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

Summary of the Ph.D. Thesis

by

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Introduction

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. In addition, 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. In some cases, premature cervical dilation contributes to premature delivery of the fetus 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. 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. 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. 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. NSAIDs have been used in pregnancy, but their adverse effects on the fetus limit their clinical importance. NOSIs exert paradox action on the uterus in pregnancy: they can enhance the cervical resistance, but they also enhance the myometrial contractions, 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-Adrenoceptor (β_2-AR) agonists have been used in tocolytic therapy for several decades. In spite of the doubts, they are still among the drugs of first choice for this aim, although their advantages over others (Ca^{2+} channel blockers, NSAIDs, magnesium and ethanol) continue to be questioned.

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

Experiments were carried out to clarify the role of pregnancy in the rat myometrial response to β-mimetics without any pretreatment with β-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**). This means that more advanced pregnancy results in a weaker action of terbutaline on myometrial contractions.

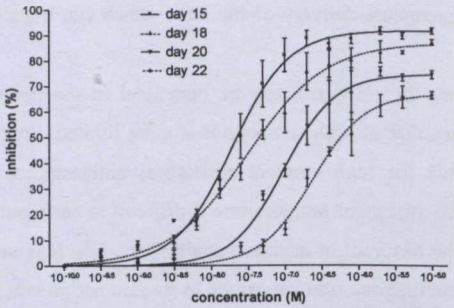

Figure 1. Changes in inhibitory effect of terbutaline on electrical field-stimulated uterine contractions in late-pregnant rat *in vitro*. Day 22 is the last day of pregnancy.

In the [^{35}S]GTPγS binding assay, 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**). 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 β-AR regulation in order to maintain cellular receptor homeostasis.

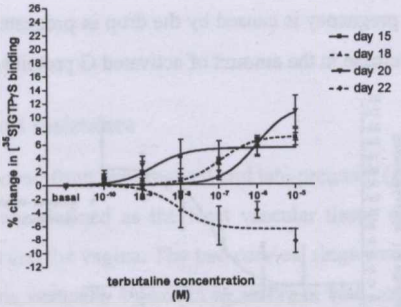

Figure 2. Change caused in [^{35}S]GTPγS binding to rat uterine membrane from days 15, 18, 20 and 22 of pregnancy by various concentrations of terbutaline

Earlier studies suggested that the presence or absence of progesterone can alter the effect of β_2-AR agonists on the myometrium in pregnancy. On the basis of the sex hormone levels at the end of pregnancy, the pregnant animals were treated with progesterone for 7 days.

The progesterone supplementation restored the weakened relaxing action of terbutaline on day 22 of pregnancy (**Fig. 3**). These data clearly demonstrate that the presence of progesterone is a determining factor for the uterine-relaxing action of terbutaline in pregnancy.

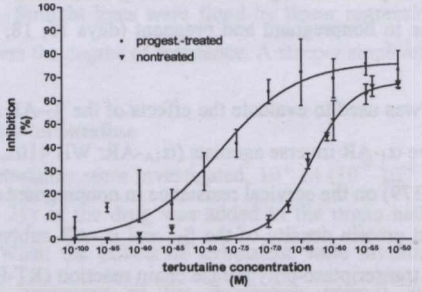

Figure 3. Effects of progesterone treatment on the contraction-inhibiting action of terbutaline in the rat myometrium *in vitro*

On the other hand, the stimulation of β_2-ARs with terbutaline followed by progesterone treatment enhanced the number of activated [^{35}S]GTPγS molecules in the myometrium in 22-day-pregnant annimals. The action of terbutaline on [^{35}S]GTPγS binding was reversed as compared with that for the nontreated samples (**Fig 4.**). 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 β_2-ARs.

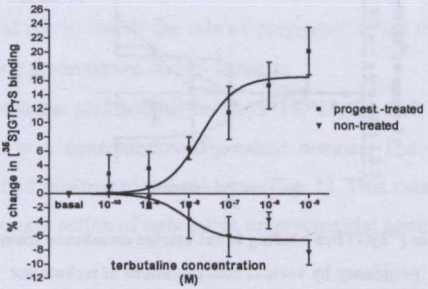

Figure 4. Effects of progesterone treatment on [^{35}S]GTPγS binding to rat uterine membrane from day 22 of pregnancy at various concentrations of terbutaline

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 β_2-AR agonist terbutaline and three subtype selective α_1-AR inverse agonists (α_{1A}-AR: WB 4101, α_{1B}-AR: AH 11110A, and α_{1D}-AR: BMY 7379) on the cervical resistance in nonpregnant and late-pregnant rats.
3. The mRNA level and protein density of the β_2- and α_1-AR subtypes were measured by means of the reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot techniques in the cervices of nonpregnant and pregnant rats.
4. To clarify the mechanisms of action of the investigated compounds on the β_2- and α_1-AR subtypes, experiments were carried out with the [^{35}S]GTPγS 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).

Methods

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. The organ bath was maintained at 37 °C and carbogen 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. 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 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.

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.

Organ bath studies with terbutaline

When the effects of terbutaline were investigated, 10^{-6} M (10^{-9}-10^{-4} M with our 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. 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 $3x10^{-9}$ - $3x10^{-5}$ M for cervices from 21-day-pregnant rats.

Investigation of the *in vitro* effects of α-AR inverse agonists

When the effects of these drugs were investigated, 10^{-6} M of the subtype-selective α_1-AR inverse agonist WB 4101 (10^{-8}-10^{-4} M, with or without 10^{-6} M phentolamine on day 22), AH

11110A, BMY 7378, or 10^{-4} and 10^{-6} M of the selective α_1-AR agonist phenylephrine (in the presence of 10^{-5} and 10^{-7} M propranolol, respectively to inhibit the action of β_2-AR) was added to the organ bath and the cervices were incubated for 5 min before stretching.

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 cervices.

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. PCR was carried out with 5 μL of cDNA, 25 μL of ReadyMix REDTaq PCR reaction mix and 50 pm of each of the forward and reverse primers. The RT-PCR products were separated on 2% agarose gels, stained with ethidium bromide and photographed under a UV transilluminator.

Western blot analysis

20 μg of protein per well was subjected to electrophoresis on 10% sodium dodecylsulfate polyacrylamide gels. Proteins were transferred from gels to nitrocellulose membranes, using a semidry blotting technique. The membranes were blocked with 5% non-fat dry milk in Tris saline buffer containing 0.1% Tween, overnight at 4 °C. . After washing, the blots were incubated for 1 h on a shaker at room temperature with β_2-, α_{1A}-, α_{1B}- and α_{1D}-AR and β-actin polyclonal antibody in the blocking buffer. Antibody binding was detected with a Western blot immunedetection kit. Quantitative analysis was performed by densitometric scanning of the gel.

[^{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 containing 20 MBq/0.05 cm^3 [^{35}S]GTPγS (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 α_1-AR inverse agonist WB 4101, AH 11110A or BMY 7378 and the subtype- nonselective α_1-AR agonist phenylephrine, in the presence of excess GDP (30 pM) in a final volume of 1 ml. On day 21, the experiment with terbutaline was also carried out in the presence of propranolol (10^{-7} M). The G_i protein-activating effect of WB 4101 was measured in the presence of 500 ng of PTX. 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 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. The percentage stimulation caused by terbutaline or the subtype-selective α_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.

Results

Isolated organ bath studies

In the isolated organ bath studies, a very limited extensibility of the cervices 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. 5**, grated bars). Terbutaline had no effect on the cervices 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. 5**, black bars).

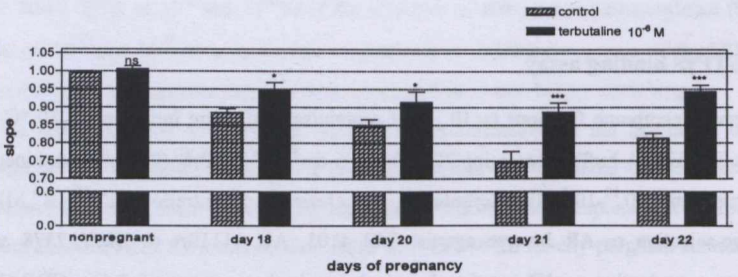

Figure 5. Effects of terbutaline on the cervical resistance of cervices from nonpregnant and late-pregnant rats *in vitro* (n=8)

The resistance is expressed as the slope of the regression lines fitted to the stress-strain curves. The Y axis is segmented into two in order to present a higher magnification of the changes in slopes.
ns: not significant, *: $p<0.05$; ***: $p<0.001$

As compared with the nontreated cervices, 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. 6**, grated bars). AH 11110A had no effect on the cervical resistance *in vitro* (**Fig. 6**, horizontally striped bars). BMY 7378 increased the cervical resistance only on day 21 (**Fig. 6**, vertically striped bars).

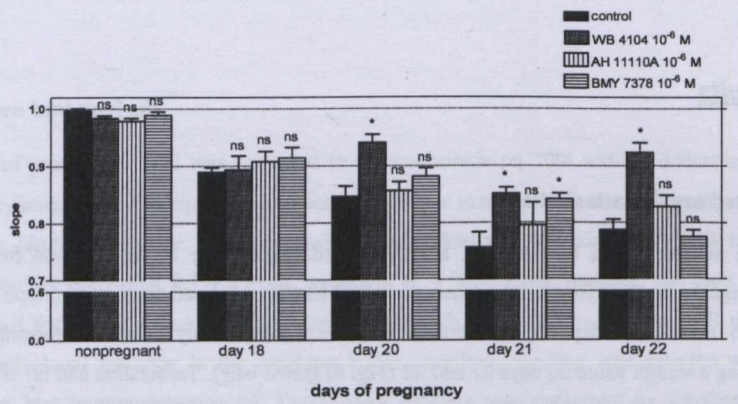

Figure 6. Effects of α_1-AR subtype-selective inverse agonists on the cervical resistance of cervices from nonpregnant and late-pregnant rats *in vitro* (n=6)

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. On each day, the level of significance relates to the comparison with the non-treated (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} on day 21, and the effect of WB 4101 was concentration-dependent in the range 10^{-8}-10^{-4} M on day 22. This concentration-response curve was shifted to the right in the presence of 10^{-7} M propranolol, and shifted to the right, without a significant change in its maximum value, in the presence of 10^{-6} M phentolamine, respectively. These data suggest the β-AR-mediated character of the action of terbutaline and the α-AR-mediated character of WB 4101 on the cervical resistance.

Neither terbutaline nor WB 4101 had an effect or the basal and precontracted cervical muscle tone on day 21 or day 22.

RT-PCR and Western blot studies

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.

Western blot analysis pointed to an approximately doubling of the optical density of the β_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.

On RT-PCR, α_{1B}-AR was not present in the nonpregnant samples, while the α_{1A}- and α_{1D}-AR mRNA contents were low. On day 18, there were increases in the mRNA levels of all cervical α_1-AR subtypes, and no further change was detected up to the end of pregnancy. The results of Western blot analysis on the levels of receptor protein expressions were in harmony with the PCR results.

[^{35}S]GTPγS binding studies

Terbutaline did not alter the [^{35}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 [^{35}S]GTPγS binding. 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. The curve for day 21 was shifted to the right without a significant change in its maximum value in the presence of 10^{-7}

M propranolol. This suggests that the activated G protein-decreasing effect of terbutaline is mediated via β-ARs.

In the GTPγS binding studies, WB 4101 caused a stimulation of [^{35}S]GTPγS binding as compared with the basal value up to day 22 of pregnancy (**Fig. 7/A**). AH 11110A elicited a nonsignificant activation of the G protein (**Fig. 7/B**). BMY 7378 led to maximum enhancement of the effect only on day 21 (**Fig. 7/C**). The [^{35}S]GTPγS-binding-increasing effect of WB 4101 was blocked by PTX (**Fig. 7/D**).

Experiments with phenylephrine

The subtype-nonselective α_1-AR agonist phenylephrine in concentrations of 10^{-6} and 10^{-4} M had no effect on the cervical resistance on day 22. The α_1-AR agonist phenylephrine caused a slight decrease in the amount of activated G -protein on day 22 (**Fig. 7/D**).

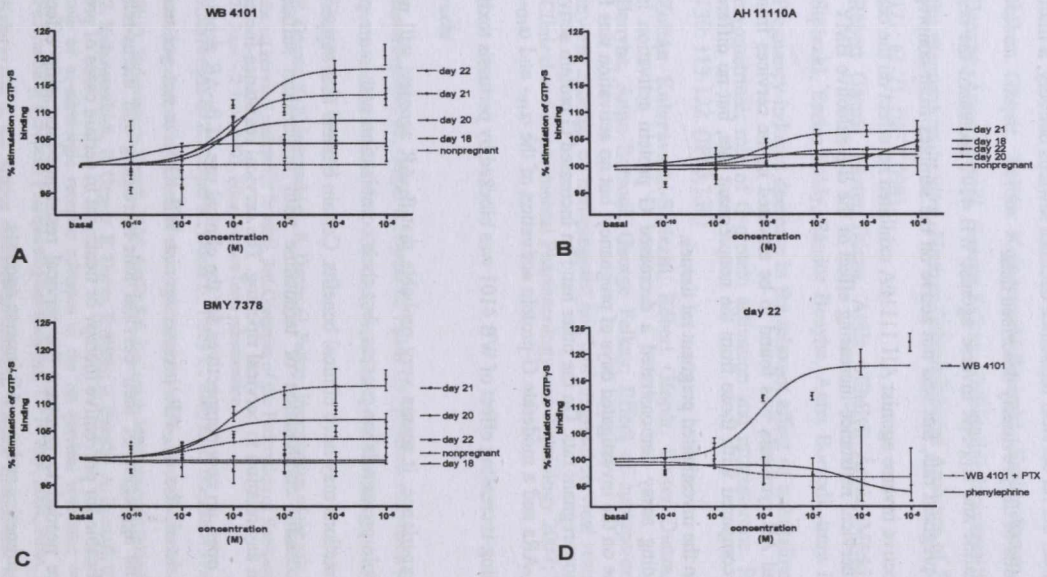

Figure 7. Change in [^{35}S]GTPγS binding following α_1-AR subtype-selective inverse agonist treatment (A, B, C), phenylephrine treatment (D), and WB 4101 treatment in the presence of PTX (D) in cervical membranes from nonpregnant and late-pregnant rats

The percentage stimulation caused by the compound was plotted against the concentration of the drug. Basal refers to the value of [^{35}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. The α_1-AR agonist phenylephrine in high concentration slightly decreased the activated G protein level on day 22. The [^{35}S]GTPγS-binding-increasing effect of WB 4101 was blocked by PTX **(D)**.

Conclusions

1. In isolated organ studies, the rat cervical resistance declines towards delivery, a finding in harmony with the results of the previously described data.
2. β_2-AR agonist terbutaline and α_{1A}-AR inverse agonist WB 4101 increased the cervical resistance in the late-pregnant rats, but was not active on the samples from nonpregnant rats. The α_{1B}-AR selective inverse agonist AH 11110A exhibited no effect on the cervical resistance, while the cervical resistance-increasing effect of the α_{1D}-selective BMY 7378 was time-limited.
3. All of the investigated AR synthesis was found to be elevated in the cervices from the late-pregnant rats as compared with those from the nonpregnant rats, but no differences were detected between the investigated pregnant rat tissues.
4. The [^{35}S]GTPγS binding assay demonstrated a decreased G protein activation in the presence of terbutaline on the investigated days of pregnancy, but no activation was found in the samples from nonpregnant rats. On the other hand an increased G protein activation was found of the α_{1A}-ARs and a moderate G-protein activation of the α_{1B}- and α_{1D}-ARs. The [^{35}S]GTPγS-binding-increasing effect of WB 4101 was blocked by pertussis toxin.

In light of the clinical experience, it seems very probable that β_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. The cervical resistance-increasing effect of terbutaline may open up new perspectives for the clinical use of β_2-AR agonists in obstetrics. On the other hand, the α_{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 β_2-mimetics and α_{1A}-AR inverse agonists.

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-Schmiedebergs 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 α_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)

Abstracts

1. **Kolarovszki-S. Z.**, Gáspár R., Ducza E., Falkay Gy.: β_2-adrenoreceptorok szerepének vizsgálata patkány cervix rezisztenciára *in vitro*. XII. Congressus Pharmaceuticus Hungaricus, 2003 május 8-10, Budapest, Magyarország (poszter)
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5. **Kolarovszki-S. Z.**: Altípus szelektív α1-adrenerg receptor antagonisták hatása a cervix rezisztenciára nem terhes és terhes patkányban *in vitro*. VII. Clauder Ottó Emlékverseny, 2004. október. 14-15, Visegrád, Magyarország (előadás)

6. **Kolarovszki-S. Z.**, Gáspár R., Ducza E., Páldy E., Benyhe S., Borsodi A., Falkay Gy.: Altípus szelektív α1-adrenerg receptor antagonisták hatása a cervix rezisztenciára nem terhes és terhes patkányban *in vitro*. Tudomány napja, 2004. november 3., Szeged, Magyarország (előadás)
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8. **Kolarovszki-S. Z.**, Gáspár R., Ducza E., Falkay Gy.: Az adrenerg rendszer szerepe a terhes patkány cervix kollagén szintézisében. Semmelweis Egyetem Doktori Iskola – Ph.D. Tudományos Napok, 2005. április 14-15, Budapest, Magyarország (előadás)
9. M. Gálik, R. Gáspár, **Z. Kolarovszki-Sipiczki**, S. Benyhe, E. Páldy, A. Borsodi and G. Falkay: Enhancement of the relaxing effects of β_2-mimetics on isolated pregnant uterus and induced preterm labor in rat. A Magyar Experimentális Farmakológia Szimpoziuma, 2005. június 6-7, Budapest, Magyarország (poszter)
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13. Gálik M., Gáspár R., Gál A., Ducza E., Minorics R., Klukovits A., **Kolarovszki-Sipiczki Z.**, Falkay Gy.: α_2-adrenerg receptorok szerepe a terhes patkány uterus kontraktilitásában. XIII. Congressus Pharmaceuticus Hungaricus, 2006 május 25-27, Budapest, Magyarország (poszter)
14. Gáspár R., Gálik M., Gál A., Ducza E., Minorics R., **Kolarovszki-Sipiczki Z.**, Falkay G.: Different roles of alpha2-adrenergic receptor subtypes in the pregnant uterine contractility in the rat. 15th World Congress of Pharmacology, July 2-7, 2006, Beijing, China (poster)