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D. DOMOKOS, E. DUCZA, G. FALKAY, R. GASPAR

ALTERATION IN EXPRESSIONS OF RhoA AND Rho-KINASES DURING PREGNANCY IN RATS: THEIR ROLES IN UTERINE CONTRACTIONS AND ONSET OF LABOUR

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

Activation of RhoA and Rho-associated kinases (ROCKs) is known to play a pivotal role in the regulation of smooth muscle contraction *via* phosphorylation of myosin-light chain and myosin phosphatase. There are few data on the RhoA and ROCKs expression levels in rat uteri. Therefore, our aim was to investigate the mRNA and protein concentration of RhoA and ROCKs in rat uterus during pregnancy, during parturition and post-partum using real time PCR and Western blot analysis. The other purpose was to evaluate the effects of the ROCK (Y-27632, fasudil and RKI 1441) and RhoA inhibitors (simvastatin) on uterine contractility in isolated organ bath experiments. The mRNA and protein levels of RhoA decreased on the 5th day of pregnancy to day 22, then a sharp increase was detected at term. The mRNA and protein concentration of ROCKs was down-regulated in the early stage of pregnancy, while it sharply increased during parturition. The RhoA-inhibitor simvastatin relaxed the uterus contractions, although its inhibitory effects were not followed by the alteration of RhoA. The strongest inhibitory effect of non-selective ROCK inhibitor fasudil was found on non-pregnant uterus, while it elicited milder relaxation on day 22, during parturition and postpartum day 1. The maximum relaxing effects of Y-27632 and RKI 1441 were altered in a proportional way with the target protein expressions. The RhoA/ROCK signalling pathway might be a potential target for the development of new tocolytic agents; however, high specificity to RhoA, ROCK I or ROCK II seems to be fundamental to the high efficacy of uterine relaxation.

Key words: *RhoA, Rho-associated kinases, pregnancy, uterus, parturition, post-partum, inhibitors of Rho-associated kinases, fasudil, simvastatin*

INTRODUCTION

Preterm birth, defined as birth before gestational week 37, is a central problem in obstetrics and the most important risk factor for perinatal morbidity and mortality (1). Effective management of this important clinical problem is currently hampered by the inability of existing tocolytic agents to arrest uterine activity safely for more than 48 hours (2). The uterus is a smooth muscle organ, which remains in a state of quiescence until parturition but it is capable of generating strong, synchronized contractions at the onset of labour. The physiological triggers that guide this transition are still poorly understood. It is also known that even obesity and adipose tissue-released hormones can lead to the alteration of sexual hormone levels in rats that may have an impact on the outcome of pregnancy and uterine function (3, 4). Thus there is an urgent need to investigate the physiological factors that may represent the potential new targets to develop new tocolytic agents for the successful treatment of premature labour.

The massive increase in intracellular calcium in uterine myocytes is likely due to depolarization caused by the IP3-stimulated smaller increase in free calcium ion from the sarcoplasmic reticulum (5). The increased level of intracellular Ca^{2+} stimulates myosin regulatory light chain kinase (MLCK), which phosphorylates the 20 kDa regulatory subunit of the myosin light chain (RLC › pRLC) and induces contractions (6). Because the rate of intracellular Ca^{2+} is not always proportional to evolved contraction and RLC phosphorylation, a second pathway has been proposed (7), which includes the activation of GTP binding Rho family member A (RhoA) and the Rho-associated kinases (ROCKs) (8). RhoA/ROCK activation leads to direct phosphorylation of RLC and to suppression of myosin phosphatase (MLCP), which potentiates the pro-contractile effects of phosphorylated RLC (9). These pathways have been studied in a variety of species and tissues. The ROCK I mRNA expression identified in the lungs, kidneys and testes was lower than that in the brain and muscles, and a high level of ROCK II mRNA was detected in the brain, muscles, heart, lungs and placenta (10).

It has been established that the activation of MLCK is dependent on the increase in cytosolic Ca^{2+}, whereas the regulation of MLCP by ROCK is essentially independent of Ca^{2+}. The fundamental role of RhoA/ROCK proteins in multiple cell signalling pathways is well established (11), however, it is still relatively unexplored in reproductive tissues. There are few and variant data outlining the ontogeny of RhoA, ROCK I and ROCK II in pregnant uterus (12, 13) and the effects of RhoA and ROCK inhibitors at different stages of pregnancy. Earlier reports have

shown that statins inhibit Rho signalling by preventing geranylgeranylation and membrane association of RhoA and block agonist-induced Rho activation (14, 15). Fasudil and Y-27632 have been reported as potent non-selective pharmacological inhibitors of ROCK, which cause relaxation of smooth muscle by competing with ATP to bind to the catalytic sites in enzymes (16, 17). RKI 1447 has a similar activity to Y-27632 and fasudil, but it is a more selective and potent ROCK inhibitor (18) and its effect has not been investigated on the smooth muscle contraction.

The objectives of the current studies were to obtain a systematic and integrated portrayal of these events in pregnant rat uteri with a combination of *in vitro* and *ex vivo* methods. 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 by using real-time PCR and Western blot analysis. The other purpose was to evaluate the effects of the ROCK (Y-27632, fasudil and RKI 1441) and RhoA inhibitors (simvastatin) on uterine contractility in isolated organ bath experiments.

MATERIAL AND METHODS

Ethical approval

The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethics Committee for Animal Research (registration number: IV/198/2013).

Housing, handling and mating of the animals

Sprague-Dawley rats (Charles-River Laboratories, Budapest, Hungary) were kept at 22 ± 3°C under a 12 h light/12 h darkness cycle; the relative humidity was 30 – 70%. The animals were maintained on a standard rodent pellet diet (Charles-River Laboratories, Budapest, Hungary) with tap

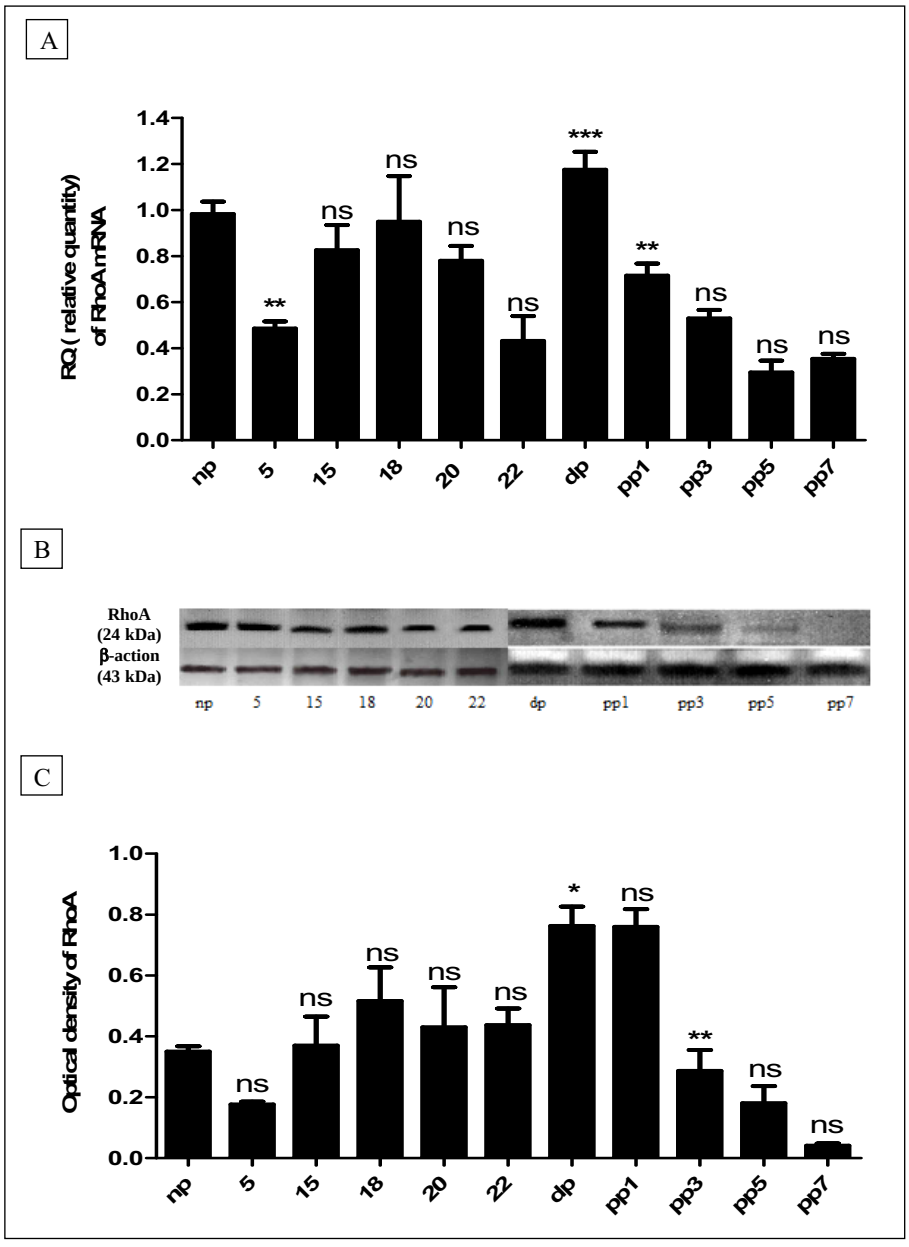

Fig. 1. Changes in mRNA (*A*) and representative protein expression (*B* and *C*) of RhoA in non-pregnant rat uterus (np) during pregnancy, during parturition (dp) and on postpartum days (pp1, 3, 5 and 7). The significances are given as compared with data on the previous day. Each bar denotes mean ± S.E.M. (standard error); n = 5. ANOVA, Tukey's test. ns: non-significant; *P < 0.05; ** P < 0.01.

water available *ad libidum*. The 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.

Real-time quantitative RT-PCR study

Tissue isolation

The animals were euthanized by CO_2 inhalation. Uterus 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, 5 and 7 days after labour). Furthermore, 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 uterine, myometrial and endometrial samples were stored at 4°C overnight in RNA later solution (Life Technologies, Budapest, Hungary), the supernatant was then removed, and the samples were stored at –70 °C until the beginning of the investigation. Total RNA was isolated from samples using the 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. Amplification of the PCR products (Life Technologies, Hungary) *RhoA* (Assay ID: Rn04219609_m1) *Roc1* (Assay ID: Rn00579490_m1), *Roc2* (Assay ID: Rn00564633_m1) and *β-actin* (Assay ID: Rn00667869_m1) as an endogenous control was performed 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 s, 60°C for 20 s. 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.

Table 1. Statistical comparisons of the mRNA (*A*) and protein (*B*) expressions of RhoA on each investigated day. Numbers show the days of pregnancy; np: non-pregnant; dp: during parturition; pp: postpartum; ns: non-significant; *P < 0.05; **P < 0.01; ***P < 0.001. ANOVA, Tukey's test.

A

	5	**15**	**18**	**20**	**22**	**dp**	**pp1**	**pp3**	**pp5**	**pp7**
np	**	ns	ns	ns	***	ns	ns	*	***	***
	5	ns	*	ns	ns	***	ns	ns	ns	ns
		15	ns	ns	*	ns	ns	ns	**	*
			18	ns	**	ns	ns	ns	**	**
				20	ns	*	ns	ns	*	ns
					22	***	ns	ns	ns	ns
						dp	**	***	***	***
							pp1	ns	ns	ns
								pp3	ns	ns
									pp5	ns
										pp7

B

	5	**15**	**18**	**20**	**22**	**dp**	**pp1**	**pp3**	**pp5**	**pp7**
np	ns	ns	ns	ns	ns	*	ns	ns	ns	ns
	5	ns	ns	ns	ns	***	***	ns	ns	ns
		15	ns	ns	ns	**	ns	ns	ns	ns
			18	ns	ns	ns	ns	ns	ns	**
				20	ns	*	ns	ns	ns	*
					22	*	ns	ns	ns	**
						dp	ns	***	***	***
							pp1	**	***	***
								pp3	ns	ns
									pp5	ns
										pp7

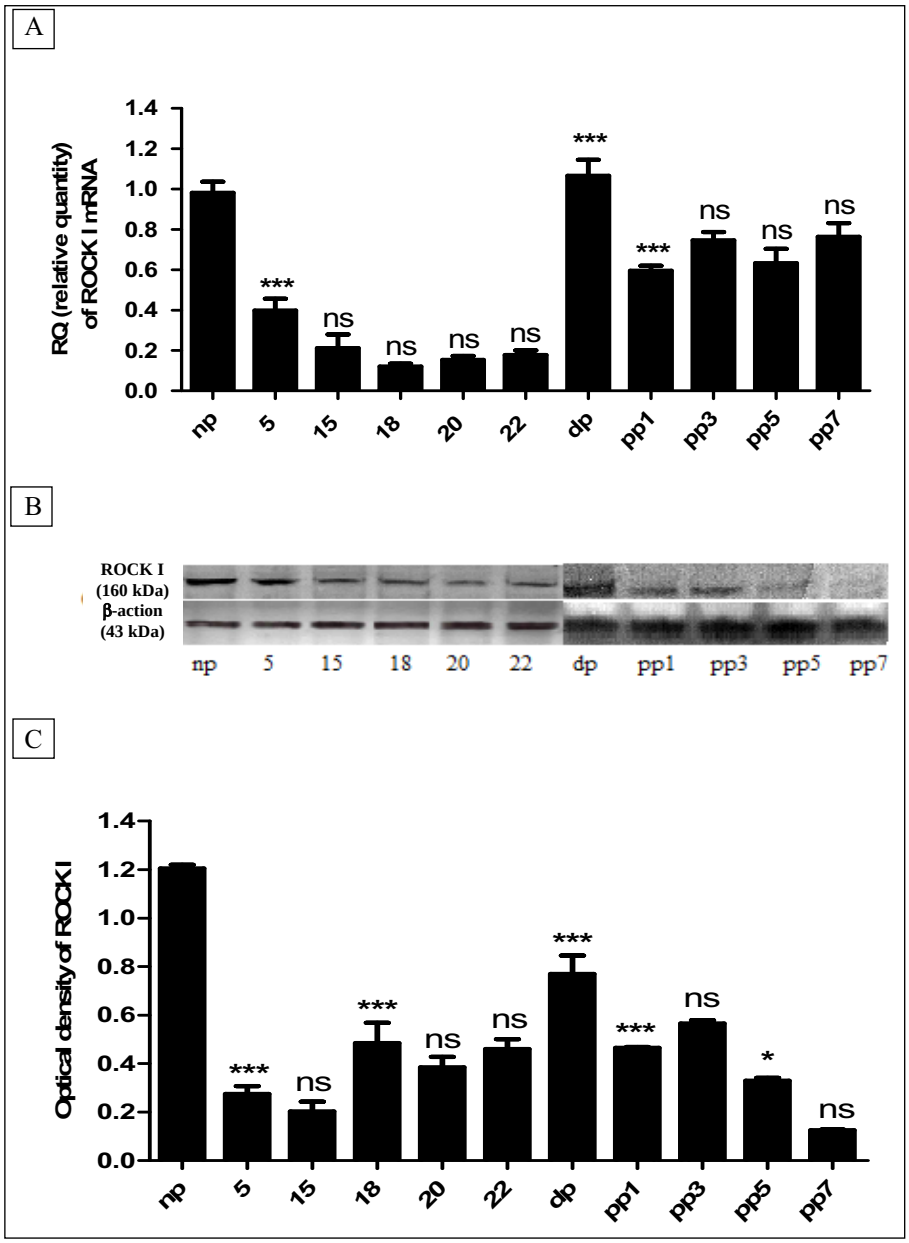

Fig. 2. The mRNA (*A*) and protein expression (*B* and *C*) of ROCK I in non-pregnant rat uterus (np) during pregnancy, during parturition (dp) and on postpartum days (pp1, 3, 5 and 7). The significances are given as compared with data on the previous day. ns: non-significant; *P <0.05; **P < 0.01; ***P < 0.001. Each bar denotes mean ± S.E.M. (standard error); n = 5. ANOVA, Tukey's test.

Western blot analysis

Samples were powdered with a Sartorius Mikro Dismembrator U (Sartorius, Gottingen, 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). Fifty µg of protein per well was subjected to electrophoresis on 4 – 12% NuPAGE Bis-Tris Gel in XCell SureLock 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 blots were incubated on a shaker with RhoA, Roc1, Roc2 and β-actin polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200) 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.

Isolated organ studies, uterus preparation

Five-mm-long uterus 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 of the post-partum period and then mounted vertically in an organ bath containing 10 ml of de Jong solution (137 mM NaCl, 3 mM KCl, 1 mM $CaCl^2$, 1 mM $MgCl^2$, 12 mM $NaHCO_3$, 4 mM NaH_2PO_4, 6 mM glucose, ph 7.4). The organ bath was maintained at 37°C, and carbogen (95% O_2 + 5% CO_2) was bubbled through it. After 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 uterine rings was measured with a gauge transducer (SG-02; Experimetria Ltd., Budapest, Hungary) and recorded with a SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd., Budapest, Hungary).

Table 2. Statistical comparisons of the mRNA (*A*) and protein (*B*) expressions of ROCK I on each investigated day. Numbers show the days of pregnancy; np: non-pregnant; dp: during parturition; pp: postpartum; ns: non-significant; *P < 0.05; **P < 0.01; ***P < 0.001. ANOVA, Tukey's test.

A

	5	**15**	**18**	**20**	**22**	**dp**	**pp1**	**pp3**	**pp5**	**pp7**
np	***	***	***	***	***	ns	***	ns	**	ns
	5	ns	**	*	ns	***	ns	***	ns	***
		15	ns	ns	ns	***	***	***	***	***
			18	ns	ns	***	***	***	***	***
				20	ns	***	***	***	***	***
					22	***	***	***	***	***
						dp	***	**	***	*
							pp1	ns	ns	ns
								pp3	ns	ns
									pp5	ns
										pp7

B

	5	**15**	**18**	**20**	**22**	**dp**	**pp1**	**pp3**	**pp5**	**pp7**
np	***	***	***	***	***	***	***	***	***	***
	5	ns	ns	ns	ns	***	ns	**	ns	ns
		15	***	ns	***	***	**	***	ns	ns
			18	ns	ns	**	ns	ns	ns	***
				20	ns	***	ns	ns	ns	**
					22	***	ns	ns	ns	***
						dp	***	ns	***	***
							pp1	ns	ns	***
								pp3	*	***
									pp5	ns
										pp7

Oxytocin-induced contractions

After the incubation period, contractions were elicited with 10^{-8} M oxytocin. In order to measure the extent of fatigue, the oxytocin-induced contractions were recorded for 30 min without inhibitors in control experiments. In experiments with the presence of Rho-kinase inhibitors RKI 1447 (Avidin Kft., Szeged, Hungary), fasudil, Y-27632 or RhoA inhibitor simvastatin (Sigma-Aldrich, Budapest, Hungary) 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 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 ± S.E.M. For statistical evaluations, data were analysed by one-way ANOVA Tukey's test.

RESULTS

The mRNA and protein expression of RhoA and ROCKs in rat uterus

Relative quantitative real-time PCR and Western blot analysis revealed that RhoA is expressed in pregnant and non-pregnant rat uteri (*Fig. 1*). The mRNA of RhoA decreased significantly in the early stage of pregnancy (day 5). Although we found an increase in the mRNA level till day 18, while a decrease till day 22, these alterations were not significant. However, a significant increase was observed during labour compared to day 22, while the mRNA expression was reduced on postpartum day 1 and remained unchanged on postpartum days 3, 5 and 7 (*Fig. 1A*). The protein expression of RhoA did not change until day 22 of pregnancy, and a significant increase was found during labour. Interestingly, the elevated RhoA protein expression did not change on postpartum day 1, but it was significantly reduced from postpartum day 3 (*Fig. 1C*). The

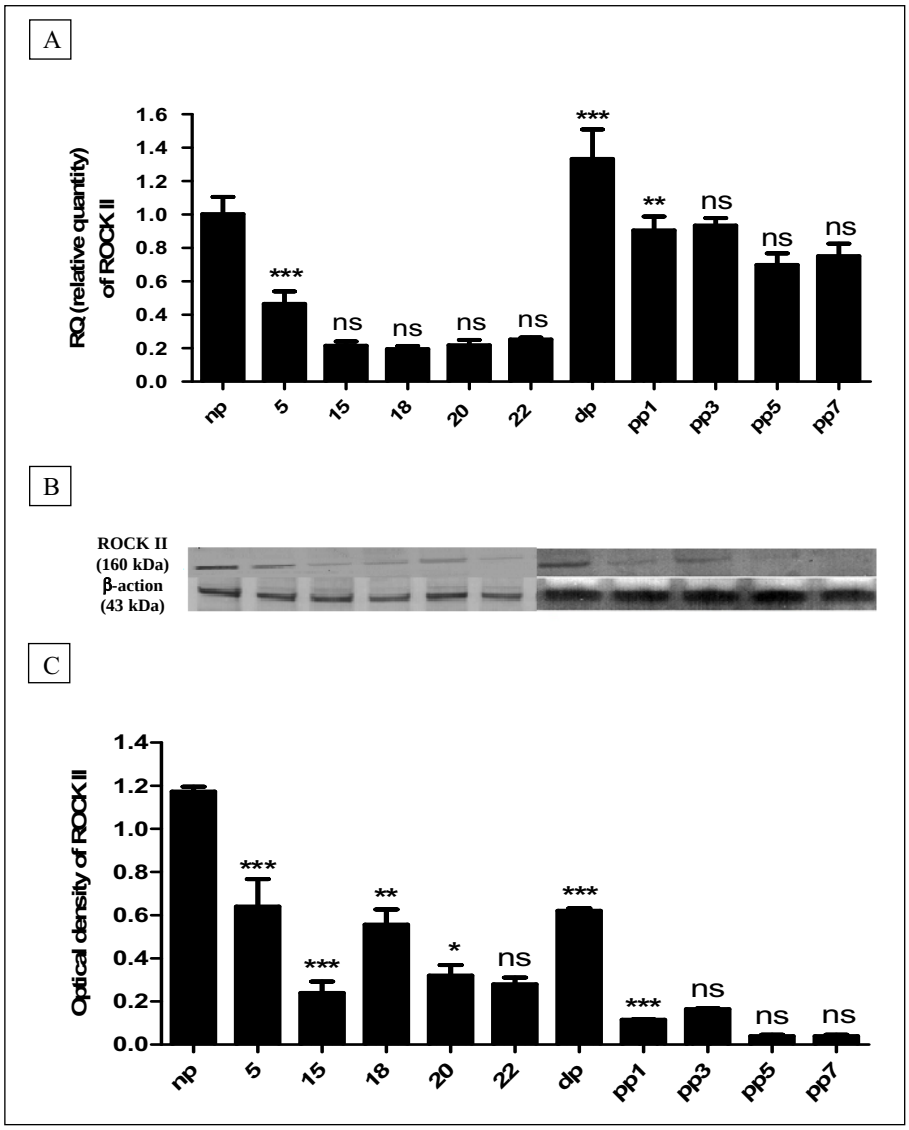

Fig. 3. The mRNA (*A*) and representative protein level (*B* and *C*) of ROCK II in non-pregnant rat uterus (np) during pregnancy, during parturition (dp) and on postpartum days (pp1, 3, 5 and 7). The significances are given as compared with data on the previous day. ns: non-significant; *P <0.05; **P < 0.01; ***P < 0.001. Each bar denotes mean ± S.E.M. (standard error); n = 5. ANOVA, Tukey's test.

complex statistical comparison (comparison to each day) reveals that characteristic alterations in mRNA and protein expressions of RhoA can be found mostly near parturition and postpartum (*Table 1A* and *1B*).

In case of ROCK I mRNA and protein expressions (*Fig. 2*) we found that the mRNA level was high in non-pregnant uteri, it was reduced by pregnancy day 5 and remained low till delivery. During labour, a 5-fold increase in the mRNA level was detected. This elevated value was reduced moderately on postpartum day 1 and remained unchanged on the remaining days of the investigated postpartum period (*Fig. 2A*). 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. In the postpartum period the alterations of ROCK I protein expression were similar to those of the mRNA expression (*Fig. 2C*). The complex statistical comparison reveals that alterations in ROCK I mRNA and protein were mostly during parturition and postpartum (*Table 2A*), although the significant changes in protein expressions were less (*Table 2B*).

A low mRNA level of ROCK II was observed from non-pregnant uteri to day 22 of pregnancy and then a marked increase was found during parturition (*Fig. 3*). On the first day of the postpartum period the ROCK II mRNA expression significantly decreased then remained unchanged till postpartum day 7 (*Fig. 3A*). The protein expression of ROCK II (*Fig. 3C*) was high in the non-pregnant uteri, but it was significantly reduced on days 5 and 15 of pregnancy. On pregnancy day 18 the protein level was elevated, then a reduced protein level was observed till delivery, while during parturition the expression of ROCK II significantly increased. On postpartum days (1, 3, 5, 7) a low protein level of ROCK II was detected. The complex statistical comparison reveals a similar pattern of alterations in mRNA and protein levels of ROCK II than that of ROCK I (*Table 3A* and *3B*).

We have measured the mRNA and protein expression of RhoA in endometrial and myometrial tissues (*Fig. 4*) separately. The RhoA mRNA expressions did not change significantly in the endometrial samples (*Fig. 4A*), but decreased in the myometrium on day 22 of pregnancy and during parturition (*Fig. 4B*). The protein expression of RhoA markedly increased in the endometrial samples on pregnancy day 22 and during parturition (*Fig. 4C*) and remained unchanged in the non-pregnant, parturient and 22 day pregnant myometrium (*Fig. 4D*).

Table 3. Statistical comparisons of the mRNA (*A*) and protein (*B*) expressions of ROCK II on each investigated day. Numbers show the days of pregnancy; np: non-pregnant; dp: during parturition; pp: postpartum; ns: non-significant; *P < 0.05; **P < 0.01; ***P < 0.001. ANOVA, Tukey's test.

A

	5	**15**	**18**	**20**	**22**	**dp**	**pp1**	**pp3**	**pp5**	**pp7**
np	***	***	***	***	***	ns	ns	ns	ns	ns
	5	ns	ns	ns	ns	***	**	**	ns	ns
		15	ns	ns	ns	***	***	***	**	***
			18	ns	ns	***	***	***	**	***
				20	ns	***	***	***	**	**
					22	***	***	***	*	**
						dp	*	*	***	***
							pp1	ns	ns	ns
								pp3	ns	ns
									pp5	ns
										pp7

B

	5	**15**	**18**	**20**	**22**	**dp**	**pp1**	**pp3**	**pp5**	**pp7**
np	***	***	***	***	***	***	***	***	***	***
	5	***	ns	**	***	ns	***	***	***	***
		15	**	ns	ns	***	ns	ns	ns	ns
			18	*	**	ns	***	***	***	***
				20	ns	**	ns	ns	*	*
					22	***	ns	ns	ns	ns
						dp	***	***	***	***
							pp1	ns	ns	ns
								pp3	ns	ns
									pp5	ns
										pp7

Contractility studies

The oxytocin-induced contractions without inhibitors were not reduced within 30 min in non-pregnant, 22 day pregnant uteri and during parturition, but they were much intense during parturition as compared with postpartum amplitudes (*Fig. 5*).

The RhoA inhibitor simvastatin inhibited the oxytocin-induced contractions in a dose dependent manner. The weakest relaxing activity was detected on postpartum day 1, while the strongest relaxing effect was measured on day 22. 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. The weakest activity was detected on postpartum day 1 (*Fig. 6*). The IC_{50} value of simvastatin was the lowest on day 22 and the highest on postpartum day 1, while these values in non-pregnant uterus and during parturition were similar (*Table 4*). Each non-selective ROCK 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. 7*). The IC_{50} values of fasudil were very similar on all investigated days (*Table 5*). The compounds Y-27632 and RKI 1447 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 (*Figs. 8* and *9*). The compounds had the highest IC_{50} values on postpartum day 1. The lowest values were measured on day 22 and during parturition with RKI 1477, while IC_{50} value for Y-27632 was the lowest in non-pregnant uteri (*Tables 6* and *7*).

DISCUSSION

Preterm birth is among the greatest challenges of obstetrician practice. Since definitive information is not available on the precise trigger mechanism of pregnant uterine contractions leading to delivery, new possible drug targets must be identified to promote the success rate of tocolytic therapy.

It has been established that the RhoA/ ROCK pathways play an important role in the uterine smooth muscle contraction (19). Several and inconsistent molecular pharmacological results have

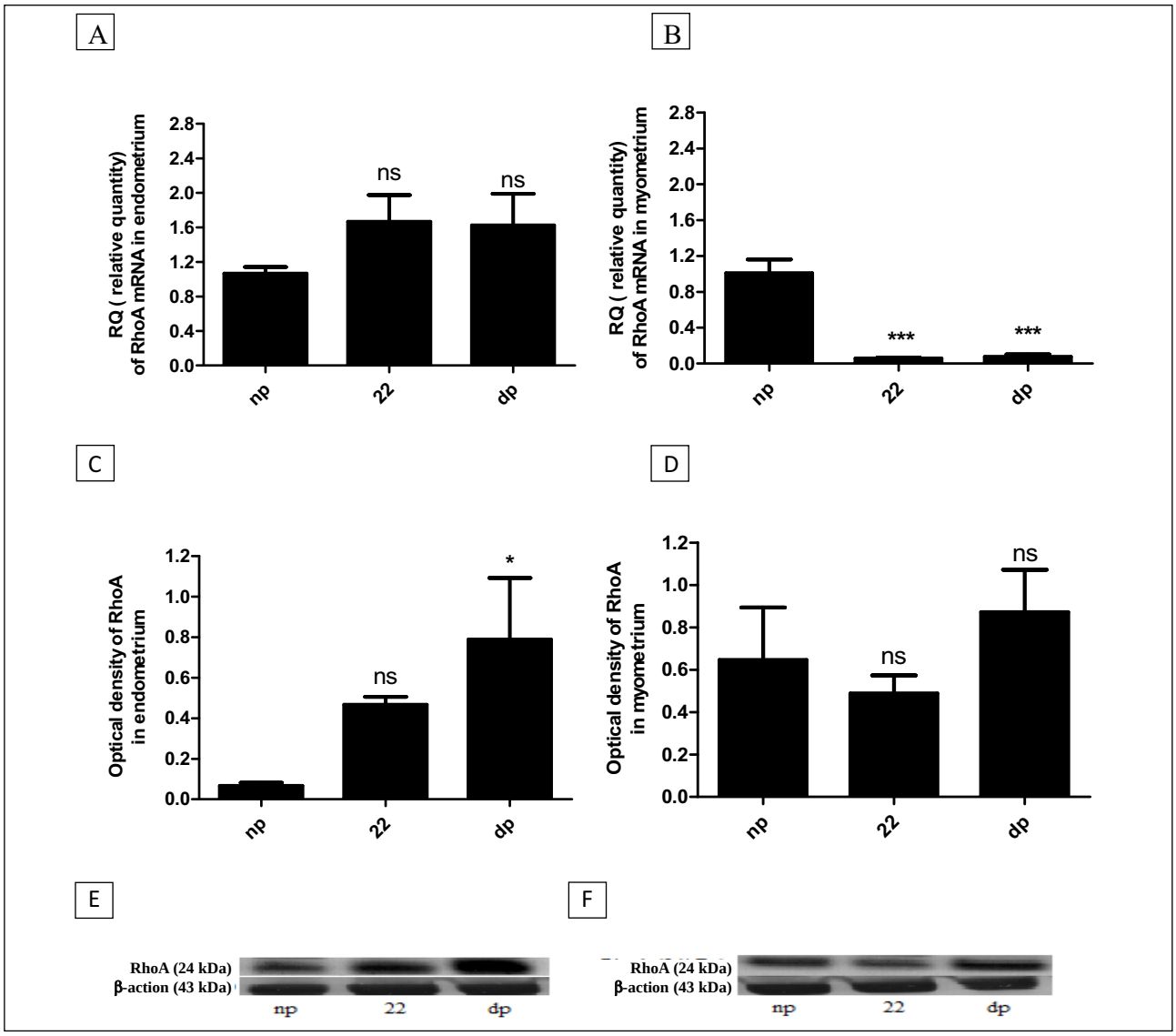

Fig. 4. Changes the mRNA expression in endometrium (*A*) and myometrium (*B*); and representative protein expression of RhoA in non-pregnant rat (np), day 22 of pregnancy (22) and during parturition (dp) in endometrium (*C*, *E*) and myometrium (*D*, *F*). The significances are given as compared with data of non-pregnant state. Each bar denotes mean ± S.E.M. (standard error); n = 5. ANOVA, Tukey's test. ns: non-significant; *P < 0.05; ***P < 0.001.

been published suggesting that RhoA and ROCKs might be involved in the increased contractility of the uterus during pregnancy (20, 21). Therefore, we investigated the RhoA/ROCKs expression in rats during pregnancy, including parturition and post-partum. We found low expression levels of ROCKs 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 known that the period for embryonic implantation in rats is between pregnancy days 4 – 7 (22). Shiokawa *et al*. demonstrated that the amount of RhoA was more pronounced in cytotrophoblast cells, whereas ROCKs were present in both cytotrophoblast and syncytiotrophoblast cells and the migration of cytotrophoblast cells has been regulated by RhoA/ROCK signalling pathway (23). Fanchin *et al*. found a significant decrease in human uterine contractility at the time of blastocyst transfers (24). If ROCKs are responsible for the contractions, the decreased expressions of ROCK I and ROCK II in the rat uterus might reduce the intensity of contraction, thereby protecting 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 after implantation and before delivery the efficacy of translation from mRNA to protein is fluctuating. The protein expressions reveal that RhoA remains unchanged from pregnancy day 5 till day 22. Both ROCK proteins showed some significant alterations between pregnancy days 5 – 22, however, the physiological significance of these changes are difficult to interpret. We suppose that the lower expression of ROCKs compared to non-pregnant uteri may contribute to the maintenance of relative quiescence in the pregnant uterus. This hypothesis is supported by the *in vitro* studies with ROCKs inhibitors, namely the compounds showed stronger relaxation of pregnant uterine contraction on those days when the expressions of ROCKs were higher. We proved that the mRNA level of

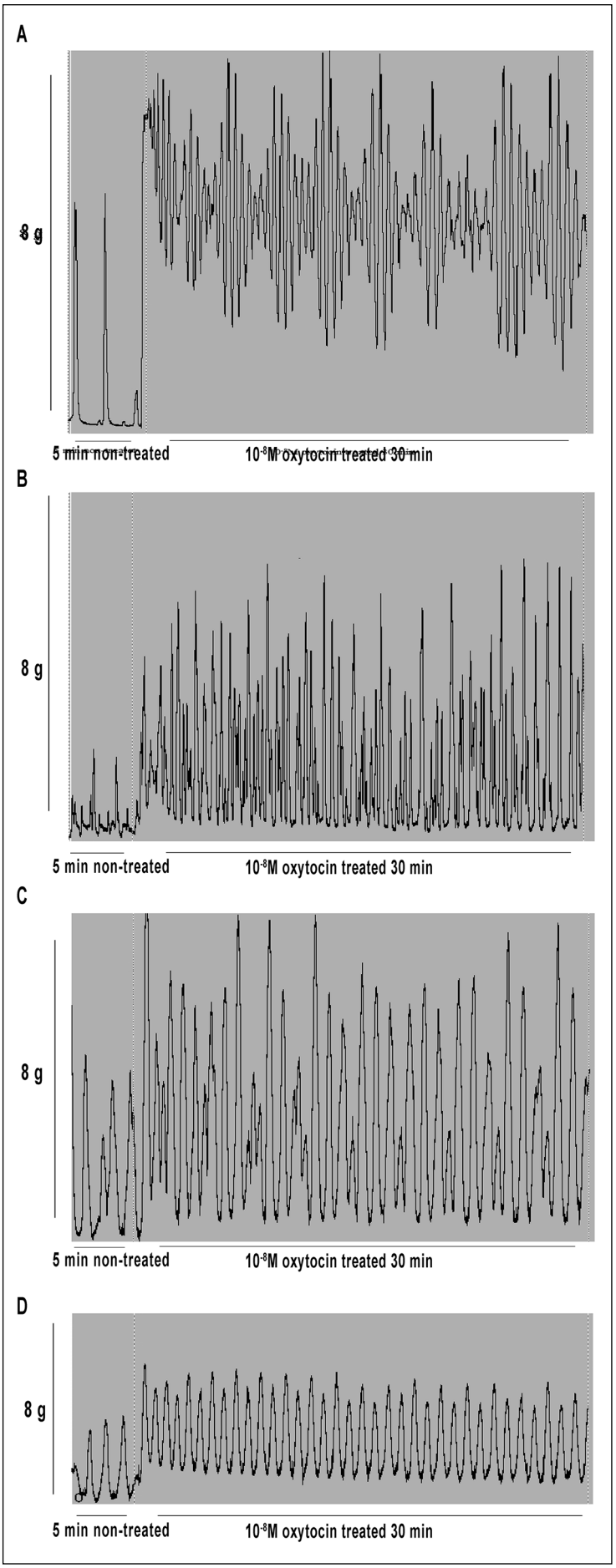

Fig. 5. Oxytocin-induced contractions without inhibitors in non-pregnant uteri (*A*), on day 22 of pregnant uteri (*B*), during parturition (*C*) and on the first day of postpartum (*D*).

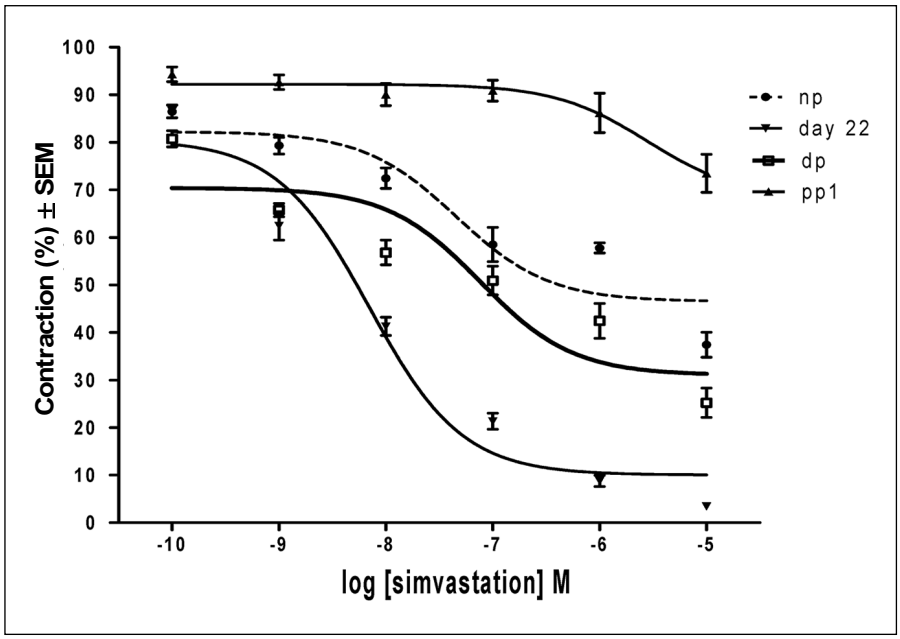

Fig. 6. Inhibitory effects of simvastatin on oxytocin-stimulated contraction in uteri *in vitro* in non-pregnant (np) rats and day 22 of pregnancy, during parturition (dp) and on the first day of the post-partum period (pp1).

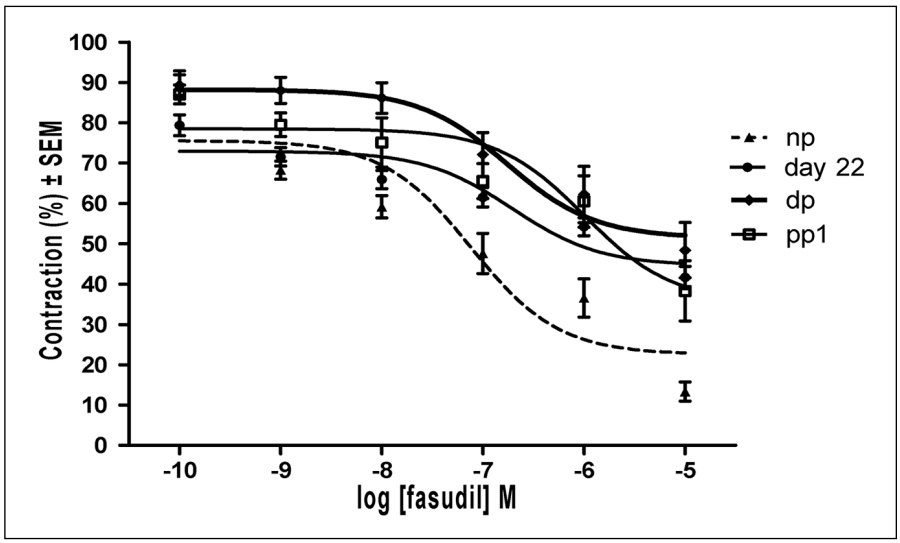

Fig. 7. The inhibitory effect of non-selective ROCK inhibitor fasudil on the non-pregnant rat uteri (np), on pregnancy day 22, during parturition (dp) and on the first day of the postpartum period (pp1).

Table 4. 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 ± S.E.M. (standard error). ANOVA, Tukey's test.

	np	**day 22**	**dp**	**pp1**
IC_{50} ± S.E.M.	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 % ± S.E.M.	58.3 ± 4.7	90.1 ± 1.1***	71.7 ± 2.2***	19.7 ± 5.5***

Table 5. 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 ± S.E.M. (standard error). ANOVA, Tukey's test.

	np	**day 22**	**dp**	**pp1**
IC_{50} ± S.E.M.	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 % ± S.E.M.	80.7 ± 2.3	58.8 ± 3.4**	57.2 ± 4.9^{ns}	71.9 ± 5.2*

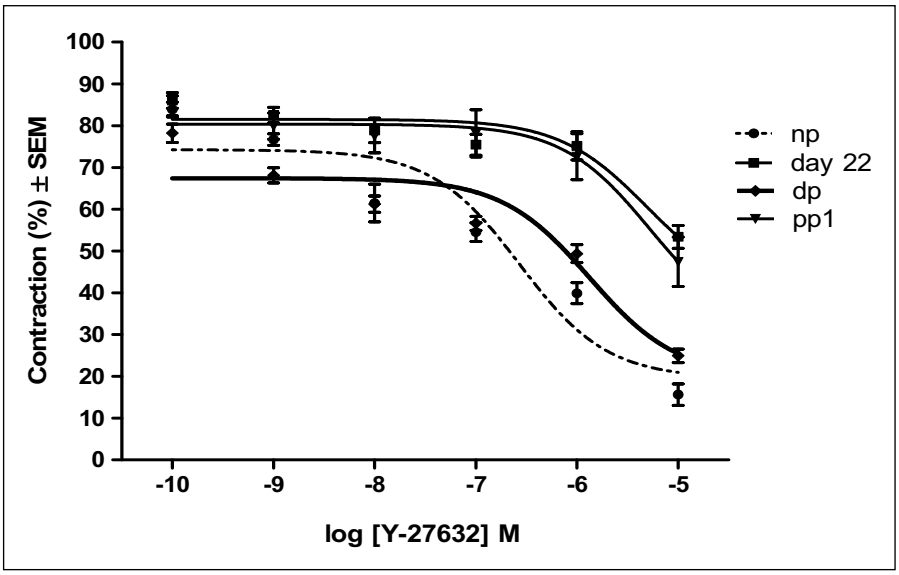

Fig. 8. The inhibitory effect of non-selective ROCK inhibitor Y-27632 on the non-pregnant rat uteri (np), on pregnancy day 22, during parturition (dp) and on the first day of the postpartum period (pp1).

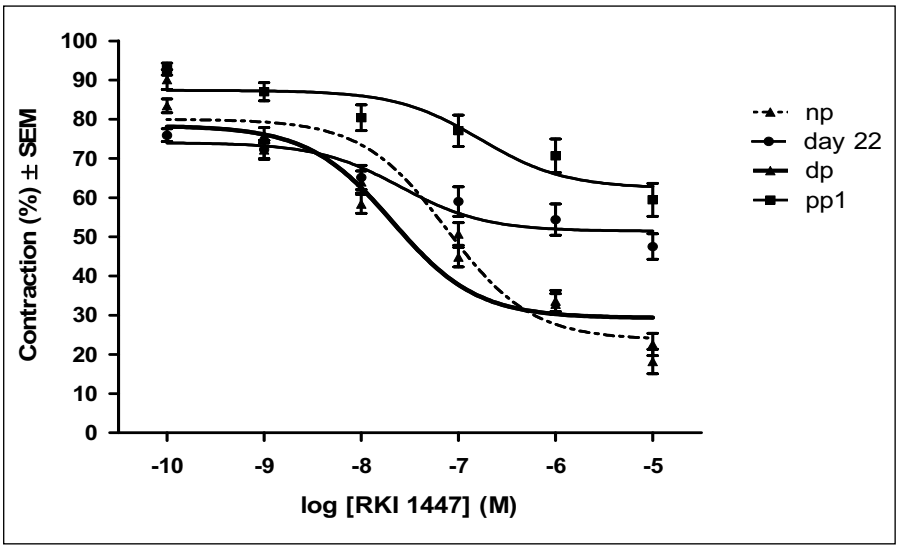

Fig. 9. The inhibitory effect of non-selective ROCK inhibitor RKI-1441 on the non-pregnant rat uteri (np), on pregnancy day 22, during parturition (dp) and on the first day of the postpartum period (pp1).

Table 6. 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 parturitiom; pp: postpartum; ns: non-significant; **P < 0.01. Each bar denotes mean ± S.E.M. (standard error). ANOVA, Tukey's test.

	np	**day 22**	**dp**	**pp1**
IC_{50} ± S.E.M.	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 % ± S.E.M.	82.3 ± 3.5	51.9 ± 8.3**	78.8 ± 2.5**	61.7 ± 8.6^{ns}

Table 7. 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 ± S.E.M. (standard error). ANOVA, Tukey's test.

	np	**day 22**	**dp**	**pp1**
IC_{50} ± S.E.M.	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 % ± S.E.M.	76.9 ± 2.8	51.0 ± 2.8***	70.8 ± 2.7**	40.7 ± 3.9***

ROCK I, ROCK II and RhoA were sharply up-regulated in the rat uterus at the onset of labour. This finding is consistent with a previously published rat study (25). We detected a sudden increase of protein expression of RhoA and ROCKs during labour, which suggests their involvement in enhanced contractility and in the initiation of delivery. It can be hypothesized that the higher expressions of RhoA/ROCKs in non-pregnant (oestrus) and delivering uteri are related to an oestrogen plasma peak in rats, because oestradiol enhances RhoA activity (26), and it has a high physiological level during both parturition and the non-pregnant oestrus period (27, 28).

We measured the RhoA expression in the endometrial and myometrial tissues separately. We found that the protein expression of RhoA markedly increased in the endometrial samples on pregnancy day 22 and during parturition. In case of the myometrium, the RhoA protein level remained unchanged. It means that the measured alterations in RhoA expression are mainly the consequences of endometrial processes at the end of pregnancy.

The roles of RhoA and ROCKs in pregnant uterine contractions can be measured in contractility studies by applying their inhibitors. An earlier study revealed that the inhibitor of RhoA prenylator induced relaxation in the anal sphincter (29). In our study, not all the pregnancy days were involved in the contractility experiments, we focused on the late-term and early postpartum pregnant uteri in comparison with the non-pregnant uterus only. Simvastatin, a hydroxy-methyl-glutaryl-coenzyme A (HMG-CoA) inhibitor is also considered as an inhibitor of RhoA. In our experiments, simvastatin elicited a relaxing effect, although its action was not consequent on the expression of RhoA protein. The RhoA expression was equivalently high during parturition and postpartum day 1, but the relaxing action of simvastatin both in maximum and IC_{50} value was the weakest on postpartum day 1 and much stronger during parturition. Additionally, the RhoA expression was the lowest on day 22; however, the simvastatin action was the best on that day. These results suggest that the pregnant uterine relaxing action of simvastatin partially depends on its RhoA inhibitory property and some additional mechanisms. The ‘statins’ were developed to block the cholesterol synthesis (30), but they have cholesterol-independent ‘pleiotropic’ effects. Statins inhibit the isoprenylation of Rho family proteins (31). The short-term statin exposition inhibits Rho signalling by preventing Rho membrane association and blocks the agonist induced Rho activation (32). Although the unprenylated Rho is able to interact with their effectors, this interaction is less potent than with prenylated Rho (33). Interestingly, the intensity of the oxytocin-induced uterine contractions during parturition and on the first day of postpartum period was different. The different oxytocin sensitivity of the uterus during parturition and postpartum day 1 might contribute to the different action of simvastatin.

We also investigated how the ROCKs inhibitor can influence the uterine contractility *in vitro*. We have selected non-selective ROCK inhibitors, fasudil, Y-27632, and RKI 1447. We merged the mRNA or protein expression data of ROCK I and ROCK II in order to get information about the whole expressions of ROCKs and to make it possible to compare the actions on contractility of non-selective blockers to their target proteins. Fasudil had quite a strong relaxing effect on the non-pregnant uterus, while it elicited moderate relaxation on pregnancy day 22, at parturition and on postpartum day 1. However, its IC_{50} values were similar on each day. Thus the action of fasudil did not follow the altered expressions of ROCKs. This phenomenon can be explained by the fact that the effect of fasudil is not strictly limited to ROCK, it has non-specific inhibitory effects on other serine/threonine kinases such as MSK1, PRK2 (34). The more specific ROCKs inhibitors Y- 27632 and RKI 1447 showed a strong relaxing effect on non-pregnant uteri and during parturition, when the mRNA and protein expression of ROCK I and ROCK II were the highest. Their maximum effects were weaker on pregnancy day 22 and postpartum day 1, when the ROCK expressions were also low. Interestingly, the affinities of these molecules (e.g. IC_{50} values) were not altered in parallel with the alteration of target proteins expressions, only their maximum effect altered in a proportional way with the expressions of the target protein (ROCKs). The total expressions of ROCKs (ROCK I and II) contribute to the regulation of uterine contractility and correlate with the inhibitory effects of specific ROCK blockers.

The complex statistical analysis (all group comparison by ANOVA Tukey’s test) showed the patterns of changes among RhoA and ROCKs expressions on different days of gestations, postpartum and non-pregnant condition. These patterns reflect that the major alterations can be detected near parturition and during the first week of postpartum period suggesting that the robust changes in RhoA/ROCKs system are correlated to labour.

We conclude that normal pregnancy suppresses ROCKs, which contributes to the low contractility during pregnancy. A sharp increase of ROCKs at the onset of labour may be a key element of enhanced contractility and the initiation of delivery. After delivery, the RhoA/ROCK system has a less importance and takes part in moderation of uterine contractility. The RhoA/ROCK signalling pathway might be a potential target for the development of new tocolytic agents. However, high specificity to RhoA, ROCK I or ROCK II seems to be fundamental to the high efficacy of uterus relaxation.

We dedicate this article to the memory of our mentor and co-worker, George Falkay, PhD, DSc. Professor George Falkay had initiated this study, but he passed away on September 08, 2016.

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REFERENCES

1. Beck S, Wojdyla D, Say L, *et al*. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. *Bull World Health Organ* 2010; 88: 31-38.
2. Kilpatrick SJ. Management of preterm labor. *Obstet Gynecol* 2012; 119: 1308-1317.
3. Smolinska N, Dobrzyn K, Kiezun M, K, Maleszka A, Kaminski T. Effect of adiponectin on the steroidogenic acute regulatory protein, P450 side chain cleavage enzyme and 3β-hydroxysteroid dehydrogenase gene expression, progesterone and androstenedione production by the porcine uterus during early pregnancy. *J Physiol Pharmacol* 2016; 67: 443-456.
4. Gregoraszczuk E, Slupecka M, Wolinski J, *et al*. Maternal high-fat diet during pregnancy and lactation had gender difference effect on adiponectin in rat offspring. *J Physiol Pharmacol* 2016; 67: 543-553.
5. Wray S, Jones K, Kupittayanant S, *et al*. Calcium signaling and uterine contractility. *J Soc Gynecol Investig* 2003; 10: 252-264.
6. Webb RC. Smooth muscle contraction and relaxation. *Adv Physiol Educ* 2003; 27: 201-206.
7. Kimura K, Ito M, Amano M, *et al*. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* 1996; 273: 245-248.

8. Somlyo AP, Somlyo AV. Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol* 2000; 522: 177-185.
9. Kureishi Y, Kobayashi S, Amano M, *et al*. Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J Biol Chem* 1997; 272: 12257-12260.
10. Nakagawa O, Fujisawa K, Ishizaki T, Saito Y, Nakao K, Narumiya S. ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett* 1996; 392: 189-193.
11. Fukata Y, Kaibuchi K, Amano M. Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends Pharmacol Sci* 2001; 22: 32-39.
12. Friel AM, Curley M, Ravikumar N, Smith TJ, Morrison JJ. Rho A/Rho kinase mRNA and protein levels in human myometrium during pregnancy and labor. *J Soc Gynecol Investig* 2005; 12: 20-27.
13. Gao J, Ding YL. Correlation between expression of RhoA and Rho kinase in uterine smooth muscle and the onset of labor. *Zhonghua Fu Chan Ke Za Zhi* 2009; 44: 345-349.
14. Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler Thromb Vasc Biol* 2001; 21: 1712-1719.
15. Eto M, Kozai T, Cosentino F, Joch H, Luscher T. F. Statin prevents tissue factor expression in human endothelial cells role of Rho/Rho-kinase and Akt pathways. *Circulation* 2001; 105: 1756-1759.
16. Asano T, Suzuki T, Tsuchiya M, *et al*. Vasodilator actions of HA1077 in vitro and in vivo putatively mediated by the inhibition of protein kinase. *Br J Pharmacol* 1989; 98: 1091-1100.
17. Shimokawa H, Rashid M. Development of Rho-kinase inhibitors for cardiovascular medicine. *Trends Pharmacol Sci* 2007; 28: 296-302.
18. Patel RA, Forinash KD, Pireddu R, *et al*. RKI-1447 is a potent inhibitor of the Rho-associated ROCK kinases with anti-invasive and antitumor activities in breast cancer. *Cancer Res* 2012; 72: 5025-5034.
19. Lee YH, Hwang MK, Morgan KG, Taggart MJ. Receptor-coupled contractility of uterine smooth muscle: from membrane to myofilaments. *Exp Physiol* 2001; 86: 283-288.
20. Niiro N, Nishimura J, Sakihara C, Nakano H, Kanaide H. Up-regulation of rho A and rho-kinase mRNAs in the rat myometrium during pregnancy. *Biochem Biophys Res Commun* 1997; 230: 356-359.
21. Tahara M, Morishige KI, Sawada K, *et al*. RhoA/Rho-kinase cascade is involved in oxytocin-induced rat uterine contraction. *Endocrinology* 2002; 143: 920-929.
22. Bullock G, Bunton TE. The Laboratory Rat. London, Academic Press 2000.
23. Shiokawa S, Iwashita M, Akimoto I, *et al*. Small guanosine triphospatase RhoA and Rho-associated kinase as regulators of trophoblast migration. *J Clin Endocrinol Metab* 2002; 87: 5808-5816.
24. Fanchin R, Ayoubi JM, Righini C, Olivennes F, Schonauer LM, Frydman R. Uterine contractility decreases at the time of blastocyst transfers. *Hum Reprod* 2001; 16: 1115-1119.
25. Taggart MJ, Arthur P, Zielnik B, Mitchell BF. Molecular pathways regulating contractility in rat uterus through late gestation and parturition. *Am J Obstet Gynecol* 2012; 207: 76. e15-e24. doi: 10.1016/j.ajog.2012.04.036
26. Oviedo PJ, Sobrino A, Laguna-Fernandez A, *et al*. Estradiol induces endothelial cell migration and proliferation through estrogen receptor-enhanced RhoA/ROCK pathway. *Mol Cell Endocrinol* 2011; 335: 96-103.
27. Downing SJ, Porter DG, Redstone CD. Myometrial activity in rats during the oestrous cycle and pseudopregnancy: interaction of oestradiol and progesterone. *J Physiol* 1981; 317: 425-433.
28. Hurn PD, Macrae IM. Estrogen as a neuroprotectant in stroke. *J Cereb Blood Flow Metab* 2000; 20: 631-652.
29. Patel CA, Rattan S. RhoA prenylation inhibitor produces relaxation of tonic smooth muscle of internal anal sphincter. *J Pharmacol Exp Ther* 2007; 321: 501-508.
30. Tobert JA. Lovastatin and beyond: the history of the HMG-CoA reductase inhibitors. *Nat Rev Drug Discov* 2003; 2: 517-526.
31. Wang CY, Liu PY, Liao JK. Pleiotropic effects of statin therapy: molecular mechanisms and clinical results. *Trends Mol Med* 2008; 14: 37-44.
32. Tatsuta M, Matsumoto Y, Nakamura H, Inoue M. Inhibition of lysophosphatidic acid-induced RhoA activation and tumor cell invasion by 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors. *Int J Oncol* 2003; 23: 1173-1178.
33. Turner SJ, Zhuang S, Zhang T, Boss GR, Pilz RB. Effects of lovastatin on Rho isoform expression, activity, and association with guanine nucleotide dissociation inhibitors. *Biochem Pharmacol* 2008; 75: 405-413.
34. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 2000; 351: 95-105.

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Author's address: Dr. Robert Gaspar, Department of Pharmacodynamics and Biopharmacy, Faculty of Pharmacy, University of Szeged, H-6701 Szeged, P.O. Box 121, Hungary.
E-mail: gaspar@pharm.u-szeged.hu