EXPRESSION, LOCALIZATION AND FUNCTION OF
THE CARNITINE TRANSPORTER OCTN2 (SLC22A5) IN
HUMAN PLACENTA

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Abbreviations:
OCTN, novel organic cation transporter; OATP-B, organic anion transporting polypeptide B;
hCG, human choriogonadotropin; MRP2, multidrug resistance protein 2; SLC, solute carrier;
TEA, tetraethylammonium
Abstract:

L-carnitine is assumed to play an important role in fetal development and there is evidence that carnitine is transported across the placenta. The protein involved in this transfer, however, has not been identified on a molecular level. We therefore characterized localization and function of the carnitine transporter OCTN2 in human placenta.

Significant expression of OCTN2 mRNA was detected in human placenta applying real-time PCR technology. Confocal immunofluorescence microscopy using an antibody directed against the carboxy terminus of OCTN2 protein revealed that it is predominantly expressed in the apical membrane of syncytiotrophoblasts. This was confirmed by co-staining of OATP-B and MRP2, which are known to be expressed mainly in the basal and apical syncytiotrophoblasts membrane, respectively. To further support this finding, we performed transport studies using basal and apical placenta membrane vesicles. We could demonstrate that the carnitine uptake into the apical vesicles was about 8 times higher compared to the basal ones. Moreover, this uptake was sodium- and pH-dependent with an apparent $K_m$-value of 21 µM and inhibited by verapamil, which is in line with published data for recombinant OCTN2. Finally, experiments using trophoblasts in cell culture revealed that expression of OCTN2 paralleled hCG production and thus is modulated by cellular differentiation.

In summary, we show expression and function of OCTN2 in human placenta. Moreover, several lines of evidence indicate that OCTN2 is localized in the apical membrane of syncytiotrophoblasts, thereby suggesting a major role in uptake of carnitine during fetal development.
Introduction

Carnitine plays an important physiological role in particular in beta-oxidation, because it facilitates long-chain fatty acid transport across the inner mitochondrial membrane. Moreover, carnitine is involved in intracellular coenzyme A homeostasis and functions as antioxidant (Bremer, 1983; Pons and De Vivo, 1995; Arduini et al., 1992). Only a few organs like brain, liver and kidney have the ability to biosynthesize carnitine (Bremer, 1983), whereas other tissues like skeletal and heart muscle, where beta-oxidation plays a major role in energy-metabolism, are highly dependent on active carnitine uptake from blood to maintain their carnitine steady-state concentration (Siliprandi et al., 1989).

Recent studies describe the organic cation transporter novel type II (OCTN2) as a high affinity uptake system for carnitine. The OCTN2 cDNA codes for 557 amino acids consisting of 12 putative transmembrane domains with a predicted molecular mass of 63 kDa (Wu et al., 1998). The transport of carnitine is sodium dependent (Tamai et al., 1998), while other compounds such as tetraethylammonium are transported by OCTN2 in a sodium independent way (Ohashi et al., 2001).

Besides its physiological function, OCTN2 is of pharmacological relevance. Drugs like verapamil, pyrilamine and beta-lactam antibiotics have been characterized as substrates of OCTN2 and/or inhibitors of carnitine transport (Ganapathy et al., 2000; Wu et al., 1999; Ohashi et al., 1999; Ohashi et al., 2001). Using screening approaches, the OCTN2 mRNA was detected in kidney, heart, skeletal muscle and placenta (Tamai et al., 1998). While OCTN2 expression in kidney seems to be mainly involved in carnitine re-absorption, expression in muscle cells is assumed to be responsible for carnitine homeostasis in this tissue. The physiological role is further supported by studies of systemic carnitine deficiency which was associated with mutations in the OCTN2 gene (Vaz et al., 1999; Lamhonwah et al., 2002).

Interestingly, previous studies indicated a limited capacity of the fetal organism for fatty acid oxidation, which is a consequence of incompletely matured carnitine biosynthesis (Novak et
al., 1981). This assumption leads to the conclusion that placental uptake of carnitine is pivotal for the fetal organism (Schmidt-Sommerfeld et al., 1981). In fact, sodium-dependent carnitine uptake has been demonstrated for the human choriocarcinoma derived JAR cell line, and brush-border syncytiotrophoblast vesicles. The protein involved in placental uptake of carnitine, however, has not been identified. (Prasad et al., 1996; Roque et al., 1996).

We therefore examined placental carnitine transport in relation to expression, localization and function of OCTN2. We assayed OCTN2 mRNA levels in term and preterm placentas, localized the protein by immunofluorescence staining to the apical membrane of syncytiotrophoblasts and determined sodium dependent carnitine uptake in basal and apical fractions of syncytiotrophoblast membrane vesicles from term and preterm placentas. Moreover, the influence of cellular differentiation on OCTN2 expression has been assessed using isolated trophoblasts.
Experimental Procedures

Human samples. Chorionic villous tissues were obtained from women undergoing caesarian and normal birth. A total of 31 samples from preterm (17) and term (14) placentas were used in the present study following written informed consent. Placentas were collected after normal vaginal deliveries and caesarian sections. Pathological samples such as gestational diabetes and preeclampsia were excluded. Samples for isolation of trophoblasts were taken from term placentas of normal deliveries. Demographic data of the placentas are summarized in table 1.

Cytotrophoblast culture. Cytotrophoblasts were isolated as described previously by Kliman et al. 1986 (Kliman et al., 1986). Placental cotyledons were prepared, minced and washed with 0.9 % saline. The tissue was then digested three times using trypsin and DNase I solved in Hanks’ balanced salt solution without Ca²⁺ and Mg²⁺ and 25 mM HEPES (pH 7.4). After incubation in a shaking water bath at 37°C, 140 ml of the cell solution were decanted and filtered through gaze and four layers of mull. Enzymatic activity in the disaggregated supernatant was stopped by centrifugation through 5 ml 90 % -FCS for 10 min at 1,000 x g. The pellet was resolved in DMEM-25 mM HEPES-DNase. After a further centrifugation step at 500 x g for 10 min with 90 % percoll the pellet was resolved in cold DMEM-25mM HEPES. Cytotrophoblasts were separated using density gradient centrifugation at 1,500 x g for 45 min on discontinuous percoll (10-70 %). Cells between the 40 % and 50 %-percoll bands were collected, washed with DMEM and plated onto 35 mm culture dishes at a density of 5 x 10⁶ cells/ dish. Cells were grown in M199 medium, supplemented with 10 % FCS, 100 units/ml penicillin/streptomycin and 5 ng/ml EGF at 37°C in a 5 % CO₂ humidified atmosphere and later on maintained in culture for five days.

RNA isolation and analysis. RNA was isolated from 17 term and 14 premature placentas (and four different cytotrophoblasts) isolations using RNeasy® Mini extraction kit (Qiagen,
Frankfurt, Germany). Villous material of the placenta was separated and stored at -80°C. After mechanical homogenization at 2,500 rpm for 2 min frozen samples were homogenized in guanidinium thiocyanate containing buffer. The isolation of RNA was performed using the kit according to the manufacturer’s instructions. Integrity of RNA was controlled by ethidium bromide staining in a formaldehyde-containing 1% agarose gel.

The isolated RNA was reversely transcribed using random hexamer primers and the TaqMan® reverse transcription (RT) kit (Applied Biosystems, Weiterstadt, Germany). The resulting cDNA was amplified by real-time PCR with intron-spanning primers and probes for human OCTN2, OATP-B and MRP2. Primer and probe oligonucleotides for OCTN2-PCR were designed based on the cDNA sequence published under GeneBank accession number AB015050. Real-time quantitative PCR for OCTN2 was performed using forward primer 5'`-aattttgagatgtttgtcgtgctg-3`, reverse primer 5'`-caagaatttctgcctccaggac-3` and probe 5`-6FAM-tccatttaggcatggagcactcc-3` for MRP2 the forward primer 5'`-ctggaacatgattcggaagc-3`, the reverse primer 5`-gaggattcccagagccgac-3` and probe 5`-6FAM-catcggagatgtgaacctggacat-3` were used. The detection of OATP-B was performed according to St. Pierre et al. 2002. For quantification of 18S rRNA a pre-developed primer and probe mix was purchased from Applied Biosystems. Analysis of OCTN2, OATP-B and MRP2 was performed using 10 ng reverse transcribed RNA, while for 18S rRNA 0.10 ng were used. A TaqMan® universal mastermix (Applied Biosystems, Weiterstadt, Germany) was used for real-time PCR reaction. PCR was performed using a real-time PCR cycler ABI Prism 7700 Sequence Detector (Applied Biosystems, Weiterstadt, Germany). For quantification of OCTN2, OATP-B, MRP2 and 18S rRNA signals were applied to a cloned standard resulting in absolute copy numbers for the respective gene.

**OCTN2-antibody.** The anti-OCTN2 antibody was raised against in rabbits against the 15 amino acids at the carboxy terminus of the deduced OCTN2 Sequence
(KDGQERPTILKSTAF). The peptide was synthesized automatically and then coupled to maleimide-activated keyhole limpet hemocyanin (Peptide Specialty Laboratories GmbH, Heidelberg, Germany). New Zealand white rabbits (Charles River, Sülzfeld, Germany) were immunized with this conjugate after followed protocol: On day 1, 5 mg of immunogen were emulsified with equal volumes of complete Freund’s adjuvants and injected subcutaneously. Boosting with 5 mg of immunogen in incomplete Freund’s adjuvants into the subcutaneous tissue was performed on day 28 and 54, respectively. Serial bleeds were collected from each animal prior the immunisation and before new boosting.

**Immunofluorescence microscopy.** Localization of OCTN2, OATP-B and MRP2 was investigated by confocal laser scanning immunofluorescence microscopy using polyclonal antibodies from rabbit raised against the 15 C-terminal amino acids of human OCTN2 and OATP-B. For MRP2 the monoclonal mouse anti-MRP2 antibody M2III-6 (Alexis Biochemicals, Heidelberg, Germany) was used.

After delivery placentas samples were fixed in formalin and paraffin sections of 2 µm were generated. The slides were deparaffinized in Xylene substitute for 10 minutes after that the paraffin sections were hydrated in alcohol at different dilutions for 5 minutes (100%, 98%, 75%, 50% and 0%). Following, sections were boiled for 10 min in 10 mM citrate buffer (pH 6.0) for OCTN2 and OATP-B staining or in 1 mM EDTA buffer (pH 8.0) for MRP2/OCTN2 double-staining. Sections were washed twice with PBS and blocked for 1 h using 5 % FCS in PBS. Following incubation with primary antibodies was performed at 4°C over night. After washing with PBS the sections were incubated for 2 h with Alexa Flour 488 labeled anti-rabbit IgG for detection of OCTN2 and OATP-B. For the MRP2/OCTN2 double-staining the Alex Flour 568 labeled anti-rabbit and the Alexa Flour 488 labeled anti-mouse antibody were used. Staining of nuclei was performed using a 1/2000 dilution of TOTO®-3 iodide with DAKO® Fluorescent Mounting Medium (DAKO Corporation, Carpinteria, CA, USA).
secondary antibodies as well as the TOTO® dye were purchased from Molecular probes (Invitrogen, Carlsbad, CA, USA). For the OCTN2 peptide competition, 50 µl 1/500 diluted antiserum were pre-incubated with 100 µg of OCTN2 peptide overnight at 4°C. After that, sections were processed as described above. The fluorescence was detected by confocal laser scanning microscopy.

**Preparation of trophoblastic vesicles from human placental tissue.** A term placenta of normal delivery was used for preparation of basal and apical vesicles according to methods described previously (Kelley et al., 1983; Booth et al., 1980). In brief, placental cotelydones were divided in two different fractions, the maternal localized part with the higher proportion of extravillous cytotrophoblasts and the fetal localized with multinuclear syncytiotrophoblasts. After several washing steps with cold PBS 20-100 g of the placental tissue were minced and then washed again. Thereafter the tissue was homogenized in incubation buffer (250 mM sucrose and 10 mM Tris/HCl, pH 7.4) supplemented with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.3 µM aprotinin and 1 µM pepstatin) using a Potter-homogenizer (20 strokes, 1000 rpm). After incubation for 1 h on ice the homogenate was centrifuged at 9,000 x g for 10 min. Supernatants were centrifuged (100,000 x g, 35 min) and the pellets were resuspended in incubation buffer and homogenized by 15 strokes with a loose-fitting Dounce B homogenizer. For separation of basal and apical membranes, MgCl₂ was added to a final concentration of 10 mM. After 10 min of incubation on ice a centrifugation step (2,200 x g, 12 min) was performed, separating the basal (pellet) and apical (supernatant) membranes. Pellets were resuspended in homogenization buffer and both fractions were homogenized using a tight-fitting Dounce B potter (30 strokes), washed by centrifugation (100,000 x g, 35 min) and homogenized again using the tight-fitting Dounce B potter (30 strokes). After centrifugation (100,000 x g, 35 min) the pellets of both membrane fractions were resuspended in 1-3 ml homogenisation buffer and the membrane suspensions
were passed 20 times through a 27-gauge needle for vesicle formation. Membrane vesicles were frozen and stored in liquid nitrogen.

To assess purity of the membrane fractions the activity of the alkaline phosphatase as marker for the apical membrane was assayed according to Pekarthy et al. (Pekarthy et al., 1972). To determine the orientation and integrity of the vesicles, the activity of the ectoenzyme nucleotide pyrophosphatase was measured in the presence and absence of detergent as described before (Bohme et al., 1994).

**Immunoblot analysis.** Crude membrane vesicle fractions were loaded onto a 10 % sodium dodecylsulfate-polyacrylamid gel after incubation in sample buffer at 95 °C for 10 min. Immunoblotting was performed using a tank blotting system (BioRad, Hercules, CA, USA) and an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Freiburg, Germany). Primary antibodies were diluted in Tris-buffered saline containing 0.05 % Tween 20 and 5 % bovine serum albumin to the following final concentrations: OCTN2 antiserum 1:1000, MRP2 monoclonal antibody (M2III-6) (Alexis Biochemicals, Heidelberg, Germany) 1:500 and OATP-B affinity purified anti-serum 1:2000. Secondary horse-radish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG antibodies (BioRad, Hercules, CA, USA) were used at a 1:2000 dilution.

**Vesicle transport studies.** Vesicles prepared from basal and apical membrane fractions were used for measuring the transport of [³H]-carnitine (specific activity: 2.96 TBq/mmol; Hartmann Analytic, Braunschweig, Germany). Transport studies were performed using a rapid filtration through nitrocellulose filters as described previously (Jedlitschky et al., 2000). 1 µg/µl total vesicular protein were incubated in the presence of 100 nM [³H]-Carnitine using an incubation buffer containing 180 mM Sucrose, 100 mM NaCl, 10 mM MgCl₂, 10 mM TrisHCl and 0.2 mM CaCl₂ in a total volume of 100 µl. In control experiments NaCl was
replaced by KCl. Aliquots of 20 µl were taken after 0.5, 1, 2.5, 5 and 10 min for time dependency studies and reaction was stopped by adding 1 ml of ice-cold KCl containing incubation buffer. For kinetic studies 20 µg vesicular probes were incubated with different concentrations of unlabeled carnitine (1 to 100 µM) and 250 nM [³H]-carnitine. For pH studies the incubation buffer was adjusted by using NaOH or HCl. Samples were filtered immediately through nitrocellulose filters (0.2 µm pore size, pre-soaked in incubation buffer) and rinsed three times with 3 ml ice-cold KCl containing incubation buffer. For detection of radioactivity filters were dissolved in 10 ml scintillation-cocktail (Rotiszint, Roth, Karlsruhe, Germany) and measured in a scintillation beta-counter (type 1409, LKB-Wallac, Turku, Finland). Rates of carnitine transport were given in fmol or pmol [³H]-carnitine x mg protein⁻¹ x min⁻¹ or as ratio of control.
Results

**OCTN2 mRNA expression.** The expression of OCTN2 was analyzed by quantitative real-time PCR in 36 samples from preterm (gestational age under 37 weeks) and term placentas. OCTN2 values were normalized to expression of 18S rRNA. OCTN2 mRNA levels were detectable in all 36 samples with copy numbers between 1,000 and 10,000. The mRNA expression normalized to 18S rRNA in preterm placentas was around 15% higher compared to term placentas (term: 1.64 x 10^-5; preterm: 1.94 x 10^-5), however, this difference was not significant. Furthermore, mRNA expression for the membrane transport proteins OATP-B and MRP2 were determined and normalized to 18S rRNA. Compared to the mRNA expression of OCTN2, expression of MRP2 was about 60% of the OCTN2. In contrast mRNA levels of OATP-B were more than 6 fold higher compared to OCTN2 (data not shown).

**Immunolocalization of OCTN2.** From immunofluorescence the OCTN2 protein appears to be expressed at the syncytiotrophoblast membrane. To further characterize the exact localization of OCTN2 in syncytiotrophoblasts placenta sections were stained with antibodies against OCTN2 (Fig.1, D), OATP-B as a basal marker (Fig.1, E) and MRP2 as apical marker (Fig.1, F). While staining of OATP-B could be assigned to the basal membrane, the double staining of OCNT2 and MRP2 revealed a co-localization in the apical membrane (yellow fluorescence). Specificity of the OCTN2 antibody was demonstrated by incubation the sections with pre-immune serum and antiserum pre-incubated with OCTN2 peptide (Fig.1, A-C).

**Transport of L-carnitine into trophoblastic membrane vesicles.** To demonstrate functional activity of OCTN2 in human placenta, transport of tritium-labeled L-carnitine was measured for apical and basal vesicle preparation (Fig.2). Membrane vesicles enriched in basal or apical
membranes were prepared from term placentas and characterized for markers as described in the methods section. Immunoblots with 25 µg protein were performed and the separation of membranes further evaluated by detection of OATP-B and MRP2 protein. As expected from previous reports (St Pierre et al., 2000; St Pierre et al., 2002) the OATP-B protein could be detected in basal and apical preparation with a significantly stronger signal for the basal fraction, while the MRP2 protein was detected mainly in the apical fraction of membrane vesicles (Fig. 2A). Furthermore the activity of the alkaline phosphatase as a marker enzyme for the apical membrane was determined. The results show an enrichment factor of 27.5 ± 11.5 for the apical fraction and 6.9 ± 0.8 for the basal one (in both cases compared to the homogenate). To assess the orientation and integrity of the vesicles, the activity of the ectoenzyme nucleotide pyrophosphatase was measured. The results show for the basal fraction 45 ± 2.5 % “right side out” vesicles and for the apical one 57 ± 5.5 % (data represent the mean value and SD for 3 preparations with triplicate determination).

The western blot analysis of OCTN2 using immunoblots with 25 µg of each vesicular protein fraction showed a significant stronger signal for the apical fraction compared to the basal one (Fig. 2A).

The corresponding carnitine transport studies into the apical fraction show a time dependent uptake with a maximum uptake after 5 min, while the basal fraction showed only little uptake of carnitine (Fig. 2B). In comparison, the maximum uptake into the apical vesicles was about 8 times higher compared to basal vesicles.

Moreover, we investigated sodium dependence of carnitine uptake into both fractions. While carnitine uptake into vesicles of the apical fraction was significantly decreased in the absence of sodium, transport of basal vesicle was affected to a minor extent (Fig.3 A). Carnitine uptake to apical vesicles was inhibited at pH 6.4 while higher values up to pH 8.4 had only a minor influence compared to physiological pH (Fig.3 B). Verapamil, a known inhibitor of OCTN2, showed also an inhibitory effect on carnitine uptake of about 70 % for 50 µM
verapamil. To determine the kinetic parameters of carnitine uptake, an apical vesicle preparation was incubated with different concentrations of unlabeled carnitine and 250 nM tritium labeled carnitine. Total transport of carnitine was calculated for each concentration and kinetic parameters were calculated from double reciprocal plot according to Lineweaver-Burk (Fig. 4). Calculated values were 21 µM for the $K_m$ and 112 pmol x min$^{-1}$ x mg$^{-1}$ protein for $V_{max}$.

**Expression of OCTN2 in differentiating cytotrophoblasts.** Cytotrophoblasts were isolated and cultured in EGF-containing medium. After two, four and six days in culture RNA from differentiated cells was isolated and OCTN2 mRNA levels were determined. The expression of OCTN2 is increasing until day four and then regressing at day six ($151 \pm 17$, $206 \pm 82$, $124 \pm 39$ percent of the 0 h value) (Fig. 5). As a biochemical marker for differentiation the hCG production of the cultured cytotrophoblasts was assessed in the supernatant, showing maximal concentration (above 14.000 U/l) at the fourth day of culture (Fig. 5).
Discussion:

In this study we examine the expression and function of OCTN2 in human term and preterm placentas. As shown previously the expression of a carnitine transporting protein in human placenta may play an important role in the carnitine supply of the growing fetus. For this reason a directed carnitine transport from the maternal to the fetal circulation is assumed to be pivotal for the fetus especially in early gestation. Indications for such a sodium-dependent carnitine transport across the apical syncytiotrophoblast membrane have been described (Prasad et al., 1996; Roque et al., 1996). Our results suggest that OCTN2 is a candidate protein mediating this transport.

We have detected significant levels of OCTN2 mRNA in placenta, the OCTN2/18S RNA ratios are in the same order of magnitude as that for MRP2. There was, however, no significant difference between term and preterm placentas indicating a role in fetal supply with carnitine during the entire pregnancy.

While the presence of OCTN2 mRNA in placenta has already been described (Wu et al., 1998), little was known about its protein expression, localization and function. To examine expression and localization of OCTN2 protein an anti-rabbit antibody against the 15 C-terminal amino acids of the human OCTN2 has been generated. The antibody displayed a well defined signal in immunofluorescence. The specificity of this signal could be demonstrated by the comparison of placenta sections incubated with the antiserum and the pre-immune serum. Furthermore, specificity of the antibody has been verified by peptide competition experiments.

In placenta the immunofluorescence signal is localized in the syncytiotrophoblast membrane and seems to be restricted to the apical membrane. This observation has been verified by double-staining with antibodies against OATP-B and MRP2, which are known to be localized in the basal and apical membrane, respectively (St Pierre et al., 2000; St Pierre et al., 2002), and by Western blot analysis (Fig. 2A).
In addition we investigated the function of placental OCTN2. OCTN2 transports carnitine as well as TEA. While the carnitine transport is sodium dependent TEA is transported in a sodium independent manner. Ohashi et al. (2001) reported that TEA uptake is enhanced by extracellular carnitine and that intracellular TEA is enforcing carnitine uptake. The authors suggest that after each carnitine transport the OCTN2 protein has to be reactivated for further uptake by transport of one molecule of TEA (Ohashi et al., 2001). We separated syncytiotrophoblast membranes in apical and basal fractions. The quality of membrane purification was tested by western blot analysis for OATP-B and MRP2 resulting in the expected bands in the respective fractions. We then measured carnitine uptake in vesicles generated from these fractions. The apical vesicles showed a significant uptake of carnitine, which was highly sodium dependent. When replacing sodium by potassium the carnitine uptake of the apical fraction was reduced to the level of the basal fraction. Further experiments showed that the observed carnitine uptake into the apical fraction exhibited kinetic parameters similar to recombinant OCTN2 and was inhibited by verapamil and unlabeled carnitine, an effect that has also been described for OCTN2-mediated carnitine uptake (Ohashi et al., 2001; Tamai et al., 1998; Wu et al., 1999). Furthermore, the apical fraction showed a pH-dependent decrease in carnitine uptake which is already known for organic cation transporter (Wu et al., 1998; Kekuda et al., 1998).

Although there are other transport proteins like OCTN1 and octn3, which transport carnitine with lower affinity than OCTN2 and in the case of octn3 in a sodium independent manner (Tein, 2003), our data concerning the protein expression and transport characteristics lead to the conclusion that OCTN2 is the major player in carnitine transport from maternal to fetal circulation.

The localization of OCTN2 in the apical membrane of the syncytiotrophoblast suggests a dominant role for carnitine uptake from maternal circulation. While it remains to be elucidated how carnitine crosses the basal membrane of syncytiotrophoblast.
Carnitine is pivotal for the fetal organism because it seems to be an important stimulator of mitochondrial respiratory chain (Huertas et al., 1992) and production of surfactant (Lohninger et al., 1990). Another issue is its potential role in energy metabolism. After delivery the energy supply changes from oxidative metabolism of carbohydrates to beta-oxidation of fatty acids as the main energy source (Arenas et al., 1998). For this process carnitine is an essential cofactor because it facilitates the transport of long-chain fatty acids across the inner mitochondrial membrane (Bremer, 1983). For this reason and because of the limited capacity especially of the early fetus for carnitine biosynthesis transplacental carnitine transport is essential for the fetal organism and after that for the first days after delivery (Nakano et al., 1989). Because of the described function and localization of OCTN2, this transporter can play an important role in transplacental carnitine transport. Therefore malfunction caused by modified expression of OCNT2 or inhibiting pharmacological agents may result in carnitine deficiency of the fetus and the newborn child similar to the systemic carnitine deficiency syndrome (SCD) described for skeletal and heart muscle (Vaz et al., 1999; Amat Di San et al., 2003).

We did not observe a significant difference between the OCTN2 mRNA level in term and preterm placentas, which indicates functional relevance of OCTN2 already for the early fetus. We could, however, show an increased expression of OCTN2 mRNA in cultured trophoblast cells, which form a multinuclear syncytium after several days in culture (Richards et al., 1994). This increase was paralleled with the hCG secretion in supernatant of cells as a biochemical marker for differentiation (Malek et al., 2001; Richards et al., 1994) and indicates a functional expression of placental OCTN2 regulated by the cytotrophoblast. These cytotrophoblast cells are progenitor cells of the syncytiotrophoblast which is assumed to play a major role in the control of placental transfer of various substances (Young et al., 2003).

Taken together, we show an apical expression of OCNT2 in syncytiotrophoblast membrane. Carnitine is transported across this membrane with characteristics similar to those described
for OCTN2. Very recently, a study on the same topic was published by Lahjouji et al. (Lahjouji et al., 2004). The results of that study concerning carnitine transport and OCTN2 expression in placenta were in line to ours. Therefore, we conclude that OCTN2 can play an important role in placental carnitine transport.
Acknowledgments

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Reference List


Footnotes:

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Legends to figures:

**Figure 1.** Immunofluorescence microscopy of OCNT2 in human placenta sections. A to C: 20x magnification, A: OCTN2 antiserum (dilution: 1/500); B: preimmune-serum (dilution 1/500); C: OCTN2 antiserum (dilution 1/500) preincubated with OCTN2 peptide (100µg). D to F: 40x magnification, D: staining of OCTN2 (dilution of antiserum 1/200), E: staining of OATP-B (dilution of affinity-purified antiserum 1/100), F: doublestaining of OCTN2 (red fluorescence) and MRP2 (green fluorescence). A to F: staining of cell nuclei with TOTO®-3 iodide (blue fluorescence; dilution 1/1000).

**Figure 2.** A: Western blot analysis for OCTN2, OATP-B and MRP2. 25 µg vesicles were blotted on a nitrocellulose membrane and detected with rabbit OCTN2 and OATP-B polyclonal antisera (dilutions 1/1000 and 1/2000) and a monoclonal mouse antibody against MRP2 (dilution 1/500). B: Time dependent uptake of carnitine into apical (○) and basal (●) syncytiotrophoblast membrane vesicle. Vesicle fractions from a term placenta were incubated with 100 nM tritium-labeled carnitine at 37°C. At indicated times uptake was stopped by adding ice cold stopping buffer, vesicles were isolated using the rapid filtration method and radioactivity was determined (n=3, data shown as mean ± SD).

**Figure 3.** Sodium and pH dependency of carnitine uptake. A: Sodium-dependent uptake of carnitine into apical and basal syncytiotrophoblast membrane vesicle. Vesicle fractions from a term placenta were incubated with 100 nM tritium-labeled carnitine at 37°C in the presence of 100 mM NaCl (closed bars) or KCl (open bars). Uptake was stopped by adding ice-cold stopping buffer after 1.5 min, vesicles were isolated using the rapid filtration method and radioactivity was determined (mean ± SD for n=3, *: significant difference with p < 0.05, Student’s T-test; ns: not significant). B: pH-dependency of carnitine uptake into apical placental membrane vesicles. Uptake of 100 nM tritium-labeled L-carnitine was measured at different extravesicular pH. Data given as mean ± SD of percent of transport at physiological pH (7.4) (n=3, * significant difference with p < 0.05, Student’s T-test).

**Figure 4.** Kinetic of carnitine uptake into apical membrane vesicles. Vesicles from a term placenta were incubated for 3 min with different carnitine concentrations and 250 nM of tritium-labeled carnitine. The kinetic constants were determined from direct curve fitting to Michaelis-Menten (A) and from double-reciprocal plot according to Lineweaver-Burk (B) (data shown as mean ± SD for n=3).

**Figure 5.** Expression of OCTN2 mRNA (bars) and concentration of secreted hCG (curve) in supernatant in cultured trophoblasts. Data were given as ratio of OCTN2/18SrRNA expression + SD (for 3 independent trophoblast isolations).
**Table 1.** Summary of demographic data on term and preterm placentas

<table>
<thead>
<tr>
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<th>Terms</th>
<th>Preterms</th>
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<tr>
<td>Mean age of the pregnant</td>
<td>27.4 ± 5.4</td>
<td>26.6 ± 5.6</td>
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<tr>
<td>Mean of gestational weeks</td>
<td>39.2 ± 1.4 weeks</td>
<td>32.2 ± 3.2 weeks</td>
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<tr>
<td>Cesarean Sections</td>
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<td>76 %</td>
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<td>Weight at birth</td>
<td>3743 ± 491 g</td>
<td>1761 ± 732 g</td>
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<td>17</td>
</tr>
</tbody>
</table>
Figure 2

A

basal fraction
apical fraction

180 kDa
85 kDa
65 kDa

MRP2
OATP-B
OCTN2

B

incubation time [min]

carnitine transport [fmol/mg protein]

0 2 4 6 8 10

0 160 330 500 660 830
Figure 3

A

![Graph showing carnitine transport](image)

- **sodium**
- **potassium**

B

![Graph showing % of transport at pH 7.4](image)

- **pH 6.4**
- **pH 7.4**
- **pH 8.4**

*Significance indicated by asterisk (*)

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**Figure 4**

**Panel A**

![Graph showing a nonlinear relationship between V (pmol/min/mg protein) and carnitine concentration (µM).](image)

**Panel B**

![Graph showing a linear relationship between 1/V (mg protein x min/pmol) and 1/(carnitine concentration) [µM⁻¹].](image)

**Km = 21 µM**

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Figure 5