Department of Pharmaceutics and Therapeutics, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan

Functional expression of equilibrative and concentrative nucleoside transporters in alveolar epithelial cells

S. Baba, R. Yumoto, M. Kawami, M. Takano*

Received February 4, 2021, accepted June 9, 2021

**Corresponding author: Prof. Mikihisa Takano, Department of Pharmaceutics and Therapeutics, Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan takanom@hiroshima-u.ac.jp*

Pharmazie 76: 416-421 (2021) *doi: 10.1691/ph.2021.1017*

Equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs) mediate the cellular uptake of nucleosides and nucleobases across the plasma membrane and play important roles in the salvage pathways of nucleotide synthesis. However, information about nucleoside transport systems in the lung alveolar epithelial cells is limited. Therefore, in the present study, we examined the function and expression of nucleoside transporters using primary cultured alveolar type II cells and transdifferentiated type I-like cells. The uptake of uridine, a substrate for ENTs and CNTs, in type II and type I-like cells was time, temperature, and concentration dependent, and was inhibited by other nucleoside transporter substrates such as adenosine. Uridine uptake in both cells was insensitive to nanomolar concentrations of NBMPR, a potent ENT1 inhibitor, while it was inhibited by higher concentrations of NBMPR, suggesting that ENT2, but not ENT1, is involved in uridine uptake in these cells. Additionally, uridine uptake was higher in the presence of Na^+ than in the absence of Na^+ and was partially inhibited by a CNT inhibitor phloridzin in these cells, suggesting that CNT is also involved in uridine uptake. In both cells, the mRNA expression of *ENT1*, *ENT2*, *CNT2*, and *CNT3* was observed. Finally, the activity of uridine uptake was considerably higher in type II cells than in type I-like cells. In addition, the mRNA expression of *ENT2*, *CNT2*, and *CNT3, but not ENT1*, was lower in type I-like cells than in type II cells. These findings would help understand the functional roles of equilibrative and concentrative nucleoside transporters in alveolar epithelial cells.

1. Introduction

Nucleoside transporters mediate the membrane transport of nucleosides, nucleobases, and their derivatives including drugs. These nucleoside transporters belong to two solute carrier (SLC) families, namely, an equilibrative nucleoside transporter (ENT) family SLC29 and a concentrative nucleoside transporter (CNT) family SLC28 (http://slc.bioparadigms.org/).

SLC29 contains four members, ENT1–ENT4. Among these ENTs, ENT1 and ENT2 are the best characterized ENTs. ENT1 and ENT2 are energy-independent facilitative transporters that can be distinguished based on their sensitivity to inhibition by S-(4-nitrobenzyl)-6-thioinosine (NBMPR). The former is considerably more sensitive to NBMPR than the latter (Baldwin et al. 2004). These transporters are localized on the plasma membrane, and they exhibit similar broad-spectrum substrate specificities for purine and pyrimidine nucleosides. In contrast to ENT1 and ENT2, ENT3 is mostly localized in intracellular compartments (Baldwin et al. 2005). ENT4 exhibits a low homology to other ENT members and may serve as a low-affinity monoamine transporter, especially in the brain (Engel et al. 2004; Baldwin et al. 2005).

SLC28 contains three members, CNT1–CNT3. CNTs are Na^+-coupled secondary active transporters, and CNT1 and CNT2 preferentially transport pyrimidine nucleosides and purine nucleosides, respectively, whereas CNT3 transports both pyrimidine and purine nucleosides (Gray et al. 2004). In contrast to CNT1 and CNT2, CNT3 may utilize H^+ gradient as a driving force, besides Na^+ gradient (Young et al. 2013).

Nucleoside transporters play an important physiological role in nucleoside uptake in the salvage pathways of nucleotide synthesis in the cells. They also regulate the extracellular concentration of adenosine available to the cell surface P1 purinergic receptors and affect several physiological processes such as cardiovascular activity and neurotransmission (King et al. 2006). In addition, nucleoside transporters have an important pharmacological role in the uptake of nucleoside and nucleobase drugs that are used to treat cancer and viral infections (Damaraju et al. 2003; Zhang et al. 2007).

The lung alveolar epithelium is comprised of two types of cells, type I and type II cells. Type I cells are thin, squamous epithelial cells that cover more than 90% of the alveolar surface area and are essential for gas exchange. On the contrary, type II cells are cuboidal epithelial cells that cover the rest of the alveolar surface area and have several important physiological functions including their role in surfactant production and secretion (Patton 1996). In addition, type II cells can proliferate and transdifferentiate into type I cells under *in-vivo* and *in-vitro* conditions, and this is important to repair the alveolar epithelium when type I cells are injured (Evans et al. 1975; Fehrenbach 2001; Ikehata et al. 2008).

We have been studying the functional expression of various membrane transporters in alveolar type I and type II epithelial cells; we found that the expression pattern of certain transporters is different between type I and type II cells. Peptide transporter 2 (PEPT2; SLC15A2) mediates the uptake of dipeptide and tripeptide as well as peptidomimetic drugs such as β-lactam antibiotics and antiviral drugs. In alveolar epithelial cells, PEPT2 was functionally expressed in alveolar type II epithelial cells, but the expression decreased with transdifferentiation. Furthermore, PEPT2 was almost completely lost after transdifferentiation into type I cells (Takano et al. 2013). On the contrary, the functional expression of P-glycoprotein (ABCB1), a primary active efflux transporter

of various toxic xenobiotics, was observed only in type I cells, but not in type II cells (Takano et al. 2016). As described above, nucleoside transporters have various important roles in cell physiology and pharmacotherapeutics. However, information regarding the function and expression of nucleoside transporters in the lung alveolar epithelial cells is limited. Therefore, in the present study, we aimed to clarify the function and expression of equilibrative nucleoside transporters ENT1 and ENT2 and concentrative nucleoside transporters CNT1, CNT2, and CNT3 in alveolar epithelial cells using primary cultured type II cells and transdifferentiated type I-like cells.

2. Investigations and results

2.1. Time, temperature, and concentration dependence of uridine uptake in primary cultured alveolar type II epithelial cells

The time course of [^{3}H]uridine uptake in alveolar type II cells was examined at 37 °C and 4 °C. The uptake at 37 °C increased almost linearly up to 30 min, and it was considerably higher than that at 4 °C (Fig. 1A). Figure 1B shows the effect of uridine concentration on its uptake clearance. The uptake clearance of uridine decreased with the increase in extracellular uridine concentration. The data are expressed as a Michaelis–Menten plot in Fig. 1C. Using a Michaelis-Menten type equation with a non-saturable component, Km (Michaelis–Menten constant), Vmax (maximum uptake rate), and Kd (diffusion constant) values were calculated as 44.9 μM, 2522.8 pmol/mg protein/10 min, and 2.5 μL/mg protein/10 min, respectively.

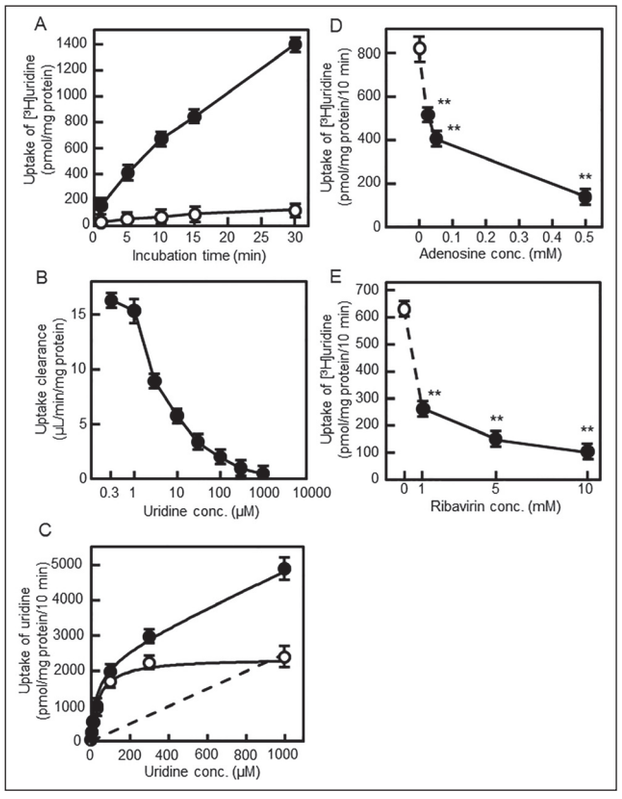

Fig. 1: General characteristics of uridine uptake in alveolar type II cells. (A) Time course of [^{3}H]uridine uptake at 37 °C (●) or 4 °C (○). (B, C) Concentration dependence of uridine uptake: (B) uptake clearance, (C) Michaelis–Menten plot; (●) total uptake, (○) transporter-mediated uptake, (dashed line) non-saturable component. (D) Effect of adenosine on [^{3}H]uridine uptake. (E) Effect of ribavirin on [^{3}H]uridine uptake. Each value represents mean ± S.E.M. (n = 3). $^{**}p < 0.01$, significantly different from each control.

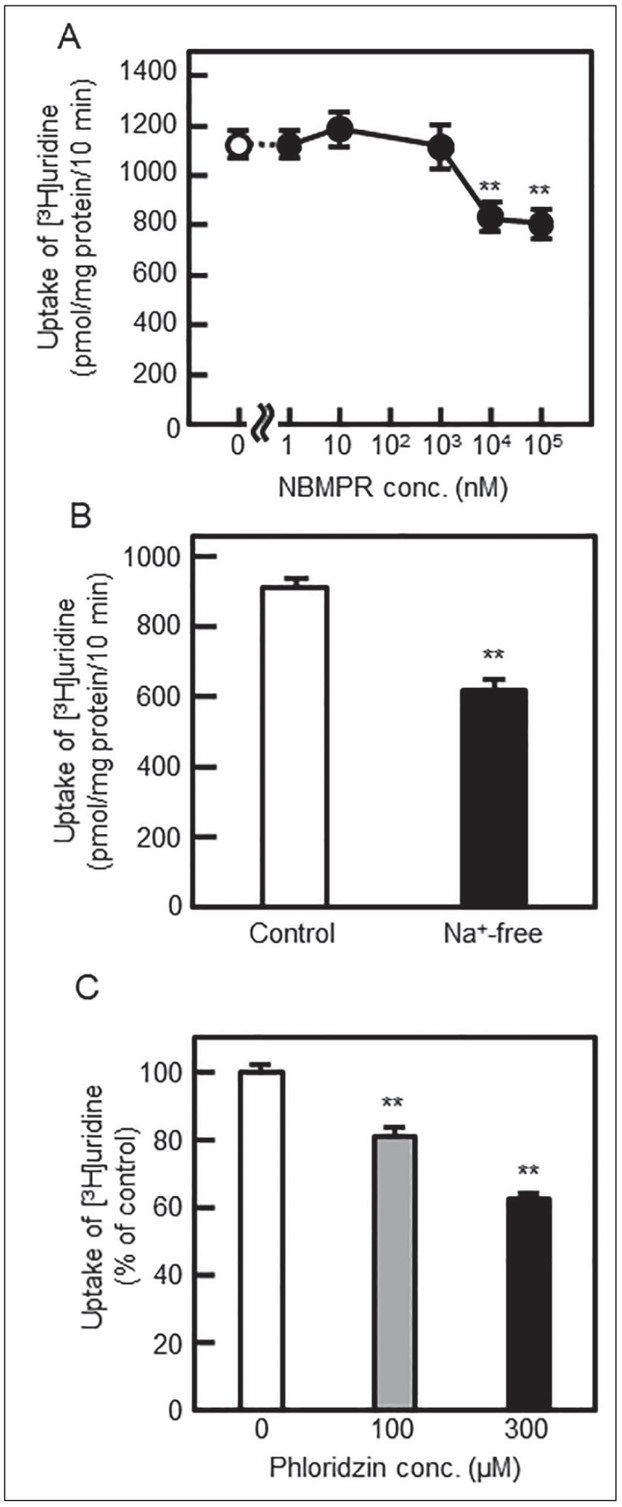

Fig. 2: Effect of NBMPR, Na^+, and phloridzin on uridine uptake in alveolar type II cells. (A) Effect of NBMPR on [^{3}H]uridine uptake. (B) Effect of Na^+ in the uptake buffer on [^{3}H]uridine uptake. Na^+-free buffer contains choline instead of Na^+. (C) Effect of phloridzin on [^{3}H]uridine uptake. Each value represents mean ± S.E.M. (n = 3). $^{**}p < 0.01$, significantly different from each control.

2.2. Effect of substrates of nucleoside transporters on uridine uptake in type II cells

The effect of adenosine and ribavirin, substrates of nucleoside transporters, on [^{3}H]uridine uptake in type II cells was examined. As shown in Fig. 1D and 1E, both compounds significantly inhibited [^{3}H]uridine uptake in a concentration-dependent manner.

2.3. Role of ENT and CNT in uridine uptake in type II cells

To understand the nucleoside transporters involved in uridine uptake in type II cells, the effect of NBMPR, a potent ENT1 inhibitor, on [^{3}H]uridine uptake was examined. [^{3}H]Uridine uptake was partially inhibited by NBMPR at concentrations higher than 10 μM, but not at nanomolar concentrations (Fig. 2A). NBMPR is known to selectively inhibit ENT1 at nanomolar concentrations, while it also inhibits ENT2 at μM concentrations, suggesting that uridine uptake in type II cells is partially mediated by ENT2, but not by ENT1. We then examined the effect of extracellular Na^+ on uridine uptake (Fig. 2B). The uptake of [^{3}H]uridine was significantly lower when Na^+ in the uptake buffer was replaced with choline (Na^+-free buffer). In addition, [^{3}H]uridine uptake was partially inhibited by phloridzin, a non-specific inhibitor of CNTs (Fig. 2C). Therefore, uridine uptake in type II cells may be mediated by ENT2 and CNTs.

2.4. mRNA expression of ENTs and CNTs in type II cells

The expression of mRNAs of ENTs and CNTs was examined by PCR analysis (Fig. 3). As a positive control, the expression of these mRNAs in the kidney was also examined. In type II cells, the mRNA expression of *ENT1*, *ENT2*, *CNT2*, and *CNT3* was observed, and the size of each band was the same as that in the kidney. The expression of *CNT1* mRNA was detected in the kidney but not in type II cells (data not shown).

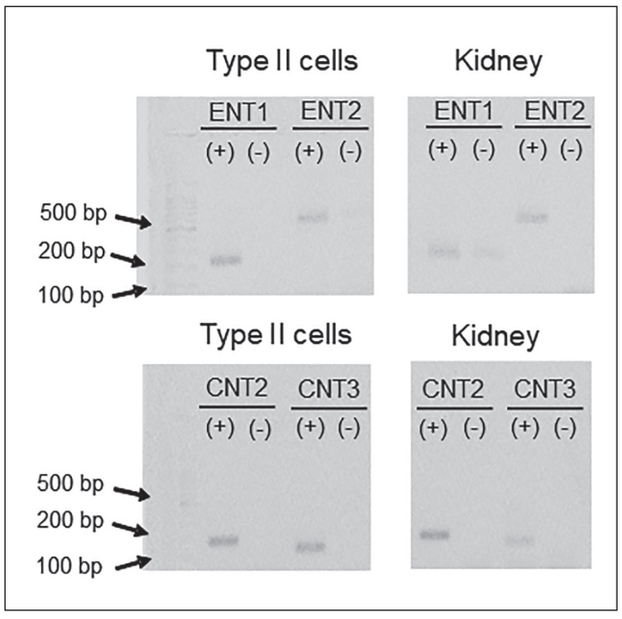

Fig. 3: mRNA expression of nucleoside transporters in alveolar type II cells. The mRNA expression of *ENT1*, *ENT2*, *CNT2*, and *CNT3* was evaluated by PCR analysis with (+) or without (-) reverse transcription. The expression of these mRNAs in the kidney was also evaluated as a positive control.

2.5. Time, temperature, and concentration dependence of uridine uptake in primary cultured alveolar type I-like epithelial cells

The time course of [^{3}H]uridine uptake in alveolar type I-like cells that were transdifferentiated from type II cells was examined (Fig. 4A). [^{3}H]Uridine uptake in type I-like cells was higher at 37 °C than at 4 °C, and the temperature-sensitive uptake increased with time. Figures 4B and 4C show the concentration dependence of uptake clearance and total uptake of uridine, respectively. As observed in type II cells, the uptake clearance of uridine decreased with the increase in uridine concentration. The values of Km, Vmax, and Kd were 107.7 μM, 1689.6 pmol/mg protein/10 min, and 9.3 μL/mg protein/10 min, respectively.

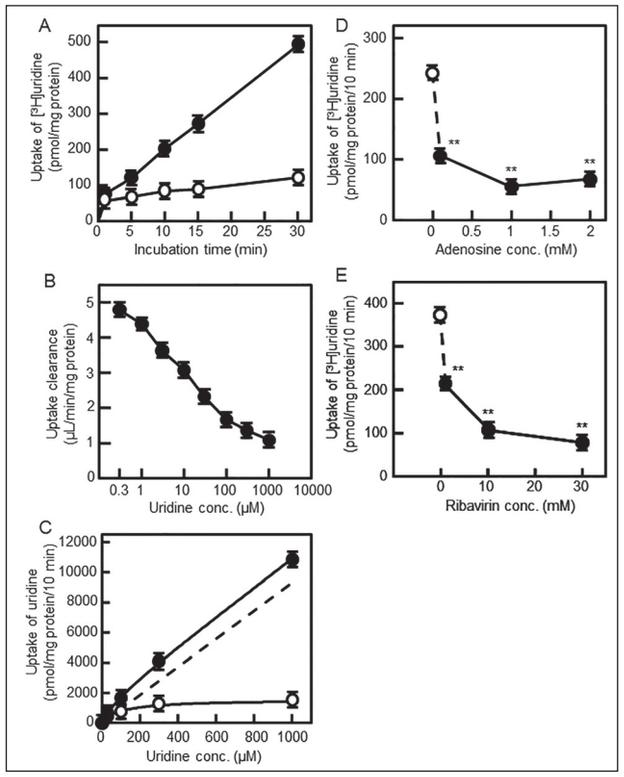

Fig. 4: General characteristics of uridine uptake in alveolar type I-like cells. (A) Time course of [^{3}H]uridine uptake at 37 °C (●) or 4 °C (○). (B, C) Concentration dependence of uridine uptake: (B) uptake clearance, (C) Michaelis–Menten plot; (●) total uptake, (○) transporter-mediated uptake, (dashed line) non-saturable component. (D) Effect of adenosine on [^{3}H]uridine uptake. (E) Effect of ribavirin on [^{3}H]uridine uptake. Each value represents mean ± S.E.M. (n = 3). $^{**}p < 0.01$, significantly different from each control.

2.6. Effect of substrates of nucleoside transporters on uridine uptake in type I-like cells

The effect of adenosine and ribavirin on [^{3}H]uridine uptake in type I-like cells was examined. As shown in Fig. 4D and 4E, both nucleoside transporter substrates potently inhibited [^{3}H]uridine uptake.

2.7. Role of ENT and CNT in uridine uptake in type I-like cells

We next examined the effect of NBMPR on [^{3}H]uridine uptake in type I-like cells (Fig. 5A). NBMPR partially inhibited [^{3}H]uridine uptake at 100 μM, but not at lower concentrations, suggesting the involvement of ENT2. Similar to the uptake in type II cells, Na^+-dependent uptake of [^{3}H]uridine and its inhibition by phloridzin were also observed in type I-like cells (Fig. 5B and 5C), although the contribution of CNTs to total uptake of uridine may be lower in type I-like cells than in type II cells.

2.8. mRNA expression of ENTs and CNTs in type I-like cells

The mRNA expression of ENTs and CNTs in type I-like cells was examined (Fig. 6). Similar to type II cells, the mRNA expression of *ENT1*, *ENT2*, *CNT2*, and *CNT3* was observed in type I-like cells, whereas *CNT1* mRNA was not detected (data not shown).

2.9. Comparison of uridine uptake activity and mRNA expression of ENTs and CNTs between type II and type I-like cells

Finally, we compared the uptake activity of uridine in type II and type I-like cells. The uptake of [^{3}H]uridine was considerably

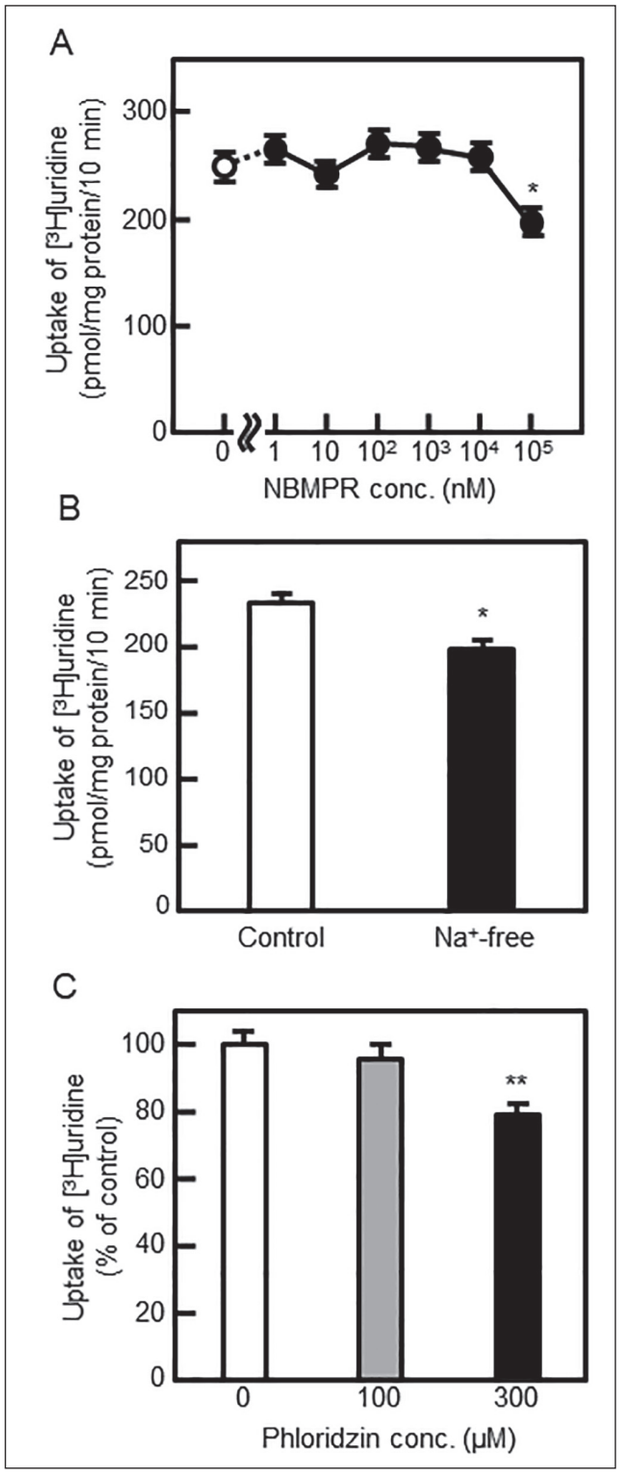

Fig. 5: Effect of NBMPR, Na^+, and phloridzin on uridine uptake in alveolar type I-like cells. (A) Effect of NBMPR on [^{3}H]uridine uptake. (B) Effect of Na^+ in the uptake buffer on [^{3}H]uridine uptake. Na^+-free buffer contains choline instead of Na^+. (C) Effect of phloridzin on [^{3}H]uridine uptake. Each value represents mean ± S.E.M. (n = 3–6). $*p < 0.05$, $**p < 0.01$, significantly different from each control.

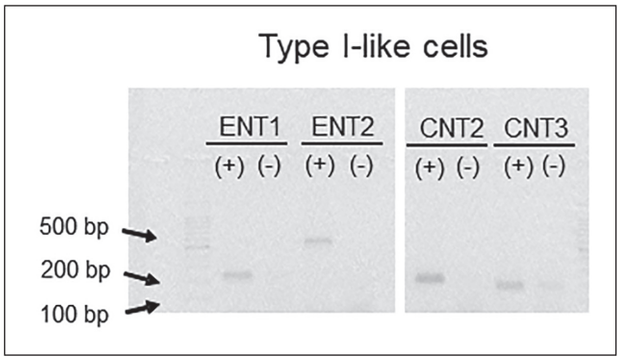

Fig. 6: mRNA expression of nucleoside transporters in alveolar type I-like cells. The mRNA expression of *ENT1*, *ENT2*, *CNT2*, and *CNT3* was evaluated by PCR analysis with (+) or without (-) reverse transcription.

higher in type II cells than in type I-like cells (Fig. 7A), and the specific uptake activity of uridine was approximately 5-fold higher in type II cells than in type I-like cells (Fig. 7B). Therefore, the uridine uptake activity of alveolar epithelial cells might decrease with the transdifferentiation of type II cells to type I-like cells. We also measured and compared the mRNA expression levels of ENTs and CNTs between type I-like and type II cells, using real-time PCR analysis (Fig. 7C). The mRNA expression levels of *ENT2*, *CNT2*, and *CNT3* were significantly lower in type I-like cells than in type II cells, corresponding to the low uridine uptake activity in type I-like cells. On the other hand, *ENT1* mRNA expression did not decrease in type I-like cells but rather increased.

3. Discussion

Here, using primary cultured alveolar epithelial cells, we examined the transport characteristics of uridine and mRNA expression of nucleoside transporters. In both type II and type I-like cells, the uptake of uridine was time, temperature, and concentration dependent. The apparent Km values of uridine uptake in the presence of Na^+ in type II and type I-like cells were 44.9 and 107.7 μM, respectively (Figs. 1C and 4C). Generally, the Km values of uridine uptake by CNTs are lower than those by ENTs (Young et al. 2013). Therefore, lower Km value in type II cells than that in type I-like cells may reflect the higher contribution of CNTs in uridine uptake in type II cells than in type I-like cells as described below. In addition, we also examined the concentration dependence of uridine uptake in the absence of Na^+, and Km values were estimated to be 162.9 and 138.0 μM in type II and type I-like cells, respectively (data not shown). In the absence of Na^+, CNTs are not functioning, and therefore these Km values may reflect the affinity of uridine to ENTs.

The uptake of uridine in type II and type I-like cells was inhibited by adenosine and ribavirin in a concentration-dependent manner, suggesting that uridine is taken up by the cells via the nucleoside transporter-mediated processes. In both cells, adenosine showed more potent inhibitory effect on uridine uptake than ribavirin, probably due to the higher affinity of adenosine to nucleoside transporters, especially ENTs, than ribavirin (Young et al. 2013). Recently, Nishimura et al. (2019) examined the role of ENTs in ribavirin uptake at the placental barrier and showed that ENT2 might contribute little to ribavirin uptake in rat placental trophoblast cells. They also observed that rat ENT1 cRNA-injected oocytes showed increased ribavirin uptake, while ENT2 cRNA-injected oocytes did not. Similar findings were observed in human hepatocytes (Fukuchi et al. 2010). Therefore, ribavirin may not be a good substrate of ENT2. On the other hand, approximately 20% inhibition of uridine uptake by 200 μM ribavirin was observed in ENT2-expressing (ENT1 knockout) HeLa cell line (Miller et al. 2021). Taken together, ribavirin may not be transported by ENT2, but may interact with and inhibit ENT2, especially at mM concentrations used in the present study. In addition, transport of ribavirin by CNT2 was suggested in rat small intestine (Mori et al. 2010). Therefore, ribavirin might also inhibit CNT-mediated uridine uptake in alveolar epithelial cells.

The uptake of uridine was also inhibited by NBMPR, but at concentrations higher than 10 μM. Yao et al. (1997) reported that uridine uptake by rat ENT1 was sensitively inhibited by NBMPR with an IC_{50} value of 4.6 nM, whereas ENT2 was relatively insensitive to NBMPR (IC_{50} value > 1 μM). Therefore, ENT mainly involved in uridine uptake in type II and type I-like cells would be ENT2. It is generally known that uridine is transported by both ENT1 and ENT2 (Baldwin 2004; Takano 2010; Yao 1997). However, the

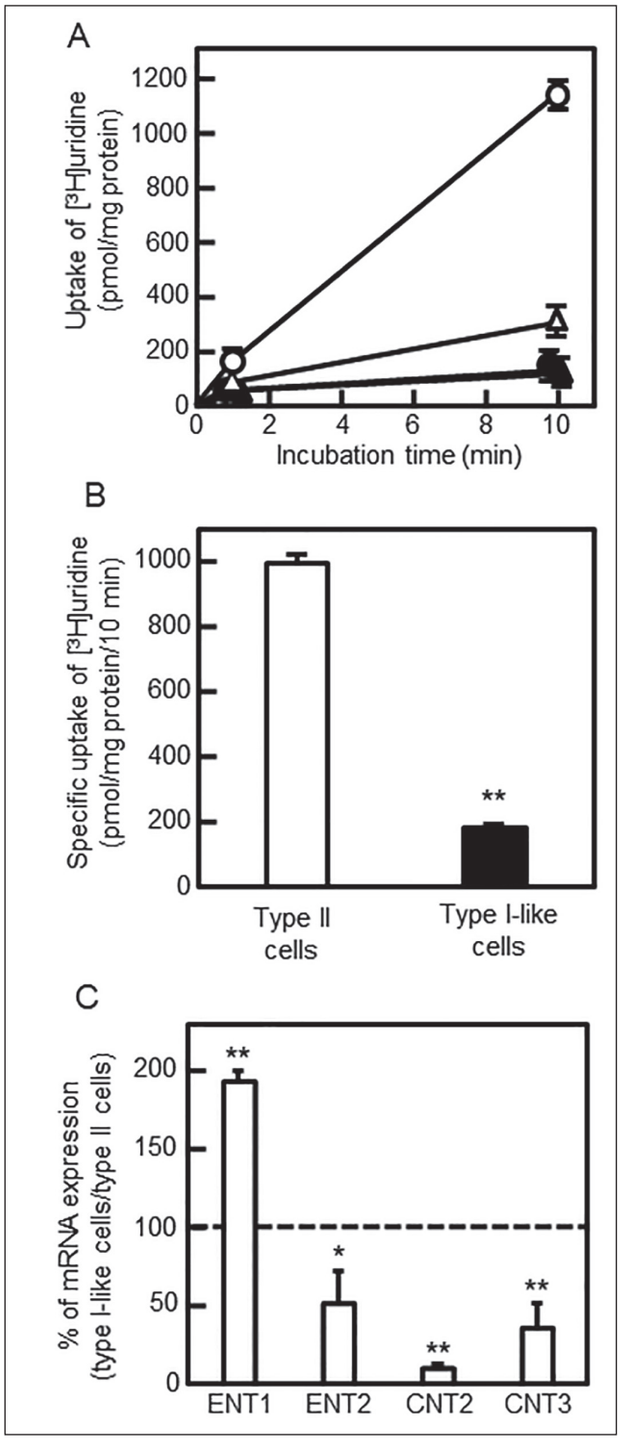

Fig. 7: Comparison of uridine uptake activity and mRNA expression of ENTs and CNTs between type II and type I-like cells. (A) Time course of [^{3}H]uridine uptake in type II (circle) and type I-like (triangle) cells at 37 °C (open symbol) or 4 °C (closed symbol). (B) Comparison of specific [^{3}H]uridine uptake (temperature-sensitive uptake) activity in type II and type I-like cells. (C) Relative mRNA expression levels (type I-like cells/type II cells) of ENTs and CNTs evaluated by real-time PCR analysis. Each value represents mean ± S.E.M. (n = 3). *$p < 0.05$, **$p < 0.01$, significantly different from the uptake in type II cells.

involvement of ENT1 in uridine uptake was not observed in this study, even though the expression of ENT1 mRNA was detected in both type II and type I-like cells. The reason is not clear at this moment, but it is reported that numerous biological mechanisms exist that decouple protein levels from mRNA levels, and therefore mRNA levels do not always correlate with functional protein levels (Fortelny et al. 2017; Selo et al. 2020). Using immunohistochemistry, Wang et al. (2021) recently reported that ENT mainly expressed in alveolar epithelial cells was ENT2 in mouse lungs.

CNTs are Na^+-coupled nucleoside transporters (Gray et al. 2004). Therefore, to clarify the possible involvement of CNTs in uridine uptake in type II and type I-like cells, the effect of extracellular Na^+ on uridine uptake was examined. In both cells, the uptake of uridine was significantly higher in the presence of Na^+. In addition, uridine uptake in the presence of Na^+ was significantly inhibited by phloridzin, a non-specific inhibitor of CNTs (Smith et al. 2004). Taken together, CNTs might be involved in uridine uptake in type II and type I-like cells, besides ENT2. As expected, the mRNA expression of ENTs (*ENT1* and *ENT2*) and CNTs (*CNT2* and *CNT3*) was detected in both cell types. In addition, the mRNA expression levels of *ENT2*, *CNT2*, and *CNT3* decreased with transdifferentiation of type II cells into type I-like cells, corresponding to the low uridine uptake activity in type I-like cells. On the other hand, *ENT1* mRNA expression did not decrease in type I-like cells. This result may support our speculation that ENT1 would not be involved in uridine uptake in alveolar epithelial cells.

Transporter-mediated uridine uptake activity in type II cells was found to be considerably higher than that in type I-like cells. Therefore, nucleoside transport activity may decrease with the transdifferentiation of type II cells to type I-like cells. A similar finding has been observed in PEPT2-mediated dipeptide transport activity (Takano et al. 2013). Such a high transport activity of nucleoside and oligopeptide in type II cells may be related to the role of type II cells as progenitors of type I cells, because the active synthesis of DNA and proteins would be needed for the proliferation and transdifferentiation of type II cells into type I cells. Further studies are needed to understand the molecular mechanisms underlying the changes in the activity of these transporters during the transdifferentiation of alveolar epithelial cells.

Based on NBMPR- and phloridzin-sensitive uridine uptake values, we attempted to estimate the contribution rates of ENT and CNT. The contribution of ENT and CNT in transporter-mediated uridine uptake was approximately 30% and 40% in type II cells, and 24% and 24% in type I-like cells, respectively. Therefore, the contribution of CNT in uridine uptake may be higher in type II cells than in type I-like cells. On the other hand, these findings may also suggest that NBMPR- and phloridzin-insensitive pathway is involved in uridine uptake in both types of cells. This point needs to be clarified further.

In conclusion, we could clarify the transport characteristics of uridine and mRNA expression of nucleoside transporters in alveolar epithelial cells. The uptake of uridine could be mediated predominantly by ENT2 as well as CNT2 and/or CNT3 in both type II and type I-like cells, although the transport activity was considerably higher in type II cells. Our study provides important information to further clarify the physiological and pharmacological roles of nucleoside transporters in the lung alveolar region.

4. Experimental

4.1. Chemicals and reagents

Dulbecco's modified Eagle medium/Ham's F-12 Nutrient Mixture (1:1) was purchased from MP Biomedicals (Solon, OH, USA). Fetal bovine serum (FBS) was purchased from Capricorn Scientific (Ebsdorfergrund, Germany). Penicillin-streptomycin and NBMPR were purchased from Wako Pure Chemical Industries (Osaka, Japan). Trypsin (1:250, powder) and Percoll were purchased from GE Healthcare Bio-Science Corp. (Piscataway, NJ, USA). Deoxyribonuclease I from bovine pancreas, adenosine, phloridzin, and uridine were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). [^{3}H]Uridine (22.5 Ci/mmol) and ribavirin were purchased from Moravek Biochemicals (Brea, CA, USA) and Tokyo Chemical Industry (Tokyo, Japan), respectively. Monarch Total RNA Miniprep Kit and ReverTra Ace® qPCR RT Master Mix were purchased from New England Biolabs Japan (Tokyo, Japan) and TOYOBO Co., Ltd. (Osaka, Japan), respectively. All other chemicals were of the highest grade commercially available.

4.2. Isolation and primary culture of rat alveolar type II and type I-like epithelial cells

Animal experiments were carried out in accordance with the Guideline for the Committee of Animal Experimentation, Hiroshima University and the Committee of Research Facilities for Laboratory Animal Science, Natural Science Center for Basic Research and Development, Hiroshima University. Alveolar type II cells were isolated

from the lungs of male Sprague-Dawley rats weighing 120–200 g as previously reported with a slight modification (Ikehata et al. 2008). In brief, the excised lungs were washed with saline through the tracheal cannula to remove macrophages and treated with 0.25% trypsin solution for 15 min at 37 °C. The minced lungs were incubated with deoxyribonuclease I (250 μg/mL) solution for 4 min at 37 °C to disperse the cells. The cell suspension was purified by discontinuous Percoll density gradient centrifugation. The isolated type II cells were cultured for 2 days after seeding at a density of 54×10^4 cells/cm^2 (used as type II cells) and for 6 days after seeding at a density of 22×10^4 cells/cm^2 (used as transdifferentiated type I-like cells). The cells were cultured in DMEM/F-12 containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in an environment of 5% CO_2/95% air, and the culture medium was changed every 2 days.

4.3. Uptake study of [^{3}H]uridine in type II and type I-like cells

The cells were preincubated in HEPES(G) buffer (145 mM NaCl, 3 mM KCl, 0.3 mM $CaCl_2$, 0.6 mM $MgCl_2$, and 5 mM HEPES with 5 mM D-glucose; pH 7.4) for 10 min at 37 °C. The cells were then incubated with 15 μM [^{3}H]uridine for 10 min at 37 °C or 4 °C without (control) or with substrates (unlabeled uridine, adenosine, and ribavirin) or inhibitors (NBMPR and phloridzin) of nucleoside transporters. After incubation, the cells were rinsed three times with ice-cold HEPES buffer with 10 μM NBMPR and 100 μM phloridzin. The cells were scraped and solubilized in 0.1 M NaOH for 30 min. Cell lysate was centrifuged at 9,700 × *g* for 3 min, and the supernatant was used for the radioactivity assay of [^{3}H]uridine using liquid scintillation counter and protein assay using the Bradford method with bovine serum albumin as the standard.

4.4. mRNA expression of nucleoside transporters in type II and type I-like cells

The total mRNA was extracted from type II and type I-like cells using the Monarch Total RNA Miniprep Kit and was reverse transcribed into cDNA using the ReverTra Ace® qPCR RT Master Mix. The PCR products were separated by electrophoresis on 2.0% agarose gels and the bands were detected using the Typhoon FLA-7000 Imaging system (GE healthcare Japan, Tokyo, Japan). mRNA isolated from rat renal cortex was used as the positive control. The PCR products were also quantified using the CFX Connect™ Real-Time PCR detection system (Bio Rad Laboratories, Inc., Hercules, CA, USA) with the Luna universal master mix qPCR Kit (New England Biolabs Inc., MA, USA), according to the manufacturer's instructions. The primer sequences for gel electrophoresis were as follows: *ENT1* sense, 5′-AGGATGCTTGGCTGACACAC-3′ and antisense, 5′-CTCTGGCTCTTGCGAACTTG-3′; *ENT2* sense, 5′-GCAATAGGACTGCGGACATCA-3′ and antisense, 5′-GCACTGCACAGAAGGAATTGA-3′; *CNT1* sense, 5′-TCTGTGTGTTCCTTGTCCTTC-3′ and antisense, 5′-GTGTAACTCAGGAAGACCTGG-3′; *CNT2* sense, 5′-AGTGCCTGTATGGCAGGAATC-3′ and antisense, 5′-GAGGCTGGACTCCTTGTCTTG-3′; and *CNT3* sense, 5′-GAAAGGTTGGGTGGCTAATGC-3′ and antisense, 5′-TGGTGTGCAGTTCTGACTTGG-3′. The primer sequences used for real-time PCR analysis were the same as above except *ENT2* sense, 5'-GCTACCACCTGGTCGGGATCA 3' and antisense, 5'-CAGGCTGCCCAGAATACGC 3'; *GAPDH* sense, 5'-AGCCCAGAACATCATCCCTG 3' and antisense, 5'-CACCACCTTCTTGATGTCATC 3'. The expression level of each mRNA was normalized to that of GAPDH mRNA, a housekeeping gene.

4.5. Statistical analysis

Data are expressed as mean±standard error of mean (S.E.M.). Statistical analyses were performed using Student's *t*-test, or one-way ANOVA followed by Tukey's test for multiple comparisons. The level of significance was set at $*p < 0.05$ or $**p < 0.01$.

Acknowledgment: We thank Ms. A. Fukushima, Hiroshima University, for her assistance in working on this study.

Conflicts of interest: None declared.

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