2-Arachidonoylglycerol impairs human cytotrophoblast cells syncytialization: Influence of endocannabinoid signalling in placental development

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A balanced cytotrophoblast cell turnover is crucial for placental development and anomalies in this process associated with gestational diseases. The endocannabinoid system (ECS) has emerged as a new player in several biological processes. However, its influence during placental development is still unknown. We report here the expression of the endocannabinoid 2-arachidonoylglycerol (2-AG) main metabolic enzymes in human cytotrophoblasts and syncytiotrophoblasts. We also showed that 2-AG induced a decrease in placental alkaline phosphatase activity, human chorionic gonadotropin secretion and Leptin mRNA levels. Moreover, 2-AG reduced giall cell missing 1 and syncytin-2 transcription and the number of nuclei in syncytiun. These effects were mediated by cannabinoid receptors and may result from 2-AG inhibition of the cAMP/PKA signalling pathway. Our data suggest that 2-AG may interfere with the biochemical and morphological differentiation of human cytotrophoblasts, through a CB receptor-dependent mechanism, shedding light on a role for the ECS in placental development.

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1. Introduction

Development of the human placenta is a highly coordinated event that is crucial for a successful pregnancy outcome. The main specialized cell type of this organ is the trophoblast, which is composed of four cell populations: cytotrophoblasts (CTs), syncytiotrophoblast (ST), extravillous trophoblasts (EvTs) and giant trophoblast cells. CTs are proliferative, mononucleated cells that differentiate into STs and EVTs during the process of placentation. EVTs accomplish invasion which, ultimately, results in the anchoring of the placenta to the uterine wall and in the remodelling of maternal arteries, decreasing the resistance to blood flow. On the other hand, STs are multinucleated, non-proliferative cells that form a continuous layer lining the chorionic villi. They have a short lifespan and are in a constant renewal, dying by apoptosis and being replaced by new cells resulting from differentiation and fusion of CTs. The ST layer is in direct contact with maternal blood and is responsible for maternal–fetal gas and nutrient exchanges and fetal protection (Gude et al., 2004; Lunghi et al., 2007). Moreover, STs have the machinery necessary to produce hormones and proteins, such as human chorionic gonadotropin (hCG), oestrogens, human placental lactogen, placental growth hormone and leptin, that regulate feto-maternal physiology and metabolism and sustain pregnancy (Malassine and Cronier, 2002). Anomalies in placental development and CT differentiation have been related to gestational diseases like preeclampsia, intrauterine growth restriction and spontaneous miscarriage (Langbein et al., 2008; Lim et al., 1997; Ruebner et al., 2010).

Despite the crucial importance of CT differentiation for the process of placentation, the signalling pathways that orchestrate this process are not well clarified. Some authors argue that the activation of early stages of the apoptotic cascade, namely, the exposure of phosphatidylinerse on the outer leaflet of plasma membrane and caspase 8 activation, are essential for CT differentiation (Gauster and Huppertz, 2010). The importance of cyclic AMP (cAMP) and protein kinase A (PKA) activation in this process is recognized (Guilbert et al., 2010; Rote et al., 2010).

The endocannabinoid system (ECS) has been suggested as an emergent intervener in physiologic and pathophysiologic cellular events in several organs and tissues. This system is constituted by the G-protein coupled cannabinoid receptors 1 and 2 (CB1, CB2), their endogenous ligands (the endocannabinoids [eCBs]) and the respective metabolic enzymes and transporters. Among the several agonists of CB receptors that have already been identified,
anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the best studied and those whose biological relevance has been widely recognized. AEA and 2-AG are mainly synthesized from membrane phospholipids by N-arachidonoylphosphatidylethanolamine-phospholipase D (NAPE-PLD) and diacylglycerol lipase (DAGL), respectively, and are degraded by the cytosolic enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively. eCBs are mainly produced “on demand” and have essentially an autocrine and paracrine action (see Fonseca et al., 2013b for review).

In the last years, eCBs have been identified as novel mediators in the complex signalling pathways that coordinate pregnancy events like implantation and decidualization (Taylor et al., 2010). However, there is a lack of evidence about the role of eCBs in placental development. In human placenta, CB receptors and AEA metabolic enzymes have already been described (Habayeb et al., 2008; Helliwell et al., 2004; Kenney et al., 1999; Park et al., 2003; Trabucco et al., 2009), but the role of 2-AG in this organ is still unknown, though the expression of its enzymatic machinery was reported in rat and baboon placentas (Brocato et al., 2013; Fonseca et al., 2012) and by our group in human cytotrophoblasts (Costa et al., 2014). In addition, we reported that 2-AG induces apoptosis in BeWo cells (a cytotrophoblast cell model), involving the apoptotic mitochondrial pathway, by a CB receptor-dependent mechanism (Costa et al., 2014).

In this work, we studied primary cultures of human cytotrophoblasts since these cells spontaneously differentiate and fuse into STs, in the presence of fetal bovine serum (Kliman et al., 1986). During differentiation, CTs aggregate and fuse, losing the expression of proteins that participate in the establishment of cell junctions, such as E-cadherin and desmoplakin. Furthermore, these differentiated cells express proteins such as placental alkaline phosphatase (pALP) and hCG. In this way, we investigated the presence of the main metabolic enzymes of 2-AG (DAGL-α and MAGL) in both human cytotrophoblasts and syncytiotrophoblast. In addition, we studied the impact of this endocannabinoid during the morphological and functional in vitro differentiation of human CTs into STs and the cellular mechanisms underlying 2-AG effects during these processes.

2. Materials and methods

2.1. Primary cultures of human cytotrophoblasts

All the procedures concerning human placental handling were performed in accordance with the Ethical Committee of Hospital S. João, Porto. In this study, we included normal term placentas (38–40 weeks of gestation) from Caucasian women living in the Porto region and aged 24–36 years old. For each assay, we isolated cytotrophoblasts from five different placentas. This isolation was performed using a modification of the Kliman’s protocol, as previously described (Keating et al., 2007). Briefly, decidual tissue was removed and villous tissue collected from at least 10 different regions homogeneously distributed in the whole placenta. The major blood vessels were discarded by fine dissection. Then, the tissue was digested in a trypsin (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) and DNase (Sigma Aldrich Co. St Louis, MO, USA)-containing solution and the obtained cells were separated in a discontinuous percoll gradient. Cytotrophoblasts were collected and seeded in 24-well plates, 21 cm² dishes or 8-well chamber slides, at densities 1 × 10⁵, 1 × 10⁶ or 4.5 × 10⁶, respectively, in DMEM/F12 medium (Sigma Aldrich Co. St Louis, MO, USA) supplemented with 10% (v/v) of fetal bovine serum (FBS) and an antibiotic–antimycotic solution (100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) (Sigma Aldrich Co. St Louis, MO, USA) and were incubated at 37 °C and 95% air/5% CO₂ humidified atmosphere.

To characterize the enzymatic machinery involved in 2-AG metabolism, the cells were collected at 12 h and 72 h of culture, corresponding to two different stages of differentiation (cytotrophoblast and syncytiotrophoblast cells, respectively), in Trizol® reagent (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) and RNA and protein were extracted according to the manufacturer’s instructions.

For the studies of cell viability and LDH release, cells were treated with 2-AG (dissolved in ethanol) for 48 h, in two different times of culture: at 12 h, to study the effects of this eCB during CT differentiation, and at 72 h, to investigate its effect in the syncytiotrophoblast. For the experiments of the differentiation markers assessment, cells were treated with 2-AG at 12 h of culture, for 48 h, to assess its effect during the differentiation process.

2.2. RT-PCR analysis

The assessment of MAGL and DAGL-α gene transcription was carried out by RT-PCR. RNA was quantified in the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), its quality evaluated with the Experion RNA StdSens Kit (Bio-Rad Laboratories, USA) and analysed with Experion analytical software (Bio-Rad Laboratories, USA). cDNA was obtained by reverse transcription of RNA using the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories, USA) 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, USA), according to the kit protocol. Table 1 resumes primer sequences and RT-PCR conditions. The specificity of PCR product amplification was assessed by analysis of the melting curve. Gene expression of DAGL-α and MAGL was normalized with two housekeeping genes (β-actin and succinate dehydrogenase subunit A, SDHA) and their analysis was achieved by the calculation of ΔΔCT values.

2.3. Western Blot analysis

Cells were harvested in lysis buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 1% Triton™X-100), at 4 °C, and the protein fraction was obtained by centrifugation at 14,000 g for 10 min, at 4 °C. For PKA phosphorylation analysis, cells were treated for 15 minutes with 2-AG, in the absence or presence of CB receptor antagonists. Protein extracts were loaded in 10% SDS-PAGE and transferred onto nitrocellulose membranes. Then, non-specific binding sites were blocked and membranes were incubated with anti-MAGL, anti-DAGL-α or phospho-PKA α/β/γ cat Thr198 (SantaCruzBiotechnology, USA; 1:100; rabbit) primary antibodies overnight, at 4 °C. Membranes were incubated at room temperature, for 1 h, with peroxidase-conjugated secondary antibody 1:1000 (anti-rabbit; Vector Laboratories, CA, USA), washed and exposed to a chemiluminescence detection kit (Super Signal West Pico; Pierce, Rockford, USA) and then to an X-ray film (Kodak XAR; Eastman Kodak, Rochester, NY). Rat brain was used as a positive control. For loading control, membranes were stripped and reincubated with anti-β-tubulin antibody (Santa Cruz Biotechnology, USA; 1:1000; rabbit). The signal intensity of DAGL-α, MAGL and p-PKA was quantified by densitometry (BIO-PROFIL Bio-1D2; Vilber Lourmat, Marne-la-Vallée, France) and the results expressed in arbitrary units, after normalization for the corresponding β-tubulin band.

2.4. Immunocytochemistry and Immunohistochemistry

For immunocytochemistry, deparaffined slides of human placenta (4 μm thick) were used. For immunohistochemistry analysis, cells were seeded in 8-well chamberslide and fixed with cold methanol at 12 h or 72 h of culture. The expression of proteins was
analysed using an avidin–biotin alkaline phosphatase complex immunohistochemical technique (Vectastain ABC kit, Vector Laboratories, CA, USA). The non-specific binding sites were blocked and slides were incubated with anti-DAGL (1:100) or anti-MAGL (1:100) antibodies at 4 °C, overnight, followed by incubation with biotinylated secondary antibody and further incubation with Vectastain ABC-AP reagent, according to the manufacturer's instructions. The reaction was developed with Sigma Fast RedTM tablets (Sigma Aldrich Co, St. Louis, MO, USA). Negative controls were performed by the replacement of the primary antibodies by rabbit IgG.

### 2.5. Cytotrophoblast cell viability and LDH release

Cytotrophoblast cells were plated in 24-well plates and treated with 2-AG (1–20 μM) at 12 h and at 72 h of culture, for 48 h. The effects of this eCB on cell viability were monitored by MTT assay. In addition, we assessed the effects of the CB receptor antagonists, AM251 and AM630 (1 μM; vehicle ethanol) (Tocris Bioscience; Bristol, England). The cells were treated in the same conditions as referred for the pALP assay. The cells were seeded in 6 well plates and treated as described for the other assays. At the end of experiment, cells were collected, centrifuged and stored at −80 °C. The impact of 2-AG in HCG production was evaluated by the quantification of the β subunit of this hormone in cell supernatants by ELFA (enzyme linked fluorescent assay) with the VIDAS HCG kit (bioMérieux SA, Marcy l’Etoile, France) using the mini-VIDAS autoanalyser (bioMérieux SA, Marcy l’Etoile, France), according to manufacturer’s protocol. The results were standardized with total protein content, calculated as μg/mg of protein and expressed as relative values compared to the control. The final results were expressed in relative values, in comparison with the untreated cells (control). For the control of the spontaneous differentiation of cytotrophoblasts into syncytiotrophoblast, ecto-pALP was assessed at different times of culture (12, 72 and 120 h).

### 2.7. Quantification of secreted β-hCG

The cells were treated in the same conditions as referred for the ecto-pALP assay. After a 48 h-treatment with 2-AG (10 μM), cell culture medium was collected, centrifuged and stored at −80 °C. The impact of 2-AG in HCG production was evaluated by the quantification of the β subunit of this hormone in cell supernatants by ELFA (enzyme linked fluorescent assay) with the VIDAS HCG kit (bioMérieux SA, Marcy l’Etoile, France) using the mini-VIDAS autoanalyser (bioMérieux SA, Marcy l’Etoile, France), according to manufacturer’s protocol. The results were standardized with total protein content, calculated as μg/mg of protein and expressed as relative values compared to the control.

### 2.8. Evaluation of leptin, glial cell missing 1 (GCM-1), HERVW-1 (syncytin-1) and HERVFRD-1 (syncytin-2) gene expression by RT-PCR

The cells were seeded in 6 well plates and treated as described for the other assays. At the end of experiment, cells were collected in TRIzol® Reagent. The assessment of RNA quality, cDNA synthesis, gene amplification and semi-quantification by qPCR were performed as described above. Primer sequences and qPCR conditions used to assess the gene expression of diacylglycerol-α (DAGL-α), monoacylglycerol lipase (MAGL), leptin, glial cell missing 1 (GCM-1), syncytin-1 and syncytin-2, succinate dehydrogenase subunit A (SDHA).

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank</th>
<th>Primer sequence (5’-3’)</th>
<th>Annealing temperature</th>
<th>Amplicon length</th>
<th>Melting temperature</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>hDAGL-α</td>
<td>NM_006133.2</td>
<td>Sense: TGGCTCTGCGGCCGTCTTAT</td>
<td>61 °C</td>
<td>130 bp</td>
<td>85.6 °C</td>
<td>(Ludanyi et al., 2008)</td>
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<tr>
<td>hMAGL</td>
<td>BC000551.2</td>
<td>Sense: CAGCGGCTCTTCTTCTTCTG</td>
<td>57 °C</td>
<td>162 bp</td>
<td>85.5 °C</td>
<td>(Ludanyi et al., 2008)</td>
</tr>
<tr>
<td>hSCG-1</td>
<td>NM_003643.3</td>
<td>Sense: GACAGGCTCTCCCTTGGAGT</td>
<td>59 °C</td>
<td>88 bp</td>
<td>77.3 °C</td>
<td></td>
</tr>
<tr>
<td>Syncytin-1 (HERVW-1)</td>
<td>NM_014590.3</td>
<td>Anti-sense: ACTACAGGCGAATTTGGACGC</td>
<td>59 °C</td>
<td>87 bp</td>
<td>77.9 °C</td>
<td></td>
</tr>
<tr>
<td>Syncytin-2 (HERVFRD-1)</td>
<td>NM_207582.2</td>
<td>Anti-sense: CCAGCTTCTCCAGGTAAGT</td>
<td>60 °C</td>
<td>215 bp</td>
<td>80.6 °C</td>
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</tr>
<tr>
<td>hLeptin</td>
<td>NM_000230.2</td>
<td>Sense: TGGCTGATCTTCTTGGT</td>
<td>60 °C</td>
<td>133 bp</td>
<td>77.6 °C</td>
<td></td>
</tr>
<tr>
<td>hβ-actin</td>
<td>NM_001101.3</td>
<td>Sense: AATCCATCATGGAAGTGACG</td>
<td>60 °C</td>
<td>234 bp</td>
<td>85.5 °C</td>
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<tr>
<td>hSDHA</td>
<td>NM_004168.2</td>
<td>Anti-sense: CTGACCTCGGAGTCCTGCTGTTG</td>
<td>58 °C</td>
<td>89 bp</td>
<td>74.8 °C</td>
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</tbody>
</table>

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 cell protein was quantified by the Bradford assay. 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 was used as a control. Equimolar concentrations of the vehicle (ethanol 0.1%) have no effects on cytotrophoblast cells (data not shown). The results were expressed in nmol of p-nitrophenol/mg of protein/minute. The final results were expressed in relative values, in comparison with the untreated cells (control). For the control of the spontaneous differentiation of cytrophoblasts into syncytiotrophoblast, ecto-pALP was assessed at different times of culture (12, 72 and 120 h).
the analysis of E-cadherin expression. Cells were seeded in chamberslides and were processed as described above. After treatment with 10 μM of 2-AG for 48 h, cells were washed with PBS and fixed with 4% paraformaldehyde. Unspecific binding sites were blocked and cells were incubated overnight, at 4 °C, with the primary antibody anti-E-cadherin (1:200; BD Biosciences, San Jose, CA, USA). Cells were washed with PBS and incubated with FITC conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology, USA; 1:200) for 1 h, at room temperature. Finally, slides were mounted in Fluoroshield™ containing DAPI (Sigma Chemical Co, St. Louis, MO, USA) and analysed in a confocal microscope, with laser excitation at 405 nm and 488 nm (Leica SP2 AOBS SE, Leica Microsystems, Wetzlar, Germany). The number of nuclei in syncytium was compared with the total number of nuclei and the resultant ratio expressed in percentage. For this analysis, a syncytium was defined as three or more nuclei inside a cytoplasm and 10 random fields were considered and counted for each treatment condition.

2.10. Cyclic AMP quantification assay

Cells were seeded in 96-well white plates and treated with 2-AG for 15 minutes, in the absence or presence of CB receptor antagonists and of pertussis toxin (PTX) 40 ng/ml (Sigma Aldrich Co. St Louis, MO, USA), a Gi protein inhibitor. cAMP levels were then assessed with the cAMP-glo™ Assay (Promega, Madison, WI, USA), according to the manufacturer’s instructions.

2.11. Statistical analysis

Statistical analysis was carried out by t-test or one or two-way ANOVA, followed by the Bonferroni post-hoc test to make pairwise comparisons of individual means when significance was indicated (GraphPad PRISM v. 6.0, GraphPad Software, Inc., San Diego, CA, USA). At least five placentas were studied and the experiments were performed in triplicate. Data were expressed as mean SEM and differences were considered to be statistically significant at p < 0.05.

3. Results

3.1. Expression of the main enzymes involved in 2-AG biosynthesis and degradation in human cytotrophoblasts and syncytiotrophoblasts

Gene transcription of the major enzymes that synthesize and hydrolyze 2-AG, DAGL-α and MAGL respectively, was investigated by RT-PCR (Fig. 1A). The transcript levels of DAGL-α and MAGL were similar in undifferentiated cytotrophoblasts (12 h of culture) and in differentiated cells (72 h of culture). Western blot and immunocytochemistry analysis corroborated RT-PCR results, revealing that...
both enzymes are expressed in the cytoplasm of cytotrophoblasts and syncytiotrophoblast (Figs 1B–D). Moreover, immunohistochemistry of placental sections revealed that both enzymes are expressed in chorionic villi (Fig. 1E).

3.2. Effects of 2-AG in cytotrophoblast cell viability and LDH release during and after differentiation into syncytiotrophoblast

To investigate the effects of 2-AG in CT differentiation, cells in the two different times of culture (12 and 72 h) were incubated with this eCB (1–20 μM), for 48 h. Treatment with 2-AG did not alter neither cell viability nor extracellular LDH levels, suggesting that this eCB is not cytotoxic, in the studied conditions (Fig. 2A, B).

3.3. Impact of 2-AG in ecto-placental alkaline phosphatase (pALP) activity during the differentiation period

The placental isoform of alkaline phosphatase is essentially expressed by the syncytiotrophoblast and its activity increases proportionally with the time of culture (Keating et al., 2009). Thus, this enzyme is frequently used as a biochemical marker to evaluate the in vitro differentiation of human cytotrophoblasts. The activity of this ecto-enzyme was assessed in cells treated at 12 h of culture with 2-AG (1–20 μM), for 48 h (Fig. 3A). We could observe that 10 and 20 μM were able to decrease significantly the ecto-pALP activity. The lowest 2-AG concentration that was able to decrease the ecto-pALP activity, 10 μM, was chosen for the experiments. To elucidate the mechanism behind this effect, cytotrophoblast cells were incubated with CB receptor antagonists. It was observed that the

![Graph A](image1.png)

**Fig. 2.** Effects of 2-arachidonoylglycerol (2-AG) in trophoblast cells’ viability (MTT assay). Cells were treated with different concentrations of 2-AG for 48 h at two different times of culture: at 12 and 72 h, to assess the effects on viability during the differentiation process and in the post-differentiation period, respectively. None of the tested concentrations was able to decrease cell viability (A) or induce LDH release to the culture medium (B).

![Graph B](image2.png)

![Graph C](image3.png)

**Fig. 3.** Effects of a 48 h-treatment with 2-arachidonoylglycerol (2-AG) in placental alkaline phosphatase (pALP) activity in human cytotrophoblasts treated at 12 h of culture. (A) 2-AG decreased pALP activity at 10 and 20 μM. (B) The reduction in pALP activity induced by 10 μM of 2-AG was reversed by AM630, a CB2 antagonist but not by the CB1 antagonist, AM251. (C) CB receptor antagonists, AM251 and AM630 (1 μM), did not decrease cytotrophoblast cell viability, in the absence or presence of 2-AG. l-Phenylalanine (L-Phe) was used as control for ecto-pALP activity (*** p < 0.001; ** p < 0.01 vs. Control; ##p < 0.01 vs. 2-AG 10 μM).
CB2 antagonist, AM630, reversed the 2-AG-induced reduction in ecto-
ALP activity. The CB1 antagonist (AM251) only attenuated 2-AG
effects by 9% (Fig. 3B). To verify that these effects were not due to
fluctuations in cell viability, we examined the effects of CB recep-
tor antagonists in the absence or presence of 2-AG (10 μM). Again,
these conditions were harmless for cytotrophoblast viability (Fig. 3C).

3.4. Effects of 2-AG in hCG secretion

The placental hormone hCG is essentially produced by the syn-
cytiotrophoblast and is crucial for different cellular processes required
for a proper pregnancy outcome. The quantification of β subunit of
this hormone revealed that 2-AG (10 μM) induced a notorious de-
crease (43%) in its secretion, after 48 h treatment (Fig. 4A). Pre-
incubation with both CB receptor antagonists reversed 2-AG effects
in 28% for AM251 and 27% for AM630, suggesting that CB1 and CB2
are implicated in the mechanism involved in 2-AG-induced de-
crease in hCG secretion.

3.5. Leptin gene transcription in 2-AG-treated cytotrophoblasts

In placenta, leptin is essentially produced by the syncy-
tiotrophoblast. The evaluation of mRNA levels revealed that
cytotrophoblasts treated with 2-AG (10 μM) for 48 h presented a
33% decrease in Leptin gene transcription, in comparison with un-
treated cells (Fig. 4B). This decrease was significantly reversed by
both AM251 and AM630, suggesting that the 2-AG effect in Leptin
gene transcription is CB receptor-dependent.

3.6. Role of 2-AG in the morphological differentiation of
human cytotrophoblasts

E-cadherin is a transmembrane protein required for cell–cell ad-
hesion. Its expression decreases in parallel with the differentiation
of the mononuclear cytotrophoblast cells into the multinucleated
syncytiotrophoblast. Human trophoblasts express two fusogenic
retroviral envelope proteins, syncytin-1 and syncytin-2, which are
encoded by HERVW-1 and HERVFRED-1, respectively. These fusogenic
proteins participate in the formation of the syncytium and its tran-
scription is regulated by GCM-1. To assess 2-AG interference in
morphological differentiation of cytotrophoblasts, we studied
the transcription of GCM-1 and also of HERVW-1 and HERVFRED-1
in cells treated with 2-AG (10 μM) and CB receptor antagonists. 2-AG
decreased the mRNA levels encoding for GCM-1 (Fig. 5A) and for
syncytin-2 (Fig. 5C), though no significant effects were detected for
syncytin-1 (Fig. 5B). The effects in GCM-1 and syncytin-2 transcription
were reversed by both CB receptor antagonists. We also observed
that 2-AG treatment reduced the number of syncytium by 22%, as
shown by the decrease in the number of nuclei within the same
cell boundary, represented by the green staining for E-cadherin
(Figs 5D, E). CB1 or CB2 receptor antagonists were also able to reverse
the decrease in syncytialization (Fig. 5D).

3.7. Influence of 2-AG in the cAMP/PKA pathway during human
cytotrophoblasts differentiation

The cyclic AMP/protein kinase A (cAMP/PKA) pathway is impor-
tant for the syncytialization process. We concluded that 2-AG (10 μM)
decreased the levels of cAMP, an effect that was reversed by both
CB receptor antagonists and also by PTX (Fig. 6A). In addition, 2-AG
diminished the phosphorylation of PKA, an effect that was also
reversed by CB receptor antagonists (Figs 6B, C).

4. Discussion

The development of placenta is a critical event for the pregnan-
cy success. In fact, impairments in the process of placentalization
resulting from abnormalities in cytotrophoblast proliferation or differ-
entiation have been associated with pregnancy complications
(Langbein et al., 2008; Lim et al., 1997; Ruebner et al., 2010).
The role of endocannabinoids during gestation is not well clarified but
an unbalanced endocannabinoid signalling has been related to ges-
tational complications, such as miscarriages (Fonseca et al., 2013a;
Taylor et al., 2010). The endocannabinoid 2-AG modulates implan-
tation (Wang et al., 2007) and decidualization (Fonseca et al., 2010)
in rodents; its importance in human placental development has been
poorly investigated, though our group described that 2-AG induces
apoptosis in BeWo cells (Costa et al., 2014).

We have previously demonstrated the expression of the main
enzymes that synthesize and hydrolyze 2-AG (DAGL-α and MAGL,
respectively), in human cytotrophoblasts (Costa et al., 2014). In this
work, we report that both human cytotrophoblasts and syncyti-
trophoblast express these enzymes in similar levels, indicating that
the expression of these molecules does not fluctuate with the dif-
ferentiation status of trophoblasts. These enzymes are required to
locally regulate 2-AG levels and their presence suggests that 2-AG
function may be relevant for placentalization. Thus, we investigated a
possible role for 2-AG during in vitro differentiation of cytotrophi-
blast into the syncytiotrophoblast, by the assessment of biochemical
and morphological differentiation markers.

We observed that exposure of cytotrophoblasts to 2-AG induced
a decrease in pALP activity, a remarkable reduction in hCG secre-
tion and a decrease in leptin transcripts, three relevant markers of
cytotrophoblast differentiation. This was accompanied by an im-
pairment in cytotrophoblast fusion, as revealed by the diminished
GCM-1 and syncytin-2 mRNA levels and E-cadherin staining. Im-
portantly, the observed decrease in the levels of differentiation
markers is not due to a reduction in cell viability. We also dem-
onstrated that the decrease in hCG secretion, Leptin, GCM-1 and
syncytin-2 mRNA levels and in the number of nuclei in syncytium
was partially reversed by both CB1 and CB2 antagonists. However,
the effect of 2-AG in pALP activity was only significantly reversed
by the CB2 antagonist.

CB receptors are G-protein coupled receptors and are mainly
coupled to Goi subunits, whose activation triggers several signal-
ing pathways, including the inhibition of adenyl cyclase.
Consequently, the decrease in cAMP levels leads to an inhibition of
cAMP-dependent protein kinase A (PKA) pathway (Demuth and Mollemann, 2006). The importance of the cAMP/PKA cascade in CTs’ morphological and biochemical differentiation is known for years. An increase in cAMP levels is required for the differentiation process and for the synthesis of hCG, in a mechanism that leads to PKA activation and phosphorylation of cAMP response element binding protein (CREB) (Keryer et al., 1998a, 1998b; Knofler et al., 1999; Milsted et al., 1987). In BeWo cells, a crosstalk between cAMP/PKA and mitogen-activated protein kinase (MAPKs) pathways was described to promote CT differentiation, by increasing hCG biosynthesis and expression of fusogenic genes (Delidaki et al., 2011). However, besides the cAMP-activated pathways, it was suggested that other cellular mechanisms may regulate hCG secretion in BeWo cells (Orendi et al., 2010). Our data revealed that 2-AG inhibits cAMP/PKA signalling pathway. Hereupon, it is likely that 2-AG-induced inhibition of CT differentiation results from the activation of CB receptors, which will consequently decrease cAMP levels, diminishing PKA activity. Moreover, we showed that 2-AG effects on cAMP levels were reversed by PTX, an inhibitor of Gi protein, which supports that CB receptors are coupled to this G protein subunit in cytotrophoblast cells and explains the decreased levels of cAMP after 2-AG treatment.

The multiple functions of hCG during pregnancy are well documented. In fact, it participates in several pregnancy events, such as stimulation of progesterone synthesis by corpus luteum, angiogenesis, immunoprotection and CT fusion and differentiation (Cole, 2012). In this way, disruption in the endocannabinoid signalling may interfere with these processes, due to the reduction of hCG production. Additionally, hCG enhances the production of leptin by the human ST, through a mechanism that involves an interplay between cAMP and MAPKs (Ge et al., 2011). This hormone has pleotropic effects in pregnancy, such as angiogenesis, stimulation of hCG secretion, CT proliferation, blastocyst-endometrium communication and implantation (Henson and Castracane, 2006). Our results revealed that 2-AG decreased leptin transcripts, suggesting that this eCB also affects the production of this protein. A linkage between leptin and ECS has already been described. In fact, this cytokine activates the promotor of FAAH in human T lymphocytes, reducing AEA levels (Maccarrone et al., 2003). Also, in leptin knockout mice, levels of AEA and 2-AG are elevated in uterus, in comparison with wild-type mice (Maccarrone et al., 2005).

Placental alkaline phosphatase is a widely used marker of syncytialization, since its expression is mainly restricted to STs. The physiological role of pALP is not clear, but there are evidences of...
its participation in the transport of maternal IgG to the fetus (Makiya and Stigbrand, 1992a, 1992b). Its activity is decreased in cytotrophoblasts isolated from intrauterine growth restriction placentas (Keating et al., 2009). Our data showed that 2-AG reduced pALP activity through CB2 activation, suggesting an impairment in CTs’ biochemical differentiation. Since the expression of this enzyme seems also to be regulated by cAMP-activated mechanisms (Orendi et al., 2010), the 2-AG-induced decrease in pALP activity may also be a result of the reduction in cAMP levels.

Since 2-AG interferes with the levels of some important proteins for the gestational course, it is suggested that deregulations in endocannabinoid signalling may negatively impact on placental development and consequently may participate in the pathophysiological mechanisms of pregnancy complications.

Moreover, our results showed that 2-AG also affects the cytotrophoblast cells fusion by a CB receptor-dependent mechanism, as observed by the reduction in the mRNA levels of the transcription factor GCM-1 and the fusogenic protein syncytin-2 and also in the number of syncytialized cells, though with no effects on syncytin-1 transcription. Syncytin-1 and -2 participate in the cell fusion and formation of the syncytiotrophoblast. Since it regulates the transcription of the syncytnis, GCM-1 is crucial for the trophoblast cell fusion process (Baczyk et al., 2009; Liang et al., 2010; Yu et al., 2002). Syncytin-2 is important for human trophoblast cells fusion (Benaîtreau et al., 2010; Chen et al., 2008; Vargas et al., 2009) and anomalies in its expression reflects the impaired trophoblast differentiation observed in some pathological conditions (Malassine et al., 2008; Vargas et al., 2011). It is described that cAMP regulates the activation of GCM-1 either by PKA-dependent (Knerr et al., 2005; Lin et al., 2011) or independent (Chang et al., 2011) mechanisms in BeWo cells. 2-AG-induced impairments in GCM-1 and syncytin-2 transcription, through the activation of both CB1 and CB2 receptors, will imply a negative impact in morphological syncytialization as corroborated by the E-cadherin staining results.

Nevertheless, our results were obtained with in vitro studies performed with supraphysiological concentrations of 2-AG, which are in agreement with those used in in vitro studies performed by others working in the endocannabinoids research field. Further studies are required to clarify the role of 2-AG and endocannabinoid signalling in placental development. On the other hand, higher 2-AG concentrations may mimic in vivo abnormal levels of this eCB that may result from an abnormal expression or activity of 2-AG metabolic enzymes.

In conclusion, our data report for the first time that 2-AG interferes with the spontaneous in vitro functional and morphological differentiation of cytotrophoblast into syncytiotrophoblast and points to a role for this eCB during placental development, through a CB receptor-dependent mechanism. In fact, the reduction in hCG secretion, Leptin gene transcription and in pALP activity suggests a negative impact of 2-AG in biochemical differentiation. Furthermore, the decrease in GCM-1 and syncytin-2 mRNA transcripts and in the number of nuclei in syncytium indicates an impairment in cytotrophoblast fusion. We propose 2-AG as a novel player in the network of hormones, proteins and other mediators that regulate cytotrophoblast cell differentiation which are essential for a successful development of the placenta.

**Author’s roles**

M.A.C.: experimental design, performance of all the experiments, data analysis and manuscript draft; E.K., B.M.F, N.A.T. and G.C.S.: experimental design, data analysis and manuscript draft.

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