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TOXICOLOGICAL SCIENCES, 177(2), 2020, 420–430

doi: 10.1093/toxsci/kfaa110
Advance Access Publication Date: 16 July 2020
Research Article

The Cannabinoid Delta-9-tetrahydrocannabinol Disrupts Estrogen Signaling in Human Placenta

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The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

ABSTRACT

Cannabis consumption is increasing worldwide either for recreational or medical purposes. Its use during gestation is associated with negative pregnancy outcomes such as, intrauterine growth restriction, preterm birth, low birth weight, and increased risk of miscarriage, though the underlying molecular mechanisms are unknown. *Cannabis sativa* main psychoactive compound, Δ^9-tetrahydrocannabinol (THC) is highly lipophilic, and as such, readily crosses the placenta. Consequently, THC may alter normal placental development and function. Here, we hypothesize alterations of placental steroidogenesis caused by THC exposure. The impact on placental estrogenic signaling was examined by studying THC effects upon the enzyme involved in estrogens production, aromatase and on estrogen receptor α (ERα), using placental explants, and the cytotrophoblast cell model BeWo. Aromatase expression was upregulated by THC, being this effect potentiated by estradiol. THC also increased ERα expression. Actions on aromatase were ERα-mediated, as were abolished by the selective ER downregulator ICI-182780 and dependent on the cannabinoid receptor CB1 activation. Furthermore, the presence of the aromatase inhibitor Exemestane did not affect THC-induced increase in ERα expression. However, THC effects on ERα levels were reversed by the antagonists of CB1 and CB2 receptors AM281 and AM630, respectively. Thus, we demonstrate major alterations in estrogen signaling caused by THC, providing new insight on how cannabis consumption leads to negative pregnancy outcomes, likely through placental endocrine alterations. Data presented in this study, together with our recently reported evidence on THC disruption of placental endocannabinoid homeostasis, represent a step forward into a deeper comprehension of the puzzling actions of THC.

Key words: Δ^9-tetrahydrocannabinol; placenta; aromatase; estradiol; estrogen receptor.

Cannabis is one of the most consumed worldwide illicit drug. Besides its recreational consumption, cannabis use for medical purposes is increasing in some European countries and states of the United States (Manthey, 2019; National Academies of Sciences and Medicine, 2017) to relieve disease-related symptoms, such as in multiple sclerosis, pediatric epilepsy, chronic pain and pain, and nausea in cancer patients undergoing chemotherapy (Elliott *et al.*, 2019; Lloyd and Cannons, 2019; Mortimer *et al.*, 2019). Moreover, cannabis use by pregnant women to relieve morning sickness is becoming a trend

(Dickson *et al.*, 2018; Roberson *et al.*, 2014). However, cannabis consumption is associated with adverse reproductive outcomes such as prematurity, intrauterine growth restriction, and low birth weight (El Marroun *et al.*, 2009; Gunn *et al.*, 2016; Hayatbakhsh *et al.*, 2012), though the molecular mechanisms underlying those effects are poorly explored. Although *Cannabis sativa* main psychoactive compound, Δ^9-tetrahydrocannabinol (THC) was discovered many years ago (Gaoni and Mechoulam, 1971) the pharmacological and toxicological effects are not fully understood, particularly in special population groups such as pregnant women. THC is highly lipophilic and as such readily crosses the placenta to reach fetal circulation. Consequently, THC may impact placental development as well as its function and influence fetal growth.

The THC biological effects are mediated by 2 members of the G-protein-coupled receptor family, cannabinoid receptor type 1 (CB1) and type 2 (CB2). CB1 is abundant in the central nervous system (CNS) and is also present in peripheral tissues including uterus. CB2 is mainly located in the spleen and immune cells being in that way involved in the modulation of inflammation, though it also exists in the CNS. Both receptors are expressed by placental trophoblast cells (Helliwell *et al.*, 2004; Maia *et al.*, 2019; Park *et al.*, 2003) and play a key role in the postimplantation period (Fonseca *et al.*, 2013), as well as, in placentation and trophoblast cell biology (Costa, 2016a).

The endocannabinoid system (ECS) is an emergent intervener in the complex signaling pathways that coordinate trophoblast cell turnover, crucial for placental development. The major endocannabinoids 2-arachidonoylglycerol and anandamide (AEA) modulate trophoblast differentiation (Costa *et al.*, 2015c) apoptosis (Costa *et al.*, 2014) and hormone production (Costa *et al.*, 2015b). Moreover, we recently demonstrated that THC disrupts placental ECS homeostasis (Maia *et al.*, 2019), reinforcing the importance of cannabinoid signaling for normal placental development.

The placenta signature cell type, trophoblasts, are the first cells to differentiate from the fertilized egg and later develop into different populations: undifferentiated cytotrophoblast (CT), with self-renewing ability and fully differentiated syncytiotrophoblast (ST) (Boss *et al.*, 2018). STs form a specialized layer, in direct contact with maternal blood, which accounts for the majority of placental hormone synthesis (Latos and Hemberger, 2016). Amongst the hormones secreted, estrogens are some of the most important, being 17-β estradiol (E2) the most abundant (Costa, 2016b). Cytochrome P450 aromatase, encoded by *CYP19A1*, is a rate limiting enzyme for E2 synthesis. This hormone plays important roles during pregnancy such as stimulation of endometrial growth and differentiation, as well as promotion of embryo implantation (Groothuis *et al.*, 2007). In addition, it is involved in angiogenesis and vasodilation leading to the establishment of the uteroplacental blood flow (Albrecht and Pepe, 2010; Corcoran *et al.*, 2014). In addition, the rise of E2 levels during term suggests its implication in uterine contractions (Di *et al.*, 2001; Welsh *et al.*, 2012). These actions are exerted through activation of estrogen receptors, ERα and ERβ (Bechi *et al.*, 2006; Bukovsky *et al.*, 2003a; Kim *et al.*, 2016). It is known that the lack of placenta ERα is correlated with abnormal placentation (Bukovsky *et al.*, 2003b), highlighting the importance of estrogen signaling during placental development.

The underlying molecular mechanisms of cannabis use on negative pregnancy outcome are not clear and endocrine alterations likely may contribute to the growth-restricted phenotype. Here, we hypothesize alterations of placental steroidogenesis caused by THC exposure. Therefore, we examined the impact of THC on placental estrogenic signaling by using placental explants and the cytotrophoblast cell model BeWo.

MATERIALS AND METHODS

Chorionic villous explants cultures. Human term placentae (38–40 weeks of gestation; $n = 10$) from Caucasian women without associated pathologies were collected under informed consent. All the procedures concerning human placental handling were performed after approval of the Ethical Committee of Centro Materno Infantil do Norte, Centro Hospitalar do Porto, Portugal. Informed-consent was obtained from all patients. Shortly, after mechanical removal of the amniotic membranes and decidua, villous explants were prepared according to a previously described protocol (Brew *et al.*, 2016) with the use of ammonium chloride (0.84% m/V) for the lysis of red blood cells. Villous explants were incubated with DMEM/F12 (Gibco/Invitrogen Corporation, California), containing 1% antibiotic/antimycotic solution (100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) and 10% Charcoal Treated Fetal Bovine Serum (FBS-CT) at 37°C and 5% CO_2 for 24 h to stabilize. Explants were treated with 10, 20, and 40 μM of THC (Lipomed AG, Switzerland) and prepared in cell culture medium for 24 h. In the end of the treatment, the explants were collected and immediately frozen for RNA and protein isolation or were fixed for histological analyses.

BeWo cell culture. BeWo cell line is a human choriocarcinoma cell line obtained from the American Type Culture Collection (ATCC, Manassas, Virginia), being a well-accepted cytotrophoblast cell model to study drug effects on placenta (Feinshtein *et al.*, 2013; Jin and Audus, 2005; Wang *et al.*, 2014). Cytotrophoblast cells are an important source of ERα expression, amongst trophoblasts, while also expressing aromatase (Hudon Thibeault *et al.*, 2019; Jeschke *et al.*, 2007; Wang *et al.*, 2006). Cells were cultured in DMEM/F12 medium supplemented with 10% (v/v) FBS-CT and a 1% antibiotic-antimycotic solution, incubated at 37°C and 95% air/5% CO_2 humidified atmosphere. For the experiments, cells were seeded in 6-well plates at density of 6×10^5 cells/well, for Western blot and real-time quantitative PCR (qRT-PCR) assay and in 24-well plates at density of 8×10^4 cells/well for E2 quantification. After adherence (24 h), cells were treated with different concentrations of THC in cell culture medium for 24 h. In the experiments exploring the involvement of aromatase, ERα, and CB1 or CB2 receptor on THC actions, Exemestane (EXE), an aromatase inhibitor (AI), (5 μM) (Amaral *et al.*, 2018) (Sequoia Research Products Ltd, Pangbourne, UK), ICI-182780 (ICI), an ER downregulator, (1 μM) (Wang *et al.*, 2006) (Sigma-Aldrich Co, Saint Louis, Missouri), AM281, CB1 receptor antagonist/reverse agonist, (0.5 μM) (Almada *et al.*, 2017) (St. Cruz Biotechnology, Dallas, TX) and AM630, CB2 receptor antagonist/reverse agonist, (0.5 μM) (Almada *et al.*, 2017) (Tocris, Bristol, UK) were preincubated for 30 min before THC treatment.

Immunohistochemistry analysis. For morphological and immunohistochemical analysis, explants were fixed in 10% (v/v) buffered formalin at room temperature for 24 h. Samples were dehydrated through a graded series of alcohols, embedded in paraffin, before transverse sections of 4 μm were mounted on (3-aminopropyl) triethoxysilane (Sigma-Aldrich Company, Madrid, Spain) coated slides.

Table 1. Primer Sequences of the Housekeeping and Target Genes

mRNA Target	Primers (5′3′)	Conditions	Accession No.
GAPDH	S-CGGGAAGCTTGTGATCAA AS-GGCATGGATGGCATGGAC	95°C, 15 s 55°C, 20 s 72°C, 20 s	NM.002046.7
ESR1	S-CCTGATCATGGAGGGTCAAA AS-TGGGCTTACTGACCAACCTG	95°C, 15 s 55°C, 30 s 72°C, 20 s	NM.001122740.1
CYP19A1	S-GACGTCGCGACTCTAAATTG AS-ACCCGGTTGTAGTAGTTGCAG	95°C, 15 s 58°C, 30 s 72°C, 20 s	NM.000103.4

Hematoxylin and eosin staining was employed to study general morphology. The ERα and aromatase expression were analyzed by immunohistochemistry using an avidin-biotin alkaline phosphatase complex, according to the manufacturer's instructions (ABC method, Vector Laboratories, Peterborough, UK). After dewaxing and rehydration, antigen recovery was performed with citrate buffer pH 6 for 4 min in the microwave. After blocking of nonspecific-binding sites, slides were incubated overnight at 4°C, with the primary antibodies (1:100) against ERα (SC-8002, St. Cruz Biotechnology, Dallas, TX), and aromatase (SC-374176, St. Cruz Biotechnology, Dallas, TX). After washing, slides were incubated with diluted biotinylated secondary antibody for 30 min, followed by incubation with ABC-alkaline phosphatase reagent. The reaction was developed by incubation with Fast Red (Sigma-Aldrich Company Ltd, Madrid, Spain). Slides were counterstained with Mayer's hematoxylin solution (Sigma-Aldrich Ltd, Madrid, Spain) and mounted in aqueous mounting medium Aquatex (Merck, Darmstadt, Germany). Negative controls were prepared by substituting the primary antiserum with an isotype-matched nonimmune IgG. Explant sections were analyzed under a bright field microscope (Eclipse E400 Nikon, Japan) equipped with image analysis NIS-Elements Documentation software (Nikon).

RNA extraction and quantitative RT-PCR. The explants for RT-PCR experiments were collected into RNAlater (Sigma-Aldrich Ltd) and stored at −20°C. Tissue samples from each treatment were submitted to homogenization with 500 µl of TRIsure reagent (Bioline, London, UK) by using a pellet pestle. BeWo cells extracts were collected in 500 µl Triple Xtractor reagent (Grisp, Portugal). One microgram of RNA was reverse-transcribed using the GRS cDNA Synthesis Mastermix (Grisp). cDNA was amplified with specific primers, using Xpert Fast SYBR Mastermix (Grisp) in MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories, Melville, New York), according to the manufacturer's protocol. The PCR conditions were, in all cases, initiated with a denaturation step at 95°C for 10 min, followed by up to 40 cycles of denaturation, annealing, and primer extension. Primer sequences are listed in Table 1. The fold change in gene expression was calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with the housekeeping genes, GAPDH and β-actin, though the presented data were calculated by using GAPDH gene normalized to each control group.

Western blotting. The expression levels of aromatase and ERα were evaluated by Western blot. Placental explants and BeWo cells were incubated with different concentrations of THC for 24 h. Explants were homogenized in homogenization buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100), containing a cocktail of protease and phosphatase inhibitors (1:100 v/v), using pellet pestle followed by 3 cycles of sonication. BeWo cells, after treatment, were collected in 120 µl lysis buffer with a cocktail of protease/phosphatase inhibitors and submitted to 1 cycle of frost/defrost. Samples were centrifuged at 10 000 × g for 10 min, supernatants were collected and protein concentration was determined by the Bradford method. Briefly, samples (25 µg) were submitted to 10% SDS-PAGE and proteins were transferred onto nitrocellulose membranes in a turbo transfer system (Trans-Blot, Bio-Rad). Primary antibodies for ERα (1:200) and aromatase (1:200) were incubated overnight at 4°C. Secondary antibodies anti-mouse (1:2000; G21040, Invitrogen, California) peroxidase-linked were incubated for 1 h and the membranes were exposed to a chemiluminescence substrate (Advansta, California). Immunoreactive bands were visualized by a ChemiDocTM Touch Imaging System (Bio-Rad Laboratories). Membranes were then stripped and incubated with anti-β-actin (1:500; SC-47778, St. Cruz Biotechnology, Dallas, TX) to control loading variations. Exe at 5 µM and ICI at 1 µM were used as positive control for aromatase and ERα degradation, respectively.

Estradiol quantification by enzyme-linked fluorescence assay. Secretion of estradiol was evaluated, in conditioned culture medium by an enzyme-linked fluorescent assay, MINI VIDAS kit (bioMérieux SA, France), according to the manufacturer's protocol.

Statistical analysis. All data are expressed as mean ± SEM. Statistical analysis was performed using 1-way ANOVA, followed by Bonferroni ad hoc posttest to make pairwise comparisons of individual means (GraphPad PRISM version 6.0; GraphPad Software, Inc, San Diego, California) when significance was indicated. Differences were considered to be statistically significant when $p < .05$.

RESULTS

Effects of THC on Aromatase and ERα Expression in Chorionic Villous Explants

THC concentrations (10, 20, 40 µM) were chosen based on previous works in the µM range, representing heavy cannabis consumers and on previous *in vitro* studies on endocrine function and cell turnover using trophoblastic cell lines or placental explants (Almada *et al.*, 2020; Chang *et al.*, 2017, 2018; Costa *et al.*, 2015a; Khare *et al.*, 2006; Lojpur *et al.*, 2019; Maia *et al.*, 2019). These concentrations did not affect the morphology and endocrine function of the explants for at least 72 h (Maia *et al.*, 2019). Similarly to our recent work concerning THC impact on AEA

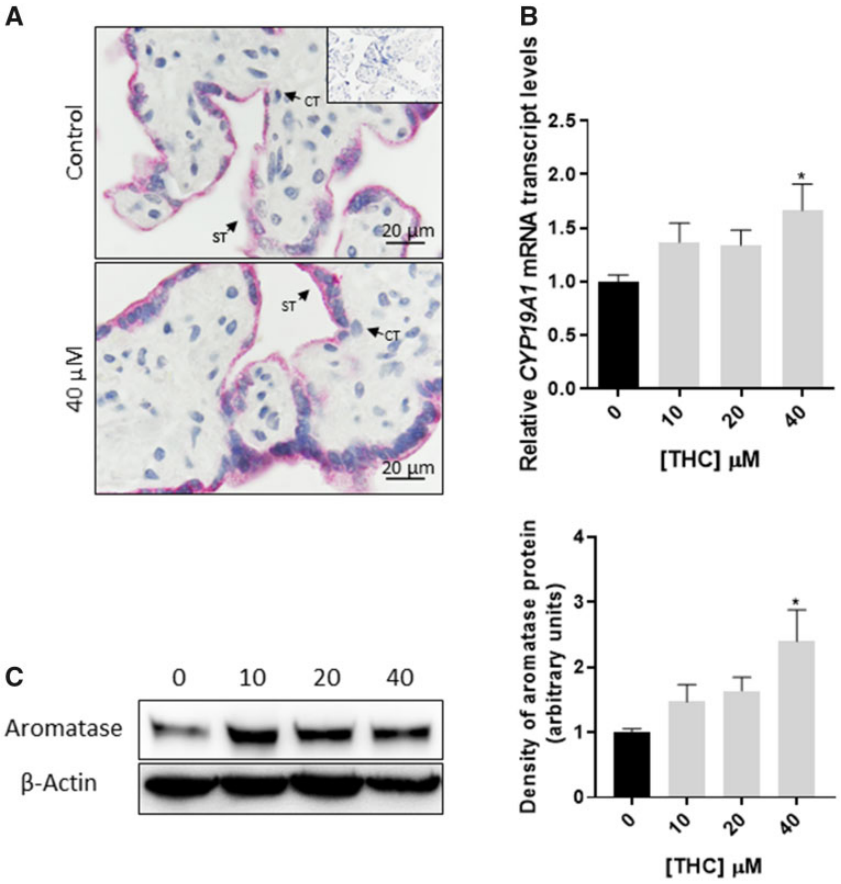

Figure 1. Impact of Δ^9-tetrahydrocannabinol (THC) on placental expression of aromatase. A, Representative immunohistochemical analysis of aromatase expression in placental explants after 24 h treatment with 40 μM THC. Arrows in sections indicate syncytiotrophoblast (ST) and cytotrophoblast (CT). Image of negative control in the small square. B, *CYP19A1* transcript levels analyzed by real-time quantitative PCR after 24 h of THC treatment at concentrations of 10, 20, and 40 μM. Values normalized to GAPDH housekeeping ($N = 10$). C, Western blot and densitometry analysis for aromatase after treatment with 10, 20, and 40 μM of THC for 24 h ($N = 10$). Representative blot normalized for the β-actin content. Significant differences between control- and THC-treated groups are denoted by $^*p < .05$.

metabolism (Maia *et al.*, 2019), we first analyzed the effect of 40 μM of THC, the highest concentration studied, on the pattern of expression of aromatase and ERα in placental explants after 24 h. Aromatase was expressed in the ST layer (Figure 1A), whereas ERα was located mainly in the nuclei of trophoblast (Figure 2A). We then investigated the impact in *CYP19A1* transcript levels and aromatase protein after a 24 h exposure to different THC concentrations (10, 20, 40 μM). The qRT-PCR results show that 40 μM THC significantly increased *CYP19A1* transcript levels (Figure 1B). Accordingly, Western blot analysis also showed that the treatment increased the expression of aromatase protein levels in placental explants (Figure 1C). The same pattern was observed for ERα mRNA and protein levels with 20 μM (Figure 2).

Effects of THC on Aromatase and ERα Expression in BeWo Cells

Similarly, to placental explants, 20 and 40 μM of THC significantly stimulated *CYP19A1* transcript levels (Figure 3A). This increase is accompanied by protein expression as verified by Western blot (Figs. 3C and 3D). Consistent with this, an increase in aromatase main product, E2, was observed upon 40 μM of THC (Figure 3B). In addition, likewise the placental explants, *ESR1* transcription was increased with 20 μM whereas the protein levels increase in response to 20 and 40 μM THC (Figure 4).

Mechanisms of Action of THC: Aromatase and ER Dependency

To test whether THC effects on aromatase and ERα expression are mediated through ERα or aromatase upregulation, BeWo cells were exposed to the selective ER downregulator ICI (Wang *et al.*, 2006) and to the AI EXE alone or in combination with THC.

EXE is a steroidal AI that induces aromatase degradation (Wang *et al.*, 2006). As expected, aromatase expression was diminished by treatment with EXE (Figure 3C). However, THC was not able to increase aromatase expression in the presence of the ER downregulator ICI, indicating the involvement of ERα. In fact, THC-induced aromatase expression is dramatically diminished when compared with THC treatment with or without ICI (Figure 3D). To explore this result, we also tested if estradiol, the ERα ligand, produced the same effect as 40 μM of THC on aromatase expression, and if, in combination they induce an even higher increase on aromatase expression. As seen in Figure 3E, estradiol caused an increase in aromatase expression, which was significantly higher when combined with 40 μM of THC. However, the combination with the steroidal irreversible AI EXE was not able to reverse THC-induced ERα expression (Figure 4C).

Role of CB Receptors in THC-mediated Effects on Aromatase and ERα

To dissect the role of CB receptors in THC-induced disruption of estrogen signaling, BeWo cells were incubated with THC in the presence or absence of either AM281 or AM630, CB1 and CB2 antagonists, respectively. AM281 was able to reverse THC effect

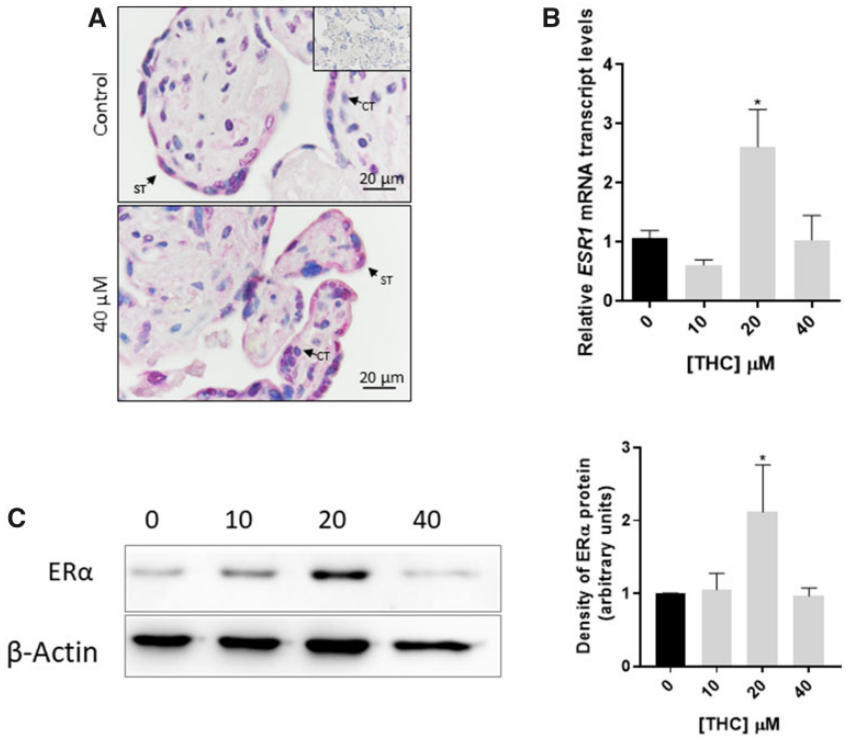

Figure 2. Impact of Δ^9-tetrahydrocannabinol (THC) in placental expression of estrogen receptor α (ERα). A, Representative immunohistochemical analysis of ERα expression in placental explants after 24 h treatment with 40 µM THC. Arrows in sections indicate syncytiotrophoblast (ST) and cytotrophoblast (CT). Image of negative control in the small square. B, *ESR1* transcript levels analyzed by real-time quantitative PCR after 24 h of THC treatment at concentrations of 10, 20, and 40 µM. Values normalized to GAPDH housekeeping ($N = 10$). C, Western blot and densitometry analysis for ERα after treatment with 10, 20, and 40 µM of THC for 24 h ($N = 10$). Representative blot normalized for the β-actin content. Significant differences between control- and THC-treated groups are denoted by $^*p < .05$.

on *CYP19A1* but not AM630 (Figure 5A). However, THC-induced increase in *ESR1* transcription was abolished by both CB receptors antagonists (Figure 5B).

DISCUSSION

Estrogen signaling is present in female reproductive events from the menstrual cycle to placental development and until parturition (Costa, 2016b). With the increase in worldwide cannabis use and the reported negative pregnancy outcomes in female consumers during gestation time, special care should be taken for alterations in normal placenta development. Our group has previously described the impact of THC in BeWo cells turnover (Costa *et al.*, 2015a) and on placental ECS homeostasis (Maia *et al.*, 2019). In addition, THC has been proven to affect proliferation, transcription, invasion, migration (Chang *et al.*, 2017) and angiogenesis (Chang *et al.*, 2018). However, little is known about THC impact on placenta endocrine function.

A disruptive estrogen signaling is associated with a plethora of placental pathologies such as preeclampsia, miscarriage, and ectopic pregnancy (Berkane *et al.*, 2017; Cui *et al.*, 2017; Fugedi *et al.*, 2014; Gebeh *et al.*, 2012, 2013; Molvarec *et al.*, 2015; Trabucco *et al.*, 2009). Recently, Anelli *et al.* (2019), reported that placental *CYP19A1* levels were significantly higher in intra uterine growth restriction (IUGR) placental tissues possibly indicating a compensatory effect to the adverse IUGR intrauterine environment.

Evidence on the ability of cannabinoids to modulate estrogen-related responses is accumulating although many questions remain unanswered. THC has been reported to exert either estrogenic or antiestrogenic effects. In breast cancer cells it has the ability to impair the expression of ERs (Takeda, 2014). Given the importance of estrogen signaling in placenta development and the lack of consensus on THC estrogenic or antiestrogenic actions, we analyzed the effect of this cannabinoid on E2 synthesis and signaling, as well as, the biochemical mechanisms behind these effects in placental explants and in the BeWo cell line. As mentioned in our previous findings on THC impact in the ECS homeostasis (Maia *et al.*, 2019), placental explants are a consensual model, used to study the impact of a given compound on cellular uptake and endocrine function (Brownbill *et al.*, 2016). Moreover, BeWo cells are a CT cell model, which express aromatase (Klempan *et al.*, 2011), as well as ERs (Wang *et al.*, 2006).

After exposure of placental explants to 40 µM of THC, we observed increased *CYP19A1* transcription and aromatase protein expression. In BeWo cells, we verified these same results, as well as an increase in E2 levels. Moreover, 20 µM of THC was able to increase *ESR1* transcript levels and protein expression in both models. However, THC at 40 µM only exerted this effect in BeWo cells. This curious discrepancy may be due to the fact that the placental explant is a more complex model with different types of trophoblast cells. However, previous studies have demonstrated that, although the placental hormones are produced in the ST, CT are the trophoblasts that have the highest ERα expression in placenta (Bukovsky *et al.*, 2003b) and, therefore may have different responses to higher THC concentrations. However, the increase in aromatase expression was reversed by pretreatment with the ER downregulator ICI whereas pretreatment with the AI EXE did not influence THC-induced increase in ERα. Thus, it is suggested that ERα is a key determinant of THC actions.

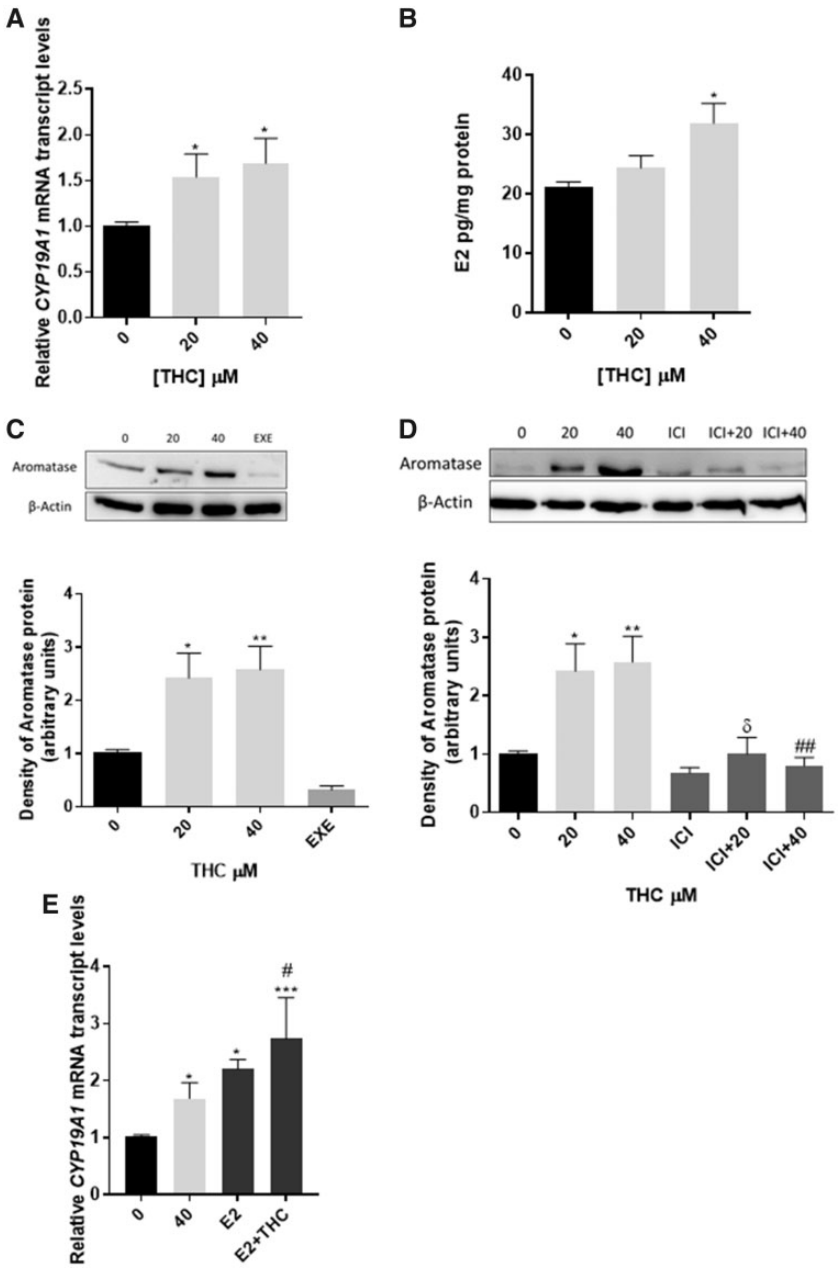

Figure 3. Impact of Δ^9-tetrahydrocannabinol (THC) on BeWo aromatase expression. A, *CYP19A1* transcript levels analyzed by real-time quantitative PCR (qRT-PCR) after 24 h THC treatment, at concentrations of 20 and 40 μM. Values normalized to GAPDH housekeeping ($N = 7$). B, Estradiol quantification by ELFA technique ($N = 7$). C, Western blot and densitometry analysis of aromatase expression after treatment with 20 and 40 μM of THC and for assessment of aromatase inhibitor Exemestane (EXE) effectiveness ($N = 7$). D, Western blot and densitometry analysis of aromatase expression upon treatment with 20 and 40 μM of THC with or without pre incubation with the estrogen receptor downregulator ICI ($N = 7$). Representative blots normalized for the β-actin content. E, Effects of the THC and estradiol (E2) combination on *CYP19A1* transcript levels by qRT-PCR ($N = 7$). Significant differences between control- and THC-treated groups and are denoted by $^{*}p < .05$, $^{**}p < .01$ and $p < .001$. Significant differences between 20 μM and ICI + 20 μM, are denoted by $\delta\ p < .05$. Significant differences between 40 μM and groups previously treated with EXE, ICI, and THC+E2, are denoted by $\#p < .05$ and $\#\#p < .01$.

The interaction between THC and estrogen signaling was first suggested in the 70 s, when the first investigators referred demasculinization or feminization properties, such as a drop in men testosterone levels (Kolodny *et al.*, 1974) and uterotrophic effect on ovariectomized rats (Solomon *et al.*, 1976). However, in the 80 s, THC was also described as having a suppressive effect on steroidogenic activity of rat granulosa cells in culture (Lewysohn *et al.*, 1984). Moreover, in MCF-7 breast cancer cells, it was reported that THC disrupts E2 signaling through the suppression of ERα effects (Takeda *et al.*, 2013). This leads to anti proliferative effects in this type of cells and to endocrine disrupting actions in normal cells, as reviewed by Takeda (2014). Because THC has "no" binding potential for ERα which can be activated by E2, the question of how THC exerted its effect on ERα is not resolved. Our data show for the first time a possible mechanism by which THC treatment affects aromatase expression through the ERα pathway. In addition, this was supported by the potentiated effect of the combination of THC with

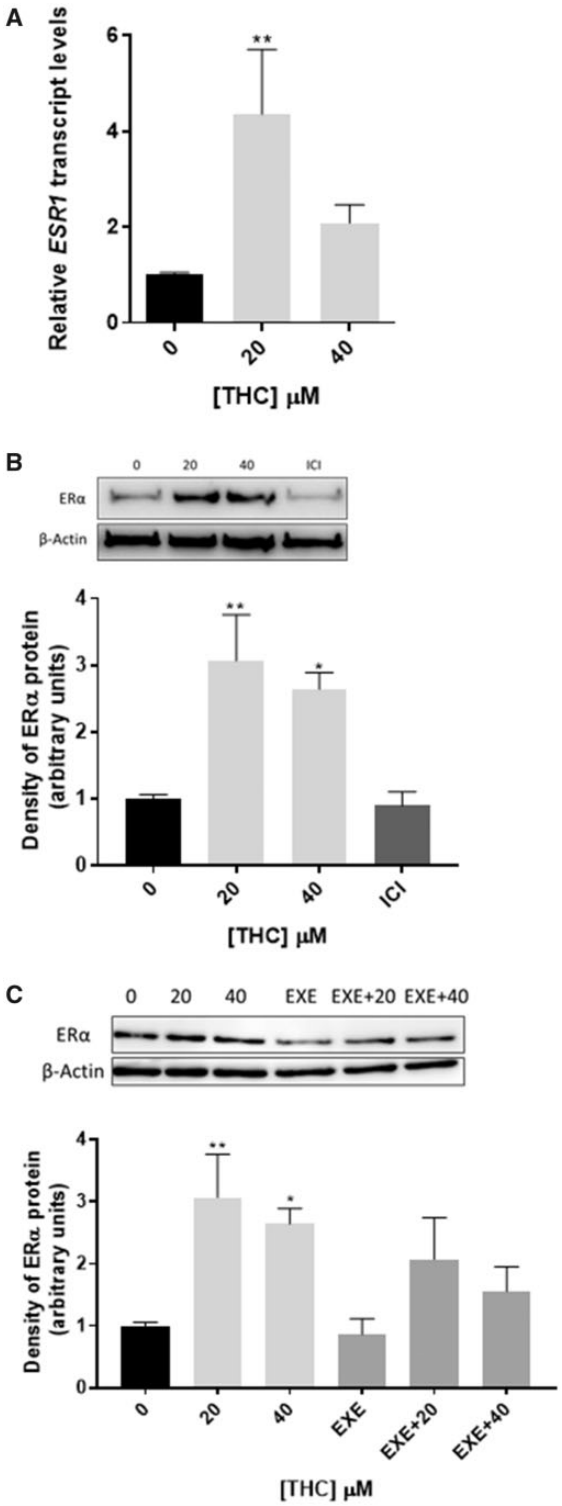

Figure 4. Impact of Δ^9-tetrahydrocannabinol (THC) on BeWo expression of estrogen receptor α (ERα). A, *ESR1* transcript levels analyzed by real-time quantitative PCR after 24 h THC treatment at concentrations of 20 and 40 µM. Values normalized to GAPDH housekeeping ($N = 7$). B, Western blot and densitometry analysis of ERα expression after treatment with 20 and 40 µM THC and assessment of the selective ER downregulator ICI effectiveness ($N = 7$). C, Western blot and densitometry analysis of ERα expression upon treatment with 20 and 40 µM of THC with or without preincubation with the aromatase inhibitor Exemestane (EXE) ($N = 7$). Representative blots normalized for the β-actin content. Significant differences between control- and THC-treated groups are denoted by $^*p < .05$ and $^{**}p < .01$.

estradiol on aromatase expression. Moreover, we also tested the hypothesis that inhibition of aromatase by the AI EXE could reverse the increase in ERα expression. However, even though a partial reversion was observed, it was not statistically significant.

THC evokes its biological activities via engagement with CB receptors CB1 and CB2. In placenta, THC induction of ERα expression may involve CB1 and CB2 activation, as the antagonists AM281 and AM630 blocked *ESR1* transcription. On the other hand, only AM281 was effective in the abrogation of THC induction of aromatase, suggesting that CB1 receptor is involved in the cannabinoid-mediated aromatase induction pathways.

Our results indicate that CB1 and CB2 contribute to the effects of THC, but other receptors may also either directly or indirectly be involved in the modulation of these receptors and THC actions. In fact, THC is able to activate the nuclear receptor, peroxisome proliferator-activated receptor gamma (O'Sullivan *et al.*, 2005) and GPR55 (Pertwee *et al.*, 2010). Both receptors are expressed in human placenta (Kremshofer *et al.*, 2015; Schaiff *et al.*, 2006). However, despite THC ability to activate a selection of receptors and ion channels, the activation of some of these receptors is dependent on THC concentration (Pertwee, 2014), which also may explain the differences in ERα expression upon treatment with 20 and 40 µM in the placental explants and BeWo transcription.

CB receptors and ERs have overlapping molecular pathways (Dobovisek *et al.*, 2016), even though their activation has mostly opposite effects in their shared pathways. In addition, CBs have the ability to form heteromers with other G-protein-coupled receptors, such as observed with dopamine receptors and GPR55. When the respective agonists stimulate both receptors, they may produce contrary effects to the ones generated by the individual parts (Bagher *et al.*, 2017). The heteromerization of GPR55 and CB2 led to a decrease in the activation of transcription factors normally activated by GPR55, however it potentiated the ERK1/2-MAPK activation (Balenga *et al.*, 2014). Despite no interaction is described between CBs and membrane ERs both are located in the lipid rafts and expressed in BeWo cells (Dobovisek *et al.*, 2016; Gambino *et al.*, 2012). It was shown previously that E2-induced genomic and nongenomic effects on leptin gene expression mediated by ERα and G-protein-coupled ER (Gambino *et al.*, 2012). However, THC increased MCF7 breast cancer cell proliferation via cannabinoid-independent signaling (Takeda *et al.*, 2008) but dependent on aromatase (Takeda *et al.*, 2009). Even though these results are opposite to the ones obtained in this work, THC actions may depend on the type of cannabinoid and non-CB receptors recruited and on the levels of endocannabinoids in the tissue or cell type (Finlay *et al.*, 2017).

Although the concentrations in study are widely used for *in vitro* assays, these are not typical levels in plasma after recreational consumption, though levels may vary for the case of sustained use of cannabinoid-containing medicines. In addition, the pharmacokinetic of THC is dependent on individual factors such as adiposity and metabolism (Heuberger *et al.*, 2015). THC due to its lipophilicity is able to cross the placenta (Grant *et al.*, 2018), and although the pharmacokinetics of THC is well-known in nonpregnant adult subjects, information about the maternal-fetal transfer of THC is scarce and the majority of studies evaluating the effects of Δ9-THC on the placenta are from animal models. Moreover, due to selective growing methods, the concentration of Δ9-THC in cannabis has increased from 3% to 12% over the last decade (ElSohly *et al.*, 2016).

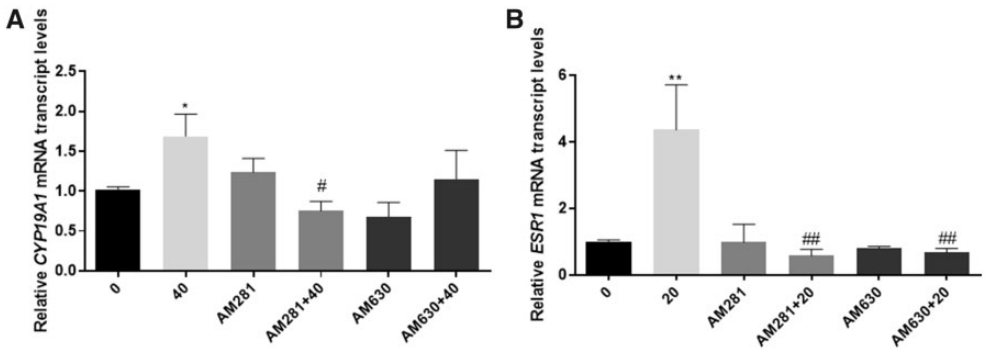

Figure 5. Dependency on cannabinoid (CB) receptors for Δ^9-tetrahydrocannabinol (THC)-mediated effects. A, *CYP19A1* transcript levels analyzed by real-time quantitative PCR after treatment with 40 μM THC with or without preincubation with cannabinoid receptor antagonists AM281 and AM630 ($N = 7$). B, *ESR1* transcript levels after treatment with 20 μM THC with or without preincubation with AM281 and AM630 ($N = 7$). Significant differences between control- and THC-treated groups are denoted by $^{*}p < .05$ and $^{**}p < .01$. Significant differences between THC-treated samples and preincubated with AM281 and AM630 are denoted by $\#p < .05$ and by $\#\#p < .01$.

Taken together our findings indicate that THC disturbs placental endocrine function as augments *ESR1* and *CYP19A1* gene transcription and thus increases E2 production. To our knowledge, this is the first time that THC effects on estrogen signaling were evaluated in term placenta as well as in trophoblast cells. Results provide insight on how THC is responsible for the negative outcomes observed in pregnant cannabis consumers. However, much work still needs to be performed in this respect to allow broadening the knowledge on cannabinoids' mechanisms of action and establishing the impact of cannabinoids in reproductive processes.

ACKNOWLEDGMENTS

The authors thank Dr Laura Pereira from the Clinical Analysis Laboratory of the Faculty of Pharmacy, Porto University for assistance with estradiol measurements.

FUNDING

FEDER Funds through the Operational Competitiveness Factors Program—COMPETE and by National Funds through FCT—Foundation for Science and Technology within the scope of the project European Regional Development Fund (PTDC/DTP-FTO/5651/2014-POCI-01-0145-FEDER-016562);
Fundação para a Ciência e a Tecnologia (BD/136105/2018 to J.M.).

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHOR CONTRIBUTIONS

G.C.-d.-S. conceived the work. J.M., M.A., and L.M. performed the experiments. D.G. and J.B. were responsible for sample collection. J.M. and B.F. analyzed the data. J.M., B.F., N.T., and G.C.-d.-S. interpreted the data. The first draft of the manuscript was written by J.M. and all authors commented on previous versions of the manuscript. All the authors have revised and approved the final version.

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