OCTN2-mediated transport of carnitine in isolated Sertoli cells

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Abstract

Carnitine is extensively accumulated in epididymis. Carnitine is also accumulated in testis at higher concentration than in the plasma and is used in spite of the presence of the blood–testis barrier. In this study, we examined the characteristics of carnitine transport in primary-cultured rat Sertoli cells, which constitute a part of the blood–testis barrier. Uptake of [³H]carnitine (11.4 nM) from the basal side of Sertoli cells was Na⁺-dependent and was significantly decreased in the presence of 10 μM (48.0 ± 7.4% of control) or 100 μM unlabeled carnitine (14.6 ± 5.7% of control). Furthermore, the uptake was significantly inhibited in the presence of 100 μM acetyl-L-carnitine, 100 μM gamma-butyrobetaine or 500 μM quinidine. In RT-PCR analysis, the high-affinity carnitine transporter OCTN2 was detected in rat whole testis tissue and primary-cultured Sertoli cells. In contrast, the low-affinity carnitine transporter ATB^{0,+} was detected in rat whole testis tissue, but not in primary cultured Sertoli cells. These results demonstrate that OCTN2 mediates carnitine supply to Sertoli cells from the circulation.

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Introduction

Carnitine is essential for fatty acid oxidation, facilitating the transfer of an acyl group into mitochondria. Acyl-carnitine ester, which can pass through the inner membrane of mitochondria, is synthesized from carnitine and acyl-coenzyme A by carnitine palmitoyltransferase I (CPT I). Acyl-carnitine ester is esterified to acyl-thioesters by CPT II and undergoes beta-oxidation in mitochondria (Ramsay *et al.* 2001). In male reproductive tissues, the concentration of carnitine is higher than that in plasma (Marquis & Fritz 1965, Casillas 1972, Jeulin & Lewin 1996), and carnitine is a key nutrient for sperm maturation (Casillas & Chaipayungpan 1979) and motility (Hinton *et al.* 1981). Studies have shown carnitine concentration to be reduced in seminal fluid of infertile patients (Matalliotakis *et al.* 2000), and improvement of sperm motility and viability of spermatozoa was obtained by treatment with carnitine and acetylcarnitine (Vicari & Calogero 2001). Carnitine is extensively accumulated in epididymis (Hinton *et al.* 1979). Additionally, carnitine is also used in testis and is accumulated in testis at high concentrations compared with plasma after administration of exogenous [³H]carnitine (Bremer 1983) in spite of the presence of the blood–testis barrier (BTB).

Sertoli cells are one of the most important constituents of the BTB, which is present between the blood

circulation and germ cells, and serves to protect germ cells from exposure to xenobiotics. The Sertoli cells also provide a route for the supply of various nutrients to germ cells to support spermatogenesis. Since hydrophilic nutrients can not readily cross the plasma membrane of Sertoli cells, selective membrane transporters for each nutrient are likely to be needed. Although carnitine is a zwitterionic hydrophilic compound at physiological pH and is essential for the maturation of sperm cells, the transport mechanism of carnitine across the BTB has not yet been clarified.

Five mammalian transporters (OCTN1, 2, 3, CT2 and ATB^{0,+}) are known to be able to transport carnitine. We established that OCTN1 and OCTN2 transport carnitine (Tamai *et al.* 1998, 2000, Yabuuchi *et al.* 1999). OCTN2 is an Na⁺-dependent, high-affinity carnitine transporter (Sekine *et al.* 1998, Tamai *et al.* 1998, 2000, Wu *et al.* 1999) and is essential for the renal reabsorption of carnitine (Yokogawa *et al.* 1999, Tamai *et al.* 2001). Loss of function of OCTN2 as a result of mutation of the OCTN2 gene leads to systemic carnitine deficiency due to inability to maintain the required carnitine level in plasma (Nezu *et al.* 1999). Enomoto *et al.* (2002) cloned a testis-specific carnitine transporter CT2 from human testis. Mouse OCTN3 is also a testis-specific carnitine transporter (Tamai *et al.* 2000), but the amino acid sequence homology between CT2 and

mouse OCTN3 is only about 32%. So, the true homologue of CT2 in laboratory animals such as mice and rats remains to be clarified. On the other hand, Nakanishi *et al.* (2001) reported that the Na⁺- and Cl⁻-coupled neutral and cationic amino acid transporter ATB^{0,+} transports carnitine with low affinity ($K_m = 0.83 \pm 0.08$ mM) compared with OCTN2 ($K_m =$ about 20 μ M). Accordingly, there are several possible carnitine transporters that may function in Sertoli cells.

In the present study, we examined the mechanism of carnitine transport across the basolateral membranes of Sertoli cells using primary-cultured, rat testis-derived Sertoli cells.

Materials and Methods

Materials

L-[³H]Carnitine (80.0 Ci/mmol) and [carboxyl-¹⁴C]inulin (2.5 mCi/g) were purchased from Amersham Biosciences Corp. (Piscataway, NJ, USA) and American Radiolabeled Chemicals (St Louis, MO, USA) respectively. Collagenase and trypsin were obtained from Sigma Chemicals Co. (St Louis, MO, USA) and Becton Dickinson Microbiology Systems (Sparks, MD, USA) respectively. All other reagents, unless otherwise noted, were purchased from Sigma Chemical Co. or Wako Pure Chemical Industries Co. (Osaka, Japan).

Preparation and primary culture of rat Sertoli cells

Sertoli cells were isolated from 20-day-old Donryu rats (Saitama Experimental Animal Supply Co. Ltd, Saitama, Japan) according to the reported method (Dorrington & Fritz 1975, Nagao 1989, Shiratsuchi *et al.* 1997). Briefly, testes were decapsulated, and seminiferous tubules were incubated in 35 ml of 0.25% collagenase in phosphate-buffered saline (PBS) for 20 min at 37°C with occasional stirring. The seminiferous tubules were washed with serum-free F12-L15 medium, and then incubated with occasional gentle pipetting in 35 ml of 0.25% trypsin in PBS for 20 min at 37°C. F12-L15 medium was composed of a 1:1 mixture of Ham's F12 medium (ICN Biomedicals Inc., Irvine, CA, USA) and L-15 medium (ICN Biomedicals Inc.), containing 15 mM HEPES, 10 unit/ml penicillin, 0.1 mg/ml streptomycin and 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). Trypsin treatment was terminated by adding 5 ml of FBS and 10 ml of F12-L15 medium containing 10% FBS. The resultant cell suspension was filtered twice through four sheets of gauze to remove cell aggregates and tissue debris, after which the cells were collected by centrifugation (300 g $\times$ 10 min). The cells were suspended in 30 ml of F12-L15 medium containing 10% FBS and washed by centrifugation (150 g $\times$ 10 min). Finally, the cells were suspended in F12-L15 medium containing 10% FBS and passed once through nylon mesh (70 μ m, BD Biosciences, Bedford,

MA, USA). The isolated testicular cells were seeded into culture dishes (#353003, BD Biosciences). Cells were grown in F12-L15 medium, containing 1 μ g/ml norepinephrine, in a humidified incubator at 32.5°C for 3 days and at 37°C for 3 days. Germ cells floating on the surface of the co-culture of testicular cells were harvested by pipetting for RT-PCR analysis at 2 and 4 days after seeding. Sertoli cells were isolated after removal of the germ cells floating on the surface of the testicular cells. About 90% of the cells adhering to the culture dish were Sertoli cells as judged from Nile red (Molecular Probes Inc., Eugene, OR, USA) staining, which is a marker for Sertoli cells (Mather *et al.* 1990). Six days after seeding, the cultures reached confluence and were used for the transport experiments and the RT-PCR analysis. Germ and Sertoli cells cultured for 2 and 4 days at 32.5°C were collected for RT-PCR analysis. For uptake experiments across the basolateral membrane of rat primary-cultured Sertoli cells, the cells were cultivated on 24-well Matrigel invasion chambers (BD Biosciences) at 32.5°C for 3 days and at 37°C for 3 days.

Carnitine transport experiments

Transport experiments in suspended primary-cultured rat Sertoli cells were performed as described in our previous study (Kato *et al.* 2005). Briefly, cells were harvested with a cell scraper and suspended in transport medium containing 137 mM NaCl, 5 mM KCl, 0.39 mM NaHCO₃, 0.44 mM KH₂PO₄, 0.95 mM CaCl₂, 0.8 mM MgSO₄, 25 mM D-glucose and 10 mM HEPES, adjusted to pH 7.4 after washing two times with the transport medium. The cell suspension was preincubated at 37°C for 20 min in the transport medium, then centrifuged. The resultant cell pellets were re-suspended in 200 μ L of transport medium containing L-[³H]carnitine to initiate the uptake. After 30 min, the cell suspension was diluted with 800 μ L of ice-cold transport medium and centrifuged immediately (7000 g $\times$ 1 min) to terminate the uptake reaction. Then, the cells were resuspended in ice-cold transport medium and obtained as the pellet after centrifugation. The resultant cell pellets were solubilized in 1 M NaOH and the cell-associated radioactivity was measured with a liquid scintillation counter (Aloka, Tokyo, Japan) using Cleasol-1 (Nacalai tesque, Kyoto, Japan) as a liquid scintillation fluid. Na⁺-free transport medium was prepared by replacing 137 mM NaCl and 0.39 mM NaHCO₃ in the standard transport medium with 137 mM N-methyl-D-glucamine (NMG)Cl or 137 mM lithium Cl and 0.39 mM KHCO₃ respectively, and was used to assess the uptake in the absence of sodium ions.

For the basolateral membrane uptake experiments using Matrigel invasion chambers (BD Biosciences), the medium in the basolateral membrane side was replaced with transport medium containing L-[³H]carnitine after preincubation in transport medium for 20 min. The apical-side medium was also replaced with transport medium. At the

designated time, the cells on the invasion chamber were rapidly washed twice with ice-cold transport medium, then solubilized in 1 M NaOH, and the cell-associated radioactivity was measured by means of a liquid scintillation counter. L-[³H]Carnitine uptake was corrected for nonspecific extracellular adhesion based on the apparent uptake of [¹⁴C]inulin, a membrane-impermeable marker.

Permeation of [¹⁴C]inulin and L-[³H]carnitine across Sertoli cells cultured on a Matrigel-coated invasion chamber

Permeation of [¹⁴C]inulin and L-[³H]carnitine was measured by using Sertoli cells cultured on a Matrigel-coated invasion chamber (BD Biosciences). After preincubation in transport medium for 20 min, radio-labeled test compounds were added to the basolateral side and the appearance of radio-activity in the apical side was measured periodically.

RNA isolation and RT-PCR

Total RNA was extracted from cultured cells with the ISO-GEN RNA extraction solution (Wako Pure Chemical Industries) according to the manufacturer's protocol. cDNA was prepared from the extracted RNA by means of reverse transcription with Improm-II reverse transcriptase (Promega, Madison, WI, USA) and oligo (dT) primers according to the manufacturer's instructions. The cDNA was used for PCR amplification under the following conditions. Different sets of primers were designed and synthesized for PCR analysis of each gene. The primer pair used for amplifying OCTN1 was 5'-ACCTCAGTGGGTACTTTGCTC-3' and 5'-CTCCGCTGTGAAGACGTACA-3', which generated a 297-bp OCTN1 PCR product. For OCTN2, 5'-TTTCGTGGGTGTGCTGATAGTCGC-3' and 5'-GTGGAAGGCGCAACAATCCCAT-3' generated a 487-bp OCTN2 PCR product. For ATB⁰⁺, 5'-AGGTGTGGGAATCACGATG-3' and 5'-GTTCACTGGGAAGTTGTCCT-3' generated a 296-bp ATB⁰⁺ PCR product. For M-CPT I, 5'-CAGGCAAAGAGACAGACTTG-3' and 5'-CTTGCAAGAGATGAGAGTGA-3' generated a 434-bp M-CPT I PCR product. For L-CPT I, 5'-ACAGGTGGTTTGACAAGTCC' and 5'-TTGATCAAGCCTTTGCCGAAA-3' generated a 331-bp L-CPT I PCR product. Reactions were carried out under the following conditions: denaturing at 94°C for 30 sec, annealing at 58°C for 30 s and extension at 72°C for 30 sec. Primer pairs used for amplifying PGK-1 were 5'-CCCTGTTGGGGTATTTGAATGGGA-3' and 5'-TTTCATGCTGTCATGAACAGTGA-3', which generated a 555-bp PGK-1 PCR product and for amplifying PGK-2 were 5'-CCCTGTTGGGGTATTTGAATGGGA-3' and 5'-TTTCTCCCTAGGAAGGACTGTGGG-3', which generated a 467-bp PGK-2 PCR product (Mizuno *et al.* 1996). The conditions of PCR for PGK-1 and PGK-2 were denaturing at 94°C for 30 s and annealing and extension simultaneously at 68°C for 2 min. PCR products were analyzed by

agarose gel electrophoresis and visualized by staining with ethidium bromide.

Western blotting for rat OCTN2 in testicular cells

For Western blotting, rabbit anti-mOCTN2 polyclonal antibody was raised against a synthetic polypeptide with the sequence TRMQKDGEESPTVLKSTAF, corresponding to the carboxyl terminal of mOCTN2 (Tamai *et al.* 2000). The COOH-terminal of the rat homologue (TRTQKDGESPTVLKSTAF) differs from the mouse sequence in two amino acid residues, T at the third and G at the eighth position, as shown in italics (Sekine *et al.* 1998, Wu *et al.* 1999), and we confirmed that the anti-mOCTN2 antibody recognized rOCTN2 (Tamai *et al.* 2001). The sample preparation and Western blot analysis were essentially the same as described previously (Tamai *et al.* 2000, 2004).

Analytical methods

Cellular protein content was determined according to the method of Lowry (Lowry *et al.* 1951) with bovine serum albumin as the standard. Cellular uptake was usually expressed as cell-to-medium ratio (μl/mg protein), which was obtained by dividing the uptake amount (pmol/mg protein) by the concentration of test compound in the transport medium (μM = pmol/μl).

All data are expressed as means ± S.E.M., and statistical analysis was performed with Student's *t*-test. The criterion of significance was taken to be *P* < 0.05.

Results

Transport of L-[³H]carnitine in primary-cultured Sertoli cells

First, we measured the uptake of carnitine by suspended, primary-cultured Sertoli cells to examine whether a carnitine transport system was active in the cells. Uptake of L-[³H]carnitine by the cells was decreased in the absence of Na⁺ compared with that in the presence of Na⁺ (Fig. 1). Therefore, it appears that an Na⁺-dependent carnitine transport system is functional in primary-cultured Sertoli cells.

Carnitine uptake at the basolateral membrane of Sertoli cells is the first step of carnitine permeation from the systemic circulation to the testis across the BTB. To evaluate carnitine uptake at the basolateral membrane, polarized Sertoli cells are required. As it is not easy to obtain monolayers of polarized Sertoli cells, we used Sertoli cells primary-cultured on the Matrigel-coated invasion chambers. First of all, we evaluated the polarization of the cells in terms of the formation of tight-junctions between the cells by measuring the permeability of a paracellular transport marker, inulin. Figure 2 shows the permeability of [¹⁴C]inulin from the basal to the apical side in the presence of Sertoli cells (0.082 ± 0.005 μl/min/cm²) and that

Figure 1 Sodium dependence of L-[³H]carnitine uptake by suspended rat primary-cultured Sertoli cells. Uptake of L-[³H]carnitine (11.4 nM) by rat primary-cultured Sertoli cells in suspension was measured for 30 min in transport buffer (pH 7.4) in the presence (open column) or absence (closed column) of Na⁺. Na⁺ was replaced with N-methylglucamine (NMG⁺). The cells were preincubated for 20 min at 37 °C in the transport buffer (pH 7.4). Uptake was expressed as cell-to-medium ratio. Each result represents the mean ± S.E.M. (*n* = 4). *Indicates a significant difference from the uptake in the presence of Na⁺ (*P* < 0.05).

in the absence of the cells ($0.425 \pm 0.050 \mu\text{L}/\text{min}/\text{cm}^2$). There is a substantial increase of the resistance to permeation in the presence of the cells owing to the formation of tight junctions. At the same time, the electrical resistance was $196.5 \pm 5.3 \Omega \cdot \text{cm}^2$ (*n* = 18) in the presence of the cells after subtracting the electrical resistance in the cell-free chamber. The decrease of permeability of [¹⁴C]inulin and the increase of electrical resistance suggested that tight junctions are formed, based on the reported criteria, including permeability of inulin (<about $0.17 \mu\text{L}/\text{min}/\text{cm}^2$) and electrical resistance (>about $40 \Omega \cdot \text{cm}^2$) for the formation of tight junctions (Onoda *et al.* 1990). Moreover, we confirmed that Na⁺-K⁺ ATPase was

Figure 2 Permeability of [¹⁴C]inulin across rat Sertoli cells primary-cultured on Matrigel-coated invasion chambers. Rat Sertoli cells were primary-cultured on Matrigel-coated invasion chambers. Permeability of [¹⁴C]inulin (74 μg/ml) was determined in the presence (closed circles) or absence (open circles) of Sertoli cells. Each result represents the mean ± S.E.M. (*n* = 4). *Indicates a significant difference from the permeability of [¹⁴C]inulin in the absence of Sertoli cells (*P* < 0.05). When the S.E.M. is not shown, it is smaller than the symbols.

localized at the apical membrane of primary-cultured Sertoli cells by immunofluorescence analysis (data not shown). This result is consistent with the reported localization of Na⁺-K⁺ ATPase in rat testicular tissue (Byers & Graham 1990). Therefore, we evaluated the basolateral membrane uptake of [³H]carnitine using the cells cultured in Matrigel-coated invasion chambers.

The concentration dependence of carnitine uptake from the basolateral membrane was measured in the range from 10 μM to 10 mM. As shown in Fig. 3, uptake of [³H]carnitine (11.4 nM) was reduced by unlabeled carnitine in a concentration-dependent manner. Figure 4a shows the uptake of [³H]carnitine in the presence and in the absence of sodium ions. When sodium ions were replaced with Li⁺ or N-methylglucamine⁺, uptake of carnitine was significantly decreased. In Fig. 4b, the inhibitory effects of carnitine analogues and a cationic drug on [³H]carnitine uptake across the basolateral membrane of the primary cultured Sertoli cells are shown. Carnitine analogues, acetylcarnitine (100 μM) and gamma-butyrobetaine (100 μM), and the cationic drug, quinidine (500 μM), significantly decreased the uptake of carnitine. These results demonstrated that carnitine uptake by Sertoli cells across the basolateral membrane is mediated by an Na⁺-dependent transporter that is sensitive to carnitine analogues and cationic drug(s).

Expression assay of carnitine transporter and carnitine palmitoyltransferase I by RT-PCR

To clarify which transporter mediates carnitine uptake by the Sertoli cells, we examined expression of previously known carnitine transporters, OCTN1, 2 and ATB⁰⁺, in rat whole testis and the primary-cultured Sertoli cells by

Figure 3 Concentration dependence of basolateral uptake of L-[³H]carnitine in rat primary-cultured Sertoli cells. Basolateral uptake of L-[³H]carnitine (11.4 nM) in rat Sertoli cells primary-cultured on Matrigel-coated invasion chambers was measured for 30 min in transport buffer (pH 7.4) in the presence (closed column) or absence (open column) of non-labeled carnitine. The cells were preincubated for 20 min at 37 °C in the transport buffer (pH 7.4). Results were obtained by subtracting the uptakes of [¹⁴C]inulin (156 μg/ml) at 30 min, and expressed as a percentage of control uptake measured in the absence of non-labeled carnitine. Each result represents the mean ± S.E.M. (*n* = 4). *Indicates a significant difference from the control (*P* < 0.05).

Figure 4 Sodium dependence and inhibitory effects of several compounds on basolateral uptake of L-[³H]carnitine in rat primary-cultured Sertoli cells. (a) Basolateral uptake of L-[³H]carnitine (11.4 nM) in rat Sertoli cells primary-cultured on Matrigel-coated invasion chambers was measured for 30 min in transport buffer (pH 7.4) in the presence (open column) or absence of Na⁺. Na⁺ was replaced with lithium (Li⁺, dotted column) or N-methylglucamine (NMG⁺, closed column). The cells were preincubated for 20 min at 37°C in the transport buffer (pH 7.4). Results were obtained by subtracting the uptakes of [¹⁴C]inulin (156 μg/ml) at 30 min, and expressed as a percentage of the uptake in the presence of Na⁺ (control). (b) Basolateral uptake of L-[³H]carnitine (11.4 nM) in rat primary-cultured Sertoli cells was measured for 30 min in transport buffer (pH 7.4) in the presence (open column) or absence (closed column) of various compounds. Concentrations of these compounds are indicated in the figure. The cells were preincubated for 20 min at 37°C in the transport buffer (pH 7.4). Results were obtained by subtracting the uptakes of [¹⁴C]inulin (156 μg/ml) at 30 min, and expressed as a percentage of the uptake in the absence of inhibitors (control). Each result represents the mean ± S.E.M. (*n* = 4). *Indicates a significant difference from the control (*P* < 0.05).

means of RT-PCR analysis. As shown in Fig. 5a, OCTN1 and OCTN2 were both expressed in rat testis and Sertoli cells. In contrast, low-affinity carnitine transporter ATB^{0,+} was detected in rat testis tissue, but not in primary-cultured Sertoli cells.

To explore the physiological role of carnitine in testicular cells, we examined expression of carnitine palmitoyl-transferase I (CPT I), which is the rate-limiting enzyme of fatty acid oxidation (McGarry & Brown 1997), in cultured Sertoli and germ cells by RT-PCR. As shown in Fig. 5b, both subtypes of CPT I, L-CPT I and M-CPT I, were detected in both cultured Sertoli and germ cells. In this culture system, phosphoglycerate kinase (PGK) mRNA

Figure 5 Expression of carnitine transporters and carnitine palmitoyl-transferase I in testicular cells evaluated by RT-PCR analysis. The specific primers described under Materials and Methods were used for determining (a) expression of carnitine transporters in testicular tissue and Sertoli cells primary-cultured for 6 weeks, and (b) expression of carnitine palmitoyltransferase I (CPT I) in rat germ cells and Sertoli cells primary-cultured for 2 and 4 weeks or 2, 4 and 6 weeks, respectively.

switches from somatic-type PGK-1 to sperm-type PGK-2 in germ cells during development across the pachytene spermatocyte stage *in vivo* (Tamaru *et al.* 1990). The expression of PGK-2 was increased in 4-day-cultured germ cells compared with 2-day-cultured germ cells (Fig. 5b). Additionally, PGK-2 was not detected in Sertoli cells (Fig. 5b), suggesting that contamination of germ cells in the obtained Sertoli cells was negligible. Therefore, it was demonstrated that both M-CPT I and L-CPT I are expressed in both germ and Sertoli cells. Furthermore, expression of OCTN2 was demonstrated in germ cells by RT-PCR analysis (data not shown) as well as Sertoli cells.

Western blotting analysis for OCTN2 in testis and Sertoli cells

We performed Western blotting analysis using anti-OCTN2 antibody to clarify the expression of OCTN2 protein in Sertoli cells (Fig. 6). A band of 70 kDa was detected in both testis and cultured Sertoli cells, and this is larger than the size of rat OCTN2 protein estimated from the amino acid sequence, 63 kDa (Wu *et al.* 1999). So, the OCTN2 in Sertoli cells might be glycosylated. Further, the size of the band corresponded to that in kidney, where rat OCTN2 is expressed (Tamai *et al.* 2001).

Figure 6 Western blotting analysis for OCTN2 in rat testis and Sertoli cells. Expression of OCTN2 in rat whole testis, primary-cultured Sertoli cells and kidney (positive control) was examined by Western blotting analysis with affinity-purified anti-OCTN2 antibody. Ten microgram aliquots of protein for rat testis, Sertoli cells and kidney were applied and analyzed.

Accordingly, it was concluded that OCTN2 protein was expressed in Sertoli cells, as well as whole testis in rat.

Discussion

We examined carnitine transport in Sertoli cells, which constitute the BTB. First, we demonstrated that the primary-cultured Sertoli cells used in the present study form tight junctions, by means of electrical resistance and inulin permeability measurements (Fig. 2), and we established the absence of contamination with spermatogenic cells by confirming the selective expression of markers for the germ cells, such as PGK gene. These results are consistent with the idea that rat Sertoli cells cultured on a Matrigel-coated invasion chamber are useful for evaluating carnitine uptake at the basolateral membrane of Sertoli cells, which is an important step in the supply of carnitine to testis.

Secondly, we demonstrated that carnitine uptake from the basolateral membrane of Sertoli cells was concentration- and Na^+ -dependent, and was inhibited by carnitine analogues and by a cationic drug. Although the concentration dependence was not analyzed kinetically, the results in Fig. 3 suggest that the half-saturation concentration for carnitine uptake was about $10 \mu\text{M}$. The estimated half-saturation concentration is comparable to the K_m values of human and rat OCTN2 for carnitine transport, $4.3 \mu\text{M}$ and $14.8 \mu\text{M}$ respectively (Tamai *et al.* 1998, Wu *et al.* 1999). Inhibitors of OCTN2, acetylcarnitine, gamma-butyrobetaine and quinidine, also significantly reduced carnitine uptake across the basolateral membrane of Sertoli cells. Furthermore, expression of OCTN2 was observed in Sertoli cells and whole testis tissues by RT-PCR and Western blotting analysis (Fig. 5a and 6). All of these observations suggested that carnitine uptake across the basolateral membrane of Sertoli cells is mediated by OCTN2.

OCTN2 is functionally expressed at blood–tissue barriers, such as the blood–brain barrier (Kido *et al.* 2001, Inano *et al.* 2003) and the blood–placenta barrier (Lahjouji *et al.* 2004, Shekhawat *et al.* 2004). Accordingly, OCTN2 may play a role in transferring carnitine across

these tissue barriers. In the present study, an isoform of OCTN2, OCTN1 was also expressed in Sertoli cells and whole testis (Fig. 5a). Human and mouse OCTN1 transported carnitine, while rat OCTN1 did not exhibit carnitine transport activity (Wu *et al.* 2000). The low-affinity carnitine transporter $\text{ATB}^{0,+}$ (Nakanishi *et al.* 2001) was not detected in Sertoli cells (Fig. 5a). These results suggested that OCTN1 and $\text{ATB}^{0,+}$ may not be involved in carnitine transport in rat Sertoli cells. OCTN3 and CT2, which are carnitine transporters expressed in mouse and human testis, may not be important for basolateral transport of carnitine in rat Sertoli cells, since Na^+ -independent carnitine transporters like OCTN3 or CT2 were not detected as shown in Fig. 4a. The lack of those types of transporters in rat Sertoli cells was not examined, since no corresponding rat molecules have been identified yet. In humans, sodium-dependent OCTN2 is expressed in testis (Tamai *et al.* 1998), and human carnitine transporter CT2, which is a sodium-independent carnitine transporter, is also expressed in testis (Enomoto *et al.* 2002). Therefore, multiple transporters that have different functionality should contribute to transport of carnitine in human Sertoli cells.

OCTN2 is a multi-specific transporter, transporting cationic compounds as well as carnitine, and various drugs, such as verapamil, mepyrmine and quinidine, which are strong inhibitors of OCTN2-mediated transport of carnitine (Ohashi *et al.* 1999, 2001, 2002). Accordingly, carnitine supply to the testis by OCTN2 might be disturbed by these cationic compounds. Furthermore, OCTN2 may mediate entry of those cationic xenobiotics to the testis from the systemic circulation, suggesting that OCTN2 might be relevant to the testicular toxicity of externally administered drugs/xenobiotics. Additionally, we examined whether carnitine which was taken up by the Sertoli cells could permeate to the apical side across Sertoli cells by measuring permeation of carnitine from the basolateral to the apical compartment by using rat Sertoli cells cultured on a Matrigel-coated invasion chamber. Permeability clearance of $[^3\text{H}]$ carnitine ($0.69 \pm 0.05 \mu\text{l}/\text{min}/\text{cm}^2$) was higher than that of $[^{14}\text{C}]$ inulin ($0.14 \pm 0.01 \mu\text{l}/\text{min}/\text{cm}^2$). Although molecular weights of carnitine and inulin were different, 161 and about 4000 respectively, and their paracellular permeabilities should be different, observed five-times higher permeability of carnitine than inulin indicates that carnitine taken up by the Sertoli cells are supplied to the seminiferous tubular fluid across the BTB.

It was reported that both M-CPT I and L-CPT I, which are rate-limiting enzymes for fatty acid oxidation (McGarry & Brown 1997), are expressed in rat and mouse testis (Brown *et al.* 1997). Adams *et al.* (1998) reported that M-CPT I is mainly expressed in spermatocytes and spermatids. In the present study, expression of M-CPT I was abundant in germ cells compared with Sertoli cells (Fig. 5b), and this result accords well with previous observations (Adams *et al.* 1998). In addition, other studies have indicated that germ cells contain the key enzyme for

oxidation of fatty acids (Bajpai *et al.* 1998), and spermatogonia and spermatocytes exhibit relatively high rates of palmitate oxidation (Lin & Fritz, 1972). Accordingly, carnitine should be essential for germ cells, though the mechanism of the supply of carnitine to germ cells has not been elucidated. OCTN2, which was expressed in germ cells, may be contributing to carnitine transport, although further studies are required to determine the relative contribution of OCTN2 in these cells.

Interestingly, we found that both L-CPT I and M-CPT I are expressed in Sertoli cells (Fig. 5b), suggesting that carnitine can be passed from the blood circulation to Sertoli cells, as well as germ cells. This speculation was supported by the findings that oxidation of fatty acids is likely to be the major energy source for Sertoli cells (Jutte *et al.* 1985) and that L-carnitine treatment significantly reduced non-esterified fatty acids in cultured rat Sertoli cells (Palmero *et al.* 2000).

In conclusion, primary-cultured Sertoli cells are a useful tool for studying transport across the basolateral membrane of Sertoli cells, and we established that uptake of carnitine at the basolateral membrane of these cells is mainly mediated by Na⁺-dependent transporter, OCTN2.

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