

Department of Pharmacodynamics and Biopharmacy
Faculty of Pharmacy, University of Szeged



**The role of ABC transporters in the regulation of
uterine contractility in rat**

Ph.D. Thesis

By

Norbert Lovász

Supervisor: Prof. George Falkay Ph.D., D.Sc.

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1. Introduction

Preterm birth defined by the World Health Organization as childbirth between 20 and 37 weeks of gestation, is the leading cause of neonatal morbidity and mortality, the incidence of which has not decreased over the last four decades despite intensive antenatal care programmes aimed at high-risk groups, the widespread use of tocolytics, and a series of other preventive and therapeutic interventions. With a view to decreasing the potentially adverse maternal and foetal events and improving the perinatal outcome, it is still a pharmacological challenge to find new therapeutic strategies, mechanisms or combinations.

The ABC (ATP-binding cassette) transporters form one of the largest families of membrane transport proteins expressed in all organisms. The ABC transporters have been extensively studied and play a vital role in many cellular processes (efflux pumps and ion channels). The efflux pumps have an important role in tissue defence through the excretion of toxic compounds and their metabolites. The ABCG2 efflux protein displays high expression levels in reproductive tissues (placenta, uterus, prostate, testis and ovary). The substrate specificity of ABCG2 is very wide, including uterus-relaxant agents (e.g. nifedipine and prazosine). Through the use of a potent inhibitor (KO134), intracellular accumulation of the substrate can be increased. A number of ABC transporters function in ion-channels as regulators; ABCC8/SUR1 and ABCC9/SUR2 are included in the K_{ATP} channels. The molecular structure of the K_{ATP} channels are different, this leads to different combinations, and creates different types of K_{ATP} channel with distinct electrophysiological properties and pharmacological sensitivities. The K^+ channel-opening compounds (KCOs) are known to be potent smooth muscle relaxants. The KCOs (diazoxide and pinacidil) are a structurally diverse group of drugs which open K_{ATP} channels in various cell types. It has been shown that different SUR subunits confer varying sensitivities to KCOs.

2. Aims of the study

The main focus of this work was to investigate the function of the ABC transporters in the regulation of the uterine contractility in the rat, from the aspects of both the efflux transporters (ABCG2) and the ion channel regulators (ABCC8/SUR1 and ABCC9/SUR2). The following aims were set:

1. Determination of the expression levels of ABCG2 in the rat uterus during gestation, and investigation of the uterus-relaxant effect of the ABCG2 substrate nifedipine in the presence of the ABCG2 inhibitor KO-134 *in vivo*.
2. Investigation of the expression of the SUR subunits of the K_{ATP} channels (ABCC8/SUR1 and ABCC9/SUR2) in the rat myometrium in non-pregnant animals and during pregnancy, and investigation of possible correlations between SUR subunit levels and the efficacy of the KCOs *in vitro*.
3. Investigation of the functional presence of the K_{ATP} channel in the myometrial relaxation induced by β_2 -AR agonists; in the presence of glibenclamide (a K_{ATP} channel blocker) and pinacidil (a K_{ATP} channel opener) in the early-pregnant (day 6) and late-pregnant rat uterus (day 22) *in vitro*, in order to find a correlation between the SUR expression and the pharmacological reactivity of the β_2 -AR agonists.

3. Materials and Methods

Animals

The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32.§). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV/01758-2/2008). Sprague–Dawley rats were kept at 22±3 °C; the relative humidity was 30–70% and the light/dark cycle was 12/12 h. The animals were maintained on a standard rodent pellet diet with tap water available *ad libitum*. They were euthanized by CO₂ inhalation.

Tissue isolation

Pregnant and non-pregnant rats were euthanized in a CO₂ chamber. Uterus tissue was rapidly removed; both horns of the uterus were sliced out. The first (cervix side) and the last (ovary side) myometrial rings were not collected. The remaining rings were washed in ice-cold saline (0.9% NaCl) and then transferred to a solution containing recombinant ribonuclease inhibitor. The samples were frozen in liquid nitrogen and stored at –70 °C until total RNA and protein extraction.

Real-time quantitative reverse transcription

Uterus tissues frozen in liquid nitrogen were mechanically homogenized. The PARIS Kit was used for total RNA and protein extraction from the tissues. The quality and the quantity of the RNA were assessed via the ratio of the absorbancies at 260 and 280 nm; all samples displayed an absorbance ratio in the range 1.6-2.0. 2 µg of total RNA and the High Capacity RNA-to-cDNA Kit were used for reverse transcription. PCR products were amplified with the TaqMan Gene Expression Master Mix and the ABI Step One Real-Time cycler. The following primers were used: assay ID Rn01476318_m1 for ABCC8/SUR1, Rn01463198_m1 for ABCC9/SUR2, Rn01639905-m1 for ABCG2, Rn00667869-m1 for β-actin and Rn99999916_s1 for GAPDH as endogenous controls. The fluorescence intensities of the probes were plotted against PCR cycle numbers. The amplification cycle exhibiting the first significant increase in the fluorescence signal was defined as the threshold cycle (C_T).

Western blot analysis

30 µg of protein per well was subjected to electrophoresis on 4-12 % NuPAGE Bis-Tris Gel in XCell SureLock Mini-Cell Units. Proteins were transferred from gels to nitrocellulose membranes, by a semidry blotting technique. Antibody binding was detected with the Western Breeze Chromogenic Western blot immune detection kit. The blots were incubated on a shaker with ABCG2, ABCC8/SUR1, ABCC9/SUR2, GAPDH and β-actin polyclonal antibody in the blocking buffer. Images were captured with the EDAS290 imaging system, and the optical density of each immunoreactive band was determined with Kodak 1D Images analysis software. Optical densities were calculated in arbitrary units after local area background subtraction.

***In vivo* contractility studies**

The method applied for the measurement of intrauterine pressure was based on the classical microballoon experiments originally described by Csapo (Csapo 1963). Throughout the experiments, the rats were anaesthetized with a combination of ketamine (36 mg/kg) and xylazine (4 mg/kg), administered intraperitoneally 24 h after the spontaneous delivery. The *in vivo* experiments were carried out on *post-partum* rats because the intrauterine pressure measurements with the Millar catheter in the pregnant animals were not sufficiently accurate: the foetus disturbed the measurement efficiency and the catheter could not be fixed appropriately. The jugular veins of the animals were cannulated for intravenous drug administration. After the cannulation, the abdominal cavity was opened and a Millar catheter fitted with a liquid-filled latex microballoon was inserted into the uterus through a small section above the cervical part. After a 45-min equilibration period, the intrauterine pressure was recorded.

***In vitro* organ studies**

Uterus preparation

Uteri were removed from non-pregnant rats in the oestrus phase (250–350 g) and from pregnant rats on day 6, 8, 18 or 22 of pregnancy. Muscle rings 5 mm long were sliced from the uterine horns and mounted vertically in an organ bath containing 10 ml of de Jongh solution. The organ bath was maintained at 37 °C, and carbogen (95% O₂ + 5% CO₂) was bubbled through it. After mounting, the rings were equilibrated for about 1 h before the experiments were undertaken, with a solution change every 15 min.

KCO studies: oxytocin-induced contractions

The uterus-relaxant effects of the KCOs pinacidil or diazoxide (10^{-8} – 10^{-4} M) were investigated on oxytocin-induced contractions alone or in the presence of glibenclamide (10^{-6} M). Following the addition of each concentration of pinacidil or diazoxide, recording was performed for 300 s. Dose-response curves (DRCs) were fitted, and AUCs were evaluated and analysed. Statistical analyses were carried out with the Prism 5.0 computer program. From the AUC values, the maximum inhibitory effects (E_{\max}) of pinacidil and diazoxide were calculated on a given day of pregnancy, and the concentrations eliciting 50% of the maximum inhibitions of uterine contraction (EC_{50}) were calculated. For statistical evaluations, data were analysed by the ANOVA Neuman–Keuls test.

KCO studies: contractions induced by electric field stimulation (EFS)

Uteri were removed from rats as described in the section on uterus preparation, except that uterus rings were mounted vertically between two platinum electrodes. Maximum rhythmic contractions were elicited with a digital, programmable stimulator, using different values of pulse width (PW, the duration of the electric field as a single stimulus) and period time (PP, the time interval between two stimuli). The uterus-relaxant action of pinacidil was investigated cumulatively on the non-pregnant, t and the 22-day-pregnant uterus on EFS-induced contractions alone, and in the presence of the Ca^{2+} -dependent K^{+} channel (K_{Ca} channel) blocker tetraethylammonium (TEA) TEA was added to the organ bath 20 min before the exposure to pinacidil. After EFS, pinacidil (10^{-8} – 10^{-4} M) was added in a cumulative manner. AUC values of 3-min periods were evaluated; the effect of pinacidil was expressed as a percentage of the contraction induced by EFS preceding the administration of the relaxing drug.

Effects of combinations of β_2 -AR agonists with pinacidil and glibenclamide on spontaneous contractions

The uterus-relaxant effects of the β_2 -AR agonists ritodrine and salmeterol (10^{-10} – 10^{-5} M) were investigated on spontaneous rhythmic contractions cumulatively, alone or in the presence of the K_{ATP} channel blocker glibenclamide (10^{-6} M) or the K_{ATP} channel opener pinacidil (10^{-9} – 10^{-7} M) after a 5-min preincubation. Following the addition of each concentration of β_2 -AR agonist, recording was performed for 300 s. DRCs were fitted, and AUC values were evaluated and analysed. Statistical analyses were carried

out with the Prism 5.0 computer program. From the AUC values, the maximum inhibitory effects (E_{\max}) of the β_2 -AR agonists on a given day of pregnancy were calculated, and the concentrations eliciting 50% of the maximum inhibitions of uterine contraction (EC_{50}) were calculated. For statistical evaluations, data were analysed with the ANOVA Neuman–Keuls test.

4. Results

ABCG₂ expression in the rat uterus

The expressions of ABCG₂ mRNA and protein were investigated in the non-pregnant, pregnant and *post-partum* rat uterus. This revealed characteristic expression during gestation: low levels of ABCG₂ were found in the non-pregnant and in the early-pregnant uterus, but on day 15 of gestation a sharp increase was observed, which reached its maximum on day 18 of pregnancy and decreased from day 20 to *post partum*. The *post-partum* levels were similar to the non-pregnant levels (**Fig. 1**).

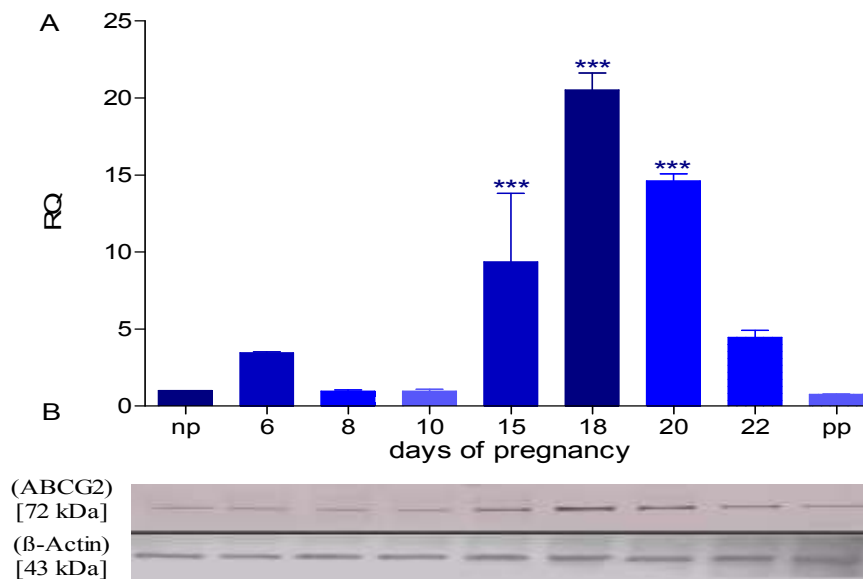


Figure 1 (A) Changes in expression of ABCG₂ mRNA during pregnancy in the rat myometrium. RQ (Relative Quantity) values on different days of pregnancy were compared with those in non-pregnant rats. *** denotes $p < 0.001$. Each value indicates the mean \pm S.E.M., $n = 5$ (np: non-pregnant, pp: *post-partum*). **(B)** Representative Western blot of ABCG₂ protein expression in non-pregnant (np), pregnant and *post-partum* (pp) rat myometrium.

Uterus-relaxing effect of nifedipine in combination with KO-134 *in vivo*

The uterus-relaxing effect of nifedipine was investigated in the *post-partum* rat uterus *in vivo* with an intrauterine pressure-measuring method. Nifedipine proved to exert a

strong relaxant effect on the spontaneous uterine contractions. Parallel administration of the ABCG₂ inhibitor KO-134 dose-dependently increased the uterus-relaxing effect of nifedipine. The ED₅₀ of nifedipine was 240 µg/kg, whereas that of its combination with 15 mg/kg KO-134 or with 30 mg/kg KO-134 was significantly lower, at 170 µg/kg and 25 µg/kg, respectively (**Fig. 2**).

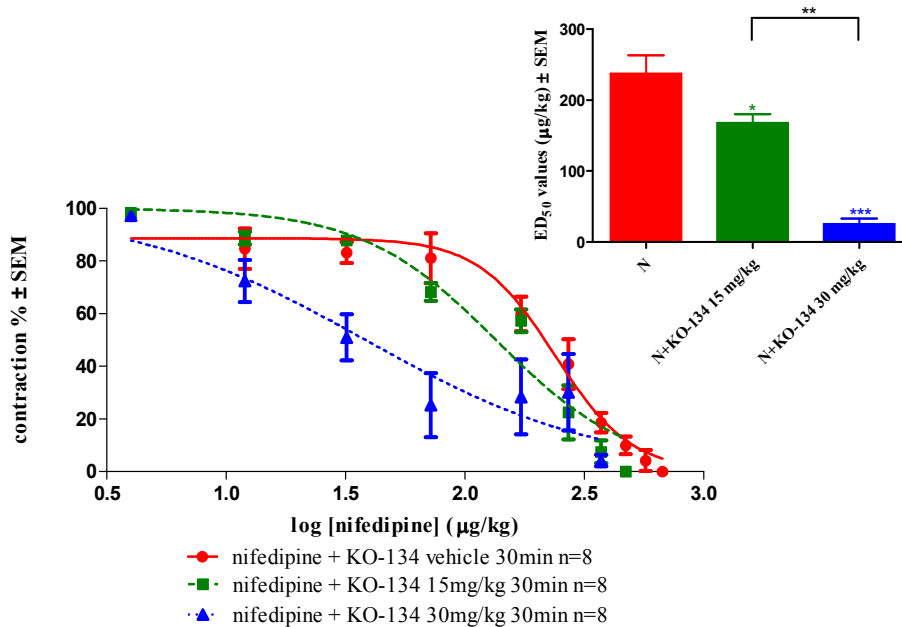


Figure 2 Uterus-relaxing effects of nifedipine (N) alone and in the presence of 15 mg/kg or 30 mg/kg doses of the ABCG₂ blocker KO-134 in the *post-partum* rat uterus *in vivo*. Insert: ED₅₀ values of nifedipine alone or in combination with KO-134 doses of 15 mg/kg or 30 mg/kg. The ED₅₀ values of the combinations were significantly lower than that of nifedipine alone. * p<0.05, ** p<0.01 and *** p<0.001. Each value denotes the mean ± S.E.M, n = 8.

ABCC8/SUR1 and ABCC9/SUR2 expression in the rat uterus

Relative quantitative real-time PCR and Western blot analysis revealed that both SUR1 and SUR2 mRNAs and proteins are expressed in the pregnant and non-pregnant rat uteri. The mRNA and protein expression of the SUR1 subtype were found to be elevated in the early stage of pregnancy (day 6), dramatically decreased from day 8 to day 12, and then remained unchanged until the end of pregnancy (**Fig. 3**). The SUR2 mRNA and protein levels did not undergo any alterations during pregnancy (**Fig. 4**). SUR1 and SUR2 were investigated on days 6 and 10 of pregnancy; samples were collected separately from implantation and interimplantation sites (**Fig. 5**).

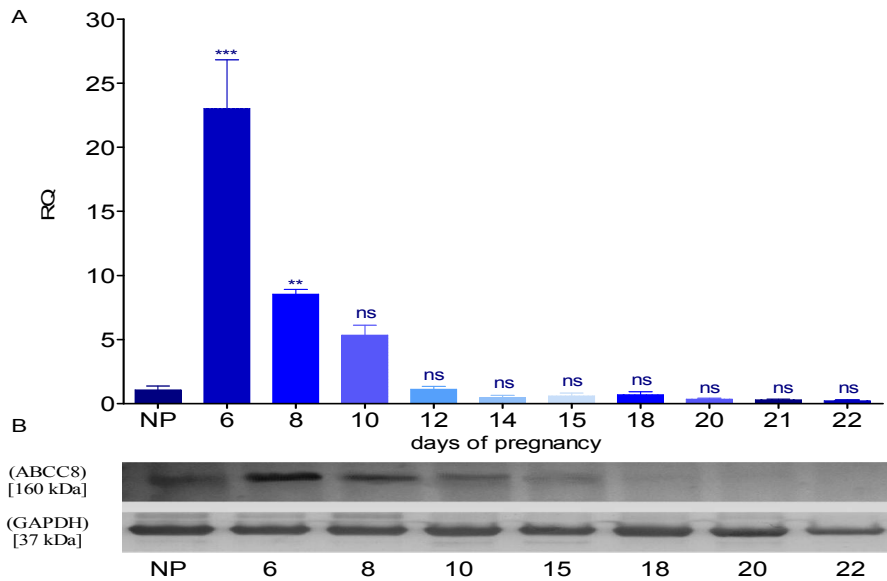


Figure 3. (A) Changes in expression of ABCC8/SUR1 mRNA during pregnancy in the rat myometrium. RQ (Relative Quantity) values on different days of pregnancy were compared with those in non-pregnant rats. ns: non-significant, ** denotes $p < 0.01$, *** $p < 0.001$. Each bar indicates the mean \pm SEM, $n = 5$. **(B)** Representative Western blot of ABCC8/SUR1 protein expression in the non-pregnant (NP) and the pregnant rat myometrium.

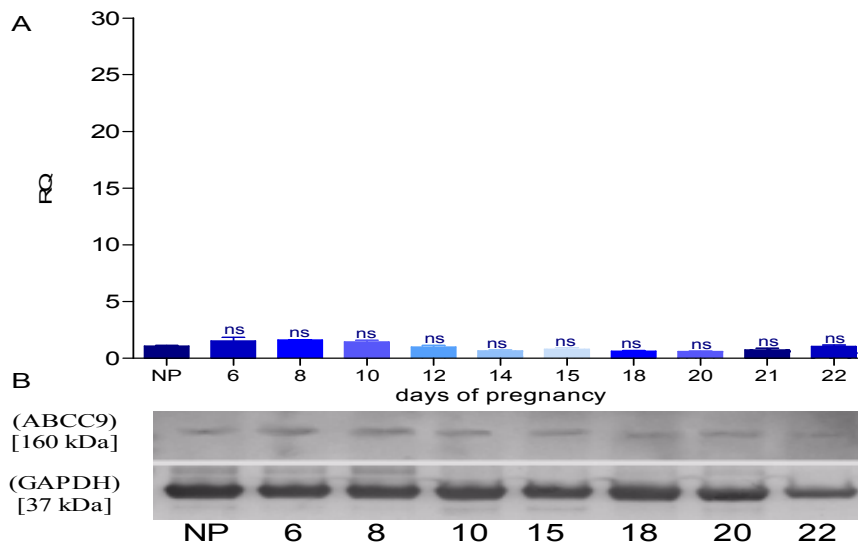


Figure 4. (A) Changes in expression of ABCC9/SUR2 mRNA during pregnancy in the rat myometrium. RQ values on different days of pregnancy were compared with those in non-pregnant rats. ns: non-significant. Each bar indicates the mean \pm SEM, $n = 5$. **(B)** Representative Western blot of SUR2 protein expression in the non-pregnant (NP) and the pregnant rat myometrium.

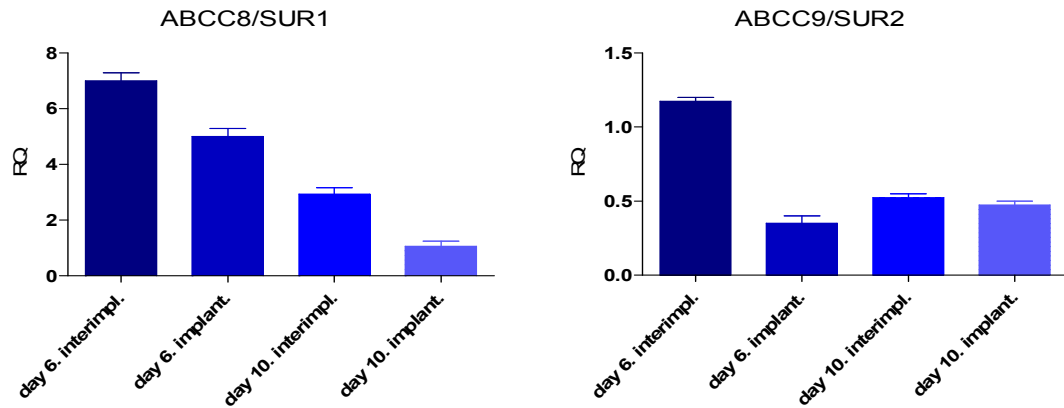


Figure 5. Change in expressions of ABCC8/SUR1 and ABCC9/SUR2 mRNA in 6-day and 10-day-pregnant animals. Samples were collected separately from implantation and interimplantation sites. Each bar indicates the mean \pm SEM, $n = 5$

Effects of the SUR-non-selective K_{ATP} channel opener diazoxide

Diazoxide in the range 10^{-8} – 10^{-4} M inhibited the oxytocin-induced contractions. The uterus-relaxant effect of diazoxide was investigated on the non-pregnant and on the 6-day, 8-day, 18-day and 22-day-pregnant rat uterus. The diazoxide-relaxant effect reached its maximum level on day 6 (60%), and was lower on days 8 and 18 (40%). Diazoxide had no significant effect on the uterine contractions in non-pregnant and term-pregnant animals. The relaxant effect was blocked by 10^{-6} M glibenclamide on day 6 of pregnancy (**Fig. 6**).

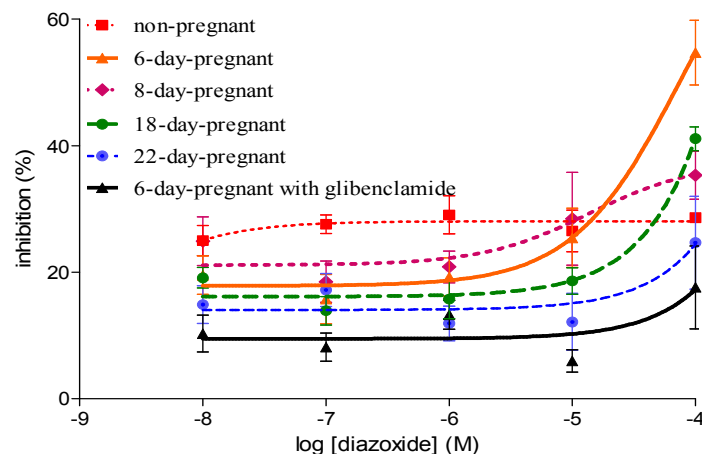


Figure 6. Uterus-relaxing effect of the K_{ATP} channel opener diazoxide (10^{-6} – 10^{-4} M) on oxytocin (10^{-6} M)-evoked rhythmic contractions in the non-pregnant and in the 6-day, 8-day, 18-day and 22-day-pregnant rat myometrium *in vitro*. Values on different days of pregnancy were compared with those in non-pregnant rats. Reversal by glibenclamide (10^{-6} M) on day 6 of pregnancy. Each value denotes the mean \pm SEM, $n = 6$.

Effects of the SUR2-selective K_{ATP} channel opener pinacidil

The oxytocin-stimulated uterine contractions of non-pregnant and of 8-day, 18-day and 22-day-pregnant rats were inhibited concentration-dependently by pinacidil in the range 10^{-8} - 10^{-4} M. The E_{max} values were elevated on days 8 and 18, but on day 22 E_{max} was significantly lower, similar to that in the non-pregnant animals. The EC_{50} values of pinacidil were significantly lower in the pregnant rat myometrium as compared to the non-pregnant myometrium (**Fig. 7**). The uterus-relaxant effect of pinacidil was blocked by glibenclamide (10^{-6} M) on days 8 and 22. The DRCs of the pinacidil were shifted to the right in the presence of glibenclamide (**Fig. 8**). The uterus-relaxant effect of pinacidil was investigated on EFS-induced contractions in non-pregnant and 22-day-pregnant rats in the presence of TEA (10^{-3} M) (**Fig. 9**). TEA significantly antagonized the uterus-relaxant effect of pinacidil on days 8 and 22 of gestation; the EC_{50} values were significantly higher in the presence of TEA.

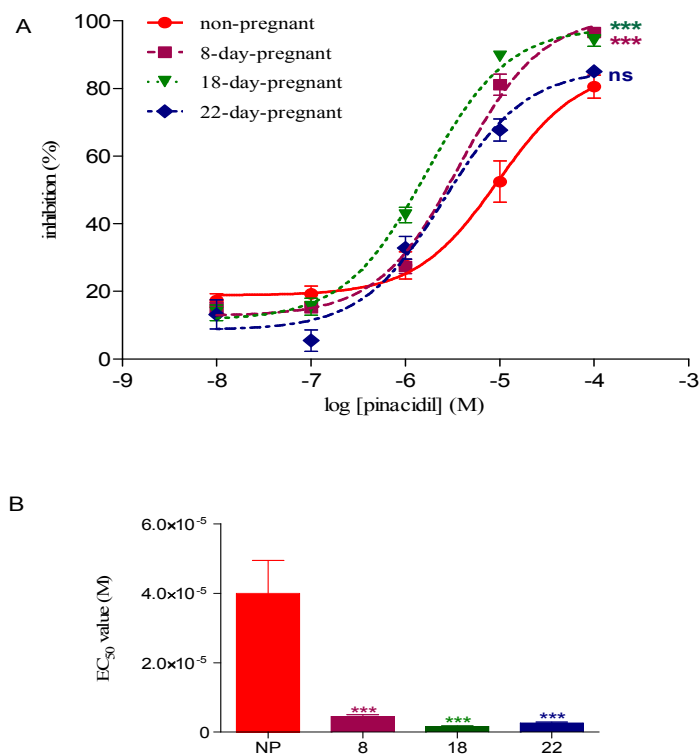


Figure 7 (A) Uterus-relaxing effect of the K_{ATP} channel opener pinacidil (10^{-8} - 10^{-4} M) on oxytocin (10^{-6} M)-evoked rhythmic contractions in the non-pregnant and in the 8-day, 18-day and 22-day-pregnant rat myometrium *in vitro*. E_{max} values on different days of pregnancy were compared with those in non-pregnant rats. **(B)** Changes in EC_{50} values of the pinacidil on oxytocin-induced contractions in the non-pregnant (NP) and in the 8-day, 18-day and 22-day-pregnant rat myometrium *in vitro*. EC_{50} values on different days of pregnancy were compared with those in non-pregnant rats. *** denotes $p < 0.001$. Each value denotes the mean \pm SEM, $n = 6$.

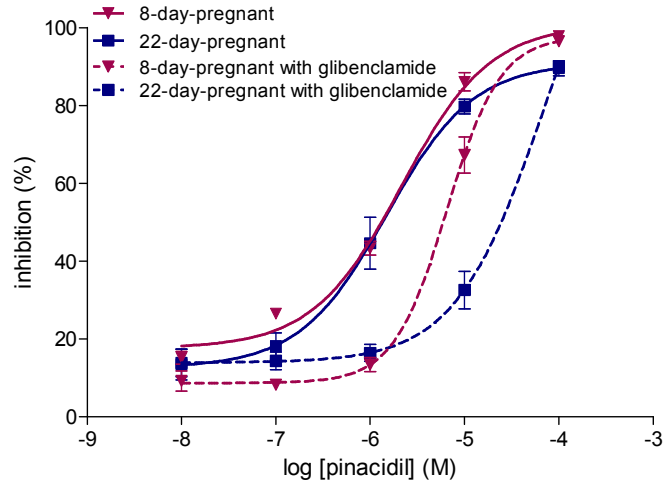


Figure 8. Uterus-relaxing effect of the K_{ATP} channel opener pinacidil (10^{-8} - 10^{-4} M) on oxytocin (10^{-6} M)-evoked rhythmic contractions on the 8-day and 22-day-pregnant rat myometrium *in vitro*; reversal by glibenclamide (10^{-6} M) on day 8 and day 22 of pregnancy. Each value denotes the mean \pm SEM, $n = 6$.

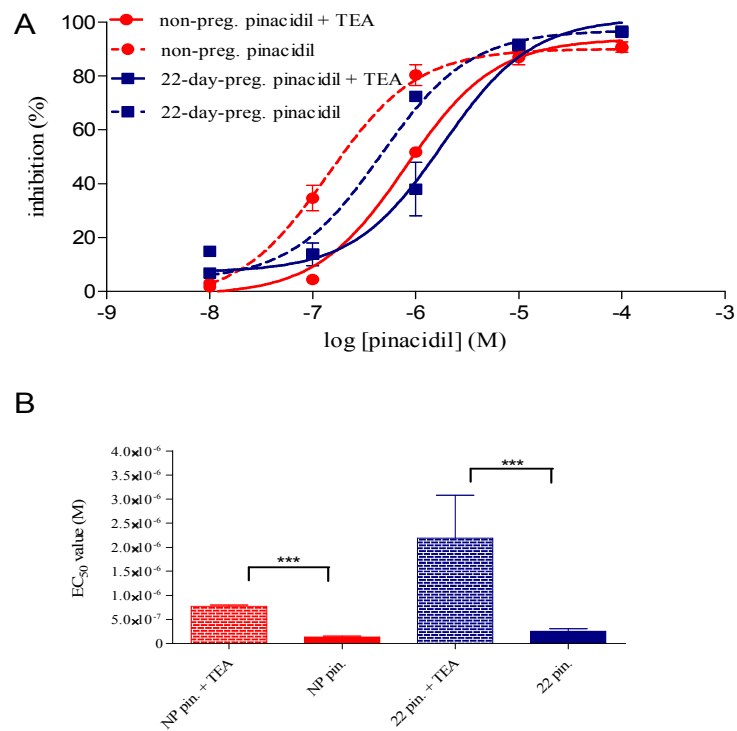


Figure 9. Uterus-relaxing effect of the K_{ATP} channel opener pinacidil (10^{-8} - 10^{-4} M) on EFS-evoked rhythmic contractions in the non-pregnant (NP pin.) and the 22-day-pregnant (22 pin.) rat myometrium *in vitro*; reversal by TEA (10^{-3} M). *** denotes $p < 0.001$. Each value denotes the mean \pm SEM, $n = 6$.

Effects of β_2 -AR agonists in the presence of the K_{ATP} channel blocker glibenclamide and the K_{ATP} channel opener pinacidil

Effects of β_2 -AR agonists on the 6-day-pregnant rat myometrium

The uterus-relaxant effects of ritodrine and salmeterol (10^{-10} - 10^{-5} M) were investigated on the 6-day-pregnant rat uterus in the presence of 10^{-6} M glibenclamide and different doses of pinacidil (10^{-9} , 10^{-8} and 10^{-7} M). Both the K_{ATP} channel blocker glibenclamide and the K_{ATP} channel opener pinacidil influenced the effects of salmeterol and ritodrine. Glibenclamide blocked the tocolytic effect of the β_2 -AR agonists, the DRCs shifted to the right, and the EC_{50} values of the β_2 -AR agonists were significantly increased in the presence of glibenclamide. Pinacidil enhanced the tocolytic effects of the β_2 -AR agonists, the DRCs were shifted to the left, and the EC_{50} values of the β_2 -AR agonists were significantly lower in the presence of pinacidil (**Figs 10 and 11**).

Effects of β_2 -AR agonists on the 22-day-pregnant rat myometrium

The uterus-relaxant effects of ritodrine and salmeterol (10^{-10} - 10^{-5} M) were investigated on the 22-day pregnant rat uterus in the presence of 10^{-6} M glibenclamide and different doses of pinacidil (10^{-9} , 10^{-8} and 10^{-7} M). Neither glibenclamide nor pinacidil was found influence the effects of the β_2 -AR agonists (**Figs 12 and 13**).

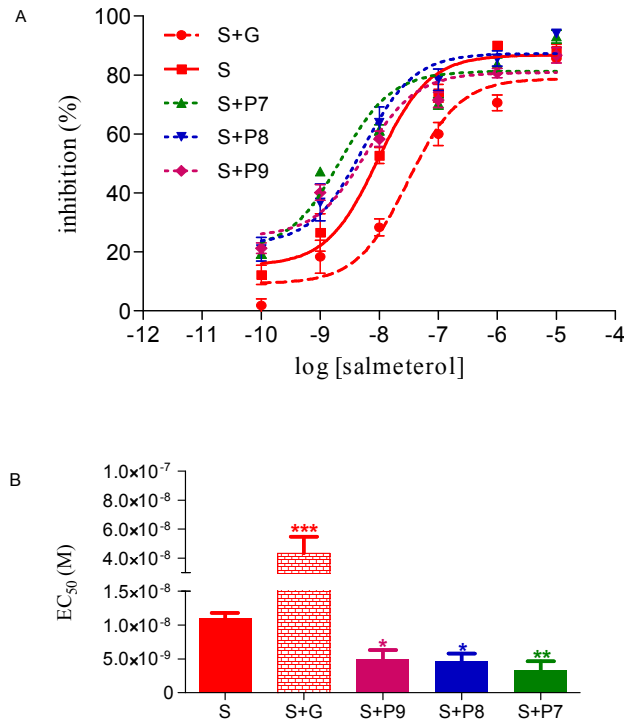


Figure 10 (A) Uterus-relaxing effect of the β_2 -AR agonist salmeterol (10^{-10} - 10^{-5} M) on the spontaneous rhythmic contractions in the 6-day-pregnant rat myometrium alone (S), reversal by glibenclamide (S+G), and in the presence of pinacidil (10^{-9} M: S+P9, 10^{-8} M: S+P8 and 10^{-7} M: S+P7) *in vitro*. **(B)** Changes in EC₅₀ values of the β_2 -AR agonist salmeterol acting on the spontaneous rhythmic contractions in the 6-day-pregnant rat, reversal by glibenclamide, and in the presence of pinacidil. The EC₅₀ values of the different combinations were compared with that for salmeterol alone. *** denotes $p < 0.001$, ** denotes $p < 0.01$ and * denotes $p < 0.05$. Each value denotes the mean \pm S.E.M, $n = 6$.

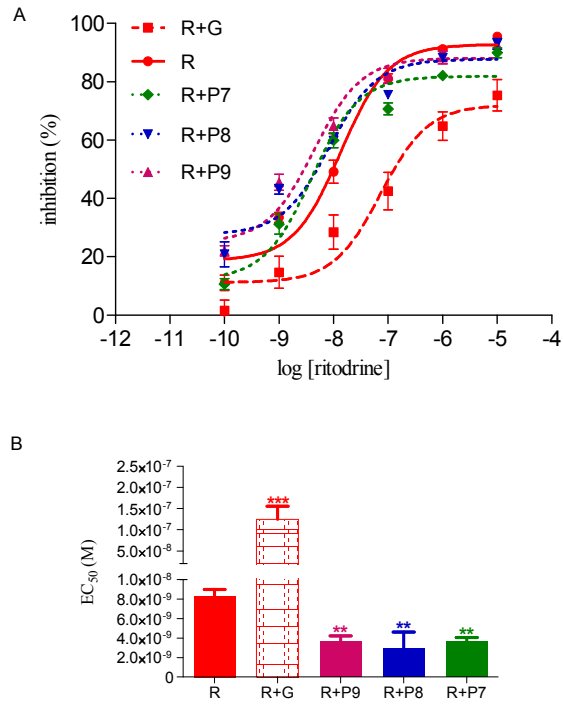


Figure 11 (A) Uterus-relaxing effect of the β_2 -AR agonist ritodrine (10^{-10} - 10^{-5} M) on the spontaneous rhythmic contractions in the 6-day-pregnant rat myometrium alone (R), reversal by glibenclamide (R+G), and in the presence of pinacidil (10^{-9} M: R+P9, 10^{-8} M: R+P8 and 10^{-7} M: R+P7) *in vitro*. **(B)** Changes in EC₅₀ values of the β_2 -AR agonist ritodrine acting on the spontaneous rhythmic contractions in the 6-day-pregnant rat, reversal by glibenclamide, and in the presence of pinacidil. The EC₅₀ values of the different combinations were compared with that for ritodrine alone. *** denotes $p < 0.001$ and ** denotes $p < 0.01$. Each value denotes the mean \pm S.E.M, $n = 6$.

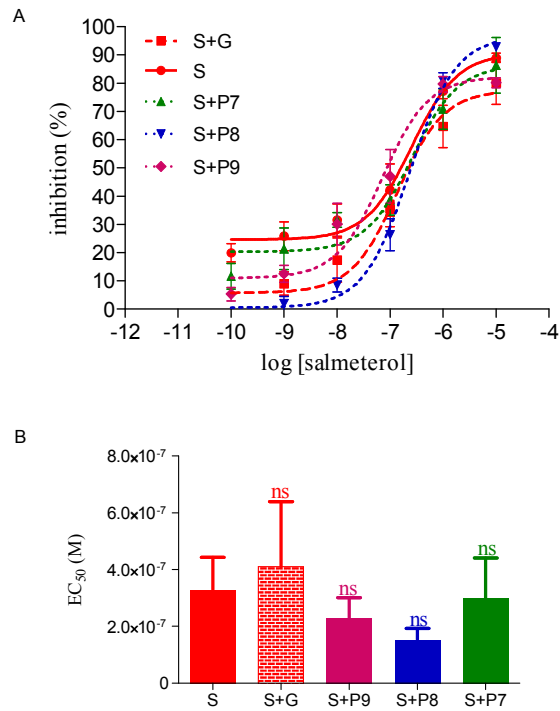


Figure 12 (A) Uterus-relaxing effect of the β_2 -AR agonist salmeterol (10^{-10} - 10^{-5} M) on the spontaneous rhythmic contractions in the 22-day-pregnant rat myometrium alone (S), reversal by glibenclamide (S+G), and in the presence of pinacidil (10^{-9} M: S+P9, 10^{-8} M: S+P8 and 10^{-7} M: S+P7) *in vitro*. **(B)** Changes in EC_{50} values of the β_2 -AR agonist salmeterol acting on the spontaneous rhythmic contractions in the 22-day-pregnant rat, reversal by glibenclamide, and in the presence of pinacidil. The EC_{50} values of the different combinations were compared with that for salmeterol alone, ns: non-significant. Each value denotes the mean \pm S.E.M, n = 6.

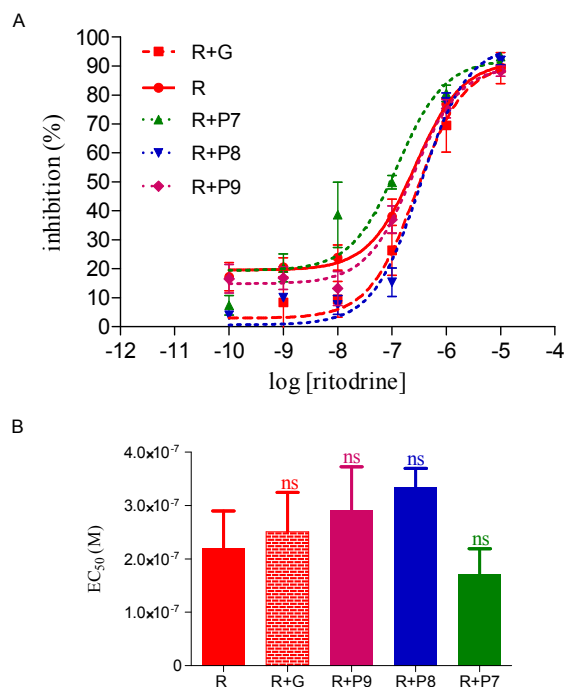


Figure 13 (A) Uterus-relaxing effect of the β_2 -AR agonist ritodrine (10^{-10} - 10^{-5} M) on the spontaneous rhythmic contractions in the 22-day-pregnant rat myometrium alone (R), reversal by glibenclamide (R+G), and in the presence of pinacidil (10^{-9} M: R+P9, 10^{-8} M: R+P8 and 10^{-7} M: R+P7) *in vitro*. **(B)** Changes in EC₅₀ values of the β_2 -AR agonist ritodrine acting on the spontaneous rhythmic contractions in the 22-day-pregnant rat, reversal by glibenclamide, and in the presence of pinacidil. The EC₅₀ values of the different combinations were compared with that for ritodrine alone, ns: non-significant. Each value denotes the mean \pm S.E.M, n = 6.

5. Discussion

The myometrial smooth muscle remains relatively quiescent throughout most of pregnancy, but at term it undergoes a transformation that results in the development of powerful rhythmic contractions. The factors regulating these painful contractions during pregnancy and labour are poorly understood. An understanding of these processes, at the cellular and molecular levels, is essential to develop novel therapeutic strategies for the management of associated clinical problems such as preterm labour, the main cause of perinatal mortality and morbidity in the developed world. Despite several agents being available to treat preterm birth, the rate of preterm birth is still increasing; tocolysis is one of the greatest challenges in obstetrical practice. New targets and new mechanisms are needed to develop sufficient tocolytic agents. ABC transporters can be one of these new targets in the treatment of preterm birth.

Several ABC transporters have been identified in the uterus, which function as efflux pumps or ion channel regulators. This work was undertaken to extend our knowledge concerning the gestational changes and functions of ABC transporters, from the aspects of both efflux transporters (ABCG2) and K_{ATP} channel regulators (ABCC8 and ABCC9) in the rat uterus.

As concerns the efflux transporters, ABCG2 is one of the most extensively studied ABC transporters; it is highly expressed in reproductive tissues (placenta, uterus and prostate) and has an important role in the tissue defence through the efflux of toxic compounds and their metabolites, thereby decreasing their intracellular concentration. Several compounds with a uterus-relaxant effect (e.g. prazosine and nifedipine) are transported by ABCG2. Nifedipine is commonly used in the therapy of PTL; it has a greater tocolytic effect with less frequent side-effects than β_2 -AR agonists (Koks *et al.*, 1998, Cararach *et al.*, 2006, Conde-Agudelo *et al.*, 2011). Our results demonstrated that there were low levels of ABCG2 in the non-pregnant and the early-pregnant uterus, but on day 15 of gestation a sharp increase was observed, leading to a maximum on day 18 and a subsequent decrease from day 20 to post-partum. The *post-partum* level was similar to that in the non-pregnant animals. Our findings are comparable to those of Cygalova *et al.* (2008), who found elevated ABCG2 levels in the rat foetus on gestational days 15, 18 and 21. It seems that corresponding expressional changes occur in the foetus and the uterus. Cygalova *et al.* (2008) concluded that the foetal and placental ABCG2 provides protection during gestation. It may be hypothesized that the

expression of the ABCG2 efflux protein in the rat uterus may also serve as a protective mechanism during gestation, functioning as a special barrier to defend the uterus and foetus from xenobiotics (*e.g.* tocolytics). From a pharmacotherapeutic aspect, it may be a relevant mechanism that can reduce the efficacy of tocolytics. Moreover, if this efflux mechanism could be blocked, then the tocolytic effect could be increased. Our *in vivo* contractility studies tend to confirm this hypothesis. The results of Zhou *et al.* (2005) and Shukla *et al.* (2006) indicated that nifedipine is transported by ABCG2. The contractility studies revealed the strong uterus-relaxant effect of nifedipine on spontaneous contractions. Although the *in vivo* experiments were carried out on *post-partum* rats, in which a low ABCG2 expression was found, our results clearly demonstrated that the combination of nifedipine with the ABCG2 blocker KO-134 significantly and dose-dependently increased the uterus-relaxing effect of nifedipine. Our findings clearly reveal that the combination of an efflux pump inhibitor with the tocolytic agent nifedipine results in an enhanced uterus-relaxing effect. In the future, ABC transporters may be new targets in drug design and development. The main problem with ABCG2 inhibitors in human use is their lack of tissue specificity, which results in undesired adverse effects. The development of a new uterus-selective ABCG2 inhibitor for human therapy appears to be a possibility of novel therapeutic relevance in the management of PTB.

Besides efflux proteins, a number of ABC transporters function in ion-channels as regulators. ABCC8/SUR1 and ABCC9/SUR2 are included in the K_{ATP} channels. Kir_{6.x} comprises the channel component of the K_{ATP} channel, while the SURs are responsible for the ATP sensitivity, pharmacological properties and trafficking of this channel. Previous studies reported that only the SUR2B subunit was involved in the K_{ATP} channels in the rat myometrium (Chien *et al.*, 1999, Sawada *et al.*, 2005). SUR1 and SUR2 mRNA transcripts were found in the human myometrium (Curley *et al.*, 2002). In contrast with Chien *et al.*, (1999) and Sawada *et al.*, (2005), our results demonstrated that both SUR subunits were expressed in the rat myometrium during gestation. Our RT-PCR and Western blot analyses revealed that the SUR1 levels were sharply elevated on day 6 of gestation and gradually decreased to term, while low SUR2 levels were found which did not change during gestation. It is well known that implantation occurs in early pregnancy (day 5-6 of pregnancy). To exclude the possibility that the elevated SUR1 levels in early pregnancy were not due to the implantation, we investigated the implantation and interimplantation sites separately in

the early pregnant stages (days 6 and 10) to determine whether there were any differences between them. The results showed that there were no differences between the implantation and the interimplantation sites, and thus the elevated SUR1 levels in the 6- and 10-day-pregnant uterus were not due to the implantation. Similarly to the results of Curley *et al.* (2002) on the human myometrium our findings indicate that the decrease in SUR1 expression in late pregnancy may facilitate the enhanced contractility of the rat myometrium. The uterus-relaxant effect of diazoxide was significantly stronger when the SUR1 expression was sharply increased on days 6 and 8 of pregnancy. Thus, the pharmacological reactivity of the non-selective diazoxide depends on the characteristic change in SUR1. In the case of SUR2, low mRNA expression and protein levels were found, which did not change during gestation. In spite of the fact that low SUR2 levels were found during gestation, a strong uterus-relaxant effect of the SUR2 agonist pinacidil was observed on the pregnant rat uterus, while the relaxant effect on the non-pregnant uterus was significantly weaker. The relaxant effect of pinacidil correlates with the SUR2 level because it remained unchanged during gestation. Pinacidil is generally accepted as a SUR2-selective K_{ATP} channel opener, but our results showed that this is questionable; the K_{Ca} channel blocker TEA, antagonized the uterus-relaxant effect of pinacidil on both the non-pregnant and the 22-day-pregnant uterus. This result confirmed that pinacidil has multiple binding sites for K^+ channels. The same results were found in the human radial artery by Gojkovic-Bukarica *et al.* (2011). Glibenclamide, a K_{ATP} channel blocker, antagonized both pinacidil and diazoxide induced-relaxation in the rat myometrium. However, it is generally accepted that glibenclamide is a selective SUR1 blocker. Glibenclamide binds to both SUR subunits, but in two different ways; SUR1 has two binding sites for blockers (sulphonylurea and benzamido), while SUR2 has only a benzamido binding site. Glibenclamide contains both sulphonylurea and benzamido moieties, and can therefore bind to SUR1 in two regions and to SUR2 in one region (Ascroft & Gribble 2000b, Ashfield *et al.*, 1999, Babenko *et al.*, 1999). Moreover, Stephan *et al.* (2006) demonstrated that glibenclamide (10^{-9} M) induced complete inhibition of the pancreatic K_{ATP} channel, whereas higher concentrations (10^{-7} M or 10^{-6} M) produced only partial and reversible inhibition of the cardiovascular K_{ATP} channels. Our results showed that these suggestions were also applicable for the rat myometrium.

In the last 20 years, the K_{ATP} channels have been extensively investigated in various tissues because they have a central role in the membrane potential regulation.

Several papers have reported that K_{ATP} channels are involved in β -AR agonist-induced smooth muscle relaxation; pulmonary vasorelaxation in the rat (Sheridan *et al.*, 1997), vasodilatation in the rat diaphragmatic microcirculation (Chang *et al.*, 1997), vasorelaxation in the rat mesenteric artery (Randall *et al.*, 1995), detruser muscle relaxation in the rat (Hudman *et al.*, 2000) and myometrial relaxation in non-pregnant buffaloes (Choudhury *et al.*, 2009). Our results clearly demonstrated that in the early-stage of gestation (day 6), when an elevated SUR1 level was observed, the β_2 -AR agonist-induced myometrial relaxation was inhibited by glibenclamide and potentiated by pinacidil. At the end of gestation (day 22), when the SUR1 level was decreased, neither glibenclamide nor pinacidil influenced the tocolytic effects of the β_2 -AR agonists. It can be concluded that the involvement of the K_{ATP} channel in the efficacy of the β_2 -AR agonist depends on the expression of the SUR1 subunit of the K_{ATP} channel. Earlier we had demonstrated that the tocolytic effects of the β_2 -AR agonists were significantly decreased towards term (days 15, 18, 20 and 22 of gestation) as compared with early gestation in the rat (Gaspar *et al.*, 2005). This phenomenon could be explained by decrease of the β_2 -AR function, which is partially controlled by β -adrenergic kinase, oestrogen/progesterone levels and G-protein-coupled receptor kinases (Ruzycky & DeLoia 1997, Simon *et al.*, 2001, Simon *et al.*, 2003). From our results, it is very likely that there are other mechanisms which cause the decreased tocolytic effect of β_2 -AR agonists at the end of gestation. The low levels of K_{ATP} channels at the end of gestation are one of the reasons for the decreased efficacy of the betamimetics. In the human myometrium, Curley *et al.* (2002) showed that the SUR1 expression was decreased in late-pregnancy as compared with non-pregnant. Moreover, low levels of the Kir_{6.1} and Kir_{6.2} subunits were determined at the end of gestation. Since, the open state of the K_{ATP} channels draws the cell membrane potential closer to the K^+ equilibrium potential, the K_{ATP} channels are closely involved in reducing cellular excitability and contractility. The low levels of the K_{ATP} channels at the end of gestation may facilitate the enhanced excitability and contractility both in the rat and in the human myometrium. The combination of betamimetics with a K_{ATP} channel opener will therefore not have any therapeutic relevance in the treatment of PTB. However, this combination may be used as a uterus relaxant in the early stage of gestation (e.g. habitual abortion).

6. Appendix

List of publications

Publications related to the Ph.D. thesis

- I. **Norbert Lovasz**, Eszter Ducza, Robert Gaspar and George Falkay. Ontogeny of sulfonyleurea-binding regulatory subunits of K_{ATP} channels in the pregnant rat myometrium. *Reproduction* 2011;142; 175–181. **IF₂₀₁₁: 3.090**
- II. **Lovász Norbert**, Ducza Eszter, Gáspár Róbert, Falkay György. A K_{ATP} csatorna szulfonilurea alegységeinek ontogenezise a terhes patkány miometriumban. *Acta Pharmaceutica Hungarica* 2010;3; 109-114.
- III. **Norbert Lovasz**, Eszter Ducza, Istvan Zupko And George Falkay. Increase of the Uterus-relaxant Effect of Nifedipine by the Abcg2 Efflux Protein Inhibitor KO134 in the Rat In Vivo. *IN VIVO* 2013; 27; 363-370. **IF₂₀₁₂: 1.219**

Abstracts

- I. **Lovász Norbert**, Minorics Renáta, Gáspár Róbert, Falkay György. Transzportfehérjék (ABCC8 és ABCC9) ontogenezise és a K_{ATP} csatornák szerepe a terhes patkány uterus kontraktilitásának szabályozásában. XIV. Congressus Pharmaceuticus Hungaricus; Budapest, 2009. november 13-15 (Poster).
- II. George Falkay, **Norbert Lovasz**, Eszter Ducza, Robert Gaspar. Variable expression of abcc8 and abcc9 transporters in the pregnant rat myometrium: the influence of gestation age. 16th World Congress on Basic and Clinical Pharmacology, 17-23 July 2010, Copenhagen, Denmark (Poster).
- III. **Lovász Norbert**, Ducza Eszter, Gáspár Róbert. ATP-szenzitív K-csatorna szulafanilurea alegységeinek (SUR1, SUR2) ontogenezise a terhes patkány uterusban. A Magyar Tudomány Ünnepe, Szeged, 2010. November 17 (Presentation).
- IV. **Norbert Lovasz**, Eszter Ducza, Istvan Zupko, George Falkay. Increased tocolytic effect of nifedipine by ABCG2 efflux protein inhibitor KO-134 in rat in vivo. Pharmaceutical Sciences for the Future of Medicines. 3rd PharmSciFair, Prague, 14-17 June, 2011 (Poster).

- V. Lovász Norbert**, Ducza Eszter, Gáspár Róbert. A KATP- transzporter fehérjék szerepe a terhes uterusz farmakológiai reaktivitásában. XVIII. Szent-Györgyi Napok, Szeged, 2011. November 14-19 (Presentation).
- VI.** George Falkay, **Norbert Lovasz**, Eszter Ducza, Istvan Zupko. Increased tocolytic effect of nifedipine by ABCG2 efflux protein inhibitor KO-134 in rat in vivo. 14th World Congress on Human Reproduction Melbourne, 30 November–3 December, 2011 (Poster).
- VII.** George Falkay, Eszter Ducza, Andrea Koncz, **Norbert Lovasz**. Role of K_{ATP} channel in the tocolytic effect of β_2 -adrenoceptor agonist terbutaline in rat myometrium, *in vitro*. 6th European Congress of Pharmacology, Granada, 17-20 July, 2012 (Poster).
- VIII.** **Norbert Lovasz**, Andrea Koncz, Eszter Ducza and George Falkay. KATP channels are involved in the tocolytic effect of β_2 agonists in pregnant rat. Society for Endocrinology BES 2013 Harrogate 18 March- 21 March 2013 (Poster).