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Low expression of fatty acid transporter 1 in the placenta mediates the reduction in fetal birth weight caused by caffeine exposure during pregnancy

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ABSTRACT: Fatty acids play extensive roles in fetal energy metabolism and various physiological processes. Insufficient maternal supply of fatty acids increases the risk of intrauterine growth retardation (IUGR). Our previous research found that prenatal caffeine exposure (PCE) can lead to IUGR, but it is unclear whether this is related to placental fatty acid transport disorder. This study systematically investigates the impact of caffeine intake during pregnancy on placental fatty acid transport function and its molecular mechanisms by establishing rat models, mice intervention models, and in vitro cell experiments. In this study, we found that PCE reduces fatty acid levels in fetal blood, accompanied by decreased expression of placental fatty acid transporter 1 (FATP1) in rats. Further studies indicate that caffeine can antagonize ADORA2A and inhibit the expression and activity of the ERK-ETS1-PXR/RXRα signaling pathway in placental trophoblast cells. Conversely, ADORA2AR agonists, ETS1 overexpression plasmids, or PXR agonists can reverse the ADORA2A receptor antagonism, the decrease in ERK-ETS1-PXR/RXRα-FATP1 expression, and the disorder of fatty acid uptake caused by caffeine. Lastly, we confirmed that PXR agonists can reverse the reduction in placental FATP1 expression, fetal fatty acid levels, and birth weight observed in mice caused by PCE. In summary, this study elucidates the placental fatty acid transport mechanism mediated by the ADORA2A-ERK-ETS1-PXR/RXRα pathway inhibition in PCE-induced IUGR model, providing crucial theoretical basis and experimental support for clinical prevention and treatment research from the perspective of placental fatty acid transport.

Keywords: intrauterine growth retardation; prenatal caffeine exposure; fatty acid transporter 1; placental development; pregnane X receptor

1. Introduction

Fatty acids are essential substances for maintaining the fluidity, permeability, and spatial structure of cell membranes [1]. During pregnancy, fetal development relies on the effective supply of nutrients from the mother. The fetus can obtain large amounts of fatty acids through maternal circulation and synthesize saturated fatty acids using maternal substrates (such as glucose), but long-chain polyunsaturated fatty acids

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(LCPUFAs) are entirely dependent on maternal supply and placental transport [2]. Clinical studies have shown that fetal blood levels of docosahexaenoic acid (DHA) and arachidonic acid (AA) are significantly reduced in cases of intrauterine growth retardation (IUGR) [3]. The levels of n-3 and n-6 LCPUFAs in the umbilical cord vessel walls of preterm infants were also significantly lower than those in term fetuses [4]. Perinatal supplementation of n-3 LCPUFAs to either the mother or newborn can reduce the incidence of preterm birth and extremely low birth weight, and exert a positive impact on the visual and neurological development of offspring [5-8]. Multiple proteins associated with fatty acid transport are expressed in the placental microvilli and basement membrane, including plasma membrane-associated fatty acid binding protein (FABPpm), fatty acid translocase (FAT, also known as CD36), and the fatty acid transport protein family (FATP1-6) [9]. These proteins collectively participate in the placental transport of fatty acids. In pregnancies with placental dysfunction, impaired maternal-fetal transfer of LCPUFAs may further compromise fetal development and long-term health outcomes [10-12]. Therefore, fatty acids and their placental transporters play a crucial role in fetal development. Research has revealed that multiple nuclear transcription factors participate in regulating the expression of placental fatty acid transporters [13]. For instance, RXR regulates the expression of FATP1 and FATP4 and promotes the uptake of long-chain fatty acids (LCFAs) by placental trophoblast cells [14-16]. The pregnane X receptor (PXR), a member of the nuclear receptor superfamily of transcription factors, participates in regulating the expression of multiple placental transporters (such as organic anion transporting polypeptides and members of the ABC-box protein family). It also plays a crucial role in protecting both mother and fetus from the effects of endogenous steroids and exogenous toxins [17-19]. However, systematic studies on the regulation of placental fatty acid transporter expression by nuclear transcription factors remain scarce at present.

Caffeine is one of the widely consumed active ingredients worldwide, commonly found in coffee, tea, soft drinks, food, and certain analgesic medications [20] Epidemiological studies have shown that maternal caffeine intake during pregnancy is widespread, and it is closely associated with premature birth, low birth weight, and an increased risk of long-term diseases in the offspring [21-23]. Our previous research has revealed that prenatal caffeine exposure (PCE) can cause placental dysfunction, induce IUGR in the fetus, and alter developmental programming across multiple organs, thereby increasing offspring susceptibility to various diseases in adulthood [23, 24]. Meanwhile, caffeine exposure significantly alters maternal and fetal lipid metabolism profiles, leading to markedly reduced levels of LCPUFAs and their metabolites in fetal blood [25]. Notably, PCE serves as a representative model for studying fetal IUGR [26, 27], reliably simulating key characteristics of placental dysfunction and abnormal fetal metabolic programming. At present, there is no report on the effects of PCE on the fatty acid transport function of the placenta. Research indicates that compared to normal fetuses, fetuses with growth retardation have lower body and subcutaneous fat reserves, and the reduced maternal-fetal exchange due to placental insufficiency may be a key factor in the limited fat production in these fetuses [28]. Therefore, this study aims to investigate

whether PCE affects the placental fatty acid transport function and thereby leads to IUGR, and to reveal its key molecular regulatory mechanism.

Based on this, this study aims to systematically explore the specific molecular mechanisms by which caffeine downregulates placental fatty acid transporters using the PCE-induced rat IUGR model and caffeine-treated human choriocarcinoma cell line (BeWo). Considering the central role of the nuclear receptor PXR in lipid metabolism regulation, we further propose that PCE may affect placental fatty acid transport by regulating the PXR signaling pathway. To verify this hypothesis, this study will use a PXR intervention mouse model to clarify the key regulatory role of PXR in the inhibition of placental fatty acid transport and fetal growth restriction caused by caffeine. By deeply analyzing the mechanism of IUGR from the new perspective of placental fatty acid transport disorder, this study aims to provide a new theoretical basis and potential targets for early intervention of fetal IUGR caused by PCE.

2. Materials and Methods

2.1 Drugs and reagents

Caffeine (CAS No. C0750) was purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). Arachidonic acid enzyme-linked immunosorbent assay (ELISA) kit (E-EL-0051c) was purchased from Elabscience Biotechnology (Wuhan, China). TRIzol reagent was purchased from Invitrogen Co. (Carlsbad, CA, USA). Total RNA reverse transcription and real-time quantitative polymerase-chain-reaction (RT-qPCR) kits were procured from Vazyme Biotech Co., Ltd. (Nanjing, China). The primers were synthesized by TIANYI HUIYUAN Biotech Co., Ltd. (Wuhan, China). Chromatin immunoprecipitation (ChIP) assay kits were purchased from Millipore Co., Ltd. (Billerica, MA, USA). A DNA purification kit was purchased from TIANGEN Biotech Co., Ltd. (Beijing, China). The pcDNA-ETS1 plasmid was constructed by GenePharma Co., Ltd. (Shanghai, China). The nuclear and cytoplasmic protein preparation kit (P1200) and BCA assay kit (P1511) were purchased from Applygen Co., Ltd. (Beijing, China). The other reagents used for experiments were of analytical grade.

2.2 Animals and treatment

Specific pathogen-free (SPF) adult Wistar rats (females: 250 ± 20 g; males: 280 ± 20 g) and SPF C57BL/6J mice (females: 20 ± 2 g; males: 23 ± 2 g) were purchased from Beijing Speifu Biotechnology Co., Ltd. Animal experiments were conducted in the Animal Experiment Center of the First Clinical College of Wuhan University (Wuhan, China). The protocol was approved by the Committee on the Ethics of Animal Experiments of Wuhan University School of Medicine (Permit No. WP20210476). All procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Chinese Animal Welfare Committee.

Wistar rats were bred as previously described [29]. The presence of sperm in the vaginal smear was designated as gestational day (GD) 0 in rats, while the detection of a vaginal plug was designated as GD0 in mice. Individually caged pregnant rats were then randomly divided into a control group and three PCE

groups. The PCE group was intragastrically administered 30, 60, 120 mg/kg·d caffeine (once daily), namely the PCE(L), PCE(M), and PCE(H) groups, whereas the control group received the same volume and frequency of saline administration from GD9 to GD20. The pregnant rats were anesthetized with isoflurane on GD20 and euthanized. All mice were divided into four groups: CON (saline, *ig*), caffeine (caffeine, 60 mg/kg·d, *ig*), pregnenolone 16α-carbonitrile (PCN, 20 mg/kg·d, *ig*), and PCN+caffeine groups, which were intragastrically administered once daily from GD 9 to GD 18. On GD18, pregnant mice were anesthetized with 2 % isoflurane and then sacrificed. There were 12 pregnant rats or mice in each group, and the litter size was 8-15. Maternal and fetal serum samples were obtained from whole blood by centrifugation at 1200 g and 4℃ for 15 min. The placenta was fixed with 4% paraformaldehyde and paraffin-embedded for subsequent H&E and CD31 staining. While the other samples were transferred to liquid nitrogen, followed by storage at −80℃. The placental tissue from three different fetal rats from each litter was randomly collected, pooled, and counted as one sample for subsequent total RNA extraction and protein extraction according to methods previously described [30].

IUGR rate per litter (%) = (number of IUGR rat fetuses per litter/ the total number of fetal rats per litter) ×100

IUGR rate per group (%) = (the sum of fetal rat IUGR rate per litter/each group of litter number) ×100

2.3 Cell culture and treatment

The human BeWo cell line is a human placental villus trophoblastic cell, which was purchased from the China Center for Type Culture Collection (Wuhan, Hubei, China). The cells were seeded in cell culture flasks containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (Gibco, St. Louis, MO, USA) and 0.1% penicillin/streptomycin, and cultured in a 5% CO_2 humidified incubator at 37℃. To evaluate the effect of caffeine on the ERK-ETS1-PXR/RXRα signaling pathway in trophoblast cells, we treated the BeWo cells with different concentrations of caffeine (0, 1, 100 μM) for 24 h and examined the expression and interactions of related proteins.

Furthermore, to confirm that caffeine mediates the low expression of FATP1 and the inhibition of long-chain fatty acid uptake through the ADORA2A-ERK-ETS1-PXR/RXRα pathway, we used the ADORA2A agonist (CGS21680, 2 μM), ERK agonist (Phorbol 12-myristate 13-acetate, PMA 5 nM), pcDNA-ETS1 plasmid (5 μg), and PXR agonist (Rifampin, 10 nM). During the specific treatment process, the BeWo cells were seeded in 6-well plates at a 5×10^3 cells/well density. After 30%-50% confluence was reached, the cells were divided into different groups and subjected to the following treatments: negative control (cells that had not undergone any drug treatment or transfection procedures), only caffeine (100 μM), only each reagent (CGS21680, PMA, pcDNA-ETS1, or rifampicin), and the combined treatment of caffeine and the above reagents. After 24 hours of treatment, all groups of cells were collected for subsequent analysis.

2.4 Arachidonic acid uptake assay

BeWo cells were cultured in 6-well plates at 1×10^5 cells/well for 12 h. The supernatant was discarded, washed twice with phosphate buffer saline (PBS), and given 2 mL of complete medium containing different concentrations of caffeine (0, 1, 10, and 100 μM) for 48 h. Six samples per group. Afterwards, the supernatant was discarded, washed twice with PBS, and 2 mL of DMEM/F-12 serum-free medium containing 100 μM arachidonic acid and 1% fatty acid-free BSA was added to each well for 24 h. The supernatant was discarded, washed twice with cold PBS containing 0.5% fatty acid-free BSA for 2 times, and washed again with PBS for 2 times. Scrape off the cells into 1 mL PBS, centrifuge at 800 g for 5 min, resuspend with 0.5 mL pure water, and prepare homogenates by ultrasonic crushing in an ice-water bath. The concentration of AA in the cell homogenates was measured by an ELISA kit, and the relative quantification of AA was performed by the BCA method to detect the total protein concentration in the homogenates.

2.5 Oleic acid intake assay

BeWo cells were cultured at 0.5×10^4 cells/well in 24-well plates. The supernatant was discarded after 12 h, and then 1 mL of complete medium containing different concentrations of caffeine (0, 1, 10, 100 μM) was added, and incubated for 48 h. Afterwards, the supernatant was discarded, washed twice with PBS, and 1 mL of DMEM/F-12 serum-free medium containing 100 μM oleic acid (OA) (complexed with 1% fatty acid-free BSA) was added to each well for 24 h. The supernatant was discarded, washed twice with cold PBS, washed twice with cold PBS containing 0.5% fatty acid-free BSA, and washed twice with cold PBS. Then, the BeWo cells were fixed with 4% paraformaldehyde for 30 min, washed twice with room temperature PBS, and stained with 0.5 mL of Oil Red O staining solution per well for 30 min. The cells were rinsed with 60% isopropanol for 5 s and washed three times with PBS. Remove the crawling slices and seal the slices with glycerol gelatin. Six samples from each group were dried, observed under a microscope, and photographed (400×). Count the number of lipid droplets in each field of view and calculate the number of lipid droplets/field area ($N/\mu m^2$).

2.6 Total RNA extract and RT-qPCR assay

Details for total RNA extraction and RT-qPCR have been described previously [31]. Placental tissue samples (50 mg) from the same position of placentas and BeWo cell samples were collected, and total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. RT-qPCR was applied to determine the mRNA expression of FATP1, FATP2, FATP4, FATP6, CD36, FABPpm, YWHAZ, and GAPDH. The sequences of the above genes are shown in Table 1. RT-qPCR was used with the ABI Step One Plus™ real-time PCR System (Applied Biosystems, Foster City, CA, USA). Relative amplicon expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Oligonucleotide primers used for RT-qPCR (rat and human).

Genes	Forward primer	Reverse primer	Annealing
Rat			
FATP1	CTTCTGGGACTTCCGTGGAC	GTGTCGTCGTAGCTCTAGCC	60℃, 30 s
FATP2	GGACGACTGCAGGAAATAC	GATGAACTCTCTCCACACATC	60℃, 30 s

FATP4	CATGCGGCCTGATGACATTG	CCGGGAGGCTGAAAACTTCT	59℃, 30 s
FATP6	CTTCAACACAGGAGACCTAATG	CCTGTATAAAGTCCAACCTTCC	60℃, 30 s
CD36	CCTCGGATGGCTAGCTGATT	AGAGCACTTGCTTCTTGCCA	60℃, 30 s
FABPpm	CAGCACCAAAAACCTGTCAGC	ACGAGGACACACTCGACAAC	60℃, 30 s
YWHAZ	TTGAGCAGAAGACGGAAGGT	CCTCAGCCAAGTAGCGGTAG	60℃, 30 s
GAPDH	GGGTGTGAACCACGAGAAAT	ACTGTGGTCATGAGCCCTTC	58℃, 30 s
Human			
FATP1	CCGGAATTGACTGTGACCACTT	CACGCAGTGCAGGGTTCA	60℃, 30 s
FATP2	TTCTATGCTGCCACTGAAGG	GGAACTCTGACGCAATATCC	60℃, 30 s
FATP4	CGCCAACAACAAGAAGATTG	GACACGTTCTCACCTTTCC	60℃, 30 s
FATP6	GAAATGAGCAGGGTTGGTGTAT	AGTGTCTCCAGTACGGTCCC	60℃, 30 s
CD36	AAACCTCCTTGGCCTGATAG	GAATTGGCCACCCAGAAACC	60℃, 30 s
FABPpm	GGAAGGAAATAGCAACAGTGG	TCCTACACGCTCACCATATAAGC	60℃, 30 s
GAPDH	GAAATCCCATCACCATCTTCCAG	ATGAGTCCTTCCACGATACCAAAG	60℃, 30 s
Mice			
FATP1	CGCTTTCTGCGTATCGTCTGCAAG	AAGATGCACGGGATCGTGTCT	60℃, 30 s
UBC	CTACAACATCCAGAAAGAGTCC	GTCTGTCTTCCTGTTTGACC	60℃, 30 s

FATP1, fatty acid transport protein 1; CD36, also known as FAT, fatty acid translocase; FABPpm, plasma membrane-associated fatty acid binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UBC, ubiquitin C.

2.7 Protein extract, Western blot, and co-immunoprecipitation (Co-IP) assays

Details for total protein, cytoplasmic protein, and nuclear protein extraction, as well as Western blotting assay, have been described previously. Briefly, homogenate tissues or cells were rinsed with ice-cold PBS, then lysed for 30 min at 4℃ in RIPA lysis buffer containing phosphatase inhibitor cocktail, followed by protein quantification using the BCA assay kit. 30 μg total protein or cytoplasmic and cytonuclear protein was loaded to each lane, isolated by SDS-PAGE (10% gels) and blotted onto PVDF membranes (Millipore, MA, USA). The PVDF membranes were blocked in 5% non-fat milk for 1 h and incubated overnight at 4℃ with the primary antibody, including PXR (1:1000, ab203132), ERK (1:500, sc-12763), phospho-ERK (1:2000, k1071447), phospho-ETS1(1:1000, A7266), RXRα (1:1000, bs1379R), GAPDH (1:5000, AC002), and H3 (1:5000, a2348). Then they were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 45 min and visualized using ECL HRP substrate (PerkinElmer Inc., Boston, Mass). Signals of antibody binding were detected by Chemi-doc Image Analyzer (Bio-Rad, Hercules, California). Relative protein levels were standardized with the GAPDH or H3 protein level and were presented as fold change of respective controls. Protein band intensities were analyzed by Image J from 6 independent bands. For the immunoprecipitation assay, the protein was incubated with 1 μg PXR or RXRα antibody overnight. Then, 40 μL 50% protein A/G agarose beads were added and continued for 4 h. Beads were resuspended and boiled with 2×SDS-PAGE loading buffer for western blotting analysis.

2.8 Immunofluorescence and immunohistochemistry staining

After 24 h of 100 μM caffeine treatment, the cells were washed three times with ice-cold PBS, fixed in 4% formaldehyde, and blocked for 30 min with 3% BSA and 2% fetal bovine serum in 0.2% Triton X-100. The cells were then incubated overnight at 4℃ with rabbit anti-PXR (1:50 dilutions) in blocking buffer. The cells were washed with PBS and incubated with 1:100 diluted Cy3-conjugated secondary antibody (GB21303, Servicebio Inc., Wuhan, China) for 60 min at room temperature. Nuclei were stained with 4,

6-diamidino-2-phenylindole (DAPI) at a 1:500 dilution for 5 min. After washing twice with PBS, the slides were sealed with an anti-fluorescence quenching agent. Fluorescence images for PXR and DAPI expression were captured using immunofluorescence microscopy (Nikon H550S, Tokyo, Japan). The integrated optic density (IOD) was measured in 5 different fields for each sample, 6 different samples of each group, using Image-Pro Plus (version 6.1, Media Cybernetics, Silver Spring, MD, USA).

Placental tissues embedded with paraffin were cut into 3 μm thick slices along the longitudinal axis, then deparaffinized and rehydrated with xylene and gradient ethanol. The slices were microwaved with citrate buffer (pH 6.0) for 15 min, soaked in 3% H_2O_2 in the dark for 25 min, and washed three times with PBS (5 min per wash). Slices were incubated in 3% BSA for 30 min, then incubated overnight with anti-PXR (1:200) antibody at 4℃. After washing with PBS 3 times, 5 min each time, slices were incubated with the secondary antibody at 37℃ for 60 min and washed with PBS. Finally, after staining it with DAB and hematoxylin, the slices were dehydrated, transparent, and sealed. The IOD was measured in 5 different fields for each sample, 5 different samples of each group, using Image-Pro Plus (version 6.1, Media Cybernetics, Silver Spring, MD, USA).

2.9 Re-chromatin immunoprecipitation (re-ChIP) assay

Re-ChIP assays were conducted to evaluate the binding levels of PXR/RXRα to the promoter region of the SLC27A1 (FATP1) gene, based on a modified version of the manufacturer's protocol. After 48 h of 100 μM caffeine treatment, the cells were washed three times with ice-cold PBS. The samples were fixed with 1% formaldehyde on a rocker at room temperature for 10 min to cross-link histones to DNA. The reaction was stopped by adding glycine at a final concentration of 125 mM. After washing with ice-cold PBS, samples (single-cell suspensions) were disaggregated using a Dounce homogenizer in lysis buffer supplemented with a protease inhibitor cocktail to aid the release of nuclei. The suspension was centrifuged at 1000 g and 4℃ for 60 s, and the nuclei were resuspended in lysis buffer. Samples were then sonicated under different conditions (for tissues: TIMER 2s, PULSE 2, AMPL 30%, 9 min; for cells: TIMER 2s, PULSE 2, AMPL 30%, 5 min) using SONICS Vibra-Cell™ (Cole-Parmer Instruments, Vernon Hills, IL) to shear DNA to lengths between 200 and 1000 base pairs. The sonicated samples of sheared DNA were then incubated with Protein G magnetic beads and divided into three aliquots: one aliquot was used for input DNA; the others were used for immunoprecipitation with anti-RXRα (1 μg, bs-20769R) and IgG (ab171870, Abcam) at 4℃ overnight on a rocker. The immunoprecipitated DNA-protein complex with beads was collected by centrifugation and washed sequentially with low-salt, high-salt, LiCl immune complex, and Tris-EDTA washing buffer solutions. The beads were eluted with 20 mM dithiothreitol at 37℃ for 30 min, and the eluents were diluted 30-fold for further incubation with the PXR antibody (1 μg, ab118336) at 4℃ overnight on a rocker. The immunoprecipitated DNA-protein complex with beads was then collected by centrifugation and washed sequentially with low-salt, high-salt, LiCl immune complex, and Tris-EDTA washing buffer solutions. Freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$) was used to elute the DNA protein complex. The samples were incubated with proteinase K (final concentration: 200 μg/mL) at

65℃ overnight. DNA fragments were further obtained via the phenol-chloroform method, ethanol precipitation, and resuspension in deionized water for RT-qPCR analysis. The sequences of the primers spanning the SLC27A1 binding region used for RT-qPCR are as follows: Primer 1: GTCTCTCTGTGTAGCCCTGG (Forward), GGAGGTGAAGTGTGGAGTCA (Reverse); Primer 2：CTGTGAGTTTGGACCCTTGC (Forward), CCCTGACTGATAACACCCCA (Reverse). The experiments were conducted in triplicate, and the levels of PXR/RXRα at the promoter region were calculated by the fold enrichment method relative to IgG antibody and normalized to the input DNA.

2.10 Statistical analysis

Data were presented as mean ± S.E.M. Statistical differences among gene expression in placentas were calculated and plotted using GraphPad Prism 6.0 (GraphPad Software, Inc., USA). Comparisons between two groups were performed using two-tailed Student's *t*-tests, and comparisons among multiple groups were performed using one-way analysis of variance (ANOVA), followed by Bonferroni's post-hoc test for multiple comparisons. The association between different targets was analyzed using Pearson's correlation method. For all tests, $P < 0.05$ was considered statistically significant.

3. Results

3.1 Low expression of FATP1 mediates the inhibition of placental fatty acid transport in rats caused by PCE

3.1.1 Effects of PCE on fatty acid transporter expression in rat placenta and its correlation with fetal birth weight

First, we examined the effect of PCE on the birth weight of male and female fetal rats. The results showed that compared with the control group, the birth weight of male fetal rats in the PCE(M) and PCE(H) groups and female fetal rats in the PCE(H) group were significantly reduced, and the IUGR rate was significantly increased (Fig 1A, 1B). A previous study [25] found that in a rat IUGR model, there were 23 and 11 differential metabolites in the fetal blood of males and females (control group *vs*. caffeine group: 120 mg/kg·d, GD9–20, *ig*). Among these, cephalin, lecithin, and oleamide, which are related to fatty acids and their metabolism, were significantly reduced in both male and female fetal blood. Additionally, AA levels were significantly decreased in female fetal blood. Fatty acids primarily rely on transmembrane transport mediated by placenta-specific transport proteins. Therefore, the normal expression of placental fatty acid transporters is directly related to the nutritional supply and growth of the fetus. Based on these findings, we further examined the effect of PCE on the mRNA expression of fatty acid transport-related genes in the placenta. The results showed that compared with the control group, the mRNA expression of FATP1, FATP2, FATP4, FABPpm, and CD36 in the male placenta of the PCE group was significantly decreased (Fig 1C-E, 1G, 1H). The mRNA expression of FATP1, FATP2, FABPpm, and CD36 in the female placenta was also significantly decreased (Fig 1C, 1D, 1G, 1H), while the expression of FATP4 and FATP6 was significantly decreased only in the PCE(L) group (Fig 1E, 1F). Pearson correlation analysis showed that in both male and female fetal rats, birth weight correlated positively only with placental FATP1 and FATP2

mRNA expression (Fig 1I, 1J), but not with FATP4, FATP6, FABPpm, or CD36 expression (Fig 1K-N). These findings indicate that PCE suppresses the mRNA expression of various fatty acid transporters in the rat placenta, and the downregulation of FATP1 and FATP2 is closely associated with reducing fetal birth weight.

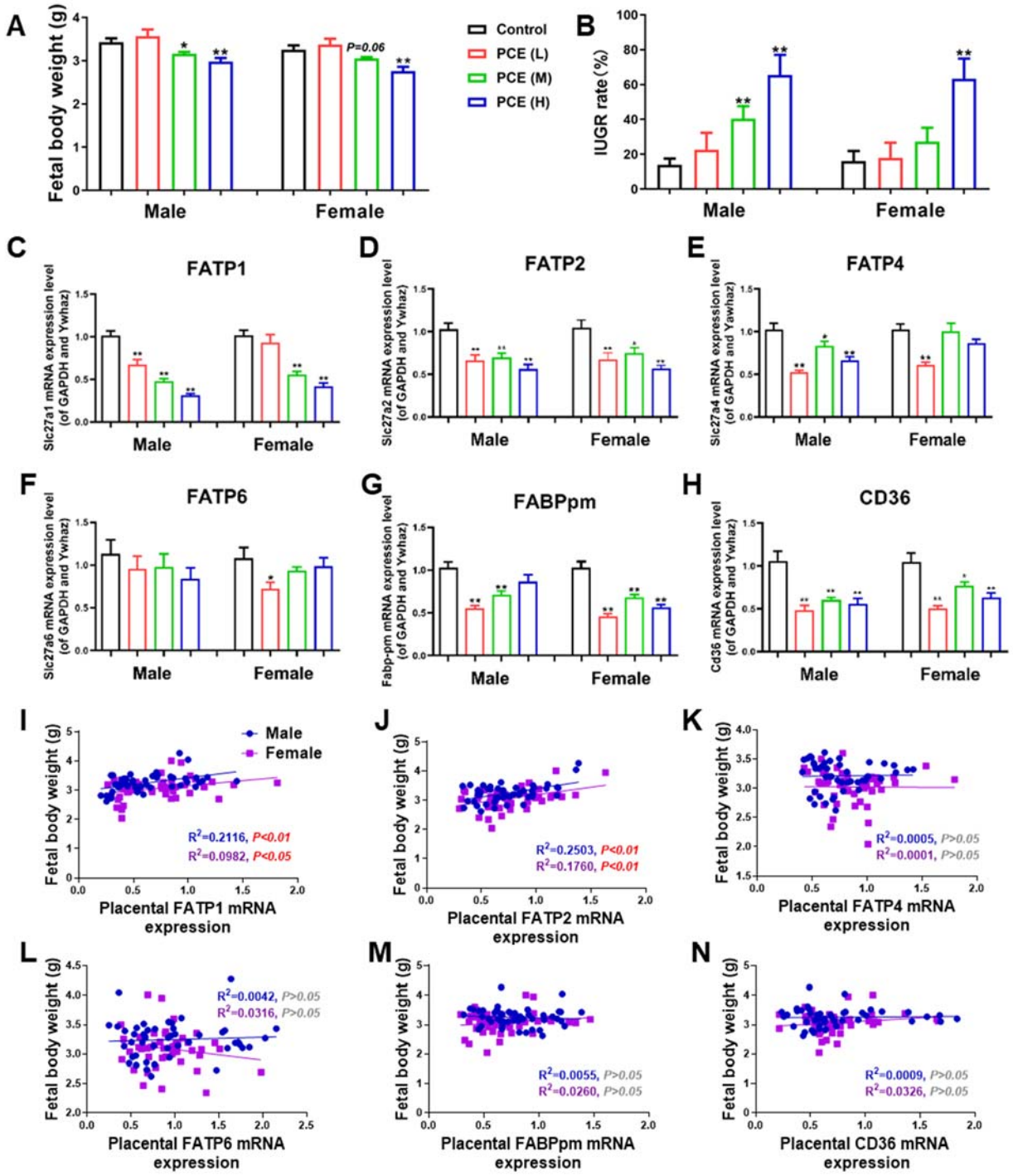


Fig 1. Low mRNA expression of placental fatty acid transporters induced by prenatal caffeine exposure (PCE) mediates low fetal weights in rats. (A) Fetal weight; (B) IUGR rate; (C-H) The mRNA expression levels of placental FATP1, FATP2, FATP4, FATP6, FABPpm and CD36; (I-N) Correlations between placental FATP1, FATP2, FATP4, FATP6, FABPpm and CD36 mRNA expression and fetal body weights. The data are presented as mean ± S.E.M., n=12 for the mRNA expression and for correlation analysis in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. control. PCE, prenatal caffeine exposure; IUGR, intrauterine growth retardation; FATP, fatty acid transport protein; FABPpm, plasma membrane-associated fatty acid binding protein; CD36, fatty acid translocase; GAPDH, glyceraldehyde phosphate dehydrogenase; PCE(L), caffeine low-dose exposure; PCE(M), caffeine medium-dose exposure; PCE(H), caffeine high-dose exposure.

3.1.2 Effects of caffeine on fatty acid transporter expression and LCFAs uptake capacity in BeWo cells

The BeWo cell line originates from malignant gestational trophoblastic carcinoma [32]. Composed of trophoblast cells and a small number of syncytiotrophoblast cells, it retains many biological characteristics of normal trophoblast cells[33, 34] and is widely used to study fatty acid transport and uptake in the placenta [35, 36]. We treated BeWo cells with different concentrations of caffeine for 48 hours to evaluate its effect on LCFAs (including OA and AA) uptake. Caffeine (1, 10, and 100 μM) significantly inhibited intracellular lipid droplet formation compared to the control group, as shown by oil red O staining (Fig 2A, B). AA uptake experiments further showed that caffeine (1, 10, and 100 μM) significantly reduced intracellular AA concentration (Fig 2C). Subsequently, we treated BeWo cells with 100 μM of caffeine to detect the expression of related fatty acid transporters. The RT-qPCR results showed that, compared with the control group, 100 μM caffeine significantly downregulated the mRNA expression of FATP1, FATP4, FABPpm, and CD36 (Fig 2D). Furthermore, different concentrations of caffeine (1, 10, and 100 μM) can significantly reduce the mRNA and protein expression of FATP1 in a concentration-dependent manner (Fig 2E, F). Finally, to clarify the role of FATP1 in placental fatty acid uptake, BeWo cells were treated with FATP1 siRNA for 48 hours. The results showed that compared with the control group, the protein expression of FATP1 was significantly decreased in the FATP1 siRNA group (Fig 2G), accompanied by a reduction in lipid droplet formation and a decrease in intracellular AA level (Fig 2H-J). The above results suggest that caffeine can inhibit the uptake of LCFAs and downregulate the expression of various fatty acid transporters, among which FATP1 may mediate the inhibition of LCFAs uptake by placental trophoblast cells induced by caffeine.

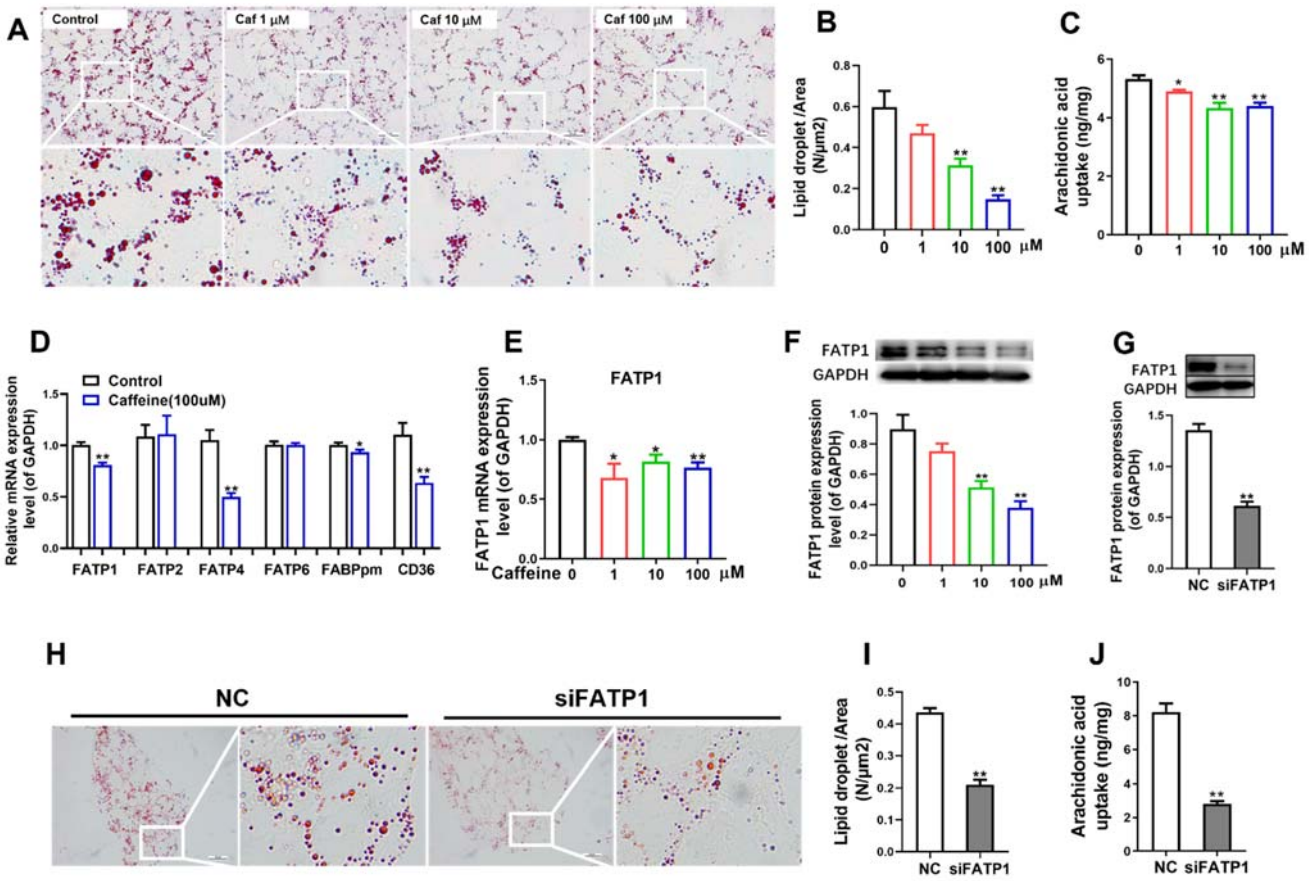


Fig 2. Effects of caffeine on the expression of fatty acid transporter and fatty acid transport in BeWo cells. (A) BeWo cells were treated with different concentrations of caffeine for 48 h and stained with oil red O (400×, scale: 25 μm, and local enlargement); (B) Number of lipid droplets; (C) Intracellular arachidonic acid level in BeWo cells treated with different concentrations of caffeine for 48 h (ELISA assay); (D) The mRNA expression of FATP1, FATP2, FATP4, FATP6, FABPpm, and CD36 in BeWo cells treated with 100 μM caffeine for 24 h; (E) The mRNA expression of FATP1 in BeWo cell treated with 1, 10, 100 μM caffeine for 48 h; (F) Protein expression of FATP1 in BeWo cell treated with 1, 10, 100 μM caffeine was detected by Western blotting; (G) FATP1 protein expression after 48 h of FATP1 siRNA treatment; (H) Oil red O staining after FATP1 siRNA treatment (400×, scale: 25 μm, and local enlargement); (I) The number of lipid droplets in BeWo cell after FATP1 siRNA treatment; (J) Arachidonic acid levels in BeWo cell after FATP1 siRNA treatment (ELISA assay). The data are presented as mean ± S.E.M., n=6 for mRNA analysis, Western blotting, and ELISA, and oil red O staining in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. control. GAPDH, glyceraldehyde-phosphate dehydrogenase; FATP, fatty acid transport protein; CD36, fatty acid translocase; FABPpm, plasma membrane-associated fatty acid binding protein.

3.2 The ADORA2A-ERK-ETS1-PXR/RXRa pathway mediates PCE-induced inhibition of FATP1 expression in rat placentas

3.2.1 PCE causes abnormal expression and activation of the ERK-ETS1-PXR/RXRα pathway in rat placentas

To investigate the potential mechanism by which PCE inhibits FATP1 expression, we first detected the protein expression levels of PXR and RXRα in the rat placentas. Immunohistochemistry and Western blot analysis results showed that compared with the control group, the total protein expression of PXR in the placentas of the PCE group was significantly decreased (Fig 3A, B), and the nuclear protein expression of PXR and RXRα was also significantly reduced (Fig 3C-E). According to previous reports, the ERK-ETS1 axis is involved in regulating PXR expression [37]. Therefore, we further evaluated the protein expression and activation status of ERK and ETS1 in the placenta. The results showed that the total protein and nuclear

protein expression of phosphorylated ETS1 (p-ETS1) were significantly decreased in male and female placentas in the PCE group (Fig 3E-G). The total protein expression of ERK1/2 remained unchanged (Fig 3E, H), while the expression of phosphorylated ERK1/2 (p-ERK1/2) was significantly reduced (Fig 3E, I). These results suggest that PCE can inhibit the protein expression and activation of ERK1/2 and ETS1, and decrease the protein expression of PXR and RXRα in the male and female placentas.

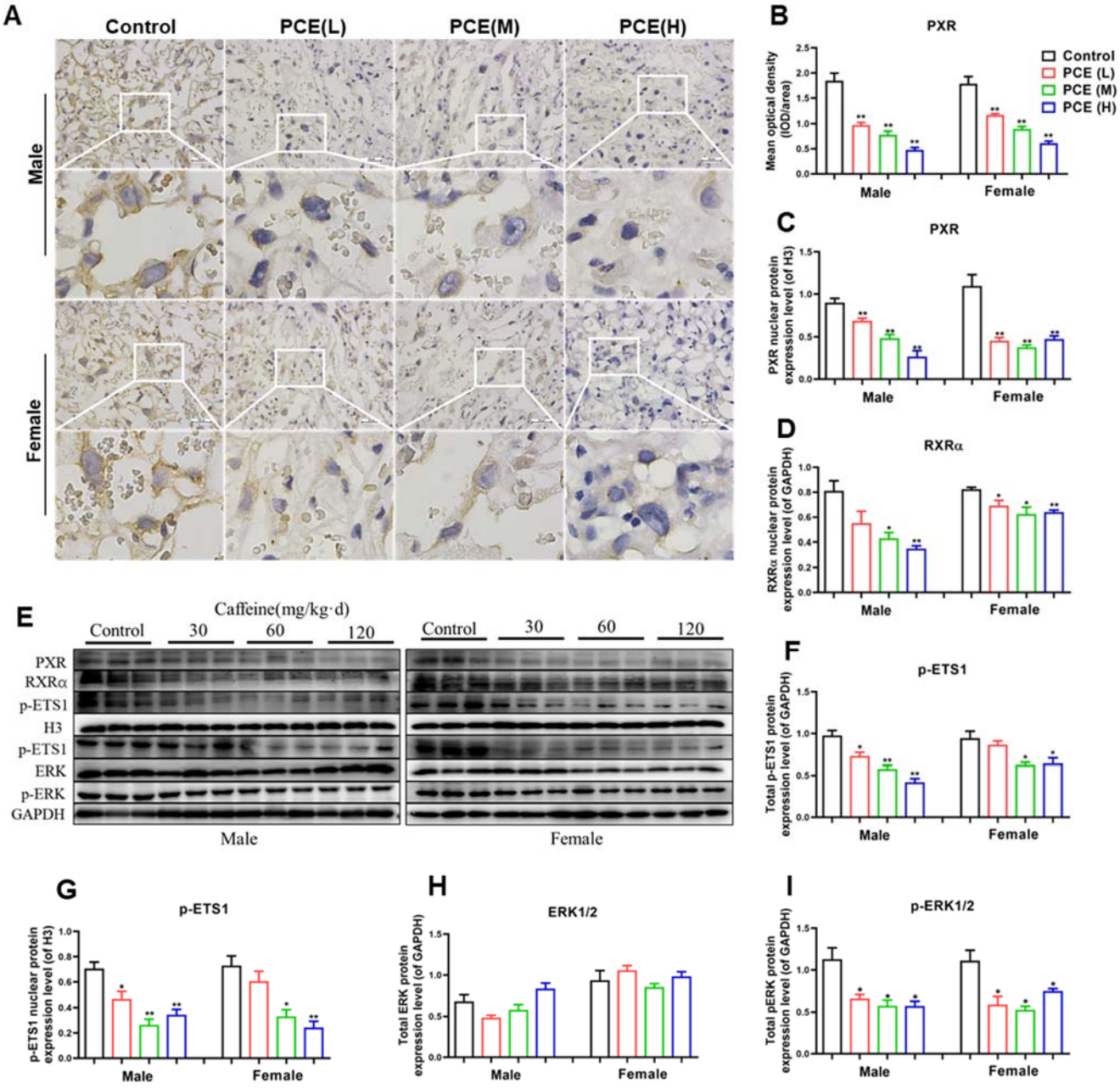
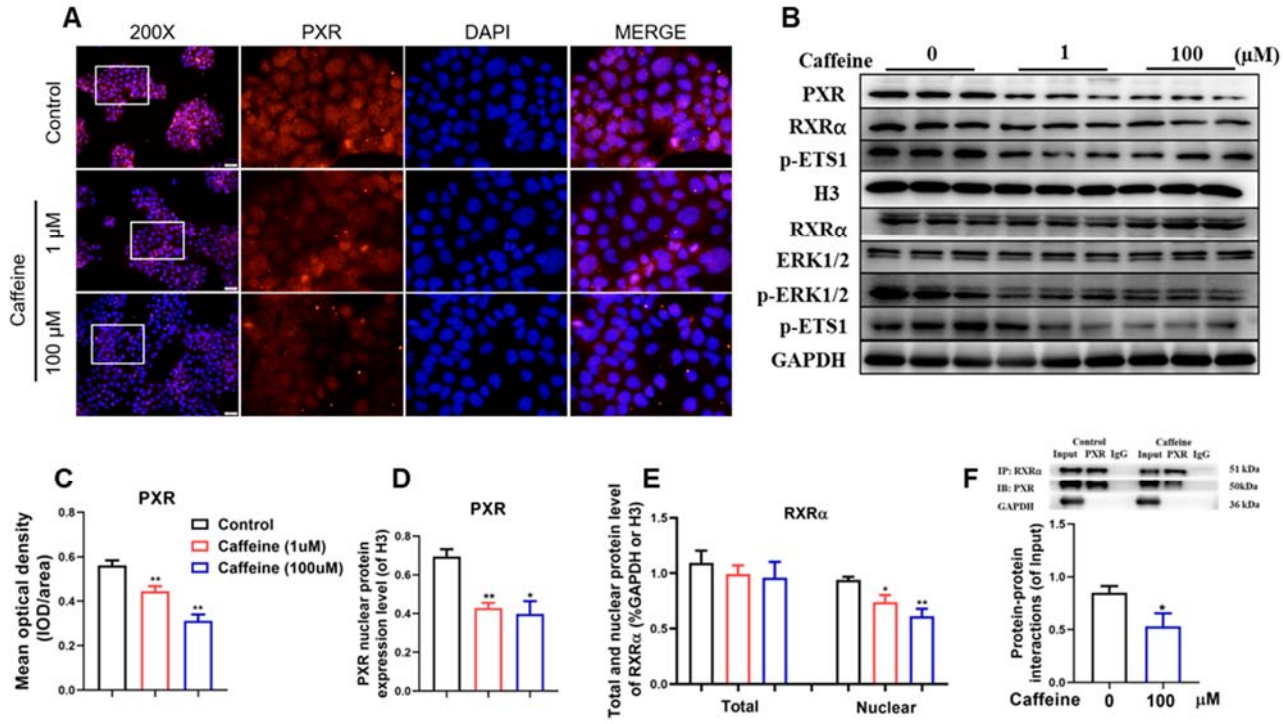


Fig 3. Effects of prenatal caffeine exposure (PCE) on the protein expression of the ERK-ETS1-PXR/RXRα pathway in rat placenta. (A) Immunohistochemical staining of PXR (400×, scale: 25 μm, and local enlargement), brown color: protein expression of PXR; (B) Mean optical density of PXR were analyzed by Image pro plus; (C) Nuclear protein level of PXR; (D) Nuclear protein level of RXRα; (E) The nuclear protein bands of PXR, RXRα, phospho-ETS1, H3, and total protein bands of phospho-ETS1, ERK1/2, phospho-ERK, GAPDH; (F, G) The total and nuclear protein levels of phospho-ETS1; (H, I) The total protein levels of ERK1/2 and phospho-ERK1/2. The data are presented as mean ± S.E.M., n=6 for protein assay, and n=5 for immunohistochemistry assay in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. control. PXR, pregnane X receptor; RXRα, retinoic acid X receptor-alpha; ETS1, E26 transformation-specific sequence 1; ERK, extracellular regulated protein kinases; GAPDH, glyceraldehyde-phosphate dehydrogenase; H3, histone 3; PCE(L), caffeine low-dose exposure; PCE(M), caffeine medium-dose exposure; PCE(H), caffeine high-dose exposure.

3.2.2 Caffeine induces abnormal protein expression and activation of the ERK-ETS1-PXR/RXRα pathway in BeWo cells

To further clarify the effect of caffeine on the ERK-ETS1-PXR/RXRα signaling pathway in trophoblast cells, we detected the expression of related protein and their interactions in BeWo cells treated with different concentrations of caffeine. The results showed that compared with the control group, treatment with caffeine (1 and 100 μM) significantly reduced the PXR fluorescence intensity (Fig 4A, C) and nuclear protein expression (Fig 4B, D). Although the total protein expression of RXRα showed no significant change, its nuclear protein content decreased significantly (Fig 4B, E). The results of the Co-IP assay demonstrated that caffeine (100 μM) could reduce the protein-protein interaction between PXR and RXRα (Fig 4F). The results of Re-ChIP further indicated that caffeine significantly inhibited the binding of the PXR/RXRα heterodimer to the FATP1 promoter region (Fig 4G, H). These results suggest that caffeine can inhibit the nuclear protein expression of PXR and RXRα in trophoblast cells, and reduce their protein interaction as well as their binding ability to the FATP1 promoter region. Finally, we examined the effect of caffeine on the protein expression and activation of ERK and ETS1. The results revealed that the total protein expression of ERK1/2 in the caffeine group remained unchanged (Fig 4B, I), while the protein expression of p-ERK1/2 decreased significantly (Fig 4B, I). Both the total protein and nuclear protein levels of p-ETS1 were significantly reduced (Fig 4B, J). In conclusion, caffeine can inhibit the activation of ERK and ETS1 in BeWo cells and decrease the protein expression and interaction of PXR/RXRα, as well as their binding to the FATP1 promoter region.



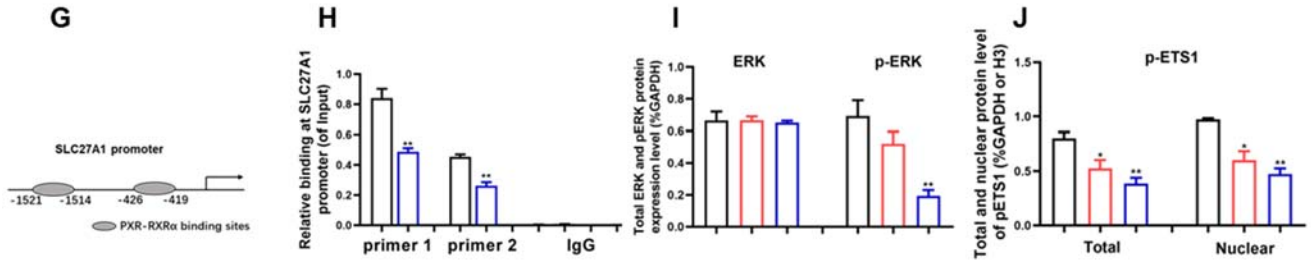


Fig 4. Effects of caffeine on the expression of the ERK-ETS1-PXR/RXRα pathway in BeWo cells. (A) Immunofluorescence staining of PXR (200×, scale: 50 μm, and local enlargement); (D) The nuclear protein bands of PXR, RXRα, phospho-ETS1, and total protein bands of RXRα, ERK1/2, phospho-ERK1/2 and phospho-ETS; (C) Mean optical density of PXR were analyzed by Image pro plus; (D) Nuclear protein level of PXR; (E) The total and nuclear protein levels of RXRα; (F) Protein–protein interactions between PXR and RXRα (Co-IP assay); (G) The binding sites of PXR/RXRα on SLC27A1 promoter; (H) The enrichment of PXR and RXRα at the SLC27A1 promoter; (I) The total protein levels of ERK1/2 and phospho-ERK1/2; (G) The total and nuclear protein levels of phospho-ETS1. The data are presented as mean ± S.E.M., n=6 for western blotting assay, immunofluorescence staining, re-chromatin immunoprecipitation, and co-immunoprecipitation assay in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. control. PXR, pregnane X receptor; RXRα, retinoic acid X receptor-alpha; ETS1, E26 transformation-specific sequence 1; ERK, extracellular regulated protein kinases; Co-IP, co-immunoprecipitation; GAPDH, glyceraldehyde-phosphate dehydrogenase; H3, histone H3.

3.2.3 The ADORA2A-ERK-ETS1-PXR/RXRa pathway mediates the low expression of placental FATP1 and the inhibition of LCFAs uptake caused by caffeine

To verify whether caffeine inhibits LCFAs uptake and reduces FATP1 expression in BeWo cells by suppressing PXR, we treated BeWo cells with caffeine alone or in combination with the PXR agonist Rifampicin. The results of the fatty acid uptake assay showed that compared with the control group, caffeine significantly inhibited the uptake of OA and AA (Fig 5A-C), while co-treatment with Rifampicin reversed the inhibitory effect of caffeine on AA and OA uptake (Fig 5A-C). In addition, caffeine (100 μM) significantly decreased the mRNA expression of FATP1, whereas treatment with Rifampicin (10 μM) restored the mRNA expression level of FATP1 (Fig 5D). These results suggest that caffeine downregulates FATP1 transcription by inhibiting PXR nuclear expression, thereby reducing the uptake of LCFAs.

To further investigate whether caffeine causes the low expression of FATP1 by inhibiting the expression of ETS1, we treated BeWo cells with caffeine alone or in combination with ETS1 overexpression plasmids. Results demonstrated that caffeine significantly inhibited the mRNA expression of FATP1 as well as the total protein expressions of p-ETS1 and PXR, whereas the ETS1 overexpression plasmid could reverse the inhibitory effect of caffeine (Fig 5E, F). Immunofluorescence analysis further demonstrated that caffeine treatment reduced the nuclear protein expression of PXR and RXRα, an effect similarly reversible by ETS1 overexpression (Fig 5G). These findings suggest that caffeine inhibits the mRNA and protein expression of ETS1, reduces the total protein level of PXR and its nuclear translocation with RXRα, thereby leading to a decrease in FATP1 mRNA expression.

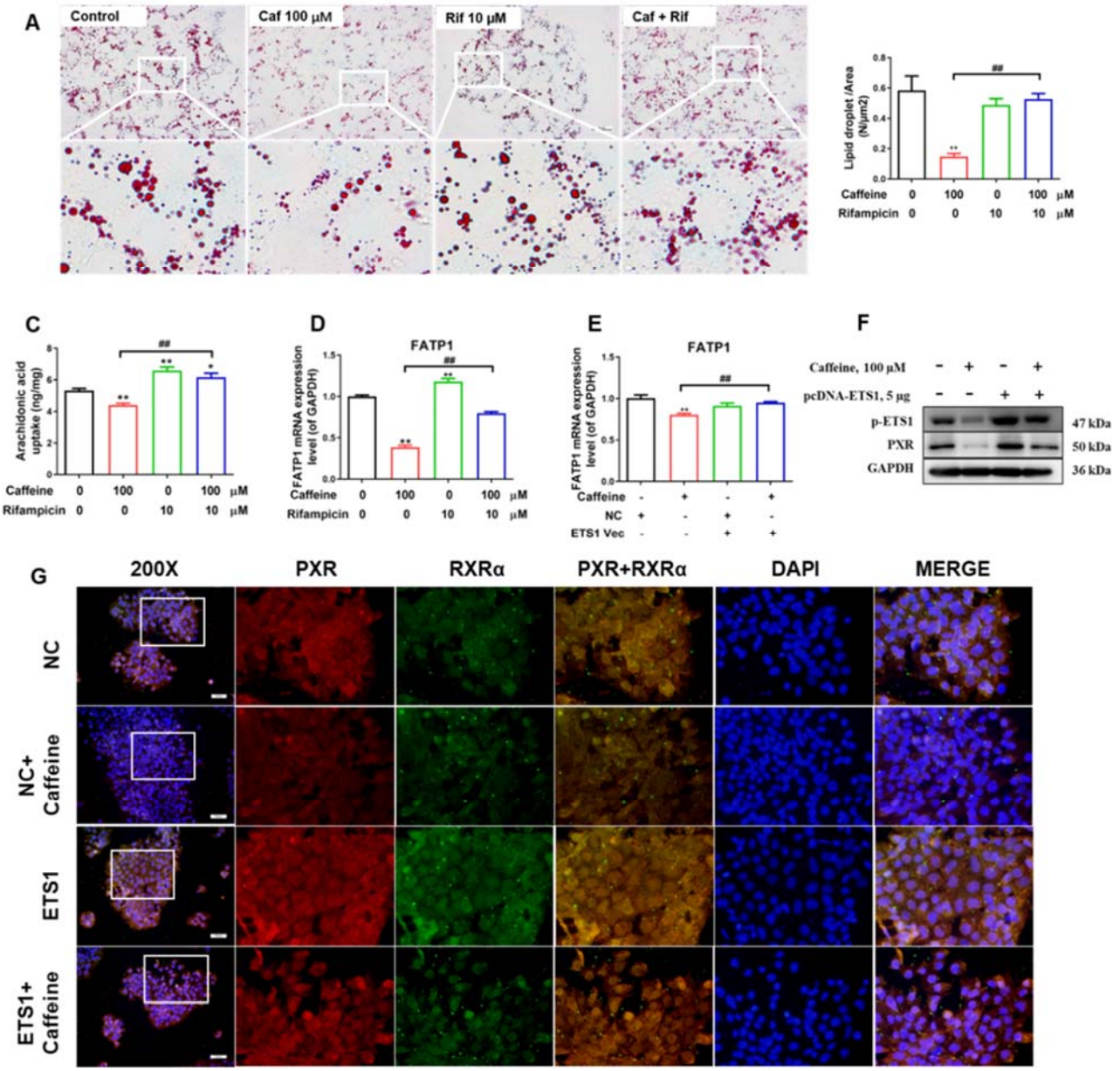
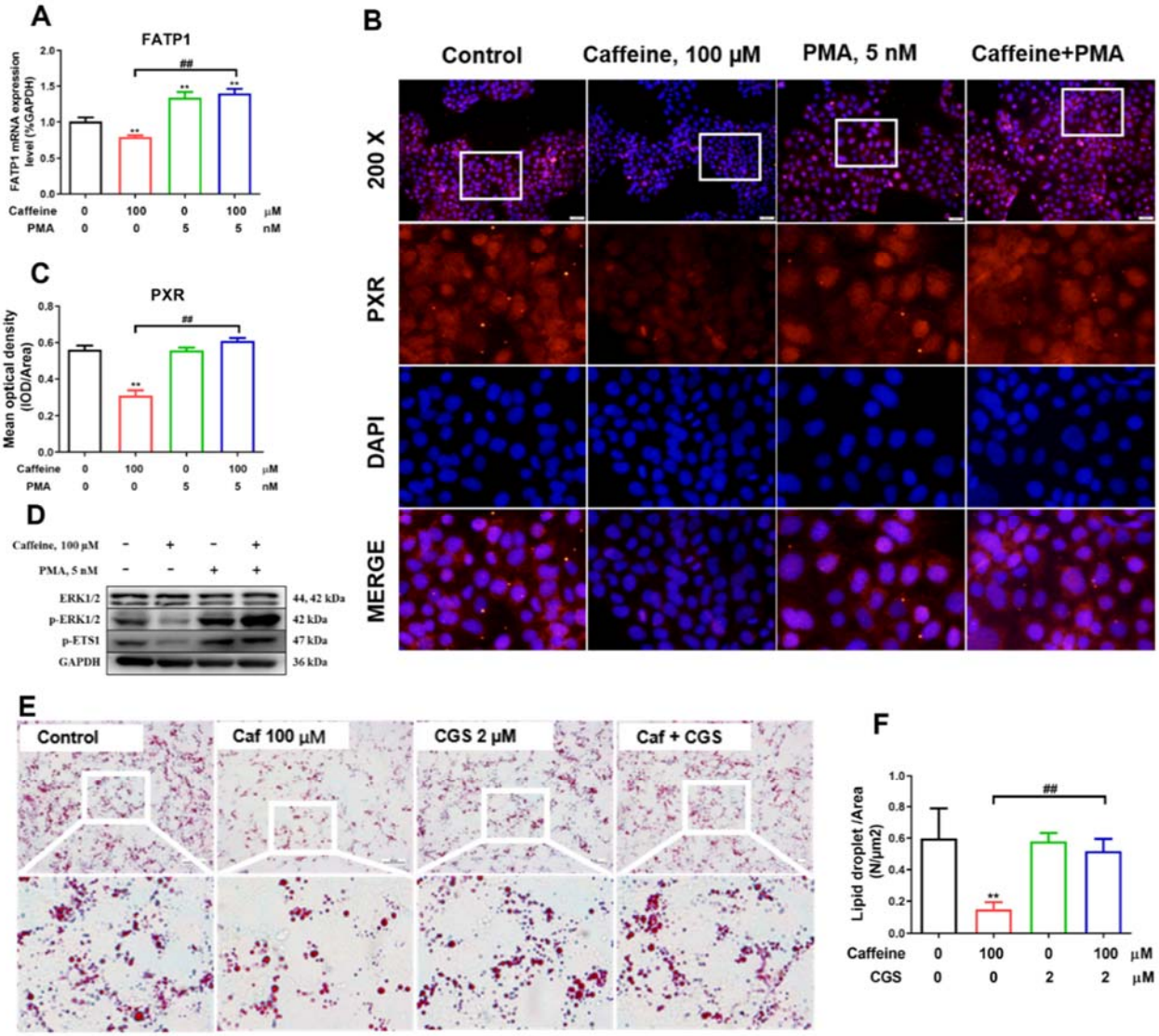


Fig 5. Effects of PXR and ETS1 intervention on the mRNA expression of FATP1 and long-chain fatty acid transport in BeWo cells. (A) Oil red O staining (400×, scale: 25 μm, and local enlargement); (B) Number of lipid droplets; (C) Arachidonic acid concentration (D) FATP1 mRNA expression in BeWo cells treated with 100 μM caffeine and/ or 10 μM Rifampicin for 24 h; (E) The FATP1 mRNA expression in BeWo cells treated with ETS1 Vec or NC; (F) The total protein bands of phospho-ETS1 and PXR and GAPDH; (G) Immunofluorescence staining of PXR and RXRα (200×, scale: 50 μm, and local enlargement) in BeWo cells treated with 100 μM caffeine and/ or 5 μg pcDNA-ETS1 for 24 h. The data are presented as mean ± S.E.M., n=6 for mRNA analysis, protein assay, immunofluorescence assay, and oil red O staining. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs*. caffeine. ETS1, E26 transformation-specific sequence 1; PXR, pregnane X receptor; RXRα, retinoic acid X receptor-alpha; FATP1, fatty acid transport protein 1; GAPDH, glyceraldehyde-phosphate dehydrogenase.

Next, to determine whether caffeine mediates the inhibition of FATP1 expression by reducing ERK phosphorylation, we treated BeWo cells with caffeine alone or in combination with the ERK agonist PMA. RT-qPCR and immunofluorescence results showed that compared with the control group, caffeine significantly inhibited FATP1 mRNA expression and PXR fluorescent protein expression (Fig 6A-C), while PMA treatment reversed the inhibitory effect of caffeine (Fig 6A-C). Meanwhile, Western blot results demonstrated that caffeine decreased the protein expression of p-ERK1/2 and p-ETS1, whereas PMA reversed the inhibitory effect of caffeine on p-ERK1/2 and p-ETS1 protein expression (Fig 6D). These

findings indicate that caffeine can downregulate FATP1 mRNA expression by inhibiting ERK phosphorylation and reducing p-ETS1 and PXR expression.

Finally, to confirm whether caffeine mediates the low expression of FATP1 and inhibition of LCFAs uptake by antagonizing ADORA2A, we treated BeWo cells with caffeine alone or in combination with the ADORA2A agonist CGS-21680. The results showed that caffeine significantly inhibited the uptake of OA and AA (Fig 6E-G) and decreased FATP1 mRNA expression (Fig 6H), while CGS-21680 reversed the inhibitory effect of caffeine (Fig 6E-H). Additionally, caffeine significantly inhibited the total protein expression of p-ERK1/2, p-ETS1, and PXR, whereas CGS-21680 could restore their expression (Fig 6I). In conclusion, caffeine can cause abnormal protein expression and activation of the ERK-ETS1-PXR pathway by antagonizing ADORA2A, leading to low mRNA expression of FATP1 and inhibition of LCFAs uptake.



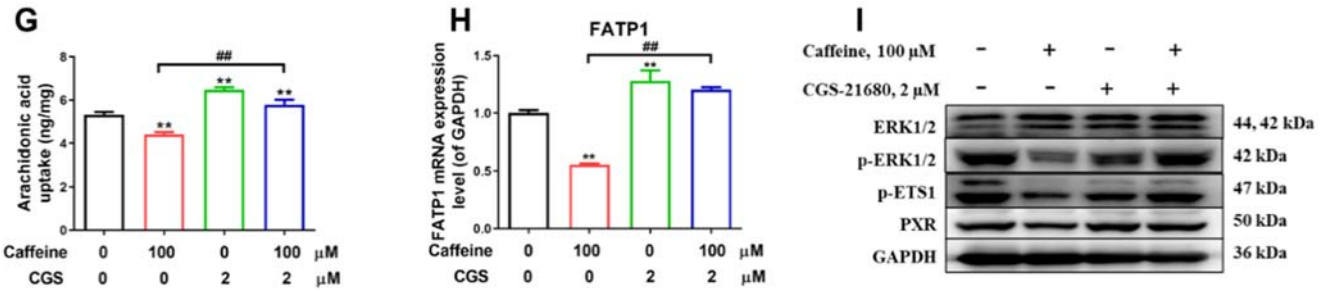


Fig 6. Effects of ERK and ADORA2A intervention on the mRNA expression of FATP1 and long-chain fatty acid transport in BeWo cells. (A) FATP1 mRNA expression; (B) Immunofluorescence staining of PXR (200×, scale: 50 μm, and local enlargement) in BeWo cells treated with 100 μM caffeine and/ or 5 nM CGS-21680 for 24 h (C) Mean optical density of PXR were analyzed by Image pro plus; (D) The total protein bands of ERK1/2 and phospho-ERK1/2 and phospho-ETS1 and GAPDH; (E) Oil red O staining (400×, scale: 25 μm, and local enlargement); (F) Number of lipid droplets; (G) Arachidonic acid concentration; (H) The FATP1 mRNA expression in BeWo cells treated with CGS21680; (I) The total protein bands of ERK1/2 and phospho-ERK1/2 and phospho-ETS1 and PXR and GAPDH in BeWo cells treated with 100 μM caffeine and/ or 2 μM CGS-21680 for 24 h. The data are presented as mean ± S.E.M., n=6 for mRNA analysis, protein assay, immunofluorescence assay, ELISA, and oil red O staining. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs*. caffeine. ERK, extracellular regulated protein kinases; ETS1, E26 transformation-specific sequence 1; PXR, pregnane X receptor; FATP1, fatty acid transport protein 1; GAPDH, glyceraldehyde-phosphate dehydrogenase; CGS, CGS-21680.

3.3 PXR agonists can reverse PCE-induced downregulation of placental FATP1 and low birth weight in mice

Lastly, to investigate whether the intervention of PXR could alleviate the inhibition of placental and fetal development caused by caffeine, we administered caffeine (60 mg/kg·d, *ig*) or the PXR agonist PCN (20 mg/kg·d, *ig*) to C57BL/6 pregnant mice from GD9 to GD18. On GD18, the pregnant mice were anesthetized and sacrificed, and the placentas and fetuses were weighed. The results showed that compared with the control group, the birth weight of fetal mice in the caffeine group was significantly reduced (Fig 7A, B), and the IUGR rate was significantly increased (Fig 7C, D). There was no significant effect on placental weight (Fig 7E, F). The fetal/placental weight ratio was decreased in male fetuses (Fig 7G) but showed no significant change in female fetuses (Fig 7H). The levels of free fatty acids in maternal male fetal serum were decreased (Fig 7I, J), with no significant change in female fetal serum (Fig 7K). Co-administration of PCN reversed the caffeine-induced low birth weight in both male and female fetal mice (Fig 7A, B) and simultaneously reversed the caffeine-induced increase in IUGR rate among female fetuses (Fig 7D). In addition, PCN can restore the levels of free fatty acids in maternal and male fetal serum (Fig 7I, J) but had no significant effect on female fetal serum (Fig 7K). Further detection revealed that compared with the control group, caffeine significantly reduced the nuclear protein expression of PXR in the male and female placentas (Fig 7L, M) and inhibited the mRNA and protein expression of FATP1 in the male placentas (Fig 7N, O, R). However, PCN intervention could reverse the inhibitory effect of caffeine on the male placentas. In the female placentas, there were no significant changes in the mRNA and protein expression of FATP1 (Fig 7P, Q, R). These results suggest that the PXR agonist PCN can reverse caffeine-induced decreases in PXR and FATP1 expression in the male placentas, as well as the reductions in free fatty acid levels in maternal and male fetal blood.

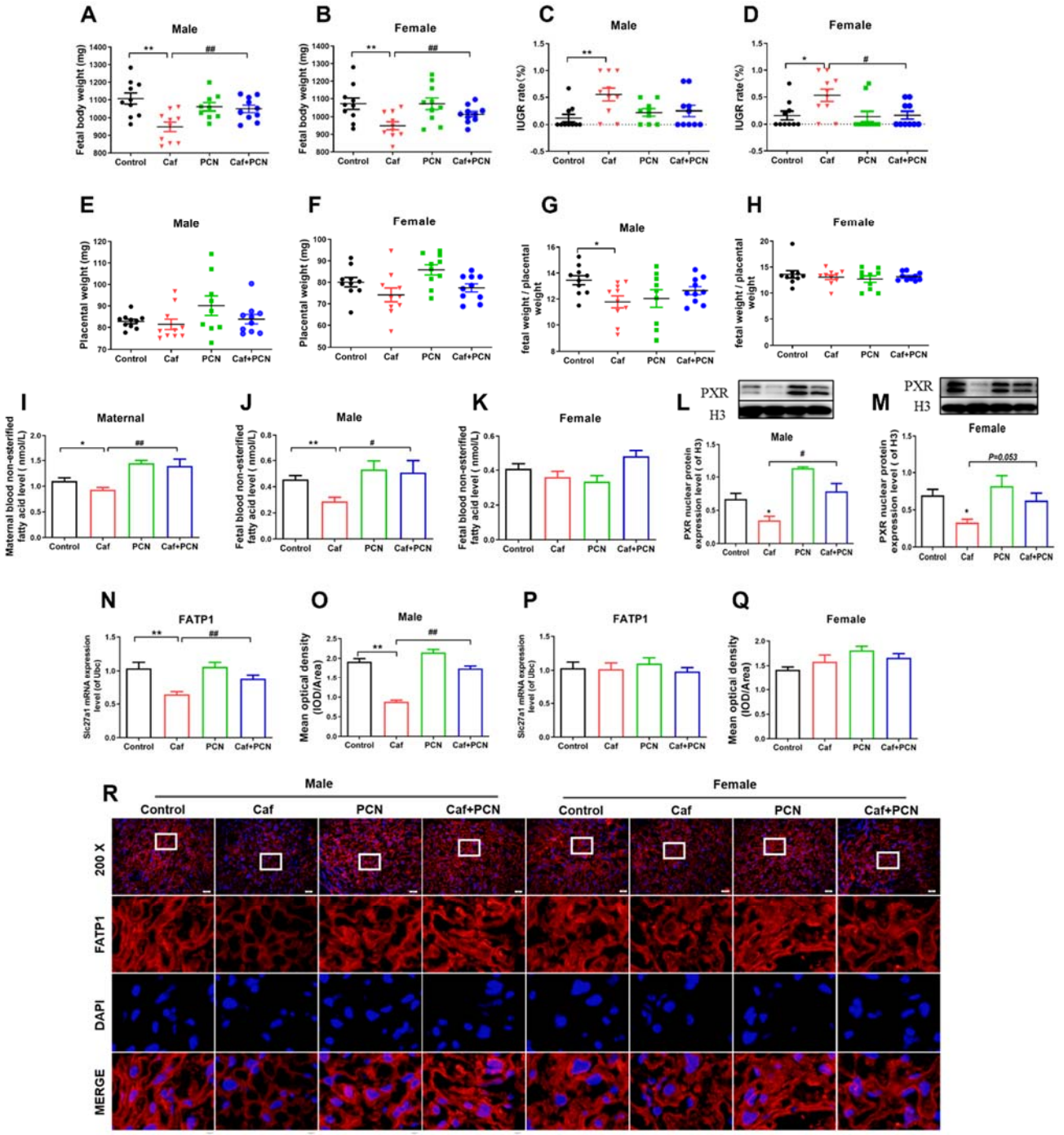


Fig 7. Effects of PCN on PCE-induced low expression of placental FATP1 and low fetal birth weight in mice. (A, B) Fetal body weight; (C, D) IUGR rate; (E, F) Placental weight; (G, H) Placental index; (I-K) The non-esterified fatty acid levels of the maternal serum, male fetal serum and female fetal serum; (L, M) Nuclear protein level of PXR in male and female placenta; (N, P) mRNA expression levels of FATP1 in male and female placenta; (O, Q) Mean optical density of FATP1 in the male and female placenta were analyzed by Image pro plus; (R) Immunofluorescence staining of FATP1 in the male and female placenta (200×, scale: 50 μm, and local enlargement). The data are presented as mean ± S.E.M. Body weights and placental weights are mean values of each litter, n=10 litters in each group; n=10 for mRNA assay and non-esterified fatty acid test; n=6 for western blotting, n=5 for immunofluorescence staining. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs*. caffeine. Caf, caffeine group; PCN, pregnenolone 16α-carbonitrile group; GD, gestational day; IUGR, intrauterine growth retardation; FATP1, fatty acid transport protein 1; Ubc, ubiquitin C; PXR, pregnane X receptor; H3, histone H3.

4. Discussion

Caffeine is widely present in beverages such as coffee, tea, and cola, and its consumption during pregnancy is very common. It is estimated that approximately 12% of pregnant women in France and 67.3%

in Japan consumed more than 200 mg of caffeine daily, exceeding the maximum recommended intake outlined in guidelines from the European Food Safety Authority and the U.S. Institute of Medicine [38, 39]. A meta-analysis indicates that approximately 18.6% of pregnant women consume as much as 350–699 mg of caffeine daily [40, 41]. Based on the dose conversion ratio between humans and rats (human: rat = 1:6.17, calculated by body surface area), a 60 kg pregnant woman consuming 300–600 mg of caffeine per day corresponds to a daily intake of 30.85–61.7 mg/kg in rats. Therefore, based on previous literature and our laboratory's prior research, we administered 30, 60, and 120 mg/kg of caffeine daily to establish the IUGR model in rats [42]. In rats, GD9 corresponds to the second trimester of human pregnancy. At this stage, the fetus's key organs and placenta have acquired their respective functions. Moreover, intervention during this period can effectively prevent premature birth and stillbirth. Additionally, fetal adipose tissue begins to form from the 14th week of human pregnancy and accumulates substantially during the mid-to-late stages of gestation. The final 10 weeks of pregnancy represent a critical window period for fetal brain demand for LCPUFAs [43]. Meanwhile, placental fatty acid transport activity is most active during the mid-to-late stages of pregnancy [44]. Therefore, this study administered caffeine to pregnant rats during GD9-20 to establish an IUGR rat model and further investigated the placental fatty acid transport mechanisms. Based on the PCE animal model, we have confirmed that caffeine exposure during pregnancy antagonizes ADORA2A, leading to the inhibition of the placental ERK-ETS1-PXR/RXRα signaling pathway, downregulation of FATP1 expression, and reduction of placental fatty acid transport, which are important mechanisms leading to fetal IUGR.

4.1 Suppression of FATP1 mRNA expression mediates reduced fatty acid transport function in the placenta of rats induced by PCE

Research indicates that placental uptake of LCFAs is mediated by specific transporters on the cell membrane [45], including the FABPpm, FAT (also known as CD36), and FATP family. FATPs constitute a complete family of transmembrane proteins comprising six members (FATP1-6) that regulate cellular uptake of LCFAs and very long-chain fatty acids [46]. Among these, FATP1 was the first FATP subtype identified in the human placenta [47]. Both FATP1 and FATP4 are highly expressed in placental tissue, playing crucial roles in the uptake of LCFAs into the placenta and their subsequent intracellular processing [9]. It has been reported that reduced levels of fatty acids (particularly LCPUFAs) in human umbilical cord blood and placental tissue are closely associated with the occurrence of IUGR and the susceptibility to long-term diseases in offspring [10, 48-50].

AA is an n-6 LCPUFA that is essential for fetal brain and optic nerve development. Its synthesis within the fetus is limited and mainly relies on active transport by the placenta [51]. Oleic amide is a long-chain fatty amide, which is synthesized through the metabolism of OA, a long-chain monounsaturated fatty acid [52]. Our previous studies revealed that PCE significantly reduces levels of saturated fatty acids, unsaturated fatty acids, and triglycerides in the blood of pregnant rats and their fetuses. Additionally, LCFAs and their metabolic derivatives (such as AA and OA) are also markedly decreased in fetal blood [25, 26, 53]. It is

suggested that there might be a disorder in the interuterine transfer of LCFAs in the rat model of IUGR caused by PCE. This study further revealed that PCE suppresses the mRNA expression of multiple fatty acid transporters (FATP1, FATP2, FATP4, FABPpm, and CD36) in the rat placenta, with the downregulation of FATP1 and FATP2 closely associated with PCE-induced low birth weight in fetuses.

It was reported that placental trophoblast cells exhibit a significantly higher uptake rate for LCFAs compared to short-chain and medium-chain fatty acids [54]. The absorption priority for LCFAs is DHA > AA > α-linolenic acid > linoleic acid > OA [36]. In this study, caffeine could significantly inhibit the uptake of OA and AA by BeWo and down-regulate the mRNA expression of FATP1, FATP4, FABPpm, and CD36 in cells. Different concentrations (1, 10, and 100 μM) of caffeine also significantly reduced the mRNA and protein expression of FATP1 in the cells. This indicates that caffeine inhibits the uptake of LCFAs by trophoblasts and the mRNA expression of multiple fatty acid transporters. A clinical study showed that human placental FATP1 expression shows a significant positive correlation with placental weight, as well as newborn birth weight, length, head circumference, chest circumference, and abdominal circumference [55]. Another study also found that the mRNA expression of FATP1 in the placenta was positively correlated with DHA levels in maternal blood, placenta, and umbilical cord blood [56]. In this study, after silencing FATP1 using siRNA interference, the formation of lipid droplets in BeWo cells decreased, and the level of AA decreased. This further confirmed the crucial role of FATP1 in the uptake of LCFAs. In summary, PCE contributes to the occurrence of IUGR by downregulating placental FATP1 expression and reducing the transport of LCFAs.

4.2 The ADORA2A-ERK-ETS1-PXR/RXRα pathway mediates the inhibition of FATP1 expression in the placenta of rats caused by PCE

RXR can form heterodimers with peroxisome proliferator-activated receptor (PPAR), jointly regulating the expression of FATP1 [14] and FATP4 [15] in placental trophoblast cells, and promoting the uptake of LCFAs. Similar to PPAR, PXR can also form heterodimers with RXR and bind to specific DNA sequences. It has been reported that PXR is involved in maintaining the lipid homeostasis of the liver [57, 58]. However, there are no reports on the regulation of placental fatty acid transporter expression by PXR at present. Using bioinformatics techniques, we predicted the presence of PXR/RXRα binding sites in the FATP1 promoter region. Furthermore, we demonstrated in BeWo cells that caffeine significantly inhibits protein interactions between PXR and RXRα, and reduces the binding of PXR/RXRα to the FATP1 promoter region. Following treatment with the PXR agonist rifampicin, the caffeine-induced downregulation of FATP1 mRNA and inhibition of OA and AA uptake in BeWo cells were significantly reversed. This suggests that caffeine inhibits the nuclear protein expression of PXR and its interaction with RXRα, thereby reducing the binding of PXR and RXRα to the promoter region of FATP1, leading to low expression of FATP1 and impaired uptake of LCFAs.

ETS1 is a transcription activator that promotes PXR mRNA expression [59] and can directly bind to PXR to enhance its transcriptional activity [60]. ERK is a serine/threonine protein kinase. Activated ERK

can phosphorylate the 38th serine residue of ETS1, thereby regulating the expression of its downstream target genes [61-64]. To date, there have been no reports indicating that the ERK-ETS1 pathway participates in regulating placental PXR expression and fatty acid uptake. In this study, we found that the expression of p-ERK1/2 and p-ETS1 (both total and nuclear protein) was significantly reduced in the placenta of PCE rats and in caffeine-treated BeWo cells. Treatment with the ERK agonist PMA reverses caffeine-induced suppression of p-ERK1/2, p-ETS1, PXR protein, and FATP1 mRNA expression. Similarly, ETS1 overexpression restores p-ETS1, PXR protein, and FATP1 mRNA expression levels. These results suggest that caffeine suppresses ETS1 activation by reducing ERK phosphorylation levels, thereby downregulating PXR and FATP1 expression in the placenta.

The physiological effects of caffeine are primarily mediated through non-specific antagonism of adenosine receptors. It has been reported that the central stimulating effect of caffeine is mainly mediated by blocking the A2A receptor [65]. Our previous research has also revealed that PCE primarily induces placental developmental toxicity in rats by antagonizing ADORA2A, rather than other subtypes [27]. In this study, to verify whether caffeine causes low mRNA expression of FATP1 and inhibition of LCFAs uptake in BeWo cells through ADORA2A, we treated the cells with the ADORA2A-specific agonist CGS-21680. We found that CGS-21680 could significantly reverse the inhibition of p-ERK1/2, p-ETS1, and PXR protein expression caused by caffeine and restore the mRNA expression level of FATP1 and the cellular uptake of OA and AA. These results indicate that caffeine disrupts the ERK-ETS1-PXR signaling pathway by antagonizing ADORA2A, thereby reducing placental FATP1 expression and LCFA uptake. In conclusion, this study confirms that the ADORA2A-ERK-ETS1-PXR/RXRα pathway plays a crucial role in the low expression of FATP1 in the placenta and inhibition of LCFAs uptake caused by PCE.

4.3 PXR agonists reverse PCE-induced inhibition of FATP1 expression in rat placenta and alleviate fetal low birth weight

PCN is a PXR-specific agonist for rodents [66]. The study found that when pregnant rats were intragastrically administered 100 mg/kg·d PCN at different stages of pregnancy, there were no significant changes in the pregnancy: mating ratio, gestation period length, number of implantation sites, and the size of the litter [67]. Previous studies have confirmed that RXR is involved in regulating the expression of FATP1 in trophoblast cells and is related to the formation of lipid droplets in mice placenta [14, 68]. Another study reported that interfering with the nuclear receptor PXR can improve lipid metabolism-related diseases [69]. This implies that the PXR agonist PCN may participate in placental lipid metabolism but does not affect pregnancy outcomes. In this study, we administered PCN (20 mg/kg·d, *ig*) to PCE (GD 9-18, 60 mg/kg·d, equivalent to the low-dose caffeine group used in the rat model) and normal mice. The results showed that PCN can reverse PCE-induced low birth weight in both male and female fetal mice. Meanwhile, PCN can also reverse the low mRNA and protein expression of FATP1 in the male placenta and the decreased levels of free fatty acids in the fetal blood. Notably, we observed a decrease in maternal free fatty acid levels in mice, and PCN intervention could reverse this effect. This indicates that, in addition to abnormal placental

structure and inhibition of fatty acid transporter expression, PCE may also affect the fetal acquisition of fatty acids by inhibiting maternal liver fatty acid synthesis. In summary, these findings not only reveal a new mechanism by which PCE interferes with placental fatty acid transport through inhibiting the PXR pathway, thereby causing fetal IUGR, but also suggest that pregnant women should strictly control their caffeine intake during pregnancy. Additionally, targeting the activation of the PXR pathway may provide a new therapeutic strategy for preventing fetal IUGR and related lipid metabolism disorders.

4.4 Limitations

Fatty acid transport across the placenta is a complex biological process that involves the coordinated action of multiple transporters, such as FATP1, FATP2, FATP4, FABPpm, and CD36. Through integrated *in vivo* and *in vitro* experiments, we identified a central role for FATP1 in caffeine-induced placental fatty acid transport dysfunction and fetal growth restriction. However, the independent contributions of other transporters, as well as possible compensatory changes that occur when FATP1 function is impaired, remain unclear. In addition, fatty acids such as AA and DHA are essential for fetal brain development and rely heavily on maternal supply and placental transport. Our previous studies showed that PCE can impair fetal hippocampal development and alter offspring behavior after birth [70]. It is therefore plausible that impaired placental transfer of key LCPUFAs may link in utero caffeine exposure to later neurobehavioral abnormalities, but this hypothesis was not directly tested in the present study. Future work should track AA and DHA accumulation in fetal and neonatal brain tissue across developmental stages and evaluate postnatal learning, memory, and behavioral phenotypes to further define the broader developmental consequences of placental fatty acid transport disruption.

5. Summary

This study found that caffeine inhibits the ERK-ETS1-PXR-RXRα signaling pathway by antagonizing ADORA2A, reduces the expression of FATP1, decreases the fetal fatty acid supply, and ultimately leads to the occurrence of IUGR (Fig 8). This study further confirmed that the PXR agonist PCN can effectively intervene in IUGR induced by PCE. In conclusion, this study revealed the placental mechanism underlying PCE-induced IUGR from the perspective of fatty acid transporter function, providing a crucial theoretical basis and experimental support for the early prevention and treatment of IUGR fetuses.

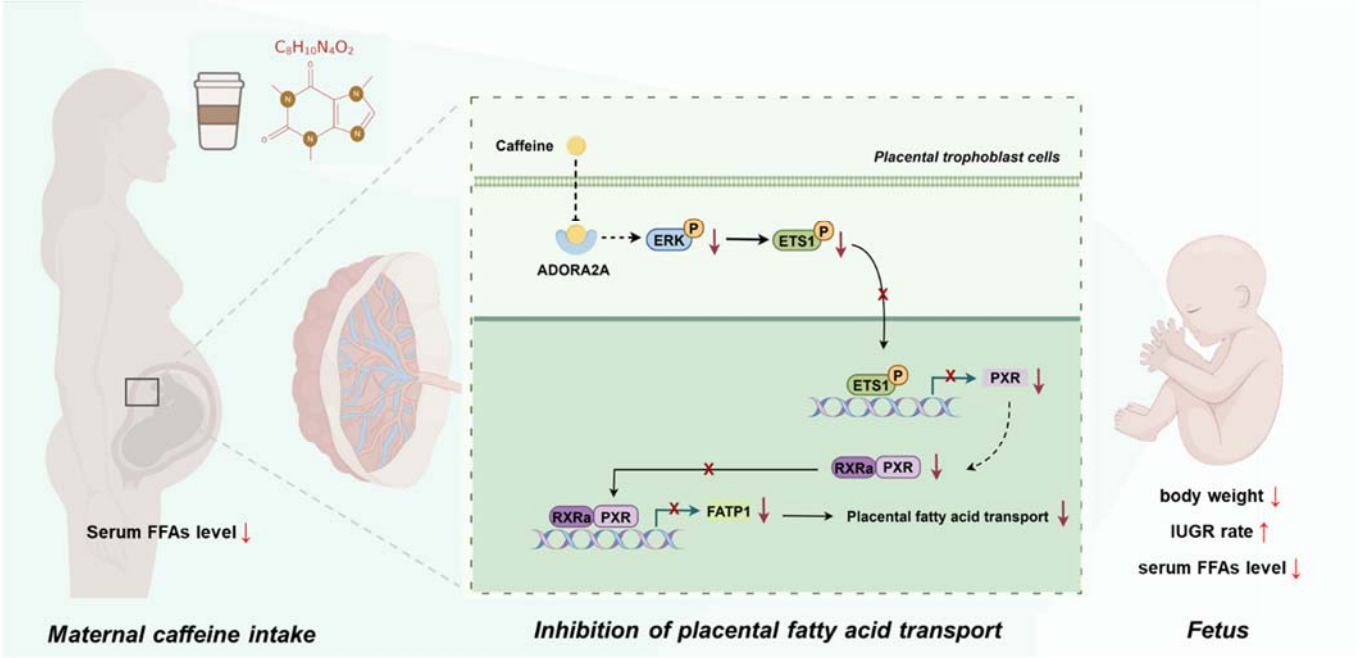


Fig 8. The placental FATP1 regulatory mechanism underlying offspring intrauterine growth retardation induced by prenatal caffeine exposure. FFAs: free fatty acids; ADORA2A: adenosine A2a receptor; ERK, extracellular regulated protein kinases; ETS1, E26 transformation-specific sequence 1; RXRα, retinoic acid X receptor-alpha; FATP1, fatty acid transport protein 1; PXR, pregnane X receptor; IUGR: intrauterine growth retardation.

Declaration of interest

The authors in preparing this manuscript did not receive any compensation from any source and declare that they have no potential conflict of interest.

Acknowledgements

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