Up-regulation of organic anion transporter 1 protein is induced by chronic furosemide or hydrochlorothiazide infusion in rat kidney

Gheun-Ho Kim1, Ki Young Na2, So-Young Kim2, Kwon Wook Joo2, Yoon Kyu Oh3, Seoung-Wan Chae4, Hitoshi Endou5 and Jin Suk Han2

1Department of Internal Medicine and 4Department of Pathology, Hallym University Hangang Sacred Heart Hospital, 2Department of Internal Medicine, Seoul National University, Clinical Research Institute of Seoul National University Hospital, 3Department of Internal Medicine, Eulji Medical College, Seoul, South Korea and 5Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, Japan

Abstract
Background. Thiazide and loop diuretics are secreted from the proximal tubule via the organic anion transport system to reach their principal sites of action. Recently, a multispecific organic anion transporter 1 (OAT1) was identified in rat kidney and was localized to the basolateral membrane of the S2 segment in the proximal tubule. We postulated that interactions between thiazide or loop diuretics and OAT1 may play a role in the adaptation to long-term diuretic use, and investigated whether OAT1 is regulated in vivo by chronic administration of diuretics at the protein level.

Methods. Semi-quantitative immunoblotting and immunohistochemistry were carried out in kidneys from male Sprague–Dawley rats using a polyclonal peptide-derived antibody to OAT1. Furosemide (12 mg/day/rat, n = 6), hydrochlorothiazide (3.75 mg/day/rat, n = 6) or vehicle (1.7% ethanolamine, n = 6) were infused subcutaneously for 7 days using osmotic minipumps. Experimental and vehicle-control rats were pair-fed, and two bottles of drinking water were provided, one containing tap water and the other containing a solution of 0.8% NaCl with 0.1% KCl.

Results. Overt diuretic responses were observed to both furosemide and hydrochlorothiazide infusions. There were no differences in body weight or creatinine clearance between the experimental and control rats. Although OAT1 protein abundance in cortical homogenates was increased by furosemide infusion (271 ± 35 vs 100 ± 15%, P < 0.05), Na-K-ATPase α1 subunit protein abundance was not affected (113 ± 14 vs 100 ± 8%, P = 0.42). Immunohistochemical localization in tissue sections confirmed a strong increase in OAT1 expression in the basolateral membrane of the S2 segment of proximal tubule. OAT1 protein abundance in cortical homogenates was also increased by hydrochlorothiazide infusion (181 ± 25 vs 100 ± 7%, P < 0.01), whereas Na-K-ATPase α1 subunit protein abundance was not affected (105 ± 4 vs 100 ± 4%, P = 0.34).

Conclusion. Chronic furosemide or hydrochlorothiazide infusion caused increases in OAT1 protein abundance in rat kidney. These results suggest that OAT1 may be up-regulated in vivo by chronic administration of diuretics at the protein level.

Keywords: furosemide; hydrochlorothiazide; organic anion transporter 1; immunoblotting; immunohistochemistry; protein abundance

Introduction

The renal proximal tubule has transport mechanisms that allow the secretion of organic anions, and p-aminohippurate (PAH) has been used as the prototypical substrate for this renal organic anion transport system [1]. The active secretory step at the basolateral membrane is carried out by a PAH/dicarboxylate antiporter, which has a broad substrate specificity [2]. Organic anions, including PAH, are taken up into proximal tubule cells from the peritubular plasma by this basol membrane transporter and are excreted into the urinary fluid through the apical membrane [3].

Recently, many organic anion transporter molecules have been cloned. Two different groups simultaneously have identified the multispecific...
organic anion transporter 1 (OAT1) from rat kidney using a functional expression cloning method [4,5]. This transporter shows characteristics that correspond to PAH transport across basolateral membrane, such as PAH/dicarboxylate exchange [6], and is localized in the basolateral membrane of S2 segments of proximal tubule in rat kidney [7].

Loop diuretics and thiazides are widely used for the clinical management of hypertension and oedema. They are weak organic acids, having a common chemical characteristic (sulfonamide diuretics), and hence are secreted by the renal organic anion transport system from the blood into the tubular lumen to reach their principal sites of action [8]. Previous studies suggest that OAT1 is probably involved in the renal tubular secretion of both thiazides and loop diuretics. Bartel et al. [9] have shown that thiazide and loop diuretics inhibit in vitro tubular uptake of PAH in single S2 segments from the proximal tubule of rabbit kidney. Recently, Uwai et al. [10] also reported that PAH uptake by OAT1-expressing Xenopus laevis oocytes was inhibited in the presence of thiazide and loop diuretics.

The molecular cloning of OAT1 provides new tools for investigating the regulation of renal tubular secretion of organic compounds at a molecular level. Despite considerable advances in understanding the basic transport pathways and mechanisms involved in the tubular secretion of organic compounds, there is still relatively little information about the regulation of this transport [11]. In addition, diuretic resistance is often encountered in clinical practice, and an interaction between diuretics and the renal organic anion transport system may play a role in the adaptation to long-term diuretic use. This study was undertaken to investigate whether OAT1 is regulated in vivo by chronic administration of diuretics at the protein level. To achieve this, we examined the effects of chronic administration of furosemide or hydrochlorothiazide on the abundance of OAT1 protein in rat kidney by using semi-quantitative immunoblotting and immunohistochemistry.

Subjects and methods

Animals and experimental protocols

We used specific pathogen-free male Sprague–Dawley rats (SLC Inc., Shizuoka, Japan) weighing 180–220 g. The animals were subjected to three different experimental protocols: chronic furosemide infusion, chronic hydrochlorothiazide infusion and sucrose water loading.

For the furosemide infusion studies, six rats were anaesthetized with enflurane (Choongwae Pharma Corp., Seoul, Korea), and osmotic minipumps (model 2ML1; Alzet, Palo Alto, CA) were implanted subcutaneously to deliver 12 mg/day of furosemide (Handok Inc., Seoul, Korea). Furosemide was dissolved in a 1.7% ethanolamine solution. Six control rats were implanted with minipumps containing vehicle (ethanolamine) alone.

The hydrochlorothiazide infusion studies were carried out in the same way as the furosemide osmotic minipump infusion protocol. Rats were infused with either 3.75 mg/day of hydrochlorothiazide (YuHan Corp., Seoul, Korea) (n=6) or vehicle (n=6) for 7 days.

The animals were placed in metabolism cages 3 days prior to the beginning of the study. Control and treated rats were chosen randomly, and all were provided with a daily fixed amount of finely ground regular rat chow (18 g/200 g BW/day) and two separate bottles of drinking water, one containing 0.8% NaCl and 0.1% KCl, and the other containing tap water. All the rats ate the entire amount of the offered rat chow and showed a steady increase in body weight throughout the study period.

The next experiment was performed to test whether the abundance of OAT1 protein is affected by the increase in urine volume per se. To achieve this, rats were divided into water-loaded (n=6) and water-restricted (n=6) groups. Water-loaded rats were provided with finely ground rat chow (18 g/200 g BW/day) plus 600 mM sucrose as the drinking fluid. For water-restricted rats, the same rat chow was made into a paste by the addition of water (18 ml/200 g BW/day). This protocol was effective in producing large differences in water intake and urine output.

Urinary measurements

Daily urinary samples were collected to measure urine volume and osmolality. Urine osmolality was measured with a cryoscopic osmometer (Osmomat 030-D-M; Gonotec, Berlin, Germany). Urinary sodium concentration was measured using an ion-selective method (System E4A; Beckman Coulter Inc., Fullerton, CA). Furosemide concentration in urine from the furosemide-infused rats was measured using high-performance liquid chromatography (HPLC).

Semi-quantitative immunoblotting

After 7 days of infusion, the rats were killed by decapitation, and kidneys were rapidly removed for dissection of the renal cortex. The cortices were placed in 10 ml of ice-cold isolation solution, containing 250 mM sucrose, 10 mM triethanolamine (Sigma, St Louis, MO), 1 µg/ml leupeptin (Sigma) and 0.1 mg/ml phenylmethylsulfonyl fluoride (Sigma) titrated to pH 7.6, and the mixture was homogenized at 15,000 r.p.m. with three strokes for 15 s using a tissue homogenizer (PowerGun 125; Fisher Scientific, Pittsburgh, PA). After homogenization, total protein concentration was measured using the bicinchoninic acid protein assay reagent kit (Sigma) and was adjusted to 2 μg/μl with isolation solution. The samples were then stabilized by adding 1 vol. of 5x Laemmli sample buffer per 4 vols of sample and by heating to 60°C for 15 min.

Initially, ‘loading gels’ were performed for each sample set. A 5 µg aliquot of protein from each sample was loaded into individual lanes, electrophoresed on 12% SDS–polyacrylamide minigels using a Mini PROTEAN® III electrophoresis apparatus (Bio-Rad, Hercules, CA), and then stained with Coomassie blue dye (G-250, Bio-Rad; 0.025% solution made in 4.5% methanol and 1% acetic acid). Selected bands from these gels were scanned (GS-700 Imaging Densitometry, Bio-Rad) to determine the density (Molecular Analyst version
1.5, Bio-Rad) and relative amounts of protein loaded in each lane. Finally, protein concentrations were ‘corrected’ to reflect these measurements.

For immunoblotting, the proteins were transferred electrophoretically from unstained gels to nitrocellulose membranes (Bio-Rad). After blocking with 5% skim milk in PBS-T (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% Tween-20, pH 7.5) for 30 min, membranes were probed overnight at 4°C with their respective primary antibodies consisting of a rabbit polyclonal peptide-derived antibody against rat renal OAT1 [7,12] and a commercial mouse monoclonal antibody for the Na-K-ATPase α₁ subunit (Upstate Biotechnology Inc., Lake Placid, NY). The secondary antibodies were goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP; Pierce, Rockford, IL) for OAT1 and goat anti-mouse IgG conjugated to HRP (Pierce) for the Na-K-ATPase α₁ subunit.

Antibody–antigen reaction sites were viewed using enhanced chemiluminescence substrate (ECL® RPN 2106; Amersham Pharmacia Biotech, Buckinghamshire, UK) before exposure to X-ray film (Hyperfilm; Amersham Pharmacia Biotech). Relative quantification of resulting immunoblot band densities was carried out by densitometry using a laser scanner (GS-700 Imaging Densitometry, Bio-Rad) and ImageQuaNT software (Molecular Analyst version 1.5, Bio-Rad).

In all cases, to confirm equality of loading among lanes, electrophoresis was run initially for the entire set of samples in a given experiment on a single 12% SDS–polyacrylamide gel, which was then stained with Coomassie blue dye (G-250, Bio-Rad; 0.025% solution made in 4.5% methanol and 1% acetic acid). These loading gels established that the above immunoblots were uniformly loaded.

Immunohistochemistry
The kidneys were perfused by retrograde perfusion via the abdominal aorta with 1% phosphate-buffered saline (PBS) to remove blood and then with periodate-lysine–paraformaldehyde (PLP; 0.01 M NaIO₄, 0.075 M lysine, 2% paraformaldehyde, in 0.0375 M Na₂HPO₄ buffer, pH 6.2) for 3 min to produce the kidney fixation. After completion of perfusion, each kidney was cut into 5 mm thick slices and immersed in 2% PLP solution overnight at 4°C. Each slice was dehydrated with a graded series of ethanol and embedded in polyester wax. The embedded pieces of kidney slices were sectioned to 5 μm thickness on a microtome (RM 2145; Leica Instruments GmbH, Nussloch, Germany).

The sections were dewaxed with a graded series of ethanol and treated with 3% H₂O₂ for 30 min to eliminate endogenous peroxidase activity. They were blocked with 6% normal goat serum (S-1000; Vector Laboratory, Burlington, CA) for 15 min. They were then incubated overnight at 4°C with their respective primary antibodies diluted in PBS. After incubation, they were washed with PBS and incubated for 30 min in biotinylated goat anti-rabbit IgG (BA-1000; Vector Laboratory) at room temperature. Next, the peroxidase standard vectastatin ABC kit (PK-4000; Vector Laboratory) was added for 30–60 min at room temperature. The sections were washed with PBS and incubated in a 3,3'-diaminobenzidine (DAB) substrate kit (SK-4100; Vector Laboratory). The sections were counter-stained with haematoxylin and the slides were mounted with Canadian balsam.

Statistics
Values are presented as means ± SEM. Quantitative comparisons between groups were made by Mann–Whitney U-tests (Statview software; Abacus Concepts Inc., Berkeley, CA). To facilitate comparisons from the semi-quantitative immunoblotting, we normalized the band density values by dividing by the mean value for the control group. Thus the mean for the control group is defined as 100%. P-values < 0.05 were considered statistically significant.

Results
Responses to furosemide infusion
In response to furosemide infusion, urine volume increased immediately and a high output was maintained throughout the 7 day infusion period (Figure 1A). The final daily urine volume collection was ~7-fold higher in furosemide-infused rats than in vehicle-infused controls. Urinary sodium excretion

![Fig. 1. Serial changes in urine output (A) and urine osmolality (B) in response to furosemide infusion. *P < 0.01 vs vehicle.](image_url)
was also markedly increased in furosemide-infused rats compared with vehicle-infused controls (10.5 ± 1.8 vs 2.5 ± 0.2 meq/day, P < 0.005). In contrast, urine osmolality was significantly decreased in response to furosemide and remained lower than controls throughout the infusion period (Figure 1B). As expected, we confirmed the presence of furosemide in the urine from furosemide-infused rats (mean, 93 mg/ml).

Figure 2A shows the immunoblot of OAT1 from renal cortical homogenates. Furosemide infusion for 7 days resulted in large increases in OAT1 protein abundance in the cortex. Normalized band densities for furosemide-infused and vehicle-infused rats were 271 ± 35 and 100 ± 15%, respectively (P < 0.05). To determine whether the increase in OAT1 abundance was selective, we carried out semi-quantitative immunoblotting for basolaterally located Na-K-ATPase α1 subunits. In contrast to OAT1, the abundance of Na-K-ATPase α1 subunit was not significantly altered by furosemide infusion (Figure 2B). Normalized band densities were 113 ± 14% for furosemide-infused rats and 100 ± 8% for vehicle-infused rats (P > 0.05). Figure 2C shows an immunoblot loading control achieved by running an identically loaded SDS-polyacrylamide gel that was stained with Coomassie blue. This contrast procedure revealed that there was little or no density variation in the major bands stained with Coomassie blue, demonstrating that the marked increased in band density seen in Figure 2A was not due to unequal loading.

Light microscopic analysis of 5μm wax sections demonstrated that there was specific immunostaining for OAT1 in S2 segments from the proximal tubules (Figure 3). High-magnification examination of the S2 segment revealed that OAT1 immunostaining was localized to the basolateral surface of proximal tubule cells, as reported earlier [7]. Chronic furosemide infusion caused strong increases in OAT1 expression in the basolateral membrane of S2 segments of proximal tubule (Figure 3).

We next examined whether the increase in OAT1 protein abundance was inducible by a short-term administration of furosemide. The OAT1 immunoblot from rat renal cortical homogenates after 3 days of furosemide vehicle infusion is shown in Figure 4A. Furosemide infusion for 3 days also resulted in a significant increase in OAT1 abundance in the cortex (139 ± 5% for furosemide-infused rats vs 100 ± 15% for vehicle-infused rats, P < 0.05). Parallel Coomassie blue-stained SDS-polyacrylamide gels demonstrated a uniform loading among all samples (data not shown).

Finally, we tested whether the increase in OAT1 protein abundance was induced by the increase in urine volume per se. As expected, sucrose water-loaded rats drank large amounts of fluid, establishing a high urine volume throughout the 7 day study period (24.5 ± 1.2 ml/day for water-loaded rats vs 2.9 ± 0.2 ml/day for water-restricted rats, P < 0.001).
OAT1 immunoblots from renal cortical homogenates of water-loaded vs water-restricted rats are shown in Figure 4B. Normalized band densities for water-loaded and water-restricted rats were 81 ± 6 and 100 ± 4%, respectively (P < 0.05). Thus, increases in urine volume caused decreases rather than increases in OAT1 protein abundance.

**Responses to hydrochlorothiazide infusion**

Hydrochlorothiazide infusion significantly increased urine volume, although this increase was not as remarkable as that induced by furosemide. Urine volume began rising on the third day of hydrochlorothiazide infusion and remained 2- to 3-fold higher than vehicle-infused controls throughout the infusion period (Figure 5A). Urine osmolality was also decreased by hydrochlorothiazide infusion by the third day and remained lower throughout the 7 day infusion period (Figure 5B). Urinary sodium excretion measured on the last day of hydrochlorothiazide infusion was significantly greater in hydrochlorothiazide-infused rats than in vehicle-infused controls (7.5 ± 0.9 vs 3.3 ± 0.6 meq/day, P < 0.05).

The immunoblot of OAT1 from renal cortical homogenates is shown in Figure 6A. Hydrochlorothiazide infusion for 7 days significantly increased OAT1 protein abundance in the cortex. Normalized band densities for hydrochlorothiazide-infused and vehicle-infused rats were 181 ± 25 and 100 ± 7%, respectively (P < 0.01). However, hydrochlorothiazide did not change the abundance of Na-K-ATPase α1 subunit protein in the same region (Figure 6B; normalized band densities: 105 ± 4% for hydrochlorothiazide vs 100 ± 4% for vehicle, P > 0.05). A parallel Coomassie-stained SDS–polyacrylamide gel demonstrated uniform loading among all samples (Figure 6C), ruling out the possibility that increases in OAT1 band density in the cortex were due to differences in loading.

**Discussion**

The proximal tubular secretion of loop diuretics and thiazides is critical for their diuretic action because it is through this mechanism that they gain access to
luminal tubular target sites in effective amounts [8]. Previous approaches using the rat or human OAT1-expressing *Xenopus laevis* oocyte system [5,6] have shown that OAT1, functioning as a PAH/dicarboxylate antiporter, mediates proximal tubular secretion of these diuretics. However, previous electrophysiological studies failed to demonstrate translocation of furosemide in *X. laevis* oocytes expressing flounder OAT1 [13], indicating that there may be species differences in substrates for OAT1. In the present study, we clarified the involvement of rat OAT1 *in vivo* in mediating tubular secretion of furosemide and hydrochlorothiazide.

We demonstrated that both furosemide and hydrochlorothiazide induced a powerful diuresis in parallel with increases in OAT1 protein abundance in rat kidney. This effect appeared to result from the diuretic agent itself, because sucrose water loading, which increased urine volume, failed to increase OAT1 protein abundance. In fact, the sucrose water loading caused decreases rather than increases in OAT1 abundance. These decreases in OAT1 in sucrose water-loaded rats were unexpected and may be related to their large increases in body weight compared with water-restricted rats. Nevertheless, it is clear that increases in OAT1 abundance induced by furosemide or hydrochlorothiazide infusion were not due to the increase in urine volume. Our results support a role for OAT1 in the renal tubular secretion of thiazides and loop diuretics at the protein level, and suggest that *in vivo* substrate stimulation up-regulates OAT1 protein.

Substrate stimulation is one of the important factors regulating OAT1 [11]. Hirsch and Hook [14,15] were the first to demonstrate that prior injection of penicillin (twice daily for 3 days) in 2-week-old rabbit pups led to an increase in the uptake of PAH by kidney cortical slices. This phenomenon was thought to be a result of increased biosynthesis of transport proteins, since pretreatment with penicillin leads to greater incorporation of leucine and glutamine into the slice proteins and to enhancement of protein content in the microsomal fraction, whereas administration of the protein synthesis inhibitor cycloheximide to nursing rats prevents substrate stimulation of PAH uptake by renal slices [16]. Thus, substrate stimulation may occur as a result of increased synthesis of the transporter protein involved in the secretion of organic compounds, and we showed that OAT1 protein abundance is increased by chronic diuretic infusion in adult rats.

In clinical practice, diuretics typically provide effective treatment for oedema when used judiciously. However, some patients become resistant to their effects. Up-regulation of OAT1 protein abundance induced by chronic administration of furosemide or thiazide diuretics may potentially counteract diuretic resistance.

An additional important finding was that increases in OAT1 in response to chronic diuretic infusion were not accompanied by generalized increases in transporter protein expression in the proximal tubule. Transport of organic anions across the basolateral membrane of renal proximal tubular cells is energetically uphill and is accomplished by a ‘tertiary’ active process. The Na-K-ATPase maintains an inwardly directed (blood-to-cell) Na\(^+\) gradient. This Na\(^+\) gradient in turn drives an Na\(^+\)/dicarboxylate co-transporter, sustaining an outwardly directed dicarboxylate gradient that is utilized by a PAH/dicarboxylate antiporter (OAT1) to move the organic anion substrate into the cell [17]. This cascade of events indirectly links OAT1 to metabolic energy and to the Na\(^+\) gradient, allowing entry of a negatively charged substrate against both its chemical concentration gradient and the electrical potential of the cell. Therefore, it is possible that OAT1 activity may change in parallel with that of Na-K-ATPase.

We found, however, that Na-K-ATPase \(\alpha_1\) subunit protein abundance did not change in response to chronic diuretic infusion. When using a newborn rabbit model, Hook and co-workers [18] found that Na-K-ATPase was not affected by penicillin pretreatment despite an increase in PAH uptake. Recently, an *in vitro* microperfusion study showed that stevioside, a metabolite of the natural sweetener stevioside, inhibited PAH transport at the basolateral...
membrane of isolated S2 segments of rabbit renal proximal tubules but had no effect on Na-K-ATPase activity [19]. Taken together with the present data, these findings suggest that furosemide and hydrochlorothiazide may have a direct stimulatory effect on OAT1 protein synthesis.

In addition to OAT1, both OAT2 and OAT3 have been identified, and these proteins are expressed in the kidney [20]. Whereas OAT2 is localized in the luminal membranes of the thick ascending limb and the collecting duct, OAT3 is localized in the basolateral membrane of the proximal tubule [20]. Further studies are warranted to verify whether other organic anion transporters, such as rOAT2 and rOAT3, are also regulated at the protein level in response to diuretic administration.

Acknowledgements. This research was supported by the Hallym Academy of Sciences, Hallym University, Korea, 2001.

Conflict of interest statement. None declared.

References


Received for publication: 25.3.02
Accepted in revised form: 20.12.02