Final report

The role of RhoA/ROCk and H2S in the regulation of pregnant rat uterine contractility and in development of new tocolytic agents

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Aims: Our project had several aims. We intended to investigate the ontogeny of RhoA/ROCK in pregnant rat myometrium and cervix to search for its role in gestation and delivery. We planned to investigate the effect of known and newly synthesized RhoA/ROCK inhibitors on in vitro myometrial contractions and the cervical resistance and on pregnant rats in vivo. Our other aim was to investigate the changes in uterine expressions of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) enzymes that are important for the endogenous production of hydrogen sulfide (H_2S) inducing relaxant action in smooth muscle. We also wanted to measure the effect of H_2S donor compounds on myometrial contractility and cervical resistance in rats.

Results and significance:

1. **Roles of RhoA and Rho-kinases in myometrial contractions and onset of labour during pregnancy in rats**

The RhoA/ROCKs expression were investigated 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. Considering that the period for embryonic implantation in rats is between pregnancy days 4-7 and the amount of RhoA is more pronounced in cytotrophoblast cells, we hypothesize that the decreased expressions of ROCK I and ROCK II in the rat myometrium might reduce the intensity of contraction, thereby protecting the implantation of the embryos.

The protein expressions reveal that RhoA remains unchanged from pregnancy day 5 till day 22, while ROCK proteins showed significant alterations between pregnancy days 5-22. 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, the compounds had stronger relaxing effect of pregnant uteri on days with higher ROCKs expressions. We also proved that the mRNA level of ROCK I, ROCK II and RhoA were sharply up-regulated in the rat uterus at the onset of labor. In parallel, we detected a sudden increase of protein expression of RhoA and ROCKs during labor. We hypothesized that the higher expressions of RhoA/ROCKs in non-pregnant (in estrus phase) and delivering uteri are related to an estrogen plasma peak in rats.

We also 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 around term.

The roles of RhoA and ROCKs in pregnant uterine contractions can be measured in contractility studies by applying their inhibitors. Simvastatin, a hydroxy-methyl-glutaryl- coenzyme A (HMG-CoA) inhibitor is also an inhibitor of RhoA. Simvastatin elicited a relaxing effect, although its action was not consequent on the expression of RhoA protein. These results suggest that the pregnant uterine relaxing action of simvastatin only partially depends on its RhoA inhibitory property and partially belongs to its pleiotropic action. We also investigated the effects of the non-selective ROCK inhibitors, fasudil, Y-27632, and RKI 1447. We merged the mRNA or protein expression data of ROCK I and ROCK II 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 a moderate relaxing effect on the non-pregnant uterus, while it elicited moderate relaxation on pregnancy day 22, at parturition and on postpartum day 1 with a similar IC50 values 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 as well. The more specific ROCKs inhibitors Y- 27632 and RKI 1447 showed a strong relaxing effect on non-pregnant uteri and during parturition, and weaker action on pregnancy day 22 and postpartum day 1 in parallel with the alteration expression of ROCK I and ROCK II. 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.

Normal pregnancy suppresses ROCKs, which contributes to the low contractility during pregnancy. A sharp increase of ROCKs at the onset of labor 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 signaling pathway might be a potential target for the development of new tocolytic agents.

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2. Uterine relaxing and ROCK inhibitory actions of newly synthetized isoquinoline derivatives in rats

We measured the efficiency of 25 original, newly synthesized isoquinoline derivatives for the Rho-kinase activity using Rho-associated kinase activity assay and determined their effects on the non-pregnant, 20-day pregnant and parturient rat myometrial contraction in vitro. The molecules were synthetized in the Institute of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Szeged by the team of Prof. Ferenc Fülöp, member of the Hungarian Academy of Sciences. The new compounds included 12 water-soluble and 12 non-water-soluble isoquinoline molecules with a basic structure of *N,N*-disubstituted 6,7-dialkoxy-phenylethylamine. As reference compounds we used Y-27632 (specific Rho-kinase inhibitor that binds to the catalytic site of both ROCK I and ROCK II and abolishes the kinase activities) and fasudil. (inhibitor of some protein kinases, e.g. PKC and MLCK, but it is more specific for

the Rho-kinase). Additionally, we also measured the relaxing activity of two well-known smooth muscle relaxants with isoquinoline structure, papaverine and drotaverine.

We found 11 isoquinoline molecules that have a lower IC_{50} values on the oxytocin-induced contraction in the non-pregnant rat uterus, but their maximal effects were weaker than those of both reference molecules. Papaverine had a higher maximum effect than Y-27632, while drotaverine had much higher IC_{50} values than our reference compounds on non-pregnant rat uterine contractions. Because both papaverine and drotaverine have a non-selective phosphodiesterase inhibitory effect and additionally block the calcium channels, their smooth muscle relaxing effects may be related in a minor way only to their ROCK inhibitory properties.

During the investigation of the ROCK II inhibitory effects of the isoquinoline molecules it was found that the inhibitory actions of two molecules (No. 218 and No.852) were like that of Y-27632, while the other 9 molecules were weaker activity.

We measured further the relaxing effects of the two most effective isoquinoline derivatives, 218 and 852, on day 20 of pregnant rat uterus and during parturition, when the significance of ROCK seems to be crucial in the process of labor. We found that 218 and 852 relaxed both the 20th-day pregnant and parturient rat uteri. Although these new derivatives do not exceed the maximum inhibitory effect of fasudil (their relaxing effect is equal to the reference molecule, which is a significant improvement in comparison with non-pregnant results), they elicit this action in one order of magnitude lower concentration compared to fasudil.

The majority of the synthesized isoquinoline derivatives have uterus relaxant effects and two of them significantly suppress the Rho-kinase mediated myosin light chain phosphorylation and have higher potency to relax the smooth muscle. These two new isoquinoline molecules may be considered as parent molecules for further development of Rho-kinase inhibitors. In the light of our results, we suggest that the isoquinoline structure has promising potential for the development of new and effective inhibitors of pregnant uterine contractions in preterm birth.

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3. The effects of RhoA and Rho-kinase inhibitors on cervical resistance in rats

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 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. 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.

For functional investigation of the roles of RhoA and ROCKs, we tested the effects simvastatin, Y-27632 and fasudil on cervical resistance. We carried out our studies only in 3 phases: non-pregnant, 20- and 22-day pregnant cervices because according to our myometrial result the most characteristic drug actions can be detected after day 18 of the gestation period. 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.

Simvastatin significantly increased the cervical resistance on pregnancy days 20 and 22. The enhanced cervical resistance can the result of the pleiotropic effect of statins that includes the inhibition of the secretion of matrix metalloproteinases (MMPs). 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 opposite as compared to myometrium.

Y-27632 and fasudil decreased the resistance of 20-day pregnant cervices but did not show such an activity on 22-day pregnant samples. Fasudil has a relaxant effect on vascular and myometrial smooth muscle while Y-27632 was shown to reduce the voltage dependent potassium (Kv) channels and muscle tone in vascular smooth muscle. Additionally, Y-27632 is a collagen synthesis inhibitor that is detectable after 30 min of incubation. 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 labor 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.

We suppose that the decrease in RhoA/ROCK expression near parturition has 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 are 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. Although simvastatin is contraindicated during pregnancy, but compounds with simvastatin-like action might be new drug candidates for preterm cervical ripening.

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4. The effects of RhoA and Rho-kinase inhibitors on uterine contractions in vivo

After the completion of the studying of in vitro effect of RhoA and ROCK inhibitors we launched an in vivo model to investigate their action on uterine contractility. Female, full term

pregnant (day 22 of pregnancy) Sprague-Dawley rats were anesthetized intraperitoneally with a combination of ketamine and xylazine solution. The jugular vein was cannulated for intravenous drug administration. A bipolar disk electrode pair was placed subcutaneously above the uterus. The abdominal skin was fixed wit surgical suture. The animals were then placed immediately onto a heated operating table to maintain the body temperature. The recorded signals were analyzed by fast Fourier transformation. The frequency of the electric activity was characterized in cpm, and the magnitude of the activity was described as maximum of power spectrum density. After recording the basal activity for 30 min, a series of doses (0.3;1.0; 3.0;10;30 mg/kg) from fasudil and Y-27632 were injected i.v. to rats in 10 min interval. In case of simvastatin, we gave a single oral dose of 50 mg /kg, and the uterine effect was detected after 30 min of administration. After the last dose and the measurement interval, blood plasma had been collected to determine the drug level in the animals by HPLC method.

We found that both fasudil and Y-27632 were reduced the pregnant uterine contraction in a dose dependent manner. Their maximum effects were about 60% decrease in uterine contraction, but the IC50 value of Y-27632 was a little bit lower than that of fasudil (0.97 mg/kg vs. 1.22 mg/kg). The single oral dose of simvastatin elicited approximately 60% reduction in uterine activity. The presence of compounds was proved from all the plasma samples having a concentration over the minimum effective limit.

These preclinical data suggest that RhoA- and ROCK-inhibitors have a quick and significant uterine relaxing effect that can be considered in the treatment of threatening preterm birth.

5. Uterine expressions of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) enzymes in non-pregnant and pregnant rats

The mRNA and protein expressions of H_2S synthetizing enzymes were also measured in non-pregnant, pregnant and postpartum rat uteri. In the myometrium we found that the mRNA expression of CBS in pregnant samples was unchanged till pregnancy day 20 as compared with the non-pregnant samples. On gestation day 20 we detected a 7-fold increase in the mRNA expression that remained high on day 22 and markedly dropped down during parturition and for the postpartum day. The protein expression in Western blot studies did not follow this pattern, we found the highest CBS expression on pregnancy day 5 and 9 (these two days were significantly higher as compared with non-pregnant uteri), but on later gestational days the expression of CBS was reduced and remained even low during and the postpartum period. In case of CSE, the mRNA expression was high on gestational days 5 and 9, but the later, including parturition and postpartum period, the expression did not exceed the level of non-pregnant samples. The myometrial protein expression of CSE was unchanged at any gestational days or postpartum days as compared with the non-pregnant condition.

In cervices, the mRNA expression of CBS remained unchanged till pregnancy day 20 as compared with non-pregnant cervix. On day 20 an almost 4-fold increase was found in the mRNA level that remained high during parturition and postpartum days 1 and 2. On postpartum day 3 a significant decrease was detected in the cervical expression of the mRNA of CBS. Contrary, in Western blot studies we found an elevation in the protein level of CBS on gestational day 18 and during parturition, but there was no significant alteration on the other days of the investigated period. In case of CSE, the RT-PCR investigations did not find any

significant changes in mRNA expressions of pregnant or postpartum samples as compared with non-pregnant cervices. However, the protein expression of CSE was almost 2-fold higher on pregnancy days 18 and 20, significantly reduced on day 22 and during parturition and raised again on postpartum day 1.

The mRNA and protein expressions of CBS and CSE were not in parallel each other and most of the cases the changes what we found seem to be irregular. It seems that the alterations of these enzymes have higher significance in the cervical tissue near term.

6. The effects of H_2S donors on the myometrial contractions and cervical resistance in pregnant rats

As the final step of our experiments within the project period, we investigated the myometrial and cervical actions of the H_2S donor GY4137 compound in our in vitro organ bath system. We investigated only the non-pregnant and the last day pregnant tissues as markedly different conditions of the uterus.

In myometrial contractility studies a concentration-response curve was fitted, the concentration range of GY4137 was between 10^{-10} and 10^{-4} M. In case of non-pregnant samples, the compound elicited a 53% relaxing effect with the EC50 value of $1.6x10^{-7}$ M. In pregnant samples the maximum relaxing effect of the compound was moderated (38%), but its EC50 value was much lower as compared with the non-pregnant uterus ($5.9x10^{-9}$ M) indicating the better sensitivity of late pregnant uterus towards the H_2S-induced relaxation.

In the cervical resistance study, we found that GY4137 did not alter the non-pregnant resistance in the cervix but elicited an extremely strong cervical resistance reducing action on the last day pregnant cervices. The resistance value for normal pregnant cervix was 0.85, while the GY4137 reduced it to 0.67). These findings suggest that although the H_2S donor molecules may have relaxing effect on the pregnant uterine contraction they can weaken the cervical resistance, thus their result may be very doubtful during preterm birth.

The influence of the changes in participants on the project

This project had dramatic and painful personal change because of the death of original principal investigator, Prof. György Falkay in September 2016, right at the beginning of the second year of the project. Because of his illness, the efficacy of the first-year activity was lower and after his death we needed a short time to continue the work. This sad event induced some delay in our work and reduced the speed of experimental work and publication, therefore some of our results are only submitted for publications and others still waiting for the completion of manuscript writings. We would like to dedicate our final report to the memory of Prof. György Falkay, who had established the reproductive pharmacological research in our team and created a scientific program for the investigation of compounds affecting pregnant uterine contractility.