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**RhoA and Rho-kinase inhibitors modulate cervical resistance: the possible role of RhoA/Rho-kinase signalling pathway in cervical ripening and contractility**

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**Abstract**

Improper cervical function may lead premature or late-term birth. The RhoA/Rho-kinase (ROCK) signalling pathway takes part in cellular functions including smooth muscle contraction. No information is available about the cervical expression of the RhoA/ROCK system during pregnancy. Our aim was to detect the mRNA and protein expression of ROCK

enzymes in rat cervixes and to evaluate the effects of RhoA/ROCK inhibitors on cervical resistance.

The mRNA and protein expressions of RhoA, ROCK I and II were measured in non-pregnant, pregnant and postpartum rat cervixes and during parturition by Real-time qPCR and Western blot. The cervical resistance modifying effects of RhoA (simvastatin) and ROCK (fasudil, Y-27632) ( $10^{-6}$ M) were investigated in tissue bath experiments.

RhoA mRNA was increased on post-partum day 3, while the RhoA protein expression was decreased near and during parturition. ROCK I mRNA and protein expressions were fluctuating with a decrease in protein expression during parturition. ROCK II mRNA and protein expressions were sharply reduced during parturition. Simvastatin increased the cervical resistance on pregnancy days 20 and 22 while Y-27632 and fasudil reduced the resistance on pregnancy days 20.

The decrease in RhoA/ROCK expression near parturition may take part in cervical ripening, especially in the final processes leading to delivery. ROCK inhibitors might be potential drug candidates to treat insufficient cervical ripening late-term pregnancies. The effect of simvastatin possibly due to its unique smooth muscle contracting activity in pregnant cervix. Compounds with simvastatin-like action might be new drug candidates for preterm cervical ripening.

Key word: cervical ripening, RhoA/ROCK pathway, pregnancy, simvastatin, fasudil, pregnancy

## 1. Introduction

The cervix undergoes extensive changes during pregnancy. This remodelling is driven by several factors including the alteration of the hormonal (Stjernholm et al., 1996) and

prostaglandin (Hertelendy and Zakar, 2004) levels, the decreased collagen concentration by the activation of matrix metalloproteinases (Ludmir and Sehdev, 2000) and cytokines (interleukin-1 $\beta$  and interleukin-8) (Winkler et al., 1998). At the end of the gestational period, the cervix is softened, shortened and dilated (Chwalisz and Garfield, 1997) contributing to the parturition in parallel with the increase in myometrium contractions. Not only premature myometrial contraction but also early cervical ripening can be responsible for premature labour (Vink et al., 2016). On the other hand, delayed or insufficient cervical ripening can be one of the reasons for post term delivery and caesarean birth especially in obese mothers (Carlson et al., 2015).

Ras homologue protein A (RhoA) is a monomer GTP-binding protein belonging to the Rho subfamily of the Ras superfamily (Ridley, 2001). Some proteins that have been identified as interacting directly with RhoA include mDia, rhotekin, citron kinase and Rho-kinases (Bustelo et al., 2007). Rho-associated coiled-coil containing kinase (ROCK) is the main effectors of RhoA (Shimokawa and Takeshita, 2005). There are two isoforms of Rho-kinase: ROCK I, and ROCK II (Fukata et al., 2001). The RhoA/Rho-kinase signalling pathway takes part in several cellular functions including cell adhesion, proliferation, motility and migration (Wettschureck and Offermanns, 2002) smooth muscle contraction (Kimura et al., 1996) and stress fibre formation (Kawano et al., 1999). The GTP-binding RhoA activates the Rho-kinases. These kinases phosphorylate the myosin phosphatase and myosin light chain. These processes together are responsible for smooth muscle contraction (Kaneko-Kawano et al., 2012). Pregnancy reduces the role of the RhoA/ROCK system in rat uterine artery contractions. The irregularity of this system may be one of the reasons for vascular-origin disorders during pregnancy including preeclampsia (Goulopoulou et al., 2012). 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 is also a part of 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.

In our previously published paper we found a significant increase in RhoA and ROCK expressions during labour. Both RhoA and ROCK inhibitors had an inhibitory action on myometrial contractions that makes them potential candidates for the treatment of premature contractions (Domokos et al, 2017). However, no information is available about the cervical expressions and pharmacological responsiveness of RhoA, ROCK I and ROCK II during pregnancy.

Our aim was to detect the mRNA and protein expression of RhoA, ROCK I and ROCK II in non-pregnant, pregnant, parturient and in post-partum rat cervixes by Real-time qPCR and Western blot analysis. Furthermore, we investigated the role of the RhoA/Rho-kinase pathway in cervical resistance using RhoA inhibitor (simvastatin) and Rho-kinase inhibitors (Y-27632 and fasudil) in non-pregnant, 20- and 22-day pregnant rat cervixes.

## 2. Material and methods

### 2.1. Ethical approval

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

### 2.2. Housing, handling and mating of the animals

Sprague-Dawley rats (Charles-River Laboratories, Budapest, Hungary) were kept under standard conditions (temperature:  $22\pm 3^{\circ}\text{C}$ , 12 h light/12 h darkness cycle; relative humidity:

30-70%), Standard rodent pellet diet (Charles-River Laboratories, Budapest, Hungary) with tap water were available *ad libidum*. The female (180-200 g) and male (240-260 g) rats were mated in a special mating cage. A timer controlled, movable metal door separated the spaces for the female and male rats. The door was opened before dawn (4.00 a.m.). Within 4-5 h after the possibility of mating, vaginal smears were taken for sperm searching under a microscope at a magnification of 1200. If the search proved positive, the female rats were separated as 1<sup>st</sup> day pregnant animals.

### 2.3. Collection of cervical tissues for RT-PCR and Western blot studies

The non-pregnant, pregnant and postpartum rats were terminated by CO<sub>2</sub> inhalation, while the foetuses were terminated by cervical dislocation. Cervical tissues were removed from non-pregnant, pregnant (gestational days 5, 15, 18, 20 and 22) and postpartum (1, 3 and 5 days after delivery) animals. During labour the cervixes were dissected always after delivery of the 3<sup>rd</sup> pup).

### 2.4. Real-time quantitative RT-PCR study

The cervical samples were 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 total RNA isolation. 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  $\beta$ -*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.

## 2.5. Western blot analysis

Cervical samples were stored at -70°C until Western blot studies. Cervices were powdered with a Sartorius Mikro Dismembrator 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% 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 monoclonal 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.

## 2.6. Cervical resistance studies

Freshly removed whole cervixes from non-pregnant (oestrus phase), 20- and 22-day pregnant rats were cut into two cervical 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 CaCl<sub>2</sub>, 1mM MgCl<sub>2</sub>, 12mM NaHCO<sub>3</sub>, 4mM NaH<sub>2</sub>PO<sub>4</sub>, 6mM glucose, pH 7.4). The organ

bath was maintained under standard condition (37°C with carbogen - 95% O<sub>2</sub> + 5% CO<sub>2</sub>—bubbling). The rings were equilibrated for about 1 hour 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<sup>-6</sup>M and the cervixes were incubated further for 5 min. Then 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. 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, the physiological decrease in cervical resistance was detectable toward the end of pregnancy (pregnancy day 22) (**Fig. 1.A-C**) (Gaspar 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 ± S.E.M. For statistical evaluations, data were analysed by one-way ANOVA Tukey's test.

### 3. Results

#### 3.1. mRNA and protein expression

Real-time qPCR and Western blot analysis revealed that RhoA and Rho-kinases are expressed in rat cervixes. The mRNA levels of RhoA were not altered till postpartum day 1 including the parturition as well. However, they were increased on postpartum day 3 as compared with the non-pregnant cervix and remained high on postpartum day 5 (**Fig. 2A**). The protein levels of RhoA were unchanged till pregnancy day 18. On pregnancy day 20, a significant decrease



was found as compared with the non-pregnant level. Such a same low level was also detected during parturition. In the postpartum period, the protein expression of RhoA slightly increased as compared with the parturient cervix (**Fig. 2B and C**).

In case of ROCK I, no change was found in mRNA expression till pregnancy day 20. On day 22 of pregnancy, a sharp increase was detected that was reduced during labour. Interestingly, on postpartum day 1, the mRNA level was raised again but this elevation was not significant as compared with the non-pregnant value. The mRNA expression was remained unchanged till post-partum day 5 (**Fig. 3A**). The protein expression of ROCK I decreased on pregnancy day 15 as compared with day 5, while a further decrease was measured on day 18 that was significant as compared even with the non-pregnant cervix. A significant increase was detected on day 22 as compared with pregnancy day 20. The protein expression of ROCK I was markedly decreased in parturient cervixes. On postpartum day 1 the protein level increased as compared with the expression during parturition. No further change was detected till postpartum day 5 (**Fig. 3B and C**).

The mRNA level of ROCK II did not change markedly from non-pregnant cervix until pregnancy day 22, then a sharp decrease was found during parturition. On postpartum day 1, the mRNA expression was sharply increased to the level of non-pregnant and 22-day pregnant cervixes (**Fig. 4A**). The alteration of the protein expression of ROCK II followed the same pattern than that of mRNA alteration (**Fig. 4B and C**).

### 3.2. Cervical resistance studies

The cervical resistances were continuously decreased towards the end of the gestational period. The RhoA inhibitor simvastatin did not alter the resistance of non-pregnant cervixes 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. The cervical resistance-increasing effect of the compound was very strong on the last day of pregnancy (**Fig.5**).

Similarly, to simvastatin, the ROCK inhibitor Y-27632 did not modified the cervical resistance in non-pregnant samples. However, it decreased the resistance of the 20-day pregnant cervix but did not alter the 22-day pregnant cervical resistance as compared with the non-treated samples (**Fig. 6**). The other ROCK inhibitor fasudil did not alter either the non-pregnant or the 22-day pregnant resistance but reduced significantly the cervical resistance in days 20 pregnancy (**Fig. 7**).

#### 4. Discussion

Adequate condition of the cervix is important for keeping the foetus inside the uterus during pregnancy and are needed to facilitate delivery. The cervix consists of an extracellular matrix including collagen, elastin, proteoglycans; and a cellular part including smooth muscle, fibroblasts, epithelium and blood vessels. The ratios of these components are modulated by several factors during pregnancy and parturition, however the regulation of cervical maturation is not yet fully clarified (House et al., 2009).

The RhoA/ROCK signalling pathway plays an important role in the regulation of several cellular processes, mainly in smooth muscle contraction by phosphorylation of the myosin phosphatase and myosin light chain (Hirano et al., 2003). Recent studies of the monomer GTP-binding RhoA and the Rho-kinases have investigated their roles in cardiovascular diseases (Loirand et al., 2006), their contributions to the invasion of cancer cells and progression (Kamai et al., 2004; Lin et al., 2007; Yoshioka et al., 1999), and their activity in uterine contraction (Taggart et al., 2012; Tahara et al., 2002). However, no study has been conducted to measure their expression and function in cervical tissue in the rat, especially

during pregnancy. Therefore, we investigated the expression of RhoA/ROCKs in rat cervix on different days of pregnancy, in the post-partum period and in non-pregnant samples. The protein expression of RhoA was significantly reduced 2 days before and during delivery, but this kind of alteration was not reflected in the mRNA expression. We found similar discrepancy between mRNA and protein expressions in case of ROCK-I, but parallel alterations were detected in case of ROCK-II. It is obvious that the protein levels of ROCKs are significantly reduced during parturition suggesting their impact on the final cervical ripening and delivery process. Furthermore, the decreased expression level of RhoA during labour correlates to the lowest degree of cervical resistance and is concordant with the result that RhoA inactivation prevents collagen-I synthesis (Kondrikov et al., 2011). Within 1 day after delivery, the RhoA and ROCK protein expressions had recovered to the level of non-pregnant expressions. These alterations suggest that the higher level of RhoA/ROCK proteins may be important for the restoration of cervical tissue that mostly involves the restoration of collagen, a process that takes several weeks even in rodents (Nallasamy et al., 2017).

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 cervical resistance. We carried out our experiments on less time points as compared with RT-PCR and Western-blot studies. The application of only 3 time points (non-pregnant, and 20- and 22-day pregnant cervixes) can be justified by 2 reasons. On one hand, according to our earlier result the most characteristic drug actions can be detected after day 18 of the gestation period (Domokos et al., 2017; Gál et al, 2009). On the other hand, the isolation of cervix for smooth muscle contractility studies is very problematic even at the last day of pregnancy (day 22), but that is practically impossible during delivery because of the lack of proper consistency of the cervix for organ bath experiments. This is also true for the early phase of postpartum period.

This is a limitation of our study, but technically we were not able to gain cervical rings for contractility studies during parturition.

Statins inhibit the geranylgeranylation and the membrane association of RhoA and furthermore, they can block the agonist induced RhoA activation (Takemoto and Liao, 2001). Interestingly, the results of the *in vitro* isolated organ bath studies showed that simvastatin significantly increased the cervical resistance on pregnancy days 20 and 22. The enhanced cervical resistance can be the result of the pleiotropic effect of statins that includes the inhibition of the secretion of matrix metalloproteinases (MMPs). MMPs have a major role in tissue destruction (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). In our experiments, the cervixes were incubated with simvastatin and the ROCKs inhibitors only for 1 h that may be too short time interval to modify MMP activity. More likely, simvastatin enhances the smooth muscle contraction in cervical tissue that leads to the increase in cervical resistance. However, this action might be independent from the RhoA-ROCK pathway and seems unique in the cervix, because simvastatin acts rather as a relaxing than a contracting agent in vascular and myometrial smooth muscles (Chen et al., 2016; Domokos et al., 2017).

Y-27632 and fasudil are potent non-selective ROCKs inhibitors that cause relaxation of smooth muscle by competing with ATP to bind to the catalytic sites in enzymes (Asano et al., 1989; Ishizaki et al., 2000). Both compounds decreased the resistance of 20-day pregnant cervixes but did not show such an activity on 22-day pregnant samples. Fasudil reduces the production of collagen while it enhances the collagenase activity in hepatic stellate cells (Fukushima et al., 2005) and these abilities might contribute to its cervical action.

Additionally, it has a relaxant effect on vascular and myometrial smooth muscle (Domokos et al., 2017; Shimomura et al., 2004). Y-27632 was shown to reduce the voltage dependent potassium (K<sub>v</sub>) channels and muscle tone in vascular smooth muscle (Li et al., 2016). This effect might contribute to the cervical resistance decreasing effect of the drug, because K<sub>v</sub> channel expressions have been proved in cervical smooth muscle (de Lera Ruiz and Kraus, 2015). But it seems more important that Y-27632 is a collagen synthesis inhibitor (Ding et al., 2012), and its action is even detectable after 30 min of incubation (Yang et al., 2014). That kind of quick action may have a crucial a role in the reduced cervical resistance found in our organ bath studies after pre-treatments with ROCK inhibitors. The fact that pregnant cervix loses its collagen content till labour also support our hypothesis that ROCK inhibitors reduce the collagen content quickly and therefore they have no action on cervical resistance on the last day of pregnancy because of the lack of cervical collagen.

Based on these results we suppose that the decrease in RhoA/ROCK expression near parturition may have a role in cervical ripening, especially in the final processes leading to delivery, and their quick raise in post-partum period indicates their function in cervical regeneration process. ROCK inhibitors further reduce the cervical resistance and they can be potential drug candidates to treat insufficient cervical ripening late-term pregnancies. The cervical resistance increasing effect of simvastatin seems to be independent from its RhoA-inhibitory action and possibly due to its unique smooth muscle contracting activity in pregnant cervix. Simvastatin is contraindicated during pregnancy, however, compounds with simvastatin-like action might be new drug candidates for preterm cervical ripening.

There are only few numbers of clinical trials, that evaluate the inhibition of Rho/Rho-kinase pathways using Rho-kinase inhibitors. Fasudil had a positive effect in preventing coronary artery spasm in patients with vasospastic angina (Masumoto et al. 2002), enhanced the forearm blood flow and reduced forearm vascular resistance in hypertensive patients

(Masumoto et al. 2001). Our results suggest that ROCK inhibitors can be potential drug candidates to treat insufficient cervical ripening, but their application in clinical practice can be recommended after proving their foetal and maternal safety.

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### **Author Contributions Section**

**Dóra Domokos:** carried out in vitro cervical resistance studies, prepared results and figures, took part in RT-PCR and Western blot studies, wrote the manuscript

**Eszter Ducza:** carried out RT-PCR and Western blot studies, prepared results and figures

**Robert Gaspar:** wrote the manuscript, supervised and organized the experiments

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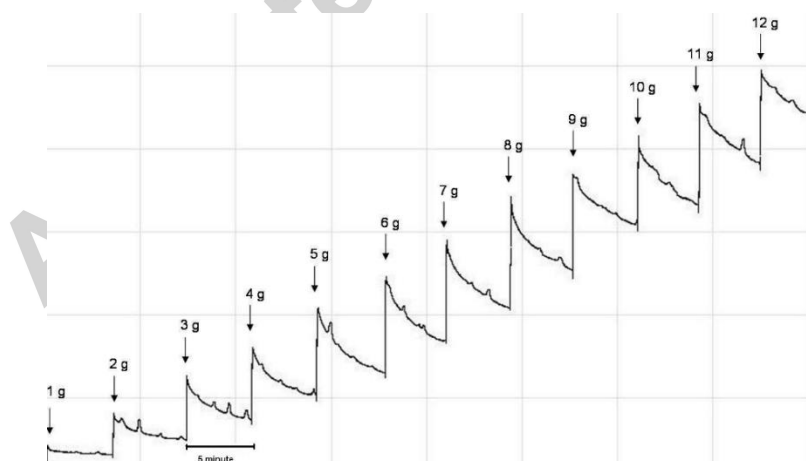
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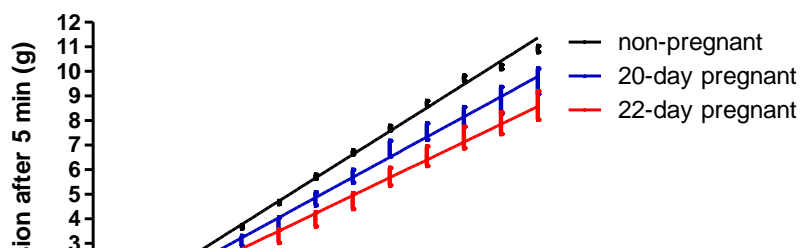
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**Figure 1**

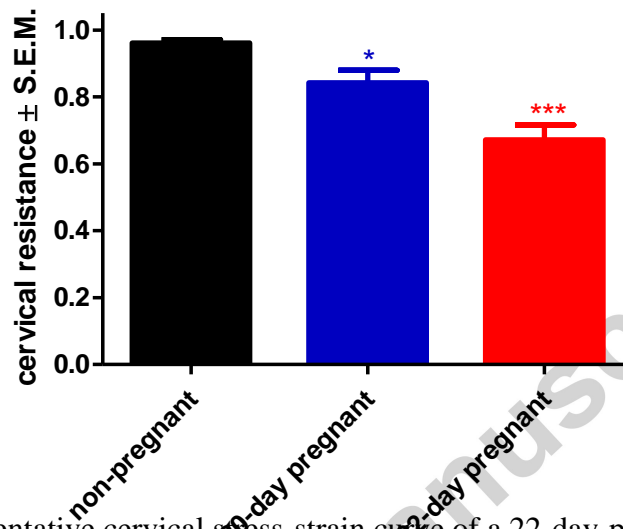
**A.**



**B.**



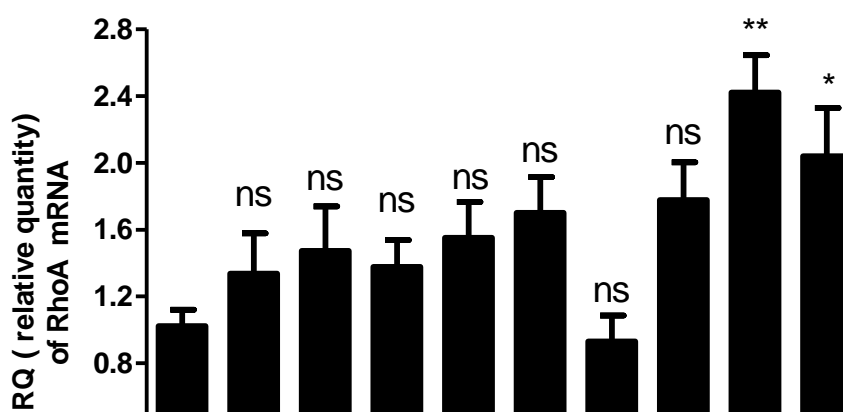
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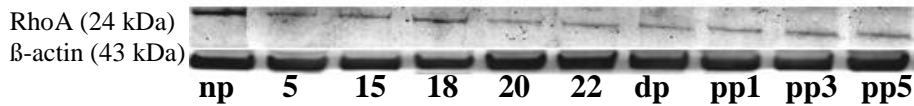
**Figure 1.A.** A representative cervical stress-strain curve of a 22-day-pregnant rat in vitro. The series of stretching and relaxation produced a saw-tooth shape record. **B.** The initial tensions were plotted against the tensions recorded after 5 min to get regression lines. The steepness of the slopes are in proportion with cervical resistance. **C.** The comparison of the cervical resistance of non-pregnant, 20-day and 22-day pregnant cervixes. \*:  $P < 0.05$ ; \*\*\*:  $P < 0.001$ . Each bar denotes mean  $\pm$  S.E.M. (standard error);  $n = 10$ . ANOVA, Tukey's test.

Figure 2

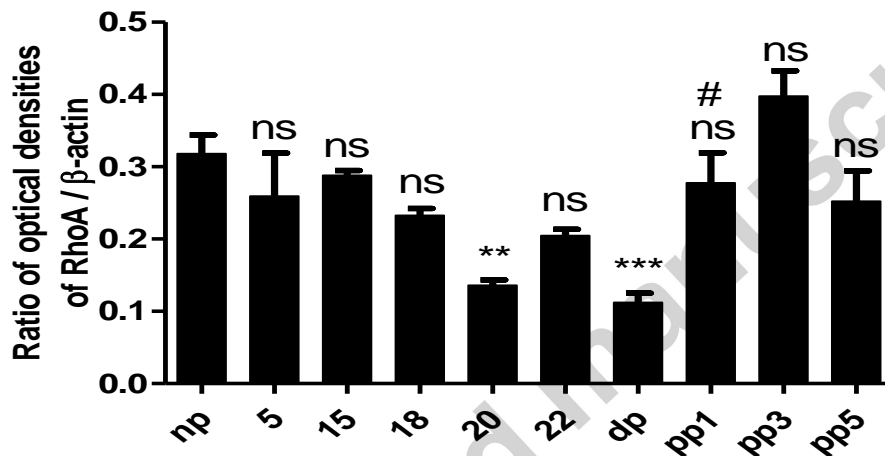
A.



B.



C.



**Figure 2.** The mRNA expression (A), a representative gel photo (B) and protein expression (C) 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  $\pm$  S.E.M. (standard error);  $n = 6$ . ANOVA, Tukey's test.

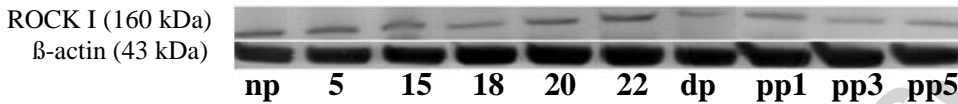
Figure 3



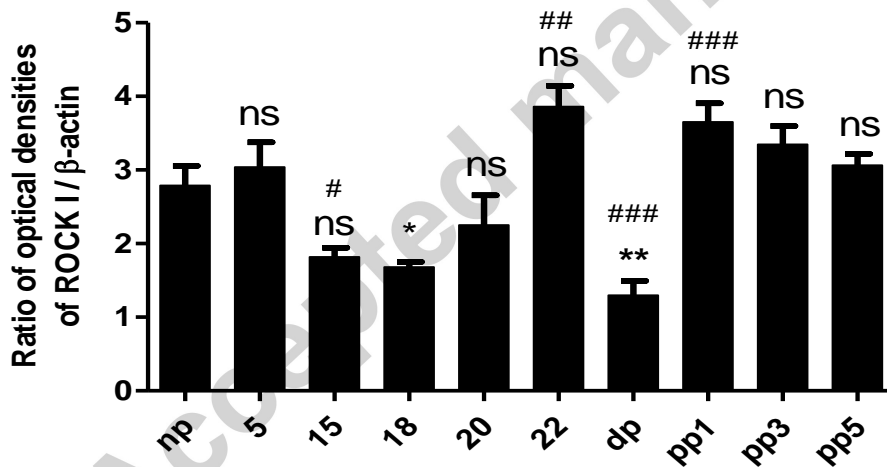


A.

B.



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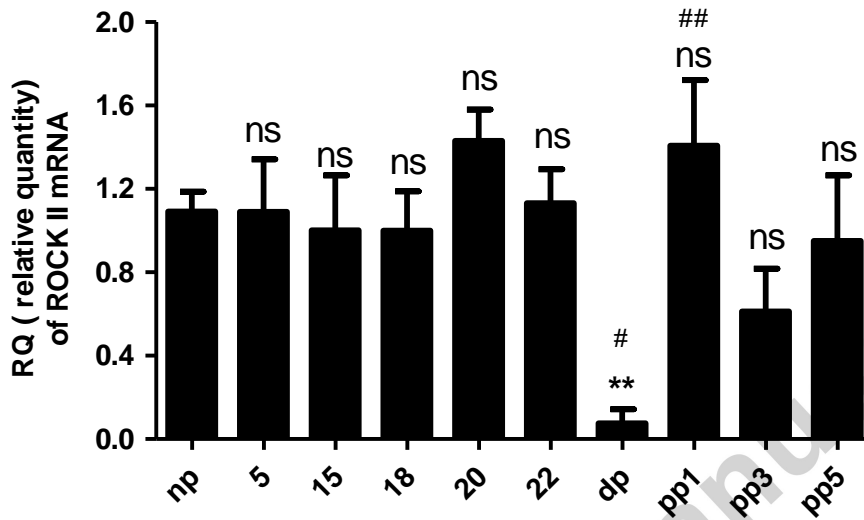


**Figure 3.** The mRNA expression (A), a representative gel photo (B) and protein expression (C) of ROCK I in non-pregnant rat cervix (np), on different days of pregnancy, during parturition (dp) and on postpartum days (pp1, pp3, pp5). The level of significance is given in two ways. Asterisks represent 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

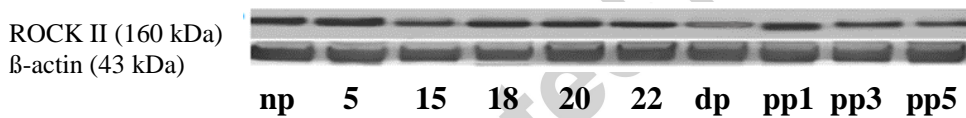
are not indicated. Each bar denotes mean  $\pm$  S.E.M. (standard error);  $n = 6$ . ANOVA, Tukey's test.

**Figure 4**

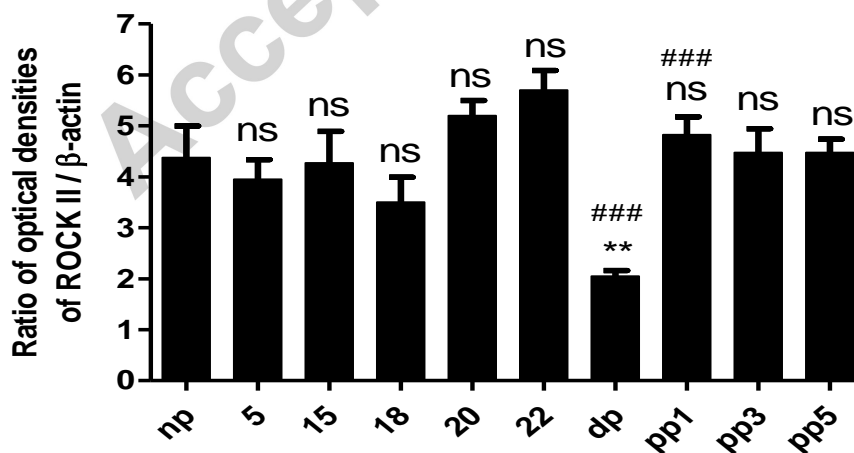
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**B.**



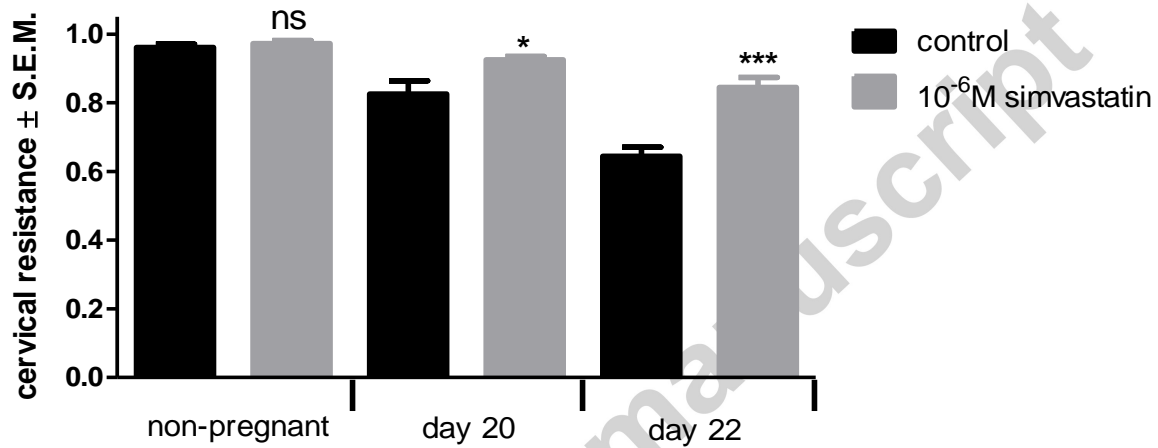
**C.**



**Figure 4.** The mRNA expression (A), a representative gel photo (B) and protein expression (C) of ROCK II in non-pregnant rat cervix (np), on different days of pregnancy, during

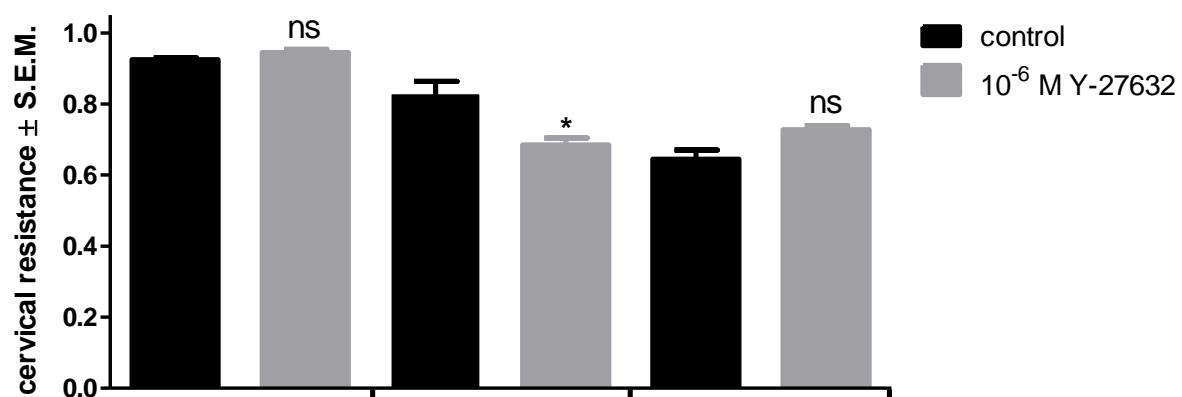
parturition (dp) and on postpartum days (pp1, pp3, pp5). The level of significance is given in two ways. Asterisks represent comparisons to data from non-pregnant cervix. ns: non-significant; \*\*:  $P < 0.01$ . Hashtags represent comparisons to the previous investigated days. #:  $P < 0.05$ ; ##:  $P < 0.01$ ; ###:  $P < 0.001$ ; non-significant changes are not indicated. Each bar denotes mean  $\pm$  S.E.M. (standard error);  $n = 6$ . ANOVA, Tukey's test.

**Figure 5**



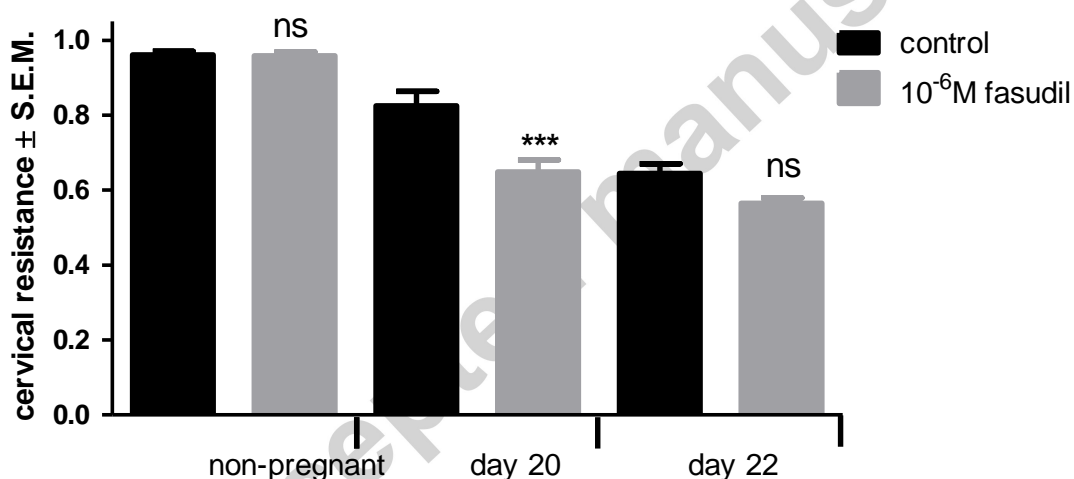
**Figure 5.** Effects of simvastatin on cervical resistance in the non-pregnant, 20- and 22-day pregnant cervixes. Simvastatin elicited significant increase in cervical resistance in pregnant rats. The significances are given as compared with non-treated (control data). ns: non-significant; \* $P < 0.05$ ; \*\*\*:  $P < 0.001$ . Each bar denotes mean  $\pm$  S.E.M. (standard error).  $n=10$ , ANOVA, Tukey's test.

**Figure 6**



**Figure 6.** Effects of Y-27632 on the resistances of the non-pregnant, 20- and 22-day pregnant cervixes. Y-27632 reduced the resistance only in 20-day pregnant cervixes. The significances are given as compared with non-treated (control) data ns: non-significant; \*P < 0.05. Each bar denotes mean  $\pm$ S.E.M. (standard error). n= 10 ANOVA, Tukey's test.

**Figure 7**



**Figure 7.** Effects of fasudil on the resistances of the non-pregnant, 20- and 22-day pregnant cervixes. Y-27632 reduced the resistance only in 20-day pregnant cervixes. The significances are given as compared with non-treated (control) data. ns: non-significant; \*\*\*P < 0.001. Each bar denotes mean  $\pm$ S.E.M. (standard error). n=10, ANOVA, Tukey's test.