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Life Sciences 71 (2002) 1623–1632

Life Sciences

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Effects of anandamide on embryo implantation in the mouse

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Received 16 January 2002; accepted 7 March 2002

Abstract

Anandamide (N-arachidonoylethanolamine), an arachidonic acid derivative, is an endogenous ligand for both the brain-type (CB1-R) and spleen-type (CB2-R) cannabinoid receptors. To investigate the possible effects of anandamide on embryo implantation in the mouse, we used a co-culture system in which mouse embryos are cultured with a monolayer of uterine epithelial cells. Our results indicate that 14 nM anandamide significantly promotes the attachment and outgrowth of the blastocysts on the monolayer of uterine epithelial cells, and those effects could be blocked by CB1-R antagonists SR141716A, but not by SR144528, a CB2-R antagonist. It suggests that the effects of anandamide on embryo attachment and outgrowth are mediated by CB1-R. However, 56 nM anandamide is capable of inhibiting the blastocyst attachment and outgrowth, we, therefore, conclude that anandamide may play an essential role at the outset of implantation.

Keywords: Anandamide; Co-culture; Embryo implantation; Mouse

Introduction

Anandamide (ANA) is one of the endogenous ligands for cannabinoid receptors, isolated from brain and peripheral tissues [1,2]. This compound binds with high affinity to brain-type (CB1-R) and spleen-type (CB2-R) cannabinoid receptors and mimics most of the effects of (−)Δ9-tetrahydrocannabinol [(−)THC], a psychoactive derivative of marijuana [3–5]. Marijuana has been reported to have adverse effects on reproduction, including retarded embryo development, fetal loss and pregnancy failure [6,7].

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PII: S0024-3205(02)01928-8

However, mouse uterus contains the highest levels of anandamide, and the levels are lower at the implantation sites, but higher at the interimplantation sites [8]. Furthermore, in mouse uterus, down-regulation of anandamide levels has been associated with uterine receptivity, while up-regulation correlates with uterine refractoriness to embryo implantation. Altogether, the above findings suggest that anandamide might play an important role during embryo implantation in the mouse.

Preimplantation mouse embryos expressed both CB1-R and CB2-R mRNA, the levels of the former in the embryo is much higher than those found in the brain [6,9]. Also, activation of CB1-R by cannabinoid ligands interferes with preimplantation embryo development, and the specific CB1 receptor antagonist might block this effect [10]. These studies suggested that embryonic arrest in response to cannabinoid might be mediated by CB1 receptor. However, both the CB1-R and CB2-R genes are expressed in the preimplantation mouse embryos, there is little information of which receptor is functional in the embryo attachment and outgrowth in the co-culture system.

Under culture conditions that plastic Petri dishes are precoated with fibronectin, 7 nM anandamide may accelerate the mouse embryonic trophoblast differentiation, but can inhibit differentiation at higher doses [11]. To further investigate the effects of anandamide on embryo implantation, in this work, an *in vitro* implantation model was established to compare the effects of different doses of anandamide on attachment and outgrowth of blastocysts on the monolayer of uterine epithelial cell, and to detect which receptor is involved in the embryo attachment and outgrowth in the co-culture system.

Materials and methods

Material

Anandamide was purchased from Sigma. SR141716 and SR144528 were gifts from Prof. Madeleine Mosse and Dr. Francis Barth (Sanofi Recherche, France), respectively. All test agents were dissolved in ethanol and diluted with Ham's F-12 medium (Gibco, Rockville, MD, USA). The final ethanol concentration was less than 0.01%.

Animal

Kunming white strain mice [12–14] (Experimental Animal Center, the Institute of Genetic Science, CAS) were housed in the animal facility of the State Key Laboratory of Reproductive Biology. Adult female mice (25–30 g, 5–8 weeks old) were mated with males of the same strain at room temperature and with a constant photoperiod (light:dark cycle, 14 h:10 h). Food and water were freely available.

Uterine epithelial cell preparation and culture in vitro

On Day 4 of pregnancy, preparation of a monolayer of uterine epithelial cells were carried out using the method reported by Zeng [15], the protocol in brief was as follows:

Female mice were injected intraperitoneally with 5 IU of pregnant mare serum gonadotropin (PMSG) and 48 h later with 5 IU of human chorionic gonadotropin (hCG). Following hCG

injection, each female was caged with a male mouse overnight. The morning when a vaginal plug was found was designated Day 1 of pregnancy. Uteri from Day 4 pregnant mice were split longitudinally to expose the epithelial cell surface, and digested with 0.6% trypsin (Sigma) solution at 4 °C for 2 h, followed by another 0.5 h at 25–30 °C. Tissues were gently shaken to dislodge the epithelium from the endometrial bed. The epithelial cells and fragments were collected by centrifugation at $500 \times g$ for 10 min. Cells were washed three times with Ham's F-12 containing 2.20 mM calcium lactate, 2.05 mM glutamine, 12.5 mM $NaHCO_3$ and 400 IU/ml gentamicin sulfate, and then resuspended in Ham's F-12 with 10% fetal calf serum (FCS, Sigma). The cell suspension adjusted to 1×10^6 cells/ml was placed on 24-well Falcon plates and incubated at 37 °C, 5% CO_2 in a humidified chamber. The culture medium was changed to remove unattached cells and cell debris after 24 h.

Embryo collection

Preimplantation blastocysts were flushed from the uterus of Day 4 pregnant mice with Hank's solution and washed three times with Ham's F-12 medium, then transferred in droplets of preheated medium (the same as that used for cell culture, except that 10% FCS was replaced by 0.4% bovine serum albumin (BSA, Sigma) and 3.67×10^{-6} M estrogen (E_2)), and incubated at 37 °C as described above.

Embryos and uterine epithelial cell co-culture

Epithelial cells harvested as described above were placed in 24-well sterile plastic plates and cultured under the same conditions as above. The next morning, a monolayer of the uterine epithelial cells had formed. After the monolayer was rinsed three times in F-12 medium, the co-culture medium was added, which contained Ham's F-12 medium supplement with 0.4% BSA and a specific concentration of anandamide. At the same time, Blastocysts pre-cultured in either culture medium or antagonist were placed in corresponding wells of monolayer of uterine epithelial cells, respectively (about 40 blastocysts per well), and their attachment and outgrowth were observed.

Criteria for attachment and outgrowth of blastocysts

At 12 h intervals, the attachment or outgrowth was observed using phase-contrast microscopy (Olympus, Japan) and defined according to the following criteria: after the plate was shaken for 20 s with one rotation per second, if the blastocyst was found to stay at the same place, this blastocyst was designated as 'attachment' (Fig. 1A); if not, it was designated as 'non-attachment' [16]. After attachment, blastocysts began to outgrow outwards. When primary giant trophoblast cells were visible around the attachment site of the attached blastocysts, we designated the blastocysts as 'outgrowth' (Fig. 1B) [17].

Anandamide treatment

To study effects of anandamide on the blastocyst attachment and outgrowth, Day 4 blastocysts were cultured in 1ml Ham's F-12 culture medium in the absence or presence of indicated concentration

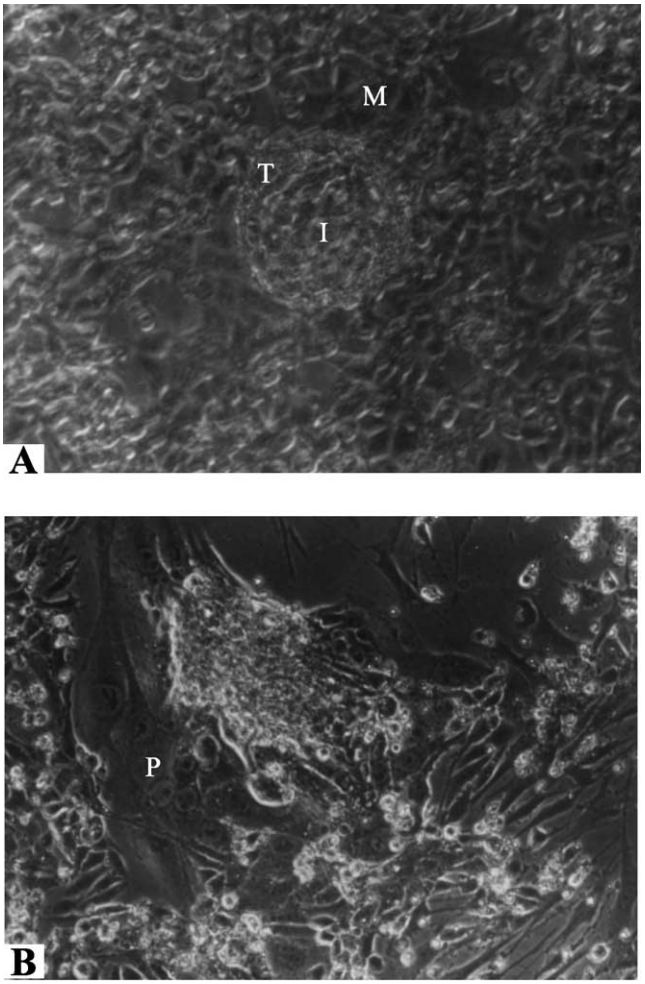

Fig. 1. Attachment and outgrowth of mouse blastocysts on a monolayer of uterine epithelial cells. (A) attached blastocysts; (B) trophectoderm outgrowth. M = monolayer of uterine epithelial cells; I = inner cell mass; T = trophoblastocyst; P = primary trohphoblast giant cell.

anandamide. Anandamide was dissolved in ethanol and diluted with Ham' F-12 culture medium. The final ethanol concentration was less than 0.1%, the control contained the same concentration of ethanol. To identify the responsible receptor subtypes mediated anandamide-induced responses, blastosysts were precultured with 8nM SR141716, a CB1-R antagonist, or SR144528, a CB2-R antagonist 1h before the culture with 14nM anandamide [9, 11]. The attachment and outgrowth of the blastocysts were observed every 12 h, the rate of attachment and outgrowth were recorded.

Statistical analysis

All experiments were repeated at least three times. All the results are shown as mean $\pm$ SE and data were analyzed using percentage t-test [12]. The value of $P < 0.05$ was considered to be significant.

Results

14 nM anandamide promotes blastocyst attachment

To determine the pharmacological effect of anandamide on blastocyst attachment, an *in vitro* co-culture system was used to test the effect of 14 nM anandamide on attachment of mouse blastocysts to the monolayers of uterine epithelial cell. Data were collected at different times (12, 24, 36 h) after hatched embryos were transferred onto the monolayer of the uterine epithelial cells. Blastocysts began to adhere to a monolayer of uterine epithelial cells after 6 h of co-culture in both the control and anandamide-treated cultures. However, different efficiencies of adhesion were observed after 12 h co-culturing. Adhesion of blastocysts treated with 14 nM anandamide had significantly higher rates of adhering to the monolayer than those of control after 12 h ($P < 0.01$) and 24 h ($P < 0.05$).

14 nM anandamide promotes blastocyst outgrowth

The outgrowth occurred after 12 h of co-culture, but there were significant differences between treatment and control groups after 48h of co-culture ($P < 0.05$) and 72 h ($P < 0.01$) (Fig. 3).

56 nM anandamide delays blastocyst attachment, preventing blastocyst outgrowth

Wang et al. [11] reported previously that higher (28 nM) anandamide might inhibit trophoblast differentiation and have adverse effects on fibronectin-binding assay (FBA) under the culturing condition that plastic Petri dishes are precoated overnight with 50ug/ml FN-120. In order to investigate whether high concentration anandamide has any effect on blastocyst attachment and outgrowth on the monolayer of uterine epithelial cells, blastocysts were cultured in medium containing 28 and 56 nM anandamide, and attachment and outgrowth rates were estimated after 12 and 48 h of culture, respectively. Our results indicate that there are no significant differences between treatment and control

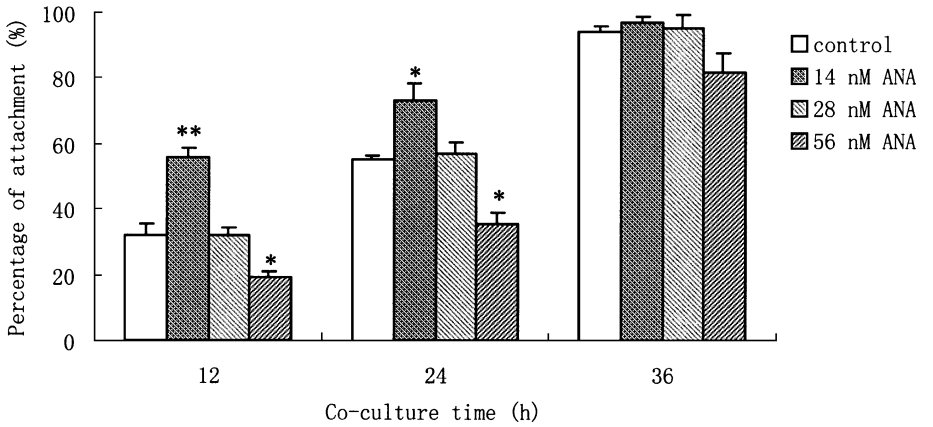

Fig. 2. Effects of 14 nM and 56 nM anandamide on the percentage of blastocyst attaching to a monolayer of uterine epithelial cells in the mouse. Results are expressed as mean ± SE of three replicates (n = 40 blastocysts per well). * and ** indicate significant differences from the control ($P < 0.05$ and $P < 0.01$, respectively).

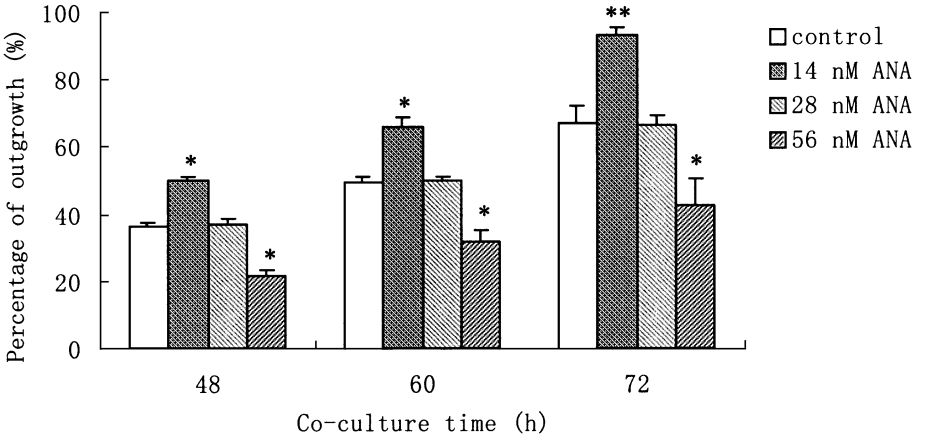

Fig. 3. Effects of anandamide on the percentage of hatched blastocysts with outgrowth on a monolayer of uterine epithelial cells in the mouse. Blastocysts from Day 4 mice were co-cultured with a monolayer of uterine epithelial cells in the medium containing a range of concentrations of anandamide. Results are expressed as mean ± SE of three replicates (n = 40 blastocysts per well). * and ** indicate significant differences from the control ($P < 0.05$ and $P < 0.01$, respectively).

culture treated with 28 nM anandamide on attachment and outgrowth. However, 56 nM anandamide decreased blastocyst attachment ($P < 0.05$) after 12, 24 hrs of co-culture. Interestingly, responses of treated and control cultures after 36 h were identical (see Fig. 2), this observation suggests that 56 nM anandamide delays but does not prevent blastocyst attachment. However, blastocyst outgrowth was inhibited after co-culture of 48, 60, 72 hrs (see Fig. 3).

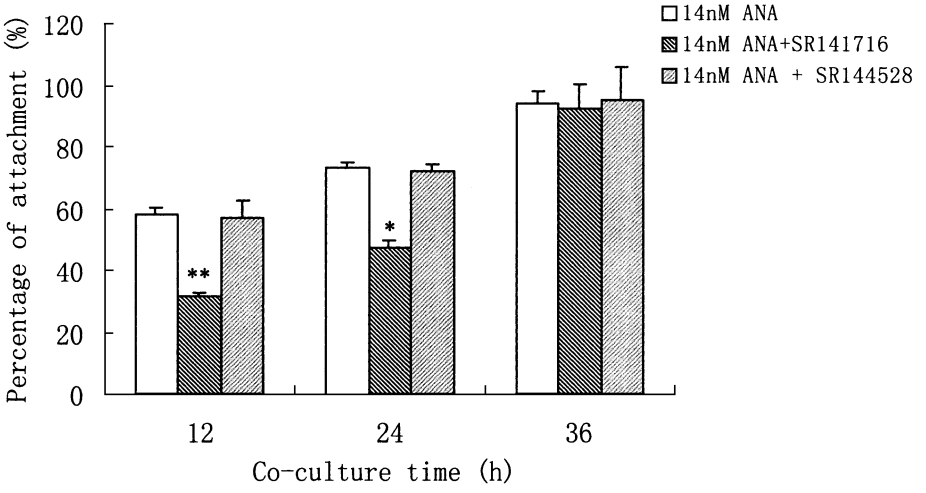

Fig. 4. Effects of SR141716 and SR144528 on the percentage of blastocysts attaching to a monolayer of uterine epithelial cells in the mouse. Results are expressed as mean ± SE of three replicates (n = 40 blastocysts per well). * and ** indicate significant differences from the control ($P < 0.05$ and $P < 0.01$, respectively).

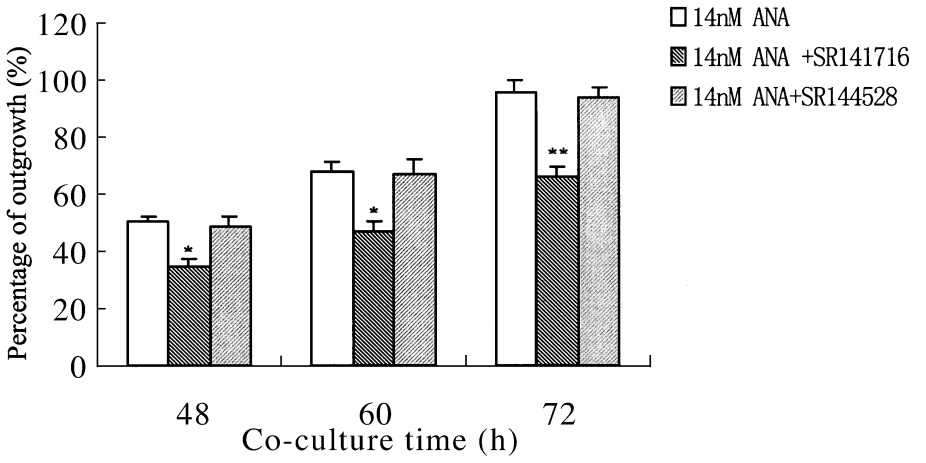

Fig. 5. Effects of SR141716 and SR144528 on the percentage of hatched blastocysts with outgrowth on a monolayer of uterine epithelial cells in the mouse. Results are expressed as mean ± SE of three replicates (n = 40 blastocysts per well). * and ** indicate significant differences from the control ($P < 0.05$ and $P < 0.01$, respectively).

Effects of 14 nM anandamide on embryo attachment and outgrowth are mediated by CB1-R, but not by CB2-R

To investigate whether both receptors are involved in anandamide-induced blastocyst attachment and outgrowth, we used specific antagonist to CB1-R (SR141716) or CB2-R (SR144528) in the *in vitro* model. After treatment of SR141716, attachment and outgrowth of blastocyst were significantly attenuated for 14 nM anandamide-treated embryos. In contrast, SR144528 did not noticeably attenuate anandamide-induced attachment and outgrowth (Figs. 4 and 5).

The results suggest that the activation of CB1-R, but not CB2-R, stimulates the attachment and outgrowth of blastocysts.

Discussion

More and more evidence indicates that anandamide, working as local mediator, is closely involved in regulation of embryo implantation [8]. The production of anandamide by the endometrium may be associated with various phase of uterine receptivity.

In this study, we show that 14 nM anandamide can significantly promote blastocyst attachment (Fig. 2) and outgrowth (Fig. 3). However, 56nM anandamide delays attachment and inhibits outgrowth of blastocysts. Wang J et al. [11] reported previously that blastocysts exhibited accelerated trophoblast outgrowth in culture to 7 nM anandamide, however with inhibition of differentiation at higher doses (28 nM anandamide). There exist some discrepancies between their results and ours, which may be due to different culture conditions. The co-culture model may provide a kind of microenvironment that is more similar to uterine environment *in vivo* than that of other models. In addition, it may be another reason that anandamide was partly hydrolyzed by fatty acid amide hydrolase (FAAH) which is a mammalian integral membrane enzyme existing primarily in uterine luminal and glandular epithelial cells [18].

The mechanism by which anandamide promotes or inhibits blastocyst attachment and outgrowth is not yet clearly understood. A key attribute of anandamide is its impact on multiple intracellular signaling pathways. Anandamide has been shown to activate focal adhesion kinase (FAK) in rat hippocampal slices in a fashion sensitive to the CB1 receptor-selective antagonist SR141716 [19]. FAK is a tyrosine kinase involved in the interaction between the integrins and actin-based cytoskeleton. Our lab demonstrated previously [20] that FAK played an important role during trophoblast cell attachment and outgrowth, and FAK could also promote the mouse ectoplacental cone (EPC) adhesion and outgrowth on fibronectin (FN). Apart from FAK, anandamide may also activate mitogen-activated protein kinase (MAPK) [21], which is involved in cell proliferation and differentiation. Similarly, low concentration of 2-arachidonoylglycerol (2-AG), by acting through CB1 receptor and phospholipase C activation, is shown to induce a transient increase in the intracellular Ca^{2+} concentration in undifferentiated N18TG2 and NG108 × 15 cells [22]. Whereas higher doses exhibit a more typical inhibition of Ca^{2+} influx [23]. It is necessary to further determine whether anandamide shares the same mechanism described as above on blastocysts.

In addition, some actions of anandamide may be not mediated by either of two known cannabinoid receptors subtype, but by CBn receptor (non-CB1/non CB2 receptor) [2]. Anandamide, in high concentrations, induces arachidonic acid release via phospholipase A_2 activation in both central and peripheral cell types [14,24] and inhibits gap junction-mediated and glutamate-triggered Ca^{2+} waves in astrocytes [25]. These effects are sensitive to pertussis toxin, but are not affected by SR141716. Therefore, it is possible that the CBn receptor, with low affinity for anandamide and high homology with CB1/CB2 receptors, is coupled to phospholipase A2 activation via adenylate cyclase inhibition and/or Gi proteins. At present time, it is still unknown whether such CBn receptor is expressed in blastocysts of the mouse.

SR141716 is considered to be a preferred antagonist to CB1-R and displays 1000-fold higher affinity for the central receptor (CB1-R) than for the peripheral receptor (CB2-R) [26,27]. Anandamide has been showed to interact equally well with CB1-R and with CB2-R [10] and SR141716 may inhibit effects of anandamide on attachment and outgrowth of blastocysts, from which we could speculate that effects of anandamide on embryo preimplantation is mediated by CB1-R. Paria et al reported that $CB1^{-/-}$ and $CB1^{-/-}/CB2^{-/-}$ double mutant embryos are resistant to anandamide with respect to embryo development and implantation *in vitro* and *in vivo*[28], which further testifies that CB1 receptor plays an important role by interaction with anandamide in co-culture system in which mouse embryos are cultured with a monolayer of uterine epithelial cells. Although the CB2-R mRNA is expressed in the mouse uterus and blasotcysts [6] and embryo development is asynchronous in $CB2^{-/-}$ mutant mice [28], its significance in preimplantation embryos remains obscure.

Because cannabinoids exerted a broad array of central and peripheral effects [29] including adverse effects on pregnancy and embryonic development [30,31], the widespread use of marijuana is a serious concern to society. Chronic administration of high doses of THC to animals lowered testosterone, impaired sperm production, motility and viability, and disrupted the ovulatory cycle [32,33]. It remain uncertain whether cannabis smoking has similar effects on human beings because the published evidence is little and inconsistent. However, several studies have suggested that women who continued to smoke cannabis during pregnancy might have a low-birthweight baby [34]. In addition, three studies have shown an increased risk of nonlymphoblastic leukaemia[35], rhabdomyosarcoma[36], and astrocytoma[37] in children whose mothers use cannabis during their pregnancies. Likely, cannabis abuse has adverse effects on reproductive functions of human beings. However, it is unknown whether such adverse action is mediated by cannabinoid receptors or by different mechanisms.

In conclusion, this study shows that anandamide plays an important role during embryo implantation in the mouse. Low concentration (14 nM) anandamide may promote attachment and outgrowth of Day 4 blastocysts on the monolayer of uterine epithelial cells, and this effect is mediated by CB1-R. Furthermore, high concentration (56 nM) delays but does not prevent blastocyst attachment and inhibit blastocyst outgrowth. These results further confirm the biphasic effects of anandamide on embryo implantation processes in the mouse.

Acknowledgements

We thank Prof. Madeleine Mosse and Dr. Francis Barth for generously providing us with SR141716 and SR144528, respectively. This research was supported by the Special Funds for Major State Basic Research Project (Grant No. G1999055903), the "Climbing Project of China" program (Grant No. 970211019-3), and the 100-Scientist-program of the Chinese Academy of Sciences.

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