Summary of Ph.D. Thesis

# The roles of $\alpha_1$-adrenergic receptor subtypes in the regulation of uterine contractility – molecular pharmacological investigations

Eszter Ducza

Department of Pharmacodynamics and Biopharmacy

University of Szeged

Szeged

2002

## Introduction

Premature birth is the principal cause of perinatal morbidity and mortality. The number of cases is high and no decrease has been experienced for many years. The authorized drugs for treatment of this state include magnesium sulfate, β-adrenergic agonists, and less often prostaglandin inhibitors, calcium-channel blockers and oxytocin-receptor antagonists. The side-effect profiles and degrees of efficacy of these drugs reveal that ideal tocolytic therapy is one of the major challenges in obstetric practice. This unsolved problem of tocolysis motivated our present research.

The spontaneous contractility of the uterine muscle is subject to heterogeneous regulation, involving oxytocin, the adrenergic system, prostaglandins, connexins and sex hormones.

The α-adrenergic receptors (α-ARs) mediate uterine contraction, while the β-ARs mediate uterine relaxation. This provides a theoretical possibility for the use of $\alpha_1$-AR blockers as tocolytic agents.

The contributions of the $\alpha_1$-AR subtypes in the regulation of the rat and human uterine contractility remain open questions. The antisense oligodeoxynucleotides provide an excellent possibility for such studies.

Traditional pharmacological methods have only a limited capacity for determination of the functional roles of receptors. The antisense effect (i.e., the selective inhibition of gene expression by antisense RNA, DNA or oligonucleotides) opened up new possibilities for the selective manipulation of living function and to defining the functional roles of receptors.

## Aims of the study

**1.** One goal of this study was to determine the changes in density and pharmacological reactivity of the $\alpha_1$-AR subtypes during late pregnancy in the rat. To demonstrate the expression of $\alpha_1$-AR subtype mRNA, we used a reverse-transcription polymerase chain reaction (RT-PCR) on days 18, 20 and 22 of pregnancy. Electric field stimulation (EFS) was applied to test the pharmacological reactivity of the rat uterus in late pregnancy.

**2.** Another aim was to detect the role of $\alpha_{1A}$-AR in the regulation of the contractility of the rat uterus in pregnancy. Because of the limitation of the animal models available for investigation of the pregnant uterine contractility, the aim was to develop a knock-down transformed animal model with which to study the role of $\alpha_{1A}$-AR. The AONs provide an excellent possibility for such studies.

The efficiency of $\alpha_{1A}$-AR antisense inhibition was indicated by a radioligand receptor binding assay and Western blot analysis. EFS was applied to test the pharmacological reactivity of the post partum isolated rat uterus.

**3.** A third goal of this study was to investigate the changes in mRNA of the $\alpha_1$-AR subtypes in the human term and non pregnant uterus by RT-PCR.

## Methods

*RT-PCR studies*

*Tissue isolation. - Rat uterus samples*

Rat uterus tissues from non pregnant animals were rapidly removed on gestation days 18, 20, and 22. The tissues were frozen in liquid nitrogen and

then stored at -70 °C until the extraction of total RNA.

*Human uterus samples*

In our investigations, non-pregnant uterus strips were cut from the fundus to the cervix and divided into parts of equal size for RT-PCR studies. The pregnant human uterus samples were from women undergoing cesarean section (cervix transversal elective) under regional anesthesia. The tissues were frozen in liquid nitrogen and then stored at -70 °C until the extraction of total RNA. Total RNA was isolated by Chomczynski and Sacchi. The total RNA was denatured and the M-MLV reverse transcriptase, RNase H Minus was added to the mixture, which was incubated at 37 °C for 60 min and then at 72°C for 10 min. PCR was carried out with cDNA, ReadyMix REDTaq PCR reaction mix and sense and antisense primer. $\alpha$-$^{32}$P-dCTP (1 μCi) was added to the above reaction mixture to quantify the amplified product. The PCR products were electrophoresed in 2% agarose gels, dried under vacuum, and placed into a PhosphorImager™ exposure cassette. Quantification was carried out by ImageQuant™ software.

*Synthesis of antisense oligodeoxynucleotides*

Three antisense oligonucleotides (AONs) and the control oligonucleotides were synthesized by Sándor Bottka in Institute of Plant Biology.

*Treatment of animals with AONs*

Post partum rats were anesthetized with urethane 2 to 3 h after labour. After laparotomy, the AON was injected into both uterine horns in a special carrier solution (pluronic gel and DOTAP). The treated uteri were

removed 24 h after delivery.

*Radioreceptor binding assays*

Radioligand binding experiments were carried out on membrane preparations of rat uterus. Saturation analysis of $\alpha_1$-ARs was performed by incubating membranes [$^3$H]prazosine with or without unlabelled phentolamine. In displacement studies the membranes were incubated [$^3$H]prazosine and unlabelled ligand (prazosine, WB4101). Incubation were started by addition of the membrane suspension to the reaction mixture. The bound radioactivity was determined in liquid scintillation counter. Nonspecific binding was determined in presence of unlabelled phentolamine.

*Western blot assay*

The protein was subjected to electrophoresis on SDS-polyacrilamide gels and were transferred from gels to nitrocellulose membranes. The membranes were blocked, than incubated with anti-$\alpha_{1A}$-AR, $\alpha_{1B}$-AR, $\alpha_{1D}$-AR polyclonal antibody. The blots were washed and incubated with peroxidase-conjugated goat anti-rabbit IgG. The antibody binding was detected with an enhanced chemiluminescence detection system and exposed on Kodak X-Omat film. Anti-β-actin monoclonal antibody was used as a control.

*Electric field stimulation*

The AON treated and control uteri were removed from rats 24 h after delivery. Muscle rings 0.5 cm long were sliced from the uterine horns and mounted vertically between two platinum electrodes in an organ bath. The organ bath was maintained at 37 °C and carbogen (95% $O_2$ / 5% $CO_2$) was bubbled through it. Maximum rhythmic contractions were elicited on

the uteri by a digital, programmable stimulator using earlier defined values of pulse width and period time and square pulses with a duration of 200 ms every 60 s, at 40 V, respectively. The tension of the myometrial rings was measured with a gauge transducer and recorded with an ISOSYS Data Acquisition System. Non-cumulative concentration-response curves to the selective $\alpha_{1A}$-antagonist 5-methylurapidil (5-MU), WB 4101, the selective $\alpha_{1B}$-antagonist cyclazosine, the selective $\alpha_{1D}$-antagonist BMY 7378 and the $\beta_2$-agonist terbutaline were constructed in each experiment. Concentration-response curves were fitted and areas under curves (AUCs) were evaluated and analyzed statistically with the Prism 2.01computer program. For statistical evaluations, data were analyzed by the ANOVA Neuman-Keuls test.

## Results

### $\alpha_1$-AR subtype mRNA expression and pharmacological reactivity in the late pregnant rat uterus

The expression of $\alpha_{1A}$-AR mRNA increased from day 18 to 22 of pregnancy in rats, while $\alpha_{1B}$-AR mRNA expression was not detectable by RT-PCR analysis. The expression of $\alpha_{1D}$-AR mRNA was highest on day 20 and decreased by day 22. The EFS-induced contraction on days 18, 20 and 22 of pregnancy was inhibited concentration-dependently by the selective $\alpha_{1A}$-antagonist 5-methylurapidil. In the measured concentration range, the selective $\alpha_{1B}$-antagonist cyclazosine had no significant action; only its highest doses displayed weak inhibitory effects on stimulated contractions. The $\alpha_{1D}$-antagonist BMY 7378 inhibited the contraction in a dose-dependent manner.

The EC50 was established from the dose-dependent curves. The $\alpha_{1A}$-AR antagonist 5-MU had EC50 values of $6.3x10^{-6}$ M, $2.9x10^{-6}$ M and $1.9x10^{-6}$ M on days 18, 20 and 22, respectively, while the EC50 of the $\alpha_{1D}$-AR antagonist BMY 7378 was $1.09x10^{-5}$ M, $4 \times 10^{-6}$ M and $3.6 \times 10^{-5}$ M on days 18, 20 and 22, respectively.

A strong correlation was found between the change in $\alpha_{1A}$-AR mRNA expression and the EC50 values of 5-MU ($r^2$=0.9712) and between the change in $\alpha_{1D}$-AR mRNA expression and the EC50 values of BMY 7378 ($r^2$=0.9936).

**Use of AONs to study the role of the $\alpha_{1A}$-ARs in the rat uterus**

Of the synthesized three AONs, 480-AON reduced the $\alpha_{1A}$-AR density in a time- and dose-dependent fashion as compared with the untreated group and the random oligonucleotide-treated control group.

The most significant changes in the density of the $\alpha_{1A}$-AR were observed 12 and 24 h after delivery. Incubation with 480-AON for 12 or 24 h caused a 58.7% or 53.0% inhibition and the $B_{max}$ values under these conditions were 12.04 ± 0.86 and 13.69 ± 0.42 fmol/mg, respectively. No significant changes in receptor density were found for the other AONs.

The $\alpha_{1A}$-selective WB 4101 identified two $\alpha_1$-adrenergic binding sites in the nontreated rat uterus, with $K_d$ values of 0.011 and 15.05 nM, while in the treated animals it bound to only one binding site ($K_i$ = 36.12 nM).

In Western blot analysis, the $\alpha_{1A}$-AR density was decreased only by 480-AON.

The AUCs of the uterine contractility indicated the significantly decreased contractility of the 480-AON-treated uterus as compared with

those of the control, the operated control, the DOTAP-treated control, the 480-missmatched AON-treated control, and the 480-scrambled AON-treated.

The EFS studies revealed that the specific $\alpha_{1A}$-blockers 5-MU and WB 4101 inhibited the rhythmic contractions by about 75% and 70% in the control uteri and by 25% and 20% in the 480-AON-treated uteri, respectively. The IC50 values for 5-MU and WB 4101 in the control uteri were $3.8 \times 10^{-5}$ and $9.5 \times 10^{-6}$, respectively. The inhibition curves of terbutaline and BMY 7378 remained unchanged for the 480-AON-treated uteri as compared with that of the control.

**Changes in $\alpha_1$-AR subtype mRNA in the human uterus**

The presence of mRNA of all $\alpha_1$-AR subtypes ($\alpha_{1A}$-, $\alpha_{1B}$- and $\alpha_{1D}$-AR) was proved in the nonpregnant human uterus; an $\alpha_{1B}$-AR mRNA predominance was detected. The maximum of the $\alpha_{1B}$-AR mRNA was found in the corpus of the uteri. The level of this receptor mRNA slowly decreased towards the fundus. The lowest expressions of $\alpha_{1A}$- and $\alpha_{1D}$-AR mRNA were observed in the cervix, but the levels of $\alpha_{1A}$- and $\alpha_{1D}$-AR mRNA continuously increased in the corpus and fundus.

$\alpha_{1A}$- $\alpha_{1B}$- and $\alpha_{1D}$-AR mRNA were detected in human uterus samples, obtained on caesarean section. A predominance of $\alpha_{1D}$-AR mRNA was found at term in the pregnant human uterus.

## Conclusions

**1.** Our findings suggest that both $\alpha_{1A}$- and $\alpha_{1D}$-ARs are involved in the regulation of the pregnant rat uterine contractility. The $\alpha_{1A}$-ARs seem to play the major role as regards the $\alpha_1$ subtypes in the late-pregnant myometrium.

**2.** We could detect no expression pharmacological reactivity of $\alpha_{1B}$-AR mRNA or in late pregnancy. This suggests that the role of $\alpha_{1B}$-AR is unimportant in the regulation of the uterus contraction in the late-pregnant rat.

**3.** We found a strong correlation between the $\alpha_{1A}$- and $\alpha_{1D}$-mRNA expressions and the pharmacological reactivity. We presume that this strong correlation could be beneficial as compared with the frequently used tocolytic agent ($\beta_2$-mimetics), where the process of receptor desensitization may decrease the effectivity of these compounds.

**4.** The local use of AONs in the uterus in an attempt to clarify the role of the receptors which influence the uterine contractility is a new method in pharmacology. This method was used to develop an $\alpha_{1A}$-AR knock-down transformed animal model, which provided experimental opportunities that were unavailable with the previously existing pharmacological methods.

**5.** In our AON studies, the important role of the $\alpha_{1A}$-AR in uterine contractility was confirmed.

**6.** In nonpregnant and pregnant human uteri, we detected the predominance of $\alpha_{1B}$-AR mRNA and $\alpha_{1D}$-AR mRNA, respectively. On the basis of mRNA synthesis, it can be presumed that the physiological role of $\alpha_1$-ARs may be of importance in pregnant human uterus contractility.

*In the light of our findings, we assume the significance of $\alpha_{1A}$-ARs and $\alpha_{1D}$-ARs in pregnant uterine contractility. We also conclude that the $\alpha_{1B}$-ARs are probably not strongly involved in the main control of myometrial contraction in pregnancy.*

*Further investigations are required to determine the exact roles of the $\alpha_1$-AR subtypes in the human pregnant uterus and to develop new, uterus-selective $\alpha_1$-adrenergic blockers as potent tocolytic agents.*

**Acknowledgements**

I would like to express my warmest thanks to my supervisor, Prof. George Falkay, for his guidance, encouragement and management of my work.

I also wish to thank my co-authors and colleagues for the pleasant cooperation and inspiring discussions.

**Full papers and abstracts related to the Ph.D. thesis**

1. E. Ducza, R. Gáspár, A. Márki, P. Gyula, S. Bottka, G. Falkay: Use of antisense oligonucleotids to verify the role of the $\alpha_{1A}$-adrenergic receptor in the contractility of the rat uterus post partum *Mol.Pharmacol 59(5) 1235-1242, 2001*

**IF.: 5.678**

2. Ducza E., Gáspár R., Márki A., Bottka S., Falkay G: Farmakológiai modell az adrenerg rendszer szerepének vizsgálatára post partum patkány uteruszon. *Acta Pharm Hung*. 71. 300-305, 2001

3. E. Ducza, R. Gáspár, G. Falkay: Alteration of $\alpha_1$-adrenergic receptor subtypes and pharmacological reactivity of the late-pregnant myometrium in the rat. *Mol Reprod and Development, 62 (3): 343-347, 2002*

**IF.: 2.535**

4. E. Ducza, R. Gáspár, G. Falkay, S. Bottka, P. Gyula: Effects of $\alpha_{1A}$-adrenergic receptor antisense oligonucleotides on post-partum rat uterus

contractility and pharmacological reactivity *Fundam. Clin. Pharm. 13(1), 247s. 1999.* **IF.: 1.265**

5. E. Ducza, R. Gáspár, Á. Márki, S. Bottka, G. Falkay: Antisense oligonucleotides: a new possibility to investigate the role of $\alpha_1$-adrenergic receptor in rat uterus contractility and pharmacological reactivity *Hum. Reprod. 15, Abstract book 1, 205. 2000.* **IF: 2.997**

6. R. Gáspár, A. Márki, E. Ducza, G. Falkay: Comparison of the effects of terbutaline and subtype selective alpha1-adrenoceptor antagonists on pregnant rat uterine contractions in vitro. *Eur J Pharm Sci 11. Suppl. 1 S109. 2000.* **IF.: 1.212**

*7.* E. Ducza, R. Gáspár, Á. Márki, G. Falkay: Alteration of α-adrenergic subtypes and pharmacological reactivity of the late-pregnant myometrium in the rat.*Fundamental&Clinical Pharmacology Suppl. 8P116, 2001* **IF.: 1.265**

8. Ducza E., Gáspár R., Falkay Gy., Bottka S.: Az antisense oligonukleotidok: új terápiás lehetőség. In vitro és in vivo farmakológiai vizsgálatok post partum patkány modellben *Gyógyszerészet, Congressus Pharmaceuticus Hungaricus XI előadásösszefoglalók, 24, 1999.*