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Effects of low oxygen levels on the expression and function of transporter OCTN2 in BeWo cells

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Abstract

Although hypoxia is normal in early pregnancy, low placental oxygen concentrations later in pregnancy are often linked to complications such as pre-eclampsia and intrauterine growth restriction. The effects of low oxygen levels on drug and nutrient uptake via the organic cation transporter OCTN2 has been studied in BeWo cells, an in-vitro model of human trophoblast. BeWo cells were cultured under 20% (control) or 2% O_2 (hypoxia) for 48 h before each experiment. In-vitro hypoxia was also simulated by the addition of $CoCl_2$ to the cell culture medium. RT-PCR indicated increased transcription of OCTN2 in BeWo cells cultured under hypoxia, but Western blots did not show a corresponding increase in the amount of OCTN2 protein in the hypoxic cells compared with control. Hypoxia resulted in significant reductions in OCTN2-mediated carnitine uptake. Decreased placental transport of carnitine may lead to symptoms of carnitine deficiency in infants from hypoxic pregnancies, whether caused by high altitude, pre-eclampsia or other factors. The OCTN1 substrate ergothioneine reversed the effects of hypoxia on carnitine transport, but identical concentrations of *N*-acetylcysteine, another water-soluble intracellular antioxidant, did not have the same effect.

Introduction

In early pregnancy, low oxygen tension is essential for normal embryonic and placental development (Caniggia et al 2000). In the first 10 weeks of gestation, maternal blood flow is limited and the placental villous tree develops in a hypoxic environment (Roh et al 2005). Between 10 and 12 weeks, the intervillous space opens and maternal blood flow increases, which leads to higher concentrations of oxygen and promotes trophoblast differentiation (Caniggia et al 2000). The oxygen pressure during the first 10 weeks is usually less than 20 mmHg (about 1% O_2) but increases to about 60 mmHg (corresponding to about 8% O_2) by 14–16 weeks. It can be as high as 80 mmHg (11% O_2) during the second trimester. Placental oxygen levels usually decrease slightly in the third trimester (Zamudio 2003).

Although hypoxia is normal in early pregnancy, low placental oxygen concentrations later in pregnancy are related to increased risks of complications (Zamudio 2003). Certain pathological conditions linked to placental hypoxia include pre-eclampsia and intrauterine growth restriction (IUGR) (Esterman et al 1997; Zamudio 2003; Roh et al 2005). Pre-eclampsia affects 2–8% of pregnancies and is associated with increased risks of maternal morbidity, perinatal death and premature delivery (Duley 2003). Suppressed syncytiotrophoblast formation has been observed in pre-eclamptic pregnancies, and in-vitro experiments involving forskolin-induced syncytialization of BeWo cells demonstrated reversibility of this suppression when oxygen levels increased from 2% to 20% (Kudo et al 2003). Smoking and cocaine abuse also appear to increase the incidence of hypoxia during pregnancy (Esterman et al 1997; Roh et al 2005).

Because of atmospheric differences in oxygen concentration, pregnancy at high altitudes may affect placental oxygenation and can exhibit placental modifications similar to those seen in pre-eclampsia and IUGR (Zamudio 2003). Placentas from high-altitude pregnancies have increased villous vascularization and thinner villous membranes than low-altitude controls. While these two adaptations can increase oxygen diffusion, nutrient transport is often reduced and down-regulation of fetal growth has been observed (Zamudio 2003). In fact, the number of babies born weighing less than 2.5 kg at full term is five times higher at high altitude than at low altitude (Zamudio 2003).

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Hypoxia during the second and third trimesters may not only result in reduced nutrient transfer, but can also affect how drugs enter the fetal circulation and how they are metabolized. During fetal hypoxia, when umbilical blood flow is reduced, a lower percentage of blood flows through the fetal liver where there is a chance for first-pass metabolism of potentially harmful substances (Rudolph 1995).

Placental glucose transport is up-regulated under hypoxic conditions. Low oxygen concentrations in the placenta result in increases in both the expression of glucose transporters (GLUTs) and glucose metabolism (Esterman et al 1997; Hayashi et al 2004). Investigations by Northern blot indicated a 3.4-fold increase in GLUT1 expression and a 10-fold increase in GLUT3 mRNA in hypoxic trophoblast compared with normoxic trophoblast (Esterman et al 1997). Hayashi et al (2004) showed that increases in GLUT1 expression are associated with the interaction of hypoxia-inducible factor-1α with a hypoxia-responsive element within the GLUT1 promoter. Despite these observed increases in GLUT1 expression in-vivo, Zamudio et al (2006) recently observed a decrease in GLUT1 density in placentas from high-altitude pregnancies.

The organic cation transporter OCTN2 is a sodium-dependent carnitine transporter present in trophoblast cells and other tissues (including brain and kidney). Carnitine is an essential nutrient involved in the oxidation of fatty acids in the mitochondria to produce energy. The transplacental transport of carnitine is significant because the fetus cannot adequately supply itself with this nutrient (Wu et al 1999), but must receive it from the maternal circulation. Low levels of carnitine may result in carnitine deficiency, a condition that primarily affects infants (Lahjouji et al 2001). Symptoms of carnitine deficiency include muscle weakness, cardiomyopathy, Reye's syndrome, hypoketotic hypoglycaemia and sudden infant death (Lahjouji et al 2001; Wang et al 2000). OCTN2 also recognizes both cationic and anionic drug molecules and can provide the means for transplacental transfer of drugs at the expense of nutrient transport (Rytting & Audus 2005).

A recent study in Korea provides evidence for the strong fetal demand for carnitine during pregnancy. This study of 50 pregnant women and 30 non-pregnant women showed that although the dietary intake of the pregnant women increased compared with non-pregnant women, plasma carnitine levels were significantly lower in pregnant women (Cho & Cha 2005). These data, together with the low urinary excretion of carnitine observed in late pregnancy, led these authors to suggest that substantial amounts of carnitine go to the fetus.

The objective of this study was to investigate the effects of low oxygen levels on OCTN2-mediated carnitine uptake into BeWo cells, an in-vitro model of human trophoblast cells (Liu et al 1997). The up-regulation of GLUT expression under hypoxic conditions has already been mentioned, and Bottalico et al (2004) observed reductions in organic cation transporter 3 (OCT3) and norepinephrine transporter mRNA expression in pre-eclamptic placentas compared with normal placentas. In this work, the expression and function of OCTN2 in BeWo cells cultured in control (20% O_2) and low-oxygen (2% O_2) conditions were investigated by RT-PCR, Western blot and carnitine uptake experiments. Carnitine is a high-affinity substrate for OCTN2, having a Km near 10 μM in BeWo cells (Rytting & Audus 2005). It should be noted that the 'control' in-vitro oxygen concentration used in this study (20% O_2) does not exactly match in-vivo placental oxygen levels, but it is consistent with in-vitro trophoblast experiments appearing in the literature (Trollmann et al 2002; Kudo et al 2003; Hayashi et al 2004, 2005).

Materials and Methods

Cell culture

Procedures for the growth and maintenance of BeWo cells in culture have been described in detail by Bode et al (2006). One significant difference in this work, however, is the culture of BeWo cells in both control and low-oxygen conditions in-vitro. For uptake studies BeWo cells were seeded in 12- or 24-well tissue culture plates, at a density of 12 500 cells per cm^2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% penicillin–streptomycin, 1% glutamine and 1% non-essential amino acids. The cells were incubated at 37°C under 5% CO_2, 95% air and saturated relative humidity. Uptake experiments took place 4 days after seeding. For the first 2 days, all cells were incubated as described above. For the final 48 h before the uptake experiment the DMEM was replaced with a modified DMEM containing 25 mM HEPES (HEPES-DMEM, Sigma-Aldrich, St Louis, MO, USA). HEPES-DMEM was used to minimize the changes in medium pH under low-oxygen conditions. Cells grown under control conditions were incubated as above, but in HEPES-DMEM. The cells grown under low-oxygen conditions for the final 48 h were incubated at 37°C, 5% CO_2, 93% N_2 and 2% O_2. The oxygen concentration in this incubator was controlled with a PRO:OX Model 110 compact oxygen controller (BioSpherix Ltd, Redfield, NY, USA). Experimental hypoxic conditions were also simulated by adding 100 or 250 μM $CoCl_2$ to the DMEM. Cobalt can interfere with the binding of oxygen to haem proteins, thus mimicking hypoxia (Taylor et al 1998). In this case, HEPES was not added to the medium. DMEM containing $CoCl_2$ was added to the cells 48 h before the uptake experiment and cells were cultured under the standard conditions (5% CO_2, 95% air) and compared with cells kept in the standard DMEM (not containing HEPES) in normal conditions. Cell viability following exposure to low oxygen concentrations or $CoCl_2$ was verified by microscopy and by trypan blue exclusion before uptake experiments, although the confluence of the cells was decreased slightly with increasing $CoCl_2$ concentrations.

RT-PCR

Total RNA was obtained from BeWo cells grown under control and low-oxygen conditions, both having HEPES-DMEM for the last 48 h. Purification of mRNA from total RNA was performed using an Oligotex mRNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RT-PCR reactions were carried out using the Access RT-PCR System from Promega (Madison, WI, USA), following the instructions of Technical Bulletin 220. The forward and reverse primers for OCTN2 were synthesized by the Biotechnology Support Facility

at the University of Kansas Medical Center. The primer sequences (5′–3′) were: OCTN2-forward primer, CTTTGCTGTTTGTCCTCTTGACCT; OCTN2-reverse primer, GCTCATTCTGCTCCATCTTCATTA (Alcorn et al 2002). Primers for β-actin were obtained from Clontech (Mountain View, CA, USA). The 100 bp DNA ladder was from Promega.

Electrophoresis of PCR products and negative controls (not shown) was carried out in 2% agarose gel, at 90 V for 1.5 h. The gel was stained in a solution containing 0.5 pg mL^{-1} ethidium bromide on a rotating platform for 45 min, followed by four 10-min washes in distilled water before viewing with a UV transilluminator.

Western blotting

Cellular lysate was obtained from BeWo cells grown under normal and low-oxygen conditions, both having HEPES-DMEM for the last 48 h, as described previously (Rytting & Audus 2005). Electrophoresis in 12% Tris-glycine gels was performed at 110 V for 2 h, followed by a 2-h 25 V transfer on ice. After overnight blocking, the membranes were incubated for 1 h with the primary antibody, OCTN2 (H-13) goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), at a 1:200 dilution. The secondary antibody was donkey anti-goat IgG–HRP (Santa Cruz Biotechnology), at a 1:10 000 dilution. The actin (C-11) HRP-conjugated goat polyclonal antibody was obtained from Santa Cruz Biotechnology. The ECL Western blotting detection reagents were obtained from Amersham Biosciences (Buckinghamshire, England).

Uptake studies

Uptake studies were carried out as described previously (Rytting & Audus 2005). Briefly, after the 48-h incubation under control or low-oxygen conditions, cells were washed and equilibrated in Hanks' balanced salt solution (HBSS) for 45 min before starting the uptake experiment by administration of radiolabelled carnitine. Uptake experiments were carried out on the laboratory bench without controlling for oxygen levels. Following each 15-, 20- or 30-min uptake study, carnitine solutions were removed by aspiration and cells were washed three times with ice-cold HBSS, followed by cell lysis and quantitation by scintillation counting. Carnitine uptake in BeWo cells was previously shown to be linear up to 30 min (Rytting & Audus 2005). Uptake data were corrected for protein content, determined using a kit from Pierce (Rockford, IL, USA) with bovine serum albumin as the standard. [^{3}H]-L-carnitine was obtained from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA), L-(+)-ergothioneine from Bachem (King of Prussia, PA, USA) and *N*-acetyl-L-cysteine from Sigma. In experiments involving ergothioneine and *N*-acetylcysteine, these substances were premixed with the radiolabelled carnitine for simultaneous addition to the cells; 500 μL of the specified concentrations in HBSS were added to the cells in 24-well tissue culture plates.

Data analysis

Data from uptake experiments were compared by single-factor one-way analysis of variance; 4–8 replicates were analysed in each case and differences were deemed statistically significant if P was less than 0.05. This method was employed in the comparison of uptake in cells cultured under low oxygen compared with control, in cells treated with $CoCl_2$ compared with control, and in cells treated with increasing concentrations of ergothioneine or *N*-acetylcysteine compared with controls.

Kinetic data were calculated using SigmaPlot software (SPSS Inc., Chicago, IL, USA). Relative intensities of Western blot bands were compared by densitometry, with subtraction of background intensity; however, statistical evaluation of the band intensities was not possible because of lack of replicates (n = 1).

Results and Discussion

In this work, low oxygen levels have been shown to reduce OCTN2-mediated carnitine uptake in the BeWo cell line, a model of the rate-limiting barrier for the maternal–fetal exchange of drugs and nutrients.

RT-PCR detection of OCTN2 in BeWo cells cultured under control and low-oxygen conditions showed a dramatic difference in the amounts of mRNA. Figure 1 shows that the band from the BeWo cells exposed to low oxygen levels (lane 2) is 8 times stronger than the band for the control BeWo cells (lane 1), even though the bands for β-actin are almost equal in intensity. While this is consistent with observations of increased GLUT1 expression under hypoxic conditions (Esterman et al 1997), Figure 2 does not show such an extreme difference between the amounts of OCTN2 protein (MW = 63 kDa) present in BeWo cell lysates from control and low-oxygen conditions. From densitometry measurements of the bands in Figure 2, the ratio of the intensity of OCTN2 to β-actin is 0.88 for lane 1 (20% O_2) and 0.37 for lane 2 (2% O_2). Taken together with the RT-PCR data, this suggests that

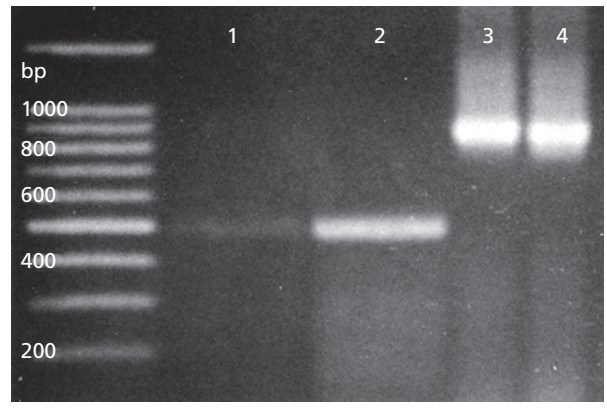

Figure 1 RT-PCR analysis of internal fragments of OCTN2 cDNAs in BeWo cells cultured under low (2%) or control (20%) oxygen levels. 150 ng mRNA was used as a template to synthesize first-strand fragments of OCTN2 cDNAs by a two-step RT-PCR process. Lanes 1 and 2 are OCTN2 band in control and low-oxygen BeWo cells, respectively; lanes 3 and 4 are β-actin band in control and low-oxygen BeWo cells, respectively. Expected product sizes: OCTN2, 474 bp; β-actin, 838 bp.

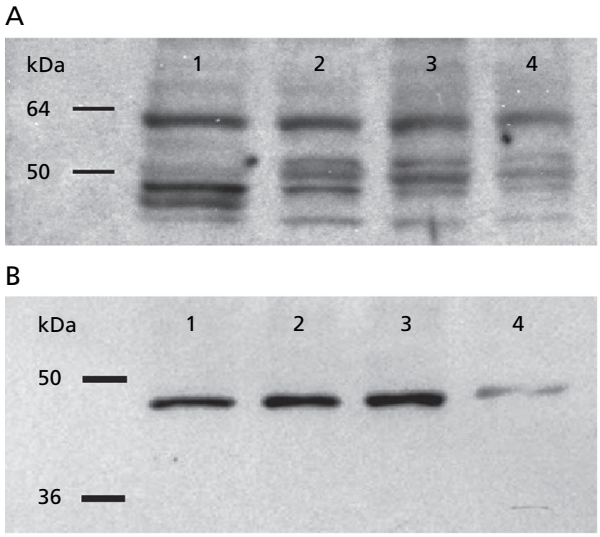

Figure 2 Western blots of OCTN2 (A) and β-actin (B) in BeWo cell lysate cultured under control (20% O_2, lane 1) or low oxygen levels (2% O_2, lane 2) for 48 h. Results are also shown for BeWo cells exposed to 100 μM $CoCl_2$ for 48 h (lane 4) compared with control (lane 3). Each lane contains 20 μg protein from each cell lysate sample. Approximate molecular weights: OCTN2, 63 kDa; β-actin, 43 kDa.

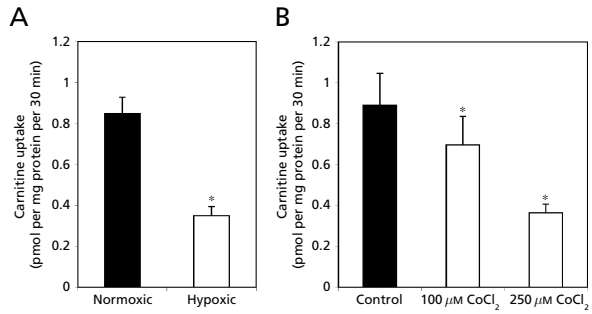

Figure 3 Uptake of 10 nM [^{3}H]-L-carnitine in BeWo cells in hypoxic or control (normoxic) conditions. BeWo cells were incubated under 20% O_2 (normoxic) or 2% O_2 (hypoxic) conditions for 48 h before the uptake study (A); or hypoxia was simulated by adding 100 or 250 μM $CoCl_2$ to the cell culture medium of BeWo cells incubated under control conditions for 48 h before the uptake study (B). Uptake was measured at 37°C for 30 min. Each point represents the mean ± s.d. for 6–8 determinations. *$P < 0.05$.

the translation of OCTN2 protein is less efficient under hypoxic conditions. This is in agreement with observations that mRNA translation is inhibited during hypoxia, probably as a response to conserve cellular energy (Koritzinsky et al 2006).

It is interesting to note some difference in the pattern of other protein bands around 50 kDa in Figure 2. One might expect that low oxygen concentrations or other differences in cell culture conditions would lead to changes in several proteins that are not the focus of this study, including some proteins that have a similarity to the sequence of the OCT antibody used in this work. It is not clear why the β-actin band from the cells treated with $CoCl_2$ shows a decreased intensity. It is not likely that $CoCl_2$ would down-regulate β-actin, as this would not be in agreement with a previous report of maintained β-actin expression on $CoCl_2$ treatment (Cho et al 2005). While $CoCl_2$ does produce some similar effects, it probably does not fully mimic hypoxia.

In addition to changes in the expression of OCTN2 under hypoxic conditions in-vitro, the function of this protein is also affected. The uptake of carnitine into BeWo cells grown under low oxygen levels is significantly lower than in control BeWo cells. Figure 3A shows that the uptake of 10 nM [^{3}H]-L-carnitine by BeWo cells cultured under 2% O_2 for 48 h is reduced to 41.1% of control (0.349 ± 0.045 vs 0.849 ± 0.079 pmol per mg protein per 30 min). This reduction is similar to the observed reduction in the ratio of OCTN2 to β-actin intensity in lane 2 of Figure 2 (to 42% of control).

When hypoxia was simulated by adding $CoCl_2$ to the medium for the last 48 h of incubation, uptake of carnitine was also significantly reduced, to 78.2 ± 15.6% and 40.9 ± 4.8% of control for 100 μM and 250 μM $CoCl_2$, respectively (Figure 3B). The decrease in carnitine uptake following exposure of the cells to 250 μM $CoCl_2$ was almost identical to the reduction observed for cells cultured under 2% O_2 (both to 41% of respective controls); the uptake values at these two conditions were not significantly different, which is also true of the two control uptake values for Figures 3A and 3B.

This decrease in OCTN2 function in hypoxic trophoblast cells is in agreement with the report by Cam et al (2005), who studied carnitine levels in 20 normal full-term neonates and 20 full-term neonates diagnosed with perinatal hypoxia–ischaemia and found that total carnitine levels were significantly lower in the hypoxic–ischaemic group than in the control group (27.8 ± 11.8 vs 43.7 ± 10.0 μg dL^{-1}).

The reduction in plasma carnitine concentrations in pregnant Korean women (attributed to the sizeable transfer of carnitine from the maternal circulation to the fetus) was described above (Cho & Cha 2005). Thiele et al (2004) compared plasma carnitine levels in 28 healthy, normotensive pregnant women and 33 women with pre-eclampsia and found that carnitine concentrations were 50% higher in the latter. This suggests that in pre-eclampsia, a condition in which placental hypoxia is assumed to exist (Zamudio 2003), the mother's carnitine concentrations are higher than normal because there is insufficient transplacental transport of carnitine to the fetus and the carnitine remains in the maternal circulation. These findings, together with the results in this work that demonstrate a reduction of OCTN2-mediated carnitine transport activity in hypoxic trophoblast, imply that newborns from hypoxic pregnancies are at a greater risk for carnitine deficiency.

Figure 4 shows carnitine uptake in control and low-oxygen BeWo cells at varying concentrations of carnitine. Uptake in hypoxic BeWo cells is 41.1%, 43.5%, 55% and 77.9% of control at 10 nM and 2, 5 and 10 μM carnitine, respectively.

We have previously shown that carnitine uptake in BeWo cells has both a saturable and non-saturable component

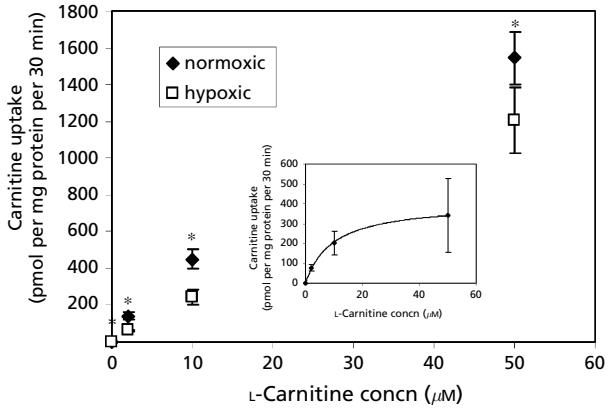

Figure 4 Uptake of carnitine at various concentrations in BeWo cells incubated under control (normoxic; 20% O_2) and hypoxic (2% O_2) oxygen concentrations for 48 h before the uptake experiment. The inset shows the difference between normoxic and hypoxic uptake values, fitted to the Michaelis–Menten equation. Uptake was measured at 37°C for 30 min. Data are mean ± s.d for 5 or 6 determinations. *$P < 0.05$.

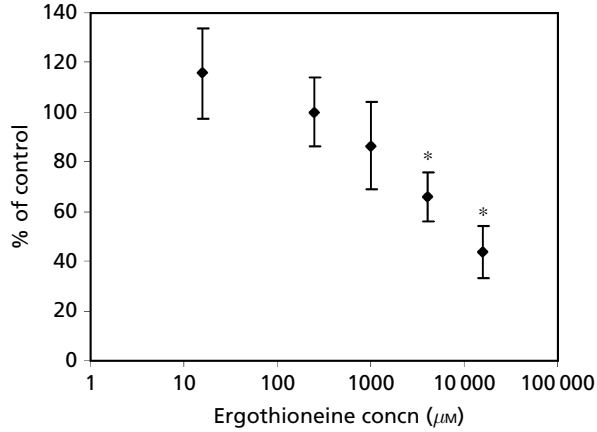

Figure 5 Carnitine uptake inhibited by various concentrations of L-ergothioneine in BeWo cells under standard conditions (20% O_2). Results of the 15-minute experiments at 37°C are presented as percent of the control situation (10 nM [^{3}H]-L-carnitine uptake in the absence of inhibitor). Data are mean ± s.d for 4 determinations. *$P < 0.05$.

(Rytting & Audus 2005). However, the plot for the low-oxygen cells in Figure 4 fits only a straight line. The saturable component – attributed to OCTN2-mediated carnitine uptake – was characterized by a Km value of 9.8 ± 2.4 μM. A non-saturable constant of 2.8 ± 0.3 μL per mg per 30 min was also determined. If the hypoxic carnitine uptake curve in Figure 4 is subtracted from the normoxic uptake curve (see Figure 4 inset), the resulting plot fits the traditional Michaelis–Menten curve, with a Km value of 9.6 ± 5.9 μM. This is in good agreement with the previously determined Km and suggests that the pathway represented by non-saturable carnitine uptake is the predominant transport mechanism under hypoxic conditions.

We have previously suggested that the observed non-saturable component of carnitine uptake might be via other transporters with a lower affinity for carnitine than OCTN2 (Rytting & Audus 2005). OCTN1 is present in placenta (and in the BeWo cell line modelling placental trophoblast) and this transporter has been shown to transport carnitine with low affinity (Tamai et al 1998; Ohashi et al 2002). To investigate whether OCTN1 might assist in the uptake of carnitine when hypoxic conditions reduce the activity of OCTN2, carnitine uptake in control and low-oxygen BeWo cells was investigated in the presence of ergothioneine, a high-affinity substrate for OCTN1 (Grundemann et al 2005), which we used as an OCTN1 inhibitor.

L-Ergothioneine does not have high affinity for OCTN2. Figure 5 shows inhibition of 10 nM [^{3}H]-L-carnitine uptake by ergothioneine; the IC50 value for the interaction of ergothioneine with OCTN2 was 6.3 ± 0.2 mM. The uptake of carnitine in BeWo cells cultured under hypoxic (2% O_2) and control conditions was studied in the presence of 100 μM (Figure 6A) and 1 mM ergothioneine (Figure 6B), which are physiologically relevant intracellular concentrations (Chaudiere & Ferrari-Iliou 1999). These concentrations are significantly lower than the IC50 value for the interaction of ergothioneine

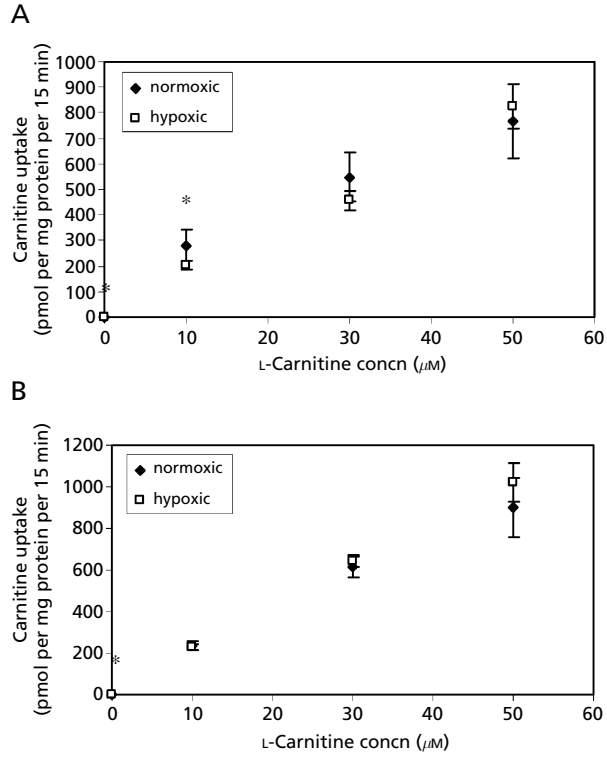

Figure 6 Uptake of carnitine at various concentrations in the presence of 100 μM ergothioneine (A) and 1 mM ergothioneine (B) in BeWo cells incubated under normoxic (20% O_2) and hypoxic (2% O_2) conditions for 48 h before the uptake experiment. Uptake was measured at 37°C for 15 min. Each point represents the mean ± s.d for 6 determinations. *$P < 0.05$.

with OCTN2 (see Figure 5) but higher than the reported affinity of OCTN1 for ergothioneine (21 μM in transfected human embryonic kidney (HEK) 293 cells (Grundemann et al 2005).

We therefore expected extensive inhibition of carnitine uptake in hypoxic BeWo cells, if in fact OCTN1 is a secondary means of carnitine transport following OCTN2. However, the presence of ergothioneine caused carnitine uptake to *increase* in low-oxygen BeWo cells. Figure 4 shows that low oxygen levels caused significant decreases in carnitine uptake at each of the concentrations studied whereas this decrease in carnitine uptake is gradually erased with increasing concentrations of ergothioneine, such that the effect of hypoxia on carnitine uptake becomes negligible (Figure 6). Even when studying 10 nM carnitine – a concentration 1000 times lower than the Km for OCTN2, at which uptake is predominantly OCTN2-mediated – the uptake of carnitine in hypoxic BeWo cells increased with increasing concentrations of ergothioneine (Figure 7A). This indicates that ergothioneine has some effect on carnitine uptake, even though the affinity of OCTN2 for ergothioneine is in the 5–10 mM range.

Figure 6 shows that ergothioneine in effect restores the carnitine transport function in a hypoxic state. With the increase in ergothioneine concentration, the plot of carnitine uptake in the low-oxygen BeWo cells becomes superimposed on the plot of uptake in the control (20% O_2) cells. It should be noted, however, that uptake in control cells in the presence of 1 mM ergothioneine is still reduced compared with the absence of ergothioneine, due to the slight inhibitory effect of ergothioneine on carnitine uptake (see Figures 5 and 7A).

Ergothioneine is found in mushrooms and is an intracellular antioxidant (Grundemann et al 2005). Antioxidant activity is one possible explanation for this ergothioneine-induced restoration of carnitine uptake in low-oxygen conditions. It has been suggested that antioxidant supplementation may reduce the incidence of pre-eclampsia in at-risk pregnancies (Holmes & McCance 2005). Because of its high mitochondrial activity, the placenta experiences high levels of oxidative stress, which is exacerbated in pre-eclampsia, IUGR and diabetes (Myatt & Cui 2004). Hypoxia may impair mitochondrial oxygen reduction, resulting in increased amounts of superoxide, leading to the apparent 'paradox of hypoxia-induced oxidative stress' (Myatt & Cui 2004).

The possibility that the effect of ergothioneine on carnitine uptake in hypoxic conditions is due to its antioxidant capabilities was investigated by studying the effect of *N*-acetylcysteine, another water-soluble intracellular antioxidant, on carnitine uptake in BeWo cells cultured under low-oxygen conditions. The uptake of 10 nM carnitine in low-oxygen BeWo cells increases in the presence of increasing concentrations of ergothioneine (Figure 7A), whereas the same concentrations of *N*-acetylcysteine did not cause any significant increase in carnitine uptake (see Figure 7B). This suggests that the antioxidant properties of ergothioneine are not responsible for the increased carnitine uptake in low-oxygen BeWo cells. It is possible that the antioxidant actions of the two compounds are sufficiently different that *N*-acetylcysteine does not serve as an appropriate model for ergothioneine in testing the hypothesis, which may influence the concentrations necessary for comparison in future studies (Aruoma et al 1989; Akanmu et al 1991; Chaudiere & Ferrari-Iliou 1999; Dekhuijzen 2004). Bisseling et al (2004) report that 50 μM *N*-acetylcysteine caused a rise in fetoplacental arterial pressure in pre-eclamptic placentas (induced by L-nitro arginine methyl ester (L-NAME)), whereas Lappas et al (2003) showed that as much as 10 mM *N*-acetylcysteine was required to significantly reduce lipopolysaccharide-stimulated phopspholipid metabolism, protease activity and

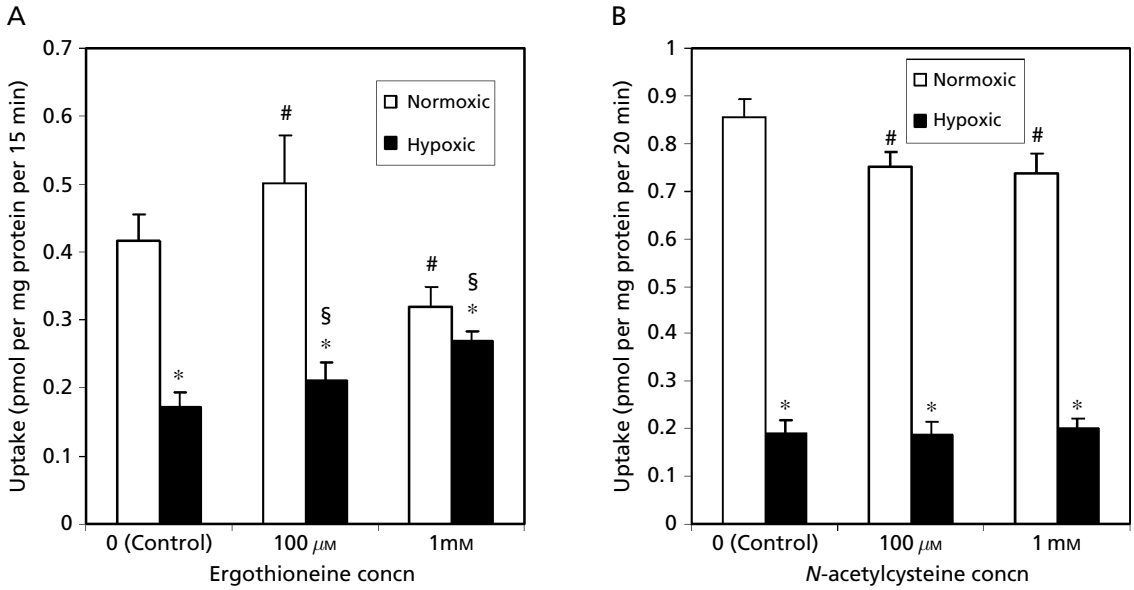

Figure 7 Uptake of 10 nM [^{3}H]-L-carnitine in BeWo cells in the absence (control) or presence of ergothioneine (A) or *N*-acetylcysteine (B). Cells were incubated under normal (20% O_2) or low-oxygen (2% O_2) conditions for 48 h before the uptake experiment. Uptake was measured at 37°C for 15 or 20 min. Each point represents the mean ± s.d for 4–6 determinations. *$P < 0.05$ for hypoxic vs normotoxic uptake at the same concentration of ergothioneine/*N*-acetylcysteine; #$P < 0.05$ for normoxic uptake at a given concentration vs control normoxic uptake; §$P < 0.05$ for hypoxic uptake at a given concentration vs control uptake.

proinflammatory cytokine release in human fetal membranes. No information can be found in the literature concerning ergothioneine in placental tissues.

If antioxidant activity is not responsible for the observed restoration of carnitine uptake in low-oxygen trophoblast cells, then additional experiments to elucidate the interactions of carnitine and ergothioneine transport and the transport functions of OCTN1 are important. Ohashi et al (2001) have described a possible tetraethylammonium–carnitine exchange mechanism in OCTN2-transfected HEK 293 cells. Similar studies using carnitine and ergothioneine would help elucidate the transport mechanisms in normoxic and hypoxic conditions. It is interesting to note that under control conditions (20% O_2), 100 μM ergothioneine caused a significant increase in carnitine uptake (see Figure 7A). The mechanisms causing this increase should also be investigated to shed light on this important issue, as the effects of ergothioneine in reversing the decreased nutrient transport under hypoxic conditions may lead to future therapeutic interventions.

Conclusions

Low oxygen concentrations in-vitro resulted in decreased transport function of OCTN2. Although RT-PCR demonstrated increased transcription of OCTN2 in BeWo cells cultured under low-oxygen conditions, the Western blots did not show a corresponding increase in OCTN2 protein in low-oxygen conditions compared with control, suggesting reduced translation efficiency under hypoxic conditions. Carnitine uptake was significantly reduced in BeWo cells cultured in low-oxygen conditions compared with cells grown in normoxic conditions. Decreased placental transport of carnitine may lead to symptoms of carnitine deficiency in infants from hypoxic pregnancies, whether caused by high altitude, pre-eclampsia or other factors. The OCTN1 substrate ergothioneine was shown to reverse the effects of low oxygen levels on carnitine transport. *N*-acetylcysteine, another water-soluble intracellular antioxidant, did not have the same effects on carnitine transport at low oxygen levels, suggesting that the effects of ergothioneine are not due to its antioxidant activity. Additional experiments are required to elucidate the mechanisms.

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