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Gene Ablation of Carnitine/Organic Cation Transporter 1 Reduces Gastrointestinal Absorption of 5-Aminosalicylate in Mice

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5-Aminosalicylic acid (5-ASA) is orally administered as a therapeutic agent for inflammatory bowel diseases, such as ulcerative colitis and Crohn’s disease. We hypothesized that the absorption of 5-ASA is mediated by the polyspecific carnitine/organic cation transporter (OCTN1/SLC22A4), based on the similarity of chemical structure between 5-ASA and other OCTN1 substrates. Therefore, we examined the involvement of this transporter in the disposition of 5-ASA in vivo by using octn1 gene knockout (octn1−/−) mice. After oral administration of 5-ASA, the plasma concentrations of 5-ASA and its primary metabolite, N-acetyl-5-aminosalicylic acid (Ac-5-ASA), in octn1−/− mice were much lower than those in wild-type mice. The time required to reach maximum plasma concentration was also delayed in octn1−/− mice. On the other hand, the plasma concentration profiles of both 5-ASA and Ac-5-ASA after intravenous administration of 5-ASA (bolus or infusion) were similar in the two strains. Uptake of 5-ASA from the apical to the basal side of isolated small-intestinal tissues of octn1−/− mice, determined in an Ussing-type chamber, was lower than that in wild-type mice. Further, uptake of 5-ASA in HEK293 cells stably transfected with the OCTN1 gene, assessed as the sum of cell-associated 5-ASA and Ac-5-ASA, was higher than that in HEK293 cells transfected with the vector alone. Overall, these results indicate that OCTN1 is involved, at least in part, in the gastrointestinal absorption of 5-ASA.

Key words organic cation transporter; 5-aminosalicylate; gastrointestinal absorption

5-Aminosalicylic acid (5-ASA) is orally administered for treatment of inflammatory bowel diseases, such as ulcerative colitis and Crohn’s disease, and is the standard first-line therapy for mild to moderate ulcerative colitis according to current treatment guidelines. Ulcerative colitis and Crohn’s disease increase the risk of colorectal cancer, and many in vitro and in vivo studies have suggested that 5-ASA inhibits proliferation of colorectal cancer cells and induces their apoptosis. Therefore, 5-ASA not only ameliorates bowel disease due to its anti-inflammatory action, but may also reduce the risk of cancer.

Since 5-ASA is well absorbed from the gastrointestinal tract, a controlled-release formulation (mesalazine) and a prodrug (sulfasalazine) of 5-ASA have been developed to deliver 5-ASA to the lower part of the gastrointestinal tract, which is a primary therapeutic target in ulcerative colitis. 5-ASA is extensively metabolized in the liver, mainly to N-acetyl-5-aminosalicylic acid (Ac-5-ASA). Controversial reports have been obtained regarding pharmacological activity of Ac-5-ASA: Ac-5-ASA had no effect on erythrocyte lipid peroxidation whereas inhibition of prostaglandin E2 synthesis by Ac-5-ASA in human rectal mucosa was reported.

5-ASA is an ionic compound at physiological pH (its pKa values are 2.6, 5.8 and 12.0), and therefore, membrane permeation of 5-ASA via simple diffusion is expected to be minimal according to pH-partition theory. Nevertheless, oral absorption of 5-ASA is quite rapid and almost complete (time to maximum plasma concentration ca. 1 h, bioavailability >75%) in humans. Thus, a transport mechanism(s) appears to be involved in gastrointestinal uptake of 5-ASA. In vitro studies using cultured cell lines heterologously transfected with transporter genes showed that 5-ASA is a substrate of organic anion transporting polypeptides (OATPs) such as OATP1B1, 1B3 and 2B1. However, it is not yet clear whether these transporters are involved in absorption and/or disposition of 5-ASA in the body.

Carnitine/organic cation transporter 1 (OCTN1/SLC22A4) is expressed ubiquitously in various organs, including kidney, liver, small intestine, brain, skeletal muscle and heart. OCTN1 recognizes both cationic and zwitterionic compounds such as tetraethylammonium, verapamil, quinidine, pyrilamine, ipratropium, oxaliplatin, methimazole, metformin, ergothioneine (ERGO), stachydrine and acetylcholine as substrates. In humans, renal tubular secretion of gabapentin is reduced in subjects with polymorphism of OCTN1 gene (L503F), indicating possible involvement of this transporter in renal disposition. OCTN1 gene knockout mice (octn1−/−) have been constructed and exhibit a marked reduction in small-intestinal absorption, tissue distribution in various organs, and renal reabsorption of ergothioneine, a typical OCTN1 substrate; these results suggest functional expression of OCTN1 in these organs of wild-type animals in vivo. Pharmacokinetic study using octn1−/− mice suggested the possible involvement of OCTN1 as an efflux system for metformin in murine small intestinal membranes. However, information on the involvement of OCTN1 in the pharmacokinetics of other therapeutic agents is still limited.

5-ASA contains carboxylic and amide groups, like many OCTN1 substrates, and so we hypothesized that 5-ASA is recognized by OCTN1. Interestingly, it is reported that OCTN1 gene expression is up-regulated in inflamed ileal mucosal segments of Crohn’s disease patients and in dextran sodium...
sulfate (DSS)-induced colitis model mice. Thus, OCTN1-mediated 5-ASA uptake could be pharmacologically important. Therefore, the purpose of the present study is to examine the involvement of OCTN1 in the pharmacokinetics of 5-ASA in vivo.

MATERIALS AND METHODS

Materials 5-ASA was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ac-5-ASA was prepared from 5-ASA and acetic anhydride according to the reported procedure. All other chemicals were commercial products of analytical grade.

Animals Male mice were used for all experiments at 6–8 weeks of age. The octn1<sup>−/−</sup> mice were generated as reported. The octn1<sup>−/−</sup> and littermates were of a mixed genetic background (C57BL/6J and 129Sv/Ev). They were maintained with free access to food and water. The present study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals in Kanazawa University.

Pharmacokinetic Studies Mice were fasted overnight with free access to water. 5-ASA was first dissolved in 0.2 N NaOH at 20mg/mL, then diluted 10 times with saline and orally administered at 10µg/kg body weight, intravenously injected as a bolus at 2 mg/kg body weight, or intravenously infused at 25 µg/min/kg body weight. At various intervals after administration, aliquots of blood were collected through the tail vein. All blood samples were immediately centrifuged to obtain plasma. Urine was collected by washing the bladder with saline through polyethylene tubing (SP31, Natsume, Tokyo, Japan). At the end of intravenous infusion of 5-ASA, mice were sacrificed, and tissues were obtained. All samples were stored at −30°C until HPLC determination.

Total body clearance (CL<sub>tot</sub>) and renal clearance (CL<sub>renal</sub>) were calculated as follows:

\[
CL_{tot} = \frac{R_{inf}}{C_{ss,plasma}} \\
CL_{renal} = \frac{V_{ss,urine}}{C_{ss,plasma}}
\]

where \(R_{inf}\), \(C_{ss,plasma}\) and \(V_{ss,urine}\) represent the infusion rate (µg/min/kg), steady-state plasma concentration (µg/ml) and steady-state urinary excretion rate (µg/min/kg), respectively. \(C_{ss,plasma}\) was estimated as the mean value of plasma concentration at 60, 90 and 120 min. \(V_{ss,urine}\) was estimated as the mean value of excretion rate from 60 to 90 min and that from 90 to 120 min.

Intestinal Transport Study in Ussing-Type Chamber The peritoneation of 5-ASA in upper region of small-intestinal tissues was assessed in an Ussing-type chamber as described previously. The transport buffer was composed of 128 mM NaCl, 5.1 mM KCl, 1.4 mM CaCl<sub>2</sub>, 1.3 mM MgSO<sub>4</sub>, 21 mM NaHCO<sub>3</sub>, 1.3 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM NaH<sub>2</sub>PO<sub>4</sub> and 5 mM d-glucose [adjusted to pH 6.0 or 7.4 for the apical or basal side, respectively], and gassed with O<sub>2</sub> before and during the transport experiment. 5-ASA was first dissolved in dimethyl sulfoxide (DMSO) and then diluted with transport buffer to give final concentrations of 5-ASA and DMSO in the donor side of 300µM and 0.3%, respectively. At the designated times, a 200µL aliquot was sampled from the acceptor side and replaced with an equal volume of prewarmed fresh buffer.

Transport Studies in HEK293/mOCTN1 Cells HEK293 cells stably transfected with mouse OCTN1 (HEK293/mOCTN1) were previously prepared. For uptake experiments, these cells were cultured in poly-l-lysine coated 12-well plates. At 24h before uptake study, sodium butyrate (final 50 µM) was added to induce expression of OCTN1. The cells were washed with transport buffer containing 125 mM NaCl, 4.8 mM KCl, 5.6 mM d-glucose, 1.2 mM CaCl<sub>2</sub>, 1.2 mM H<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, and 25 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) (adjusted to pH 7.4 with 1 N NaOH) twice. Cells were preincubated at 37°C for 10 min, followed by aspiration of the medium and addition of fresh medium containing 150 µM 5-ASA or 1 mM Ac-5-ASA. The concentration of each compound was set to be the lowest value for the observed uptake to be above detection limit in HPLC. At the designated times, the medium was aspirated, and the cells were washed with ice-cold transport buffer 3 times, and then solubilized with 0.2 N NaOH at room temperature overnight and subjected to HPLC determination.

Measurement of 5-ASA and Ac-5-ASA by HPLC Pre-column derivatization using propionic anhydride was performed to determine 5-ASA and Ac-5-ASA as described previously with some modifications. Briefly, to 10 or 20 µL of plasma and diluted urine samples, 10 µL of water, 20 or 10 µL of internal standard, respectively, and 2 µL of propionic anhydride were added. The samples were then gently mixed for 20 min at room temperature, and 50 µL of 6% perchloric acid was added to precipitate protein. Tissue samples were mixed with the same volume of internal standard and three volumes of phosphate buffered saline (PBS), and homogenized, followed by derivatization with 2 µL of propionic anhydride and protein precipitation with perchloric acid. After centrifugation at 15000 rpm and 4°C for 5 min, the supernatant was subjected to HPLC. The HPLC system consisted of a constant-flow pump (LC-10Avp, Shimadzu, Kyoto, Japan), a fluorimetric detector (RF-10A XL, Shimadzu), an automatic sample injector (SIL-10A, Shimadzu), and an integrator (CLASS-VP, Shimadzu). The reversed-phase column (Cosmosil 5C18-AR-II, 4.6×150mm; Nacalai Tesque) was maintained at 40°C in a column oven (CITO-10Avp, Shimadzu). The mobile phase was a mixture of 20 mM sodium phosphate buffer (pH 2.5) and methanol (77:23), and the fluorometric detector was set at 355 nm (excitation) and 515 nm (emission). The flow rate was 1.0 mL/min. 4-Aminosalicylic acid (4-ASA) was used as an internal standard.

Data Analysis The statistical significance of differences was determined by means of Student’s t-test or one-way ANOVA with the Bonferroni test, and \(p<0.05\) was regarded as denoting a significant difference.

RESULTS

Effect of octn1 Gene Deletion on Plasma Concentration of 5-ASA after Oral Administration To investigate possible involvement of OCTN1 in intestinal absorption of 5-ASA, 5-ASA was orally administered to wild-type and octn1<sup>−/−</sup> mice (Fig. 1). The plasma concentration of 5-ASA after oral administration in octn1<sup>−/−</sup> mice was significantly lower than that in wild-type mice, the difference being greater at the early phase after administration (Fig. 1A). In octn1<sup>−/−</sup> mice, the peak of plasma concentration was also lower, and
the time of peak plasma concentration tended to be delayed compared with that in wild-type mice (Fig. 1A). Since 5-ASA is metabolized to Ac-5-ASA by N-acetyltransferase (NAT),\textsuperscript{29} we also measured the plasma concentration of Ac-5-ASA (Fig. 1B). The plasma concentration of Ac-5-ASA in octn1\textsuperscript{−/−} mice was also lower than that in wild-type mice (Fig. 1B).

**Minimal Effect of octn1 Gene Deletion on Systemic Elimination of 5-ASA** Next, to examine the possible role of OCTN1 in systemic elimination of 5-ASA, 5-ASA was intravenously administered to wild-type and octn1\textsuperscript{−/−} mice (Fig. 2). In contrast to the results after oral administration (Fig. 1), the plasma concentrations of both 5-ASA and Ac-5-ASA after an intravenous bolus or during constant intravenous infusion were similar in the two strains (Fig. 2), showing comparable systemic elimination (represented as CL\textsubscript{tot,plasma}; Table 1). Tissue distribution and urinary excretion were further examined (Fig. 3). Concentrations of 5-ASA in liver and kidney at the end of the infusion, and urinary excretion of 5-ASA during the intravenous infusion were below the detection limit in both strains, probably because of rapid metabolism of 5-ASA (see Discussion). On the other hand, the tissue-to-plasma concentration ratio (Kp) of Ac-5-ASA in both liver and kidney at the end of 5-ASA infusion showed no significant difference between wild-type and octn1\textsuperscript{−/−} mice (Figs. 3A, B).
The \( V_{\text{ss,urine}} \) values of Ac-5-ASA were also similar in the two strains (60–80% of the infusion rate of 5-ASA; Fig. 3C), indicating substantial metabolism of 5-ASA and recovery as Ac-5-ASA in the urine. \( CL_{\text{tot}} \) and \( CL_{\text{renal}} \) for Ac-5-ASA showed little difference between the two strains (Table 1).

**Intestinal Membrane Permeation of 5-ASA Was Reduced in \( octn1^{-/-} \) Mice**  
To directly investigate the membrane permeability of 5-ASA in small intestine, permeation of 5-ASA was next examined in an Ussing-type chamber. We used upper region of small intestinal tissues because regional differentiation was not clearly observed in functional expression of OCTN1 in mouse small intestine assessed as the uptake of \( [\text{H}] \)ERGO from the apical side. The permeation of 5-ASA in the absorptive direction (from apical to basal side) in intestine of \( octn1^{-/-} \) mice was significantly lower than that in wild-type mice (Fig. 4A), whereas that in the secretory direction (from basal to apical side) was similar in wild-type and \( octn1^{-/-} \) mice (Fig. 4B). Intestinal permeation of 5-ASA in the two strains was much higher in the absorptive direction than in the secretory direction (Fig. 4).

**Uptake Study of 5-ASA and Ac-5-ASA in OCTN1-Transfected Cell Lines**  
We next investigated whether 5-ASA and/or Ac-5-ASA are substrates of OCTN1. During incubation of 5-ASA with HEK293/mOCTN1 cells, Ac-5-ASA was detected in both HEK293/mOCTN1 cells and medium, indicating substantial metabolism of 5-ASA in HEK293 cells. Therefore, uptake of 5-ASA was measured as the sum of 5-ASA and Ac-5-ASA in the cells, and Ac-5-ASA in the medium. On this basis, time-dependent uptake of 5-ASA was observed in both HEK293/mOCTN1 and HEK293/mock cells, the 5-ASA uptake in the former case being slightly higher than that in wild-type mice.

### Table 1. Pharmacokinetic Parameters of 5-ASA and Ac-5-ASA Estimated during Intravenous Infusion (25 \( \mu \)g/min/kg) of 5-ASA in Wild-Type and \( octn1^{-/-} \) Mice

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>( octn1^{-/-} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( CL_{\text{tot,plasma}} ) (mL/min/kg)( ^a )</td>
<td>54.9±7.1</td>
<td>50.0±4.9</td>
</tr>
<tr>
<td>( CL_{\text{renal,5-ASA}} ) (mL/min/kg)( ^b )</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>( CL_{\text{renal,Ac-5-ASA}} ) (mL/min/kg)( ^d )</td>
<td>15.9±1.3</td>
<td>19.6±1.4</td>
</tr>
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</table>

\( ^a \) Data were expressed as the mean±S.E.M. (\( n=5–6 \)).  
\( ^b \) Total clearance of 5-ASA.  
\( ^c \) Renal clearance of 5-ASA.  
\( ^d \) Renal clearance of Ac-5-ASA.

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**Fig. 3. Tissue-to-Plasma Concentration Ratio and Urinary Excretion Rate of Ac-5-ASA during Intravenous Infusion of 5-ASA**  
Tissue-to-plasma concentration ratio (\( K_p \)) (A, B) and steady-state urinary excretion rate (\( V_{\text{ss,urine}} \)) (C) of Ac-5-ASA in wild-type (closed column) and \( octn1^{-/-} \) mice (open column) were determined at the end of intravenous infusion of 5-ASA (25 \( \mu \)g/min/kg) for 120 min. Each value represents the mean±S.E.M. (\( n=5–6 \)).

**Fig. 4. Membrane Permeation of 5-ASA in Small Intestine of Wild-Type and \( octn1^{-/-} \) Mice**  
The permeation of 5-ASA (300 \( \mu \)M) from the apical to basal side (absorptive direction; A) and that from basal to apical side (secretory direction; B) were measured in an Ussing-type chamber using small intestinal tissues obtained from wild-type (○) and \( octn1^{-/-} \) mice (○). Each value represents the mean±S.E.M. (\( n=4–5 \)). When error bars are not shown, they were smaller than the symbols. *Significantly different from wild-type mice (\( p<0.05 \)).
the latter (Fig. 5A). Uptake of Ac-5-ASA was also measured during incubation with Ac-5-ASA, and the uptake in HEK293/mOCTN1 was higher than that in HEK293/mock cells.

**DISCUSSION**

Although 5-ASA is orally administered for treatment of ulcerative colitis and Crohn’s disease, the mechanism of its intestinal uptake has not been established. Here, we found that the plasma concentration of 5-ASA after oral administration in octn1−/− mice was much lower than that in wild-type mice, and there was a similar difference in the plasma concentration of its metabolite, Ac-5-ASA, between the two strains. On the other hand, there was no marked difference in plasma concentration of 5-ASA or Ac-5-ASA after intravenous administration, suggesting that systemic elimination of 5-ASA is similar in the two strains. Since renal excretion of 5-ASA was negligible, systemic elimination may occur predominantly via the liver. Furthermore, Clint of 5-ASA in both strains (Table 1) was close to the hepatic plasma flow rate (ca. 50 mL/min/kg body wt), which may therefore be the rate-limiting step in systemic elimination of 5-ASA.

To confirm the role of OCTN1 in intestinal uptake of 5-ASA, we used an Ussing-type chamber system to examine transport of 5-ASA across small-intestinal tissues. We found that the membrane permeability of 5-ASA in the absorptive direction in octn1−/− mice was lower than that in wild-type mice, in accordance with the lower plasma concentration of 5-ASA in octn1−/− mice after oral administration. Since OCTN1 is expressed on apical membrane of small intestinal epithelial cells in humans and mice, it seems likely that the reduction in plasma 5-ASA profile in octn1−/− mice is due to loss of OCTN1 function at the intestinal plasma membrane. This is the first evidence that octn1 gene product mediates gastrointestinal absorption of 5-ASA in mouse small intestine.

However, it should be noted that 5-ASA is orally absorbed to some extent even in octn1−/− mice, indicating that other mechanisms besides OCTN1 are involved in absorption of 5-ASA. OATPs are expressed in human intestinal epithelial cells and may have a role in drug absorption. Indeed, OATP2B1 recognizes 5-ASA as a substrate. Therefore, plural transporters are likely to contribute to intestinal membrane permeability of 5-ASA. On the other hand, 5-ASA (mesalamine; mesalazine) is classified as a class 2 compound in the Biopharmaceutics Drug Disposition Classification System (BDDCS), and the BDDCS predicts that the effect of efflux transporter(s) could be predominant in the intestinal absorption of class 2 drugs. This is inconsistent with the present finding that uptake transporter(s) mediate absorption of 5-ASA. However, the BDDCS does not reliably predict the effect of transporters. Also, there may be species differences in the mechanisms of intestinal membrane permeation of 5-ASA.

To directly demonstrate recognition of 5-ASA by OCTN1, we performed uptake studies in HEK293/mOCTN1 cells. However, 5-ASA was markedly metabolized in HEK293 cells. Addition of NAT inhibitors such as fisetin (100 µM) and quer cetin (50 µM) reduced the formation of Ac-5-ASA (data not shown), suggesting functional expression of NAT in HEK293 cells. Nevertheless, these inhibitors also reduced uptake of a typical OCTN1 substrate, [3H]ergothioneine. Therefore, since it was difficult to estimate OCTN1 function, the uptake of 5-ASA was assessed as the sum of 5-ASA and Ac-5-ASA in the cells, and Ac-5-ASA in the medium in the present study (Fig. 5A). On this basis, the uptake of 5-ASA in HEK293/mOCTN1 cells was slightly higher than that in HEK293/mock cells (Fig. 5A), suggesting a direct interaction of OCTN1 with 5-ASA. Rapid metabolism in cell lines is generally problematic in studies to estimate transport function. König et al. addressed this issue by measuring OATP-mediated 5-ASA transport based the uptake of total radioactivity derived from [3H]5-ASA in HEK293 cells stably transfected with OATP genes. Further studies may be required to estimate the contributions of individual transporters to membrane permeation of 5-ASA in the body.

5-ASA has an anti-inflammatory effect at pathological lesions in inflammatory bowel diseases. Interestingly, gene expression of OCTN1 and accumulation of its typical substrate ergothioneine are up-regulated in inflamed intestinal tissues of both Crohn’s disease patients and gastrointestinal inflammation model mice, compared with those in non-inflamed tissues. Therefore, the present finding regarding possible association of OCTN1 with 5-ASA disposition may imply that OCTN1 contributes to the delivery of 5-ASA to inflamed tissues.
intestinal tissues. Further studies are required to examine possible association of this transporter with pharmacological activity of 5-ASA.

Uptake of Ac-5-ASA in HEK293/mOCTN1 was slightly higher than that in HEK293/mock cells (Fig. 5B). In addition, Kp of Ac-5-ASA in kidney tended to be lower in octn1−/−mice, whereas Kp[urine] of Ac-5-ASA tended to be higher in octn1+/−mice (Fig. 3). These results can be explained by partial involvement of OCTN1 in renal reabsorption of Ac-5-ASA although further studies are required to examine this hypothesis.

In conclusion, deletion of the octn1 gene reduces gastrointestinal absorption of 5-ASA in mice. Therefore, OCTN1 could be at least partially involved in small-intestinal uptake of 5-ASA. Further investigation is needed to establish the molecular mechanism of the interaction between 5-ASA and OCTN1.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


8) Zhao LN, Li JY, Yu T, Chen GC, Yuan YH, Chen QK. 5-Aminosalicylates reduce the risk of colorectal neoplasia in patients with ulcerative colitis: an updated meta-analysis. PLoS ONE, 9, e94208 (2014).


