Expression of adrenergic receptors in mouse preimplantation embryos and ovulated oocytes

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Abstract

Epinephrine and norepinephrine can play an important role in basic developmental processes such as embryogenesis and morphogenesis, regulating cell proliferation, differentiation and migration. We showed that β -adrenergic receptors can mediate the effects of catecholamines on preimplantation embryos in our previous work. In the present study, we designed specific oligonucleotide primers which can distinguish among all members of the α -adrenergic receptor family, and showed (using RT-PCR) that the α 2C-adrenergic receptor is transcribed in ovulated oocytes, 8- to 16-cell morulae and expanded blastocysts. We did not detect the α 2C-adrenoceptor transcript in 4-cell embryos. Our immunohistochemical study showed the presence of α -2C-adrenoceptor protein in ovulated oocytes, 8- to 16- cell embryos and blastocysts, but the signal in 4-cell embryos was weak, and probably represents remaining protein of maternal origin. We did not detect any other α -adrenergic receptor in preimplantation embryos and oocytes. Exposure of mouse preimplantation embryos to the α 2-adrenergic agonist UK 14 304 led to significant reduction of the embryo cell number, and the effect was dose dependent. Our results suggest that epinephrine and norepinephrine could affect the embryo development in the oviduct via adrenergic receptors directly and support the opinion that maternal stress can influence the embryo even in very early pregnancy.

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Introduction

Epinephrine and norepinephrine are well-known neurotransmitters and hormones that play an essential role in the regulation of the cardiovascular system and energy metabolism, especially in the case of stress or increased energy need. In addition to these well-known effects, epinephrine and norepinephrine can also play an important role in basic developmental processes such as embryogenesis and morphogenesis, regulating cell proliferation, differentiation, and migration (for review, see Buznikov *et al.* 1996, Pendleton *et al.* 1998, Weiss *et al.* 1998, Herlenius & Lagercrantz 2001). The essential role of epinephrine and norepinephrine in mouse fetal development has been clearly demonstrated, when mice lacking the capability to synthesize these compounds died *in utero* or shortly after birth (Kobayashi *et al.* 1995, Thomas *et al.* 1995, Zhou *et al.* 1995).

In our previous work, we demonstrated the possibility of direct influence of epinephrine and norepinephrine on the very early embryo (before its implantation into the uterus) via three subtypes of β -adrenergic receptors (Čikoš *et al.* 2005). The β -adrenoceptors belong, together with α 1- and α 2- adrenoceptors, to the adrenergic receptor family of G protein-coupled receptors. To

transduce extracellular signals, the receptors couple to heterotrimeric GTP-binding (G) proteins which activate various effectors. The β -adrenoceptors couple predominantly to Gs proteins (G proteins which stimulate adenylyl cyclase activity), α 1-adrenoceptors couple predominantly to Gq proteins (G proteins which stimulate phospholipase C activity) and α 2-adrenoceptors couple predominantly to Gi proteins (G proteins which inhibit adenylyl cyclase activity). Signaling pathways leading from adrenergic receptors then regulate the activity of metabolic enzymes, ion channels or transcription factors (for review see, Watson & Arkinstall 1994, Saunders & Limbird 1999). Moreover, the adrenergic receptors can activate mitogen-activated protein kinases and thereby influence fundamental cellular processes (Alblas *et al.* 1993, DeGraff *et al.* 1999, Schramm & Limbird 1999, Kim *et al.* 2002, Shizukuda & Buttrick 2002, Pullar & Isseroff 2003).

To ascertain whether epinephrine and norepinephrine could influence the preimplantation embryo also via α -adrenergic receptors, in the present study we examined the expression of all subtypes of α 1- and α 2- adrenergic receptors in mouse embryos at various stages of preimplantation development.

Materials and Methods

Informatics analysis and primer design

Nucleotide sequences of mouse adrenergic receptors were obtained from public databases (GenBank, available at <http://www.ncbi.nlm.nih.gov/> and GPCRDB: Information system for G protein-coupled receptors, available at <http://www.gpcr.org/7tm/>). mRNA nucleotide sequences of $\alpha 1A$ -, $\alpha 1B$ -, $\alpha 1D$ -, $\alpha 2A$ -, $\alpha 2B$ -, and $\alpha 2C$ - adrenergic receptors (GenBank accession numbers: AF031431, XM_126326, S80044, M99377, M94583, M97516) were aligned using Clustal W (Higgins *et al.* 1996) and Blast 2 algorithms (Altschul *et al.* 1997). The regions which do not exert significant nucleotide homology among the members of the α -adrenergic receptor family were selected for location of primers. The primers were designed using the program Primer 3 (available at http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and analysis of appropriate PCR products for recognition sites of restriction enzymes was performed using the program Webcutter 2.0 (available at <http://rna.lundberg.gu.se/cutter2/>).

Embryo recovery

All animal experiments were approved by the Ethical Committee of the Institute of Animal Physiology SAS, Košice. Female mice (ICR strain, Velaz, Prague, Czech Republic; 4–5 weeks old) underwent superovulation treatment by i.p. injection of 5 IU of serum gonadotropin (Folligon, Intervet International Bv. Boxmeer, Holland), followed 46 h later by administration of 5 IU of human chorionic gonadotropin (hCG, Organon, Oss, Holland). The mice were killed by cervical dislocation 24 h after hCG and unfertilized oocytes (at metaphase II) were isolated by flushing from the oviduct. To obtain preimplantation embryos, females were mated with males of the same strain overnight (mating was confirmed by identification of a vaginal plug), killed by cervical dislocation (57-, 72-, or 98 h after hCG) and the embryos (4-cell embryos, 8–16-cell morulae and expanded blastocysts) were isolated by flushing from the oviduct or uterus. Oocytes and embryos were washed in several drops of flushing-holding (FHM) medium (Lawits & Biggers 1993) containing 1% BSA and pooled according to their morphology. Cumulus cells were removed with 0.1% hyaluronidase (Sevac, Prague, Czech Republic).

RT-PCR

Total RNA was extracted from batches of 100–150 mouse preimplantation embryos and unfertilized oocytes. The RNA was also isolated from mouse tissues known to be rich in α -adrenergic receptors (brain, heart and liver), which served as positive controls. TRIzol Reagent (Invitrogen Life technologies) was used according to the manufacturer's instructions. Contaminating DNA in RNA preparations was

digested by incubation for 15 min at 22 °C with amplification grade DNase I (Invitrogen Life technologies) in 20 mM Tris–HCl pH 8.4, 2 mM MgCl₂ and 50 mM KCl. The DNase I reaction was stopped by ethanol precipitation. RNA pellets were dissolved in water and denatured by incubation for 15 min at 65 °C.

The RNA was reverse transcribed at 42 °C for 1 h in 20 μ l containing 200 units of Superscript II RNase H[−] Reverse Transcriptase (Invitrogen Life technologies), 4 μ M oligo dT18, 50 mM Tris–HCl pH 8.3, 3 mM MgCl₂, 75 mM KCl, 10 mM DTT, 500 μ M dNTPs (dATP, dTTP, dCTP, dGTP), 40 units RNase OUT (Recombinant RNase Inhibitor, Invitrogen Life technologies) and 0.5 μ g acetylated BSA. The reaction was terminated by heating at 95 °C for 5 min. To check for the presence of genomic DNA contamination in the RNA preparations, reverse transcriptase negative controls (no reverse transcriptase in the reaction) were carried out in parallel, using half from each RNA sample. The cDNA preparations were then cleaned by ethanol precipitation (Liss 2002) and the cDNA pellets were diluted in an appropriate amount of 10 mM Tris (pH 8.3) so that 1 μ l of the cDNA corresponded to 2.5 embryo/oocyte equivalents.

PCR amplification was carried out in the presence of 0.5 μ M of each oligonucleotide primer (see Table 1), 50 mM KCl, 10 mM Tris–HCl pH 8.3, 2 mM MgCl₂, 0.2 mM dNTPs (dATP, dTTP, dCTP, dGTP), and 0.05 units/ μ l Taq DNA polymerase (Invitrogen Life Technologies). One microliter of cDNA was amplified in 25 μ l PCR mix. For $\alpha 1B$ -, 1D-, 2A-, and 2B- adrenoceptors, an initial denaturation step at 95 °C for 2 min was followed by 40 cycles at 94 °C for 30 s and 68 °C for 90 s. For $\alpha 1A$ -, and 2C- adrenoceptors an initial denaturation step at 95 °C for 2 min was followed by 40 cycles at 94 °C for 30 s, 63 °C for 45 s, and 72 °C for 45 s. To check for the presence of cross contamination, the reaction with water instead of cDNA was performed concurrently (blank reaction). Detection of β -actin transcript using β -actin primers (5'-GTGGGCCGCTCTAGG-CACCAA-3' and 5'-CTCTTTGATGTACGCACGATTTC-3', give a 539 bp PCR product; Temeles *et al.* 1994) served as a control for RNA integrity and the RT-PCR process.

The PCR products were analyzed using electrophoresis on a 2% agarose gel stained with SYBR Green I (Sigma). A 100 bp DNA ladder and a 25 bp DNA ladder (Invitrogen Life technologies) were used as markers to determine the size of the PCR products. To confirm the amplification of the correct sequences, we digested the obtained PCR products with appropriate restriction enzymes – amplification reactions were ethanol precipitated, and DNA pellets were dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and digested with the restriction enzyme in the appropriate reaction buffer.

Immunostaining

Preimplantation embryos and oocytes were isolated as described above and the zona pellucida was removed with 0.5% pronase in KSOM at 37 °C. Zona-free oocytes

Table 1 Primer sequences and locations on gene, predicted size of amplicons.

Gene	Primer sequence (5' - 3')	Primer location	Amplicon size (bp)
$\alpha 1A$	FP: CAGATGGAGTCTGTGAATGGAA	COOH, exon 2	185
	RP: AATGGTTGGAAGTGGTGATT	COOH, exon 2	
$\alpha 1B$	FP: ATACCTGGGTCGTGGAACG	COOH, exon 2	140
	RP: GGAGCTTGAAAGTGAAGAGTGG	COOH, exon 2	
$\alpha 1D$	FP: AGCACTACGCCGAGCCTC	3 CL, exon 1	127
	RP: TGCTACTCTGTGCCCTGGATT	3 CL, exon 1	
$\alpha 2A$	FP: TTCTTTTTCACCTACAGCTCA	6 TM	112
	RP: TGTAAGATAACAGGGTTCAGCGA	7 TM	112
$\alpha 2B$	FP: ACCTTCCCTTGCTGACTGTACT	3'UTR	112
	RP: TGGGAGGGAGGTATTCTAATCA	3'UTR	
$\alpha 2C$	FP: GGCTGTGAAGTATAGGGCTTTAG	3'UTR	105
	RP: ATAGGAAGTCAGCCCTTGCTC	3'UTR	

FP, forward primer; RP, reverse primer; COOH, a sequence coding receptor carboxy-terminus; 3 CL, a sequence coding potential 3rd cytoplasmic loop; 6 TM, a sequence coding potential 6th transmembrane region; 7 TM, a sequence coding potential 7th transmembrane region; 3'UTR, untranslated sequence at the 3' end of mRNA; bp, base pairs.

and embryos were washed thrice in PBS/BSA (PBS containing BSA; Sigma–Aldrich), fixed in methanol at 10 °C (Sigma–Aldrich) for 5 min, then washed in PBS/BSA, followed by washing in PBS/BSA/SAP (PBS/BSA containing 0.05% saponin (SAP); Sigma–Aldrich). Non-specific immunoreactions were blocked with 2.25% normal donkey serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 0.1% saponin in PBS for 45 min at room temperature. The oocytes and embryos were incubated with the primary antibody raised against the $\alpha 2C$ -adrenergic receptor (affinity-purified goat polyclonal antibody, $\alpha 2C$ -AR C-20, Santa Cruz Biotechnology, 2 µg/ml) in PBS/BSA/SAP at 4 °C overnight. After extensive washing in PBS/BSA/SAP, specific secondary antibody coupled with fluorescein (donkey anti-goat IgG-FITC, Santa Cruz Biotechnology, 2 µg/ml) was used to visualize primary antibody reactions (30 min at room temperature). Afterwards, oocytes and embryos were washed in PBS/BSA/SAP and PBS/BSA, mounted on glass slides in UltraCruz mounting medium (Santa Cruz Biotechnology, contains DAPI for cell nuclei staining), sealed with coverslips and observed using an epifluorescent microscope (BX 51 Olympus, Tokyo, Japan).

Negative control groups of oocytes and embryos were incubated without the primary antibody or without the primary and secondary antibody, or with the primary antibody preadsorbed with an excess (20 times by weight) of the immunizing peptide according to the manufacturer's protocol (Blocking peptide, $\alpha 2C$ -AR C-20 P, Santa Cruz Biotechnology). Oocytes and embryos

in each experimental group (incubation with primary antibody) were evaluated by comparison with control groups of oocytes and embryos.

Embryo culture and morphological evaluation

The four-cell embryos were isolated by flushing from the oviduct in FHM medium 57 h after hCG. They were washed thrice in KSOM culture medium, transferred into 30 µl drops of KSOM media supplemented with UK 14 304 (Sigma–Aldrich) dissolved in DMSO or with the equivalent volume of DMSO alone and cultured in a humidified atmosphere with 5.0% CO₂ at 37 °C for 60 h. Three doses of UK 14 304 were used: UK 14 304 was dissolved in DMSO and added to a KSOM culture medium (Lawits & Biggers 1993) at final concentrations of 0.1, 1, and 10 µM at the beginning of the incubation period and then 20 and 40 h later. The control groups of embryos were cultured in the presence of the equivalent amounts of solvent (DMSO) added into the KSOM at the same time as UK 14 304 (the compounds were added in the volume of 1 µl).

After the 60 h of incubation, the embryos were stained with Hoechst 33 342 (20 µg/ml; Sigma–Aldrich) for 10 min at 37 °C, washed, sealed with coverslips, and observed using an epifluorescent microscope (BX 51 Olympus). The number of cell nuclei was determined in all embryos. Each embryo was assigned to one of the four classes according to the cell number (developmental stage). Following classes were established: embryos with cell number less than 16, embryos with cell number between 17 and 32, embryos with cell number between 33 and 64, and embryos with cell number 65 and more. The distribution of embryos among the four developmental classes was then analyzed.

To eliminate experimental bias, at least three independent series were performed in each experimental group (the three UK 14 304 doses and the control group) and the results were pooled. The following total numbers of embryos (*n*) were examined: 10 µM UK 14 304 *n* = 139; 1 µM UK 14 304 *n* = 113; 0.1 µM UK 14 304 *n* = 140; DMSO controls *n* = 148.

The One-way ANOVA followed by Duncan's test was used for evaluating the embryo cell number, while the chi-squared test was used for analyzing embryo distribution. Differences of *P* ≤ 0.05 were considered significant.

Results

Primer design

We located the primers for α -adrenergic receptors mainly in regions encoding a portion of the receptor outside of transmembrane domains, which are less conserved than transmembrane domains. The primers for $\alpha 1D$ -adrenoceptor also detect an alternative splice variant coding longer adrenoceptor isoform (GenBank accession number XM_141369). The primers for $\alpha 1A$ -adrenoceptor do not detect two potential alternative

transcripts lacking a part of the coding sequence from the second exon (GenBank unfinished high-throughput cDNA sequences AK085653 and AK042759, lastly modified in October 2006). Sequences and locations of the primers are shown in Table 1.

Amplification reaction for each primer pair was optimized using positive controls – mouse tissues known to be rich in the receptor (Fig. 1a). The identity of each PCR product was verified by digestion with appropriate restriction enzymes according to the sequence information: digestion of the α 1A-adrenoceptor PCR product (185 bp) with Alu I gave 112 and 73 bp DNA fragments, digestion of the α 1B-adrenoceptor PCR product (140 bp) with Alu I gave 102 and 34 bp DNA fragments (the remaining 4 bp DNA fragment is not detectable on 2% agarose gel), digestion of the α 1D-adrenoceptor PCR product (127 bp) with Hpa II gave 87 and 40 bp DNA fragments, digestion of the α 2A-adrenoceptor PCR product (112 bp) with Hpa II gave 73 and 39 bp DNA fragments, digestion of the α 2B-adrenoceptor PCR product (112 bp) with Taq I gave 74 and 38 bp DNA fragments, digestion of the α 2C-adrenoceptor PCR product (105 bp) with Alu I gave 81 and 24 bp DNA fragments, and digestion with Mbo II gave 67 and 38 bp DNA fragments (Fig. 2).

Expression of α -adrenergic receptor mRNAs

Mouse oocytes and preimplantation embryos at various developmental stages (four-cell embryos, 8- to 16-cell

Figure 1 RT-PCR with primers for α -adrenergic receptors. (a) RT-PCR with primers for α -adrenergic receptors in mouse positive control tissues. Agarose gels with separated PCR products are shown. Primers detecting α 1A- (lane B, the 185 bp PCR product) and α 1B- (lane C, the 140 bp PCR product) adrenoceptors were used with heart cDNA, primers detecting α 1D- (lane D, the 127 bp PCR product), α 2A- (lane E, the 112 bp PCR product), and α 2C- (lane G, the 105 bp PCR product) adrenoceptors were used with brain cDNA, primers detecting α 2B-adrenoceptor (lane F, the 112 bp PCR product) were used with liver cDNA. Lanes A and H, molecular weight markers (MWM). The sizes of the MWM in base pairs (bp) are indicated to the left and the right of the panel respectively. (b) RT-PCR analysis of α 2C-adrenoceptor mRNA expression in mouse oocytes and preimplantation embryos. Agarose gels with separated PCR products (the 105 bp PCR product corresponds to the α 2C-adrenoceptor) are shown. Lanes: B, oocytes; D, four-cell embryos; F, 8- to 16- cell embryos; H, blastocysts; J, positive control tissue (brain); C, E, G and I, corresponding reverse transcriptase negative controls; K, blank reaction. Lanes A and L, molecular weight markers (MWM). The sizes of the MWM in base pairs (bp) are indicated to the left and the right of the panel respectively.

morulae and expanded blastocysts) were examined by RT-PCR using reaction conditions which were optimized for each primer pair in the positive control tissues before examination. An amount of cDNA corresponding to 2.5 embryo/oocyte equivalents was amplified in 40 cycles and analyzed on agarose gel.

We detected a PCR product corresponding to the α 2C-adrenergic receptor (105 bp) in oocytes, 8- to 16-cell morulae, and expanded blastocysts. No PCR product was detected in four-cell embryos or in the reactions where reverse transcriptase or cDNA were omitted (Fig. 1b). Digestion of the 105 bp PCR product with appropriate restriction enzymes produced DNA fragments of the expected sizes: digestion with Alu I gave 81 and 24 bp DNA fragments and digestion with Mbo II gave 67 and 38 bp DNA fragments (Fig. 2).

PCR products corresponding to other α -adrenergic receptor mRNAs were not detected in the oocytes and embryos, even after an increase of cDNA amount in PCR or after an additional 25 cycles of reamplification (data not shown).

The embryos produced a PCR fragment corresponding to the β -actin mRNA at all developmental stages, thus confirming the integrity of the RNA and the RT-PCR process (data not shown).

Immunohistochemical study

The immunoreactive α 2C-adrenergic receptor was identified in oocytes, 8- to 16-cell morulae, and blastocysts, but the signal was weak in four-cell embryos (Fig. 3). The specificity of the signal was confirmed using several controls: the immunostaining intensity was significantly reduced in controls incubated with the primary antibody preadsorbed with the immunizing peptide (Fig. 3) or in controls incubated without the

Figure 2 Digestion of the PCR products with restriction enzymes. Lanes: B, digestion of the α 1A-adrenoceptor PCR product with Alu I (112 + 73 bp DNA fragments); C, digestion of the α 1B-adrenoceptor PCR product with Alu I (102 + 34 bp DNA fragments); D, digestion of the α 1D-adrenoceptor PCR product with Hpa II (87 + 40 bp DNA fragments); E, digestion of the α 2C-adrenoceptor PCR product with Alu I (81 + 24 bp DNA fragments); F, digestion of the α 2C-adrenoceptor PCR product with Mbo II (67 + 38 bp DNA fragments); G, digestion of the α 2A-adrenoceptor PCR product with Hpa II (73 + 39 bp DNA fragments); H, digestion of the α 2B-adrenoceptor PCR product with Taq I (74 + 38 bp DNA fragments). Lanes A, E, and J, molecular weight markers (MWM). The sizes of the MWM in base pairs (bp) are indicated to the left and the right of the panel respectively.

primary antibody or without the primary and secondary antibody (data not shown).

Effect of UK 14 304 on embryo development

Table 2 shows the analysis of embryo distribution after incubation of the mouse preimplantation embryos with different doses of the $\alpha 2$ -adrenoceptor agonist UK 14 304. Highly significant changes ($P < 0.001$) in embryo distribution with an increased proportion of embryos with lower cell numbers and decreased proportion of embryos with higher cell numbers were found after the UK 14 304 treatment. Incubation of the embryos with UK 14 304 led to significant reduction of the mean embryo cell number in comparison with the control embryos and the response was dose dependent (Fig. 4).

Discussion

We showed that β -adrenergic receptors are expressed in mouse oocytes and preimplantation embryos in our previous work (Čikoš *et al.* 2005). To extend our knowledge to all members of the adrenergic receptor family, we proceeded to examine the expression of α -adrenergic receptors in mouse preimplantation embryos and oocytes. We detected the $\alpha 2C$ -adrenoceptor transcript in oocytes, 8- to 16-cell embryos, and blastocysts, but not in four-cell embryos. These results suggest that ovulated oocytes express the $\alpha 2C$ -adrenoceptor and that transcription of the embryonal $\alpha 2C$ -adrenoceptor gene begins around the 8- to 16-cell stage. In accordance with the results of the RT-PCR experiment, our immunohistochemical study showed

Figure 3 Immunohistochemical localization of the $\alpha 2C$ -adrenoceptor in mouse oocytes (A, B and C), four-cell embryos (D, E and F), 8- to 16-cell embryos (G, H and I), and blastocysts (J, K and L). Representative images are shown. A, D, G and J: Cell nuclei stained with DAPI; B, E, H and K: Oocytes/embryos incubated with the antibody against the $\alpha 2C$ -adrenoceptor (identical samples as in images A, D, G and J); C, F, I and L: Oocytes/embryos incubated with the antibody against the $\alpha 2$ -adrenoceptor preadsorbed with the immunizing peptide. The bar, 10 μ m.

Table 2 Embryo distribution after incubation of mouse embryos with UK 14 304.

Embryo cell number	Control	UK 0.1 μ M	UK 1.0 μ M	UK 10.0 μ M
<16 cells ^a	10.1	24.3	25.7	30.9
17–32 cells ^a	17.6	17.2	18.6	27.3
33–64 cells ^a	41.9	46.4	46.9	35.3
>65 cells ^a	30.4	12.1 ^{*b}	8.8 ^{*b}	6.5 ^{*b}
Number of embryos	148	140	113	139

The four-cell mouse embryos were incubated in the presence of indicated concentrations of UK 14 304 (UK) for 60 h. Each embryo was then assigned to one of the four classes according to the cell number (<16 cells, 17–32 cells, 33–64 cells, >65 cells) and the distribution of embryos among the four developmental classes was analyzed.

^aThe number of embryos from each experimental group (Control, UK 0.1, UK 1.0, UK 10.0 μ M) belonging to the indicated developmental classes is expressed as a percentage. ^bStatistical difference between the control group and UK 14 304 treated groups: * $P<0.001$.

the presence of α 2C-adrenoceptor protein in ovulated oocytes, 8- to 16- cell embryos, and blastocysts, but the signal in four-cell embryos was weak and probably represents the remaining protein of maternal origin. We did not detect any other α -adrenergic receptor in preimplantation embryos and oocytes.

Taken together, the results of our previous work (Čikoš *et al.* 2005) and the results of the present study indicate that two types of adrenergic receptors are expressed in mouse ovulated oocytes and preimplantation embryos. The receptors are under the control of the same natural ligands (epinephrine, norepinephrine) but they couple primarily to G proteins with opposing actions on adenylyl cyclase activity (β -adrenoceptors to Gs, α 2-adrenoceptors to Gi). Co-stimulation of the two receptors in the same cell might be expected to

Figure 4 Embryo cell number after incubation with UK 14 304. The four-cell mouse embryos were incubated in the presence of indicated concentrations of UK 14 304 (UK) for 60 h and then the cell number was estimated. Values are arithmetical means $\pm$ S.E.M. Numbers of embryos (n): 10 μ M UK 14 304 $n=139$; 1.0 μ M UK 14 304 $n=113$; 0.1 μ M UK 14 304 $n=140$; Control $n=148$. ^aStatistical difference between the control group of embryos and the UK 14 304-treated groups: *** $P<0.001$. ^bStatistical difference between the group of embryos treated with 10 μ M UK 14 304 and the embryos treated with the two lower doses of UK 14 304: * $P<0.05$, ** $P<0.01$.

antagonize each other's signaling. However, the ability of a Gi protein-coupled receptor to decrease cAMP production in the cell is not automatic; it depends, for example, on the isoform of adenylyl cyclase present in the cell (for review, see Cooper 2003). Moreover, it has been shown that under certain circumstances α 2-adrenoceptors can even stimulate cAMP production in some cell types, and the effect of α 2-adrenoceptors on cAMP production appears to be dependent on agonist concentration (Duzic & Lanier 1992, Pepperl & Regan 1993, Eason & Liggett 1995, Pohjanoksa *et al.* 1997). An interesting cross-talk between α 2-adrenoceptors and β 2-adrenergic receptor has been shown in pregnant rat myometrium with different receptor interactions at mid-pregnancy and in late pregnancy. By themselves, α 2-adrenoceptor agonists did not significantly influence adenylyl cyclase activity in myometrial cells, but they were able to modulate the enzyme activity after β 2-adrenoceptor stimulation (Mhaouty *et al.* 1995, Limon-Boulez *et al.* 2001). The possibility of cross-talk between α 2- and β -adrenoceptors has also been shown in brain cells (Atkinson & Minneman 1992, Shivachar & Eikenburg 1999). These results indicate that simultaneous activation of β - and α 2- adrenergic receptors in the same cell need not necessarily have a contradictory effect on cAMP production; the signaling pathway employed by the receptor depends on the cell type and physiological status as well as on the intensity of receptor stimulation by a particular agonist.

To examine the effect of α 2C-adrenoceptor activation on the development of mouse preimplantation embryos, we exposed the embryos to three doses of the α 2-adrenergic agonist UK 14 304. We found that the percentage of embryos which reached higher developmental stages was significantly reduced after the exposure of the embryos to UK 14 304. Estimation of the mean embryo cell number revealed that the effect of UK 14 304 was dose dependent and led to significantly lower numbers of cells in UK 14 304-treated embryos in comparison with control embryos. These results indicate that activation of α 2C-adrenoceptor in mouse preimplantation embryos can inhibit cell proliferation, similarly as shown for β -adrenergic receptors (Čikoš *et al.* 2005). The influence of adrenergic receptors on fundamental cellular processes has been demonstrated in various mammalian cell types, particularly regulation of cell proliferation, migration, and apoptosis by activated β - and α 2- adrenoceptors (Kennedy *et al.* 1983, Seuwen *et al.* 1990, Wang & Limbird 1997, Slotkin *et al.* 2000, Cussac *et al.* 2002, Kim *et al.* 2002, Shizukuda & Buttrick 2002, Pullar & Isseroff 2003, Zhu *et al.* 2003). Cell responses to stimulation of adrenergic receptors are mediated via various signaling pathways. The β -adrenoceptors coupled predominantly to Gs proteins regulate the activity of metabolic enzymes, ion channels, or transcription factors. The α 2-adrenoceptors coupled primarily to Gi proteins inhibit the

activity of adenylyl cyclase and voltage-gated Ca^{2+} channels and activate receptor-operated K^{+} channels (for review see, Watson & Arkinstall 1994, Saunders & Limbird 1999). Moreover, stimulation or inhibition of mitogen-activated protein kinases by activated adrenergic receptors has been shown in several cell types utilizing a variety of signaling pathways (Alblas *et al.* 1993, DeGraff *et al.* 1999, Schramm & Limbird 1999, Lindquist *et al.* 2000, Hutchinson *et al.* 2002, Shizukuda & Buttrick 2002, Pullar & Isseroff 2003).

Natural ligands for adrenergic receptors, epinephrine and norepinephrine, have been detected in the oviductal fluid of some species. Concentrations of these catecholamines varied with the region of the oviduct and with the stage of the estrous cycle (Khatchadourian *et al.* 1987, Way *et al.* 2001). The possible sources of norepinephrine and epinephrine in the oviductal fluid could be nerves, blood circulation or graafian follicles (Helm *et al.* 1982, Fernandez-Pardal *et al.* 1986, Kannisto *et al.* 1986, Itoh *et al.* 2000, Kotwica *et al.* 2003). Moreover, it was speculated that catecholamines could be produced also by the early embryo itself (Burden & Lawrence 1973, Sadykova *et al.* 1990). Epinephrine and norepinephrine present in oviductal fluid could influence the oviduct epithelium via adrenergic receptors which have been shown in oviduct epithelial cells of several species (Tolszczuk & Pelletier 1988, Dickens *et al.* 1993, Einspanier *et al.* 1999). Increased fluid formation, effects on Cl^{-} ion transport and electrical potential differences have been shown after treatment of rabbit oviductal cells with agonists of adrenergic receptors (Dickens *et al.* 1993, Dickens & Leese 1994). Besides, the effects on the oviduct epithelium, adrenoceptor agonists can also influence the functioning of spermatozoa in the oviduct, including capacitation and acrosome reaction (Way & Killian 2002, Adeoya-Osiguwa *et al.* 2006).

It is well known that circulating levels of epinephrine and norepinephrine are highly elevated during stress and trauma. Animal experiments have convincingly demonstrated that prenatal maternal stress can significantly affect the pregnancy outcome (for review see Mulder *et al.* 2002). Some results suggest that maternal stress can negatively impact on the embryo even at very early stages of its development. For instance, it has been shown that litter sizes of female hamsters stressed during early pregnancy were significantly smaller than those of controls, with fetal loss occurring between days 5 and 10 of pregnancy (Pratt & Lisk 1989). In humans, it has been demonstrated that pregnancies characterized by increased maternal cortisol (commonly used stress marker) during the first three weeks after conception are more likely to result in spontaneous abortion. (Nepomnaschy *et al.* 2006).

Our results indicate that, besides β -adrenergic receptors, the $\alpha_2\text{C}$ -adrenoceptor is also expressed in mouse oocytes and preimplantation embryos. We also found that ligands for the receptors are capable of

affecting the preimplantation embryo development *in vitro*. These results suggest that epinephrine and norepinephrine could directly affect embryo development in the oviduct via adrenergic receptors and support the opinion that maternal stress can influence the embryo even in very early pregnancy.

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