Interactions of Human Organic Anion Transporters and Human Organic Cation Transporters with Nonsteroidal Anti-Inflammatory Drugs

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ABSTRACT

The purpose of this study was to elucidate the interactions of human organic anion transporters (hOATs) and human organic cation transporters (hOCTs) with nonsteroidal anti-inflammatory drugs (NSAIDs) using cells stably expressing hOATs and hOCTs. NSAIDs tested were acetaminophen, acetylsalicylate, salicylate, diclofenac, ibuprofen, indomethacin, ketoprofen, mefenamic acid, naproxen, piroxicam, phenacetin, and sulindac. These NSAIDs inhibited organic anion uptake mediated by hOAT1, hOAT2, hOAT3, and hOAT4. By comparing the IC50 values of NSAIDs for hOATs, it was found that hOAT1 and hOAT3 exhibited higher affinity interactions with NSAIDs than did hOAT2 and hOAT4. HOAT1, hOAT2, hOAT3, and hOAT4 mediated the uptake of either ibuprofen, indomethacin, ketoprofen, or salicylate, but not acetylsalicylate. Although organic cation uptake mediated by hOCT1 and hOCT2 was also inhibited by some NSAIDs, hOCT1 and hOCT2 did not mediate the uptake of NSAIDs. In conclusion, hOATs and hOCTs interacted with various NSAIDs, whereas hOATs but not hOCTs mediated the transport of some of these NSAIDs. Considering the localization of hOATs, it was suggested that the interactions of hOATs with NSAIDs are associated with the pharmacokinetics and the induction of adverse reactions of NSAIDs.

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been widely used for their anti-inflammatory and analgesic properties. The indications of NSAIDs are broadening from rheumatic diseases and various pain states, such as cancer pain, and biliary and colic pain, to include possibly Alzheimer’s disease and colon cancer prevention (Day et al., 2000). Table 1 shows the chemical structures of NSAIDs tested in the current study. Although all of these NSAIDs are weak organic acids, they are grouped in several classes based on their chemical structures. Although the chemical diversity yields a broad range of pharmacokinetic characteristics (Frust and Munster, 2000), they have some general properties in common. NSAIDs have been shown to induce various forms of adverse drug reactions including adverse gastrointestinal effects (Day et al., 2000), renal dysfunction and nephrotoxicity (Day et al., 2000), and rhabdomyolysis (Ross and Hoppel, 1987; Leventhal et al., 1989; Delrio et al., 1996).

The secretion of numerous organic anions and cations, including endogenous metabolites, drugs, and xenobiotics, is an important physiological function of the renal proximal tubule. The process of secreting organic anions and cations through the proximal tubule cells is achieved via unidirectional transcellular transport involving the uptake of organic anions and cations into the cells from the blood across the basolateral membrane, followed by extrusion across the brush-border membrane into the proximal tubule fluid (Pritchard and Miller, 1993). Recently, cDNAs encoding the human organic anion transporter (hOAT) family have been successively cloned, including hOAT1 (Reid et al., 1998; Hosoyamada et al., 1999), hOAT2 (Y. Kobayashi, unpublished observation), hOAT3 (Cha et al., 2001), and hOAT4 (Cha et al., 2000). The human organic cation transporters (hOCTs) isolated thus far are hOCT1 (Gorboulev et al., 1981; Wood et al., 1985; Purcell et al., 1991; Day et al., 2000), adverse neurological effects (Hoppman et al., 1991; Day et al., 2000), and rhabdomyolysis (Ross and Hoppel, 1987; Leventhal et al., 1989; Delrio et al., 1996).

ABBREVIATIONS: NSAID, nonsteroidal anti-inflammatory drug; hOAT, human organic anion transporter; hOCT, human organic cation transporter; rOAT1, rat organic anion transporter 1; PGE2, prostaglandin E2; PGF2α, prostaglandin F2α; PAH, para-aminobenzoic acid; S1, S2, S3, the first, second, and third segments of the proximal tubule; oatp, organic anion-transporting peptide; NPT1, human-type I sodium-dependent inorganic phosphate transporter.
Materials and Methods

**Materials.** [14C]Para-aminobenzoic acid (PAH) (1.86 GBq/mmol), [3H]PGF2α (6808 GBq/mmol), [3H]estrone sulfate (1961 GBq/mmol), and [14C]TETA (2.035 GBq/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). [3H]Acetalsaliclylate (2.04 GBq/mmol), [14C]salicylic acid (2.05 GBq/mmol), [3H]ibuprofen (0.5 GBq/mmol), [3H]indomethacin (0.74 GBq/mmol), and [3H]ketoprofen (1.11 GBq/mmol) were purchased from Murumachi Chemicals (Tokyo, Japan). NSAIDs were obtained from Sigma-Aldrich (St. Louis, MO). Other materials used included fetal bovine serum, trypsin, and geneticin from Invitrogen (Carlsbad, CA); recombinant epidermal growth factor from Wako (Osaka, Japan); insulin from Shiizu (Shizuoka, Japan); RITC 80-7 culture medium from Iwaki Co. (Tokyo, Japan); and TTX-50 from Promega (Madison, WI).

**Cell Culture.** S2 cells were established by culturing the microdissected S2 segment derived from transgenic mice harboring the temperature-sensitive simian virus 40 large T-antigen gene. The establishment and characterization of S2 hOAT1, S2 hOAT2, S2 hOAT3, S2 hOAT4, S2 hOCT1, and S2 hOCT2 were reported previously (Enomoto et al., 2002; Kimura et al., 2002; Takeda et al., 2002). Briefly, the full-length cDNAs of hOAT1, hOAT2, hOAT3, hOAT4, hOCT1, and hOCT2 were subcloned into pcDNA 3.1 (Invitrogen), a mammalian expression vector. S2 hOAT1, S2 hOAT2, S2 hOAT3, S2 hOAT4, S2 hOCT1, and S2 hOCT2 were obtained by transfecting S2 cells with pcDNA3.1-hOAT1, pcDNA3.1-hOAT2, pcDNA3.1-hOAT3, pcDNA3.1-hOAT4, pcDNA3.1-hOCT1, and pcDNA3.1-hOCT2 coupled with pcSV2neo, a neomycin resistance gene, using TTX-50 according to the manufacturer’s instructions. S2 cells transfected with pcDNA3.1 lacking an insert and pSV2neo were designated as S2 pcDNA 3.1 (mock) and used as control. These cells were grown in a humidified incubator at 33°C and under 5% CO2 using RITC 80-7 medium containing 5% fetal bovine serum, 10 mg/ml transferrin, 0.08 U/ml insulin, 10 ng/ml recombinant epidermal growth factor, and 400 mg/ml geneticin. The cells were subcultured in a medium containing 0.05% tryossin-EDETA solution (containing 137 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 4 mM NaHCO3, 0.5 mM EDTA, and 5 mM Hepes; pH 7.2) and used for 25–35 passages. Clonal cells were isolated using a cloning cylinder and screened by determining the optimal substrate for each transporter, i.e., [14C]PAH for hOAT1 (Hosoyamada et al., 1999), [3H]PGF2α for hOAT2 (Enomoto et al., 2002), [3H]estrone sulfate for hOAT3 and hOAT4 (Cha et al., 2000, 2001), and [3H]TETA for hOCT1 and hOCT2 (Gorboulev et al., 1997, Zhang et al., 1998).

**Uptake Experiments.** Uptake experiments were performed as previously described (Enomoto et al., 2002; Kimura et al., 2002; Takeda et al., 2002). The S2 cells were seeded in 24-well tissue culture plates at a density of 1 × 10⁵ cells/well. After the cells were cultured for 2 days, the cells were washed three times with Dulbecco’s modified phosphate-buffered saline solution (containing 137 mM NaCl, 3 mM KCl, 8 mM NaHPO4, 1 mM KH2PO4, 1 mM CaCl2, and 0.5 mM MgCl2, pH 7.4) supplemented with 5.5 mM glucose and then preincubated in the same solution in a water bath at 37°C for 10 min. The cells were then incubated in a solution containing either 30 μM [14C]acetylaseiciclylate, 30 μM [14C]salicylate, 500 nM [3H]ibuprofen, 5 μM [3H]indomethacin, or 50 nM [3H]ketoprofen at 37°C for up to 30 min. The uptake was stopped by the addition of ice-cold Dulbecco’s modified phosphate-buffered saline solution, and the cells were washed three times with the same solution. The cells in each well were lysed with 0.5 ml of 0.1 N sodium hydroxide and then washed three times with the same solution. The cells in each well were suspended in 0.5 ml of 0.1 N hydroxide and then washed three times with the same solution. The cells in each well were suspended in 0.5 ml of 0.1 N hydroxide and then washed three times with the same solution.

**Inhibition Study.** To evaluate the inhibitory effects of NSAIDs on organic anion uptake mediated by hOAT1, hOAT2, hOAT3, and hOAT4, and organic cation uptake mediated by hOCT1 and hOCT2, the cells were incubated in a solution containing either [14C]PAH for 2 min (hOAT1), [3H]PGF2α for 20 s (hOAT2), [3H]estrone sulfate for 2 min (hOAT3 and hOAT4), or [3H]TETA for 5 min (hOCT1 and hOCT2).
Effects of NSAIDs on Organic Anion Uptake Mediated by hOATs. We examined the inhibitory effects of various concentrations of NSAIDs on the organic anion uptake mediated by hOAT1, hOAT2, hOAT3, and hOAT4. Figure 1 shows the effects of diclofenac on the organic anion uptake mediated by hOAT1, hOAT2, hOAT3, and hOAT4. Diclofenac inhibited the organic anion uptake mediated by hOAT1 (Fig. 1A), hOAT2 (Fig. 1B), hOAT3 (Fig. 1C), and hOAT4 (Fig. 1D) in a dose-dependent manner (*P < 0.001, **P < 0.01, and ***P < 0.05 versus control). Similarly, all of the other NSAIDs tested dose dependently inhibited the organic anion uptake mediated by hOAT1, hOAT2, hOAT3, and hOAT4 (data not shown). The IC50 values are listed in Table 2.

Effects of NSAIDs on Organic Cation Uptake Mediated by hOCTs. Since anionic drugs such as PGE2 and PGF2α were recently shown to be transported by not only hOATs but also hOCTs (Kimura et al., 2002), we examined the effects of 0.5 mM (Fig. 2A) and 2 mM (Fig. 2B) concentrations of NSAIDs on the organic cation uptake mediated by hOCT1 and hOCT2. When the concentration of NSAIDs was set at 0.5 mM (100-fold higher than the substrate concentration), among the various NSAIDs tested, diclofenac, ibuprofen, indomethacin, ketoprofen, mefenamic acid, and sulindac significantly inhibited hOCT1-mediated TEA uptake (Fig. 2A; *P < 0.001 and ***P < 0.05 versus control), and indomethacin, naproxen, piroxicam, and sulindac significantly inhibited hOCT2-mediated TEA uptake (Fig. 2A; *P < 0.001, ***P < 0.01, and ***P < 0.05 versus control). In contrast, when the concentration of inhibitor was set at 2 mM (400-fold higher than the substrate concentration), as shown in Fig. 2B, all NSAIDs except salicylate significantly inhibited TEA uptake mediated by hOCT1 (*P < 0.001 and ***P < 0.01 versus control), and all NSAIDs except acetylsalicylate significantly inhibited TEA uptake mediated by hOCT2 (*P < 0.001, ***P < 0.01 and ***P < 0.05 versus control).

NSAID Uptake Mediated by hOATs and hOCTs. To determine whether hOATs and hOCTs mediate the uptake of NSAIDs, we evaluated the uptake activities of either [14C]acetylsalicylate, [14C]salicylate, [3H]ibuprofen, [3H]indomethacin, or [3H]ketoprofen by hOATs and hOCTs. The uptake rates of [14C]acetylsalicylate by hOAT1, hOAT2, hOAT3, and hOAT4 were not higher than those by mock (Fig. 3A; N.S.); those of [14C]salicylate by hOAT1, hOAT2, hOAT3, and hOAT4 were 1.98-, 1.75-, 2.04-, and 1.49-fold higher than those by mock (Fig. 3B; *P < 0.001 versus mock); those of [3H]ibuprofen by hOAT1 and hOAT3 but not hOAT2 and hOAT4 were 1.38- and 1.74-fold higher than those by mock (Fig. 3C; *P < 0.001 and ***P < 0.01 versus mock); those of [3H]indomethacin by hOAT1 and hOAT3 but not hOAT2 and hOAT4 were 1.47- and 1.18-fold higher than those by mock (Fig. 3D; *P < 0.001 and ***P < 0.01 versus mock); and those of [3H]ketoprofen by hOAT1, hOAT3, and hOAT4 but not hOAT2 were 1.75-, 1.39-, and 1.23-fold higher than those by mock (Fig. 3E; *P < 0.001 and ***P < 0.01 versus mock). For reference, hOAT1-mediated PAH uptake, hOAT2-mediated PGF2α uptake, and hOAT3- and hOAT4-mediated estrone sulfate uptake were 16-, 16.7-, 37-, and 31-fold higher, respectively, than those by control (Enomoto et al., 2002; Takeda et al., 2002). In contrast to hOATs, hOCT1 and hOCT2 did not mediate the transport of various NSAIDs tested in the current study (data not shown).

Discussion

hOAT1 and hOAT3 have been shown to mediate the transport of NSAIDs, antitumor drugs, histamine H2-receptor antagonist, prostaglandins, diuretics, angiotensin-converting enzyme inhibitors, and β-lactam antibiotics (Hosoyamada et al., 1999; Cha et al., 2001). Some differences in characteristics exist between hOAT1 and hOAT3, such as substrate specificity and localization: hOAT1 is localized at the basolateral side of the S2 segment of the proximal tubule (Hosoyamada et al., 1999), whereas hOAT3 is localized at the first, second, and third segments (S1, S2, and S3) of the proximal tubule (Cha et al., 2001). In addition, hOAT1, but not hOAT3, exhibits transport properties as an exchanger (Hosoyamada et al., 1999; Cha et al., 2001). HOAT2, also shown to be localized at the basolateral side of the proximal tubule, mediates the transport of organic anions including salicylate and PGF2α (Enomoto et al., 2002). HOAT4 localization: hOAT1 is localized at the basolateral side of the S2 segment of the proximal tubule (Hosoyamada et al., 1999; Cha et al., 2001). Some differences in characteristics exist between hOAT1 and hOAT3, such as substrate specificity and localization: hOAT1 is localized at the basolateral side of the S2 segment of the proximal tubule (Hosoyamada et al., 1999), whereas hOAT3 is localized at the first, second, and third segments (S1, S2, and S3) of the proximal tubule (Cha et al., 2001). In addition, hOAT1, but not hOAT3, exhibits transport properties as an exchanger (Hosoyamada et al., 1999; Cha et al., 2001). HOAT2, also shown to be localized at the basolateral side of the proximal tubule, mediates the transport of organic anions including salicylate and PGF2α (Enomoto et al., 2002). HOAT4
also mediates the apical transport of various anionic drugs in the proximal tubule (Babu et al., 2002); however, this transporter exhibits relatively narrow substrate recognition compared with hOAT1 and hOAT3 (Cha et al., 2000). HOCT1 is mainly localized in the liver and mediates polyspecific pH-independent transport of organic cations. In contrast, hOCT2 is mainly localized in the kidney and mediates pH-independent, electrogentic, and polyspecific transport of organic cations (Gor-
boulev et al., 1997). Using stable cell lines, we have elucidated the interactions of hOATs and hOCTs with various NSAIDs.

By comparing the IC50 values of NSAIDs among hOATs, it was found that hOAT1 and hOAT3 generally exhibited high affinity for NSAIDs. In contrast, hOAT2 exhibited the lowest affinity for ibuprofen, indomethacin, ketoprofen, and naproxen; hOAT4 exhibited the lowest affinity for diclofenac, mefenamic acid, and phenacetin; hOAT2 and hOAT4 exhibited the lowest affinity for acetylsalicylate, salicylate, and sulindac. Thus, hOAT2 and hOAT4 generally appear to exhibit the lowest affinity for NSAIDs among the hOATs.

By comparing the IC50 values of various NSAIDs for hOAT1-mediated PAH uptake with the Ki values for rOAT1-mediated PAH uptake (Apiwattanakul et al., 1999), it was found that the IC50 values of acetylsalicylate, salicylate, indomethacin, naproxen, phenacetin, and piroxicam for hOAT1 were similar to the Ki values for rOAT1 (within 3-fold difference; Zhang et al., 1998), whereas those of acetylsalicylate and ibuprofen were different (more than 3-fold). It is reported that when the substrate concentration is low compared with the Km value, Ki values will be identical with the IC50 values despite the mechanism of inhibition (Cheng and Prusoff, 1973). In this regard, all of the experiments in the current study were performed using substrate concentrations less than the Km values. In addition, in contrast to the fact that rOAT1 mediated the uptake of acetylsalicylate (Apiwattanakul et al., 1999), hOAT1 did not exhibit acetylsalicylate uptake activity. Although there was a difference in the expression system between the results of hOAT1 and rOAT1, i.e., cultured cells versus oocyte expression system, some interspecies differences between human and rat appear to exist for the interactions of OAT1 with some of the NSAIDs. Similarly, Morita et al. (2001) have demonstrated that the Ki values of ketoprofen, diclofenac, and ibuprofen for salicylate uptake in LLC-PK1 cells stably expressing rOAT2 were 1.84, 49.3, and 155 μM, respectively, whereas the IC50 values for hOAT2 in the current study were 400, 14.3, and 692 μM, respectively. Thus, there appears to exist a significant difference between human and rat in the interactions of OAT2 with some of NSAIDs.

In a previous study using oocytes expressing rOAT1 (Apiwattanakul et al., 1999), we also found that all hydrophobic NSAIDs potently inhibited PAH uptake, whereas hydrophilic NSAIDs inhibited PAH uptake to a lesser degree. As shown in Table 2, this tendency was true not only for PAH uptake by hOAT1, but also for PGF2α uptake by hOAT2 and estrone sulfate uptake by hOAT3 and hOAT4.

Unexpectedly, some of the NSAIDs inhibited organic cation uptake mediated by hOCT1 and hOCT2, although these two transporters did not mediate the uptake of NSAIDs. The transport of substrates by carrier proteins consists of three processes: substrate binding, translocation, and dissociation. Thus, it was suggested that some of the NSAIDs inhibited TEA binding with hOCT1 and hOCT2 molecules; however, they were not translocated by hOCT1 and hOCT2. The results showing the inhibitory effect of NSAIDs on TEA uptake mediated by hOCT1 and hOCT2 do not contradict the fact that NSAIDs possess anionic moieties. This is due to the assumption that the structures of the binding sites of OATs and OCTs are quite similar, except for the charge recognition sites (Sekine et al., 1999). Similarly, we have previously demonstrated that hOATs and hOCTs interacted with PGF2α and PGF2α, which possess anionic moieties (Kimura et al., 2002).

As demonstrated in the urinary excretion rate of unchanged drugs in Table 1, NSAIDs are highly metabolized in the liver, some by phase I and phase II mechanisms, and others by direct glucuronidation (phase II alone) (Frust and Munster, 2000). In addition, NSAIDs including acetylsalicylate, acetaminophen, sulindac, and diclofenac have been shown to induce liver injury (Zimmerman 1981; Wood et al., 1985; Purcell et al., 1991). In this regard, hOAT2 was shown to be localized at the basolateral side of the liver (Y. Kobayashi, unpublished observation). In the current study, hOAT2 interacted with all of the NSAIDs tested and mediated the transport of some of these NSAIDs. Thus, it was suggested that hOAT2 mediates the uptake of NSAIDs in the basolateral side of the hepatocyte, leading to the metabolism of NSAIDs or the induction of liver injury.

The significant aspects of the interactions of hOATs with NSAIDs in the kidney are suggested to be as follows. The first is to mediate the urinary excretion of NSAIDs. So far, renal handling of salicylate has been studied extensively in animal experiments (Ferrier et al., 1983; Schild and Roch-Ramel, 1988). In humans, as shown in Table 1, 2–30% of administered salicylate is eliminated by urinary excretion in the unchanged form. In the current study, we found that hOAT1 and hOAT3 interacted with salicylate and mediated its transport. Thus, it was suggested that hOAT1 and hOAT3 mediate the uptake of salicylate in the basolateral membrane of the proximal tubule in humans. The second is associated with the induction of renal papillary necrosis. In humans, NSAIDs, including ibuprofen and mefenamic acid, were reported to induce renal papillary necrosis (Robertson et al., 1980; Shah et al., 1981). The mechanism of the induction of renal papillary necrosis has been postulated to be as follows: the accumulation of NSAIDs and the subsequent secretion of these drugs into the lumen lead to high concentrations of these drugs in papillary tips, thereby causing renal papillary necrosis. In the current study, we found that hOAT1 and hOAT3 mediated the transport of ibuprofen and hOAT1, hOAT2, hOAT3, and hOAT4 interacted with ibuprofen and mefenamic acid. Thus, NSAIDs transport mediated by hOAT1, hOAT2, hOAT3, and hOAT4 may be associated with the induction of renal papillary necrosis.

NSAIDs have been shown to induce various forms of adverse neurological effects including cognitive dysfunction, confusion, somnolence, behavioral disturbances, seizures and dizziness (Hoppman et al., 1991; Day et al., 2000). In this regard, we found that hOAT3 mRNA was expressed in the kidney, some by phase I and phase II mechanisms, and others by direct glucuronidation (phase II alone) (Frust and Munster, 2000). In addition, NSAIDs including acetylsalicylate, acetaminophen, sulindac, and diclofenac have been shown to induce liver injury (Zimmerman 1981; Wood et al., 1985; Purcell et al., 1991). In this regard, hOAT2 was shown to be localized at the basolateral side of the liver (Y. Kobayashi, unpublished observation). In the current study, hOAT2 interacted with all of the NSAIDs tested and mediated the transport of some of these NSAIDs. Thus, it was suggested that hOAT2 mediates the uptake of NSAIDs in the basolateral side of the hepatocyte, leading to the metabolism of NSAIDs or the induction of liver injury.
Interactions of hOATs and hOCTs with NSAIDs


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