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Faculty of Pharmacy

Department of Pharmacodynamics and Biopharmacy

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

Ph.D Thesis

Judit Bóta

Supervisor:

Róbert Gáspár Ph.D.

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Contents

List of publications

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.
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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.
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III Hajagos-Tóth J, **Bóta J**, Ducza E, Samavati R, Borsodi A, Benyhe S, Gáspár R.
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2. Presentations related to the Ph.D. thesis

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Progeszteron kezelés hatása az alfa-adrenerg receptorok működésére vemhes patkány uteruszban
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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)

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
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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)

Bóta J, Hajagos-Tóth J, Ducza E, Gáspár R.
Progeszteron kezelés hatása az α-adrenerg receptor altípusok működésére vemhes patkány uteruszban
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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
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List of abbreviations

AR: adrenergic receptor

AC: adenylate cyclase

cAMP: cyclic adenosine monophosphate

E2: 17β-estradiol

EC_{50}: half maximum effective concentration

E_{max}: maximum possible effect

GTPγS: guanosine-5'-O-(γ-thio)triphosphate

IBMX: 3-isobutyl-1-methylxanthine

NA: noradrenaline

P4: progesterone

PKA: protein kinase A

PKC: protein kinase C

PLC: phospholipase C

PG: prostaglandine

PTB: preterm birth

PTX: pertussis toxin

Tris-HCl: tris(hidroxymethyl)aminomethane hydrochloride

1. Introduction

1.1 The role of the adrenergic system in the uterine function

The physiology of uterine quiescence and contractility is very complex. Myometrial contraction is regulated by a number of factors, such as ion channels, transmitters, female sexual hormones and the adrenergic receptor (AR) system. Dysregulation of the myometrial contractility can lead to either preterm or slow-to-progress labor. It is therefore crucial to understand the mechanisms that regulate uterine contractility in order to prevent or treat the pathological processes related to the pregnant myometrium (Kamel, 2010; Blanks *et al*,, 2007; Wray, 2015; Illanes *et al.*, 2014).

It has been established that the rat myometrium has rich adrenergic innervation and these activations modulate uterine contraction, so it plays an important role in the control of uterine contractility (Papka *et al.*,1985; Borda *et al.*, 1979). A number of studies have been made to employ drugs that affect the adrenergic system and thereby myometrial contractility (Mihalyi *et al.*, 2003; Lopez Bernal, 2007).

The α_1-, α_2- and β_2-ARs subtypes are found in human and rat myometrium (Kolarovszki-Sipiczki *et al.*, 2007; Gáspár *et al.*, 2007; Pedzińska-Betiuk *et al.*, 2011), therefore pregnancy may alter the signal transduction processes of some ARs (Zhou *et al.*, 2000).

1.1.1. The β-adrenergic receptors

The β-ARs were divided into β_1-, β_2-, and β_3-ARs. All of three β-ARs are found in pregnant myometrium (Liu *et al.*, 1998; Dennedy *et al.*, 2001). Activation of the β_2-ARs leads to stimulation of the release of the G-protein and enhance the GTP-dependent adenylate cyclase (AC) activity. AC catalyzes the conversation of ATP to cyclic adenosine monophosphate (cAMP) which actives the cAMP-dependent protein kinase A (PKA) and leads to myometrial relaxation. In addition PKA induces hyperpolarisation, inhibits phospholipase C (PLC), reduces gap junction permeability and inhibits myosin light chain kinase (Blanks *et al.*, 2007). Because of the myometrial relaxing effect β_2-AR agonists are used as tocolytic agents in clinical practice (Dowell *et al*. 1994). A number of studies proved that most β_2-mimetics can put off labor at least 48 hours (Oei, 2006). β-AR mimetics may have several maternal (tachycardia, pulmonary oedema, hypokalemia, sodium retention) and fetal (respiratory distress syndrome, intracranial bleeding and neonatal jaundice) side effects, mainly in

consequence with their high therapeutic doses used for uterus-relaxing action (Gyetvai *et al.*, 1999; Papatsonis *et al.*, 2000). Nowadays there are drugs with similar efficacy and fewer side effects (e.g.: nifedipine) (Abramovici *et al.*, 2012), therefore the use of β_2-AR mimetics has been the subject of intensive debate in the literature and practice.

Earlier findings suggest that pregnancy itself may alter the myometrial action of adrenergic drugs. It is known that the β_2-agonists have weaker action on myometrial contractions at the end of pregnancy in the mouse uterus (Cruz *et al.*, 1990) and the uterus-relaxing effect of terbutaline spontaneously decreased on electrical field-stimulated samples towards the end of the pregnancy in rats. Terbutaline decreased the amount of activated myometrial G-protein in [^{35}S]GTPγS binding assay on the last day of pregnancy (Gáspár *et al.*, 2005). It is presumed that the reason of this phenomenon is the reduced level of progesterone (P4) level, because P4 increases the synthesis of β_2-ARs during pregnancy (Roberts *et al.*, 1989; Dowell *et al.*, 1994; Engstrom *et al.*, 2001).

1.1.2. The α_1-adrenergic receptors

The α-ARs have been subdivided into α_1- and α_2-AR subtypes. The α_1-AR family has three subtypes: α_{1A}-, α_{1B}- and α_{1D}-ARs (Hieble *et al.*, 1995). These receptors participate in many essential physiological functions, such as sympathetic neurotransmission, myocardial inotropy and chronotropy, modulation of hepatic metabolism, uterine contraction, vascular tone and contraction of smooth muscle in the genitourinary system (García-Sáinz *et al.*, 1999). Stimulation of all three subtypes leads to activation of the $G_{q/11}$ signalling pathway, which results in the activation of PLC, generates inositol triphosphate and diacylglycerol, leading to muscle contraction through mobilization of intracellular calcium and activation of protein kinase C (PKC) (Michelotti *et al.*, 2000). Although they activate the same G protein signalling pathway, they have different functional roles according to their different organ distributions (Piascik *et al.*, 2001; Schmitz *et al.*, 1981). However, it has also been demonstrated that pertussis toxin (PTX)-sensitive G proteins can participate in the mediation of α_1-adrenergic actions (García-Sáinz *et al.*, 1999).

The α_1-AR agonists elicit contractions in the myometrium (Ducza *et al.* 2002, Ducza *et al.* 2005). All of the α_1-AR subtypes are found in the early-pregnant rat uterus, with a predominance of the α_{1A}-ARs, while the α_{1B}-ARs were not detected in the late-pregnant (day 18-22) rat uterus (Ducza *et al.* 2009). The mRNA expression of the α_{1A}-ARs was highest on

day 22 of pregnancy, which suggests that they are involved in the increase in uterine contractility at the end of gestation (Ducza *et al*. 2002). It has been proved that α_1-AR antagonists alone or in combination with sex hormones (17β-estradiol (E2), P4) induce a significant decrease in the uterine activity of the rat *in vitro* (Gáspár et al., 1998.), similar to the effects of β_2-AR agonists.

A description of the pharmacological effects of the α_1-AR ligands suggests that several antagonists appear to have inverse agonist properties at these receptors. They are thought to reduce the functional activity of the receptors below the baseline activity observed in the absence of any ligands. The α_{1A}-AR antagonist WB 4101 and the α_{1D}-AR antagonist BMY 7378 are inverse agonists at concentrations of 10^{-8} to 10^{-5} M (Noguera *et al*. 1996, Rossier *et al*. 1999). The resistance-increasing effect of α_1-AR inverse agonists on the different days of gestation in the rat has also been clarified (Kolarovszki-Sipiczki *et al.*, 2007).

1.1.3. The α_2-adrenergic receptors

The α_2-ARs were initially identified as presynaptic receptors inhibiting the release of neurotransmitters in isolated tissues *in vitro* (Starke et al., 1975). The α_2-ARs have been divided into three groups (Knaus *et al.,* 2007; Civantos Calzada *et al.*, 2001), including α_{2A}-, α_{2B}- and α_{2C}-AR subtypes. All of the three receptor subtypes are coupled to the PTX-sensitive G_i-protein α-subunit (Karim F et al. 2000) and decreases the activities of AC and the voltage-gated calcium currents, activates the receptor-operated potassium currents and MAP kinase activity (Wang Q *et al.*, 2012). The stimulation of these receptors leads to presynaptic feedback inhibition of noradrenaline (NA) release on the adrenergic neurons (Knaus E *et al.*, 2007), and mediates vasoconstriction, increased blood pressure and nociception.

Differences in the receptor subtypes and their various localizations are thought to be responsible for their different roles and various physiological functions, and especially in the cardiovascular and central nervous systems (Gyires *et al.*, 2009). Stimulation of α_{2A}-ARs could be linked with bradycardia, hypotension (MacMillan *et al.*, 1996) and consolidation of working memory (Wang *et al.*, 2007), while α_{2B}-ARs has opposite effect for hypotension (Link *et al.,* 1996) and are essential for placenta vascular development (Philipp *et al.*, 2002).

α_2-ARs were identified as feedback regulators of adrenal catecholamine release (Brede *et al.*, 2003), an essential pathway to limit the progression of heart diseases (e.g. cardiac hypertrophy and failure) (Lymperopoulos *et al.*, 2007). The α_2-ARs can mediate inhibitory

effects, such as the suppression of neurotransmitter and hormone release, and stimulatory effects, such as the aggregation of platelets and the contraction of smooth muscles (e.g. the myometrium) (Taneike *et al.*, 1995).

Furthermore all of them have been identified in both pregnant and non-pregnant myometrium, and were shown to take part in both increased and decreased myometrial contractions (Bouet-Alard R *et al,*. 1997; Gáspár *et al.*, 2007). Under certain circumstances α_2-ARs can couple not only to G_i-proteins but to G_s-proteins, resulting in activation of AC (Offermann S *et al.*, 2003). Pregnancy has been proved to induce a change in the G_i/G_s-activating property of α_2-AR in rats, resulting in a differential regulation of myometrial AC activity at mid-pregnancy versus term (Mhaouty *et al.*, 1995). The α_{2B}-ARs were shown to predominate and mediate contraction in last-day-pregnant animals as decreases the intracellular cAMP level, while the stimulation of the myometrial α_{2A}- and α_{2C}-ARs leads to an increase in the cAMP level, and mediates only weak contractions (Gáspár *et al,*. 2007).

1.2. The effect of sexual steroid hormones for pregnant uterine contractility

Ovarian steroids play a key role in the coordination of reproductive function in female mammals. The development and function of female reproductive tissues are regulated by two major sex steroid hormones (E2, P4) (Clarke *et al.*, 1990).

Throughout most of the gestational period the uterus should remain quiescent to allow the fetus to grow, and change to be very active just before the onset of labor. It is known that P4 is responsible for uterine quiescence (Maggio *et al.*, 2014; Norwitz *et al.*, 2015), while E2 have a major role in myometrial contractions (Kamel *et al.*, 2010; Renthal *et al.*, 2015). The regulation of uterine activity by sex steroids includes prostaglandin (PG) production, $PGF_{2\alpha}$ receptor function, oxytocin receptors and acting cytosolic receptors through a genomic process and G-protein-coupled pathways (Okabe *et al.*, 1999., Cohen-Tannoudji *et al.*, 1995). During pregnancy P4 enhances relaxation by increasing the G_s-coupled β_2-AR cAMP cascade, decreases the amount of PG and oxytocin and increases the release of calcitonin which promotes the storage of calcium and decreases free calcium level (Ding *et al.*, 1994). The level of P4 decreases at the onset of labour, while E2 level increases abruptly, resulted in a sudden rise in uterine activity in response to α_l-AR agonist, oxytocin, and $PGF_{2\alpha.}$ All these excitatory agents are coupled to a phosphoinositide-specific PLC and increases intracellular free calcium, which mediate uterine contractions by G protein of the Gq/G11 family. In

human term myometrium, AC activity can be inhibited by α_2-AR agonists functionally linked to a PTX-sensitive G_i protein (Cohen-Tannoudji *et al.*, 1995).

The P4 level normally declines at term prior to the development of labor and it is therefore used to alone or in combination with β-AR agonists is applied successful to prevent threatening preterm birth (PTB) (Mick *et al.*, 2015; Gáspár *et al.*, 2005). It was shown, that P4 therapy is useful in selected patient populations decreasing the incidence of PTB and in some studies reducing the rate of neonatal morbidities (Maggio and Rouse 2014). The reducing P4 levels are maintained by injections of the hormons, animals (rats and rabbits) do not go into labour (Garfield, 2012). In clinical trials gestagens reduced the risk of delivery before 37 gestational week in case of increased risk of spontaneous PTB (Mackenzie et al. 2006).

1.2.1. The effect of 17β-estradiol for adrenergic system

The E2 predominance at the end of pregnancy is known to increase the number of α-ARs (Legrand *et al*., 1987), promotes contraction (Roberts *et al.*, 1989) and the sensitivity of the α_1-ARs (Riemer *et al*. 1987a; 1987b). E2 increases α_1-AR expression and the linkage of the receptor to PLC. In addition E2 also uncouples the β-AR from AC (Roberts *et al.*, 1989). A predominance of E2 decreases the levels of β-AR-mediated G_s proteins and cAMP (Riemer *et al*. 1988).

E2 increases evoked NA release in the hypothalamus of female rodents, in part by reducing the ability of α_2-ARs to act as negative feedback inhibitors of NA release, uncoupling the receptor from G protein and stabilize α_2-AR phosphorylation by inhibiting receptor internalization and dephosphorylation. (Ansonoff *et al.*, 2001). E2 pretreatment increased the mRNA expression of the α_{2A}-ARs in the spinal cord (Thomson *et al.*, 2008), which could contribute to the higher prevalence of pain syndromes in women. On the other hand, E2 was shown to increase the smooth muscle expression of α_{2C}-ARs and the cold-induced constriction of cutaneous arteries (Eid *et al.*, 2007). In addition, E2 stimulates the NA release in the hypothalamus due to the decreased coupling of the α_2-ARs to G protein (Ansonoff *et al.*, 2001).

E2 treatment can modulate the effect of β_2-AR agonists. It was described that E2 treatment alone or in combination with P4 reduced maximal relaxation effect of isoproterenol on isolated uterine strips whereas P4 alone had no effect on this parameter. The reduction was accompanied by an enhanced β_2-AR mRNA concentration (Engstrom *et al.*, 2001).

1.2.2. The effect of progesterone for adrenergic system

P4 promotes myometrium relaxation, in which the ARs play an important role. It was observed that E2/P4 ratio influences the ratio of α_1/ß-ARs, which implies with the increasing E2 level will increase the number of α_1-ARs. In late pregnant rat myometrium the density of α_1-ARs 25 to 50 times more than the density of ß-ARs. It suggests the high density of α_1-ARs causes uterus contraction (Gáspár *et al.*, 2001).

The presence or absence of P4 can alter the effect of β_2-AR agonists on the pregnant myometrium (Dowell *et al.*, 1994; Engstrom *et al.*, 2001) and sex hormones play a role in the regulation of G-proteins in the myometrium (Elwardy-Merezak *et al.*, 1994; Cohen-Tannoudji *et al.*, 1995). A predominance of P4 increases the synthesis of β_2-ARs, the number of activated G_s-proteins and the cAMP level during pregnancy (Engstrom *et al*. 2001, Roberts *et al.* 1989; Nimmo 1995). It was observed that the effects of the β_2-ARs agonists may be decreased at the end of pregnancy in consequence of the drop in plasma P4 level. Gáspár *et al.* (2005) found that P4 pretreatment inverted the dose-dependent decrease in the amount of activated G-protein of β_2-ARs by terbutaline and a stronger inhibitory action of it on late pregnant myometrial contractions. It is presumed that the effects of β_2-AR agonists in tocolytic therapy may possibly be potentiated with P4. Gálik et al. (2008) investigated the combination of P4 and β_2-AR agonists on rat myometrium. They showed that gestagens can enhance the effect of β_2-AR agonists on hormonally-induced rat preterm birth model.

2. 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 in pregnant rats:

1. Investigation of the role of the α_1-AR and α_2-AR subtypes by subtype-specific antagonists after *in vivo* 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

3. Materials and methods

Animal investigations were carried out with the approval of the Hungarian Ethical Committee for Animal Research (permission numbers: IV/01758-0/2008 and 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.§).

3.1. Housing and handling of the animals

Sprague-Dawley rats were purchased from INNOVO Kft. (Gödöllő, Hungary) and were housed under controlled temperature (20-23 °C), in humidity- (40-60%) and light- (12 h light/dark regime) regulated rooms. The animals were maintained on a standard rodent pellet diet (INNOVO Kft., Gödöllő, Hungary), with tap water available *ad libitum*.

3.2. 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. An electric engine-controlled, movable metal door separated the rooms between the male and female rats. Since rats are usually active at night, the separating door was opened before dawn. 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. Female rats with positive smear and those in whom the smear was not taken due to vaginal sperm plug were regarded as first-day pregnant animals.

3.3. In vivo sexual hormone treatments of the rats

Pretreatment of the pregnant animals with E2 (Sigma-Aldrich, Budapest, Hungary) was started on day 18 of pregnancy. E2 was dissolved in olive oil. The animals were injected subcutaneously with 5 μg/kg of E2 once a day for 4 days (Hódi *et al.* 2014).

The P4 (Sigma-Aldrich, Budapest, Hungary) pretreatment of the pregnant animals was started on day 15 of pregnancy. P4 was dissolved in olive oil and injected subcutaneously every day up to day 21 in a dose of 0.5 mg/0.1 ml (Hajagos-Tóth *et al.* 2009).

On day 22, the uterine samples were collected, and contractility and molecular pharmacological studies were carried out.

3.4. RT-PCR studies

Tissue isolation: Rats (250-350 g) were sacrificed by CO_2 asphyxiation. Fetuses rats were sacrificed by immediate cervical dislocation. The uterine tissues from pregnant animals (n=6-8 in each experiment) (tissue between two implantation sites) were rapidly removed and placed in RNAlater Solution (Sigma-Aldrich, Budapest, Hungary). The tissues were frozen in liquid nitrogen and then stored at -70 °C until the extraction of total RNA.

Total RNA preparation from tissue: Total cellular RNA was isolated by extraction according to the procedure of Chomczynski and Sacchi (1987). After precipitation with isopropanol, the RNA was washed with 75% ethanol and then resuspended in diethyl pyrocarbonate-treated water. RNA purity was controlled at an optical density of 260/280 nm with BioSpec Nano (Shimadzu, Kyoto, Japan); all samples exhibited an absorbance ratio in the range 1.6-2.0. RNA quality and integrity were assessed by agarose gel electrophoresis.

Reverse transcription and amplification of the PCR products were performed by using the TaqMan RNA-to-C_T^{TM} 1-Step Kit (Life Technologies, Budapest, Hungary) and the ABI StepOne Real-Time cycler. RT-PCR amplifications were performed at 48 °C for 15 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. The generation of specific PCR products was confirmed by melting curve analysis. Table 1 contains the assay IDs for the used primers. 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).

TaqMan assays	**Assay ID** (Life Technologies, Hungary)
α_{1A}-AR	Rn00567876_m1
α_{1D}-AR	Rn00577931_m1
α_{2A}-AR	Rn00562488_s1
α_{2B}-AR	Rn00593312_s1
α_{2C}-AR	Rn00593341_s1
β-actin	Rn00667869_m1

Table 1: Assay IDs of the applied primers.

3.5. Western blot analysis

20 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, with use of the iBlot Gel Transfer System (Life Technologies, Budapest, Hungary). The antibody binding was detected with the WesternBreeze Chromogenic Western blot immune detection kit (Life Technologies, Budapest, Hungary). The blots were incubated on a shaker with α_{1A}-AR, α_{1D}-AR, α_{2A}-AR, α_{2B}-AR, α_{2C}-AR and β-actin polyclonal antibody (Santa Cruz Biotechnology, California, 1:200) in the blocking buffer. Molecular weight markers identified the bands of the given α-AR proteins in the myometrium. Images were captured with the EDAS290 imaging system (Csertex Ltd., Budapest, Hungary), and the optical density of each immunoreactive band was determined with Kodak 1D Images analysis software (Csertex Ltd., Budapest, Hungary). Optical densities were calculated in arbitrary units after local area background subtraction.

3.6. Isolated organ studies

Uteri were removed from rats (250-350 g) on day 22 of pregnancy (n=8-12 in each experiment). 5-mm-long muscle rings were sliced from both horns of the uterus (2-2 rings dissected from the centre of each horn) they were trimmed of fat, the foeto-placental units were removed and mounted vertically in an organ bath containing 10 ml de Jongh solution (composition: 137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM NaH_2PO_4, 6 mM glucose, pH=7.4). The temperature of the organ bath was maintained at 37 °C, and carbogen (95% O_2 + 5% CO_2) was perfused continuously through the bath. After mounting, the uterine rings were equilibrated for 60 min before experiments were started, with a buffer change every 15 min. 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).

α_1-adrenergic receptors

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. Following the addition of each concentration of NA, recording was performed for 300 s.

α_2-adrenergic receptors

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 doxasosin (10^{-6} M) in order to exclude β-adrenergic and α_1-adrenergic action. The selective α_{2A}-AR subtype antagonist BRL 44408, the $\alpha_{2B/C}$-AR subtype antagonist ARC 239 (each 10^{-7} M), α_{2C}-AR subtype antagonist spiroxatrine, 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} (maximum possible effect) and EC_{50} (half maximum effective concentration) values were calculated from the AUC values. Statistical evaluations were performed by using the ANOVA Dunnett test or the two-tailed unpaired t-test. NA, propranolol, yohimbine and BMY 7378 spiroxatrine were purchased from Sigma-Aldrich, Budapest, Hungary; and WB 4101, BRL44408, ARC239 was purchased from Tocris Bioscience, Bristol, UK.

3.7. Measurement of uterine cAMP accumulation

Uterine tissue samples from 22-day-pregnant rats were incubated in an organ bath (10 ml) containing de Jongh solution (37 °C, perfused with carbogen). 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. After stimulation, the samples were immediately frozen in liquid nitrogen and stored until the extraction of cAMP (Hajagos-Tóth

et al. 2015). Frozen tissue samples were then ground, weighed, homogenized in 10 volumes of ice-cold 5% trichloroacetic acid and centrifuged at 1000*g* for 10 min. The supernatants were extracted with 3 volumes of water-saturated diethyl ether. After drying, the extracts were stored at −70 °C until the cAMP assay. Uterine cAMP accumulation was measured with a commercial cAMP Enzyme Immunoassay Kit (Cayman Chemical, USA); tissue cAMP levels were expressed in pmol/mg tissue.

3.8. [^{35}S]GTPγS binding assay

Uteri were removed (n=5 in each experiment) and homogenized in 20 volumes (w/v) of ice-cold buffer (10 mM Tris-HCl, 1 mM EDTA, 0.6 mM $MgCl_2$, and 0.25 M sucrose, pH 7.4) with an Ultra Turret T25 (Janke & Kunkel, Staufen, Germany) homogenizer, and the suspension was then filtered on four layers of gauze and centrifuged (40,000*g*, 4 °C, 20 min). After centrifugation, the pellet was resuspended in a 5-fold volume of buffer. The protein contents of the samples were diluted to 10 mg protein/sample. Membrane fractions were incubated in a final volume of 1 ml at 30 °C for 60 min in Tris-EGTA buffer (pH 7.4) composed of 50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, containing 20 MBq/0.05 cm^3 [^{35}S]GTPγS (0.05 nM) (Sigma Aldrich, Budapest, Hungary), together with increasing concentrations (10^{-9}–10^{-5} M) of NA. WB 4101, BMY 7378, BRL 44408, ARC 239 and spiroxatrine were used in a fixed concentration of 0.1 μM. 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. Total binding was measured in the absence of the ligands, non-specific binding was determined in the presence of 10 μM unlabeled GTPγS and subtracted from total binding. The difference represents basal activity. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/B filters with Brandel M24R Cell harvester. Filters were washed three times with 5 ml ice-cold buffer (pH 7.4), and the radioactivity of the dried filters was detected in UltimaGold™ MV scintillation cocktail with Packard Tricarb 2300TR liquid scintillation counter (Zádor et al 2014). The [^{35}S]GTPγS binding experiments were performed in triplicate and repeated at least three times.

G_i protein was inhibited with pertussis toxin (Sigma Aldrich, Budapest, Hungary) in a concentration of 500 ng/ml after the addition of protein and GDP to the Tris-EGTA buffer 30 min before [^{35}S]GTPγS.

4. Results

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

4.1.1. 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 (**Fig. 1a**) nor the P4 (**Fig. 1c**) pretreatment changed the mRNA expression. The results of Western blot analysis at the level of protein expression revealed no change, correlating with the PCR results (**Fig. 1b, d**).

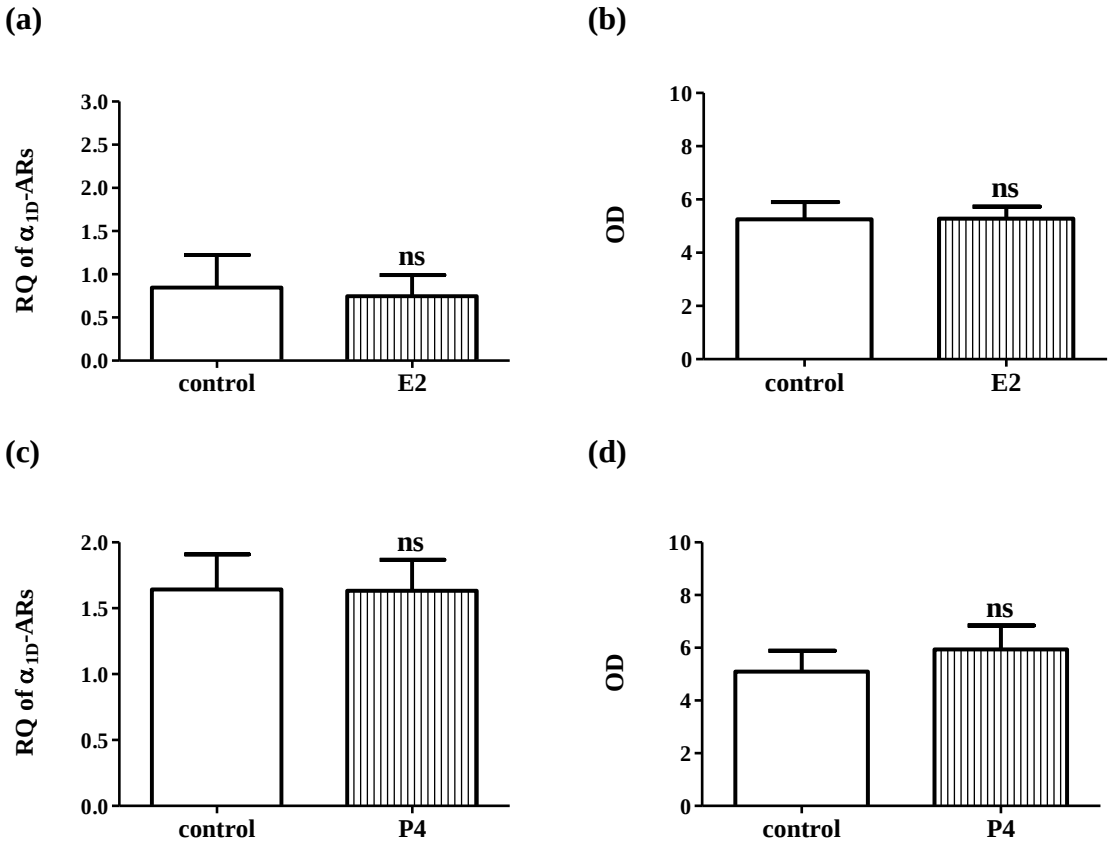

Fig. 1. Changes in the myometrial mRNA and protein expressions of the α1D-ARs after E2 pretreatment (**a, b**) or P4 pretreatment (**c, d**) in 22-day-pregnant rat uteri. The antibody binding was expressed as optical density (OD) data (A) for α_{1A}-AR. The y-axis shows the ratio of α_1-AR/ β-actin protein optical density. The statistical analyses were carried out with the two-tailed unpaired t-test. (RQ: relative quantity)

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

After E2 pretreatment, the expression of the α_{1A}-AR subtype mRNA was significantly decreased (**Fig. 2a**), whereas there was no change after P4 pretreatment (**Fig. 2c**). The results of Western blot analysis at the protein expression level reinforced the PCR results (**Fig. 2b, d**).

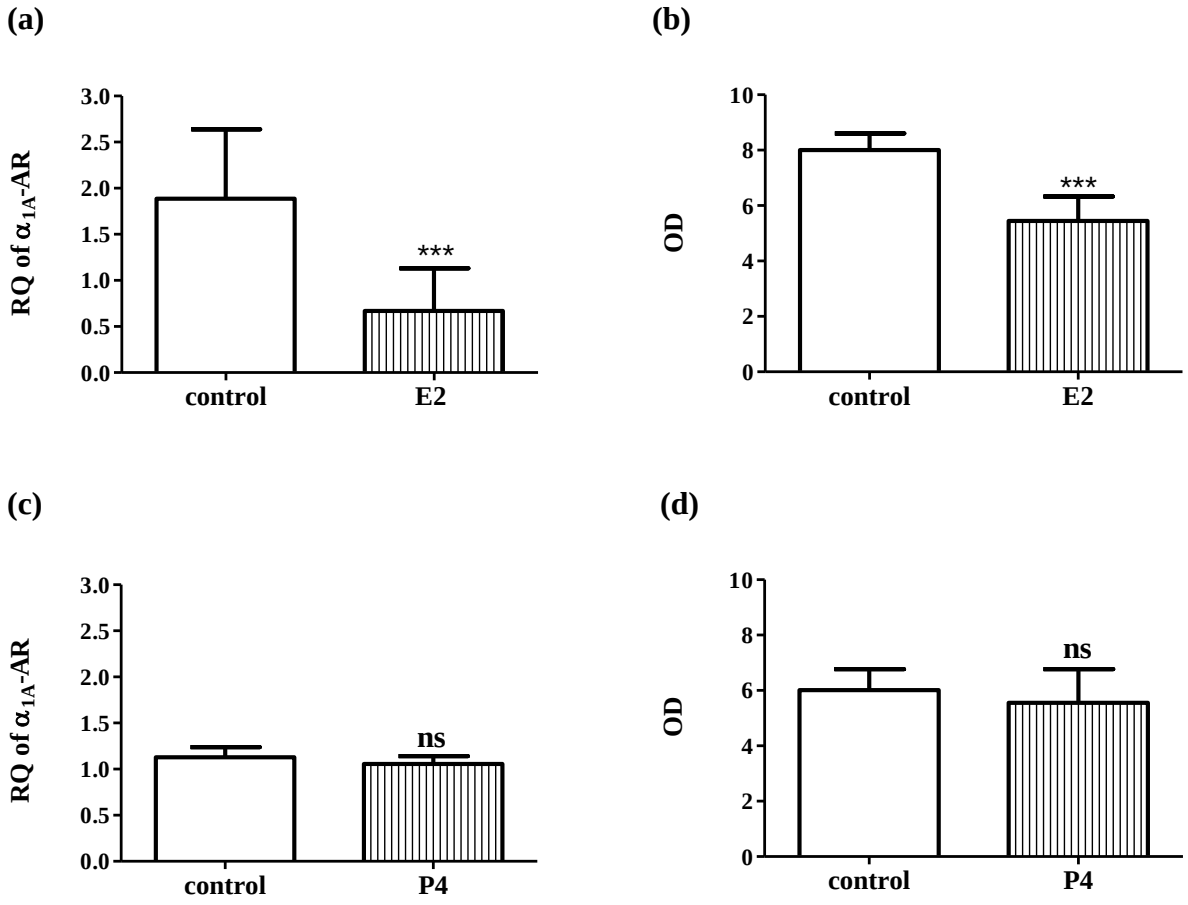

Fig. 2. Changes in the myometrial mRNA and protein expressions of the α_{1A}-ARs after E2 pretreatment (**a, b**) or P4 pretreatment (**c, d**) in 22-day-pregnant rat uteri. The antibody binding was expressed as optical density (OD) data (A) for α_{1A}-AR. The y-axis shows the ratio of α_1-AR/ β-actin protein optical density.The statistical analyses were carried out with the two-tailed unpaired t-test. ***P<0.001(RQ: relative quantity)

4.1.3. Effects of α_1-adrenergic receptor 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. 3a**). In the presence of the α_{1A}-AR antagonist and α_{1D}-AR antagonist the NA concentration-response curve was shifted to the right. In the presence of the α_{1A}-AR antagonist WB 4101, the maximum contractile effect of NA did not change, but the concentration-response curve was shifted to the right as compared with the control. After E2 pretreatment, the NA concentration-response curve was further shifted to the right (**Fig. 3b**), while the maximum contracting effect remained unchanged. The P4 pretreatment (**Fig. 3b**) reduced the maximum contractile effect of NA to one third in the presence of the α_{1A}-AR antagonist. There were no changes in the EC_{50} values.

In the presence of the α_{1D}-AR antagonist BMY 7378, the NA concentration-response curve was shifted to the right relative to the control and there were no significant changes in the maximum contractile effect of NA. 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. 3c**).

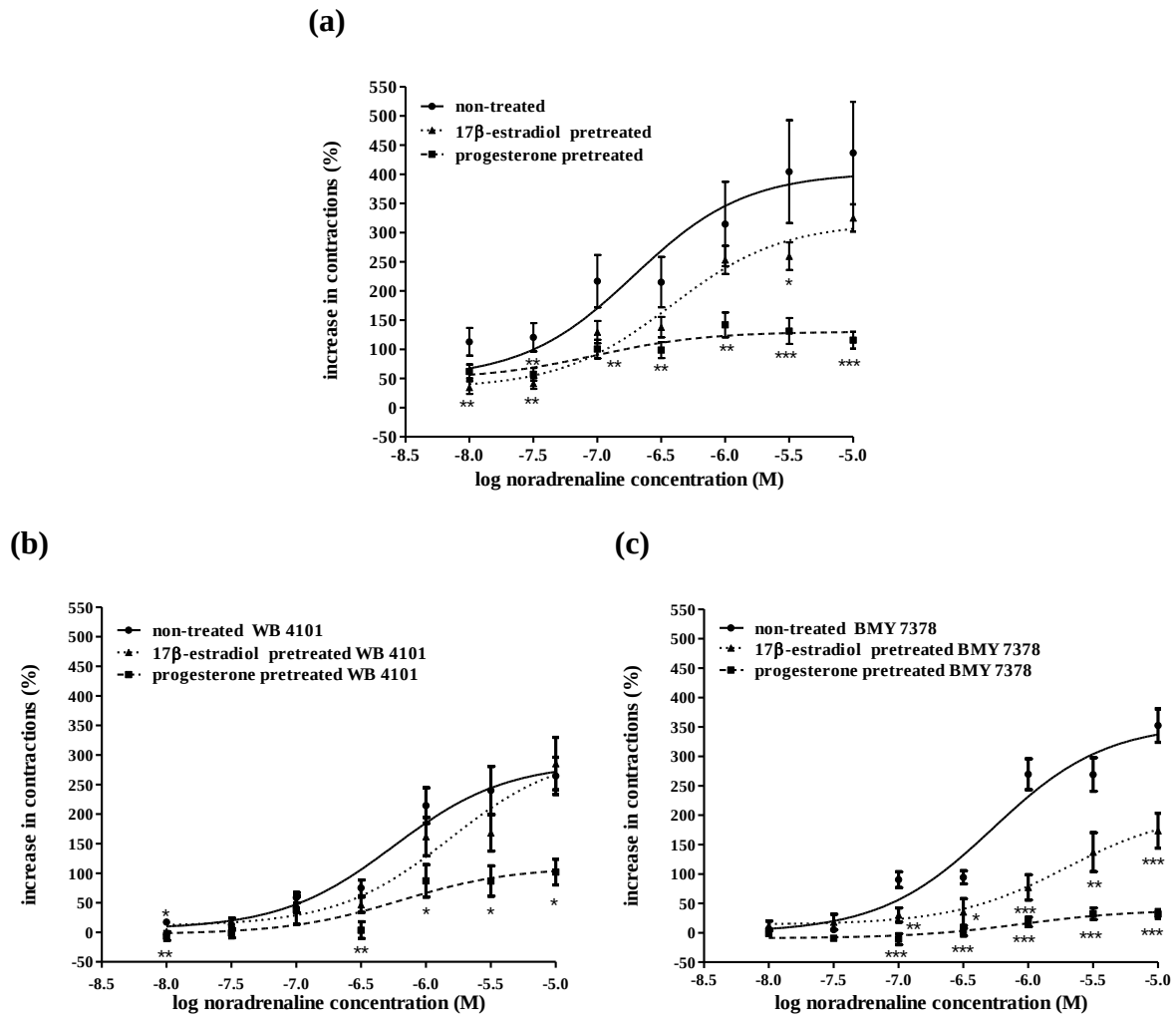

Fig.3. Effects of the subtype-selective α_{1A}-AR antagonist WB 4101 and the α_{1D}-AR antagonist BMY 7378 on the NA-evoked contractions in the 22-day-pregnant rat myometrium after P4 or E2 pretreatment. The studies were carried out in the presence of the β-AR antagonist propranolol (10^{-5} M) and the 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 the α_{1A}-AR antagonist WB 4101 (**b**) or the α_{1D}-AR antagonist BMY 7378 (**c**) in an isolated organ bath. The change in contraction was calculated via the area under the curve and expressed in % ± S.E.M. The statistical analyses were carried out with the ANOVA Dunnett test. *P<0.05; **P<0.01; ***P<0.001

4.1.4. Effects of subtype-selective α_1-adrenergic receptor antagonists on miometrial [^{35}S]GTPγS binding level

In the presence of WB 4101, NA slightly increased the [^{35}S]GTPγS binding (**Fig. 4a**). After E2 pretreatment, there was no change, but after P4 pretreatment the extent of [^{35}S]GTPγS binding was enhanced.In the presence of BMY 7378, NA slightly stimulated the [^{35}S]GTPyS binding as compared with the basal value (**Fig. 4b**). After E2 pretreatment, there was no difference in the maximum of [^{35}S]GTPyS binding. After P4 pretreatment, NA caused a noteworthy increase in [^{35}S]GTPyS binding.

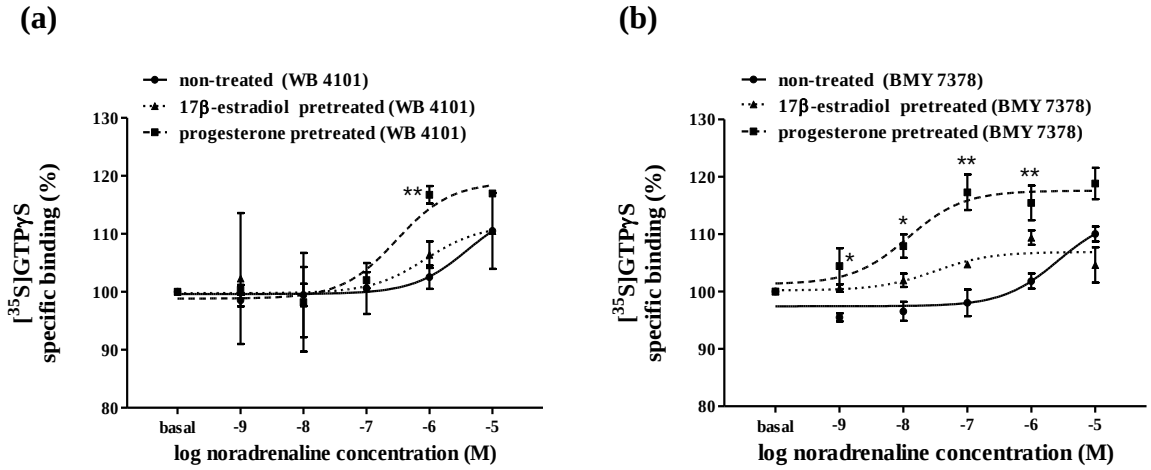

Fig. 4. Changes induced by various concentrations of NA in [^{35}S]GTPγS binding in the presence of WB 4101 (**a**) or BMY 7378 (**b**) following pretreatment with E2 or P4. In all cases, the β-ARs and the α_2-ARs were inhibited by propranolol and yohimbine. Basal refers to the level of [^{35}S]GTPγS binding without substance. The statistical analyses were carried out with the ANOVA Dunnett test. *P<0.05; **P<0.01.

4.1.5. Effects of subtype-selective α_1-adrenergic receptor antagonists on miometrial [^{35}S]GTPγS binding level in the presence of pertussis toxin

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]GTPyS binding after E2 or P4 pretreatment (**Fig. 5a**). In the presence of BMY 7378, NA did not stimulate the [^{35}S]GTPyS binding, either in high concentration or after E2 pretreatment. However, NA reduced the [^{35}S]GTPγS binding after P4 pretreatment (**Fig. 5b**).

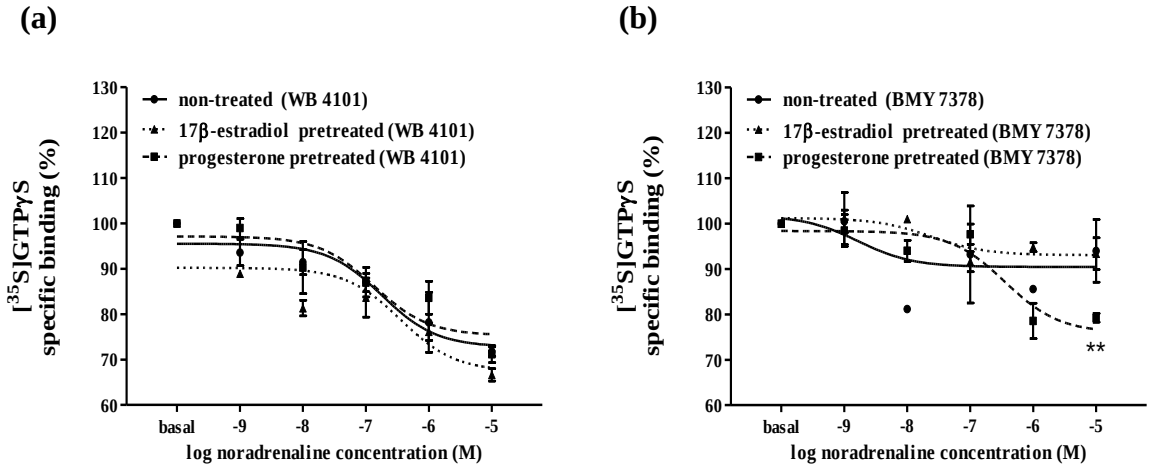

Fig. 5. The effects of PTX on the changes induced by various concentrations of NA in [^{35}S]GTPγS binding in the presence of WB 4101 (**a**) or BMY 7378 (**b**) following pretreatment with E2 or P4. In all cases, the β-ARs and the α_2-ARs were inhibited by propranolol and yohimbine. Basal refers to the level of [^{35}S]GTPyS binding without substances. The statistical analyses were carried out with the ANOVA Dunnett test. **P<0.01.

4.2. Effects of 17β-estradiol pretreatment on the myometrial α_2-AR subtypes

4.2.1. The myometrial mRNA expressions of the α_2-adrenergic receptors after 17β-estradiol pretreatment

The mRNA expression of all α_2-AR subtypes (**Fig. 6a,b,c**) were significantly decreased after E2 pretreatment compared to the non-treated uteri.

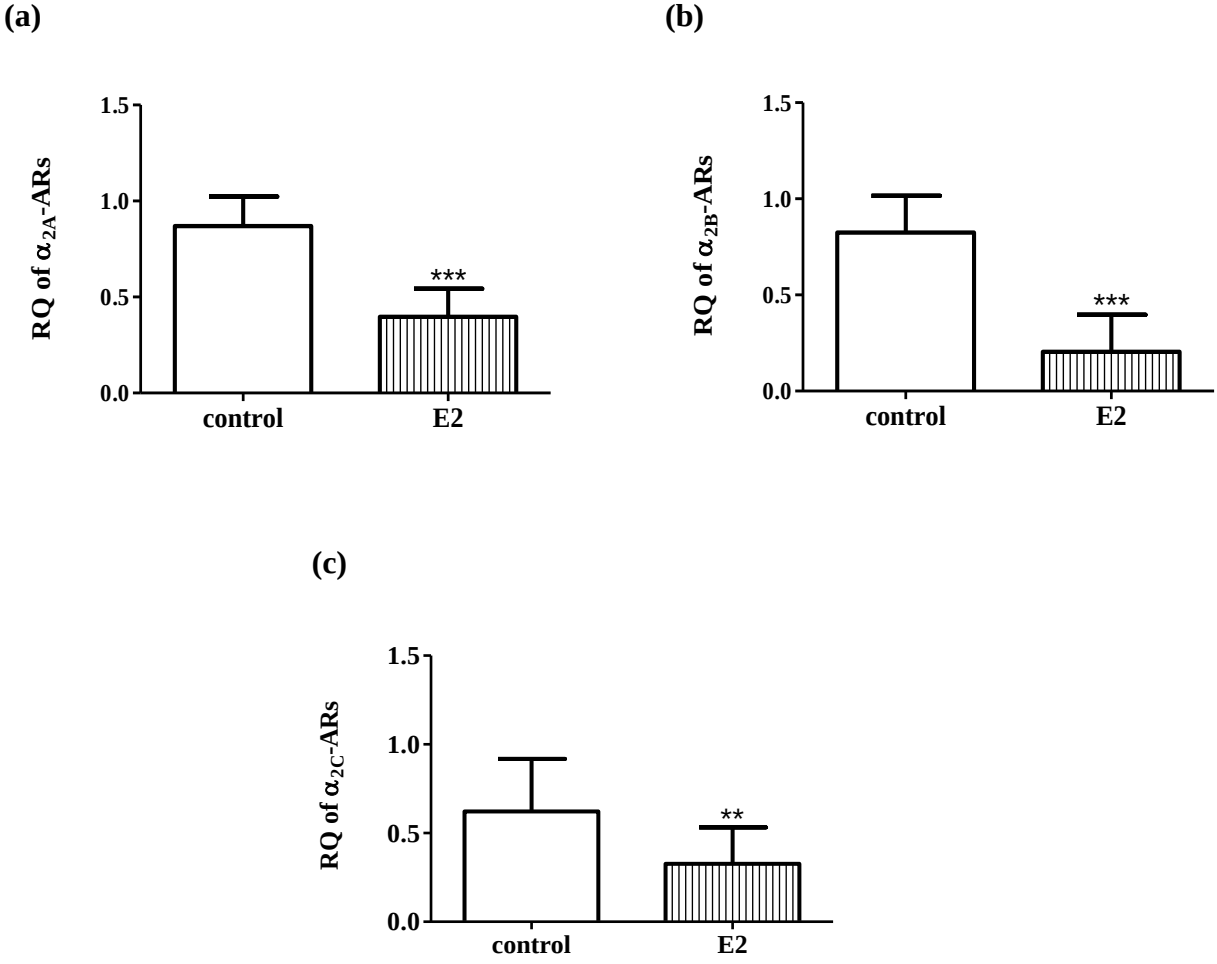

Fig. 6. Changes in the myometrial mRNA and protein expression of the α_{2A}- (**a**), α_{2B}- (**b**) and α_{2C}-ARs (**c**) after E2 pretreatment. The statistical analyses were carried out with the two-tailed unpaired t-test. (RQ: relative quantity) ** P=0.01; *** P<0.001

4.2.2. The myometrial protein expressions of the α_2-adrenergic receptors after 17β-estradiol pretreatment

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 (**Fig. 7a, b, c**).

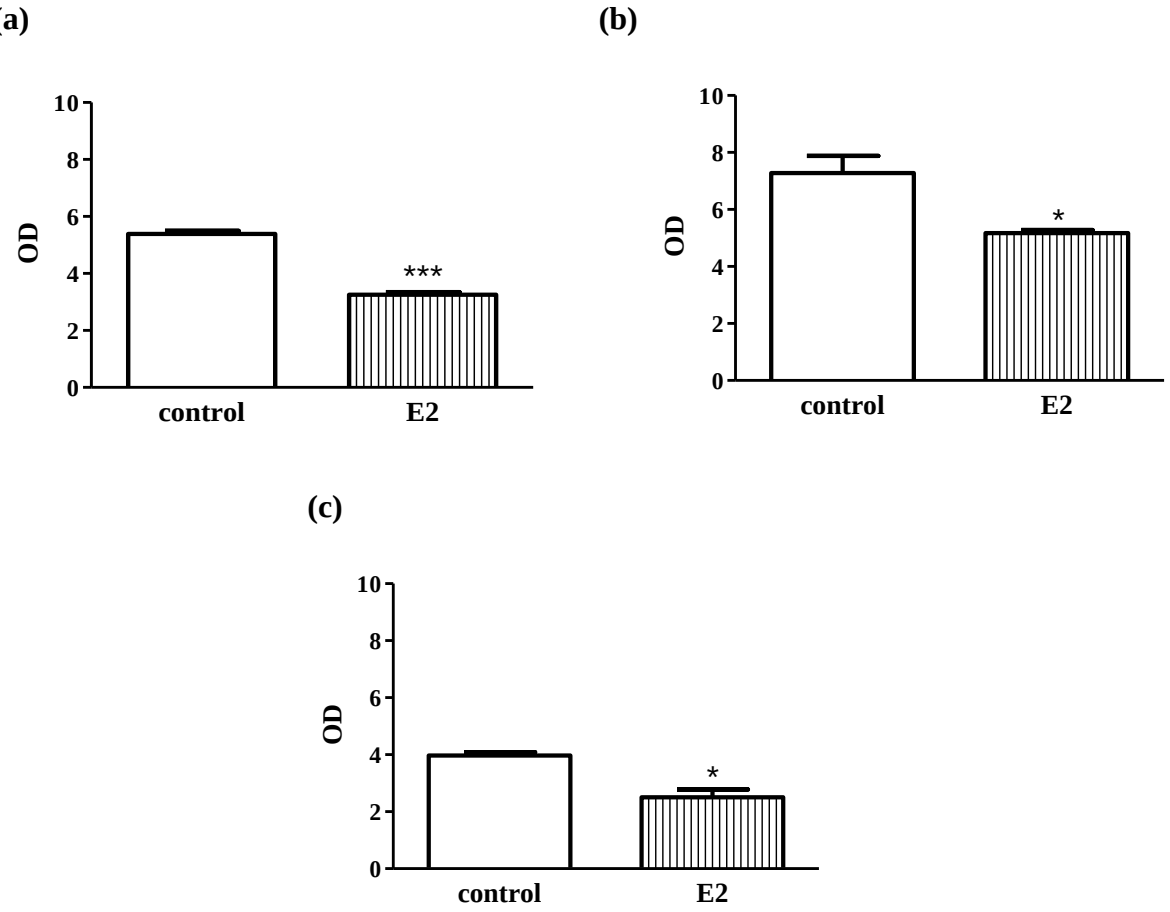

Fig.7. Changes in the α_2-AR levels in the 22-day pregnant rat myometrium after E2 pretreatment. The antibody binding was expressed as optical density (OD) data (**a**) for α_{2A}-, (**b**) for α_{2B} and (**c**) for α_{2C}-ARs. The y axis shows the ratio of α_2-AR/ β-actin protein optical density. The statistical analyses were carried out with the two-tailed unpaired t-test. * $P<0.05$; *** $P<0.001$

4.2.3. 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. 8a**). After E2 pretreatment, the myometrial contracting effect of NA was decreased.

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. 8b**). 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. 8b**), 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. 8b**), 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. 8b**), but decreased it compared to the BRL 44408+spiroxatrine treated control.

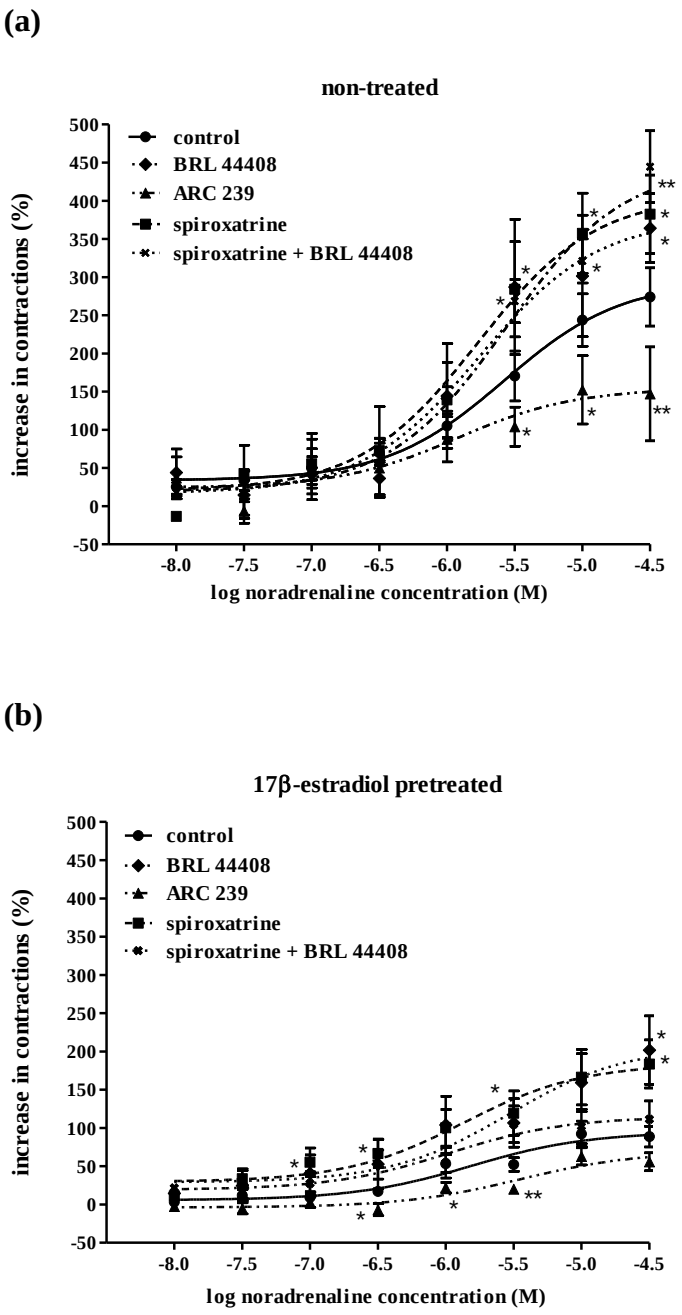

Fig. 8. Effects of the subtype-selective α_{2A}-AR antagonist BRL 44408, $\alpha_{2B/C}$-AR antagonist ARC 239, and the α_{2C}-AR antagonist, spiroxatrine on the NA-evoked contractions in the 22-day-pregnant rat myometrium (**a**), after E2 pretreatment (**b**). The studies were carried out in the presence of the β-AR antagonist, propranolol (10^{-5}M), and the α_1-AR antagonist, doxazosin (10^{-7}M) in each case. The change in contraction was calculated via the area under the curves and expressed in % ± S.E.M. The statistical analyses were carried out with ANOVA Dunnett test. *P< 0.05; **P< 0.01; ***P< 0.001.

4.2.4. Effects of subtype-selective α_2-adrenergic receptor antagonists on miometrial cAMP level after 17β-estradiol pretreatment

E2 pretreatment increased the myometrial cAMP level (**Fig. 9**) 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. However, it did not change the cAMP level in the presence of the spiroxatrine + BRL 44408 combination.

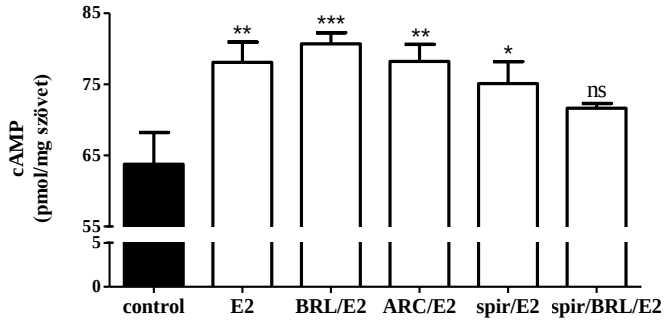

Fig.9. Effects of the subtype-selective α_{2A}-AR antagonist BRL 44408, the $\alpha_{2B/C}$-AR antagonist ARC 239 and the α_{2C}-AR antagonist spiroxatrine on the myometrial cAMP level (pmol/mg tissue ± S.D.) in the presence of IBMX (10^{-3} M) and forskolin (10^{-5} M) (control) in the 22-day-pregnant rat (n = 6) after E2 pretreatment. The statistical analyses were carried out with ANOVA followed by Dunnett's Multiple Comparison Test. *P<0.05; **P<0.01; ***P<0.001.

4.2.5. 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-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. 10a**).

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. 10b**).

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 1×10^{-9} M. In the presence of PTX, E2 pretreatment abolished the [^{35}S]GTPγS binding-inhibitory effect of NA (**Fig. 10c**).

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. 10d**).

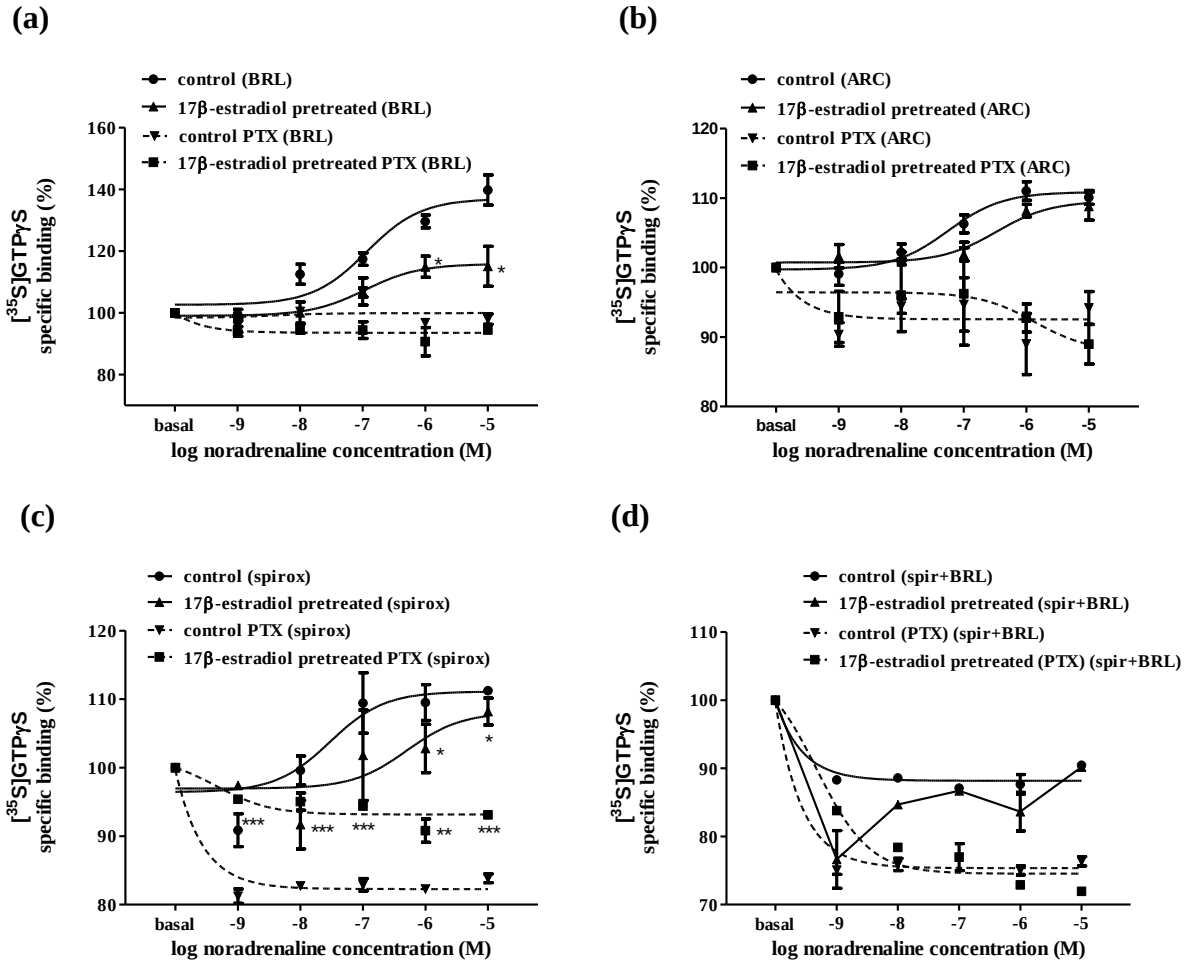

Fig. 10. Changes induced by various concentrations of NA in [^{35}S]GTPγS binding in the presence of subtype-selective α_{2A}-antagonist BRL 44408 (**a**), the $\alpha_{2B/C}$- antagonist ARC 239 (**b**), the α_{2C}- antagonist spiroxatrine (**c**) and the BRL 44408-spiroxatrine combination (**d**) following pretreatment with E2. In all cases, the β-ARs and the α_1-ARs were inhibited by propranolol and doxazosin. Basal refers to the level of [^{35}S]GTPγS binding without substance. The statistical analyses were carried out with the ANOVA Dunnett test. *P<0.05; **P<0.01; ***P<0.001

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

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

The mRNA expression of each α_2-AR subtype (**Fig. 11a,b,c**) was significantly increased after P4 pretreatment as compared with the non-treated uteri.

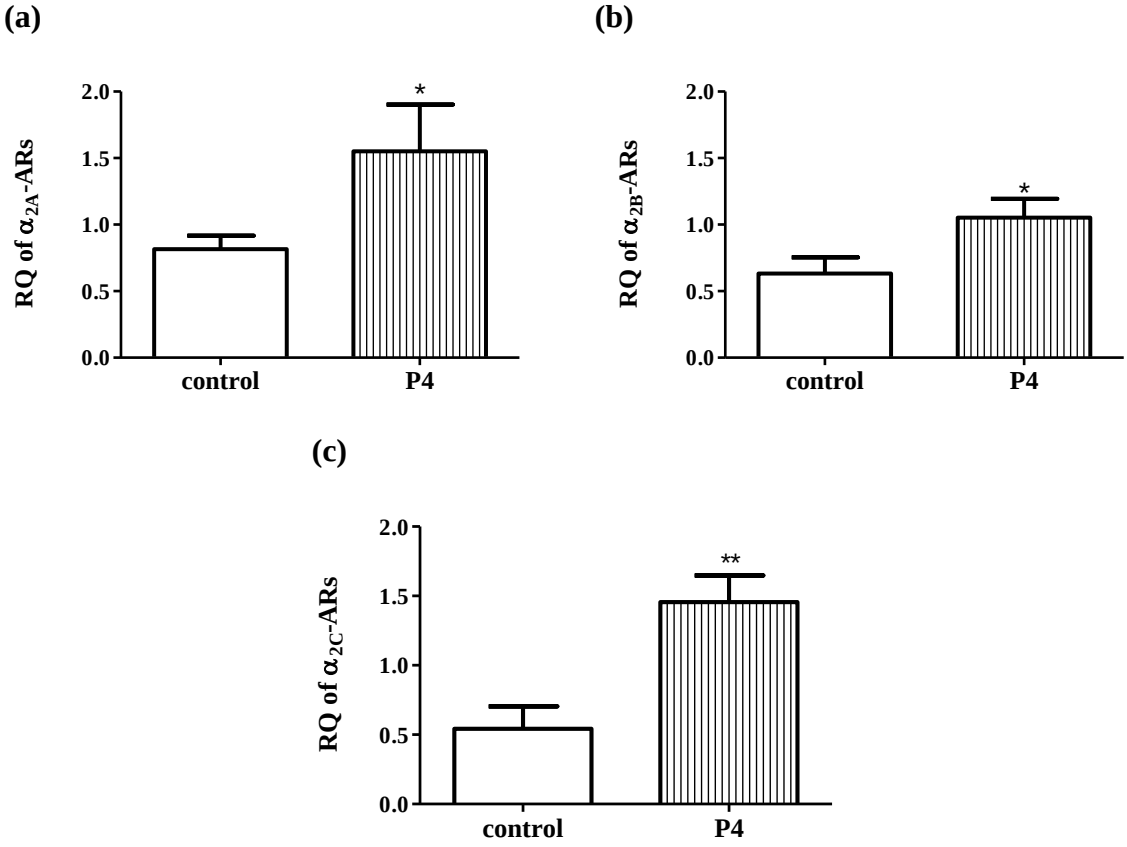

Fig. 11. Changes in the myometrial mRNA expressions of the α_{2A}-ARs (**a**), α_{2B}-ARs (**b**) and α_{2C}-ARs (**c**) after P4 pretreatment in 22-day-pregnant rat uteri. The statistical analyses were carried out with the two-tailed unpaired t-test. * P<0.1; ** P<0.01; *** P<0.001(RQ:relative quantity)

4.3.2. The myometrial protein expressions of the α_2-adrenergic receptors after progesterone pretreatment

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. 12a, b, c**).

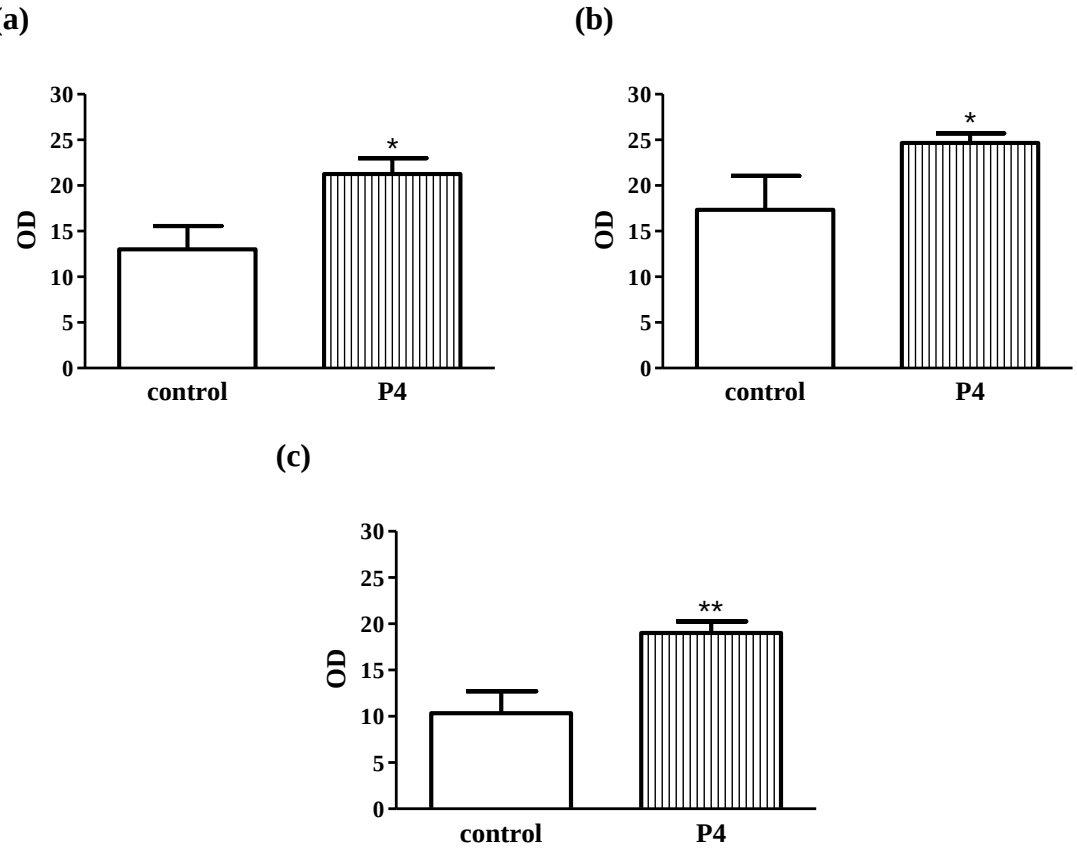

Fig. 12. Changes in the α_2-AR levels in the 22-day pregnant rat myometrium after P4 pretreatment. The antibody binding was expressed as optical density (OD) data (**a**) for α_{2A}-, (**b**) for α_{2B}-and (**c**) for α_{2C}-ARs. The y axis shows the ratio of α_2-ARs /β-actin protein optical densities. The statistical analyses were carried out with the two-tailed unpaired t-test .*P<0.1; ** P<0.01.

4.3.3. 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. 13a**). After P4 pretreatment, the myometrial contracting effect of NA was decreased (**Fig 13b**).

In the presence of the α_{2A}-AR antagonist BRL 44408, P4 pretreatment decreased the NA-evoked contractions as compared with the P4-treated control (**Fig. 13b**). BRL 44408 enhanced the NA-induced contractions, this being markedly reduced by P4 pretreatment **(Fig. 13a,b).**

In the presence of the $\alpha_{2B/C}$-AR antagonist 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. 13b**). ARC 239 reduced the NA-induced contractions, which were decreased further by P4 pretreatment **(Fig. 13a,b**).

P4 pretreatment decreased the maximum contracting effect of NA in the presence of spiroxatrine as compared with the P4-treated control (**Fig. 13b**). Spiroxatrine enhanced the NA-induced contractions, which were enormously reduced by P4 pretreatment **(Fig. 13a,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. 13b**). The combination of the two compounds increased the NA-induced contractions, which were reduced by P4 pretreatment **(Fig. 13a,b**).

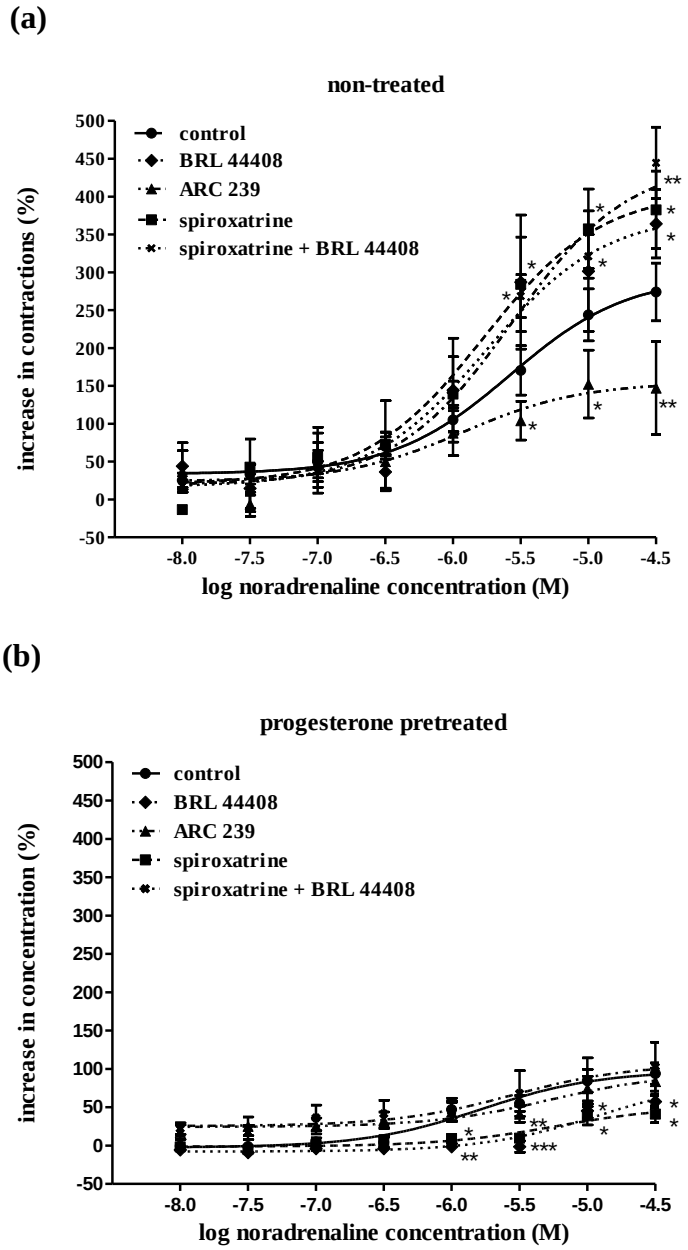

Fig. 13. Effects of the subtype-selective α_{2A}-AR antagonist BRL 44408, the $\alpha_{2B/C}$-AR antagonist ARC 239 and the α_{2C}-AR antagonist spiroxatrine on the NA-evoked contractions in the 22-day-pregnant rat myometrium (**a**) and after P4 pretreatment (**b**). The studies were carried out in the presence of the β-AR antagonist propranolol (10^{-5} M) and the α_1-AR antagonist doxazosin (10^{-7} M) in each case. The change in contraction was calculated via the area under the curve and expressed in % ± S.E.M. The statistical analyses were carried out with the ANOVA Dunnett test. *P< 0.05; **P < 0.01; ***P < 0.001.

4.3.4. Effects of subtype-selective α_2-adrenergic receptor antagonists on miometrial cAMP level after progesterone pretreatment

P4 pretreatment increased the myometrial cAMP level (**Fig. 14**) 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.

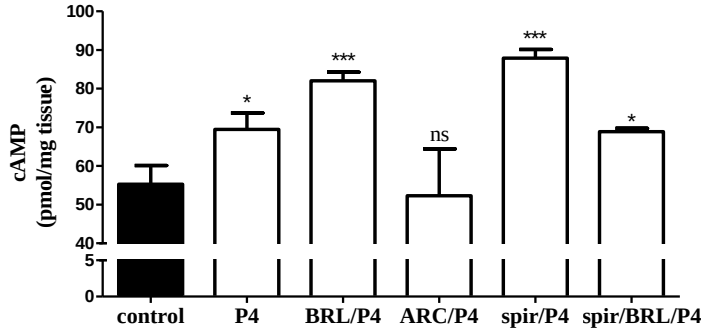

Fig.14. Effects of the subtype-selective α_{2A}-AR antagonist BRL 44408, the $\alpha_{2B/C}$-AR antagonist ARC 239 and the α_{2C}-AR antagonist spiroxatrine on the myometrial cAMP level (pmol/mg tissue ± S.D.) in the presence of IBMX (10^{-3} M) and forskolin (10^{-5} M) (control) in the 22-day-pregnant rat after P4 pretreatment. The statistical analyses were carried out with ANOVA followed by Dunnett's Multiple Comparison Test. *P < 0.05; **P < 0.01; ***P < 0.001.

4.3.5. 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-trated 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. 15a**).

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. 15b**).

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. 15c**).

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. 15d**).

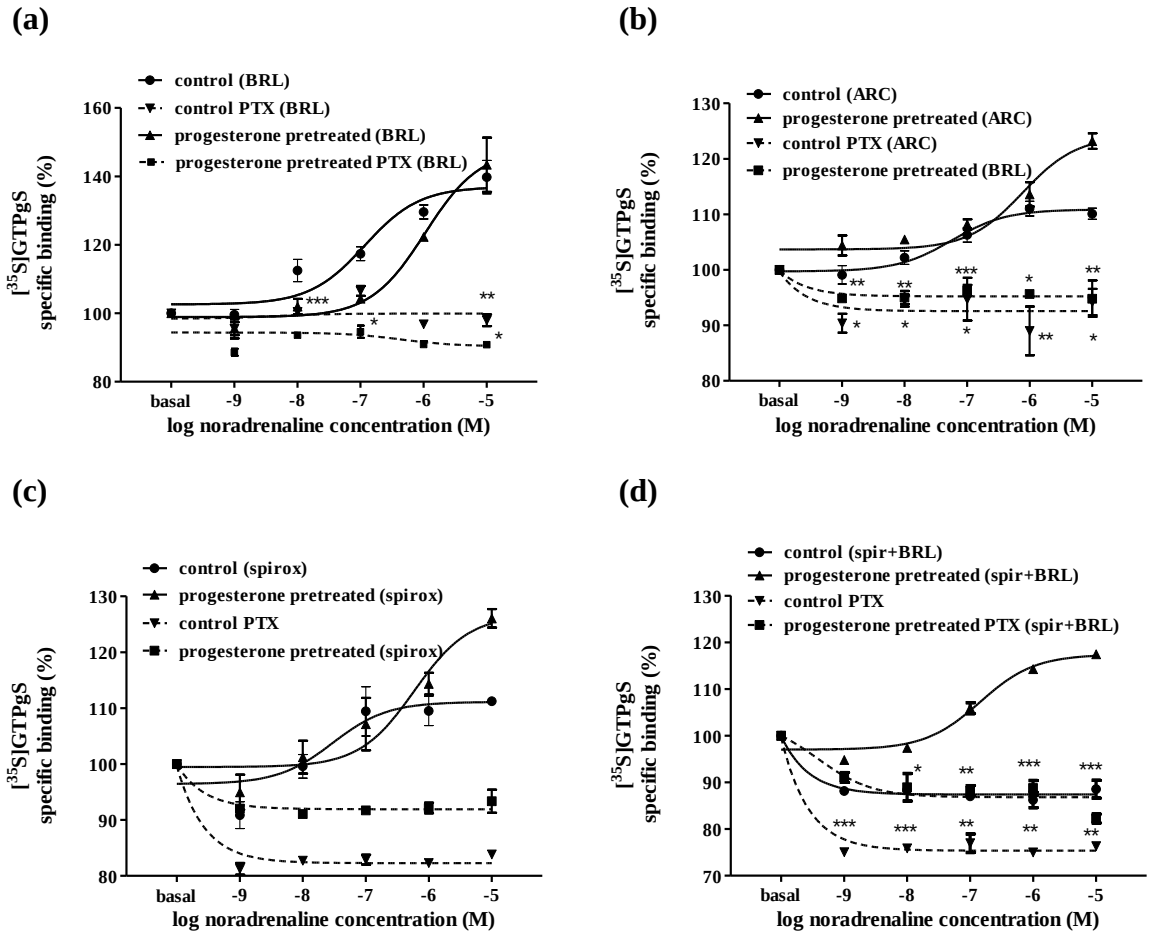

Fig. 15. Changes induced by various concentrations of NA in [^{35}S]GTPγS binding in the presence of the subtype-selective α_{2A}-AR antagonist BRL 44408 (**a**), the $\alpha_{2B/C}$-AR antagonist ARC 239 (**b**), the α_{2C}-AR antagonist spiroxatrine (**c**) and the spiroxatrine + BRL 44408 combination (**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. The statistical analyses were carried out with the ANOVA Dunnett test. **P < 0.01; ***P < 0.001.

5. Discussion

A number of comprehensive experiments have been performed previously to investigate the roles of the α- and β-ARs in the control of myometrial contraction, 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. Since sexual hormones and the adrenergic system have major role in myometrial contractions during human gestation, 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*. The α_1-AR-selective action of NA was provided by the application of the α_2-blocker yohimbine, while α_2-AR selective action was provided in the presence of α_1-AR antagonist doxazosine and in all cases we used the β-AR antagonist propranolol to block β-AR.

5.1. α_1-adrenergic receptors and the female sexual hormones

In case of α_1-ARs it was demonstrated that the α_{1B}-ARs cannot be detected in the late-pregnant rat uterus (Ducza *et al*. 2002). Therefore we focused on the α_{1A}-ARs and α_{1D}-ARs, and investigated the roles of the α_1-AR subtypes in myometrial contractility in the presence of subtype-selective antagonists with NA-stimulated contraction.

E2 pretreatment slightly decreased the maximum contracting effect of NA through the α_1-ARs, but it shifted the NA concentration-response curve to the right, indicating the weaker sensitivity of the receptors to NA. This is in contrast with the earlier finding (Legrand *et al*. 1987), which demonstrated that E2 predominance prior to birth (6 hours) causes a sharp increase in the density of α_1-ARs. Since we treated the animals with E2 for 4 days prior to the last day of gestation, and the physiological hormonal changes were also allowed to develop, we revealed the consequences of an extraordinary E2 predominance on the α_1-ARs subtypes.

P4 pretreatment reduced the maximum contracting effect of NA by more than a half, in harmony with earlier observations (Anesini and Borda 2003). 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.

When the α_{1A}-AR blocker WB 4101 was added to the system, NA stimulated only the α_{1D}-ARs. Neither E2 nor P4 changed the mRNA and protein expression of the α_{1D}-ARs as compared with the control values. Nevertheless, this does not explain why P4 decreased the myometrial contracting effect of NA.

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. To find an explanation, we carried out [^{35}S]GTPγS binding studies. α_1-ARs are mainly coupled to $G_{q/11}$ protein (Berridge 1993), while the [^{35}S]GTPγS binding assay measures the total level of G protein activation following antagonist occupation of the G protein-coupled receptor (Kolarovszki-Sipiczki *et al*. 2007). In the presence of the selective α_{1A}-antagonist WB 4101, NA moderately increased the [^{35}S]GTPγS binding and there were no differences after E2 pretreatment in comparison with the control values. However, P4 increased the [^{35}S]GTPγS binding induced by NA via the α_{1D}-ARs, which may contribute to the decreased myometrial contracting effect. Earlier studies demonstrated that the α_1-ARs can be coupled to G_i protein in some cases (Gurdal *et al*. 1997, Otani *et al*. 2001), and therefore we investigated the [^{35}S]GTPγS binding in the presence of PTX. The inhibitory action of PTX is specific for the G_i protein and allows a distinction from other G protein-mediated signal transduction pathways. 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. P4 pretreatment did not change the protein expression of the α_{1A}-ARs as compared with the control values, but after E2 pretreatment the protein expression was decreased, which can explain the decreased myometrial contracting effect of NA after E2 pretreatment, but does not explain the effect of P4. E2 pretreatment decreased the maximum contracting effect of NA and increased the EC_{50} values.

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. For further clarification, we performed [^{35}S]GTPγS binding studies in the case of the α_{1A}-ARs. NA slightly stimulated the [^{35}S]GTPγS binding. The presence of E2 did not alter the [^{35}S]GTPγS binding-stimulating effect of NA, whereas P4 increased it. 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.

5.2. α_2-adrenergic receptors and 17β-estradiol

E2 pretreatment decreased the mRNA and protein expression of the myometrial α_2-AR subtypes and also decreased the NA-evoked myometrial contraction through the α_2-ARs, which was similar to our earlier findings with the α_{1A}-ARs. According it means that E2 influences the expression of α_2-ARs differently in various tissues, as it increases the expression of the receptors in the spinal cord and cutaneous arteries (Thompson *et al.* 2008, Eid *et al.* 2007).

According to the isolated organ bath studies 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.

To find an explanation about the weaker myometrial contractions via the α_{2B}-AR subtype after E2 pretreatment, we measured the myometrial cAMP level, as the changes in the cAMP level are involved in the myometrial effect of the α_2-ARs. E2 pretreatment increased the myometrial cAMP level, which also proves the decreased myometrial contracting effect of NA through the α_2-ARs. It did not modify the cAMP level through the α_{2A}-ARs, which is in harmony with our previous study (Gáspár *et al.* 2007). However, it increased the myometrial cAMP level through the α_{2B}-ARs, which can explain the weaker myometrium contracting effect of NA.

The α_2-ARs can couple not only to the G_i protein α-subunit, but under certain circumstances, also to G_s proteins (Offermanns 2003). E2 was also shown to decrease the coupling of the α_2-ARs to G protein (Ansonoff *et al.* 2001). To find an explanation for the cAMP changes, we measured the myometrial [^{35}S]GTPγS binding of the α_2-AR subtypes after E2 pretreatment and in the presence of PTX, whose inhibitory action is specific for the G_i 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 (with spiroxatrine). 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 (Ansonoff *et al.* 2001). This process did not change myometrial contraction as compared with the hormone treated control.

5.3. 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, which was like our earlier findings with the α_1-ARs.

In the isolated organ bath studies, 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.

To find an explanation of the weaker myometrial contractions via the α_{2B}-AR subtype after P4 pretreatment, we measured the myometrial cAMP level, since the changes in the cAMP level are involved in the myometrial effect of the α_2-ARs. P4 pretreatment increased the myometrial cAMP level, which additionally proves the decreased myometrial contracting effect of NA through the α_2-ARs. It did not alter the cAMP level through the α_{2A}-ARs, which is in harmony with the result of the isolated organ bath studies that NA did not influence the myometrial contractions via these receptors after P4 pretreatment. However, it increased the myometrial cAMP level through the α_{2B}-ARs, which can explain the weaker myometrium-contracting effect of NA in the presence of BRL 44408 (stimulation via α_{2B}- and α_{2C}-ARs), spiroxatrine (stimulation via α_{2A}- and α_{2B}-ARs) and the spiroxatrine + BRL 44408 combination (stimulation via α_{2B}-AR).

The literature indicates that the G_i/G_s-activating property of α_2-AR in rats changes during gestation, resulting in differences in the regulation of myometrial AC activity at mid-pregnancy versus term [Mhaouty *et al.* 1995]. We therefore measured whether P4 can modify the myometrial [^{35}S]GTPγS binding of the α_2-AR subtypes in the presence of the G_i protein blocker PTX at the end of pregnancy. 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. To confirm this hypothesis, we measured the myometrial [^{35}S]GTPγS binding of the α_{2B}-AR subtype in the presence of the spiroxatrine + BRL 44408 combination. P4 reversed the effect of NA on [^{35}S]GTPγS binding in the presence of PTX and also reversed the [^{35}S]GTPγS binding-stimulating effect of NA. This result suggests that, in of predominance of P4, the α_{2B}-ARs are coupled, at least partially, to G_s protein, which leads to the activation of AC and decreases the NA-induced myometrial contraction via these receptors.

6. 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.

The expression of the α_{1A}-ARs is highly E2-sensitive, as it was decreased after E2 pretreatment in contrast with a literature report. P4 pretreatment does not have any effect on the mRNA and protein expressions of either the α_{1A}-ARs or the α_{1D}-ARs, it does have an impact on the G protein coupling, leading to decreased myometrial contraction via the G_i protein. However, in a predominance of E2, their effects are less dependent on the G_i protein pathway.

E2 decreases the expressions of the α_2-AR subtypes and leads to increased uterine cAMP level. It does not modify the myometrial relaxing effect via the α_{2A}- and α_{2C}-ARs. In case of these receptors we suppose that the E2 treatment mainly induce the activation of $\beta\gamma$ subunit of G_i protein, increasing the smooth muscle cAMP level [Zhou *et al.* 2000]. In case of α_{2B}-ARs E2 alters the myometrial contracting effect of NA by reduced coupling of the receptor to G_i protein.

We conclude that P4 increases the expression of each α_2-AR subtype, and reduces the NA-induced myometrial contractions via the totality of these receptors. P4 blocks the G-protein coupling and cAMP production via the α_{2A}-ARs. In the case of the α_{2C}-ARs, we presume that P4 treatment mainly induces the activation of the $\beta\gamma$ subunit of the G_i protein, eliciting an increase in the smooth muscle cAMP level [Zhou *et al.* 2000]. In the case of the α_{2B}-ARs, G_s coupling is a determining factor in the function of the receptors after P4 treatment, which leads to an increased cAMP level and decreased myometrial contraction.

Based on these results we suppose that the alteration of female sex hormone during pregnancy alters the contractility of the myometrium via α-AR subtypes. Any dysregulation in this system may lead to contractility disorders and even preterm birth. The subtype selective agonist or antagonists of α-AR subtypes may have the potency to reduce premature contractions.

7. References

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