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Original

Interaction of Human Renal Organic Anion Transporter OAT1 and OAT3 with Flavonoids

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SUMMARY

An antioxidant effect of the flavonoids in food has been widely noticed as it may prevent the oxidative stress-associated chronic diseases including ischemic heart disease and diabetes mellitus. Flavonoids which are distributed widely in plants are classified into a flavonol, flavanol, flavanone, flavone, isoflavone and anthocyanidin. Of which quercetin, a flavanol, presents ubiquitously as a quercetin glycoside. Most of flavonoids including quercetin which are absorbed in the intestine enter the circulatory blood after they are converted to the conjugated metabolites, and are rapidly eliminated by the kidney into urine. Most studies have focused on the intestinal absorption of the flavonoids which may regulate a physiological function and efficacy of the flavonoids, however their elimination routes at the kidney have remained unknown. This study aims to clarify molecular mechanisms of renal elimination of the flavonoids by inhibiting their transports in view of regulation of their bioavailability in the body. By using the cells stably expressing organic anion transporters 1 (OAT1) and 3 (OAT3) which had been previously established by us, the inhibitory effects of extracellular flavonoids (0.01, 0.1, 1, 10, 100 uM) on the uptake of representative substrates, *para*-aminohippuric acid (PAH) for OAT1 and estrone sulfate (ES) for OAT3 were investigated. In results, the flavonoids including quercetin, kaempterol, apigenin, luteolin and naringenin inhibited the OAT1 and OAT3-mediated organic anion transports. Inhibitions by genistein and daidzein were only shown in the OAT1 transport and rutin neither inhibited the transport of OAT1 nor OAT3. OAT1 and OAT3 which exist at the basolateral side of the renal proximal tubules are known as the opening site for the uptake of organic anions and the present results suggested OATs may be a renal elimination route for the circulatory flavonoids.

Key Words : kidney, transporter, organic anions, flavonoids, proximal tubules

INTRODUCTION

Flavonoids are distributed widely in plants and are classified into flavonol, flavanol, flavanone, flavone, isoflavone and anthocyanidin[1)]. Intake of the flavonoids is mainly through a diet and it is estimated to be 100 mg per day. An antioxidant effect of the flavonoids in food

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has been paid attention as it may prevent the oxidative stress-associated chronic diseases including ischemic heart disease and diabetes mellitus. Most studies have focused on the intestinal absorption and elimination of the flavonoids[1], however their elimination routes at the kidney have remained unknown.

Organic anion transporters are present in the renal proximal tubular cells and play an important role on elimination of xenobiotics including drugs[2]. In 1997 the author's research group had succeeded in identifying the renal specific organic anion transporter 1 (OAT1) for the first time in the world[3], and has identified many molecules starting with OAT1. That is, we have identified the genes of OAT1, OAT2 and OAT3 which are expressed at the basolateral membrane and those of OAT4 and OATv1 which are expressed at the luminal membrane in the proximal tubules[4]. Among which, OAT1 and OAT3 at the basolateral membrane are considered important as the first step of an uptake from the blood when the drug undergoes tubular excretion.

This study aimed to clarify molecular mechanisms of renal elimination of the flavonoids and used the cells stably expressing OAT1 and OAT3 (S2-hOAT1 and S2-hOAT3) which we have already established. We investigated to what extent the extracellular flavonoids inhibit a cellular uptake of *para*-aminohippuric acid (PAH) or estrone sulfate (ES), a representative substrate of each transporter, as an indicator.

METHODS

Materials

[^{14}C] PAH (1.86 GBq/mmol) and [^{3}H] ES (1961 GBq/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA). Flavonols including quercetin, kaempterol, rutin (quercetin-3-*O*-rutinoside) and quercetin-3-*O*-β-glucoside (Q3G), flavones including apigenin and luteolin, flavanones including naringenin and isoflavons including genistein and daidzein and other reagents were purchased from Sigma-Aldrich Japan (Tokyo) (Figure 1).

Cell culture

S2 cells used in this study, which are derived from the renal proximal tubules in mice and stably express human organic anion transporters 1 (hOAT1) and 3 (hOAT3) (S2-hOAT1 and S2-hOAT3), have been previously established[5]. S2-hOAT1 and S2-hOAT3 cells were grown in a RITC 80-7 medium including 5 % FBS, 10 mg/ml transferrin, 0.08 U/ml insulin, 10 ng/ml recombinant epidermal growth factor and 400 μg/ml geneticin at 37°C in 5% CO_2. Cells were subcultured by treatment with a 0.05% trypsin-EDTA solution (137 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 4 mM $NaHCO_3$, 0.5 mM EDTA, 5 mM HEPES, pH 7.2) and used for 25-35 passages.

Measurement of transport activity of organic anions

Cells were seeded on a 24-well tissue culture plate at a density of 1×10^5 cells/well. Two days after culturing, the cells were washed three times with a Dulbecco's modified phosphate-buffered saline (D-PBS : 137 mM NaCl, 3 mM KCl, 8 mM $NaHPO_4$, 1 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4) supplemented with 5.5 mM glucose, pre-incubated in the same solution at 37℃ for 10 min and then the transport activity of radioisotope labelled organic anions was measured. Flavonoids (0.1, 1, 10, 100 μM) were added to the D-PBS containing the radioisotope labelled organic anions ([^{14}C] PAH, 5 μM ; [^{3}H] ES, 50 nM) and they were added to a supernatant of the cells and the cells were incubated for 2 min. Then, a cellular uptake of the radioisotope labelled ones was determined by using a liquid scintillation counter.

Statistical Analysis

The experiment was conducted two to three times in combination of the flavonoids and S2-hOAT1 or S2-hOAT3 cells and the IC_{50} value was obtained from each experiment. Thus, data was expressed as mean ±SD where a number of the experiment was three and mean was only shown for the experiment which number was two. Two sample *t* test with Welch's correction was conducted for the comparison of the IC_{50} values of Quercetin between S2-hOAT1 and S2-hOAT3 cells. A *P* value of less than 0.05 was considered statistically significant. Regarding with Q3G, kaempferol, apigenin, luteolin and naringenin the statistical analysis could not be made as the number of the experiment of the S2-hOAT1 or S2-hOAT3 cells was two. The statistical analysis was not applicable for rutin, genistein and daidzein for they showed no

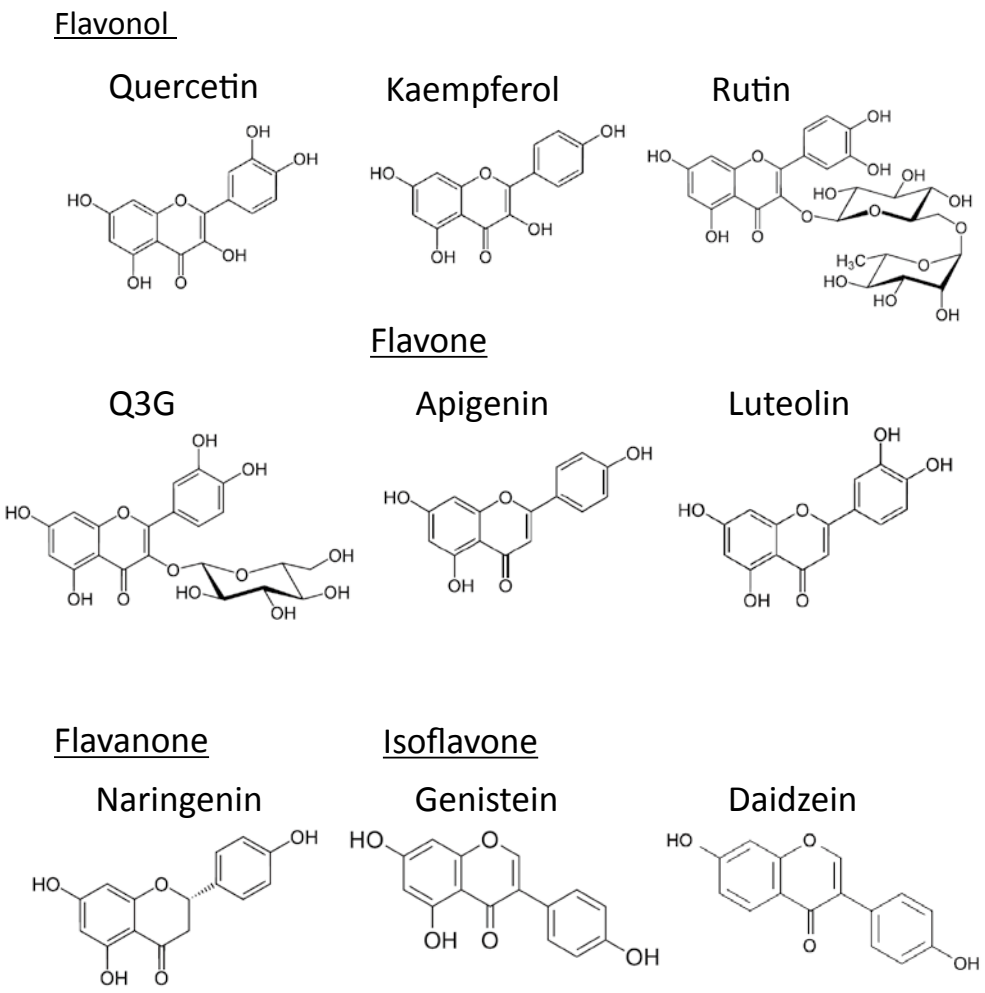

Figure 1
Chemical structures of flavonoids used in this study.

inhibition in both cells.

RESULTS

Inhibitory effect of the flavonoids on organic anion transport in the S2-hOAT1 cells

Inhibition studies for an uptake of [^{14}C] PAH by the flavonoids were conducted by using the S2-hOAT1 cells in order to investigate effects of the flavonoids on the hOAT1-mediated organic anion transport. As shown in Figure 2, eight flavonoids except for rutin inhibited a PAH transport dose dependently. Table 1 shows the IC_{50} values. Mean IC_{50} values of apigenin and luteolin, flavones, were the lowest and those of kaempferol and quercetin, flavonols, followed next in order. That of Q3G, a quercetin glycoside, was higher than that by quercetin aglycon. The mean IC_{50} values of naringenin, a flavanone, and genistein and daidzein, isoflavones, were high more than five and ten times those of flavones, respectively.

Inhibitory effect of the flavonoids on organic anion transport in the S2-hOAT3 cells

Next, inhibition studies for an uptake of [^{3}H] ES by the flavonoids were conducted by using the S2-hOAT3 cells in order to investigate effects of the flavonoids on the hOAT3-mediated organic anion transport. As shown in Figure 3, 50% inhibitions were not achieved by rutin, genistein and daidzein up to 100 μM concentration. Six flavonoids except for those mentioned before inhibited the hOAT3-mediated ES transport dose dependently. The mean IC_{50} values were shown in Table 1. Apigenin and luteolin, flavones, demonstrated the lowest values. Quercetin and kaempferol, flavonols, showed similar values. Inhibition

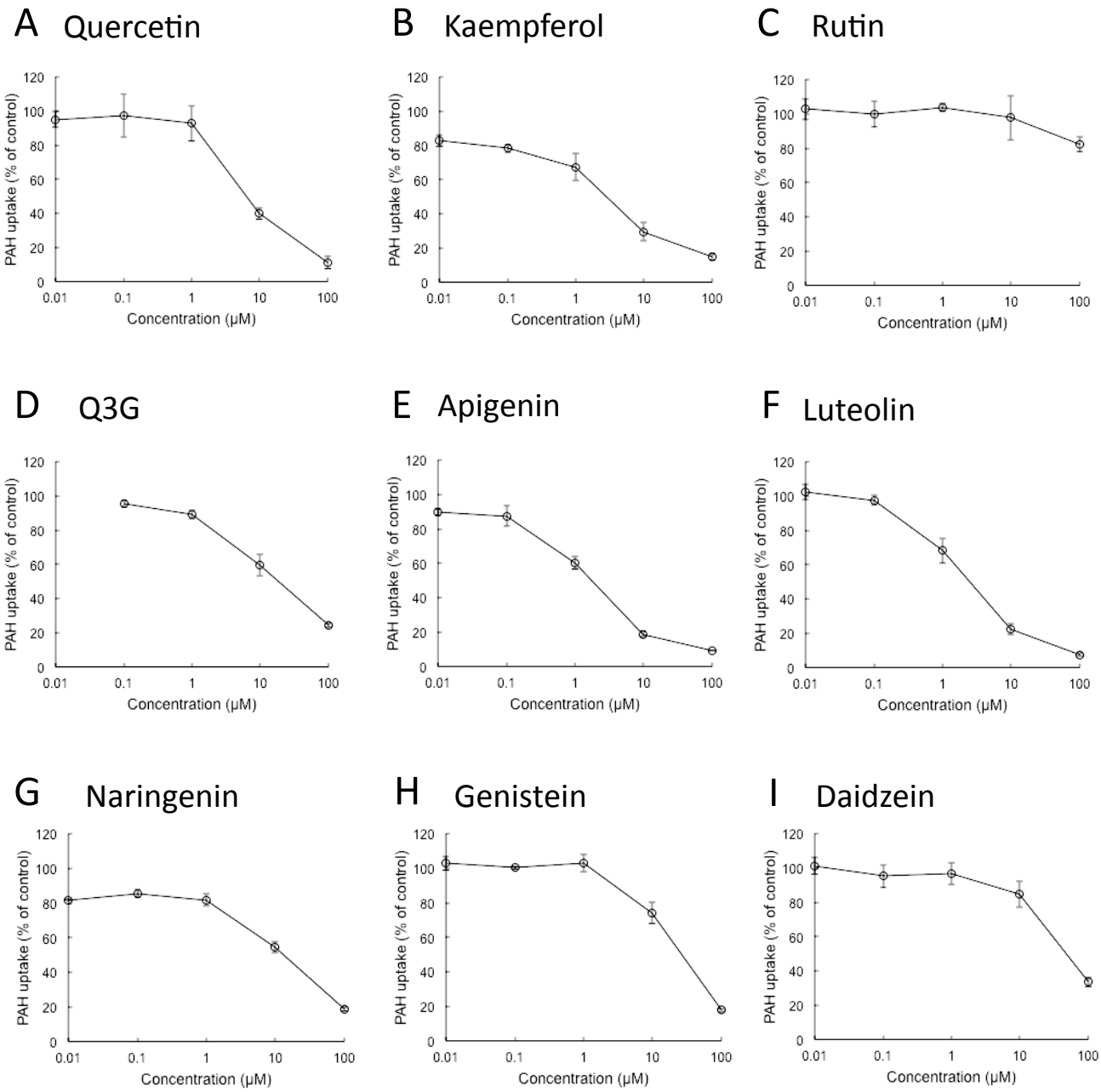

Figure 2

Inhibitory effects of flavonoids on PAH uptake mediated by S2-OAT1 cells. Quercetin (**A**), kaempterol (**B**), rutin (**C**), Q3G (**D**), apigenin (**E**), luteolin (**F**), naringenin (**G**), genistein (**H**) or daidzein (**I**) (0.1, 1, 10, 100 μM for each flavonoid) was added to the S2-hOAT1 cells with D-PBS containing a radioisotope labelled PAH (5 μM) and the cells were incubated for 2 min. Each value represents the mean ± S.E. of eight to twelve monolayers from two to three separate experiments.

by Q3G, a quercetin glycoside, was higher than that by quercetin aglycon and a difference of the IC_{50} values were more than five times. The mean IC_{50} value of naringenin, a flavanone, was slightly higher than those of flavones, but an inhibitory effect of naringenin was demonstrated.

Comparison of the IC_{50} values of quercetin between the S2-hOAT1 cells and the S2-hOAT3 cells

As shown in Table 1, IC_{50} values of Quercetin were 6.430 ± 1.343 and 2.828 ± 0.142 μM for hOAT1 and hOAT3, respectively ($P<0.05$). The results indicated quercetin has a stronger inhibitory effect on the

Table 1 IC_{50}s of flavonoid inhibition for hOAT1 and hOAT3

Flavonoids	(MW)	hOAT1 (μM)	hOAT3 (μM)	*P* value
Flavonol	Quercetin (302.2)	6.430 ± 1.343	2.828 ± 0.142	0.042
	Q3G (464.4)	18.667	15.841 ± 2.451	CA
	Kaempferol (286.2)	2.958	2.604 ± 0.250	CA
	Rutin (610.5)	NI	NI	NA
Flavone	Apigenin (270.2)	1.785	1.651 ± 0.298	CA
	Luteolin (286.2)	2.522	1.124 ± 0.304	CA
Flavanone	Naringenin (272.3)	13.475	2.163 ± 0.150	CA
Isoflavone	Genistein (270.2)	28.587 ± 3.607	NI	NA
	Daidzein (254.2)	47.697 ± 9.386	NI	NA

MW : Molecular Weight, NI : No Inhibition, CA : Could not be analyzed. NA : Not applicable. Data was expressed as mean ± SD where a number of the experiment was three and mean was only shown for the experiment which number was two. Two sample *t* test with Welch's correction was conducted regarding with Quercetin between S2-hOAT or S2-hOAT3 cells.

hOAT3-mediated organic anion transport than that on the hOAT1-mediated one.

DISCUSSION

In this study, we examined the interaction of flavonoids with renal basolateral organic anion transporters OAT1 as well as OAT3 *in vitro* to clarify the molecular mechanism of renal flavonoids excretion. Using OAT1- and OAT3-stably expressing mammalian cells named S2-hOAT1 and S2-hOAT3, we evaluated the inhibitory effects of flavonoids against OAT1-mediated PAH uptake and OAT3-mediated ES uptake and found that both OAT1 and OAT3, major molecule for basolateral entrance pathway for organic anions in kidneys, interacted with flavonoids to various degree.

Organic anion transporters (OATs), classified as SLC22 family[4] with twelve transmembrane domains, are mainly expressed in the kidney and the liver and contirbute to the excretion of endo- and exogenous organic anions less than 500 (M.W.). In the kidney, OATs are expressed mainly in the proximal tubule : OAT1, OAT2, and OAT3 are localized at the basolateral membrane while OAT4 is localized at apical membrane in human[4]. OAT1, OAT3, and OAT4 are known to utilize intracellular TCA cycle intermediates such as α-ketogulutarate, a dicarboxylate, as a driving force for organic anion uptake into the cells[4]. Based on their intracellular localization, both OAT1 and OAT3 are known to function as basolateral entrance pathways for organic anions in the case of tubular drug secretion.

Flavonoids are dietary polyphenols found particularly in fruits and vegetables. Quercetin, one of the major flavonols, is ingested 20-100 mg per day by dietary intake[6], and its peak plasma concentration reached 1-2 μM after ingestion[7,8]. Based on the analysis using human colonic cell line Caco-2, flavonoids

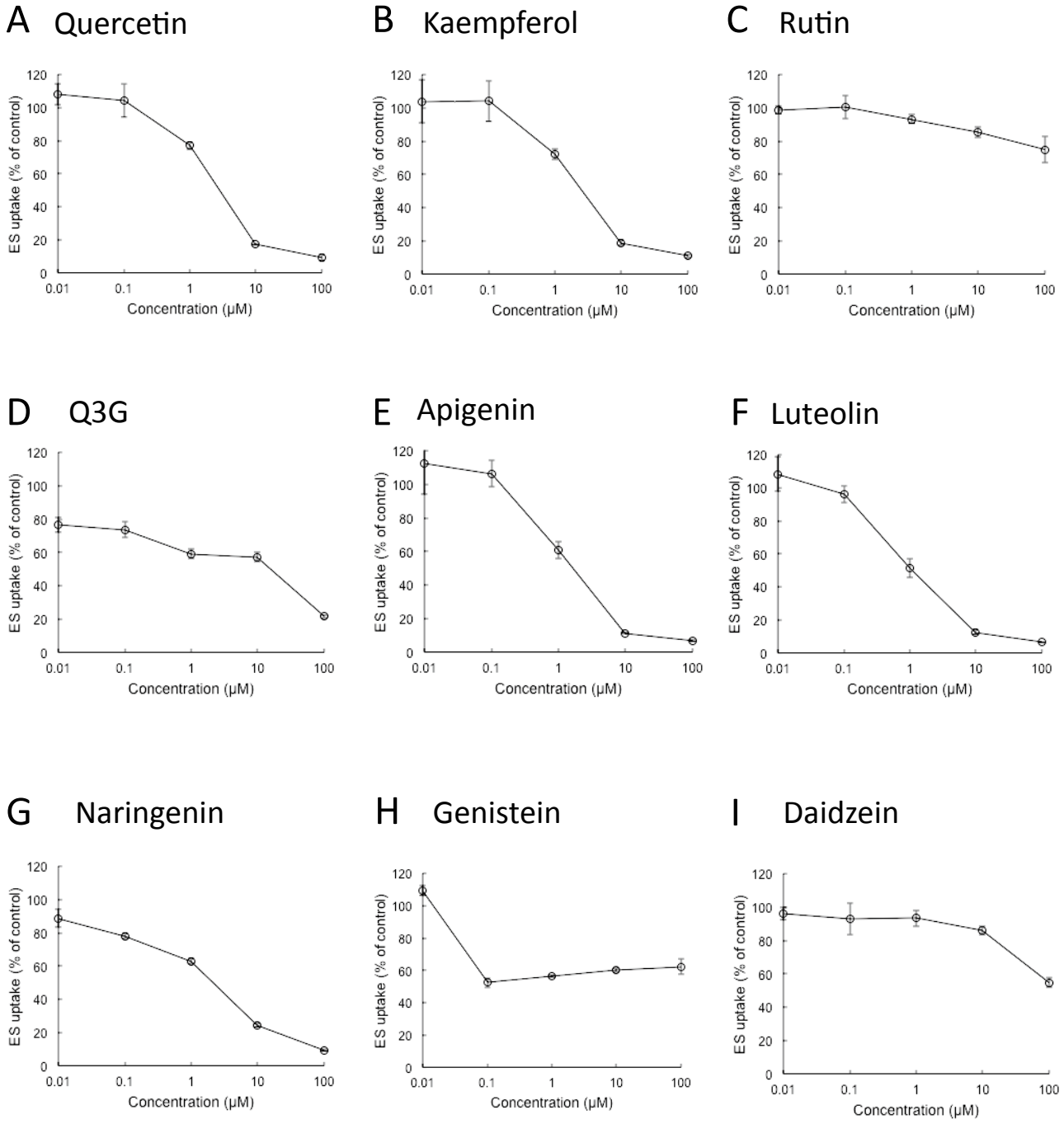

Figure 3

Inhibitory effects of flavonoids on ES uptake mediated by S2-OAT3 cells. Quercetin (**A**), kaempterol (**B**), rutin (**C**), Q3G (**D**), apigenin (**E**), luteolin (**F**), naringenin (**G**), genistein (**H**) or daidzein (**I**) (0.1, 1, 10, 100 μM for each flavonoid) was added to the S2-hOAT3 cells with D-PBS containing a radioisotope labelled ES (50 nM) and the cells were incubated for 2 min. Each value represents the mean ± S.E. of eight to twelve monolayers from two to three separate experiments.

such as quercetin are thought to be absorbed transcellularlly from the intestinal lumen to blood circulation via membrane transporters[9]. To date, several transporters such as sodium-coupled glucose transporter (SGLT1)[10] and multidrug resistance-associated protein (MRP2)[11] are detected as responsible transporters for the membrane permeation of flavonoids in the intesitinal epithelial cells and both SGLT1 and MRP2 are shown to mediate the transport of quercetin 4'-O-glucoside (Q4G)[10]. This Q4G is taken

up by SGLT1 at the luminal membrane and partly excreted by MRP2 at the same side. Intracellular enzyme β-glucosidase hydrolyze Q4G and create quercetin aglycon, then it passes serosal membrane and enter into the blood[1].

Flavonoids exist mostly in the blood and their Cmax is 0.5-2.5 μM[8]. Among flavonoids, it is reported that genistein and daidzein are excreted from the kidney[12]. This result indicated the importance of the kidney as a flavonoids excretory pathway from the body. It is well known that organic anion transporters (OATs) function as renal excretory pathway for xenobiotics and molecular weights of flavonoids tested in this study (from daidzein 254.2 to rutin 610.5 shown in Table 1) matches to the range of substrate recognition by OATs[4]. Thus, in this study, we aimed to clarify molecular mechanisms of renal elimination of flavonoids focusing on basolateral organic anion transporters OAT1 and OAT3 as entrance pathways for flavonoids.

As shown in Fig. 2 and 3, both OAT1 and OAT3 interacted with several flavonoids including flavonol, flavone, flabvanone, and isoflavone in a different manner. Quercetin showed the stronger inhibition against both OAT1 and OAT3 than its monoglycoside Q3G and its diglycoside rutin indicating that these transporters recognize mainly quercetin aglycone, not its glycoside. This pattern is different from those of SGLT1[10] and MRP2[11]. IC_{50}s of quercetin and kaempferol (flavonol), apigenin and luteolin (flavone) against both OAT1 and OAT3 are 1.1-6.4 μM (Table 1). These flavonoids are thought to be the substrates for them because these are compatible with their blood concentration. IC_{50}s of naringenin (flavanone) may be different in OAT1 and OAT3, indicating that it would be a better substrate for OAT3 but not for OAT1. Furthermore, genistein and daidzein (isoflavone) did not demonstrate over 50% inhibition against OAT3 and its IC_{50}s against OAT1 were more than 10 μM suggesting that these two isoflavone are not the transport substrates of OATs and OATs do not contribute to the renal excretion of isoflavone. For their renal excretion, transporters other than OATs may contribute to it. Generally speaking, OATs interacted with several flavonoids examined in this study, indicating that their role in renal flavonoids excretion as basolateral entrance pathway summarized in Fig. 4.

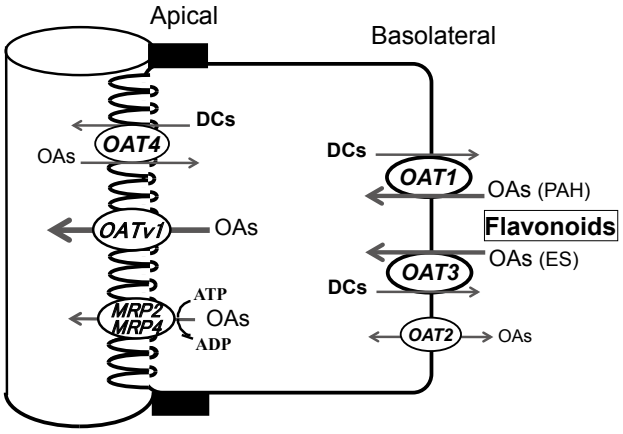

Figure 4

Proposed model of flavonoids excretion in renal tubular cells. OAT1 and OAT3 may function as an entrance pathway for flavonoids in the proximal tubular cells. OAT : organic anion transporter, MRP : multidrug resistance-associeted protein, OAs : organic anions, DCs : dicarboxylates, PAH : para-aminohippuric acid, ES : estrone sulfate.

With comparison of IC_{50}s between OAT1 and OAT3, the affinity of flavonol/flavone and that of flavanone/isoflavone may be different. This may lead not only to control the bioavailability of flavonoids in the body through inhibitng its renal tubular transport, but also to provide important structure-activity informtion for searching the seed of developing drugs that specifically inhibit OAT1 and/or OAT3. Further study is necessary to obtain such information useful for drug discovery.

CONCLUSION

To elucidate the molecular mechanism of renal excretion of flavonoids, we focused on organic anion transporters. Using the cells stably expressing OAT1 and OAT3 (S2-hOAT1 and S2-hOAT3), we investigated the inhibitory effects of flavonoids on a cellular uptake of PAH or ES, a representative substrate of each transporter, as an indicator. Among flavonoids tested in this study, rank order of the mean IC_{50}s for OAT1 are as follows : flavones (apigenin, luteolin) < flavonols (quercetin, kaempferol) <flavanone (naringenin) <isoflavone (genistein, daidzein). Those for OAT3 are as follows : flavones (apigenin, luteolin) < flavanone (naringenin) <flavonols (quercetin, kaempferol). Particularly in the case of quercetin, IC_{50}s of

aglycon is smaller than those of glycosides for both transporters.

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Conflict of Interest

No COI for all authors.

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