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Distribution, Molecular Structure and Functional Analysis of Carnitine Transporter (SLC22A5) in Canine Lens Epithelial Cells

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Abstract: While carnitine has been reported to have an anti-oxidative role on the ocular surface, there has been no report on the existence of a carnitine transporter (SLC22A5) in the lens. Therefore, we investigated the carnitine transport activity of canine lens epithelial cells (LEC) and determined the molecular structure of canine *SLC22A5*. The carnitine transport activity was 7.16 ± 0.48 pmol/mg protein/30 min. Butyrobetaine, the analogue of carnitine, reduced 30% of the activity at 50 μ M. A coding sequence of canine carnitine transporter was 1694 bp long and was predicted to encode 557 amino acid polypeptides. The deduced amino acid sequence of canine carnitine transporter showed >80% similarity to that of mouse and human. Western blot analysis detected the band at 60 kDa in the membrane of lens epithelial cells. The high content of carnitine in the lens is possibly transported from aqueous humor by SLC22A5.

Key words: carnitine, cataract, lens epithelial cells, oxidative stress, SLC22A5

Introduction

Many amino acid transport systems are distinguished based on differences in their substrate-selectivity, ion-dependence, pH sensitivity, and kinetics and regulatory properties, using membrane vesicle preparation or cultured cells [3, 4]. The carnitine (3-hydroxy-4-*N*-trimethylaminobutyric acid)/organic cation transporters are classified as organic ion transporters in the subfamily of 22A, which belongs to the solute carrier (SLC) transporter superfamily that includes organic cation transporters (OCTs), organic anion transporters (OATs) and carnitine/organic cation transporters (OCTNs). OCTNs are unique in recognizing zwitterionic carnitine as an endogenous substrate, and they also recognize various xenobiotics, mainly cationic compounds. Among them,

OCTN2 (SLC22A5) is an Na-dependent, high-affinity carnitine transporter, which maintains levels in serum by functioning as a reabsorption transporter after glomerular filtration in kidney [2, 20, 25]. Carnitine is a small, water-soluble molecule that has important physiological roles, including involvement in the β -oxidation of fatty acids by facilitating the transport of long-chain fatty acids across the mitochondrial inner membrane as their acylcarnitine esters, and modulation of intracellular CoA homeostasis [2, 20]. Carnitine deficiency causes severe pathological symptoms such as cardiomyopathy and muscle weakness [14, 24, 28, 31].

Lens epithelial cells (LECs) are the progenitors of the lens fibers *in vivo* and undergo a developmental transition into fiber cells of the lens cortex, a process characterized by distinct biochemical and morphologic chang-

(Received 4 March 2014 / Accepted 28 May 2014 / Published online in J-STAGE 22 July 2014)

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Table 1. Sequences of oligonucleotides used in this study

Primer		Sequence (5'-3')	Expected Product (bp)
Oligonucleotide for cloning of canine			
s1	sense	SLC22A5 GCAGCTGGAGCAGGAGAGCTG	1,096
a1	anti	AGGCGGGATGCTGTGGAGCTGAC	
human <i>SLC22A5</i> : AK313230			
GSP1 (for 5'RACE)	anti	CCTGGTAGCGTCAGTGCCAGCAGCAGC	
GSP2 (for 3'RACE)	sense	TCCATGGCCACAGCCCTCTTCCTGGGC	
Oligonucleotide for RT-PCR transcript			
canine <i>SLC22A5</i>	sense	CGGGGCAGCTCTCAGACAGGTTTGCC	321
	anti	CCTGGTAGCGTCAGTGCCAGCAGCAGC	
<i>GAPDH</i> (AB038240)	sense	ATC ACC ATC TTC CAG GAG CGA GA	192
	anti	GTC TTC TGG GTG GCA GTG ATG G	

(accession number) is indicated.

es such as the synthesis of crystallin proteins, cell elongation, loss of cellular organelles, and disintegration of the nucleus. It is generally understood that primary damage to LECs may cause abnormal differentiation of epithelial cells to lens fibers, which is eventually expressed as an opacity of lens tissues, i.e., a cataract. Notably, concentration of carnitine was reported to be highest in the lens among ocular tissues [19], and a dramatic depletion of lenticular carnitine was reported one of the early events in streptozocin-induced cataract in rat [18]. In addition, acetyl-L-carnitine, which is naturally produced by the enzyme acetyl carnitine transferase [9], prevents selenite-induced and L-buthionine-(S,R)-sulfoximine-induced cataractogenesis in animal models [6, 7]. It was reported that human corneal and conjunctival epithelia possessed distinct carnitine transport activity by *SLC22A5*, suggesting an osmoregulatory role of these tissues [32]. In spite of the significance of carnitine on the ocular surface, there has been no report on carnitine transport activity in LECs. Previously, we developed the canine lens epithelial cell line and reported several of its characteristics [12]. In this study, we examined the carnitine transport activity of this cell line, determined its molecular structure, and investigated the distribution of canine *SLC22A5*.

Materials and Methods

Animal and cell samples

All experiments were performed according to the guidelines of The Laboratory Animal Care Committee of Azabu University, and were in compliance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research

Institutions. The canine lens epithelial cell line originating from mature cataract was maintained as described previously [12]. Several tissues were obtained from a 5-year-old male Japanese Shiba dog after deep anesthesia by pentobarbital.

Measurement of carnitine transport activity

Radioactive (^3H -)carnitine was purchased from American Radiolabeled Chemicals (St Louis, MO, USA). Carnitine uptake was measured as described previously [16]. Simply, the cells were plated in 5×10^5 cells/6-well plate 24 h before the experiment. After washing the cells, the medium containing 100 μM carnitine with radioisotope (185 kBq/ml) was added and incubated at 37°C. To study the effect of butyrobetaine, the analogue of carnitine, on transport activity, 50 μM butyrobetaine was included in an incubation medium containing radio-labeled carnitine. Uptake was terminated by washing with ice-cold phosphate-buffered saline. After solubilizing the cells with 1% SDS, the radioactivity was measured with a liquid scintillation counter and protein content was determined by Micro BCA method.

Determination of cDNA sequence of canine carnitine transporter

The cDNA sequence of canine carnitine transporter was determined as described previously [15]. Briefly, total RNA from the lens epithelial cell line was isolated using an RNA extraction solution (Isogen, Nippon Gene, Tokyo, Japan). The primers used in this study are shown in Table 1. The primer set (s1 and a1) for the amplification of a partial canine *SLC22A5* cDNA was prepared from the conserved region sequences between humans and mice (DDBJ accession No. AK313230 and

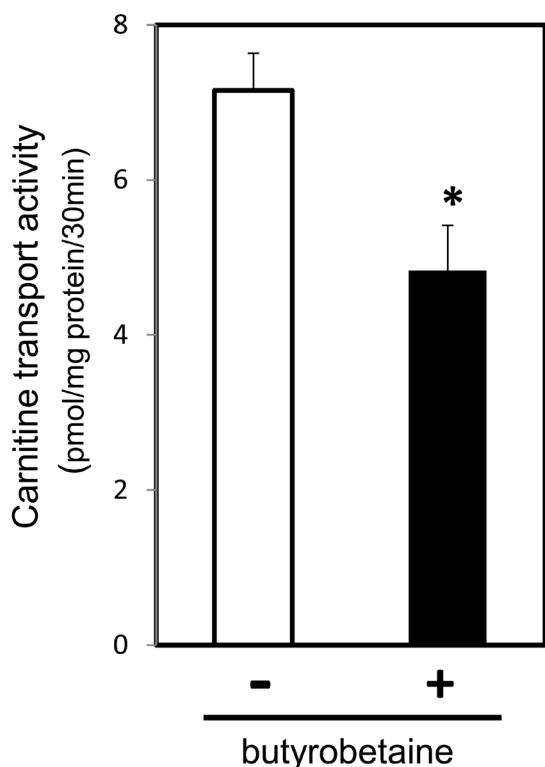


Fig. 1. Carnitine transport activity of LEC in the presence (closed column) or absence (open column) of 50 μ M butyrobetaine. The experiment was performed in triplicate using 6-well plates. The values are means and SD of 4 individual experiments. Statistical analysis was performed by use of Welch's *t* test. The criterion significance was taken to be $P < 0.05$ (*).

BC031118, respectively). RT-PCR amplification was performed employing a SuperscriptIII first cDNA system kit (Invitrogen, Carlsbad, CA, USA) with Hot start Ex Taq DNA polymerase (Takara Bio, Otsu, Japan). The band was excised from the agarose gel and purified using a Wizard SV gel clean-up system (Promega, Tokyo, Japan). The extracted and purified DNA was cloned into a pCR II-TOPO cloning vector (Invitrogen) and sequenced with a BigDye terminator kit ver.3 (Applied Biosystems, Carlsbad, CA, USA). The nucleotide sequence obtained exhibited high similarities (>85%) to human *SLC22A5* cDNA sequences. In order to determine the 3' and 5' regions of cDNA, RACE methods were carried out using a SMARTer RACE cDNA amplification kit (Clontech, Mountain View, CA, USA) and a set of canine *SLC22A5* gene-specific primers (GSP1 and GSP2).

RT-PCR analysis of carnitine mRNA in canine tissues

In order to examine the expression of carnitine transporter mRNA in various organs of a dog, we performed RT-PCR using newly designed primers specific to canine carnitine transporters (GSP1 and GSP2: Table 1). RT-PCR conditions for *SLC22A5* were as follows: 35 cycles of three steps; 94°C for 15 sec, 68°C for 15 sec, and 72°C for 20 sec, while conditions for GAPDH were 30 cycles of three steps; 94°C for 15 sec, 60°C for 15 sec, and 72°C for 20 sec with Hot start Ex Taq DNA polymerase.

Western blot analysis

To investigate the expression of carnitine transporter protein in the membrane, anti-canine *SLC22A5* serum was prepared with the peptide antigen designed according to a C-terminus amino acid sequence of canine carnitine transporter (Fig. 3. amino acid 537–550, C-GHTRMLKDSESSSI). Peptide conjugated with key-hole limpet hemocyanin (KLH) was purchased from the laboratory of Operon (Tokyo, Japan). Anti-canine carnitine transporter serum was purified by passage over an affinity column of the peptide antigen. Cell membranes of the tissues for Western blot analysis were prepared as reported by Denker *et al.* [5]. In brief, the cells were homogenized at 4°C in a buffer containing 0.1 M KCl, 5 mM Na₂HPO₄ pH 7.5, 0.75 mM Na-EGTA pH 7.5, 1 mM DTT, 5 mM MgCl₂, 200 μ g/ml phenylmethylsulfonyl fluoride, and 4 μ g/ml leupeptin. Homogenates were centrifuged for 10 min to remove debris. The 1 ml supernatant was laid over a 5 ml sucrose solution containing 0.8 mM sucrose and 2 mM Na-EGTA, and was centrifuged at 32,000 g for 40 min. Protein concentration of the pellet was determined by the BCA method, and was used for Western blot analysis as previously described [15]. The membrane-rich fraction was simply solubilized, electrophoresed into 12% polyacrylamide gels, and immunoblotted with a chemiluminescence autoradiograph. The membrane-rich fraction was then treated with a primary antibody against C-terminus of canine *SLC22A5*, followed by a secondary antibody (anti-rabbit IgG (H+L) goat IgG Fab' HRP, $\times 20,000$, Seikagaku Corp., Tokyo, Japan). The *SLC22A5* protein was detected with an ECL plus a chemiluminescence detection system (GE Healthcare Bioscience, Chalfont, UK) and exposed to an x-ray film. To test the specificity of anti-serum, 1 ml of the purified anti-serum was absorbed with 1 mg of the peptide (C-GHTRMLKDSESSSI) at 4°C over night.

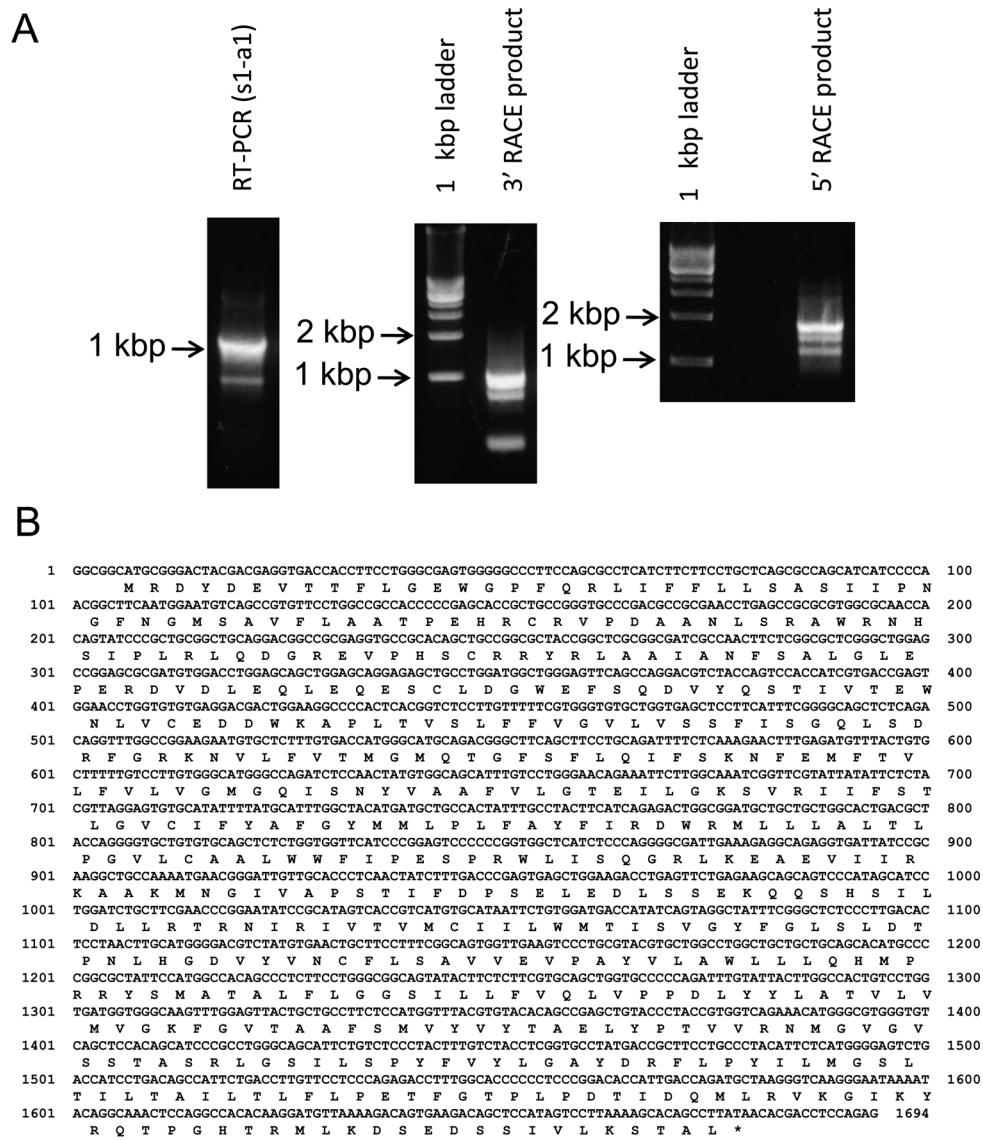


Fig. 2. PCR-based cloning of 5'- and 3'-stretched cDNA clones for canine carnitine transporter (A). Electrophoresis of RT-PCR products of canine *SLC22A5* using primers (s1 and a1) well conserved between humans and rodents (A) (left). 5' and 3' RACE products with the primers shown in Table 1 (right). Nucleotide and deduced amino acid sequence of canine are shown (B). Full-length canine carnitine transporter cDNA was 1694 bp in length and contained an entire open reading frame of 1671 bp, encoding canine carnitine transporter of 557 amino acids. The termination codon is asterisked.

This pre-absorbed anti-serum was also used for Western blot analysis.

Results

Carnitine transport activity of the canine lens epithelial cell line as indicated in Fig. 1. The transport activity was 7.16 ± 0.48 nmol/mg protein/30 min, while the presence of $50 \mu\text{M}$ butyrobetaine, the specific inhibitor

of carnitine transporter, reduced transport activity by 30%. Figure 2 shows the electrophoresis of RT-PCR products of canine carnitine transporters using primers (s1 and a1), that were well-conserved between human and rodent. The nucleotide sequence comprising 1,096 bp showed high similarities to rat carnitine cDNA sequences. The primers used for RACE were prepared from the sequence details listed in Table 1. An obtained nucleotide sequence corresponding to a full-length canine carnitine

dog	1:	MRDYDEVTFITLGEWGPQRLLIFLLSASII	PNGFNGMSAVFLAATPEHRCRVPDAANLSSAWRNH	SIPLRQLQDREVP	PHSCRRYRLAAIANFSALGLEPE	100
human	1:	MRDYDEVTAFLGEWGPQRLLIFLLSASII	PNGFTGLSSVFLIATPEHRCRVPDAANLSSAWRNHT	VPLRLRDGREVP	PHSCRRYRLATIANFSALGLEPG	100
mouse	1:	MRDYDEVTAFLGEWGPQRLLIFLLSASII	PNGFNGMSIVLAGTPEHRCVLPHTVNLSSAWRNH	SIPLETKDRQVQP	KCRRYRLATIANFSELGLEPG	100
		*****	*****	*****	*****	*****
dog	101:	RDVDLEQLEQESCLDGEWFSQDVYQSTIVTEWNLV	CEDDWDKAPLTVSLFFVGVVLSFISGQLSDRFRGR	KNVLFVMTGMQ	TGFSFLQIFSKNFEMFTVLF	200
human	101:	RDVDLQLEQESCLDGEWFSQDVLSTIVTEWNLV	CEDDWDKAPLTVSLFFVGVVLLGSFISGQLSDRFRGR	KNVLFVMTGMQ	TGFSFLQIFSKNFEMFVVLF	200
mouse	101:	RDVDLEQLEQESCLDGEWYDKDVFVLSLTIIVTEW	DLVCKDDWAPLTVSLFFVGVVLMGSFISGQLSDRFRGR	KNVLFVMTGMQ	TGFSFLQVFSVNFEMFTVLF	200
		****	*****	*****	*****	*****
dog	201:	VLVGMGQISNYVAAFVLTGEILGKSVRIIFSTL	GVCIIFYAFGYMMLPLFAYFIRDWRMLLLAL	TLPGVLCALW	WVFPESPRWLISQGR	KEAEVIRKA
human	201:	VLVGMGQISNYVAAFVLTGEILGKSVRIIFSTL	GVCIIFYAFGYMMLPLFAYFIRDWRMLLLAL	TMPGVLCALW	WVFPESPRWLISQGR	FEEAEVIRKA
mouse	201:	VLVGMGQISNYVAAFVLTGEILSKSIRIIFATL	GVCIIFYAFGMVLPFLFAYFIRDWRMLLLAL	TPGVLCALW	WVFPESPRWLISQGR	IKAEAEVIRKA
		*****	*****	*****	*****	*****
dog	301:	AKMNGIVAPSTIFDPSELEDLSSEKQOSSH	SILDLLRTRNIRIVTVMCIILWMTISVGYFGL	SLDTPNLHGD	VYVNCFLSAVVEFPAYVLA	WLLQHMPPR
human	301:	AKANGIVVPSITIFDPSELQDLSKQOSSH	NIIDLLRTRNIRIVTVMCIILWMTISVGYFGL	SLDTPNLHGD	IVNCFLSAMVEFPAYVLA	WLLQYLPPR
mouse	301:	AKINGIVAPSTIFDPSELQDLSNTPKQHH	IYDLIRTRNIRIVTVMCIILWMTISVGYFGL	SLDTPNLHGD	IYVNCFLLAAVEFPAYVLA	WLLQYLPPR
		**	****	*****	*****	*****
dog	401:	YSMATALFLGGSVLLFMQVLPDLYLATVLM	VMGKFGVTAAFSMVYVYTAELYPTVVRNMG	VGSVSTASRLG	SILSPYFVYLGA	YDRFLPYILMGSLTI
human	401:	YSMATALFLGGSVLLFMQVLPDLYLATVLM	VMGKFGVTAAFSMVYVYTAELYPTVVRNMG	VGSVSTASRLG	SILSPYFVYLGA	YDRFLPYILMGSLTI
mouse	401:	YSISAALFLGGSVLLFMQVLPSELFYLS	TALVMGKFGITSAFMSVYVYTAELYPTVVRNMG	VGSVSTASRLG	SILSPYFVYLGA	YDRFLPYILMGSLTI
		**	*****	*****	*****
dog	501:	<u>LTAILTLFLPETFGTPLPDTIDQMLRVKGI</u>	<u>KYRQTPGHTRMLKDESDSSIVLKSTAL</u>			557
human	501:	<u>LTAILTLFLPESFGTPLPDTIDQMLRVKGM</u>	<u>KHRKTPSHTRMLKDGQERPTILKSTAF</u>			557
mouse	501:	<u>LTAILTLFFPESFGVPLPDTIDQMLRVKGI</u>	<u>QWQIQSQTRMQKDGESPTVLKSTAF</u>			557
		*****	*****	*****	*****	*****

Fig. 3. Amino acid sequences of canine *SLC22A5* were compared with those of human and mouse. Multiple sequence alignments were performed using the Genetyx program (ver. 10). Asterisks and dots indicate identical residues and conservative substitutions, respectively. Amino acid sequence used for preparation of anti-canine *SLC22A5* serum was overlined.

transporter cDNA was 1,719 bp in length (DDBJ accession number AB923814) and contained an entire open reading frame of 1,671 bp, encoding a canine carnitine transporter of 557 amino acids and having a theoretical mass of 63 kDa (Fig. 2B). Comparison of cDNA sequence of obtained in this study and Genbank XM_855641 (putative canine *SLC22A5*) indicated the difference in 3 nucleotide (nt945, 949 and 951) and 1 amino acid (aa315: phenylalanine and serine). Figure 3 shows a comparison of a canine carnitine transporter with those reported from humans and rodents. The deduced amino acid sequence of the carnitine transporter showed very close similarities of 82% to those of mouse and human. RT-PCR analysis of carnitine transporter expression in various canine tissues is indicated in Fig. 4. A single distinct band was observed in all the samples examined. Western blot analysis using antiserum against C-terminus of canine carnitine transporter detected a band at 60 kDa in the LEC (Fig. 5). This band disappeared when a primary antibody was pre-absorbed with C-terminus peptides.

Discussion

Cataracts are one of the most significant ophthalmologic diseases in veterinary medicine. It is well-under-

stood that dogs are more prone to develop cataracts than other domestic animals.

The lens undergoes major oxidative stress because it is constantly exposed to light and oxidants [23, 27, 29]. The first line of defense against oxidation stress is constituted by radical scavenging antioxidants that reduce the oxidative insult. For example, glutathione, ascorbic acid and taurine, which are all highly represented in lens tissue, exert protective effects in an *in vitro* model of a diabetic cataract [11, 21, 30]. Among ocular tissues, carnitine was highest in the lens [19], and the carnitine level of lenses with deep opacification was lower than that of a normal lens, suggesting its protective role in the development of cataract [8]. In fact, carnitine protected against damage produced by gamma radiation by increasing the activity of the enzyme super oxide dismutase, and by scavenging free radicals that were generated by ionizing radiation [13]. While carnitine has been reported to have an anti-oxidative or anti-radical role in the ocular surface, there has not been a report on the evidence of the existence of carnitine transporter in lens.

In this study, we conducted a functional analysis of carnitine transport activity in LECs and revealed the certain butyrobetaine-sensitive components of carnitine transport activities. We determined a full-length cDNA sequence of canine *SLC22A5* based on a conserved

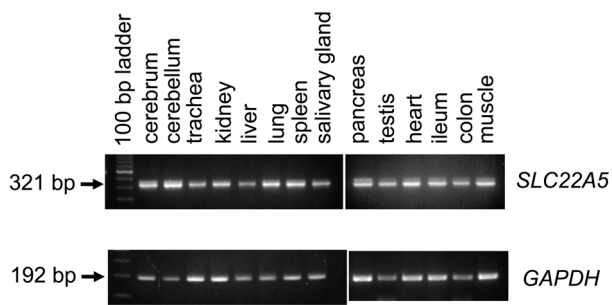


Fig. 4. RT-PCR analysis of mRNA of canine *SLC22A5* in various dog tissues (A). Integrity of RNA was examined by glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

cDNA sequence among mammals. The deduced amino acid sequence of canine *SLC22A5* showed high similarities to those of human and mouse. RT-PCR analysis confirmed that carnitine transporter was detected all tissues examined. Expression of human *SLC22A5* was reported to be ubiquitous, but it was more so in kidney, muscle, and heart, and less so in pancreas, liver, lung, brain, and small intestine, by Northern blot analysis [26]. Precise evaluation of canine *SLC22A5* may be necessary using Real-time RT-PCR analysis. Butyrobetaine reduced only 30% of the carnitine transport activity of LEC. There was reportedly other carnitine transporters, for example *SLC22A16* [1]. Therefore, other carnitine transporters may possibly exist in LECs. While the canine cDNA sequence that possessed high homology with *SLC22A5* was clarified in this study, there is no evidence of this putative canine *SLC22A5* transport carnitine. Next, we will examine the functional analysis of canine *SLC22A5* by transfection study.

Recently, Peluso *et al.* reported that carnitine safeguarded the chaperone activity of α -crystallin, which prevents protein aggregation in the lens by decreasing the protein posttranslational modifications induced by oxidative stress [17]. Posttranslational modifications of crystallin, consequent to aging or diabetes, resulted in conformational changes and aggregation of the proteins and led to lens opacification [10]. As the fluid of the ocular surface contains certain a level of carnitine [22], carnitine in lens may possibly be transported by *SLC22A5* from aqueous humor to protect crystallins from oxidative stresses. In summary, we revealed the carnitine transport activity in LECs, determined the molecular structure of canine *SLC22A5*, and investigated its distribution in several tissues. To our knowledge, this is the

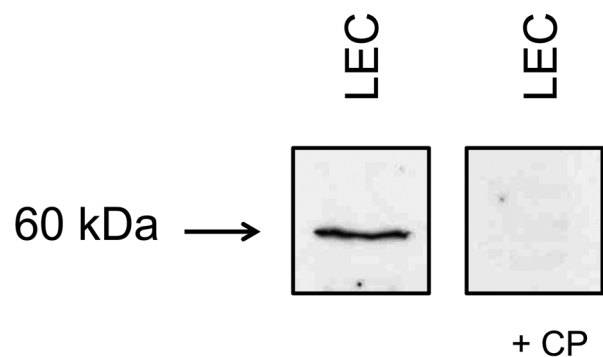


Fig. 5. Western blot analysis of *SLC22A5* expression using anti-serum against the C-terminus of canine *SLC22A5* peptides. CP represents antiserum pre-absorbed with its corresponding antigenic peptides.

first report on carnitine transport activity in LECs. The data in this paper may facilitate the study of the carnitine metabolism and the function in lens.

Acknowledgments

This study was supported in part by a Grant-in-aid to HO from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 19580376), and by a research project grant awarded by the Azabu University Research Services Division.

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