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Characterisation of the endocannabinoid system in rat haemochorial placenta

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ABSTRACT

Trophoblast cells that comprise the placenta play a crucial role in the complex cross-talk between fetus and maternal tissues. Although anandamide and 2-arachidonoylglycerol, the best studied endocannabinoids, affect trophoblast attachment and outgrowth, the functional significance of the endocannabinoid system in the development of placenta has not been established. We investigated the correlation between endocannabinoid levels and the pattern of expression of the receptors and metabolic enzymes of the endocannabinoid system during rat placental development. Here, we showed that all the endocannabinoid machinery is dynamically expressed in the functionally distinct basal and labyrinth zones of the rat placenta. Indeed, endocannabinoid levels are shown to increase with the progression of pregnancy. Together, these data support a role for the endocannabinoid system in normal placental function and evidence for a potential novel cellular target for the deleterious action of cannabis-derived compounds during the second half of pregnancy.

1. Introduction

The Δ^9-tetrahydrocannabinol (THC), principal psychoactive ingredient of the marijuana plant, Cannabis sativa, mimics the effects of the endogenous ligands named endocannabinoids, which binds to and activates cannabinoid receptors. Besides cannabinoid receptors and endocannabinoids, the endocannabinoid system (ECS) also comprises the specific molecular machinery for the synthesis, transport, and inactivation of the ligands [1].

Two arachidonate derivatives, N-arachidonoylethanolamine or anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are considered to be the major endocannabinoid ligands for the endocannabinoid receptors [2,3]. Although other endocannabinoid-like molecules, such as N-oleoylethanolamine (OEA) and N-palmitoylethanolamine (PEA) are considered inactive at these receptors, they are commonly referred to as 'entourage compounds' because they are degraded by the same enzyme protecting AEA from degradation [4].

Classically, AEA is thought to be produced by the cleavage of a membrane lipid precursor, N-arachidonoylphosphatidylethanolamine (NAPE), by the endocannabinoid-specific enzyme NAPE-phospholipase D (NAPE-PLD), whilst 2-AG is produced by the combined actions of phospholipase C, and diacylglycerol lipase (DAGL), resulting in the production of 1,2-diacylglycerol as an intermediate product [5]. The classical view that endocannabinoids are synthesized "on demand" is currently being challenged, as new evidence indicates that there may be other controlling mechanisms for local and peripheral endocannabinoid levels, including intracellular transport and storage in specific reservoirs [6–8].

Nevertheless, once synthesized, endocannabinoids are thought to be released and act either on the cannabinoid receptors of the cells surrounding the site of production, or are rapidly internalized and hydrolysed. AEA hydrolysis is mediated by the enzyme fatty acid amide hydrolase (FAAH) to arachidonic acid and ethanolamine. Minor catabolic routes involve lipoxygenases, cytochromes P450, and primarily cyclooxygenase-2 (COX-2), resulting novel bioactive lipid derivatives [9]. Although FAAH, α-β-hydrolase 6 (ABHD6) and 12 (ABHD12) can also catabolise 2-AG, the enzyme monoacylglycerol lipase (MAGL) is considered to be the main catabolic enzyme for this molecule, resulting in the production of arachidonic acid and glycerol [5,10].

The cannabinoid receptor family currently includes two main receptor types, CB_1 and CB_2, although a third, the orphan receptor, GPR55, has also been suggested [11]. Initially, CB_1 was primarily found in the brain [12] and CB_2 was predominantly described in cells of the immune system [13], though several groups, including ours, have demonstrated expression of both receptors throughout the body [14,15]. Cannabinoid receptors belong to the Gi/o

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family of seven transmembrane G-protein coupled receptors (GPCRs) with an extracellular amino terminus and an intracellular carboxyl terminus. CB_1 receptors can also act through Gs or Gq11 proteins [16]. Furthermore, there are many reports of the ability of cannabinoid ligands to activate non-cannabinoid receptors providing new molecular targets for cannabinoid research. Among these is the transient receptor potential vanilloid type-1 receptor (TRPV1), a non-selective ligand-gated ion channel activated by heat, acidic pH and capsaicin, the active component of chilli peppers [17]. In fact, the action of AEA on TRPV1 has been suggested as evidence for AEA being one of the natural endogenous ligands for TRPV1 and consequently, has been proposed as the prototypical endovanilloid [18].

Although Cannabis sativa consumption is often associated with adverse pregnancy outcomes, including miscarriage and prematurity [19], the underlying mechanisms remain unclear. What is known is that endocannabinoid signalling has been reported to be critical for embryo-uterine cross-talk and, consequently, for successful blastocyst activation and implantation. Low levels of AEA in the receptive uterus and of CB_1 in activated blastocysts are beneficial for implantation, whilst higher levels are deleterious [20–22].

Although the general placenta architecture varies considerably among mammalian species, their basic morphology, the main cell types and functions, as well as, the molecular mechanisms underlying placental development are well conserved across species [23]. Rat, like human, presents a highly invasive type of placenta, the haemochorial, with deep invasiveness of trophoblast cells. Although the rat diverges from humans in the implantation process, it is an appropriate model for understanding the placenta morphological development.

Recently, we demonstrated that the rat maternal decidua is a potential target for cannabinoid action [14,24,25], and concluded that endocannabinoids could potentially affect the development of the placenta. Here, we investigate the potential role of the ECS on the rat placenta in the second half of pregnancy, by measuring the levels of AEA, 2-AG and the endocannabinoid-like compounds, OEA and PEA to verify the presence of endocannabinoid ligands in placental tissues during placenta development. In addition, the spatial and temporal expression patterns of all the components of ECS, including the eicosanoid-related enzyme COX-2 were examined, to determine if the placenta is potentially responsive to endocannabinoids.

2. Materials and methods

2.1. Animals and tissue preparation

The animal protocol was in accordance to the guidelines of the Ethics Committee and was approved by the institutional review board of the Institute of Molecular and Cellular Biology, Oporto University. The experiments were performed in compliance with the European legislation on the use of laboratory animals.

Female Wistar rats (Charles River Laboratories, Barcelona, Spain) were mated and the day on which spermatozoa were found in the morning vaginal smears was designated as the day 1 of pregnancy. On days 14, 16 and 19 of pregnancy, animals ($n = 5$) were sacrificed, uterine horns collected and implantation units were fixed in 10% (v/v) buffered formalin and processed for routine paraffin histology. Tissues were sectioned (4 μm) and stained using hematoxylin and eosin staining (H&E) to determine the general morphology and to identify the different cell types present.

For tissue preparation foetus were removed from implantation units and placenta (fetal portion) was separated from maternal tissues by blunt dissection.

2.2. Western blotting

Following separation, placentas were homogenized (1:1) in homogenisation buffer (20 mM HEPES buffer, 2 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$) supplemented with 1 mM phenylmethylsulphonylfluoride (PMSF) and aprotinin (1%). The homogenates were centrifuged at 700 × *g* for 10 min at 4 °C and the supernatants re-centrifuged at 12,000 × *g* for 30 min at 4 °C. The resulting supernatants were stored at −80 °C. Protein concentrations in the tissue homogenates were measured using the Bradford assay (Bio-Rad, Laboratories Melville, NY, USA). Proteins were separated by SDS-PAGE (8–12%) under reducing conditions, and transferred onto nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Blots were probed by overnight incubation at 4 °C with antibodies against CB_1 (1:100; sc-20754; Santa Cruz, CA, USA), CB_2 (1:100; sc-25494; Santa Cruz, CA, USA), TRPV1 (1:100; sc-12498; Santa Cruz, CA, USA), NAPE-PLD (1:100; 10005430; Cayman Chemicals, MI, USA), FAAH (1:100; sc-26427; Santa Cruz, CA, USA), COX-2 (1:100; 160126; Cayman Chemicals, MI, USA), DAGL (1:300; a gift from Dr. Juan Suárez) or MAGL (1:100; 100035; Cayman Chemicals, MI, USA) diluted in 5% non-fat milk solution. After incubation, membranes were washed with PBS (0.1% Tween 20) and incubated for one hour at room temperature with a secondary anti-rabbit or anti-goat peroxidase-conjugated IgG in blocking solution. Immunoreactive bands were visualized using an enhanced chemiluminescence system (Super Signal West Pico; Pierce, Rockford, USA) and exposure to X-ray film (Kodak XAR; Eastman Kodak, Rochester, NY, USA). Membranes were stripped and probed again with a rabbit anti-β-tubulin antibody (1:500; Santa Cruz, CA, USA) for densitometric quantification and normalisation to β-tubulin expression. Positive control tissues were spleen for CB_2, day 22 pregnant uterus for COX-2, and rat brain was used for the other antigens.

2.3. Quantitative PCR analysis

Between 50 and 100 mg of placenta from each day were homogenized directly in the homogenisation buffer (Qiagen RNAEasy kits; Qiagen, Hilden, Germany) and the RNA purified through Qiagen columns according to the manufacturer's instructions. RNA quality was assessed using ethidium-stained gels and a 260 nm/280 nm ratio. RNA with a 260/280 ratio of 1.8 and above was reverse-transcribed using avian myelomablastosis reverse transcriptase (Promega Corp. Southampton, UK) as previously described [26] (Table 1).

For quantitative PCR (Q-PCR), 10 pmol/μl of gene-specific primers, with 1 μl of cDNA as template, were used in a SYBR green system (Roche Diagnostics, Lewes, UK) within a Roche Lightcycler 1.2. The PCR conditions were started with a denaturation step at 95 °C for 10 min followed by up to 50 cycles of denaturation, annealing and primer extension, as previously described [14,24,27]. Standard curves of diluted cDNA pools were constructed for each gene target and the expression levels corrected for the levels of rat β-actin using the $2^{-\Delta\Delta Ct}$ method [28] and normalised to levels determined on day 14 of pregnancy.

2.4. Extraction of N-acylethanolamines and 2-arachidonoylglycerol

An internal standard solution containing 12.5 pmol/ml AEA-d8, 12.5 pmol/ml OEA-d2, 25 pmol/ml PEA-d4 and 25 pmol/ml 2-AG-d8 internal standards were added to pre-weighed placentae samples (100 mg) collected for this purpose. Lipids were then extracted by homogenisation of the tissues in chloroform/methanol 2:1 (v/v; 2 ml) and centrifugation (800 × *g* for 30 min at room temperature) to allow phase separation. The lower organic phase was recovered, dried under a constant stream of nitrogen, reconstituted in 80 μl of acetonitrile, and transferred to an HPLC sample vial ready for UPLC–MS/MS analysis, as described previously [29,30].

2.5. Quantification of N-acylethanolamines and 2-arachidonoylglycerol

The quantification of the AEA, OEA, PEA and 2-AG was achieved using a UPLC–MS/MS system composed of an Acquity UPLC in line with a Quattro Premier tandem mass spectrometer (Waters Ltd., Elstree, UK), as described previously [29–31]. In brief, separation was accomplished with an Acquity UPLC BEH C18 column (2.1 mm × 50 mm; Waters Ltd.) maintained at 40 °C. The two mobile phases consisted of 2 mM ammonium acetate containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). Gradient conditions were as follows: 0–0.5 min, 80% A; 1.5 min, 0% A; 2.5 min, 80% A and then re-equilibrated at 80% A until 3.5 min. For 2-AG, the mobile phases consisted of aqueous 70 μM silver acetate (A) and methanolic 70 μM silver acetate (B). Gradient conditions were as follows: 0–0.2 min, 40% A; 3.0 min, 0% A; 3.25 min, 0% A and 3.5 min 40% A. Quantification of analytes was achieved using tandem electrospray mass spectrometry in positive ion mode (ES+) with the products being monitored in multiple reaction monitoring mode. The transitions employed for AEA and for AEA-d8 were m/z 348.3 > 61.9 and m/z 356.3 > 62.9; for OEA and OEA-d2 were m/z 326.5 > 61.9 and 328.2 > 61.9; for PEA and PEA-d4 were 300.5 > 61.9 and 304.2 > 61.9; and for 2-AG were 485.13 > 411.16 and 493.13 > 419.16 for $[2AG]Ag^+$ and $[2\text{-}AG\text{-}d8]Ag^+$, respectively. The injection volumes for samples and standards were 7 μl for N-acylethanolamines and 5 μl for 2-arachidonoylglycerol and eight-point calibration curves in triplicate were performed.

2.6. Immunohistochemistry

Immunohistochemistry was achieved using an avidin–biotin alkaline phosphatase complex immunohistochemical technique (Vectastain ABC kit, Vector Laboratories, CA, USA). After dewaxing, rehydration and blocking of non-specific binding sites, slides were incubated overnight with the respective antibodies at 4 °C (1:100 for all the antibodies, except for DAGL which was 1:500). Subsequently, slides were incubated with biotinylated secondary antibody and then with