The roles of the α_1-adrenergic receptor subtypes in rat embryonic implantation

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Objective: To focus on the possible roles of α_1-adrenergic receptors (α_1-ARs) in rat embryonic implantation.
Design: Laboratory study.
Setting: Animal and pharmacology laboratory at Department of Pharmacodynamics and Biopharmacy, University of Szeged, Hungary.
Animal(s): Pregnant and nonpregnant Sprague-Dawley rats.
Intervention(s): Uterus tissues were collected during the peri-implantation period.
Main Outcome Measure(s): We used a reverse transcription–polymerase chain reaction (RT-PCR) and Western blotting to demonstrate the expressions of mRNAs and the protein expressions of the α_1-AR subtypes in the early-pregnant uterus. Electric field stimulation was applied to test the pharmacologic reactivity of the α_{1A}-AR, and the physiologic role of this receptor was tested in a knock-down transformed animal model using an antisense oligonucleotide that elicits sequence-selective inhibition of the α_{1A}-AR gene expression.
Result(s): The presence of all α_1-AR subtypes (α_{1A}, α_{1B}, and α_{1D}) was proved, with a predominance of α_{1A}-AR. The maximal expression of the α_{1A}-AR was attained on the day of implantation. The selective α_{1A} antagonist 5-methylurapidil inhibited the contraction in a dose-dependent manner. The number of implantation sites was decreased (~75%) in the α_{1A}-AR knock-down transformed rats.
Conclusion(s): We assume that the α_{1A}-AR predominance plays a crucial role in embryonic implantation in the rat. (Fertil Steril® 2009;91:1224–9. ©2009 by American Society for Reproductive Medicine.)

Key Words: α_1-adrenergic receptors, rat, implantation, antisense oligonucleotide

Adrenergic signal transduction is involved in many aspect of pregnancy. The β-adrenergic receptors (β-ARs) are involved in uterine relaxation, which is reflected in clinical practice by frequent application of β_2-agonist as tocolytic agents. Stimulation of α_1-ARs results in vasoconstriction and uterine contraction; stimulation of α_2-ARs results in inhibition of norepinephrine release and it may also contribute to uterine contraction. Smooth muscle tone depends, at least in part, on the relative activity of the AR system, and abnormal functioning of these receptors may be involved in preterm labor and the pathophysiologic mechanisms of preeclampsia or gestational hypertension (1).

Our earlier experiments revealed a predominance of the α_1-ARs at the end of pregnancy and postpartum (2–4) and the density and pharmacologic reactivity indicated that α_{1A}-AR seems to play the major role in the late-pregnant uterus function (5). This article focuses on the roles of the α_1-ARs in implantation.

Implantation is a complex process in which the embryo makes close contact with the maternal endometrium during the establishment of pregnancy. Successful implantation requires precise coordination between the embryo and the uterus under the influence of ovarial steroids. Generalized uterine swelling and progressive closure of the uterine lumen position the blastocyst immediately adjacent to the luminal epithelium, with eventual attachment of the embryo to the uterus on day 5 of pregnancy in the rat (6).

The aim of this study was to determine the changes in expression of the α_1-AR messenger RNA (mRNA) subtypes near the implantation period in the rat. To demonstrate the expressions of the α_1-AR subtypes, mRNAs, and proteins, we used a reverse transcription–polymerase chain reaction (RT-PCR) and Western blotting. Electric field stimulation was applied to test the α_1-AR functions through the uterine contractions at the time of implantation. Finally, an α_{1A}-AR knock-down transformed animal model was set up with an antisense oligodeoxynucleotide (AON) to prove the crucial role of the α_{1A}-AR subtype in blastocyte implantation.

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MATERIALS AND METHODS

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 number IV/1813-1/2002). Sprague-Dawley rats (Charles-River Laboratories, Budapest, Hungary) were kept at 22° ± 3°C; the relative humidity was 30%–70% and the light/dark cycle was 12/12 hours. They were maintained on a standard rodent pellet diet (Charles-River Laboratories) with tap water available ad libitum. The animals were sacrificed by CO_2 inhalation.

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. Because rats are usually active at night, the separating door was opened before dawn. Within 4–5 hours 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 ×1,200. If the search proved positive, or when smear taking was impossible because of an existing vaginal sperm plug, the female rats were separated and were regarded as first-day pregnant animals.

RT-PCR Studies

Tissue isolation Female Sprague-Dawley rats (250–300 g) were anesthetized with sodium pentobarbital (1 g/kg intraperitoneally [IP]). Uterus tissues from nonpregnant and pregnant animals were rapidly removed and dissected in ice-cold saline (0.9% NaCl) containing 2 units/mL of recombinant ribonuclease inhibitor (RNasin; Promega, London, United Kingdom). The tissues were frozen in liquid nitrogen and then stored at −70°C until the extraction of total RNA.

Total RNA preparation Total cellular RNA was isolated by extraction with acid guanidinium thiocyanate-phenol-chloroform according to the procedure of Chomczynski and Sacchi (7). After precipitation with isopropanol, the RNA was treated with RNase-free DNase I for 30 minutes at 37°C, re-extracted with phenol, precipitated with ethanol, washed with 75% ethanol, and then resuspended in diethyl pyrocarbonate-treated water, and the RNA concentration was determined by optical density measurement at 260 nm.

RT-PCR The RNA (0.5 μg) was denatured at 70°C for 5 minutes in a reaction mixture containing 20 units of RNase inhibitor (Invitrogen, Budapest, Hungary), 200 μM dNTP (Sigma-Aldrich, Budapest, Hungary), 20 μM oligo(dT) (Invitrogen) in 50 mM Tris-HCl at pH 8.3, 75 mM KCl, and 5 mM $MgCl_2$ in a final reaction volume of 19 μL. After the mixture had cooled to 4°C, 20 units of M-MLV Reverse Transcriptase, RNase H Minus (Promega) was added, and the mixture was incubated at 37°C for 60 minutes and then at 72°C for 10 minutes.

The PCR assay was carried out with 5 μL of complementary DNA (cDNA), 25 μL of ReadyMix REDTaq PCR reaction mix (Sigma-Aldrich), and 50 pmol sense and antisense primers. The sequences of the primers were as reported by Scofield et al. (8).

The PCR assay was performed with PCR Sprint thermal cycler (Hybaid Corp., London, United Kingdom) with the following cycle parameters: after initial denaturation at 95°C for 3 minutes, the reactions were taken through 35 cycles of 1 minute at 94°C, 1 minute of annealing at 54°C (α_{1B}-AR and α_{1D}-AR) or 50°C (α_{1A}-AR) and 72°C for 2 minutes. After the last cycle, incubation was continued for 10 minutes at 72°C, followed by lowering of the temperature to 4°C. Simultaneously, we performed RT-PCR for housekeeping gene GAPDH (9) in the uterus as a positive, internal control.

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., Budapest, Hungary). For statistical evaluations, data were analyzed by analysis of variance (ANOVA) with the Neuman-Keuls test.

Western blot analysis

Protein per well (20 μg) was subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels in Series Standard Dual Cooled Units (BioRad, Budapest Hungary). Proteins were transferred from gels to nitrocellulose membranes (Scheicher and Schuell, Dassel, Germany), using a semidry blotting technique (BioRad). The membranes were blocked with 5% nonfat dry milk in Tris saline buffer (50 mM Tris, pH 7.4, 200 mM NaCl) containing 0.1% Tween, overnight at 4°C. After washing, the blots were incubated for 1 hour at room temperature on a shaker, with α_{1A}-AR, α_{1B}-AR, and α_{1D}-AR and β-actin polyclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) in the blocking buffer. The antibody binding was detected with a Western-Breeze Chromogenic Western blot immundetection kit (Invitrogen). Digital images were captured with the EDAS290 imaging system (KODAK, Invitrogen), and the optical density of each immunoreactive band was determined with Kodak 1D Images analysis software. Optical densities were calculated as arbitrary units after local area background subtraction, normalized to the density of the β-actin immunoreactivity, and reported as fold induction relative to the control.

Uterus Preparation and Electric Field Stimulation

Uteri were removed from rats (200–250 g) on days 4, 5, 6, or 7 of pregnancy. Muscle rings 0.5 cm long were sliced from the uterine horns and mounted vertically between two platinum electrodes in an organ bath containing 10 mL of de Jongh solution (137 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM NaH_2PO_4, 6 mM

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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 hour before experiments were undertaken, with a solution change every 15 minutes. The initial tension was set to about 1.25 g, which was relaxed to about 0.5 g at the end of equilibration. Maximal rhythmic contractions were elicited with a digital, programmable stimulator (ST-02; Experimetria U.K. Ltd., London, United Kingdom), as described earlier (2). Briefly, different values of pulse width (the duration of the electric field as a single stimulus) and period time (the time interval between two stimuli) were used at 40 V for 240 seconds, The shortest interval time was sought with which to elicit rhythmic contractions. After identification of this value, the pulse width was gradually increased as much as possible at the constant time interval to maintain the rhythmic contractions. The stimulation parameters are shown in Table 1. The tension of the myometrial rings was measured with a gauge transducer (SG-02; Experimetria U.K. Ltd.) and recorded with an ISOSYS Data Acquisition System (Experimetria U.K. Ltd.). Noncumulative concentration–response curves to the α_{1A}-antagonist/inverse agonist 5-methylurapidil (5-MU) (Sigma-Aldrich) were constructed in each experiment. After the addition of a concentration of 5-MU, recording was performed for 240 seconds. After this period, the electric field was switched off and the tissues were washed three times and left to rest for 5 minutes. Concentration–response curves were fitted and areas under curves (AUCs) were evaluated and analyzed statistically with the Prism 4.0 (GraphPad Software, San Diego, CA) computer program. From the AUC values, the EC_{50} and E_{max} values were calculated (EC_{50} = concentration of terbutaline eliciting 50% of the maximal [100%] inhibition of uterine contractions; E_{max} = maximal inhibitory effect of terbutaline on a given day of pregnancy.) For statistical evaluations, data were analyzed by the Neuman-Keuls ANOVA test.

Treatment of Animals With Antisense Oligodeoxynucleotides

The α_{1A}-AON (Invitrogen) was mixed with *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP; Boehringer-Mannheim, Mannheim, Germany) and 20% F127 pluronic gel (Sigma-Aldrich). The solution was maintained in liquid form at 4°C before injection. The effective sequence and dose–response analysis of α_{1A}-AON were reported earlier (10). Pregnant animals (n = 12) on day 4 under sodium pentobarbital anesthesia were treated with α_{1A}-AON (50 nmol/200 μL). The animals in the positive control group (n = 8) were treated only with the vehicle mixture and the negative control group (n = 8) received no injection. An incision was made in the lower abdomen and the AON solution was injected from prechilled syringes into the luminal space of each uterine horn. The AON sample was administered in two steps at two locations along the length of the horn. The incision was then closed, and the animals were returned to their cages. Uteri were used for further investigation on day 10 of pregnancy.

RESULTS

To determine the distribution of the α_1-AR subtype mRNAs in the pregnant rat uterus, total RNAs from each tissue were reverse transcribed. The resulting complementary DNAs (cDNA) were amplified by PCR using a set of primers specific for each α_1-AR cDNA sequence. The successful normalization of RNA amounts during the RT step was verified by amplification of a fragment of the reference standard GAPDH cDNA in all of the samples analyzed. The presence of the mRNA of all α_1-AR subtypes (α_{1A}, α_{1B}, and α_{1D}) was proved and a predominance of α_{1A}-AR mRNA was detected (Fig. 1A). The maximal expression of α_{1A}-AR mRNA was attained on the day of implantation (day 5). Lower expressions of α_{1B}-AR and α_{1D}-AR mRNAs were demonstrated in the rat uterus. We did not observe any regularity between the expressions of α_{1B}-AR and α_{1D}-AR mRNAs.

The results of the Western blot analysis correlated with those of the RT-PCR analysis. The expression of the α_{1A}-AR protein was the highest on days 5–6 of pregnancy (Fig. 1B).

The selective α_{1A}-antagonist 5-MU inhibited the electric field stimulation -contraction in a dose-dependent manner. The EC_{50} value of the compound was shifted to the left on day 6 and to the right on day 7. The maximal inhibitory effect

TABLE 1

The pulse widths and period times for in vitro electric field stimulation eliciting maximal rhythmic contractions in early-pregnant rat uterine rings (n = 8).

	Days of pregnancy			
EFS parameters	**4**	**5**	**6**	**7**
PW (ms)	75.2 ± 2.5	78.6 ± 9.4	80.5 ± 10.3	70.3 ± 20.5
PER (s)	20.3 ± 3.4	15.0 ± 2.9	18.2 ± 2.7	20.0 ± 8.5

Note: EFS = electric field stimulation; PW = pulse width [ms ± SEM]; PER = period time [s ± SEM].

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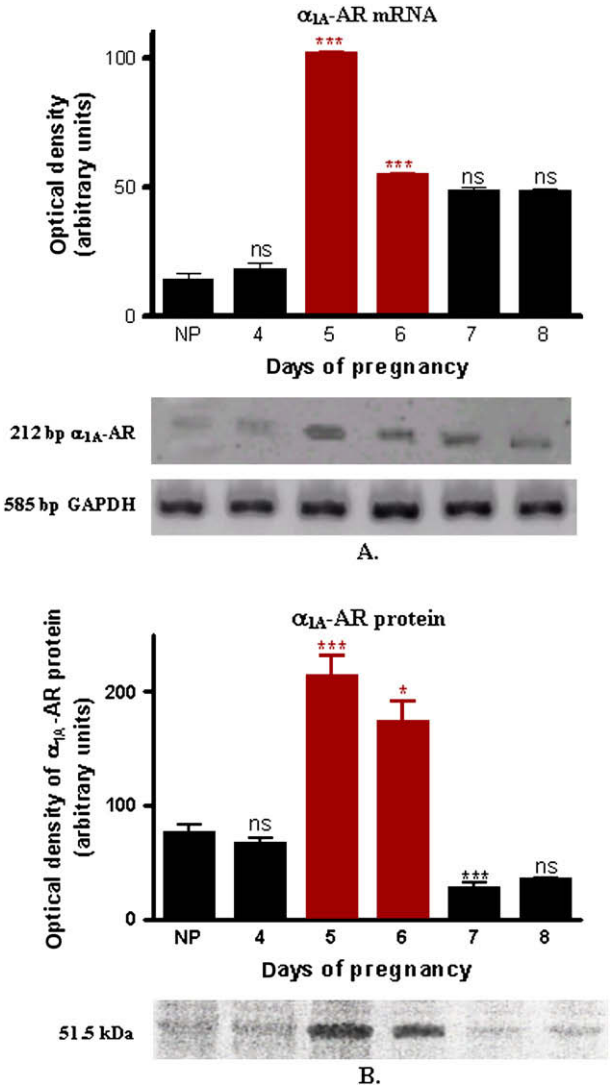
FIGURE 1

The changes in messenger RNA (mRNA) expression (**A**) and the protein level (**B**) of α_{1A}-AR in the nonpregnant and the early-pregnant rat uterus, measured by reverse transcription–polymerase chain reaction (RT-PCR) and Western blot, as described in the Materials and Methods section. (not significant = $P>.05$; *$P<.05$; ***$P<.001$ compared with the data on the previous day). Each bar represents the mean ± SD, n = 6.



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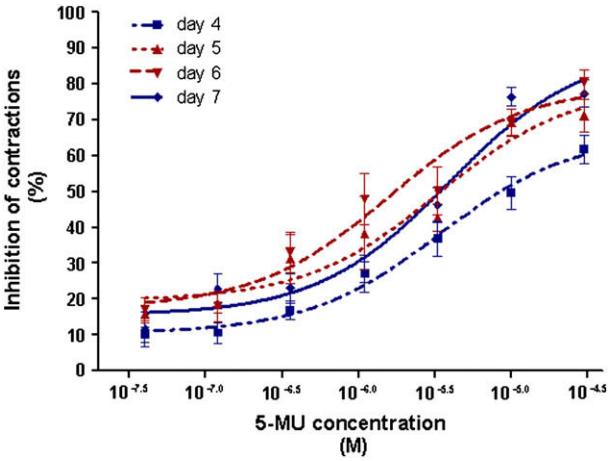
of 5-MU was increased on day 5 and remained unchanged up to day 7 (Fig. 2).

The role of the α_{1A}-AR in implantation was investigated with α_{1A}-AON. We detected a decreased expression of α_{1A}-AR mRNA (Fig. 3), and the number of implantations also decreased after α_{1A}-AON treatment (~75%) compared to the vehicle-treated and untreated control animals (Fig. 4).

FIGURE 2

Inhibitory effects of the α_{1A}-antagonist 5-methylurapidil (5-MU) on electric field-stimulated contractions on different days of early pregnancy in the isolated rat uterus in vitro. The EC_{50} (concentration of terbutaline eliciting 50% of the maximal [100%] inhibition of uterine contractions) values were significantly shifted on days 5 and 6; the maximal inhibitory effect was increased on day 5, and remained unchanged up to day 7.



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DISCUSSION

A crucial stage in pregnancy is the implantation of the embryo into the endometrium. In the rat, this event occurs at the end of day 5 of pregnancy and is regulated by several endogenous factors (e.g., steroid hormones, cytokines, and growth factors) (11). However, the tissue distribution and the role of the adrenergic receptors in the pregnant rat uterus in this period are unknown.

The present study is the first to illustrate the alteration in expression of the mRNAs, of the α_1-AR subtypes, and the physiologic functions in the early-pregnant rat uterus, especially during the time of embryonic implantation.

All the α_1-AR subtypes (α_{1A}, α_{1B}, and α_{1D} with their mRNAs and proteins) were found in the early-pregnant rat uterus, with a predominance of α_{1A}-AR. The presence of the other two subtypes was very limited. The expression of the α_{1A}-AR protein was significantly increased on days 5 and 6, suggesting a special role for this receptor subtype on these days. Because this is the period of implantation in the rat, we presumed that this sharp increase in the receptor number may play a role in the control of the implantation process. Our studies were focused only on the role of the α_{1A}-AR in the early-pregnant rat uterus.

Then we determined the function of these receptors, and therefore investigated their roles in the control of uterine smooth muscle contractility. Electric field-stimulated uterine

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FIGURE 3

The changes in α_{1A}-AR messenger RNA (mRNA) expression after α_{1A}-AR antisense oligodeoxynucleotide treatment in the rat myometrium, measured by reverse transcription–polymerase chain reaction as described in the Materials and Methods section. The relative amounts of the receptor subtype mRNAs are indicated by the optical densities of the bands (not significant = $P>.05$; *$P<.05$ compared with the data on the previous day). Each bar represents the mean ± SD, n = 6.

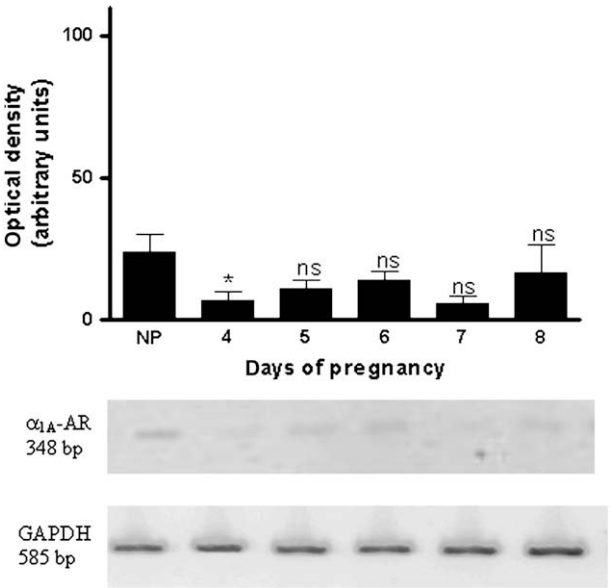

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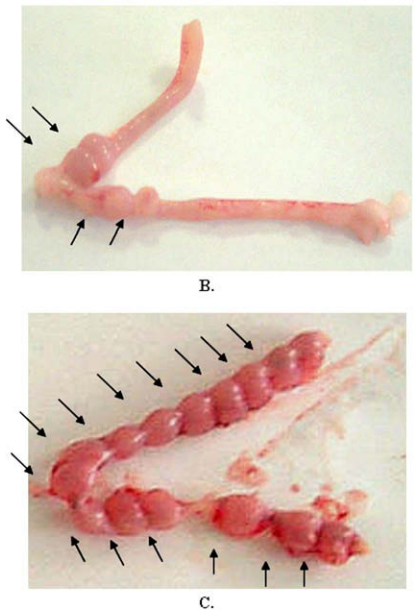
FIGURE 4

The effect of α_{1A}-AR antisense oligodeoxynucleotide (AON) on implantation in rats (**A**). The absence of implantation sites (*arrows*) (**B**) in the AON-treated rat uteri and the presence in the control uteri (**C**) were seen on day 10.

	AON-treated (mean±SEM)	Vehicle-treated (DOTAP) (mean±SEM)	Control (mean±SEM)
Number of implantations	*4.0 ± 1.0* (*n=12*)	*13.8 ± 1.2* (*n=8*)	*15.5 ± 1.9* (*n=8*)

A.



B.

C.

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contractions were inhibited by the α_{1A}-AR inverse agonist 5-MU (12). The EC_{50} values indicated that the ligand–receptor interaction is strongest on day 6 (the lowest EC_{50} value), whereas the maximal inhibitory effect was reached on day 5 and remained high up to day 7. Accordingly, at near implantation not only the number but also the sensitivity and function of the α_{1A}-AR are moderately elevated, which justifies the assumption that these receptors may play an important role in the control of this period of pregnancy.

The contractility study was important in proving the existence of a functioning α_{1A}-AR, but gave no direct information about its role in implantation. Therefore, an α_{1A}-AR knock-down transformed animal model was developed, using AONs. The major advantage of the antisense effect (i.e., the selective inhibition of gene expression by antisense RNA, DNA, or oligonucleotides) instead of a conventional drug effect is that binding of the oligonucleotide drug to its receptor (mRNA) occurs through a highly predictable and well-characterized set of rules (Watson-Crick base pairing) (13). Antisense inhibition, although highly specific, does not result in a total knock-out of the gene expression, but rather in a partial decrease of the expression of the α_{1A}-AR, which impedes the embryonic implantation. Treatment with DOTAP (vehicle) was ineffective; the obtained result being purely the consequence of the AON effect. The significant decrease in the number of implanted embryos in the AON-treated uteri suggests "negative" pharmacologic evidence of the role of the α_{1A}-AR in the implantation process. This means that, with the lack of α_{1A}-AR, a determining mechanism is blocked, probably causing a poor adherence of the embryos to the endometrium.

The α_{1A}-AR expression is significantly higher on day 5 of pregnancy. In earlier studies we proved that the number of estrogen receptors (ERs) (α) was elevated at the time of implantation (day 5 of pregnancy) in the rat (14). From the strong correlation, it was hypothesized that both receptors should be involved in the control of implantation. The elevated level of ERα mRNA on day 5 may be related to the blastocyst implantation, because estrogen (E) is essential in the induction of implantation. It may be assumed that E induces epithelial proliferation in the endometrium through

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the ERα, which is necessary for blastocyst attachment by the end of day 5. These findings are in accord with those of a previous investigation, which demonstrated that implantation was not observed in ER knock-out mice (15). Our theory was based on earlier experimental results of a heterologous regulation between E and adrenergic systems (16). In addition, we proved a strong correlation between the ERα and α_{1B}-AR mRNA expressions, in the pregnant human uterus, whereas other investigators found E cross-talk to the α_{1B}-AR in HEK-293-transfected cells (17, 18).

In the light of our findings, we presume that α_{1A}-AR has a crucial role in embryonic implantation in the rat, whereas the other two α_1-AR subtypes are not involved in this process. We assume that this role of the α_{1A}-AR is under the control of E hormones. This special function of the α_{1A}-AR may offer a new pharmacologic possibility with which to promote successful implantation.

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