High levels of anandamide, an endogenous cannabinoid, block the growth of sheep preimplantation embryos by inducing apoptosis and reversible arrest of cell proliferation

M.Y. Turco¹, K. Matsukawa¹, M. Czernik¹, V. Gasperi¹, N. Battista¹, L. Della Salda¹, P.A. Scapolo¹, P. Loi¹, M. Maccarrone¹,²,³† and G. Ptak¹,³†

¹Department of Comparative Biomedical Sciences, University of Teramo 64100, Teramo, Italy; ²European Centre for Brain Research (CERC)/IRCCS S. Lucia Foundation, 00143 Rome, Italy
³Correspondence address. Tel: +39-0861-266837; Fax: +39-0861-411285; E-mail: gptak@unite.it (G.P.)/Tel: +39-0861-266875; Fax: +39-0861-266877; E-mail: mmaccarrone@unite.it (M.M.)

BACKGROUND: The process of implantation is mediated by various molecules, one of which is anandamide (AEA), a lipid signalling ligand belonging to the family of endocannabinoids. AEA exerts its effects on implantation by binding to the Type 1 Cannabinoid Receptor (CB1-R), expressed in both blastocysts and uterus. We wanted to know whether the endocannabinoid signalling system was present also in the sheep reproductive tract and which kind of effect(s) AEA had on the development of sheep blastocysts in vitro. METHODS: We analysed the expression and activity of the endocannabinoid system in sheep reproductive tracts and blastocysts. Hatched sheep blastocysts were then exposed to AEA and its effect(s) were determined by TUNEL assay and by measuring the rate of necrosis and 5-bromo-deoxyuridine incorporation. RESULTS: We show that the AEA signalling system is present in sheep and that high concentrations of AEA induce apoptosis and inhibit cell proliferation via a CB1-R-dependent mechanism. Indeed, AEA effects were blocked when sheep blastocysts were cultured in the presence of the CB1-R antagonist SR161417A. Moreover, AEA inhibition of cell proliferation was reversible, as arrested embryos resumed a normal growth rate upon AEA removal from the medium. CONCLUSIONS: Our results suggest that disturbed regulation of AEA signalling via CB1-R may be associated with pregnancy failure. AEA could lower the quality of blastocysts by inducing apoptosis and inhibiting cell proliferation, thus making them incompetent for implantation.

Keywords: anandamide; endocannabinoids; embryo development; implantation; sheep

Introduction

Successful cross-talk between the embryo and receptive uterus must take place to allow implantation and establish pregnancy. This process involves a sequence of finely regulated events that ultimately lead to adhesion and intimate contact between the blastocyst trophectoderm (TE) and the maternal endometrium. The cross-talk is mediated by many factors, whose exact roles and pathways have not yet been fully determined. One of these factors is N-arachidonoyl-ethanolamine (anandamide, AEA), a long-chain, unsaturated fatty acid-derivative that belong to a family of lipid mediators described as the endocannabinoid system (Mechoulam, 2002; De Petrocellis et al., 2004; Bari et al., 2006). This endogenous ligand was identified as a result of studies on the effects of Δ⁹-tetrahydrocannabinol (THC), the active constituent of marijuana, as AEA and THC share their main target, i.e. the G-protein-coupled receptor, Type 1 Cannabinoid Receptor (CB1-R) (Mastuda et al., 1990).

The use of illicit drugs, particularly marijuana, among women has raised serious concerns about their effects on pregnancy (Sherwood et al., 1999; Fergusson et al., 2002). A significant number of females exposed to THC have reported unsuccessful pregnancies due to failure of implantation or spontaneous abortion (Maccarrone et al., 2000a,b), lower infant birth weight (Fergusson et al., 2002) and cognitive deficits of the offspring (Fried et al., 2003). These data and others suggest that the endocannabinoid system is operative in the reproductive apparatus (for a comprehensive review see Wang et al., 2006a). Indeed, studies conducted in the mouse have shown that the main receptor of AEA, CB1-R, is expressed and functional in mouse uterus (Das et al., 1995), oviducts and embryos (Yang et al., 1996; Paria et al., 1998). AEA is synthesized in the uterus and oviducts of pregnant mice (Paria et al., 1996), where its levels are, by far, the...
highest found in any mammalian tissue (Schmid et al., 1997). Moreover, AEA levels are spatiotemporally regulated during pregnancy, as they are significantly lower in the receptive uterus and at the implantation site than in a non-receptive uterus and at inter-implantation sites (Schmid et al., 1997). This finding may indicate that low levels of AEA are beneficial, while high levels are detrimental for implantation. In addition, there is a coordinated down-regulation of uterine AEA levels and blastocyst CB1-R expression prior to implantation in mice, suggesting that this ligand-receptor signalling system may locally help in regulating the ‘time-window’ of implantation (Paria et al., 2001).

In mouse blastocysts, AEA activates cannabinoid receptors in the TE to initiate two distinct signalling systems in a dose-dependent manner. Low concentrations of AEA (7–14 nM) stimulate, via CB1-R, the mitogen-activated protein kinase signalling cascade that promotes embryo growth and differentiation leading to zona hatching and trophoblast outgrowth, two essential events which allow the blastocyst to make contact with the uterine luminal epithelium. In contrast, high concentrations of AEA (28 nM) have adverse effects on blastocyst development as they close, via CB1-R, the voltage-operated N-type calcium channels and consequently inhibit trophoblast outgrowth (Wang et al., 1999).

AEA undergoes a selective uptake through a purported membrane transporter (Battista et al., 2005), followed by intracellular hydrolysis by fatty-acid amidase hydrolase (FAAH), to regulate its levels (McKinney and Cravatt, 2005). On the other hand, AEA is hydrolyzed by FAAH, to regulate its levels (McKinney and Cravatt, 2005). FAAH seems particularly relevant for the regulation of AEA activity in the reproductive system, and it is considered a gate-keeper of fertility in both humans (Maccarrone et al., 2000a,b; Maccarrone and Finazzi-Agro`, 2004) and mice (Wang et al., 2006b). FAAH is expressed in the endometrial epithelium of humans and mice and is also found in circulating lymphocytes (Maccarrone et al., 2000a,b, 2001). FAAH expression has been shown to change during the peri-implantation period in the mouse uterus and embryos (Paria et al., 1999). Decreased levels of FAAH in maternal lymphocytes and, as a consequence, increased levels of AEA were reported to be associated with early pregnancy loss in humans (Maccarrone et al., 2000a,b). FAAH activity is stimulated by important factors that favour human fertility: progesterone, a hormone essential for the maintenance of pregnancy, and Th2 cytokines produced by peripheral T lymphocytes (Maccarrone et al., 2001). Furthermore, the release of another cytokine, which favours embryo implantation and survival, the leukaemia inhibitory factor (LIF) (Ptak et al., 2006), is regulated by AEA (Maccarrone et al., 2001) and Th2 cytokines (Stewart and Cullinan, 1997). Overall, these data suggest that AEA is intimately involved in the multifaceted hormone-cytokine network responsible for embryo–uterine interactions (Maccarrone and Finazzi-Agro`, 2004).

In order to understand how AEA is involved in the regulation of the implantation ‘time window’, it is necessary to study how it affects blastocyst development. So far, studies on the endocannabinoid system have not been conducted in many mammalian embryos, except for the mouse (Paria et al., 1999, 1998, 2001, 2002). Since mouse embryos have a short preimplantation period (4.5 days), with blastocyst-stage embryos invariably implanting into the uterus within 24 h upon hatching from their zonae pellucida, the use of an alternative model characterized by a longer preimplantation period, such as the sheep, would be more useful to investigate the role of AEA, during this developmental stage. Like other ruminant species, the sheep presents a non-invasive, synepithelialchorial implantation which is very different from that of the mouse. Indeed, unlike in the mouse, once hatched from the zonae pellucida, sheep blastocysts continue differentiating and do not implant until Day 16 of pregnancy (Spencer et al., 2004). Importantly, the timing of development of sheep embryos to the blastocyst stage is similar to that of human embryos (i.e. 6–7 days), and the long gap between blastocyst hatching and attachment to the uterine wall gives a unique opportunity to study how AEA influences implantation of the embryo.

In this work, we first examined whether the endocannabinoid system is expressed in the sheep reproductive tract and blastocysts, and then analysed the effect(s) of 28 nM AEA on the in vitro development of sheep blastocysts.

**Methods**

All chemicals, unless otherwise indicated, were obtained from Sigma Chemicals Co. (St Louis, MO, USA). N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazolecarboxamide (SR141716A) was a kind gift of Sanofi-Aventis Recherche (Montpellier, France). NArPE was synthesized from arachidonic acid and phosphatidyethanolamine, as reported (Fezza et al., 2005). [3H]AEA (205 Ci/mmol) and [3H]CP55,940 (126 Ci/mmol) were purchased from Perkin–Elmer Life Sciences, Inc. (Boston, MA, USA). [3H]NArPE (200 Ci/mmol) was from ARC (St Louis, MO, USA).

**Detection of the endocannabinoid system in the sheep reproductive tract**

**Collection of sheep reproductive tracts**

During the breeding season (September–January), oestrus was detected in cyclic females with the help of a vasectomized ram. Then, females were slaughtered at two separate (middle and late) stages of the oestrous, and their reproductive organs were collected for analysis. The middle stage (Days 8–12 of the oestrous cycle) normally corresponds to the time of blastocyst development and elongation in the uterus. The late stage of the oestrus cycle (Days 15 and 16) (in the absence of viable embryos in the uterus) is characterized by luteal regression and growth of ovulatory follicle(s).

**Analysis of AEA synthesis, hydrolysis and endogenous levels**

Synthesis of AEA through the activity of N-acyl-phosphatidylethanolamine (NAPE)-hydrolyzing phospholipase D (E.C. 3.1.4.4; NAPE-PLD) was measured in uterus homogenates (100 μg/test), using 100 μM [3H]NArPE as reported (Fezza et al., 2005). NAPE-PLD activity was
expressed as picomoles of $[^{3}H]$AEA released per minute per milligram protein.

Hydrolysis of $[^{3}H]$AEA by FAAH (E.C. 3.5.1.4; FAAH) was quantified in uterus homogenates (10 µg/test), incubated at pH 9.0 with 5 µM $[^{3}H]$AEA (Maccarrone et al., 2005). The release of $[^{3}H]$arachidonic acid from $[^{3}H]$AEA was evaluated through reversed phase-high performance liquid chromatography (RP-HPLC) and was expressed as picomoles per minute per milligram protein (Maccarrone et al., 2005).

To evaluate the concentration of endogenous AEA, uteri were homogenized with an UltraTurrax T25 in 50 mM Tris–HCl, 1 mM EDTA (pH 7.4), and 1 mM phenylmethylsulfonyl fluoride buffer. Lipids were extracted and the organic phase was freeze-dried (Maccarrone et al., 2005). Dry pellets were resuspended in 20 µl of methanol and analysed by high performance liquid chromatography with fluorimetric detection, as reported (Maccarrone et al., 2005). AEA content was expressed as picomoles per milligram protein.

**Receptor binding assay**

Membrane fractions were prepared from uteri as reported (Maccarrone et al., 2005) and were stored at -80°C for no longer than 1 week. Membrane fractions were used in rapid filtration assays with the synthetic cannabinoid $[^{3}H]$CP55,940 (200 pM), and non-specific binding was determined in the presence of 1 µM ‘cold’ agonist (Maccarrone et al., 2005). Receptor binding was expressed as femtomole of $[^{3}H]$ligand bound per milligram of protein.

**Western blot analysis of uterine expression**

The expression of CB1 and CB2 receptors (CB1-R and CB2-R) was assessed in uterus homogenates (20 µg/lane) by western blot analysis, using rabbit anti-CB1-R or anti-CB2-R polyclonal antibodies (each diluted 1:250), and goat anti-rabbit alkaline phosphatase conjugates (diluted 1:2000) as secondary antibody (Maccarrone et al., 2005). Mouse brain and mouse spleen homogenates (20 µg/lane) were used as positive controls for CB1-R or CB2-R, respectively (Maccarrone et al., 2005). FAAH expression was determined in uterus homogenates (20 µg/lane) with the same western blotting procedure, using rabbit anti-FAAH antibody. Purified FAAH (1 µg/lane) was used as a positive control. Non-immune rabbit serum (Primm S.r.l., Milan, Italy) was used as a control for the specificity of the polyclonal antibodies (Maccarrone et al., 2005). Rabbit anti-CB1-R and anti-CB2-R polyclonal antibodies were purchased from Cayman Chemicals (Ann Arbor, MI, USA), whereas the rabbit anti-FAAH polyclonal antibody was prepared by Primm S.r.l. Goat anti-rabbit alkaline phosphatase conjugates were from Bio-Rad (Hercules, CA, USA), whereas purified FAAH was a kind gift from Dr Benjamin F. Cravatt (The Scripps Research Institute, La Jolla, CA, USA).

**CB1-R detection in blastocysts**

Expression of CB1-R in sheep blastocysts was demonstrated by indirect immunofluorescence. Blastocysts were washed in 0.4% polyvinylpyrrolidone (PVP) in PBS, transferred onto coverslips and air-dried. They were fixed in 4% paraformaldehyde (PFA) at room temperature for 15 min, followed by treatment in 0.1% Triton X-100 under the same conditions. Blastocysts were then incubated with the primary antihuman CB1-R (N-terminal) polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (1:100) at 4°C overnight. After washing in 0.1% BSA/PBS, blastocysts were incubated with the secondary goat anti-rabbit IgG antibody conjugated to FITC (1:500) at room temperature for 1 h. Propidium iodide (PI) (0.5–1.0 µg/ml) was used to counterstain cells. As a negative control, some blastocysts were processed in the same way but for the absence of the primary antibody. Human lymphocytes were isolated from peripheral blood and fixed on slides to be used as a positive control. Images were captured with a Zeiss LSM 510 confocal scanning laser microscope.

**Analysis of AEA effect on blastocysts**

**In vitro maturation, fertilization and culture of sheep embryos**

Methods for in vitro embryo production were adapted from those previously described (Ptak et al., 1999, 2002, 2003). Ovaries were obtained from the abattoir and transported in PBS (Gibco Life Technologies, Rockville, MD, USA) containing 5 ml/l gentamycin (Gibco). Cumulus–oocyte complexes (COCs) were collected by aspiration through a 21G needle fitted to a syringe. Oocytes with at least 2–3 layers of compact cumulus cells and uniform granular cytoplasm were matured in bicarbonate-buffered TC1-199, supplemented with 2 mM l-glutamine, 100 µM cysteamine, 0.3 mM sodium pyruvate, 5 µg/ml FSH (Ovagen, ICP, Auckland, New Zealand), 5 µg/ml LH, 1 µg/ml 17-β-estradiol and 10% fetal bovine serum (Gibco), yielding an osmolality of 275 mOsm. COCs were incubated in 5% CO2 at 38.5°C for 24 h.

Then the partially denuded, mature oocytes were transferred into 50 µl drops of bicarbonate-buffered synthetic oviductal fluid (SOF) enriched with 20% (v/v) heat-inactivated oestrous sheep serum, 2.9 mM calcium lactate and 16 µM isotretinoin. Ram semen was thawed and washed in SOF with 4 mg/ml bovine serum albumin (BSA) at 20 g for 5 min. Fertilization was carried out at a final concentration of 5 x 10⁶ oocytes/ml and fertilized oocytes were left in 5% CO2 at 38.5°C overnight.

Presumptive zygotes were transferred into 20 µl drops of SOF enriched with 1% (v/v) minimum essential medium (MEM) with non-essential amino acids (Gibco Life Technologies), 2% (v/v) basal medium eagle (BME) with essential amino acids, 1 mM glutamine and 8 mg/ml BSA and covered with mineral oil washed in SOF. Embryos were cultured in a humidified atmosphere of 5% CO2, 7% O2 and 88% N2 at 38.5°C. On Day 3, the medium was changed to SOF with 1.4 mM glucose and on Day 5, 10% of charcoal-stripped FBS was added.

**Experimental design**

At Day 7, embryos, which had developed to the blastocyst stage, were transferred into base medium made up of 50% (v/v) TC1-199, 50% (v/v) DMEM (Gibco) with 1 mM l-glutamine, 0.3 mM sodium pyruvate, 5 µl/l gentamycin and 10% of charcoal-stripped FBS. They were then divided randomly into the following three groups: control group, embryos grown in base medium alone; AEA group, in which 28 nM AEA and 20 nM SR141716A, an antagonist of CB1-R, were added. All embryos were incubated in 5% CO2, 7% O2, 88% N2 at 38.5°C for 48 h, and only hatched blastocysts were subsequently used for the different experimental procedures. The concentration of 28 nM of AEA was chosen according to Wang et al. (1999), who reported that 28 nM of AEA inhibited trophoblast differentiation while 7–14 nM AEA accelerated trophoblast growth.

**TUNEL assay**

The TUNEL (Terminal Deoxynucleotidyl Transferase-mediated dUTP nick-end labelling) assay, to quantify apoptosis, was performed according to the manufacturer’s instructions (Qiogene Inc., Carlsbad, CA, USA).

**Viability assay**

To determine the rate of cell necrosis, blastocysts were treated with 0.5 mg/ml PI for 5 min at 38.5°C and counterstained with Hoechst 33342 for 10 min at room temperature. Blastocysts were then
mounted on slides and observed under a fluorescent microscope (Nikon, Tokyo, Japan).

*BrdU* incorporation

Measurement of the proliferation rate was conducted using 5-bromo-deoxyuridine (BrdU), a thymidine analogue, which is incorporated only by cells undergoing DNA synthesis, as described by Campbell et al. (1993). Blastocysts were incubated in 100 μM of BrdU in a humidified atmosphere of 5% CO2, 7% O2 and 88% N2 at 38.5°C for 4–6 h. Blastocysts were then washed in 0.4% PVP in PBS, transferred to coverslips, air dried, and finally fixed in methanol at –30°C for 20 min. Blastocysts were treated at room temperature with 0.1% Triton X-100 for 2 min and then with 4 M hydrochloric acid for 30 min. Slides were incubated with mouse monoclonal anti-BrdU (1:10) at 4°C overnight and then with a secondary rabbit anti-mouse IgG antibody (Chemicon International Inc., Temecula, CA, USA) (1:200) at room temperature for 2 h. After treatment with Hoechst 33,342, coverslips were mounted and observed.

Reversibility assay

After the 48 h incubation with AEA, to evaluate the reversibility of the effect of AEA on cell proliferation, the control and AEA + SR1 groups were transferred into fresh base medium. On the other hand, the AEA group was divided into two sets: one in which AEA was left and the other in which AEA was replaced by fresh base medium. All embryos were incubated for another 48 h, followed by analysis of BrdU incorporation, as described above.

Statistical analysis

Statistical analysis was performed by one-way ANOVA and only differences with *P* < 0.05 were considered significant. Results are presented as mean ± SEM of at least five experimental trials. Biochemical data are reported as means ± SD of at least three independent experiments each performed in duplicate. Statistical analysis of the biochemical data was performed with the non-parametric Mann–Whitney *U* test using the InStat 3 programme (GraphPAD Software for Science, San Diego, CA, USA). Only differences with *P* < 0.05 were considered significant.

Results

The endocannabinoid system in the sheep reproductive tract

The main components of the endocannabinoid system that metabolize or bind to AEA (i.e. FAAH, NAPE-PLD and CB1-R) were expressed and functional in sheep uteri. Levels of activity of the two AEA-metabolizing enzymes varied according to the period of the oestrous cycle (middle and late) (Table I). Specifically, FAAH hydrolytic activity decreased approximately by half during the late period (from 222 ± 20 to 102 ± 10 pmol/min/mg protein), whereas NAPE-PLD activity almost doubled during the late compared with the middle period of the oestrous cycle (from 40 ± 3 to 25 ± 3 pmol/min/mg of protein). FAAH endogenous levels in the uterus increased about 2-fold (from 1.05 ± 0.10 to 1.73 ± 0.14 pmol/mg protein) during the late period of the oestrous cycle. These findings suggest that both a reduced hydrolysis and an increased synthesis contributed to the observed increase in AEA uterine concentration. Western blot analysis further demonstrated the presence of CB1-R, but not of CBR-2, and the presence of FAAH in the sheep uterus (Fig. 1A). CB1-R expression was almost identical in the middle and late stages of the ovulatory cycle, whereas FAAH expression was higher in the middle than in the late oestrus period (Fig. 1A), consistently with the data on the enzymatic activity (Table I). The lack of commercially available anti-NAPE-PLD antibodies did not allow us to extend our analysis to this protein.

CB1-R expression in sheep blastocysts

With an antibody raised against the N-terminal part of human CB1-R, we showed that in hatched sheep blastocysts CB1-R was localized mainly in TE cells (Fig. 1B).

The effect of AEA on sheep blastocyst development

Apoptosis

The rate of apoptosis was significantly higher in hatched blastocysts exposed to AEA (5.4 ± 0.4%) than that in controls (2.2 ± 0.4%) (*P* < 0.05; Table II). On the contrary, the group, in which blastocysts were co-incubated with AEA and SR141716A, showed rates of apoptosis comparable to those

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### Table I. The endocannabinoid system in uterus during the middle (Days 8–12) and late (Day 17) oestrous cycle of sheep.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Middle oestrous cycle</th>
<th>Late oestrous cycle</th>
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<tbody>
<tr>
<td>FAAH activity^1</td>
<td>222 ± 20</td>
<td>102 ± 10^2</td>
</tr>
<tr>
<td>Endogenous AEA^2</td>
<td>1.05 ± 0.10</td>
<td>1.73 ± 0.14^3</td>
</tr>
<tr>
<td>NAPE-PLD activity^3</td>
<td>25 ± 3</td>
<td>40 ± 3^3</td>
</tr>
<tr>
<td>CB1-R binding^4</td>
<td>47 ± 5</td>
<td>44 ± 5</td>
</tr>
</tbody>
</table>

^1Expressed as picomole per minute per milligram protein (substrate was 5 μM [3H]AEA). ^2Expressed as picomole per milligram protein. ^3Expressed as picomole per minute per milligram protein (substrate was 100 μM [3H]NArPE). ^4Expressed as femtomole per milligram protein (ligand was 200 μM [3H]CP55 940). *P* < 0.01 versus middle oestrous cycle. ^5P* < 0.05 versus middle oestrous cycle.

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### Figure 1: Immunolocalization of CB1-R in sheep uterus and blastocysts.

(A) Western blot analysis of CB1-R, CB2-R and FAAH in a sheep uterus. Mouse brain, spleen or purified FAAH were used as positive controls. (B) CB1-R expression in hatched sheep blastocysts. CB1-R positive cells are in green (FITC) and nuclei in red. (Top panel) CB1-R positive blastocysts. (Middle panel) Blastocysts incubated without primary antibody (negative control). (Bottom panel) Human lymphocytes (positive control).
of the control group (2.8 ± 0.4%). Moreover, the distribution of apoptotic cells appeared to differ in the three groups. They were localized mainly in the inner cell mass (ICM) in the control and AEA+SR1 groups, while in AEA-exposed blastocysts they were present both in the ICM and TE (Fig. 2).

**Viability**
AEA did not appear to influence the rate of necrotic cells in hatched blastocysts, as the three groups did not show any significant difference in the percentage of PI-positive cells (Table II, Fig. 3).

**Proliferation**
AEA had an inhibitory effect on cell proliferation in hatched blastocysts, as the rate of cells undergoing DNA synthesis was significantly lower in the AEA group (19.2 ± 2.9%) in comparison to the control (27.8 ± 2.1%) and AEA+SR1 groups (29.5 ± 1.3%) (P < 0.05) (Table II, Fig. 4).

**Reversibility of AEA anti-proliferative effect**
We then asked whether AEA inhibition of cell proliferation could be reversed. With this aim, after exposure to AEA for 48 h, we split the AEA group into two. In the first half, we replaced AEA with fresh medium, whereas in the second half, we left AEA for another 48 h. At the end of this second period of incubation, we checked again the rate of BrdU incorporation. The rate of cell proliferation in blastocysts, in which AEA was removed, was not different from that of the control group (Table III). On the other hand, in the group in which blastocysts were kept continuously in AEA, the number of proliferating cells was decreased (Table III).

### Table II. Effect of AEA on cell proliferation (BrdU uptake), apoptosis (TUNEL assay) and viability (PI staining) in sheep blastocysts.

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Treatment</th>
<th>Treatment/total cells (mean ± SEM)</th>
<th>%, positive cells</th>
</tr>
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<tbody>
<tr>
<td>Cell proliferation</td>
<td>AEA</td>
<td>19.2 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AEA + SR141716A</td>
<td>29.5 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>27.8 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>AEA</td>
<td>5.4 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AEA + SR141716A</td>
<td>2.8 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2.2 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Viability</td>
<td>AEA</td>
<td>4.7 ± 0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AEA + SR141716A</td>
<td>4.0 ± 0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>5.4 ± 0.4</td>
<td></td>
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</table>

AEA, anandamide. One-way ANOVA. <sup>a,b</sup>Values with different superscripts within same column differed significantly (P < 0.05).

Figure 2: Effect of AEA on programmed cell death in sheep blastocysts.
Nuclei were stained using Hoechst 33342 (left column). Apoptotic nuclei were detected by TUNEL assay (TUNEL positive cells in green, middle column). Merged images are shown in the right column. We observed a higher number of apoptotic cells in the AEA group compared to controls (control and AEA+SR1 groups). Blastocysts treated with staurosporine (an inducer of apoptosis) were used as a positive control. All blastocysts used for the analysis were hatched.

Figure 3: AEA does not influence the rate of necrosis in sheep blastocysts.
Hatched blastocysts were stained with PI to check for cell viability (PI-positive, necrotic cells are in pink). Comparable rates of necrosis were observed in all groups independently of the treatment.

Figure 4: Cell proliferation is inhibited by exposure to high doses of AEA.
BrdU-positive cells are in green. The AEA group showed fewer proliferating cells than the control and AEA+SR1 groups. The left column shows nuclear staining by Hoechst 33342, while the proliferating, BrdU-positive cells are indicated by FITC in the right column.
Table III. Reversibility of the effect of AEA on cell proliferation in sheep blastocysts assessed by BrDU incorporation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% positive cells/total cells mean ± SEM</th>
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<tbody>
<tr>
<td>AEA 96 h</td>
<td>15.1 ± 1.1*a</td>
</tr>
<tr>
<td>AEA 48 h/−AEA 48 h</td>
<td>29.0 ± 3.2*b</td>
</tr>
<tr>
<td>Control 96 h</td>
<td>33.7 ± 4.1*b</td>
</tr>
</tbody>
</table>

AEA, anandamide. One-way ANOVA. a,bValues with different superscripts within same column differed significantly (P < 0.05).

Discussion

In this study, we show that the endocannabinoid system, i.e., AEA, its receptor (CB1-R) and its metabolic enzymes (NAPE-PLD and FAAH), is present and active in the sheep reproductive system. Indeed, sheep uterus and blastocysts express CB1-R, the main trigger of AEA-mediated signalling, as already shown in mouse uterus and blastocysts (Yang et al., 1996). These data suggest that endocannabinoids may be considered a conserved system among mammalian species (i.e., mouse, humans and sheep) despite their different implantation mechanisms. Our results indicate that in sheep, like in other mammals (Wang et al., 2006a), the biological activity of AEA is subjected to a ‘metabolic control’ in order to lower its concentration during the stage that corresponds to the development of the blastocyst. Indeed, the synthetic (NAPE-PLD) and hydrolytic (FAAH) enzymes regulate the effects triggered AEA, by controlling its endogenous tone.

Various signalling pathways are regulated by the G-protein-coupled receptor CB1-R, leading to different cellular responses. Indeed, AEA has been reported to have an anti-proliferative effect in human breast carcinoma cells, causing arrest at the G1/S transition (De Petrocellis et al., 1998). In lymphocytes, AEA seems to be involved in inhibiting proliferation and inducing apoptosis (Schwarz et al., 1994). Here, we show that these two fundamental cell processes, apoptosis and proliferation, are similarly influenced also in sheep blastocysts by AEA, at a concentration of 28 nM. In elucidating, the nature of the effects of AEA on sheep blastocysts, it was of fundamental importance to distinguish between necrosis and apoptosis. Necrosis is accidental cell death caused by cellular injury that affects large groups of cells, leading to an inflammatory response (Wyllie et al., 1980). In contrast, apoptosis is a regulated, physiological type of cell death that does not release cell debris. We show that the necrotic index of blastocysts was not influenced by the addition of AEA, indicating that AEA does not have a generic toxic effect, but rather a specific, CB1-R-mediated effect, which activates selective signalling pathways leading to apoptosis.

In preimplantation embryos, the presence of a certain number of apoptotic cells is physiological (Hardy, 1997). It is an active and necessary process that helps in removing selectively damaged or abnormal blastomeres/cells, thus allowing the continuation of embryo development (Hardy et al., 2003). However, despite these beneficial effects, apoptosis may have overall detrimental effects if the number of apoptotic cells is elevated too much compared with that of normal cells (Byrne et al., 1999; Levy et al., 2001). Depending on this ratio, an embryo will either continue or arrest its development, and so the incidence of death cells can be used as an indicator of the embryo’s quality (Maddox-Hyttel et al., 2003).

The exposure of sheep blastocysts to 28 nM AEA increased the number of apoptotic cells. The observation that the CB1-R antagonist SR16417A effectively blocked the effect of AEA, confirmed that CB1-R mediates this apoptotic effect. Moreover, we observed that, in control blastocysts, most apoptotic cells appeared to be localized in the ICM, consistent with previous observations in mouse, human (Hardy, 1997) and bovine blastocysts (Byrne et al., 1999; Gjorret et al., 2003). On the other hand, in AEA-exposed blastocysts, apoptotic cells were randomly distributed in both ICM and TE. The increased apoptotic index in the TE, which gives rise to extraembryonic tissues, suggests the participation of AEA in implantation. It has been proposed that both the embryo and the maternal reproductive tract produce ‘survival factors’ that regulate apoptosis in the preimplantation embryo (Brison and Schultz, 1997). Perhaps, AEA, at physiological doses, is one of these ‘survival factors’ that influence the fate of the preimplantation embryo by affecting implantation and therefore, the establishment of pregnancy.

It is believed that embryos with a larger number of cells are more likely to implant and to give rise to viable offspring (Van Soom et al., 1997). Thus, the rate of cell proliferation, as for apoptosis could also be considered an indicator of embryo quality. In this study, we show that blastocysts treated with high doses of AEA have fewer proliferating cells. The CB1-R antagonist effectively blocked the inhibitory effect of AEA, confirming its specific action through CB1-R. This finding suggests that high concentrations of AEA prevent implantation of embryos by blocking their development. Interestingly, a similar cell proliferation inhibition/arrest takes place in embryos from mammals that possess the ability to undergo dormancy. Several species can delay implantation using this mechanism, including mice. It has been reported that during dormancy, the uterus contains higher levels of AEA than the receptive uterus in which down-regulation of AEA levels normally occurs (Paria et al., 2001). In mice, the maintenance of high levels of exogenously administered cannabinoids blocks uterine receptivity for implantation, and as a consequence, dormant blastocysts are present in the uteri (Paria et al., 2001, 2002). The relatively high dose of AEA (Wang et al., 2003) used in our experiments specifically decreased the proliferation rate of sheep blastocysts, similarly to that which happens in dormant mouse blastocysts.

Synchronized development of the embryo to the blastocyst stage and of the uterus to the receptive state happens only during a limited time span called the ‘window’ of implantation (Paria et al., 1993). AEA seems to play an important role in regulating this process by synchronizing trophoblast differentiation and uterine receptivity in mouse and humans (Wang et al., 2006a). The coordinated development of embryo and uterus is a key, common step for the establishment of pregnancy among various mammals in spite of their dissimilar types of implantation mechanisms. A large number of pregnancy losses in mammalian species are due to death of the preimplantation embryo or implantation failure (King, 1991).
Such a high rate of failure during the early stages of pregnancy suggests the existence of selective mechanisms that arrest gestation when conditions are less than optimal (Wilcox et al., 1988).

In this study, we show also that the AEA effect on cell proliferation can be reversed, as the transfer of AEA-exposed blastocysts into fresh, AEA-free medium allowed the resumption of growth with a proliferation rate comparable to that of control embryos. This observation indicates an important ability of these embryos to recover and to resume normal growth. This feature is crucial for embryos from species with delayed implantation; however it can also be relevant for any embryo if the suboptimal conditions for its development could be overcome. In theory, the rescue of an embryo which did not start to implant on time due to the presence of high uterine AEA concentrations is possible by transferring to an adequately synchronized foster mother. Another possibility could be the attenuation of the adverse uterine condition (high AEA concentration), thus making feasible subsequent recovery and implantation of the embryo to the uterus of the mother.

In conclusion, unbalanced signalling by AEA, via CB1-R, may be associated directly or indirectly with the failure of pregnancy, although it is yet unclear how AEA signalling could be triggered to participate in cell growth or death (Guzman, 2005; Maccarrone, 2006). Indeed, by inducing apoptosis and inhibiting cell proliferation it lowers the quality of blastocyst, thus making them incompetent for implantation. On the other hand, restoration of proliferation in blastocysts, thus making them incompetent for implantation.

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