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Hypoxia-Mediated Down-Regulation of OCTN2 and PPARα Expression in Human Placentas and in BeWo Cells

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Abstract: The objective of the present study was to investigate the effects of hypoxia on placental expression of OCTN2 and PPARα. OCTN2 and PPARα expression in the human placenta in the presence or absence of preeclampsia was examined by immunohistochemical (IHC) analysis, Western blotting, and quantitative polymerase chain reaction (qPCR). Effects of hypoxia on the expression of OCTN2 and PPARα in human placental explants and human choriocarcinoma BeWo cells were examined by Western blotting and qPCR analyses. IHC, Western blot, and qPCR studies showed that OCTN2 and PPARα protein and mRNA levels were lower in syncytiotrophoblasts from preeclamptic human placentas than in those from normal placentas. Hypoxic treatment caused a decrease in OCTN2 and PPARα expression in human placental explants and in BeWo cells. WY14643, a PPARα agonist, caused an increase in OCTN2 expression in BeWo cells under hypoxic conditions. In conclusion, under hypoxic conditions, placental OCTN2 is down-regulated through PPARα-mediated pathways.

Keywords: OCTN2; PPARα; Preeclampsia; Hypoxia; BeWo cells

Introduction

The etiology of preeclampsia, a hypertensive and inflammatory disorder of human pregnancy, leading to maternal and perinatal morbidity and death, is multifactorial and remains poorly understood. Placental oxidative stress induced by hypoxia-reoxygenation has been proposed as a possible etiological factor in preeclampsia.[1] Nonetheless, abnormal maintenance of chronic hypoxia may be critical in preeclampsia, since it can alter human cytotrophoblast differentiation/invasiveness and change gene expression profiles.[2,3] Hypoxia regulates the expression of a number of genes that enable cells to adapt to this stress condition.[4] The production of hypoxia-inducible factor 1α (HIF-1α) in response to

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hypoxia can increase the expression of a variety of genes, including TGFβ3, which switches trophoblast differentiation toward an invasive phenotype, the extravillous trophoblast.[5] However, a persistent increase in HIF-1α in the syncytiotrophoblast, villous cytotrophoblasts, and fetoplacental vasculature may lead to insufficient trophoblast invasion of the maternal spiral arteries, contributing to the development of preeclampsia.[6] Hypoxia during preeclampsia can cause impaired syncytialization, as in BeWo cells and in human placental cotyledons; it down-regulates the expression of syncytin, a protein linked to syncytialization.[7−9]

The plasma carnitine concentration in pregnant women with preeclampsia is about 50% higher than that in normotensive healthy pregnant women.[10] Carnitine is essential for mitochondrial fatty acid oxidation and can regulate immune networks.[11,12] Active placental carnitine transfer can lead to significantly higher carnitine levels in cord blood than in the maternal blood,[13] which is important to the fetus for carnitine reserves, metabolic functions, and tissue development.[14] The higher carnitine concentrations in pregnant women with preeclampsia suggest a decrease in placental carnitine transfer.

OCTN2 (SLC22A5), a high-affinity carnitine transporter, is the primary transporter responsible for placental transfer of carnitine from the mother to the fetus and also interacts with a number of therapeutic agents, including cardiovascular drugs[15] and anticonvulsants.[16] OCTN2 is located in the brush-border membrane of syncytiotrophoblasts of the human placenta[17] and of human choriocarcinoma BeWo cells.[7] It was previously reported that hypoxia results in a significant reduction in carnitine uptake in BeWo cells, but RT-PCR (reverse transcription polymerase chain reaction) indicated increased transcription of OCTN2, whereas Western blots did not show a significant change in the amount of OCTN2 protein.[18] Thus, the mechanism involved in the hypoxia-induced regulation of OCTN2 is unknown, and its clinical implications need to be examined.

Recently, several studies have demonstrated that peroxisome proliferator-activated receptor α (PPARα) can directly upregulate OCTN2 expression in rodents.[19−21] However, it is not known whether it also regulates placental OCTN2 expression in humans under normoxic and hypoxic conditions. In the present study, we examined OCTN2 and PPARα protein levels in human placentas from normal pregnancies and from pregnancies with preeclampsia. Human placental explants and BeWo cells were also used as in vitro models to study the regulatory role of hypoxia in PPARα and OCTN2 expression.

Experimental Section

Clinical Tissue Collection. Human placental tissues from normal term placentas (males, $n = 7$; females, $n = 3$) and

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Table 1. Clinical Characteristics for the Normal and Preeclamptic Placentas Collected in the Study[a]

	maternal age (years)	gestation (weeks)	infant weight (g)	placental weight (g)	systolic/diastolic pressure (mmHg)
normal term placentas	37 ± 4	37.6 ± 2.1	3102 ± 544	525 ± 69	117 ± 6/72 ± 5
preeclamptic placentas	33 ± 3[b]	33.4 ± 4.3[c]	1696 ± 651[d]	410 ± 67[e]	169 ± 4/107 ± 10[d]

[a] The data are the mean ± SD. [b] $P < 0.05$. [c] $P < 0.01$. [d] $P < 0.0001$. [e] $P < 0.001$ compared to the normal term placentas.

preeclamptic placentas (males, $n = 7$; females, $n = 4$) were obtained from the Department of Obstetrics and Gynecology, National Taiwan University Hospital. All human placental tissues were collected by Cesarean section. The use of human placentas for this study was approved by the local hospital Ethics Committee, and written consent was obtained from the patients before the collection of samples. Details of the maternal age, gestation week, new-born body weight, and systolic-diastolic pressure are listed in Table 1. Preeclampsia was defined as pregnancy-induced hypertension (blood pressure ≥140/90 mmHg) and proteinuria (≥300 mg/24 h) in women who were normotensive before 20 weeks of gestation and had no other underlying clinical problems, such as renal disease. The severity of preeclampsia was assessed by the frequency and intensity of abnormalities, including a blood pressure ≥160/110 mmHg, proteinuria ≥2.0 g/24 h or ≥ +2 dipstick, a platelet count less than 100,000/μL, and elevated alanine aminotransferase or aspartate aminotransferase levels.

Immunohistochemistry. Placental tissue sections were obtained by dissecting a segment (1.5 cm square, 0.5 cm thick) from the maternal part of the placenta and were fixed in 4% (w/v) paraformaldehyde/phosphate-buffered saline (PBS) at 4 °C. The sections were then deparaffinized in xylene and rehydrated in a series of graded alcohols. After quenching endogenous peroxidase activity with 1% H_2O_2 in PBS for 10 min at room temperature, the sections were rinsed three times with PBS and incubated with 5% nonfat milk in PBS for 30 min at room temperature to reduce nonspecific binding, then for 16 h at 4 °C with goat antihuman OCTN2 antibodies (1:200; Santa Cruz Biotechnology, CA, USA) or rabbit antihuman PPARα antibodies (1:100; Lifespan Biosciences, WA, USA) diluted in 5% nonfat milk in PBS. Specific immunostaining was revealed using the supersensitive link-label IHC detection system (Bio Genex, CA, USA). The section was incubated with biotinylated antigoat IgG or antirabbit IgG antibody for 1 h at room temperature in the buffer provided in the kit, washed, then incubated with streptavidin peroxidase for 1 h at room temperature, and followed by interaction with the DAB (3,3-diaminobenzidine) liquid substrate system (Sigma, MO, USA) for 1 min. All sections were counterstained with hematoxylin for 1 min and mounted with UltraKitt (J.T. Baker, Deventer, Netherlands). Negative controls were performed by replacing the primary antibodies with control IgG at the same concentration. The intensity of immunohistochemical (IHC) staining was scored as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (intense staining). For each specimen, an HSCORE value was derived using the formula: HSCORE $= P(i + 1)$, where i represents the intensity score and P the corresponding percentage of cells.[22] Five different areas were evaluated at 200× magnification by two independent observers blinded to the source of the samples.

Tissue Culture. Placental tissue sections were obtained by dissecting eight segments (1 cm square, 0.5 cm thick) from the villous part of the placenta. The villous tissues were then minced and cultured at 37 °C in F12K medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1% antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of amphotericin B), and 15% fetal bovine serum (Hyclone, Utah, USA) under normoxic conditions (95% atmospheric air and 5% CO_2) or hypoxic conditions (93% N_2 and 5% CO_2); hypoxia was induced in an anaerobic chamber (PROOX model 110, BioSpherix, NY, USA).

Cell Culture. The BeWo cell line (BCRC # 60073) was continuously cultured in 85% Ham's F12K medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1% antibiotics (100 U/mL of penicillin, 100 μg/mL of streptomycin, and 250 ng/mL of amphotericin B), and 15% fetal bovine serum (Hyclone, Utah, USA). The cells were maintained in 75 cm^2 or 25 cm^2 flasks at 37 °C under normoxic conditions (95% atmospheric air and 5% CO_2) or hypoxic conditions (94.5% N_2 and 5% CO_2); hypoxia was induced in an anaerobic chamber (PROOX model 110, BioSpherix, NY, USA). The oxygen concentration in the medium under normoxic and hypoxic conditions was 125.5 ± 10.8 and 33.2 ± 6.7 mmHg, respectively ($n = 5$). The culture medium was changed every 2 days and the culture split every 4 days. For subculturing, the cells were removed enzymatically (0.25% trypsin/EDTA (ethylenediaminetetraacetic acid)), split 1:4, and subcultured in a T75 flask. The expression of syncytin and OCTN2 was investigated after treatment with 100 μM forskolin (Sigma, MO, USA) for 72 h. To study the effect of hypoxia on syncytin and OCTN2 expression, the cells were incubated with or without 100 μM forskolin for 24 h under normoxic conditions, then with forskolin for 48 h under normoxic or hypoxic conditions. To investigate the role of PPARα in OCTN2 expression, the cells were incubated with 100 μM WY14643 (Sigma, MO, USA) for the last 0, 6, 12, or 24 h of the 48 h incubation under normoxic and hypoxic conditions.

Preparation of Crude Membrane, Lysate, and Nuclear Fractions. To prepare crude membranes, placental tissues were first homogenized at 4 °C in 0.01 M Tris (tris(hy-

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droxymethyl)aminomethane) buffer using a Waring blender (model MC1, Waring, CT, USA). Crude membranes were prepared from the placental tissue homogenate and BeWo cells according to a previously described method.[7] To prepare tissue lysates, placental tissues were ground in liquid nitrogen in a prechilled mortar and pestle and mixed with RIPA buffer (150 mM NaCl, 50 mM Tris, 1 mM EGTA (ethylene glycol tetraacetic acid), 1% NP-40) containing protease inhibitors (Roche Diagnostics, Germany). After centrifugation of the mixture at 20,800 g for 30 min at 4 °C, the supernatant was taken as the lysate. To extract nuclear proteins, BeWo cells freshly scraped from T75 flasks were washed with ice-cold PBS and incubated for 10 min on ice in lysis buffer (10 mM HEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid), pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT (dithiothreitol), and 0.2 mM PMSF (phenylmethanesulfonylfluoride)), and the lysate was centrifuged at 425 g for 5 min at 4 °C. The pellet was then incubated for 20 min at 4 °C in high-salt extraction buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, and 0.2 mM PMSF) and centrifuged at 20,817 g for 5 min at 4 °C, and the supernatant was taken as the nuclear extract. The protein content was determined using the DC protein assay (Bio-Rad, CA, USA) with bovine serum albumin as the standard.

Western Blot Analysis. An aliquot of crude membranes, lysate, or nuclear extract was diluted with 4X loading buffer (200 mM Tris-HCl, 1.43% 2-mercaptoethanol, 8% SDS (sodium dodecylsulfate), 0.4% bromophenol blue, 40% glycerol) to a protein concentration of 1−2 μg/μL and the proteins (10−30 μg/lane) separated by electrophoresis on a 8−10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences, Buckinghamshire, UK). Nonspecific binding was blocked by incubation for 1 h at room temperature with 5% skim milk in TNT buffer (10 mM Tris, 154 mM NaCl, 0.2% v/v Tween 20, pH 7.4) and the membrane incubated overnight at 4 °C with goat antibodies against human OCTN2, rabbit antibodies against human syncytin or human RXRα (all from Santa Cruz Biotechnology, CA, USA), or mouse antibodies against human HIF-1α (Novus Biologicals, CO, USA), human HIF-2α (Novus Biologicals, CO, USA), or human PPARα (Abcam, Cambridge, England) in TNT buffer containing 5% skim milk. Immunoblotting for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin was performed using mouse antibody against human GAPDH (Biodesign International, ME, USA) or human β-actin (Sigma-Aldrich, MO, USA), respectively, as loading controls. The membrane was then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated donkey antigoat IgG antibodies (Santa Cruz Biotechnology, CA, USA) for OCTN2, HRP-conjugated goat antirabbit IgG antibodies (Cedarlane, Ontario, Canada) for syncytin or RXRα, or HRP-conjugated goat antimouse IgG antibodies (Jackson ImmunoResearch Laboratories, PA, USA) for GAPDH, HIF-1α, HIF-2α, or PPARα, all diluted in TNT buffer. A bound antibody was detected using Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, MA, USA) and a Kodak X-OMAT 2000 developing machine.

Quantitative Polymerase Chain Reaction (qPCR). Total RNA was isolated from human placental specimens and BeWo cells (2×10^5 cells) by the acid phenol−guanidinium−chloroform method using a TRIzol reagent (Invitrogen, CA, USA). First-strand cDNA was synthesized from the total RNA using an oligo$(dT)_{12-18}$ primer and the ImProm-II reverse transcription system (Promega, CA, USA) according to the manufacturer's protocol. qPCR was used for the quantification of OCTN2 and PPARα mRNA levels. GAPDH was used as the internal control. The primer sequences for the PCR amplification of OCTN2, PPARα, and GAPDH were: OCTN2 primer set (forward, 5′-GTTCCAAGAAGCAGCAGTCC-3′; reverse, 5′-ATGCTGGGACTTCAACCATC-3′), PPARα primer set (forward, 5′-GCTTTGGCTTTACGGAATACCA-3′; reverse, 5′-TGAAAGCGTGTCCGTGATGA-3′), and GAPDH primer set (forward, 5′-GAAGGTGAAGGTCGGAGT-3′; reverse, 5′-GAAGATGGTGATGGGATTTC-3′). The cDNA (1 μL) was mixed with 7 μL of sterile deionized distilled water, 10 μL of brilliant SYBR green reagent (Stratagene, CA, USA), and OCTN2 forward/reverse primers (1 μL each), PPARα forward/reverse primers (1 μL each), or GAPDH forward/reverse primers (1 μL each). All steps were performed in an ice bath to retain the activity of the reagents and cDNAs. The program for PCR was denaturation (95 °C, 3 min) followed by 40 cycles of 95 °C for 1 min, 52 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 10 min. The process and signal collection were carried out on a qPCR system Mx3000 (Stratagene, CA, USA). The relative quantity of OCTN2 or PPARα mRNA normalized to GAPDH mRNA was analyzed using MxPro Software (Stratagene, CA, USA).

Cellular Uptake of Carnitine. To measure cellular uptake, BeWo cells (2.5×10^4 cells) were grown on collagen-coated 24 well Nunclon multidishes (1.9 cm^2 culture area, polystyrene, Nunc, Roskilde, Denmark) and were used for experiments 5 days after subculturing. To estimate the kinetic properties, the cells were incubated for 30 min with 14 nM ^{3}H-labeled carnitine (ARC, MO, USA), then washed with 0.5 mL of ice-cold ECF buffer (140 mM NaCl, 5.4 mM KCl, 1.2 mM $MgSO_4$, 1.4 mM $CaCl_2$, 10 mM HEPES, 10 mM D-glucose, pH 7.4), and solubilized in 0.5 mL of 1% Triton X-100, and the solution was transferred to a vial containing 5 mL of Ready Protein (Beckman, CA, USA). Radioactivity was measured by liquid scintillation counting using an external standard method for quench correction. The protein content of the cells was determined using the DC protein assay (Bio-Rad, CA, USA) with bovine serum albumin as the standard. Uptake data were expressed as the amount of carnitine taken up per milligram of protein. Each uptake study was performed in triplicate and the mean value used as the data from one experiment.

Statistical Analysis. Statistical differences were evaluated by the analysis of variance (ANOVA) or Student's *t* test, with a level of significance of 0.05. Pairwise comparisons

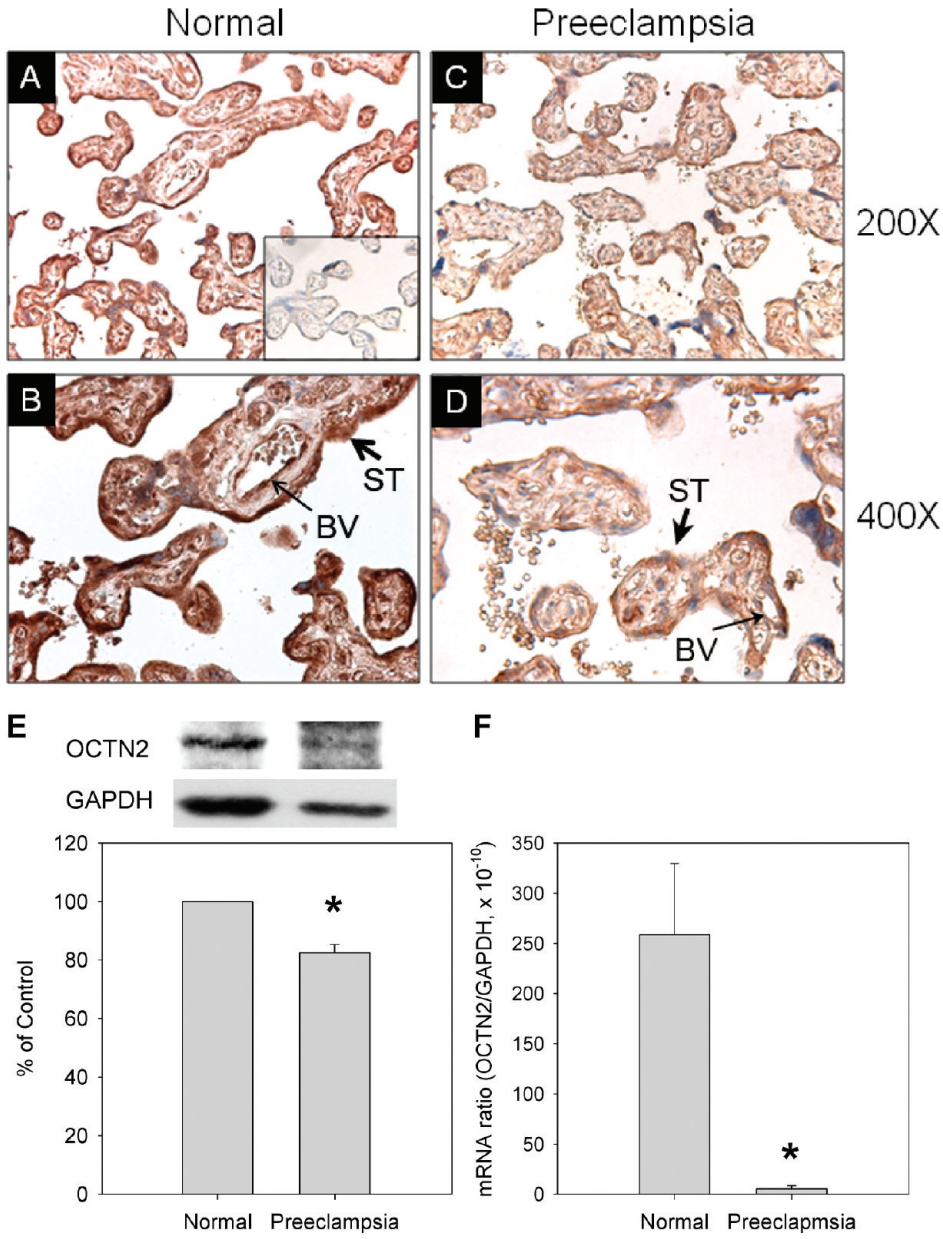

Figure 1. OCTN2 expression in human normal term placentas and preeclamptic placentas. (A−D) IHC analysis (n = 7 for normal placentas; n = 11 for preeclamptic placentas). Representative images are shown. The magnification is 200× in A and C and 400× in B and D. OCTN2 is expressed in syncytiotrophoblasts (ST, thick arrow) and blood vessels (BV, thin arrow). The inset at the lower right corner of panel A is the negative control. (E) Western blots (n = 3 for normal placentas; n = 3 for preeclamptic placentas). (F) qPCR (n = 4 for normal placentas; n = 7 for preeclamptic placentas). The asterisk indicates a significant difference ($p < 0.05$) compared to normal placentas.

between groups were made using Fisher's least significant difference test.

Results

OCTN2 and PPARα Expression in Human Placentas. Normal term placentas (n = 7) and preeclamptic placentas (n = 11) were immunostained for OCTN2 and PPARα and the intensity of staining scored as described in the Experimental Section. As shown in Figure 1A−D, immunohistochemical (IHC) analysis detected OCTN2 in syncytiotrophoblasts and blood vessels. OCTN2 levels were significantly ($p < 0.001$) lower in preeclamptic placentas than in normal placentas (HSCORE values of 294.5 ± 46.9 and 54.5 ± 16.4 in normal and preeclamptic placentas, respectively; mean ± SEM). The results of Western blots (Figure 1E) and qPCR (Figure 1F) corroborated the IHC findings. As shown in Figure 2A−D, IHC showed that PPARα was also detected in syncytiotrophoblasts and that its expression was signifi-

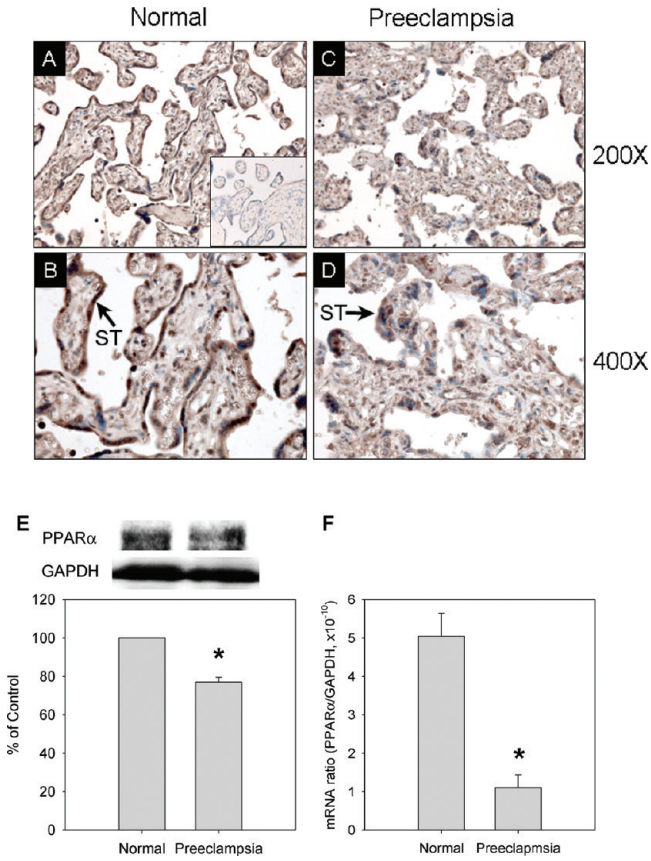

Figure 2. PPARα expression in human normal term placentas and preeclamptic placentas. (A−D) IHC analysis ($n = 7$ for normal placentas; $n = 11$ for preeclamptic placentas). Representative images are shown. The magnification is 200× in A and C and 400× in B and D. PPARα is expressed in the syncytiotrophoblast (ST, thick arrow). The inset at the lower right corner of panel A is the negative control. (E) Western blots ($n = 3$ for normal placentas; $n = 3$ for preeclamptic placentas). (F) qPCR ($n = 4$ for normal placentas; $n = 7$ for preeclamptic placentas). The asterisk indicates a significant difference ($p < 0.05$) compared to normal placentas.

cantly ($p < 0.001$) lower in preeclampsia (HSCORE values of 280.5 ± 38.4 and 24.0 ± 5.2 in normal and preeclamptic placentas, respectively; mean ± SEM), and this was corroborated by Western blots (Figure 2E) and qPCR (Figure 2F). We hypothesized that hypoxia might play a role in the placental expression of these proteins, so further studies were performed to investigate the effects of hypoxia on placental protein expression in cultured human placental explants and human choriocarcinoma BeWo cells as in vitro experimental models.

Expression of OCTN2, PPARα, and RXRα in Cultured Placental Explants under Hypoxic Conditions. Tissues from normal term placentas ($n = 3$) were used for explant cultures. As shown in Figure 3A, Western blots showed a significant decrease in OCTN2 levels in the membrane fraction of placental explants after 48 h of hypoxia. Levels of PPARα (Figure 3B) and its obligate heterodimeric partner, RXRα, in cell lysates (Figure 3C) were also decreased after 48 h of hypoxia. qPCR results showed that mRNA levels for OCTN2 (Figure 3D) or PPARα (Figure 3E) were consistent with the protein results.

Expression of Syncytin and OCTN2 in BeWo Cells in Response to Forskolin Treatment and Hypoxia. Given that syncytin levels are correlated with the progression of syncytialization, syncytin protein was measured in the membrane fraction of BeWo cells with or without treatment with 100 μM forskolin, a protein kinase A activator used to induce syncytialization, under normoxic or hypoxic conditions. Although syncytin levels increased with forskolin treatment, this increase was blocked under hypoxic conditions (Figure 4A), suggesting that induction of syncytialization was impaired by hypoxia. Figure 4B shows that, in accordance with previous findings,[7] OCTN2 protein expression in the membranes of BeWo cells was up-regulated by more than 2-fold by long-term forskolin treatment and this effect was reduced by about 35% by hypoxia, similar to the hypoxia-induced reduction seen in the absence of forskolin.

Expression of HIF1-α, HIF-2α, PPARα, and RXRα in BeWo Cells under Hypoxic Conditions. To examine the expression of hypoxia-inducible factors (HIFs), the key transcription factors regulated by hypoxia, levels of HIF-1α and HIF-2α protein in the nuclear fraction of BeWo cells was examined by Western blotting under hypoxic conditions. Levels of both HIF-1α (Figure 5A) and HIF-2α (Figure 5B) were significantly increased at 4 h of hypoxia, and this increase was maintained up to 12 h of hypoxia; then levels decreased to near control levels. Expression of PPARα (Figure 5C) and RXRα (Figure 5D) was also examined and was found to remain unchanged for 12 h and then showed a significant decrease after 24 h (PPARα) or 48 h (PPARα and RXRα) of hypoxia.

Effects of a PPARα Agonist on OCTN2 Expression in BeWo Cells with or without Hypoxia. Although PPARα has been demonstrated to regulate OCTN2 expression in rat liver cells,[18−20] whether it regulates human placental OCTN2 expression under normoxic or hypoxic conditions is not clear. To investigate the role of PPARα in OCTN2 expression in BeWo cells, cells were incubated for 48 h under normoxic or hypoxic conditions with or without addition of the PPARα agonist WY14643 (100 μM) for the last 4 h of treatment, and then OCTN2 mRNA and protein levels were examined. The qPCR results showed that OCTN2 mRNA levels were significantly decreased under hypoxic conditions and were increased by WY14643 treatment under both normoxic and hypoxic conditions (Figure 6A). Similar findings were observed for OCTN2 protein levels in a Western blot study of the effect of different times of exposure to WY14643. Under normoxic conditions, WY14643 increased OCTN2 expression by about 1.4-fold after 6 h of incubation, and levels remained high up to at least 24 h (Figure 6B); OCTN2 induction was also seen as early as 4 h of WY14643 incubation

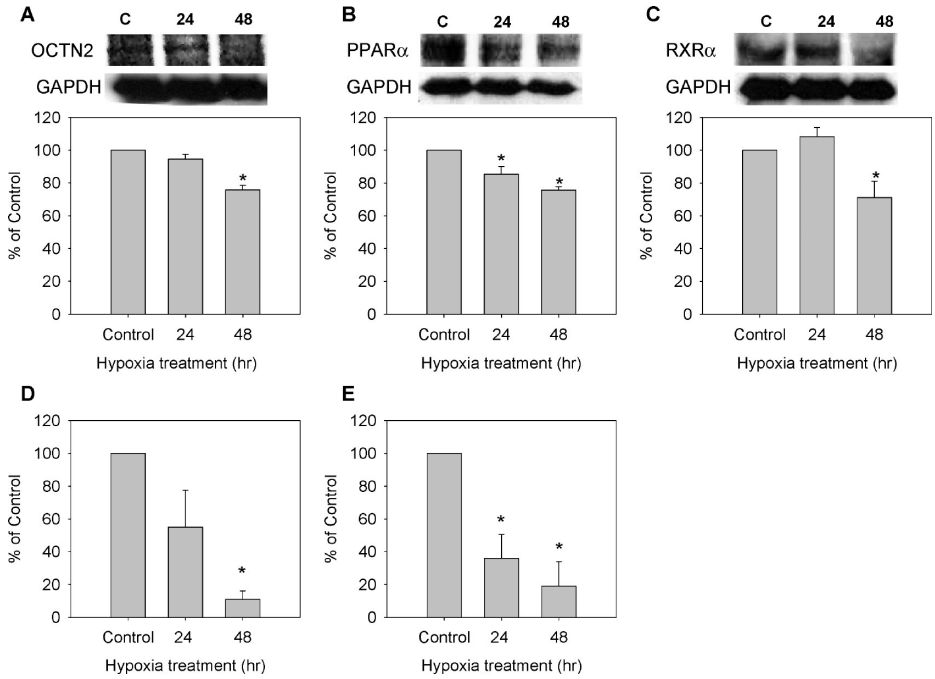

Figure 3. Protein and mRNA levels in placental explants subjected to normoxia or hypoxia. (A−C) Protein levels of (A) OCTN2 in the membrane fraction, (B) PPARα in the lysate, or (C) RXRα in the lysate of placental explants under normoxic or hypoxic conditions for 24 or 48 h. (D and E) Effects of hypoxia on (D) OCTN2 mRNA and (E) PPARα mRNA levels measured by quantitative PCR. The data are the mean ± SEM for three different preparations. The asterisk indicates a statistically significant difference ($p < 0.05$) compared to the untreated control.

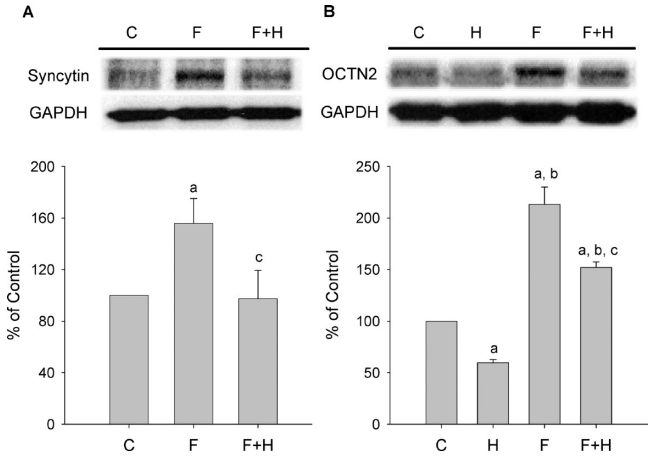

Figure 4. (A) Syncytin expression in the membrane fraction of control BeWo cells and BeWo cells treated with forskolin in normoxia or hypoxia (B) OCTN2 expression in the membrane fraction of BeWo cells under normoxic or hypoxic conditions with or without forskolin treatment. The cells were incubated in normoxic {"C"} for 72 h, in hypoxic ("H") medium for the last 48 h, or in normoxic medium containing 100 μM forskolin for 24 h, then in normoxic ("F") or hypoxic ("F+H") medium containing 100 μM forskolin for 48 h. The data are the mean ± SEM for three different preparations. A statistically significant difference ($p < 0.05$) is indicated compared to the untreated control ("a"), hypoxic control ("b"), or forskolin-treated normoxic control ("c").

(data not show; no shorter incubation was tested), indicating a rapid regulatory response of OCTN2 expression by PPARα. Although 48 h of hypoxia reduced OCTN2 expression, the addition of 100 μM WY14643 for the final 6 h incubation increased OCTN2 levels by about 2-fold. However, the induction effect of WY14643 decreased after 12 h or longer treatment (Figure 6C).

Carnitine Uptake by BeWo Cells. Carnitine uptake was measured to determine whether OCTN2 activity was altered in the presence of WY14643 or hypoxia. As shown in Figure 7, consistent with the protein level results, carnitine uptake was significantly decreased by 50% by 48 h of hypoxia. WY14643 treatment in the final 6 h of incubation resulted in a 20% increase in carnitine uptake under normoxic conditions and a 80% increase under hypoxic conditions.

Discussion

In this study, our aim was to identify the molecular mechanisms involved in the regulation of carnitine uptake in the preeclamptic placenta. We showed that OCTN2 and PPARα mRNA and protein levels were significantly reduced in syncytiotrophoblasts in the preeclamptic human placenta compared to the normal placenta. The results using placental explants and BeWo cells further demonstrated that hypoxia plays an important role in the regulation of placental OCTN2 and PPARα expression. Under hypoxic conditions, OCTN2 protein levels and carnitine uptake were decreased, as were levels of PPARα and its obligatory DNA-binding partner, RXRα.

OCTN2 is expressed in the syncytiotrophoblasts of the human placenta[17] and on the apical side of BeWo cells.[7] It was previously reported that hypoxia results in a reduction of carnitine uptake in BeWo cells, but the effects of hypoxia

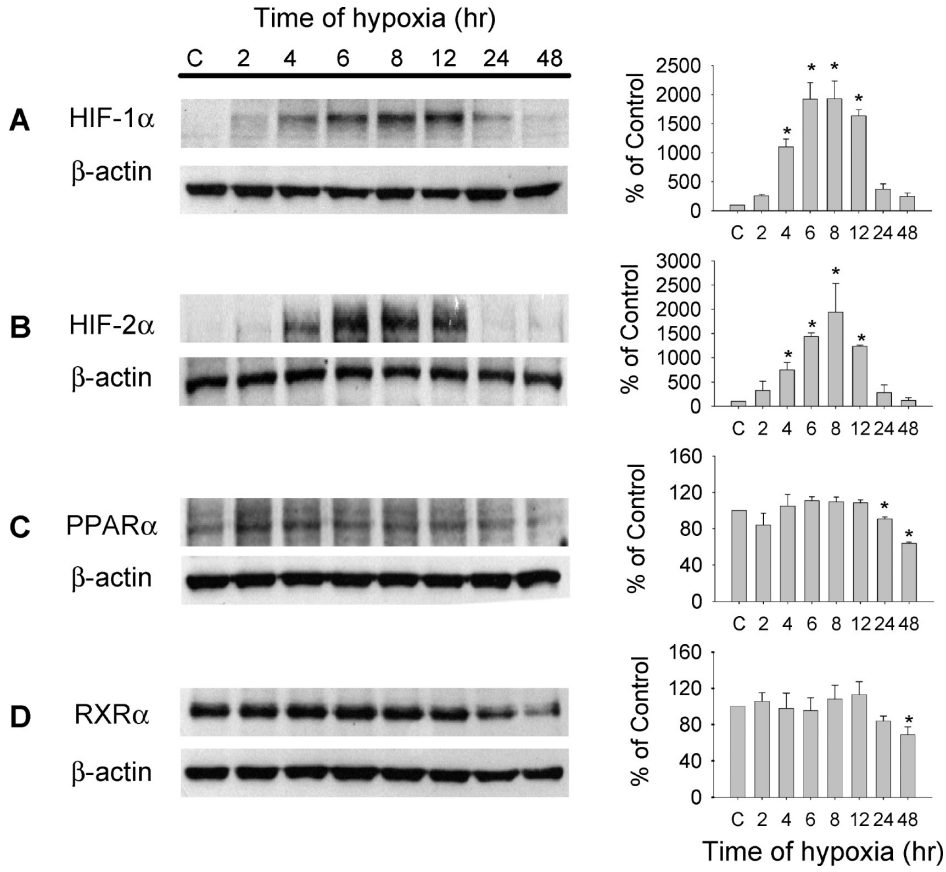

Figure 5. Expression of (A) HIF1α, (B) HIF2α, (C) PPARα, or (D) RXRα protein in the nuclear fraction of BeWo cells after different periods of hypoxia. The data are the mean ± SEM for three different preparations. The asterisk indicates a statistically significant difference ($p < 0.05$) compared to the control.

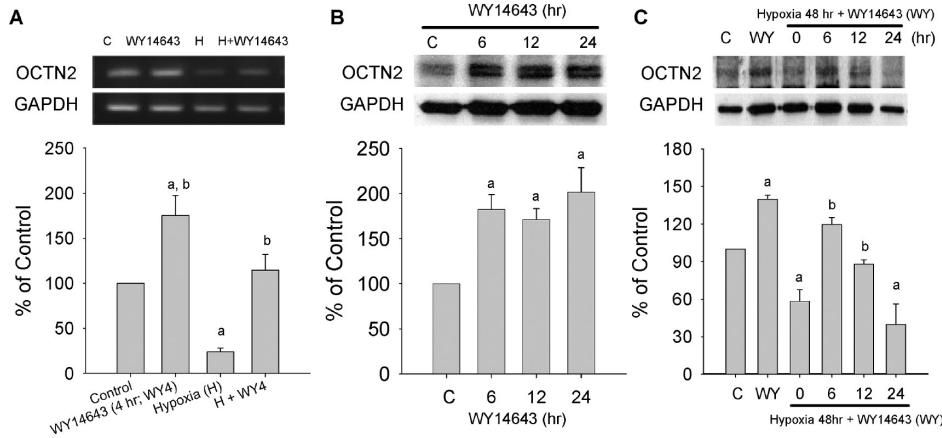

Figure 6. (A) qPCR results for OCTN2 mRNA levels in BeWo cells after 48 h of normoxic or hypoxic conditions with or without treatment with 100 μM WY14643 during the last 4 h. (B and C) Effect of WY14643 on OCTN2 protein expression in BeWo cells under normoxic or hypoxic conditions. The cells were incubated under normoxic (B) or hypoxic (C) conditions for 48 h with 100 μM WY14643 being added for the last 0, 6, 12, or 24 h. The data are the mean ± SEM of 3−5 different preparations. "a" and "b" indicate a statistically significant difference ($p < 0.05$) compared to the untreated normoxic control or untreated hypoxic control, respectively.

on OCTN2 protein and mRNA levels were inconsistent.[18] In the present study, we demonstrated that OCTN2 mRNA and protein levels were reduced in human placental explants and in BeWo cells under hypoxic conditions. This is the first report of a reduction in OCTN2 levels in the syncytiotrophoblasts of human preeclampsia placentas. The down-regulation of OCTN2 in preeclampsia may cause a decrease in placental carnitine active transfer from the mother to the fetus and result in higher maternal plasma carnitine levels, as reported in pregnant women with preeclampsia.[10] Given that carnitine is important to the fetus for carnitine reserves, metabolic functions, and tissue development,[14] the decrease

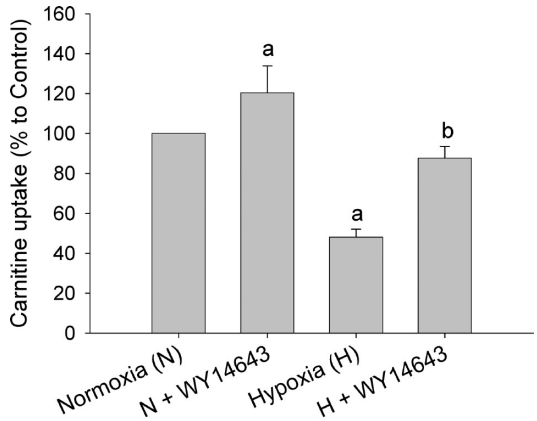

Figure 7. ^{3}H-carnitine uptake in BeWo cells after 48 h of normoxia or hypoxia with or without WY14643 treatment for the final 6 h. The data are the mean ± SEM for three experiments, each in triplicate. "a" and "b" indicate a statistically significant difference ($p < 0.05$) compared to the untreated normoxic control or untreated hypoxic control, respectively.

in placental carnitine transfer during preeclampsia may partly lead to neonatal morbidity or mortality.

In contrast to the repressive effects of hypoxia on OCTN2 expression, treatment with the PPARα agonist WY14643 increased OCTN2 expression after a short (6 h) incubation under both normoxic and hypoxic conditions. Likewise, forskolin, an adenylate cyclase activator, also increased OCTN2 expression under both conditions. Given that activation of the PKA pathway can increase PPARα activity,[23] the forskolin-induced up-regulation of OCTN2 is probably mediated by PPARα. Information on placental PPARα expression in preeclampsia is limited. Our present study showed reduced expression of PPARα protein in preeclamptic placentas. Although no placental abnormality is observed in PPARα null mice, PPARα deficiency results in an increased abortion rate.[24] In addition, PPARα can regulate the expression of many genes involved in cellular fatty acid transport and oxidation. We therefore propose that the reduction in PPARα levels in the placenta may cause a reduction in fatty acid transport and limit fatty acid supply to the fetus, which may then, at least in part, lead to higher fatty acid levels in preeclampsic women.[25,26] However, the effects of hypoxia on other transporters/enzymes that might be regulated by PPARα deserve further attention.

Until now, the signaling pathway involved in hypoxia-induced expression and activity of PPARα has been poorly characterized. It has been demonstrated that, in T84 intestinal epithelial cells, hypoxia down-regulates PPARα and induces HIF-1α expression and that antisense depletion of HIF-1α abolishes this down-regulation of PPARα, suggesting that PPARα is regulated through a HIF-1α-dependent mechanism.[27] However, another study in rat neonatal cardiac myocytes showed that hypoxia decreases the formation of the PPARα/RXR complex and its binding to target DNA by decreasing nuclear and cellular levels of RXRα without altering nuclear levels of PPARα.[28] Our present study demonstrated that protein levels of both PPARα and RXRα were decreased in human placental explants and BeWo cells cultured under hypoxic conditions. These results suggest the possibility that the hypoxia-induced regulation of PPARα may be tissue-specific. In our study, levels of both HIF-1α and HIF-2α were increased after 4 h of hypoxia, but decreased at 24 h. Since the interaction between HIF and its binding site in target genes is rapid, the half-life of association and dissociation being less than 1 min,[29] the delay between the increase in HIF levels and the decrease in PPARα levels suggests that intermediate regulators may control PPARα expression in BeWo cells.

In conclusion, the reduction in placental OCTN2 levels caused by hypoxia provides a possible explanation for the decrease in placental carnitine transfer seen in preeclampsia, leading to higher carnitine levels on the maternal side. The down-regulation of PPARα in the preeclamptic placenta is important not only because of its role in the hypoxia-mediated down-regulation of OCTN2 but also because of its diverse roles in gene regulation and fatty acid metabolism.

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