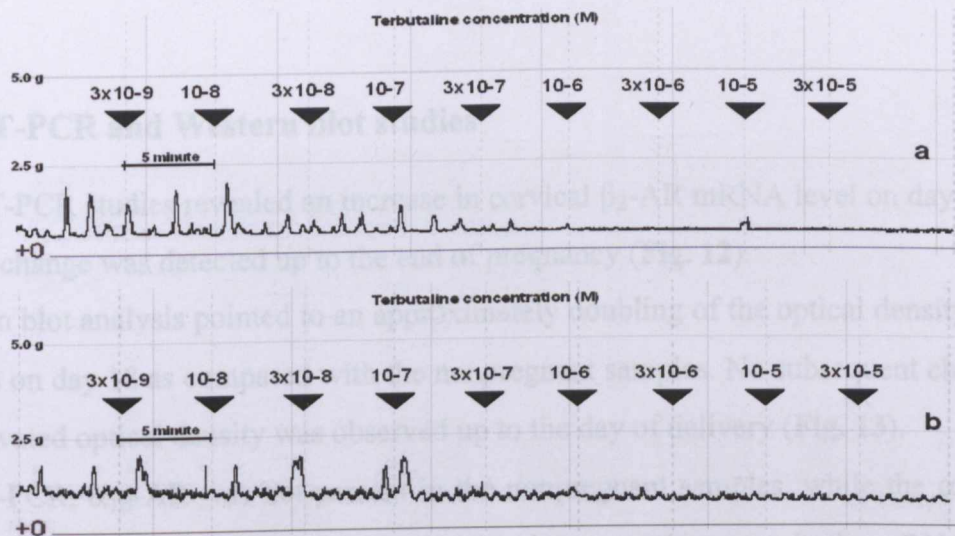
A.



B.

**Figure 9. (A) Effect of propranolol on cervical resistance-increasing action in cervixes from 21-day-pregnant rats. (B) Effect of phentolamine on cervical resistance-increasing action of WB 4101 in cervixes from 22-day-pregnant rats**

The effect of terbutaline and WB 4101 on the cervical resistance was expressed as the slope difference, which was calculated by subtraction of the average slope (day 21: 0.7473, day 22: 0.7867) of the nontreated samples from the slope of the treated ones.



**Figure 10. Effects of terbutaline on smooth muscle tone in basal (a) and precontracted (b) cervixes on day 21 of pregnancy *in vitro***

(a) In the basal experiments, the pretension of the cervixes 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. (b) The pretension of precontracted samples was 5 g, which had decreased to about 1.2-1.5 g at the end of the incubation period.



**Figure 11. Effects of WB 4101 on smooth muscle tone in basal (A) and precontracted (B) cervixes on day 22 of pregnancy *in vitro***

(A) In the basal experiments, the pretension of the cervixes was 2 g at the beginning of the incubation period. This tension was decreased to about 0.5-0.6 g after 1 h. The doses of WB 4101 were added every 5 min. (B) The pretension of the precontracted samples was 5 g, which had decreased to about 1.7-1.9 g at the end of the incubation period.

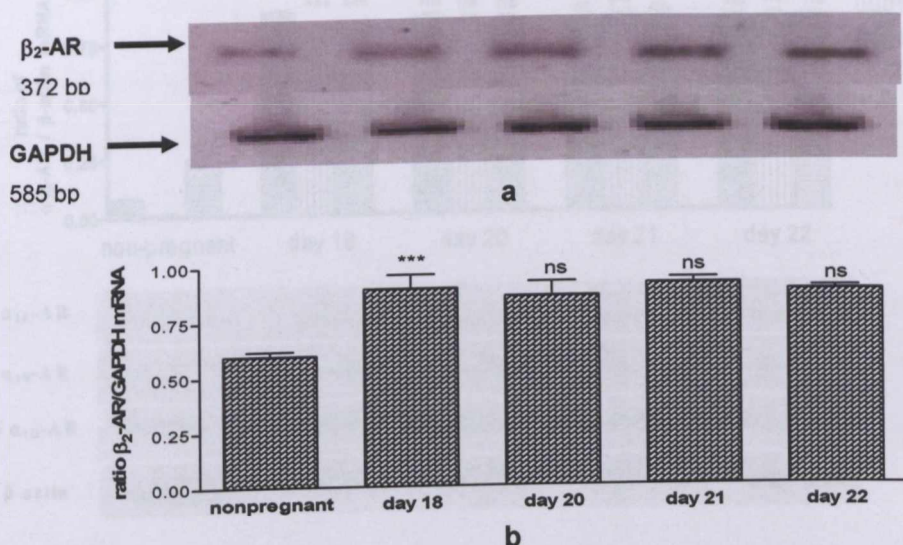
## 5.2. RT-PCR and Western blot studies

The RT-PCR studies revealed an increase in cervical  $\beta_2$ -AR mRNA level on day 18. No further change was detected up to the end of pregnancy (**Fig. 12**).

Western blot analysis pointed to an approximately doubling of the optical density of the  $\beta_2$ -ARs on day 18 as compared with the nonpregnant samples. No subsequent change in this elevated optical density was observed up to the day of delivery (**Fig. 13**).

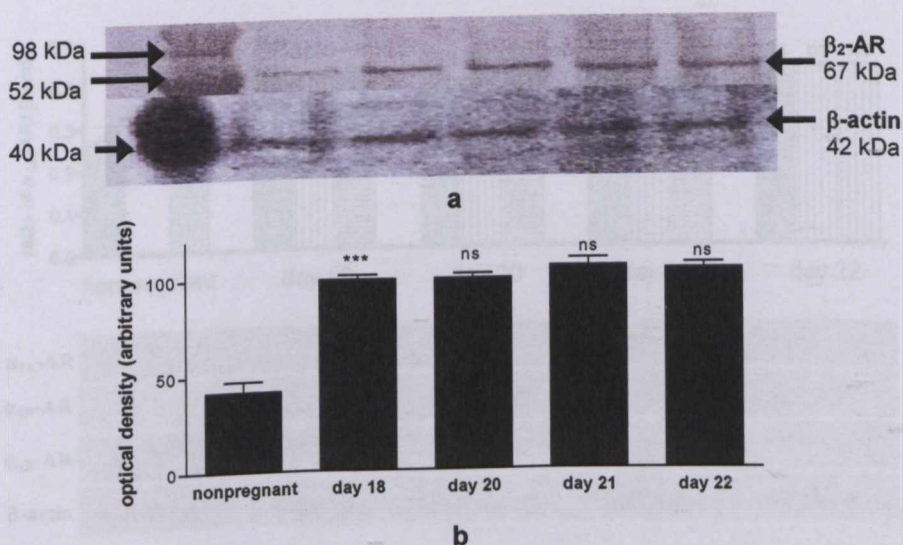
On RT-PCR,  $\alpha_{1B}$ -AR was not present in the nonpregnant samples, while the  $\alpha_{1A}$ - and  $\alpha_{1D}$ -AR mRNA contents were low. On day 18, there were increases in the mRNA levels of all cervical  $\alpha_1$ -AR subtypes, and no further change was detected up to the end of pregnancy (**Fig. 14**). The results of Western blot analysis on the levels of receptor protein expressions were in harmony with the PCR results (**Fig. 15**).





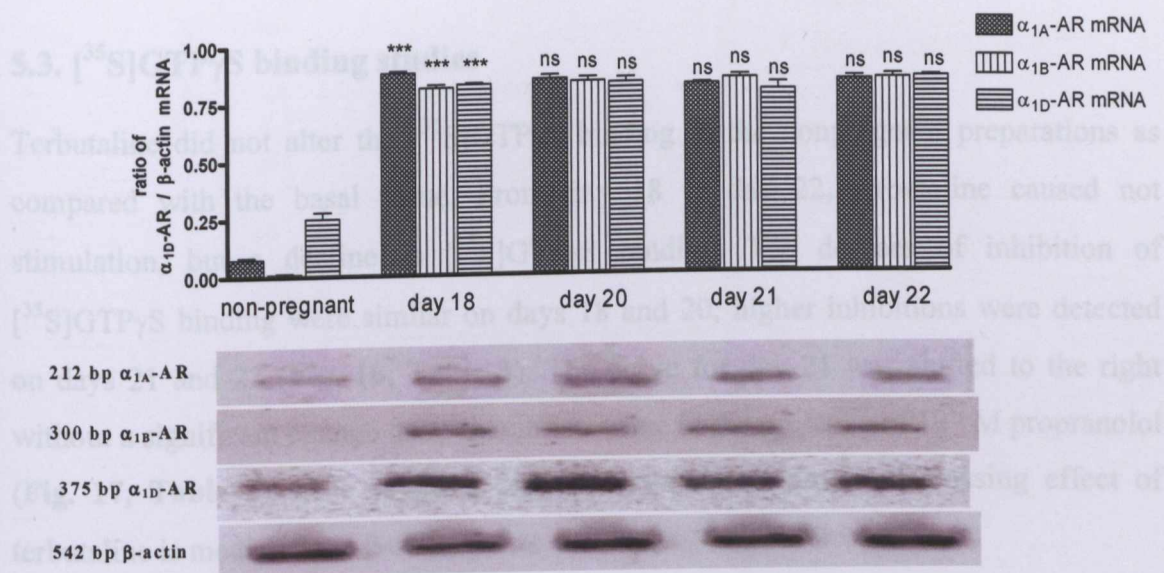
**Figure 12.** Change in mRNA level of  $\beta_2$ -AR in cervices from nonpregnant and late-pregnant rats (n=6)

(a)  $\beta_2$ -AR RT-PCR and glyseraldehyde-3-phosphate dehydrogenase (GAPDH) products from the cervical total RNA of a nonpregnant animal and a pregnant animal on days 18, 20, 21 and 22 of pregnancy. (b) The result was expressed as a ratio of the optical densities of  $\beta_2$ -AR/GAPDH. The level of significance relates to the comparison with the previous investigated day.  
ns: not significant, \*\*\*:  $p < 0.001$



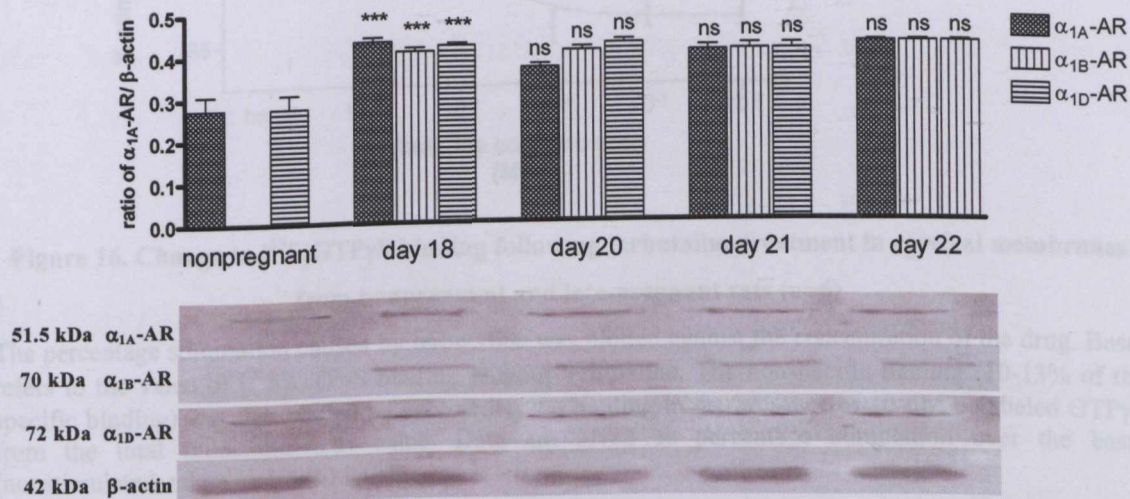
**Figure 13.** Change in  $\beta_2$ -AR level in cervices from nonpregnant and late-pregnant rats (n=6)

(a) The  $\beta_2$ -AR and  $\beta$ -actin Western blot products from cervices from nonpregnant and 18, 20, 21 and 22-day-pregnant rats. The antibody binding was detected with an enhanced chemiluminescence detection system, (b) and expressed as optical density (semiquantitative) data. The level of significance relates to the comparison with the previous investigated day.  
ns: not significant, \*\*\*:  $p < 0.001$



**Figure 14. Change in mRNA level of  $\alpha_1$ -AR in cervices from nonpregnant and late-pregnant rats (n=6)**

Each band contains RNA from the cervix of one animal. Semiquantitative analysis was performed by densitometric scanning of the gel and the result was expressed as the ratio of the optical densities of  $\alpha_1$ -AR/GAPDH. The grated bars show the  $\alpha_{1A}$ -AR, the horizontally striped bars the  $\alpha_{1B}$ -AR and the vertically striped bars the  $\alpha_{1D}$ -AR mRNA level. The level of significance relates to the comparison with the level on the previous investigated day.  
ns: not significant, \*\*\*: p<0.001



**Figure 15. Change in  $\alpha_1$ -AR level in cervices from nonpregnant and late-pregnant rats (n=6)**

The  $\alpha_1$ -AR and  $\beta$ -actin Western blot products from cervices from nonpregnant and 18, 20, 21 and 22-day-pregnant rats. The 51.5, 70, 72 and 42 kDa Western blot products are the  $\alpha_{1A}$ -AR,  $\alpha_{1B}$ -AR,  $\alpha_{1D}$ -AR and  $\beta$ -actin, respectively. The antibody binding was detected with an enhanced chemiluminescence detection system, and expressed as the ratio of the optical densities of  $\alpha_1$ -AR/ $\beta$ -actin. The level of significance relates to the comparison with the previous investigated day.  
ns: not significant, \*\*\*: p<0.001



5.3. [<sup>35</sup>S]GTPγS binding studies

Terbutaline did not alter the [<sup>35</sup>S]GTPγS binding in the nonpregnant preparations as compared with the basal value. From day 18 to day 22, terbutaline caused not stimulation, but a decline in [<sup>35</sup>S]GTPγS binding. The degrees of inhibition of [<sup>35</sup>S]GTPγS binding were similar on days 18 and 20; higher inhibitions were detected on days 21 and 22 (Fig. 16, Table 3). The curve for day 21 was shifted to the right without a significant change in its maximum value in the presence of 10<sup>-7</sup> M propranolol (Fig. 17, Table 3). This suggests that the activated G protein-decreasing effect of terbutaline is mediated via β-ARs.

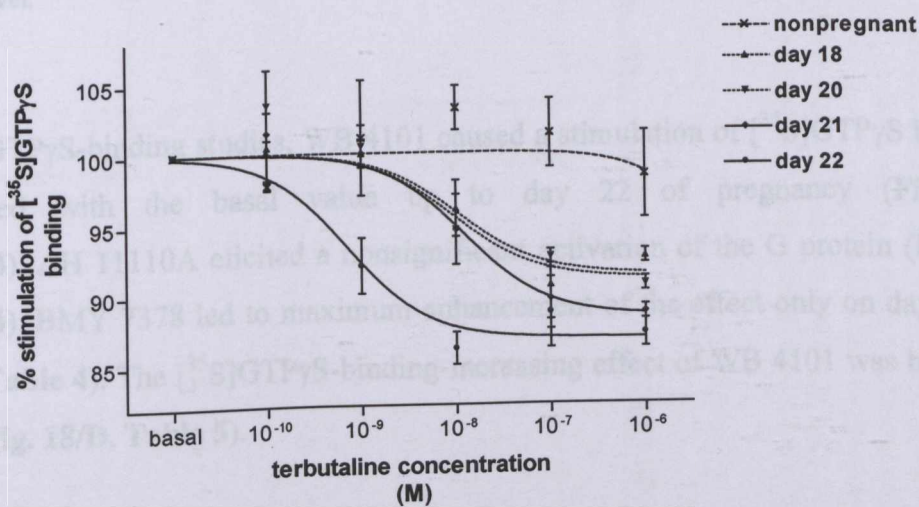
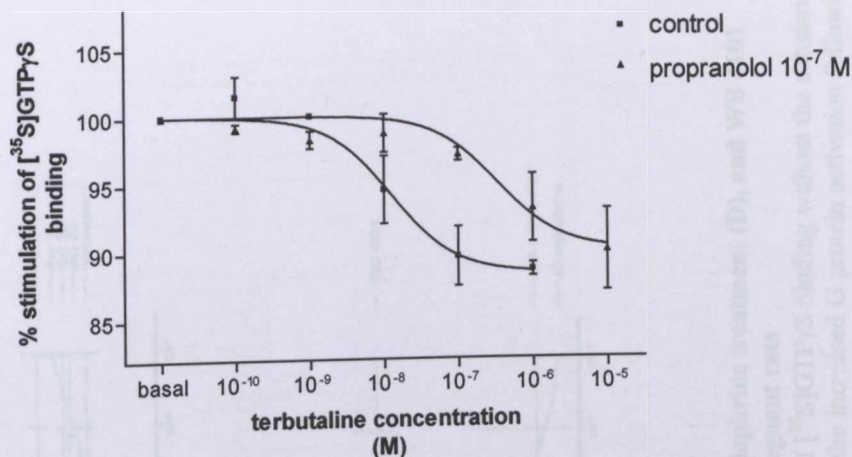


Figure 16. Change in [<sup>35</sup>S]GTPγS binding following terbutaline treatment in cervical membranes from nonpregnant and late-pregnant rats (n=6)

The percentage stimulation caused by terbutaline was plotted against the concentration of the drug. Basal refers to the value of [<sup>35</sup>S]GTPγS binding without terbutaline. The nonspecific binding (10-13% of the specific binding) was determined by subtracting the binding in the presence of 10 μM unlabeled GTPγS from the total (nonstimulated) value. Data are given as percentage stimulation over the basal (nonstimulated, taken as 100%) level.





**Figure 17. Effect of propranolol on G protein-activating action of terbutaline in cervixes from 21-day-pregnant rats (n=6)**

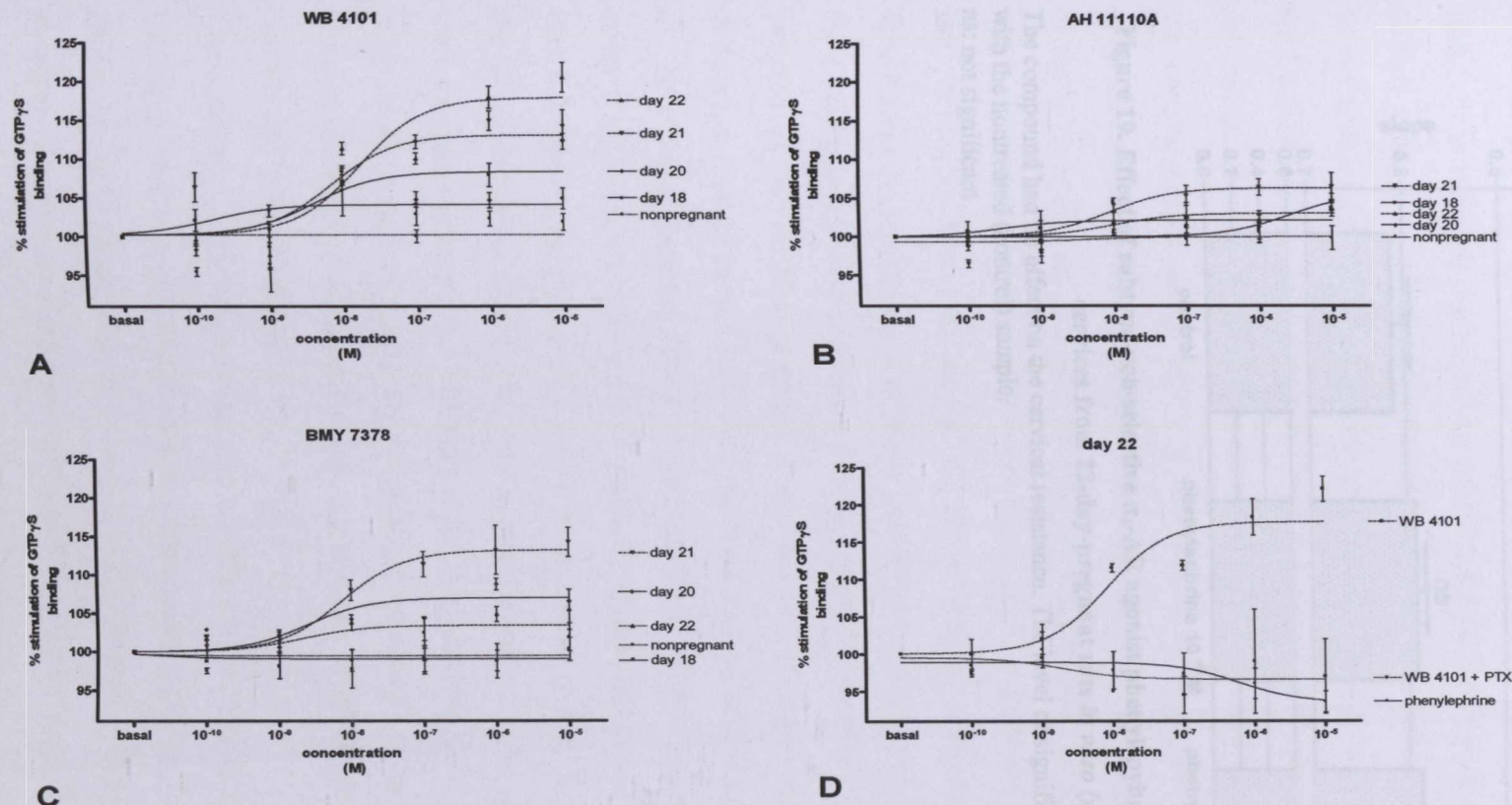
Terbutaline decreased the  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding of cervix membranes from 21-day-pregnant rats in a concentration-dependent manner (control curve). Basal refers to the value of  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding without terbutaline. Data are given as percentage stimulation/inhibition over the basal (nonstimulated, taken as 100%) level.

In the  $\text{GTP}\gamma\text{S}$ -binding studies, WB 4101 caused a stimulation of  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding as compared with the basal value up to day 22 of pregnancy (**Fig. 18/A, Table 4**). AH 11110A elicited a nonsignificant activation of the G protein (**Fig. 18/B, Table 4**). BMY 7378 led to maximum enhancement of the effect only on day 21 (**Fig. 18/C, Table 4**). The  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ -binding-increasing effect of WB 4101 was blocked by PTX (**Fig. 18/D, Table 5**).

#### 5.4. Experiments with phenylephrine

The subtype-nonspecific  $\alpha_1$ -AR agonist phenylephrine in concentrations of  $10^{-6}$  and  $10^{-4}$  M had no effect on the cervical resistance on day 22 (**Fig. 19**).

The  $\alpha_1$ -AR agonist phenylephrine caused a slight decrease in the amount of activated G protein on day 22 (**Fig. 18/D, Table 5**).



**Figure 18. Change in [<sup>35</sup>S]GTPγS binding following α<sub>1</sub>-AR subtype-selective inverse agonist treatment (A, B, C), phenylephrine treatment (D), and WB 4101 treatment in the presence of PTX (D) in cervical membranes from nonpregnant and late-pregnant rats**

The percentage stimulation caused by the compound was plotted against the concentration of the drug. Basal refers to the value of [<sup>35</sup>S]GTPγS binding without the substance. Data are given as the percentage stimulation over the basal (nonstimulated, taken as 100%) level. (A) The rising curves indicate the increased G protein activation following the addition of WB 4101 to the cervical membrane preparations. (B) AH 11110A caused nonsignificant stimulation; this G protein activation does not seem to be sufficient to increase the cervical resistance. (C) BMY 7378 increased the G-protein activation significantly only on day 21. (D) The α<sub>1</sub>-AR agonist phenylephrine in high concentration slightly decreased the activated G protein level on day 22. The [<sup>35</sup>S]GTPγS-binding-increasing effect of WB 4101 was blocked by PTX.



8. Discussion

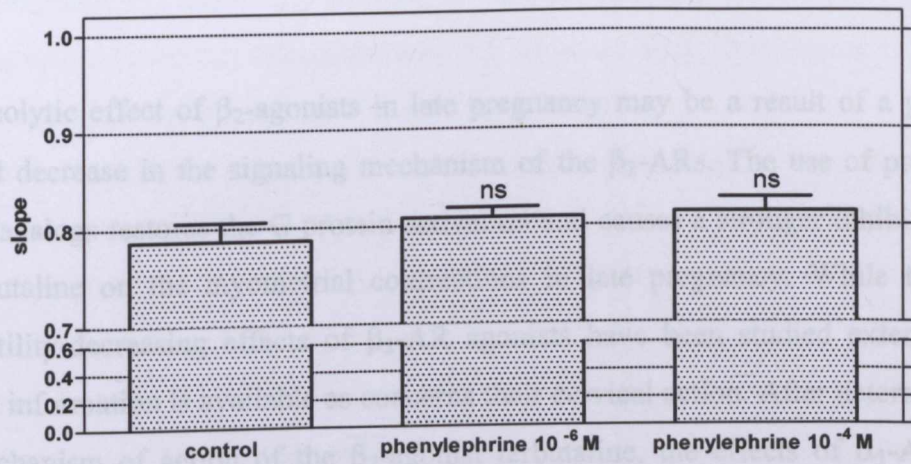


Figure 19. Effect of subtype-non-selective  $\alpha_1$ -AR agonist phenylephrine on cervical resistance of cervixes from 22-day-pregnant rats *in vitro* (n=6)

The compound had no effect on the cervical resistance. The level of significance relates to the comparison with the nontreated (control) sample.

ns: not significant

of these receptors as well as the effects of these drugs have been investigated (Shi et al., 2002; Düzcan et al., 2002); however, no study has previously been carried out of the effects of subtype-selective  $\alpha_1$ -AR inverse agonists on the resistance of cervical smooth muscle from pregnant rats. To know more, the changes in cervical resistance during pregnancy were investigated first. Isolated organ studies revealed that the rat cervical resistance decreases towards delivery, a finding in harmony with the results of others (Shi et al., 1999; Vederholov 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/1 min (Shi et al., 1999). 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 opening 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. The compounds (terbutaline and the  $\alpha_1$ -AR inverse agonists) administered *in vitro* to the organ both were not active on the nonpregnant samples. As regards the late-pregnant rat cervix, only terbutaline and the  $\alpha_{1A}$ -AR selective WB 4101 effected a considerable increase in cervical resistance to mechanical stretching. The  $\alpha_{1B}$ -AR selective AH 11110A exhibited no effect on the cervical resistance, while the cervical resistance-increasing effect of the  $\alpha_1$ -selective BMY 7378 was time-limited, manifested only on day 21. At this latter time, the measured increases in slope exceeded 0.1, i.e. very high

## 6. Discussion

The tocolytic effect of  $\beta_2$ -agonists in late pregnancy may be a result of a pregnancy-induced decrease in the signaling mechanism of the  $\beta_2$ -ARs. The use of progesterone and its analogs restores the G protein activation and causes a stronger inhibitory action of terbutaline on the myometrial contractions in late pregnancy. While the uterine contractility-decreasing effects of  $\beta_2$ -AR agonists have been studied extensively, no reliable information is available as concerns their cervical action. After determination of the mechanism of action of the  $\beta_2$ -agonist terbutaline, the effects of  $\alpha_1$ -AR inverse agonists on the cervical resistance were characterized in pregnant rats *in vitro*. The roles of these receptors as concerns the uterine activity and the effects of these drugs have been investigated in detail (Ducza *et al.*, 2001; Ducza *et al.*, 2002); however, no study has previously been carried out of the effects of subtype-selective  $\alpha_1$ -AR inverse agonists on the resistance of cervical smooth muscle from pregnant rats. To know more, the changes in cervical resistance during pregnancy were investigated first.

Isolated organ studies revealed 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/1 min (Shi *et al.*, 1999). 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.

The compounds (terbutaline and the  $\alpha_1$ -AR inverse agonists) administered *in vitro* to the organ bath were not active on the nonpregnant samples. As regards the late-pregnant rat cervix, only terbutaline and the  $\alpha_{1A}$ -AR-selective WB 4101 elicited a considerable increase in cervical resistance to mechanical stretching. The  $\alpha_{1B}$ -AR selective AH 11110A exhibited no effect on the cervical resistance, while the cervical resistance-increasing effect of the  $\alpha_{1D}$ -selective BMY 7378 was time-limited, manifested only on day 21. At this latter time, the measured increases in slope exceeded 0.1, i.e. very high

values in our system, the difference in slope between the results for the cervixes from the nonpregnant and the 22-day-pregnant nontreated samples, for example, being 0.18. These results indicate that terbutaline and WB 4101 are very potent agents, eliciting an acute increase in cervical resistance to mechanical stretching.

It is known that the smooth muscle content of the cervix is quite low, and it reduced further by pregnancy-induced apoptosis (Leppert and Yu, 1994). Because of the low smooth muscle content of the cervix, the possibility that the effects of the investigated compounds are not mediated by  $\beta_2$ -ARs (terbutaline) and  $\alpha_{1A}$ -ARs (WB 4101) was considered. In order to create a concentration-response curve, cervical samples from 21-day-pregnant rats were used for terbutaline and cervical samples from 22-day-pregnant rats were used for WB 4101, because the difference between the nontreated and treated resistances was highest in these cases. In the organ bath studies, the cervical resistance-increasing action of the drugs was concentration-dependent, and could be antagonized with the nonselective  $\beta$ -AR-blocker propranolol (terbutaline) or the nonselective  $\alpha$ -AR-blocker phentolamine (WB 4101). Inhibition of the effect of WB 4101 with phentolamine is a further evidence for the inverse agonist property of WB 4101, because true antagonists (e.g. phentolamine) can antagonize the effects of inverse agonists (Leppert and Yu, 1994). Accordingly, the cervical resistance-increasing property of terbutaline and WB 4101 is predominantly related to these ARs. However, these drugs were inefficient on the basal cervical muscle tone when gradual stretching was omitted.

This effect of terbutaline or an  $\alpha_{1A}$ -AR inverse agonist on smooth muscle was unusual and the differences in the cervical tone-increasing effects of  $\alpha_1$ -AR inverse agonists were also unidentified. To clarify this action, the changes in the  $\beta_2$ -AR and  $\alpha_1$ -AR subtype mRNA were determined by RT-PCR. The receptor synthesis was found to be elevated in the cervixes from the late-pregnant rats as compared with those from the nonpregnant rats, but no differences were detected between the investigated pregnant rat tissues. The same held true for the  $\beta_2$ -AR and  $\alpha_1$ -AR subtype protein densities determined by Western blot analysis. Although these methods were only semiquantitative (the changes in optical density were followed in both methods), higher amounts of the  $\beta_2$ -ARs and  $\alpha_1$ -AR subtypes were detected in the cervixes from the late-pregnant rats than in those from the nonpregnant animals. These differences themselves partially explain the inefficiency of terbutaline and WB 4101 in the cervixes from the

nonpregnant rats, but give no explanation for the inefficiency of AH 11110A and BMY 7378 on the cervixes from the pregnant rats.

For further information, [ $^{35}\text{S}$ ]GTP $\gamma$ S-binding studies were carried out; this method is most useful for investigation of the activation of G $_i$ -coupled receptors, such as the  $\beta_2$ -ARs. The  $\alpha_1$ -ARs are mainly coupled to the G $_{q/11}$  protein (Minneman, 1988; Berridge, 1993). On the other hand, it has been proved that the  $\alpha_1$ -ARs can at times be coupled to G $_0$  or G $_i$  proteins (Gurdal *et al.*, 1997; Otani *et al.*, 2002) and in these cases [ $^{35}\text{S}$ ]GTP $\gamma$ S binding is applicable.

The [ $^{35}\text{S}$ ]GTP $\gamma$ S-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 [ $^{35}\text{S}$ ]GTP $\gamma$ S replaces endogenous guanosine triphosphate and binds to the  $\alpha$  subunit of the G protein (G $_{\alpha}$ ). The  $\gamma$ -thiophosphate bond is resistant to the hydrolysis of G $_{\alpha}$  by GTPase. The labeled G $_{\alpha}$  subunits therefore accumulate and can be measured by counting the amount of  $^{35}\text{S}$  incorporated (Harrison and Traynor, 2003).

Surprisingly, in our studies,  $\beta_2$ -AR occupancy by terbutaline resulted in a moderate decrease in [ $^{35}\text{S}$ ]GTP $\gamma$ S binding as compared with the basal value in the pregnant samples, while no effect was found in the cervixes from the nonpregnant animals, indicating the lack of further G protein activation. This latter phenomenon is quite strange, but not unique, because uncoupled  $\beta_2$ -ARs have already been reported in the aorta of 24-month-old rats (Gurdal *et al.*, 1995). It seems very probable that the relaxing action mediated through  $\beta_2$ -AR is not necessary in the myometrium of nonpregnant rats. 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 [ $^{35}\text{S}$ ]GTP $\gamma$ S binding caused by  $\beta_2$ -AR agonists on different preparations, but none of them reported a fall in [ $^{35}\text{S}$ ]GTP $\gamma$ S level (Cerione *et al.*, 1985; Garnier *et al.*, 1999). This decrease means that  $\beta_2$ -AR stimulation reduces the level of activated G protein in the cervix from pregnant rat. The degrees of inhibition in [ $^{35}\text{S}$ ]GTP $\gamma$ S binding ( $E_{\text{max}}$  values) were in harmony with the increases in cervical resistance elicited by terbutaline. On day 21, the action of terbutaline was antagonized by propranolol, which provides further evidence for the  $\beta$ -AR-mediated drug effect. The shift to the left in the terbutaline dose-response

curve and the decrease in  $EC_{50}$  on day 22 indicate the higher sensitivity of the  $\beta$ -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 ( $G_i$ ) can not be excluded, because protein kinase A-regulated  $G_i$ -coupled  $\beta_2$ -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]GTP $\gamma$ S binding because the  $G_s$  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]GTP $\gamma$ S measurements (Mumby, 1997).

In our studies of  $\alpha_{1A}$ -AR occupancy, WB 4101 resulted in a moderate increase in [ $^{35}$ S]GTP $\gamma$ S binding as compared with the basal value in the pregnant samples, while no effect was found in the cervixes from the nonpregnant rats. Stimulation of  $\alpha_{1B}$ -AR had no effect, and  $\alpha_{1D}$ -AR stimulation had only a time-limited effect in the [ $^{35}$ S]GTP $\gamma$ S-binding study. In subsequent experiments, WB 4101 was investigated further, while the other two inverse agonists were discarded.

Drugs that behave as inverse agonists commonly decrease both the [ $^{35}$ S]GTP $\gamma$ S-binding level and the G protein activation, while real agonists elicit the opposite effects (Zhu *et al.*, 2000). On the other hand, as we demonstrated in the [ $^{35}$ S]GTP $\gamma$ S-binding studies, the  $\beta_2$ -agonist terbutaline behaves as an inverse agonist in the cervix of pregnant rat cervix. Accordingly, it may be concluded that the G protein-activating properties of agonists and inverse agonists may be reversed in the cervix of pregnant rat.

In order to acquire more evidence in this respect, the effect of a real agonist was also tested in our system. Phenylephrine had no effect on the cervical resistance *in vitro*, but in high concentration it decreased the activated G protein level. These results support our theory that the activation of G protein in the cervical adrenergic system by an agonist or an inverse agonist is controlled in an opposite way as compared with the myometrium (see below). The inefficiency of phenylephrine on the cervical resistance can be explained by the fact that it has no G-protein-moderating action.

It is known that the catalytic A subunit ( $S_1$ ) of PTX transfers the adenosyl diphosphate (ADP) ribosyl moiety of nicotinamide adenine dinucleotide (NAD) to the membrane-bound regulatory protein  $G_i$  that normally inhibits the adenylate cyclase. This inhibitory action of PTX is special to the  $G_i$  protein, and is appropriate for distinguishing between

the different G protein-mediated signal transductions. In our studies, PTX inhibited the [<sup>35</sup>S]GTPγS-binding-increasing (and hence the G protein-activating) effect of WB 4101, and consequently we assume that the effects of WB 4101 are at least, partially connected with PTX-sensitive G proteins, presumably G<sub>i</sub> proteins, although an interaction with G<sub>q/11</sub> protein (G<sub>q/11</sub>) can not be excluded. We therefore assume that these changes in level of activated G proteins mediate an intracellular process which is not sufficient to alter the smooth muscle basal tone, but which can provide a stronger resistance to stretching forces.

These facts lead us to conclude that terbutaline and WB 4101 has a unique smooth muscle resistance-increasing effect against mechanical stretching on the isolated cervix from pregnant rat, which can be explained by the unusual decreased (terbutaline) and increased (WB 4101) level of activated G protein. To the best of our knowledge, this is the first example of a G protein-inhibiting effect of terbutaline, and G protein-activating effect of an α<sub>1</sub>-AR inverse agonist compound.



## 7. Conclusions

In light of the clinical experience, it seems very probable that  $\beta_2$ -AR agonists will not be sufficient to stop the whole preterm labor 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  $\beta_2$ -AR agonists in obstetrics. On the other hand, the  $\alpha_{1A}$ -AR inverse agonist WB 4101 is not yet used in obstetrical practice. In the aggregate of their cervical tone-increasing and uterus-relaxant effect, they might be applicable for preventive therapy or treatment in certain cases of preterm labor. Further studies are needed to compare the cervical resistance-increasing effects of several clinically used  $\beta_2$ -mimetics and  $\alpha_{1A}$ -AR inverse agonists.

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10. Appendix

**Table 1.** EC<sub>50</sub> and E<sub>max</sub> values of terbutaline cervical resistance-increasing effect on day 21 pregnant rats *in vitro* (n=6)

	EC <sub>50</sub> ± SEM (M)	E <sub>max</sub> ± SEM
terbutaline	4.9 x 10 <sup>-8</sup> ± 1.3 x 10 <sup>-8</sup>	0.14 ± 0.02
terbutaline + propranolol 10 <sup>-7</sup> M	4.8 x 10 <sup>-7</sup> ± 1.1 x 10 <sup>-7</sup> (**)	0.13 ± 0.03 (ns)

EC<sub>50</sub>: concentration of terbutaline eliciting half of E<sub>max</sub>  
E<sub>max</sub>: maximum slope difference increasing effect of terbutaline. Slope difference is calculated by the subtraction of the average slope (0.7473) of non-treated samples from the treated ones. SEM: standard error of mean, ns: not significant, \*\*: p<0.01

**Table 2.** EC<sub>50</sub> and E<sub>max</sub> values of WB 4101 cervical resistance-increasing effect on 22-day-pregnant rats *in vitro* (n=6)

	EC <sub>50</sub> ± SEM (M)	E <sub>max</sub> ± SEM
WB 4101	5.3 x 10 <sup>-8</sup> ± 1.8 x 10 <sup>-8</sup>	0.10 ± 0.01
WB 4101 + phentolamine 10 <sup>-6</sup> M	3.7 x 10 <sup>-6</sup> ± 2.2 x 10 <sup>-6</sup> (***)	0.11 ± 0.01 (ns)

EC<sub>50</sub>: concentration of WB 4101 eliciting half of E<sub>max</sub>,  
E<sub>max</sub>: maximum slope difference-increasing effect of WB 4101 (the slope difference is calculated by subtraction of the average slope (0.7867) for the non-treated samples from that for the treated ones),  
SEM: standard error of mean,  
ns: not significant, \*\*\*: p<0.001

**Table 3.** EC<sub>50</sub> and E<sub>max</sub> values of terbutaline [<sup>35</sup>S]GTPγS binding-inhibitory effect on cervix membranes from late-pregnant rat preparations (n=6)

Day of pregnancy	Terbutaline		Terbutaline + Propranolol 10 <sup>-7</sup> M	
	EC <sub>50</sub> ± SEM (M)	E <sub>max</sub> ± SEM (%)	EC <sub>50</sub> ± SEM (M)	E <sub>max</sub> ± SEM (%)
18	1.1 x10 <sup>-8</sup> ± 0.4 x10 <sup>-8</sup>	8.8 ± 0.8	-	-
20	9.6 x10 <sup>-9</sup> ± 1.8 x10 <sup>-9</sup> (ns)	9.1 ± 1.0 (ns)	-	-
21	1.3 x10 <sup>-8</sup> ± 0.5 x10 <sup>-8</sup> (ns)	11.8 ± 0.9 (*)	3.3 x10 <sup>-7</sup> ± 0.8 x10 <sup>-7</sup> (**)	10.1 ± 1.9 (ns)
22	7.1 x10 <sup>-10</sup> ± 0.9 x10 <sup>-10</sup> (***)	13.3 ± 1.1 (ns)	-	-

EC<sub>50</sub>: inhibitory concentration of terbutaline eliciting half of E<sub>max</sub>  
E<sub>max</sub>: maximum inhibitory effect of terbutaline on a given day of pregnancy  
SEM: standard error of mean

The level of significance in the column of **Terbutaline** relates to the comparison with the previous investigated day. The level of significance of E<sub>max</sub> values on day 21 and 22 was \* and \*\*, as compared with non-pregnant value, respectively.  
The level of significance in the column of **Terbutaline + Propranolol** relates to the comparison with values of 21 day cervices with **Terbutaline** column (ns: not significant, \*: p<0.05; \*\*: p<0.01); \*\*\*: p<0.001).

**Table 4.** E<sub>max</sub> values of [<sup>35</sup>S]GTPγS binding-stimulatory of subtype-selective α<sub>1</sub>-AR inverse agonists on cervix membranes from late-pregnant rat preparations (n=6)

Day of pregnancy	WB 4101	AH 11110A	BMY 7378
	E <sub>max</sub> ± SEM (%)	E <sub>max</sub> ± SEM (%)	E <sub>max</sub> ± SEM (%)
non-pregnant	102.8 ± 0.6	99.3 ± 0.5	99.5 ± 0.4
18	105.0 ± 0.6 (ns)	104.8 ± 3.7 (ns)	99.1 ± 0.3 (ns)
20	108.4 ± 0.5 (*)	102.6 ± 1.0 (ns)	107.0 ± 0.4 (ns)
21	113.2 ± 0.5 (***)	106.3 ± 0.8 (ns)	113.2 ± 0.5 (***)
22	117.5 ± 0.7 (***)	103.0 ± 0.6 (ns)	103.4 ± 0.4 (ns)

E<sub>max</sub>: maximum stimulatory effect on a given day of pregnancy,  
SEM: standard error of mean.  
The level of significance relates to the comparison with the previous investigated day. (ns: not significant, \*: p<0.05; \*\*\*: p<0.001).

**Table 5.**  $E_{max}$  values of [ $^{35}$ S]GTP $\gamma$ S binding-stimulatory or inhibitory effects of subtype-selective  $\alpha_1$ -AR antagonist and subtype-nonselective agonist on cervix membranes from day 22 pregnant rat preparations (n=6)

Day of pregnancy	phenylephrine	WB 4101	WB 4101 + PTX
	$E_{max} \pm SEM (\%)$	$E_{max} \pm SEM (\%)$	$E_{max} \pm SEM (\%)$
22	93.5 $\pm$ 2.2	117.7 $\pm$ 1.2	96.6 $\pm$ 1.6

PTX : pertussis toxin  
 $E_{max}$ : maximum stimulatory effect on a given day of pregnancy,  
SEM: standard error of mean.



## 11. List of publications

### 10.1. Publications related to the Ph.D. thesis

1. Róbert Gáspár, **Zoltán Kolarovszki-Sipiczki**, Eszter Ducza, Eszter Páldy, Sándor Benyhe, Anna Borsodi, George Falkay: Terbutaline increases the cervical resistance of the pregnant rat *in vitro*. Naunyn-Schmiedeberg's Archives of Pharmacology 2005 Jan; 371: 61-71. (IF: 2,098)
2. Róbert Gáspár, Eszter Ducza, Attila Mihályi, Árpád Márki, **Zoltán Kolarovszki-Sipiczki**, Eszter Páldy, Sándor Benyhe, Anna Borsodi, Imre Földesi, George Falkay: Pregnancy-induced decrease in the relaxant effect of terbutaline in the late-pregnant rat myometrium: role of G-protein activation and progesterone. Reproduction 2005 July; 130: 113-122. (IF: 3,136)
3. **Zoltán Kolarovszki-Sipiczki**, Róbert Gáspár, Eszter Ducza, Eszter Páldy, Sándor Benyhe, Anna Borsodi, George Falkay: Effect of subtype-selective  $\alpha_1$ -adrenoceptor inverse agonists on non-pregnant and late pregnant cervical resistance *in vitro* in the rat. Clinical and Experimental Pharmacology and Physiology 2007; 34: 42-47. (IF: 1,437)

### 10.2. Abstracts

1. **Kolarovszki-S. Z.**, Gáspár R., Ducza E., Falkay Gy.:  $\beta_2$ -adrenoreceptorok szerepének vizsgálata patkány cervix rezisztenciára *in vitro*. XII. Congressus Pharmaceuticus Hungaricus, 2003 május 8-10, Budapest, Magyarország (poszter)
2. R. Gáspár, **Z. Kolarovszki-S.**, E. Ducza, G. Falkay: Investigation of the role of  $\beta_2$ -adrenergic receptors in the rat cervical resistance *in vitro*. 3rd Congress of the Federation of European Pharmacological Societies, 28 June – 2 July, 2003, Nice, France (oral presentation)
3. **Kolarovszki-S. Z.**, Gáspár R., Ducza E., Páldy E., Benyhe S., Borsodi A., Falkay Gy.: Terbutalin hatása a cervix rezisztenciára terhes és nem terhes patkányban *in vitro*. Semmelweis Egyetem Doktori Iskola – Ph.D. Tudományos Napok, 2004. április 8-9, Budapest, Magyarország (előadás)

4. **Z. Kolarovszki-S.**, R. Gáspár, E. Ducza, E. Páldy, S. Benyhe, A. Borsodi, G. Falkay: Investigation of the role of  $\alpha_1$ -adrenergic receptor subtypes in the rat cervical resistance *in vitro*. 4th Meeting of the Federation of European Pharmacological Societies, 7 – 10 July, 2004, Porto, Portugal (poster)
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