Short Communication

Interactions of Human Organic Anion Transporters With Aristolochic Acids

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Abstract. Aristolochic acids (AAs), contained in Chinese herbal preparations, have been considered to induce nephropathy. In order to elucidate the molecular mechanisms of AA-induced nephrotoxicity, we have elucidated the interaction of human organic anion transporters (hOATs) with AAs using their stable cell lines. AA-I and AA-II inhibited organic anion uptake by hOAT1, hOAT3, and hOAT4 in dose-dependent manners. Treatment of hOAT3 with AA-I resulted in a significant reduction in viability compared with that of mock, which was rescued by the organic anion transport inhibitor probenecid. In conclusion, hOAT3-mediated AA-I uptake may be associated with the induction of nephrotoxicity.

Keywords: aristolochic acid, organic anion transporter, aristolochic acid nephropathy

In 1993, Vanherweghem et al. reported renal failure cases called Chinese herbs nephropathy caused by Chinese medicine taken for a slimming regime (1). Over the past several years, a considerable number of studies on this disease have been carried out. Chinese herbs nephropathy appears to include two clinical aspects: a rapidly progressive interstitial nephritis and adult-onset Fanconi syndrome (2). Aristolochic acids (AAs) contained in Chinese herbal preparations are suspected of causing nephrotoxicity (3). In this regard, the term "aristolochic acid nephropathy" has recently been used instead of Chinese herbs nephropathy. AAs are a mixture of structurally similar nitrophenanthrene carboxylic acids, with AA-I and AA-II being the major AAs (4). In 1964, AAs were shown to be nephrotoxic in humans (5); and in 1982, they were shown to be strong carcinogens in rodents (6). Previous in vitro and in vivo experiments have revealed that renal proximal tubules are the target of AAs (7, 8). Recently, a relation between AAs and Balkan endemic nephropathy has also been reported (9). However, how AAs are taken up into proximal tubules remains unknown.

The excretion of numerous organic anions (OAs), including endogenous metabolites, drugs, and toxins, is an important physiological function of renal proximal tubules (10). The process of excreting OAs through proximal tubular cells is achieved via unidirectional transcellular transport, involving the uptake of OAs into cells from the blood across the basolateral membrane, followed by extrusion across the apical membrane into the tubular lumen. To date, four isoforms of human organic anion transporters (hOATs: hOAT1 – hOAT4) have been identified in the kidneys (10).

Since AAs have anionic moieties (4), they are classified as OAs and they need transporters for their membrane permeation. However, the precise mechanism for that has not been elucidated yet. Among the renal hOATs, hOAT1 and hOAT3 are thought to play major roles in the basolateral uptake of anionic compounds in proximal tubules because of their broad substrate selectivities (10). hOAT2, also localized at the basolateral side of proximal tubules, mediates the transport of some OAs such as sali-

cylate and prostaglandin $F_{2\alpha}$ (10). hOAT4 mediates OA transport at the apical side of proximal tubules; however, this transporter exhibits relatively narrow substrate selectivity compared with those of hOAT1 and hOAT3 (10). To clarify the molecular mechanisms of AA-induced nephrotoxicity, we examined the interactions of hOATs with AAs using cells derived from the second portion of the proximal tubule (S_2) that stably express hOATs (S2 hOATs). We also investigated the effects of AAs on the viability of S_2 cells stably expressing hOAT1 and hOAT3.

[^{14}C]p-Aminohippurate (PAH) (1.86 GBq/mmol) and [^{3}H]estrone sulfate (ES) (1961 GBq/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA, USA). AAs (1:1 mixture of AA-I and AA-II) were obtained from Biomol Res. Lab. (Plymouth Meeting, PA, USA). Probenecid was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Other materials used in this study were the same as those previously reported (11).

AAs were subjected to preparative high-performance liquid chromatography (HPLC) to obtain AA-I (22 mg) and AA-II (19 mg) and were identified by comparing ^{1}H-nuclear magnetic resonance spectroscopy and mass spectrometry spectral data with reported data (12). Preparative HPLC was performed with CH_3CN-H_2O-AcOH (300:300:1) on a CAPCELL PACK C18 AG120 column (Shiseido, Tokyo) employing a UV monitoring flow system (400 nm) at a flow rate of 10.0 ml/min.

Uptake experiments using S2 hOATs (S2 hOAT1, S2 hOAT2, S2 hOAT3, and S2 hOAT4) were performed as previously described (11). For kinetic analysis, S2 hOAT1 and S2 hOAT3 were incubated at 37˚C in a solution containing either [^{14}C]PAH (hOAT1) or [^{3}H]ES (hOAT3) at different concentrations in the absence or presence of AA-I for 2 min. Based on the OA uptake under each condition, double-reciprocal plot analyses were performed as previously described (13). When the inhibition was competitive, Ki values were calculated by the following equation:

Ki = concentration of AA-I / [(Km for PAH or ES with AA-I / Km for PAH or ES without AA-I) − 1]

Cell viability was assessed as described elsewhere (13). S2 hOAT1, S2 hOAT3, and mock cells were incubated in a solution with or without 10 or 50 μM AA-I or AA-II in the absence or presence of 1 mM probenecid for 48 h at 33°C. After the incubation, 1 ml of 0.5% MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) was added to the medium, and the cells were further incubated for 4 h. The cells were then lysed in isopropanol/HCl solution; subsequently, the optical density was measured at 570 nm using that at 630 nm as a reference (Beckman, DU640).

Data are expressed as means ± S.E.M. Statistical dif-

ferences were determined using one-way analysis of variance with Dunnett's post-hoc test. Differences were considered significant at $P < 0.05$.

First, we examined the inhibitory effects of AA-I and AA-II on OA uptake in S2 hOATs. Both AA-I and AA-II significantly inhibited OA uptake mediated by hOAT1 (A), hOAT3 (B), and hOAT4 (C) in dose-dependent manners (Fig. 1). In contrast, neither AA-I nor AA-II exhibited significant inhibitory effects on OA uptake mediated by hOAT2 (data not shown). IC_{50} values (μM) of AA-I and AA-II were 0.44 ± 0.08 and 1.06 ± 0.09 for hOAT1, 0.65 ± 0.08 and 1.28 ± 0.18 for hOAT3, and 61.3 ± 8.35 and 72.0 ± 9.32 for hOAT4, respectively. Inhibition by AAs in the basolateral isoforms hOAT1 and hOAT3 was stronger than that in the apical isoform hOAT4, suggesting that hOAT1 and hOAT3 are responsible for the entrance of AAs into proximal tubules. In addition, inhibitory effects of AA-I were stronger than those of AA-II in all three OAT isoforms tested. This seems compatible with the previous finding by Shibutani et al. that AA-I is more nephrotoxic than AA-II in rodents (14).

Next, to further elucidate the effects of AA-I on hOAT1 and hOAT3, inhibitory kinetics was analyzed. Lineweaver-Burk plots of the effects of AA-I on hOAT1- and hOAT3-mediated OA uptake demonstrated that AA-I inhibited OA uptake by hOAT1 (A) and hOAT3 (B) in a competitive manner (Fig. 2). Ki values (μM) were 0.80 ± 0.15 for hOAT1 and 0.84 ± 0.10 for hOAT3.

Then we examined the effects of AA-I at 10 and 50 μM on the viability of S2 hOAT1, S2 hOAT3, and mock cells in the absence or presence of probenecid, a potent OA transport inhibitor (12). Exposure of S2 hOAT3 to AA-I (50 μM) for 48 h resulted in a significant decrease in the viability of S2 hOAT3 compared with mock cells (n = 4, **$P < 0.01$ vs. mock) (Fig. 3). The decrease in viability was rescued by the addition of probenecid (1 mM). No significant change in viability by exposure of both S2 hOAT1 and S2 hOAT3 to AA-II was observed (data not shown), also supporting the previous finding by Shibutani et al. that AA-I is solely responsible for AA-induced nephrotoxicity (14). Although exposure of S2 hOAT1 to AA-I (50 μM) for 48 h resulted in a significant decrease in viability, 1 mM probenecid could not recover the reduced viability (data not shown).

In the current study, AA-I and AA-II significantly inhibited hOAT1- and hOAT3-mediated OA uptake. The interaction of AAs with both hOAT1 and hOAT3 does not indicate that AAs are transport substrates for both hOAT1 and hOAT3. In this regard, unfortunately, we could not determine the intracellular AA-I and AA-II contents according to the method previously described
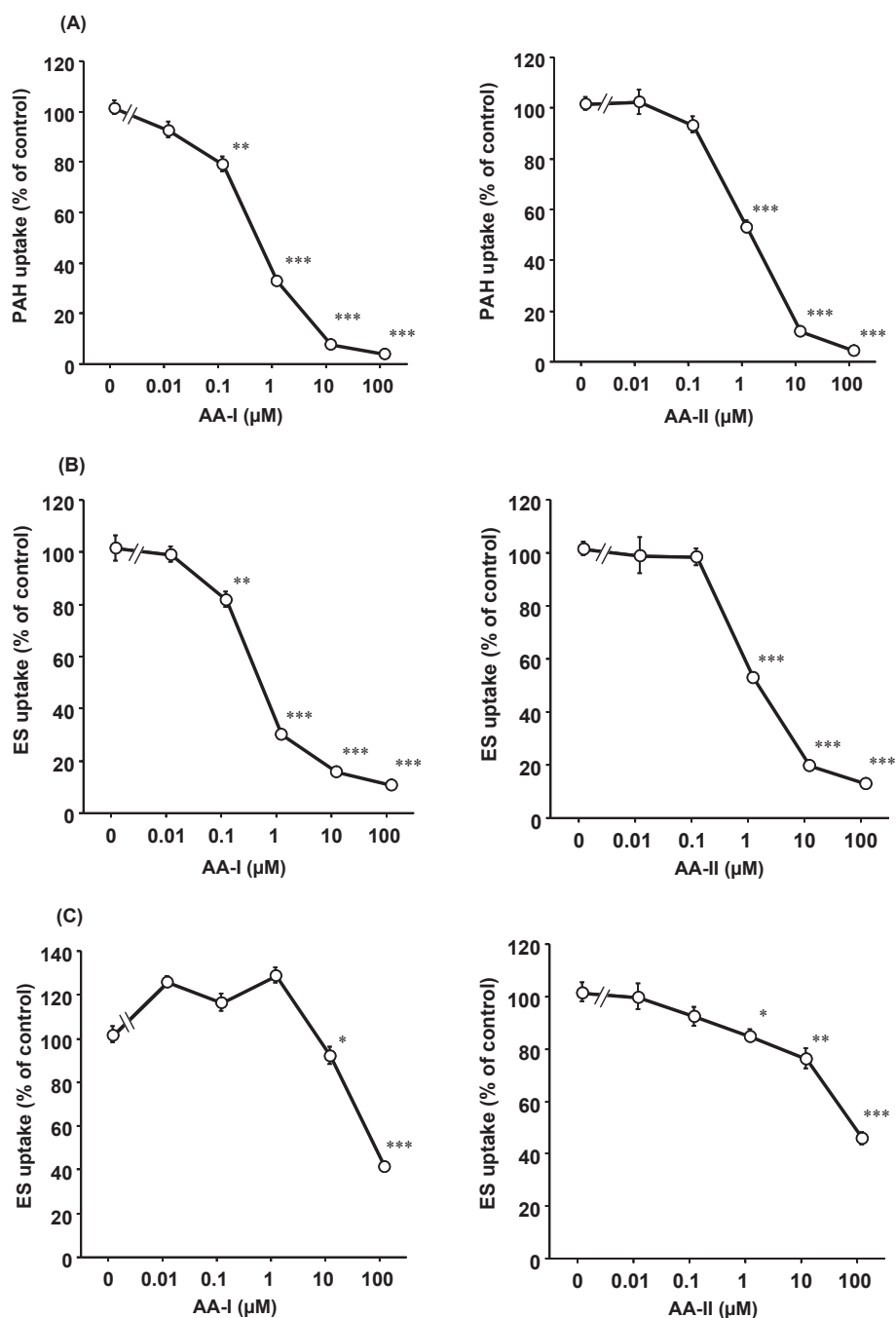
Fig. 1. Effects of various concentrations of AA-I and AA-II on OA uptake mediated by hOATs. S2 hOAT1 (A), S2 hOAT3 (B), and S2 hOAT4 (C) were incubated at 37°C in a medium containing 5 μM [^{14}C]PAH for 2 min (hOAT1) and 50 nM [^{3}H]ES for 2 min (hOAT3 and hOAT4) in the absence or presence of various concentrations of AA-I or AA-II. Each value represents the mean ± S.E.M. of six monolayers from two separate experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. control.

(2). This may be because the intracellular AA content was too small to be determined by this method. However, we suggest that hOAT1 and hOAT3 are involved in the basolateral uptake of AAs in proximal tubules in vivo based on the following reasons: 1) hOAT1 and hOAT3 are major hOATs mediating the basolateral uptake of

various anionic compounds (10); 2) hOAT1 and hOAT3 exhibit high-affinity interactions with AA-I and AA-II; and 3) the recent finding that AA-I and AA-II are excreted in rat urine (15) indicates that anionic AAs are transcellularly transported by membrane transporters at both sides of the proximal tubules, and since we found no
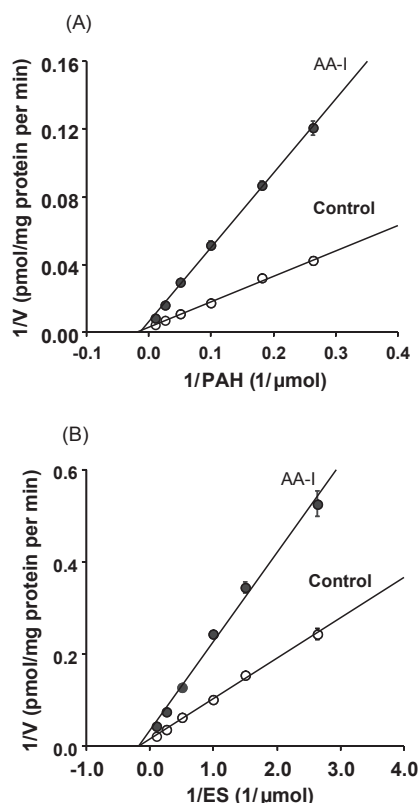
Fig. 2. Kinetic analysis of the effects of AA-I on OA uptake mediated by hOATs. S2 hOAT1 (A) and S2 hOAT3 (B) were incubated in a medium containing various concentrations of [^{14}C]PAH (hOAT1) and [^{3}H]ES (hOAT3) in the absence or presence of AA-I at 1 μM (hOAT1 and hOAT3) for 2 min at 37°C. Lineweaver-Burk plot analyses were performed. Each value represents the mean ± S.E.M. of four monolayers of one typical experiment from two separate experiments.

Fig. 3. Effects of AA-I on the viability of S2 hOAT3 and mock in the absence or presence of probenecid. S2 hOAT3 and mock cells were cultured in a solution with or without AA-I (10 or 50 μM) in the absence or presence of probenecid (1 mM) for 48 h at 33°C. Each value represents the mean ± S.E.M. of four monolayers of one typical experiment from two separate experiments. **$P < 0.01$ vs. mock and #$P < 0.05$ vs. hOAT3.

qualitative difference in the transport properties of OAT1 and OAT3 between humans and rats with a few exceptions (10), we can extrapolate this finding to humans.

hOAT3 seems to be the most probable candidate that contributes to the membrane permeation of AA-I because the addition of AA-I significantly reduced the viability of S2 hOAT3 compared with mock cells and because probenecid, a potent inhibitor of hOAT3, rescued the AA-I-induced effect as shown in Fig. 3. Lack of probenecid recovery in S2 hOAT1 may indicate that AAs are also transported by hOAT1 using a binding site different from PAH, a prototypical substrate for OAT1. Thus, AA-I seems to be the transport substrate for hOAT3 and the uptake of AA-I via hOAT3 may lead to subsequent nephrotoxicity.

In conclusion, hOAT1 and hOAT3 exhibit high-affinity interaction with AAs, and hOAT3-mediated AA-I uptake may be associated with AA-induced nephrotoxicity.
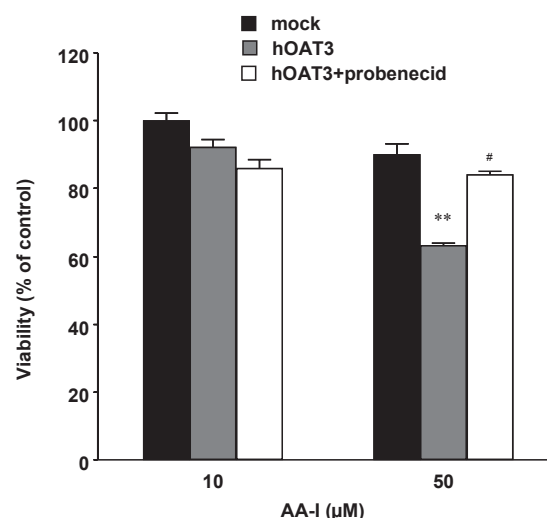
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