Department of Pharmacodynamics and Biopharmacy
Faculty of Pharmacy, University of Szeged
Supervisor: Prof. George Falkay Ph.D., D.Sc.

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

Ph.D. Thesis

By
Norbert Lovász

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List of abbreviations

ABC transporter: ATP-binding cassette transporter

ABCC8: ATP-binding cassette, sub-family C, member 8

ABCC9: ATP-binding cassette, sub-family C, member 9

ABCG2: ATP-binding cassette, sub-family G, member 2

ATP: adenosine-5'-triphosphate

AUC: area under curve

ß$_2$-AR: ß$_2$-adrenergic receptor

CCB: Ca^{2+}-channel blocker

DHPs: dihydropyridine-type Ca^{2+}-channel blockers

DRC: dose-response curve

EFS: electric field stimulation

K_{ATP} channel: ATP-sensitive K^+ channel

K_{Ca}: Ca^{2+}-dependent K^+ channel

KCOs: K^+ channel-opening compounds

Kir: K^+ inward rectifier

NBD: nucleotide binding domain

P: progesterone

PGs: prostaglandins

PTB: preterm birth

PTL: preterm labour

SUR: sulphonylurea receptor

SUR1: sulphonylurea-binding regulatory subunit 1

SUR2: sulphonylurea-binding regulatory subunit 2

TEA: tetraethylammonium

TMD: transmembrane domain

1. Introduction

1.1. Epidemiology and consequences of preterm birth

Preterm birth (PTB), 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. In the USA, the rate of PTB is 12-13%, in Europe and other developed country the reported rates are generally 5-9% (Goldenberg *et al.*, 2008, Reich 2012). The incidence of preterm labour (PTL) in Hungary is still around 8% (Blencowe *et al.*, 2012).

The exact causes of PTB are unsolved. In fact, the cause of 50% of the causes of PTB is never determined. Several risk factors for PTB have been established: previous repeated uterine and cervical anomalies, second trimester abortion, multiple pregnancy, precocious foetal endocrine activation, *in vitro* fertilization, intrauterine inflammation/infection, gestational bleeding, abnormal placentation, urogenital infection, maternal medical complications, a low socio-economic status and a low body mass index before conception (Hillier *et al.* 1995, Moutquin 2003, Hendler *et al.* 2005, Haas 2006, Banhidy *et al.* 2007, Pennell *et al.* 2007, Simhan & Caritis 2007). Other factors, such as the maternal age, physical workload, inadequate prenatal care, psychosocial stress, sexual activities, drug and alcohol abuse, smoking and maternal weight gain, are still under evaluation (Berkowitz *et al.*, 1998, Parazzini *et al.*, 2003). Children who are born prematurely have higher rates of cerebral palsy, sensory deficits, neuroinflammation, learning disabilities and respiratory illnesses as compared with children born at term (Moster *et al.*, 2008). The morbidity associated with PTB often extends to later life, resulting in enormous physical, psychological and economic costs (Petrou *et al.*, 2003, Petrou 2005).

1.2. Tocolytic therapy

Several agents are used clinically as tocolytics, including magnesium sulphate, β_2-adrenergic receptor (β_2-AR) agonists, oxytocin receptor antagonist atosiban, progesterone (P), prostaglandin (PG) synthesis inhibitors, nitric oxide (NO) donors and

calcium (Ca^{2+}) channel blockers (CCBs), but the efficacy of the current modes of pharmacological treatment has been questioned (Kim & Shim 2006). Despite the research to develop drugs to inhibit myometrial contractions, there has been no reduction in the incidence of PTB for more than 30 years. Present therapies cannot prevent PTB, but at best provide sufficient delay in order to attempt treatments that ameliorate the consequences of prematurity. According to Husslein & Quartarolo (2003), the main rationale for the use of these drugs is to delay delivery for at least 48 hours in order to allow time for the effective treatment with corticosteroids, or transfer of the pregnant mother to a specialized high-risk obstetrical unit.

Magnesium sulphate has been used intravenously for the treatment of eclamptic convulsions, and for seizure prophylaxis in the setting of suspected preeclampsia (Graham 1998, Omu *et al.,* 2008). It has been widely used as a tocolytic in the USA and for more than 30 years was often the first-line tocolytic, but it has rarely been used for this purpose in Europe (Morgan *et al.,* 2008, Mercer & Merlino 2009). The therapeutic serum concentrations of Mg^{2+} for the prevention and treatment of seizures have not been rigorously determined; ,minimum levels of 4 mEq/l (4.8 mg/dl) have been suggested based ont he basis of clinical experience, rather than a formal dose-response evaluation (Salinger *et al.,* 2013). Various mechanisms of action have been proposed, such as Mg^{2+}competing with Ca^{2+} and thereby affecting multiple intracellular pathways, but the exact role remains controversial. According to a 2010 Cochrane review, magnesium sulphate is not effective in delaying birth or preventing PTB, because there was not enough evidence to show any difference between Mg^{2+} maintenance therapy and either placebo or no treatment (Han *et al.,* 2010).

NO donors (e.g. glyceryl trinitrate) have been used as a patch on the abdomen for cervical ripening, labour induction and tocolysis. NO is formed from L-arginine by the action of NO synthetase. NO increases levels of cyclic guanosine monophoshate and protein kinase G and can thereby affect several pathways associated with relaxation. According to Arrowsmith *et al.* (2010), NO donors did not delay labour or improve the neonatal outcome as compared with placebo or on alternative tocolytic.

The PGs play an important role in the onset and maintenance of labour. Indomethacin was first used for tocolysis in 1974 (Zukerman *et al.,* 1974), but despite the favourable results, most studies have limited the duration of indomethacin use because of the development of oligohydramnios, an increased risk of necrotizing

enterocolitis and renal failure and constriction of the ductus arteriosus Botalli (Giles & Bisits, 2007, Sood *et al.,* 2011).

Competitive antagonists of the oxytocin receptor (atosiban, barusiban, epelsiban and retosiban) have been shown to inhibit the uterotonic action of oxytocin completely in a competitive and dose-dependent manner and to inhibit oxytocin-mediated PG release. Atosiban was the first compound that was directly developed for the management of PTB. Although atosiban has been extensively studied in randomized, controlled trials, there is still controversy about its effectiveness and long-term safety (Kinsler *et al.,* 1996, Papatsonis *et al.,* 2005, Kim *et al.,* 2006).

β_2-AR agonists were considered the drugs of choice for the treatment of threatening PTB on the basis of randomized controlled trials and several subsequent meta-analyses, which showed that β-agonists delay PTB for at least the required 48 hours. Although there are convincing data indicating the effective prolongation of pregnancy, β-mimetics have the most undesirable side-effect profile of all currently employed tocolytics (Pryde *et al.,* 2001, Oei 2006). The most serious reported side-effects associated with the administration of β_2-AR agonists as tocolytics are pulmonary oedema, hypotension and tachycardia.

CCBs, initially proposed as tocolytics in the 1980s, have had a recent resurgence. These agents act to inhibit Ca^{2+} influx across the cell membrane, thereby decreasing the tone in the smooth muscle vasculature (Tan *et al.,* 2006). They were originally introduced to treat hypertension. Comparative trials with β-agonists have revealed a more favourable neonatal outcome and better prolongation of gestation (Papatsonis *et al.,* 1997, Koks *et al.,* 1998). Compared with other tocolytic agents, CCBs significantly delay birth (7 days) when used before 34 weeks. Most of these trials involved the comparison of nifedipine with ritodrine. However, no placebo-control trials have addressed the acute management of PTL, and there is uncertainty about the optimum form, dose and route of administration for CCBs (Arrowsmith *et al.,* 2010).

Supplemental treatment with P has been studied to prevent PTB and birth and as an adjunct to treat acute PTL (Meis *et al.,* 2003, da Fonseca *et al.,* 2003, Conde-Agudelo *et al.,* 2013). It has been demonstrated to reduce the risk of recurrent PTB when used prophylactically, but has not been thoroughly investigated as an adjunct to tocolytic drugs on the human myometrium. The primary action of P is thought to be mediated by its interaction with the intracellular nuclear P receptor, but actions via a plasma membrane receptor have recently been discovered. It may also have an anti-

inflammatory effect, which aids its tocolytic action. Through its binding to nuclear receptors, P alters gene expression, bringing about long-term changes in the contractile phenotype of the myometrium. P inhibits phosphodiesterase PDE4, thereby increasing the level of cAMP (Arrowsmith *et al.,* 2010).

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.

1.3. ABC transporters, general overview

The ABC (ATP-binding cassette) transporters form one of the largest families of membrane transport proteins expressed in all organisms. The family is characterized by a conserved structure of ABC binding domains (containing the "ATP-binding cassette" motif) and transmembrane domains. In mammals, the functional ABC protein contains two nucleotide binding domains (NBDs) and and two transmembrane domains (TMDs). The four domains can be present in one polypeptide chain (full transporters) or might be set up by homo- or heterodimerization of two polypeptides containing one of each domain (half-transporters). Each TMD consists of six transmembrane α-helixes (6-12) which span the membrane (**Fig. 1**). Two TMDs together form a pore-like structure creating a channel across the membrane. The TMD probably has two conformations: an open and a closed conformation. The changes in the conformations are regulated by ATP hydrolysis which is responsible for the translocation of the compound across the cell membrane.

The human ABC transporter family consists of 49 members, divided into 7 subfamilies, from A to G, based on similarity in gene structure, the order of the domains and sequence homology. To date, 16 ABC genes have been linked to inherited diseases, such as Tangier disease (ABCA1), Dubin-Johnson syndrome (ABCC2), pseudoxanthoma elasticum (ABCC6) and cystic fibrosis (ABCC7) (Dean 2005).

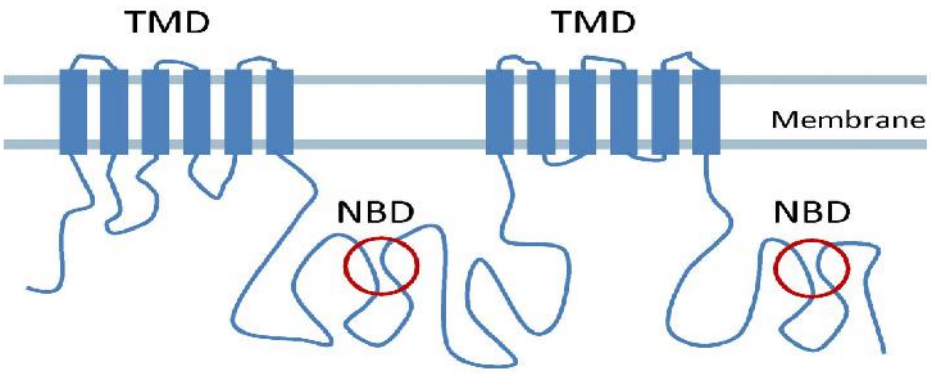


Figure 1 Typical structure of the ABC transporters. The TMDs each contain 6 transmembrane segments and the NBDs contain the Walker A and Walker B (red circle) motifs. ABC half-transporters consist of one TMD and one NBD that combine to form a functional unit upon translation. Adapted from Kavishe *et al.* (2009).

The ABC transporter family has been extensively studied, and its members play a vital role in many cellular processes. ABC transporters are responsible for the multidrug resistance of cancer cells (ABCB1, ABCC1 and ABCG2) (Hyde *et al.,* 1990), but may also be capable of transporting several substrates such, as metal ions, peptides, amino acids, sugars and a large number of hydrophobic compounds and metabolites across the plasma membrane and also intracellular membranes. ABC transporters have an important role in tissue defence through the excretion of toxic compounds and their metabolites (Dean *et al.,* 2001). The expression levels of ABC transporters are high in tissues which have a barrier function; the brain, kidney, intestine, placenta, liver and lung. The expression levels of the transporters are tightly regulated, emphasizing their importance in organ protection (Leslie *et al.,* 2005).

1.4. The role of ABC transporters in the uterus

Several ABC transporters have been identified in human reproductive tissues (placenta, uterus, prostate and testis); ABCA 1, 4, 5-10, ABCB 5, 7, 8, ABCC 1, 5, 7-10, ABCD 2-4 and ABCF 1-3 (Langmann *et al.,* 2003). These transporters are mainly efflux proteins. The most extensively studied ABC efflux transporters are the ABCB1 (P-glikoprotein/MDR1), ABCC1 (MRP1) and ABCG2 (BCRP/MXR).

The ABCG2 efflux protein displays high expression levels in reproductive tissues such as the placenta (Maliepaard *et al.,* 2001a) and uterus (Langmann *et al.,*

2003), and somewhat lower expression levels in the prostate, testis and ovary (Doyle *et al.*, 1998). ABCG2 transports various compounds through the cell membrane: **Table 1** shows the compounds transported by ABCG2, and **Table 2** presents ABCG2 inhibitors.

Table 1 Compounds transported by the ABCG2 efflux protein.

$ABCG_2$ substrates		**References**
chemotherapeutic agents	mitoxantrone, topotecane, irinotecane, methotrexate, imatinibe	Ding *et al.* (2010)
antiviral agents	lamivudine, zidovudine, abacavir	Krishnamurthyet & Schuetz (2006), Pal *et al.* (2011)
antibiotics	ciprofloxacin, ofloxacin, norfloxacin, erythromycin, rifampicin, nitrofurantoin	Robey *et al.* (2009), Hutson *et al.* (2010), Hahnova-Cygalova *et al.* (2011)
Ca^{2+} channel blockers	nifendipine	Shukla *et al.* (2006), Zhou *et al.* (2005)
HMGCoA reductase inhibitors	rosuvastatin, pitavastatin, cerivastatin	Huang *et al.* (2006), Robey *et al.* (2009)
others	cimetidine folic acid dipyridamole	Staud & Pavek (2005), Robey *et al.* (2009)

Table 2 ABCG2 inhibitors.

$ABCG_2$ inhibitors		**References**
flavonoids	apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin, naringenin, silymarin	Morris & Zhang (2006)
Ca^{2+} channel blockers	nicardipine, niguldipine, nitrendipine, verapamil	Zhou *et al.* (2005), Heinrich *et al.* (2006)
oestrogens	oestrone, 17β-oestradiol	Imai *et al.* (2003) and (2005)
fumitremorgin C analogues	KO-132 KO-134 KO-143 mycotoxin fumitremorgin C demethoxyfumitremorgin C	Allen *et al.* (2002), Rabindran *et al.* (2000), van Loevezijn *et al.* (2001)
others	elacridar (GF120918) tariquidar (XR9576) novobiocin etposide cyclosporine-A HER tyrosine kinase inhibitor (CI1033) camptothecin analogues (GF120918)	de Bruin *et al.* (1999), Staud & Pavek (2005), Heinrich *et al.* (2006), Erlichman *et al.* (2001), Maliepaard *et al.* (2001b)

As concerns the dihydropyridine-type Ca^{2+} channel blockers (DHPs), Zhou *et al.* (2005) reported that, apart from nifedipine, they enhance intracellular mitoxantrone accumulation in a concentration-dependent manner. Shukla *et al.* (2006) found that DHPs are transported by ABCG2 and also determined the effect of DHPs on the

ATPase activity of ABCG2; both nicardipine and nifedipine stimulated ATP hydrolysis by the transporter, the maximum stimulation by nifedipine proving equal to or greater than that obtained with prazosine, a known substrate of ABCG2. Moreover, they established that fumitremorgin C inhibited nifedipine-stimulated ATPase activity in a concentration-dependent manner. Ca^{2+}-channel antagonists are known to abolish the intracellular Ca^{2+} transients and myometrial contractions (Forman *et al.,* 1979). The Ca^{2+}-channel blocker most commonly used in the onset of PTL is nifedipine. In a recent review (Conde-Agudelo *et al.,* 2011), nifedipine was described as superior to $ß_2$-AR agonists and magnesium sulphate for tocolysis and was associated with less frequent side-effects than $ß_2$-AR agonists (Koks *et al.,* 1998, Cararach *et al.,* 2006).

Although the vast majority of the ABC transporters are energy-dependent transporters, the family also contains examples of channel gating by ATP binding and hydrolysis. These proteins are called ion channel regulators and are also members of the ABC transporter family; the cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7) and ATP-dependent K^+ channel regulators, such as the sulphonylurea receptors SUR1/ABCC8 and SUR2/ABCC9. The ion channels, including the K^+ channels, are central to the regulation of the cell membrane potential and contractility of the smooth muscle (Wray 1993). The opening of these channels results in an outward flow of K^+, drawing the cell membrane potential closer to the K^+ equilibrium potential, and thereby reducing cellular excitability and contractility (Khan *et al.,* 2001). There are several types of K^+ channels: the large-conductance Ca^{2+}- and voltage-sensitive K^+ channel (BK_{Ca} channel), the ATP-sensitive K^+ channel (K_{ATP} channel), the Shaker-like voltage-gated K^+ channel (Kv channel), and small-conductance Ca^{2+}-sensitive K^+ channels (SK channel) (Brainard *et al.,* 2007). K_{ATP} channels were first discovered in cardiac myocytes (Noma 1983) and later in many other tissues, including pancreatic ß-cells, skeletal muscle, smooth muscle, the brain, pituitary, kidney and mitochondria. By linking the cell metabolic state to the membrane potential, K_{ATP} channels regulate a variety of cellular functions, including insulin secretion from pancreatic ß-cells, the excitability of skeletal muscle and neurones, K^+ recycling in the renal epithelia, and cytoprotection in cardiac and brain ischaemia (Inagaki & Seino 1998, Yokoshiki *et al.* 1998). Several papers have reported that K_{ATP} channels are involved in the smooth muscle relaxation induced by β-AR agonists; 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).

K_{ATP} channels are large hetero-octameric complexes containing four subunits from the inwardly rectifying K^+ channel family ($Kir_{6.x}$: $Kir_{6.1}$ or $Kir_{6.2}$) and four regulatory SUR subunits from the ABC transporter family ABCC8 (SUR1) and ABCC9 (SUR2). SUR2 has two different isoforms, SUR2A and SUR2B; these are splicing variants. Both subunits (SURs and $Kir_{6.x}$) are necessary for the channel function. $Kir_{6.x}$ comprises the K^+ channel component of the K_{ATP}, while the SURs are responsible for the ATP sensitivity, pharmacological properties, and trafficking of this channel (Aguilar-Bryan *et al.*, 1998, Gross *et al.*, 1999, Bryan *et al.*, 2004, Teramoto 2006, Ko *et al.*, 2008) (**Fig. 2.**).

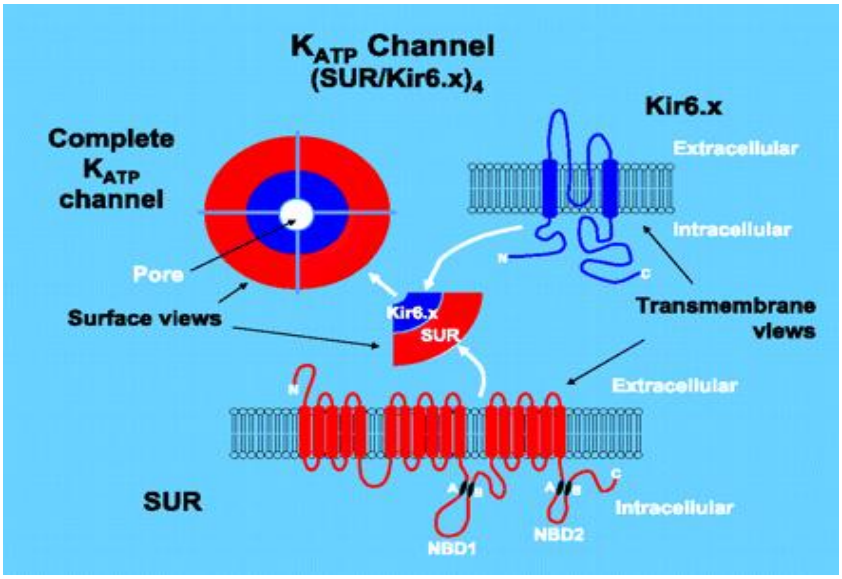


Figure 2 Diagramatic representation of K_{ATP} channel structure. K_{ATP} channels regulate the transport of K^+ through cell membranes. They are formed by the combination of two types of subunit, the pore-forming inwardly rectifying subunit ($Kir_{6.X}$) and the regulatory SUR. Transport of K^+ *via* the $Kir_{6.X}$ pore is controlled externally *via* K^+ channel regulators binding to SUR nucleotide binding sites (NBD1 and NBD2) on the internal surface. Each channel consists of an outer ring of four SUR receptor subunits and an inner ring of four pore-forming subunits, $Kir_{6.X}$ (complete channel, surface views). Each subunit is a transmembrane protein (transmembrane views). Adapted from Shorter *et al.* (2008).

The molecular structure of the K_{ATP} channels is different, due to the heterologous expression of $Kir_{6.x}$ and the SUR subunits. This leads to different combinations, and creates different types of K_{ATP} channel with distinct electrophysiological properties and pharmacological sensitivities that reflect the various K_{ATP} channels in native tissues. While $Kir_{6.2}$/SUR1 constitutes the pancreatic ß-cell type (Inagaki *et al.,* 1995), the cardiac-type K_{ATP} channels consist of $Kir_{6.2}$/SUR2A (Inagaki *et al.,* 1996), and $Kir_{6.2}$/SUR2B probably constitutes the non-vascular smooth muscle type. The vascular smooth muscle-type K_{ATP} channel comprises $Kir_{6.1}$/SUR2B (Yamada *et al.,* 1997). $Kir_{6.1}$ and SUR2B mRNA transcripts have been identified in the rat myometrium (Chien *et al.,* 1999, Sawada *et al.,* 2005). Investigations on the human myometrium indicated that the major K_{ATP} channel is composed of $Kir_{6.1}$ and SUR2B and that down-regulation of this channel may facilitate the myometrial function (Curley *et al.,* 2002). K^+ channel-opening compounds (KCOs) are known to be potent smooth muscle relaxants and have been reported to be potent inhibitors of non-pregnant uterine contractions (Novakovic *et al.,* 2007). The KCOs, including diazoxide, pinacidil, cromakalim and nicorandil, are a structurally diverse group of drugs which open K_{ATP} channels in various cell types (Aschroft & Gribble, 2000a). It has been shown that different SUR subunits confer varying sensitivities to KCOs. For example, $Kir_{6.2}$/SUR1 channels are activated strongly by diazoxide, but not by pinacidil, $Kir_{6.2}$/SUR2A channels are activated by pinacidil and cromakalim, but only weakly by diazoxide, while $Kir_{6.2}$/SUR2B channels are activated by diazoxide, pinacidil and cromakalim (Inagaki *et al.,* 1995, Isomoto *et al.*. 1996, Babenko *et al.,* 1998, Gribble *et al.,* 1998, D'Hahan *et al.,* 1999).

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

3.1. Housing and handling of the 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 (SPRD) rats (Charles-River Laboratories, Hungary) 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 (Charles-River Laboratories, Hungary) with tap water available *ad libitum*. They were euthanized by CO_2 inhalation.

3.2. Mating of the animals

Mature female (180–200 g) and male (240–260 g) 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.

3.3. Tissue isolation

Pregnant and non-pregnant SPRD rats were euthanized in a CO_2 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 (RNalater, Life Technologies, Hungary). The samples were frozen in liquid nitrogen and stored at –70 °C until total RNA and protein extraction.

3.4. Real-time quantitative reverse transcription

Uterus tissues frozen in liquid nitrogen were mechanically homogenized. The PARIS Kit (Protein and RNA Isolation System, Life Technologies, Hungary) 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 (Life Technologies, Hungary) were used for reverse transcription. PCR products were amplified with the TaqMan Gene Expression Master Mix (Life Technologies, Hungary) and the ABI Step One Real-Time cycler. The following primers were used: assay ID Rn01476318_ml for ABCC8/SUR1, Rn01463198_m1 for ABCC9/SUR2, Rn01639905-m1 for ABCG2, Rn00667869-m1 for ß-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).

3.5. Western blot analysis

30 μg of protein per well was subjected to electrophoresis on 4-12 % NuPAGE Bis-Tris Gel (Life Technologie, Hungary) in XCell SureLock Mini-Cell Units (Invitrogen, Hungary). Proteins were transferred from gels to nitrocellulose membranes (Scheicher and Schuell, Germany), by a semidry blotting technique (BioRad, Hungary). Antibody binding was detected with the Western Breeze Chromogenic Western blot immune detection kit (Invitrogen, Hungary). The blots were incubated on a shaker with ABCG2, ABCC8/SUR1, ABCC9/SUR2, GAPDH and ß-actin polyclonal antibody (Santa Cruz Biotechnology, California, 1:200) in the blocking buffer. Images were captured with the EDAS290 imaging system (KODAK, Invitrogen, Hungary), 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.

3.6. *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 (Isosys Data Acquisition System, Experimetria Ltd., U.K.). The effect of the administered drug was assessed by expressing the integrated tension relating to a 5-min period; areas under the curves (AUCs) of 5-min periods were evaluated; expressed as a percentage in terms of the AUC of the spontaneous contractions preceding the administration of the relaxing drugs.

3.7. *In vitro* organ studies

3.7.1. 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 (composition: 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM NaH_2PO_4, 6 mM glucose, pH=7.4). The organ bath was maintained at 37 °C, and carbogen (95% O_2 + 5% CO_2) 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. The initial tension of the preparation was set to about 1.25 g, which was relaxed to about 0.5 g at the end of equilibration. The tension of the myometrial rings was measured with a gauge transducer (SG-02; Experimetria Ltd., Budapest, Hungary) and recorded with a SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd., Budapest, Hungary).

3.7.2. KCO studies: oxytocin-induced contractions

The uterus-relaxant effects of the KCOs pinacidil or diazoxide (10^{-8}–10^{-4} M) (Sigma-Aldrich, Budapest, Hungary) were investigated on oxytocin-induced contractions alone or in the presence of glibenclamide (10^{-6} M) (Sigma-Aldrich, Budapest, Hungary). 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 (Graphpad Software Inc. San Diego, CA, USA) 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.

3.7.3. 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 (ST-02, Experimetria U.K. Ltd.), 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, the 8-day, the 18-day 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) (Sigma-Aldrich, Budapest, Hungary) on non-pregnant and 22-day-pregnant animals. 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. EFS parameters were as follows; non-pregnant and 8-day-pregnant (PP: 30 s, PW: 50 ms), 18-day-pregnant (PP: 18 s, PW: 75 ms) and 22-day-pregnant (PP: 25 s, PW: 150 ms).

3.7.4. Effects of combinations of ß$_2$-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) (Sigma-Aldrich, Budapest, Hungary) 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

4.1. $ABCG_2$ 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 (days 6, 8, and 10), 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. 3**).

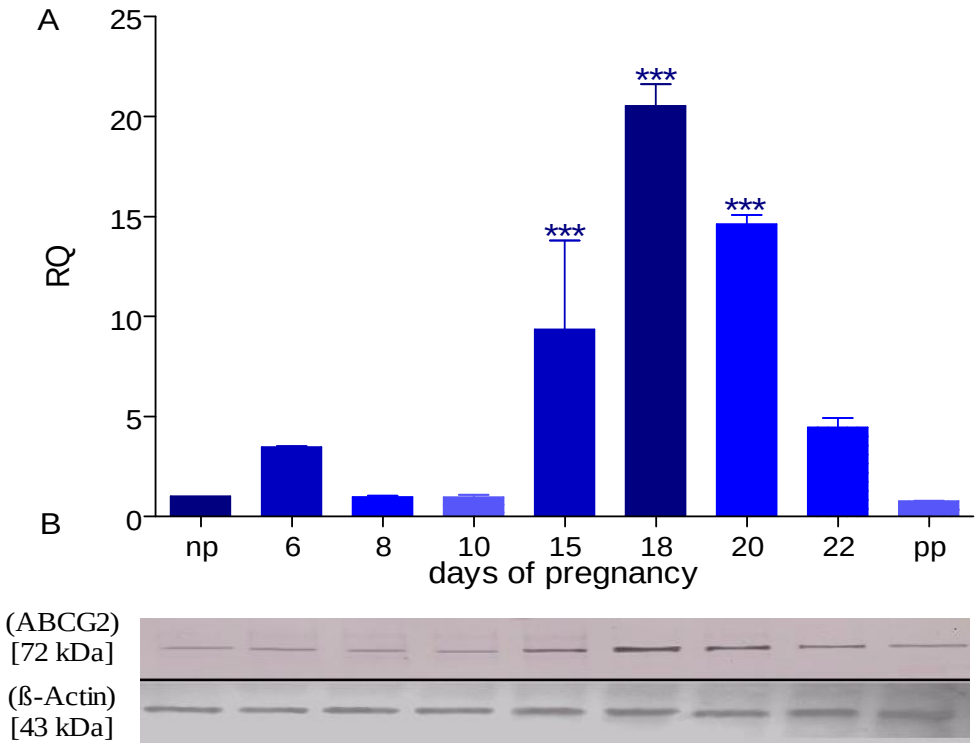


Figure 3 (A) Changes in expression of ABCG2 mRNA during pregnancy in the rat myometrium. RQ (Relative Quantity) values on different days of pregnancy were compared with those in non-pregnant rats. *** denotes $p < 0.001$. Each value indicates the mean ± S.E.M, n = 5 (np: non-pregnant, pp: *post-partum*). **(B)** Representative Western blot of ABCG2 protein expression in non-pregnant (np), pregnant and *post-partum* (pp) rat myometrium.

4.2. 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 $ABCG_2$ inhibitor KO-134 dose-dependently increased the uterus-relaxing effect of nifedipine. The ED_{50} 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. 4**). **Figure 5** shows the experimental procedures.

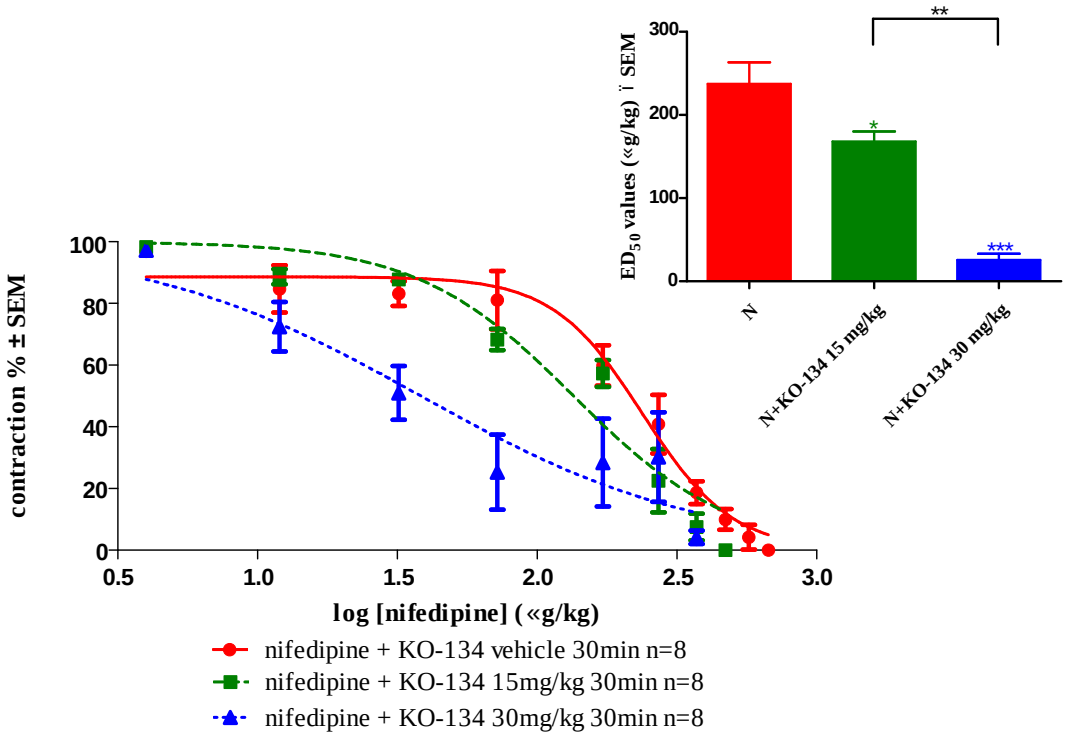
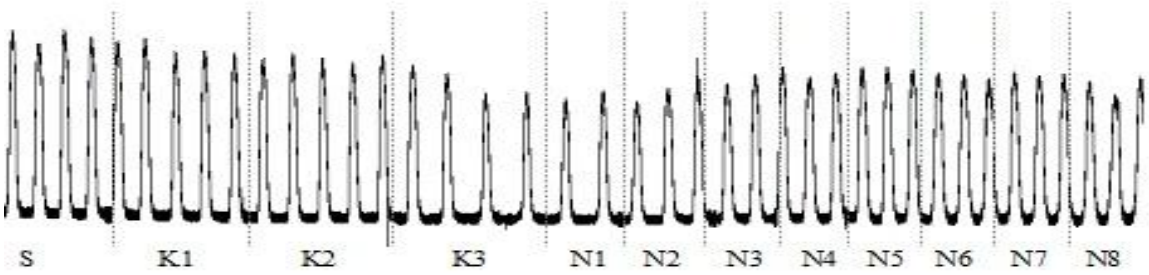


Figure 4 Uterus-relaxing effects of nifedipine (N) alone and in the presence of 15 mg/kg or 30 mg/kg doses of the $ABCG_2$ blocker KO-134 in the *post-partum* rat uterus *in vivo*. Insert: ED_{50} values of nifedipine alone or in combination with KO-134 doses of 15 mg/kg or 30 mg/kg. The ED_{50} values 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.

A

Incubation period - spontaneous contraction	KO-134 vehicle KO-134, 15 and 30 mg/kg	Nifedipine (N) doses, at 5-min intervals (µg/kg)									
45 min	30 min	N 4	N 8	N 20	N 40	N 100	N 100	N 100	N 100	N 100	N 100

B



C

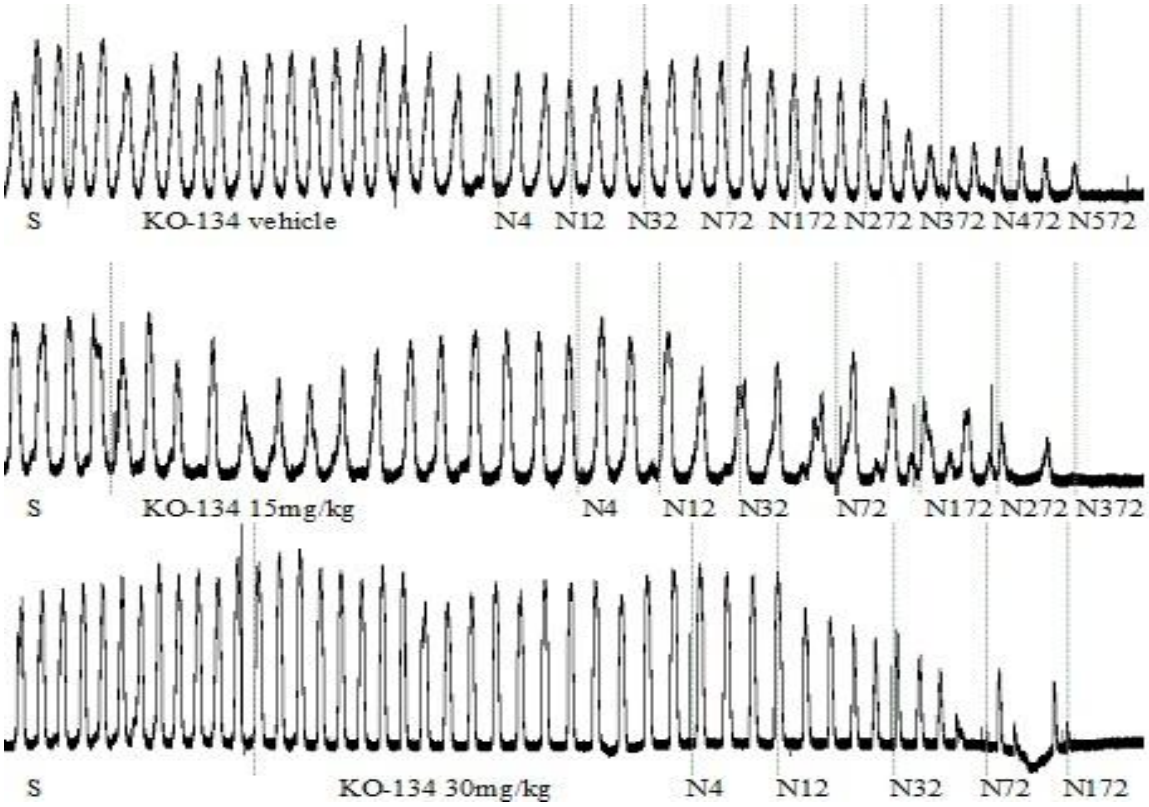


Figure 5 (A) Experimental procedures. **(B)** Solvent controls for KO-134 and nifedipine. S: spontaneous contractions**,** K1, K2, K3: KO-134 vehicle (DMSO:chremophor:saline, 2:1:7, v/v/v), N1-N8: nifedipine vehicle (PEG 400:DMSO:saline, 3:3:10, v/v/v).

(C) Representative patterns of intrauterine pressure change in the presence of KO-134 vehicle + nifedipine or KO-134 15 mg/kg or 30 mg/kg + nifedipine. Under the patterns, the cumulative nifedipine doses are indicated (N4 = 4 µg/kg, ... N572 = 572 µg/kg).

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

Relative quantative 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. 6**). The SUR2 mRNA and protein levels did not undergo any alterations during pregnancy (**Fig. 7**). SUR1 and SUR2 were investigated on days 6 and 10 of pregnancy; samples were collected separately from implantation and interimplantation sites (**Fig. 8**).

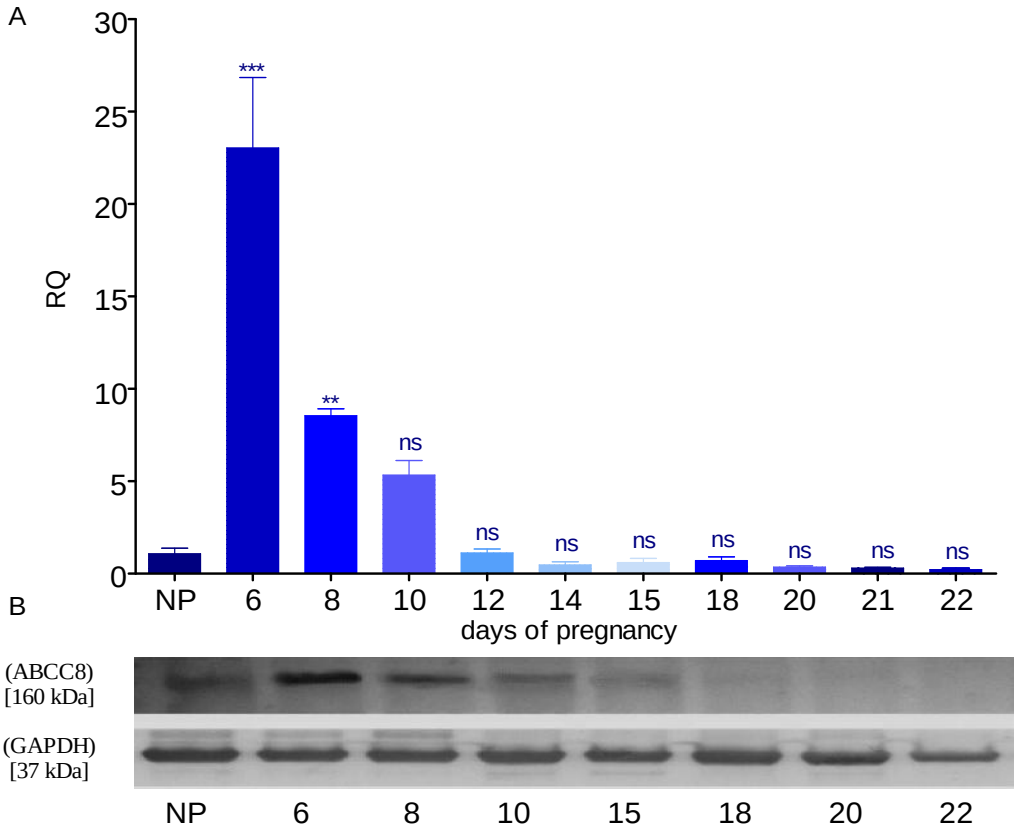


Figure 6. (**A)** Changes in expression of ABCC8/SUR1 mRNA during pregnancy in the rat myometrium. RQ (Relative Quantity) values on different days of pregnancy were compared with those in non-pregnant rats. ns: non-significant, ** denotes $p< 0.01$, *** $p<0.001$. Each bar indicates the mean ± SEM, n = 5. (**B)** Representative Western blot of ABCC8/SUR1 protein expression in the non-pregnant (NP) and the pregnant rat myometrium.

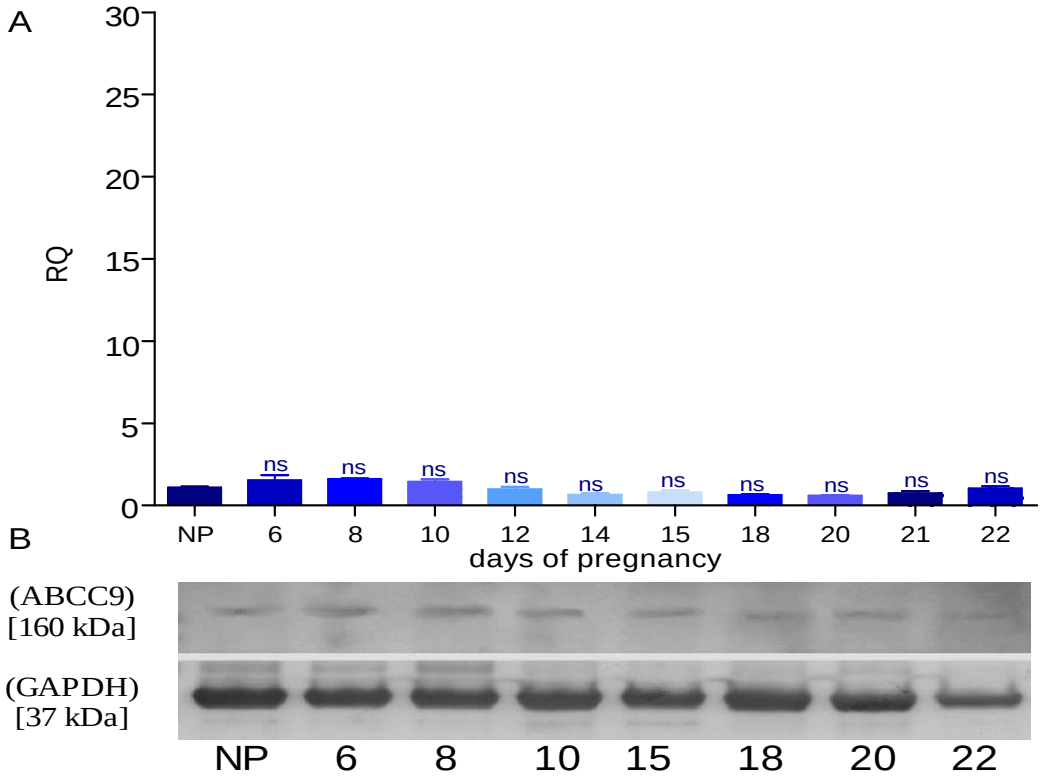


Figure 7. (**A)** Changes in expression of ABCC9/SUR2 mRNA during pregnancy in the rat myometrium. RQ values on different days of pregnancy were compared with those in non-pregnant rats. ns: non-significant. Each bar indicates the mean ± SEM, n = 5. (**B)** Representative Western blot of SUR2 protein expression in the non-pregnant (NP) and the pregnant rat myometrium.

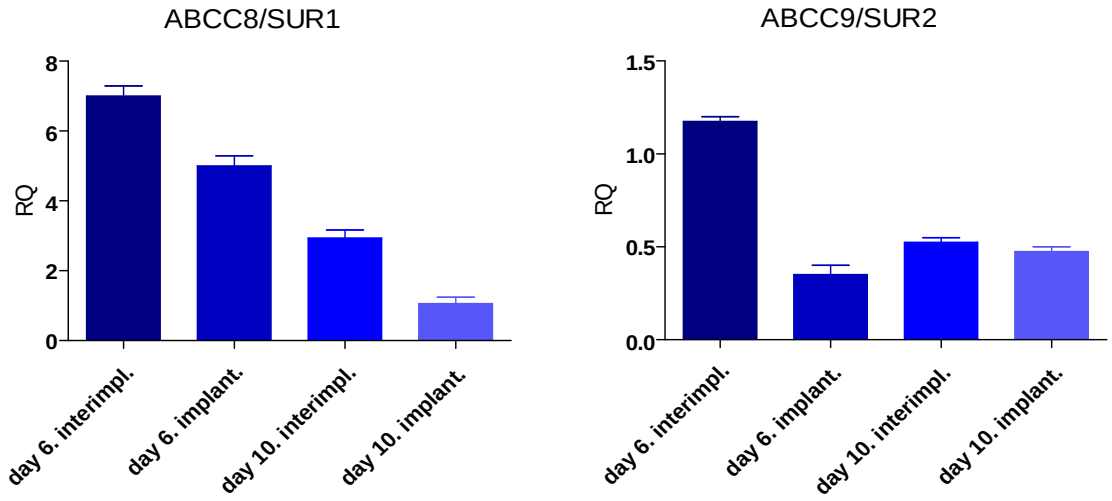


Figure 8. Change in expressions of ABCC8/SUR1 and ABCC9/SUR2 mRNA in 6-day and 10-day-pregnant animals. Samples were collected separately from implantation and interimplantation sites. Each bar indicates the mean ± SEM, n = 5

4.4. Effects of the SUR-non-selective K_{ATP} channel opener diazoxide and the K_{ATP} channel blocker glibenclamide

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. 9**).

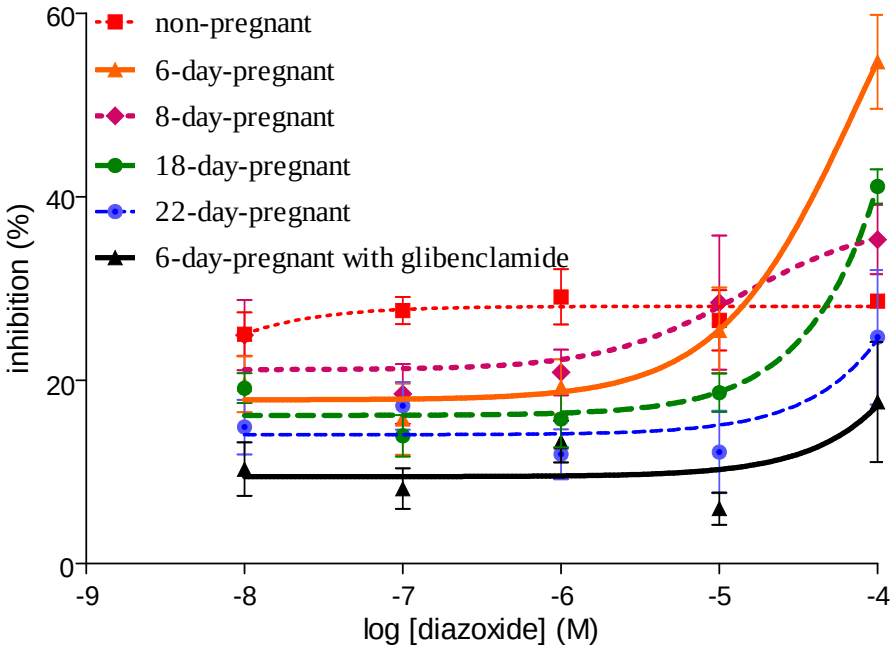


Figure 9. Uterus-relaxing effect of the K_{ATP} channel opener diazoxide (10^{-6}-10^{-4} M) on oxytocin (10^{-6} M)-evoked rhythmic contractions in the non-pregnant and in the 6-day, 8-day, 18-day and 22-day-pregnant rat myometrium *in vitro*. Values on different days of pregnancy were compared with those in non-pregnant rats. Reversal by glibenclamide (10^{-6} M) on day 6 of pregnancy. Each value denotes the mean ± SEM, n= 6.

4.5. Effects of the SUR2-selective K_{ATP} channel opener pinacidil, the K_{ATP} channel blocker glibenclamide and the K_{Ca} channel blocker TEA

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 (**Fig. 10 A**). The EC_{50} values of pinacidil were significantly lower in the pregnant rat myometrium as compared to the non-pregnant myometrium (**Fig. 10 B**). 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. 11**). The uterus-relaxant effect of pinacidil was investigated on EFS-induced contractions in non-pregnant and in 8-day, 18-day and 22-day-pregnant rats (**Fig. 12**), and reversal by TEA (10^{-3} M) on the non-pregnant and the 22-day pregnant uterus (**Fig. 13**). The EFS-induced contractions were inhibited by pinacidil, but there were no differences between the E_{max} and EC_{50} values on either gestational days. 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.

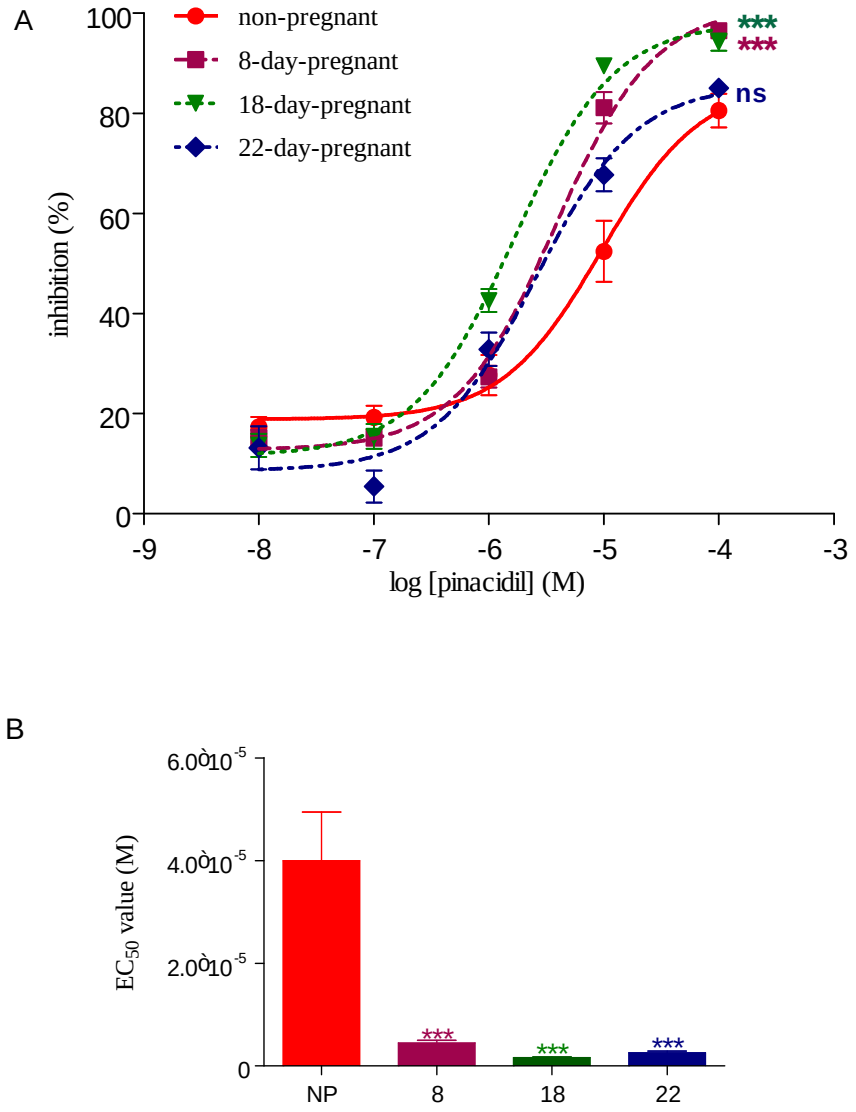


Figure 10 (**A**) Uterus-relaxing effect of the K_{ATP} channel opener pinacidil (10^{-8}-10^{-4} M) on oxytocin (10^{-6} M)-evoked rhythmic contractions in the non-pregnant and in the 8-day, 18-day and 22-day-pregnant rat myometrium *in vitro*. E_{max} values on different days of pregnancy were compared 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 ± SEM, n = 6.

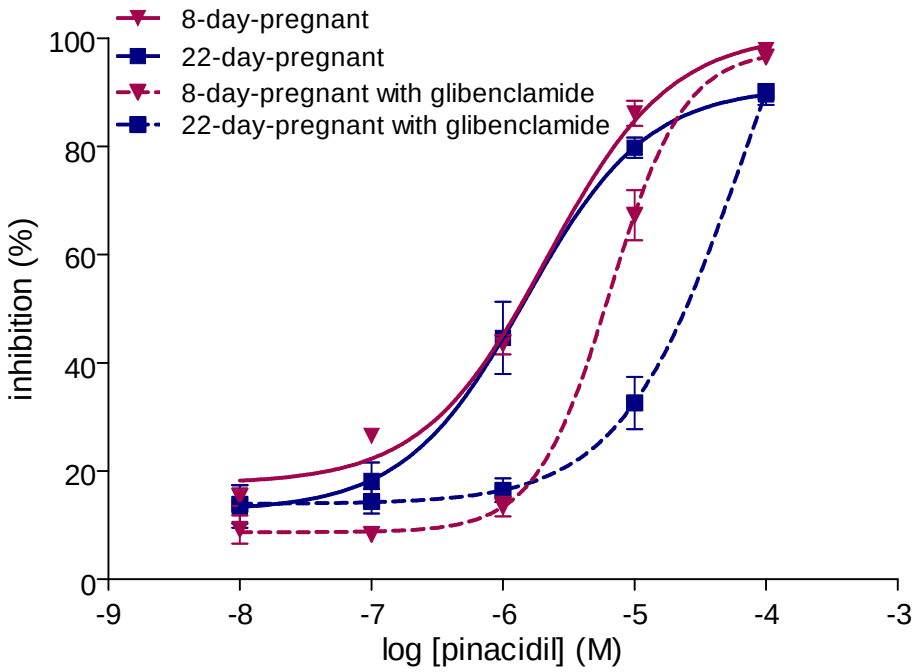


Figure 11. Uterus-relaxing effect of the K_{ATP} channel opener pinacidil (10^{-8}-10^{-4} M) on oxytocin (10^{-6} M)-evoked rhythmic contractions on the 8-day and 22-day-pregnant rat myometrium *in vitro*; reversal by glibenclamide (10^{-6} M) on day 8 and day 22 of pregnancy. Each value denotes the mean ± SEM, n= 6.

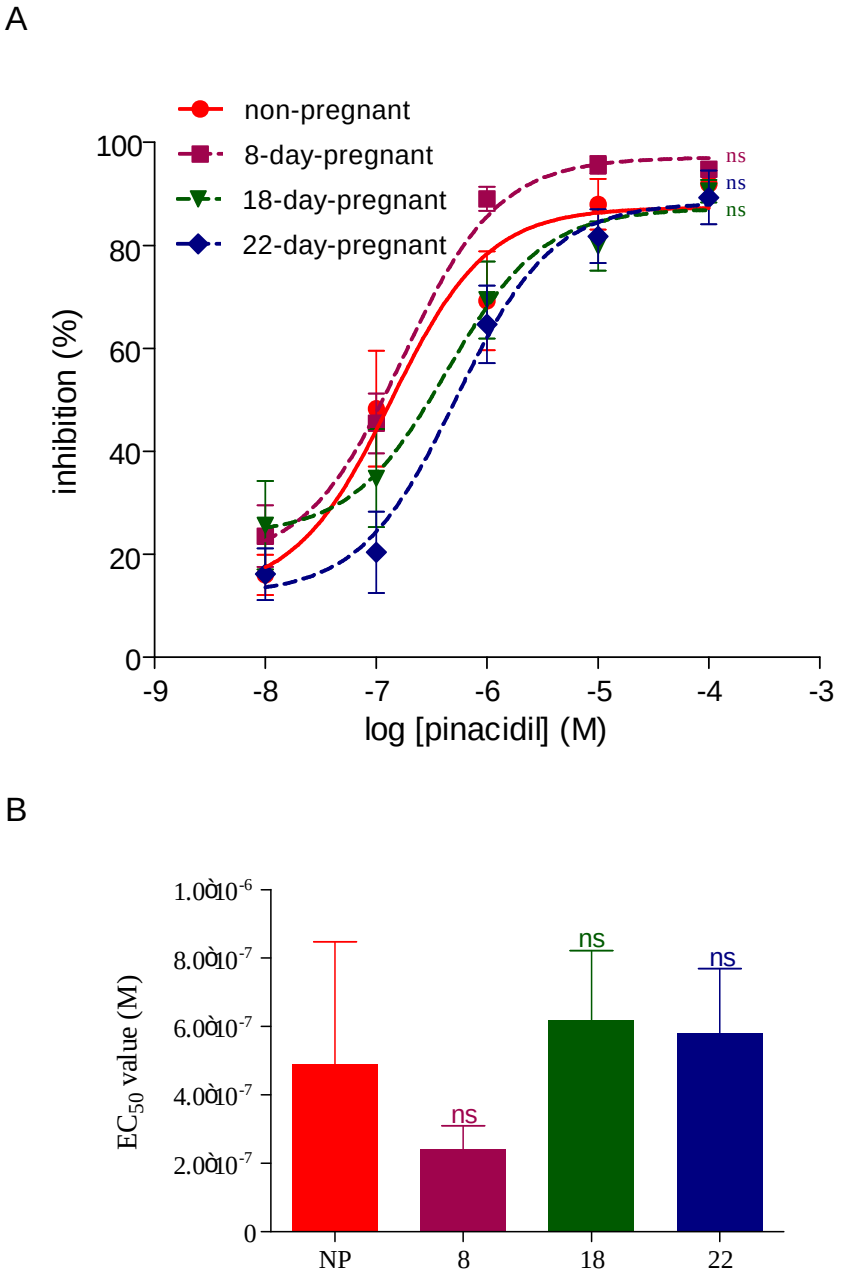


Figure 12. **(A)** Uterus-relaxing effect of the K_{ATP} channel opener pinacidil (10^{-8}-10^{-4} M) on EFS-evoked rhythmic contractions in the non-pregnant and in the 8-day, 18-day and 22-day-pregnant rat myometrium *in vitro*. (**B**) Changes in EC_{50} values of pinacidil on EFS-induced contractions 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. ns: non-significant. Each value denotes the mean ± SEM, n = 6.

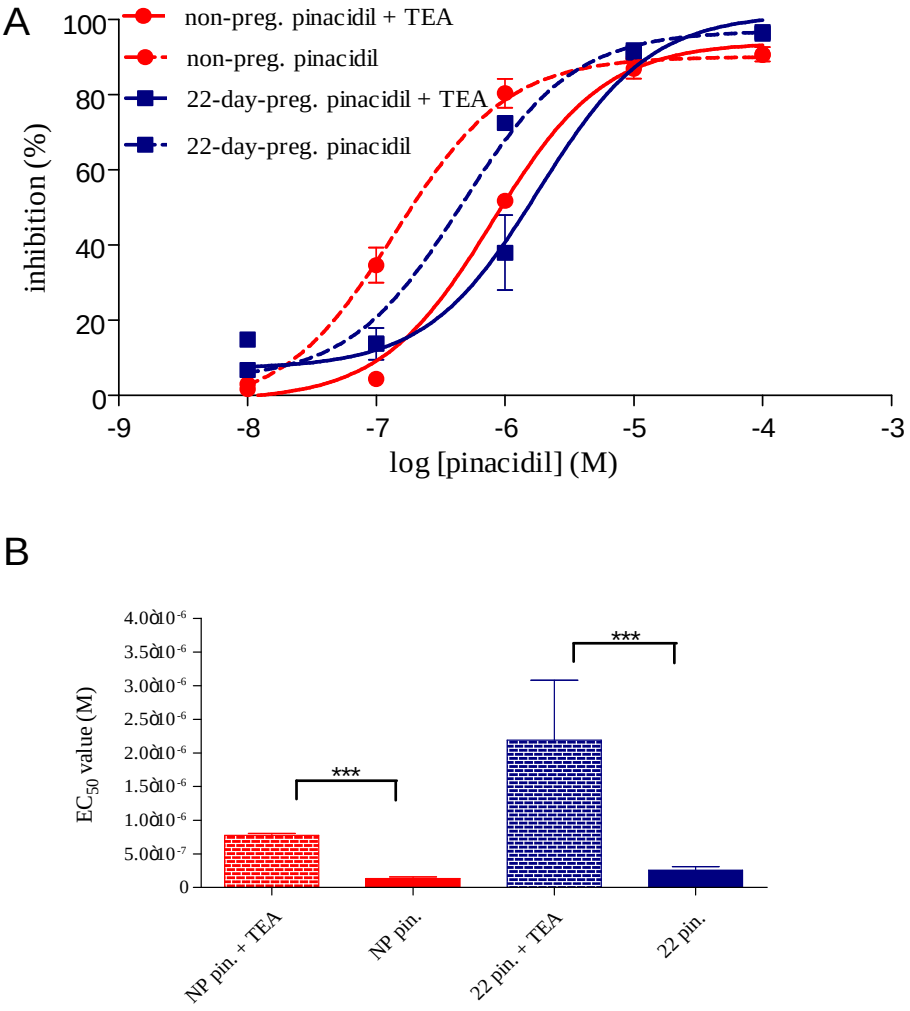


Figure 13. Uterus-relaxing effect of the K_{ATP} channel opener pinacidil (10^{-8}-10^{-4} M) on EFS-evoked rhythmic contractions in the non-pregnant (NP pin.) and the 22-day-pregnant (22 pin.) rat myometrium *in vitro*; reversal by TEA (10^{-3} M). *** denotes $p<0.001$. Each value denotes the mean ± SEM, n = 6.

4.6. Effects of ß$_2$-AR agonists in the presence of the K_{ATP} channel blocker glibenclamide and the K_{ATP} channel opener pinacidil

4.6.1. 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 agonsits were significantly lower in the presence of pinacidil (**Figs 14 and 15**).

4.6.2. 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 16 and 17**).

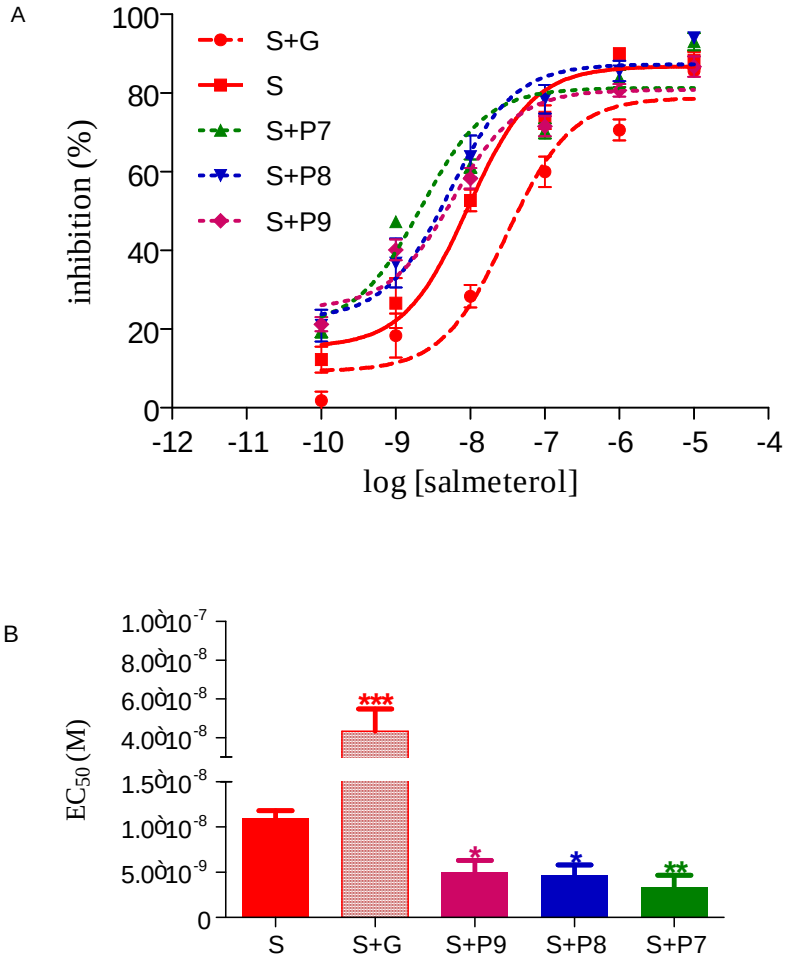


Figure 14 (A) Uterus-relaxing effect of the β_2-AR agonist salmeterol (10^{-10}-10^{-5} M) on the spontaneous rhythmic contractions in the 6-day-pregnant rat myometrium alone (S), reversal by glibenclamide (S+G), and in the presence of pinacidil (10^{-9} M: S+P9, 10^{-8} M: S+P8 and 10^{-7} M: S+P7) *in vitro*. **(B)** Changes in EC_{50} 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 EC_{50} 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 ± S.E.M, n = 6.

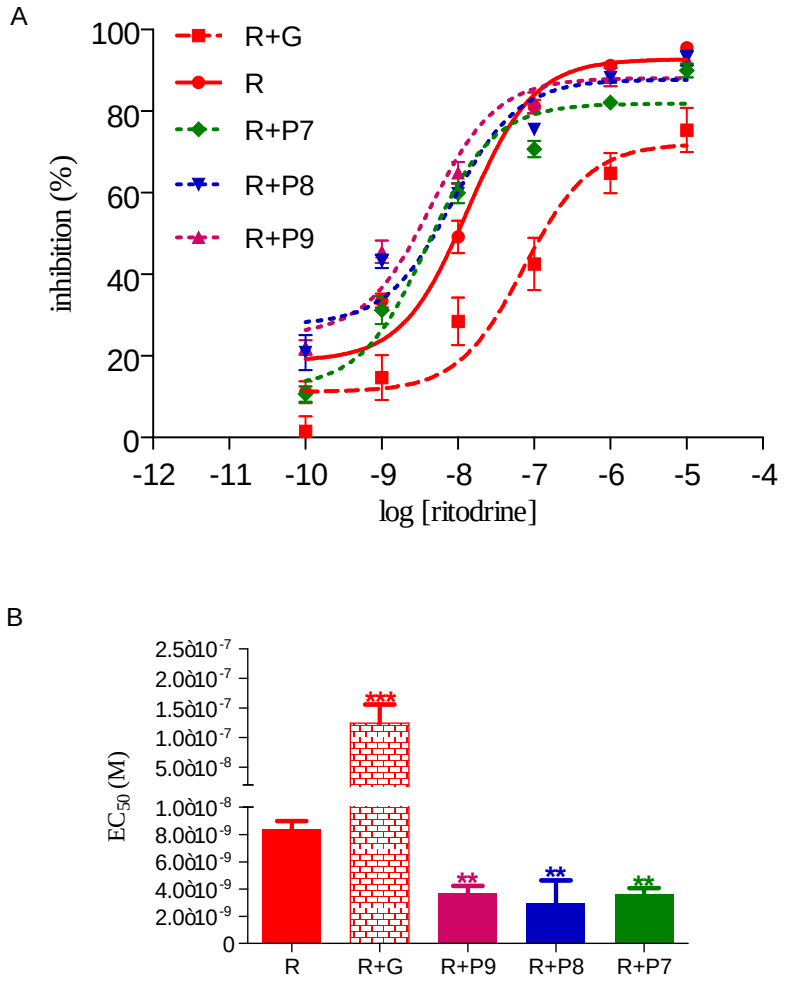


Figure 15 (A) Uterus-relaxing effect of the β_2-AR agonist ritodrine (10^{-10}-10^{-5} M) on the spontaneous rhythmic contractions in the 6-day-pregnant rat myometrium alone (R), reversal by glibenclamide (R+G), and in the presence of pinacidil (10^{-9} M: R+P9, 10^{-8} M: R+P8 and 10^{-7} M: R+P7) *in vitro*. **(B)** Changes in EC_{50} 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 EC_{50} 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 ± S.E.M, n = 6.

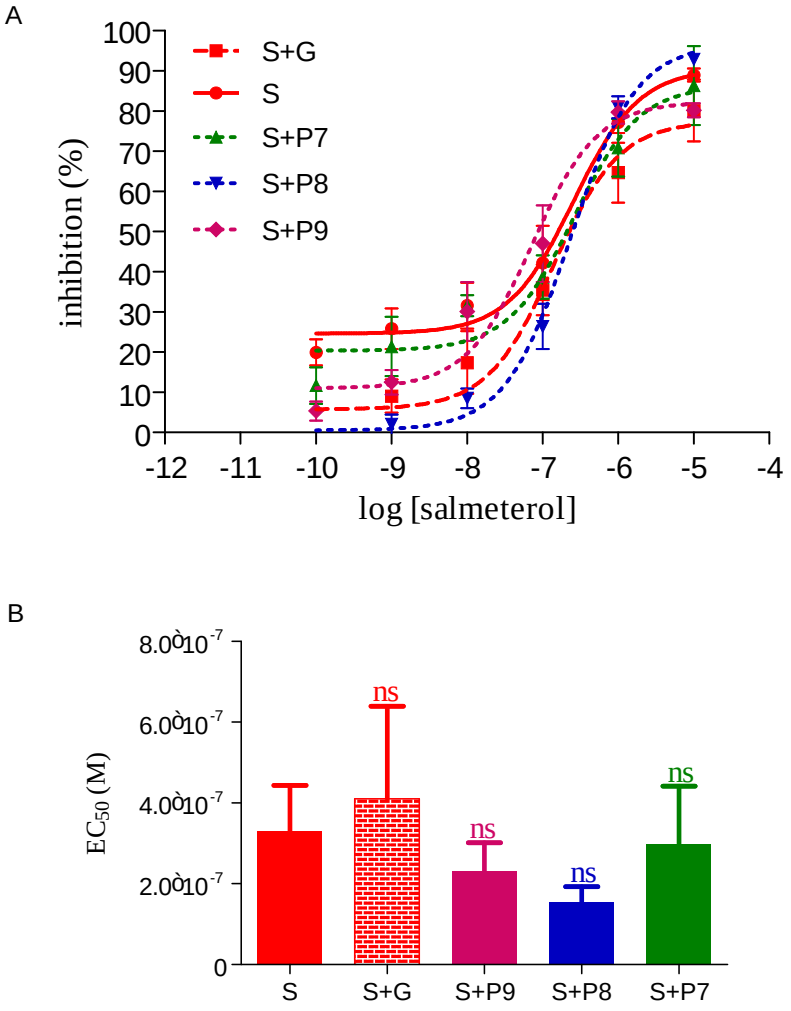


Figure 16 (A) Uterus-relaxing effect of the β_2-AR agonist salmeterol (10^{-10}-10^{-5} M) on the spontaneous rhythmic contractions in the 22-day-pregnant rat myometrium alone (S), reversal by glibenclamide (S+G), and in the presence of pinacidil (10^{-9} M: S+P9, 10^{-8} M: S+P8 and 10^{-7} M: S+P7) *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 ± S.E.M, n = 6.

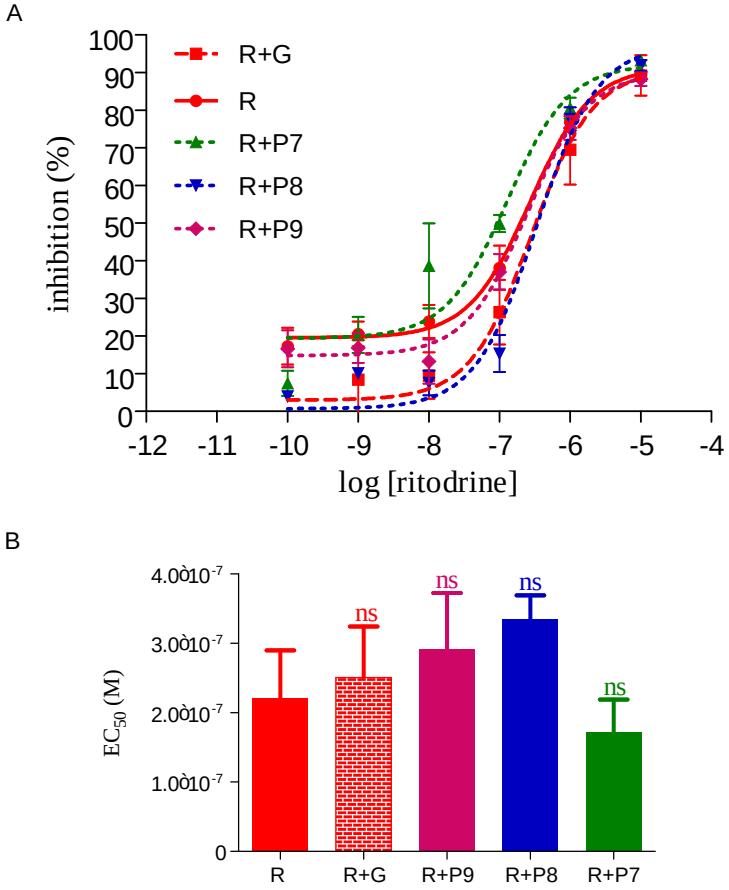


Figure 17 (A) Uterus-relaxing effect of the β_2-AR agonist ritodrine (10^{-10}-10^{-5} M) on the spontaneous rhythmic contractions in the 22-day-pregnant rat myometrium alone (R), reversal by glibenclamide (R+G), and in the presence of pinacidil (10^{-9} M: R+P9, 10^{-8} M: R+P8 and 10^{-7} M: R+P7) *in vitro*. **(B)** Changes in EC_{50} 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 EC_{50} values of the different combinations were compared with that for ritodrine alone, ns: non-significant. Each value denotes the mean ± 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 PTL, the main cause of perinatal mortality and morbidity in the developed world. Despite several agents being available to treat PTB, the rate of PTB 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 PTB.

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 $ß_2$-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 bothmthe 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 (Aschroft & 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 $ß_2$-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 $ß_2$-AR agonists. It can be concluded that the involvement of the K_{ATP} channel in the efficacy of the $ß_2$-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 $ß_2$-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 $ß_2$-AR function, which is partially controlled by ß-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 $ß_2$-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. Summary

PTL and prematurity remain the main causes of perinatal morbidity and mortality. Despite the recent developments in perinatology and prenatal care, the mechanisms of induction of preterm uterine contractility are still unknown. Tocolysis is one of the greatest challenges in obstetrical practice. The rate of PTB is generally 8-10% in the world despite the use of tocolytic therapy. New therapeutic targets are needed to reduce the PTB rate. In the light of our results, we can conclude that one of these new targets can be the ABC transporters.

1. ABCG2 showed a characteristic expression during gestation; at term it functions as a special barrier to protect the foetus by the efflux of xenobiotics. The *in vivo* studies showed that the ABCG2 substrate nifedipine had a potent uterus-relaxing effect which was dose-dependently potentiated by an ABCG2 blocker KO-134. 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.

2. The SUR subunits of the K_{ATP} channels also showed a characteristic expression during gestation. The *in vitro* studies revealed that both KCOs had a uterus-relaxant effect; diazoxide was effective only on those days when the SUR1 subunit displayed the highest level, while the uterus-relaxant effect of pinacidil was independent of the gestation time. In the future, the development of a 'pinacidil-like' uteroselective K_{ATP} channel opener may be of therapeutic relevance in the treatment of PTB.

3. The $ß_2$-AR agonist and pinacidil combination showed that the K_{ATP} channels are involved in the uterus-relaxant effect of $ß_2$-AR agonists. This combination proved to be potent in early gestation, while it had no benefits at term. On the basis of these findings, the therapeutic application of both the $ß_2$-AR agonist and a K_{ATP} channel opener can not be suggested as a promising tocolytic agent. However, this combination may be of value as a uterus relaxant in the early stage of gestation.

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8. Appendix

8.1. List of publications

8.1.1. Publications related to the Ph.D. thesis

I. **Norbert Lovasz**, Eszter Ducza, Robert Gaspar and George Falkay. Ontogeny of sulfonylurea-binding regulatory subunits of K_{ATP} channels in the pregnant rat myometrium. Reproduction 2011;142; 175–181. **IF_{2011}: 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. **IF_{2012}: 1.219**

8.1.2. Abstracts

I. **Lovász Norbert**, Minorics Renáta, Gáspár Róbert, Falkay György. Transzportfehérjék (ABCC8 és ABCC9) ontogenezise és a KATP 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 ß2-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 ß2 agonists in pregnant rat. Society for Endocrinology BES 2013 Harrogate 18 March- 21 March 2013 (Poster).

9. Acknowledgements

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I also would like to thank my co-authors and colleagues in the Department of Pharmacodynamics and Biopharmacy for the pleasant co-operation.

I.

REPRODUCTION
RESEARCH

Ontogeny of sulfonylurea-binding regulatory subunits of K_{ATP} channels in the pregnant rat myometrium

N Lovasz, E Ducza, R Gaspar and G Falkay

Department of Pharmacodynamics and Biopharmacy, Faculty of Pharmacy, University of Szeged, PO Box 121, H-6701 Szeged, Hungary

Correspondence should be addressed to G Falkay; Email: falkay@pharm.u-szeged.hu

Abstract

ATP-sensitive potassium channels (K_{ATP} channels) are composed of sulfonylurea receptors (SURs) and potassium inward rectifiers ($Kir_{6.x}$) that assemble to form a large octameric channel. This study was designed to examine the expression and role of sulfonylurea-binding regulatory subunits 1 (SUR1 (ABCC8)) and 2 (SUR2 (ABCC9)) of the K_{ATP} channels in the pregnant rat myometrium with particular regard to the contractility. RT-PCR and western blot analyses were performed to detect the presence of SUR1 and SUR2. The SUR1 levels were markedly increased in the early stages of pregnancy. The highest level was detected on day 6 of pregnancy, whereas in the late stages, the levels of SUR1 were significantly decreased. The SUR2 level remained unchanged throughout pregnancy. The SUR non-selective diazoxide and the SUR2-selective pinacidil inhibited oxytocin-induced contractions. Glibenclamide, a K_{ATP} channel blocker, antagonized both pinacidil- and diazoxide-induced relaxations. It was established that SURs are responsible for pharmacological reactivity of K_{ATP} channel openers. We conclude that both SURs are involved in the K_{ATP} channel in the pregnant rat myometrium. It may further be concluded that 'pinacidil-like' K_{ATP} channel openers may be of therapeutic relevance as tocolytic agents in the future.

Reproduction (2011) **142** 175–181

Introduction

The factors regulating myometrial function during pregnancy and labor are poorly understood. An understanding of these processes, at the cellular and molecular levels, is essential if novel therapeutic strategies are to be developed for the management of associated clinical problems such as preterm labor, the main cause of perinatal mortality and morbidity in the developed world (Byrne & Morrison 2002). The ion channels, including the potassium (K^+) channels, are central to the regulation of the cell membrane potential and contractility of the smooth muscle (Wray 1993). The opening of these channels results in an outward flow of K^+, drawing the cell membrane potential closer to the K^+ equilibrium potential and thereby reducing cellular excitability and contractility (Khan *et al*. 2001). There are several types of K^+ channels: the large-conductance calcium- and voltage-sensitive K^+ channel (BK_{Ca} channel), the ATP-sensitive K^+ channel (K_{ATP} channel), the Shaker-like voltage-gated K^+ channel (Kv channel), and small-conductance calcium-sensitive K^+ channels (SK channel; Brainard *et al*. 2007). K_{ATP} channels were first discovered in cardiac myocytes (Noma 1983) and later in many other tissues including pancreatic β-cells, skeletal muscle, smooth muscle, brain, pituitary, kidney, and mitochondria. By linking the cell metabolic state to the membrane potential, K_{ATP} channels regulate a variety of cellular functions, including insulin secretion from pancreatic β-cells, the excitability of skeletal muscle and neurones, K^+ recycling in the renal epithelia, and cytoprotection in cardiac and brain ischemia (Inagaki & Seino 1998, Yokoshiki *et al*. 1998). K_{ATP} channels are large hetero-octameric complexes containing four subunits from the inwardly rectifying K^+ channel family ($Kir_{6.x}$: $Kir_{6.1}$ or $Kir_{6.2}$) and four regulatory sulfonylurea receptor (SUR) subunits from the ATP-binding cassette (ABC) transporter family ABCC8 (SUR1) and ABCC9 (SUR2). SUR2 has two different isoforms, SUR2A and SUR2B; these are splicing variants. Both subunits (SURs and $Kir_{6.x}$) are necessary for the channel function. $Kir_{6.x}$ comprises the K^+ channel component of the K_{ATP}, whereas the SURs are responsible for the ATP sensitivity, pharmacological properties, and trafficking of this channel (Aguilar-Bryan *et al*. 1998, Gross *et al*. 1999, Bryan *et al*. 2004, Teramoto 2006, Ko *et al*. 2008). The molecular structure of the K_{ATP} channels is different due to the heterologous expression of $Kir_{6.x}$ and the SUR subunits. This leads to different combinations and creates different types of K_{ATP} channel with distinct electrophysiological

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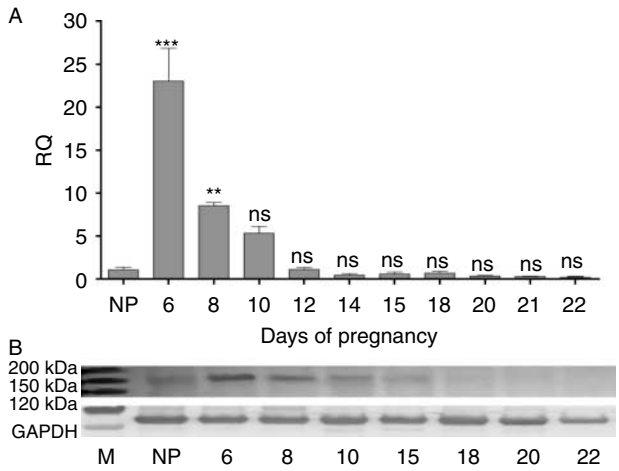

Figure 1 (A) Changes in expression of *Sur1* mRNA during pregnancy in the rat myometrium. RQ values on different days of pregnancy were compared with those in non-pregnant rats. ns, non-significant; $^{**}P<0.01$, $^{***}P<0.001$. Each bar indicates the mean ± S.E.M., $n=5$. (B) Representative western blot of SUR1 protein expression in the non-pregnant (NP) and the pregnant rat myometrium.

properties and pharmacological sensitivities that reflect the various K_{ATP} channels in native tissues. Although $Kir_{6.2}$/SUR1 constitutes the pancreatic β-cell type (Inagaki *et al*. 1995), the cardiac-type K_{ATP} channels consist of $Kir_{6.2}$/SUR2A (Inagaki *et al*. 1996) and $Kir_{6.2}$/SUR2B probably constitutes the non-vascular smooth muscle type. The vascular smooth muscle-type K_{ATP} channel comprises $Kir_{6.1}$/SUR2B (Yamada *et al*. 1997). *$Kir_{6.1}$* and *Sur2b* mRNA transcripts have been identified in the rat myometrium (Chien *et al*. 1999, Sawada *et al*. 2005). There are no reports of the expression of SURs in the rat myometrium during gestation. Investigations on the human myometrium indicated that the major K_{ATP} channel is composed of $Kir_{6.1}$ and SUR2B and that downregulation of this channel may facilitate the myometrial function (Curley *et al*. 2002). Those authors were unable to delineate the exact time at which the downregulation occurs because of the ethical constraints; it was not possible to carry out serial sampling. K^+ channel-opening compounds (KCOs) are known to be potent smooth muscle relaxants and have been reported to be potent inhibitors of non-pregnant uterine contractions (Novakovic *et al*. 2007). The KCOs including diazoxide, pinacidil, cromakalim, and nicorandil are a structurally diverse group of drugs that open K_{ATP} channels in various cell types (Ashcroft & Gribble 2000*a*). It has been shown that different SUR subunits confer varying sensitivities to KCOs. For example, $Kir_{6.2}$/SUR1 channels are activated strongly by diazoxide, but not by pinacidil, $Kir_{6.2}$/SUR2A channels are activated by pinacidil and cromakalim, but only weakly by diazoxide, whereas $Kir_{6.2}$/SUR2B channels are activated by diazoxide, pinacidil, and cromakalim (Inagaki *et al*. 1995, Isomoto *et al*. 1996, Babenko *et al*. 1998, Gribble *et al*. 1998, D'Hahan *et al*. 1999).

The objectives of this study were to investigate the expression of the SUR subunits of the K_{ATP} channels in the rat myometrium in non-pregnant animals and during pregnancy and to investigate possible correlations between SUR protein levels and the effectiveness of KCOs.

Results

mRNA and protein expression assays

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 days 8 to 12, and then remained unchanged until the end of pregnancy (Fig. 1A–B). The SUR2 mRNA and protein levels did not undergo any alterations during pregnancy (Fig. 2A–B).

Effects of SUR non-selective K_{ATP} channel opener diazoxide and K_{ATP} channel blocker glibenclamide

Diazoxide in the range 10^{-8}–10^{-4} M inhibited the oxytocin-induced contractions. The uterus-relaxant effect of diazoxide was investigated in non-pregnant and in 6-, 8-, 18-, and 22-day pregnant rat uteri. The diazoxide-relaxant effect reached its maximum

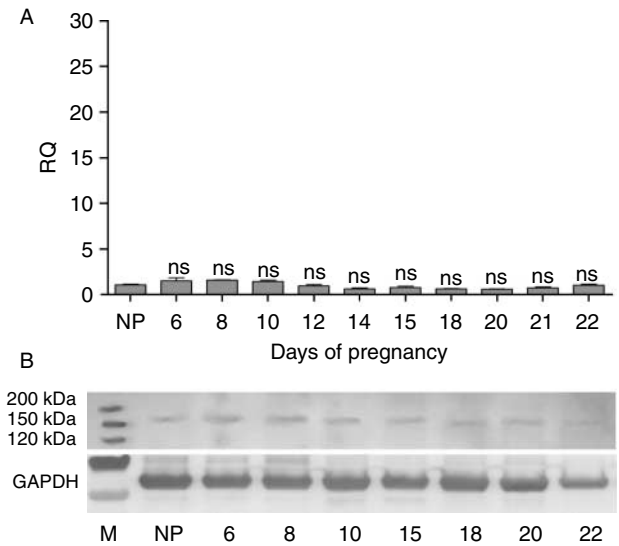

Figure 2 (A) Changes in the expression of *Sur2* mRNA during pregnancy in the rat myometrium. RQ values on different days of pregnancy were compared with those in non-pregnant rats. ns, non-significant. Each bar indicates the mean ± S.E.M., $n=5$. (B) Representative western blot of SUR2 protein expression in the non-pregnant (NP) and the pregnant rat myometrium.

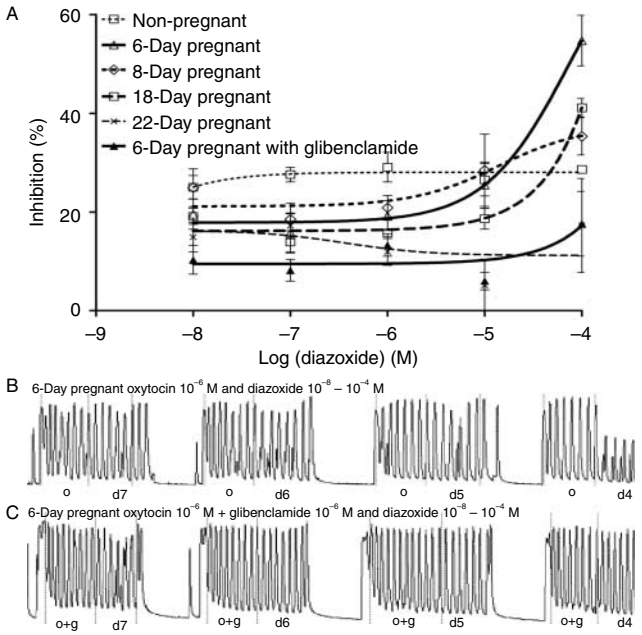

Figure 3 (A) Uterus-relaxing effect of the K_{ATP} channel opener diazoxide (10^{-8}–10^{-4} M) on oxytocin (10^{-6} M)-evoked rhythmic contractions in the non-pregnant and in the 6-, 8-, 18-, and 22-day pregnant rat myometrium *in vitro*. Values on different days of pregnancy were compared with those in non-pregnant rats. Reversal by glibenclamide (10^{-6} M) on day 6 of pregnancy. Each value denotes the mean ± S.E.M., $n=6$. (B) Representative non-cumulative patterns for 6-day pregnant uterus contractions. The effect of diazoxide on oxytocin (o)-induced contractions and (C) in the presence of glibenclamide (o+g).

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 (Fig. 3). The relaxant effect was blocked by glibenclamide 10^{-6} M on day 6 of pregnancy (Fig. 3).

Effect of SUR2-selective K_{ATP} channel opener pinacidil and K_{ATP} channel blocker glibenclamide and Ca-dependent K^+ channel blocker tetraethylammonium

The oxytocin-stimulated uterine contractions of non-pregnant and of 8-, 18-, and 22-day pregnant rats were inhibited concentration dependently by pinacidil in the range 10^{-8}–10^{-4} M (Fig. 4). The EC_{50} values of pinacidil were significantly lower in the pregnant rat myometrium compared with the non-pregnant stage (Fig. 5). 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 (Fig. 4). The uterus-relaxant effect of pinacidil was blocked by glibenclamide 10^{-6} M on days 8 and 22 (Fig. 6). The uterus-relaxant effect of pinacidil was investigated on electric field stimulation (EFS)-induced contractions in the presence of tetraethylammonium (TEA; 10^{-3} M) on non-pregnant and 22-day pregnant uterus (Fig. 7).

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 labor are poorly understood. K_{ATP} channel activation has been shown to decrease the uterine tone and this is a target for the inhibition of uterine activity in the treatment of preterm labor (Piper *et al.* 1990, Brainard *et al.* 2007). K_{ATP} channels are composed of two different subunits: SURs and $Kir_{6.x}$. It has been established that the SURs are responsible for the pharmacological reactivity of the KCOs on each K_{ATP} 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). Curley *et al.* (2002) found *SUR1* and *SUR2* mRNA transcripts in the human myometrium. This study was undertaken to extend our knowledge concerning the gestational changes of SUR1 and SUR2 of the K_{ATP} channels in the rat uterus. In contrast to Chien *et al.* (1999) and Sawada *et al.* (2005), our results clearly demonstrated that both SUR-binding regulatory subunits were expressed in the rat myometrium during gestation. Our findings demonstrate that there is a fivefold downregulation in *Sur1* mRNA level in

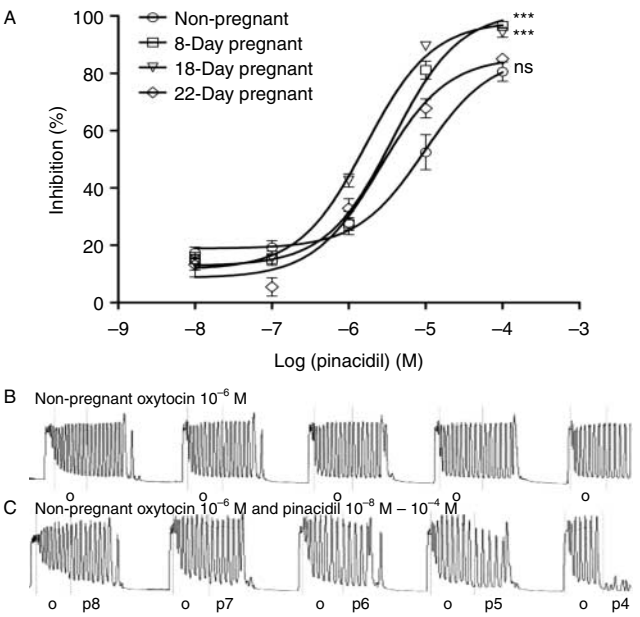

Figure 4 (A) Uterus-relaxing effect of the K_{ATP} channel opener pinacidil (10^{-8}–10^{-4} M) on oxytocin (10^{-6} M)-evoked rhythmic contractions in the non-pregnant and in the 8-, 18-, and 22-day pregnant rat myometrium *in vitro*. E_{max} values on different days of pregnancy were compared with those in non-pregnant rats. ***Denotes $P<0.001$, ns, non-significant. Each value denotes the mean ± S.E.M., $n=6$. (B) Representative non-cumulative patterns for non-pregnant oxytocin (o)-treated uterus contractions and (C) the effect of pinacidil on oxytocin-induced contractions.

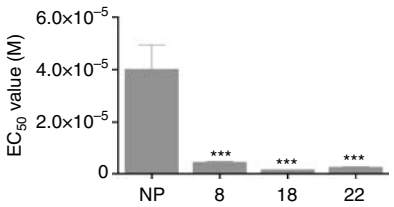

Figure 5 Changes in EC_{50} values of the K_{ATP} channel opener pinacidil on oxytocin-induced contractions in the non-pregnant (NP) and in the 8-, 18-, and 22-day pregnant rat myometrium *in vitro*. EC_{50} values on different days of pregnancy were compared with those in non-pregnant rats. ***$P<0.001$. Each value denotes the mean±S.E.M., $n=6$.

the rat myometrium in late pregnancy compared with the non-pregnant myometrium and ~80-fold decrease relative to early stages (days 6–8) of pregnancy. Similar 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.

We have demonstrated that KCOs (diazoxide and pinacidil) are potent relaxants of the non-pregnant and pregnant rat uterus and are antagonized by glibenclamide. Diazoxide non-selectively activates K_{ATP} channels containing SUR1 or SUR2 (Inagaki *et al.* 1996, Seino & Miki 2003). Pinacidil selectively activates K_{ATP} channels containing SUR2 subunits (Yokoshiki *et al.* 1998). 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 low SUR2 levels, a strong uterus-relaxant effect of the SUR2 agonist pinacidil was observed on the pregnant rat uterus, whereas 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, but the difference between the pregnant and the non-pregnant stages on oxytocin-induced contractions is not clearly understood. The Ca-dependent K^+ channel (K_{Ca} channel) blocker TEA antagonized the uterus-relaxant effect of pinacidil on non-pregnant and 22-day pregnant uterus. This result confirms that the 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 relaxations. However, it is generally accepted that glibenclamide is a selective SUR1 blocker. Our results showed that glibenclamide selectivity in the pregnant rat myometrium is questionable. Ashfield *et al.* (1999) and Babenko *et al.* (1999) reported that both SUR subunits can bind K_{ATP} channel blockers, but in two different ways. Whereas SUR1 has two binding sites for blockers (sulfonylurea and benzamido), SUR2 has only a benzamido-binding site. Glibenclamide contains both sulfonylurea and benzamido moieties, and it can, therefore, bind to SUR1 in two regions and to SUR2 in one region (Ashcroft & Gribble 2000*b*). Stephan *et al.* (2006) demonstrated that glibenclamide (10^{-9} M) induced complete inhibition of the pancreatic K_{ATP} channel, whereas higher concentrations (10^{-7} or 10^{-6} M) produced only partial and reversible inhibition of the cardiovascular K_{ATP} channels. These studies clearly revealed that glibenclamide is a non-selective SUR blocker. It is very likely that this mechanism exists in the pregnant rat myometrium.

In conclusion, this study provides the ontogeny of the SUR-binding regulatory subunits of K_{ATP} channels in the pregnant rat myometrium. It has been established that the SUR-binding regulatory subunits play important roles in the pharmacological reactivity of KCOs. The relaxant effect of diazoxide was significantly stronger when the SUR1 expression was sharply increased on days 6 and 8 of pregnancy and did not show any appreciable effect on those gestation days when SUR1 was downregulated.

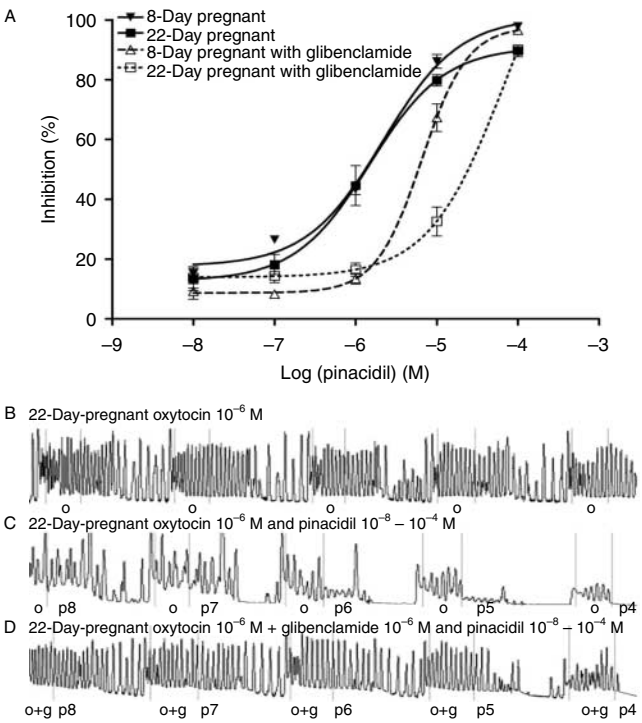

Figure 6 (A) Uterus-relaxing effect of the K_{ATP} channel opener pinacidil (10^{-8}–10^{-4} M) on oxytocin (10^{-6} M)-evoked rhythmic contractions on the 8- and 22-day pregnant rat myometrium *in vitro*; reversal by glibenclamide (10^{-6} M) on days 8 and 22 of pregnancy. Each value denotes the mean±S.E.M., $n=6$. (B) Representative non-cumulative patterns for 22-day pregnant oxytocin (o)-treated uterus contractions. (C) The effect of pinacidil on oxytocin-induced contractions and (D) in the presence of glibenclamide (o+g).

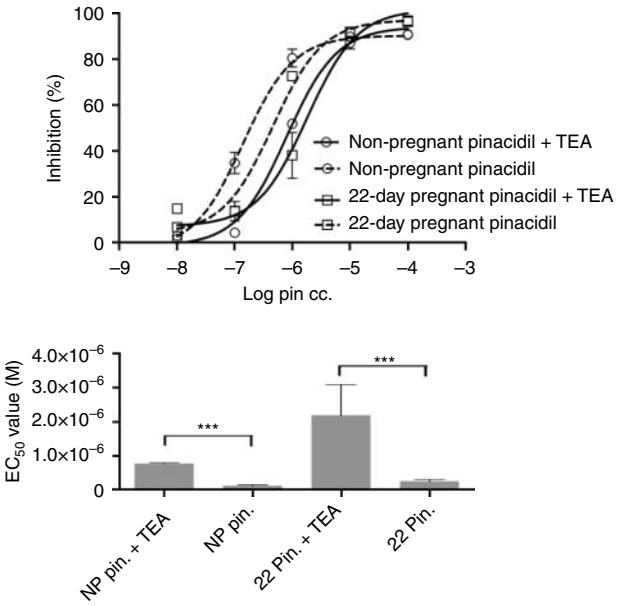

Figure 7 Uterus-relaxing effect of the K_{ATP} channel opener pinacidil (10^{-8}–10^{-4} M) on EFS-evoked rhythmic contractions in the non-pregnant (NP pin.) and the 22-day pregnant (22 pin.) rat myometrium *in vitro*, reversal by TEA (10^{-3} M). ***$P<0.001$. Each value denotes the mean±S.E.M., $n=6$.

The downregulation of SUR1 expression in the rat uterus may contribute to the enhanced contractility associated with the onset of labor. As the uterus-relaxant effect of the SUR2-selective pinacidil on the K_{ATP} channel is independent of the gestational age, it can be concluded that the development of 'pinacidil-like' uteroselective K_{ATP} channel openers may be of novel therapeutic relevance in the management of preterm labor in the future. However, the main problem with KCOs is lack of specificity, resulting in undesired adverse effects. Further research in this field may shed light on the development of new drugs acting via K^+ channels. Whether newly developed KCOs will exhibit significant selectivity for the uterus remains to be seen.

Materials and Methods

Housing and handling of the 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 Ethics Committee for Animal Research (registration number: IV/01758-2/2008). Sprague–Dawley rats (Charles-River Laboratories, Budapest, Hungary) were kept at 22±3 °C; the relative humidity was 30–70% and maintained on a 12 h light:12 h darkness cycle. The animals were maintained on a standard rodent pellet diet (Charles-River Laboratories) with tap water available *ad libitum*. They were killed by CO_2 inhalation.

Mating of the animals

Mature female (180–200 g) and male (240–260 g) 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. Because 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×. 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.

Real-time quantitative RT-PCR

Uterus tissues were separated and frozen in liquid nitrogen and the tissue was mechanically homogenized. The PARIS Kit (Protein and RNA isolation system; Life Technologies, Budapest, Hungary) was used for total RNA and protein extraction from the tissues.

The quality and the quantity of the RNA were assessed at A 260/280, and all samples displayed an absorbance ratio in the range 1.6–2.0. Two micrograms of total RNA and the High Capacity RNA-to-cDNA Kit (Life Technologies) was used for RT. PCR products were amplified with the TaqMan Gene Expression Master Mix (Life Technologies) and the ABI StepOne Real-Time cycler. The following primers were used: assay ID Rn01476318_ml for *Abcc8/Sur1* and Rn01463198_m1 for *Abcc9/Sur2* and Rn99999916_s1 for *Gapdh* as endogenous control. 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

Protein (30 μg per well) was subjected to electrophoresis on 4–12% NuPAGE Bis-Tris Gel (Life Technologies) in XCell SureLock Mini-Cell Units (Invitrogen). Proteins were transferred from gels to nitrocellulose membranes (Scheicher and Schuell, Dassel, Germany) by a semi-dry blotting technique (Bio-Rad). The antibody binding was detected with the WesternBreeze Chromogenic western blot immune detection kit (Invitrogen). The blots were incubated on a shaker with SUR1, SUR2, and GAPDH polyclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany, 1:200) in the blocking buffer. Images were captured with the EDAS290 imaging system (KODAK, Invitrogen), and the optical density of each immunoreactive band was determined with Kodak 1D Images analysis software. Optical densities were calculated as arbitrary units after local area background subtraction.

Isolated organ studies

Uterus preparation

Uteri were removed from non-pregnant rats in the estrus 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 (composition: 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM NaH_2PO_4, 6 mM glucose, pH 7.4). The organ bath was maintained at 37 °C, and carbogen (95% O_2 + 5% CO_2) 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. The initial tension of the preparation was set to about 1.25 g, which was relaxed to about 0.5 g at the end of equilibration. The tension of the myometrial rings was measured with a gauge transducer (SG-02; Experimetria Ltd, Budapest, Hungary) and recorded with a SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd).

KCO studies

Oxytocin-induced contractions

Contractions were elicited with 10^{-6} M oxytocin and non-cumulative dose-response curves were constructed in each experiment in the presence of pinacidil or diazoxide (10^{-8}–10^{-4} M; Sigma–Aldrich). Following the addition of each concentration of pinacidil or diazoxide, recording was performed for 300 s. Concentration-response curves were fitted and area under curve (AUC) were evaluated and analyzed. Statistical analyses were carried out with the Prism 5.0 (Graphpad Software, Inc., San Diego, CA, USA) 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 analyzed by the ANOVA Neuman–Keuls test.

Contractions induced by EFS

Uteri were removed from rats as described in the 'uterus preparation' section, except that uterus rings were vertically mounted between two platinum electrodes. Maximum rhythmic contractions were elicited with a digital, programmable stimulator (ST-02, Experimetria UK Ltd.), 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 and the 22-day pregnant uterus on EFS-induced contractions alone and in the presence of the K_{Ca} channel blocker 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 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. EFS parameters were as follows: non-pregnant (PP: 30 s, PW: 50 ms) and 22-day pregnant (PP: 25 s, PW: 150 ms).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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A K_{ATP} csatorna szulfonilurea alegységeinek ontogenezise a terhes patkány miometriumban

LOVÁSZ NORBERT, DUCZA ESZTER, GÁSPÁR RÓBERT, FALKAY GYÖRGY

Szegedi Tudományegyetem Gyógyszerésztudományi Kar, Gyógyszerhatástani és Biofarmáciai Intézet, Szeged, Eötvös utca 6. – 6720
Levelezési cím: falkay@pharm.u-szeged.hu

Summary

Lovasz, N., Ducza, E., Gaspar, R., Falkay, G.: ***Ontogeny of sulphonylurea-binding regulatory subunits of K_{ATP} channels in the pregnant rat myometrium***

K_{ATP} channels are composed of sulphonylurea receptors (SURs) and potassium inward rectifiers ($Kir_{6.x}$) that assemble to form a large octameric channel. This study was designed to examine the expression and role of sulphonylurea-binding regulatory subunits 1 [SUR1 (ABCC8)] and 2 [SUR2 (ABCC9)] of the K_{ATP} channels in the pregnant rat myometrium with particular regard to the contractility. RT-PCR and Western blot analysis were performed to detect the presence of SUR1 and SUR2. The SUR1 levels were markedly increased in the early stages of pregnancy. The highest level was detected on day 6 of pregnancy, while in the late stages the levels of SUR1 were significantly decreased. The SUR2 level remained unchanged throughout pregnancy. The SUR-non-selective diazoxide and the SUR2-selective pinacidil inhibited oxytocin-induced contractions. Glibenclamide, a K_{ATP} channel blocker, antagonized both pinacidil and diazoxide-induced relaxations. It was established that SURs are responsible for pharmacological reactivity of K_{ATP} channel openers. We conclude that, both SURs are involved in the K_{ATP} channel in the pregnant rat myometrium. It may further be concluded that "pinacidil-like" K_{ATP} channel openers may be of therapeutic relevance as tocolytic agents in the future.

Keywords: *pregnant rat uterus, potassium channels, SUR, potassium channel openers*

Összefoglalás

Az ATP szenzitív K^+-csatornákat (K_{ATP}) szulfonilurea (SUR) és befelé irányuló K^+-csatorna fehérje ($Kir_{6.x}$) alegységek alkotják hetero-oktamer szerkezetben. Jelenlegi kísérleteink célja a K_{ATP} csatorna SUR alegységeinek vizsgálata (SUR1/ABCC8 és SUR2/ABCC9) a terhes patkány uterus kontraktilitásának szabályozása kapcsán. A SUR1 és SUR2 alegységek vizsgálatára RT-PCR és western blot technikát alkalmaztunk. A SUR1 alegység expressziója esetén karakterisztikus változást találtunk: a terhesség elején jelentősen megemelkedett, míg a terhesség végéhez közeledve folyamatosan csökkent. A SUR2 alegység expressziója esetén nem tapasztaltunk változást a terhesség alatt. Korábbi vizsgálatok megerősítették azt, hogy a SUR alegységek felelősek a K_{ATP} csatorna agonisták farmakológiai reaktivitásáért. A nem szelektív SUR agonista, diazoxid és a SUR2 szelektív, pinacidil gátolta az oxytocin indukálta uterus kontrakciókat. Glibenklamid, K_{ATP} csatorna antagonista gátolta mind a diazoxid, mind a pinacidil által kiváltott uterus relaxációt.

Eredményeink alapján kimondható, hogy mindkét SUR alegység kimutatható a patkány uterusban terhesség alatt. Az in vitro vizsgálatok eredményei alapján a jövőben egy „pinacidil-szerű" K_{ATP} csatorna agonista fontos szerepet játszhat a korai fájástevékenység és koraszülés terápiájában, mint tokolítikum.

Kulcsszavak: *terhes patkány uterus, kálium csatorna, SUR, kálium csatorna agonista.*

Bevezetés

Az ABC-transzporterek (*ATP-binding casette transporters*, ATP-kötő kazetta transzporterek) az egyik legnagyobb és legősibb fehérjecsalád tagjai. Képviselői megtalálhatók minden létező taxonban a prokariótáktól az emberig. Ezek a transzmembrán fehérjék számos anyag membránon való átjutását végezhetik, a sejtmembránon vagy a sejt belső membránjain keresztül. Nevüket a sajátos szerkezetű ATP-kötő régióról kapták, amelyeken belül jól elkülöníthető szekvencia-motívumok találhatók. Jelenlegi ismereteink szerint valamennyi ABC transzporter működéséhez két alegység szükséges; egy ATP-kötő úgynevezett nukleotid kötő (NBD) régió, és hat membránon átérő fehérjeszakaszt tartalmazó, transzmembrán (TMD) régió. Az NBD a citoplazma felől foglal helyet, szerepe az ATP hidrolízise, amely az energiát szolgáltatja transzport folyamatokhoz. A TMD a membránban foglal helyet, szerepe az ioncsatorna képzés, a tulajdonsága alapvetően meghatározza a transzportált anyagok jellegét. A transzportfehérjék szerepe nem egységes; a prokariótákban elsősorban az influx mechanizmus jellemző, míg az eukariótákban mind az influx és efflux. Az efflux során a citoplazmában elhelyezkedő NBD-on történő ATP hidrolízisből keletkező energia a szubsztrátot át-

nyomja a TMD által kialakított csatornán. Az influx során a TMD-nek nemcsak az ioncsatorna képzés a szerepe, hanem a szubsztrát megkötése is. A tudomány jelenlegi állása szerint mintegy 49 (2009) humán ABC transzportfehérjét ismerünk, melyeket hét alcsaládba soroljuk ABCA-tól ABCG-ig. Az egységes nevezéktan megteremtésében a Human Genome Organization (HUGO) fontos szerepet játszik. Az ABC transzporterek nevezéktanával kapcsolatosan az alábbi cím nyújt segítséget: http://www.genenames.org/genefamily/abc.html. A 49 humán ABC transzporterből 46 tölt be valódi transzporter funkciót, a maradék három; ABCC7, ABCC8 és ABCC9 ioncsatorna regulátorként működik [1]. Az ioncsatornáknak, ezen belül a K^+ csatornáknak alapvető szerepe van a membránpotenciál alakulásában, ezáltal számottevően befolyásolják a celluláris folyamatokat. Különböző K^+ csatornákat ismerünk; kalciumfüggő-, feszültségfüggő- és ATP-szenzitív K^+ csatornákat (K_{ATP}) [2]. Az ABC transzporterek közül két fehérje az ABCC8 és az ABCC9 játszik szerepet a K_{ATP}-csatornák felépítésében. Ezekre a csatornákra a hetero-oktamer szerkezet jellemző; 4 db Kir.6.x alegységet (befelé irányuló K^+ csatorna fehérje) és 4 db szulfonilurea (SUR) alegységet tartalmaznak. A Kir.6 alegységek két altípusa ismert a Kir 6.1 és Kir 6.2, melyek a csatorna belső részén foglalnak helyet, szerepük a csatornaképzés. A SUR alegységek az ABC transzporterek családjába tartozó ABCC8 és ABCC9 vagy más néven SUR1 és SUR2. A SUR2-nek két izoformja ismert: SUR2A és SUR2B. A SUR alegységek meghatározzák a csatorna farmakológiai tulajdonságait és felelősek az ATP kötődésért. Azonban ahhoz, hogy K_{ATP}-csatorna funkcionáljon mind a Kir és a SUR alegységek szükségesek[3, 4, 5]. A K_{ATP}-csatornák szerkezete nem egységes az alegységek változatos expressziója miatt. Így változatos molekuláris szerkezetű és eltérő farmakológiai tulajdonságokkal rendelkező K_{ATP}-csatornákat kapunk; a hasnyálmirigy ß-sejtjeiben Kir 6.2 /SUR1 [6], a szívben Kir 6.2 /SUR2A [7], a vaszkuláris simaizomban Kir 6.1 /SUR2B, míg a nem vaszkuláris simaizomban Kir 6.2 / SUR2B [8] felépítésű K_{ATP}-csatornák találhatók. A K_{ATP}-csatornák nyitása K^+ kiáramláshoz vezet, mely a membránpotenciált a K^+ egyensúlyi potenciálja felé viszi, mely szöveti kontraktilitás csökkenéshez vezet. A K_{ATP}-csatorna nyitását serkentő anyagok (KCO) – mint például a diazoxid és pinacidil – jó simaizom relaxáló hatással bírnak. A simaizom relaxáló hatásukat már bizonyították nem terhes patkány uteruson [9]. A hatásukban azonban alapvető különbségek mutatkoznak; míg a diazoxid olyan csatornákon képes hatni, amelyek vagy SUR1-t vagy SUR2-t tartalmaznak, addig a pinacidil csupán csak akkor hatásos, ha a csatornában SUR2 található [10, 11].

Kísérleteink során célul tűztük ki a K_{ATP}-csatornák SUR alegységeinek meghatározását a terhességi idő függvényében, valamint lehetséges összefüggések keresését a KCO (pinacidil és diazoxide) farmakológiai reaktivitása és a K_{ATP}-csatornák felépítése között.

Anyagok és módszerek

Az állatkísérleteket a Szegedi Tudományegyetem Munkahelyi Állatetikai Bizottságának és a Csongrád Megyei Mezőgazdasági, Szakigazgatási Hivatal Élelmiszerlánc Biztonsági és Állategészségügyi Igazgatóság engedélyével végeztük (engedélyszám: IV./01758-21/10082).

Állatkísérletek

Az állatok pároztatása

Ivarérett nőstény (180-200 g) és hím (240-260 g) Sprague-Dawley patkányokat pároztattunk. A pároztatás kezdetétől számított 4-5 órán belül a nőstény állatoktól hüvelykenetet vettünk és mikroszkóp alatt hímivarsejteket kerestünk. Amennyiben a keresés pozitív eredménnyel zárult, akkor az állatot elkülönítettük, mint az 1. napos vemhes nőstényt.

Real-Time PCR analízis

A PCR vizsgálatokat nem terhes valamint 6, 8, 10, 12, 14, 15, 18, 20, 21, 22 napos terhes uterusokkal végeztük, valamint a terhesség 6. illetve 10. napján implantációs és interimplantációs helyeket külön gyűjtve is feldolgoztuk. A mintákat folyékony nitrogénben fagyasztva mechanikusan porítottuk Sartorius Mikro Dismembrator segítségével. Ezután a mintákból PARIS Kit (Life Technolgies, Hungary) segítségével RNS-t és fehérjét izoláltunk. Az RNS mennyiségi és minőségi meghatározása nanodrop technika alkalmazásával (Biospec Nano, Shimadzu Biotech) történt. A reverz transzkripciós (RT) lépésben 2 μg RNS-t és High Capacity RNA-to-cDNA Kitet (Life Technolgies, Hungary) használtunk. A felsokszorozás TaqMan Gene Expression Master Mix (Life Technolgies, Hungary) alkalmazásával ABI StepOne Real-Time

cycler gépen történt. A következő primereket használtuk: assay ID ABCC8/SUR1: Rn01476318_m1, ABCC9/SUR2: Rn01463198_m1 és endogén kontrollként a gliceraldehid-3-foszát dehidrogenázt (GAPDH): Rn99999916_s1.

Western blot analízis

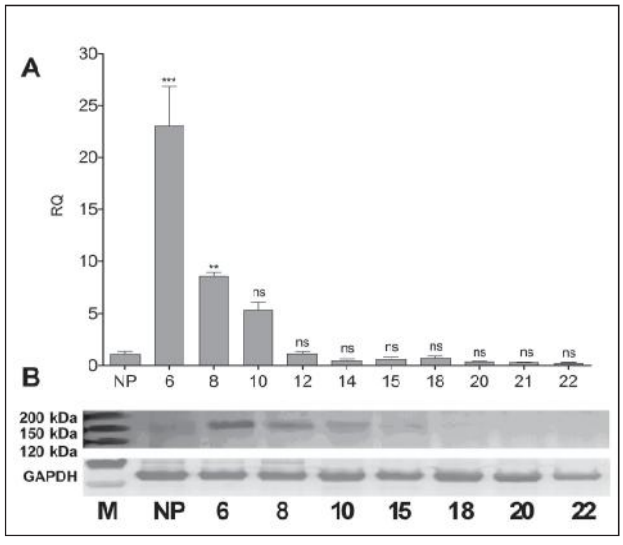
Mintánként 30 μg fehérjét 4-12%-os NuPAGE Bis-Tris Gel (Life Technologie, Hungary) gélen elektroforézisnek vetettünk alá. A fehérjét a nitrocellulóz (Scheicher and Schuell, Germany) membránra helyeztük át, félszáraz blottoló eljárással (Bio Rad). Mosás után a membránt szobahőmérsékleten SUR1, SUR2 és GAPDH poliklonális antitestekkel (Santa Cruz Biotechnology, California, 1:200), blokkoló pufferben 1 órán át inkubáltuk. Az immunreaktív sávokat WesternBreeze Chromogenic Western blot immune detection kit (Invitrogen, Hungary) segítségével láthatóvá tettük, majd elektronikusan rögzítettük az EDAS290 imaging system (KODAK, Invitrogen, Hungary) segítségével. Az optikai denzitás meghatározása Kodak 1D Images software-rel történt.

Uterus preparálása, KCO hatásának vizsgálata oxytocin indukálta kontrakciókra

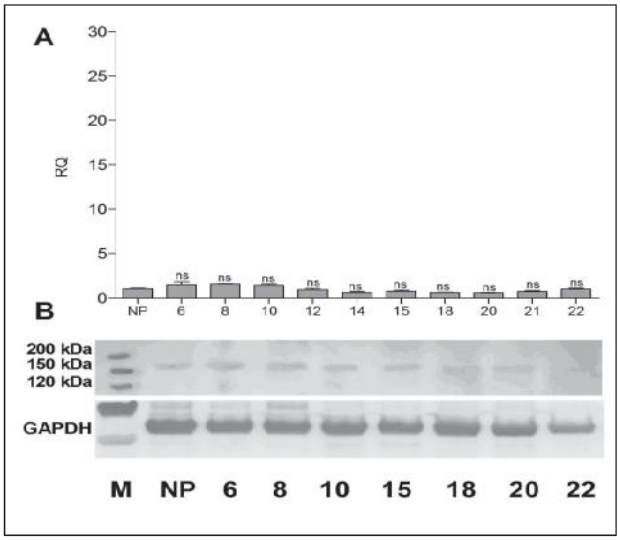
Kísérleteink során nem terhes valamint 6, 8, 18 és 22 napos terhes patkány uterusokat használtunk. Az állatok CO_2-al történő leölése után az uterusokból 5 mm hosszúságú gyűrűket metszettünk. A preparátumokat karbogénnel átáramoltatott de Jongh oldatot tartalmazó, 37 °C-os szervfürdőbe helyeztük (de Jongh oldat összetétele mM-ban: 137 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 12 $NaHCO_3$, 4 NaH_2PO_4, 6 glükóz, pH: 7,4). Az inkubálási periódus (4 x 15perc) letelte után az uterus kontrakciókat oxytocinnal (10^{-6} M) váltottuk ki, majd megkezdtük a pinacidil és diazoxid (10^{-8}-10^{-4} M) adagolását. Az adagolást nem kumulatív módon végeztük. Vizsgálatainkat K_{ATP}-csatorna blokkoló glibenklamid (10^{-6} M) jelenlétében is elvégeztük 6, 8 és 22 napos terhes uteruson. A pinacidil és diazoxid (Sigma-Aldrich, Hungary) gátló hatását az oxytocin által kiváltott ritmikus kontrakciókhoz viszonyítottuk és a kontrakciós görbék alatti területet értékeltük a kontroll AUC-hez viszonyítva, 5 percet értékelve. A görbék regisztrálását, az adatok rögzítését és feldolgozását ISOSYS DataAcquisition System (Experimenta Kft., Hungary) segítségével végeztük. Az eredmények statisztikai elemzését a Prism 5.0 (GraphPad Software, USA) segítségével ANOVA Newman-Keuls teszttel végeztük.

Pinacidil hatásának vizsgálata EFS indukálta kontrakciókra

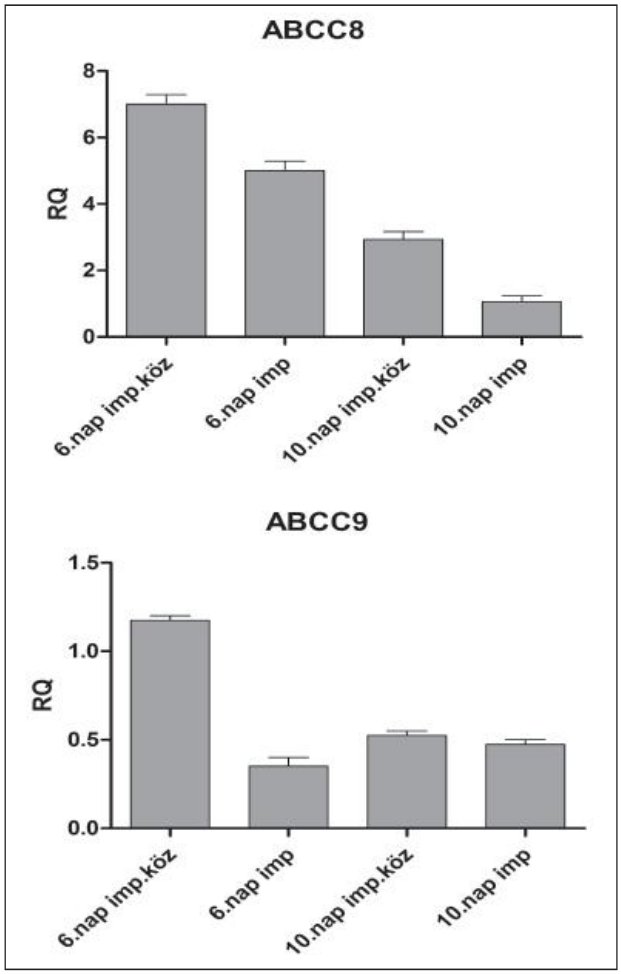
A pinacidil hatását EFS (Elektromos Térerő Ingerlés) indukálta kontrakciókon is megvizsgáltuk nem terhes valamint 22 napos terhes uterusokon. Az uterus preparálása a fent említett módon történt, azzal a kivétellel, hogy ebben az esetben a szöveteket egy speciális szervtartóra rögzítettük, amelyen két platina elektród található. A szövetet a két elektród közé erősítettük, majd a kontrakciókat elektromos térerőingerléssel indukáltuk. Ebben az esetben a pinacidil hatásának vizsgálatát kumulatív módon végeztük 20 perces TEA (tetraetil-ammónium) (10^{-3} M) előinkubálás után. A kísérlet paraméterei a következők voltak; PW: 30 s (egy elektromos stimulus hossza) és PP: 50 ms (két elektromos stimulus közt eltelt idő). A pinacidil gátló hatását az EFS által kiváltott ritmikus kontrakciókhoz viszonyítottuk és a kontrakciós görbék alatti területet értékeltük a kontroll AUC-hez viszonyítva. A görbék regisztrálása, az adatok rögzítése és feldolgozása, az eredmények statisztikai elemzése az előzőekben leírtak szerint történt.



1. ábra: ***(A):*** *SUR1/ABCC8 mRNS expressziója a terhességi napok függvényében. Az RQ (Relative Quantity) értékek statisztikai összehasonlítást a nem terhes állapotban kapott értékekhez viszonyítva tüntettük fel; ns: nem szignifikáns,**: p<0.01; ***: p<0.001, S.E.M: standard error of mean, n = 5.* ***(B): (felül)*** *SUR1/ABCC8 fehérje expressziója terhességi napok függvényében (M: marker, NP: nem terhes),* ***(alul)*** *endogén kontroll Gliceraldehid-3-foszát dehidrogenázt (GAPDH) [37kDa].*



*2. ábra: (**A):** SUR2/ABCC9 mRNS expressziója a terhességi napok függvényében. Az RQ (Relative Quantity) értékek statisztikai összehasonlítást a nem terhes állapotban kapott értékekhez viszonyítva tüntettük fel; ns: nem szignifikáns, S.E.M: standard error of mean, n = 5.*
*(**B): (felül)** SUR2/ABCC9 fehérje expressziója terhességi napok függvényében (M: marker, NP: nem terhes), **(alul)** endogén kontroll Gliceraldehid-3-foszát dehidrogenázt (GAPDH) [37kDa].*



3. ábra: Az SUR1/ABCC8 és az SUR2 /ABCC9 expressziója a terhesség 6. és 10. napján az implantációs és interimplantációs helyeket külön vizsgálva. S.E.M: standard error of mean n = 5

Eredmények

Real-Time PCR és Western blot analízis

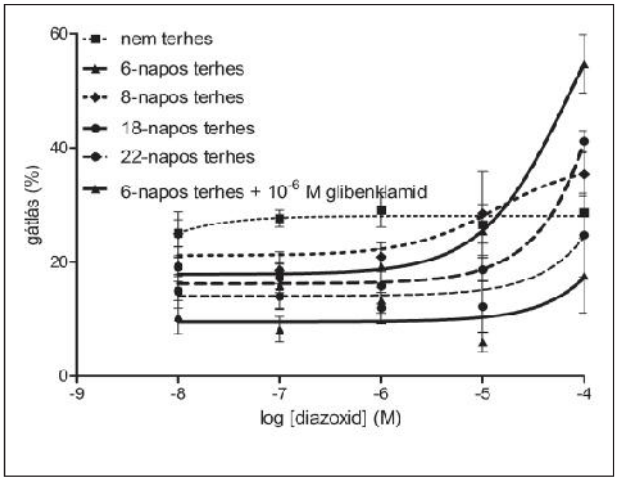
A Real-Time PCR és Western blot analízis igazolta, hogy mindkét SUR alegység kimutatható a nem terhes és a terhes patkány uterusban. A SUR1 esetén mind az mRNS és a fehérje szintje drámaian megemelkedett a terhesség elején (6. nap) majd csökkent a 8. naptól a 12. napig és utána változatlan maradt a terhesség végéig (*1. ábra*). A SUR2 esetén nem tapasztaltunk számottevő változást a terhesség folyamán (*2. ábra*). A SUR1 esetén tapasztalt markáns emelkedés kapcsán a 6. illetve a 10. terhességi napokon az implantációs és az interimplantációs helyeket külön gyűjtve is megvizsgáltuk mindkét SUR alegység kapcsán (*3. ábra*).

A nem szelektív SUR agonista diazoxid uterus relaxáló hatása

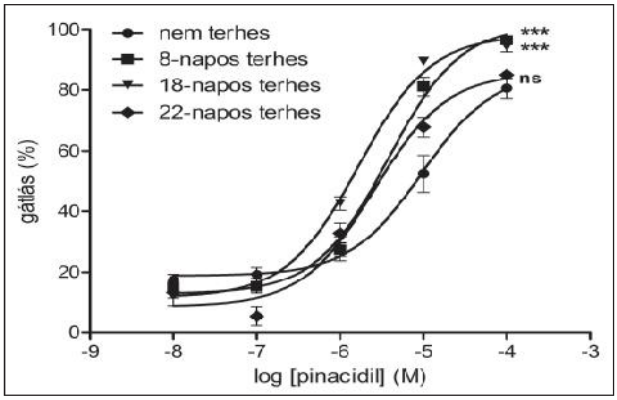
A diazoxid uterus-relaxáló hatását oxytocin indukálta kontraciókon vizsgáltuk nem terhes valamint 6, 8, 18 és 22 napos terhes uterusokon 10^{-8}-10^{-4} M dózistartományban. A diazoxid kontrakció gátló hatása a terhesség 6. napján volt a legerősebb (60%), majd a 8. és 18 napon csökkent (40%). Azonban hatástalannak bizonyult a nem terhes valamint a 22 napos terhes uteruson. A terhesség 6. napján tapasztalt kontrakció gátló hatást 10^{-6} M glibenklamiddal blokkolni tudtuk (*4. ábra*).

A SUR2 szelektív agonista pinacidil uterus relaxáló hatása

A pinacidil (10^{-8}-10^{-4} M) dózisfüggően gátolta az oxytocin indukálta kontrakciókat a nem terhes valamint 8, 18 és 22 napon terhes uteruson (*5. ábra*). Az EC_{50} értékeket tekintve hatása kifejezettebb volt a terhes uterusokon a nem terheshez viszonyítva (*6. ábra*). Azonban az E_{max} értékeket tekintve a terhesség 8. és 18. napján volt a leghatásosabb, míg a 22. napon a nem terheshez hasonló értéket tapasztaltunk (5. ábra). A pinacidil uterus-relaxáló hatása a terhesség 8. illetve 22. napján blokkolható volt 10^{-6} M glibenklamiddal (*7. ábra*). Az EFS indu-



4. ábra: A nem szelektív SUR agonista diazoxid (10^{-8}-10^{-4} M) kontrakció gátló hatása oxytocin (10^{-6} M) stimulálta kontrakciókra a nem terhes és a terhesség 6., 8., 18., és 22. napján, valamint glibenklamid (10^{-6} M) jelenlétében a terhesség 6. napján. S.E.M: standard error of mean, n = 6

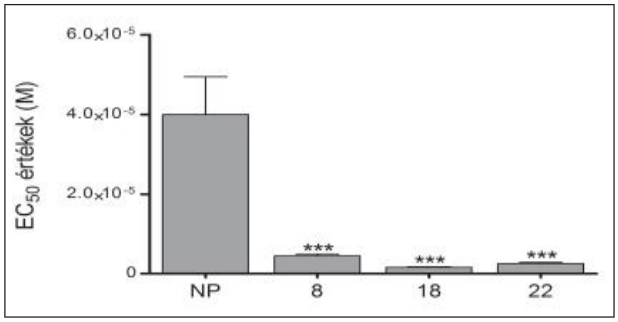


*5. ábra: A SUR2 szelektív pinacidil (10^{-8}-10^{-4} M) kontrakció gátló hatása izolált uterus gyűrűn (10^{-6} M) oxytocinnal indukált kontrakciókra a nem terhes, 8, 18 és 22 napos terhes patkány uteruson. Az E_{max} értékek statisztikai összehasonlítást a nem terhes állapotban kapott értékekhez viszonyítva tüntettük fel; ns: nem szignifikáns, ***: p<0.001. S.E.M: standard error of mean, n = 6.*

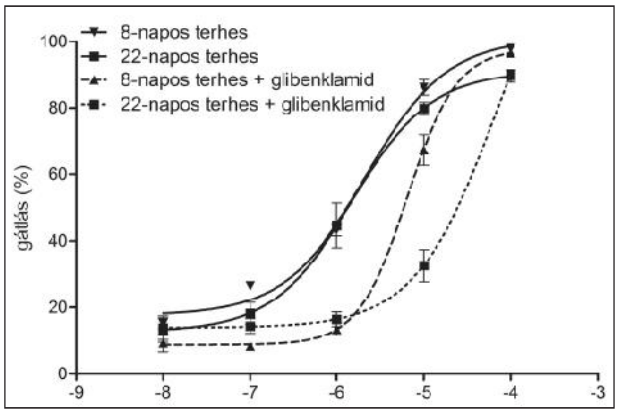
kálta kontrakciókat a pinacidil szintén dózisfüggően gátolta, míg 10^{-3} M TEA jelenlétében a dózis-hatás görbék signifikánsan jobbra tolódtak (*8. ábra*).

Az eredmények értékelése, következtetések

A terhesség során végbemenő folyamatok eredményeként az uterus struktúrája drámai változáson megy keresztül. A terhesség végén, a fájástevékenység beindulásáig, a miometrium kontraktilitása enyhén fokozódik, majd szüléskor ugrásszerűen megnő. A K_{ATP}-csatornák nyitásával az uterus kontraktilitása csökkenthető, így olyan vegyületek melyek K_{ATP}-csatornákat nyitnak fontos sze-

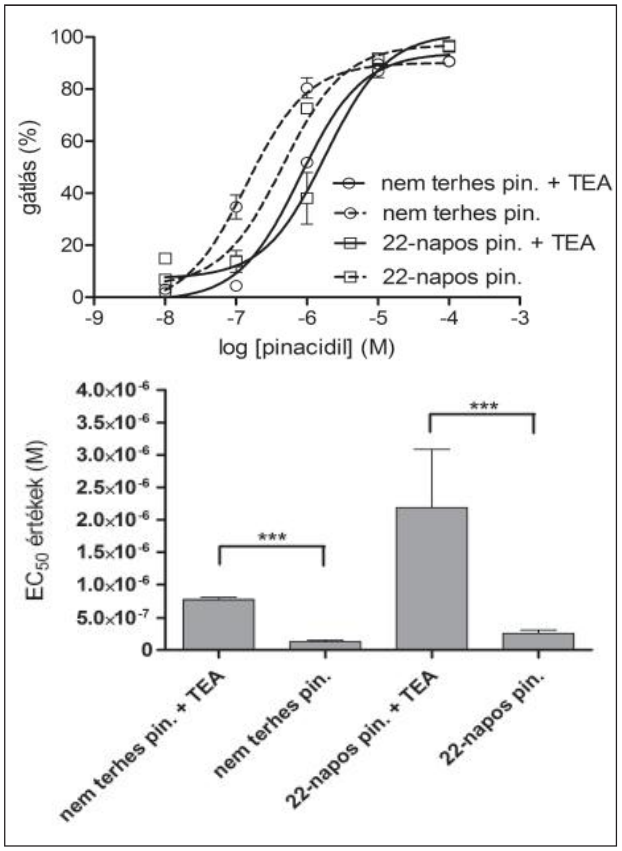


*6. ábra: A SUR2 szelektív pinacidil (10^{-8}-10^{-4} M) uterusra gyakorolt relaxáló hatásának Ec_{50} értékei (10^{-6} M) oxytocin jelenlétében nem terhes (NP), 8, 18 és 22 napos terhes patkány uteruson. A statisztikai összehasonlítást a nem terhes állapotban kapott értékekhez viszonyítva tüntettük fel. S.E.M: standard error of mean; ***: p<0.001, n=6.*



7. ábra: A SUR2 szelektív pinacidil (10^{-8}-10^{-4} M) kontrakció gátló hatása izolált uterus gyűrűn (10^{-6} M) oxytocinnal indukált kontrakciókra 8 és 22 napos terhes patkány uteruson (10^{-6} M) glibenklamid jelenlétében. S.E.M: standard error of mean, n = 6.

repet játszanak a korai fájástevékenység és koraszülés megakadályozásának terápiájában [12]. A K_{ATP}-csatornák felépítésében szerepet játszó ABC transzporterek (ABCC8/SUR1 és ABCC9/SUR2) alapvetően meghatározzák a csatorna farmakológiai tulajdonságait. Mivel a K_{ATP}-csatornák felépítése nem egységes, így elengedhetetlen ismernünk az uterusban lévő K_{ATP}-csatornák felépítését. Korábbi közlemények azt igazolták, hogy SUR2B alegység található a patkány uterusban[13, 14]. *Curley* és mtsai [15], SUR1 és SUR2 mRNS izoláltak a humán miometriumban. A mi eredményeink azt igazolják, hogy mindkét alegység expresszálódik a patkány uteursban a terhesség folyamán és a SUR1 esetén karakterisztikus változás mutatható ki. Hasonlóan *Curley* és mtsai eredményeihez [15], mi is SUR1 csökkenést tapasztaltunk a terhesség végéhez közeledve. Feltehetően a SUR1



*8. ábra: A SUR2 szelektív pinacidil (10^{-8}-10^{-4} M) kontrakció gátló hatása izolált uterus gyűrűn EFS indukált kontrakciókra nem terhes és 22-napos terhes patkány uteruson (10^{-3} M) tetraetil-ammónium (TEA) jelenlétében. Az EC_{50} értékek statisztikai összehasonlítást a pinacidil hatásához viszonyítva tüntettük fel. SEM: standard error of mean; ***: p<0.001, n = 6.*

csökkenése illetve eltűnése az uterus kontraktilitásának fokozódását facilitálhatja.

A KCO (diazoxid és pinacidil) jelentős uterus-relaxáló hatással bírnak, mely hatás kivédhető glibenklamid adásával. A diazoxid nem szelektív SUR agonistaként míg a pinacidil SUR2 szelektív agonistaként képes hatni a K_{ATP}-csatornákon. A diazoxid hatása szoros összefüggést mutat a SUR1 expressziójának változásával, mivel a legerősebb diazoxid hatást akkor találtuk, amikor a SUR1 expresszió legmagasabb volt. Az is megerősítésre került, hogy az extrém magas SUR1 expresszió a terhesség 6. napján nem a vaszkularizációnak betudható, mivel az implatációs és interimplantációs helyeket külön vizsgálva nem tapasztaltunk jelentős különbséget sem SUR1 sem a SUR2 expressziója között. A SUR2 kapcsán nem tapasztunk olyan szintű változást, mint a SUR1 esetén, de a SUR2 szelektív pinacidil meglepően erőteljes uterus-reláxáló hatással bírt a terhesség bármely időszakában. A glibenklamidal történő vizsgálatok a 8. illetve a 22. napon sem hoztak választ kérdésünkre, hogy mitől ilyen erős a pinacidil hatása. A glibenklamid blokkoló hatásában különbség mutatkozott a 8. és 22. napon, de ennek az okát magyarázni nem tudjuk. Azonban a TEA-val végzett kísérletek bebizonyították, hogy a pinacidil feltehetően nemcsak a K_{ATP}-csatornákon keresztül hat, hanem TEA szenzitív K^+ csatornák is involválva vannak a hatásában. Hasonlóan *Bukarica* és mtsai (2011) [16] humán artérián kapott eredményeihez, a pinacidil hatása a mi vizsgálataink során is gátolható volt TEA-val. Így feltehetően, a humán artérián, úgy patkány uterusban is a pinacidil K_{ATP}-csatornákhoz való szelektivitása megkérdőjelezhető, amint azt mindkét esetben a TEA-val végzett kísérletek bizonyították.

Eredményeink alapján elmondhatjuk, hogy mindkét SUR alegység kimutatható a patkány uterusban terhesség alatt. A K_{ATP}-csatornák SUR alegységei felelősek a csatorna farmakológiai reaktivitásáért. A SUR nem szelektív diazoxid uterus-relaxáló hatása akkor a legerősebb, amikor a SUR1 expressziója a legmagasabb. A SUR1 downregulációja feltehetően elősegíti az uterus kontraktilitásának fokozódását a terhesség végéhez közeledve. A SUR2 szelektív pinacidil uterus-relaxáló hatása a terhesség bármely szakaszában vizsgálva erőteljesnek bizonyult, mely hatásban feltehetően nemcsak K_{ATP}-csatornák, hanem TEA szenzitív K^+ csatornák is involválva vannak. Eredményeink alapján, a közeljövőben egy uterus szelektív ''pinacidil szerű'' K_{ATP}-csatorna opener kifejlesztése indokolt lehet, a koraszülés és korai fájástevékenység megakadályozásának terápiájában.

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III.

in vivo 27: 363-370 (2013)

Increase of the Uterus-relaxant Effect of Nifedipine by the Abcg2 Efflux Protein Inhibitor KO134 in the Rat *In Vivo*

NORBERT LOVASZ, ESZTER DUCZA, ISTVAN ZUPKO and GEORGE FALKAY

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

Abstract. *Background/Aim: High Abcg2 (ATP-Binding Cassette Transporter Subfamily G, Member-2) levels have been found in reproductive tissues, such as the placenta and uterus. 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. Nifedipine, commonly used in acute tocolytic therapy, exerts a greater tocolytic effect and has fewer side-effects than β_2-adrenergic receptor agonists. The aims of the present study were to investigate the expression of Abcg2 in the rat uterus during gestation and the uterus-relaxant effect of nifedipine in the presence of the Abcg2 inhibitor KO134. Materials and Methods: Real-time Polymerase Chain Reaction (PCR) and western blot analyses were performed to detect the levels of Abcg2 during gestation in the rat. The uterus-relaxant effect of nifedipine in vivo was investigated by the intra-uterine pressure measuring method, described by Csapo. Results: Low levels of Abcg2 were found in non-pregnant animals and early-pregnancy (days 6, 8 and 10), but on day 15 of gestation, a sharp increase in Abcg2 levels was observed, which reached its maximum on day 18 and later decreased until the end of gestation. The post-partum levels were similar to those in non-pregnant rats. The in vivo contractility studies revealed that nifedipine had a strong uterus-relaxant effect on spontaneous contractions, and that this effect was significantly and dose-dependently increased by the Abcg2 blocker KO134. Conclusion: The administration of efflux pump inhibitors in combination with tocolytic agents may be of novel therapeutic relevance in the management of pre-term labour.*

Correspondence to: Professor George Falkay, Ph.D., D.Sc., Department of Pharmacodynamics and Biopharmacy, Faculty of Pharmacy, University of Szeged, H-6701, P.O. Box 121, Szeged, Hungary. Tel: +36 62341974, e-mail: falkay@pharm.u-szeged.hu

Key Words: Premature labour, rat uterus, nifedipine, tocolysis, Abcg2 inhibition, ontogeny of Abcg2.

Preterm birth, defined by the World Health Organization as childbirth between 20 and 37 weeks of pregnancy, is a major determinant of neonatal mortality and morbidity and has long-term adverse consequences for health. The exact causes and aetiologies of preterm birth are not known. Its incidence, now exceeding 12% of all births in the USA, is constantly increasing despite major improvements in medical (especially perinatal) care facilities and extensive medical research. Its annual costs reached 26.2 billion US dollars in 2005, imposing a huge public burden (1). With a view to reducing the potentially adverse maternal and foetal events and improving perinatal outcome, it is a pharmacological challenge to find new therapeutic strategies. Ca^{2+}-channel blockers are known to abolish intracellular Ca^{2+} transients and myometrial contractions (2). The Ca^{2+}-channel blocker most commonly used in the onset of preterm labour is nifedipine.

The ATP-binding cassette (ABC) transporters, expressed in all organisms, form one of the largest families of membrane transport proteins. These transporters are responsible for multi-drug resistance (3, 4), may be also capable of transportation across the plasma membrane and intracellular membranes (5). They play important roles in tissue defence through the excretion of toxic compounds (6). The expression levels of these transporters are tightly-regulated, emphasizing their importance in organ protection (7).

The efflux pump protein ABC subfamily G member-2 (Abcg2) is highly expressed in reproductive tissues such as the placenta (8) and uterus (9), and at somewhat lower levels in the prostate, testis and ovary (10). Abcg2 transports various compounds through the cell membrane (Table I). A number of Abcg2 inhibitors have been reported (Table II).

Regarding the Ca^{2+}-channel blockers of the dihydropyridine type (DHPs), Zhou *et al.* (11) reported that apart from nifedipine, they enhance intracellular mitoxantrone accumulation in a concentration-dependent manner. Shukla *et al.* (12) demonstrated that DHPs are transported by Abcg2, and determined the effects of DHPs on the ATPase activity of Abcg2; nifedipine stimulated ATP hydrolysis by the transporter, maximum stimulation proving equal to or greater than that achieved with prazosine, a known substrate of Abcg2.

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Table I. *Compounds transported by the ABC-Transporter subfamily G member-2 (Abcg2) transporter.*

Abcg2 substrates		References
Chemotherapeutic agents	Mitoxantrone, topotecan, irinotecan, methotrexate, imatinib	(13)
Anti-viral agents	Lamivudine, zidovudine,	(14)
	abacavir	(15)
Antibiotics	Ciprofloxacin, ofloxacin,	(16)
	norfloxacin, erythromycin,	(17)
	rifampicin, nitrofurantoin	(18)
Ca^{2+}-channel blockers	Nifedipine	(11, 12)
HMGCoA reductase inhibitors	Rosuvastatin, pitavastatin,	(19)
	cerivastatin	(16)
Others	cimetidine, folic acid,	(20)
	dipyridamole	(16)

Table II. *ABC-Transporter subfamily G member-2 (Abcg2) inhibitors.*

Abcg2 inhibitors		References
Flavonoids	Apigenin, biochanin A, chrysin, genistein, kaempferol, hesperetin, naringenin, silymarin	(21)
Ca^{2+}-channel blockers	Nicardipine, niguldipine,	(11)
	nitrendipine, verapamil	(22)
Oestrogens	Oestrone, 17β-oestradiol	(23, 24)
Fumitremorgin C analogues	KO132, KO134, KO143,	(25)
	mycotoxin fumitremorgin C,	(26)
	demethoxyfumitremorgin C	(27)
Others	Elacridar (GF120918),	(28)
	Tariquidar (XR9576), Novobiocin,	(20)
	Etposide, Cyclosporine-A,	(22)
	HER tyrosine kinase inhibitor (CI1033),	(29)
	Camptothecin analogues (GF120918)	(30)

Moreover, they established that fumitremorgin C inhibited nifedipine-stimulated ATPase activity in a concentration-dependent manner. These results confirmed that nifedipine is transported by Abcg2. It may, therefore, be hypothesized that a combination of nifedipine with the Abcg2 blocker KO134 will result in an increase in the efficacy of nifedipine.

The aims of the present study were to determine the levels of Abcg2 during gestation in the rat, and to investigate the uterus-relaxant effect of nifedipine in the presence of the Abcg2 inhibitor KO134.

Materials and Methods

Drugs. Nifedipine and KO134 were purchased from Sigma-Aldrich Ltd, Budapest, Hungary. Nifedipine was dissolved in polyethylene-glycol 400: dimethyl-sulfoxide: saline (3:3:10, v/v/v) and KO134 in dimethyl-sulfoxide: cremophor: saline (2:1:7, v/v/v).

Housing and handling of the animal. 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 (Charles-River Laboratories, Budapest, Hungary) 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 (Charles-River Laboratories) with tap water available *ad libitum*.

Mating of the animals. Mature female (180-200 g) and male (240-260 g) 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 obtained 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.

Tissue isolation. Non-pregnant, pregnant (6th, 8th, 10th, 15th, 18th, 20th and 22th) and *post-partum* Sprague-Dawley rats (weight: 250-300 g) were euthanized in a CO_2 chamber. Uterine tissue was rapidly removed; both horns of the uterus were excised. The first

(cervical side) and the last (ovarian 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 (RNALater, Life Technologies, Budapest, Hungary). The samples were frozen in liquid nitrogen and stored at −70°C until total RNA and protein extraction.

Real-time quantitative reverse transcription-Polymerase Chain Reaction (PCR). Uterine tissues frozen in liquid nitrogen and were mechanically homogenized. The PARIS Kit (Protein and RNA Isolation System; Life Technologies) was used for total RNA and protein extraction from the tissues. The High Capacity RNA-to-cDNA Kit (Life Technologies) was used for reverse transcription. PCR products were amplified with the TaqMan Gene Expression Master Mix (Life Technologies) and a ABI StepOne Real-Time cycler (50°C hold 2 min, 95°C hold 10 min, than 40 cycle 95°C 15 sec and 60°C 1min). The following primers were used: assay ID Rn01639905-m1 for *Abcg2*, and Rn00667869-m1 for *β-actin* as endogenous control. 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. Thirty micrograms of protein per well was subjected to electrophoresis on 4-12% NuPAGE Bis-Tris Gel (Life Technologies) in XCell SureLock Mini-Cell Units (Invitrogen, Budapest, Hungary). Proteins were transferred from gels to nitrocellulose membranes (Scheicher and Schuell, Dassel, Germany) by a semi-dry blotting technique (BioRad, Budapest, Hungary). The blots were incubated on a shaker with polyclonal antibodies against Abcg2 and β-actin (Santa Cruz Biotechnology, CA, USA; 1:200) in the blocking buffer. Antibody binding was detected with the WesternBreeze Chromogenic Western Blot Immune Detection Kit (Invitrogen). Images were captured with the KODAK EDAS290 imaging system (Invitrogen), and the optical density of each immunoreactive band was determined with Kodak 1D Image 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 intra-uterine pressure was based on the classical microballoon experiments originally described by Csapo (31-33). The *in vivo* experiments were carried out on post-partum rats because the intra-uterine pressure measurements with a Millar catheter in the pregnant animals were not sufficiently accurate: the foetus disturbed the measurement efficiency and the catheter could not be fixed appropriately. Throughout the experiments, the rats were anaesthetized with a combination of ketamine (36 mg/kg) and xylazine (4 mg/kg), administered intra-peritoneally 24 h after the spontaneous delivery. 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 incision above the cervical part. After a 45-min equilibration period, the intrauterine pressure was recorded with S.P.E.L. Advanced ISOSYS Data Acquisition System (Experimetria, Budapest, Hungary). The effect of nifedipine was assessed by expressing the integrated tension relating to a 5-min period. Areas under the curves (AUCs) of 5-min periods were evaluated and the effect of nifedipine was expressed as a percentage in terms of the AUC of the spontaneous contractions preceding the administration of the relaxing drug. The experimental procedures and the patterns of intrauterine pressure change are presented in Figure 1.

Statistical analyses. All experiments were carried out on at least 8 animals and each reported value is given as the mean±S.E.M. All curve fittings, data calculations and statistical analyses were performed with the Prism 5.0 computer software (Graph Pad Software Inc, San Diego, CA, USA). Group comparisons were performed by one-way ANOVA tests with the Newman-Keuls post test.

Results

Abcg2 expression in the rat uterus. The expressions of Abcg2 mRNA and protein were investigated in non-pregnant, pregnant and post-partum rat uterus. This revealed a characteristic expression during gestation: low levels of Abcg2 were found in the non-pregnant and the early-pregnant uterus (days 6, 8 and 10), but on day 15 of gestation, a sharp increase was observed, a maximum was reached on day 18 of gestation, and the level then decreased from day 20 to post-partum. The post-partum levels were similar to the non-pregnant levels (Figures 2 and 3).

Uterus-relaxing effect of nifedipine in vivo. The uterus-relaxing effect of nifedipine was investigated in the post-partum rat uterus *in vivo* with an intra-uterine pressure measuring method. Nifedipine proved to exert a strong relaxant effect on the spontaneous uterine contractions. Parallel administration of the Abcg2 inhibitor KO134 dose-dependently increased the uterus-relaxing effect of nifedipine. The effective dose fifty percent (ED_{50}) of nifedipine was 240 μg/kg, whereas that of its combination with 15 mg/kg KO134 or with 30 mg/kg KO134 were significantly lower ($p<0.001$) at 170 μg/kg and 25 μg/kg, respectively (Figure 4 A and B).

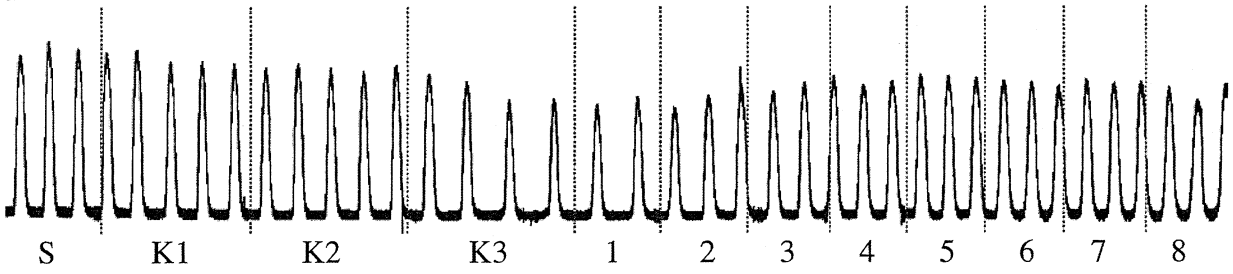
Discussion

ABC transporters play important roles in the absorption, distribution and elimination of many compounds, potentially resulting in multi-drug resistance and therapy failure. The level of expression of these transporters is tightly-regulated, emphasizing their importance in organ protection. Abcg2, a recently identified ABC transporter, is highly expressed in reproductive tissues (placenta, uterus and prostate) and has an important role in tissue defence through the efflux of toxic compounds and their metabolites, thereby reducing their intracellular concentrations. Several compounds with a uterus-relaxant effect (*e.g.* prazosine and nifedipine) are transported by Abcg2. The blocking of Ca^{2+}-channels has been shown to reduce uterine tone and this is a target for the inhibition of uterine activity in the treatment of pre-term labour.

A

Incubation period, spontaneous contraction	KO134 vehicle, KO134 15 or 30 mg/kg	Injected nifedipine doses, at 5-min intervals (μg/kg)									
45 min	30 min	4	8	20	40	100	100	100	100	100	100

B



C

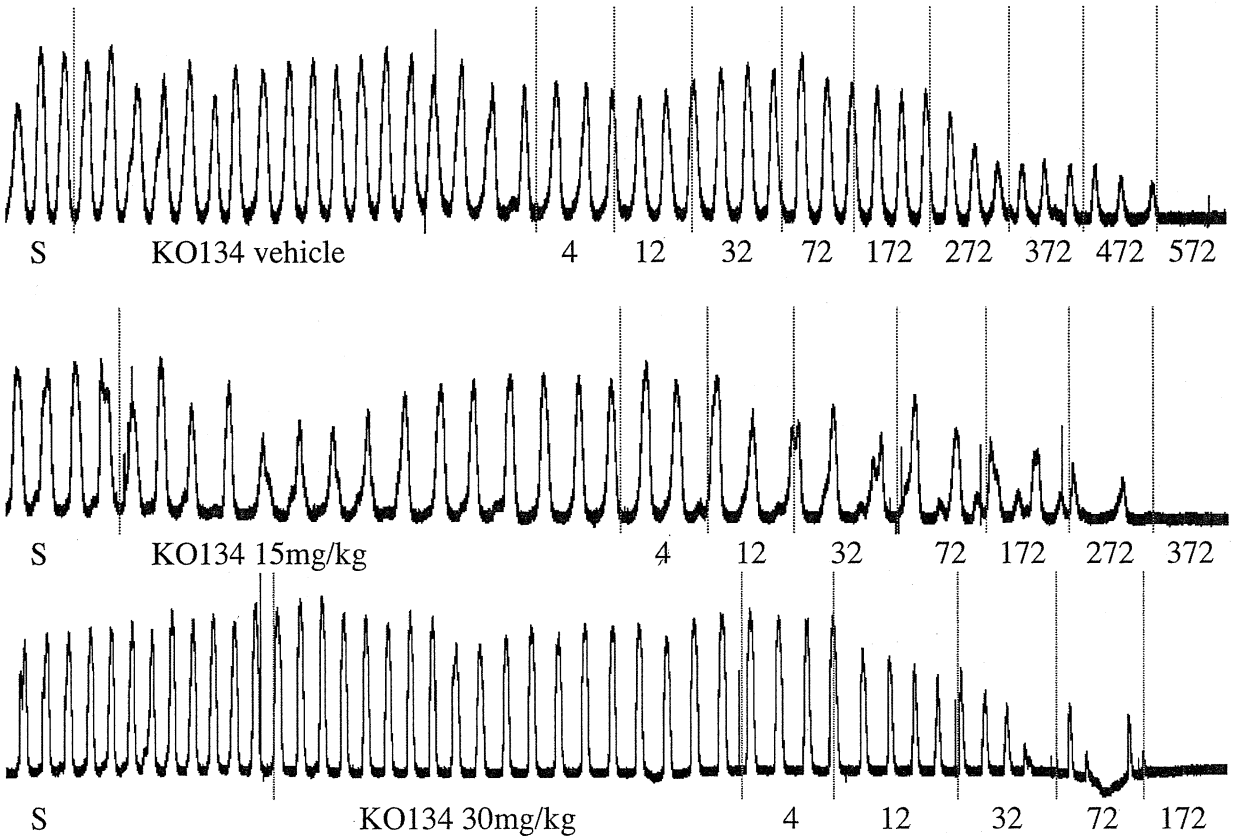


Figure 1. *A Experimental procedure. Representative patterns of intra-uterine pressure change in the presence of B solvent controls for KO134 and nifedipine. S: Spontaneous contractions, K1, K2, K3: KO134 vehicle (DMSO:cremophor:saline, 2:1:7, v/v/v), 1-8: nifedipine vehicle (PEG 400:DMSO:saline, 3:3:10, v/v/v), C KO134 vehicle plus nifedipine, and KO134 at 15 mg/kg or 30 mg/kg plus nifedipine. Under the patterns for C, the cumulative nifedipine doses are indicated.*

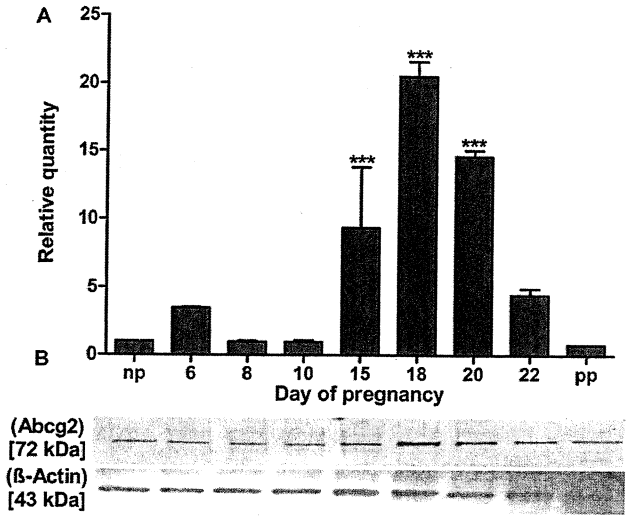


Figure 2. *A: Changes in expression of ABC-Transporter subfamily G member-2 (Abcg2) mRNA during gestation in the rat myometrium. Values for relative quantity on different days of gestation were compared with that in non-pregnant rats (np). ***Denotes p<0.001 compared to np. Each value indicates the mean±S.E.M, n=5. B Representative Western blot of Abcg2 protein expression in non-pregnant, pregnant and post-partum (pp) rat myometrium, with β-Actin as endogenous control.*

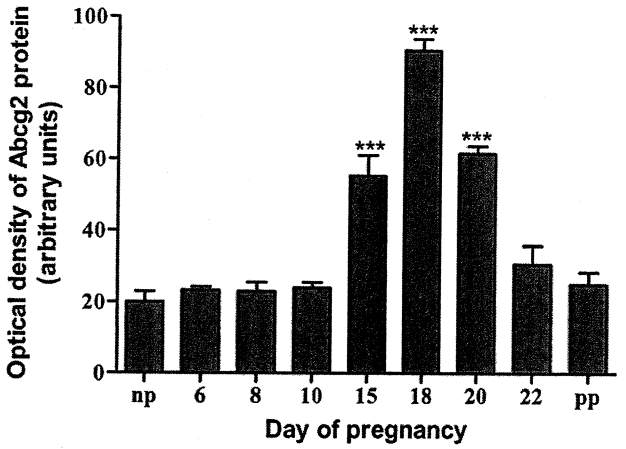


Figure 3. *Densitometric analysis of ABC-Transporter subfamily G member-2 (Abcg2) western blot data (data show on Figure 2B). Densitometric values on different days of gestation were compared with that in non-pregnant rats. ***Denotes p<0.001. Each value indicates the mean±S.E.M, n=5. np: Non-pregnant, pp: post-partum.*

Nifedipine is commonly used in the therapy of preterm labour. Nifedipine has been reported to be superior to β_2-adrenergic receptor agonists and magnesium sulfate for tocolysis (34) and to be associated with less frequent side-effects than β_2-adrenergic receptor agonists (35, 36).

A number of studies have been conducted regarding the expression of Abcg2 in various tissues from different species, but according to our knowledge this is the first publication on the expression of Abc2 in the rat uterus during gestation. Low levels of Abcg2 were found 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.* (36), 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.* (36) 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 pharmaco-therapeutic 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.* (11) and Shukla *et al.* (12) 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 KO134 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 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 preterm delivery.

Conflicts of Interest

All the Authors declare that they have no conflicts of interest.

Acknowledgements

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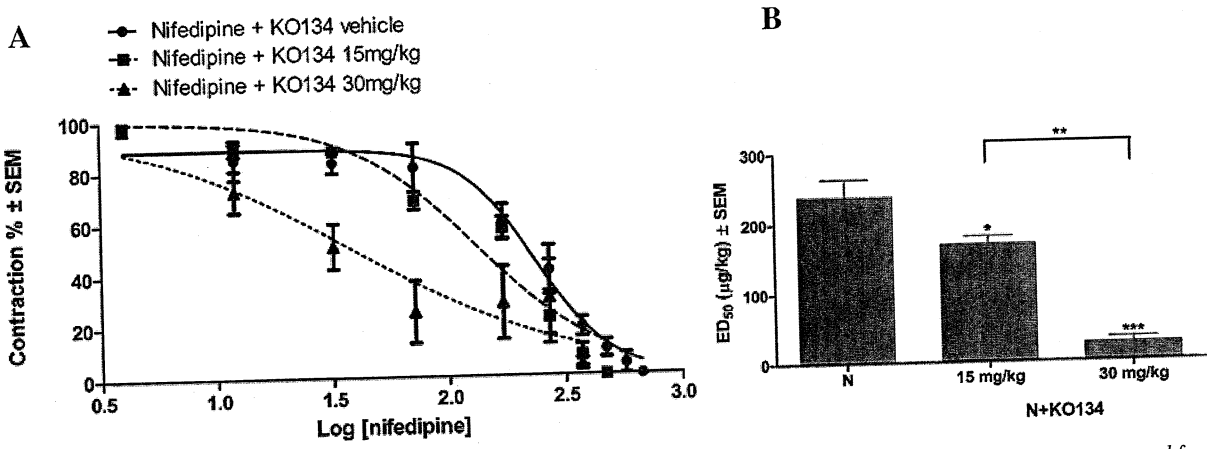

Figure 4. *A Uterus-relaxing effects of nifedipine (N) alone and in the presence of 15 mg/kg or 30 mg/kg doses of the ABC-Transporter subfamily G member 2 (Abcg2) blocker KO134 in the post-partum rat uterus in vivo. B Effective dose fifty percent (ED50) values for nifedipine alone and in combination with KO134 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.*

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