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Regulation of Protein Expression and Function of OCTN2 in Forskolin-Induced Syncytialization in BeWo Cells

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ABSTRACT

Placental OCTN2 is a high-affinity carnitine transporter that can interact with a number of therapeutic agents. The process of syncytialization is associated with the expression of a variety of genes. However, the association between syncytialization and OCTN2 expression is not yet clear. Given that forskolin induces BeWo cells to undergo biochemical and morphological differentiation, the purpose of the present study was to investigate whether the function and expression of OCTN2 are influenced by forskolin treatment during syncytialization.

The forskolin-induced differentiation of BeWo cells was validated by secretion of β-human chorionic gonadotropin (β-hCG) and syncytin expression. Cellular localization of OCTN2 was analyzed by confocal microscopy. Expression of OCTN2 and the modular proteins PDZK1, PDZK2, NHERF1 and NHERF2 was analyzed by Western blotting and carnitine uptake by BeWo cells was estimated and the kinetic properties of uptake measured. The results showed that forskolin treatment increased β-hCG secretion and syncytin expression, suggesting induction of syncytialization. Confocal images of BeWo cells showed the localization of OCTN2 in the brush-border membrane. OCTN2 protein expression was upregulated in isolated brush-border membranes by long-term forskolin treatment, but the V_m for carnitine uptake was unchanged, although the K_m increased. PDZK1, NHERF1 and NHERF2 protein expression in the brush-border membrane was downregulated by forskolin treatment, whereas PDZK2 levels remained unchanged.

In conclusion, protein expression and function of OCTN2 in BeWo cells can be regulated by forskolin treatment. While the presence of forskolin results in an increase in OCTN2 protein expression, the increase in uptake capacity may be compensated by the decreased expression of PDZK1, NHERF1 or NHERF2.

1. Introduction

The placenta is a unique and transient organ that is crucial for the growth and survival of the developing embryo and fetus. The syncytiotrophoblast of the human placenta is the epithelial layer which has differentiated from the underlying cytotrophoblasts and mediates the exchange of nutrients, therapeutic agents, toxins, and drugs of abuses between the maternal and fetal circulations. Although the mechanisms controlling syncytiotrophoblast formation are still far from clear, syncytin, a protein encoded by a human endogenous retrovirus-W envelope protein gene, is increased during syncytialization [1–3]. An increase in syncytin levels has been associated with glial cell missing a (GCMa) protein [4], the expression of which is mediated by PKA and CREB-binding protein [5,6]. Biochemical and morphological trophoblast differentiation can be stimulated by 8-Br-cAMP or forskolin, an adenylate cyclase activator [7–9].

The fetus and neonate cannot synthesize adequate amount of carnitine [10] and the active transfer of carnitine from the mother to the fetus is important to the fetus for the maturation of carnitine reserves, metabolic functions, and tissue development [11]. Symptoms of carnitine deficiency may include cardiomyopathy, cardiac arrest, Reye's syndrome, hypoglycemia, and sudden infant death [12,13]. The active placental carnitine transfer may lead to significant higher carnitine levels in cord blood than those in the maternal blood [14]. Under physiological concentrations (<20 μM), placental OCTN2 (SLC22A5), a high-affinity carnitine transporter, is the primary transporter that is responsible for the transfer of L-carnitine from the mother to the fetus. OCTN2 also interacts with a number of therapeutic agents, including cardiovascular drugs [15], β-lactam antibiotics [16], and anticonvulsants [17]. OCTN2 is

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located in the brush-border (i.e., apical) membrane of intestinal and renal proximal tubular epithelial cells of rodents [18,19] and syncytiotrophoblasts of human placenta [20]. While the sorting mechanism of OCTN2 is far from clear, OCTN2 targeting to the apical surface of the renal plasma membrane is suggested to be associated with its interaction with PDZK1, a PDZ domain-containing protein [21]. The same study also showed that the uptake activity of OCTN2 can be regulated by PDZK1. It was later demonstrated that coexpression of PDZK2 and OCTN2 increased the uptake of carnitine by OCTN2 with minimal effect on its substrate recognition specificity [22]. Other PDZ domain-containing proteins, NHERF1 and NHERF2, also interact with OCTN2 [23].

Given the expression of OCTN2 in placental syncytiotrophoblast, the formation of syncytiotrophoblast or syncytialization may have significant impact on the function and expression of OCTN2. It was reported that plasma carnitine concentrations in pregnant women with preeclampsia increased by about 50% compared with normotensive healthy pregnant women, suggesting a decrease in placental carnitine transfer [24]. Several studies have demonstrated the association between preeclampsia and impaired syncytialization [25–27]. Although the process of syncytialization involves the expression of a variety of genes [28–30], the association between syncytialization and OCTN2 expression is not yet clear. The purpose of this study was to investigate whether the function and/or expression of OCTN2 is influenced in forskolin-induced syncytialization. Human placental choriocarcinoma BeWo cells were used as a model to study the kinetic properties and protein levels of OCTN2 in the absence and presence of forskolin. The results showed that, as well as inducing syncytialization, forskolin-induced OCTN2 protein expression and changed its function.

2. Methods

2.1. BeWo cell culture

The BeWo cell line (BCRC # 60073) was purchased from the Bioresource Collection and Research Center, Hsinchu, Taiwan. The cells were 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 amphotericin B) and 15% fetal bovine serum. The cells were routinely maintained in 75-cm^2 Falcon flasks at 37 °C in a 5% CO_2 and 95% humidity atmosphere. The culture medium was changed every 2 days and the culture split every 4 days. For sub-culturing, the cells were removed enzymatically (0.25% trypsin/EDTA), split 1:3, and subcultured in a T75 flask.

2.2. Measurement of human chorionic gonadotropin

Human chorionic gonadotropin (β-hCG) was measured using an immunoassay kit with a β-hCG standard (MICRO-ELISA BETA hCG, Leinco Technologies, USA) according to the manufacturer's protocol.

2.3. Immunocytochemistry and confocal microscopy

BeWo cells (2.5×10^4 cells) were cultured on a chamber slide (Nalge Nunc International, USA), fixed for 10 min at 37 °C in 4% *p*-formaldehyde in phosphate-buffered saline (PBS), and permeabilized for 20 min on ice in 0.2% Triton X-100 in PBS. After PBS washes, the cells were blocked with 4% bovine serum albumin in PBS for 1 h at room temperature. The cells were then incubated overnight at 4 °C with a 1:50 dilution of goat anti-human OCTN2 antibody (Santa Cruz Biotechnology, USA) in 1% bovine serum albumin, followed by incubation for 60 min at room temperature with a 1:250 dilution of rhodamine-conjugated rabbit anti-goat IgG antibody (Rockland, USA). After PBS washes, the cells were also stained with DAPI (Sigma–Aldrich, USA) for 3 min, then observed using a Zeiss confocal microscope and analyzed using Zeiss LSM Image Browser software.

2.4. Preparation of crude membranes and brush-border membranes

All steps were at 4 °C. The cells ($1–2 \times 10^7$ confluent cells) were trypsinized and the cell suspension centrifuged at 1000*g* for 10 min. To prepare crude membranes, the cell pellet was homogenized in 0.01 M Tris buffer containing protease inhibitors (Roche Diagnostics, Germany), the homogenate centrifuged at 10,000*g* for 15 min, and the resulting supernatant centrifuged at 100,000*g* for 60 min. The pellet was resuspended in Tris buffer and was used for Western blot analysis. Brush-border membranes (BBMs) were prepared as described by Lin et al. [31] with modifications. In brief, the cell pellet was homogenized in 15 ml of buffer containing 10 mM mannitol, 2 mM HEPES/Tris (pH 7.0), and protease inhibitors, then 150 μl of 1 M calcium chloride was added and the mixture stirred slowly for 15 min, then centrifuged at 756*g* for 10 min. The supernatant was centrifuged at 31,000*g* for 15 min, the pellet resuspended in 5 ml of buffer containing 100 mM mannitol and 20 mM HEPES/Tris (pH 7.4), and the suspension centrifuged at 48,400*g* for 20 min and the final pellet resuspended and used for Western blot analysis. The protein content was determined using the DC protein assay (Bio-Rad) with bovine serum albumin as the standard. The purity of the isolated BBMs was estimated by measuring alkaline phosphatase (ALP) activity using an ALP liquicolor assay kit (Human, Germany).

2.5. Western blot analysis

An aliquot of crude membrane or BBM protein was diluted with 4× loading buffer (200 mM Tris–HCL, 1.43% (v/v) 2-mercaptoethanol, 8% (w/v) SDS, 0.4% (w/v) bromophenol blue, 40% glycerol (v/v)) to a protein concentration of 1–2 μg/μl and the proteins (15–25 μg/lane) separated by electrophoresis on a 10–12% sodium dodecylsulfate (SDS)–polyacrylamide gel and transferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences, 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 or rabbit antibodies against human syncytin (both from Santa Cruz Biotechnology, USA), mouse antibody against human PDZK1 (Abcam, England), rabbit antibodies against human PDZK2 (Abcam, England), rabbit antibodies against human NHERF1 (Alpha Diagnostic, USA) or goat antibodies against NHERF2 (Abcam, England) in TNT buffer containing 5% skim milk. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin was blotted by mouse antibody against human GAPDH (Biodesign International, USA) or human β-actin (Sigma–Aldrich, USA), respectively, to indicate the consistency of sample loading. The membrane was then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated donkey anti-goat IgG antibodies (Santa Cruz Biotechnology, USA) for OCTN2 and NHERF2, HRP-conjugated goat anti-rabbit IgG antibodies (Cedarlane, Canada) for syncytin, PDZK2 and NHERF1, or HRP-conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch Laboratories, USA) for GAPDH and PDZK1, all diluted in TNT buffer. Bound antibody was detected using Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, USA) and a Kodak X-OMAT 2000 developing machine.

2.6. Cellular uptake and transcellular flux

Forskolin was added to the culture medium at a final concentration of 10 or 100 μM in a final concentration of 0.1% DMSO; control cultures received 0.1% DMSO. 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, Denmark) and were used for experiments 5 days after sub-culturing.

To measure the time-dependency of cellular uptake, cells were incubated with 50 nM ^{3}H-carnitine (American Radiolabeled Chemicals, USA) in the presence of 140 mM sodium or 140 mM *N*-methyl-D-glucamine for 5–120 min. To estimate the kinetic properties, cells were incubated for 30 min with 1–100 μM unlabeled carnitine and 50 nM ^{3}H-labeled carnitine, then were 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), solubilized in 0.5 ml of 1% Triton X-100, and the solution transferred to a vial containing 5 ml of Ready Protein (Beckman Coulter, USA).

To measure transcellular flux, BeWo cells (5×10^4 cells) were seeded on a polycarbonate insert (10 mm diameter, 0.4 μm, Nunc, Denmark) coated with type I rat tail collagen. The culture medium was changed every day and the cells used for experiments 7–9 days after sub-culturing, at which point the transepithelial electrical resistance was about 70–90 ohms cm^2. Transcellular flux was measured by adding 0.5 ml of ECF buffer containing 50 nM ^{3}H-carnitine and 800 nM ^{14}C-mannitol (PerkinElmer, USA) to the upper (apical) or lower (basolateral) chamber, depending upon the direction of transport studied. Samples (50 μl) from the basolateral side (for apical-to-basolateral transport) or the apical side (for basolateral-to-apical transport) were taken after 5–120 min and mixed with 5 ml of Ready Protein. Transcellular uptake of ^{3}H-carnitine was estimated by subtracting the ^{14}C-mannitol uptake.

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) with bovine serum albumin as the standard.

2.7. Data analysis

The rate of substrate uptake was analyzed using a nonlinear regression model, $V = ((V_m C)/(K_m + C))$ with a nonsaturable component, *KC*, where *V* represents the uptake rate; V_m, the maximum uptake rate; *C*, the carnitine concentration; K_m, the Michaelis constant; and *K*, the first order constant. The selection of the model for data interpretation was on the basis of the goodness of fit evaluated by Model Selection Criteria (MSC), the coefficient of determination, and the standard