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Investigation of the effect of the RhoA and Rho-kinases for the rat myometrium and cervix

Ph.D. Thesis Summary

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Introduction

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 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.

The phosphorylated or dephosphorylated state of myosin light chain play a key role in the smooth muscle contraction and relaxation. In previous reports, a Ca^{2+} independent pathway has been identified, which includes the GTP binding RhoA and ROCKs. 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 characterized is the Rho-kinase (ROCK), which is a serine/threonine protein kinase. Two isoforms of ROCK have been identified, ROCK I or ROKβ and ROCK II or ROKα. The GTP-bound RhoA activates the Rho-kinases leading to the phosphorylation of regulatory myosin- binding subunit of myosin phosphatase which results in the inhibition of the phosphatase activity. Furthermore, the Rho-kinases phosphorylate direct the myosin light chain. These mechanisms promote the enhancement of the phosphorylated myosin light chain and induce smooth muscle contraction. Therefore, the RhoA/Rho-kinase mediated pathway can be a possible target for therapeutic interventions in various diseases.

Aims

There are little and inconsistent reports about the expression levels of RhoA and Rho- kinases in myometrium and no information about their role in regulation of cervical function, therefore

1. 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
2. the other purpose was to evaluate the roles of the RhoA and Rho-kinases on uterine contractility and cervical resistance in isolated organ bath experiments *in vitro* by using RhoA inhibitor simvasvatin and Rho-kinase inhibitors Y-27632, fasudil
3. our aim was to analyse the efficiancy of newly synthetized isoquinoline derivatives on the pregnant and non-pregnant uterine contractions *in vitro*, and to test their effect for Rho-kinase activity by using Rho-kinase activity assay.

Materials and methods

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).

Mating

The mature female (180-200 g) and male (240-260 g) rats were mated in a special mating cage. 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. If the search proved positive, the female rats were separated and were regarded as first-day pregnant animals.

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 (after delivery of the 3rd pup) and post-partum (1, 3 andb5 days after labour). Total RNA was isolated from samples using the TRI Reagent. 1 µg mRNA per sample was amplified with the SensiFAST Probe HiROX One-Step Kit and the ABI StepOne Real-Time cycler. The relative quantities were used for statistical analysis. For Western blot analysis, the samples were homogenized in a RIPA Lysis Buffer. 50 µg of protein per well was subjected to electrophoresis on 4−12% NuPAGEBis-Tris Gel in XCellSureLock Mini-Cell Units.

Proteins were transferred from gels to nitrocellulose membranes using the iBlot Gel Transfer System. Antibody binding was detected with the WesternBreeze Chromogenic Western Blot Immundetection Kit. The optical density of each immunoreactive band was determined with Kodak 1D Images analysis software.

Rho-kinase activity assay

The Rho-kinase activity was measured by an immunoassay recommending by the manufacturer. The plate was pre-coated with recombinant myosin phosphatase target subunit 1 (MYPT1). The absorbance was measured on a 96-well plate reader SpectroStarNano at 450 nm. The absorbance signal reflects the relative amount of ROCK activity.

In vitro contractility study

The uterus rings were removed from non-pregnant rats in the oestrus phase and from rats on pregnancy day 22, during labour (after births of 3 pup) and on the first day of the post-partum period. The tissue samples were mounted vertically in an organ bath. The rhythmical uterine contractions were elicited with 10^{-8} M oxytocin. Cumulative dose-response curves were constructed in the concentration range of 10^{-10} -10^{-5} M with the presence of Rho-kinase inhibitors or RhoA inhibitor. The tension of the uterine rings was measured with a gauge transducer and recorded with a SPEL Advanced ISOSYS Data Acquisition System For statistical evaluations, data were calculated by using Prism 5.0 software and analysed by one-way ANOVA Tukey's test and unpaired t-test..

In vitro cervical resistance study

The cervices from non-pregnant (oestrus phase), 20- and 22-day pregnant rats mounted in organ bath. Evaluation of the cervical resistance was carried out such that the initial tension of the cervix was represented versus the stretch after 5 min with and without inhibitors. The cervical 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. Straight lines were fitted by linear regression and the slopes of the lines were used to express the degree of resistance. The tension was recorded by ISOSYS software. Data were calculated by using Prism 5.0 software and the statistical analysis were carried out by one-way ANOVA Dunett test.

Results

The mRNA and protein expressions of RhoA and Rho-kinases in the rat myometrium

The mRNA of RhoA decreased significantly in the 5th day of pregnancy, then slightly increased till day 18, while a gradually but not significantly decrease was detected till day 22. A marked increase was observed during labour, while the mRNA expression was reduced on postpartum day 1 and remained unchanged on postpartum days (**Fig.1 A**). The protein expression of RhoA did not change during pregnancy, but a significant increase was measured during labour. (**Fig.1 B**).

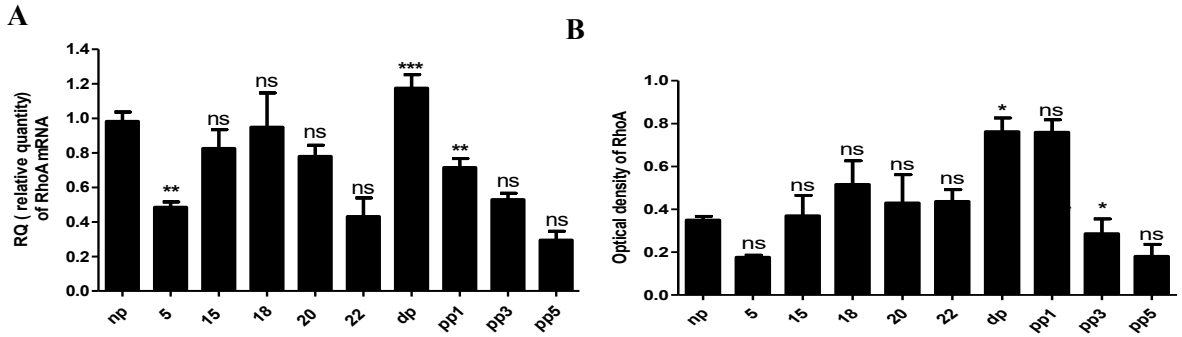

Figure 1. The mRNA (A) and protein (B) expression of RhoA in non-pregnant myometrium (*np*), during pregnancy, during parturition (*dp)* and on postpartum days (*pp*1, 3, 5 and 7). *ns:non-significant*; * $p < 0.05$; ** $p < 0.01$.

Low mRNA levels of ROCK I and II was measured till delivery. During labour, a ~5-fold increase of the expression levels 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. 2 and 3 A**). The protein expression of ROCK I and ROCK II were high in the non-pregnant myometrium that were decreased in pregnancy day 5 and increased on pregnancy day 18. The protein level of ROCK I remained unchanged till delivery, where a marked increase was detected (**Fig. 2 B**). The protein expression of ROCK II reduced on pregnancy day 20 then notably increased during parturition (**Fig. 3 B**).

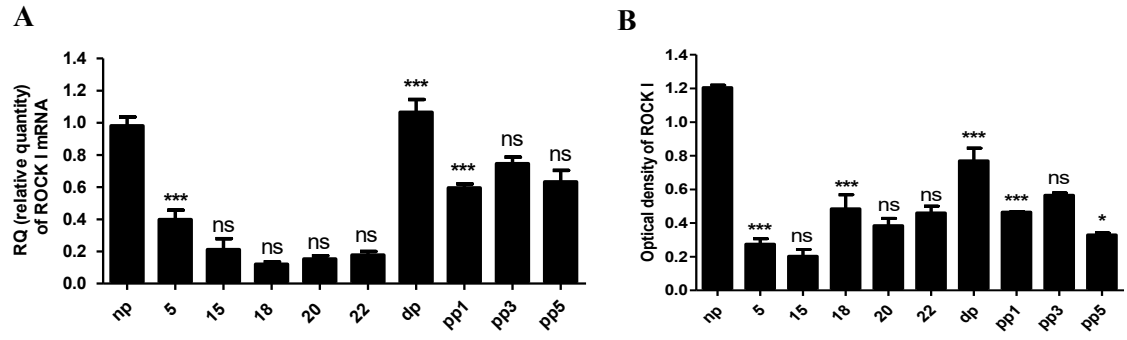

Figure 2. The mRNA (A) and protein expression (B) of ROCK I in non-pregnant rat uterus (*np*) during pregnancy, during parturition (*dp*) and on postpartum days (*pp*1, 3, 5 and 7). *ns:non-significant*; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

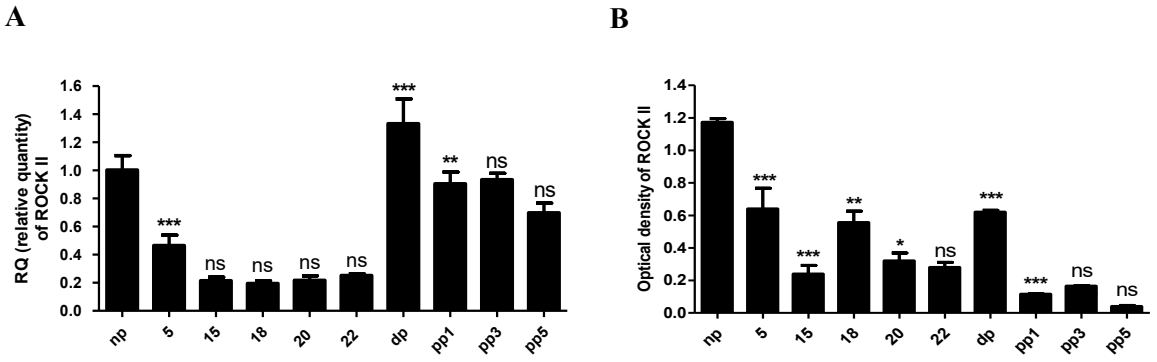

Figure 3. **The mRNA (A) and protein expression (B) of ROCK II** in non-pregnant rat uterus (*np*) during pregnancy, during parturition (*dp*) and on postpartum days (*pp*1, 3, 5 and 7). *ns:non-significant*; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

The effects of RhoA inhibitor simvastatin and Rho-kinase inhibitors -27632, fasudil and RKI1447 for rat uterine contractility *in vitro*

Simvastatin (10^{-10}-10^{-5} M) inhibited the oxytocin-induced uterine contractions in a dose-dependent manner. The weakest relaxing activity and IC_{50} value were found on postpartum day 1, while the strongest relaxing effect and the lowest IC_{50} were measured on day 22 of pregnancy (**Table 1**).

	np	**d22**	**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. np: non-pregnant; dp during parturition; pp: postpartum; *ns: non-significant*; * $p < 0.05$; *** $p < 0.001$.

Each non-selective Rho-kinase inhibitor inhibited the oxytocin induced contractions dose-dependently (10^{-10}-10^{-5} M). Fasudil had the strongest inhibitory effect on non-pregnant uteri. The IC_{50} values of fasudil were very similar on all investigated days (**Table 2**).

	np	**d22**	**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. np: non-pregnant; dp: during parturition; pp:postpartum. *ns: non-significant*; * $p < 0.05$; ** $p < 0.01$. Each bar denotes mean ± SEM (standard error).

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. Y-27632 (**Table 3**) and RKI 1447 (**Table 4**) had the highest IC_{50} values on postpartum day 1.

	np	d22	dp	pp1
IC_{50} ± SEM	2.7e-007 ± 3.6e-007	4.9e-006 ± 4.3e-007ns	1.3e-006 ± 3.7e-007ns	5.3e-006 ± 2.0e-007ns
Inhibition% ±SEM	82.3 ± 3.5	51.9 ± 8.3**	78.8 ± 2.5**	61.7 ± 8.6ns

Table 3 The maximum inhibition and the IC_{50} values of Y-27632. .np: non-pregnant; dp: during parturition; pp: postpartum. *ns:non-significant*; ** $p < 0.01$. Each bar denotes mean ± SEM (standard error).

	np	d22	dp	pp1
IC_{50} ± SEM	7.6e-008 ± 2.1e-008	2.5e-008 ± 3.0e-009ns	2.1 e-008 ± 2.4e-008ns	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. np: non-pregnant; dp: during parturition; pp: postpartum. *ns:non-significant*; ** $p < 0.01$; *** $p < 0.001$. Each bar denotes mean ± SEM (standard error).

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

The efficacies of 13 water-soluble and 12 non-water-soluble isoquinolines 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. 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 (**Fig. 4**).

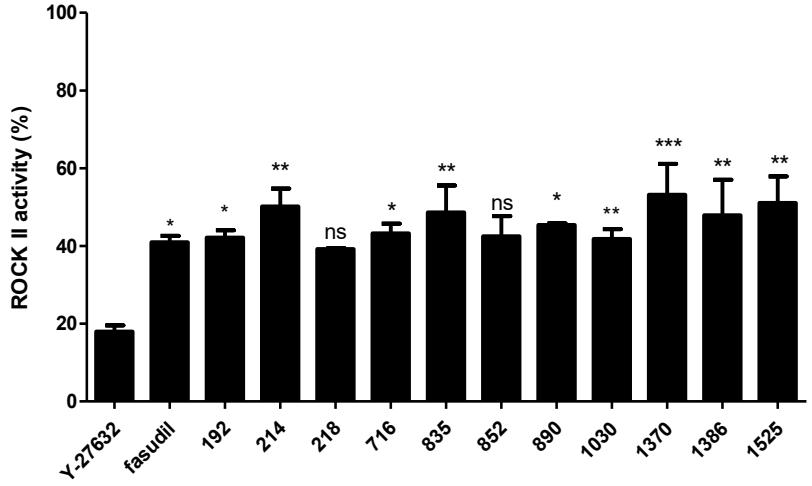

Figure 4. The inhibitory effects of the isoquinoline derivatives for ROCK II activity. The significances are given as compared with Y-27632. Each bar denotes mean ± SEM (standard error). ns: non-significant; * $p < 0.05$; ** $p < 0.01$ *** $p < 0.001$

The IC_{50} values of 218 (**Fig. 5 A**) and 852 (**Fig. 5 B**) were markedly lower than those of fasudil on day 20 of the pregnant rat uterus (when the expression of ROCKs were low). Moreover, 218 and 852 relaxed the rat uteri during parturition (when the expressions of ROCKs were high) with greater potency as compared with fasudil (**Fig. 6 A and B**).

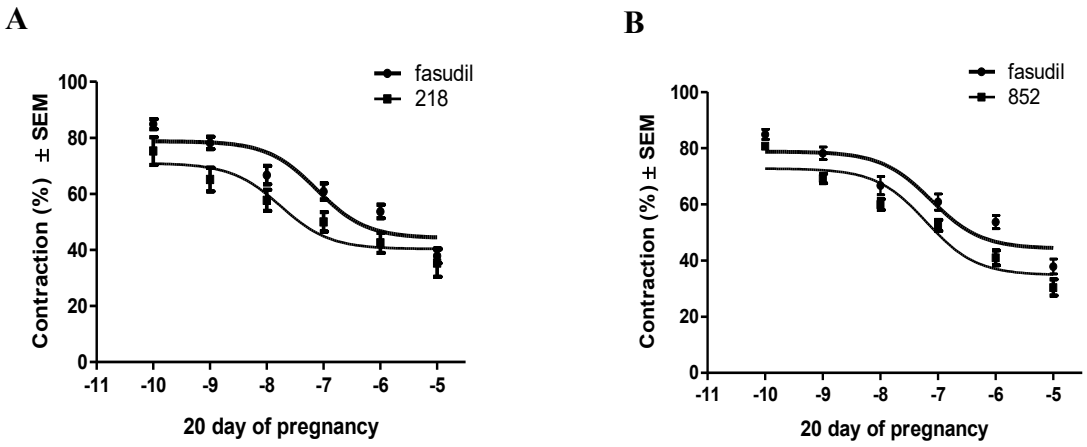

Figure 5. **The inhibitory effects of 218 (A) and 852 (B)** isoquinoline derivatives on the 20th-day pregnant rat uterus. S.E.M standart error

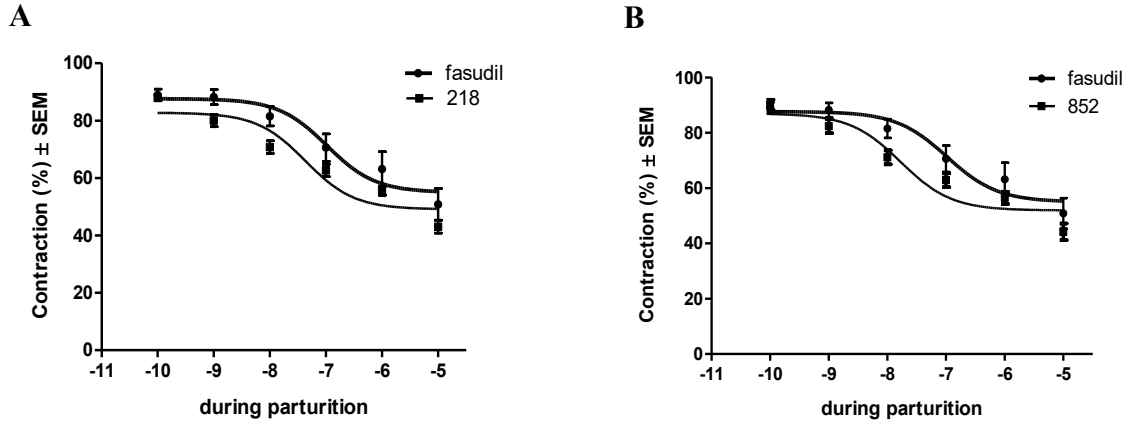

Figure 6. **The inhibitory effects of 218 (A) and 852 (B)** isoquinoline derivatives during parturition in rat uterus. . S.E.M standart error

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

The mRNA expression of RhoA did not change markedly during pregnancy and postpartum (**Fig11 A**). The protein level increased the first day after delivery. (**Fig. 7 B).**

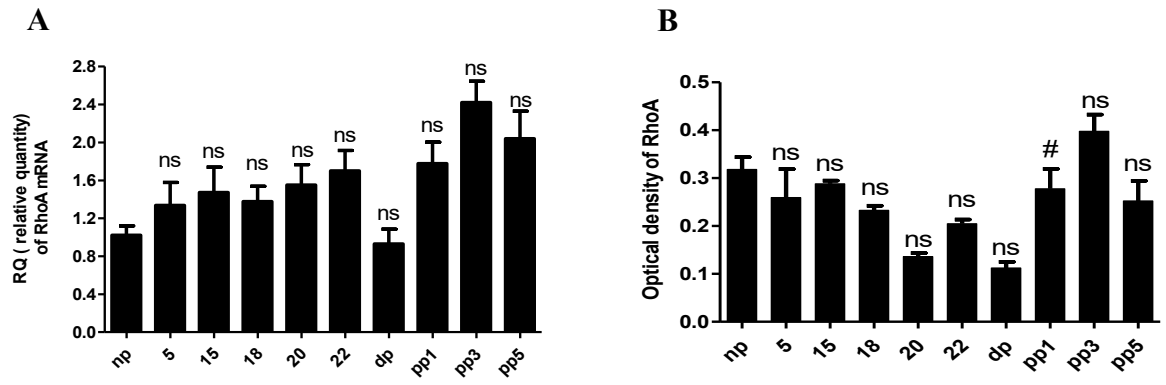

Figure 7. The mRNA (A) and protein expression (B) of RhoA in non-pregnant rat cervix (*np*), on different days of pregnancy, during delivery (*dp*) and on postpartum days (*pp1, pp3, pp5*). *ns: non-significant*; *: $p<0.05$.

No significant change was found in mRNA expression of ROCK I and ROCK II till pregnancy day 20. On pregnancy day 22, a sharp increase was measured in the ROCK I mRNA level. A marked decrease was observed in the mRNA expression of ROCK I and ROCK during labour compared with day 22. On postpartum day 1, the mRNA levels of Rho-kinases were raised again and remained high till post-partum day 5 (**Fig. 8 and 9 A**).

The protein level of ROCK I reduced on pregnancy day 15, then remained unchanged till delivery. A marked increase was detected on pregnancy day 22 then decreased in parturient cervices (**Fig. 8 B**). The alteration of the protein expression of ROCK II followed the same pattern of mRNA alterations (**Fig. 9 B).**

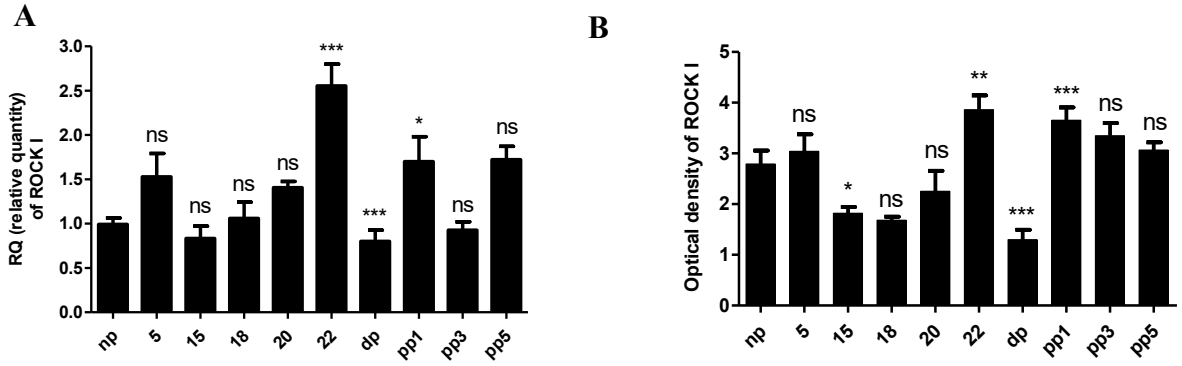

Figure 8. **The mRNA (A) and protein expression (B) of ROCK I** in non-pregnant rat cervix (*np*), on different days of pregnancy, during parturition (*dp*) and on postpartum days (*pp1, pp3, pp5*). ns: non-significant; *: p<0.05; **: p< 0.01; ***: p< 0.001.

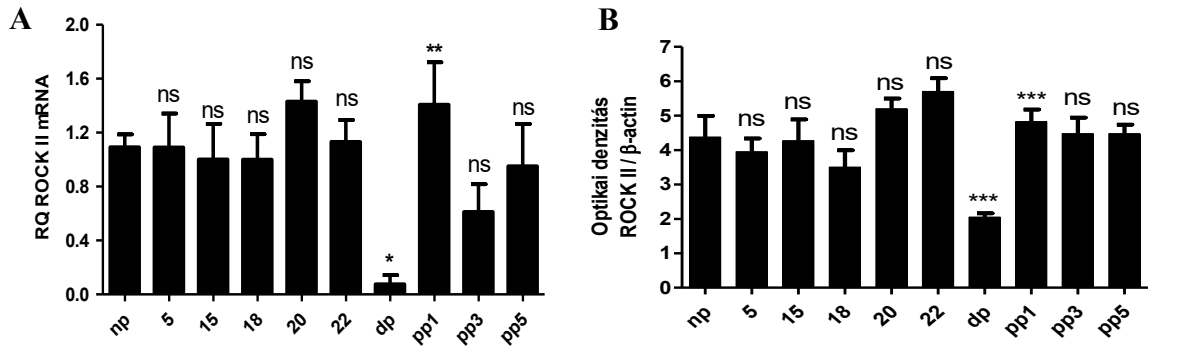

Figure 9. **The mRNA (A) and protein expression (B) of ROCK II** in non-pregnant rat cervix (*np*), on different days of pregnancy, during parturition (*dp*) and on postpartum days (*pp1, pp3, pp5*). ns: non-significant; *: p<0.05; **: p< 0.01; ***: p< 0.001.

Effects of RhoA inhibitor simvastatin and Rho-kinase inhibitors Y-27632 and fasudil for cervical resistance

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.

Simvastatin enhanced the cervical resistance in days 20 and 22 of pregnancy compared with the non-treated values (**Fig.10**).

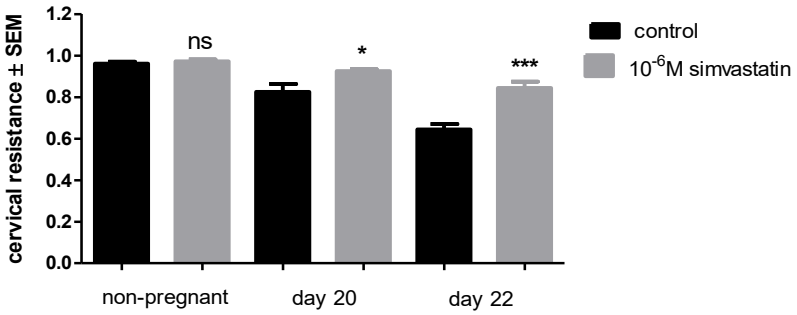

Figure 10. Effects of simvastatin on cervical resistance in the non-pregnant, 20- and 22-day-pregnant cervices. ns: non-significant; *p< 0.05;***: p < 0.001. Each bar denotes mean ±S.E.M. (standard error).

The Rho-kinase inhibitor Y-27632 decreased the resistance of the 20-day pregnant cervix, but did not alter the non-pregnant and 22-day pregnant cervical resistances (**Fig. 11**).

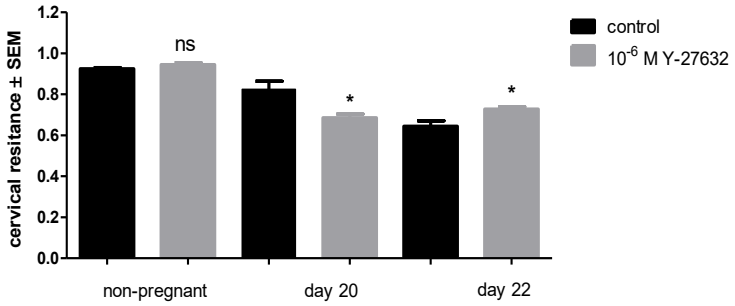

Figure 11.. **Effects of Y-27632 on the resistances** of the non-pregnant, 20- and 22-day pregnant cervices. ns: non-significant; *p < 0.05. Each bar denotes mean ±S.E.M. (standard error).

Fasudil reduced the cervical resistances in days 20 and 22 of pregnancy (**Fig. 12**).

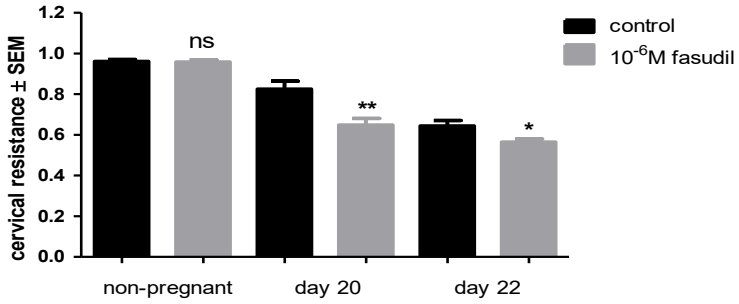

Figure 12. **Effects of fasudil on the resistances** of the non-pregnant, 20- and 22-day pregnant cervices. ns: non-significant; *p< 0.05; **p< 0.01. Each bar denotes mean ±S.E.M. (standard error).

Discussion

Activation of RhoA and Rho-associated kinases is known to play a pivotal role in the regulation of smooth muscle contraction. Inhibition of the RhoA and ROCKs might result in the uterine relaxation, therefore they can be possible targets for tocolytic therapy.

The Real time PCR analysis show the mRNA level of RhoA and ROCKs decreased in the 5th day pregnant uterua, which is the time of the implantation period. It was determined that the human uterine contraction is decreased at the time of blastocyst transfers. 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 during pregnancy. 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. The high levels of RhoA and Rho-kinases at the onset of labour proposing their contribution to enhanced contractility and the initiation of labour. The mRNA and protein level of RhoA markedly decrease at term in the cervix which is concordant with the result that RhoA inactivation prevents collagen-I synthesis and contributes to the smooth muscle relaxation. Furthermore, the ROCK I and ROCK II levels also declined at term suggesting their impact on the final cervical ripening and delivery process. 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. We tested the effects of the RhoA inhibitor simvastatin. Statins have a cholesterol-independent „pleiotropic” effect, they are able to inhibit the synthesis of isoprenoids, which are necessary for membrane translocation and activation of small GTPases RhoA. 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. 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.

The cervical resistance was increased by simvastatin on pregnancy days 20 and 22, when the RhoA 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). It was 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. The ROCK inhibitors fasudil, Y-27632, and RKI 1447 act as competitors against ATP to bind to the catalytic sites in enzymes resulting in smooth muscle relaxation. 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. 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. Fasudil is able to suppress the collagen secretion and increased the collagenase activity in hepatic stellate cells. It was found that Y-27632 inhibits the voltage dependent potassium (Kv) channels and reduces the muscle tone in vascular smooth muscle. But it was proved that Y-27632 has a collagen synthesis inhibitory effect. These abilities may play an important role in the decreased cervical resistance on pregnancy day 20. 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.

Isoquinolines relaxed the smooth muscles e.g. drotaverine hydrochloride reduces the duration of labour by affecting cervical dilation, while papaverine decreases the tonus and the contractile activity of smooth muscles. We measured the efficiency of 11 isoquinoline molecules for ROCK II activity and 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.

We conclude that ROCKs are suppressed during pregnancy in uterine tissue to maintain relaxed state of the uteri. The higher levels of Rho-kinase in cervical tissues till delivery may contribute to closed position of cervix. 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. Furthermore, the isoquinoline structure can be a promising drug candidate for the development of effective inhibitors of Rho-kinases.

Publications related to the Ph.D. thesis

1 Domokos D., Ducza E., Falkay G. 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.

2 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. IF: 2,478

3 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. IF: 2,442

4 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. IF: 3,04

Abstracts related to the Ph.D. thesis

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.

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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.