Functional Characterization of Ergothioneine Transport by Rat Organic Cation/Carnitine Transporter Octn1 (slc22a4)

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It has been reported that organic cation/carnitine transporter 1 (OCTN1) is associated with rheumatoid arthritis and Crohn's disease. Additionally, we reported that OCTN1 is expressed in hematopoietic cells, and is associated with proliferation and differentiation of erythroid cells. However, physiological role of OCTN1 is still unclear. Ergothioneine, an anti-oxidant, was recently reported to be a good substrate of human OCTN1. However, the transport characteristics of ergothioneine in rat remains to be clarified. The present study, is to further investigate the role of rat Octn1 on transport of ergothioneine in rat Octn1 transfected cells and natively expressing cell line PC12 derived from rat adrenal pheochromocytoma. [^{3}H]Ergothioneine uptake by rat Octn1 stably transfected HEK293 cells was saturable, sodium dependent with 1 : 1 stoichiometry of ergothioneine, and pH dependent. Since ergothioneine was reported to presumably play a protective role against oxidative stress-induced apoptosis in PC12 cells, its transport in this cell line was investigated. The expression of rat Octn1 and a saturable and Na^+-dependent transport of ergothioneine were observed in PC12 cells, suggesting that ergothioneine transport in this cell line may be mediated by rat Octn1. These findings suggested that rat Octn1 may act as a survival factor by taking up ergothioneine to suppress oxidative stress in this cell line. In conclusion, functional characteristics of ergothioneine transport by rat Octn1 is similar to that of human OCTN1 and it is suggested that rat Octn1 is important by transporting anti-oxidant ergothioneine in PC12 cells, though its role *in vivo* is to be investigated.

Key words organic cation/carnitine transporter 1; ergothioneine; transporter; anti-oxidant; PC12; carnitine

Organic cation/carnitine transporter OCTN1 (*SLC22A4*) shows a broad tissue distribution and is the first pH-dependent organic cation transporter.[1,2] The OCTN family consists of three members, that are, OCTN1 and OCTN2 (*SLC22A5*) in mice, rats, and humans, and Octn3 in mice. Among them, OCTN2 and Octn3 exhibit predominant carnitine transport activity in Na^+-dependent and independent manner, respectively, while OCTN1 has relatively low carnitine transport activity. Taking these findings into consideration, OCTN1 may have a different physiological role from OCTN2 or Octn3.[3—5] Based on expression of OCTN1 in kidney, we previously found that OCTN1 was localized on the apical membrane of renal proximal tubular epithelial cells and suggested that it is involved in the tubular secretion of cationic compounds.[6] Furthermore, based on preferential expression of OCTN1 in erythroid cells,[7] we also suggested that its physiological role is closely associated with differentiation and/or growth of blood cells,[8] while its physiological substrates have not been clearly identified yet.

Interestingly, there are several reports on the relationship of OCTN1 with autoimmune diseases, which may be useful to clarify the physiological roles of OCTN1. The genetic polymorphisms of OCTN1 gene are associated with chronic inflammatory diseases such as rheumatoid arthritis (RA)[9] and Crohn's disease (CD).[10,11] On the other hand, we recently found an increase of human OCTN1-mRNA expression by inflammatory cytokine *via* nuclear factor-κB (NF-κB) activation.[12] Moreover, ergothioneine was found to be a good substrate of human OCTN1,[13] and ergothioneine exhibited stimulatory effects on proliferation of human OCTN1-overexpressing Caco-2 cells that are derived from human colonic carcinoma.[14] In addition, some cases of RA patient are associated with a high level of ergothioneine in red blood cells.[15] Ergothioneine is a unique naturally occurring antioxidant that is abundant in most plants and animals and plays dual roles in both energy regulation and protecting cells from oxidative stress, while its function has not been fully understood.[16] Since ergothioneine is not synthesized in human, it is needed to be taken from dietary sources.[17]

Although the information on the characteristics of OCTN1 has been accumulated, its physiological role has not been determined yet. Wu *et al.* reported that rat Octn1 mediates Na^+-independent and pH-dependent transport of a prototypical organic cation tetraethylammonium (TEA).[18] Rat Octn1 interacts with a variety of structurally diverse organic cations such as desipramine, dimethylamiloride, cimetidine, procainamide, and verapamil, while carnitine, a zwitterion, interacts with rat Octn1 with a low affinity, suggesting that rat Octn1 is a multispecific organic cation transporter.[18] In addition, human OCTN1 is an Na^+-independent and pH-dependent transporter of TEA[1,2] and Na^+- and pH-dependent transporter of ergothioneine.[13,14]

Until now, most studies of OCTN1 were done for human OCTN1,[1,2,6,13] while the studies on OCTN1 in experimental animals, which are useful for elucidation of roles of OCTN1, are very limited.[18] Furthermore, in order to investigate physiological role of OCTN1, it is useful to obtain more information using the cells that natively expressing OCTN1. Accord-

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ingly, in the present study we investigated the characteristics of rat Octn1, mainly focusing on ergothioneine transport by rat Octn1-transfected cells. Furthermore, to explore physiological roles of rat Octn1, ergothioneine transport in natively expressing cell line, PC12 cells, was investigated.

MATERIALS AND METHODS

Chemicals [^{3}H]Ergothioneine (3.7 GBq/mmol) was custom made by Moravec Biochemicals (Brea, CA, U.S.A.). Unlabeled ergothioneine and other reagents for cell culture and transport experiments were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Sigma-Aldrich (St. Louis, MO, U.S.A.).

Cell Culture HEK293 and PC12 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA, U.S.A.) and from RIKEN BioResource Center (Tsukuba, Japan), respectively. HEK293 cells and PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, U.S.A.) and DMEM/F12 1 : 1 medium (Sigma-Aldrich), respectively, containing 10% (v/v) fetal bovine serum (FBS) (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin, and at 37 °C in an atmosphere of 5% CO_2.

Establishment of Rat Octn1-Expressing HEK293 Cell Line The cDNA of rat Octn1 was cloned by reverse transcription polymerase chain reaction (RT-PCR) from rat liver as follows. Primers for PCR that are specific for rat Octn1 were designed on the basis of the sequence information of GenBank accession number AF169831. The isolated rat Octn1 cDNA was checked by sequencing and was subcloned to the plasmid vector pcDNA3.1 (Invitrogen). HEK293 cells were transfected with pcDNA3.1 plasmid vector containing rat Octn1 (pcDNA3.1/*rOctn1*) to establish stably rat Octn1-expressing cells according to the calcium phosphate precipitation method.[19] After 3-week cultivation with 600 μg/ml G418 (Sigma-Aldrich), several single colonies were obtained and examined for rat Octn1 expression by both PCR analysis and [^{3}H]ergothioneine transport activity. Obtained HEK293 cells were routinely grown in DMEM containing 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G418 in a humidified incubator at 37 °C in an atmosphere of 5% CO_2. HEK293 cells transfected with plasmid vector alone (pcDNA3.1) were used to obtain the background activity (Mock).

Transport Experiments For the transport experiments of [^{3}H]ergothioneine by HEK293 cells, the cells were harvested and suspended in transport medium containing 125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES (pH 7.4). The cell suspension and transport medium containing a radiolabeled ergothioneine were preincubated separately for 20 min and then mixed to initiate the uptake reaction. At appropriate times, 200-μl aliquots of the mixture were withdrawn, and the cells were separated from the transport medium by centrifugation in a microtube containing a mixture of silicone oil and liquid paraffin with a density of 1.03 on the layer of 3 N KOH. The resultant cell pellets solubilized in 3 N KOH were neutralized with HCl, and the associated radioactivity was quantitated in a liquid scintillation counter (Aloka, Tokyo, Japan).

Reverse Transcription Polymerase Chain Reaction Total RNA was prepared from PC12 cells using ISOGEN (Nippon Gene, Toyama, Japan) and its content was determined by measuring the absorbance at 260 nm. mRNA of Octn1 was detected by RT-PCR. Single-strand cDNAs were constructed using an oligo(dT) primer (Invitrogen) and Im-Prom-II^{TM} reverse transcriptase (Promega, Madison, WI, U.S.A.). The PCR for rat Octn1 was performed with the following specific primers: rat Octn1 sense ACCTCAGTGGGTTACTTTGCTC, rat Octn1 anti-sense CTCCGCTGTGAAGACGTACA. Amplification condition for PCR was denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s through a total of 28 cycles. The PCR products were separated by electrophoresis in 2% agarose. The fluorescence intensity of each band after staining with ethidium bromide was detected using Light Capture^{TM} (Atto Co., Tokyo, Japan).

Analytical Methods Cellular protein content was determined according to the method of Bradford using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) with bovine serum albumin as a standard.[20] Cell-to-medium ratio (μl/mg protein) was obtained by dividing the uptake amount in the cells by the concentration of test compound in the transport medium. To estimate kinetic parameters for saturable transport and the stoichiometry between Na^+ and ergothioneine, the uptake rate was fitted to the following equations by means of nonlinear least-squares regression analysis using MULTI program.[21]

$$v = V_{max} \cdot [s]/(K_m + [s]) \quad (1)$$

$$v' = V_{max} \cdot [Na^+]^n/(K^n_{mNa+} + [Na^+]^n) \quad (2)$$

where v and v' and [s] are the uptake rates and concentration of ergothioneine, respectively. K_m, V_{max}, and n are the half-saturation concentration (Michaelis constant), the maximum uptake rate, and the Hill coefficient, respectively. All data were expressed as the mean±S.E.M., and statistical analysis was performed by Student's t-test. The criterion of significance was set at the level of $p<0.05$.

RESULTS

Ergothioneine Uptake by Rat Octn1 in HEK293 Cells The time course of [^{3}H]ergothioneine uptake by HEK293 cells stably transfected with rat Octn1 or expression vector pcDNA3.1 alone was examined (Fig. 1). [^{3}H]Ergothioneine (1 μM) uptake at pH 7.4, expressed as the cell-to-medium ratio, increased time-dependently in rat Octn1-transfected cells for 2 min and attained steady-state uptake by 10 min, whereas uptake by HEK293 cells transfected with pcDNA3.1 vector alone (designated as Mock) exhibited a negligible increase of uptake over 10 min. Thus, the uptake at 30 s was routinely used for the initial uptake rate measurement in the subsequent studies.

Next, we estimated K_m value of ergothioneine uptake by rat Octn1. The relationship between the initial uptake rates and concentrations of ergothioneine is shown in Fig. 2. Ergothioneine uptake by rat Octn1 obtained after subtraction of the uptake by Mock cells, was saturable at concentration over 50 μM (Fig. 2A). The Eadie–Hofstee plot gave a single straight line, suggesting the participation of a single saturable

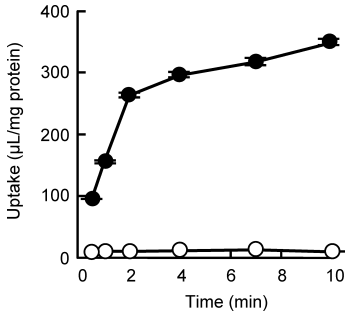

Fig. 1. Time Course of [^{3}H]Ergothioneine Uptake by Rat Octn1 Expressing HEK293 Cells

Uptake of [^{3}H]ergothioneine (1 μM) by rat Octn1-transfected (closed) and pcDNA3.1 vector-transfected (open, Mock) HEK293 cells was measured at pH 7.4 and 37 °C. The results are shown as means±S.E.M. (n=4).

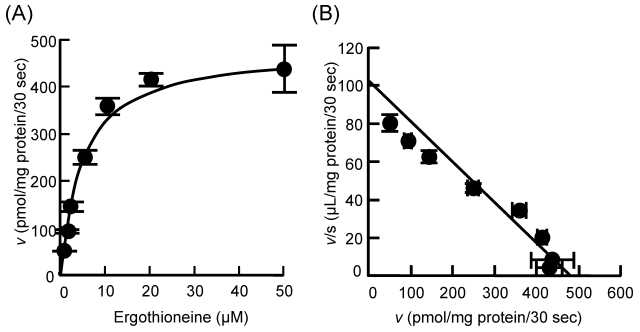

Fig. 2. Concentration Dependence of [^{3}H]Ergothioneine Uptake by Rat Octn1 Expressing HEK293 Cells

(A) Uptake of ergothioneine by rat Octn1 expressing HEK293 cells was measured at pH 7.4 and 37 °C for 30 s, and is shown as the values after subtraction of the Mock uptake. The solid line represents the uptake calculated by the kinetic parameters K_m and V_{max} as described in Results. (B) Rat Octn1-mediated uptake was analyzed by means of the Eadie–Hofstee plot. The results are shown as means±S.E.M. (n=4).

component for ergothioneine transport by rat Octn1 (Fig. 2B). By nonlinear least-squares regression analysis of the result in Fig. 2A, the kinetic parameters, K_m and V_{max}, evaluated according to Eq. 1 were 4.64±0.67 μM and 501±34 pmol/mg protein/30 s, respectively.

Na$^+$- and pH-Dependence of Ergothioneine Uptake by Rat Octn1-Expressing HEK293 Cells When extracellular Na$^+$ was replaced with *N*-methyl-D-glucamine (NMG$^+$) at equimolar concentration, the uptake was significantly decreased to <3% of control (data not shown). Therefore, the Na$^+$-concentration dependence of rat Octn1-mediated [^{3}H]ergothioneine uptake was characterized to obtain the stoichiometry between ergothioneine and sodium ions. As shown in Fig. 3, the uptake rate of [^{3}H]ergothioneine was increased with increasing concentration of Na$^+$ and the estimated Hill coefficient (n) and K_{mNa+} according to Eq. 2 were 0.90±0.11 and 27.4±8.4 mM, respectively.

The pH dependence of rat Octn1-mediated [^{3}H]ergothioneine uptake in HEK293 cells is shown in Fig. 4. When pH in the transport medium was acidic (pH 5.0 or 5.5), rat Octn1-mediated [^{3}H]ergothioneine uptake by HEK293 cells was significantly decreased to approximately 35 to 55% of that at neutral or alkaline pH, whereas the uptake by Mock cells was not affected by changes in medium pH value (Fig. 4).

Characteristics of Ergothioneine Uptake by PC12 Cells

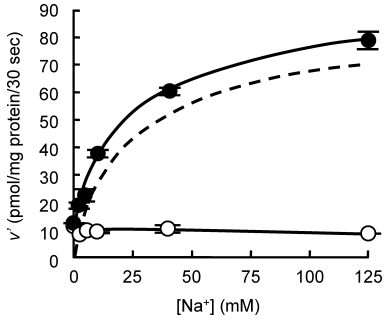

Fig. 3. Na$^+$-Concentration Dependence of [^{3}H]Ergothioneine Uptake by Rat Octn1 Expressing HEK293 Cells

[^{3}H]Ergothioneine (1 μM) uptake at various concentrations of Na$^+$ from 2.5 to 125 μM by rat Octn1-transfected (closed) and pcDNA3.1 vector-transfected (open, Mock) HEK293 cells was measured at pH 7.4 and 37 °C for 30 s. Rat Octn1-mediated uptake shown by dotted line was obtained by subtracting the uptake by Mock cells from that by rat Octn1-transfected cells. Osmolality of the medium was adjusted by adding appropriate concentrations of *N*-methylglucamine chloride. The sum of NaCl and *N*-methylglucamine chloride concentration in the medium was maintained at 125 mM, and Na$^+$ concentration was changed from Na$^+$-free to 125 mM. The results are shown as means±S.E.M. (n=4).

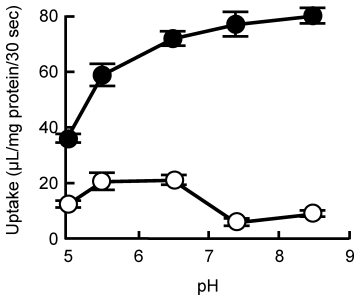

Fig. 4. Effect of pH on [^{3}H]Ergothioneine Uptake by Rat Octn1 Expressing HEK293 Cells

Uptake of [^{3}H]ergothioneine (1 μM) by rat Octn1-(closed) and pcDNA3.1 vector-transfected (open) HEK293 cells was measured at 37 °C for 30 s. The results are shown as means±S.E.M. (n=4).

It has been reported that ergothioneine inhibit apoptosis induced by the oxidant stress in PC12 cells.[22] Since the intracellular uptake of ergothioneine is presumed to be due to the ergothioneine transporters such as OCTN1, PC12 cells may express the ergothioneine transporters. To clarify the mechanism of ergothioneine transport in PC12 cells, which are useful to understand physiological roles of rat Octn1, we investigated the expression of rat Octn1 and the uptake of ergothioneine in PC12 cells.

First of all, rat Octn1 mRNA was detected in PC12 cells by RT-PCR (Fig. 5). Then, the time course of ergothioneine uptake in PC12 cells in the presence or absence of Na$^+$ was studied as shown in Fig. 6. [^{3}H]Ergothioneine uptake was increased linearly up to 2 min in PC12 cells in the presence of Na$^+$, while such an increase in the uptake was not observed in the absence of Na$^+$. Thus, the uptake at 2 min was used for evaluation of the initial uptake rate. The concentration dependence of ergothioneine uptake by PC12 cells is shown in Fig. 7. Saturable ergothioneine uptake was estimated by subtracting an apparent [^{3}H]ergothioneine uptake in the presence of high concentration of unlabeled ergothioneine (200 μM) to exclude [^{3}H]ergothioneine adsorption to cell surface and the result is shown in Fig. 7. The Eadie–Hofstee plot of saturable uptake of ergothioneine by PC12 cells, indicated an involve-

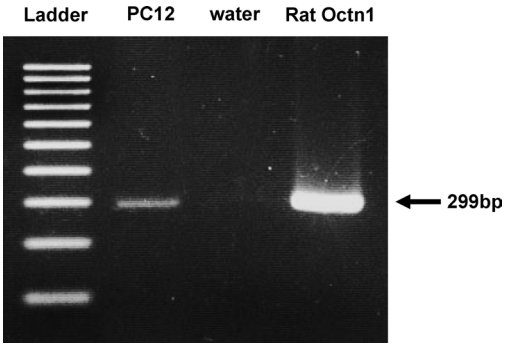

Fig. 5. Expression of the Gene for Rat Octn1 in PC12 Cells by RT-PCR Method

The reaction condition and specific primers were described in Materials and Methods. RT-PCR products were analyzed by agarose gel electrophoresis followed by staining with ethidium bromide. The specific product from rat Octn1-cDNA was 299 bp in size as shown by arrow as positive control.

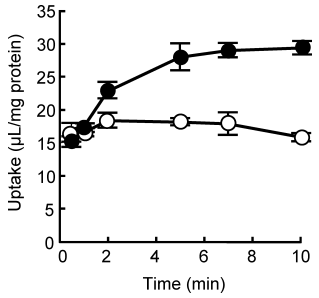

Fig. 6. Time Course and Na^+-Dependence of [^{3}H]Ergothioneine Uptake by PC12 Cells

Uptake of [^{3}H]ergothioneine (3 μM) by PC12 cells was measured at pH 7.4 and 37 °C. Uptake of ergothioneine by PC12 cells in the presence (closed) or absence of Na^+ (open) were studied. Osmolality of the medium was adjusted by adding appropriate concentrations of *N*-methylglucamine chloride. The results are shown as means±S.E.M. (n=4).

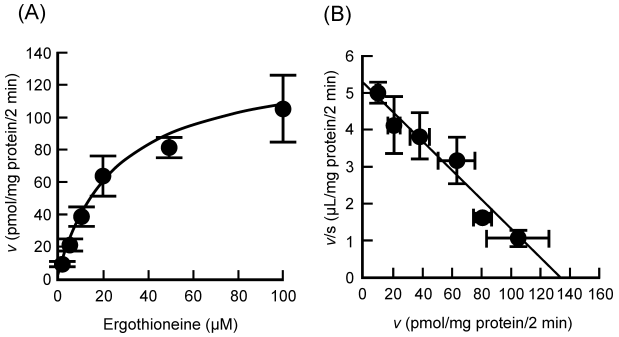

Fig. 7. Concentration-Dependence of [^{3}H]Ergothioneine Uptake by PC12 Cells

(A) Uptake of increasing concentrations of ergothioneine from 2 to 100 μM was measured at pH 7.4 and 37 °C for 2 min. The apparent uptakes were obtained after subtraction of the uptake value in the presence of 200 μM unlabeled ergothioneine. (B) The uptake was analyzed by means of the Eadie–Hofstee plot. The results are shown as means±S.E.M. (n=3 or 4).

ment of a single transport system (Fig. 7B). The obtained kinetic parameters for K_m and V_{max} according to Eq. 1 were 25.5±8.9 μM and 133.7±37.3 pmol/mg protein/2 min, respectively.

DISCUSSION

OCTN1 was associated with rheumatoid arthritis and Crohn's disease.[9—11] Furthermore, we previously suggested that OCTN1 is expressed preferentially in erythroid cells.[7,8] Accordingly, OCTN1 is considered to be a physiologically important transporter in several cells such as blood cells, whereas its physiological roles remain to be clarified. As substrates of OCTN1, organic cations such as TEA and carnitine were demonstrated considering from the closely related transporter family members such as organic cation transporters OCTs and carnitine transporter OCTN2.[1,2] Then, a more recently identified substrate ergothioneine, known as an anti-oxidant, exhibited Na^+-dependent and high transport activity by human OCTN1, suggesting that it is a physiologically important substrate of OCTN1,[13] while no clear relationship between ergothioneine and above mentioned diseases or cellular effect has been investigated. Although it is important to elucidate physiological roles of OCTN1, the related information in experimental animals, which are useful to study the roles, is limited.[18] For the first time, the present study characterized the transport of ergothioneine by rat Octn1 using established HEK293 cells stably expressed with rat Octn1 and the cell line PC12 that natively expresses rat Octn1.

First of all, we established rat Octn1 stably expressing HEK293 cells to characterize ergothioneine transport. Functional expression of rat Octn1 in rat Octn1-cDNA transfected HEK293 cells were confirmed by RT-PCR analysis and ergothioneine uptake assays. Then, one cell line that exhibited high transport activity was selected and used for the following functional analysis of the transport of ergothioneine (Fig. 1). Kinetic analysis of the initial uptake of ergothioneine by rat Octn1 showed a straight line in Eadie–Hofstee plot with the K_m value of 4.64 μM (Fig. 2), which is close to that of human OCTN1 (21 μM).[13] The rat Octn1-mediated transport of ergothioneine exhibited clear Na^+ dependence with the transport stoichiometry of 1 : 1 of ergothioneine and Na^+ from the Hill coefficient, 0.90 (Fig. 3). Furthermore, this ergothioneine uptake was pH dependent with a maximum uptake activity at approximately pH 7.4 (Fig. 4). These characteristics were similar to those of human OCTN1.[13,14] On the other hand, transport of carnitine, which is one of physiological substrate for OCTN1, exhibited different affinity between rat and human OCTN1-transfected cells.[10,18] Based on this observation, it was suggested that ergothioneine may be more specific physiological substrate of OCTN1 than carnitine, because substrate recognition for ergothioneine was strongly conserved in both species with high affinity.

In order to investigate the physiological roles of Octn1 in rat tissue, the ergothioneine transport was characterized in PC12 cells that were derived from rat adrenal pheochromocytoma. In addition, recently, it was reported that ergothioneine has a protective effect against the hydrogen-peroxide induced apoptosis in PC12 cells.[22] Therefore, we demonstrated whether rat Octn1 is functionally expressed in PC12 cells. The expression of rat Octn1 in PC12 cells was clarified by RT-PCR method (Fig. 5). In addition, PC12 cells exhibited Na^+-dependent ergothioneine uptake (Fig. 6), and the kinetic parameter of ergothioneine uptake by PC12 cells (K_m) was comparable with that of rat Octn1 (Fig. 7). Since the negligible transport was observed in the absence of Na^+, the uptake of ergothioneine by PC12 cells could be accounted for by rat Octn1. Moreover, we recently reported that the in-

creased apoptosis in K562 cells was associated with the suppressed expression of human OCTN1 with siRNA method.[8] Accordingly, we hypothesized that the OCTN1-mediated uptake of ergothioneine is important in protective function against oxidative damage and rat Octn1 may be involved in the uptake of the anti-oxidant by PC12 cells.

We previously reported that mRNA expression of human OCTN1 was up-regulated by inflammatory cytokines, TNF-α and IL-1β, *via* NF-κB activation.[12] In addition, reactive oxidant species (ROS), such as hydrogen peroxide, underwent an activation of NF-κB.[23] On the other hand, ergothioneine had an inhibitory effect on ROS or TNF-α induced NF-κB activation.[24] Accordingly, it was thought that the physiological role of OCTN1 is to mediate the uptake of ergothioneine, which reduces the production of ROS and the resultant inflammatory response. So, it was suggested that ergothioneine supplementation has a beneficial effect on chronic inflammatory disease and oxidative stress-derived disease.[22,24] Since it has been shown that some cases of RA patients were associated with a high level of ergothioneine in red blood cells and the ergothioneine level was linearly correlated with an OCTN1 mRNA level,[15] the tissue distribution of ergothioneine may be controlled by OCTN1. In addition, K_m value of ergothioneine transport by the genetic valiant of OCTN1, which is observed at high frequency in CD patient, was higher than that of the wild-type, suggesting lower transport efficiency by the variant.[14] Accordingly, the tissue distribution of ergothioneine may be altered in these situations (namely, SNPs and change of expression level of OCTN1) that could cause the alteration of OCTN1-mediated transport of ergothioneine.

In conclusion, the present study characterized the transport of ergothioneine as a physiological substrate of rat Octn1 by using rat Octn1-transfected HEK293 cells and PC12 cells. Ergothioneine transport by rat Octn1 is very similar to that of human OCTN1. Although functional similarity of ergothioneine transport between in rat Octn1-transfected cells and PC12 cells was observed, it is essential to further examine the effect of siRNA or specific inhibitor of Octn1 to conclude the involvement of Octn1 in ergothioneine transport in PC12 cells. However, it was thought that rat Octn1 is involved in ergothioneine transport in PC12 cells, which may be related to the protective effect of ergothioneine in PC12 cells against oxidative stress. So, this study suggested that rat Octn1 is important by transporting anti-oxidant ergothioneine and further studies should be essential to clarify the physiological role of rat Octn1 in oxidative stress condition and inflammatory diseases.

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