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

Summary of the Ph.D. Thesis

By

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Introduction

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, and even appears to be increasing slightly when the data from the U.S.A. Currently, a preterm infant is born every 3 minutes and 11 seconds in the U.S.A.

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%). 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.

Unfortunately, the efficacy of current pharmacological treatments for the management of PTB is often questioned. The β_2 -adrenergic receptor (β_2 -AR) agonists are preferably used for tocolysis, mainly in European countries but 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.

Another problem is that the myometrial responsiveness to the β_2 -agonists decreases towards the end of pregnancy. The potential explanation of the pregnancy-induced desensitization may be the decreasing level of progesterone (P). 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 inverts 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. 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 roles of the α_2 -ARs in the regulation of myometrial contractility are not fully understood. Three α_2 -AR subtypes (α_{2A} -AR, α_{2B} -AR and α_{2C} -AR) have been detected in the human myometrium. The cellular mechanisms underlying the contractile action of α_2 -AR activation are not well understood. Recent studies have demonstrated that the α_2 -AR subtypes play different roles in the contractility of the pregnant rat uterus.

Aims

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. 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*.

Materials and Methods

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.

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

Induction of PTL

The animals were treated with mifepristone (3 mg/0.1 ml) and PGE₂ (0.5 mg/animal) on day 19 of pregnancy. Mifepristone was suspended in olive oil and given as a subcutaneous (sc.) injection at 9 a.m. At 4 p.m, PGE₂ 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 was dissolved in a 1:1 methanol - water mixture. An Alzet osmotic pump 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. The dose of salmeterol was administrated 130 µg/day/animal.

P or 17 α -hydroxyprogesterone caproate (17P) 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 with an Ultra-Turrax T15 homogenizer and centrifuged (20 000xg, 40 min, 4 °C). The pellets were resuspended and centrifuged as previously described and stored at -70 °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 and 100 µl unlabelled ligand (dihydroalprenolol) for non-specific binding, or 100 µl incubation buffer for total binding. At the end of the incubation, the bound radioligand was separated from the residual free radioligand by rapid filtration on a Brandell cell harvester through Whatman GF/B filters. The bound radioactivity was determined in a HighSafe scintillation cocktail in a Wallac 1409 liquid scintillation counter.

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 \pm SEM. The amount of β_2 -AR protein (B_{max}) was calculated by Scatchard transformation of the saturation curves. Statistical analysis was carried out as mentioned above.

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 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 (pH = 7.4). The temperature of the organ bath was maintained at 37 °C, and carbogen (95% O₂ + 5% CO₂) 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, respectively. Contractions were elicited with 25 mM KCl, and the effects of terbutaline (10^{-12} – 10^{-7} M) were tested. Concentration-response curves were fitted and analysed statistically with the Prism 4.0 computer program.

For statistical evaluations, data were analysed by means of the ANOVA Newman-Keuls test.

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 (1×10^{-8} – 3×10^{-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). The material containing the α_2 -AR antagonist ARC 239 was left to incubate for 20 min before the administration of contracting agents.

The evaluation and statistical analysis were carried out as described previously. The data were analysed by two-tailed unpaired *t* tests.

Results

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.

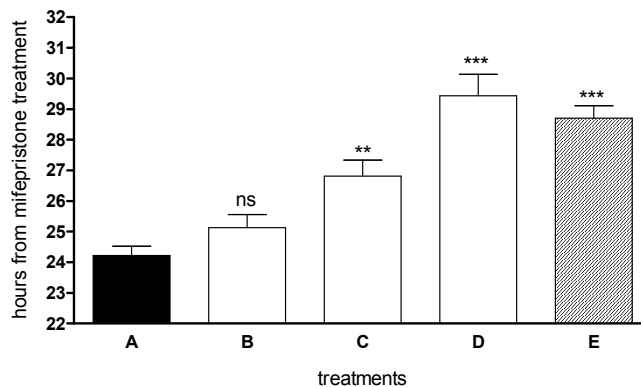


Fig. 1. The effects of progesterone, salmeterol and combined gestagen - salmeterol treatments on hormone-induced preterm labour in the rat. The treatments were started on gestation day 15.

The bar graphs show means \pm 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 ($p > 0.05$).

The results were similar when the treatments were started on one or other days 16-18 of pregnancy. 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.

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.

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.

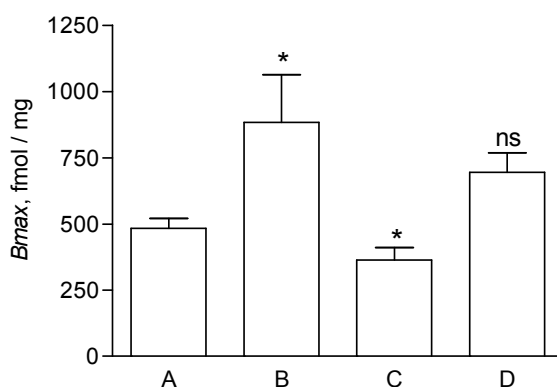


Fig. 2. The change in β_2 -adrenergic receptor density is affected by salmeterol and/or gestagen treatment in hormone-induced preterm labour in the rat on day 20 of pregnancy. The salmeterol and gestagen treatments started on pregnancy day 15.

The bar graphs show means \pm 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 ($p<0.05$).

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 compared with the control 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.

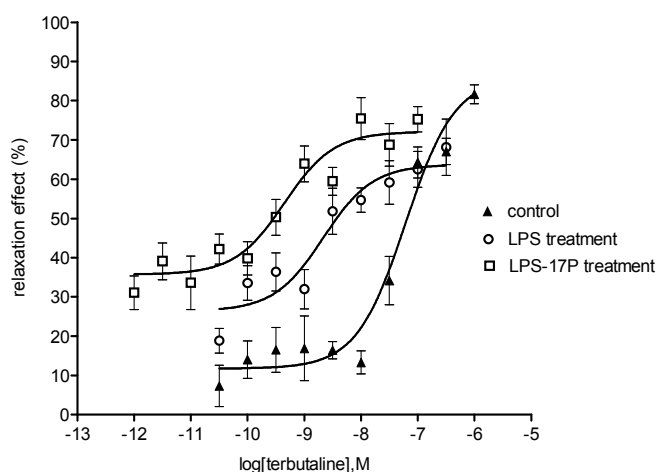


Fig. 6. Uterus-relaxing effect of terbutaline on KCl-induced contractions in non-treated, LPS -treated and LPS – 17P-treated rats on day 20 of pregnancy.
The bar graphs show means \pm SEM.

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.

Discussion

Some authors have demonstrated that P treatment enhances the effects of β_2 -mimetics *in vitro*. Furthermore, the combination of micronized P to β -mimetic treatment reduced the uterine activity more quickly than β_2 -mimetic treatment alone; however, there was no effect as concerns the prolongation of pregnancy. 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.

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.

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 density of β_2 -ARs was not altered significantly by the salmeterol – 17P combination as compared with the control PTL group, 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.

We investigated the efficacy of β_2 -mimetic and gestagen treatments on LPS-induced PTL, *in vitro*. In these experiments, we used terbutaline instead of salmeterol. 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. 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.

One trend of research relates to the search for new targets for tocolytic therapy. 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.

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; 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*. We assume that the putative therapeutic combination of a β_2 -AR agonist and a gestagen can enhance the efficacy of human tocolytic therapy. 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.

Furthermore, the $\alpha_{2B/C}$ -antagonist ARC 239 significantly decreased the contractile response to noradrenaline in tissues from hormone-induced PTL in rats. The $\alpha_{2B/C}$ -AR antagonists open up a new potential mechanism of action to overcome premature contractions in pregnancy.

Publications related to the Ph.D. thesis

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