Placenta 36 (2015) 69–76

Contents lists available at ScienceDirect

Placenta

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

The endocannabinoid anandamide induces apoptosis in cytotrophoblast cells: Involvement of both mitochondrial and death receptor pathways

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ARTICLE INFO

Article history:
Accepted 21 October 2014

Keywords:
Anandamide
Apoptosis
Cytotrophoblasts
Endocannabinoid signalling
Placenta

ABSTRACT

Introduction: A balanced proliferation, apoptosis and differentiation in trophoblast cells of the human placenta is crucial for a proper placental development. Alteration in trophoblast apoptosis and differentiation are associated with gestational-related complications, such as preeclampsia, intrauterine growth restriction or miscarriages. The endocannabinoids (eCBs) have been recognized as new interveners in pregnancy events such as implantation and decidualization. However, their importance in placentation is poorly understood. We hypothesise that these novel lipid mediators may intervene in cytotrophoblast apoptosis and, concomitantly, have a role during placental development.
Methods: primary human cytotrophoblasts (hCTs) and the human trophoblast-like choriocarcinoma cell line BeWo cells were exposed to Anandamide (AEA). It was investigated the cellular pathways involved in cell death, by the assessment of cell morphology, caspases activity, mitochondrial membrane potential ($\Delta\psi m$), reactive oxygen/nitrogen species (ROS/RNS) and western blot of cleaved Poly (ADP-ribose) polymerase 1 (PARP-1), truncated Bid (t-Bid) and IκB-α.
Results: AEA decreased hCTs viability and induced morphological features of apoptosis (chromatin condensation and fragmentation), caspase-3/7 activation and PARP-1 cleavage. In BeWo, AEA also increased the activities of caspase-3/7 and 9, induced loss in $\Delta\psi m$ and production of ROS/RNS. These effects were reversed by either CB1 or CB2 antagonists, whereas the increase in caspase-3/7 activity was only reversed with CB2 blockage. AEA-treated cells showed increased caspase-8 activation and formation of t-Bid, suggesting the interplay between intrinsic and extrinsic apoptotic pathways. AEA also increased IκB-α expression, a NF-κB regulatory protein.
Conclusion: Our results highlight the importance of eCBs in cytotrophoblast cell apoptosis and indicate that a crosstalk between intrinsic and extrinsic apoptotic pathways is involved in AEA-induced effects.

1. Introduction

The placenta is a specialized organ with vital functions, such as nutrient and gas exchange, immunomodulation and protein biosynthesis. The main placental cells, the trophoblasts, are subdivided in different cell types: cytotrophoblasts, syncytiotrophoblast and extravillous trophoblasts (EVTs). Cytotrophoblasts are mononuclear cells that proliferate, fuse and differentiate into other types of trophoblasts. The syncytiotrophoblast is a multinucleated layer that produces several hormones and proteins and is in direct contact with maternal blood, allowing the mother-fetus communication. The EVTs have invasive properties and are involved in the uterine blood vessels remodelling [1]. A coordinated proliferation, differentiation and apoptosis of trophoblasts is required for a proper placental development, and any disturbance in these processes is associated with gestational complications [2–7].

The Endocannabinoid System (ECS) has emerged as a key modulator of multiple physiological and pathophysiological processes, including reproductive events like implantation and decidualization. This system comprises the two cannabinoid receptors CB1 and CB2, their endogenous ligands (endocannabinoids-eCBs), the enzymes involved in eCBs biosynthesis and degradation and a putative membrane transporter (See Ref. [8] for review).

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http://dx.doi.org/10.1016/j.placenta.2014.10.011
0143-4004/

Anandamide (AEA) or *N*-arachidonoylethanolamine was identified in 1992 in pig brain [9] and is currently the best studied eCB. It is synthesized from membrane phospholipids in a two-step reaction catalyzed by the enzymes *N*-acyltransacylase and *N*-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD). After exerting its effect, it is hydrolyzed inside the cell, mainly by Fatty Acid Amide Hydrolase (FAAH), an inner cellular membrane enzyme. This eCB induces apoptosis in several cell types by activating different receptors and cell signalling pathways. In fact, it was described that AEA-induced apoptosis involve the activation of its main targets, the CB receptors, triggering several downstream pathways, including activation of mitogen-activated protein kinases (MAPKs), increase of ceramide levels or intracellular Ca^{2+} and generation of oxidative stress [10–14].

The importance of cannabinoid signalling homoeostasis was only been recently recognized. In fact, a proper AEA tone is required for embryo transport along the mouse oviduct [15] and low levels of this eCB are required in implantation sites and are mainly regulated locally by the enzyme FAAH (see Ref. [16] for review). Nevertheless, information about the role of AEA during the period of placental development is lacking. It was reported that CB receptors and AEA main metabolic enzymes are expressed in first trimester and term placentas [17–20]. Also, in preeclamptic placentas, NAPE-PLD expression was increased whereas FAAH was decreased in comparison to normal placentas, suggesting a function for eCBs in the pathophysiology of preeclampsia [21]. It was also described that AEA decreases the viability of BeWo cells, via CB2 receptor [20]. Moreover, we have recently demonstrated that the other major eCB, 2-arachidonoylglycerol (2-AG), has the ability to induce apoptosis in BeWo cells through a CB receptor- and mitochondrial-dependent mechanism [22].

Here, we investigate the impact of AEA on cytotrophoblasts viability and explore the cellular mechanisms triggered by this endocannabinoid. We hypothesise that this major endocannabinoid may integrate the network of hormones, cytokines and other molecules that regulate cytotrophoblast proliferation and apoptosis.

2. Materials and methods

All chemicals were from Sigma–Aldrich Co. (St. Louis, MO, USA), except: Anandamide, AM251 and AM630 (Tocris Bioscience, Bristol, UK); 3, 3′-dihexyloxacarbocyanine iodide ($DiOC_6$) (Gibco/Invitrogen Corporation, Carlsbad, CA, USA); Z-VAD-fmk (BD PharMingen, San Diego, CA); CytoTox 96 nonradioactive cytotoxicity assay kit, Caspase-Glo® (Promega, Madison, WI, USA); ^{3}H-thymidine (Amersham, Aylesbury, UK); percoll (GE Healthcare, Buckinghamshire, UK); and WesternBright™ ECL HRP substrate (Advansta, Menlo Park, USA).

2.1. Isolation and culture of human cytotrophoblasts

All the procedures were performed in accordance with the Ethical Committee of Hospital S. João, Porto (authorization n° 237-13). For each assay, cytotrophoblasts were isolated from five different term placentas from clinically normal pregnancies, according to a modification of the Kliman's protocol, as previously described [23]. Briefly, decidual tissue was removed, villous tissue was collected from at least 10 different regions homogeneously distributed in the whole placenta, and the major blood vessels were discarded by fine dissection. Then, the tissue was digested in a trypsin and DNAse-containing solution and the obtained cells were separated in a discontinuous percoll gradient, at 1200 g. The cytotrophoblasts were collected and incubated at 37 °C and 95% air/5% CO_2 humidified atmosphere, in DMEM/F12 medium supplemented with 10% (v/v) FBS and antibiotic–antimycotic solution (100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericine B). To evaluate the purity of the cytotrophoblast cultures, cells were fixed with a 4% paraformaldehyde solution and immunostained with anti-cytokeratin-7 and anti-vimentin antibodies. Around 95% of the cells were cytokeratin-7 positive, which corresponds to the epithelial hCT cells. For the experiments, cells were plated in 6- and 96-well plates or 8-well chamber slides, at densities 4.5×10^5, 1.5×10^5 or 5×10^5 cells/well, respectively.

2.2. BeWo cell culture conditions

The human choriocarcinoma cell line BeWo (ATCC, Manassas, VA, USA) was incubated at 37 °C and 95% air/5% CO_2 humidified atmosphere, in DMEM/F12 medium supplemented with 10% (v/v) FBS and an antibiotic–antimycotic solution. For the experiments, cells were used between 78 and 90 passages and seeded in 96 or 6-well plates and 8-well chamber slides, at densities 1×10^4, 2×10^5 and 3×10^4 cells/well, respectively. At least five independent experiments performed in triplicate for each assay.

For the investigation of cellular pathways triggered by AEA, BeWo cells were pre-treated for 30 min with CB1 and CB2 antagonists, AM251 or AM630 (1 μM), respectively. AEA, AM251 and AM630 were dissolved in ethanol. Equimolar concentrations of the vehicle did not induce any effects on all the parameters (data not shown).

2.3. Cell viability and cytotoxicity assays

The hCTs and BeWo cells were plated in 96-well plates and, after adhesion, incubated in DMEM/F12 medium with 1% FBS and 1% antibiotic solution, in the absence or presence of AEA (1–25 μM) for 12 and 24 h (hCTs) or for 24, 48 and 72 h (BeWo cells). The yellow tetrazole MTT (0.5 mg/ml final concentration) was added and cells were incubated for 2 h 30 min, at 37 °C. The resultant purple formazan was extracted by a solution of Dimethylsulfoxide (DMSO):isopropanol (3:1) and quantified by spectrophotometry, at 540 nm, in a Multiscan Ascent microplate reader. The activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) released into the culture medium was assessed by the CytoTox 96 nonradioactive cytotoxicity assay kit, according to the manufacturer's instructions.

2.4. Morphological studies

The alterations in cell morphology induced by AEA (15 μM) treatment of hCTs for 24 h or AEA (10 μM) treatment of BeWo for 48 h were analyzed by Giemsa and Höechst staining. These concentrations were selected according to the viability assays of each cellular model. After the treatment, cells plated in 8-well chamber slides were fixed with a 4% paraformaldehyde solution, stained with Giemsa and analyzed under light microscopy. For Höechst staining, cells were exposed to 0.5 mg/ml Höechst 33342 for 20 min and examined under a fluorescence microscope (Eclipse E400, Nikon, Japan) equipped with an excitation filter with maximum transmission at 360/40 nm.

2.5. Incorporation of 3H-thymidine

The impact of AEA in BeWo cells proliferation was evaluated by the quantification of ^{3}H-thymidine incorporation in the absence or presence of AEA (1–25 μM) for 24 and 48 h ^{3}H-thymidine (0.5 μCi final concentration) was added to each well, 8 h before the end of the incubation time. Cells were frozen/thawed twice, harvested with a semi-automated cell harvester (Skatron Instruments, Lier, Norway). After the addition of scintillation cocktail, the incorporation of ^{3}H-thymidine was quantified in a scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA, USA).

2.6. Determination of caspase-3/7, 8 and 9 activities

The activity of caspases was determined by luminescence assays by using caspase-Glo®-3/7, 8 or 9 assays, according to manufacture instructions. Cells were seeded in 96-well white plates and exposed to 15 μM of AEA for 20 h (in case of hCTs) or to 10 μM of AEA for 36 h (in case of BeWo cells). The plates were incubated for 1 h at room temperature and the resultant luminescence was measured in a Microplate Luminometer (BioTek Instruments, Winooski, VT, USA) and presented as in relative light units (RLU). A negative control was performed by co-incubation of AEA with a specific caspase inhibitor, Z-VAD-fmk (20 μM) and a positive control was performed with the apoptosis inductor staurosporine (STS; 100 nM) added 12 h before the end of experiment.

2.7. Evaluation of mitochondrial membrane potential ($\Delta\psi m$) and intracellular reactive oxygen and nitrogen species (ROS/RNS)

For the assessment of $\Delta\psi m$ and ROS/RNS production, BeWo cells were seeded in 96-well black plates and treated with AEA (10 μM) for 36 h or 48 h, respectively. For $\Delta\psi m$ studies, cells were washed and incubated with $DiOC_6$ 100 nM, for 20 min, at 37 °C, in the dark. For the evaluation of ROS/RNS production, cells were washed and incubated with the fluorescent probe 2, 7-Dichlorodihydrofluorescein diacetate (DCDHF-DA), for 1 h, at room temperature. For both assays, the resulting fluorescence was measured in a Microplate Fluorimeter (BioTek Instruments, Vermont, USA) (excitation-485 ± 10 nm; emission 530 ± 12.5 nm). The positive controls for $\Delta\psi m$ or ROS/RNS production were the mitochondrial depolarizing carbonyl cyanide m-chlorophenylhydrazone (CCCP; 10 μM) or H_2O_2 (200 μM), respectively. The results were expressed in relative fluorescence units (RFU).

2.8. Western Blot analysis

Western Blot was used for the assessment of cleaved PARP-1 in hCTs and Bid/t-Bid and IκB-α (Table 1). BeWo cells were seeded in 6 well plates and incubated in the absence or presence of AEA alone or pre-incubated with CB receptors antagonists.