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Different roles of alpha2-AR subtypes in the uterine function in vitro in the rat

Ph.D. Thesis

by

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CONTENTS:

List of publications

Full papers and abstrats related to the Ph.D. thesis

I. **Gál Adrienn,** Kolarovszki-Sipiczki Zoltán, Gálik Márta, Ducza Eszter, Minorics Renáta, Klukovits Anna, Falkay György, Gáspár Róbert: Az ARC 239 hatása a miometrium és a cervix működésére patkányban, in vitro. Acta Pharmaceutica Hungarica 2009; 79(2). 75-80.

II. **Adrienn Gál,** Eszter Ducza, Renáta Minorics, Anna Klukovits, Márta Gálik, George Falkay and Róbert Gáspár: The roles of α_2-AR subtypes in the control of cervical resistance in the late-pregnant rat. European Journal of Pharmacology 2009; 615(1-3). 193-200. **IF.: 2,787**

III. Róbert Gáspár, **Adrienn Gál,** Márta Gálik, Eszter Ducza, Renáta Minorics, Zoltán Kolarovszki-Sipiczki, Anna Klukovits and George Falkay: Different roles of α_2-AR subtypes in non-pregnant and late-pregnant uterine contractility in vitro in the rat. Neurochemistry International 2007; 51(5). 311-318. **IF.: 3,228**

IV. **A. Gál,** R. Gáspár, E. Ducza, R. Minorics, M. Gálik, A. Klukovits and G. Falkay: The roles of α_2-AR subtypes in the control of cervical resistance in the late-pregnant rat. 2nd Meeting of the Diczfalusy Foundation: Prevention in Women´s Health, September 30-October 1, 2008, Szeged, Hungary

V. **A Gál,** R. Gáspár, M. Gálik, E. Ducza, R. MInorics, Z. Kolarovszki-Sipiczki, A. Klukovits and G. Falkay: Different roles of alpha2-adrenergic receptor subtypes in the pregnant uterine contractility *in vitro* in the rat. The First Conference of PhD Students in Medicine and Pharmacy, July 9-11, 2008, Targu Mures, Romania

VI. Gáspár R., Gálik M., **Gál A.,** Ducza E., Minorics R., Kolarovszki-Sipiczki Z., Falkay G.: Different roles of alpha2-adrenergic receptor subtypes in the pregnant uterine contractility in the rat. 15th World Congress of Pharmacology, July 2-7, 2006, Beijing, China

VII. Gálik M., Gáspár R., **Gál A.,** Ducza E., Minorics R., Klukovits A., Kolarovszki-Sipiczki Z., Falkay Gy.: α_2-adrenerg receptorok szerepe a terhes patkány uterus

kontraktilitásában. XIII. Congressus Pharmaceuticus Hungaricus, 2006 május 25-27, Budapest, Magyarország

VIII. Gáspár R., Kolarovszki-Sipiczki Z., Gál A., Minorics R., Gálik M., Ducza E., Falkay G.: Alpha2-AR subtypes as new targets for tocolytic therapy. 1st International Symposium in Hot topics & Controversies in Perinatal Medicine, June 16-19, 2005, Rhodes Island, Greece

Other publications and abstracts

IX. Renáta Minorics, Róbert Gáspár, **Adrienn Gál,** Anna Klukovits and George Falkay: Progesterone decreases the relaxing effect of the β_3-adrenergic receptor agonist BRL 37344 in the pregnant rat myometrium. Reproduction 2009; 138(2):383-390. **IF.: 3,073**

X. Maria N. Modica, Giuseppe Romeo, Loredana Salerno, Valeria Pittalà, Maria A. Siracusa, Ilario Mereghetti, Alfredo Cagnotto, Tiziana Mennini, Róbert Gáspár, **Adrienn Gál**, George Falkay, Márta Palkó, Gábor Maksay, Ferenc Fülöp: Synthesis and receptor binding of new thieno[2,3-d]-pyrimidines as selective ligands of 5-HT(3) receptors. Archive der Pharmazie 2008; 341. 333-343. **IF.:1,429**

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XII. R. Minorics, R. Gáspár, **A. Gál** and G. Falkay: Can the β_3-adrenergic receptor be a novel target in the tocolytic therapy? 2nd Meeting of the Diczfalusy Foundation: Prevention in Women´s Health, September 30 - October 1, 2008, Szeged, Hungary

XIII. Minorics R**,** Gáspár R, **Gál A**, Klukovits A, Falkay G.: Progesterone decreases the relaxing effect of BRL 37344, a β_3-adrenergic receptor agonist in the pregnant rat myometrium. Prevention in Women's Health 2nd Meeting of the Egon & Ann Diczfalusy Foundation 2008, September 30 - October 1, Szeged, Hungary.

XIV. R. Minorics, R. Gáspár, **A. Gál** and G. Falkay: Can the β_3-adrenergic receptor be a novel target in the tocolytic therapy? EACPT 2007, August 28 – September 2, Amsterdam, The Netherlands

List of abbreviations

Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature: Nomenclature and symbolism for Amino Acids and Peptides (J Biol Chem 1984; 219: 345-373)

cAMP	cyclic adenosine monophosphate
EC_{50}	half of the maximum effect
E_{max}	maximum effect
GAPDH	glyseraldehyde-3-phosphate dehydrogenase
G protein	heterotrimeric guanine nucleotide binding regulatory protein
GTPγS	guanosine-5'-O-(γ-thio)triphosphate
IC	intracellular
NA	noradrenaline
PGE_2	Prostaglandine E_2
PTX	pertussis toxin
RT-PCR	reverse transcriptase – polymerase chain reaction
s.c.	subcutaneous
Tris-HCl	tris(hidroxymethyl)aminomethane

1. Introduction

1.1. Anatomy and function of the uterus

The uterus is a major female hormone-responsive reproductive sex organ of most mammals, including humans. The human uterus is composed of 2 basic parts, the fundus and the cervix. The fundus is composed of myometrium, and the endometrium. The myometrium predominantly consists of smooth muscle cells, and supporting stromal and vascular tissue. The cervix (or neck of the uterus) is the lower, narrow portion of the uterus where it joins with the top end of the vagina.

The uterus has a spontaneous contractile activity, however, during pregnancy the progesterone secreted from the placenta suppresses the activity of the uterus, keeping the foetus within the uterus. At this time, the cervix remains firm and non-compliant. This physical integrity is critical so that the developing foetus can remain in the uterus until the appropriate time of the delivery. At term, however, the uterine contractions become more frequent and regular, while the cervix dilates, softens and becomes more distensible, a process called cervical ripening. These alterations facilitate a timely passage of the foetus at parturition. Under the effect of myometrial contractions, the cervix passively dilates and is pulled over the presenting foetal part. The exact process of these changes is not clear. The complex mechanism is controlled by a heterogeneous regulation, involving physiological factors such as oxytocin, the adrenergic system, prostaglandins, connexin and sex hormones (Fu et al., 1996; Roberts et al., 1989; Miyoshi et al., 1996). An understanding of these processes, at the molecular and cellular level, is essential to developing novel therapeutic strategies for management of associated clinical problems such as preterm labour that accounts for 70% of neonatal mortality and 75% of neonatal morbidity (Challis et al., 2001).

1.2 The role of the adrenergic system in the uterine function

It has been clearly established that the adrenergic system plays a major role in the uterine function. Thus, a number of attempts have been made to employ drugs that affect the adrenergic system in the treatment of myometrial contractility and cervical

resistance disorders, with special attention to premature labour. Compounds that decrease the myometrial contractions and increase the cervical resistance can be beneficial in the prevention of premature complications, but the number of such compounds is quite limited.

1.2.1. The role of the β-adrenoceptors in the uterine function

Currently, β_2-adrenergic-receptor (β_2-AR) agonists are still one of the most frequently used tocolytics in Europe (they are not routinely used in the US), although their therapeutic significance in premature labour is constantly questioned, especially when they are administered in prolonged therapies (Lampert et al., 1993; Katz and Farmer, 1999). They may have several maternal and foetal side-effects, mainly in consequence with their high therapeutic doses used for uterus-relaxing action. Up to 25 fatal cases have been described following pulmonary oedema. The availability of at least as effective other tocolytics with less side effects have resulted in the suggestion to omit the β-agonists (King et al., 2004; Anotayanonth et al., 2004).

The β-ARs were initially divided into β_1- and β_2-ARs defined in terms of agonist potencies. Further experimentation using β-antagonists exposed another receptor subtype which appeared to be insensitive to typical β-adrenoceptor antagonists, this was classified as β_3-adrenoceptor (Gauthier *et al.*, 1996). Pharmacological evidence is emerging in support of a further receptor subtype β_4-AR (Sarsero *et al.*, 1998), although as yet there are no selective compounds for this particular subtype. Both β_1- and β_2-ARs coexist in the myometrium with a higher proportion of the β_2-subtype (approximately 85% of the total β-AR population). All the β-AR couples via G_s to myometrial adenylyl cyclase, increases intracellular levels of cyclic adenosine monophosphate (cAMP) and activates cAMP-dependent protein kinases. This leads to myometrial relaxation through effects on intracellular Ca^{2+} concentration and myosin light chain kinase.

Besides the expansive side-effects of the β_2-agonists, the potential effectiveness of β_2-AR agonists has been the subject of intensive debate in the literature. Some articles claim that most β_2-mimetics can put off labour for 48-72 h (Katz et al. 1999), while others conclude that their duration of action is only 24-48 h (Higby *et al.* 1993). Nevertheless, it has been stated that β_2-agonist treatment does not influence the preterm

delivery rate and the perinatal outcome (Sciscione *et al.* 1998). Some earlier findings suggest that pregnancy itself may alter the myometrial action of adrenergic drugs. It is known that the myometrial responsiveness of the β_2-agonists decreases at the end of pregnancy in the mouse (Cruz et al., 1990). Gáspár et al. (2005) demonstrated that the uterus-relaxing effect of terbutaline spontaneously decreased on electrical field-stimulated samples towards the end of the pregnancy in the rat. In addition, the terbutaline decreased the amount of activated myometrial G-protein in [^{35}S]GTPγS binding assay on last day of pregnancy. The authors presumed that the effects of β_2-adrenergic receptor agonists in tocolytic therapy may possibly be potentiated with progesterone.

As regards the role of the β_2-ARs in the control of cervical resistance, it has been proved that the terbutaline at 10^{-6} M enhances the cervical resistance of the late-pregnant samples on the β_2-ARs *in vitro* from day 18 to day 22, but did not alter the resistance of the non-pregnant samples. This cervical resistance-increasing effect was concentration dependent and antagonized with propranolol on day 21. Based on [^{35}S]GTPγS studies, they presumed that the cervical resistance-increasing effect of terbutaline is a consequence of its G-protein activation-decreasing property via β_2-ARs, which finally leads to an increased muscle resistance against mechanical stretching (Gáspár et al., 2005).

1.2.2. The role of the α-adrenoceptors in the uterine function

In the rat, besides the β_2-ARs, the α_1-adrenergic receptors (α_1-ARs) have been found to have a great impact on myometrial contractility (Legrand et al., 1986; Zupkó et al., 1997) and the cervical resistance (Kolarovszki-Sipiczki et al., 2007).

The α-ARs have been subdivided into α_1 and α_2 subtypes. All α-ARs are the members of the G-protein coupled receptor superfamily. α_1-ARs are coupled through the G_p/G_q mechanism. The α_1-AR agonists elicit contractions in the smooth muscles via the increase in the intracellular inositol phosphate and Ca^{2+} levels (Michelotti et al., 2000). It has been proved that α_1-AR antagonists induce a significant decrease in the uterine activity of the rat, both in vitro and in vivo (Gáspár et al., 1998; Zupkó et al., 1997), similar to the effects of β_2-AR agonists. Ducza et al. (2002) proved that α_{1A}- and α_{1D}-

ARs are involved in the regulation of the pregnant uterine contractility, but the α_{1A}-AR seems to play the major role in late-pregnant myometrial contraction. 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).

As concerns the α_2-ARs, Kovacs and Falkay (1993) demonstrated the existence of functionally distinct α_2-ARs in the human cervix, similarly as in pregnant rabbits, which suggests the importance of α_2-ARs in the cervical contractility; however, we have no scientific data as concerns the role of the α_2-ARs in the control of myometrial contractions and cervical resistance.

1.2.3. The α_2-adrenoceptors

The results of functional, structural and radioligand-binding studies have led to the α_2-being subdivided into α_{2A}, α_{2B} and α_{2C} subtypes (Bylund et al., 1994). 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 term "autoreceptors" has been introduced for those receptors that are "sensitive to the neuron's own transmitter." In contrast to autoreceptors, non-adrenergic α_2-ARs (heteroreceptors) are modulated by neurotransmitters derived from neighbouring neurons (Bylund et al., 1994).

For the α_2-ARs, mouse models with targeted deletions of the individual subtypes have greatly advanced the understanding of the physiological role and the therapeutic potential of these receptors (Gilsbach and Hein, 2008). Several studies claimed that the differences in the receptor subtypes and their various localizations are thought to be responsible for their different roles. Activation of α_{2A}-ARs could be linked with bradycardia and hypotension (MacMillan et al., 1996), sedation (Lakhlani et al., 1997), and consolidation of working memory (Wang et al., 2007). In contrast, α_{2B}-ARs counteracted the hypotensive effect of α_{2A}-ARs (Link et al., 1996) and were 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 cardiac hypertrophy and failure in experimental models (Lymperopoulos et al., 2007) and in humans with congestive heart failure

(Small et al., 2002). The α_2-ARs can mediate a variety of cell functions, including 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).

The cellular mechanisms underlying the action of α_2-AR activation are not well understood. The α_2-ARs are coupled to a class of the PTX sensitive G_i protein (Karim et al., 2000; Pohjanoksa et al., 1997), whose activation leads to the inhibition of the adenylate cyclase enzyme, the reduction of the intracellular cAMP concentration, the inhibition of voltage-gated Ca^{2+} channels (VGCCs) (Boehm 1999 and Leão and von Gersdorff 2002), and the activation of inwardly rectifying K^+ channels (Bylund 1992, Li et al. 2005). and finally, the physiological answer. (Ruffolo et al., 1991). However, some articles claimed that the α_2-ARs can also couple not only the G_i-protein α-subunit, but under certain circumstances to G_s-proteins leading to activation of adenylyl cyclase (Eason et al. 1992, Offermanns, 2003). Studies on recombinant α_2-ARs expressed in different cell lines have demonstrated that each receptor is capable of coupling to several signal transduction systems (Regan et al., 1992). It has also been shown that all three subtypes have the potential to couple physically and functionally not only to G_i but also to G_s in membranes from CHO (Chinese Hamster Ovary) cells (Eason et al., 1992).

The role of α_2-ARs is not fully clear from the aspect of myometrial contractility and cervical resistance. Three α_2-AR subtypes (α_{2A}-AR, α_{2B}-AR and α_{2C}-AR) have been identified in the human myometrium, while only α_{2A}-AR and α_{2B}-AR were found in the rat uterus (Bouet-Alard et al., 1997). To date there has been no investigation of the roles of the different α_2-AR subtypes in the control of uterine function. The existence of α_2-AR subtype-selective compounds offers a good possibility for determination of the functions of the given subtypes.

2. Aims

1. The first aim of the study was to identify the myometrial and the cervical α_2-AR subtype mRNA and protein expressions by using RT-PCR and Western-blot techniques, respectively.

2. By isolated organ methods our aim was to investigate the role of the α_2-AR subtypes in the noradrenaline evoked myometrial contractions (day 22 of pregnancy) and the cervical resistance (gestation days 18, 20, 21 and 22) by subtype-specific antagonists *in vitro*. Additionally, while the changes in the intracellular cAMP is crucial in the control of smooth muscle contractions and relaxations, our aim was to measure the cAMP release after receptor stimulation by noradrenaline in the presence of the subtype-specific α_2-AR antagonists.

3. Moreover, to investigate the effect of the drugs on overstimulated myometrial samples by using induced labour model.

3. Methods

3.1. Housing and handling of the animals

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.§). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration numbers: IV/1813-1/2002), which is in harmony with the control of the European Union. Sprague-Dawley rats (Charles-River Laboratories, Hungary) were kept at 22 ± 3 °C; the relative humidity was 30-70% and the light/dark cycle was 12/12 h. The animals were maintained on a standard rodent pellet diet (Charles-River Laboratories, Hungary) with tap water available *ad libitum.* They were sacrificed by CO_2 inhalation.

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. A metal door, which was movable by a small electric engine, separated the rooms for the male and female animals. A timer controlled the function of the engine. Since rats are usually active at night, the separating door was opened before dawn. Within 4-5 h after the possibility of mating, vaginal smears were taken from the female rats, and a sperm search was performed under a microscope at a magnification of 1200 times. If the search proved positive, or if smear taking was impossible because of an existing vaginal sperm plug, the female rats were separated and were regarded as first-day pregnant animals.

3.3. Organ samples

The contractility was investigated *in vitro* in non-pregnant, last day pregnant (day 22 of pregnancy) and labour-induced 20-day-pregnant rat myometrial samples. The cervical resistance experiments were carried out on rat cervical samples from the 18-, 20-, 21- and 22-days of pregnancy.

3.4. RT-PCR studies

Tissue isolation. Uterine tissues from non-pregnant and 22-day-pregnant animals, cervical tissues from 18-, 20-, 21- and 22-day-pregnant animals were rapidly removed and the embryonic tissues were separated. The samples were frozen in liquid nitrogen and then stored at -70 ºC until total RNA extraction.

Total RNA preparation. Total cellular RNA was isolated by extraction with acid guanidinium thiocyanate-phenol-chloroform by the procedure of Chomczynski and Sacchi (1987). After precipitation with isopropanol, the RNA was washed 3 times with ice-cold 75% ethanol and then dried. The pellet was resuspended in 100 µl DNase- and RNase-free distilled water. The RNA concentrations of the samples were determined from their absorbances at 260 nm.

RT-PCR. The RNA (0.5 µg) was denatured at 70 ºC for 5 min in a reaction mixture containing 20 µM oligo(dT) (Hybaid Corp., UK), 20 U RNase inhibitor (Hybaid Corp., UK), 200 µM dNTP (Sigma-Aldrich, Hungary) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl and 5 mM $MgCl_2$ in a final reaction volume of 20 µl. After the mixture had been cooled to 4 ºC, 20 U MMLV reverse transcriptase (GIBCO, UK) and Ribonuclease Inhibitor (Sigma-Aldrich, Hungary) were added, and the mixture was incubated at 37 ºC for 60 min.

The PCR was carried out with 5 µl cDNA, 25 µl ReadyMix REDTaq PCR reaction mix (Sigma-Aldrich, Hungary), 2 µl 50 pM sense and antisense primers of the α_2-AR subtypes (GeneBank accession no. NM012739 for α_{2A}; NM138505 for α_{2B}; NM138506 for α_{2C}) and 16 µl DNase- and RNase-free distilled water. The coupling temperatures and numbers of cycles for the different α_2-AR subtypes were as follows: 57 ºC, 32 cycles for α_{2A}; 56 ºC, 32 cycles for α_{2B}; and 59 ºC, 36 cycles for α_{2C}-ARs.

The PCR was performed with a PCR Sprint thermal cycler (Hybaid Corp., UK). After the initial denaturation at 95 ºC for 5 min, the reactions were taken through the previously determined number of cycles for each α_2-AR subtype: 60 s at 95 ºC, 60 s at the appropriate coupling temperature, and 60 s at 72 °C, followed by lowering of the temperature to 4 ºC. This PCR protocol furnished optimized conditions and linear phase amplification for each of the primer sets employed. The optimum number of cycles for each set of primers was determined by performing kinetic analyses.

The RT-PCR products were separated on 2% agarose gels, stained with ethidium bromide and photographed under a UV transilluminator. Semiquantitative analysis was

performed by densitometric scanning of the gel with Kodak EDAS290 (Csertex Ltd., Hungary).

3.5. Western blotting studies

20 μg of protein per well was subjected to electrophoresis on 10% sodium dodecylsulfate polyacrylamide gels in Series Standard Dual Cooled Units (BioRad, Hungary). Proteins were transferred from gels to nitrocellulose membranes (Scheicher and Schuell, Germany), using a semidry blotting technique (BioRad, Hungary). The membranes were blocked overnight at 4 ^{o}C with 5% non-fat dry milk in Tris saline buffer (50 mM Tris, pH 7.4, 200 mM NaCl) containing 0.1% Tween. After washing, the blots were incubated for 1 h at room temperature on a shaker, with α_{2A}-, α_{2B}-, and α_{2C}-AR and β-actin polyclonal antibody (Santa Cruz Biotechnology, California, USA, 1:200) in the blocking buffer. Immunoreactive bands were visualized with the WesternBreeze Chromogenic Western blot immune detection kit (Invitrogen, Hungary) and quantified. Protein bands were identified via the MagicMark Western Protein Standard (Invitrogen, Hungary).

3.6. Isolated organ studies

3.6.1. Measurement of myometrial contractility

Myometrial tissue samples were removed from non-pregnant (180-200 g), 22-day-pregnant (270-350 g), and labour-induced 20-day-pregnant rats (240-300 g). Muscle rings 5 mm long were sliced from the uterine horns and mounted in an organ bath (8 parallels) containing 10 ml de Jongh solution (in mM: 137 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 12 $NaHCO_3$, 4 NaH_2PO_4, 6 glucose, pH: 7.4). The organ bath was maintained at 37 °C, and carbogen (95% O_2 + 5% CO_2) was bubbled through it. After mounting, the rings were equilibrated for about 1 h before experiments were undertaken, with a solution change every 15 min. The initial tension was set to about 1.25 g, which was relaxed to about 0.5 g at the end of equilibration. The tension of the myometrial rings was measured and recorded with a gauge transducer and an S.P.E.L. Advanced ISOSYS Data Acquisition System (Experimetria Ltd, Hungary), respectively. Contractions were elicited with noradrenaline (10^{-8}–$3x10^{-5}$ M) and cumulative concentration-response

curves were constructed in each experiment in the presence of propranolol (10^{-5} M) and doxazosin (10^{-7} M) in order to avoid β- and α_1-adrenergic actions. α_2-AR antagonists (BRL 44408 for α_{2A}, ARC 239 for $\alpha_{2B/C}$ and spiroxatrine for α_{2C}) (each 10^{-7} M) were left to incubate for 20 min before the administration of contracting agents. Following the addition of each concentration of noradrenaline, 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, USA) computer program. From the AUC values, E_{max} and EC_{50} values were calculated (E_{max}: The maximum contracting effect of noradrenaline alone or in the presence of an α_2-AR antagonist; EC_{50}: the concentration of noradrenaline alone or in the presence of an α_2-AR antagonist which elicits half of the maximum contracting effect of noradrenaline.).

3.6.2. Measurement of cervical resistance

Cervical tissues were removed from late-pregnant (gestational day 18, 20, 21 or 22) rats. The cervix was defined as the least vascular tissue with two parallel lumina between the uterine horns and the vagina. The two cervical rings were separated and mounted with their longitudinal axis vertically by hooks in an organ bath containing 10 ml de Jongh buffer The lower sides of the cervices were fixed to the bottom of the tissue holders in the organ chambers, while the upper parts were hooked to gauge transducers (SG-02, Experimetria Ltd, Hungary). The initial tension was set to about 1.00 g.

After incubation, the cervical resistance was investigated by gradual increase of the tension in the tissues, as described previously (Gáspár et al., 2005; Kolarovszki-Sipiczki et al., 2007). The cervices were stretched in incremental steps and allowed to relax for 5 min. After every 5 min the next initial tension was set, in 1-g steps between 1 and 12 g. The tension was increased manually via the control screw of the gauge transducer. The precise initial tension and the relaxation of the cervices were followed with an online computer, using the S.P.E.L. Advanced Isosys Data Acquisition System (Experimetria Ltd, Hungary). The resultant stress-strain curve had a saw-tooth shape (**Fig 1**).

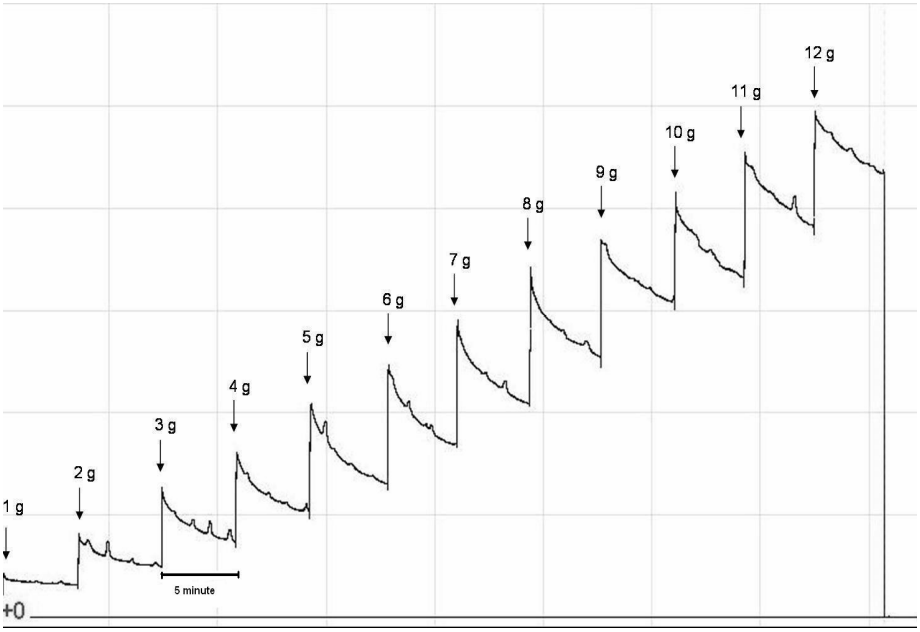

Figure 1. Representative stress-strain curve of a 22-day-pregnant rat cervix in vitro

The cervices were stretched in incremental steps and allowed to relax for 5 min. After every 5 min, the next initial tension value was adjusted. The series of stretching and relaxation resulted in a saw-tooth shape. The initial tensions were plotted against the tensions recorded after 5 min to create regression lines

In the evaluation of the cervical resistance, the initial tension of the cervix was plotted versus the stretch after 5 min. Straight lines were fitted by linear regression and the slopes of the lines were used to express the degree of resistance. A steeper slope reflected higher resistance.

When the effects of the α_2-AR subtypes were investigated, the samples were incubated for 10 min with 10^{-6} M of the subtype-selective α_2-AR antagonist in the presence of propranolol (10^{-5} M) and doxazosine (10^{-6} M). Control values were registered in the presence of or without noradrenaline. Noradrenaline (10^{-5} M) was added to the organ bath and the contents were left to incubate for 5 min before stretching.

When the effect of noradrenaline was investigated on 18-day-pregnant cervices, cumulative concentration-response curves of noradrenaline (10^{-8} - 10^{-4} M) were constructed in the presence of or without the antagonists (BRL 44408 or spiroxatrine).

When the effect of the G_i-protein inhibitor pertussis toxin (PTX) was investigated on 18- and 20-day-pregnant cervices, the samples were pretreated with PTX (400 ng/ml) for 2 hours (Hansen et al., 2003) before stretching. Control values were registered in the

presence of or without noradrenaline. The data were analysed with the Prism 4.00 (GraphPad Software, U.S.A) computer program.

3.6.3. Induction of premature labour in pregnant rat

The experimental premature labour procedure was carried out according to the model of Rechberger et al. (1996). Briefly, 19-day-pregnant rats were treated with s.c. antiprogesterone (mifepristone) at 3 mg/animal at 9.00 a.m. At 4.00 p.m., prostaglandin E_2 (0.5 mg/animal) was administered intravaginally. Our preliminary results had revealed that, after this treatment, the pregnant animals delivered between 9.00 and 10.00 a.m. on day 20 of pregnancy. Accordingly, the animals were sacrificed and uterine tissues were removed at 9.00 a.m., ensuring that the pregnant myometrium was very close to, but not after delivery.

3.7. cAMP studies

3.7.1. Preparation and treatment of myometrial and cervical samples

Tissue samples from non-pregnant and 22-day-pregnant rats were incubated in an organ bath (10 ml) containing de Jongh solution (see above) at 37 °C, perfused with a mixture of 95% oxygen and 5% CO_2. Isobutylmethylxanthine (10^{-3} M), doxazosin, propranolol and the investigated subtype-selective α_2-AR antagonists were incubated with the tissues for 20 min, and noradrenaline was then added for 10 min to stimulate cAMP generation.. At the end of the noradrenaline incubation period, forskolin (10^{-5} M) was added for another 10 min, as described by Roberts et al. (1998). The non-specific phosphodiesterase inhibitor 3-isobutyl-1-methylxantine (IBMX) was used to block the degradation of the generated intracellular cAMP (Schlageter et al., 1980), while forskolin was added to enhance the activity of adenylyl cyclase (Seamon and Daly, 1986).

When the effect of the G_i-protein inhibitor PTX on the cAMP accumulation was investigated, the samples were pretreated with PTX (400 ng/ml) for 2 h (Hansen et al., 2003) before the IBMX incubation. Control values were registered in the presence of or without noradrenaline. After stimulation, the samples were immediately frozen and

stored in liquid nitrogen until the extraction of cAMP. After this, the samples were immediately frozen and stored in liquid nitrogen until cAMP extraction.

The samples were next ground under liquid nitrogen weighed, homogenized in 10 volumes of ice-cold 5% trichloroacetic acid and centrifuged at 600 *g* for 10 min. The supernatant was extracted with 3 volumes of water-saturated diethyl ether. After drying, the extracts were stored at -70 °C until the cAMP assay.

3.7.2. Detection of cAMP Enzyme Immunoassay Kit

Myometrial and cervical cAMP accumulation was measured with a commercial cAMP Enzyme Immunoassay (EIA) Kit (Sigma-Aldrich, Hungary). Briefly, the kit uses a polyclonal antibody to cAMP to bind, in a competitive manner, the cAMP in the sample or an alkaline phosphatase molecule that has cAMP covalently attached to it. On a secondary antibody-coated microwell plate, the cAMP-antibody and the alkaline phosphatase-antibody complexes are conjugated. Following the addition of *p*-nitrophenyl phosphate, a substrate of alkaline phosphatase, the *p*-nitrophenol generated can be determined via its yellow colour at 405 nm. The more intense the colour, the lower the amount of intracellular cAMP.

3.8. Materials

Noradrenaline, 3-isobutyl-1-methylxanthine (IBMX), forskolin and spiroxatrine (8-[(2,3-dihydro-1,4-benzodioxin-2-yl)methyl]-1-phenyl-1,3,8-triazaspiro[4,5]decan-4-one) were purchased from Sigma–Aldrich, Hungary; BRL 44408 (2-[2H-(1-methyl-1,3-dihydroisoindole)methyl]-4,5-dihydroimidazole) and ARC 239 (2-[2,4-(O-methoxyphenyl)piperazin]-1-yl dihydrochloride from Tocris, UK; and PTX from Izinta Ltd., Hungary. Doxazosin was donated by Pfizer Hungary Ltd.

Noradrenaline, BRL 44408, ARC 239 and spiroxatrine were dissolved in distilled water to give a 10 mM stock solutions, which were stored at –18 °C and freshly diluted in distilled water before the beginning of the experiment. PTX was dissolved in a sodium phosphate buffer containing (in mM) 100 Na_2HPO_4, 50 NaCl, pH 7.0. IBMX and forskolin were freshly dissolved in ethanol. The final organ bath concentration of ethanol (which had no quantifiable effects) did not exceed 0.05%.

3.9. Statistical analyses

All experiments were carried out on at least 6 animals. Statistical analyses were performed with ANOVA, followed by the Neuman-Keuls test, except the myometrial contractility studies, when data were analysed by two-tailed unpaired *t* tests.

4. Results

4.1. RT-PCR studies

RT-PCR studies revealed the mRNAs of all three α_2-AR subtypes in both the non-pregnant and the 22-day-pregnant rat myometrium. In the non-pregnant animals, there was a slight predominance of α_{2A}-AR mRNA (**Fig. 2a, b**), while in the pregnant myometrium a strong α_{2B}-AR mRNA predominance was found (**Fig. 2c, d**).

In case of the cervix, all the three α_2-AR subtype mRNAs were expressed each day in the cervical samples; however, differences were detected between the levels of expression on the different days. The mRNA expressions of each subtype were found to be elevated on days 20 and 21. On day 22, however, the α_{2A}- and α_{2B}-AR mRNA levels remained unchanged, while the expression of α_{2C}-AR mRNA was lower (**Fig. 3a-f**).

4.2. Western blotting studies

The Western blotting analysis gave a result for the non-pregnant myometrium similar to that from the RT-PCR studies. In the pregnant myometrium, the predominant α_2-AR subtype protein was the α_{2B}-AR (**Fig 4a, b**), while the optical density of the α_{2A}-AR protein was significantly lower than that of the α_{2C}-AR (**Fig 4c, d**).

In case of the cervical samples the analysis revealed the presence of the proteins of all three α_2-AR subtypes on each investigated day. The protein expressions of the α_2-AR subtypes were significantly increased on day 21, whereas decreases were observed on day 22 (**Fig. 5a-f**).

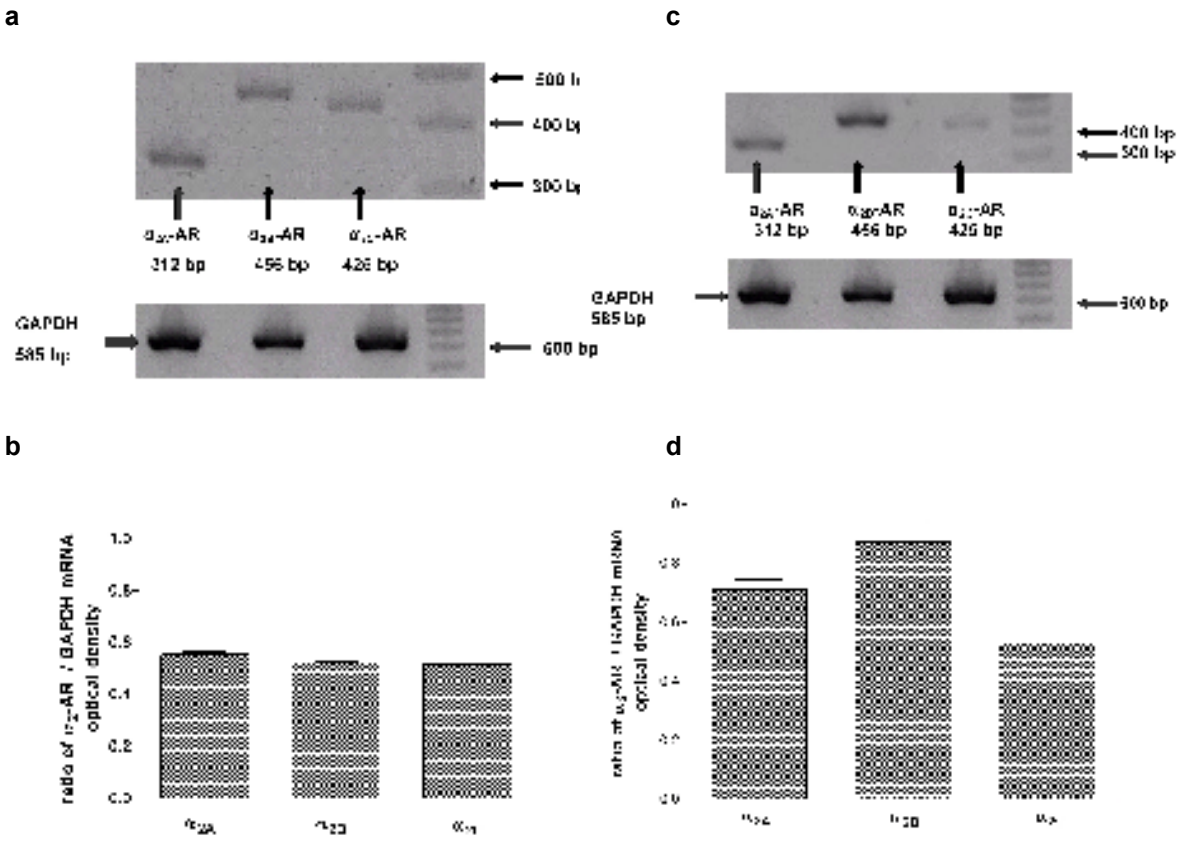

Figure 2. The Changes in mRNA levels of the α_2-AR subtypes in non-pregnant and 22-day-pregnant rat myometrium (n = 6).

α_2-AR reverse transcription-polymerase chain reaction and GAPDH products from the total myometrial RNA of a non-pregnant **(a)** animal and a 22-day-pregnant animal **(c).** The result was expressed in the ratio of the optical densities of α_2-AR/GAPDH mRNA **(b and d)**. The levels of significance by ANOVA followed by the Neuman–Keuls test: α_{2A}-AR vs. α_{2B}-AR, $p < 0.01$; α_{2A}-AR vs. α_{2C}-AR, $p < 0.01$; α_{2B}-AR vs. α_{2C}-AR in the non-pregnant and α_{2B}-AR vs. α_{2A}-AR, $p < 0.05$; α_{2B}-AR vs. α_{2C}-AR, $p < 0.001$; α_{2A}-AR vs. α_{2C}-AR, $p < 0.01$ in the 22-day-pregnant myometrium.

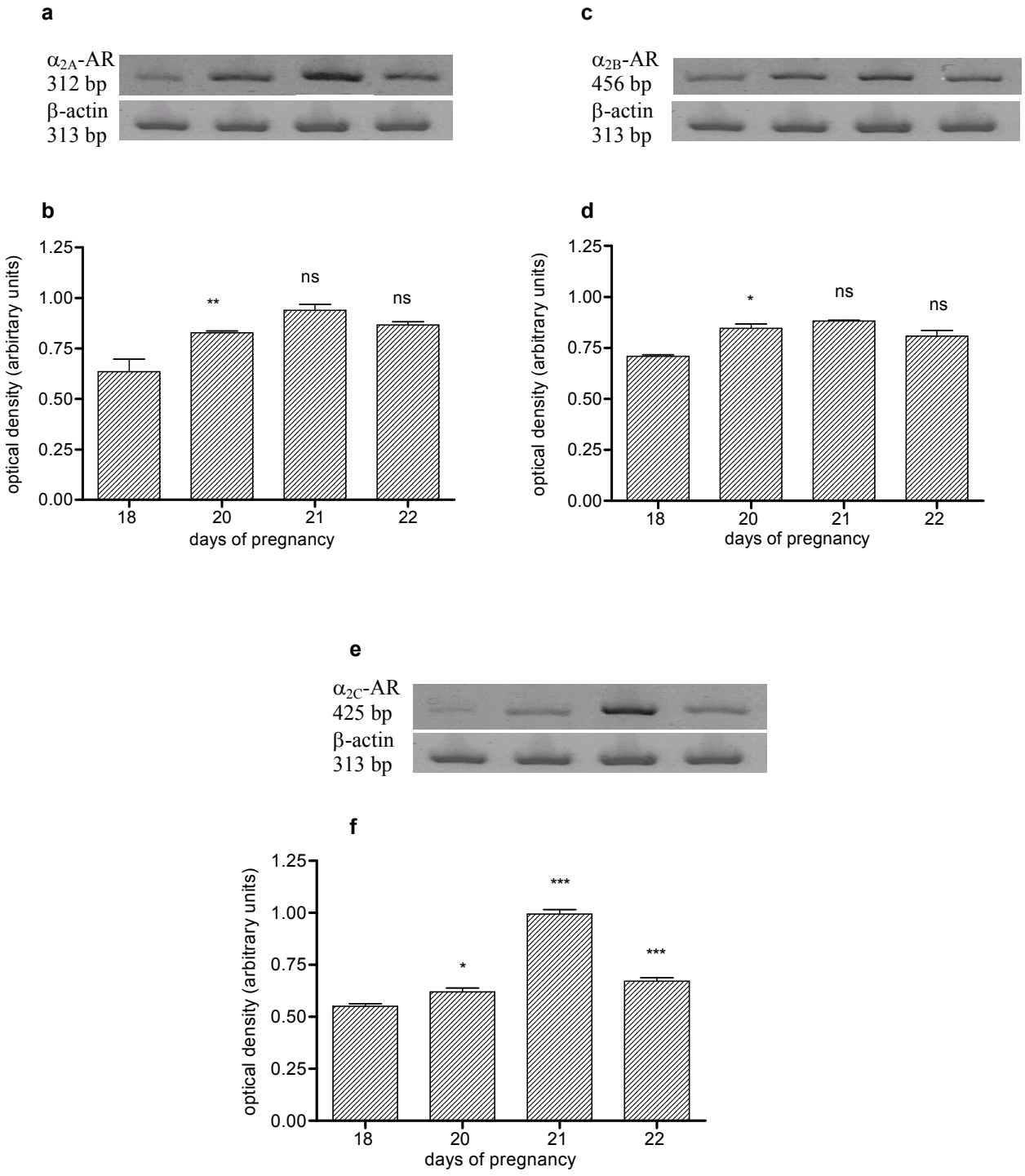

Figure 3. Changes in mRNA levels of the α_2-AR subtypes in 18-, 20-, 21- and 22-day-pregnant rat cervices (n = 6).

α_2-AR reverse transcription-polymerase chain reaction and GAPDH products from the total myometrial RNA of a pregnant animal on days 18, 20, 21 and 22 of pregnancy **(a** for α_{2A}, **c** for α_{2B} and **e** for α_{2C}**).** The result was expressed in the ratio of the optical densities of α_2-AR/GAPDH mRNA (**b** for $\alpha_{2A,}$ **d** for α_{2B} and **f** for α_{2C}). The level of significance for ANOVA followed by the Neuman–Keuls test relates to the comparison with the previous investigated day. ns: not significant, *p < 0.05; ** p < 0.01; *** p <0.001.

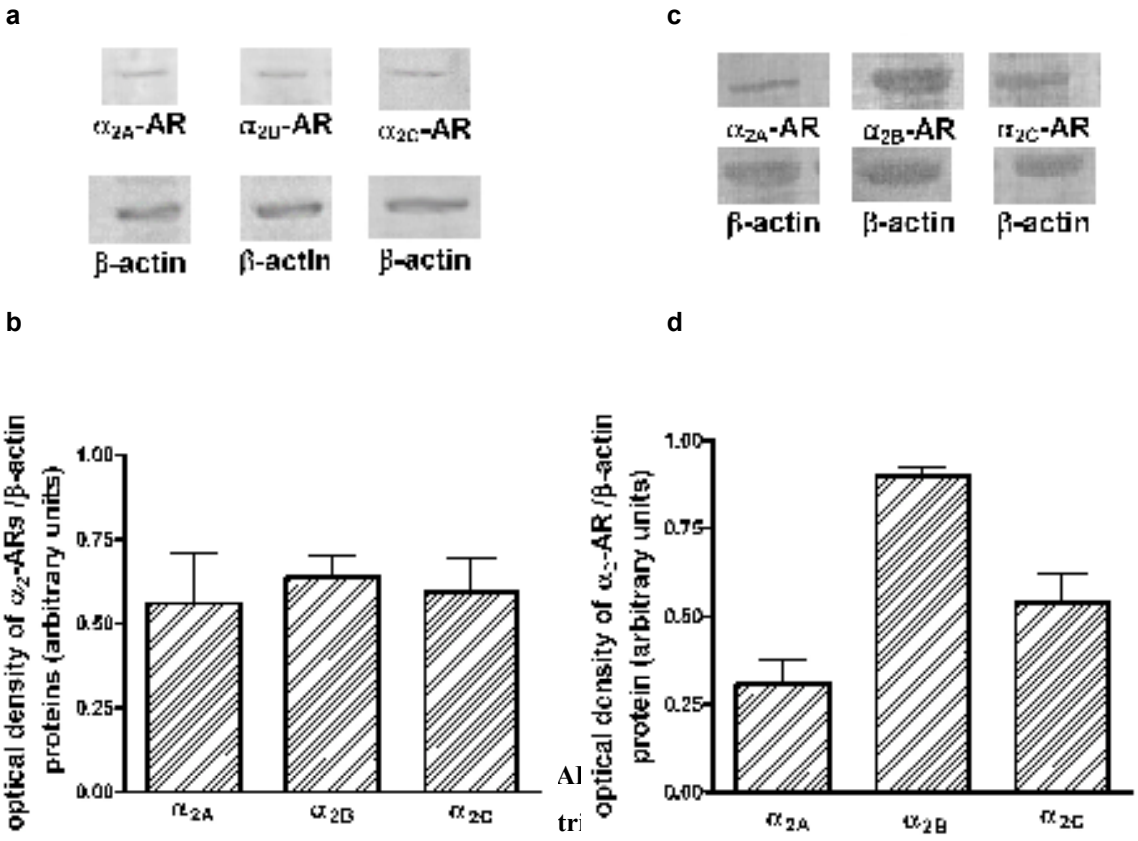

Figure 4. Changes in protein levels the α_2-AR subtypes in non-pregnant and 22-day-pregnant rat myometrium (n = 6).

The α_2-AR and β-actin Western blotting products from non-pregnant and 22-day-pregnant rat myometrial samples (**a** for non-pregnant and **c** for 22-day-pregnant). The antibody binding was detected with an enhanced chemiluminescence detection system, and expressed as optical density (semiquantitative) data (**b** for non-pregnant and **d** for 22-day-pregnant). The level of significance for ANOVA followed by the Neuman–Keuls test: α_{2A}-AR vs. α_{2B}-AR, $p > 0.05$; α_{2A}-AR vs. α_{2C}-AR, $p > 0.05$; α_{2B}-AR vs. α_{2C}-AR, $p > 0.05$ in the non-pregnant and α_{2B}-AR vs. α_{2A}-AR, $p < 0.001$; α_{2B}-AR vs. α_{2C}-AR, $p < 0.01$; α_{2A}-AR vs. α_{2C}-AR, $p < 0.001$ in the 22-day-pregnant myometrium.

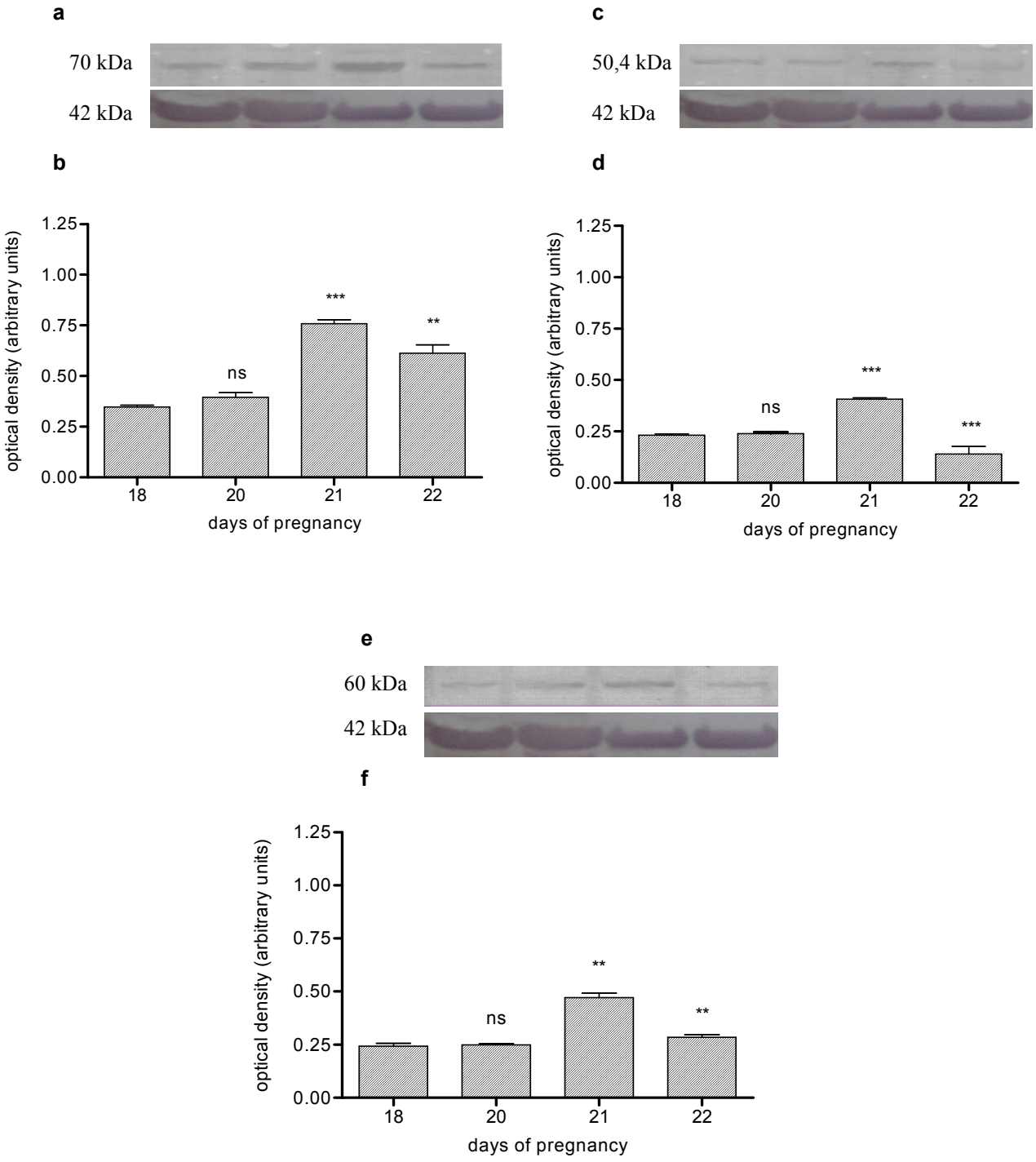

Figure 5. Changes in protein levels the α_2-AR subtypes in 18-, 20-, 21- and 22-day-pregnant rat cervices (n=6).

The α_2-AR and β-actin Western blotting products from 18-, 20-, 21- and 22-day-pregnant rat cervices (**a** for $\alpha_{2A,}$ **c** for α_{2B} and **e** for α_{2C}). The antibody binding was detected with an enhanced chromogenic detection system, and expressed as optical density (semiquantitative) data (**b** for $\alpha_{2A,}$ **d** for α_{2B} and **f** for α_{2C}). The level of significance for ANOVA followed by the Neuman–Keuls test relates to the comparison with the previous investigated day. ns: not significant, **p < 0.01; *** p < 0.001.

4.3. Isolated organ studies

4.3.1. Effects of α_2-AR subtype antagonists on the non-pregnant and 22-day-pregnant myometrial contractions

Noradrenaline in the concentration range 10^{-8} - $10^{-4.5}$ M did not exert a contractile effect on the non-pregnant uterine rings, whereas vivid contractions were elicited by 25 mM KCl (data not shown). In the 22-day-pregnant myometrium, noradrenaline concentration-dependently increased the contractions, and these were slightly increased by the α_{2A}-AR antagonist BRL 44408 (**Fig 6**). The $\alpha_{2B/C}$-AR antagonist ARC 239 significantly decreased the maximum effect of noradrenaline (**Fig 7**), while the α_{2C}-AR antagonist spiroxatrine enhanced the noradrenaline-induced contractions (**Fig 8**). The combination BRL 44408 + spiroxatrine also caused an increase in the maximum myometrium-contracting effect of noradrenaline (**Fig 9**). The EC_{50} and E_{max} values of the curves are listed in **Table 1** (see Chapter 10).

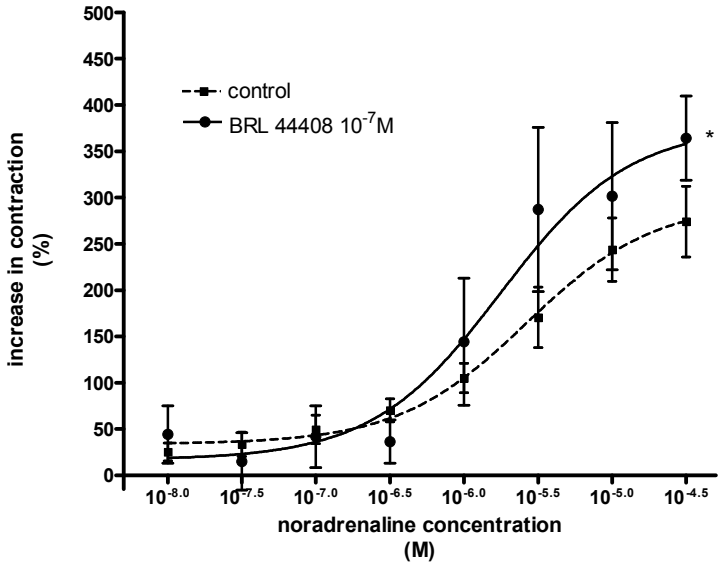

Figure 6. Effect of the subtype-selective α_{2A}-AR antagonist BRL 44408 on the noradrenaline-evoked contractions (control) in the 22-day-pregnant rat myometrium in an isolated organ bath (n = 8).

The studies were carried out in the presence of the β-AR antagonist propranolol (10^{-5} M) and the α1-AR antagonist doxazosin (10^{-7} M). 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 the two-tailed unpaired t-test. *$p < 0.05$.

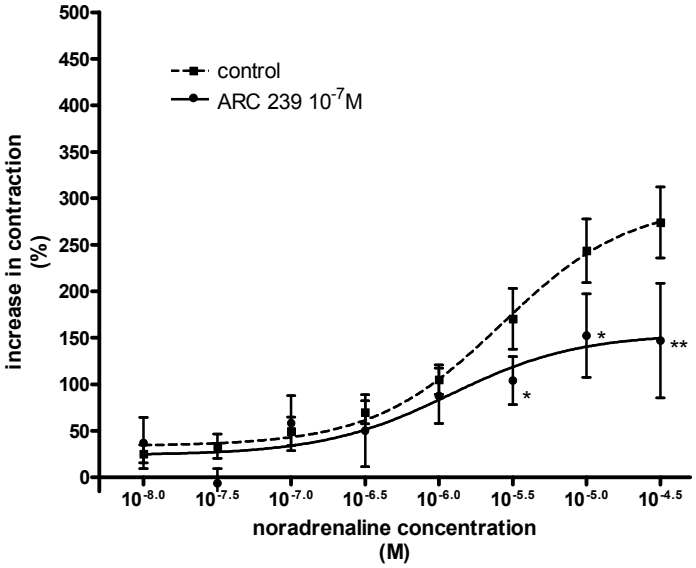

Figure 7. Effect of the subtype-selective $_{\alpha 2B/C}$-AR antagonist ARC 239 on the noradrenaline-evoked contractions (control) in the 22-day-pregnant rat myometrium in an isolated organ bath (n = 8).

The studies were carried out in the presence of the β-AR antagonist propranolol (10^{-5} M) and the α1-AR antagonist doxazosin (10^{-7} M). 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 the two-tailed unpaired t-test. *p < 0.05; **p < 0.01.

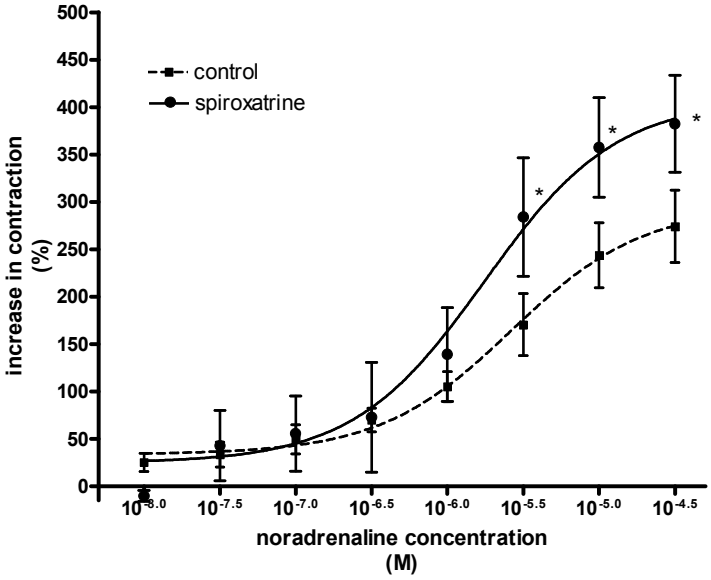

Figure 8. Effect of the subtype-selective α_{2C}-AR antagonist spiroxatrine on the noradrenaline-evoked contractions (control) in the 22-day-pregnant rat myometrium in an isolated organ bath (n = 8).

The studies were carried out in the presence of the β-AR antagonist propranolol (10^{-5} M) and the α1-AR antagonist doxazosin (10^{-5} M). 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 the two-tailed unpaired t-test. *p < 0.05.

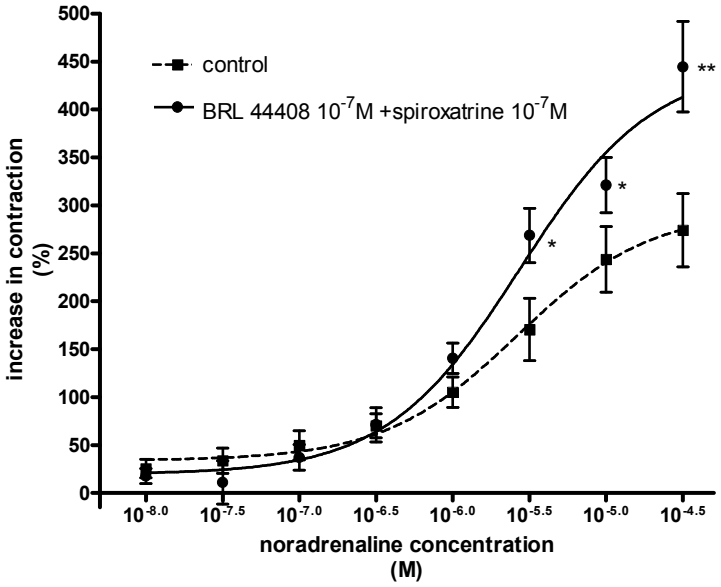

Figure 9. Effects of the subtype-selective α_{2A}-AR antagonist BRL 44408 and α_{2C}-AR antagonist spiroxatrine on the noradrenaline-evoked contractions (control) in the 22-day-pregnant rat myometrium in an isolated organ bath (n = 8).

The studies were carried out in the presence of the β-AR antagonist propranolol (10^{-5} M) and the α1-AR antagonist doxazosin (10^{-7} M). 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 the two-tailed unpaired t-test. *$p < 0.05$; **$p < 0.01$.

4.3.2. Isolated organ studies with myometrium from hormonally-induced preterm birth

Noradrenaline ($10^{-8.5}$-10^{-5} M) enhanced the contractions of labour-induced uterine rings, although its effect was less than that in the 22-day-pregnant animals. ARC 239 blocked the noradrenaline-evoked contractions (**Fig 10**). The EC_{50} and E_{max} values of the curves are presented in **Table 2** (see Chapter 10)

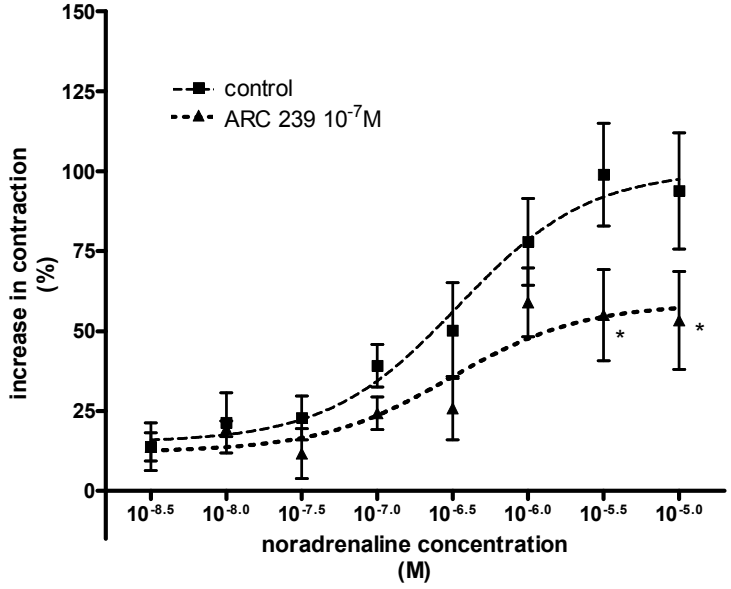

Figure 10. Effect of the subtype-selective $\alpha_{2B/C}$-AR antagonist ARC 239 on the noradrenaline-evoked contractions (control) in the labour-induced rat myometrium in an isolated organ bath (n = 8).

The studies were carried out in the presence of the β-AR antagonist propranolol (10^{-5} M) and the α1-AR antagonist doxazosin (10^{-7} M). 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 the two-tailed unpaired t-test. *$p < 0.05$.

4.3.3. Effects of α_2-AR subtype antagonists on the cervical resistance

We found that on day 18 noradrenaline enhanced the cervical resistance, both alone (stimulation of all α_2-AR subtypes) and also in the presence of ARC 239 (α_{2A}-AR stimulation), while BRL 44408, spiroxatrine and their combination blocked the effect of noradrenaline (**Fig. 11a**). On day 20, however, noradrenaline, both alone and in the presence of ARC 239 decreased the cervical tone, while similarly as on day 18, BRL 44408, spiroxatrine and their combination again blocked the effect of noradrenaline (**Fig. 11b**). On day 21, the resistance-increasing action of noradrenaline was maintained only in the presence of spiroxatrine ($\alpha_{2A/B}$-AR stimulation) (**Fig. 11c**). Noradrenaline, however, did not alter the cervical resistance in the presence of or without the antagonists on day 22 (**Fig. 11d**).

4.3.4. Effects of BRL 44408 and spiroxatrine on the cervical resistance-increasing dose-response curve of noradrenaline

The cervical resistance-increasing effect of noradrenaline was concentration-dependent in the range 10^{-8}-10^{-4} M on day 18. In the presence of 10^{-6} M BRL 44408 the effect of the noradrenaline was decreased. In the presence of 10^{-6} M spiroxatrine the effect of noradrenaline was also antagonized; its cervical resistance-increasing effect was totally blocked, even at high doses (**Fig. 12**).

4.3.5. Effects of α_2-AR subtype antagonists on cervical resistance in the presence of PTX

PTX significantly decreased the cervical tone as compared with the control level without PTX on day 18. PTX also decreased the resistance-increasing effect of noradrenaline, both alone and in the presence of ARC 239 (**Fig. 13a**). On day 20, PTX did not alter the basal cervical resistance or the effects of noradrenaline and noradrenaline + ARC 239 on the cervical tone (**Fig. 13b**).

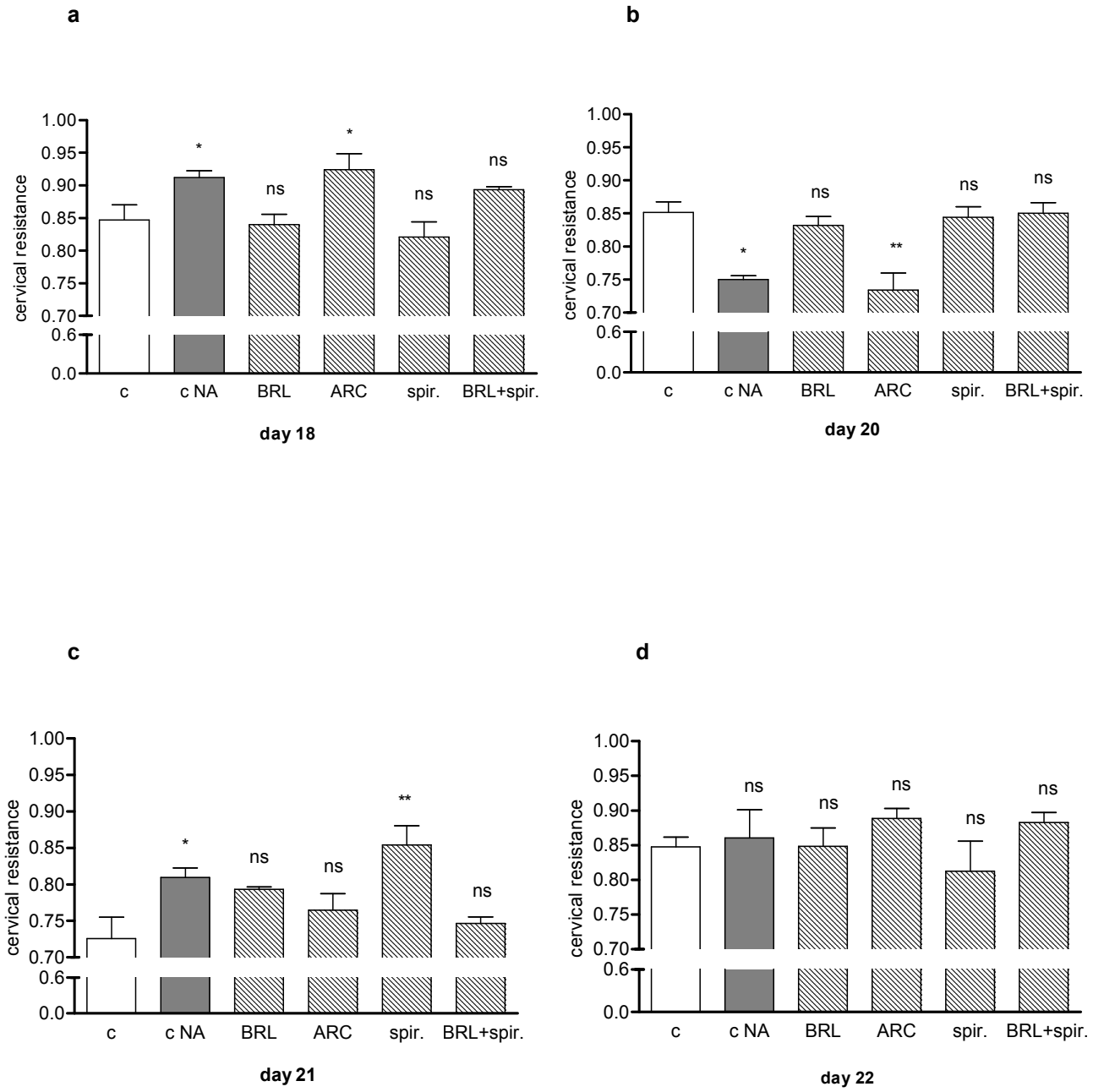

Figure 11. Effects of α_2-AR subtype antagonists on the resistance of 18-, 20-, 21- and 22-day-pregnant rat cervices in vitro (n=8) (a, b, c and d).

The resistance is expressed as the slope of the regression line fitted to the stress–strain curves. The y axis is segmented into two in order to present a higher magnification of the changes in slopes. White bars show the slopes from the control without noradrenaline, grey bars those from the control with noradrenaline, and striped bars those from the subtype-selective α_2-antagonist-treated cervical samples *in vitro*. On each day, the level of significance relates to the comparison with the control sample. * $P<0.05$; ** $P<0.01$

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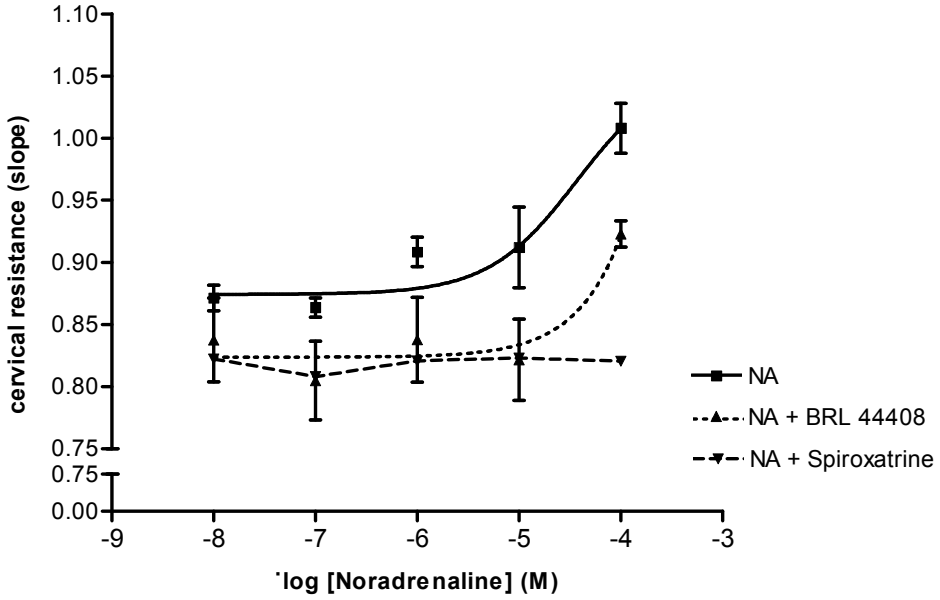

Figure 12. Effects of BRL 44408 and spiroxatrine on the cervical resistance-increasing action of noradrenaline in 18-day-pregnant cervices (n=8).

The presence of BRL 44408 significantly shifted the noradrenaline curve to the right. The EC_{50} values BRL 44408 1.8×10^{-5} M and 9.8×10^{-5} M without and in the presence of BRL 44408, respectively. In the presence of spiroxatrine, the effect of noradrenaline was totally blocked in the investigated concentration range.

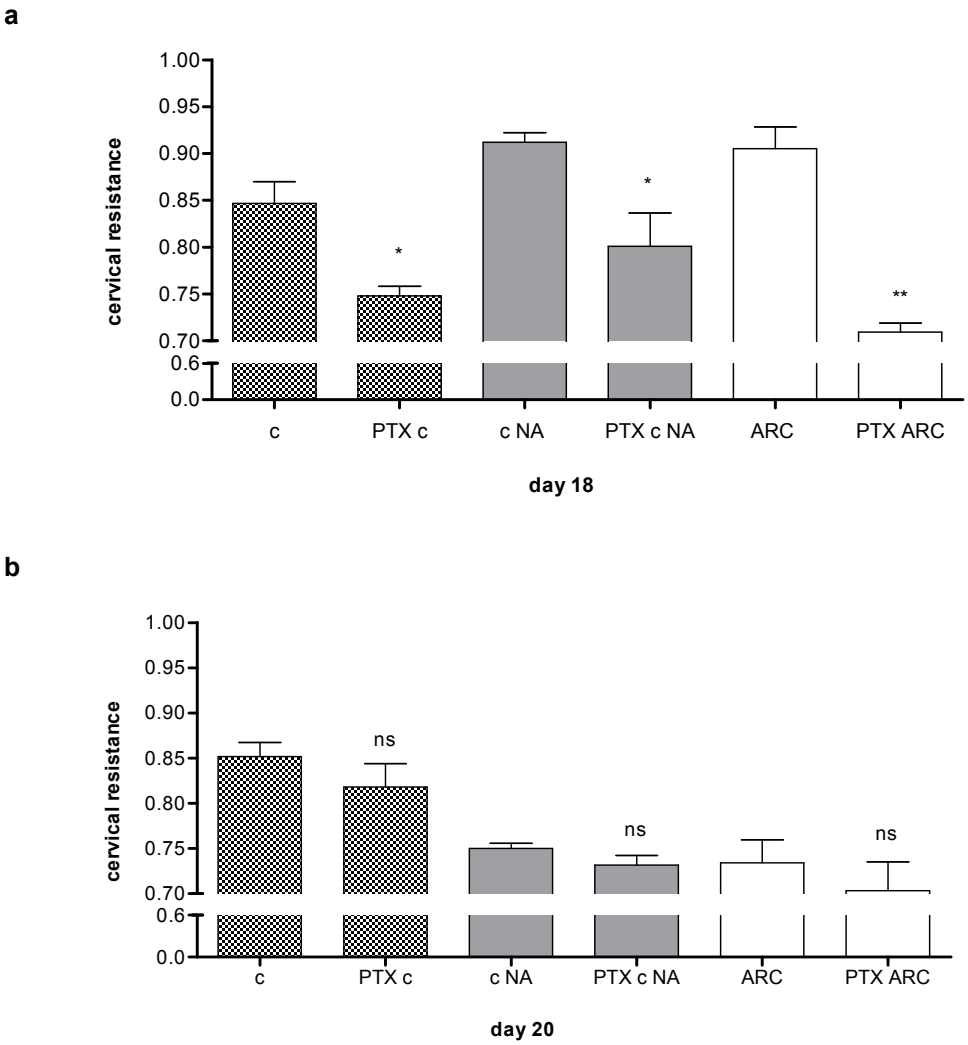

Figure 13. Effects of α_2-AR subtype antagonists on the resistance of 18- (a) and 20- (b) day-pregnant rat cervices in the presence or in the absence of pertussis toxin in vitro (n=8).

The resistance is expressed as the slope of the regression line fitted to the stress–strain curves. The Y axis is segmented into two in order to present a higher magnification of the changes in slopes. Checked bars show the slopes from the samples without noradrenaline, grey bars those from the samples with noradrenaline, and white bars those from the subtype-selective $\alpha_{2B/C}$-antagonist ARC 239-treated cervical samples *in vitro*. On each day, the level of significance relates to the comparison with the control (c) sample without pertussis toxin (c, c NA and c ARC 239). * $P<0.05$; ** $P<0.01$.

4.4. cAMP studies

4.4.1. Effects of subtype-selective α_2-AR antagonists on myometrial cAMP level

In the non-pregnant uterine tissue, BRL 44408, ARC 239, spiroxatrine, and the combination BRL 44408 + spiroxatrine did not influence the amount of cAMP produced in the presence of $3x10^{-6}$ M noradrenaline (data not shown). In the pregnant uteri, ARC 239 was able to increase the cAMP level produced by noradrenaline, while BRL 44408, spiroxatrine and the combination BRL 44408 + spiroxatrine caused significant decreases in the amount of myometrial cAMP (**Fig 14**).

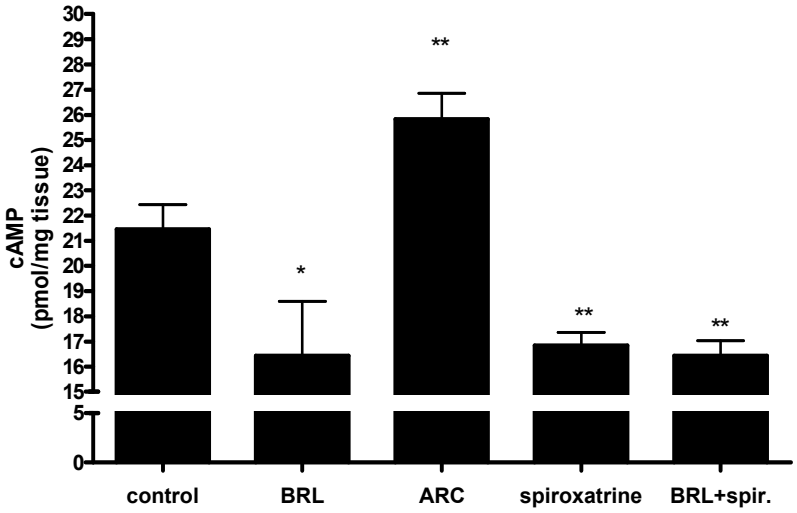

Figure 14. Effects of the subtype-selective α_2-AR antagonists on the myometrial cAMP level (pmol/mg tissue ± S.E.M.) stimulated by noradrenaline in the presence of isobutylmethylxanthine and forskolin (control) in the 22-day-pregnant rat (n = 6).

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). cAMP production was increased by IBMX (10^{-3} M) and forskolin (10^{-5} M). The antagonist concentrations were 10^{-7} M in each case. The statistical analyses were carried out with ANOVA followed by the Neuman–Keuls test. *$p < 0.05$; **$p < 0.01$.

4.4.2. Effects of subtype-selective α_2-AR antagonists on cervical cAMP level

In the cAMP enzyme immunoassay studies, noradrenaline treatment significantly decreased the intracellular cAMP production on day 18, and the same effect was found in the presence of ARC 239 (α_{2A}-AR stimulation) (**Fig. 15a**). An elevated cAMP accumulation was seen on day 20 both by noradrenaline alone and in the presence of ARC 239 (**Fig. 16a**). On day 21, noradrenaline decreased the level of intracellular cAMP, and an analogous effect was displayed in the presence of spiroxatrine ($\alpha_{2A/B}$-AR stimulation) (**Fig. 17a**). Nevertheless, the antagonists did not influence the amount of cAMP in the presence of noradrenaline on day 22.

4.4.3. Effects of subtype-selective α_2-AR antagonists on cervical cAMP level in the presence of PTX

The cAMP accumulation studies with PTX pretreatment were carried out in those cases when noradrenaline and the subtype-selective antagonists significantly altered both the cervical resistance and the cAMP level. PTX pretreatment substantially enhanced the intracellular cAMP accumulation (**Figs. 15b, 16b and 17b**). Besides the increased cAMP values, on days 18 and 21, all of the effects of noradrenaline alone and in the presence of ARC 239 (**Fig. 15b**) and spiroxatrine (**Fig. 17b**) were eliminated. However, on day 20, PTX treatment did not alter the effects of noradrenaline alone or together with ARC 239 (**Fig. 16b**).

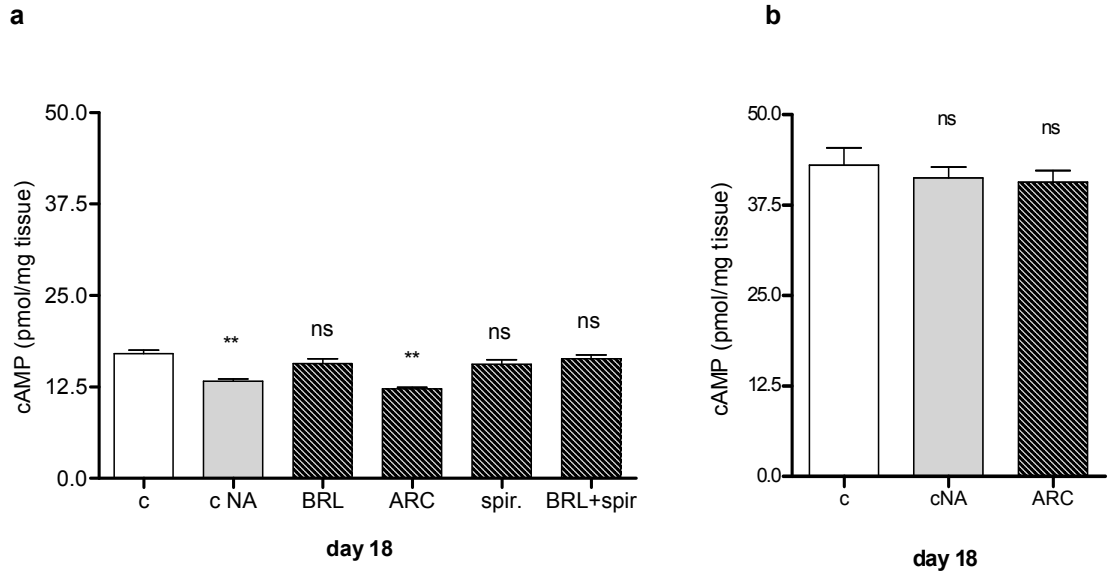

Figure 15. Effects of the subtype-selective α_2-AR antagonists on the cervical cAMP level (pmol/mg tissue ± S.E.M.) in the presence (a) or in the absence of pertussis toxin (b) on day 18 of gestation.

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^{-6} M). cAMP production was increased by IBMX (10^{-3} M) and forskolin (10^{-5} M). White bars show the values from the control without noradrenaline, grey bars those from the control with noradrenaline, and striped bars those from the subtype-selective α_2-antagonist-treated cervical samples *in vitro*. The statistical analyses were carried out with the unpaired t-test or ANOVA, followed by the Neuman–Keuls test. ** P< 0.01.

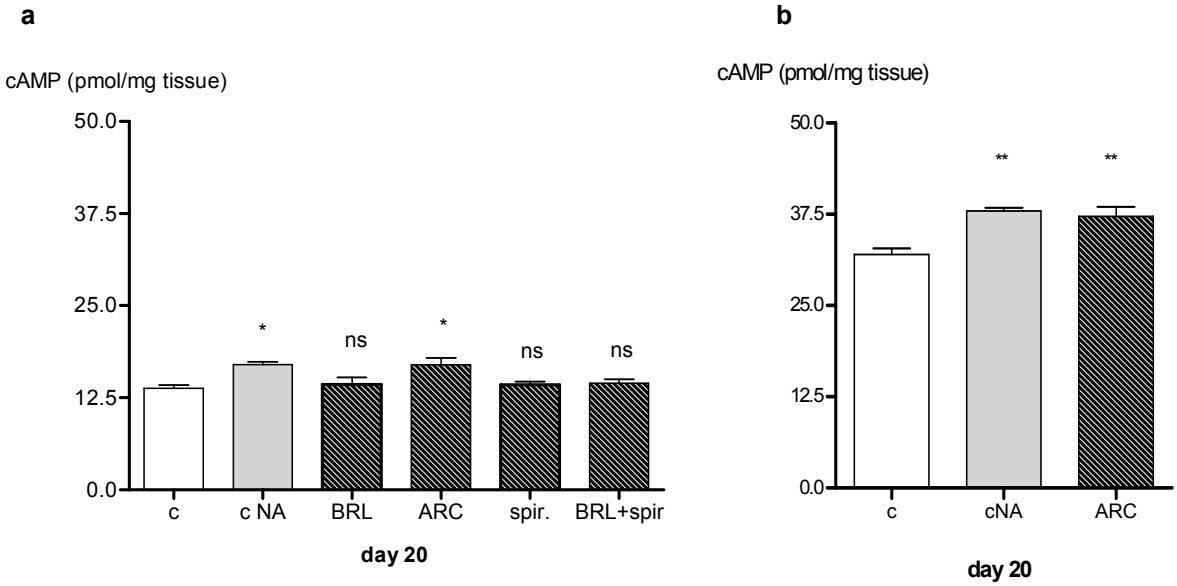

Figure 16. Effects of the subtype-selective α_2-AR antagonists on the cervical cAMP level (pmol/mg tissue ± S.E.M.) in the presence (a) or in the absence of pertussis toxin (b) on day 20 of gestation.

The statistical analyses were carried out with the unpaired t-test or ANOVA, followed by the Neuman–Keuls test. * P< 0.05, ** P< 0.01.

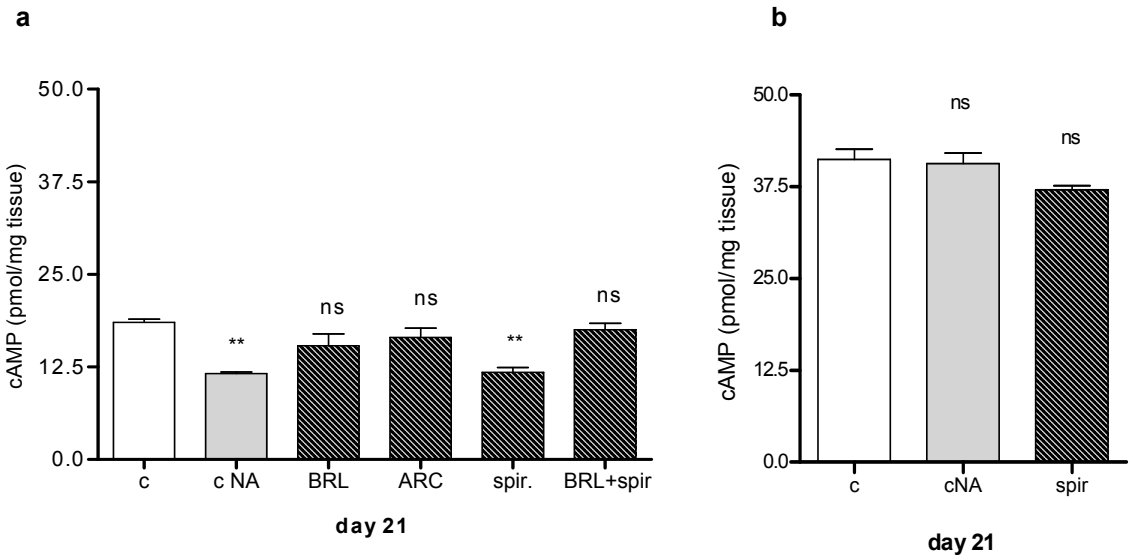

Figure 17. Effects of the subtype-selective α_2-AR antagonists on the cervical cAMP level (pmol/mg tissue ± S.E.M.) in the presence (a) or in the absence of pertussis toxin (b) on day 21 of gestation.

The statistical analyses were carried out with the unpaired t-test or ANOVA, followed by the Neuman–Keuls test. * $P< 0.05$, ** $P< 0.01$.

5. Discussion

The exact mechanism(s) of the initiation of labour are still poorly understood. So far numerous extensive experiments have been carried out to investigate the roles of the α_1- and β_2-ARs in the control of the uterine function, however, no study has been performed related to the effect of the α_2-AR subtypes in the labour. The main focus of our study was to clarify the role of these subtypes in the control of myometrial contractions and cervical resistance in the late pregnant rat, *in vitro*.

We proved the presence of all three α_2-AR subtypes in both the non-pregnant and the 22-day-pregnant rat myometrium and in the cervical samples during the final period of pregnancy (days 18, 20, 21 and 22). An earlier study did not detect α_{2C}-AR in the rat myometrium, though in that work (Bouet-Alard et al., 1997) a radioligand-binding technique was used, which has a lower specificity as compared with our RT-PCR and Western blotting techniques, using α_2-AR subtype-specific primers and polyclonal antibodies, respectively.

To determine which of the three known α_2-AR subtypes is involved in the regulation of myometrial contractions and cervical resistance, subtype-selective antagonists were used alone or in combination. The three subtype-selective compounds offered a possibility to investigate the results of the stimulation of only one or two α_2-AR subtypes. BRL 44408, ARC 239 and spiroxatrine were revealed earlier to exhibit selectivity for α_{2A}-, $\alpha_{2B/C}$- and α_{2C}-ARs, respectively (Corboz et al., 2003; Uhlen et al., 1994). Each of these compounds displays various affinities for the 5-HT_{1A} receptors (Foong and Bronstein., 2009) and spiroxatrine also exerts effects in the dopaminergic system (Chu et al. 1999.), but these effects are likely to have only a low impact on the uterine contractions and the cervical resistance. Although molecules with higher subtype selectivity would be desired to make more precise investigations, these three compounds (in appropriate concentrations) currently offer the best possibility to investigate the results of the stimulation of the individual α_2-AR subtypes by noradrenaline with passable precision (Corboz et al., 2005; Gáspár et al., 2007; Wikberg-Mattson and Simonsen, 2001).

The functional consequences of the stimulation of the different α_2-AR subtypes were detected (isolated organ bath studies) in parallel with the generation of intracellular

cAMP, while the changes in the tissue cAMP level are crucial in the control of the smooth muscle contraction and relaxation (Pierce et al., 2002). In the presence of the subtype-selective antagonists, we found that the changes brought about in the myometrial contractions and the cervical resistance by noradrenaline were in complete accordance with the alterations in the intracellular cAMP levels.

5.1. Myometrial contractions

The roles of α_2-AR subtypes in myometrial contractions were investigated via the effects of subtype-selective antagonists on the noradrenaline-stimulated contractions. Noradrenaline was ineffective on the non-pregnant uteri, whereas KCl enhanced the contractions. These results suggest no coupling between the α_2-ARs and adenylyl cyclase, which was supported by cAMP studies, where the presence of the antagonist did not alter the tissue cAMP production. This finding reaffirmed the earlier report on the lack of connection between α_2-ARs and contractions in the non-pregnant rat myometrium (Kyozuka et al., 1988).

Noradrenaline elicited contractions in the late-pregnant uteri, which were mediated via the α_2-ARs because of the presence of α_1- and β-AR blockers (doxazosin and propranolol). BRL 44408 blocked the α_{2A}-ARs, and hence noradrenaline could stimulate only the α_{2B}- and α_{2C}-ARs. The simultaneous stimulation of these two receptors mildly increased the contractions and decreased the intracellular cAMP level. In the presence of ARC 239, only the α_{2A}-ARs remained free; the stimulation of this subtype decreased the effect of noradrenaline, with a rise in the myometrial cAMP level. Spiroxatrine blocked the α_{2C}-ARs, and thus the α_{2A}- and α_{2B}-ARs were stimulated by the agonist, and an increase in contraction and a decrease in the cAMP level were found. The combination BRL 4408 + spiroxatrine blocked the α_{2A}- and α_{2C}-ARs; the stimulation of the free α_{2B}-ARs also increased the uterine contractions and decreased the amount of tissue cAMP. The presence of the antagonists did not alter the EC_{50} values of the contracting dose - response curves of noradrenaline, indicating that the compound had the same affinity for each of the α_2-ARs.

These results suggest that in the late-pregnant myometrium α_{2A}-ARs mediate only weak contractions, which can be regarded as relaxation as they are compared with the effect of noradrenaline on all the three receptor subtypes. The α_{2B}-ARs are responsible for strong contractions. The α_{2C}-ARs also seem to decrease the contractions, because the

contracting effect mediated through the α_{2B}-ARs was significantly increased when α_{2C}-ARs were blocked by spiroxatrine. The extent of the increase in contraction was lower on the simultaneous stimulation of the α_{2B}- and α_{2C}-ARs than in the case of the α_{2B}- and α_{2A}-ARs, which can be explained by the higher density of the α_{2C}-ARs as compared with the α_{2A}-ARs.

The α_2-ARs are classically described as coupling to the PTX-sensitive G_i-protein α-subunit (Karim and Roerig, 2000; Pohjanoksa et al., 1997), but under certain circumstances α_2-ARs can also couple to G_s-proteins, leadings to activation of adenylyl cyclase (Eason et al., 1992; Offermanns, 2003). On the other hand, it has been proved that pregnancy is able 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 adenylyl cyclase activity at mid-pregnancy versus term (Mhaouty et al., 1995). The different functions of the postsynaptic α_2-AR subtypes inside the same tissue seem to be unique in the pregnant uterus. Although there are some other tissues in which stimulation of the α_2-AR subtypes results in opposite effects, e.g. in the vasculature, the stimulation of α_{2A}-ARs and α_{2C}-ARs causes relaxation and contraction, respectively, but in this case the α_{2A}-ARs are localized presynaptically, while the α_{2B}-ARs are located on the postsynaptic surface (Phillip et al., 2002).

Another question is why the effect of BRL 44408 + spiroxatrine did not exceed the contraction-increasing effect of spiroxatrine alone. Although an increasing tendency in the maximal contractions and a very slight decrease in the tissue cAMP level were observed when the combination was used, these changes were not significant as compared with the effect of spiroxatrine. It is known that the α_2-ARs are also prone to be involved in the processes of homo- and heterodimerization. It has additionally been proved that, when both α_{2A}- and α_{2C}-ARs are expressed, there is a greater likelihood that the two receptors will form heterodimers than homodimers. The α_{2C}-ARs alter α_{2A}-AR signalling by forming oligomers (Hein, 2006; Small et al., 2006). This means that these two receptor subtypes probably function together, although the details of their cooperation are not well known. If the α_{2C}-AR is also able to alter the function of the α_{2A}-ARs in the uterine smooth muscle, this cooperation might give a partial explanation for the similar maximum effects and changes in tissue cAMP levels. Thus, if α_{2C}-AR is blocked, the function of α_{2A}-AR might be modified independently from its blockade.

The uterus-contracting effect of noradrenaline can be explained as a resultant effect mediated by the AR subtypes. Because of the α_{2B}-AR predominance at the end of pregnancy, the contraction is the main resultant effect, which is altered by the other two subtypes, mediating a decrease in the intensity of noradrenaline-induced contractions.
ARC 239 had a marked relaxing effect on the noradrenaline-stimulated uterine contractions; it was therefore tested on the myometrium from the induced labour model. This test was designed to investigate the effects of this compound on overstimulated uterine tissue which is very close to delivery (within 1 h). Its effect on the noradrenaline-evoked contractions was also convincing; it can be promising in the therapy of premature labour. The increased sensitivity of labour-induced uteri to noradrenaline (where the EC_{50} value was 10 times lower than in normal pregnancy) may mean a further advantage for α_2-AR blockers in the inhibition of premature contractions.

5.2. Cervical resistance

As concerns the roles of the α_2-AR subtypes on the cervical resistance, on day 18, in the presence of ARC 239 only the α_{2A}-ARs remained free; the stimulation of this subtype by noradrenaline increased the resistance with a reduction in the cervical cAMP level, while stimulation of the same on day 20 resulted in the opposite effect. The roles of the α_{2A}-ARs were supported by the fact that the α_{2A}-AR antagonist BRL 44408 was able to block the cervical effects of noradrenaline. On the other hand, in the presence of spiroxatrine (an α_{2C}-AR inhibitor) the cervical-increasing effect of noradrenaline disappeared. Since we are not able to produce clear α_{2C}-AR stimulation with the available antagonists, we can only presume that, similarly to the pregnant myometrium, the α_{2C}-ARs are also involved in the control of the cervical resistance evoked by noradrenaline. This theory is supported by the fact that the dual blockade of the α_{2A}- and α_{2C}-ARs (BRL 44408 + spiroxatrine) inhibited the cervical effect of noradrenaline. The different effects mediated through the α_{2A}-ARs (and probably the α_{2C}-ARs) on the different days of pregnancy were surprising; however, based on our previous experiences, we may suggest that the α_{2A}-ARs (and possibly α_{2C}-ARs) can couple to both G_i- and G_s-proteins not only in the pregnant myometrium but also in the pregnant cervical samples. In order to clarify the different G-protein activation, we repeated the investigations in the presence of pertussis toxin. PTX is a well-known inhibitor of G_i-

proteins. On day 18, in the presence of PTX, the basic cAMP production and the cervical resistance were enhanced and decreased, respectively. The effects of noradrenaline alone (non-selective α_2-AR stimulation) and in the presence of the $\alpha_{2B/C}$-AR blocker ARC 239 (selective α_{2A}-AR stimulation) were eliminated by PTX. This indicates the predominance of G_i-coupling of α_{2A}-ARs (and possibly α_{2C}-ARs) on this day of pregnancy.

On day 20, the PTX pre-treatment did not alter the basic cervical tone, the cAMP level or the effects of noradrenaline alone and in the presence of ARC 239. These results suggest that on this day there is a G_s-protein predominance in the coupling of α_{2A}- (and α_{2C}-) ARs. However, similarly as on day 18, there was no effect of noradrenaline in the presence of spiroxatrine or the combination of BRL 44408 + spiroxatrine, indicating the possible involvement of α_{2C}-ARs too. Our results have shown that the effects mediated through these α_{2A}- and α_{2C}-AR subtypes are consequently the same; hence, we presume the previously mentioned dimerization of these subtypes in the pregnant rat cervix.

On day 21, we detected a cervical resistance-increasing effect of noradrenaline. Surprisingly, this effect could be maintained in the presence of spiroxatrine alone. This means that the effect of noradrenaline is probably mediated through the α_{2A}- and α_{2B}-ARs, while the α_{2C}-ARs are not involved in the cervical resistance-increasing effect. After PTX treatment, the effects of noradrenaline either alone or in the presence of spiroxatrine ($\alpha_{2A/B}$-AR stimulation) disappeared. Accordingly, on day 21, the agonist action may be mediated predominantly by coupling to G_i-proteins.

On 22 day of gestation, however, noradrenaline had no effect on the cervical resistance in the presence of the antagonists. Although we have found a decrease in the α_2-AR subtypes relative to day 21, these changes alone do not give a satisfactory answer as concerns the lost receptor function alone. The cAMP studies give us good reason to suppose that the α_2-ARs are uncoupled from the G-proteins on this day as opposed to the pregnant myometrium, when strong noradrenaline action was found on that day.

Interestingly, we have also proved that the block of predominant α_2-AR subtypes (α_{2A}-ARs by BRL 44408 and α_{2C}-ARs by spiroxatrine on day 18) resulted in a strong decrease in the noradrenaline dose - response curve, especially in the case of spiroxatrine. This furnished further evidence of real subtype-selective antagonism between the agonist and the antagonist.

6. Conclusions

Based on these facts, we can conclude that the α_2-AR subtypes play different roles in the contractility of the rat uterus. In non-pregnant animals, they are not involved in the control of myometrial contractions. In last-day-pregnant animals, the α_{2B}-ARs predominate and mediate contraction, while the α_{2A}- and α_{2C}-ARs decrease the contractile response to noradrenaline.

	Contractile response		
	α_{2A}	**α_{2B}**	**α_{2C}**
day 22	⇩	⇧	⇩

In the cervix the α_{2A}- and α_{2C}-ARs were responsible for the developing action on day 18 and 20, while the α_{2A}- and α_{2B}-ARs were found to be responsible for the action on day 21. There was no significant effect of noradrenaline alone and in the presence of the subtype selective antagonists on day 22. Our results lead us to presume a parallel G_i/G_s-coupling of α_{2A}- and α_{2C}-ARs in the pregnant rat cervix, which functions in an alternate way depending on the day of pregnancy. The α_2-AR subtypes may possibly cooperate with the other subtypes (heterodimerization) for the appropriate control of cervical resistance near term.

	α_{2A}		**α_{2B}**		**α_{2C}**	
	G protein coupling	resistance	G protein coupling	resistance	G protein coupling	resistance
day 18	G_i	⇧	-	-	G_i	⇧
day 20	G_s	⇩	-	-	G_s	⇩
day 21	G_i	⇧	G_i	⇧	-	-
day 22	-	-	-	-	-	-

If preterm labour results from myometrial and cervical incompetence, it seems to make sense to block the myometrial contractions and to increase the mechanical strength of the cervix to arrest cervical opening and to prevent preterm delivery. In the light of our experiences it seems very probably that the blockade of the α_2-AR subtypes will not be sufficient to stop the whole preterm labour process, but their combination with more

potent inhibitor drugs may have clinical benefits. Further attempts need to identify the factor(s) underlying the molecular mechanisms leading to the differential regulation of adenylyl cyclase activity at late pregnancy. Understanding these processes may bring closer the revealing of the reasons of premature labour.

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10. Appendix

Table 1. Changes in the 22-day pregnant uterus-contracting effect of noradrenaline (EC_{50} and E_{max} values) in the presence of subtype-selective α_2-AR antagonists (10^{-7} M)

	Stimulated α_2-ARs	EC_{50} ± S.E.M. (M)	E_{max} ± S.E.M. (%)
Noradrenaline (control)	α_{2A}-, α_{2B}- and α_{2C}-ARs	$2.6 \times 10^{-6} \pm 0.8 \times 10^{-6}$	295.1 ± 30.3
Noradrenaline + BRL 44408	α_{2B}- and α_{2C}-ARs	$1.8 \times 10^{-6} \pm 0.9 \times 10^{-6}$	377.0 ± 51.8
Noradrenaline + ARC 239	α_{2A}-ARs	$1.2 \times 10^{-6} \pm 1.3 \times 10^{-6}$	154.4 ± 34.5
Noradrenaline + spiroxatrine	α_{2A}- and α_{2B}-ARs	$1.8 \times 10^{-6} \pm 1.1 \times 10^{-6}$	408.0 ± 41.5
Noradrenaline + BRL 44408 + spiroxatrine	α_{2B}-ARs	$2.7 \times 10^{-6} \pm 1.0 \times 10^{-6}$	446.8 ± 34.8

EC_{50}: the concentration of noradrenaline alone or in the presence of an α_2-AR antagonist which elicits half the maximum contracting effect of noradrenaline.

E_{max}: maximum contracting effect of noradrenaline alone or in the presence of the α_2-AR antagonists.

Table 2. Changes in the labour-induced uterus-contracting effect of noradrenaline (EC_{50} and E_{max} values) in the presence of α_{2BC}-AR antagonist ARC 239 (10^{-7} M)

	Stimulated α_2-ARs	EC_{50} ± S.E.M. (M)	E_{max} ± S.E.M. (%)
Noradrenaline (control)	α_{2A}-, α_{2B}- and α_{2C}-ARs	$3.4 \times 10^{-7} \pm 1.6 \times 10^{-7}$	100.3 ± 10.4
Noradrenaline + ARC 239	α_{2A}-ARs	$3.1 \times 10^{-7} \pm 1.2 \times 10^{-7}$	58.6 ± 8.7

EC_{50}: the concentration of noradrenaline alone or in the presence of ARC 239 which elicits half the maximum contracting effect of noradrenaline.

E_{max}: maximum contracting effect of noradrenaline alone or in the presence of ARC 239.

I.

II.

III.