NEUROTOXIC PYRIDINIUM METABOLITES OF HALOPERIDOL ARE SUBSTRATES OF HUMAN ORGANIC CATION TRANSPORTERS

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ABSTRACT:

Two neurotoxic pyridinium metabolites of haloperidol, 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxybutyl]pyridinium ion (HPP⁺) and 4-(4-chlorophenyl)-1-[4-(fluorophenyl)-4-hydroxybutyl]-pyridinium ion (RHPP⁺), are formed in the liver and found in the brain. To understand how these neurotoxic pyridinium metabolites are distributed in the brain, HPP⁺ and RHPP⁺ were evaluated as substrates for human organic cation transporters (hOCTs). Both HPP⁺ and RHPP⁺ were accumulated in Caco-2 cells, and these accumulations were significantly inhibited by pretreatment with the hOCT inhibitors verapamil, cimetidine, phenoxybenzamine, and corticosterone. The contribution of each hOCT was evaluated based on measurements of the intracellular concentrations of haloperidol pyridinium metabolites.

The antipsychotic drug haloperidol is biotransformed into several metabolites, including reduced haloperidol and a number of pyridinium metabolites, such as HPP⁺ [4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxybutyl]pyridinium ion] and RHPP⁺ (a reduced form of HPP⁺), in humans (Eyles et al., 1994; Igarashi et al., 1995). Haloperidol pyridinium metabolites are structurally similar to the neurotoxin MPP⁺ (1-methyl-4-phenylpyridinium; Fig. 1), which is known to cause neurodegeneration, and have been reported as having similar or more potent neurotoxic effects, e.g., inhibition of mitochondrial respiration, irreversible inhibition of tyrosine hydroxylase, and depletion of dopamine and serotonin (Bloomquist et al., 1994; Rollema et al., 1994; Fang et al., 1995). The formation of toxic pyridinium metabolites of haloperidol is mediated primarily by hepatic cytochrome P450 3A (Igarashi et al., 1995), although it has not been clearly established how the cationic metabolite HPP⁺ formed in peripheral tissues becomes localized to the brain (Igarashi and Castagnoli, 1992; Igarashi, 1998).

HPP⁺ has been detected in the brains of patients who have chronically received haloperidol (Bloomquist et al., 1994). Furthermore, HPP⁺ shows a moderate brain uptake index, which indicates that it may permeate the blood-brain barrier. These results support the hypothesis that HPP⁺ is formed mainly in the liver, circulates in the bloodstream, and enters the brain, where it induces damage to brain dopaminergic neurons (Kawashima et al., 2002, 2004).

Recent reports have suggested that MPP⁺ is a high-affinity substrate for the human organic cation transporters hOCT1, hOCT2, and hOCT3 (Hayer-Zillgen et al., 2002). The hOCT1 and hOCT2 forms are expressed predominantly in the liver and kidneys, respectively (Gorboulev et al., 1997), whereas hOCT3 (also known as extraneuronal monoamine transporter), which also has a high affinity for monoamines, is highly expressed in the placenta (Verhaagh et al., 1999). Recently, it has been reported that hOCT3 may play a significant role in the disposition of cationic neurotoxins and neurotransmitters in the brain (Wu et al., 1998). Because MPP⁺ is metabolically stable as a charged molecule and shows low-level diffusion across the cytoplasmic membrane, it accumulates exclusively in nerve cells, in a distribution process that may be mediated by hOCTs (Russ et al., 1999).
Although HPP⁺ is less potent than MPP⁺ in the dopaminergic system, these compounds display comparable toxic effects on the serotonergic system. Considering the structural similarities between MPP⁺ and both haloperidol metabolites, it seems likely that these metabolites represent substrates for hOCTs.

In the present study, we evaluated whether the toxic haloperidol pyridinium metabolites HPP⁺ and RHPP⁺ are substrates for hOCTs. In addition, we characterized HPP⁺ and RHPP⁺ uptake into hOCT-overexpressing MDCK cells to clarify the potential contribution of hOCTs to the disposition of these two metabolites.

Materials and Methods

Chemicals and Reagents. Verapamil, cinetidine, phenoxbenzamine, and corticosterone were purchased from Sigma Chemical Co. (St. Louis, MO). The HPP⁺ and RHPP⁺ were generous gifts from Dr. Neal Castagnoli, Jr. (Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA). MPP⁺ (3.21 TBq/mmol) and tetraethylammonium bromide, [1-14C]-, TEA (185 MBq/mmol) were purchased from PerkinElmer (Boston, MA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), trypsin, and Lipofectamine 2000 were purchased from Gibco-BRL (Invitrogen, Carlsbad, CA).

Cell Culture and Stable Transfection of hOCT1, hOCT2, and hOCT3. Caco-2 cells were maintained in a humidified atmosphere of 5% CO₂ in air and were grown in DMEM that was supplemented with 20% FBS, 2 mM l-glutamine, 1% nonessential amino acids, and 100 U/ml penicillin-streptomycin. The medium was changed every second day. For the uptake assay, 5 × 10⁵ Caco-2 cells were seeded on 12-well plastic cell culture clusters. The uptake studies were usually performed 7 to 9 days after the cells formed a monolayer. MDCK cells were maintained in a humidified atmosphere of 5% CO₂ in air and were grown in DMEM that was supplemented with 10% FBS, 2 mM l-glutamine, and 100 U/ml penicillin-streptomycin. For the construction of stable hOCT1-, hOCT2-, and hOCT3-expressing cells, the MDCK cells were transfected with pcDNA3.1 plasmids that carried the genes for hOCT1, hOCT2, and hOCT3, respectively, using Lipofectamine 2000. The stable cells were selected for resistance to 800 ng/ml genetin (G418; Life Technologies, Karlsruhe, Germany) for 3 weeks (Hayer-Zillgen et al., 2002). The expression levels of hOCT1, hOCT2, and hOCT3 were verified in functional assays and Western blots. For the uptake assay, 5 × 10⁵ cells were seeded on 12-well plastic cell culture clusters. The uptake assay was performed after monolayer formation.

Plasmids. The plasmids pEXO-hOCT1 and pEXO-hOCT2, which contain the hOCT1 and hOCT2 cDNAs, respectively, were kindly provided by Dr. Kathleen M. Giacomini (University of California, San Francisco, CA). The hOCT1- and hOCT2-coding regions were amplified from these plasmids using polymerase chain reaction and Pfu DNA polymerase. The following specific primers were designed based on the nucleotide sequences for human OCT1 and OCT2 (GenBank accession nos. NM_003057 and NM_003058): hOCT1 forward, 5’-GCCGGTACCATGCCCCAGGTGATGACATTC-3’ and reverse, 5’-GCCGGTACCATGCCCCAGGTGATGACATTC-3’; and hOCT2 forward, 5’-GCCGGTACCATGCCCCAGGTGATGACATTC-3’ and reverse, 5’-GCCGGTACCATGCCCCAGGTGATGACATTC-3’.
bated with 5% nonfat dry milk in Tris-buffered saline/Tween 20 for 2 h. The membranes were then incubated with polyclonal antibodies against OCT1, OCT2, and OCT3 (Santa Cruz Biotechnology), an anti-actin antibody (Cell Signaling Technology, Beverly, MA) overnight at 4°C. After rinsing with TBST (1.37 M NaCl, 0.3% Tween 20, 200 mM Tris Cl, pH 7.6), the membranes were incubated with horseradish peroxidase-labeled anti-goat IgG antibody or anti-rabbit IgG antibody (Santa Cruz Biotechnology) and visualized using the Enhanced Chemiluminescence (ECL) System (Santa Cruz Biotechnology).

Data Analysis. Uptake was calculated as the cell/medium ratio based on the intracellular uptake per microgram of cell protein per min (dpm/min/µg protein or multiple reaction monitoring/min/µg protein) relative to the initial drug concentration. The \( K_m \) and \( V_{max} \) values for substrate uptake were estimated by nonlinear least-squares regression analysis to the Michaelis-Menten equation using the WinNonlin software (Pharsight, Mountain View, CA). Transporter-mediated HPP\(^+\) or RHPP\(^+\) uptake was calculated by subtracting the uptake levels in mock cells from the net uptake in transfected cells for the kinetic study. Statistical significance was analyzed using the Student’s \( t \) test, and \( p < 0.05 \) was considered to be statistically significant. The reproducibility of the results in the present study was confirmed by two or three subsequent experiments, and all the data are expressed as the mean ± S.E.M. of three replicates.

Results

Intracellular Accumulations of HPP\(^+\) and RHPP\(^+\) in Caco-2 Cells. Because Caco-2 cells express several drug transporters, including hOCT1 and hOCT3 (Martel et al., 2001), hOCT-mediated cellular uptake of \([\text{H}]\text{HPP}\)^\(^+\), which is a well known hOCT substrate, was determined in Caco-2 cells, as a positive control. Caco-2 cells were incubated with 200 nM \([\text{H}]\text{HPP}\)^\(^+\) for various periods of time in the absence or presence of 100 µM verapamil, a known hOCT inhibitor. The cellular uptake of \([\text{H}]\text{HPP}\)^\(^+\) increased nonlinearly for up to 90 min (Fig. 2A). In the presence of verapamil, the intracellular concentration of \([\text{H}]\text{HPP}\)^\(^+\) in Caco-2 cells was significantly reduced by 2-fold within 90 min (Fig. 2A). When the haloperidol metabolites 10 µM HPP\(^-\) and RHPP\(^+\) were incubated under the same conditions as for \([\text{H}]\text{HPP}\)^\(^+\), the intracellular concentrations of HPP\(^-\) and RHPP\(^+\) showed no proportional increases, although saturation was not complete until the cells were incubated for 90 min (Fig. 2, B and C). However, the intracellular accumulations of HPP\(^-\) and RHPP\(^+\) in Caco-2 cells were significantly reduced by approximately 2-fold in the presence of verapamil for 90 min (Fig. 2A). When the haloperidol metabolites 10 µM HPP\(^-\) and RHPP\(^+\) were incubated under the same conditions as for \([\text{H}]\text{HPP}\)^\(^+\), the intracellular concentrations of HPP\(^-\) and RHPP\(^+\) were also significantly reduced by the addition of other inhibitors of OCT1, OCT2, and OCT3, i.e., phenoxybenzamine, cimetidine, and corticosterone (Fig. 3).

Intracellular Accumulations of HPP\(^+\) and RHPP\(^+\) in hOCT1-, hOCT2-, and hOCT3-Expressing MDCK Cells. To further characterize hOCT-mediated uptake of HPP\(^+\) and RHPP\(^+\), the intracellular concentrations of these metabolites were evaluated in MDCK cells that transiently overexpressed hOCT1, hOCT2, and hOCT3. Twenty-four hours after transfection of the MDCK cells with each of the hOCTs, 200 nM \([\text{H}]\text{HPP}\)^\(^+\), 10 µM HPP\(^-\), or 10 µM RHPP\(^+\) were added for 30 min at 37°C in the absence or presence of 50 µM phenoxybenzamine (Fig. 4). The activities of the overexpressed hOCTs were confirmed by the 2- to 3-fold higher \([\text{H}]\text{HPP}\)^\(^+\) uptake into the hOCT1-, hOCT2-, and hOCT3-overexpressing cells com-
OCT1, hOCT2, and hOCT3 proteins have molecular sizes in the 50 kDa range. In the present study, HPP uptake was concentration-dependent, with estimated values of 50.92, 2.79, and 2.23 pmol/min/µg protein for hOCT1, hOCT2, and hOCT3, respectively (Fig. 7, A–C) (Table 1). RHPP \({\text{H}}^{+}\) accumulated in the hOCT1- and hOCT3-overexpressing MDCK cells, with estimated \(K_m\) values of 5.15 and 8.21 µM and \(V_{\text{max}}\) values of 1230.9 and 1348.6 pmol/min/µg protein for hOCT1 and hOCT3, respectively (Fig. 7, D and E) (Table 1). These results suggest that both HPP \({\text{H}}^{+}\) and RHPP \({\text{H}}^{+}\) are substrates for hOCTs, with the exception of RHPP \({\text{H}}^{+}\) for hOCT2.

Discussion

Chronic use of the antipsychotic drug haloperidol causes adverse neurodegenerative events in patients. This toxicity has been attributed to the distribution of the toxic haloperidol metabolites HPP \({\text{H}}^{+}\) and RHPP \({\text{H}}^{+}\) into the brain, with consequent neuronal cell death. Haloperidol can be biotransformed into toxic metabolites by the action of cytochrome P450 3A4 (CYP3A4), which is a major hepatic drug-metabolizing enzyme. Because CYP3A4 expression is rare or limited in the brain, it has been postulated that haloperidol metabolites are formed in peripheral tissues and subsequently transported into the brain. However, to date there have been no reports of transporters for haloperidol metabolites, which might lend support to the notion of unique distribution patterns for these neurotoxic cationic compounds.

In the present study, we identified the transporters for HPP \({\text{H}}^{+}\) and RHPP \({\text{H}}^{+}\) as hOCTs. Haloperidol metabolites are structurally similar to the neurotoxin MPP \({\text{H}}^{+}\) (Fig. 1), which is an agent of neurodegeneration. Structurally, these compounds share a highly cationic pyridinium headgroup. Functionally, they exhibit similar brain toxicities in terms of neuronal cell death, attributable to the inhibition of mitochondrial function. Initially, we investigated the transport of haloperidol metabolites into Caco-2 cells, which express various kinds of transporters and are able to transport organic cations across membranes via two distinct \(\text{Na}^+\)-independent transporters, hOCT1 and hOCT3 (Martel et
processes using hOCT inhibitors. Therefore, we investigated the levels of accumulations, we hypothesized that hOCTs mediate the transport of haloperidol metabolites. Considering the similarities between MPP⁺ and haloperidol metabolites, we hypothesized that hOCTs mediate the transport of haloperidol metabolites. Therefore, we investigated the levels of accumulation of HPP⁺ and RHPP⁺ in Caco-2 cells and inhibited these transport processes using hOCT inhibitors.

The intracellular uptake of HPP⁺ and RHPP⁺ in Caco-2 cells showed nonlinear saturation (Fig. 2, B and C). Pretreatment with hOCT inhibitors, such as verapamil (Martel et al., 2000), cimetidine (Zhang et al., 1997; Grundemann et al., 1998), phenoxybenzamine, and corticosterone (Hayer-Zillgen et al., 2002), significantly reduced haloperidol metabolite uptake (Figs. 2 and 3). These compounds markedly inhibited hOCT function, although their effects on other transporters have not been fully investigated. Furthermore, phenoxybenzamine is a very specific inhibitor of hOCTs and has no known inhibitory effects on other transporters. Therefore, the inhibition of haloperidol metabolite uptake by these hOCT inhibitors suggests that hOCTs mediate the transport of these drugs.

The role of hOCTs in haloperidol metabolite uptake was demonstrated in MDCK cells that overexpressed hOCT1, hOCT2, or hOCT3. Cells that overexpressed hOCT1 and hOCT3 accumulated HPP⁺ and RHPP⁺, and the specificity of transport was verified by pretreatment with hOCT inhibitor (Fig. 4). HPP⁺ was accumulated to high levels in MDCK cells that stably expressed the hOCTs (Fig. 6). Interestingly, hOCT2-expressing cells accumulated HPP⁺ but not RHPP⁺. These results indicate that each hOCT has a different substrate spectrum.

Human organic cation transporter systems have been studied primarily in the kidney and liver because these organs play major roles in the elimination of xenobiotics (Pritchard and Miller, 1993; Ulrich, 1994). The hOCT1 and hOCT2 isoforms are primarily expressed in the liver and kidney, respectively, and to a lesser degree in the small intestine (Gorboulev et al., 1997; Busch et al., 1998; Zhang et al., 1998). These two hOCTs exhibit considerable overlap in terms of substrate specificity, as revealed by MPP⁺ and HPP⁺. The neuronal toxicity of haloperidol results from the toxic products of its metabolism, which is mediated mainly by CYP3A4 and partially by another P450 isofrom, CYP2D6. CYP3A4 is highly expressed in the liver and intestine. Thus, haloperidol can be metabolized by CYP3A4 in hepatocytes and intestinal epithelia and then transported into the bile duct and bloodstream through the actions of hOCT1 and hOCT2. The metabolites can be eliminated in the urine through hOCTs, which are highly expressed in the kidney. The contributions of hOCT1 and hOCT2 to the hepatic, intestinal, and renal transport of haloperidol metabolites need to be studied further in vivo to develop a better understanding of haloperidol disposition.

Several cationic neurotoxins and neurotransmitters are good substrates for hOCT1 and hOCT2 (Pritchard and Miller, 1993; Ulrich, 1994) and, given the potential roles of these transporters in the
handling of neurotoxins and neurotransmitters, attempts have been made to detect the expression of hOCT1 and hOCT2 in the brain (Grundemann et al., 1994; Gorboulev et al., 1997; Zhang et al., 1998). Although hOCT1 and hOCT2 mRNAs were not detected in the brain by Northern blot analysis (Gorboulev et al., 1997; Zhang et al., 1997), the results of studies using more sensitive reverse transcription-polymerase chain reaction methods have indicated that hOCT2-specific transcripts are present in the brain (Gorboulev et al., 1997; Grundemann et al., 1997). The neuronal expression of hOCT2 has been suggested by in situ hybridization and immunocytochemistry results (Busch et al., 1998). Assuming that hOCT2 is expressed in the blood-brain barrier, even at a low level, it may contribute to the distribution of haloperidol into the brain. Given that hOCT2 has been shown to transport HPP<sup>+</sup> in an effective manner in the present study, the HPP<sup>+</sup> formed in peripheral tissues may be taken up into the brain by the hOCT2 transporter.

Another hOCT isoform, hOCT3, was first identified as the corticosterone-sensitive extraneuronal catecholamine transporter and has

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**TABLE 1**

<table>
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<tr>
<th>Compounds</th>
<th>Kinetic Parameters</th>
<th>hOCT1</th>
<th>hOCT2</th>
<th>hOCT3</th>
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<tr>
<td>HPP&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt; (pmol/min/µg protein)</td>
<td>0.99 ± 0.05</td>
<td>2.79 ± 0.13</td>
<td>2.23 ± 0.16</td>
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<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt; (µM)</td>
<td>282.1 ± 4.7</td>
<td>256.1 ± 11.3</td>
<td>400.2 ± 15.2</td>
<td></td>
</tr>
<tr>
<td>RHPP&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt; (pmol/min/µg protein)</td>
<td>5.15 ± 0.63</td>
<td>8.21 ± 0.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt; (µM)</td>
<td>1230.9 ± 24.8</td>
<td>1348.6 ± 36.3</td>
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**FIG. 7.** Concentration dependence of HPP<sup>+</sup> and RHPP<sup>+</sup> accumulations in MDCK cells that overexpress hOCT1, hOCT2, and hOCT3. HPP<sup>+</sup> was applied to the apical side of MDCK cells that stably express hOCT1 (A), hOCT2 (B), or hOCT3 (C), together with various concentrations of HPP<sup>+</sup> (0, 0.1, 0.5, 1, 2, 3, 5, 10, and 15 µM) for 15 min at 37°C. RHPP<sup>+</sup> was applied to the apical side of MDCK cells that stably express hOCT1 (D) or hOCT3 (E), together with various concentrations of RHPP<sup>+</sup> (0, 0.1, 0.5, 1, 2, 3, 5, 10, or 15 µM) for 15 min at 37°C. The cellular concentrations of HPP<sup>+</sup> and RHPP<sup>+</sup> were measured by LC/MS/MS. Uptake was saturable and fitted to the Michaelis-Menten curve. Exponential saturation curves were fitted to the experimental data. Linear coefficient r² = 0.9990. The data are presented as arithmetic mean ± S.E.M. (n = 6). The insert shows an Eadie-Hofstee plot of HPP<sup>+</sup> and RHPP<sup>+</sup> uptake, which was used to determine the kinetic parameters. V, velocity; V/S, velocity per concentration of HPP<sup>+</sup> or RHPP<sup>+</sup>.
been detected in the brain cortex as well as in liver and heart tissues (Grundemann et al., 1998). The ability of hOCT3 to interact with cationic neurotoxins and neurotransmitters and the expression of hOCT3 in the brain suggest that hOCT3 plays a role in the distribution of haloperidol metabolites into the brain. Considering that hOCT3 is an effective transporter of HPP⁺ and RHPP⁺, it is possible that hOCT3 contributes to the transport of these neurotoxic metabolites. Further in vivo studies are required to investigate whether hOCT3 mediates the distribution of haloperidol pyridinium metabolites into the brain.

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References


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