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

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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 β2-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.

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. Ca2+-channel blockers are known to abolish intracellular Ca2+ transients and myometrial contractions (2). The Ca2+-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 Ca2+-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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Key Words: Premature labour, rat uterus, nifedipine, tocolysis, Abcg2 inhibition, ontogeny of Abcg2.

0258-851X/2013 $2.00+.40
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/EEC) 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.

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

<table>
<thead>
<tr>
<th>Abcg2 substrates</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Chemotherapeutic agents</td>
<td>Mitoxantrone, topotecan, irinotecan, methotrexate, imatinib (13)</td>
</tr>
<tr>
<td>Anti-viral agents</td>
<td>Lamivudine, zidovudine, abacavir (14)</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Ciprofloxacin, ofloxacin, norfloxacin, erythromycin, rifampicin, nitrofurantoin (16)</td>
</tr>
<tr>
<td>Ca2+-channel blockers</td>
<td>Nifedipine (11, 12)</td>
</tr>
<tr>
<td>HMGCoA reductase inhibitors</td>
<td>Rosuvastatin, pitavastatin, cerivastatin (19)</td>
</tr>
<tr>
<td>Others</td>
<td>cimetidine, folic acid, dipyridamole (20)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Abcg2 inhibitors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>Apigenin, biochanin A, chrysir, genistein, kaempferol, hesperetin, naringenin, silymarin (21)</td>
</tr>
<tr>
<td>Ca2+-channel blockers</td>
<td>Nicardipine, nifedipine, nitrendipine, verapamil (22)</td>
</tr>
<tr>
<td>Oestrogens</td>
<td>Oestrone, 17β-oestradiol (23, 24)</td>
</tr>
<tr>
<td>Fumitremorgin C analogues</td>
<td>KO132, KO134, KO143, myco toxin fumitremorgin C, demethoxyfumitremorgin C (25, 26)</td>
</tr>
<tr>
<td>Others</td>
<td>Elacridar (GF120918), Tarquidur (XR9576), Novobiocin, Etposide, Cyclosporine-A, HER tyrosine kinase inhibitor (CI1033), Camptothecin analogues (GF120918) (27, 28, 29, 30)</td>
</tr>
</tbody>
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364
The effect of nifedipine was expressed as a percentage in terms of the area under the curves (AUCs) of 5-min periods were evaluated and the patterns of intrauterine pressure change are presented in Figure 1.

**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 Run01639905-m1 for Abcg2, and Run00667869-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 (Ct).

**Western blot analysis.** Thirty micrograms of tissue 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 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 means±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 (ED50) 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 Ca2+ 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.
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.
Nifedipine is commonly used in the therapy of preterm labour. Nifedipine has been reported to be superior to β₂-adrenergic receptor agonists and magnesium sulfate for tocolysis (34) and to be associated with less frequent side-effects than β₂-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 expression 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

This work was supported by the New Hungary Development Plan (TÁMOP-4.2.1/B-09/1/KONV-2010-0005).
References

1 Institute of Medicine (US) Committee on Understanding Premature Birth and Assuring Healthy Outcomes, 2007.


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 means ± S.E.M, n=8.


