University of Szeged

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The effects of female sexual hormones on α_1- and α_2- adrenergic receptor subtypes in the pregnant rat myometrium

Ph.D. Thesis Summary

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Szeged

2017

University of Szeged

Graduate School of Pharmaceutical Sciences

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Introduction

The adrenergic system and female sexual steroid hormones play an important role in the control of uterine contractility.

The α_1-adrenergic receptor (AR) family has 3 subtypes (α_{1A}, α_{1B}, α_{1D}). Stimulation of all 3 subtypes results in activation of the Gq/11 signalling pathway, involving activation of phospholipase C, generation of the second messenger inositol triphosphate and diacylglycerol, and mobilization of intracellular calcium, which leads to smooth muscle contraction. Although all three α_1-AR subtypes activate the same G-protein signalling pathway, their different tissue distributions suggest that they play distinct functional roles. It was demonstrated that the α_{1B}-ARs cannot be detected in the late-pregnant rat uterus therefore we focused on the α_{1A}- and α_{1D}-ARs.

The α_2-AR family has 3 subtypes too: α_{2A}, α_{2B}, α_{2C}. The differences in the receptor subtypes and their various localizations are thought to be responsible for their different roles. Stimulation of the myometrial α_{2A}- and α_{2C}-ARs leads to an increase in the cAMP level, and mediate only weak contractions, which can be regarded as relaxation as they are compared with the effect of noradrenaline (NA). Stimulation of the α_{2B}-ARs decreases the intracellular cAMP level and results in increased contractions.

The in vivo progesterone (P4) pretreatment increased the myometrial relaxing effect of the β_2-agonist terbutalin, and the synthesis of the β_2-AR. The effect of the β_2-AR agonists decreases at the end of pregnancy in consequence of the reduced plasma P4 level which results in a significant decrease in the amount of activated G-proteins coupled to β-ARs and the decreased amount of G-protein.

Aims

Although female sexual hormones have significant actions on the adrenergic receptors, no information has been available about their impact on the myometrial expressions and functions of α-AR subtypes. The main focus of our study was to investigate the role of the α-AR subtypes in the late pregnant rat uterus after sexual hormone pre-treatment. The following aims were set:

1. Investigation of the role of the α_1-AR and α_2-AR subtypes by subtype-specific antagonists after *in vivo* 17β-estradiol (E2) and P4 pre-treatment with isolated organ studies.
2. Our further aim was to identify of the myometrial α_1-AR and α_2-AR subtypes mRNA and protein expressions after female sexual hormone pretreatment by using RT-PCR and Western blot techniques in 22-day-pregnant rats.
3. Moreover, to investigate the changes of second messenger system of α_1-AR and α_2-AR after E2 and P4 pretreatment.

Materials and methods

All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (permission number: IV/198/2013). 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 (XXVIII. tv. 32.§).

Mating of the animals

Mature female (180-200 g) and male (240-260 g) Sprague-Dawley rats were mated in a special mating cage in the early morning hours. Within 4-5 h after the possibility of mating, copulation was confirmed by the presence of a copulation plug or vaginal smears. In positive cases, the female rats were separated and regarded as first-day-pregnant animals.

In vivo sexual hormone treatments of the rats

Pretreatment of the pregnant animals with E2 was started on day 18 of pregnancy. The animals were injected subcutaneously with 5 μg/kg of E2 once a day for 4 days.

The P4 pretreatment of the pregnant animals was started on day 15 of pregnancy. P4 was injected subcutaneously every day up to day 21 in a dose of 0.5 mg/0.1 ml.

E2 and P4 were dissolved in olive oil. On day 22, the uterine samples were collected, and contractility and molecular pharmacological studies were carried out.

RT-PCR studies

RT-PCR studies were carried out on 22-day-pregnant rat uterus preparations (n=6-8 in each experiment). Total cellular RNA was isolated by extraction according to the procedure of Chomczynski and Sacchi (1987). Reverse transcription and amplification of the PCR products were performed by using the TaqMan RNA-to-C_T^{TM} Step One Kit and an ABI StepOne Real-Time cycler. The amplification of β-actin served as an internal control. All samples were run in triplicate. The fluorescence intensities of the probes were plotted against PCR cycle numbers. The amplification cycle displaying the first significant increase in the fluorescence signal was defined as the threshold cycle (C_T).

Western blot analysis

Sample proteins was subjected to electrophoresis on NuPAGE Bis-Tris Gel in XCell SureLock Mini-Cell Units. Proteins were transferred from gels to nitrocellulose membranes using the iBlot Gel Transfer System. Antibody binding was detected with the Western Breeze® Chromogenic immunodetection kit. Images were captured with the EDAS290 imaging system, and the optical density of each immunoreactive band was determined with Kodak 1D Images analysis software. Optical densities were calculated in arbitrary units after local area background subtraction.

Isolated organ studies

Uteri were removed from rats (250-350 g) on day 22 of pregnancy (n=8-12 in each experiment). Muscle rings were sliced from both horns of the uterus and mounted vertically in an organ bath. The initial tension of the preparation was set to ~1.5 g, which had relaxed to ~0.5 g by the end of the equilibration period. The tension of the myometrial 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).

Contractions were elicited with NA (10^{-8} to 10^{-5} M) and cumulative concentration-response curves were constructed in each experiment in the presence of propranolol (10^{-5} M) and yohimbine (10^{-6} M) in order to exclude β-adrenergic and α_2-adrenergic action. The selective α_{1A}-AR subtype antagonist WB 4101, the α_{1D}-AR subtype antagonist BMY 7378 (each 10^{-7} M), propranolol and yohimbine were left to incubate for 5 min before the administration of

contracting agents. In α_2-ARs studies the selective α_{2A}-AR subtype antagonist BRL 44408, the $\alpha_{2B/C}$-AR subtype antagonist ARC 239, α_{2C}-AR subtype antagonist spiroxatrine (each 10^{-7} M), propranolol and doxasosine were left to incubate for 20 min before the administration of contracting agents. Following the addition of each concentration of NA, recording was performed for 300 s.

Concentration-response curves were fitted and areas under curves (AUCs) were evaluated and analysed statistically with the Prism 4.0 (Graphpad Software Inc. San Diego, California, USA) computer program. E_{max} and EC_{50} values were calculated from the AUC values. Statistical evaluations were performed by using the ANOVA Dunnett test or the two-tailed unpaired t-test.

Measurement of uterine cAMP accumulation

Uterine tissue samples from 22-day-pregnant rats were incubated in an organ bath. 3-isobutyl-1-methylxanthine (IBMX) (10^{-3} M), doxazosin (10^{-7} M), propranolol (10^{-5} M) and the investigated subtype-selective α_2-AR antagonists (each 10^{-7} M) were incubated with the tissues for 20 min, and NA (3×10^{-6} M) were added to the bath for 10 min. At the end of the NA incubation period, forskolin (10^{-5} M) was added for another 10 min. Uterine cAMP accumulation was measured with a commercial cAMP Enzyme Immunoassay Kit (Cayman Chemical, USA).

[^{35}S]GTPγS binding assay

WB 4101, BMY 7378, BRL 44408, ARC 239 and spiroxatrine were used in a fixed concentration of 0.1 μM. NA was added to increasing concentrations (10^{-9}–10^{-5} M). For the blocking of β-ARs, propranolol and α_1-AR antagonist, doxasosin or α_2-AR antagonist, yohimbine were used in a fixed concentration of 10 μM. G_i protein was inhibited with pertussis toxin in a concentration of 500 ng/ml.The radioactivity of the dried filters was detected in UltimaGold^{TM} F scintillation cocktail with a Packard Tricarb 2300TR liquid scintillation counter.

Results

Effects of 17β-estradiol and progesterone pretreatment on the myometrial the function of α_1-adrenergic receptor subtypes

The myometrial mRNA and protein expressions of the α_{1D}-adrenergic receptors after 17β-estradiol or progesterone pretreatment

In the case of the α_{1D}-AR subtype mRNA, neither the E2 nor the P4 pretreatment changed the mRNA expression. After E2 pretreatment, the expression of the α_{1A}-AR subtype mRNA was significantly decreased (**Fig. 1a**), whereas there was no change after P4 pretreatment. The results of Western blot analysis at the protein expression level reinforced the PCR results (**Fig. 1b**).

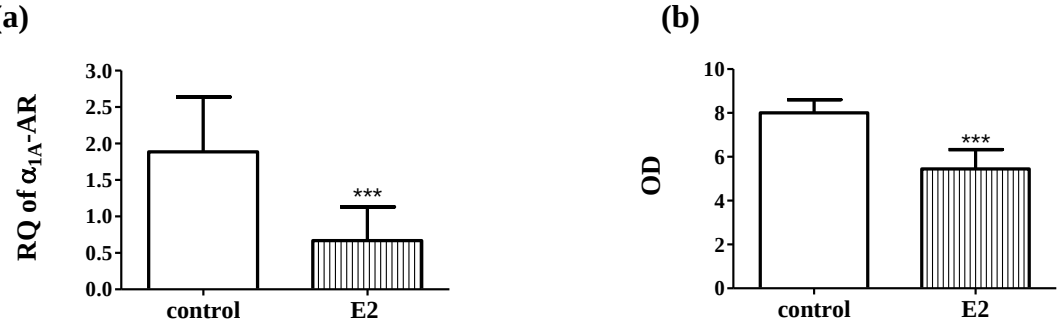


Fig. 1. Changes in the myometrial mRNA and protein expressions of the α_{1A}-ARs after E2 pretreatment **(a, b)** in 22-day-pregnant rat uteri. ***p<0.001(RQ:relative quantity)

Effects of α_1-AR subtype antagonists on the 22-day pregnant myometrial contractions

In the 22-day-pregnant myometrium, NA increased the myometrial contractions concentration-dependently (10^{-8}-10^{-5} M). After E2 pretreatment, the NA concentration-response curve was shifted to the right, and there was a moderate decrease in the myometrial contracting effect of NA. After P4 pretreatment, the maximum contractile effect of NA was significantly decreased (**Fig. 2a**). In the presence of the α_{1A}-AR antagonist (WB 4101) or α_{1D}-AR antagonist (BMY7378) the NA concentration-response curve was shifted to the right. In the presence of WB 4101 after E2 pretreatment, the NA concentration-response curve was further shifted to the right, while the maximum contracting effect remained unchanged. The P4 pretreatment reduced the maximum contractile effect of NA to one third in the presence of the α_{1A}-AR antagonist (**Fig. 2b**).

In the presence of BMY 7378, the NA concentration-response curve was shifted to the right relative to the control. After E2 pretreatment, the maximum contractile effect of NA was reduced, this being more marked after P4 pretreatment. The E2 treatment also shifted the EC_{50} value to the right (**Fig.2c**).

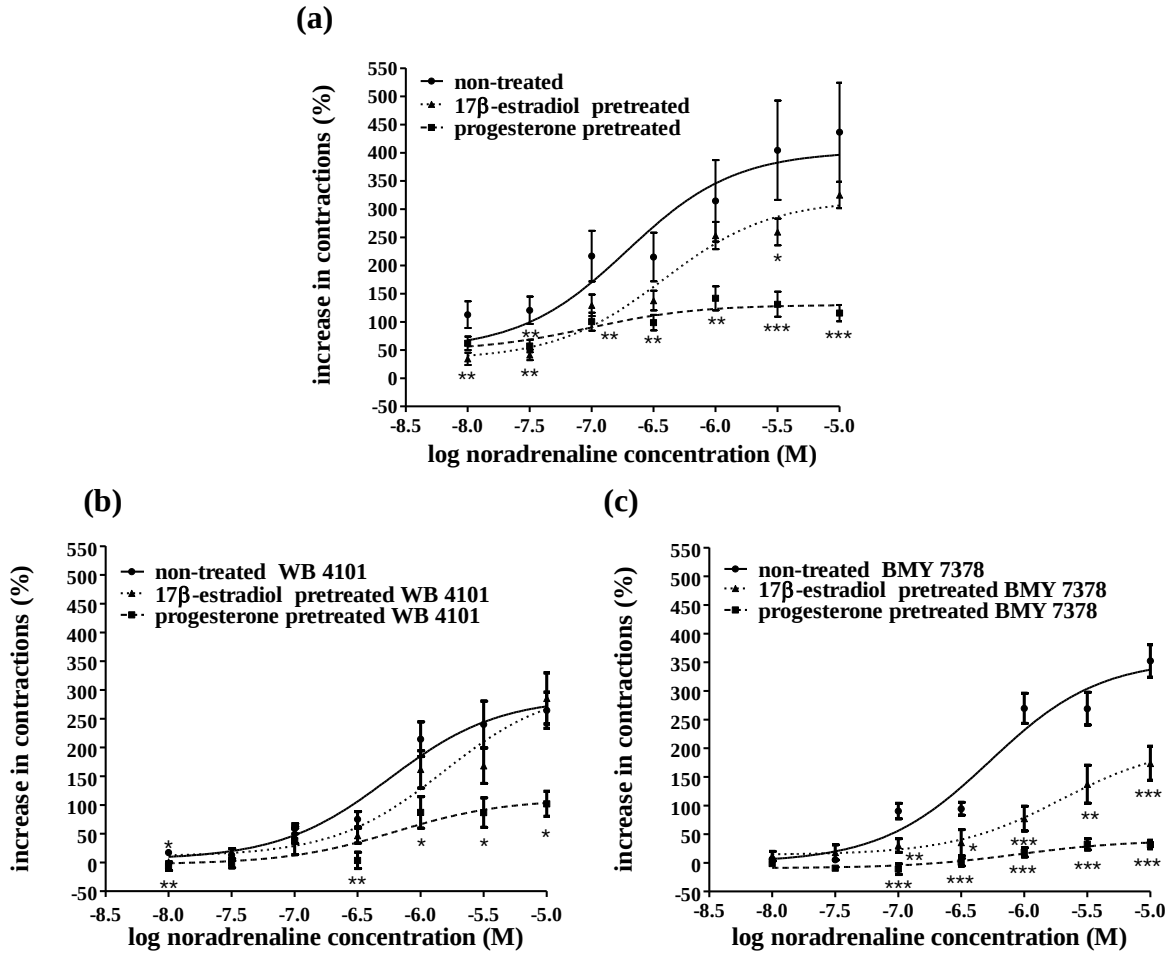


Fig.2. Effects of the subtype-selective α_{1A}-AR antagonist WB 4101 and the α_{1D}-AR antagonist BMY 7378 on the NA-evoked contractions in the 22-day-pregnant rat myometrium after P4 or E2 pretreatment. The studies were carried out in the presence of the β-AR antagonist propranolol (10^{-5} M) and the α_2-AR antagonist yohimbine (10^{-6} M) in each case and in the absence of α_1-antagonists **(a)** or in the presence of WB 4101 **(b)** or BMY 7378 **(c)** in an isolated organ bath. *p<0.05; **p<0.01; ***p<0.001

Effects of subtype-selective α₁-adrenergic receptor antagonists on myometrial [^{35}S]GTPγS binding level in the absence or presence of pertussis toxin

In the presence of WB 4101, NA slightly increased the [^{35}S]GTPγS binding (**Fig. 3a**). P4 pretreatment enhanced the extent of [^{35}S]GTPγS binding compared with the non-treated value. In the presence of BMY 7378, NA slightly stimulated the [^{35}S]GTPγS binding, while after P4 pretreatment, NA caused a noteworthy increase in [^{35}S]GTPγS binding (**Fig. 3b**).

In order to distinguish the G protein-mediated signal transduction pathways, we inhibited the G_i protein with PTX. In the presence of WB 4101, NA decreased the [^{35}S]GTPγS binding after E2 or P4 pretreatment (**Fig. 3c**). In the presence of BMY 7378, NA reduced the [^{35}S]GTPγS binding only after P4 pretreatment (**Fig. 3d**).

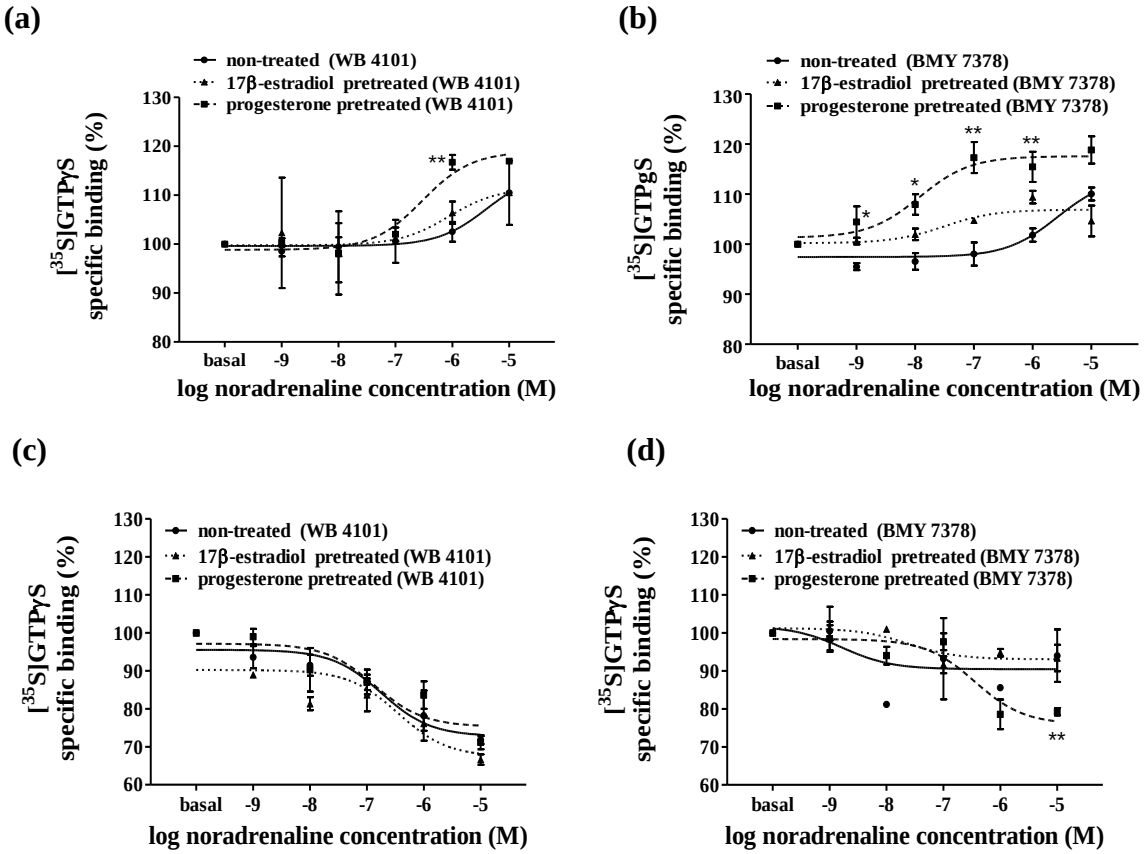
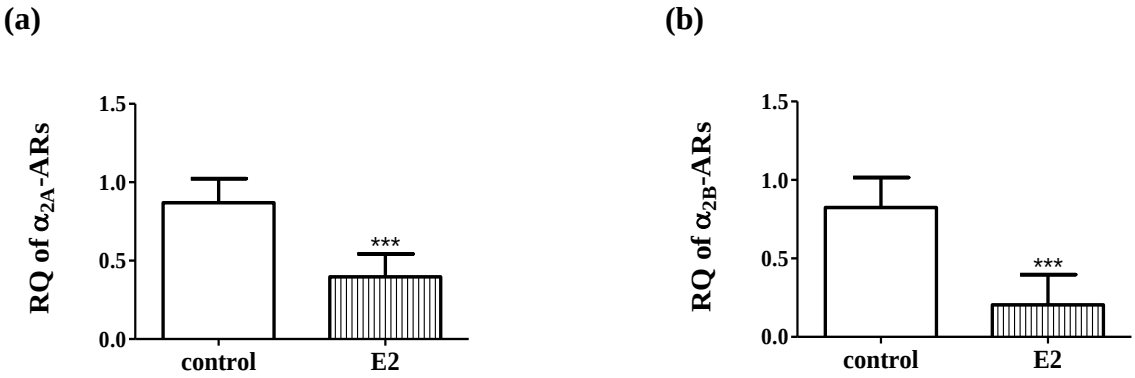


Fig. 3. Changes induced by various concentrations of NA in [^{35}S]GTPγS binding in the presence of WB 4101 **(a, c)** or BMY 7378 **(b, d)** following pretreatment with E2 or P4 in the absence **(a, b)** or presence **(c. d)** of PTX . In all cases, the β-ARs and the α_2-ARs were inhibited by propranolol and yohimbine. Basal refers to the level of [^{35}S]GTPγS binding without substance. *p<0.05; **p<0.01.

Effects of 17β-estradiol pretreatment on the myometrial α_2-adrenergic receptor subtypes

The myometrial mRNA and protein expressions of the α_2-adrenergic receptors after 17β-estradiol pretreatment

The mRNA expression of all α_2-AR subtypes (**Fig. 4 a,b,c**) were significantly decreased after E2 pretreatment compared to the non-treated uteri. The results of Western blot analysis at the level of protein expression revealed significant decrease in each α_2-AR subtypes, correlating with the PCR results.



(c)

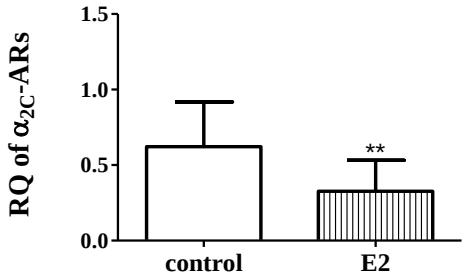


Fig. 4. Changes in the myometrial mRNA expression of the α_{2A}- (**a**), α_{2B}- (**b**) and α_{2C}-ARs (**c**) after E2 pretreatment. The statistical analyses were carried out with the two-tailed unpaired t-test. (RQ: relative quantity) ** $p<0.01$; *** $p<0.0001$

Effects of α_2-adrenergic receptor subtype antagonists on the 22-day pregnant myometrial contractions after 17β-estradiol pretreatment

In the 22-day-pregnant myometrium, NA in the concentration range of 10^{-8} to $10^{-4.5}$ M increased the myometrial contractions (**Fig. 5a**). After E2 pretreatment, the myometrial contracting effect of NA was decreased (**Fig. 5b**).

In the presence of the α_{2A}-AR antagonist BRL 44408, E2 pretreatment increased the NA evoked contractions compared to the E2-treated control (**Fig. 5b**). However, it decreased the myometrial contracting effect of NA compared to the BRL 44408-treated control.

In the presence of the $\alpha_{2B/C}$-AR antagonist ARC 239, E2 pretreatment decreased the myometrial contractions compared to the E2-treated control (**Fig. 5b**), and decreased it compared to the ARC 239-treated control.

In the presence of spiroxatrine, E2 increased the maximum contracting effect of NA compared to the E2-treated control (**Fig. 5b**), but decreased it compared to the spiroxatrine-treated control.

In the presence of the combination of BRL 44408 and spiroxatrine, E2 did not modify the maximal myometrial contracting effect of NA compared to the E2-treated control (**Fig. 5b**), but decreased it compared to the BRL 44408+spiroxatrine treated control.

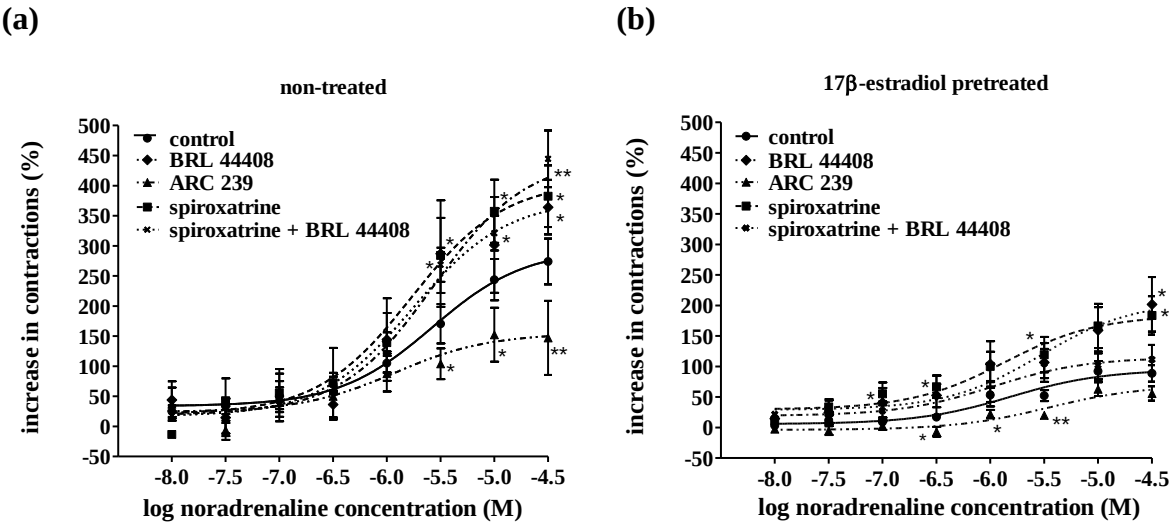


Fig. 5. Effects of the subtype-selective α_{2A}-AR antagonist BRL 44408, $\alpha_{2B/C}$-AR antagonist ARC 239, and the α_{2C}-AR antagonist, spiroxatrine on the NA-evoked contractions in the 22-day-pregnant rat myometrium (**a**), after E2 pretreatment (**b**). The studies were carried out in the presence of the β-AR antagonist, propranolol (10^{-5}M), and the α_1-AR antagonist, doxazosin (10^{-7}M) in each case. *p< 0.05; **p< 0.01; ***p< 0.001.

Effects of subtype-selective α2-adrenergic receptor antagonists on miometrial cAMP level after 17β-estradiol pretreatment

E2 pretreatment increased the myometrial cAMP level (**Fig. 6**) produced in the presence of NA. E2 pretreatment also increased the myometrial cAMP level in the presence of NA and BRL 44408, ARC 239 and spiroxatrine.

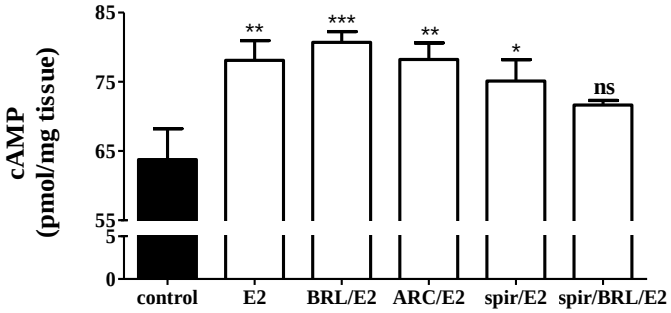


Fig.6. Effects of the subtype-selective α_2-AR antagonists (BRL 44408, ARC 239, spiroxatrine) on the myometrial cAMP level (pmol/mg tissue ± S.D.) in the presence of IBMX (10^{-3} M) and forskolin (10^{-5} M) (control) in the 22-day-pregnant rat after E2 pretreatment. *p<0.05; **p<0.01; ***p<0.001.

Effects of subtype-selective α2-adrenergic receptor antagonists on miometrial [35S]GTPγS binding level in the absence or in the presence of pertussis toxin on the non-trated or 17β-estradiol pretreated uterine tissues

In the presence of BRL 44408, NA increased the [^{35}S]GTPγS binding, and it was significantly decreased after E2 pretreatment. In the presence of PTX, the [^{35}S]GTPγS binding-stimulating effect of NA ceased, and E2 pretreatment did not modify this effect (**Fig. 7a**).

In the presence of ARC 239, NA moderately increased the [^{35}S]GTPγS binding similarly to E2 pretreatment. In the presence of PTX, NA slightly decreased the [^{35}S]GTPγS binding, which was not changed after E2 pretreatment (**Fig.7b**).

In the presence of spiroxatrine, NA increased the [^{35}S]GTPγS binding and it was slightly decreased after E2 pretreatment. In the presence of PTX, however, NA decreased the [^{35}S]GTPγS binding below the basal level from a concentration of 1 x 10^{-9} M. In the presence of PTX, E2 pretreatment abolished the [^{35}S]GTPγS binding-inhibitory effect of NA (**Fig. 7c**).

In the presence of spiroxatrine+BRL 44408 combination, NA inhibited the [^{35}S]GTPγS binding, and E2 caused further inhibition in the [^{35}S]GTPγS binding of NA and abolished the dose-dependency of NA action. In the presence of PTX, the spiroxatrine+BRL 44408 combination dose-dependently inhibited in the [^{35}S]GTPγS binding of NA similarly to E2 pretreatment (**Fig. 7d**).

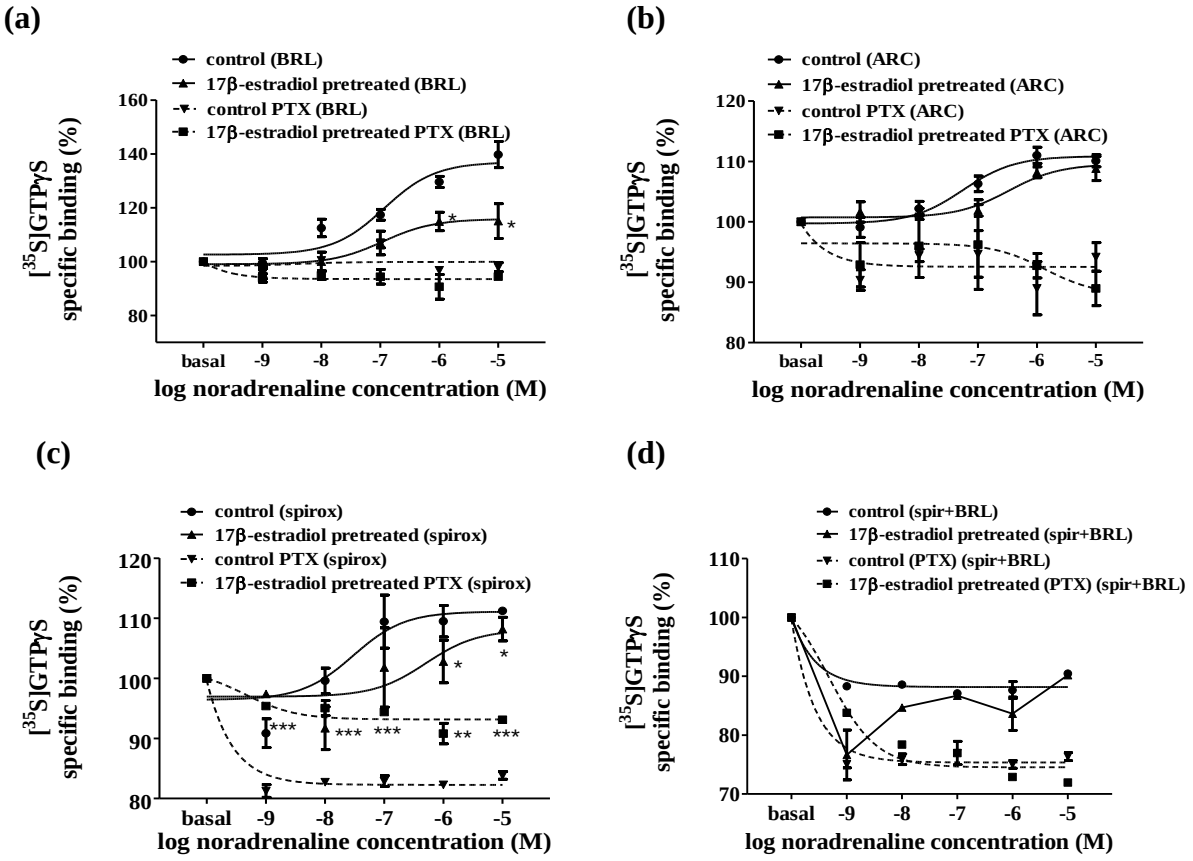


Fig. 7. Changes induced by various concentrations of NA in [^{35}S]GTPγS binding in the presence of subtype-selective α_{2A}-antagonist BRL 44408 **(a)**, the $\alpha_{2B/C}$- antagonist ARC 239 **(b)**, the α_{2C}- antagonist spiroxatrine **(c)** and the BRL 44408-spiroxatrine combination **(d)** following pretreatment with E2. In all cases, the β-ARs and the α_1-ARs were inhibited by propranolol and doxazosin. *p<0.05; **p<0.01; ***p<0.001

Effects of progesterone pretreatment on the myometrial α_2-adrenergic receptor subtypes

The myometrial mRNA expressions of the α_2-adrenergic receptors after progesterone pretreatment

The mRNA expression of each α_2-AR subtype (**Fig. 8 a,b,c**) was significantly increased after P4 pretreatment as compared with the non-treated uteri. The results of Western blot analysis at the level of protein expression revealed a significant increase in each α_2-AR subtype, which correlated with the PCR results.

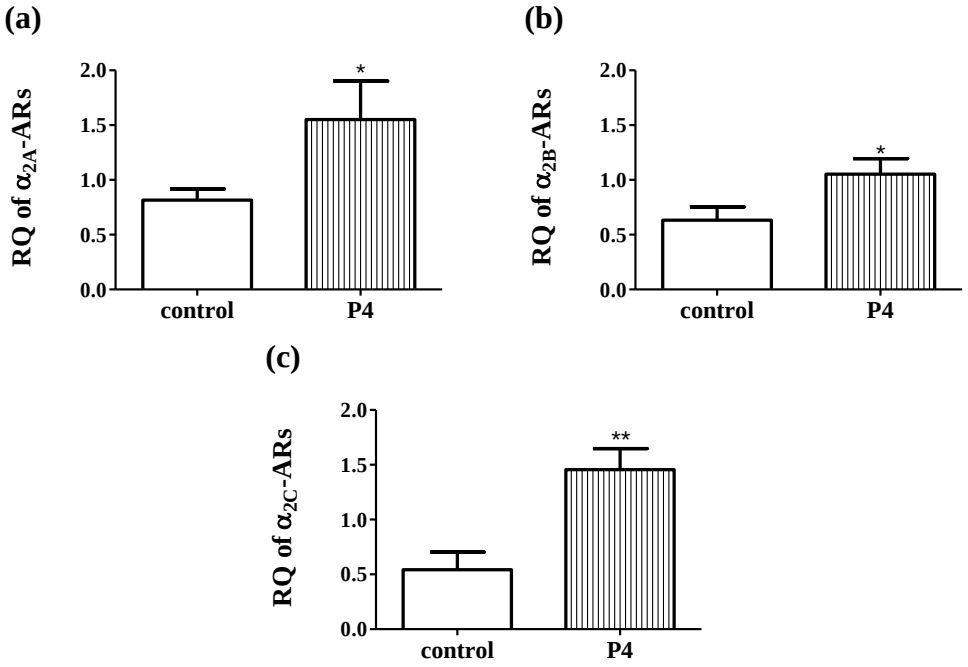


Fig. 8. Changes in the myometrial mRNA expressions of the α_{2A}-ARs (**a**), α_{2B}-ARs (**b**) and α_{2C}-ARs (**c**) after P4 pretreatment in 22-day-pregnant rat uteri. * $p<0.1$; ** $p<0.01$; *** $p<0.001$.

Effects of α_2-adrenergic receptor subtype antagonists on the 22-day pregnant myometrial contractions after progesterone pretreatment

In the 22-day-pregnant myometrium, NA in the concentration range of 10^{-8} to $10^{-4.5}$ M increased the myometrial contractions (**Fig. 9a**). After P4 pretreatment, the myometrial contracting effect of NA was decreased (**Fig 9b**).

In the presence of BRL 44408, P4 pretreatment decreased the NA-evoked contractions as compared with the P4-treated control (**Fig. 9b**). BRL 44408 enhanced the NA-induced contractions, this being markedly reduced by P4 pretreatment **(Fig. 9a,b).**

In the presence of ARC 239, P4 pretreatment did not modify the myometrial contracting effect of NA relative to the P4-treated control. The concentration-response curve was very flat, the difference between the minimum and the maximum effect was less then 20% (**Fig.**

9b). ARC 239 reduced the NA-induced contractions, which were decreased further by P4 pretreatment **(Fig. 9a,b**).

P4 pretreatment decreased the maximum contracting effect of NA in the presence of spiroxatrine as compared with the P4-treated control (**Fig. 9b**). Spiroxatrine enhanced the NA-induced contractions, which were enormously reduced by P4 pretreatment **(Fig. 9a,b**).

In the presence of the combination of spiroxatrine + BRL 44408, P4 pretreatment did not modify the maximum myometrial contracting effect of NA in comparison with the P4-treated control (**Fig. 9b**). The combination of the two compounds increased the NA-induced contractions, which were reduced by P4 pretreatment **(Fig. 9a,b**).

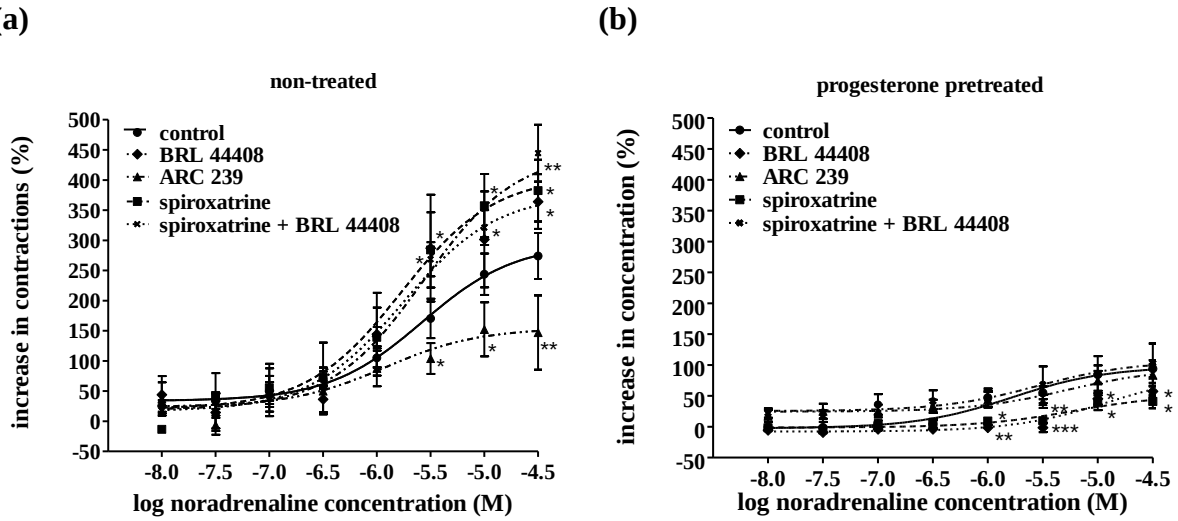


Fig. 9. Effects of the subtype-selective α_2-AR antagonists (BRL 44408, ARC 239 and spiroxatrine) on the NA-evoked contractions in the 22-day-pregnant rat myometrium (**a**) and after P4 pretreatment (**b**). The studies were carried out in the presence of the β-AR antagonist propranolol (10^{-5} M) and the α_1-AR antagonist doxazosin (10^{-7} M) in each case. *p< 0.05; **p < 0.01; ***p < 0.001.

Effects of subtype-selective α2-adrenergic receptor antagonists on miometrial cAMP level after progesterone pretreatment

P4 pretreatment increased the myometrial cAMP level (**Fig. 10**) produced in the presence of NA, as increased in the presence of BRL 44408, spiroxatrine and the spiroxatrine + BRL 44408 combination. However, ARC 239 did not modify the amount of myometrial cAMP after P4 pretreatment.

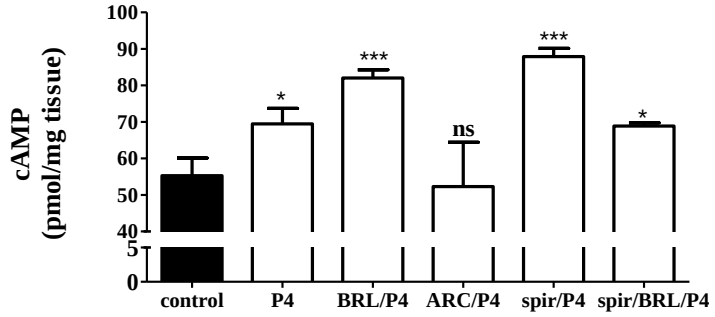


Fig.10. Effects of the subtype-selective α_2-AR antagonists (BRL 44408, ARC 239 and spiroxatrine) on the myometrial cAMP level (pmol/mg tissue ± S.D.) in the presence of IBMX (10^{-3} M) and forskolin (10^{-5} M) (control) in the 22-day-pregnant rat (n = 6) after P4 pretreatment. *p < 0.05; **p < 0.01; ***p < 0.001.

Effects of subtype-selective α_2-adrenergic receptor antagonists on miometrial [^{35}S]GTPγS binding level in the absence or in the presence of pertussis toxin on the non-treated or progesterone pretreated uterine tissues

In the presence of BRL 44408, NA increased the [^{35}S]GTPγS binding, which was slightly decreased after P4 pretreatment. In the presence of PTX, the [^{35}S]GTPγS binding-stimulating effect of NA ceased, and it was decreased further after P4 pretreatment (**Fig. 11a**).

In the presence of ARC 239, NA moderately increased the [^{35}S]GTPγS binding and it was more elevated after P4 pretreatment. In the presence of PTX, the [^{35}S]GTPγS binding-stimulating effect of NA ceased, which was not modified even by P4 pretreatment (**Fig. 11b**).

In the presence of spiroxatrine, NA slightly increased the [^{35}S]GTPγS binding and it was more elevated after P4 pretreatment. In the presence of PTX, however, NA elicited a decline in the [^{35}S]GTPγS binding, to below the basal level from a concentration of 1 x 10^{-9} M. In the presence of PTX, P4 pretreatment blocked the [^{35}S]GTPγS binding-inhibitory effect of NA (**Fig. 11c**).

In the presence of the spiroxatrine + BRL 44408 combination, NA inhibited the [^{35}S]GTPγS binding, but it was significantly increased after P4 pretreatment. In the presence of PTX, the spiroxatrine + BRL 44408 combination caused a dose-dependent inhibition in the [^{35}S]GTPγS binding of NA, but the inhibition was reduced after P4 pretreatment (**Fig. 11d**).

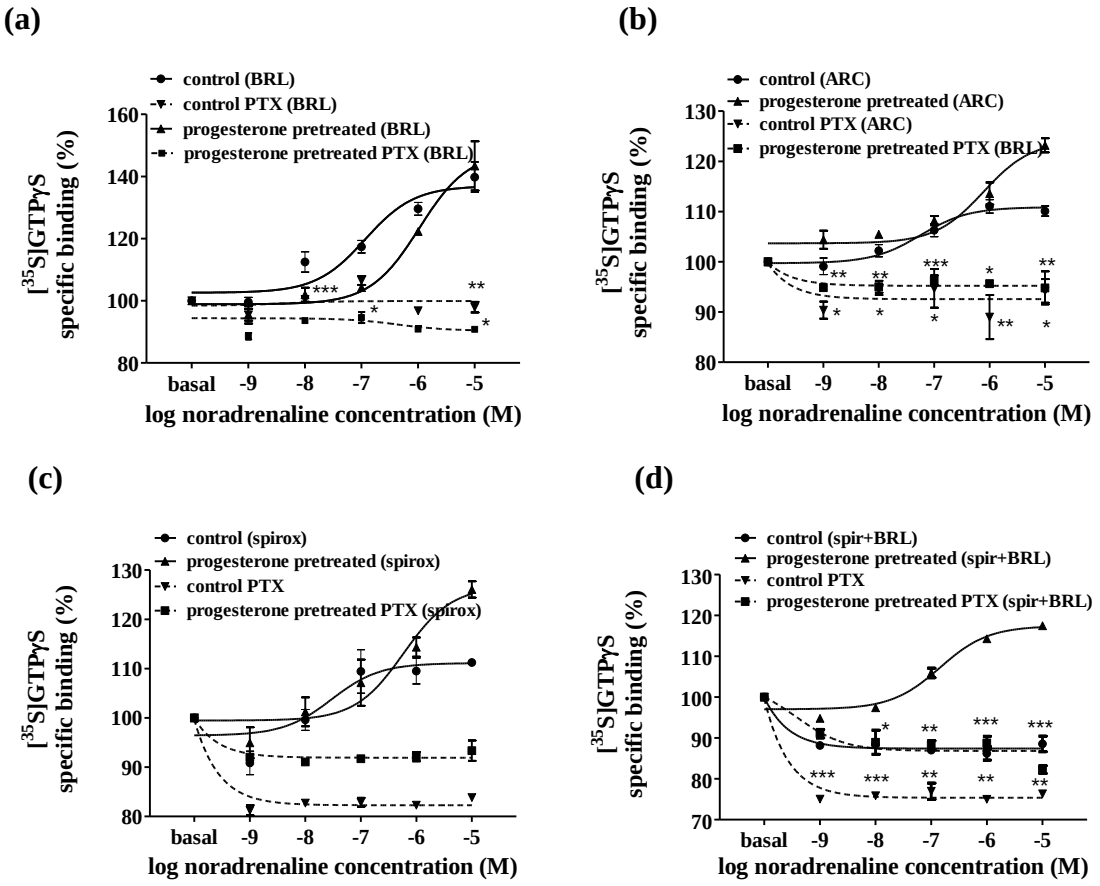


Fig. 11. Changes induced by various concentrations of NA in [^{35}S]GTPγS binding in the presence of BRL 44408 **(a)**, ARC 239 **(b)**, spiroxatrine **(c)** and the spiroxatrine + BRL 44408 combination **(d)** following pretreatment with P4. In all cases, the β-ARs and the α_1-ARs were inhibited by propranolol and doxazosin. Basal refers to the level of [^{35}S]GTPγS binding without substance. **p < 0.01; ***p < 0.001.

Discussion

Sexual hormones and the adrenergic system have major role in myometrial contractions during human gestation. But to date there have been no studies that have focused on the influence of E2 and P4 on the function of the α_1-and α_2-ARs in the pregnant rat myometrium, therefore the main focus of our study was to investigate the effect of E2 and P4 on the function and expression of the α_1-and α_2-ARs subtypes in the late pregnant rat myometrium, *in vitro*.

α_1-adrenergic receptors and the female sexual hormones

The α_{1B}-ARs cannot be detected in the late-pregnant rat uterus, therefore we investigated the roles of the α_{1A}-ARs and α_{1D}-ARs subtypes in myometrial contractility. E2 pretreatment slightly decreased the effect of NA through the α_1-ARs, indicating the weaker sensitivity of the receptors to NA. P4 pretreatment reduced the maximum contracting effect of NA. In the presence of subtype-specific α_1-AR blockers, the effect of NA was reduced, confirming that both the α_{1A}-ARs and the α_{1D}-ARs are involved in myometrial contraction.

Neither E2 nor P4 changed the mRNA and protein expression of the α_{1D}-ARs. E2 pretreatment did not cause any changes, while P4 pretreatment reduced the maximum myometrial contracting effect and the EC_{50} values of NA via the α_{1D}-ARs. α_1-ARs are mainly coupled to $G_{q/11}$ therefore to find an explanation, we carried out [^{35}S]GTPγS binding studies. In the presence of WB 4101, NA moderately increased the [^{35}S]GTPγS binding and only P4 increased the [^{35}S]GTPγS binding via the α_{1D}-ARs, which may contribute to the decreased myometrial contracting effect. In the presence of PTX and WB 4101, the [^{35}S]GTPγS binding-stimulating effect of NA turned to inhibition, this was most marked in the presence of P4. This result suggests, that in a predominance of P4, the α_{1D}-ARs are coupled, at least partially, to G_i protein, which leads to a reduction of the NA-induced myometrial contraction via these receptors.

In the presence of the α_{1D}-AR blocker BMY 7378 NA could stimulate only the α_{1A}-ARs. The protein expression decreased only after E2 pretreatment, which can explain the decreased myometrial contracting effect of NA after E2 pretreatment. The myometrial contracting effect of NA was decreased, indicating a lower contractile response in the absence of the α_{1D}-ARs. Additionally, P4 pretreatment diminished the myometrial contracting effect of NA. The [^{35}S]GTPγS binding was stimulated slightly by NA while the presence of P4 increased the stimulating effect of NA: PTX reversed the stimulation to inhibition in the presence of P4, which means that G_i coupling is a determining factor in the function of the α_{1A}-ARs after P4

treatment. This provides an explanation why NA did not induce myometrial contraction after P4 pretreatment.

α_2-adrenergic receptors and 17β-estradiol

E2 pretreatment decreased the mRNA and protein expression of the myometrial α_2-AR subtypes and decreased the NA-evoked myometrial contraction through the α_2-ARs. According to the isolated organ bath studies and the changing of myometrial cAMP level E2 pretreatment decreased the NA-evoked myometrial contraction via the α_2-ARs, although it did not modify the myometrial relaxing effect via the α_{2A}-ARs. However, it abolished the myometrial contraction increasing effect via the α_{2B}-ARs. Since there are no available antagonists to produce only α_{2C}-AR stimulation (ie,. $\alpha_{2A/B}$-AR blockers), we can only presume that E2 did not modify the myometrial relaxing effect via the α_{2C}-ARs.

The α_2-ARs can couple not only to the G_i protein α-subunit, but under certain circumstances, also to G_s proteins. E2 was also shown to decrease the coupling of the α_2-ARs to G protein. In the presence of PTX, E2 did not modify the [^{35}S]GTPγS binding of the α_{2A}-ARs, but it reversed the effect of NA on [^{35}S]GTPγS binding via α_{2A}- and α_{2B}-ARs. According to these findings E2 modifies the coupling of α_{2B}-ARs, but does not change the G protein binding of the α_{2A}-ARs. To prove this hypothesis, we measured the myometrial [^{35}S]GTPγS binding of the α_{2B}-AR subtype in the presence of spiroxatrine+BRL 44408. E2 decreased the amount of activated G-protein, which is probably a consequence of E2-induced uncoupling of α_{2B}-ARs from the G proteins.

α_2-adrenergic receptors and progesterone

P4 pretreatment increased the mRNA and protein expression of the myometrial α_2-AR subtypes, but decreased the NA-evoked myometrial contraction through the α_2-ARs.

P4 pretreatment ceased the NA-evoked myometrial contraction through the α_2-ARs, although it practically ceased the myometrial contracting effect of the NA through the α_{2A}-ARs. Additionally, it abolished the myometrial contraction-increasing effect through the α_{2B}-ARs, and reversed the myometrial contracting effect in the presence of BRL 44408 and in the presence of spiroxatrine. Since there are no available $\alpha_{2A/B}$-AR blockers to produce only α_{2C}-AR stimulation, we can only presume that P4 maintained the myometrial relaxing effect through the increased number and function of α_{2C}-ARs. The changing of myometrial cAMP level was in harmony with isolated organ bath studies.

The G_i/G_s-activating property of α_2-AR in rats changes during gestation, resulting in differences in the regulation of myometrial adenylate cyclase activity at mid-pregnancy versus

term. P4 did not modify the [^{35}S]GTPγS binding of the α_{2A}-ARs. However, via the α_{2A}- and α_{2B}-ARs (with spiroxatrine), P4 reversed the effect of NA on the [^{35}S]GTPγS binding in the presence of PTX and also increased the [^{35}S]GTPγS binding-stimulating effect of NA. These findings indicate that P4 modifies the coupling of α_{2B}-ARs, but not the G protein binding of the α_{2A}-ARs. The changing of the [^{35}S]GTPγS binding confirm this hypothesis in the presence of the spiroxatrine + BRL 44408 combination, too. This result suggests that, in of predominance of P4, the α_{2B}-ARs are coupled, at least partially, to G_s protein.

Conclusion

In the light of our results, it can be concluded that the functions of the α_1- and α_2-AR subtypes are influenced differently by the female sexual steroid hormones.

A limitation of our study is that we did not carry out any studies on human myometrium, and there might be differences in the function of the rat and human myometrial α_1-AR and α_2-AR subtypes. However, our present findings give a better understanding on the complex physiology of changes during pregnancy. We suppose that either subtype-specific agonists or antagonists can be used as a target for drugs against abnormal myometrial contractility. The application of various α-AR subtypes antagonists cannot completely the process of preterm birth, however selective α_1-AR and α_2-AR subtypes antagonist in combination with progesterone may have clinical significance.

Appendix

1. Publications related to the Ph.D. thesis

I Bóta J, Hajagos-Tóth J, Ducza E, Samavati R, Borsodi A, Benyhe S, Gáspár R. The effects of female sexual hormones on the expression and function of α_{1A}- and α_{1D}-adrenoceptor subtypes in the late-pregnant rat myometrium.
European Journal of Pharmacology 769: pp. 177-184. (2015) *[IF: 2.730]*

II Hajagos-Tóth J, **Bóta J**, Ducza E, Csányi A, Tiszai Z, Borsodi A, Samavati R, Benyhe S, Gáspár R. The effects of estrogen on the α_2- adrenergic receptor subtypes in ratuterine function in late pregnancy in vitro.
Croatian Medical Journal 57:(2) pp. 100-109. (2016) *[IF: 1.483]*

III Hajagos-Tóth J, **Bóta J**, Ducza E, Samavati R, Borsodi A, Benyhe S, Gáspár R.
The effects of progesterone on the alpha2-adrenergic receptor subtypes in late-pregnant uterine contractions in vitro.
Reproductive Biology and Endocrinology 14(1) pp. 33. (2016) *[IF: 2.147]*

2. Presentations related to the Ph.D. thesis

I Bóta J.
Progeszteron kezelés hatása az alfa-adrenerg receptorok működésére vemhes patkány uteruszban
Scientific Students' Associations Conference (TDK), Szeged, Hungary, 2013 (Oral presentation)

II Hajagos-Tóth J, **Bóta J**, Ducza E, Samavati R, Benyhe S, Gáspár R.
The effect of progesterone on the expression and function of the different α_2-adrenergic receptor subtypes in late pregnant rat myometrium
FEPS, Kaunas, Lithuania 2015 (Oral presentation)

III Hajagos-Tóth J, **Bóta J**, Ducza E, Samavati R, Benyhe S, Borsodi A, Gáspár R.
The effect of oestrogen on the expression and function of the different α_2-adrenergic receptor subtypes in late pregnant rat myometrium
RECOOP TriNet Meeting, Prague, Czech Republic 2015 (Oral presentation)

IV Hajagos-Tóth J, **Bóta J**, Ducza E, Csányi A, Tiszai Z, Samavati R, Borsodi A, Benyhe S, Gáspár R.
The effects of estrogen on the α_2-adrenergic receptor subtypes in rat uterine function in late pregnancy in vitro
Bridges in Life Sciences 11th Annual Scientific Conference, Prague, Czech Republic 2016 (Oral presentation)

V Bóta J, Hajagos-Tóth J, Ducza E, Gáspár R.
Progeszteron kezelés hatása az α-adrenerg receptor altípusok működésére vemhes patkány uteruszban
XV. Congressus Pharmaceuticus Hungaricus, Budapest, Hungary, 2014. (Poster)

VI Bóta J, Hajagos-Tóth J, Ducza E, Samavati R, Benyhe S, Borsodi A, Gáspár R. Alteration in the expression and function of α_1-adrenergic receptor subtypes in late pregnant rat uterus by progesterone and oestrogen pretreatment
FEPS, Kaunas, Lithuania 2016 (Poster)