Molecular and Cellular Endocrinology 411 (2015) 167–176

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Anandamide restricts uterine stromal differentiation and is critical for complete decidualization

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ARTICLE INFO

Article history:
Received 6 November 2014
Received in revised form 24 March 2015
Accepted 27 April 2015
Available online 7 May 2015

Keywords:
Anandamide
Uterus
Decidualization
Pseudopregnancy

ABSTRACT

The major endocannabinoid, anandamide (AEA), is widely distributed in the body, especially in the reproductive tissues, where it is implicated in early pregnancy events, particularly during implantation period. Although AEA is synthesized in decidual cells and showed to induce apoptosis through CB1 receptor, its roles in decidualization remain to be elucidated. This study examined the effect of AEA in the progression of decidualization both in vitro and in vivo and explored the involvement of COX-2 in its action. To determine the function of AEA during this differentiation process, we employed a primary culture system in which undifferentiated stromal cells isolated from pregnant rat uterus undergo decidualization. AEA treatment markedly interfered with the differentiation program, as revealed by α2-macroglobulin (α2-MG) expression and alkaline phosphatase activity. Additionally, it was evaluated the effects of AEA in decidual establishment in the pseudopregnant rat model. The abundance of AEA in the uterine lumen disrupted the decidualization process accompanied by a decreased expression of COX-2 and VEGF. It was also observed that uterine lumen, which failed the progression of decidualization in response to AEA, also presented lower expression of NAPE-PLD and FAAH. Thus, the mechanisms by which AEA inhibits decidualization can be either via direct actions on stromal cell differentiation within the reproductive tract system or by the inhibition of COX-2 derived products and, consequently, the vascular remodeling required to proper decidualization. In addition, the previous observations showing that higher AEA levels in pre-implantation sites are hostile to blastocyst survival may result from problems in decidual cell reaction more than with implantation failure.

1. Introduction

Endocannabinoids emerged as important mediators in various physiological functions, including reproduction (Karasu et al., 2011). The major endocannabinoid, anandamide (AEA), besides widely distributed in the body, is present in high amounts in the reproductive tissues and is a relevant mediator synchronizing embryo development, transport and implantation (Karasu et al., 2011). Additionally it was demonstrated that AEA induces decidual cell apoptosis through CB1 receptor, involving this molecule in maternal tissue remodeling in the fetoplacental unit (Fonseca et al., 2009, 2013).

Besides cannabinoid receptors (CB1 and CB2), AEA may also activate the transient receptor potential vanilloid type 1 (TRPV1) and the orphan G-protein-coupled receptor 55 (GPR55) (Pertwee et al., 2010). The efficiency of endocannabinoids depends on their metabolism by the target organ. It has been demonstrated that fatty acid amide hydrolase (FAAH), the enzyme that catalyses AEA hydrolysis to arachidonate and ethanolamine, is crucial for controlling AEA levels or function (Maccarrone et al., 2002). Although FAAH expression and activity were detected in human and mouse uterine epithelium, during the peri-implantation period, in mouse, FAAH activity is higher at the implantation sites than at the inter-implantation sites creating a suitable AEA environment for implantation (Wang et al., 2007).

One of the key processes in uterine receptivity is the progesterone-mediated differentiation of endometrial stromal cells into decidual cells, which is crucial for embryo survival through the early stages of implantation (Brosens and Gellersen, 2006). Contrary to humans, where decidualization occurs spontaneously during normal menstrual cycle, in rodents, the implanting blastocyst triggers the differentiation of the fibroblast stromal cells of endometrium into decidual cells (Fonseca et al., 2012). This differentiation process involves intense modifications in gene expression, cellular morphology and function. This process is also accompanied by secretion of various cytokines and hormones, being α2-macroglobulin (α2-MG), a protease inhibitor involved in the regulation of placental trophoblast invasion, the major product secreted by rat decidual tissue (da Silva et al., 1996; Gellersen et al., 2007). Besides α2-MG

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http://dx.doi.org/10.1016/j.mce.2015.04.024
0303-7207/

expression, an increase in alkaline phosphatase (ALP) activity in the uterine stroma is associated with the decidual cell reaction, characteristic of an increased metabolic activity (Christie, 1966). In rodents, decidualization can also be induced in hormone-primed animals, either by trauma or by intraluminal injection of oil (Rankin et al., 1979).

Implantation and decidualization is associated to an increase in endometrial permeability and angiogenesis. Cyclooxygenase derived prostanoids participate in the angiogenesis during implantation and decidualization by regulating the vascular endothelial growth factor (VEGF) signaling pathway (Matsumoto et al., 2002).

In fact, the involvement of prostaglandins (PGs) in the progression of decidualization is well known. The use of an inhibitor of cyclooxygenase (COX-2), the rate-limiting enzyme in PG synthesis, has detrimental effects on decidualization, whereas the supplementation of prostaglandin E2 (PGE2) or prostaglandin F2α (PGF2α) restores it (Hamilton and Kennedy, 1994; Kennedy, 1985; Kennedy and Lukash, 1982). Furthermore, COX-deficient mice also revealed multiple failures in several reproductive events during early pregnancy that include implantation and decidualization (Lim et al., 1997).

The molecular mechanisms of decidualization are still poorly understood and various critical factors controlling differentiation and decidua-specific hormone expression remain unrevealed. To address the function of AEA in decidualization, it was used as a primary culture system in which undifferentiated stromal cells isolated from pregnant rat uterus undergo decidualization. In addition, to evaluate the effects of AEA in the decidualization process, it was used an in vivo deciduoma model, mimicking the reaction of the uterus during blastocyst implantation (Finn and Keen, 1963).

2. Materials and methods

2.1. Animal care and use

All animal studies were performed with Direção-Geral de Alimentação e Veterinária (DGAV) approval and according to the European legislation on the use of laboratory animals. Sexually mature female rats (Wistar rats with 10–12 wk from Charles River Laboratories, Barcelona, Spain) were used. For experiments involving pregnant rats, the day on which a vaginal plug was detected is referred to as day 1 of pregnancy.

2.2. Isolation of stromal cells and induction of decidualization in vitro

Rat endometrial stromal cells (RES) were isolated and cultured as previously described (Matsumoto et al., 2009). RES were isolated from uterine horns of pregnant rats (day 5), which were dissected longitudinally to expose the uterine lumen and incubated with 0.5% trypsin (Invitrogen Corporation, Carlsbad, CA, USA) at 4 °C for 1 h. The partially digested tissues were then washed twice in Hank's balanced salt solution (HBSS) and placed in HBSS containing 1 mM EDTA, DNase I (200 U/ml) (Roche Diagnostics GmbH, Mannhein, Germany) and 0.05% trypsin at 37 °C for 30 min. Non-digested tissue was removed by filtration through a 60-μm mesh and endometrial stromal cells were collected by centrifugation at 200 *g* for 10 min.

The isolated cells were seeded in 96-well plates, 24-well plates, 6-well plates or 8-well chamber slides, at densities 5×10^4, 2.5×10^5, 7.5×10^5, or 7.5×10^5, respectively. Cells were then cultured in Dulbecco's modified Eagle's Medium-F12 medium (DMEM-F12; Gibco/Invitrogen Corporation, Carlsbad, CA, USA), containing 10% charcoal-stripped fetal bovine serum (FBS; Gibco/Invitrogen Corporation, Carlsbad, CA, USA) and supplemented with antibiotic–antimycotic solution (200 U/ml penicillin G, 0.5 μg/ml amphotericine B and 200 μg/ml streptomycin) (Gibco/Invitrogen Corporation, Carlsbad, CA, USA). After a 2 h attachment period, the medium was removed and replaced with fresh medium to eliminate epithelial cells. Cell cultures were maintained at 37 °C in humidified air with 5% CO2.

Decidualization of RES was induced by incubating confluent cells in DMEM-F12 medium containing 2% charcoal-stripped FBS with 100 nM medroxyprogesterone acetate (MPA) (Sigma Chemical Co, St. Louis, MO, USA) and 0.5 mM 8-bromo-cAMP (cAMP) (Sigma Chemical Co, St. Louis, MO, USA), in the presence or absence of varying concentrations of AEA (0.1–25 μM) (Tocris Bioscience, Bristol, UK) for 24–72 h. To study the involvement of cannabinoid receptors in AEA effects, cells were pre-exposed, for 30 minutes, to 1 μM of AM251, AM281 (CB1 antagonists) and AM630 (CB2 receptor antagonist) (AM251 and AM630 were from Tocris Bioscience, Bristol, UK and AM281 from Santa Cruz Biotechnology, Dallas, Texas, USA). The involvement of TRPV1 receptor was studied by pre-incubating cells with 1 μM of capsazepine (CPZ) or 20 nM of 5′-iodoresiniferatoxin (5′-iRTX) (both from Tocris Bioscience, Bristol, UK), two TRPV1 antagonists. Medium was replaced every 24 h. Purity of cultures was evaluated by cellular morphology and α2-MG expression was used as a decidual marker.

For morphological studies, cells were grown on chamber slides and observed by phase contrast microscopy and after Giemsa staining, whereas for Western blot and Q-PCR analysis, experiments were performed in 6-well plates.

2.3. Cell viability

RES were seeded in a 96-well culture plate in the absence or presence of AEA (0.1–50 μM) for 24, 48 and 72 h. After incubation, MTT (0.5 mg/ml final concentration; Sigma) assay was performed and lactate dehydrogenase (LDH) release was measured using the CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. For decidualized cells, after 48 and 72 h of in vitro decidualization, AEA was added (0.1–50 μM) and MTT assay and LDH release were performed. Cell viability and LDH release were analyzed by calculating the mean of five independent experiments performed in triplicate and expressed as a percentage of the untreated control cells.

2.4. Decidual alkaline phosphatase activity assay

The functional activity of the decidual cells was evaluated by measuring the alkaline phosphatase (ALP) activity as previously described (Costa et al., 2014). Briefly, cells from 24-well plates were rinsed with buffer and then incubated at 37 °C for 1 h with the pALP substrate p-nitrophenilphosphate, 2.5 mM (Sigma Chemical Co, St. Louis, MO, USA), in the presence of 100 mM $MgCl_2$ (Sigma Chemical Co, St. Louis, MO, USA). The reaction was stopped by the addition of ice-cold 0.02 M NaOH solution to the extracellular medium, and the absorbance quantified at 405 nm, in a Multiscan Ascent microplate reader. The p-nitrophenol (pNP) formed was determined by interpolation in a calibration curve of pNP (Sigma Chemical Co, St. Louis, MO, USA). Cells were lysed and protein was quantified by Bradford assay. A negative control was assessed by the incubation with L-phenylalanine (2 mM; Sigma Chemical Co, St. Louis, MO, USA), a pALP inhibitor, for 30 minutes before the addition of pALP substrate. The final results were expressed in relative values, in comparison with the untreated cells (control).

2.5. Artificially induced deciduoma

To generate deciduoma, mature female rats were mated with vasectomized males of the same strain to induce pseudopregnancy (day 1 is the day of detection of copulatory plug). In the first group