The endocannabinoid anandamide impairs *in vitro* decidualization of human cells

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Abstract

Endocannabinoids (eCBs) are endogenous mediators that along with the cannabinoid receptors (CB1 and CB2), a membrane transporter and metabolic enzymes form the endocannabinoid system (ECS). Several eCBs have been discovered with emphasis on anandamide (AEA). They are involved in several biological processes such as energy balance, immune response and reproduction. Decidualization occurs during the secretory phase of human menstrual cycle, which involves proliferation and differentiation of endometrial stromal cells into decidual cells and is crucial for the establishment and progression of pregnancy. In this study, a telomerase-immortalized human endometrial stromal cell line (St-T1b) and non-differentiated primary cultures of human decidual fibroblasts from term placenta were used to characterize the ECS using immunoblotting and qRT-PCR techniques. It was shown that St-T1b cells express CB1, but not CB2, and that both receptors are expressed in HdF cells. Furthermore, the expression of fatty acid amide hydrolase (FAAH), the main degrading enzyme of AEA, increased during stromal cell differentiation. AEA inhibited cell proliferation, through deregulation of cell cycle progression and induced polyploidy. Moreover, through CB1 binding receptor, AEA also impaired cell differentiation. Therefore, AEA is proposed as a modulator of human decidualization. Our findings may provide wider implications, as deregulated levels of AEA, due to *Cannabis sativa* consumption or altered expression of the metabolic enzymes, may negatively regulate human endometrial stromal cell decidualization with an impact on human (in)fertility.

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Introduction

Human endometrial stromal cells (HESCs) undergo waves of proliferation and differentiation into morphologically and functionally distinct cells called decidual cells. This process, named decidualization, occurs in women during the secretory phase of the menstrual cycle and prepares a receptive uterus for implantation. The decidual process is triggered by sustained activation of the second messenger cyclic adenosine monophosphate (cAMP) and the protein kinase A (PKA) pathway, which in turn sensitizes stromal cells to progesterone (Gellersen & Brosens 2014). In addition to uterine angiogenesis and homeostasis (Weimar *et al.* 2013), decidual cells participate in embryo recognition (Norwitz *et al.* 2001, Teklenburg *et al.* 2010), regulation of trophoblast invasion and modulation of immune and oxidative stress response at feto–maternal interface (Gellersen & Brosens 2003). Impaired decidualization, as a result of poor hormone responsiveness and/or impaired endometrial stromal cell growth and/or differentiation, is often associated with implantation failure, unexplained infertility, recurrent

spontaneous miscarriage and pregnancy disorders, such as preeclampsia or intrauterine growth retardation and premature birth (Strowitzki *et al.* 2006).

The endocannabinoids (eCBs), in particular anandamide (AEA) and 2-arachidonoylglycerol (2-AG), the best studied eCBs, are emergent modulators of several pathophysiological processes. Besides the endogenous cannabinoids, the endocannabinoid system (ECS) comprises the cannabinoid receptors (CB1 and CB2), the enzymes involved in the biosynthesis and degradation of eCBs, and a purported transporter (Fonseca *et al.* 2013b). AEA is mainly synthesized by *N*-arachidonoylphosphatidylethanolamine hydrolysing phospholipase D (NAPE-PLD), and, subsequently, hydrolysed to arachidonic acid and ethanolamine by the enzyme fatty acid amide hydrolase (FAAH) (Fonseca *et al.* 2013b). The latter is considered the major metabolic gatekeeper for AEA uterine levels throughout menstrual cycle and pregnancy (Maccarrone & Finazzi-Agro 2004, Habayeb *et al.* 2008). eCBs modulate basic biological processes,

including the choice between cell proliferation, death and differentiation (Fonseca *et al.* 2013a). In the rat model, it has been demonstrated that AEA interferes with decidual cell turnover by inducing apoptosis (Fonseca *et al.* 2013b) and limits uterine stromal cell differentiation (Fonseca *et al.* 2015). Nevertheless, the underlying mechanisms that govern the process of human endometrial decidualization and the involvement of the eCBs in this process remain poorly understood. Therefore, in this work, we aimed to investigate the role of the main eCB, AEA on *in vitro* decidualization process in two cell culture models, an immortalized human endometrial stromal cell line (St-T1b) and human decidual fibroblasts (HdF) derived from human term placenta. The ECS was characterized in non-differentiated and in cells undergoing decidualization, and the role of AEA in these cells was investigated.

Materials and methods

St-T1b cell line culture

The St-T1b was kindly supplied by Dr Birgit Gellersen from Endokrinologikum Hamburg, Hamburg, Germany. This cell line was obtained from uterine biopsy samples at the time of hysterectomy from benign gynaecological disorders and immortalized with human telomerase (hTERT). This cell line displays the typical morphological features of proliferative endometrial fibroblasts and expresses the appropriate lineage-specific markers such as vimentin and CD90 while being CK7-negative (Samalecos *et al.* 2009). Cells were maintained at 37°C with 5% CO₂ in DMEM/F-12 medium (Gibco Invitrogen Corporation, CA, USA) supplemented with 10%

charcoal-stripped foetal bovine serum (CT-FBS) (Gibco Invitrogen Corporation), 1 nM 17 β -oestradiol (E₂) (Sigma-Aldrich), 1 μ g/mL insulin (Sigma-Aldrich) and 2% penicillin–streptomycin–amphotericin B (Gibco Invitrogen Corporation). Initially, cells were incubated for 24 h in complete medium and then experiments were performed in DMEM/F-12 with 2% CT-FBS.

Primary cultures of HdF

HdFs isolated from term decidua are undifferentiated fibroblast-like cells of the endometrial stroma (Richards *et al.* 1995). The protocol for the isolation of fibroblast cells from term decidua was based on Richards *et al.* (1995). Caesarean or vaginal delivery normal human placentas (38–40 weeks of gestation) from Caucasian women, living in Porto region and aged 24–36 years old, were obtained from Centro Materno-Infantil do Norte, Porto. All the procedures using term placentas were performed according to the ethics committee of Centro Materno-Infantil do Norte. The decidua basalis was scraped from the chorionic membrane, dissected into small pieces and enzymatically digested in PBS with collagenase (1 mg/mL) for 1 h at 37°C with gentle shaking. The suspension was then centrifuged at 260g for 6 min. The pellet was resuspended with ammonium chloride (0.84%) solution for red blood cell lysis at 37°C. The resulted suspension was filtered through a 40 μ m nylon mesh and centrifuged at 260g for 10 min. The isolated cells were maintained at 37°C with 5% CO₂ in DMEM/F-12 medium supplemented with 10% CT-FBS, 1 nM E₂, 1 μ g/mL insulin and 2% penicillin–streptomycin–amphotericin B. After 24 h, the non-adherent cells were discarded and the adherent cells were grown to confluence and sub-cultured. The purity of primary HdF was confirmed by immunocytochemical analysis for the cytoskeletal proteins vimentin and cytokeratin-7, a fibroblast and an epithelial cell marker respectively (Fig. 1).

Figure 1 Study of the decidualization process in St-T1b and HdF cells after 3 and 6 days of exposure to decidualization stimulus. Morphological studies by Giemsa staining of St-T1b cell line, both in non-differentiated (non-dif) (A) and in cells exposed for 6 d to the differentiation stimulus (dif) (B). Similar to St-T1b cells, in HdF, non-differentiated cells (C) maintain the fibroblastic spindle shape, whereas after 6 days of exposure to decidual treatment, differentiated cells (D) became larger and with rounded nucleus. The purity of primary HdF was confirmed by immunocytochemical analysis of the expression of the cytoskeletal proteins vimentin (E) and cytokeratin-7 (F) respectively a fibroblast and an epithelial cell marker. Negative control obtained by omitting primary antibody is represented in the square. The steady-state transcript levels of *IGFBP1* and *PRL* were evaluated by qRT-PCR and shown to significantly increase in both St-T1b (G) and HdF cells (H), after 3 and 6 days of decidualization treatment. (* $P < 0.05$ vs Non-dif) ($n = 3$; two-way ANOVA, Tukey's multiple comparisons).

Differentiation treatment

In vitro differentiation was induced in minimal medium MM1 (DMEM/F-12 medium supplemented with 2% CT-FBS and 2% penicillin–streptomycin–amphotericin B) containing 1 µM medroxyprogesterone acetate (MPA; Sigma-Aldrich, MO, USA), 10 nM E₂ and with 0.5 mM 8-bromoadenosine 3', 5'-cAMP (Sigma-Aldrich) or 0.05 mM cAMP, for St-T1b and HdF respectively. The differentiation process was induced for 3 or 6 days (d), and the medium was changed every 3 d. Cell differentiation was confirmed by morphological analysis and mRNA levels of prolactin (PRL) and the insulin-like growth factor binding protein-1 (IGFBP1), the main secretory products of decidual cells, were evaluated by qRT-PCR. In order to investigate the role of AEA during the decidualization process, cells were treated with the differentiation medium (MM1 + cAMP, E₂ and MPA) in the presence or absence of AEA (10 µM) (Tocris Bioscience, Minneapolis, MN, USA). To explore the role of cannabinoid receptors, cells were pre-incubated with AM281, selective CB1 receptor antagonist/inverse agonist (1 µM; Tocris Bioscience) and AM630, CB2 antagonist/inverse agonist (1 µM; Tocris Bioscience) for 30 min before the addition of AEA (10 µM). At this concentration, the antagonists *per se* did not affect neither the decidual response nor the cell viability. Non-differentiated cells treated with MM1 were used as a control. Cells from four to ten sub-passages were used in the studies.

Cell viability and rate of DNA synthesis

HdF and St-T1b cells were plated at a density of 2.5×10^3 cells/well in 96-well plates and treated with AEA (10 µM) in non-differentiated medium (minimal medium, MM1: DMEM/F-12 medium supplemented with 2% CT-FBS and 2% penicillin–streptomycin–amphotericin B) and with

differentiation medium (MM1 + cAMP, E₂ and MPA). Cell viability was assessed by MTT assay after 6 d of treatment. The yellow tetrazole MTT (0.5 mg/mL final concentration) (Sigma-Aldrich) was added and cells were incubated at 37°C for 3 h. The resultant purple formazan was dissolved in a solution of dimethylsulphoxide:isopropanol (3:1) and spectrophotometrically quantified at 540 nm. To study the effects of AEA on DNA synthesis, [³H]-thymidine incorporation assay was performed. In the last 32 h of each exposure time, [³H]-thymidine (0.5 µCi) (Amersham) was added to cells. Cells were harvested and, after addition of a scintillation cocktail, [³H]-thymidine incorporation was determined in a scintillation counter (LS 6500; Beckman Instruments). Results are expressed as relative percentage of the untreated control cells. The results are the mean of at least three independent experiments carried out in triplicate.

Morphological studies

Morphological alterations induced upon differentiation treatment were evaluated by Giemsa staining. Both cell types were plated at a density of 1.5×10^4 cells/well in 24-well culture plate, and cells were exposed to differentiation treatment for 6 d. After treatment, cells were fixed with 4% p-formaldehyde in PBS for 20 min at 4°C and stained with Giemsa for 30 min. Cells were observed under the microscope Eclipse E400, Nikon equipped with image analysis software LeicaQwin (Cambridge, UK).

qRT-PCR analysis

The assessment of steady-state transcript levels was carried out by qRT-PCR. For these studies, cells were seeded at a cellular density of 5×10^4 cells/well in six well culture plate.

Table 1 Primer sequences for control and target genes. Detailed description of all genes tested, primer pairs' sequences and Q-PCR conditions.

Symbol	Accession number	Description	Primers (5'–3')	Amplification conditions [§]	T _m (°C)	Reference
IGFBP1	NM_000596.2	<i>Homo sapiens</i> insulin-like growth factor binding protein 1	S: GAGATAACTGAGGAGGAG AS: CCAAAGGATGGAATGATC	95°C, 03 s 59°C, 30 s 72°C, 90 s	78	(Samalecos <i>et al.</i> 2009)
PRL	NM_001163558.2	<i>Homo sapiens</i> prolactin	S: CTTTCATCCAGAAGTACCCT AS: TCTTCCAGATATTGGCTT	95°C, 03 s 60°C, 30 s 72°C, 90 s	80	
CB1	NM_001160259.1	<i>Homo sapiens</i> cannabinoid receptor 1	S: CTTCCACAGAAATCCC AS: TACCTTCCCATCCTCAGA	95°C, 03 s 62°C, 30 s 72°C, 90 s	87	Habayeb <i>et al.</i> (2008)
CB2	NM_001841.2	<i>Homo sapiens</i> cannabinoid receptor 2	S: CGTGGCTGTGCTCTATCTGA AS: ATCTCGGGGCTTCTTCTTT	95°C, 03 s 62°C, 30 s 72°C, 90 s	87	Habayeb <i>et al.</i> (2008)
FAAH	NM_001441.2	<i>Homo sapiens</i> fatty acid amide hydrolase	S: GGCCGTCAGCTACACTATGC AS: ATCAGTCGCTCCACCTCCC	95°C, 03 s 59°C, 30 s 72°C, 90 s	87	Habayeb <i>et al.</i> (2008)
NAPE-PLD	NM_001122838.1	<i>Homo sapiens</i> N-acyl phosphatidylethanolamine phospholipase D	S: AGATGGCTGATAATGAGAA AS: TTCTCCTCCACCAGTC	95°C, 03 s 58°C, 30 s 72°C, 90 s	72	
GAPDH	NC_000012.12	<i>Homo sapiens</i> glyceraldehyde-3-phosphate dehydrogenase	S: AGAACATCATCCCTGCCTC AS: GCCAAATTCGTTGTCATACC	95°C, 03 s 55°C, 30 s 72°C, 90 s	78	Habayeb <i>et al.</i> (2008)

[§]Cycling conditions were as follows: an initial step at 95°C 3 min for enzyme activation in all cases, followed by up to 40 cycles of denaturation, annealing and primer extension as described; *The primers for *IGFBP1*, *PRL* and *NAPE-PLD* were designed in-house using Primer Premier (version 5.0 software; Premier Biosoft International, Palo Alto, CA; www.premierbiosoft.com).

After treatment, the cells were collected in TRIzol reagent (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) and RNA and protein were extracted according to the manufacturer's instructions. St-T1b and HdF cells were treated with the differentiation medium for 3 and 6 d. To study the role of the AEA and the involvement of CB receptors on the decidualization process, cells were treated with differentiation medium in the absence or presence of AEA (10 µM) plus CB1/CB2 antagonists for 6 d. RNA was quantified in the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE, USA), its quality was evaluated with the Experion RNA StdSens Kit (Bio-Rad Laboratories) and was analysed with Experion analytical software (Bio-Rad Laboratories). cDNA was obtained by reverse transcription of RNA using the iScript Select cDNA Synthesis Kit (Bio-Rad Laboratories) and it was subsequently amplified with specific primers, using KAPA SYBR FAST qPCR Master Mix 2x Kit (Kapa Biosystems, Woburn, MA, USA) in MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories), according to the kit protocol. Table 1 shows the primer sequences and the qRT-PCR conditions. The specificity of PCR product amplification was assessed by analysis of the melting curve. Gene expression was normalized with two housekeeping genes, GAPDH and β -2 microglobulin. As both genes were stable, analysis was achieved by the calculation of $\Delta\Delta CT$ values using β -2 microglobulin as reference. The results are the mean of five independent experiments carried out in triplicate.

Immunocytochemistry

For immunocytochemistry studies, cells were seeded in 24-well plate on glass-coverslips, at a cellular density of 1.5×10^4 cells/well and fixed with cold methanol after treatment with differentiation stimulus for 6 d. The expression of proteins was analysed using an avidin–biotin alkaline phosphatase complex technique (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA). To prevent non-specific binding, slides were incubated in blocking serum (normal serum) and slides were incubated with primary antibodies vimentin (1:500, sc 6260; Santa Cruz Biotechnology) and cytokeratin-7 (1:100, M 7018; Dako) overnight at 4°C. It was followed by incubation with biotinylated secondary antibody and with Vectastain ABC-AP reagent, according to the manufacturer's instructions. The reaction was developed Sigma Fast Red™ tablets (Sigma-Aldrich). Mayer's haematoxylin solution (Sigma-Aldrich) was used as counterstaining and slides were mounted in Aquamount medium (BDH Laboratory Supplies, PI, UK). Negative controls were performed by the replacement of the primary antibodies by rabbit IgG. The results are the mean of three independent experiments.

Cell cycle analysis

Cells were plated at a density of 5×10^4 cells in six well dishes and exposed to the differentiation medium in the absence or presence of AEA (10 µM), plus CB1/CB2 antagonists for 6 d. After trypsinization, cells were centrifuged (260 g, 5 min, 4°C), resuspended in PBS and fixed in 70% cold ethanol. After fixation, cells were centrifuged and resuspended in 0.5 mL

DNA staining solution (5 µg/mL propidium iodide (PI), 0.1% Triton X-100 and 200 µg/mL DNase-free RNase A) overnight at 4°C. DNA content was analysed by flow cytometry based on the acquisition of 40,000 events (with a threshold of 100,000) in a BD Accuri C6 (Becton–Dickinson Jose, CA, USA) equipped with BD Accuri C6 software. Detectors for the three fluorescence channels (FL-1, FL-2 and FL-3) and for forward (FSC) and side (SSC) light scatter were set on a linear scale. Debris and cell aggregates were gated out, and singlets and doublets were analysed using a two-parameter plot of FL-2-Area to FL-2-Width of PI fluorescence. The anti-proliferative effects were indicated by the percentage of cells in G_0/G_1 , S and G_2/M phases of cell cycle.

The results are the mean of three independent experiments carried out in triplicate.

Western blotting

St-T1b cells and HdF (both non-differentiated and undergoing differentiation) were treated in the presence or absence of AEA (10 µM) in six well plates, at a cellular density of 5×10^4 cells/well. Cell extracts were prepared in a potassium phosphate buffer containing a cocktail of protease inhibitors [AEBF (4-(2-aminoethyl) benzenesulphonyl fluoride hydrochloride) at 104 mM, aprotinin at 80 µM, bestatin at 4 mM, E-64 at 1.4 mM, leupeptin at 2 mM and pepstatin A at 1.5 mM] (Sigma-Aldrich). Protein concentrations were measured by Bradford assay. Samples (20 µg) were run on 10% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking with 0.5% non-fat milk in TBS/Tween 80 for 1 h, membranes were incubated with the primary antibody (Table 2) at 4°C overnight. Membranes were then washed and incubated with rabbit anti-goat IgG or goat anti-rabbit IgG horseradish peroxidase-conjugated antibody and detected by enhanced chemiluminescence Western Bright™ ECL and exposed to X-ray film. β -tubulin was used as a loading control. To quantify and compare protein levels, the density of each band was measured by densitometry. The results are the mean of three independent experiments.

Evaluation of mitochondrial transmembrane potential ($\Delta\psi_m$)

For mitochondrial transmembrane potential ($\Delta\psi_m$) assessment, non-differentiated and cells undergoing decidualization were

Table 2 Summary of antibodies used for the Western blotting procedures. Antibodies were from Santa Cruz Biotechnology.

Protein	SDS-Page (%)	Primary antibody		Secondary antibody		Reference
		Source	Dilution	Source	Dilution	
CB1	10	Rabbit	1/100	Goat	1/1000	sc-20754
CB2	10	Rabbit	1/100	Goat	1/1000	sc-25494
NAPE-PLD	10	Goat	1/100	Rabbit	1/1000	sc-163118
FAAH	10	Goat	1/100	Rabbit	1/1000	sc-26427
Cyclin B1	10	Rabbit	1/200	Goat	1/1000	sc-752
pAkt	10	Rabbit	1/100	Goat	1/1000	sc-7985-R
Total Akt	10	Rabbit	1/200	Goat	1/1000	sc-8312
β -tubulin	10	Rabbit	1/500	Goat	1/1500	h-235

treated with or without AEA (10 μ M) and stained with DiOC₆(3) (30 nM) for 30 min. PI (5 μ g/mL) was added before analysis to discriminate among viable cells that stain only with DiOC₆(3) (DiOC₆(3)+/PI⁻), early apoptotic cells that lost the ability to accumulate DiOC₆(3), and therefore lost mitochondrial transmembrane potential (DiOC₆(3)-/PI⁻), and late apoptotic/necrotic cells that stain only with PI (DiOC₆(3)-/PI⁺). As positive control, cells were incubated with 15 μ M of the mitochondrial depolarizant agent carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (Sigma-Aldrich). The $\Delta\Psi_m$ was analysed by flow cytometry based on the acquisition of 20,000 events (with a threshold of 100,000) in a BD Accuri C6 (Becton–Dickinson Jose, CA, USA) equipped with BD Accuri C6 software. Detectors for forward (FSC) and side (SSC) light scatter were set on a linear scale, while the three fluorescence channels (FL-1, FL-2 and FL-3) were set on the logarithmic scale. FL-1 was used to measure DiOC₆(3) at green fluorescence and FL-2 and FL-3 to measure PI red fluorescence. The results are the mean of three independent experiments carried out in triplicate.

Statistical analysis

Statistical analysis was performed using analysis of variance *t*-test or two-way ANOVA followed by Tukey's test for multiple comparisons (GraphPad PRISM version 4.0; GraphPad Software). All numerical data are expressed as mean $\pm$ S.E.M. and differences were considered to be statistically significant when $P < 0.05$.

Results

Expression of decidual markers in decidualizing St-T1b and HdF cells

In order to study the differentiation process, two endometrial cell culture systems were used, a telomerase-immortalized St-T1b and a primary culture of HdFs. In both St-T1b and HdF cell models, differentiation was induced for 3 and 6 days (d). The differentiation process was assessed by the steady-state

transcript levels of specific decidual markers, the prolactin (PRL) and the insulin-like growth binding protein-1 (IGFBP1) and by morphological analysis (Fig. 1). Giemsa staining showed that non-differentiated cells from St-T1b cell line (Fig. 1A) and HdF cells (Fig. 1C) maintained the elongated fibroblastic spindle shape, whereas decidualizing cells became larger and with rounded nucleus in both St-T1b cell line (Fig. 1B) and HdF cells (Fig. 1D). As PRL and IGFBP1 are widely used biochemical markers of decidualization, mRNA transcript levels were analysed in non-differentiated and in differentiating cells after 3 and 6 d of differentiation stimulus by qRT-PCR. In both St-T1b (Fig. 1G) and HdF cells (Fig. 1H), the mRNA levels of the decidual markers were significantly increased, particularly in cells induced to decidualize for 6 d. Therefore, the subsequent studies were conducted in cells undergoing differentiation for 6 d.

Characterization of the ECS in St-T1b and HdF cells

The expression of major enzymes involved in AEA metabolism, mRNA transcript and protein levels of NAPE-PLD, FAAH and cannabinoid receptors, CB1 and CB2, were investigated by qRT-PCR and Western blotting analysis respectively (Fig. 2). In St-T1b cells (Fig. 2A and D), mRNA and protein levels of CB1 receptor were not affected upon differentiation, and it has been found that these cells do not express CB2 receptor. No differences were observed in NAPE-PLD expression, whereas FAAH expression was upregulated in cells undergoing differentiation. In HdF cells, a fully functional ECS biochemical machinery was found (Fig. 2B and E). CB1 mRNA transcript and protein levels were upregulated in differentiating cells, whereas CB2 expression remained unaffected. Regarding the metabolic enzymes, NAPE-PLD expression remained constant during *in vitro* differentiation, while FAAH was upregulated at mRNA transcript and protein levels.

Figure 2 Characterization of the endocannabinoid system (ECS) by western blotting and qRT-PCR in non-differentiated (non-dif) and differentiating cells (dif) treated for 6 days with differentiation stimulus. mRNA transcript levels of the CB receptors and the main metabolic enzymes of AEA in St-T1b cell line (A) and HdF cells (B). (C) Protein levels of the principal members of the ECS and western blotting quantification in both St-T1b (D) and HdF (E) cells. In HdF, CB1 expression was increased in cells undergoing decidualization. St-T1b cell line does not express CB2 receptor. (PC, positive control) (* $P < 0.05$ vs non-dif) ($n = 3$; two-way ANOVA, Tukey's multiple comparisons).

Figure 3 Effect of AEA in non-differentiated (non-dif) and differentiating cell (dif) viability and proliferation. Differentiation cells were treated with decidual stimulus (cAMP, MPA and E₂) in the absence or presence of AEA (10 μM). Cell viability and the rate of DNA synthesis were assessed by MTT and [³H]-thymidine incorporation assay respectively. AEA did not affect non-differentiated and differentiating St-T1b cells viability (A). In St-T1b cell line, AEA decreased the rate of DNA synthesis (B). In HdF cells, AEA did not affect viability of non-differentiated and of cells undergoing differentiation (C), but significantly decreased DNA synthesis in non-differentiated and in differentiating HdF cells (D). (**P* < 0.05 vs non-dif; #*P* < 0.05 vs dif) (*n* = 5; *t*-test).

AEA effect on human endometrial cell viability and cell proliferation

Non-differentiated cells or cells exposed to the differentiation medium were cultured in the presence or absence of AEA (10 μM) for 6 d, and cell viability was assessed by MTT assay. In the culture conditions used, AEA did not affect cell viability of non-differentiated nor of cells undergoing *in vitro* differentiation, in both St-T1b (Fig. 3A) and HdF (Fig. 3C) cell models. Therefore, this concentration was chosen as, on the conditions used, it does not affect cell viability, and, as it was reported previously, impairs *in vitro* stromal cell differentiation in the rat model.

The decidualization process involves extensive proliferation and differentiation of HESCs; thus, to investigate the role of AEA on the rate of DNA synthesis, [³H]-thymidine incorporation was examined in both non-differentiated and differentiating cells, in the presence or absence of AEA (10 μM) for St-T1b (Fig. 3B) and HdF (Fig. 3D) cell models. In non-differentiated cells, AEA caused a decrease in [³H]-thymidine incorporation of 28% in St-T1b and of 30% in HdF cells compared with non-treated cells. As expected, cell proliferation was reduced in differentiating cells compared with non-differentiated cells. However, in cells undergoing differentiation, AEA treatment markedly decreased the rate of DNA synthesis by 40% compared with differentiating cells, in both cell models.

In order to understand the anti-proliferative effect, AEA impact on cell cycle progression was explored (Table 3). In non-differentiated cells, AEA treatment led to a significant cell cycle arrest at G₂/M phase, both in St-T1b (12.37%) and in HdF cells (34.38%) compared with non-differentiated cells (8.51% and 30.16%). Upon differentiation, stromal cells cease to proliferate; thus, the differentiation stimulus caused a significant increase in cells arrested at the G₀/G₁ phase and a decrease in the cellular content at the S phase, in both cell models. In St-T1b cells exposed to AEA during differentiation treatment, a decrease in the cellular content at the G₀/G₁ phase compared with cells undergoing differentiation (84.77% vs 92.15% respectively) and a significant arrest

Table 3 Impact of AEA on cell cycle progression in St-T1b and HdF cells. Analysis of singlets (*n*) and duplets (2*n*) in FL2-A and FL2-W fluorescence channels are presented. The data represents *n* = 3, 2-way ANOVA, Tukey's multiple comparisons.

	Non-dif	Non-dif (AEA)	Dif	Dif (AEA)	AEA + AM281	AEA + AM630
St-T1b cells						
G ₀ /G ₁	84.59 ± 1.48	82.00 ± 0.80	92.15 ± 0.65*	84.77 ± 1.70 [‡]	90.13 ± 0.92 [§]	
S	6.90 ± 2.34	5.63 ± 1.49	1.63 ± 0.42*	1.88 ± 0.41	1.83 ± 0.70	
G ₂ /M	8.51 ± 1.40	12.37 ± 0.99*	6.22 ± 0.62	13.38 ± 1.48 [‡]	8.04 ± 0.54 [§]	
<i>n</i>	98.73% ± 0.29	96.02% ± 0.36	98.68% ± 0.44	96.67% ± 0.49	96.96% ± 0.14	
2 <i>n</i>	0.63% ± 0.41	1.28% ± 0.65*	0.66% ± 0.39	1.78% ± 0.64 [‡]	1.54% ± 0.14 [‡]	
HdF cells						
G ₀ /G ₁	65.05 ± 0.58	62.13 ± 0.52	70.51 ± 1.20*	55.32 ± 1.74 [‡]	67.20 ± 2.07 [§]	57.20 ± 2.05
S	5.45 ± 0.66	3.49 ± 0.32	2.00 ± 0.45	4.29 ± 1.21	4.63 ± 0.43	4.95 ± 0.32
G ₂ /M	30.16 ± 1.04	34.38 ± 0.26*	26.91 ± 0.60	40.40 ± 1.12 [‡]	28.17 ± 1.64 [§]	37.85 ± 0.94
<i>n</i>	94.24% ± 1.02	90.91% ± 0.95*	95.25% ± 0.60	88.26% ± 1.87 [‡]	89.00% ± 1.39 [‡]	88.00% ± 1.92 [‡]
2 <i>n</i>	5.76% ± 0.66	9.10% ± 1.02*	4.74% ± 0.20	11.74% ± 1.90 [‡]	11.00% ± 1.64 [‡]	12.00% ± 1.04 [‡]

**P* < 0.05 vs Non-dif; [‡]*P* < 0.05 vs Dif; [§]*P* < 0.05 vs Dif (AEA).

Figure 4 AEA treatment induced an increase in bi-nucleated cells. St-T1b cells undergoing differentiation (A). In St-T1b cells undergoing differentiation, AEA treatment led to the appearance of bi-nucleated cells (arrows) as shown in Giemsa staining. Representative FACS plots from flow cytometric studies through analysis of FL2-A and FL2-W fluorescence channels, representing singlets (n) and duplets (2n) in differentiating cells (C) and in differentiating cells treated with AEA (D). In HdF cells, a significant accumulation of bi-nucleated cells in AEA-treated cells was observed through Giemsa staining when comparing differentiated cells (E) and differentiated AEA-treated cells (F). FACS plots of differentiating HdF cells (G) and cells exposed to differentiation stimulus in the presence of AEA (H). Arrows indicate the presence of bi-nucleated cells. (n: singlets; 2n: duplets, analysed by FL2-A and FL2-W channels).

at G_2/M phase when compared with differentiating cells have been found. A similar pattern of cell cycle distribution was found in HdF cells. Consistently, in HdF cells, during *in vitro* differentiation, AEA-induced a decrease in the cellular content at G_0/G_1 phase when compared with differentiating cells (55.32% vs 70.51% respectively), and a significant cell cycle arrest at G_2/M phase (40.40 vs 26.91% respectively). Analysis of decidualizing cells treated with AEA plus AM281, a selective CB1 antagonist, showed a cell cycle distribution similar to differentiating cells, such as G_0/G_1 arrest and a decrease in G_2/M phase, in both cell

models. The effect of AEA in the presence of AM630, a selective CB2 antagonist, was also studied in HdF cells, although no significant difference in cell cycle distribution patterns was observed when compared with AEA-treated differentiating cells.

Unexpectedly, it was noticed by Giemsa staining that in AEA-treated cells, there was an increased number of bi-nucleated cells (Fig. 4B and F). The flow cytometric study, through analysis of singlets (n) and duplets (2n) in FL-2A and FL-2W channels, confirmed the increased number of bi-nucleated cells (2n) upon AEA treatment (Fig. 4D and H). In both non-differentiated and differentiating cells, AEA treatment induced at least a two-fold increase in the number of bi-nucleated cells, in St-T1b and HdF cells. In contrast to St-T1b, approximately 6% of non-differentiated HdF cells were 2n (Table 3). In addition, it was observed that pre-incubation with CB1 receptor antagonist did not prevent the appearance of bi-nucleated cells in both cell models.

To clarify the mechanism underlying AEA-induced retention at G_2/M , the expression of cyclin B1, a key regulator of G_2/M transition was investigated by Western blotting. As shown in Fig. 5A and B, in AEA-treated St-T1b and HdF cells respectively cyclin B1 protein levels were not altered neither in non-differentiated nor in cells undergoing decidualization. To further explore the signalling pathway underlying AEA-induced effect, Akt activation was investigated by Western blotting. AEA reduced Akt phosphorylation in both non-differentiated and differentiating cells compared with respective controls, in both cell models (Fig. 5). In addition, as in rats, it has been suggested that mitochondria play a crucial role in decidual polyploidization (Ma *et al.* 2011). The impact of AEA in mitochondrial transmembrane potential ($\Delta\Psi_m$) was studied by flow cytometry on non-differentiated and in cells undergoing differentiation. However, in both cell types and in St-T1b and HdF cells (Table 4), AEA did not induce any alteration in the $\Delta\Psi_m$.

AEA interferes with cell differentiation

To explore the role of AEA upon the differentiation process, both St-T1b and HdF cells were induced to differentiate in the absence or presence of AEA (10 μ M) for 6 d, and mRNA expression of *IGFBP1* and *PRL* genes was analysed by qRT-PCR. As expected, mRNA levels of both genes were significantly higher in decidualizing cells compared with non-differentiated cells. However, as shown in Fig. 6, the transcript levels of both decidual markers were significantly decreased in cells induced to differentiate in the presence of AEA, to similar levels of those found in non-differentiated cells. To investigate the influence of cannabinoid receptors, cells were exposed to the differentiation stimulus in the presence of AEA (10 μ M) and pre-incubated with a CB1 antagonist (AM281) or a CB2 antagonist (AM630). In both St-T1b (Fig. 6A) and HdF (Fig. 6B) cells, pre-incubation with

Figure 5 Effect of AEA on cyclin B1 and Akt protein levels by Western blotting. Cyclin B1 expression was not altered by AEA treatment in both non-differentiated and cells undergoing differentiation in St-T1b cell line (A) and HdF cells (B). However, AEA reduced the activation of Akt in both non-differentiated and cells undergoing decidualization in St-T1b cell line (C) and HdF cells (D). (* $P < 0.05$ vs non-dif; # $P < 0.05$ vs dif) ($n = 3$, two-way ANOVA, Tukey's multiple comparisons).

AM281 significantly counteracted the AEA reduction on decidual marker gene expression to levels similar of control decidualizing cells. The contribution of CB2 in the case of HdF was also studied, although the expression of the genes encoding the decidual markers was not affected.

Discussion

Decidualization involves extensive stromal cell proliferation and differentiation, which is followed by programmed cell death, although the underlying molecular mechanisms remain unclear. eCBs are novel lipid mediators and may be important players in the molecular network composed by hormones, cytokines and growth factors that orchestrates human decidualization. It is known that AEA mediates several cellular events ranging from cell proliferation,

differentiation and apoptosis, dependent on the biological system/environment and cellular context (Fonseca *et al.* 2013b). It was recently shown that AEA levels are lower in cells undergoing differentiation compared with non-differentiated cells (Almada *et al.* 2016). Nevertheless, its role upon the human decidualization is not yet disclosed. In rats, it was described previously that the metabolic enzymes expressed in decidual cells are the main controllers of eCB levels throughout pregnancy (Fonseca *et al.* 2010). In addition, AEA induces apoptosis in rat primary decidual cell cultures (Fonseca *et al.* 2013a, Almada *et al.* 2015) and interferes with decidualization process (Fonseca *et al.* 2015). Herein, a novel role for AEA as a modulator of human decidualization was proposed, through an effect on both endometrial stromal cell proliferation and differentiation.

In this study, the ECS was characterized in St-T1b and HdF cells, in both non-differentiated cells and in cells undergoing differentiation. As reported previously, HdF cells isolated from term decidua are considered undifferentiated fibroblast-like cells (Richards *et al.* 1995). On the other hand, it was also reported that St-T1b cell line expresses the appropriate phenotypic endometrial stromal cell markers and their decidual response closely mimics that of primary cultures (Samalecos *et al.* 2009). Both models have been used to investigate *in vitro* decidualization in several studies (Richards *et al.* 1995, Samalecos *et al.* 2009).

In opposite to St-T1b cell line, CB1 expression was upregulated in HdF decidualizing cells, which is in

Table 4 Effect of AEA on mitochondrial transmembrane potential ($\Delta\psi_m$) in St-T1b and HdF cells. Viable cells that stain only with DiOC₆(3) (DiOC₆(3)/PI⁻). Early apoptotic cells that lost mitochondrial transmembrane potential and therefore the ability to accumulate DiOC₆(3) (DiOC₆(3)/PI⁻).

	Non-dif	Non-dif (AEA)	Dif	Dif (AEA)
St-T1b cells				
Viable	97.45 ± 0.40	97.21 ± 0.23	96.35 ± 0.20	97.14 ± 0.18
$\Delta\psi_m$ loss	2.55 ± 0.40	2.79 ± 0.20	3.65 ± 0.20	2.86 ± 0.19
HdF cells				
Viable	97.00 ± 0.33	96.83 ± 0.33	98.01 ± 0.28	98.11 ± 0.31
$\Delta\psi_m$ loss	3.00 ± 0.33	3.17 ± 0.33	1.99 ± 0.28	1.89 ± 0.30

Figure 6 mRNA transcript levels of specific human decidual marker genes. Upon AEA treatment, *IGFBP1* and *PRL* expression was significantly decreased in St-T1b cell line (A) and HdF cells (B). AEA induced decreased in mRNA expression of both markers reverted by CB1 antagonist, AM281, in both St-T1b and HdF cells. In HdF, no reversion was observed in the case of AM630, the CB2 antagonist. (* $P < 0.05$ vs non-dif; # $P < 0.05$ vs dif; ^a $P < 0.05$ vs dif (AEA) ($n = 5$, two-way ANOVA, Tukey's multiple comparisons).

agreement with a previous study (Moghadam *et al.* 2005). In this regard, it is possible that the endocrine environment to which HdF cells have been exposed may influence CB1 expression. In addition, it was shown that FAAH expression was upregulated during the decidualization process *in vitro*, whereas NAPE-PLD expression remained constant in both cell models. These findings are consistent with the expression of the metabolic enzymes in human endometrium during the menstrual cycle, in which FAAH protein levels are higher in the secretory phase, which corresponds to the differentiation of stromal cells, compared with the proliferative phase (Taylor *et al.* 2010). Moreover, our findings further support the recognized role of FAAH,

the main AEA degrading enzyme, as a metabolic gatekeeper decreasing AEA uterine levels and promoting the implantation process (Maccarrone & Finazzi-Agro 2004).

Although St-T1b cells did not express CB2 receptor, HdF cell model express both cannabinoids receptors. Moreover, as it was herein shown in HdF model, CB1 receptor appears to be mediating AEA effects during *in vitro* decidualization, rather than CB2 that did not revert the inhibitory effect of this eCB. Therefore, these cell models complement each other to study the role of the AEA during this process. Nevertheless, since St-T1b does not express CB2, it cannot be used by itself to investigate the effect of other eCBs that might also activate CB2 during *in vitro* decidualization.

In addition, it was demonstrated that AEA inhibited endometrial cell proliferation supported by a reduction on the rate of DNA synthesis, cell cycle arrest at the G₂/M phase and accumulation of bi-nucleated cells. The latter features were observed in non-differentiated and in cells undergoing decidualization, in both cell models, suggesting that it is not cell specific. In addition, it was found that AEA treatment did not affect cyclin B1 expression, whereas it reduced phosphorylation of Akt, a signalling pathway involved in cell survival and proliferation (Song *et al.* 2005). In fact, it has been reported that diminished Akt phosphorylation is associated with inhibition of actin polymerization and consequently cytokinesis failure, appearance of multi-nucleated cells and cell growth inhibition (Matesic *et al.* 2006). Moreover, as described previously, the accumulation of bi-nucleated cells and cell cycle arrest at the G₂/M phase may also result from aberrant mitosis and failed cytokinesis, or defects in mitotic spindle checkpoint (Jordan & Wilson 2004, Lee *et al.* 2009, Kundu *et al.* 2014). Therefore, it may be suggested that although cells progress through G₂/M phase, they may fail to undergo cytokinesis, probably due to a direct effect of AEA.

Interestingly, the association of polyploidy with the decidualization process has already been reported. In rodents, endoreduplication (polyploidy) of endometrial stromal cells is well established (Tan *et al.* 2002, Tan *et al.* 2004, Das 2009). In fact, it has been reported that decidual polyploidy develops as mono- or bi-nucleated cells due to blockage in G₂/M phase of the cell cycle, as a result of a mitotic cycle without cytokinesis (Sroga *et al.* 2012). Moreover, decidua with insufficient polyploidy limits implantation (Das 2009, Sroga *et al.* 2012), whereas aberrant decidual polyploidy was also shown to be detrimental to this process (Gao *et al.* 2015). In human decidualization, the presence of bi-nucleated cells has been reported by Tang *et al.* (1993, 1994) and is a topic of increasing recognition in the literature; however, their significance has never been addressed. Nevertheless, in the conditions used, AEA-induced bi-nucleation in both non-differentiated and in

cells undergoing decidualization and was independent of CB1 receptor activation.

Decidualization is a key event for successful implantation, in which endometrial stromal cells cease to proliferate and undergo a differentiation process into decidual cells, being the latter induced by an increase in intracellular cAMP levels. In this study, it was demonstrated that AEA significantly decreased the decidual markers transcript levels, *PRL* and *IGFBP1*, upon *in vitro* decidualization, in a mechanism dependent on CB1 activation. As CB1 activation is known to inhibit adenylate cyclase, it is likely that the AEA inhibitory effect on decidualization is mediated through CB1-induced decrease in cAMP production. In addition, it was shown that AEA prevented cell cycle arrest at G₀/G₁ phase during *in vitro* decidualization, which may hamper cell differentiation. Reinforcing the involvement of CB1 receptor on the inhibitory effect caused by AEA, it was shown that the pattern of cell cycle distribution of cells treated with a selective CB1 antagonist (AM281) plus AEA was similar to the untreated cells undergoing differentiation. Thus, our findings suggest that AEA may be a negative modulator of human stromal cell differentiation. Moghadam *et al.* (2005) showed previously that WIN55,212-2, a full CB1 agonist, inhibited decidualization through CB1 by triggering an apoptotic pathway. However, in our conditions, AEA effect was not accompanied by cell death. Despite in rats, it has been suggested that mitochondria may play a role in polyploidization, no alterations in the mitochondrial transmembrane potential were observed.

Understanding the molecular signature that underlies the role of AEA on endometrial stromal cells further elucidates the cellular and molecular basis that governs decidualization. Two major breakthroughs are highlighted in this study. On the one hand, AEA inhibits decidual stromal cell proliferation and leads to the appearance of bi-nucleated cells, which may consequently impair stromal cell differentiation. On the other hand, AEA also act as a negative modulator of endometrial stromal cell differentiation through a CB1-dependent mechanism. Moreover, it is widely accepted that AEA levels are mainly regulated by FAAH activity. Altogether, our findings corroborate that a balance tone of AEA uterine levels is crucial for human decidualization, and thus elevated levels of this eCB, due to cannabinoids consumption or to FAAH deregulation, may impair this process. We also suggest that AEA may be a central tie bridging both steps of the decidual program, stromal cell proliferation and differentiation. Decidualization is a remarkable feature of human reproduction. It is known that impaired decidualization process is associated with a variety of reproductive disorders, ranging from infertility, recurrent miscarriage, utero-placental interaction disorders. Nowadays, failure of the endometrium to achieve a receptive state is considered as a major cause of infertility as well as the rate-limiting

step in assisted reproductive technology. Therefore, unveiling the molecular mechanisms that underpin the decidualization process is of great importance to improve reproductive outcome.

Declaration of interests

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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