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ABSTRACT

While studies have demonstrated that the main psychoactive component of *cannabis*, Δ9-tetrahydrocannabinol (Δ9-THC) alone induces placental insufficiency and fetal growth restriction, the underlying mechanisms remain elusive. Given that both (i) endoplasmic reticulum (ER) stress in pregnancy and (ii) gestational exposure to Δ9-THC leads to placental deficiency, we hypothesized that Δ9-THC may directly induce placental ER stress, influencing trophoblast gene expression and mitochondrial function. BeWo human trophoblast cells treated with Δ9-THC (3–30 μM) led to a dose-dependent increase in all ER stress markers and CHOP; these effects could be blocked with CB1R/CB2R antagonists. Moreover, expression of ER stress-sensitive genes ERRγ, VEGFA, and FLT-1 were increased by Δ9-THC, and abrogated with the ER stress inhibitor TUDCA. Δ9-THC also diminished mitochondrial respiration and ATP-coupling due to decreased abundance of mitochondrial chain complex proteins. Collectively, these findings indicate that Δ9-THC can directly augment ER stress resulting in aberrant placental gene expression and impaired mitochondrial function.

1. Introduction

Cannabis is the most frequently used illicit drug in the world having an estimated 140 million consumers; with the impending shift towards legalizing recreational cannabis use this number of users is only expected to rise [1]. Among pregnant women in North America, recent studies indicate nearly 1 in 5 women (18–24 years) were using cannabis during pregnancy [2]. Moreover, while the rates of smoking cannabis in pregnancy have steadily increased from 2002 to 2014, it has also been reported that more women would use cannabis in pregnancy if it were legalized [3]. Women who continue to use cannabis while pregnant may do so to mitigate nausea, anxiety, and lack of appetite [4]. For example, a survey of women in Vancouver, Canada found that 77% of cannabis use during pregnancy was related to the common conception that it will reduce nausea symptoms [4]. While systematic reviews and meta-analyses demonstrate that maternal cannabis use results in low birth weight outcomes, these studies are confounded by sociodemographic influences and the fact that many cannabis users also use other drugs (*e.g.* tobacco) [5–9]. The major psychoactive cannabinoid in cannabis, Δ9-tetrahydrocannabinol (Δ9-THC) can cross the placenta and animal studies indicate that 10% of maternal concentrations can be found in fetal plasma, with 2–5 times higher concentration in fetal tissues [10–12]. This is of great concern considering that due to selective growing methods, the concentration of Δ9-THC in cannabis has increased from 3% to 12% over the last decade [13]. In animal models, fetal exposure to Δ9-THC alone during gestation leads to placental insufficiency and fetal growth restriction, but the underlying mechanisms remain incompletely understood [14,15].

The molecular targets of action for Δ9-THC in the placenta are the two G-coupled cannabinoid receptors CB1R and CB2R, part of the endocannabinoid system [16,17]. CB1R and CB2R are found in heart, embryo, uterus, placenta, peripheral blood lymphocytes while CB1R is found predominantly in central nervous system [17]. The endocannabinoid system has a role in reproduction from the early stages of fertilization, embryo implantation, and placentation [18]. In the pla-

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centa, cannabinoid receptors have been detected in early first and third trimester placental tissues [18]. Interestingly, the levels of endogenous cannabinoid anandamide decrease gradually during pregnancy as high systemic levels of endogenous cannabinoids impede normal pregnancy progression [18,19]. Δ9-THC in cannabis is able to bind to these same cannabinoid receptors and activate them in a similar manner as endogenous cannabinoids.

The majority of knowledge evaluating the effects of Δ9-THC alone on the placenta and fetal development arises from animal studies. In rodents, exposure of pregnant dams to Δ9-THC during gestation led to placental insufficiency and low birth weight offspring [14,15]. In human BeWo placental trophoblasts, Δ9-THC impairs proliferation, invasion and migration [14,20]. However, the mechanism of action linking Δ9-THC exposure and fetal growth restriction mediated *via* placental insufficiency remain incompletely understood.

Recent studies have suggested that endoplasmic reticulum (ER) stress plays a role in etiology of intrauterine growth restriction. The ER is responsible for many functions within the cell, one of which being protein synthesis [21]. ER stress is induced from an accumulation of misfolded and unfolded proteins in the ER lumen [21]. To restore ER homeostasis, the uncoupled protein response (UPR) is activated through three signaling pathways: ATF6, IRE1α, and PERK [22]. The first pathway involves increases in chaperone proteins such as glucose regulated protein 78 (GRP78) which are responsible for enhancing and assisting with protein folding [22]. The second pathway, IRE1 is involved in further upregulation of chaperone proteins and protein degradation, in part due to increases in the expression of the transcription factor, spliced XBP-1 [21]. Finally, the PERK pathway (*i.e.* increases in ATF4) is involved in the attenuation of protein translation *via* phosphorylation of eukaryotic initiation factor (P-eIF2α) in order to reduce the amount of new proteins entering the ER lumen [22]. However, if UPR fails to alleviate ER stress, downstream apoptotic pathways are activated, mediated through C/EBP-homologous protein/Gadd153 (CHOP), which has been associated with compromised placental development and function (*i.e.* IUGR, gestational diabetes, and preeclampsia) *in vivo* and *in vitro* [23–28]. Moreover, during mouse pregnancy, when pregnant dams were administered a single dose of tunicamycin (which inhibits N-linked glycosylation) to pharmacologically induce ER stress, this led to lower placental and fetal weight outcomes due in part to placental abnormalities in nutrient transport and in the aberrant expression of genes involved in angiogenesis [29]. Together, this research suggests that induction of ER stress is one driver of placental insufficiency and fetal growth restriction [29].

Given that (i) treatment with Δ9-THC during pregnancy *in vivo* leads to placental deficiency and fetal growth restriction [14,15,30]; and that (ii) ER stress is associated with both placental dysfunction and impeded fetal growth [29], we hypothesized that administration of Δ9-THC to placental cells will induce ER stress leading to aberrant trophoblast gene expression. The aim of this study was to further understand the cellular mechanism of previously reported Δ9-THC-induced placental insufficiency by directly investigating the relationship between Δ9-THC exposure and placental ER stress in human BeWo trophoblast cells. BeWo cells are a choriocarcinoma cell line derived from first trimester villous trophoblasts which have been widely used as an *in vitro* model to examine the effects of drugs (*i.e.* Δ9-THC, ethanol, nicotine) on placental function [14,20,31,32]. Given the integration of the ER and mitochondria in the placenta [33], we also sought to examine if Δ9-THC induced ER stress was also associated with placental mitochondrial dysfunction.

2. Materials and methods

2.1. Cell culture

BeWo human trophoblast cell line was used as a model of the human placenta *in vitro*. The BeWo cell line derives from a human gestational choriocarcinoma, and is a known cellular model of the human trophoblast, having been widely used as an *in vitro* model for drug (*i.e.* Δ9-THC) studies [14,20,31]. Cells were maintained (passages 8–18) at 37 °C and 5% CO_2/95% atmospheric air in cell culture incubator. Cells were cultured in 75-cm^2 flasks in F-12 K Nutrient medium (Gibco) with 10% fetal bovine serum (Gibco) and 1% P/S.

2.2. Δ9-THC treatments

To test the effect of Δ9-THC on ER stress in BeWo cells, the cells were plated on a 12-well plate with 2×10^5 cells per well 1 mL of F-12 K Nutrient medium and were seeded for 24 h prior treatment. The medium was changed to one that contained increasing concentrations (*e.g.* 3–30 μM) of Δ9-THC (dissolved in final concentration of 0.1%(v/v) ethanol, Cayman Chemicals, Ann Arbor, MI) for 24 h. The vehicle consisted of culture medium containing 0.1%(v/v) ethanol. The doses of Δ9-THC were chosen based on previous pharmacokinetic studies which determined equivalent doses to those found in the serum of cannabis users [20,34]. The 24-hour time-point allowed for detection of changes in the mRNA and protein involved in the UPR which indicated activation of placental ER stress, as we have demonstrated previously with nicotine, a known trigger of ER stress [35].

2.3. CB1R and CB2R antagonist treatments

To block the effects of Δ9-THC on the cannabinoid receptors, cells were treated with CB1R and CB2R antagonists SR141716 and SR144528 respectively. BeWo cells were plated onto 12-well plate and were pre-treated with 1 μM SR141716, 0.1 μM SR144528, or both for 1 h and then treated with 15 μM Δ9-THC solution for 24 h. We chose 15 μM as a mid-range dose and that did not affect cellular viability in BeWo cells [14,20]. These concentrations of the SR141716 and SR144528 have been previously demonstrated to ameliorate the detrimental effects of Δ9-THC on migration in both BeWo and human umbilical vein endothelial cells (HUVEC) cells but alone had no significant effect on BeWo cell migration [14,36].

2.4. Amelioration of ER stress - TUDCA treatments

To prevent Δ9-THC-induced ER stress, we used tauroursodeoxycholic acid (TUDCA); TUDCA has been shown to relieve ER stress in several cell and tissue types, including the placenta [35,37]. After seeding cells in 12-well plate, cells were pretreated for 1 h with 100 μM of TUDCA (Sigma-Aldrich), and then treated with 15 μM Δ9-THC for 24 h. This dose of TUDCA has been effective to ameliorate nicotine-induced ER stress and UPR activation in rat Rcho-1 trophoblast giants cells after 24 h[35]. Additionally, BeWo cells were also treated with 15 μM 11−COOH-THC (Sigma-Aldrich), the inactive THC metabolic, to assess its potential effects on ER stress.

2.5. RNA extraction and real time-polymerase chain reaction

Total RNA was extracted from cells using TRIzol reagent (Invitrogen). Chloroform (Sigma-Aldrich) was added to the solution, and then centrifuged at 12,500 rpm. Supernatant was transferred to a fresh tube with an equal volume of isopropanol (Sigma-Aldrich) and centrifuged again at 12,500 rpm. Total RNA was then collected from the

pellet and dissolved in DEPC-treated water. Deoxyribonuclease I, Amplification Grade (Invitrogen) was added to the RNA to digest contaminating single- and double-stranded DNA. Four micrograms of RNA were reverse-transcribed to cDNA using random hexamers and Superscript II Reverse Transcriptase (Invitrogen). Primer sets directed against gene targets of interest were designed through National Center for Biotechnology Information's primer designing tool and generated *via* Invitrogen Custom DNA Oligos (Table 1). Quantitative analysis of mRNA expression was performed *via* RT-PCR using fluorescent nucleic acid dye Sso-Fast EvaGreen supermix (BioRad) and BioRad CFX384 Real Time System. The cycling conditions were 95 °C for 10 min, followed by 43 cycles of 95 °C for 15 s and 60 °C for 30 s and 72 °C for 30 s. The cycle threshold was set so that exponential increases in amplification were approximately level between all samples. Relative fold changes were calculated using the comparative cycle times (Ct) method, normalizing all values to the geometric mean of the housekeeping gene, GAPDH. GAPDH was determined as a suitable housekeeping gene using algorithms from GeNorm, Normfinder, BestKeeper, and the comparative ΔCt method to place it as the most stable housekeeping gene from those tested (*e.g.* β-actin, 18S ribosomal RNA) [38–41]. Given all primer sets had equal priming efficiency, the ΔCt values for each primer set were calibrated to the average of all control Ct values, and the relative abundance of each primer set compared with calibrator was determined by the formula $2^{\Delta\Delta Ct}$, in which ΔΔCt was the normalized value.

2.6. Protein extraction and Western blot

BeWo cells were homogenized in RIPA buffer (50 mM Tris−HCL, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.25% $C_{24}H_{39}NaO_4$, supplemented with phosphatase inhibitors (20 mM NaF, 40 mM Na-pyrophosphate, 40 mM Na_3VO_4, 200 mM β-glycerophosphate disodium salt hydrate), and a protease inhibitor cocktail (Roche)). The solution was sonicated at 30% amplitude for 5 s total, 1 s per pulse. It was then mixed in a rotator for 10 min at 4 °C and centrifuged at 16,000 *g* for 20 min at 4 °C. The resulting supernatant was collected as the total cellular protein extract and quantified by colorimetric DC protein assay (BioRad). Loading samples were prepared with fresh total protein extract (avoiding repeated freeze-thaw cycles), NuPAGE LDS Sample Buffer (4X) (Invitrogen), NuPAGE Reducing Agent (10X) (Invitrogen), and deionized water, and heated at 70 °C for 10 min to denature the proteins. Proteins (20 μg/well) were separated by size *via* gel electrophoresis in gradient polyacrylamide gels (Novex), and transferred onto polyvinylidene difluoride membrane (Millipore). Membranes were blocked in 1x Tris-buffered saline-Tween 20 buffer with 5% non-fat milk (blocking solution), and then probed using primary antibodies (Table 2) of the protein targets of interest, diluted in the blocking solution. Secondary antibodies (Table 3) were used to detect the species-specific portion of the primary antibody, diluted in the blocking solution. Immuno-reactive bands were visualized using Super-Signal West Dura Chemiluminescent Substrate (Thermo Scientific). Relative band intensity was calculated using ImageLab software (BioRad) and normalized to the quantified total protein on each respective membrane, as determined through Ponceau staining[42].

2.7. Measuring oxygen consumption rate using Seahorse mitochondrial flux analyses

To measure mitochondrial function in BeWo cells, we employed the Seahorse XFe24 analyzer to assess the oxygen consumption rate (OCR). Cells were seeded onto XFe24 seahorse plates at a density of 1×10^5 cells per well and incubated with culture medium alone or culture media treated with 15μM Δ9-THC for 24 h. Culture media were exchanged with unbuffered Seahorse XF base medium supplemented with 10 mM D-glucose, 2 mM L-glutamine, and 2 mM sodium pyruvate for one hour prior to assay. After baseline OCR measurements (*i.e.* basal respiration), we calculated different indices of mitochondrial function from OCR measurements by the sequential injection of selective electron transport chain inhibitors and uncoupling agents, including oligomycin (1.5 ug/mL), 2,4-dinitrophenol (DNP) (50μM), a mixture of rotenone (0.5μM) and antimycin A (0.5μM). The OCR values were normalized to the amount of DNA content from each well using fluorescence Hoescht's stain. Briefly, cell lysates samples are diluted in 2x Hoescht ,33342 dye assay mix (0.0324 mM Hoescht Dye, 2 mM NaCl, 2 mM NaH_2PO_4) and fluorescence was measured at 360 nm excitation with460 nm emission filters. DNA content was determined *via* a standard curve. Addition of oligomycin reduces OCR and reflects respiration independent of oxidative phosphorylation. Addition of DNP stimulates electron transport chain activity and maximal OCR (*e.g.* maximal respiration) and exposes a reserve capacity for respiration (*e.g.* spare respiratory capacity). Addition of Rotenone/Antimycin A (*e.g.* mitochondrial chain complex inhibitors) indicates OCR independent of electron transport chain activity (*i.e.* proton leak). Each experiment was performed with 6 biological replicates from 1 experiment, and repeated 3 more times.

2.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 software. All results were expressed as means of normalized values ± SEM. All experiments were replicated four times (n = 4). The significance of the differences ($p < 0.05$) between normalized mean values were then evaluated using one-way ANOVA followed by Tukey's post-test or, where indicated, an unpaired Student's *t*-test.

Table 1
Forward and reverse sequences for the primers used for quantitative Real-Time PCR.

Gene	Forward	Reverse	GenBank/Reference
Grp78	TGTCTTTTGTCAGGGGTCTTT	CACAGTGGTGCCTACCAAGA	NM_005347
Spliced Xbp1	CCGCAGCAGGTGCAGG	GGGGCTTGGTATATATGTGG	NM_005080.3
Atf6	TTGACATTTTTGGTCTTGTGG	GCAGAAGGGGAGACACATTT	XM_011509309.1
Atf4	CTATACCCAACAGGGCATCC	GTCCCTCCAACAACAGCAAG	NM_001675.4
CHOP	TGATGCTCCCAATTGTTCATG	TCGCCGAGCTCTGATTGAC	NM_001195053.1
VEGFA	ATGACGAGGGCCTGGAGTGTG	CCTATGTGCTGGCCTTGGTGAG	NM_001287044.1
ERRγ	CTGACGGACAGCGTCAACC	GGCGAGTCAAGTCCGTTCTG	NM_001438
FLT-1	ACAATCAGAGGTGAGCACTGCAA	TCCGAGCCTGAAAGTTAGCAA	JX512442
FLK-1	GTGTCAGAATCCCTGCGAAGTA	GAAATGGGATTGGTAAGGATGA	NM_002253
NDI	TGGGTACAATGAGGAGTAGG	GGAGTAATCCAGGTCGGT	MH449555.1
GAPDH	AGGTCCACCACTGACACGTT	GCCTCAAGATCATCAGCAAT	NM_002046

Table 2
Western Blot antibodies, dilutions used in experiments, and company and catalogue information.

Antibody name	Source	Dilution	Company (#Catalogue)
GRP78	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, Massachusetts (#C50B12)
ATF4	Mouse monoclonal	1:500	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA (#sc-390063)
CHOP	Mouse monoclonal	1:500	Cell Signaling Technology Inc., Danvers, Massachusetts (#L63F7)
P-eIF2α [Ser 51]	Rabbit monoclonal	1:1000	Cell Signaling Technology Inc., Danvers, Massachusetts (3597)
CITRATE SYNTHASE	Rabbit polyclonal	1:1000	donated by Dr. Sandeep Raha at McMaster University (Hamilton, ON, Canada)
OXPHOS	Mouse monoclonal	1:500	Abcam (ab110413)

Table 3
Western Blot secondary antibodies, dilutions used in experiments, and company and catalogue information.

Antibody name	Dilution	Company (#Catalogue)
Donkey Anti-Rabbit IgG (H + L)	1:10000	Jackson ImmunoResearch Laboratories, West Grove, PA, USA (#711-001-003)
Donkey Anti-Mouse IgG (H + L)	1:5000	Jackson ImmunoResearch Laboratories, West Grove, PA, USA (#715-001-003)

3. Results

3.1. Δ9-THC induced ER stress in a dose-dependent manner

BeWo cells were treated with vehicle or increasing doses of Δ9-THC (3–30 μM) for 24 h as previously performed [14,20]. Δ9-THC treatment led to the activation of pathways in the UPR in a concetration-dependent manner. At 24 h the steady state mRNA levels of the ER stress markers GRP78 (Fig. 1A), spliced-XBP1 (Fig. 1C), ATF6 (Fig. 1E), and ATF4 (Fig. 1F) were significantly increased by Δ9-THC at 15 and 30 μM compared to vehicle control. The protein levels of GRP78 (Fig. 1B), phosphorylated eIF2α [Ser51] (Fig. 1D), and ATF4 (Fig. 1G) were also increased at 15 and 30 μM compared to vehicle. Downstream the UPR pathway, the steady-state mRNA and protein levels of CHOP, a transcription factor activated during chronic ER stress to upregulate apoptotic pathways, was also significantly increased at 24 h (Fig. 1I).

3.2. Pre-treatment with CB1R/CB2R antagonist abrogated Δ9-THC-induced ER stress in BeWo cells

To identify whether Δ9-THC mediated its effects on ER stress through its cannabinoid receptors, BeWo cells were pretreated for 1 h with 1 μM SR141716 (CB1R antagonist) and 0.1 μM SR144528 (CB2R antagonist) and then treated with Δ9-THC (15 μM) for 24 h. Δ9-THC significantly increased the steady-state mRNA levels of all ER stress markers (*i.e.*, GRP78, spliced-XBP1, ATF6, ATF4, and CHOP) at 24 h (Fig. 2A-E); and this effect of Δ9-THC was completely blocked with CB1R/CB2R antagonists, except for ATF6 mRNA (Fig. 2C), which was partially blocked. Interestingly, treatment with BeWo cells with equimolar concentrations of 11−COOH-THC, the major Δ9-THC metabolite, had no effects on any ER stress markers after 24 h (Fig. 2A-E).

3.3. Pre-treatment with TUDCA alleviated the effects of Δ9-THC-induced ER stress and downstream ER stress target genes in BeWo cells

To determine whether Δ9-THC-induced ER stress could be prevented, the bile acid TUDCA, previously demonstrated to ameliorate placental ER stress (*i.e.* suppress PERK pathway) was employed [25,35]. BeWo cells were pretreated for 1 h with 100 μM of TUDCA and then treated with Δ9-THC (15 μM) for 24 h. Δ9-THC significantly increased steady-state mRNA levels of ER stress markers (*i.e.*, GRP78, spliced-XBP1, ATF6, ATF4, and CHOP) at 24 h while TUDCA pretreatment completely abrogated this effect (Fig. 3A-E). There was no effect on ER stress markers with TUDCA pretreatment alone (Fig. 3A-E). To examine whether Δ9-THC mediated ER stress may result in impaired placental function, we next examined if established ER stress target genes were altered downstream. After 24 h, Δ9-THC increased the steady-state levels of ERRγ and VEGFA mRNA (Fig. 3F-G), two genes demonstrated to be up-regulated by ER stress [43–45]. Moreover, pretreatment with TUDCA abrogated the effects of Δ9-THC (Fig. 3F-G). TUDCA also blocked the stimulatory effect of Δ9-THC on the expression of FLT-1 (VEGFR-1, Fig. 3H), while FLK-1 (VEGFR-2) mRNA could not be detected (data not shown). It is noteworthy that treatment of BeWo cells with COOH-THC had no effect on ERRγ, VEGFA, or Flt-1 mRNA levels compared to vehicle (Fig. 3F-H).

3.4. Δ9-THC treatment decreases mitochondrial respiration and ATP coupling efficiency in BeWo cells

Given the links between ER stress and mitochondrial dysfunction in trophoblast cells [33], we next investigated if Δ9-THC impairs mitochondrial respiration using the XFe24 Seahorse analyzer to measure OCR (Fig. 4A). After 24 h in culture, 15 μM Δ9-THC significantly lowered basal respiration by 50% (Fig. 4B, $p < 0.001$), maximal respiration by 25% (Fig. 4C, $p < 0.05$), ATP-synthase-linked respiration by 50% (Fig. 4E, $p < 0.001$), and ATP coupling efficiency by 25% (Fig. 4G, $p < 0.05$) in BeWo cells. However proton leak (Fig. 4D) and mitochondrial spare capacity (Fig. 4F) was not different between vehicle and Δ9-THC treated cells.

3.5. Δ9-THC treatment decreases the expression of mitochondrial complex proteins in BeWo cells

Since mitochondrial respiration and ATP coupling efficiency was diminished by Δ9-THC, we next pursued if decreases in mitochondrial number, mass, and mitochondrial complex proteins might account for mitochondrial dysfunction. After 24 h in culture, 15 μM Δ9-THC did not significantly alter the mtDNA expression of ND-1 (Fig. 5A), an indicator of total mitochondrial number [46,47]. However, Δ9-THC decreased citrate synthase protein levels by 25% (Fig. 4B, $p < 0.05$), a marker of total mitochondrial mass, compared to vehicle controls [48]. In addition, 15 μM Δ9-THC led to lower protein levels of ATP5 A (part of mitochondrial protein complex V, 50% decrease, Fig. 5D, $p < 0.05$), MTCO1 (part of mitochondrial protein complex IV, 50% decrease, Fig. 5E, $p < 0.05$), UQCRC2 (part of mitochondrial complex III, 30% decrease, Fig. 5F, $p < 0.05$), and NDUFB8 (part of mitochondrial complex I, 30% decrease, Fig. 5H, $p < 0.05$). SDH8 (part of mitochondrial complex II) protein levels were not significantly different between vehicle and Δ9-THC-treated cells (Fig. 5G).

4. Discussion

In this study, we demonstrated that bioactive Δ9-THC (and not its inactive metabolite) can directly augment ER stress in a human trophoblast cell line as indicated through the dose-dependent increases in

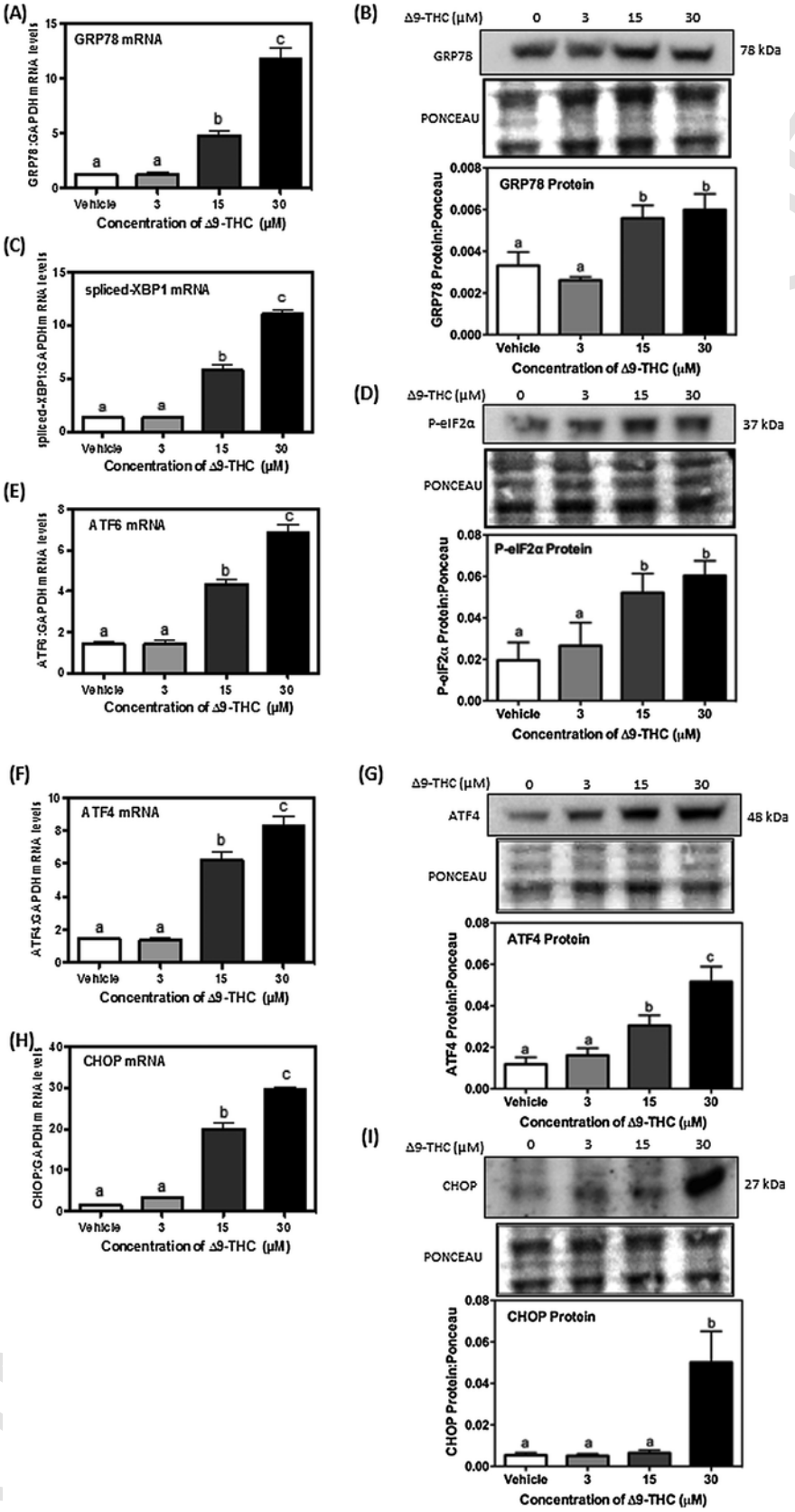

Fig. 1. Δ 9-THC increases ER stress in human BeWo trophoblast cells. Real-time qPCR of human BeWo cells were treated with either vehicle or Δ9-THC (3–30 μM) for 24 h. Total RNA was extracted and reverse-transcribed to cDNA and normalized to GAPDH. For protein analysis, specific targeted protein bands were detected by respective antibodies *via* Western blot and normalized by PONCEAU staining. **A.** GRP78 mRNA, **B.** GRP78 protein, **C.** splied-XBP1 mRNA, **D.** pEIF2α [Ser51], **E.** ATF6 mRNA, **F.** ATF4 mRNA, **G.** ATF4 protein, **H.** CHOP

mRNA, and **I.** CHOP protein. All values were expressed as mean ± SEM (N = 6/group). Significant differences between treatment groups determined by 1-way ANOVA. Different letters represent means that are significantly different from one another according to Tukey's posttest ($P < .05$).

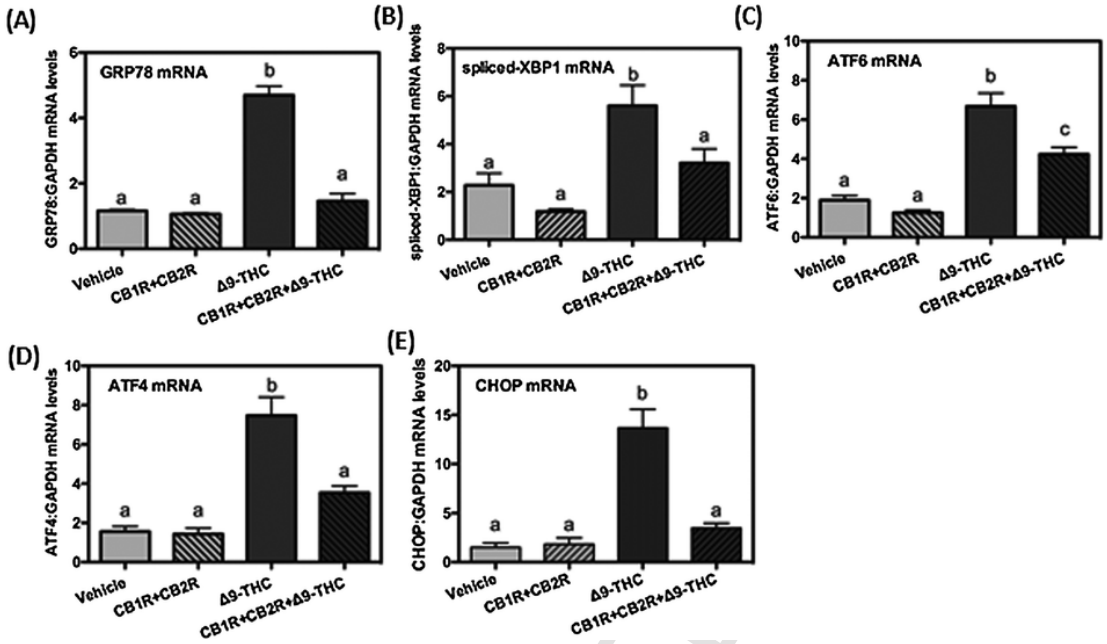

Fig. 2. Pretreatment with CB1R/CB2R antagonists abrogates Δ9-THC-induced ER stress. Real-time qPCR of human BeWo cells were treated with 15 μM Δ9-THC with or without 1 μM SR141716 and 0.1 μM SR144528 antagonists. Total RNA was extracted and reverse-transcribed to cDNA. **A.** GRP78 mRNA, **B.** splied-XBP1 mRNA, **C.** ATF6 mRNA, **D.** ATF4 mRNA, and **E.** CHOP mRNA. All values were expressed as mean ± SEM (N = 3/group). Significant differences between treatment groups determined by 1-way ANOVA. Different letters represent means that are significantly different from one another according to Tukey's posttest ($P < .05$).

ER stress markers GRP78, spliced-XBP1, ATF6, ATF4, and CHOP. The involvement of cannabinoid receptors CB1R and CB2R was further verified with the inhibition of Δ9-THC-induced ER stress using CB1R/CB2R antagonists. This is the first time Δ9-THC has been demonstrated to induce ER stress in a non-neuronal cell type [49]. We further revealed that Δ9-THC, through increases in ER stress, can augment the expression of key ER-stress sensitive genes ERRγ, VEGFA, and FLT-1, suggesting possible alterations in placental function. Given the links between ER stress in placenta and mitochondrial dysfunction [33], it also interesting that Δ9-THC led to impaired mitochondrial respiration and ATP coupling efficiency, in part due to decreased mitochondrial complex proteins.

Since ER stress and placental deficiency have been observed to precede intrauterine growth restriction, the presence of augmented ER stress in human BeWo placental cells reveals a potential mechanism through which Δ9-THC in pregnancy may result in adverse placental and fetal outcomes [23–26]. Employing concentrations of Δ9-THC that are found to be equivalent to those doses in the serum of cannabis users, this led to the activation of three major branches in the ER stress pathway [20,34]. Increased steady-state mRNA levels of ATF6 proposes involvement of the ATF6 branch, which downstream would lead to further expression of ER chaperone proteins, including GRP78 [21]. Likewise, increased mRNA expression of spliced-XBP1 indicates the activation of IRE-1 pathway implicated in downstream upregulation of ER chaperones and protein degradation [21]. Δ9-THC also increased the levels of phosphorylated eIF2α [Ser51] suggesting activation of the PERK pathway and protein translation attenuation as another means of trying to relieve ER stress. The decrease in protein translation may explain why Δ9-THC is able to diminish both proliferation and migration in BeWo cells [14,20]. It should be noted that for the current and published Δ9-THC studies in BeWo cells, the experiments were conducted in 21% atmospheric O_2, which leads to ˜0.5–9% dissolved oxygen concentrations depending on the depth of media [14,20,50]. While Δ9-THC induced ER stress in BeWo cells with similar oxygen concentrations, further studies are warranted to address if alterations in oxygen concentrations influences the degree of ER stress given hypoxia is a distinct trigger of placental ER stress [24,51]. Moreover, the potential role for ER stress on Δ9-THC induced impairment of invasion and migration in extravillous trophoblasts (*e.g.* HTR8/SNneo cells) should also be explored [14,36].

Under conditions of prolonged ER stress that is not resolved, the UPR switches to initiate apoptosis [52]. CHOP, regulated by both ATF4 and ATF6, is known to be involved in ER stress induced apoptosis and amplifies various downstream apoptotic pathways [52]. We observed that Δ9-THC also augmented both the mRNA and protein levels of ATF4, leading to increased CHOP levels in BeWo cells, suggesting apoptosis has been initiated. The Δ9-THC induction of protein attenuation (*e.g.* phosphorylated eIF2α [Ser51]) and CHOP in BeWo cells may shed some light to the observed decrease in placental and fetal weights derived from pregnant mouse dams treated with Δ9-THC during gestation [14]. Further studies are warranted to investigate more downstream apoptotic markers (*i.e.* Caspases 3, 9, 12, and BCL-2) to evaluate the extent of Δ9-THC- induced ER stress apoptosis. It is noteworthy that the ER stress observed was specific to active Δ9-THC given equimolar concentrations of COOH-THC did not exert any significant changes in hallmarks of the UPR.

Considering that Δ9-THC directly augmented placental ER stress, we were next interested in investigating the effects of Δ9-THC on critical placental target genes known to be altered by ER stress. We demonstrated that the Δ9-THC, but not its major metabolite COOH-THC, induces the expression of ERRγ, VEGFA, and FLT1 (VEGFR-1) in BeWo cells; and that this could be ameliorated with inhibition of ER stress (*e.g.* TUDCA). Interestingly, it is well established that the expression of the nuclear receptor ERRγ is increased by ER stress-induced ATF6, and that ERRγ itself can bind to the promoter of *ATF6* to up-regulate its own expression, in a positive feed-forward loop [43]. It is also possible that Δ9-THC may directly induce ERRγ expression given activation of CB1R has been shown to induce hepatic

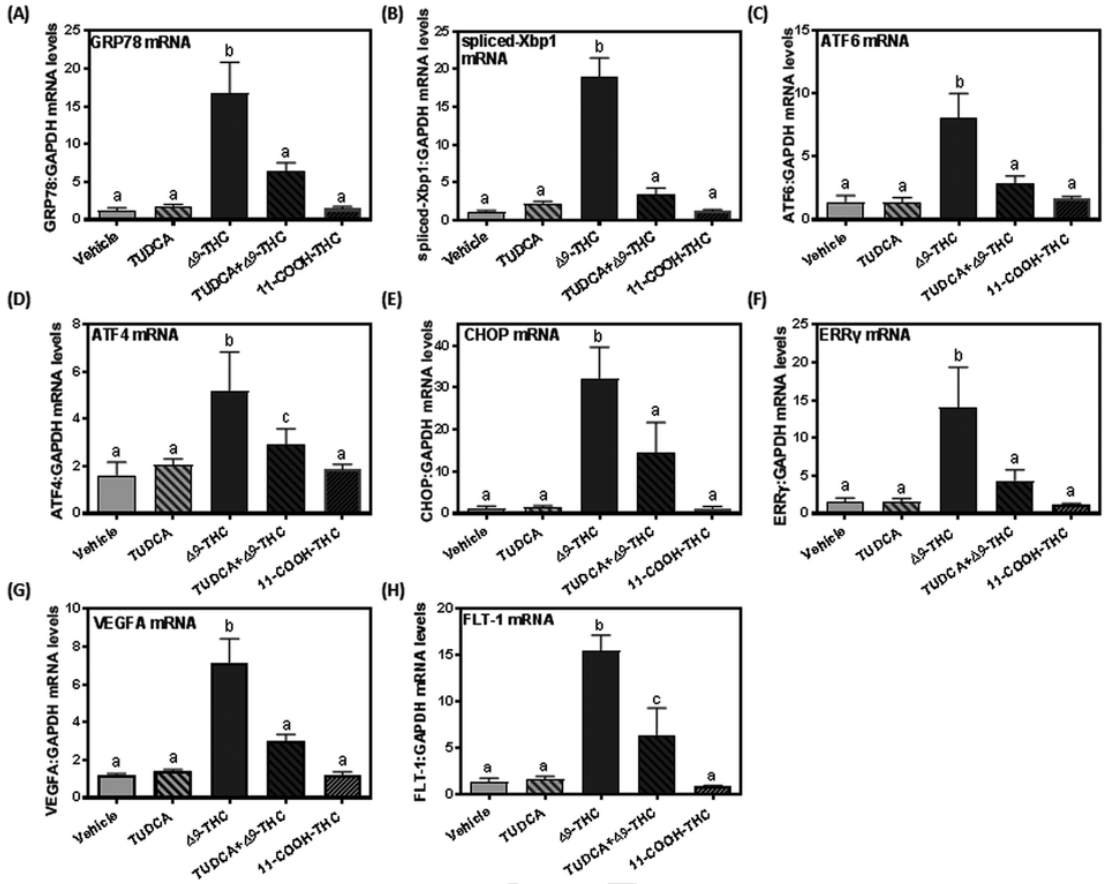

Fig. 3. Pre-treatment with TUDCA alleviated the effects of Δ9-THC-induced ER stress. Real-time qPCR of human BeWo cells were pretreated with 100 μM of TUDCA for 1 h and then treated with 15 μM Δ9-THC for 24 h. In addition, cells were also treated with 15 μM 11−COOH-THC for 24 h. Total RNA was extracted and reverse-transcribed to cDNA. **A.** GRP78 mRNA, **B.** spliced-Xbp1 mRNA, **C.** ATF6 mRNA, **D.** ATF4 mRNA, **E.** CHOP mRNA, **F.** ERRγ mRNA, **G.** VEGA mRNA, and **H.** FLT-1 (VEGFR-1) mRNA. All values were expressed as mean ± SEM (N = 3/group). Significant differences between treatment groups determined by 1-way ANOVA. Different letters represent means that are significantly different from one another according to Tukey's posttest ($P < .05$).

ERRγ expression [53]. Previous studies have demonstrated that activation of placental ERRγ leads to elevated maternal blood pressure which is noteworthy considering the expression of ERRγ is higher in preeclamptic placentas, a condition which is also associated with augmented ER stress in the placenta [27,28,54]. Paradoxically, the elevated ERRγ did not improve mitochondrial function in Δ9-THC-treated cells, despite its known role to promote mitochondrial biogenesis during trophoblast differentiation [55]. This may be due to the fact that these BeWo cells were not induced to differentiate in this experimental paradigm. With respect to VEGFA and FLT-1 (VEGFR-1), it has been established that elevated ER stress increases VEGFA expression *via* ATF4 and XBP-1 binding to the proximal promoter of *VEGFA*, while ER stress inhibitors can diminish VEGFA expression [44,45,56,57]. Interestingly, pharmacological induction of ER stress by tunicamycin in pregnancy results in higher protein levels of Vegfa in the spongiotrophoblast layer of the mouse placenta, while lipopolysaccharide-induced ER stress also leads to augmented placental Vegfa expression [29,58]. It should be noted that in those studies the protein levels of placental Flt-1T were not examined. In BeWo cells, the Δ9-THC-induction of VEGFA and FLT-1 may occur as a compensation to indirectly relieve ER stress given VEGFA promotes XBP1 splicing and angiogenesis in endothelial cells [59]. Certainly future rodent studies are warranted to examine if maternal exposure to Δ9-THC *in vivo* leads to placental zone-specific alterations in ER stress and downstream proliferation and angiogenesis. Given that TUDCA can relieve placental ER stress induced by nicotine, high glucose, and now Δ9-THC, it will also be of great interest to examine if TUDCA *in vivo* can prevent the adverse effects of maternal Δ9-THC exposure on trophoblast function and fetal development [14,25,35].

Mitochondria are responsible for providing the majority of cellular energy in the form of ATP. The inner membranes of these organelles provide the various proteins required for ATP synthesis, and *via* the transfer of proteins in the electron transport chain, ATP synthesis occurs due to oxidative phosphorylation [60]. Impaired placental mitochondrial function has been associated with fetal growth restriction and preeclampsia [61–65]. Given the close approximation of the ER and mitochondria in punctate sites known as the mitochondrial-associated ER membrane (MAM), it has been suggested integrated signaling occurs between both organelles [66]. As calcium transporters and ion channels are concentrated in the MAM, calcium-mediated ER signals can influence both mitochondrial electron transport chain activity and ATP production to meet the protein synthesis demands of the ER [33]. Since ER and mitochondrial functions are coupled *via* MAMS, so too is ER and mitochondrial stress [67,68]. After discovering that Δ9-THC induced ER stress in BeWo cells, we further demonstrated that mitochondrial oxidative metabolism was impaired. Specifically, Δ9-THC elicited decreases in basal and maximal mitochondrial respiration, ATP-synthase-linked respiration, and ATP coupling efficiency. This Δ9-THC induced defect in the electron transport chain activity may be attributed to the observed decreases in the abundance of mitochondria complex proteins (*e.g.* I, III, IV, and V) and mitochondrial mass (as reflected by the decrease in citrate synthase expression) [48]. In other cell types, activation of the cannabinoid receptors can reduce mitochondrial function. [69–72]. For example, Δ9-THC or synthetic CBR re-

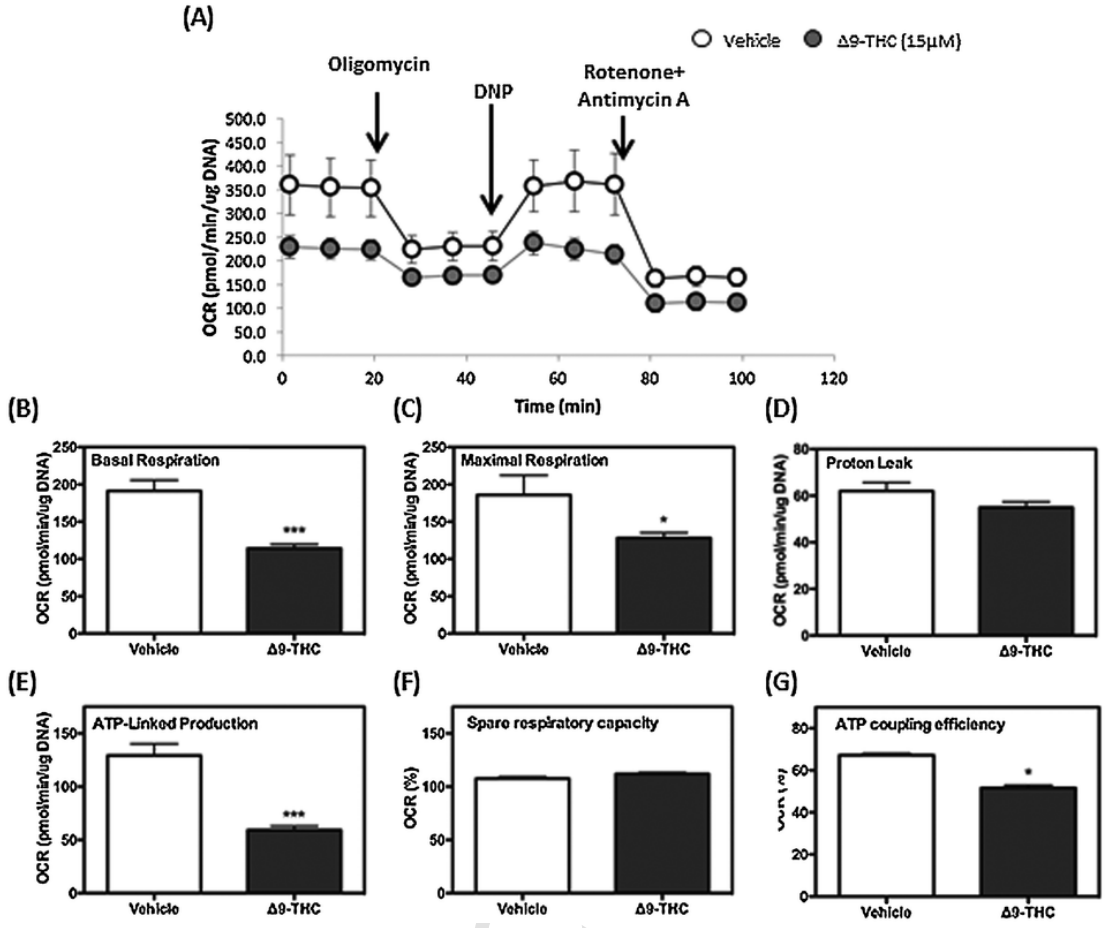

Fig. 4. Δ9-THC decreases mitochondrial function in cultured BeWo human trophoblast cells. Seahorse XFe24 analyzer was used to measure the oxygen consumption rate of cultured BeWo trophoblasts. Cells were seeded onto XFe24 seahorse plates at 1×10^5 cells per well and incubated with culture medium alone or culture media treated with 15μM Δ9-THC for 24 h. Culture media were exchanged with unbuffered Seahorse media supplemented with 10 mM D-glucose, 2 mM L-glutamine, and 2 mM sodium pyruvate for one hour prior to assay. To measure different oxygen consumption rate (OCR) parameters selective inhibitors were injected, oligomycin (1.5 ug/mL), 2,4-Dinitrophenol (DNP, 50μM), rotenone (0.5μM) and antimycin A (0.5 μM). Addition of oligomycin reduces OCR and reflects respiration independent of oxidative phosphorylation. Addition of DNP stimulates electron transport chain activity and maximal OCR. Addition of Rotenone/Antimycin A (mitochondrial chain complex inhibitors) indicates OCR independent of mitochondrial metabolism. Mitochondrial spare capacity was calculated from the change in OCR following DNP-stimulated maximal respiration. The OCR values were normalized to the amount of DNA content from each well using fluorescence Hoescht's stain. Data are presented as the mean of 6 biological replicates from 1 experiment, repeated 3 more times (see **Supplemental** Fig. 1 **for experiments 2-4**). Mean ± SEM, Significance was assessed by Student's *t*-test (*P < 0.05). **A.** Representative oxygen consumption rate (OCR) tracing. **B.** Basal respiration, **C.** Maximal respiration, **D.** Proton leak, **E.** ATP-linked production, **F.** Spare respiratory capacity, **G.** ATP coupling efficiency.

ceptor agonists (*e.g.* ARA and HU 210) in the H460 human lung cell line diminished mitochondrial oxygen consumption, complex I, and complex II/III activity [69]. Further studies are warranted to assess the specific contributions of Δ9-THC-induced ER stress towards to mitochondrial dysfunction observed. To date there is some debate regarding placental insufficiency and type of mitochondrial dysfunction. In small for gestational age (SGA) pregnancies, placentae exhibited lower mitochondrial mass (*e.g.* decreased citrate synthase activity), while another study demonstrated that intrauterine growth restricted (IUGR) placentae had higher mitochondrial content, oxygen consumption, and mitochondrial complex protein activity (*e.g.* complex I,III, and IV) [61,62]. In IUGR pigs the placental mitochondrial content was diminished, while in a rabbit model of fetal growth restriction, decreases in placental mitochondrial complex II, and II/III was observed without any effects on mitochondrial content [64,73]. The observed ability of Δ9-THC to impede mitochondrial function *in vitro* may account for the placental insufficiency (*e.g.* decreased placental and fetal weights) observed *in vivo* given pharmacological inhibition of mitochondrial function in the mouse preimplantation embryo led to lower mitochondrial respiration rate, ATP synthesis, and placental growth [14,74]. Collectively, this would suggest that Δ9-THC could reduce placental size and efficiency *via* defects to *both* ER and mitochondrial function.

In conclusion, this study has demonstrated for the first time that Δ9-THC directly induces ER stress in the placenta. With the use of human BeWo placental cells, we were able to provide insight on the effects of Δ9-THC exposure on placental ER stress, and its downstream effects linked to aberrant trophoblast gene expression and associated mitochondrial dysfunction. Moreover, we revealed that Δ9-THC-induced ER stress can be alleviated with TUDCA supplementation, demonstrating a potential therapeutic option for treating the negative effects of maternal cannabis exposure. Further studies are warranted to address whether Δ9-THC leads to ER stress *in vivo* and if this mediates the placental insufficiency which has been previously reported and/or if Δ9-THC-induced placental ER stress has long-term effects on offspring health. Moreover, given the strong links between Δ9-THC, ER and mitochondrial dysfunction, it will also be critical to further examine if exposure to Δ9-THC during gestation impairs the development of other fetal organs as diminished fetal cardiac mitochondrial function is observed in a rabbit model of placental-insufficiency induced IUGR [73]. With the increasing world-wide use of cannabis in pregnancy and higher concentrations of Δ9-THC, these studies are imperative to fur-

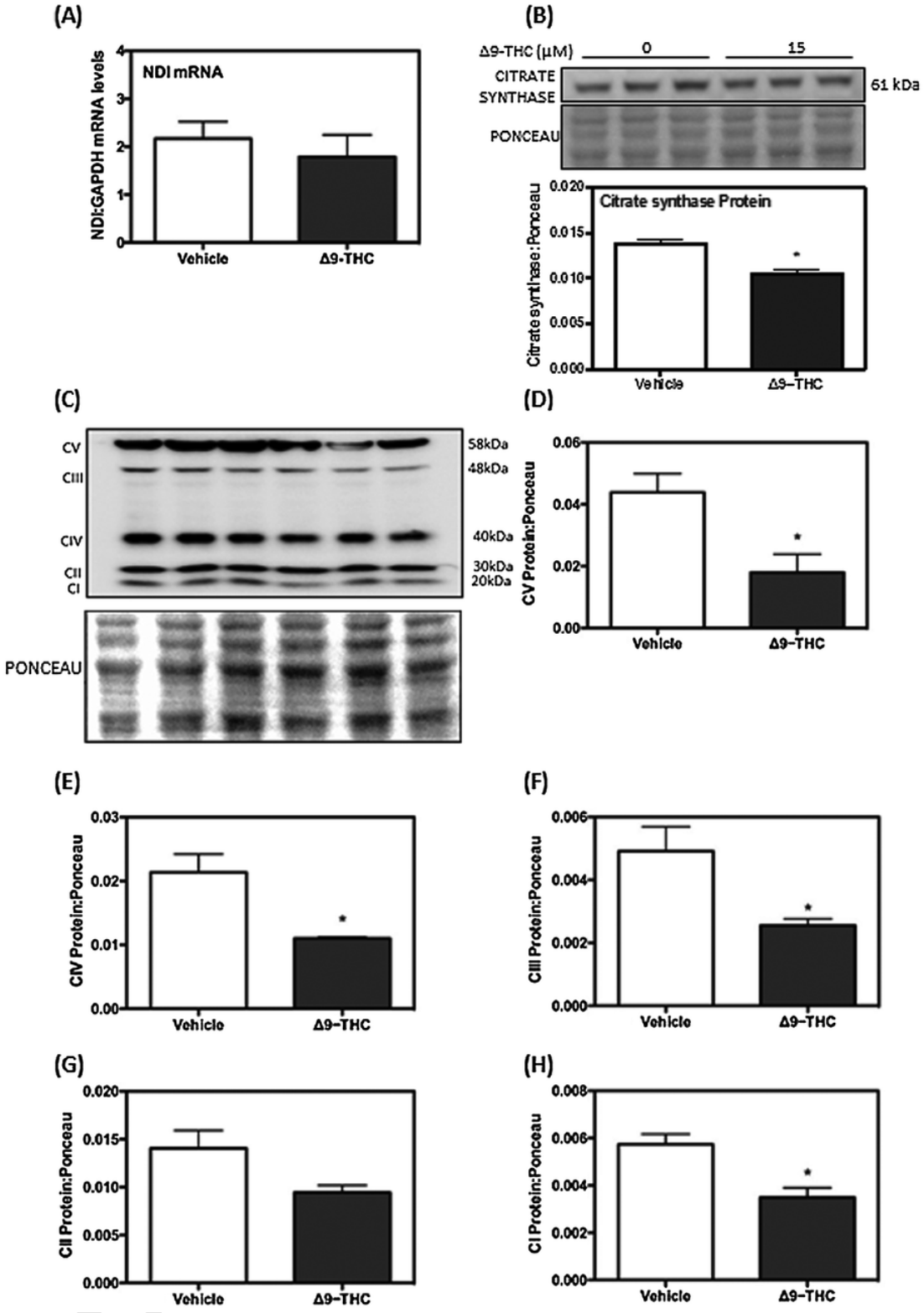

Fig. 5. Δ9-THC Decreases Expression of Citrate Synthase and Mitochondrial Complex Proteins in Cultured BeWo Human Trophoblast Cells. Cells were with culture medium alone or culture media treated with 15 μM Δ9-THC for 24 h. **A.** Total RNA was extracted and reverse-transcribed to cDNA and **A.** NADH:Ubiquinone oxidoreductase core subunit V1 was normalized to GAPDH. For proteins analysis, cell lysates were extracted and run on an immunoblot for **B.** Citrate Synthase. **C.** Representative OXPHOS Mitochondrial Complex Proteins (I–V) Blot. **D.** Complex V protein, **E.** Complex IV protein, **F.** Complex III protein, **G.** Complex II protein, and **H.** Complex I protein. All proteins were normalized by PONCEAU staining. Mean ± SEM, Significance was assessed by Student's *t*-test (*P < 0.05), N = 3/group.

ther understand the risk of Δ9-THC exposure on fetal and postnatal outcomes.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.reprotox.2019.04.008.

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