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Investigation of the RhoA and Rho-kinases in the rat myometrium and cervix

Ph.D. Thesis

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Table of contents

List of abbreviations

Table of contents

List of abbreviations

BMI: body mass index

MLC: miosin- light chain

MLCK: miosin- light chain kinase

MLCP: miosin-phosphatase

mRNA: messenger RNA

PCR: polimerase chain reaction

PG: prostaglandin

PTB: Preterm birth

PTL: preterm labour

RhoA: Rho family member A

ROCKs: Rho- associated kinases

cGMP: cyclic guanosine monophoshate

PK: protein kinase

cAMP: cyclic adenosine monophosphate

NO: nitric oxide

ADRB2: β_2-adrenoceptor agonists

P: progesterone

GEF: guanine nucleotide exchange factor

GAP: GTP-ase activating ptoteins

GDI: guanine nucleotide dissociation inhibitors

PKN: Protein kinase N

p140mDia: Mammalian homolog of Drosophila diaphanous

RBD: Rho-binding domain

PH: pleckstrin-homology

AA: arachidonic acid

1. Introduction

1.1 Classification, epidemiology and cause of preterm birth

Preterm birth (PTB) is defined by WHO (1976) as the births before 37 weeks of gestation or fewer than 259 days since the first day of last menstrual period of a women. PTB is the major cause of neonatal morbidity and mortality, which has been not reduced over the past 20-30 years despite intensive antenatal and postnatal care programs, the comprehensive administration of tocolytics, and a series of preventive and therapeutic interventions. The exact causes of PTB are still unknown. Several risk factors for PTB have been reported: previous low birth weight or preterm delivery, repeated second trimester abortion, uterine and cervical abnormalities, *in vitro* fertilization, multiple pregnancy, maternal chronic medical conditions (e.g. diabetes, hypertension, anaemia, asthma, thyroid diseases), periodontal diseases, gestational bleeding, abnormal placentation, urinary tract infection, social and environmental status, smoking, alcohol consumption, maternal low body mass index (BMI) and genetic risk (Moutquin 2003, Haas 2006, Pennell et al. 2007, Blencowe et al. 2013). Bacterial vaginosis, HIV and syphilis are also associated with a higher incidence of PTB (Gravett et al. 2010). Other factors including maternal age, parity, drug abuse, heredity, sexual activities, excessive physical workload, stress, inadequate prenatal care, and maternal weight gain are still misjudged (Berkowitz et al. 1998, Muglia and Katz 2010). The mortality rate of preterm babies increases with decreasing gestational age. Preterm births account for 70% of neonatal deaths and more than 75 % of the preterm infants, who are born alive, suffer from cerebral palsy, sensory deficits, neuro-inflammation, learning disabilities and respiratory diseases in later life compared with children born at term; resulting in enormous physical, psychological and economic costs (Petrou et al. 2003 and 2005, Papatsonis 2005).

1.2 Tocolytic therapy

PTB is the central problem in obstetrics and the principal risk factor for perinatal morbidity and mortality. Several agents are used for the tocolytic therapy such as magnesium sulfate; prostaglandin inhibitors (e.g. indomethacin, ketorolac); nitrates (e.g. nitroglycerine); β-mimetics (e.g. ritodrine, terbutaline); calcium channel blockers (e.g. nifedipine); oxytocin receptor blockers (e.g. atosiban), and progesterone. but there are many controversies in establishing efficacy and safety of the treatment (Kim et al. 2006, Witcher 2002).

Furthermore, these agenst are not able to reduce the frequencies of preterm birth within 2-7 days and are not effective for the improvements of newborn mortality and morbidity.

Magnesium sulphate plays an essential role in glycolysis, oxidative phosphorylation, protein synthesis and membrane integrity (Pasternak et al. 2010). The exact mechanism of action of magnesium sulphate as a tocolytic agent is partially known and controversial. It has been proposed that magnesium is able to modulate the calcium uptake and bind to the smooth muscle cells, thereby regulates multiple intracellular pathways (Mohan et al. 2006). Crowther et al. (2014) reported that magnesium sulphate had no effect for preventing PTB. Furthermore applying of magnesium sulphate is associated with the increased risk of newborn mortality. Intravenous magnesium administration can cause peripheral vasodilation, and the maternal side effects involves in nausea, vomiting, headache and palpitations. In higher dose (over the recommended therapeutic concentration), magnesium sulphate can lead to respiratory failure, hypotension, heart block and cardiac arrest (Kumar et al. 2013).

Prostaglandins play an important role in the uterine contraction by resulting an increase of intracellular Ca level and contributing to the activation of myosin light chain kinase (Asboth et al. 1996). COX enzymes regulate the production of PG, therefore inhibiton of COX activity lead to a decreased level of PG and reduced uterine activity. Indomethacin, that ables to binding to COX reversibly, is a frequently used drug for tocolysis. Despite the favourable results, several studies have limited the use of indometacin because of the development of neonatal adverse effects including oligohydramnios, increased risk of ductus arteriosus closure, necrotizing enterocolitis and renal failure (Norton et al. 1993); and maternal adverse effects such as gastric ulcer and asthma (Caritis 2005).

Nitric oxide (NO) is a forceful vasodilatator and present in myometrial cells. NO increases cyclic guanosine monophoshate (cGMP) and protein kinase (PK) G levels, thereby promote uterine relaxation. It was reported contradictory results of effectivity of NO donors. The results of the transdermal nitroglycerin administration showed better tocolytic effects than placebo and it was found out that nitroglycerin able to delay delivery for two days (Hubinont and Debiève 2011). According to the results of five randomized controlled trials, nitroglycerin did not delay labour and not improve neonatal outcome compared to placebo or alternative tocolytic (Arrowsmith 2010).

The most commonly used drugs for the treatment of preterm labour are the betamimetics. Ritodrine and terbutaline were introduced as selective β_2-adrenoceptor (ADRB2) agonists and

ritodrine hydrochloride was approved for use in preterm labour from 1980. β_2-AR agonists able to reduce the intracellular cyclic adenosine monophosphate (cAMP) concentration and promote the myometrial relaxation based on randomised controlled trials and several subsequent meta-analyses. β_2-AR agonists were more effective than placebo for delaying preterm labour for two days, but there have no benefit for long-term treatment and perinatal mortality and morbidity rate (Anotayanonth et al. 2004). Unfortunately, it was reported significant maternal side effects associated with the administration of β_2-AR agonists is tachycardia, dyspnoea, hypokalemia, hyperglycemia, and chest pain.

Oxytocin receptor antagonists (atosiban, barusiban, epelsiban and retosiban) able to completely inhibit the uterotonic action of oxytocin in a competitive and dose-dependent manner and to downregulate oxytocin receptors and to inhibit oxytocin-mediated prostaglandin synthesis (Bernal 2007). Atosiban was the first drug which was directly developed to manage PTB and the only drug which is used in clinical practice. Three multinational, multicentre, double-blind, randomised, controlled trials was reported that atosiban had similar tocolytic effect and it was associated with fewer maternal cardiovascular side effects comparison to the conventional beta-agonist therapy (Worldwide Atosiban versus Beta-agonists Study Group 2001) even so there is still controversy about its effectiveness and long-term safety (Witcher 2002).

Supplemental treatment with progesterone (P) has been reported to prevent preterm labour (PTL) and birth (Meis et al. 2003, Fonseca et al. 2003). Progesterone is an essential steroid hormone which was secreted by the corpus luteum and by the placenta after 8 weeks of gestation. P has a physiological effect on uterine quiescence via regulation the intracellular calcium level, prostaglandin production and myometrial oxytocin receptor concentration (Renzo et al. 2005). Although progesterone treatments seem effective in patients with risk of preterm birth, collection more data in large randomised controlled trials are needed for confirmation its benefit in the prevention of preterm delivery.

Ca^{2+} channel antagonists (have been used increasingly in tocolytic therapy. Those agents that inhibit the calcium channels able to block directly the transfer of calcium ions across the cell membrane and from the sarcoplasmic reticulum. These actions lead to a decrease in intracellular free calcium and inhibition of myosin light-chain kinase–mediated phosphorylation resulting in myometrial relaxation (Simhan and Caritis 2007). Nifedipine is the most commonly used drug for inhibition of preterm labour and had a similar tocolytic

effect compared with β adrenergic receptor agonists, but no placebo-controlled trials have adressed the efficacy and safety of nifedipine for this indication.

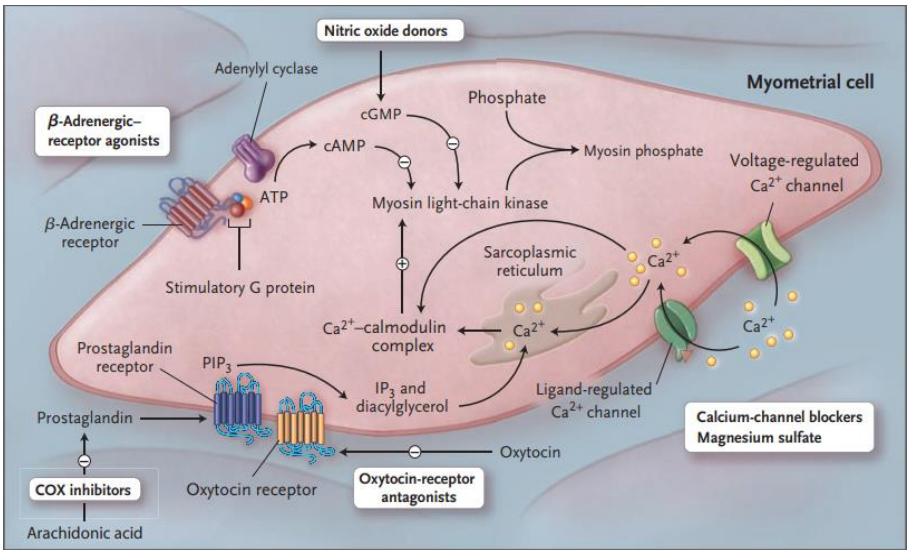


Figure 1. Sites of action of the commonly used tocolytic agents (Simhan and Caritis 2007).

It is a pharmacological challenge to find new effective therapeutic strategies, mechanisms or combinations to reducing the potentially maternal and foetal adverse effects and improving the perinatal outcome. The ideal tocolytic agent should be easy to administer, inexpensive, without significant maternal, fetal or neonatal side effects, and effective at postponing preterm birth.

1.3 RhoA and Rho-kinases, general overview

Rho is small GTP bindig protein, which is one of the major families of Ras superfamily. Rho was discovered in 1985 in *Aplysia* as a Ras homolog protein. Rho-like proteins were identified in all eukaryota species (Hall 2012). The Rho family members are molecular switches, they able to modulate the signal transduction pathways via interchange of their conformational states. The GDP-bound form of Rho is inactive and the active state is exist, when bound GTP (Ridley 2001). The Rho family can be subdivided into six families by based on the primary amino acid sequence, structural motifs and biological function: the *RhoA-related* subfamily such as RhoA, RhoB and RhoC; the *Rac1-related* subfamily including Rac1, Rac2, Rac3 and RhoG; the *Cdc42-related* subfamily with members of Cdc42, TC10, TCL, Chp/Wrch-2 and Wrch-1; the *Rnd subfamily* such as Rnd1, Rnd2, and RhoE/Rnd3; the *RhoBTB subfamily*; and the recently described *Miro subfamily* (Wennerberg and Der 2004). The RhoA-related proteins exhibit significant amino acid sequence identity (~85%), but they have any functional differences. The activation of Rho family members are regulated by

several factors and proteins. The ratio of the GDP/GTP form of RhoA is regulated by the activity of guanine nucleotide exchange factor (GEF) and the GTP-ase activating ptoteins (GAP). GEF promotes the dissociation of GDP and facilitates the GTP binding to RhoA. GAPs enhance the GTP hydrolysis rate and increase the GTPase activity of RhoA. Furthermore, an other molecules. the guanine nucleotide dissociation inhibitors (GDI) play a role in the regulation of GDP/ GTP-bound state of RhoA by inhibiting of the exchange of GTP and the hydrolysis of GTP, thereby the RhoA don't able to bind to the membranes and to activate their effectors (Wettschureck and Offermanns 2002) (**Figure 2**).

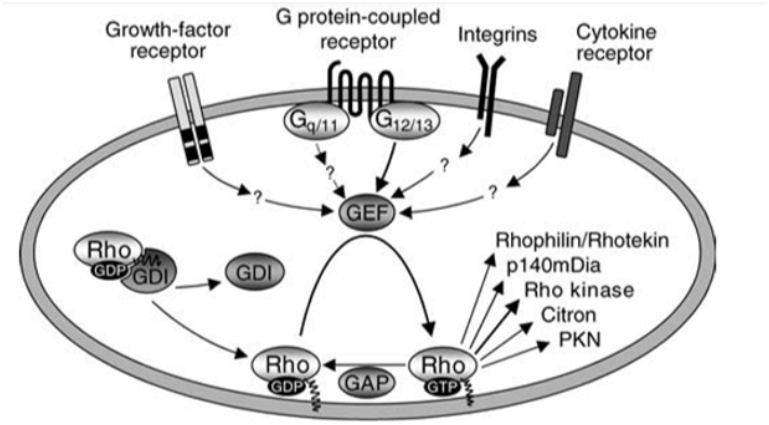


Figure 2. Regulation of Rho activity. Guanine nucleotide exchange factor (GEF) proteins catalyze the exchange of GDP for GTP on Rho GTPases resulting in their activation. GTPase-activation proteins (GAP) and GDP-dissociation inhibitors (GDI) have a negative regulatory role causing the inactivation of Rho. PKN Protein kinase N; p140mDia Mammalian homolog of Drosophila diaphanous; citron citron-kinase (Wettschureck and Offermanns 2002).

The small RhoA takes part in several cellular function including cell adhesion, cell motility, migration, and cytokinesis. Several effectors of RhoA have been discovered, but the first and the best characterized RhoA effector is the Rho-kinase (Loirand et al. 2006). Rho-kinase (ROCK) is a serine/threonine protein kinase. Two isoforms of ROCK have been identified, ROCK I or ROKβ and ROCK II or ROKα. ROCK-1 and ROCK-2 are highly homologous, the identity of amino acid sequence is 65%; the RBD is 58% and the kinase domain is 92% (Nakagawa et al. 1996). Human ROCKs genes are located on chromosome 18 (18q11.1) and chromosome 2 (2p24), respectively. The sequences of ROCKs involve in a kinase domain at the N terminal end, a Rho-binding domain (RBD) in the middle portion and a pleckstrin-homology (PH) domain with a cysteine-rich domain at the C terminal end (Amano et al. 2000). The PH domain is a split into two region by insertion of cysteine-rich region. GTP

bound Rho able to interact with the RBD domain and activates the phosphotransferase activity of Rho-kinase.

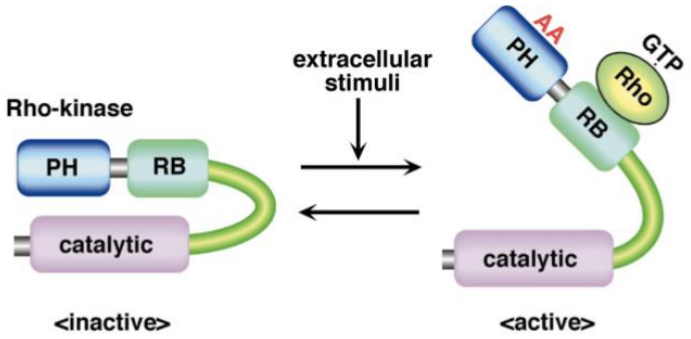


Figure 3. Model for the regulation of Rho-kinase. RB: Rho-binding domain; PH: pleckstrin-homology domain; AA: arachidonic acid (Amano et al. 2000).

The activation of Rho-kinases depend on the conformational state of RhoA. However, the stimulatory effect of activated RhoA on the activity of ROCKs is limited. Molecules such as arachidonic acid or sphingosine phosphorylcholine are able to provoke the activation of ROCK independently of RhoA.

The Rho-kinases expressed in different part of the human body. It has been reported that, ROCK II is highly expressed in the brain and slightly in the lung. ROCK I is expressed hardly in the brain, skeletal muscles, lung, kidney, pancreas and medium level in the placenta and liver (Matsui et al. 1996).

1.4 The RhoA/Rho-kinase pathway in the smooth muscle

The contraction or relaxation state of the smooth muscle depends on the interaction between actin and myosin. This process is regulated by the activity of myosin light chain kinase (MLCK) and myosin phosphatase (MLCP). MLCK is responsible for the phosphorylation of the myosin light chain (MLC), thereby MLCK takes part in the initiation of the contraction. MLCP able to dephosphorylate the MLC resulting in relaxation. One of the major mechanisms of the smooth muscle contraction is depend on the massive increase in intracellular calcium. Calcium ions bind to calmodulin and activate MLCK, which phosphorylates the 20 kDa regulatory subunit of the myosin light chain (MLC → pMLC) and contributes to activation of contraction (Webb, 2003). In addition, a second pathway has been identified (Kimura et al. 1996), which is independent of Ca^{2+} and includes the GTP binding RhoA and ROCKs (Somlyo and Somlyo 2000). The active, GTP-bound RhoA activates their

effectors leading to the phosphorylation of regulatory myosin- binding subunit of MLCP which results in the inhibition of the phosphatase activity (Taggart et al. 2012). Furthermore, the Rho-kinases phosphorylate direct the myosin light chain. These mechanisms promote the enhancement of the phosphorylated MLC and induce smooth muscle contraction (**Figure 4**.)

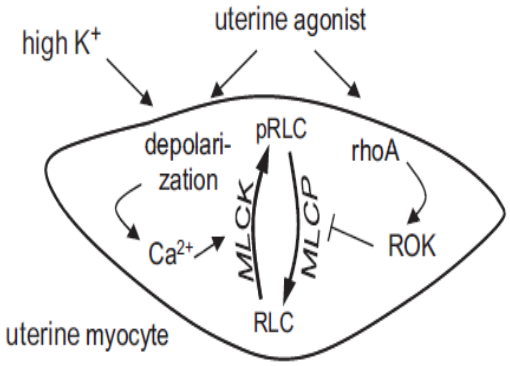


Figure 4. RhoA/Rho-kinase signal pathway in the uterine myocyte (Taggart et al. 2012)

Pathological activity of RhoA and Rho-kinase in smooth muscle leads to hypertension (Uehata et al. 1997) asthma (Chiba et al. 2010), thrombin-stimulated vascular smooth muscle migration and endothelial contraction. Therefore, the Rho/Rho-kinase mediated pathway can be a possible target for therapeutic interventions in various diseases.

RhoA/Rho-kinase pathway can be inhibited at several stages: at the receptor by antagonists to the activating agonist, by hydrolysis of bound GTP to GDP facilitated by GAPs, by complexation of free RhoA with GDI, through deactivation of Rho-kinases by inhibitors. Simvastatin, a HMG-CoA reductase inhibitor ables to block the synthesis of geranylgeranyl pyrophosphate, which is needed to posttranslational lipidation, membrane localization, and function of RhoA (Park et al. 2002).

Y-27632 as a specific inhibitor of ROCKs able to block the agonist-induced contraction of vascular and bronchial smooth muscles by inhibiting the Ca senzitization and blocks the Rho-kinase activity by competing with ATP for binding to the kinase (Ishizaki et al. 2000). The other non isoform selective Rho-kinase inhibitor is the fasudil, which is the only ROCK inhibitor that is applied in clinical practice and has been used in Japan to treat cerebral vasospasm (Asano et al. 1989). Fasudil inhibits the Rho-kinase mediated myosin light chain phosphorylation and suppresses the myosin phosphatase activity (Fukata et al. 2001). The development of fasudil began the functional research of quinoline or isoquinoline derivatives by chemical screening to measure their vasodilatory activity in normal and spastic arteries.

Several preclinical data suggest the benefits of ROCK inhibition in cardiovascular diseases, therefore many biotechnology and pharmaceutical companies are synthetized and developed ROCK inhibitors. RKI-1447 has a similar inhibitory activity to Y-27632 and fasudil. It inhibits ROCKI and ROCKII dose-dependence with IC_{50} values of 14.5 nM and 6.2 nM (Patel et al. 2012).

There are few and variant data about the expression of RhoA, ROCK I and ROCK II in pregnant uterus (Moran et al. 2002, Gao et al. 2009) and cervix, as well as the effects of RhoA and ROCK inhibitors at different stages of pregnancy.

2. Aims

- The aim of our study was to investigate the ontogeny of the mRNA and protein expression of RhoA, ROCK I, and its isoform, ROCK II in pregnant rat uteri and cervix by using real-time PCR and Western blot analysis.

- The other purpose was to evaluate the effects/roles of the RhoA and Rho-kinases (Y-) on uterine contractility and cervical resistance in isolated organ bath experiments *in vitro* by using RhoA inhibitor simvasvatin and ROCK inhibitors Y-27632, fasudil and RKI 1447.

- Our further aim was to analyse the effects/efficiancy of newly synthetized isoquinoline derivatives on the pregnant and non-pregnant uterine contractions *in vitro*, and to test their actions on ROCK activity as compared with well-known reference compounds.

3. Materials and Methods

3.1. Housing and handling of the animals

All experiments involving animal subjects were carried out with the approval of the National Scientific Ethical Committee on Animal Experimentation (permission number: IV/198/2013). The animals were treated in accordance with the European Communities Council Directives (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII). Sprague–Dawley 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

The mature female (180-200 g) and male (240-260 g) rats were mated in a special mating cage. A movable metal door separated the spaces for the female and male animals. A timer controlled the function of the engine that moved the separating door. Because rats are usually active at night, the 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 a vaginal sperm plug was visible, the female rats were separated and were regarded as first-day pregnant animals.

3.3. Tissue isolation

The animals were euthanized in a CO_2 chamber. Uterus and cervix tissues were rapidly removed from 5- , 15-, 18-, 20- and 22-day –pregnant animals, non-pregnant ones, as well as those in labour and post-partum (1, 3 and 5 days after labour). During labour the tissues were dissected always after delivery of the 3rd pup). The cervix side and the ovary side uterine rings were not collected. The remaining tissue samples 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. We also investigated the non-pregnant, day 22 pregnant and parturient endometrium and myometrium separately. The endometrium was removed and collected by gentle and careful scraping of the inner surface of the uterine horns

with the blunt edge of a scalpel. The endometrial samples were also stored at 4°C overnight in RNAlater solution (Life Technologies, Budapest, Hungary), the supernatant was then removed, and the samples were stored at -70 °C until the beginning of the investigation.

3.4. Real time quantitative polymerase change reaction study

The uterine and cervical tissues were frozen in liquid nitrogen, then these were mechanically homogenized. Total RNA was isolated from samples using TRI Reagent (Molecular Research Centre, Inc., Cincinnati, OH, USA). The quantities of RNA were assessed via the ratio of the absorbance at 260 and 280 nm; all samples exhibited ratios in the 1.6-2.0 range. The following primers were used for amplification of the PCR products: Rn04219609_m1 for RhoA, Rn00579490_m1 for Roc1, Rn00564633_m1 for Roc2 and Rn00667869_m1 for ß-actin as an endogenous control (Life Technologies, Hungary). PCR products were amplified with the SensiFAST Probe HiROX One-Step Kit (Bioline, Csertex Ltd., Hungary) and the ABI StepOne Real-Time cycler. The following conditions were used for amplification: 45°C for 10 min, 95°C for 2 min and 40 cycles of 95°C for 5 sec, 60°C for 20 sec. The fluorescence intensities of the probes were plotted against PCR cycle numbers. The amplification cycle displaying the first significant increase in the fluorescence signal was defined as the threshold cycle (C_T).

3.5. Western blot analysis

Samples were powdered with a Sartorius MikroDismembrator U (Sartorius, Göttingen, Germany) and homogenized in a RIPA Lysis Buffer combined with PMSF solution, sodium orthovanadate solution and protease inhibitor cocktail solution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). 50 µg of protein per well was subjected to electrophoresis on 4−12% NuPAGEBis-Tris Gel in XCellSureLock Mini-Cell Units (Life Technologies, Budapest, Hungary). Proteins were transferred from gels to nitrocellulose membranes using the iBlot Gel Transfer System (Life Technologies, Budapest, Hungary). Antibody binding was detected with the WesternBreeze Chromogenic Western Blot Immundetection Kit (Life Technologies, Budapest, Hungary). The incubation with RhoA, Roc1, Roc2 and β-actin polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200) were made on a shaker in the blocking buffer. The optical density of each immunoreactive band was determined with Kodak 1D Images analysis software (Carestream Health, Inc., Rochester, NY, USA). Optical densities were calculated as arbitrary units after local area background subtraction.

3.6. Rho-kinase activity assay

The Rho-kinase activity was measured by an immunoassay recommending by the manufacturer (Merck Ltd., Hungary). All compounds were added in 200 μM final concentration to the assay. Briefly, 50 μl samples containing 10 mUnit of active ROCK II with and without inhibitors were added into a 96-well plate, that was pre-coated with recombinant myosin phosphatase target subunit 1 (MYPT1). After 30 min incubation at 30 °C, the plate was washed three times with 1X Wash buffer (combination of 20X TBS, 20% Tween® and distilled water), then incubated with 100 μl/well of anti-phospho- MYPT1 antibody for 1 h at room temperature. After washing, 100 μl/well of HRP-conjugated secondary antibody was added and incubated for 1 h at room temperature. After the addition of 100 μl/well of chromogenic substrate reagent, the reaction was terminated by adding 100 μl/well of the stop solution. The absorbance was measured on a 96-well plate reader (SpectroStarNano, BMG Labtech, Germany) at 450 nm. The absorbance signal reflects the relative amount of ROCK activity. Each data point was performed in triplicate and the assay was repeated twice.

3.7. In vitro organ bath study

3.7.1. Uterus preparation

5-mm-long uterine rings were removed from non-pregnant rats in the oestrus phase (180-200 g) and from rats on pregnancy day 22, during labour and on the first day after the parturition and then mounted vertically in an organ bath. The bath contain 10 ml of de Jong solution (137mM NaCl, 3mM KCl, 1mM CaCl2, 1mM MgCl2, 12mM NaHCO3, 4mM NaH2PO4, 6mM glucose, ph 7.4) and was maintained at 37 ºC, and carbogen (95% O_2 + 5% CO_2) was bubbled through it. After being mounted, the rings were equilibrated for about 1 h with a change of solution every 15 min. The initial tension was set at 1.5 g. The tension of the myometrial rings was measured with a gauge transducer (SG-02) and recorded with a SPEL Advanced ISOSYS Data Acquisition System (MDE Ltd., Budapest, Hungary).

3.7.2. Oxytocin-induced contractions study

3.7.2.1. Contraction with presence simvastatin, fasudil, Y-27632 and RKI 1447

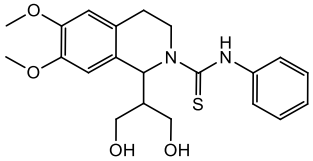
After the incubation period, uterine contractions were elicited with 10^{-8} M oxytocin. To measure the extent of fatigue, the oxytocin-induced contractions were recorded for 30 min without inhibitors in control experiments. Cumulative dose-response curves were constructed in the concentration range of 10^{-10} -10^{-5} M with the presence of Rho-kinase inhibitors RKI

1447 (Avidin Kft., Szeged, Hungary), fasudil, Y-27632 or RhoA inhibitor simvastatin (Sigma-Aldrich, Budapest, Hungary). The effects of each concentration of the inhibitors were recorded for 300 s. The areas under the curves (AUC) were evaluated and analysed, then concentration-response curves were fitted. The maximum inhibition (Imax) and the concentration to elicit 50% of the maximum inhibitions of uterine contraction (IC_{50}) were calculated with the Prism 5.0 (Graphpad Software, Inc., San Diego, CA, USA) computer programs. All the experiments were carried out on at least 6 animals, and the values are given as means ± SEM. For statistical evaluations, data were analysed by one-way ANOVA Tukey's test.

3.7.2.2. Contraction with presence of newly synthetized isoquinolines

25 isoquinoline derivatives (13 water-soluble and 12 non-water-soluble) were selected from the Departmental Compound Library of the Institute of Pharmaceutical Chemistry, University of Szeged, including some *N,N-disubstituted 6,7-dialkoxy-phenylethylamines*. The uterus preparation was carried out by the above described method.

Uteri were removed as previously described from non-pregnant, day 20 of pregnancy and during parturiton. We measured the effects of the newly synthetized isoquinoline derivatives, fasudil and Y-27632 on the uterine contractions precontracted with 10^{-8} M oxytocin. Cumulative dose-response curves were constructed in the concentration range of 10^{-10} -10^{-5}M. The effects of each concentration of the inhibitors were recorded for 300 s. The maximum inhibitory effects (Emax) of isoquinoline derivatives, fasudil and Y-27632 were calculated from the changes of the AUC values, while the half maximal inhibitory concentration (IC_{50}) values were determined as the concentration required to reach the halfway point between the top and bottom plateaus of the inhibitory curve. All these values were calculated with the Prism 5.0 (Graphpad Software, Inc., San Diego, CA, USA) computer program. For statistical evaluations, data were analyzed with unpaired t-test or ANOVA, Tukey's Multiple Comparison Test.



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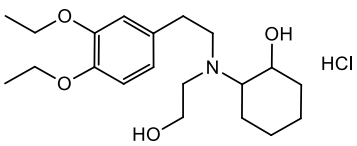

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3.7.3. Cervical resistance study

The whole cervical tissues were removed freshly from non-pregnant (oestrus phase), 20- and 22-day pregnant rats. The tissues were cut into two rings and mounted with their longitudinal axis vertically by hooks in an organ bath containing 10 ml de Jongh buffer (137mM NaCl, 3mM KCl, 1mM CaCl2, 1mM MgCl2, 12mM NaHCO3, 4mM NaH2PO4, 6mM glucose, ph 7.4). The organ bath was maintained under standard condition (37ºC with carbogen - 95% O_2 + 5% CO_2–bubbling). The rings were equilibrated for about 1 h with a change of buffer solution every 15 min. The initial tension was set at 1.0 g. RhoA inhibitor (simvastatin) or Rho-kinase inhibitors (Y-27632, fasudil) (Sigma-Aldrich, Budapest, Hungary) were added to the organ bath in a concentration of 10^{-6}M and the cervices were incubated for 5 min. Then the rings were stretched in incremental steps and allowed to relax for 5 min. After every 5 min, the next initial tension was set, in 1.0 g steps between 1.0 g and 12.0 g. The tension was increased manually via the control screw of a gauge transducer and recorded by ISOSYS software (MDE Heidelberg, Waldorf, Germany). Evaluation of the cervical resistance was carried out such that the initial tension of the cervix was represented versus the stretch after 5 min. Straight lines were fitted by linear regression and the slopes of the lines were used to express the degree of resistance. A steeper slope reflected a higher resistance (Gáspár et al., 2005; Gál et al, 2009).

All the experiments were carried out on at least 6 animals, and the values are given as means ± SEM. For statistical evaluations, data were analysed by one-way ANOVA Dunett test.

4. Results

4.1. Expression of RhoA, ROCK I and ROCK II in the rat uterus

4.1.1. Alteration of RhoA in the uterus

The mRNA and protein expression of RhoA and Rho-kinases were investigated in the non-pregnant, pregnant and postpartum rat uterus. The mRNA level of RhoA decreased in the 5th day of pregnancy and slightly increased till day 18, while a decrease was detected till day 22, however, these alterations were not significant. During labour a sharp increase was found in mRNA expression compared to day 22, then reduced on postpartum day 1 and remained unchanged on postpartum days 3, 5 and 7 (**Fig. 5A**). The protein level of RhoA did not change significantly until day 22 of pregnancy, while a marked increase was found during labour. Interestingly, the elevated RhoA protein expression were unchanged on postpartum day 1, but it was significantly reduced from postpartum day 3 (**Fig. 5B**).

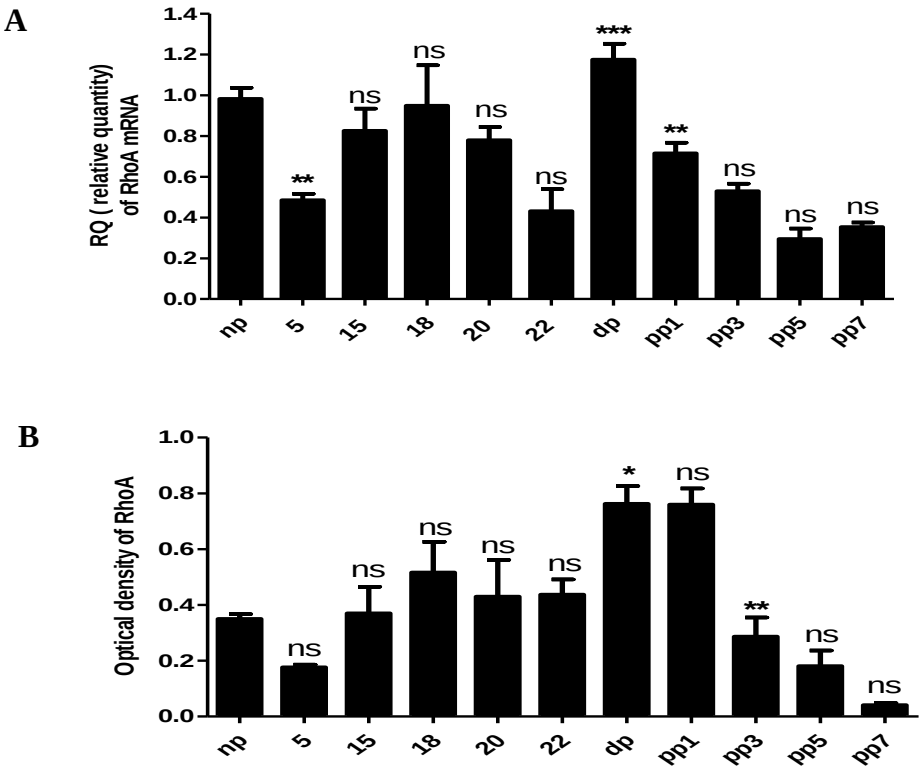

Figure 5. Changes in mRNA (**A**) and protein level (**B**) of RhoA in non-pregnant rat uterus (*np*) during pregnancy, during parturition (*dp*) and on postpartum days (*pp*1, 3, 5 and 7). The significances are given as compared with data on the previous day. Each bar indicates the mean ± SEM (standard error); n=6. ANOVA, Tukey's test.*ns:non-significant*; * $p < 0.05$; ** $p < 0.01$.

The mRNA and protein expression of RhoA were also investigated in endometrial and myometrial tissues (**Fig. 6**) separately. The mRNA expressions of RhoA did not change significantly in the endometrial samples (**Fig. 6A**), but decreased in the myometrium on 22th of pregnancy and during parturition (**Fig.6B**). Interestingly, the protein level of RhoA markedly increased in the endometrial samples during delivery (pregnancy day 22 and during parturition) (**Fig. 6C**) and remained unchanged in myometrium (Fig. **6D**).

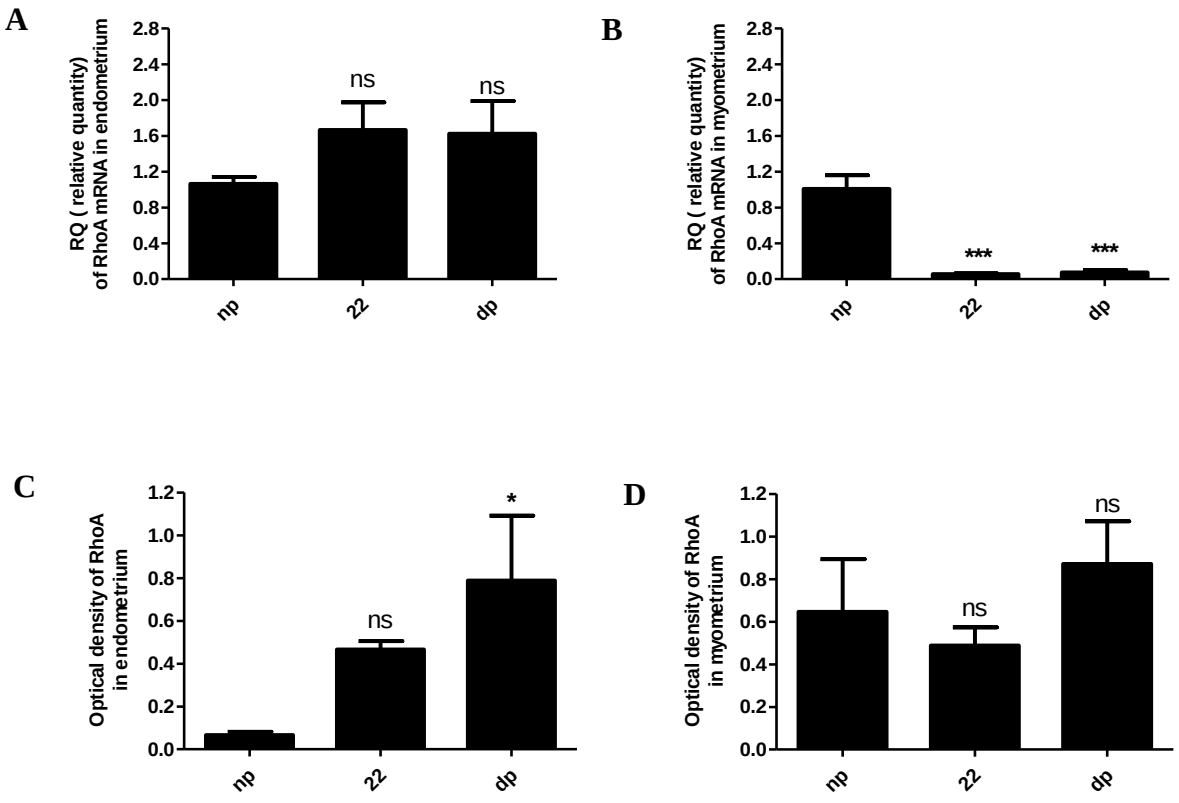

Figure 6. Changes the RhoA mRNA and protein expression in endometrium and myometrium in non-pregnant rat (*np*), day 22 of pregnancy (*22*) and during parturition (*dp*) in endometrium (**C**, **E**) and myometrium (**D**, **F**). The significances are given as compared with data of non-pregnant state. Each bar denotes mean ± SEM (standard error); n=5.ANOVA, Tukey's test. *ns: non-significant*; * $p < 0.05$; *** $p < 0.001$.

4.1.2. Alteration of ROCK I and ROCK II in the uterus

We found that the mRNA level of ROCK I was high in non-pregnant uterus that was decreased in pregnancy day 5 and remained low till parturition. However a 5-fold increase was detected during labour and that was reduced moderately on postpartum day 1 and remained unchanged on the postpartum period (**Fig. 7A**). The protein expression of ROCK I

followed a similar pattern till pregnancy day 18 to that of mRNA expression. On pregnancy day 18 the protein expression was elevated, and a further increase was detected during labour. The further alterations of ROCK I protein expression after labour were similar to those of the mRNA expression (**Fig. 7B**).

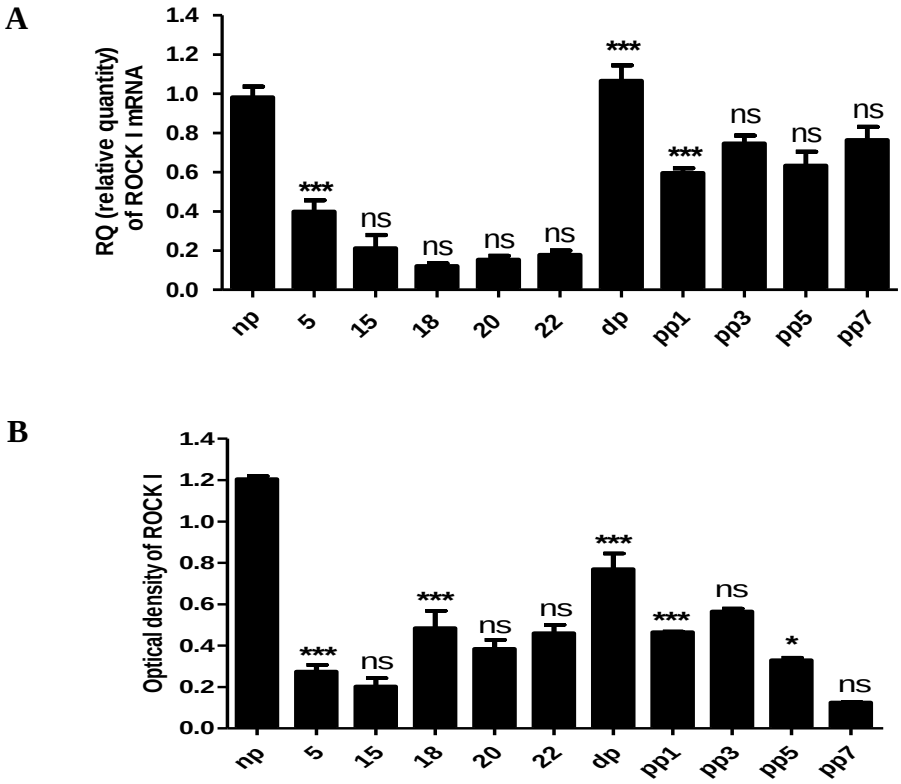

Figure 7. The mRNA (**A**) and protein expression (**B**) of ROCK I in non-pregnant rat uterus (*np*) during pregnancy, during parturition (*dp*) and on postpartum days (*pp*1, 3, 5 and 7). The significances are given as compared with data on the previous day. *ns:non-significant*; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Each bar indicates the mean ± SEM (standard error); n=6. ANOVA, Tukey's test.

In case of ROCK II, a low mRNA level was observed from non-pregnant uteri to day 22 of pregnancy and then a sharp increase was found during parturition (**Fig. 8)**. On the first day after delivery, the ROCK II mRNA expression significantly decreased then remained unchanged till postpartum day 7 (**Fig. 8A**). Interestingly, the protein expression of ROCK II was high in the non-pregnant uteri, that was significantly reduced on pregnancy days 5 and 15 (**Fig.8B**). On day 18 the protein level was increased, then a reduced protein level was measured till delivery, while during parturition the protein expression of ROCK II significantly enhanced. On postpartum days (1, 3, 5, 7) low protein levels were detected.

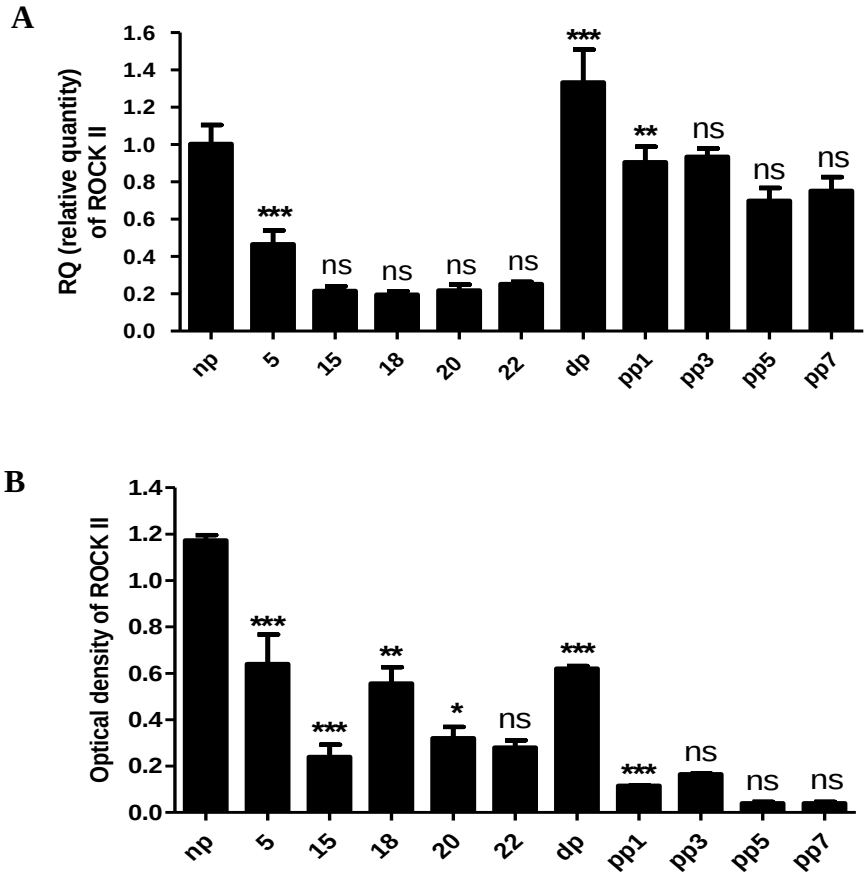

Figure 8 The mRNA (**A**) and protein level (**B** and **C**) of ROCK II in non-pregnant rat uterus (*np*) during pregnancy, during parturition (*dp*) and on postpartum days (*pp*1, 3, 5 and 7). The significances are given as compared with data on the previous day.*ns:non-significant*; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.Each bar indicates mean ± SEM (standard error); n=6. ANOVA, Tukey's test.

4.2. Effects of RhoA inhibitor simvastatin and Rho-kinase inhibitors y-27632, fasudil and RKI1447 in vitro

Simvastatin (10^{-10}-10^{-5} M) inhibited the oxytocin-induced uterine contractions in a dose-dependent manner. The weakest relaxing activity was found on postpartum day 1, while the

strongest relaxing effect was measured on day 22 of pregnancy. Simvastatin elicited a similar relaxing effect on non-pregnant uterus and during parturition, but its activity was lower as compared with day 22 of pregnant uterus (**Fig. 9**).

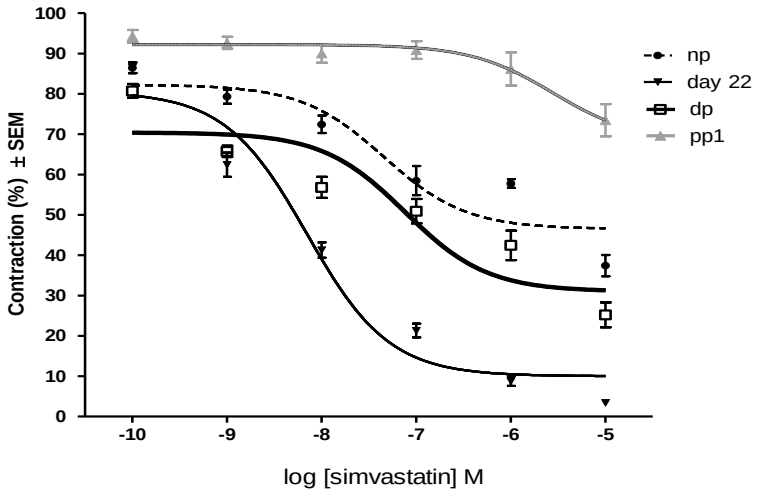

Figure 9 Inhibitory effects of simvastatin on oxytocin-stimulated contraction in myometria *in vitro* in non-pregnant (*np*) rats and day 22 of pregnancy, during parturition (*dp*) and on the first day of the post-partum period (*pp1*).

The IC_{50} value of simvastatin was the lowest on pregnancy day 22 and the highest on postpartum day 1, while these values in non-pregnant uterus and during parturition were similar (**Table 1**).

	np	day 22	dp	pp1
IC_{50} ± SEM	4.6e-008 ± 9.3e-007	7.0e-009 ± 2.4e-009^{ns}	7.6e-008 ± 1.8e-008^{ns}	2.8e-006 ± 1.4e-007*
Inhibition% ± SEM	58.3 ± 4.7	90.1 ± 1.1***	71.7 ± 2.2***	19.7 ± 5.5***

Table 1 The maximum inhibition and the IC_{50} values of simvastatin. The significances are given as compared with data on the previous day.np: non-pregnant, dp: during parturition, pp: postpartum; ns: non-significant; * $p < 0.05$; *** $p < 0.001$. Each bar denotes mean ± SEM (standard error). ANOVA, Tukey's test.

Each non-selective Rho-kinase inhibitor (fasudil, Y-27632 and RKI 1447) inhibited the oxytocin induced contractions dose-dependently. Fasudil had the strongest inhibitory effect on

non-pregnant uteri, while it elicited similar relaxation on day 22, during parturition and postpartum day 1 (**Fig. 6**).

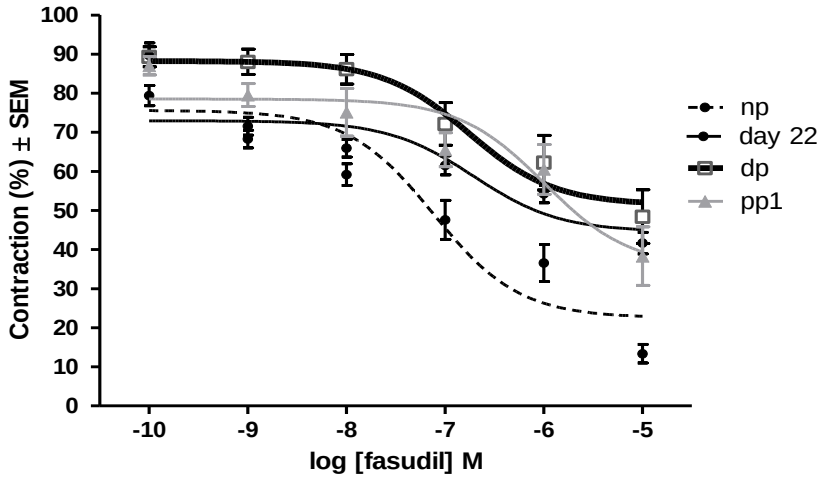

Figure 6 The inhibitory effect of non-selective ROCK inhibitor fasudil on the non-pregnant rat uteri (*np*), on pregnancy day 22, during parturition (*dp*) and on the first day of the postpartum period (*pp1*).

The IC_{50} values of fasudil were very similar on all investigated days (**Table 2**).

	np	day 22	dp	pp1
IC_{50} ± SEM	7.7e-008± 1.6e-008	2.0e-007± 4.0e-008^{ns}	1.7e-007± 2.4e-008^{ns}	1.0e-006± 1.4e-007^{ns}
Inhibition% ± SEM	80.7 ± 2.3	58.8 ± 3.4**	57.2 ± 4.9^{ns}	71.9 ± 5.2*

Table 2 The maximum inhibition and the IC_{50} values of fasudil. The significances are given as compared with data on the previous day.np: non-pregnant; dp: during parturition; pp:postpartum. ns: non-significant; * $p < 0.05$; ** $p < 0.01$. Each bar denotes mean ± SEM (standard error). ANOVA, Tukey's test.

The compounds Y-27632 (**Fig. 7**) and RKI 1447 (**Fig. 8**) showed a very similar action: they had a strong relaxing effect on non-pregnant uteri and during parturition, while their action was moderated on day 22 and postpartum day 1.

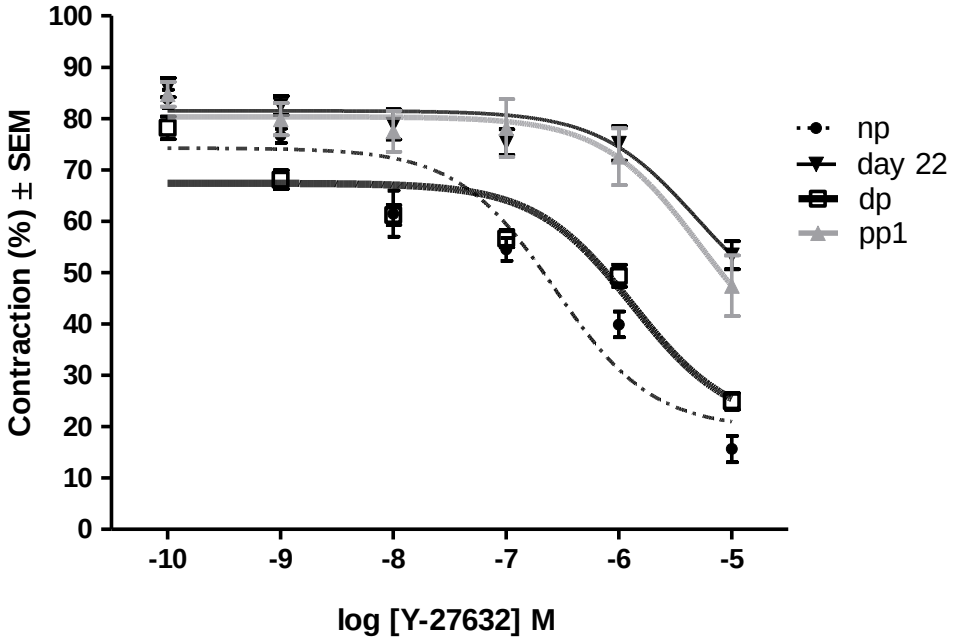

Figure 7 The inhibitory effect of non-selective ROCK inhibitor Y-27632 on the non-pregnant rat uteri (*np*), on pregnancy day 22, during parturition (*dp*) and on the first day of the postpartum period (*pp1*).

Y-27632 had the highest IC_{50} values on postpartum day 1. The lowest values of Y-27632 (**Table 3**) were measured in non-pregnant uteri.

	np	day 22	dp	pp1
IC_{50} ± SEM	2.7e-007 ± 3.6e-007	4.9e-006 ± 4.3e-007^{ns}	1.3e-006 ± 3.7e-007^{ns}	5.3e-006 ± 2.0e-007^{ns}
Inhibition% ± SEM	82.3 ± 3.5	51.9 ± 8.3**	78.8 ± 2.5**	61.7 ± 8.6^{ns}

Table 3 The maximum inhibition and the IC_{50} values of Y-27632. The significances are given as compared with data on the previous day.np: non-pregnant; dp: during parturition; pp: postpartum.ns: non-significant; ** $p < 0.01$. Each bar denotes mean ± SEM (standard error). ANOVA, Tukey's test.

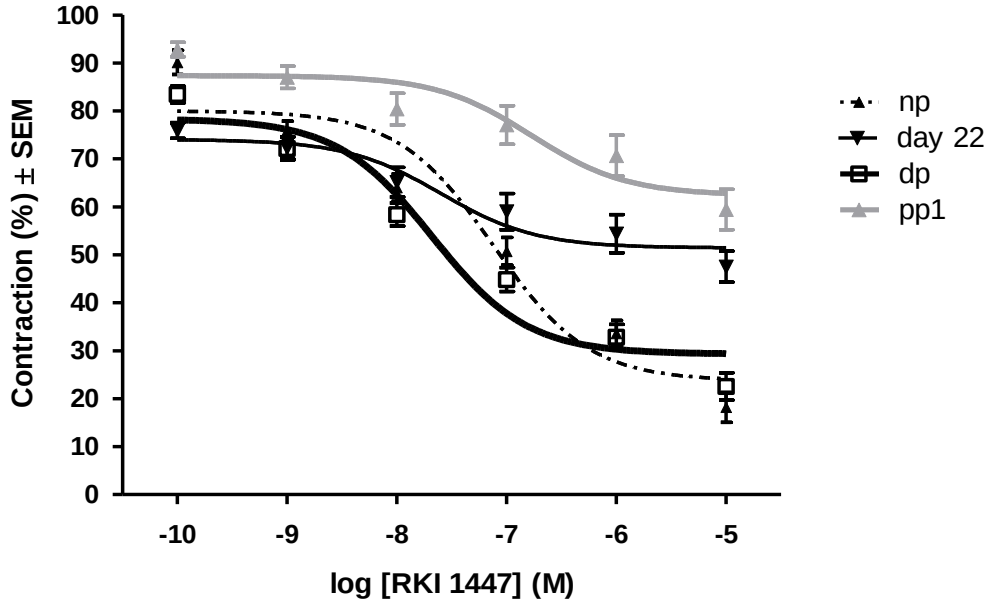

Figure 8 The inhibitory effect of non-selective ROCK inhibitor RKI-1441 on the non-pregnant rat uteri (*np*), on pregnancy day 22, during parturition (*dp*) and on the first day of the postpartum period (*pp1*).

The highest IC_{50} values of RKI 1441 were found on postpartum day 1, while lowest values were measured on day 22 and during parturition (**Table 4**).

	np	day 22	dp	pp1
IC $_{50}$ ± SEM	7.6e-008 ± 2.1e-008	2.5e-008 ± 3.0e-009^{ns}	2.1 e-008 ± 2.4e-008^{ns}	1.7e-007± 3.8e-007**
Inhibition% ± SEM	76.9 ± 2.8	51.0 ± 2.8***	70.8 ± 2.7**	40.7 ± 3.9***

Table 4 The maximum inhibition and the IC_{50} values of RKI-1447. The significances are given as compared with data on the previous day.np: non-pregnant; dp: during parturition; pp: postpartum.ns: non-significant; ** $p < 0.01$; *** $p < 0.001$. Each bar denotes mean ± SEM (standard error). ANOVA, Tukey's test.

4.3. Effects of newly synthetized isoquinoline derivatives for the uterine contraction in vitro

We have synthesized 13 water-soluble and 12 non-water-soluble isoquinoline molecules. Their efficacies were investigated on the non-pregnant rat uterine contractions. The IC_{50} values of 11 from among the 25 derivatives were significantly lower on the oxytocin-induced contraction compared with Y-27632 and fasudil (**Table 5, 6**), although their maximal inhibitory effects were weaker than those of Y-27632 and fasudil. Interestingly, the IC_{50} values of papaverine and drotaverine were less pronounced than those of Y-27632 (**Table 5)** and fasudil, but they showed similar maximal effects to the control molecules.

Table 5

	IC_{50} ± SEM	E_{max} ± SEM
Y-27632	5.05e-007 ± 1.19e-007	82.31 ± 3.49
fasudil (/)	7.81e-007 ± 2.32e-007 ^{ns}	87.37 ± 1.51 ^{ns}
192	1.89e-008 ± 4.64e-009 ****/****	49.57 ±1.46 *******/ ***
214	9.81e-008 ±2.91e-008 ***/***	51.72 ± 1.98 *******/ ***
218	**4.18e-008 ± 1.18e-008 **/****	**46.3 ± 3.42** ***/ ***
716	9.02e-008 ± 2.13e-008 ****/***	45.57 ± 3.06 *******/ ***
724	6.1e-007 ± 2.39e-007 **^{ns}** /^{ns}	58.76 ± 1.94 *******/ ***
890	3.71e-008 ± 1.52e-008 ****/***	47.95 ± 1.76 *******/ ***
1030	2.42e-008 ± 4.76e-009 ****/***	47.33 ± 2.07 *******/ ***
1370	8.24e-009 ± 1.38e-009 ****/****	43.67 ± 1.06 *******/ ***
1374	7.88 e-007 ± 1.96e-007 **^{ns}**/^{ns}	57.25 ± 4.52 *******/ ***
1386	3.22e-008 ± 1.37e-008 ****/****	54.53 ± 2.89 *******/ ***
1525	1.33e-008 ± 2.09e-009 ****/****	50.38 ± 1.63 *******/ ***
1634	1.17e-006 ± 1.93e-007 ***/**^{ns}	82.51 ± 3.44 **^{ns}**/ ^{ns}
3641	1.12e-006 ± 8.23e-007 **^{ns}**/^{ns}	59.78 ± 7.04 *******/ ***
papaverine	4.2e-007 ± 1.07e-007 **^{ns}**/^{ns}	98.52 ± 0.78 ***/** ^{ns}
drotaverine	1.94e-006 ± 4.51e-007 ****/***	88.79 ± 8.83 **^{ns}**/ ^{ns}

Table 6

	IC_{50} ± SEM	E_{max} ± SEM
y 27632	5.05e-007 ± 1.19e-007	82.31 ± 3.49
fasudil	7.81e-007 ± 2.32e-007 ^{ns}	87.37 ± 1.51$^{ns/ns}$
28	1.91e-007 ± 1.0e-007 **^{ns}**/^{ns}	52.83 ± 2.15 ***/***
787	7.23e-007 ± 1.24e-007 **^{ns}**/^{ns}	76.64 ± 5.72 ^{ns}/^{ns}
835	8.45e-008 ± 2.04e-008 ****/ **	54.46 ± 5.78 ***/ ***
842	2.63e-007 ± 1.18e-007 **^{ns}**/^{ns}	66.41 ± 6.48 ^{ns}/ **
852	**1.58e-007 ± 3.25e-008 */** *	**52.52 ± 1.89 ***/** ***
854	2.78e-007 ± 1.29e-007 **^{ns}**/^{ns}	53.31 ± 2.22 ***/ ***
858	1.19e-006 ± 1.37e-007 ****/^{ns}	83.55 ± 2.02 ^{ns}/^{ns}
1390	4.95e-007 ± 1.84e-007 **^{ns}**/^{ns}	54.53 ± 1.78 ***/ ***
1521	5.22e-007 ± 3.37e-007 **^{ns}**/^{ns}	55.1 ± 3.09 ***/ ***
1649	2.58e-007 ± 1.09e-007 **^{ns}**/^{ns}	53.2 ± 1.71 ***/ ***
2263	1.25e-006 ± 2.06e-007 *****/^{ns}	72.23 ± 2.54 ^{ns}/ ^{ns}
2295	1.08e-006 ± 5.4e-007 **^{ns}**/^{ns}	84.71 ± 7.51 ^{ns}/^{ns}

Table 5 and 6. Half maximal inhibitory concentration (IC_{50}) values and the maximal inhibitory effects (Emax) of the water and non-water soluble isoquinoline derivatives. The IC_{50} was determined as the concentration required to reach the halfway point between the top and bottom plateaus of the inhibitory curve. Significances are compared to the values of Y-27632 or fasudil (/). Statistical analysis was carried outwith ANOVA, Tukey's Multiple Comparison Test. ns: non-significant; * $p < 0.05$; ** $p < 0.01$ *** $p < 0.001$

According to the significant IC_{50} values, we measured the effects of isoquinoline molecules on ROCK II activity. We found 2 isoquinolines out of 11 compounds (218 and 852) that have ROCK II inhibiting activity similar to that of Y-27632 (**Figure 9**).

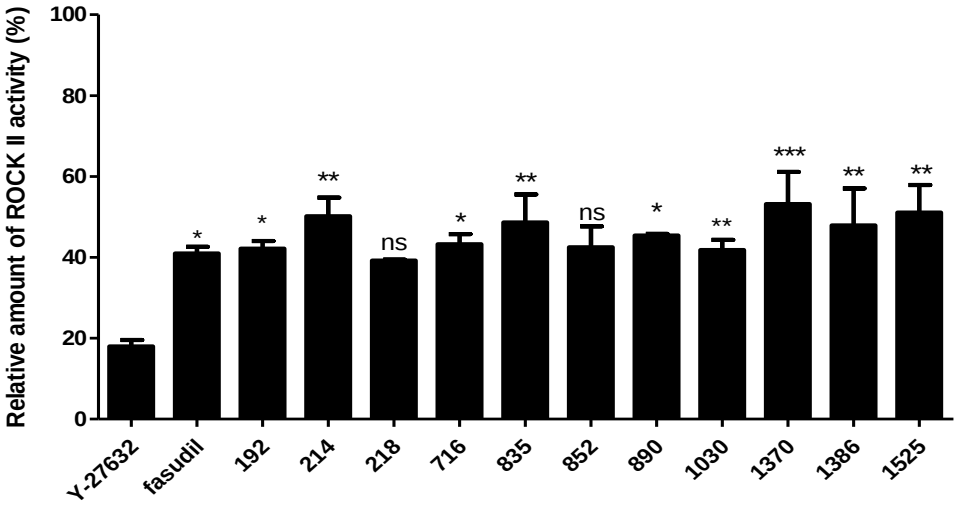

Figure 9. The inhibitory effects of the isoquinoline derivatives for ROCK II activity. The significances are given as compared with Y-27632. Each bar denotes mean ± SEM (standard error). Statistical analysis was carried out with ANOVA, Tukey's Multiple Comparison Test. ns: non-significant; * $p < 0.05$; ** $p < 0.01$ *** $p < 0.001$ as compared with the inhibitory effect of Y-27632.

We measured the relaxing effects of two isoquinoline derivatives, 218 and 852, on day 20 of the pregnant rat uterus (when the expression of ROCKs were low). We found that the inhibitory IC_{50} values of 218 (**Figure 10A**) and 852 (**Figure 10B**) were markedly lower than those of fasudil.

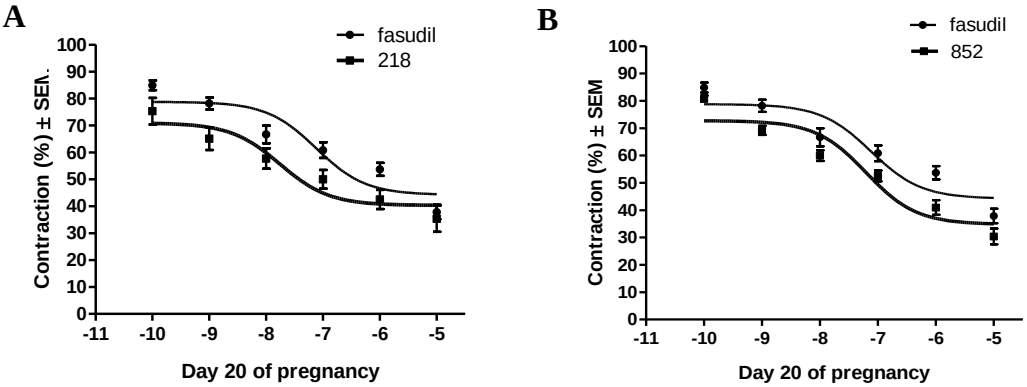

Figure 10. The inhibitory effects of 218 (**A**) and 852 (**B**) isoquinoline derivatives on the 20th-day pregnant rat uterus. Statistical analysis was carried out with ANOVA, t-test

Moreover, 218 and 852 relaxed the rat uteri during parturition (when the expressions of ROCKs were high) with greater potency as compared with fasudil (**Figure 11A and B**).

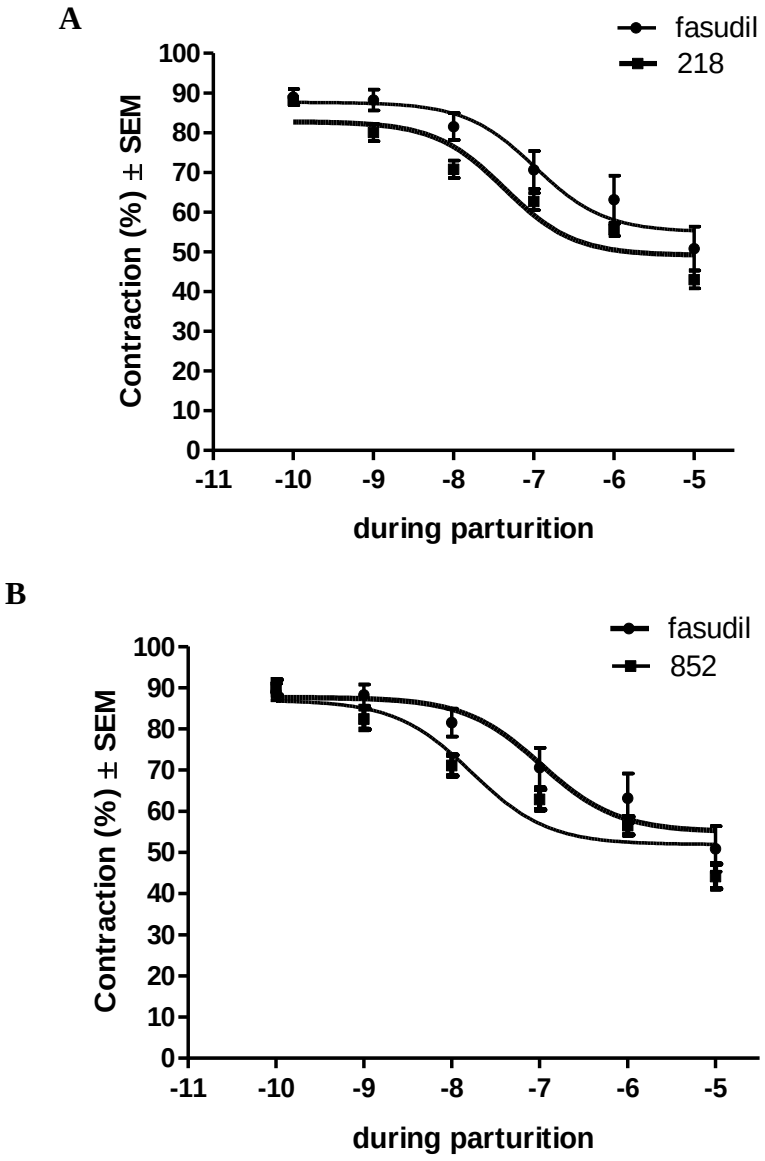

Figure 11. The inhibitory effects of 218 (**A**) and 852 (**B**) isoquinoline derivatives during parturition in rat uterus. Statistical analysis was carried out with ANOVA, t-test.

4.4. Expression of RhoA, ROCK I and ROCK II in rat cervix

4.4.1. The mRNA and protein expression of RhoA in cervix

The mRNA levels of RhoA did not change till postpartum day 1. However, an increased level was detected on postpartum day 3 as compared with the non-pregnant cervix and they were high on postpartum day 5 (**Figure 12A**). The protein levels of RhoA were unchanged till pregnancy day 18. On pregnancy day 20 and during parturition, a significant decrease was found as compared with the non-pregnant level. In the postpartum period, the protein expression of RhoA slighly increased compared with the parturient cervix (**Figure 12B**).

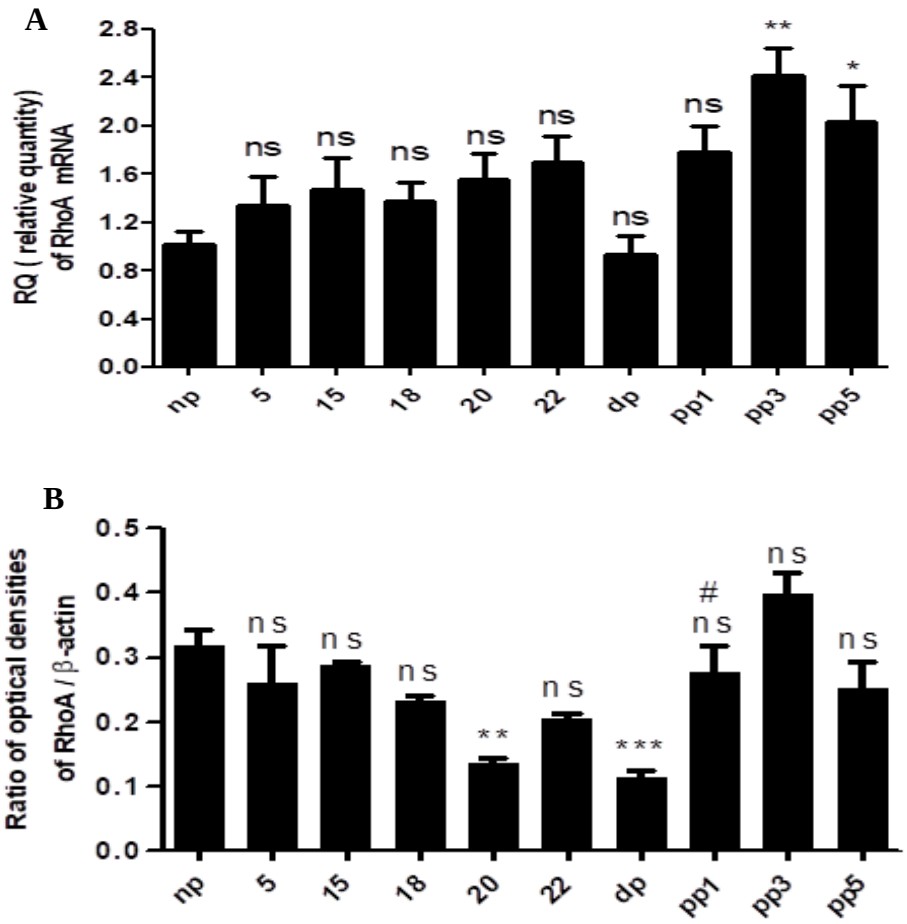

Figure 12. The mRNA (**A**) and protein expression (**B**) of RhoA in non-pregnant rat cervix (*np*), on different days of pregnancy, during parturition (*dp*) and on postpartum days (*pp1*, *pp3*, *pp5*). The level of significance is given in two ways. Asterisks represent comparisons to data from non-pregnant cervix. ns: non-significant; *: $p<0.05$; **: $p< 0.01$; ***: $p< 0.001$. Hashtags represent comparisons to the previous investigated days. #: $p<0.05$; non-significant changes are not indicated. Each bar denotes mean ± S.E.M. (standard error); n = 5. ANOVA, Tukey's test.

4.4.2. The mRNA and protein expression of ROCK I and ROCK II in the cervix

No change was found in mRNA expression of ROCK I till pregnancy day 20. On pregnancy day 22, a sharp increase was measured that was decreased during labour compared with day 22. Interestingly, on postpartum day 1, the mRNA level was raised again and remained high till post-partum day 5 (**Fig. 13A**). The protein level of ROCK I reduced on pregnancy day 15 as compared with day 5, while a further decrease was measured on day 18 compared with the non-pregnant cervix. A marked increase was detected on pregnancy day 22 compared with day 20 that was markedly decreased in parturient cervical tissue. On postpartum day 1 the protein level raised as compared with the level during parturition. No further change was detected till postpartum day 5 (**Fig. 13B**).

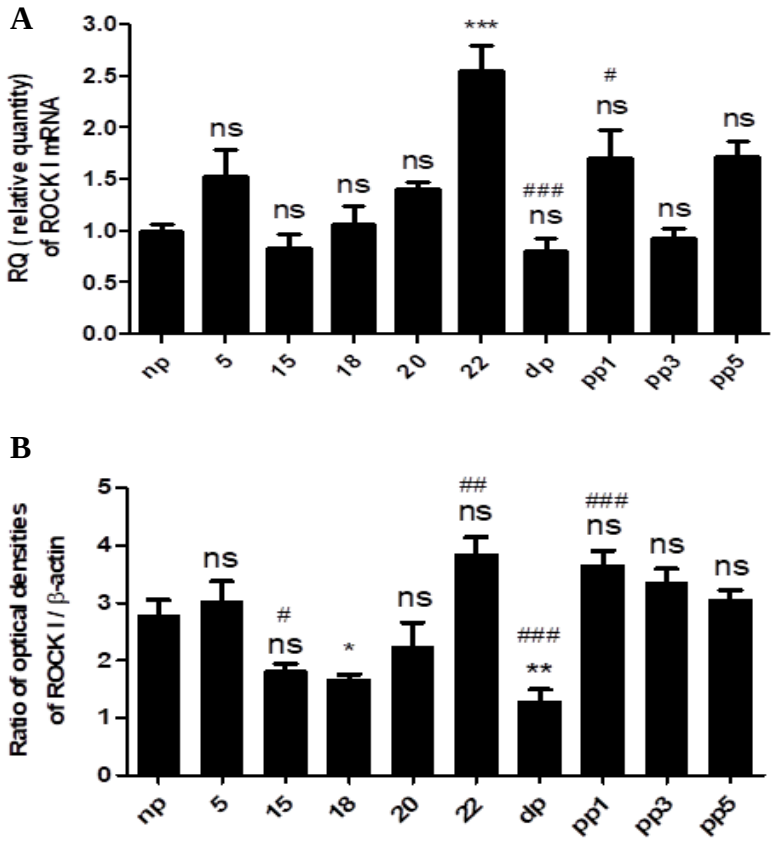

Figure 13. The mRNA (A) and protein expression (B) of ROCK I in non-pregnant rat cervix (*np*), on different days of pregnancy, during parturition (*dp*) and on postpartum days (*pp1, pp3, pp5*). The level of significance is given in two ways. Asterisks represent comparisons to data from non-pregnant cervix. ns: non-significant; *: p<0.05; **: p< 0.01; ***: p< 0.001. Hashtags represent comparisons to the previous investigated days. #: p<0.05; ##: p< 0.01; ###: p< 0.001; non-significant changes are not indicated. Each bar denotes mean ± S.E.M. (standard error); n = 5. ANOVA, Tukey's test.

The mRNA level of ROCK II did not change significantly from non-pregnant cervix until pregnancy day 22, then a sharp decrease was detected during parturition. On postpartum day 1, thecexpression was markedly increased to the level of non-pregnant and 22-day pregnant cervices (**Fig. 14A**). The alteration of the protein expression of ROCK II followed the same pattern of mRNA alterations (**Fig. 14B**).

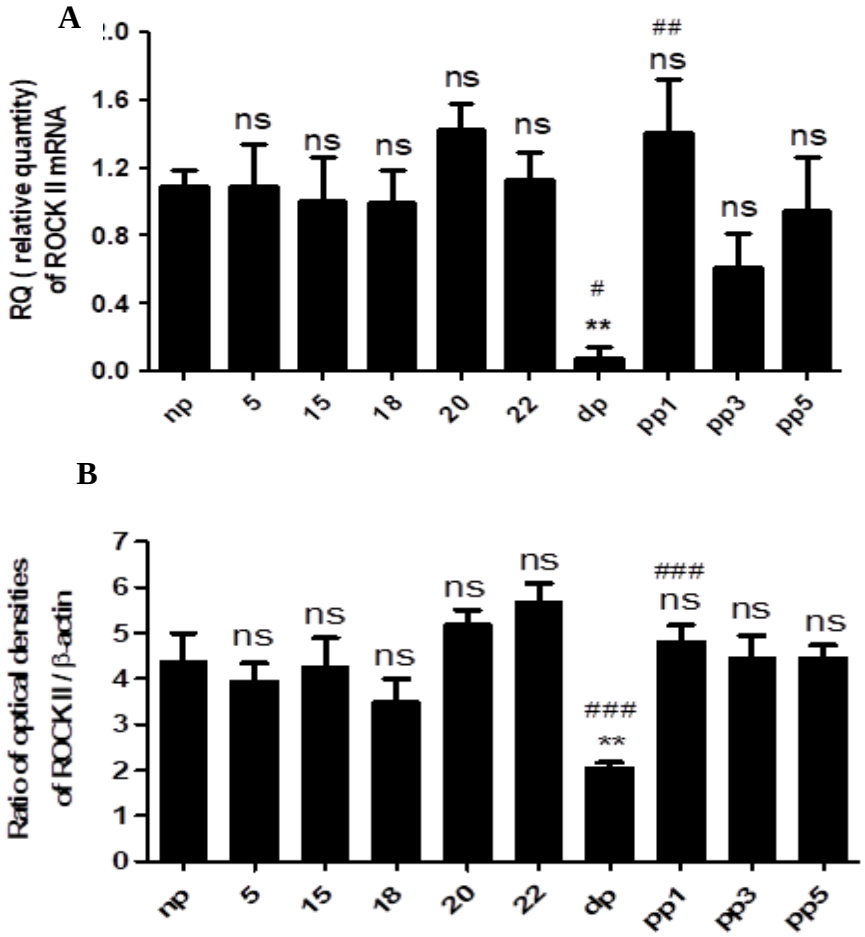

Figure 14. The mRNA expression (A) and protein expression (B) of ROCK II in non-pregnant rat cervix (np), on different days of pregnancy, during parturition (dp) and on postpartum days (pp1, pp3, pp5). The level of significance is given in two ways. Asterisks represent comparisons to data from non-pregnant cervix. ns: non-significant; **: $p < 0.01$. Hashtags represent comparisons to the previous investigated days. #: $p<0.05$; ##: $p < 0.01$; ###: $p < 0.001$; non-significant changes are not indicated. Each bar denotes mean ± S.E.M. (standard error); n = 5. ANOVA, Tukey's test.

4.5. Effects of RhoA inhibitor simvastatin and Rho-kinase inhibitors Y-27632 and fasudil

The cervical resistances were continuously decreased towards the end of the gestational period. Neither the RhoA inhibitor simvastatin nor ROCK inhibitors (Y-27632 and fasudil) significantly altered the resistance of non-pregnant cervices at a concentration of 10^{-6}M. However, simvastatin enhanced the cervical resistance in days 20 and 22 of pregnancy compared with the non-treated values (**Fig.15**).

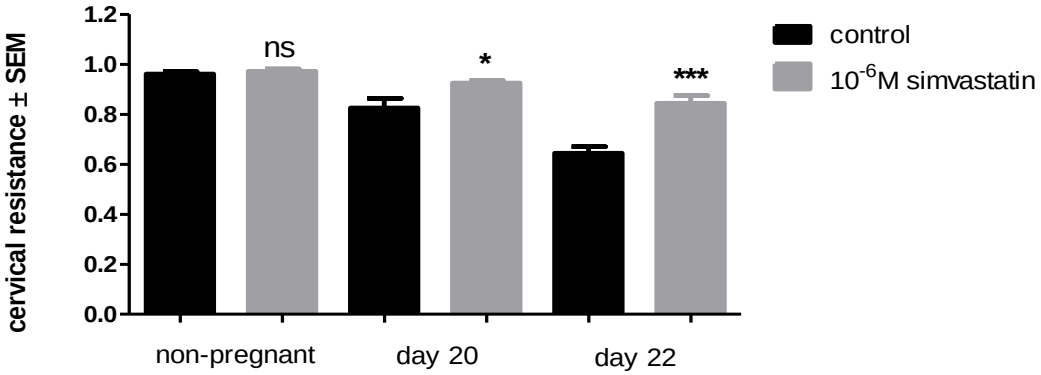

Figure 15 Effects of simvastatin on cervical resistance in the non-pregnant, 20- and 22-day-pregnant cervices. Evaluation of the cervical resistance was carried out such that the initial tension of the cervix was represented versus the stretch after 5 min. Straight lines were fitted by linear regression and the slopes of the lines were used to express the degree of resistance. The significances are given as compared with non-treated (control data). ns: non-significant; *p< 0.05;***: p < 0.001. Each bar denotes mean ±S.E.M. (standard error). ANOVA, Tukey's test.

The Rho-kinase inhibitorY-27632 decreased the resistance of the 20-day pregnant cervix, but did not alter the non-pregnant and 22-day pregnant cervical resistances as compared with the non-treated samples (**Fig. 16**).

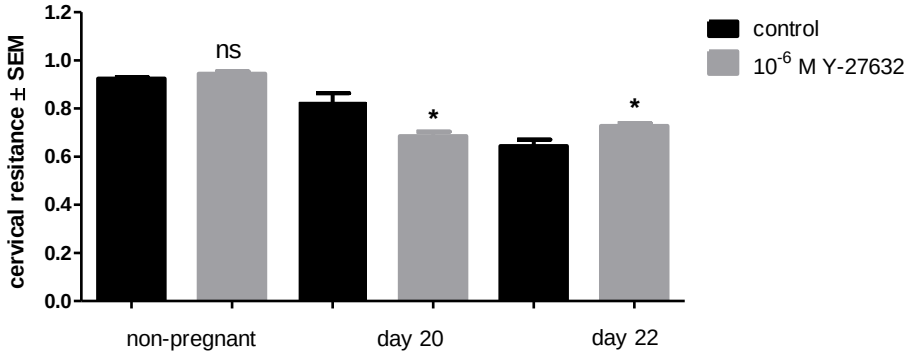

Figure 16. Effects of Y-27632 on the resistances of the non-pregnant, 20- and 22-day pregnant cervices. Evaluation of the cervical resistance was carried out such that the initial tension of the cervix was represented versus the stretch after 5 min. Straight lines were fitted by linear regression and the slopes of the lines were used to express the degree of resistance. The significances are given as compared with non-treated (control) data ns: non-significant; *p < 0.05. Each bar denotes mean ±S.E.M. (standard error). ANOVA, Tukey's test.

Fasudil did not alter the non-pregnant resistance, but reduced significantly the cervical resistances in days 20 and 22 of pregnancy (**Fig. 17**).

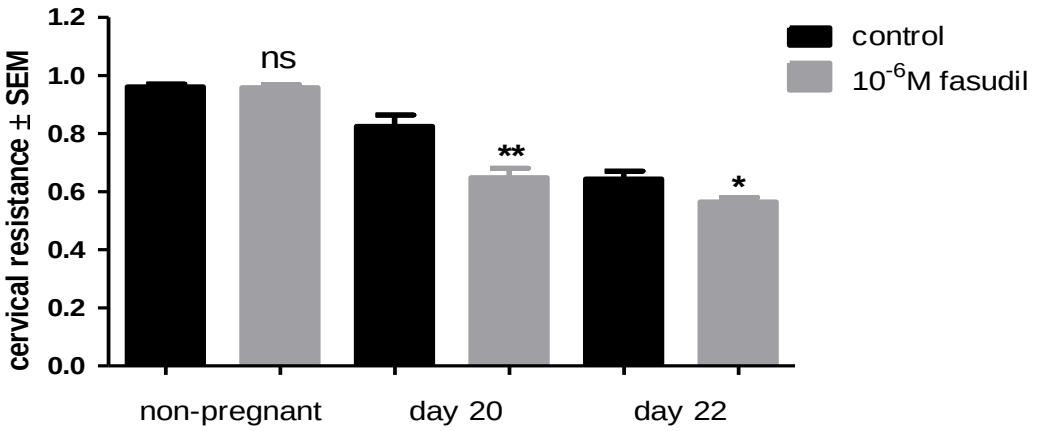

Figure 17. Effects of fasudil on the resistances of the non-pregnant, 20- and 22-day pregnant cervices. Evaluation of the cervical resistance was carried out such that the initial tension of the cervix was represented versus the stretch after 5 min. Straight lines were fitted by linear regression and the slopes of the lines were used to express the degree of resistance. The significances are given as compared with non-treated (control) data. ns: non-significant; *p< 0.05; **p< 0.01. Each bar denotes mean ±S.E.M. (standard error). ANOVA, Tukey's test.

5. Discussion

The normal conformational and physiological changes of the uterus and cervix are necessary to be maintained the quiescent state throughout pregnancy as well as the initiation of powerful rhythmic contractions at term. The precise trigger mechanism of pregnant uterine contractions and cervix dilatation are yet unknown. An understanding of these processes, at cellular and molecular levels, is essential to identify new possible drug targets to develop potent and efficacous tocolytic agents.

Several studies have investigated the contribution of the monomer GTP-binding RhoA and the Rho-kinases in cardiovascular diseases (Loirand et al., 2006), in the invasion of cancer cells and progression (Kamai et al., 2004; Lin et al., 2007; Yoshioka et al., 1999), and in uterine contraction. There are little and inconsistent reports outlining the role of RhoA and Rho- kinases in uterine contractility (Niiro et al., 1997; Tahara et al., 2002; Taggart et al.,2012) and no information about their role in regulation of cervix maturation or dilatation during pregnancy.

Therefore, we examined the expressions of RhoA and ROCKs and we have found that the mRNA level of RhoA and ROCKs decreased in the uterus on day 5 of pregnancy; however, the protein expression of RhoA was unchanged on this day as compared with the non-pregnant level. It is well known that the implantation period in rats is between pregnancy days 4-7 (Bullock and Bunton, 2000). Fanchin et al. (2001) determined that the human uterine contraction is decreased at the time of blastocyst transfers. Furthermore, Shiokawa et al. (2000 and 2002) found that the human decidual cells express RhoA and the migration of cytotrophoblast cells has been regulated by RhoA/ROCK signalling pathway. Furthermore, Rho GTPases contribute to the regulation of the invasion of the embryo into the endometrial stroma (Grewal et al., 2008) via modulating actin, myosin, and microtubule dynamics (Etienne-Manneville and Hall 2002). The decreased expressions of ROCKs in the rat uterus might reduce the intensity of contraction, thereby facilitating the implantation of the embryos. The mRNA and protein expressions of RhoA and ROCKs were not always in parallel between pregnancy days 5-15, suggesting that the efficacy of translation from mRNA to protein is fluctuating. The protein expression of RhoA was unchanged from pregnancy day 5 till day 22, while the ROCK proteins showed some significant alterations suggesting that, the active RhoA can contribute to cellular events without activation of Rho-kinases. The lower

expression of ROCKs may contribute to the maintenance of relative quiescence in the pregnant uterus. We detected that the mRNA and protein levels of RhoA and Rho-kinases were markedly up-regulated in the rat uterus at the onset of labour proposing their contribution to enhanced contractility and the initiation of labour. The higher expressions of RhoA and ROCKs in non-pregnant (oestrus) and delivering uteri can be related to an oestrogen plasma peak in rats. The oestradiol secretion are high in oestrus phase in the rat, while the level markedly decreases at night of 5^{th} day of pregnancy and reaches the peak on the day of parturition (Yoshinaga et al., 1969; Downing et al., 1981). The RhoA expressed in both endometrial and myometrial tissues. Furthermore, high protein expression was measured in the endometrial samples on pregnancy day 22 and during parturition comparing to the non-pregnant samples but remained unchanged in the myometrium suggesting that the alterations in RhoA expression are mainly the consequences of endometrial processes at the end of pregnancy.

The cervix is a dense fibrous organ which acts as a mechanical barrier to keep the fetus inside the uterus during pregnancy. At the end of gestational period the cervical tissue is softensed and dilated (Chwalisz and Garfield, 1997) to facilitate the delivery. This process is called maturation, and regulated by several factors including the hormonal changes (Stjernholm et al., 1996) and prostaglandin amounts (Hertelendy and Zakar, 2004), the degradation of collagen network by the activation of matrix metalloproteinases (Ludmir and Sehdev, 2000). We measured that the mRNA level of RhoA slightly increased during pregnancy in rat cervix that followed a decrease at term. Interestingly, the protein expression of RhoA was significantly reduced on pregnancy day 20 and during delivery, but this alteration was not completely reflected the mRNA expression. In case of ROCK I and ROCK II, the expression levels sharply declined at term suggesting their impact on the final cervical ripening and delivery process. The decreased cervical resistance correlates to the low level of RhoA, which is concordant with the result that RhoA inactivation prevents collagen-I synthesis (Kondrikov et al., 2011) and contributes to the smooth muscle relaxation. Within 1 day after delivery, the RhoA and ROCK protein expressions had recovered to the level of non-pregnant level suggesting that the higher amount of RhoA/ROCK proteins may be important for the reconstruction of cervical tissue. Additionally, the RhoA-ROCK system is involved in apoptotic processes in several organs (e.g. kidney, liver, heart) (Wang et al., 2018; Ding et al, 2016; Niermann et al., 2016). The apoptotic process contributes to cervical ripening at the end

of pregnancy (Hassan et al., 2006), thus this is another reason to investigate the RhoA-ROCK system in cervical ripening.

For functional investigation of the roles of RhoA and ROCKs, we tested the effects of the RhoA inhibitor simvastatin and Rho-kinase inhibitors Y-27632 and fasudil on uterine contractility and cervical resistance. The hydroxy-methyl-glutaryl- coenzyme A (HMG-CoA) inhibitors (statins) were developed to inhibit the cholesterol synthesis (Tobert, 2003), but they have cholesterol-independent „pleiotropic" effects. Statins like simvastatin can inhibit the synthesis of isoprenoids, which are necessary for membrane translocation and activation of small GTPases RhoA (Wang et al., 2008; Tatsuta et al., 2003). It was previously reported that the inhibitor of RhoA caused relaxation in the anal sphincter (Patel and Rattan, 2007) and decreased the vascular contractility via increasing endothelial nitrogen monoxide production in rabbit artery (Shiga et al., 2005). Our *in vitro* organ bath study focused on the late-term (during parturition) and early postpartum (postpartum day 1) uteri in comparison with the non-pregnant uterus only, because our previous results showed that the most characteristic drug actions can be determined after day 18 of the gestation period (Domokos et al., 2017; Gál et al, 2009).

We found that simvastatin relaxed the rat uterine contraction, although its action was not consequent on the expression of RhoA protein. The RhoA level was equivalently high during parturition and postpartum day 1, but the relaxing action of simvastatin both in maximum and IC_{50} value was the slowest on postpartum day 1 and much stronger during parturition. The RhoA expression level was low on day 22, while the simvastatin inhibitory action was the largest on that day. Interestingly, the intensity of the oxytocin-induced uterine contractions during parturition and on the first day of postpartum period was different, which might contribute to the different action of simvastatin. Furthermore, the relaxing action of simvastatin partially depends on its RhoA inhibitory property. It can be supported that the unprenylated Rho is able to interact with their effectors, but this interaction is less potent than with prenylated Rho (Turner et al., 2008).

The cervical resistance was increased by simvastatin on pregnancy days 20 and 22, when the protein expression was low. The enhanced cervical resistance can be resulted by the pleiotropic effect of statins that is associated with the inhibition of the secretion of matrix metalloproteinases (MMPs) (Wong et al., 2001). MMPs play a major role in collagen network degradation (Luan et al., 2003) and collagen synthesis (Spinale, 2002; Strauss et al., 1996).

Although simvastatin was reported to decrease MMP-9 activity in human endothelial cells, this action was detected after 12 h of preincubation with the compound, only (Izidoro-Toledo et al., 2011). Ispanovic et al. (2008) reported that RhoA suppresses MMP-2 and MT1-MMP expression and MMP-2 activation. These suggest that simvastatin may have an RhoA independent effect in cervical tissue that leads to the increase in cervical resistance and seems unique in the cervix, because simvastatin acts rather as a relaxing than a contracting agent in vascular (Chen et al., 2016) and – as we proved – in myometrial smooth muscles.

We also examined the effects of ROCK inhibitors on the uterine contractility. We have used non-selective ROCK inhibitors, fasudil, Y-27632, and RKI 1447. Y-27632 and fasudil are potent non-selective ROCKs inhibitors, they act as competitors against ATP to bind to the catalytic sites in enzymes (Asano et al., 1989; Ishizaki et al., 2000) resulting in smooth muscle relaxation. We combined the mRNA or protein expression data of ROCK I and ROCK II in order to provide information about the whole expressions of ROCKs to compare the actions of inhibitors to their target proteins. Fasudil had a strong inhibitory effect on the non-pregnant uterus, while it elicited moderate relaxation on 22 day pregnant, parturient and postpartum uterus. However, the IC_{50} values of fasudil were similar on each day and its action did not follow the expression levels of ROCKs. It can be explained that the effect of fasudil is not strictly attached to ROCK activity, it has non-specific inhibitory effects on other serine/threonine kinases such as MSK1, PRK2 (Davies et al., 2000). The actions of more specific ROCKs inhibitors Y- 27632 and RKI 1447 reflected the alteration of ROCKs expression. They had large relaxing effect on non-pregnant uteri and during parturition, when the expression levels of ROCKs were the highest. The maximum inhibitory effects of Y-27632 and RKI 1447 were weaker on pregnancy day 22 and postpartum day 1, when the ROCKs levels were also low. Interestingly, their IC_{50} values were not followed the alteration of target proteins expressions suggesting that both of ROCK I and II expressions together can contribute to the regulation of uterine contractility and correlate with the inhibitory effects of specific ROCK blockers.

Y-27632 and fasudil decreased the resistance of 20-day pregnant cervices but had no significant effects for the resistance of 22-day pregnant samples. Fukushima et al. (2005) reported that fasudil suppresses the collagen secretion and increased the collagenase activity in hepatic stellate cells suggesting its contribution to the cervical action showes relaxant effect on vascular and myometrial smooth muscle (Shimomura et al., 2004). Li et al. (2016) found that Y-27632 inhibits the voltage dependent potassium (Kv) channels and reduces the muscle

tone in vascular smooth muscle. This ability might be associated with the cervical resistance decreasing effect of the drug, because Kv channel expressions have been confirmed in smooth muscle part of cervix (de Lera Ruiz and Kraus, 2015). But it was proved that Y-27632 has a collagen synthesis inhibitory effect (Ding et al., 2012), and its action can be detected after 30 min of incubation (Yang et al., 2014). Its quick effect may play an important role in the alteration of cervical resistance found in our in vitro organ bath examinations after pre-treatments with ROCK inhibitors. We hypothesized that ROCK inhibitors reduce the collagen concentration quickly during pregnancy, but they have no action on parturient cervical resistance because cervix loses its collagen content till labour. According of our results we suppose that the RhoA/ROCK signalling pathway may contribute to the cervical ripening, mainly in the final processes leading to delivery, and their sharp increase in post-partum period reveal their role in cervical remodelling process.

We analysed the influences of some newly synthesized isoquinolines on the non-pregnant rat uterine contraction related to the ROCK activity as compared with action of fasudil and Y-27632. It was known that isoquinolines relaxed the smooth muscles e.g. drotaverine hydrochloride reduces the duration of labour by affecting cervical dilation (Sharma et al., 2001), while papaverine decreases the tonus and the contractile activity of smooth muscles (Huddart and Saad, 1980). Our results showes that 11 isoquinoline molecules had a lower IC_{50} values on the oxytocin-induced uterine contraction in the non-pregnant rat, but their maximal inhibitory effects were weaker than those of both reference molecules. Papaverine had a stronger maximum inhibitory effect than Y-27632, while drotaverine had higher IC_{50} values than our reference compounds on non-pregnant uterine contractions. Papaverine and drotaverine have a non-selective phosphodiesterase inhibitory effect and are able to block the calcium channels, therefore their observed relaxing effects on uterine contraction may be less associated with the ROCK inhibitory properties. After the testing of the efficiency of the isoquinoline molecules for ROCK II activity, we found that the inhibitory actions of two molecules (218 and 852) were similar to that of Y-27632. We also tested their effect on day 20 of pregnancy and during parturition and we found equal relaxing effect to the reference molecule, which is a significant development in comparison with non-pregnant results.

6. Summary

Preterm birth is the major cause of neonatal morbidity and mortality. Despite the recent developments in perinatology and prenatal care, the mechanisms of preterm uterine contractility are still unknown It is a pharmacological challenge to find new effective therapeutic mechanisms, targets to reducing the preterm delivery rate and improving the perinatal outcome.

Based on our results, we can conclude that:

1. Normal pregnancy suppresses ROCKs in uterine tissue till parturition to maintain relaxed state of the uteri.
2. The higher levels of ROCK in cervical tissues till delivery may contribute to closed position of cervix.
3. A sharp increase of ROCKs in the uterus and the marked decrease in the cervix at the onset of labour may be a key element of enhanced contractility and the initiation of delivery with dilation of cervix.
   3.1. Y27632 had a large relaxing effect on non-pregnant uteri and during parturition, when the expression levels of ROCKs were the highest.
   3.2. Simvastatin enhanced the cervical resistance, therefore compounds with simvastatin-like action might be new drug candidates for preterm cervical ripening.
4. The RhoA/Rho-kinase signal pathway can be a new target for the development of new tocolytic agenst, but the high specificity of RhoA and Rho-kinase inhibitors seems to be essential for the high efficacy of normal uterine and cervical function. The isoquinoline structure can be promising for the development of new and effective inhibitors of ROCKs.

7. References

1. Amano, M., Fukata, Y. and Kaibuchi, K. (2000). Regulation and functions of Rho-associated kinase. *Experimental cell research*, *261*(1), 44-51
2. Anotayanonth, S., Subhedar, N. V., Neilson, J. P. and Harigopal, S. (2004). Betamimetics for inhibiting preterm labour. *Cochrane Database of Systematic Reviews*, (4).
3. Arrowsmith, S., Kendrick, A. and Wray, S. (2010). Drugs acting on the pregnant uterus. *Obstetrics, Gynaecology & Reproductive Medicine*, *20*(8), 241-247.
4. Asano T., Suzuki T., Tsuchiya M., Satoh S., Ikegaki I., Shibuya M., Suzuki Y. and Hidaka H. (1989) Vasodilator actions of HA1077 in vitro and in vivo putatively mediated by the inhibition of protein kinase. *Br J Pharmacol*. 98(4), 1091-1100.
5. Asboth, G., Phaneuf, S. Y. L. V. A. I. N., Europe-Finner, G. N., Toth, M., and Bernal, A. L. (1996). Prostaglandin E2 activates phospholipase C and elevates intracellular calcium in cultured myometrial cells: involvement of EP1 and EP3 receptor subtypes. *Endocrinology*, *137*(6), 2572-2579.
6. Bernal A. L. (2007). The regulation of uterine relaxation. In *Seminars in cell & developmental biology* 18(3) 340-347, Academic Press.
7. Berkowitz G. S., Blackmore-Prince C., Lapinski R. H. and Savitz D. A. (1998) Risk factors for preterm birth subtypes. *Epidemiol* 9(3) 279– 285.
8. Blencowe H., Cousens S., Chou D., Oestergaard M., Say L.,Lawn, J. et al. (2013). Born too soon: the global epidemiology of 15 million preterm births. *Reproductive health*, *10*(1), S2.
9. Bullock G. and Bunton T. E. (2000) The Laboratory Rat. .*Academic Press*. London, UK.
10. Caritis S. (2005) Adverse effects of tocolytic therapy. *British Journal of Obstetrics and Gynaecology*, 112(1) 74– 78.
11. Chen Y., Zhang H., Liu H., Cao A. (2016) Mechanisms of simvastatin-induced vasodilatation of rat superior mesenteric arteries. Biomed Rep. 5(4), 491-496
12. Chiba, Y., Matsusue, K., & Misawa, M. (2010). RhoA, a possible target for treatment of airway hyperresponsiveness in bronchial asthma. *Journal of pharmacological sciences*, *114*(3), 239-247.

13. Chwalisz K. and Garfield R. E. (1997). Regulation of the Uterus and Cervix during Pregnancy and Labor Role of Progesterone and Nitric Oxide. *Annals of the New York Academy of Sciences*, *828*(1), 238-253.
14. Crowther C. A., Brown J., McKinlay C. J. and Middleton, P. (2014). Magnesium sulphate for preventing preterm birth in threatened preterm labour. *The Cochrane Library*.
15. da Fonseca E. B., Bittar R. E., Carvalho M. H. (2003) Prophyalactic administration of progesterone by vaginal suppository to reduce the incidence of spontaneous preterm birth in women at increased risk: a randomized placebo-controlled, double-blind study. *Am J Obstet Gynecol* 188:419-424.
16. Davies S. P., Reddy H., Caivano M., Cohen P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochemical Journal*. 351(1):95-105
17. de Lera R. M., Kraus R. L. (2015) Voltage-gated sodium channels: Structure, function, pharmacology, and clinical indications. J Med Chem. 58(18), 7093-7118.
18. Ding W. Y., Ti Y., Wang J., Wang Z. H., Xie G.L., Shang Y.Y et al. (2012) Prostaglandin F2α facilitates collagen synthesis in cardiac fibroblasts via an F-prostanoid receptor/protein kinase C/Rho kinase pathway independent of transforming growth factor β1. *Int J Biochem Cell Biol.* 44(6), 1031.
19. Ding R., Han J., Zhao D., Hu Z., Ma X. (2016) Pretreatment with Rho-kinase inhibitor ameliorates lethal endotoxemia-induced liver injury by improving mitochondrial function. *Int Immunopharmacol*. 40: 125-130.
20. Domokos D., Ducza E., Falkay G., Gaspar R. (2017) Alteration in expressions of RhoA and Rho-kinases during pregnancy in rats: their roles in uterine contractions and onset of labour. *J Physiol Pharmacol.* 68(3), 439-451.
21. Downing S. J., Porter D. G., Redstone C. D. (1981) Myometrial activity in rats during the oestrous cycle and pseudopregnancy: interaction of oestradiol and progesterone. *The Journal of physiology*. 317:425-433
22. Etienne-Manneville S. and Hall A. (2002). Rho GTPases in cell biology. *Nature*, *420*(6916), 629.
23. Fanchin R., Ayoubi J. M., Righini C., Olivennes F., Schönauer L. M., Frydman R. (2001) Uterine contractility decreases at the time of blastocyst transfers. *Human Reproduction*. 16(6),1115-1119.

24. Fukata, Y., Kaibuchi, K., & Amano, M. (2001). Rho–Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends in pharmacological sciences*, *22*(1), 32-39.
25. Fukushima M., Nakamuta M., Kohjima M., Kotoh K., Enjoji M., Kobayashi N., Nawata H., (2005) Fasudil hydrochloride hydrate, a Rho-kinase (ROCK) inhibitor, suppresses collagen production and enhances collagenase activity in hepatic stellate cells. *Liver Int*. 25(4), 829-838
26. Gál A., Ducza E., Minorics R., Klukovits A., Gálik M., Falkay G., Gaspar R. (2009) The roles of alpha2-adrenoceptor subtypes in the control of cervical resistance in the late-pregnant rat. *Eur J Pharmacol*. 615(1-3), 193-200.
27. Gao, J., & Ding, Y. L. (2009). Correlation between expression of RhoA and Rho kinase in uterine smooth muscle and the onset of labor. *Zhonghua fu chan ke za zhi*, *44*(5), 345-349.
28. Gaspar R., Kolarovszki-Sipiczki Z., Ducza E., Páldy E., Benyhe S., Borsodi A., Falkay G. (2005) Terbutaline increases the cervical resistance of the pregnant rat in vitro. *Naunyn Schmiedebergs Arch Pharmacol*. 371(1), 61-71.
29. Gravett M. G., Rubens C. E., Nunes T. M. (2010) Global report on preterm birth and stillbirth (2 of 7): discovery science. *BMC Pregnancy Childbirth*. 2-10(1).
30. Grewal S., Carver J. G., Ridley A. J. and Mardon H. J. (2008). Implantation of the human embryo requires Rac1-dependent endometrial stromal cell migration. *Proceedings of the National Academy of Sciences*.
31. Haas D. M. (2006) Preterm birth in clinical evidence. London: BMJ Publishing Group
32. Hall A. (2012) Rho family GTPases. *Biochemical Society Transaction*, 40(6)1378-1382.
33. Hassan S. S., Romero R., Haddad R., Hendler I., Khalek N., Tromp G., Diamond M. P., Sorokin Y., Malone J. Jr. (2006) The transcriptome of the uterine cervix before and after spontaneous term parturition. *Am J Obstet Gynecol*. 195(3), 778-86.
34. Hertelendy F. and Zakár T. (2004). Prostaglandins and the myometrium and cervix. *Prostaglandins, leukotrienes and essential fatty acids*, 70(2), 207-222
35. Hubinont C. and Debiève F. (2011). Prevention of preterm labour: 2011 update on tocolysis. *Journal of pregnancy*. Article ID 941057
36. Huddart H. and Saad K. H. (1980). Papaverine-induced inhibition of electrical and mechanical activity and calcium movements of rat ileal smooth muscle. *Journal of Experimental Biology*, *86*(1), 99-114.

37. Ishizaki T., Uehata M., Tamechika I., Keel J., Nonomura K., Maekawa M., Narumiya S., (2000) Pharmacological properties of Y-27632, a specific inhibitor of rho-associated kinases. *Mol Pharmacol*. 57(5), 976-983
38. Ispanovic, E., Serio, D. and Haas, T. L. (2008). Cdc42 and RhoA have opposing roles in regulating membrane type 1-matrix metalloproteinase localization and matrix metalloproteinase-2 activation. *American Journal of Physiology-Cell Physiology*, 295(3), C600-C610.
39. Izidoro-Toledo T.C., Guimaraes D.A., Belo V.A., Gerlach R.F., Tanus-Santos J.E. (2011) Effects of statins on matrix metalloproteinases and their endogenous inhibitors in human endothelial cells. *Naunyn Schmiedebergs Arch Pharmacol*. 383(6), 547-554
40. Kamai T., Yamanishi T., Shirataki H., Takagi K., Asami H., Ito Y. and Yoshida K. I. (2004). Overexpression of RhoA, Rac1, and Cdc42 GTPases is associated with progression in testicular cancer. *Clinical cancer research*, 10(14), 4799-4805.
41. Kim A. and Shim J. Y. (2006) Emerging tocolytics for maintenance therapy of preterm labour: oxytocin antagonists and calcium channels blockers. *Br J Pharmacol* 113 (s3), 113-115.
42. Kimura K., Ito M., Amano M., et al. (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase).*Science.* 273(5272):245-248.
43. Kondrikov D., Caldwell R. B., Dong Z. and Su Y. (2011). Reactive oxygen species-dependent RhoA activation mediates collagen synthesis in hyperoxic lung fibrosis. *Free Radical Biology and Medicine*, 50(11), 1689-1698.
44. Kumar K., Al Arebi A. and Singh, I. (2013). Accidental intravenous infusion of a large dose of magnesium sulphate during labor: A case report. *Journal of anaesthesiology, clinical pharmacology*, 29(3), 377.
45. Li H., Shin S. E., Kim H. W., Kim H. S., Jung W. K., Ha K. S., Han E. T., Hong S. H., Choi I. W., Bae Y. M., Firth A. L., Bang H., Park W. S. (2016) Y-27632, a Rho-associated protein kinase inhibitor, inhibits voltage-dependent K+ channels in rabbit coronary arterial smooth muscle cells. *Pharmacology*. 98(5-6), 220-227.
46. Lin M. T., Lin B. R., Chang C. C., Chu C. Y., Su H. J., Chen S. T. et al. (2007). IL-6 induces AGS gastric cancer cell invasion via activation of the c-Src/RhoA/ROCK signaling pathway. *International journal of cancer*, *120*(12), 2600-2608.
47. Loirand G., Guérin P. and Pacaud P. (2006). Rho kinases in cardiovascular physiology and pathophysiology. *Circulation research*, *98*(3), 322-334.

48. Luan Z., Chase A. J., Newby A. C. (2003) Statins inhibit secretion of metalloproteinases-1,-2,-3, and-9 from vascular smooth muscle cells and macrophages. *Arterioscler Thromb Vasc Biol.* 23(5), 769-775.
49. Ludmir J. and Sehdev H. M. (2000). Anatomy and physiology of the uterine cervix. *Clinical obstetrics and gynecology*, *43*(3), 433-439.
50. Matsui T., Amano M., Yamamoto T., Chihara K., Nakafuku M., Ito M. et al. (1996). Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *The EMBO journal*, 15(9), 2208-2216.
51. Meis P. J., Klebanoff M., Thorm E. (2003) Prevention of recurrent preterm delivery by 17-alpha hydroxyprogesterone caproate. *N Engl J Med*, 349 1299.
52. Mohan A. R.and Phillip R. B. (2006) Drugs acting on the pregnant uterus. *Current Obstetrics and Gynaecology*, 16(3), 174-180.
53. Moran, C. J., Friel, A. M., Smith, T. J., Cairns, M., & Morrison, J. J. (2002). Expression and modulation of Rho kinase in human pregnant myometrium. *Molecular human reproduction*, *8*(2), 196-200.
54. Moutquin J. M. (2003) Classification and heterogeneity of preterm birth. *BJOG* ,110 20 30-33.
55. Muglia L. J. and Katz M. (2010) The enigma of spontaneous preterm birth. *N Engl J Med*., 362: 529-535.
56. Nakagawa O, Fujisawa K, Ishizaki T, Saito Y, Nakao K, Narumiya S. (1996) ROCK-I and ROCK-II,two isoforms of Rho-associated coiled-coilforming protein serine/threonine kinase in mice. *FEBS Lett*er, 392:189–193.
57. Niermann C., Gorressen S., Klier M., Gowert N.S., Billuart P., Kelm M., Merx M. W., Elvers M. (2016) Oligophrenin1 protects mice against myocardial ischemia and reperfusion injury by modulating inflammation and myocardial apoptosis. *Cell Signal*. 28(8), 967-978.
58. Niiro N., Nishimura J., Sakihara C., Nakano H., Kanaide H. (1997) Up-regulation of rho A and rho-kinase mRNAs in the rat myometrium during pregnancy. *Biochemical and biophysical research communications*, 230(2):356-359.
59. Norton M. E., Merrill J., Cooper B. A., Kuller J. A., Clyman R. I. (1993) Neonatal complications after the administration of indomethacin for preterm labour. *N Engl J Med*, 329:1602-7.
60. Papatsonis D. N. M. (2005) International Congress Series, 1279: 251–270.

61. Pasternak K., Kocot J. and Horecka A. (2010) Biochemistry of magnesium. *Journal of Elementology* ,15(3), 601-616.
62. Park, H. J., Kong, D., Iruela-Arispe, L., Begley, U., Tang, D., & Galper, J. B. (2002). 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors interfere with angiogenesis by inhibiting the geranylgeranylation of RhoA. *Circulation research*, *91*(2), 143-150.
63. Patel C. A. and Rattan S. (2007) RhoA prenylation inhibitor produces relaxation of tonic smooth muscle of internal anal sphincter. *Journal of Pharmacology and Experimental Therapeutics*, 321(2):501-508.
64. Patel, R. A., Forinash, K. D., Pireddu, R., Sun, Y., Sun, N., Martin, M. P., ... & Sebti, S. M. (2012). RKI-1447 is a potent inhibitor of the Rho-associated ROCK kinases with anti-invasive and anti-tumor activities in breast cancer. *Cancer research*, canres-0954.
65. Pennell C. E., Jacobsson B., Williams S. M., Buus R. M., Muglia L. J., Dolan S. M., Morken N. H., Ozcelik H., Lye S. J. and PREBIC Genetics Working Group. (2007) Genetic epidemiologic studies of preterm birth: guidelines for research. *Am J Obstet Gynecol*, 196: 107-18.
66. Petrou S., Mehta Z., Hockley C., Cook-Mozaffari P., Henderson J., Goldacre M. (2003) The impact of preterm birth on hospital inpatient admissions and costs during the first 5 years of life. *Pediatrics*, 112: 1290-7.
67. Petrou S. (2005) The economic consequences of preterm birth during the first 10 years of life. *BJOG*, 112(1), 10-5.
68. Renzo G. C., Rosati A., Mattei A., Gojnic M. and Gerli S. (2005). The changing role of progesterone in preterm labour. *BJOG: An International Journal of Obstetrics and Gynaecology*, 112(s1), 57-60.
69. Ridley A. J. (2001). Rho family proteins: coordinating cell responses. *Trends in cell biology*, 11(12), 471-477.
70. Sharma J. B., Pundir P., Kumar A. and Murthy N. S. (2001). Drotaverine hydrochloride vs. valethamate bromide in acceleration of labor. *International Journal of Gynecology and Obstetrics*, 74(3), 255-260.
71. Shimomura E., Shiraishi M., Iwanaga T., Seto M., Sasaki Y., Ikeda M., Ito K. (2004) Inhibition of protein kinase C-mediated contraction by Rho kinase inhibitor fasudil in rabbit aorta. *Naunyn Schmiedebergs Arch Pharmacol*. 370(5), 414-422.

72. Shiga N., Hirano K., Hirano M., Nishimura J., Nawata H. and Kanaide H. (2005). Long-term inhibition of RhoA attenuates vascular contractility by enhancing endothelial NO production in an intact rabbit mesenteric artery. *Circulation research*, 96(9), 1014-1021.
73. Shiokawa S., Sakai K., Akimoto Y., Suzuki N., Hanashi H., Nagamatsu S. et al. (2000) Function of the small guanosine triphosphate-binding protein RhoA in the process of implantation. *The Journal of Clinical Endocrinology and Metabolism*, 85(12), 4742-4749.
74. Shiokawa S., Iwashita M., Akimoto I., et al. (2002) Small guanosine triphospatase RhoA and Rho-associated kinase as regulators of trophoblast migration. *The Journal of Clinical Endocrinology and Metabolism,* 87(12):5808-5816.
75. Simhan H. N. and Caritis S. N. (2007). Prevention of preterm delivery. *New England Journal of Medicine*, 357(5), 477-487.
76. Somlyo A. P. and Somlyo A. V. (2000) Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *The Journal of Physiology,* 522(2):177-185.
77. Spinale F. G. (2002) Matrix metalloproteinases: regulation and dysregulation in the failing heart. *Circ Res*, 90(5), 520-530
78. Stjernholm Y., Sahlin L., Åkerbergb S., Elinder A., Eriksson H. A., Malmström A. and Ekman G. (1996). Cervical ripening in humans: potential roles of estrogen, progesterone, and insulin-like growth factor-I. *American journal of obstetrics and gynecology*, 174(3), 1065-1071.
79. Strauss B. H., Robinson R., Batchelor W. B., Chisholm R. J., Rav, G., Natarajan M. K. et al (1996) In vivo collagen turnover following experimental balloon angioplasty injury and the role of matrix metalloproteinases. *Circ Res*, 79(3), 541-550.
80. Taggart M. J., Arthur P., Zielnik B., Mitchell B. F. (2012) Molecular pathways regulating contractility in rat uterus through late gestation and parturition. *American journal of obstetrics and gynecology*, 207(1), 76-e15.
81. Tahara M., Morishige K. I., Sawada K., et al. (2002) RhoA/Rho-kinase cascade is involved in oxytocin-induced rat uterine contraction. *Endocrinology*, 143(3):920-929.
82. Tatsuta M., Matsumoto Y., Nakamura H., Inoue M. (2003) Inhibition of lysophosphatidic acidinducedRhoA activation and tumor cell invasion by 3-hydroxy-

3-methylglutaryl-coenzyme A reductase inhibitors. *International journal of oncology*, 23:1173-1178.

83. Tobert, J. A. (2003) Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors. *Nature Reviews Drug Discovery*, 2(7):517-526.
84. Turner S. J., Zhuang S., Zhang T., Boss G. R., Pilz R. B. (2008) Effects of lovastatin on Rho isoform expression, activity, and association with guanine nucleotide dissociation inhibitors. *Biochemical pharmacology*, 75(2):405-413.
85. Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., ... & Narumiya, S. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature*, *389*(6654), 990.
86. Wang C. Y., Liu P. Y., Liao J. K. (2008) Pleiotropic effects of statin therapy: molecular mechanisms and clinical results. *Trends in molecular medicine*, 14(1):37-44.
87. Wang Y., Zhang H., Yang Z., Miao D., Zhang D. (2018) Rho kinase inhibitor, fasudil, attenuates contrast-induced acute kidney injury. *Basic Clin Pharmacol Toxicol*, 122(2), 278-287.
88. Webb R. C. (2003) Smooth muscle contraction and relaxation.*Advances in physiology education*, 27(4):201-206.
89. Wennerberg K. and Der C. J. (2004). Rho-family GTPases: it's not only Rac and Rho (and I like it). *Journal of cell science*, 117(8), 1301-1312.
90. Wettschureck N. and Offermanns S. (2002). Rho/Rho-kinase mediated signaling in physiology and pathophysiology. *Journal of molecular medicine*, 80(10), 629-638.
91. WHO: WHO: recommended definitions, terminology and format for statistical tables related to the perinatal period and use of a new certificate for cause of perinatal deaths. Modifications recommended by FIGO as amended October 14, 1976. *Acta Obstet Gynecol Scand*. 1977, 56: 247-253.
92. Witcher P. S. (2002). Treatment of preterm labor. *The Journal of perinatal & neonatal nursing*, 16(1), 25-46.
93. Wong B., Lumma W. C., Smith A. M., Sisko J. T., Wright S. D. and Cai, T. Q. (2001). Statins suppress THP-1 cell migration and secretion of matrix metalloproteinase 9 by inhibiting geranylgeranylation. *Journal of leukocyte biology*, 69(6), 959-962.
94. Worldwide Atosiban versus Beta-agonists Study Group Participants are listed in. (2001). Effectiveness and safety of the oxytocin antagonist atosiban versus beta-

adrenergic agonists in the treatment of preterm labour. *BJOG: An International Journal of Obstetrics and Gynaecology*, *108*(2), 133-142.

95. Yang R., Chang L., Liu S., Jin X., Li Y. (2014) High glucose induces Rho/ROCK-dependent visfatin and type I procollagen expression in rat primary cardiac fibroblasts. *Mol Med Rep*. 10(4),1992-1998.
96. Yoshinaga K., Hawkins R. A. and Stocker J. F. (1969). Estrogen secretion by the rat ovary in vivo during the estrous cycle and pregnancy. *Endocrinology*, 85(1), 103-112.
97. Yoshioka K., Nakamori S. and Itoh K. (1999). Overexpression of small GTP-binding protein RhoA promotes invasion of tumor cells. *Cancer research*, 59(8), 2004-201

8. Appendix

8.1. List of publication

8.1.1. Publications related to the Ph.D. thesis

Domokos Dóra, Ducza Eszter, Falkay György. Ras homologous A / Rho-associated coiled-coil containing protein kinase rendszer a terhes patkány uterusban: új target potenciális tocolyticumok fejlesztésére. Magyar Nőorvosok Lapja, 2015; 78:(5) pp. 250-256.

Domokos, D., Ducza, E., Falkay, G., & Gáspár, R. (2017). Alteration in expressions of RhoA and Rho-kinases during pregnancy in rats: their roles in uterine contractions and onset of labour. *Journal of Physiology and Pharmacology*, *68*(3), 439-451.

Domokos, D., Fülöp, F., Falkay, G., & Gáspár, R. (2018). Effects of newly synthetized isoquinoline derivatives on rat uterine contractility and ROCK II activity. *Bioorganic & Medicinal Chemistry Letters*, *28*(3), 466-469.

Domokos, D., Ducza, E., & Gáspár, R. (2019). RhoA and Rho-kinase inhibitors modulate cervical resistance: The possible role of RhoA/Rho-kinase signalling pathway in cervical ripening and contractility. *European Journal of Pharmacology*, *843*, 27-33.

8.1.2. Abstracts

Domokos Dóra, Ducza Eszter, Lovász Norbert, Falkay György. A RhoA és Rho-kinázok változásának és farmakológiai reaktivitásának vizsgálata terhes patkány uteruson. Gyógyszerészet 58:(5) pp. 296-308. Congressus Pharmaceuticus Hungaricus Budapest XV. Budapest, Magyarország: 2014.04.10-12.

Domokos D, Ducza E, Falkay G. A RhoA / Rho - kinázok expressziójának és farmakológiai reaktivitásának vizsgálata terhes patkány uteruson. III. Interdiszciplináris Doktorandusz Konferencia 2014.04.15-17.

Domokos D, Fülöp F, Falkay G. Inhibitory effect of original synthetized isoquinoline derivatives for the rat uterus contraction. Bridges in Life Sciences 10th Annual Scientific Conference, Wroclaw, Poland; 2015. 04.16-19.

Domokos D, Ducza E, Gaspar R. RhoA inhibitor and Rho-kinase inhibitors modulate the cervical resistance: RhoA/ Rhokinase signal pathway may play a role in the cervical remodeling and smooth muscle contractility. 8th RECOOP Annual Project Review Meeting, Zagreb, Croatia, 2017. 10.19-21.

Domokos D, Ducza E, Gaspar R. Uterine expressions and pharmacological influences of RhoA and Rho-kinases during pregnancy in rats. Joint meeting of the Federation of European Physiological Societies and the Austrian Physiological Society, Vienna, Austria, 2017.09.13-15.

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