Department of Pharmacodynamics and Biopharmacy
University of Szeged, Faculty of Pharmacy
Supervisor: Prof. George Falkay Ph.D., D.Sc.

An investigation of the effects of β_2-mimetics, gestagens and an α_2-antagonist in premature labour model in the rat

Ph.D. Thesis

By
Márta Gálik

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II. **Márta Gálik**; Róbert Gáspár, Ph.D; Zoltán Kolarovszki-Sipiczki; George Falkay D.Sc. Gestagen treatment enhances the tocolytic effect of salmeterol in hormone-induced preterm labor in the rat *in vivo*. Am J Obstet Gynecol; 2008 Mar;198(3): 319-5.

III. **Gálik M**., Dr. Gáspár R., Dr. Klukovits A., Dr. Márki Á., Prof. Falkay Gy. A salmeterol tocolyticus hatásának fokozása gestagennel. Magyar Nőorvosok Lapja 2008; 71: 37-43.

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V. Gáspár R., Kolarovszki-Sipiczki Z., Gál A., Minorics R., **Gálik M**., Ducza E., Falkay G.: α_2-adrenergic receptor subtypes as new targets for tocolytic therapy. 1st Hot Topics & Controversies in Perinatal Medicine; Rhodes Island, Greece, June 16-19, 2005,

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IX. **Gálik Márta**, Kolarovszki-Sipiczki Zoltán: Szalmeterol tokolitikus hatásának fokozása gesztagénnal patkány koraszülési modellen. VIII. Clauder Ottó Emlékverseny; Budapest, 2007. április 12-13.

X. R. Gáspár, **M. Gálik**, Z. Kolarovszki-Sipiczki, E. Ducza, Á. Márki, S. Benyhe, E. Páldy, A. Borsodi, G. Falkay. Gestagens enhances the relaxing effect β_2-adrenergic receptor agonists on pregnant rat myometrium: old drugs with new perspective in therapy. 8^{th} Congress of the European Association for Clinical Pharmacology and Therapeutics; Amsterdam, August 29-September 1, 2007.

List of abbreviations

PTB: preterm birth

PTL: preterm labour

β_2-AR: β_2-adrenergic receptor

P: progesterone

PR: P receptor

PR-A: P receptor A

PR-B: P receptor B

PR-C: P receptor C

IC: intracellular

PGs: prostaglandins

ERα: oestrogen receptor-α

cAMP: cyclic adenosine monophosphate

α-ARs: α-adrenergic receptors

α_1-AR: α_1-adrenergic receptor

α_2-AR: α_2-adrenergic receptor

VDCCs: voltage-dependent Ca^{2+} channels

α_{2A}-AR: α_{2A}-adrenergic receptor

α_{2B}-AR: α_{2B}-adrenergic receptor

α_{2C}-AR: α_{2C}-adrenergic receptor

LPS: lipopolysaccharide

PGE_2: prostaglandine E_2

sc.: subcutaneous

17P: 17α-hydroxyprogesterone caproate

ip.: intraperitoneally

AUCs: areas under curves

1. Introduction

1.1. Epidemiology and consequences of preterm birth

The World Health Organization defines preterm birth (PTB) as the delivery of an infant between 20 and 37 weeks of gestation. The incidence of preterm labour (PTL) in developed European countries and in the U.S.A. is still around 5-7% and 12.5%, respectively, (Haram *et al*., 2003; Reedy, 2007), and even appears to be increasing slightly when the data from the U.S.A. between 1980 and 2000 are considered (ACOG, 2003). Currently, a preterm infant is born every 3 minutes and 11 seconds in the U.S.A. (Reedy, 2007).

The aetiology of PTL is unclear. Epidemiological studies have identified several risk factors for PTB, such as a prior PTB, black race, teenage or older mothers, those of low education and of low socioeconomic status, cigarette smoking, a heavy and/or stressful occupation, a low maternal pregnancy body mass index, and poor or excessive weight gain. Medical and obstetrical complications including multifoetal pregnancy, gestational or pre-existing diabetes, (essential or pregnancy-induced) hypertension, placenta previa and placental abruption, poly- or oligohydramnions, abdominal surgery in the late second or third trimester, and systemic or regional infection (such as asymptomatic bacteruria) are also strongly related to PTB (Wen *et al*., 2004).

The mechanisms involved in many of these risk factors are poorly understood. Some of them may not be related to PTB directly, but may act as a trigger of an underlying cause or an intermediate step in a sequence of events leading to PTB (Wen *et al*., 2004). It is supposed that the primary risk factor for PTB is a previous PTL. Hoffman *et al*. found that a woman who had undergone one PTL had twice the risk of another, and with three or more PTLs, the risk in a subsequent pregnancy was five times higher than that for women who did not have a previous history of PTL (Hoffman *et al*., 1984).

Although the survival of premature newborns has been improved dramatically by the major advances in medical technology in industrialized countries over recent decades, PTB is still one of the major reasons for neonatal mortality (70%) and

morbidity (75%) (Challis *et al*., 2001). One of the causes of the high morbidity is the treatment of preterm infants with extremely low birth weight (Goldenberg *et al*., 1998). Short-term morbidities associated with PTB are respiratory distress syndrome, intraventricular haemorrhage, periventricular leukomalacia, necrotizing enterocolitis, bronchopulmonary dysplasia, sepsis and patent ductus arteriosus. Long-term morbidities include cerebral palsy, mental retardation and retinopathy of prematurity (Goldenberg, 2002).

The total cost for hospital newborn care only in the U.S.A. is currently $35.7 billion annually. Infants with a diagnosis of PTB or low birth weight account for nearly $18.1 billion a year (March of dimes web site). In Hungary, the cost of the clinical care of an infant born before 29 weeks of gestation exceed 3.9 million HUF (Vida *et al*., 2007). Preterm newborns are often the most expensive population for health care insurance companies (Jones *et al*., 2002).

1.2. Tocolytic therapy

Unfortunately, the efficacy of current pharmacological treatments for the management of PTB is often questioned. The use of magnesium sulphate was described by Steer in 1977 (Steer *et al*., 1977). Its efficacy has been disputed (Goldenberg, 2002; Tan *et al*., 2006), but it is widely used for tocolysis, particularly in the U.S.A. The Ca^{2+} channel blocker nifedipine proved to be as effective as ritodrine in delaying PTB, but the side-effects that occurred led to the discontinuation of this therapy (Ferguson *et al*., 1990). However, nifedipine seems to be safe as regards its effect on the uteroplacental circulation. On the other hand, there is thought to be a lack of evidence of its superiority versus placebo that may fully attest to its safety and efficacy (Oei, 2006). Indomethacin was first used to inhibit human PTL by Zukerman et al. (Zukerman *et al*., 1974). Despite the favourable results, most studies have limited the duration of indomethacin use to 24-48 h because of the development of oligohydramnios, constriction of the ductus arteriosus and an increased risk of necrotizing enterocolitis. Indomethacin can be used as a second-line tocolytic agent in the event of a danger of early PTB (Goldenberg, 2002). The oxytocin receptor antagonist atosiban is effective in diminishing uterine contractions; moreover, the value of atosiban therapy has been found to be similar to that of β_2-

mimetic therapy, but with considerably fewer side-effects (The Worldwide Atosiban Versus Beta-agonists Study Group, 2001). It may be useful in delaying delivery for 24-48 h in the setting of PTL (Goldenberg, 2002).

The β_2-adrenergic receptor (β_2-AR) agonists are preferably used for tocolysis, mainly in European countries. In the U.S.A., these agents have been set aside in tocolytic therapy, because they have many maternal and foetal side-effects. Their effectiveness has also been the subject of intensive debate in the literature. Some articles claim that most β_2-AR agonists can put off labour for 48-72 h (Katz *et al*., 1999), while others conclude that the duration of their action is only 24-48 h (Higby *et al*., 1993). Another problem is the desensitization of the β_2-ARs in tocolytic therapy.

At present, the realistic aim of tocolytic therapy is not to prevent PTB, but rather to delay delivery so as to allow that interventions aimed at reducing neonatal mortality and morbidity, such as the use of antenatal corticosteroids, and/or transfer of the women at risk to a clinic.

1.3. The role of progesterone (P) in uterine contraction

The uterine smooth muscle belongs in a broad class of smooth muscles termed phasic smooth muscle. Phasic smooth muscle is characterized by the generation of action potentials, spontaneous contractile activity that is independent of extrinsic innervations, and the presence of gap junctions that serve for the electric coupling of adjoining myocytes (Garfield, 1990).

Changes occur in the excitability of the myometrium during pregnancy, which becomes maximal at the time of parturition (Csapo, 1977). It was concluded that the uterus must pass through some ‘priming process’ (others have referred to this ‘the preparatory phase of labour’) before it can respond to uterotonic agents. Some articles suggest that this ‘priming process’ and the change in the P level are linked.

P is a steroid hormone that plays an essential role in each step of human pregnancy. P acts via the P receptor (PR), a member of the family of ligand-activated nuclear transcription regulators. Human PRs have three isoforms: the 116 kDa PR-B, the 94 kDa PR-A and a 60 kDa PR-C (Wei *et al*., 1997; Giangrande *et al*., 1999). The binding of P to PR-B results in the activation of P-responsive genes, while PR-A

acts as a modulator or repressor of the PR-B function. This concept appears to be questioned by experiments with transgenic mice, which suggest that PR-A and PR-B have overlapping, rather than antagonistic actions in fertility and uterine function (Mulac-Jericevic *et al*., 2004). The existence of a functional PR-C isoform has been postulated, but the data are conflicting.

In early pregnancy, P produced by the corpus luteum is critical for the maintenance of early pregnancy until the placenta takes over this function at 7-9 weeks of gestation. The name of the hormone comes from its action: **proge**stational **ster**oid horm**one**. It is well known that P is required for implantation, but its role in late pregnancy is less clear. It has been proposed that P may be important in maintaining uterine quiescence in the second half of pregnancy by limiting the production of stimulatory prostaglandins (PGs) and inhibiting the expression of contraction-associated protein genes (ion channels, oxytocin and PG receptors, and gap junctions) within the myometrium (Norwitz *et al*., 1999; Challis *et al*., 2000). Furthermore, P regulates the intracellular (IC) Ca^{2+} concentration by stimulating the secretion of the peptide hormone calcitonin, which is postulated to lower the free Ca^{2+} level in the uterus, thereby preventing contraction (Ding *et al*., 1994).

Labour at term most likely represents a release from the inhibitory mechanisms in the myometrium, rather than an active process of uterine stimulation. Csapo and Cousins described a relative P deficiency and an increase in the oestradiol-17β / P ratio in patients with PTL (Csapo *et al*., 1974; Cousins *et al*., 1977). On the other hand, systemic P withdrawal may not correlate directly with the onset of labour in humans. There is increasing evidence to suggest that P exerts its influence indirectly via a 'functional' withdrawal at the level of the uterus. The onset of labour at term is associated with an elevation in the myometrial PR-A/PR-B expression ratio, resulting in the 'functional' withdrawal of P action (Sfakianaki *et al*., 2006). Interestingly, expression of the oestrogen receptor-α (ERα) increases in parallel with the PR-A/PR-B expression ratio in non-labouring myometrium (Cermik *et al*., 2001; Leonhardt *et al*., 2003). These findings suggest that functional oestrogen activation and functional P withdrawal are linked. P probably decreases the myometrial oestrogen responsiveness by the inhibition of ERα expression (Sfakianaki *et al*., 2006). Such an interaction could explain why the human uterus is refractory to the high levels of circulating oestrogens for most of pregnancy. At term, the functional P withdrawal removes the suppression of myometrial ERα expression,

leading to an increase in myometrial oestrogen responsiveness. Oestrogen can then act to transform the uterus into a contractile phenotype.

It is also known that the oestrogen-P ratio is responsible for the expression of myometrial α-and β-ARs (Gáspár *et al*., 2001). A higher oestrogen-P ratio has been found to result in a higher α-and β-AR ratio.

1.4. ARs

The adrenergic system plays an important role in the control of uterine contractility. Three β- ARs subtypes (β_1-, β_2- and β_3-AR) have been characterized by pharmacological, biochemical and molecular biological cloning approaches. They belong in the G protein-coupled receptor superfamily. The existence of β_4-AR has been reported in the mammalian heart (Kaumann**,** 1997); it has not been detected yet in the human uterus.

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). The β_2-ARs are mainly postsynaptic and are located on a number of tissues, including the blood vessels, the bronchi, the gastrointestinal tract, the skeletal muscle, the liver and the mast cells. It couples via G_s protein to myometrial adenylate cyclase, increases the IC levels of cyclic adenosine monophosphate (cAMP) and activates cAMP-dependent protein kinases. This leads to myometrial relaxation through effects on the IC Ca^{2+} concentration and myosin light chain kinase. Because of their role in relaxation, the β_2-ARs are still one of the main targets of tocolytic therapy.

On the other hand, it is known that the myometrial responsiveness to the β_2-agonists decreases towards the end of pregnancy (Cruz *et al*., 1990). The uterus-relaxing effect of terbutaline on electric field-stimulated contractions decreases towards the end of the pregnancy in the rat (Gáspár *et al*., 2005). Moreover, in the [^{35}S]GTPγS binding assay, terbutaline decreases the amount of activated myometrial G-protein on the last day of pregnancy.

The potential explanation of the pregnancy-induced desensitization may be the decreasing level of P. Earlier studies reported that the presence or absence of P can alter the effect of β_2-AR agonists on the pregnant myometrium (Dowell *et al*., 1994; Engstrom *et al*., 2001). During pregnancy, the β_2-ARs are selectively up-

regulated by P at a gene transcription level (Vivat *et al*., 1992). P has been shown to stabilize the high-affinity state of the receptor by increasing the coupling of the β_2-ARs to adenylate cylase (Cohen-Tannoudji *et al*., 1991). A correlation between the decreased function of the β_2-ARs and P withdrawal has been reported: *in vivo* P pretreatment favourably affects the level of the β_2-ARs, and also enhances the uterus-relaxing effect of terbutaline *in vitro*. Additionally, P treatment invertes the dose-dependent decrease in the amount of activated G-protein of β_2-ARs by terbutaline on day 22 of pregnancy in the rat (Gáspár *et al*., 2005). In parallel, has been reported that *in vitro* P increases the uterus-relaxing effect of ritodrine by reducing 50% of the maximum response, amplitude and frequency of the myometrial contractions in the isolated human pregnant myometrium (Chanrachakul *et al*., 2005). These results suggest that the P and β_2-agonists in combination might be a promising possibility for the tocolytic therapy in the future.

The β_3-ARs have recently been investigated as a potential target for tocolysis. They had not been detected previously, probably because of the low affinity of [^{125}I]iodocyanopindolol, a ligand for the β_3-ARs, this ligand being commonly used for β-AR-binding studies. The level of expression of β_3-AR transcripts is increased in the near-term human myometrium, in contrast with the β_2-ARs (Bardou *et al*., 2000). The modulation of β_2- and β_3-AR expression and their function is probably related to hormonal changes. Recent results on brown adipocytes demonstrated that oestradiol and P are able to modify β_3-AR affinity and density (Malo *et al*., 2001). Furthermore, the β_3-AR subtype is resistant to the agonist-induced desensitization near term as compared with β_2-ARs (Rouget C *et al*., 2004). Further difference between the β_2- and β_3-ARs is in the effects of their agonists: activation of the β_3-ARs did not reveal any change in the human metabolism, such as the plasma glucose, insulin or K^+ levels (Haesler *et al*., 1994; Goldberg *et al*., 1995). Despite the benefits of the β_3-ARs, their use as tocolytic drugs is limited; because almost all that is known about the pathophysiological role of this receptor subtype involves merely considerations based predominantly on preclinical studies (Vytenis *et al*., 2004). Further human studies are needed because the effects of the β_3-ARs on the cardiovascular system are controversial at the moment (van Baak *et al*., 2002; Gauthier *et al*., 2007).

The α-ARs have been classified as α_1 and α_2 subtypes. The α_1-ARs are considered to be responsible for the excitatory effects on the catecholamines in

several species, e.g. the rabbit, rat and guinea-pig. The α_1-AR agonists elicit contractions in the smooth muscles via increases in the IC inositol triphosphate and Ca^{2+} levels (Michelotti *et al*., 2000). The roles of the α_2-ARs in the regulation of myometrial contractility, however, are not fully understood. Three α_2-AR subtypes (α_{2A}-, α_{2B}- and α_{2C}-AR) have been detected in the human myometrium, while only α_{2A}-AR and α_{2B}-AR were found in the rat uterus (Bouet-Alard *et al*., 1997). Resently, all three α_2-AR subtypes have been reported in both the non-pregnant and the 22-day-pregnant rat uteri (Gáspár *et al*., 2007).

The cellular mechanisms underlying the contractile action of α_2-AR activation are not well understood. The α_2-ARs are coupled with a class of heterotrimeric GTP-binding proteins known as G_i, and activation of the receptors decreases the adenylate cyclase activity (Ruffolo *et al*., 1991). The inhibition of basal cAMP generation enhances smooth muscle contractions. On the other hand, some data suggest that other mechanisms are also involved in α_2-AR-induced contractions. The activation of α_2-ARs is linked with the stimulation of phospholipase A_2 in some cell types (Fraser, 1991; Nebigil *et al*., 1992), which releases arachidonic acid from the membrane phospholipids and induces contractions in the vascular smooth muscle (Jancar *et al*., 1987). Phospholipase A_2 has been proposed as a modulator of the Ca^{2+} sensitivity of the contractile elements, acting by inhibiting myosin light chain phosphatase (Gong *et al*., 1992; Karaki *et al*., 1997). Furthermore, recent studies indicated the α_2-AR-mediated activation of voltage-dependent Ca^{2+} channels (VDCCs) in the smooth muscles (Lepretre *et al*., 1994; ZhuGe *et al*., 1997). This finding suggests the following cascade of events for α_2-AR activation in the porcine myometrium: α_2-AR activation → G_i activation → opening of the VDCCs → on increased Ca^{2+} influx → on increased IC concentration of Ca^{2+} → smooth muscle contraction.

The differences in the receptor subtypes and their various localizations are thought to be responsible for their different roles. It is known that the α_2-ARs affect a number of behavioural functions in the central nervous system. The α_{2C}-AR subtype has been demonstrated to inhibit the processing of sensory information in the central nervous system in the mouse (Scheinin *et al*., 2001). In contrast, the stimulation of α_{2A}-ARs and α_{2B}-ARs may actually improve the spatial working memory in mice (Bjorklund *et al*., 2001). The α_2-ARs are important regulators in the cardiovascular system. The α_{2B}-ARs are responsible for the initial hypertensive phase, whereas

long-acting hypertension is mediated by the α_{2A}-ARs (Link *et al*., 1996; Altman *et al*., 1999). Recent studies have demonstrated that the α_2-AR subtypes play different roles in the contractility of the pregnant rat uterus (Gáspár *et al*., 2007).

2. Aims

The main focus of our study was to enhance the tocolytic effect of β_2-mimetics and to test the possible effect of the most potent subtype-selective α_2-antagonist in a PTL model. To date no extensive experiments have been carried out to investigate the *in vivo* and *in vitro* uterus-relaxing effect of a combination of gestagens and β_2-mimetics in the rat, and no data are available on the *in vitro* effect of the $\alpha_{2B/C}$-antagonist ARC 239 on highly contractible uterine tissue from PTL. Accordingly, the following aims were set:

1. To adapt a reproducible PTL model in the rat in order to investigate the *in vivo* efficacy of the β_2-AR agonist salmeterol, gestagens and a combination of salmeterol and gestagens.

2. To determine the density of β_2-ARs by radioligand binding assay in the hormone-induced PTB model after salmeterol and/or gestagen treatment.

3. To test the efficacy of single β_2-AR agonist or combined β_2-AR agonist – gestagen treatments in a lipopolysaccharide (LPS-) -induced PTL model in the rat *in vitro*.

4. And finally, to investigate the effect of the $\alpha_{2B/C}$-antagonist ARC 239 in noradrenaline-induced contractions in the hormone-induced PTL model *in vitro*.

3. Materials and Methods

Housing and mating 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 number: IV/1813-1/2002). 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. They were maintained on a standard rodent pellet diet (Charles-River Laboratories, Hungary) and tap water, both available *ad libitum.*

Mature female (180-200 g) and male (240-260 g) Sprague-Dawley rats were mated in a special mating cage. Vaginal smears were taken from the female rats and a sperm search was performed under a microscope at a magnification of 1200 x. When the smear proved positive, the female rats were separated and were regarded as first-day pregnant animals.

3.1. Determination of the effects of salmeterol and gestagens in a hormone-induced PTL model

Induction of PTL

PTL was induced according to Rechberger *et al*. (1996). Briefly, the animals were treated with mifepristone (3 mg/0.1 ml) and PGE_2 (0.5 mg/animal) on day 19 of pregnancy, which may correlate approximately to gestation weeks 34-35, if we consider that the duration of pregnancy in the rat and the human is 22 and 280 days, respectively. Mifepristone was suspended in olive oil and given as a subcutaneous (sc.) injection at 9 a.m. At 4 p.m, PGE_2 was applied intravaginally. The time of delivery of the first foetus was noted as the duration in hours from the time of mifepristone administration.

Animal treatments

Salmeterol xinafoate (Sigma Aldrich, Budapest, Hungary) was dissolved in a 1:1 methanol - water mixture. An Alzet osmotic pump (Model 2ml1; DURECT Corp., Cupertino, U.S.A.) loaded with salmeterol xinafoate solution or the vehicle was inserted sc. into the back skin of rats on one or other of days 15-18 of pregnancy, under isoflurane anaesthesia (Burton's narcotic apparatus). The dose of salmeterol was administrated 130 μg/day/animal. Pefloxacine was used in a dose of 8 mg/0.1 ml/animal to prevent infections.

P (Sigma Aldrich, Budapest, Hungary) or 17α-hydroxyprogesterone caproate (17P; donated by Richter Gedeon Nyrt, Budapest, Hungary) was suspended in olive oil and was injected sc. in a dose of 0.5 mg/0.1 ml/day from the day of pump insertion until day 20 of pregnancy.

Experimental design

Group A was the control group, while Group B was treated with P, Group C with salmeterol, Group D with the combination of salmeterol and P, and Group E with the combination of salmeterol and 17P. There were 8 rats in each group. All the animals were operated on for osmotic minipump insertion and were treated with sc. injections. The osmotic minipumps contained salmeterol or vehicle, while the sc. injection contained gestagens or the vehicle.

The salmeterol/gestagen/vehicle treatments started on different days (15, 16, 17 or 18) of pregnancy, with the exception of the animals in Group E, where the treatment always started on day 15 of pregnancy.

Statistical analysis was carried out with the ANOVA Newman-Keuls test.

Radioligand binding assay

Animals were treated with 17P and/or salmeterol from days 15 to 20 of pregnancy. PTL was induced on day 19 of pregnancy as described previously. Uterine tissues were removed on day 20 of pregnancy and were homogenized in buffer (20 mM $NaHCO_3$) with an Ultra-Turrax T15 homogenizer (Janke&Kunkel, IKA-Labortechnik, Germany) and centrifuged (20000xg, 40 min, 4 ^{o}C). The pellets were resuspended and centrifuged as previously described and stored at -70 ^{o}C.

The reaction mixture contained 100 µl membrane preparation (~0.5 mg/ml protein), 100 µl tritiated β_2-AR selective ligand [^{3}H]ICI 118,551 with a specific activity of 18,8 Ci/mmol (Tocris, U.K.) and 100 µl unlabelled ligand (dihydroalprenolol, Sigma-Aldrich, Hungary) for non-specific binding, or 100 µl incubation buffer (consisting of 0.05 M Tris-HCl, 0.01 M $MgCl_2$, and 2.5% ethanol, pH = 7.42) for total binding. At the end of the incubation (25 ^oC 50 min), the bound radioligand was separated from the residual free radioligand by rapid filtration on a Brandell cell harvester (SEMAT, U.K.) through Whatman GF/B filters (SEMAT, U.K.) and washed with 3x10 ml ice-cold buffer (Tris-HCl, pH=7.42). The bound radioactivity was determined in a HighSafe scintillation cocktail in a Wallac 1409 liquid scintillation counter (Turku, Finland).

Saturation analysis of β_2-ARs was performed with 0.5 -10 nM [^{3}H]ICI 118,551 in the presence or absence of 1 µM unlabelled dihydroalprenolol. Specific binding was determined by subtracting the non-specific binding from the total binding. All assays were carried out at least 3 times in duplicate and values are given as means ± SEM. The amount of β_2-AR protein (*Bmax*) was calculated by Scatchard transformation of the saturation curves. Statistical analysis was carried out as mentioned above.

3.2. Determination of the effects of terbutaline and 17P in the uteri of rats challenged with LPS

LPS-induced PTL model

The pregnant animals were divided into three groups: a non-treated, an LPS-treated and an LPS-17P-treated group.

The LPS (*E. coli* endotoxin 055:B5; 62.5 µg; Sigma-Aldrich, Hungary) was dissolved in physiological saline and was injected intraperitoneally (ip.) on days 18 to 20 of pregnancy. 17P was suspended in olive oil and was injected sc. in a dose of 0.5 mg/0.1 ml/day on the days of LPS treatment.

The non-treated group received physiological saline ip. and olive oil sc.

Isolated organ bath studies with terbutaline

Uteri were removed from intact and treated rats on day 20 of pregnancy. Muscle rings 5 mm long were sliced from the uterine horns and mounted in an organ bath 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 temperature of 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 1 h before the experiments were undertaken; the buffer was refreshed every 15 min. The initial tension was set at 1.5 g. The tension of the myometrial rings was measured with a gauge transducer and areas under curves (AUCs) were evaluated with the S.P.E.L. Advanced ISOSYS Data Acquisition System (Experimetria Ltd, Hungary), respectively. Contractions were elicited with 25 mM KCl, and the effects of terbutaline (10^{-12} – 10^{-7} M) were tested. Following the addition of each concentration of terbutaline, recording was performed for 300 s. Concentration-response curves were fitted and analysed statistically with the Prism 4.0 (GraphPad Software, U.S.A.) computer program. The E_{max} and EC_{50} values were calculated (E_{max}: maximum relaxing effect of terbutaline on KCl-induced contractions; EC_{50}: the concentration of terbutaline which elicits half of E_{max}). For statistical evaluations, data were analysed by means of the ANOVA Newman-Keuls test.

3.3. Determination of the effects of the $\alpha_{2B/C}$ antagonist ARC 239 in the hormone-induced PTL model

The uterine tissues were removed from hormone-induced PTL rats at 9.00 a.m. on day 20 of pregnancy, ensuring that the pregnant myometrium was very close to, but not after delivery.

Isolated organ bath studies with the $\alpha_{2B/C}$ antagonist ARC 239

The tissue preparation and incubation were performed as described above. After the incubation, contractions were elicited with noradrenaline ($1x10^{-8}$–$3x10^{-5}$ M; Sigma-Aldrich, Hungary) and cumulative concentration-response curves were constructed in each experiment in the presence of propranolol (10^{-5} M) and doxazosin (10^{-7} M; donation of Pfizer Hungary Ltd., Hungary) in order to prevent β- and α_1-AR

stimulation-evoked responses. The material containing the α_2-AR antagonist ARC 239 (Tocris, U.K.) was 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. The evaluation and statistical analysis were carried out as described previously. The E_{max} and EC_{50} values were calculated. (E_{max}: maximum contracting effect of noradrenaline alone or in the presence of $\alpha_{2B/C}$-AR antagonist; EC_{50}: The concentration of noradrenaline alone or in the presence of the $\alpha_{2B/C}$-AR antagonist which elicits half of E_{max}). For statistical evaluations, data were analysed by two-tailed unpaired *t* tests.

4. Results

4.1. The effects of salmeterol and gestagens in the hormone-induced PTL model

In Group A (control), PTL occurred within 24 h after mifepristone treatment, at about 9 a.m. on pregnancy day 20.

In Group B (P), the treatment started on day 15 of pregnancy, and it was not effective in delaying the time of PTB. In Group C (salmeterol), the effect of the treatment was significant; the PTB was delayed by 2.41 ± 0.52 h. In the Group D (salmeterol – P combination), the treatment delayed the PTL by 5.24 ± 0.69 h (Fig. 1).

The results were similar when the treatments were started on one or other days 16-18 of pregnancy (Fig. 2-4). In each case, Groups D and Group E (combined therapy) were more effective than those in Groups B and C (monotherapy). The difference in efficacy between Groups C and D was most expressed for the treatment started on day 15 (Fig. 1).

In Group E, the PTB-delaying effect of salmeterol – 17P treatment was very similar to that of the salmeterol – P combination. The difference between the two combinations was not significant (Fig. 1).

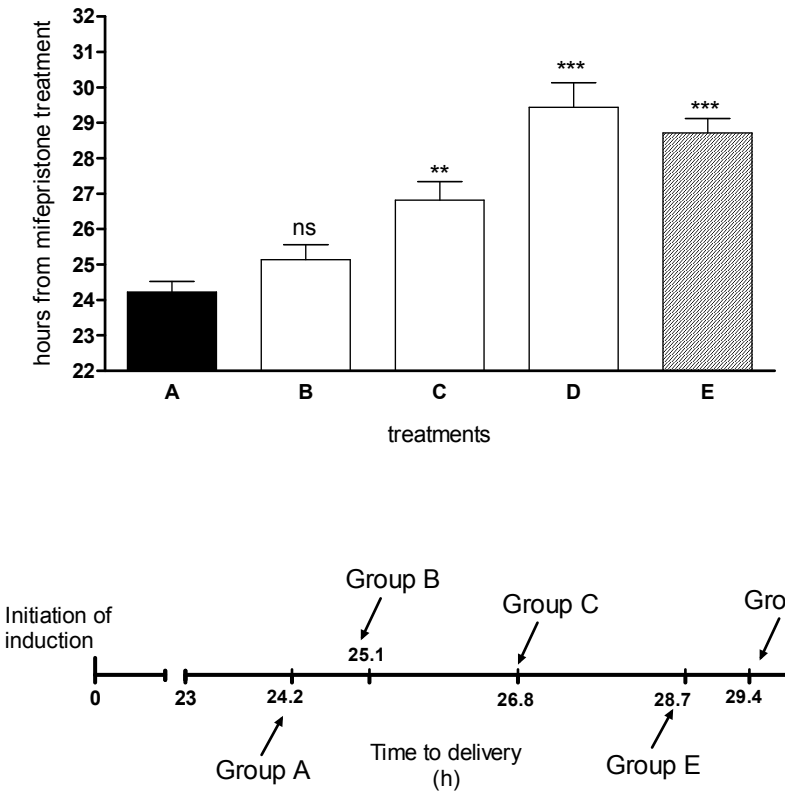


Fig. 1. The effects of progesterone (P; 0.5 mg/0.1 ml), salmeterol (130 µg/day) and combined gestagen - salmeterol treatments on hormone-induced preterm labour (PTL) in the rat (n = 8 for each group). The treatments were started on gestation day 15.

a. **A**: Control PTL group; **B**: P-treated; **C**: salmeterol-treated; **D**: salmeterol – P combination-treated; **E**: salmeterol – 17α-hydroxyprogesterone caproate (17P) combination-treated animals.

The bar graphs show means ± SEM. The effects were compared with the results on the control group. ns: not significant; ** $p<0.01$; *** $p<0.001$.

The difference in efficacy between the treatments reflected in Groups C and D was significant ($p<0.01$). The Group E combination was as effective as the Group D combination ($p>0.05$).

b. The time to delivery from the initiation of PTL (mifepristone treatment). The longest delay was caused by the combination of salmeterol - P (5.2 h).

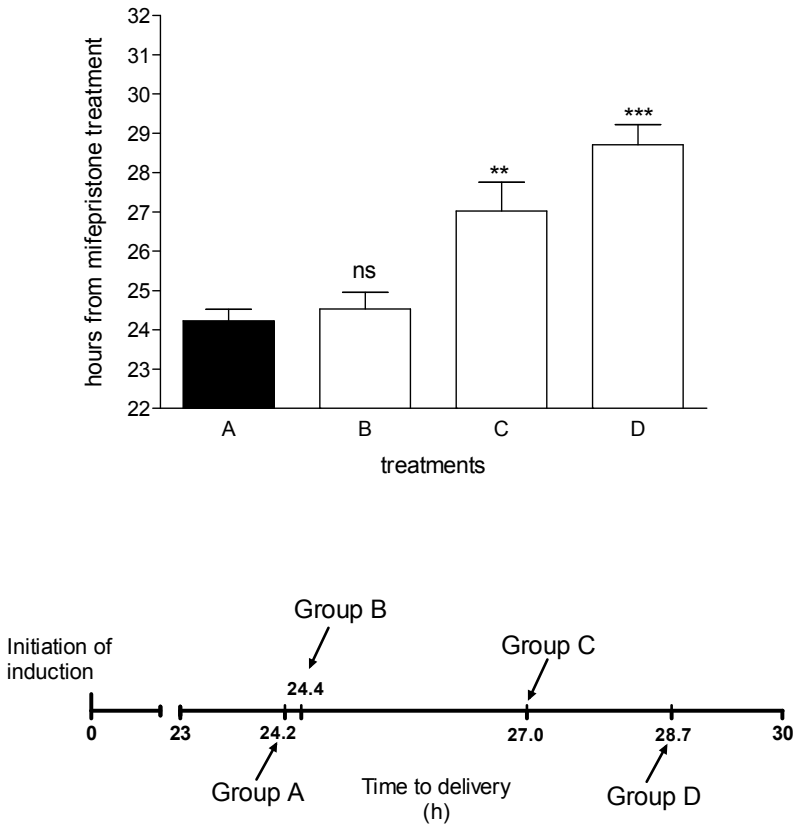


Fig. 2. The effects of progesterone (P; 0.5 mg/0.1 ml), salmeterol (130 µg/day) and combined P - salmeterol treatments on hormone-induced preterm labour (PTL) in the rat (n = 8 for each group). The treatments were started on gestation day 16.

a. **A**: Control PTL group; **B**: P-treated; **C**: salmeterol-treated; **D**: salmeterol – P combination-treated animals.

The bar graphs show means ± SEM. The effects were compared with the results on the control group. ns: not significant; ** $p<0.01$; *** $p<0.001$.

The difference in efficacy between the treatments reflected in Groups C and D was significant ($p<0.05$).

b. Time to delivery from the initiation of PTL (mifepristone treatment). The longest delay was caused by the combination of salmeterol - P (4.5 h).

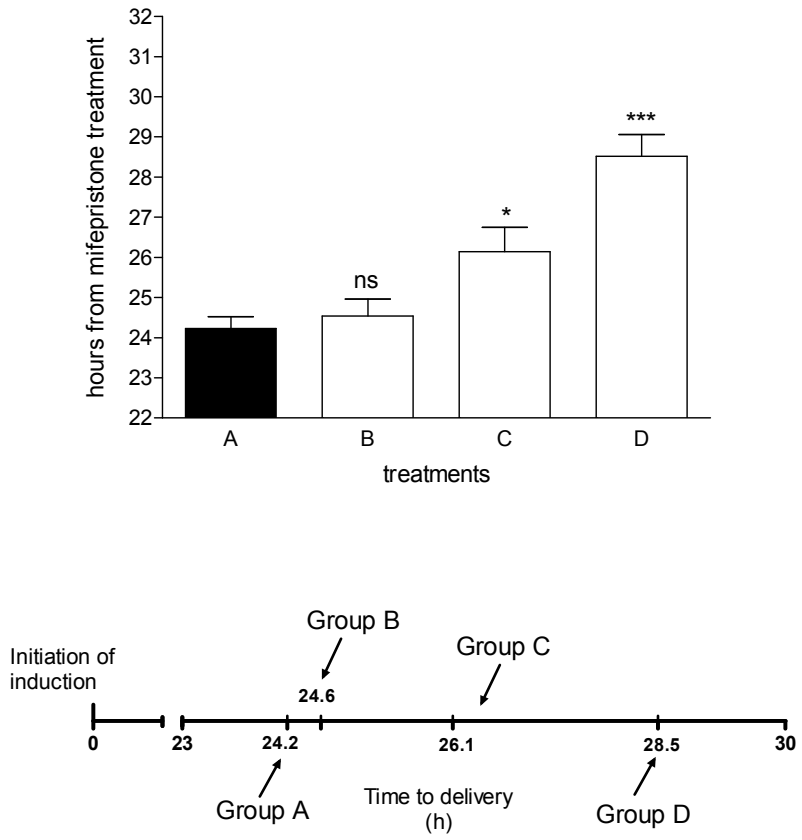


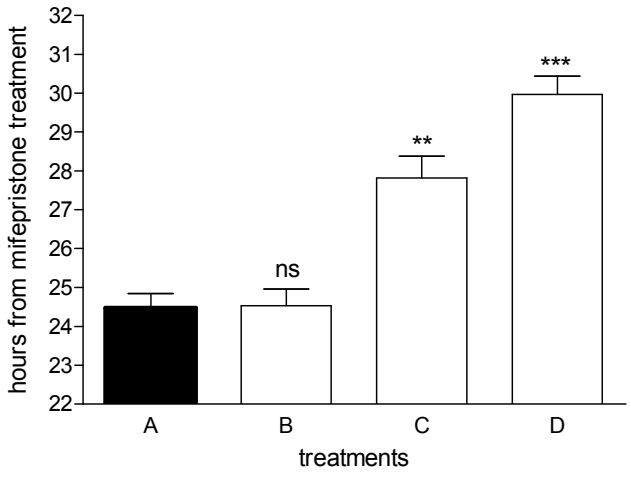
Fig. 3. The effects of progesterone (P; 0.5 mg/0.1 ml), salmeterol (130 µg/day) and combined P - salmeterol treatments on hormone-induced preterm labour (PTL) in the rat (n = 8 for each group). The treatments were started on gestation day 17.

a. **A**: Control PTL group; **B**: P-treated; **C**: salmeterol-treated; **D**: salmeterol – P combination-treated animals.

The bar graphs show means ± SEM. The effects were compared with the results on the control group. ns: not significant; * $p<0.05$; *** $p<0.001$.

The difference in efficacy between the treatments reflected in Groups C and D was significant ($p<0.05$).

b. The time to delivery from the initiation of PTL (mifepristone treatment). The longest delay was caused by the combination of salmeterol - P (4.3 h).



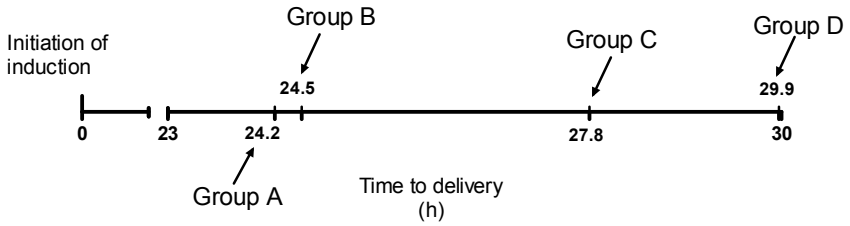


Fig. 4. The effects of progesterone (P; 0.5 mg/0.1 ml), salmeterol (130 μg/day) and combined P - salmeterol treatments on the hormone-induced preterm labour (PTL) in the rat (n = 8 for each group). The treatments were started on gestation day 18.

a. **A**: Control PTL group; **B**: P-treated; **C**: salmeterol-treated; **D**: salmeterol – P combination-treated animals.

The bar graphs show means ± SEM. The effects were compared with the results on the control group. ns: not significant; ** $p<0.01$; *** $p<0.001$.

The difference in efficacy between the treatments reflected in Groups C and D was significant ($p<0.05$).

b. The time to delivery from the initiation of PTL (mifepristone treatment). The longest delay was caused by the combination of salmeterol - P (5.8 h).

Radioligand binding assay

The β_2-AR density was enhanced by 17P treatment as compared with the control group ($p<0.05$). In contrast, the salmeterol treatment decreased the amount of β_2-ARs ($p<0.05$). However, the gestagen – salmeterol combination did not affect the receptor density significantly (Fig. 5).

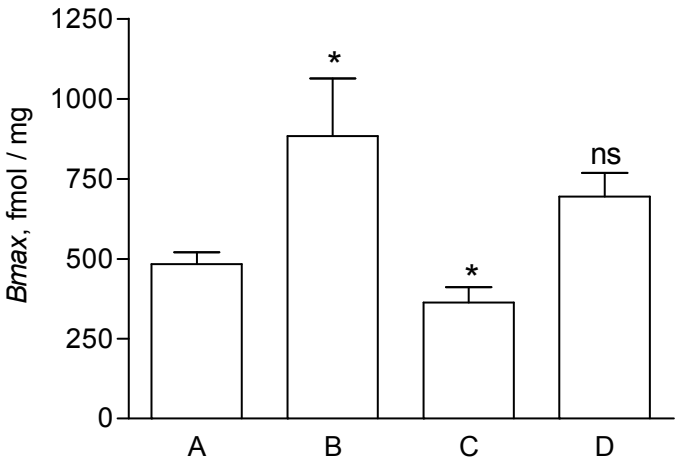


Fig. 5. The change in β_2-adrenergic receptor (β_2-AR) density is affected by salmeterol (130 µg/day) and/or gestagen (0.5 mg/0.1 ml) treatment in hormone-induced preterm labour (PTL) in the rat on day 20 of pregnancy (n = 6 for each group). The salmeterol and gestagen treatments started on pregnancy day 15.

A: Control PTL group; **B**: 17α-hydroxyprogesterone caproate (17P-) -treated; **C**: salmeterol-treated; **D**: salmeterol – 17P combination-treated animals.

The bar graphs show means ± SEM. The effects were compared with the results on the control group. ns: not significant; * $p<0.05$.

The difference in efficacy between the treatments reflected in Groups B and C was significant ($p<0.01$). The difference in β_2-AR density between the Groups C and D was significant ($p<0.05$).

4.2. The effects of terbutaline and gestagens in an LPS-induced PTL model *in vitro*

The KCl-induced contractions were inhibited by terbutaline in a concentration-dependent manner. The efficacy of terbutaline was enhanced in LPS-treated rat. The concentration-response curve was shifted to the left. The effect of terbutaline was further improved by 17P treatment started in parallel with the LPS treatment (Fig. 6). The EC_{50} and E_{max} values of the curves are presented in Table I.

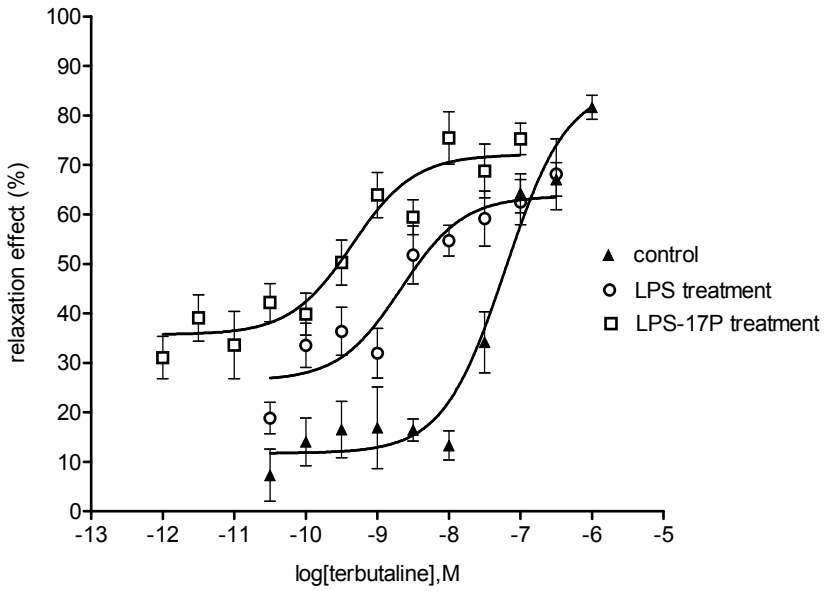


Fig. 6. Uterus-relaxing effect of terbutaline on KCl-induced contractions in non-treated, lipopolysaccharide (LPS; 62.5 µg) -treated and LPS (62.5 µg) – 17α-hydroxyprogesterone caproate (17P-; 0.5 mg/0.1 ml) -treated rats on day 20 of pregnancy (n = 6).

The bar graphs show means ± SEM.

Table I. Changes in EC_{50} and E_{max} values of terbutaline on KCl-induced contractions in non-treated, lipopolysaccharide (LPS; 62.5 µg) -treated and LPS (62.5 µg) –17α-hydroxyprogesterone caproate (17P-; 0.5 mg/0.1 ml) -treated rats on day 20 of pregnancy.

The effects were compared with the results on the control physiologic 20-day-pregnant rat group. SEM: standard error of mean; ns: not significant; * $p<0.05$; ** $p<0.01$.

E_{max}: maximum inhibitory effect of terbutaline on KCl-induced contractions. EC_{50}: the concentration of terbutaline which elicits half of E_{max}.

Group	***EC*$_{50}$** (M ± SEM)	***E*$_{max}$** (% ± SEM)
Non-treated	$3.4x10^{-8} \pm 9.8x10^{-8}$	84.13 ± 5.2
LPS-treated	$2.1x10^{-9} \pm 6.0x10^{-9}$ (*)	63.72 ± 3.7 (ns)
LPS-17P-treated	$1.8x10^{-10} \pm 4.6x10^{-10}$ (**)	71.25 ± 2.7 (ns)

4.3. The effect of ARC239 in the hormone-induced PTL model *in vitro*

ARC 239 was able to block the noradrenaline-evoked contractions in the hormone-induced PTL model in the rat (Fig. 7). The EC_{50} and E_{max} values of the curves are presented in Table II.

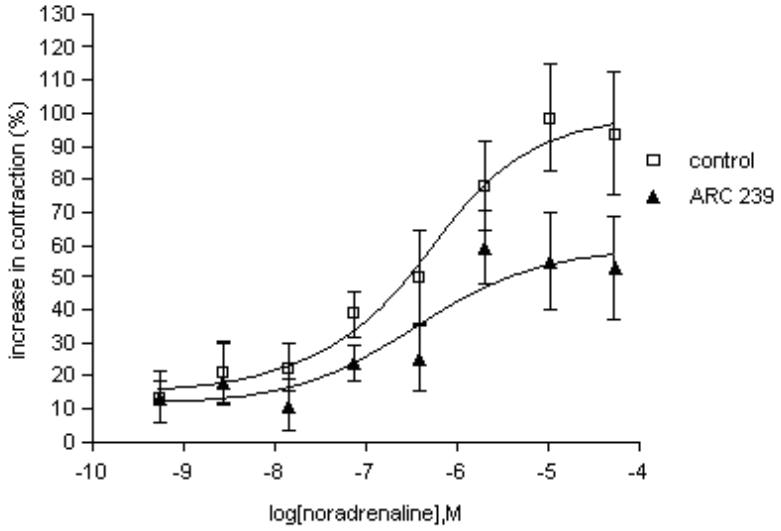


Fig. 7. Effect of the subtype-selective $\alpha_{2B/C}$-AR antagonist ARC 239 on the noradrenaline-evoked contractions (control) in hormone-induced premature labour (PTL) in the rat (n = 8). The studies were carried out in the presence of the β-adrenergic receptor (AR) antagonist propranolol (10^{-5} M) and the α_1-AR antagonist doxazosin (10^{-7} M). The change in contraction was calculated via the area under the curves and expressed as % ± SEM. ARC 239 at 10^{-7} M decreased the maximum contracting effect of noradrenaline.

Table II. Changes in the labour-induced uterus-contracting effect of noradrenaline (EC_{50} and E_{max} values) in the presence of the $\alpha_{2B/C}$-adrenergic receptor (AR) antagonist ARC 239 (10^{-7} M).
The effect of ARC 239 was compared with the noradrenaline-evoked contractions. SEM: standard error of mean; ns: not significant; ** p<0.01.
E_{max}: maximum contracting effect of noradrenaline alone or in the presence of ARC 239. EC_{50}: the concentration of noradrenaline alone or in the presence of ARC 239 which elicits half of E_{max}.

Group	***EC*$_{50}$** (M ± SEM)	***E*$_{max}$** (% ± SEM)
Noradrenaline (control)	$3.4x10^{-7} \pm 1.6x10^{-7}$	100.3 ± 10.4
Noradrenaline + ARC 239	$3.1x10^{-7} \pm 1.2x10^{-7}$ (ns)	58.6 ± 8.7 (**)

5. **Discussion**

PTB is the one of the greatest challenges in obstetrical practice. The currently used medications are not able to stop or sufficiently delay the process of the PTB.

In the U.S.A., the therapeutic guidelines were recently extended with the prophylactic use of gestagens for late PTB. The results of the first randomized controlled trial of P for the prevention of PTL in women at increased risk were published (Papiernik *et al*., 1970). Others focused attention on 17P, for its use was associated with a substantially reduced rate of recurrent PTB in high-risk women as compared with placebo in gestation of less than 37 weeks (36.7% vs. 54.9%) (Meis *et al*., 2003). After these publications, the use of 17P for patients with previous PTL was proposed (American College of Obstetricans and Gynecologists, 2003). The results of Meis *et al*. were soon corroborated by many studies, and the safety of 17P in pregnancy was demonstrated in several animal and clinical investigations (Mason *et al*., 2005; Mackenzie *et al*., 2006). Accordingly, the submission of a New Drug Application was announced with the U.S.A. Food and Drug Administration for Gestiva™, containing 17P. This drug might be appropriate for a reduction of the incidence of late PTL (delivery in gestation weeks 34-36), which accounts for 74% of preterm infants (Davidoff *et al*., 2006; Hickok *et al*., 2006). However, the use of gestagen agents is proposed mainly for the prevention of PTL in pregnant women and their benefit in active PTL has not been demonstrated.

P and its derivative 17P have come into prominence as a consequence of other findings. Some authors have demonstrated that P treatment enhances the effects of β_2-mimetics *in vitro* (Gáspár *et al*., 2005; Chanrachakul *et al*., 2005). The combination of micronized P to β-mimetic treatment reduces the uterine activity more quickly than β_2-mimetic treatment alone; however, there is no effect as concerns the prolongation of pregnancy (Noblot *et al*., 1991). These results led us to test the efficacy of salmeterol – gestagens (P or 17P) treatment on hormone-induced PTL in the rat *in vivo*. Our model was very effective, with good reproducibility, because all the animal labour underwent within one hour, on the day following mifepristone treatment. We used the same *in vivo* P dose that had proved effective in increasing the effect of the β_2-agonist in an earlier study (Gáspár *et al*., 2005). An *in*

vivo dose for the tocolytic effect of salmeterol was not available for the rat, and we therefore chose the dose demonstrated to be able to induce muscular growth in the rat, indicating the systemic effect of the drug (Moore *et al*., 1994).

Interestingly, the gestagen treatment alone did not prevent the hormone-induced PTL, although the P antagonist mifepristone in combination with PGE_2 could elicit PTL. Salmeterol treatment alone was effective in delaying PTL, and this effect was enhanced by its combination with gestagens, independently of the first day of treatment: even one day of gestagen treatment before the administration of mifepristone and PGE_2 potentiated the effect of salmeterol. This result means that the synergistic effect of gestagens can develop rapidly.

We presumed that the improved efficacy of the combination is a consequence of an increase either in the myometrial β_2-AR density or in the amount of activated G-proteins. The radioligand binding assay demonstrated that the number of β_2-ARs was significantly elevated by 17P treatment as compared with the control PTL group. The salmeterol treatment decreased the density of β_2-ARs, which was in harmony with the clinical observations. Desensitization is one of the most frequent problems with β_2-mimetics in tocolytic therapy. The density of β_2-ARs was not altered significantly by the salmeterol – 17P combination, and thus the greater efficacy of the combination could be explained by the increased amount of activated G-proteins coupled to β-ARs, caused by the gestagens, as found earlier (Gáspár *et al*., 2005).

There is a growing body of evidence that infection of the deciduae, foetal membranes and amniotic fluid is associated with 25% of PTBs (Romero *et al*., 1988). We attempted to demonstrate the *in vivo* effects of salmeterol and/or gestagen in an LPS-induced PTL model, which is widely accepted as an appropriate animal test for human PTB. Unfortunately, the LPS model does not yield good reproducibility; the majority of the pregnant rats were not able to deliver the foetuses and intrauterine death often occurred (Elovitz *et al*., 2004). Accordingly, we investigated the efficacy of β_2-mimetic and gestagen treatments only *in vitro*. In these experiments, we used terbutaline instead of salmeterol to determine the efficacy of the combination of gestagen and a β_2-mimetic applied.

Although the clinical efficacy of the β_2-mimetics in cases of chorioamnionitis seems to be limited (Nowak *et al*., 1998), the efficacy of terbutaline was enhanced in the event of tissue inflammation in the rat as compared with the control uterus on day 20 of pregnancy. The increase in the effect of terbutaline might be explained by

the enhanced signal transduction of the β_2-ARs (elevation of the cAMP level; unpublished data). This result appears to confirm the finding that the peripheral blood cells of patients with rheumatoid arthritis produce more cAMP on β_2-AR agonist stimulation than do cells of healthy controls (Lombardi *et al*., 1999). On the other hand, if the pregnant rats were treated with 17P in parallel with LPS, the effectiveness of terbutaline was significantly enhanced as compared with the control and the LPS-treated group. The gestagen increased the effect of β_2-AR on the inflamed uterus.

One trend of research relates to the search for new targets for tocolytic therapy besides the β_2-mimetics. It has been presumed that there is no relation between α_2-ARs and myometrial contractions in non-pregnant rats (Kyozuka, 1988), but noradrenaline elicits contractions in late-pregnant uteri via the α_2-ARs *in vitro*. The results of studies of subtype-selective α_2-AR-mediated effects with selective antagonists suggest that the α_{2B}-ARs are responsible for strong contractions, whilst the α_{2A}- and α_{2C}-ARs seem to decrease the contracting effect of noradrenaline (Gáspár *et al*., 2007).

In view of these results, the effect of the $\alpha_{2B/C}$-ARs antagonist ARC 239 on the over-stimulated myometrium was investigated in the hormone-induced PTB model *in vitro*. These tissues have been found to display increased sensitivity to noradrenaline: the EC_{50} value was 10 times lower than in normal pregnancy (Gáspár *et al*., 2007); however, the maximum contraction effect of noradrenaline was almost half that on the last day of pregnancy. The maximum contracting effect of noradrenaline was decreased by about 50% in the presence of ARC 239, proving to be equally effective in late pregnancy and in the PTB model.

All these results led us to conclude that the tocolytic efficacy of the β_2-mimetics may be enhanced significantly by combination with gestagens both under hormone-induced conditions *in vivo* and in the LPS-induced PTB model *in vitro*. Furthermore, the $\alpha_{2B/C}$-antagonist ARC 239 significantly decreased the contractile response to noradrenaline in tissues from hormone-induced PTL in rats. These uterine-relaxing effects can be explained by a marked elevation of the cAMP level (Gáspár *et al*., 2007).

To the best of our knowledge, our study was the first to test the tocolytic effect of a β_2-mimetic - gestagen combination on two PTL models and an α_2-AR subtype-selective antagonist as a putative therapeutic target in PTL in the rat.

6. Conclusions

In the light of these results, we assume that the putative therapeutic combination of a β_2-AR agonist and a gestagen can enhance the efficacy of human tocolytic therapy. However, it is very difficult to transpose such a result from an animal study into human practice. We presume that the delay of more than 5 h caused by the salmeterol – gestagen combination in rat PTB is very promising for human trials. If we consider that both β_2-mimetics and gestagens are well known as concerns their pharmacokinetics and toxicity, the expected therapeutic risk of their combination is relatively low.

The $\alpha_{2B/C}$-AR antagonists open up a new potential mechanism of action to overcome premature contractions in pregnancy. Further studies are needed to investigate the effects of ARC 239 in an *in vivo* model, which requires clarification of its pharmacokinetic parameters.

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