Imperatorin is Transported through Blood-Brain Barrier by Carrier-Mediated Transporters

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Abstract

Imperatorin, a major bioactive furanocoumarin with multifunctions, can be used for treating neurodegenerative diseases. In this study, we investigated the characteristics of imperatorin transport in the brain. Experiments of the present study were designed to study imperatorin transport across the blood-brain barrier both in vivo and in vitro. In vivo study was performed in rats using single intravenous injection and in situ carotid artery perfusion technique. Conditionally immortalized rat brain capillary endothelial cells were as an in vitro model of blood-brain barrier to examine the transport mechanism of imperatorin. Brain distribution volume of imperatorin was about 6 fold greater than that of sucrose, suggesting that the transport of imperatorin was through the blood-brain barrier in physiological state. Both in vivo and in vitro imperatorin transport studies demonstrated that imperatorin could be transported in a concentration-dependent manner with high affinity. Imperatorin uptake was dependent on proton gradient in an opposite direction. It was significantly reduced by pretreatment with sodium azide. However, its uptake was not inhibited by replacing extracellular sodium with potassium or N-methylglucamine. The uptake of imperatorin was inhibited by various cationic compounds, but not inhibited by TEA, choline and organic anion substances. Transfection of plasma membrane monoamine transporter, organic cation transporter 2 and organic cation/carnitine transporter 2/1 siRNA failed to alter imperatorin transport in brain capillary endothelial cells. Especially, tramadol, clonidine and pyrilamine inhibited the uptake of [3H]imperatorin competitively. Therefore, imperatorin is actively transported from blood to brain across the blood-brain barrier by passive and carrier-mediated transporter.

Key Words: Imperatorin, Alzheimer’s disease, Blood-brain barrier, Proton coupled antiporter

INTRODUCTION

Imperatorin is isolated from the root of Angelica dahurica. It is a major bioactive furanocoumarin (Baek et al., 2000). It has long been recognized that imperatorin exhibits many biological properties such as anticancer (Kozioł and Skalicka-Woźniak, 2016), antibacterial (Stavri and Gibbons, 2005) anti-inflammatory (Abad et al., 2001) and HIV replication-inhibiting activities (Sancho et al., 2004). It is also therapeutically helpful for disorders with high anxiety level and memory impairment (Budzynska et al., 2012).

Alzheimer’s disease (AD) and Parkinson’s disease (PD) are neurodegenerative diseases characterized by cholinergic dysfunction with cholinergic deficiency in the brain (Kozioł and Skalicka-Woźniak, 2016). Imperatorin as an inhibitor of acetylcholinesterase (AChE) might be useful for treating AD and PD (Kim et al., 2002; Sigurdsson and Gudbjarnason, 2007). It has a small molecular weight (270 g/mol) and a large value of log P (3.65). Recently, it has been reported that imperatorin is highly passed through the blood-brain barrier (BBB) according to in vivo (oral administration) and in vitro permeability data using LC-MS/MS analysis method (Lili et al., 2013). However, the characteristics of imperatorin transport through the BBB remains unknown. Thus, it is important to investigate the transport characteristics of imperatorin to predict the effect of imperatorin on AD and PD.

BBB is formed by three cellular elements (astrocytes, pericytes and endothelial cells) at the lining of the tight junction. It expresses multiple transporters, which can influence the BBB permeability of their substrates (Ohtsuki and Terasaki, 2007). These transporters can mediate the blood-to-brain influx for nutrient and other essential molecules as well as the
brain-to-blood efflux to eliminate metabolites and neurotoxic compounds from brain (Ohtsuki and Terasaki, 2007). Several influ-
x and efflux drug transporter are expressed at the BBB, in-
cluding sodium-independent glucose transporter (GLUT1/Sli-
c2a1), monocarboxylate transporter 1 (MCT1/Slc16a1), amino
acid transporter, organic anion transporter 3 (Oat3/Slc22a8),
organic anion-transporing polypeptides (Oatps/Sloc) and
multidrug resistance-associated protein (Mrps/ABCC) (Oht-
suki and Terasaki, 2007). Organic cation transporters (Oct1-3/Slc22a1-3), high-affinity choline transporter (ChT/Slc5a7),
organic cation/carinaline transporters 1-2 (Octn1-2/Slc22a4-5),
plasma membrane monooamine transporter (Pmat/Slc29a4),
and multidrug and toxin extrusion protein (Mate/Slc47a) are
involved in the influ and efflux transport of various cationic
drugs (Okura et al., 2002; Roth et al., 2011), tramadol (Kitamura
et al., 2008), oxy-

Radioisotope and reagents

Radiolabeled compound [3H]imperatorin (3.7 Ci/mmol) was
purchased from American Radiolabeled Chemical, Inc (St.
Louis, MO, USA). Unlabeled compounds such as impera-
torin, tramadol hydrochloride, pyrilamine maleate salt, verapamil
hydrochloride, quinidine, nicotine, clonidine hydrochloride,
and multidrug resistance-associated protein (Mrps/ABCC) (Oht-
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drugs (Okura et al., 2002; Roth et al., 2011), tramadol (Kitamura
et al., 2008), oxy-
dergic and opioids such as pyrilamine (Okura
et al., 2008; Roth et al., 2011), tramadol (Kimura et al., 2014), nicotine (Cistermino et
al., 2013) and clonidine (Andr et al., 2009) are transported by
the proton coupled antipporter. However, the molecular nature
of this antipporter remains unknown. It has been reported that
this transporter is dependent on energy and oppositely direct-
ed proton gradient. However, it is independent on membrane
potential or sodium (Shimomura et al., 2013).

The objective of the present study was to investigate the
transport mechanism of imperatorin across the BBB using in
vivo intravenous injection (IV) and in situ internal carotid artery
perfusion (ICAP) techniques. To clarify the functional properties
of imperatorin influ at the BBB and its interaction with several
transporters of substrates, in vitro uptake studies, Real-Time
PCR and siRNA transfection were performed using conditionally
immortalized rat brain capillary endothelial cells (TR-BBB cells).

MATERIALS AND METHODS

Radioisotope and reagents

Radiolabeled compound [3H]imperatorin (3.7 Ci/mmol) was
purchased from American Radiolabeled Chemical, Inc (St.
Louis, MO, USA). Unlabeled compounds such as impera-
torin, tramadol hydrochloride, pyrilamine maleate salt, verapamil
hydrochloride, quinidine, nicotine, clonidine hydrochloride,
1-Methyl-4-phenylpyridinium ion (MPP+) and other comp-
ounds were purchased from Sigma Aldrich (St. Louis, MO,
USA).

Animals

Male Sprague-Dawley rats (SD rats, 7 weeks, 250-350
was purchased from Koatech Inc (Pyeongtaek, Korea).
All animal experiments were approved by the Commit-
tee of the Ethics of Animal Experimentation of Sookmyung
Women’s University (Seoul, Korea; Approval No.: SMWU-
IACUC-16017-014).

In vivo brain uptake study

Intravenous injection technique (Pharmacokinetic): Pharmacokinetic parameters and brain uptake of [3H]impara-
torin were investigated in rats following a single IV injections
according to previous reports (Partridge et al., 1994; Lee et
al., 2014). SD rats were anesthetized ketamine/xylazine (100
mg/kg and 2 mg/kg; Yuhan, Seoul, Korea). [3H]Imperatorin
(1.35 µM) was injected to the left femoral vein of SD rat. Fol-
lowing administration, blood samples (0.3 mL) were collected
via polyethylene 50 (PE 50) tube implanted in the left femo-
ral artery at 0.25-60 min. At 60 min after injection, brain and
other organs were collected. Organ samples were solubilized
with solunene-350 (PerkinElmer, Waltham, MA, USA) and
radioactivity was counted by using a Tri-Carb liquid scintilla-
tion counter (Tri-Carb 2810TR; PerkinElmer) with ultima gold
(PerkinElmer).

Plasma radioactivity (dpm/ml) was converted to the per-
centage of injected dose (ID) per milliliter (ml). The %ID/ml was
fit to a bi-exponential equation (1):

\[
\%ID/ml = A_1 e^{-k_{12}} + A_2 e^{-k_{22}}
\]  

(1)

The intercepts (A1 and A2) and the slopes (k1 and k2) were
used to compute the pharmaco kinetic parameters.

Pharmacokinetic parameters were computed as described
previously (Lee et al., 2014) to obtain the area under the plas-
ma concentration curve (AUC) at 60 min.

The BBB permeability-surface area (PS) product or organ
clearance (µl/min/g) was determined using the following equa-
tion (2):

\[
PS_{product} = \frac{V_D - V_0}{C_p(t)} AUC(t)
\]  

(2)

where V0 is the terminal brain/plasma ratio or the brain volume
of distribution, and V0 is the plasma volumes for the respective
organisms and C0 (t) is the terminal plasma concentration (%ID/
ml). The terminal brain uptake, expressed as %ID/g brain, was
calculated from the PS (µl/min/g) and the 60-min plasma AUC
(%ID min/ml) using the following equation (3):

\[
%ID/g(t) = PS_{product} \times AUC(t)
\]  

(3)

Brain uptake index method (BUI): BUI technique was
performed as previously (Suzuki et al., 2002). After rat was
anesthetized with ketamine, the common carotid ar-
tery was injected with 200 µl Ringer-HEPES buffer contain-
ing [3H]imperatorin (2.5 µCi) with or without unlabeled inhibitor
compound and [14C]n-butanol (0.5 µCi) used as an internal
reference compound. Rat was decapitated 15 s after injection
and cerebrum were dissolved in solunene-350. Their radioactiv-
ity was performed using Tri-Carb Liquid Scintillation Coun-
ters. The distribution characteristic of [3H]imperatorin were
equipped using the percentage of [3H]imperatorin uptake relative
to [14C]n-butanol that was expressed by Eq (4):

\[
BUI(\%) = \frac{\text{[3H]} \text{dpm in the brain}}{\text{[14C]} \text{dpm in the injectate solution}} \times 100
\]  

(4)

Internal carotid artery perfusion technique: ICAP tech-
nique was performed as reported previously (Takasato et
al., 1984; Lee and Kang, 2016). SD rats were anesthetized with
ketamine/xylazine (100 mg/kg and 2 mg/kg). [3H]Imperatorin
(270 nm) with or without unlabeled imperatorin and inhibitor
compounds were diluted in KHB and perfused into the internal
carotid artery at a flow rate of 4 mL/min for 15 sec using
micro-syringe pump. To examine [3H]imperatorin transport on
pH alteration, HCl or NaOH was added to KHB in some exper-
iments after gassing to bring the pH to 6.40, 7.40 or 8.40.
The total imperatorin concentration in perfusate or incubation buffer, \( C_{\text{tot}} \) (mM) was determined using the following equation (5):

\[
V_d(\mu L/g) = \frac{[\text{brain(dpm)}/\text{brain(g)}]}{[\text{perfusate(dpm)}/\text{perfusate(µL)}]}
\]

The BBB permeability surface area (PS) product was calculated using the following equation (5):

\[
PS(\mu L/min/g) = \frac{V_d(\mu L/g)}{t \text{ (min)}}
\]

where \( V_d \) was the brain volume of the [3H] compound and \( t \) was the perfusion time (15 Sec).

For the concentration-dependency experiment (André et al., 2009), the flux of [3H]imperatorin was calculated from the flux (\( J_n \), nmol/min/g of brain) by given equation (6):

\[
J_n = PS \times C_{\text{tot}}
\]

The [3H]imperatorin brain flux (\( J_n \)) or cellular velocity (\( \mu \)mol/min/g) was described as saturable (Michaelis-Menten term). A passive unsaturable component has been measured with equation (7):

\[
J_{\text{pass}} = \frac{V_{\text{max}} \times C_{\text{tot}}}{K_{m}+C_{\text{tot}}} + K_{\text{passive}} \times C_{\text{tot}}
\]

where \( C_{\text{tot}} \) (mM) was the total imperatorin concentration in perfusate or incubation buffer, \( V_{\text{max}} \) (µmol/min/g) is the maximal velocity of transport, and \( K_{m} \) (mM) of imperatorin was the concentration at the half-maximal carrier velocity. \( K_{\text{passive}} \) (µL/min/g) was an unsaturable component representing the rate transport by passive diffusion. Data were fitted using nonlinear regression analysis.

[3H]Imperatorin uptake study in TR-BBB cells: TR-BBB cells were cultured according to a previously reported method (Terasaki and Hosoya, 2001; Kang et al., 2002). For in vitro uptake study, [3H]imperatorin transport in TR-BBB cells was performed according to a previously described method (Kang et al., 2002). Cells were then incubated with 200 µL transport buffer containing 135 mM [3H]imperatorin with or without selected compounds at 37°C for a designed time. Aliquots were collected to count the radioactivity using the Tri-Carb Liquid Scintillation Counter. Cellular protein content was determined with a DC protein assay kit (Bio-Rad Laboratories Co, Hercules, CA, USA) as a standard. [3H]Imperatorin uptake was expressed as cell-to-medium (µL/mg protein) ratio as follows: radioactivity (dpm/µL) in the sample per milligram cell protein (dpm/mg protein).

The initial uptake of imperatorin was measured for 5 min For kinetic studies, the Michaelis-Menten constant (\( K_{m} \)) and the maximum uptake rate (\( V_{\text{max}} \)) of [3H]imperatorin were estimated using the following equation (6):

\[
V = V_{\text{max}} \times C/(K_{m}+C) + K_{c}\times C
\]

where \( V \) and \( C \) were the initial uptake rate of [3H]imperatorin at 5 min and the concentration of imperatorin, respectively. \( V_{\text{max}} \) was the maximum uptake rate for the saturable component, and \( K_{c} \) was the first order constant for non-saturable component respectively.

\[
V_{\text{max}}/K_{c} \text{ (µL/min/mg protein)} = \text{calculated as the uptake clearance for saturable transport compound. The saturable component of imperatorin was plotted by non saturable uptake from total uptake in Eadie-Hofstee plot.}
\]

The inhibitory constant (\( K_{i} \)) was in the presence of 2 mM tramadol, clonidine or pyrilamine. It was calculated from the following equation (9):

\[
V = V_{\text{max}}\times C/[K_{m}\times(1+I/K_{i})+C]+K_{c}\times C
\]

where I was the concentration of each mutual inhibitory effects of compound, as the inhibitor concentration.

Energy, sodium ion and membrane potential dependency of imperatorin uptake by TR-BBB cells were determined as described previously (Kitamura et al., 2014). The uptake was evaluated using a radioactive measurement and was used to 20 min pre-incubation with 0.1% of sodium azide (NaN₃) and 25 µM of rotenone (dissolved in the transport buffer containing 0.2% DMSO) which were metabolic energy inhibitor. In this experiment, 10 mM D-glucose in the ECF buffer was replaced by 10 mM 3-O-methylglucose to reduce metabolic energy. To assess sodium ion dependency, the uptake was measured under sodium ion-free condition by replacing NaCl in ECF buffer with NMG²⁺. The uptake also performed under membrane-disrupted condition by replacing of sodium ion with KCl followed by treatment with 10 µM valinomycin (transport buffer containing 0.2% DMSO) for 10 min. In order to evaluate the effect of proton gradient on imperatorin uptake by TR-BBB cells, cells were simultaneously treated with 10 µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, a protonophore). FCCP was dissolved in transport buffer containing 0.10% DMSO. For extracellular pH (pHₑ) dependent, [3H]imperatorin uptake at pH 6.4 and pH 8.4. To examine the effect of intracellular pH (pHi) dependent, cells were pre-treated with 30 mM of ammonium chloride (NH₄Cl) for 30 min to reduce pHi, and simultaneously treated with 30 mM NH₄Cl to increase pHi (Okura et al., 2008).

RNA interference analysis

For gene silencing of a set of four siRNAs (GE Healthcare Dharmaco, Inc., Landsmeer, Netherlands) specific for rOctn2, rPmat, rOctn1, rOct2 and negative control were used in TR-BBB cells, including target sequences of Octn2, rPmat, rOctn1, rOct2 and negative control. TR-BBB cells were seeded onto collagen-coated 6- and 24-well plates at a density of 1×10⁵ cells/cm². At 24 h after seeding, siRNAs specific for Octn2, Pmat, rOctn1and rOct2 (200 nM) or negative control siRNA (control) were transfected into TR-BBB cells using Lipofectamine® 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s protocol. Cells were used for quantitative real-time PCR and [3H]imperatorin uptake was analyzed at 48 h after the initiation of transfection (Lee and Kang, 2016).

Statistical analysis

All data were expressed as means ± standard error of the means (SEM). Statistical analysis of data was performed by one-way ANOVA analysis of variance followed by Dunnett’s (post hoc test) for single and multiple comparisons, respectively. Statistically significant was considered at p<0.05.
**RESULTS**

*In vivo* brain uptake of $[^3H]$imperatorin across the blood-brain barriers

First, brain uptake and pharmacokinetic parameters of imperatorin across the BBB were examined following IV injection of $[^3H]$imperatorin. Time course result of $[^3H]$imperatorin clearance from blood in SD rats was shown in Fig. 1. Pharmacokinetic parameters by analyzing data in Fig. 1 are listed in Table 1A. Plasma AUC and BBB PS products of $[^3H]$imperatorin were $22 \pm 1\%$ ID/min/mL and $3.94 \pm 0.54 \mu$L/min/g, respectively (Table 1A). Thus the BBB PS product of $[^3H]$imperatorin was 12 fold higher than BBB PS product of sucrose ($0.32 \pm 0.52 \mu$L/min/g) (Lee and Kang, 2016). The volume of distribution ($V_D$) of $[^3H]$imperatorin ($392 \pm 30 \mu$L/g) (data not shown) (Lee and Kang, 2016). The clearance of $[^3H]$imperatorin was 615 ± 3 μL/g after perfusion (data not shown) (Lee and Kang, 2016). The clearance of $[^3H]$imperatorin by other peripheral tissues such as heart, liver, lung and kidney is shown in Table 1B. Uptake of $[^3H]$imperatorin by the brain in rats was 0.086 ± 0.008%ID/g (Table 1B). These results indicated that imperatorin was actively taken up into the brain across the BBB under physiological condition.

In order to confirm the blood-to-brain transports mechanism, imperatorin was also measured with the ICAP technique. The brain volume distribution (corrected $V_D$) value of $[^3H]$imperatorin was 615 ± 3 μL/g after perfusion (data not shown). The $V_D$ was 60 fold greater than the $V_D$ of $[^4C]$sucrose (68 ± 9 μL/g) (data not shown) (Lee and Kang, 2016). The clearance of $[^3H]$imperatorin by other peripheral tissues such as heart, liver, lung and kidney was also significantly decreased by pH 6.4, while brain $V_D$ of $[^3H]$imperatorin was increased at pH 8.4 compared to that of the control (Fig. 2). A decrease in the pH can lead to inversion of the proton gradient that favors the movement of the proton out of cells. The brain transport of $[^3H]$imperatorin was significantly increased in mannitol Na+-free buffers compared with control (Fig. 2). However brain transport of $[^3H]$imperatorin was markedly decreased in carbonate-free HEPES. Therefore, imperatorin is actively transported from blood to the brain across the BBB. It depends on opposite direction of proton gradient.

Brain influx of imperatorin was found to be concentration dependent (Fig. 3). The used concentrations were reflected unsaturated and saturated conditions (lower than 0.25 mM, Fig. 3 insert). The plot of influx against total imperatorin concentration yielded an apparent $K_m$ of 0.18 mM and $V_{max}$ of 0.50 μmol/min/g. Unsaturated apparent component ($K_m$) certainly reflected passive diffusion. Nonlinear regression analysis gave $K_m$ of $3.1 \times 10^{-17}$ μL/min/g representing a very small value of total brain imperatorin influx. This suggests that involvement of influx transporter in luminal side of the BBB transport of imperatorin. Cationic drug such as tramadol, pyrilamine, clonidine and verapamil strongly inhibited $[^3H]$imperatorin uptake in rat brain based on ICAP (Table 2). The uptake of $[^3H]$imperatorin was also strongly inhibited by other cationic drug such as MPP+ (Pat3 substrate) (Okura et al., 2011), ALC, and L-carnitine (Kido et al., 2001). However, some cationic compounds in Table 2 such as TEA (substrate of OCTs, Oct1, and Oct2) (Tamai et al., 2004; Ohta et al., 2006) and choline (ChT substrate) (Kang et al., 2005) did not affect $[^3H]$imperatorin transport in the brain. Moreover, $[^3H]$imperatorin uptake was not changed by organic anion such as PAH (Oat3 substrate) (Hosoya et al., 2009) or 6-Mercaptourine either (6-Mp,Oat3 and Mrps substrate) (Hosoya et al., 2009; Lee et al., 2011). In addition, the BUI value of $[^3H]$imperatorin was 50.4%. The BUI of $[^3H]$imperatorin was significantly inhibited by unlabeled imperatorin (1 mM), 10 mM of verapamil, nicotine, pyrilamine, clonidine (Table 3). However $[^3H]$imperatorin uptake was not changed by 10 mM of TEA and PAH. These results indicate that imperatorin across the BBB and in vivo brain uptake of its may be also involved in carrier-mediated transport system.

![Fig. 1. Clearance from plasma of $[^3H]$imperatorin for up to 60 min after single intravenous injection in SD rats. Each point represents the mean ± SEM (n=3 rats).](image-url)

**Table 1.** Pharmacokinetic parameters (A) and brain volume of distribution (B) of $[^3H]$imperatorin after single intravenous (IV) injection in SD rats

| Organ | Organ clearance (μL/min/g) | Uptake (%ID/g) |
|-------|--------------------------|----------------|
| Brain | 3.94 ± 0.5               | 0.086 ± 0.008  |
| Heart | 73.7 ± 5.9               | 1.62 ± 0.10    |
| Liver | 54.7 ± 2.5               | 1.21 ± 0.08    |
| Lung  | 76.4 ± 3.4               | 1.68 ± 0.03    |
| Kidney| 168 ± 17                 | 3.70 ± 0.33    |

Parameters computed from the plasma radioactivity profile in Fig 1. $V_D$, BBB PS products, %ID/g and pharmacokinetic parameters were estimated after IV injection of $[^3H]$imperatorin (1.35 μM) at 60 min in SD rats. Each value represents mean ± SEM (n=3). $T_{1/2}$: half-life; AUC: area under the curve of plasma concentration; CL: clearance; MRT: mean residence time; $V_{d,ss}$: plasma volume of distribution at steady state.
In vitro characteristics of the [3H]imperatorin transport mechanism by TR-BBB cells

To determine the mechanism of [3H]imperatorin uptake into brain, TR-BBB cell lines were used as a rat in vitro model of BBB. Uptake of [3H]imperatorin was time-dependent. It was increased linearly until 5 min at pH 7.4 (Fig. 4A). Therefore, uptake was evaluated at 5 min in the following kinetic and inhibition studies. The effect of pH on [3H]imperatorin uptake by TR-BBB cells was examined by incubating ECF-buffer containing [3H]imperatorin at pH 6.4, 7.4 or 8.4. [3H]imperatorin uptake was significantly reduced at pH 6.4, while it was markedly increased at pH 8.4 compared to that of the control (Fig. 5A). To confirm the result of pH alteration, the effect of pH, alteration on [3H]imperatorin uptake by TR-BBB cells was continuously examined. [3H]imperatorin uptake was reduced when pH became alkalized that induced by 30 mM NH₄Cl, while was increased at intracellular acidification condition induced by 20 min pretreatment of NH₄Cl (Fig. 5B). To test the driving force of [3H]imperatorin uptake, pretreatment with protonophore, FCCP, was performed for 10 min. FCCP decreased [3H]imperatorin uptake compared to the control (Table 4). These results suggest that the uptake of imperatorin is driven by an oppositely directed proton gradient. [3H]imperatorin uptake was also markedly reduced by pretreatment with metabolic inhibitors such as rotenone or sodium azide compared to that in the control. In contrast, uptake of imperatorin was not significantly decreased by replacement of sodium with N-methylglucamine or potassium chloride. Moreover, uptake of imperatorin was not changed by 10 min treatment with valinomycin, a potassium ionophore (Table 4). These results indicate that imperatorin uptake through the BBB is transported by proton coupled antiporter and it is energy dependent, but

Table 2. Inhibitory effect of various compounds on [3H]imperatorin brain uptake following internal carotid artery perfusion in SD rats

| Inhibitor | Concentration (mM) | Brain volume of distribution (Vd, % of control) |
|-----------|--------------------|-----------------------------------------------|
| Control   | 100 ± 0            |                                               |
| Imperatorin| 1                  | 17.2 ± 1.7***                                |
| Tramadol  | 1                  | 79.2 ± 3.8***                                |
| Paeonol   | 1                  | 56.8 ± 7.1***                                |
| Verapamil | 1                  | 15.1 ± 2.3***                                |
| Pyrilmamine| 1                 | 59.2 ± 2.6***                                |
| Clonidine | 1                  | 69.5 ± 1.5***                                |
| MPP+      | 1                  | 56.3 ± 5.8***                                |
| ALC       | 1                  | 58.1 ± 1***                                  |
| L-Carnitine| 1                 | 79.2 ± 5.3***                                |
| TEA       | 1                  | 103 ± 5                                      |
| Choline   | 1                  | 99.1 ± 3.3                                   |
| PAH       | 1                  | 94.4 ± 7.1                                   |
| 6-MP      | 1                  | 102 ± 2                                      |

Inhibition of [3H]imperatorin brain uptake by other compounds 1 mM was evaluated using internal carotid artery perfusion technique at rate of 4 ml/min for 15 sec in SD rats, pH7.4. Each value represents mean ± SEM (n=3~7). ***p<0.001, significantly different from the control.

Table 3. Brain uptake index (BUI) of [3H]imperatorin

| Inhibitor | Concentration (mM) | BUI (%) |
|-----------|--------------------|--------|
| Control   | 50.4 ± 1.8         |        |
| Imperatorin| 1                  | 24.9 ± 3.4*** |
| Verapamil | 10                 | 22.1 ± 3.8*** |
| Nicotine  | 10                 | 27.6 ± 0.0*** |
| Pyrilmamine| 10                | 29.1 ± 1.7*** |
| Clonidine | 10                 | 33.9 ± 5.5** |
| TEA       | 10                 | 48.6 ± 4.5 |
| PAH       | 10                 | 58.5 ± 6.3 |

A mixture of [3H]imperatorin was injected into the common carotid artery in the presence or absence of various inhibitors. Rats were decapitated at 15 s after injection. Each value represents the mean ± SEM (n=3~6). **p<0.01, ***p<0.001; significantly different from control.
unlabeled imperatorin (0.01~1 mM) was examined. Calculation for kinetic parameters of imperatorin revealed Michaelis-Menten constant (Km) of 59 μM and a maximum rate of uptake velocity (V max) of 2.22 nmol/mg protein/min (Fig. 4B). Non-saturable uptake clearance (Kd) of imperatorin was 17 μL/(mg protein×min).

The transporter(s) responsible for imperatorin uptake into TR-BBB cells was determined by testing the inhibitory effect of several compounds clearly related to BBB transporters. Uptake of [3H]imperatorin was strongly inhibited by several cat-ionic drugs such as tramadol, pyrilamine, diphenhydramine, clonidine, nicotine, verapamil (Kubo et al., 2013), and quinine (Table 5). Organic cation, MPP+, ALC, and L-carnitine moderately inhibited [3H]imperatorin uptake. However some compounds as shown in Table 4, TEA, choline, PAH, estrone-3-sulfate (Oatps) (Sai et al., 2006) and 6-Mp failed to inhibit [3H]imperatorin uptake.

In vivo and in vitro results showed that imperatorin uptake was inhibited by MPP+, L-carnitine, and ALC. Therefore imperatorin uptake could be mediated by Pmat or Octn2. To evaluate whether imperatorin transport was mediated by Pmat and Octn2, siRNA transfection was performed to knockdown rPmat or rOctn2 in TR-BBB cells. Quantitative real-time PCR analysis showed that rPmat, Oct2, Octn2 and rOctn1 siRNA decreased mRNA expression levels of rPmat and Octn2 to 33.5%, 59.4%, 60.6% and 47.8%, respectively, compared to control siRNA (data not shown). Moreover, [3H]MPP+ and [3H]ACL uptake were significantly inhibited by rPmat and rOctn2 siRNA, respectively (Fig. 6A, 6B). In contrast, [3H]imperatorin uptake was not significantly affected by rPmat, rOct2, or rOctn1 siRNA transfection (Fig. 6C, 6D).

The plots of [3H]imperatorin uptake with or without 1 mM of tramadol, pyrilamine or clonidine intersected at the ordinate axis. These results demonstrate that tramadol, pyrilamine and
**Table 5.** Inhibitory effects of selected compounds on [3H]imperatorin uptake by TR-BBB cells

| Compound          | Concentration (mM) | Relative Uptake (% of Control) | Predicted Log D |
|-------------------|--------------------|--------------------------------|-----------------|
| Control           | 1                  | 100 ± 3                        |                 |
| + Imperatorin     | 1                  | 44.7 ± 2.6***                  | 2.98            |
| + Tramadol        | 10                 | 71.7 ± 4.7**                   | 0.29            |
| + Paeonol         | 1                  | 35.4 ± 4.7***                  | 2.3             |
| + Paeonol         | 10                 | 46.8 ± 2.1***                  |                 |
| + Verapamil       | 1                  | 40.8 ± 1.6***                  |                 |
| + Pyrllamine      | 1                  | 66.2 ± 8.5***                  | 2.46            |
| + Quinidine       | 1                  | 65.3 ± 3.7***                  | 0.76            |
| + Clonidine       | 1                  | 65.4 ± 2.8***                  | 0.98            |
| + Nicotine        | 1                  | 66.2 ± 2.3***                  | 1.60            |
| + Diphenhydramine | 1                  | 68.3 ± 3.7***                  | -0.62           |
| + MPP+            | 1                  | 81.3 ± 3.9*                    | -0.29           |
| + ALC             | 1                  | 84.9 ± 1.8*                    | -3.43           |
| + L-Carnitine     | 1                  | 86.4 ± 5.6                     | -4.13           |
| + TEA             | 1                  | 107 ± 10                       | -3.26           |
| + Choline         | 1                  | 100 ± 5                        | -4.14           |
| + PAH             | 1                  | 90.3 ± 5.7                     | -3.69           |
| + E,S             | 1                  | 103 ± 6                        | -1.40           |
| +6-MP             | 1                  | 127 ± 9                        | -3.09           |

The uptake of [3H]imperatorin by TR-BBB cells was measured in the absence (control) or presence of compounds at 37°C for 5 min. Each value represents the mean ± SEM (n=3–4). *p<0.05, **p<0.01, ***p<0.001, significantly different from the control.

**DISCUSSION**

Neurodegenerative diseases such as Alzheimer’s disease (AD) and Parkinson’s disease (PD), were found that deficient of Ach based on the activity studies of the acetylcholinesterase (AChE) (Kozioł and Skalicka-Woźniak, 2016). Imperatorin, a furanocoumarin derivative, has been documented to have many pharmacological properties for possible drug development. It has been examined as an AChE inhibitor with prospects of AD and PD therapy (Senol et al., 2011; Budzynska et al., 2015). However, transport mechanism of imperatorin into the brain remains unclear. In this present study, the function of imperatorin transport across the BBB was investigated using in vivo and in vitro approaches.

In vivo analysis, plasma pharmacokinetics of imperatorin in SD rat are shown in Table 1 and Fig. 1. Results indicated that imperatorin was eliminated rapidly. This rapid systemic clearance is principally due uptake by other organs (Table 1). BBB PS products of imperatorin was 20-fold greater than that of sucrose by IV injection in previous report (Wu and Partridge, 1999). In addition, brain uptake of imperatorin has been reported to be the same as 1%ID/g of morphine (0.0810 ± 0.0005) (Wu et al., 1997). Following IV injection result, Vd of [3H]imperatorin in rat brain was nearly 6 fold higher than the Vd of [14C]sucrose, a maker for vascular space (Table 1B) (Lee and Kang, 2016). This result suggests that imperatorin might be actively transported to brain through the BBB under physiological state. Moreover, the Vd of [3H]imperatorin in rat brain following ICAP technique was 60 fold greater than the Vd of [14C]sucrose (Pardridge et al., 1994). This Vd value was similar to that of 4-phenylbutyrate readily taken up by the brain through the monocarboxylate transporter 1 (MCT1) (Lee and Kang, 2016). It was also similar to that of taurine mediated by taurine transporter (TAUT) in BBB (Kang, 2000; Kang et al., 2002). Our results suggest that imperatorin is actively and highly transported through the BBB to the brain.

High BBB permeability of imperatorin in mice has been reported in previous studies (Lili et al., 2013). However, the mechanism of imperatorin transport by brain is unknown. In vivo [3H]imperatorin uptake was shown to be concentration dependent and saturable in this study (Fig. 3). The apparent BBB Michaelis-Menten (Km) parameter for imperatorin transport in the rat brain (pH 7.4) agreed well with the in vivo Km value in clonidine (proton coupled antiporter substrate) mouse using in situ brain perfusion (0.6 mM) (André et al., 2009). The unsaturated component (Ke) confirms that passive diffusion of imperatorin is negligible. These data suggest that imperatorin uptake by brain is regulated by specific carrier-mediated transporter with imperatorin concentration under 250 µM. Based on in vivo transport mechanism study, imperatorin uptake was significantly increased at pH 6.4 and after intracellular acidification induced by mannitol (Fig. 2). On the other hand, decreased imperatorin transport was observed at lower pH (pH 6.4) and after intracellular alkalinization induced by HEPES (Fig. 2). Similar results have been reported in previous studies (André et al., 2008; Sadiq et al., 2011; Cisternino et al., 2013; Chaply et al., 2014). These results indicate that imperatorin transport in rat brain is proton dependent, but oppositely di-
Imperatorin transport into brain is mediated by sodium-dependent but pH-dependent carrier-mediated transport. Moreover, it was unaffected by OCTs, Mates, Octn1, Oat3 or Mrps substrates (Table 3). These results support that a carrier-mediated transport process specific for cationic compounds such as Pmat, Octn2 and proton coupled antiporter is possibly involved in the transport of imperatorin to the brain.

Fig. 7. Lineweaver-Burk plots of [3H]imperatorin uptake by TR-BBB cells showing competitive inhibition by tramadol, clonidine and paeonol. [3H]imperatorin uptake was performed at 37°C for 5 min in the presence (●) or absence (○) of 1 mM tramadol (A), 1 mM clonidine (B) or 1 mM pyrilamine (C). Each point presents the mean ± SEM (n=3).

It is important to identify the function of imperatorin uptake through BBB. Based on in vitro cellular uptake study, imperatorin uptake was shown to be sodium- and pH-dependent independent manner because it was not affected by the replacement of sodium with NMG⁺-, or KCl as well as valinomycin (Table 4). In contrast, it was energy dependent. Moreover, the magnitude of imperatorin uptake at pH 7.4 was significantly higher compared to that at pH 6.4 but lower than that at pH 8.4 (Fig. 5A). Imperatorin uptake by TR-BBB cells was increased after intracellular acidification but decreased after intracellular alkalization (Fig. 5B). It was also significantly inhibited by FCCP, a protonophore (Table 4). Our in vivo and in vitro results were in agreement with many previous reports showing that the functional transport characteristics of imperatorin through BBB are similar to the characteristics of proton coupled antiporter substrates (Okura et al., 2008; André et al., 2009; Sadiq et al., 2011; Cisternino et al., 2013; Shimomura et al., 2013). The involvement of proton coupled antiporter in the brain transport of several lipophilic cationic drugs such as pyrilamine, tramadol (Kitamura et al., 2014), nicotine (Cisternino et al., 2013; Tega et al., 2013), diphenhydramine (Cisternino et al., 2013), clonidine (Chapy et al., 2015) has already been reported in human and mouse BBB as well as in rat BBB. Our in vivo and in vitro studies also revealed that [3H]imperatorin uptake was markedly inhibited by tramadol, pyrilamine, nicotine, clonidine, and diphenhydramine (Table 2, 5). Especially, [3H]imperatorin uptake was competitively inhibited by tramadol, pyrilamine, nicotine, clonidine, and diphenhydramine (Table 2, 5). In vivo and in vitro results also showed that imperatorin uptake was inhibited by substances that high lipophilicity (Fig. 8). These data indicated that an influx transporter that related proton coupled antiporter might play a major role in imperatorin transport. However, in vivo and in vitro results also showed that imperatorin uptake was inhibited by MPP⁺, ALC and L-carnitine (Table 2, 5). Octn2 has been found in rat brain capillary endothelial cells. It involved in the transport of L-carnitine and acetyl-L-carnitine from the circulating blood to the brain across the BBB. It is mediated by sodium-dependent but pH-
Fig. 8. A comparison of the relative inhibitory effect (% of control) and lipophilicity (log D). (A) In vitro results of compounds from Table 2. (B) In vivo results of compounds from Table 4. Compounds were classified into group I (closed circles, paeonol, primary, secondary and tertiary amines), group II (open square, quaternary amines), and group III (open circles, organic anion drugs). The star represents unlabeled imperatorin.

independent (Kang et al., 2002; Miecz et al., 2008) Pmat was found to be highly expressed in TR-BBB13 cells and MPP⁺ was known as a substrate of Pmat. It is energy independence but membrane potential and pH dependent (Okura et al., 2011). Thus, they are different from the characteristics of imperatorin uptake by TR-BBB cells identified in this study. In addition, [³H]imperatorin uptake was not reduced in 200 nM of rPmat and rOctn 2 knockdown cells (Fig. 6C). These results suggest that rPmat and rOctn2 do not make significant contribution to imperatorin uptake in TR-BBB cells. Furthermore, our in vivo and in vitro results showed organic cation such as TEA (classic substrate of sodium-dependent Octs, Octn1 and Maters) had no significant effect on imperatorin uptake by TR-BBB cells and rOctn2 siRNA transfection did not affect imperatorin uptake (Fig. 6D) (Ohta et al., 2006; Hiasa et al., 2007). Notably, Octn1 and Maters are known polyspecific organic cation transporters and proton antiporators, both of which are transporters are mediated by membrane potential dependence (Koepsell et al., 2007; Terada and Inui, 2008). In addition, [³H]Imperatorin uptake was not significantly inhibited by choline, as classic substrate of sodium-dependent transport system, ChT (Kang et al., 2005). There results suggest that Octs, Octn1, Maters, and ChT may not be transport independent in TR-BBB cells.

Lili et al. (2013) have demonstrated that imperatorin is not a substrate or inhibitor for the P-glycoprotein (P-gp) because the efflux ratio for imperatorin is 0.5 (if the value is larger than 3.0, it is a substrate of P-gp) and its IC₅₀ value is higher than 90 μM. Our studies also suggested that P-gp or organic anion transporter might not play major roles in imperatorin transport at the BBB because [³H]imperatorin uptake into rat brain and by TR-BBB cells were not significantly inhibited by 6-MP, a substrate of Oat3 and Mrps (Table 2, 5) (Deguchi et al., 2000; Mori et al., 2004; Lee et al., 2011). Furthermore, [³H]imperatorin uptake was not significantly affected by with PAH (a substrate of Oat3) (Hosoya et al., 2009; Roth et al., 2012) and estrone-3-sulfate (a substrate of Oatps) (Sai et al., 2006; Roth et al., 2012). Especially, our in vivo and in vitro results showed that [³H]imperatorin uptake was driven by an oppositely directed proton gradient (Fig. 2, 5). Oat3, Oatps and P-gp transport were involved in proton-dependent transport system (Sai et al., 2006; Lee et al., 2011; Fujii et al., 2013).

Based on its neuronal protective effect and its high BBB permeability, imperatorin might be a useful therapeutic candidate for treatment of neurological disorders. It also can be distributed into many regions in rat brain such as cortex, striatum, and hippocampus (Zhang et al., 2011). Lee et al. (2016) have demonstrated that imperatorin can protect cerebellar granule cells against perfluorohexane sulfonate-induced neuronal apoptosis via inhibiting NMDA receptor/intracellular calcium-mediates ERK pathway, suggesting that imperatorin also might be a useful therapeutic candidate for treatment of neurological disorders involving excitotoxicity and neuronal damage. Additional investigation is necessary to characterize the uptake of imperatorin by neurons and astrocytes. Our in vivo and in vitro experiment was showed same results. Therefore the transport properties of imperatorin are not affected by metabolism or protein binding effect. However, the investigation of it metabolism and protein binding effect is need for the next experiment using high performance liquid chromatography.

Our results demonstrated that the transport mechanism of imperatorin through the BBB involved a proton coupled antiporter. The carrier-mediated system could be responsible for the brain uptake of imperatorin at the BBB which is much more important pharmacokinetically than passive diffusion. These findings suggest that imperatorin might have clinical application as an optimal pharmacotherapy for CNS diseases.

CONFLICT OF INTEREST

There are no conflict of interest.

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