Effects of furosemide on renal calcium handling

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Submitted 20 January 2007; accepted in final form 20 July 2007

Lee C-T, Chen H-C, Lai L-W, Yong K-C, Lien Y-III. Effects of furosemide on renal calcium handling. Am J Physiol Renal Physiol 293: F1231–F1237, 2007.—Furosemide is a loop diuretic agent that decreases sodium reabsorption by competing for the chloride site of sodium potassium chloride cotransporter (NKCC2) in the thick ascending limb of loop of Henle (TALH) (29). By diminishing sodium reabsorption, the concentrating process that occurs in the medulla is thus impaired and fluid loss ensues. Long-term furosemide treatment is usually indicated primarily for its natriuretic action for the treatment of a variety of clinical conditions such as hypertension, edematous status, and chronic renal insufficiency (6).

Administration of furosemide not only elicits diuresis but is also associated with adverse effects, such as volume contraction, alterations in electrolyte and acid-base homeostasis, glucose intolerance, and lipid abnormality (11). It has been well established that furosemide and other loop diuretics promote urinary calcium and magnesium excretion (9). About 20% ultrafiltered calcium and 60–80% magnesium are reabsorbed in the TALH. Loop diuretics reduce the lumen-positive trans-epithelial voltage and consequently diminish paracellular transport of calcium and magnesium (12). Patients with Bartter syndrome caused by NKCC2 mutation manifest similar electrolyte disturbances (30). Because the calciiuric effect is rapid and effective, furosemide has been used as a first-line therapy for patients with hypercalcemia in the absence of volume contraction (2).

The distal convoluted tubule (DCT) is an important nephron segment for renal handling of salt, calcium, and magnesium (3). Recently, the novel apical calcium channel specifically expressed in DCT was identified (14). Soon after that, another calcium channel was cloned and found to be expressed in the DCT and also in more distal tubules (23, 26). Subsequent studies have revealed that these channels exhibit characteristics of the superfamily of transient receptor potential (TRP) and are renamed as TRPV5 (TRP vailloid) and TRPV6 (15). With their colocalization with intracellular calcium binding proteins, such as calbindin-D28k, and the basolateral transporters, such as sodium/calcium exchanger and calcium pump, these transport molecules constitute the fundamental machinery for calcium transport in the DCT (15). In the present study, we investigated the acute and chronic effects of furosemide, and effects of the combination of furosemide and thiazides, on renal calcium handling and expression of calcium channels and calcium binding proteins in the kidney.

MATERIALS AND METHODS

Animals

Male C57/BL6 mice, 6–8 wk old weighing ~20 g, were used. All animals were allowed free access to both food and drinking water. The contents of food included 0.3% sodium, 1.2% calcium, and 0.16% magnesium. Animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arizona and the Chang-Gong Memorial Hospital, and all animal procedures were performed according to the IACUC policy.

Study Design

Acute effects of furosemide. The mice (n = 4) were administered with a single dose of furosemide (15 mg/kg), or furosemide with chlorothiazide (25 mg/kg), by intraperitoneal injection and killed 4 h later. Urine samples were collected in metabolic cages. The whole kidney was used for total RNA isolation.

Chronic effects of furosemide. The mice were divided into five groups (n = 6–8 in each group): 1) control group, 2) furosemide...
group (15 mg/kg) every 12 h for 3 days, 3) furosemide with salt supplement group (same dosage as group 2 and drinking water with 0.8% NaCl and 0.1% KCl was offered), 4) salt supplement group (mice were given drinking water with 0.8% NaCl and 0.1% KCl but no furosemide treatment), and 5) coadministration of furosemide and chlorothiazide group (with same dosage as in acute experiment every 12 h for 3 days). On the fourth day, urine samples were collected in metabolic cages, and blood samples were collected for hematocrit and biochemical measurements. The kidneys were harvested for isolation of total RNA or protein and for immunofluorescence study.

**Gentamicin treatment.** To verify the effects of furosemide-induced hypercalciuria on DCT calcium transport molecules, we added a gentamicin-treated group (n = 8 mice) as a hypercalciuric control because gentamicin can induce hypercalciuria without renal tubular damages (25). The mice were administered with a single subcutaneous dose of gentamicin (40 mg/kg) daily for 4 days. The collection of urine, blood, and kidney samples was performed as described in the furosemide experiments.

**Biochemical Assays and Hematocrit**

Creatinine and calcium levels were measured in urine and blood samples according to manufacturer’s protocols as described previously (19). The urinary excretion of calcium was expressed as calcium-to-creatinine (Ca/Cr) ratio. Hematocrit was measured before and after treatment, with diuretics as an indicator of volume status.

**Total RNA Isolation and cDNA Synthesis**

The whole kidney was homogenized, and total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription for first-strand cDNA synthesis was performed with a reverse transcription system (SuperScript system, Invitrogen).

**Real-Time PCR**

The mRNA levels of apical calcium channels TRPV5 and TRPV6, calbindin-D28k, and calbindin-D9k were measured by quantitative real-time RT-PCR as described previously (20). The first-strand cDNA was subject to real-time PCR using the Cepheid Smart Cycle II system (Cepheid, Sunnyvale, CA) and a fluorescent dye (SYBR green; Molecular Probes, Eugene, OR) as a tracer. cDNA was subjected to real-time PCR using the Cepheid Smart Cycle II system (Cepheid, Sunnyvale, CA) and a fluorescent dye (SYBR green; Molecular Probes, Eugene, OR) as a tracer. The abundance of studied protein was measured.

**Immunoblotting Study**

The frozen renal tissue was homogenized at 4°C in protein lysis buffer solution containing protease inhibitor cocktail (Roche, Penzberg, Germany). Immunoblotting for quantification of calbindin-D28k was performed as described previously (21). The primary antibody was goat anti-mouse calbindin-D28k monoclonal antibody (1:2,000, Sigma). After washing and blocking were completed, the membrane was incubated with goat anti-rabbit antibody conjugated with horseradish peroxidase. The abundance of studied protein was then quantified by densitometric analyses. The changes in protein abundance are presented as percentages of control animal values. β-Actin was used as the internal control in this study.

**Statistical Analysis**

All values are expressed as means ± SE. Statistical analyses of the data were performed with SPSS software. Unpaired Student’s t-tests were used to compare differences between two groups. To determine the significant difference among controls, furosemide-treated, furosemide plus salt supply, salt only, and furosemide plus chlorothiazide groups, one-way ANOVA and Tukey’s test were used. P < 0.05 was considered statistically significant for all tests.

**RESULTS**

**Acute Effects of Furosemide**

A significant increase in urinary calcium excretion was observed 4 h after a single dose treatment of furosemide (urine Ca/Cr: 1.98 ± 0.18 vs. 0.21 ± 0.09; P < 0.05). Coadministration of chlorothiazide partially reversed furosemide-induced hypercalciuria (urine Ca/Cr: 1.16 ± 0.05; P < 0.05 vs. control and furosemide only). This increased calcium excretion in the furosemide group was accompanied with elevated mRNA abundance of TRPV5 and TRPV6 (174 ± 10% and 183 ± 9% of control; both P < 0.05) and calbindin-D28k (222 ± 13%; P < 0.05) but no significant change in calbindin-D9k (144 ± 10%) mRNA abundance. Significant upregulation was observed in mice treated with furosemide plus chlorothiazide (TRPV5: 181 ± 5%; TRPV6: 169 ± 8%; calbindin-D28k: 179 ± 10%, and calbindin-D9k: 158 ± 5%; all P < 0.05).

**Chronic Effects of Furosemide**

After administration of furosemide for 3 days, a fourfold increase in urinary calcium excretion was observed (Table 2). A similar increase in urinary excretion of calcium was also observed in mice with salt supplement only; however, the increase was less in the latter group. The calciiuretic effect of furosemide is decreased in mice by the addition of chlorothiazide (urine Ca/Cr: 0.81 ± 0.09 for furosemide only and 0.47 ± 0.15 for furosemide plus chlorothiazide; P < 0.05). No
significant changes in serum creatinine or calcium levels were found in any of the treatment groups. There was a significant increase in hematocrit and decrease in body weight in the furosemide-treated and furosemide plus chlorothiazide-treated groups compared with the control animals. The hematocrit values and weight loss (4% in furosemide group and 3.4% in furosemide plus chlorothiazide group) were not significantly different between these two groups. The hematocrit values of mice in the furosemide plus salt or salt only groups were not different from control mice. They also showed no significant weight changes after treatment (Table 2).

The increases in calcium excretion in furosemide only, salt only, and furosemide plus salt groups are associated with a significant increase in mRNA abundance of TRPV5 (170 ± 10%), TRPV6 (210 ± 9%, 182 ± 10, 194 ± 10% of the control, respectively), calbindin-D28k (310 ± 14%, 290 ± 12%, 264 ± 10% of the control, respectively), and calbindin-D9k (220 ± 13%, 254 ± 9%, 230 ± 9% of the control, respectively). In the furosemide plus chlorothiazide group, except for calbindin-D9k, all of the other transport molecules showed a >50% increase in gene expression: 171 ± 8% for TRPV5, 170 ± 11% for TRPV6, and 167 ± 7% of control for calbindin-D28k (all P < 0.05 vs. control), with a marginal increase found in calbindin-D9k (140 ± 6% of control; Fig. 1).

To assess whether protein expression was also increased, we performed immunofluorescence studies with antibodies against TRPV5 and calbindin-D28k. Figure 2 shows that there are more TRPV5- and calbindin-D28k-positive tubules in kidneys from mice treated with furosemide, furosemide with salt supplementation, salt alone, and furosemide with chlorothiazide. Semiquantitative studies showed significant increases in both TRPV5 protein (furosemide only: 210 ± 9%, furosemide plus salt: 201 ± 12%, salt only: 223 ± 7%, furosemide plus chlorothiazide: 190 ± 7% vs. controls; all P < 0.05; Fig. 2, top) and calbindin-D28k protein (furosemide only: 212 ± 10%, furosemide with salt: 227 ± 9%, salt only: 197 ± 12%, furosemide plus chlorothiazide: 185 ± 10% vs. controls; all P < 0.05; Fig. 2, bottom).

The increased protein expression of calbindin-D28k was further confirmed by immunoblotting studies (Fig. 3). Furosemide treatment was associated with increased abundance of calbindin-D28k (170 ± 6% of control; P < 0.05). A similar increase was observed in furosemide with salt, salt alone, and coadministration with furosemide and chlorothiazide groups (169 ± 5%, 189 ± 8% and 173 ± 6%, respectively; all P < 0.05; Fig. 3).

Effects of Gentamicin

Gentamicin injection produced marked calciuria (urine Ca/Cr: 2.0 ± 0.11 vs. 0.19 ± 0.03; P < 0.05) without changing serum calcium (9.0 ± 0.1 mg/dl) or creatinine (0.41 ± 0.9 mg/dl) levels significantly. Similar to furosemide, gentamicin

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Table 2. Effects of diuretics on body weight, hematocrit, and serum and urine chemistry

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<th>Salt</th>
<th>Furosemide + Chlorothiazide</th>
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<td>Body weight after treatment, g</td>
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<td>Hematocrit, %</td>
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<td>Urinary calcium-to-creatinine ratio</td>
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</table>

Values are means ± SE. *P < 0.05 vs. before treatment; †P < 0.05 versus control animals; ‡P < 0.05 vs. furosemide treatment.

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Fig. 1. Gene expression of TRPV5 (A), TRPV6 (B), calbindin-D28k (C), calbindin-D9k (D) among the 5 groups of mice: control (Ctrl), furosemide treatment (F), furosemide treatment with salt supplementation (F+S), salt supplementation only (S), and coadministration with furosemide and chlorothiazide (F+C). TRPV5 and TRPV6, transient receptor potential vanilloid 5 and 6; CBD-28k and CBD-9k: calbindin-D28k and calbindin-D9k. *P < 0.05 compared with the controls.

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Fig. 2. Immunofluorescent staining of representative kidney sections using anti-TRPV5 (top) and anti-CBD-28k (bottom) antibodies in the 5 treatment groups (Ctrl, F, F+S, S, and F+C). There are more positive-staining tubules in all treated groups than shown in the control kidney. Bottom right of top and bottom show results of semiquantitative studies. *P < 0.05 compared with the controls.
treatment increased mRNA expression of TRPV5 (175 ± 6%), TRPV6 (160 ± 8%), calbindin-D28k (180 ± 5%), and calbindin-D9k (168 ± 6%; all P < 0.05 vs. control). Immunofluorescence studies (Fig. 4) showed that gentamicin treatment was associated with increased abundance of TRPV5 (170 ± 9%) and calbindin-D28k (196 ± 8%; both P < 0.05 vs. control). The increased protein expression of calbindin-D28k was further confirmed by immunoblotting studies (Fig. 5). Gentamicin treatment was associated with increased abundance of calbindin-D28k (183 ± 9% of control; P < 0.05).

DISCUSSION

The major finding in the present study is that furosemide-induced hypercalciuria is accompanied by an increased abundance of calcium transporters in the DCT for both acute and chronic treatments. The immediate response illustrates the rapidness of the reaction in DCT transport. In the chronic experiments, furosemide upregulates the expression of TRPV5, TRPV6, calbindin-D28k, and calbindin-D9k, and these effects are not affected by the volume status of the mice.

Fig. 3. Immunoblotting results of CBD-28k protein in the 5 treatment groups (Ctrl, F, F+S, S, and F+C). The relative CBD-28k protein abundance is presented as CBD-28k-to-β-actin ratio. The ratio of control animals is set as 100%. *P < 0.05 compared with the controls.

Fig. 4. Immunofluorescence studies demonstrate that gentamicin (GM) treatment increases TRPV5 and CBD-28k (CBD) in the kidney. Ctrl, control mouse.

Fig. 5. Immunoblotting results of CBD-28k protein. The relative CBD-28k protein abundance is presented as CBD-28k-to-β-actin ratio. The ratio of control animals is set as 100%. *P < 0.05 compared with the controls.
As furosemide inhibits NKCC2, the lumen-positive charge is reduced because of diminished active sodium reabsorption and potassium recycling. Consequently, the paracellular transport of calcium in the TALH is reduced and more calcium is delivered into downstream segments beyond the TALH. In vivo micropuncture study has shown that intraluminal furosemide perfusion in the TALH resulted in a load-dependent increase in absolute calcium reabsorption in superficial distal tubules (27). In the presence of increased solute load, a two- to threefold increases in calcium binding proteins, calbindin-D9k and calbindin-D28k, TRPV5, and TRPV6 were observed in our study. It has been proposed that vitamin D might mediate the furosemide-induced increase in calbindin-D28k (28). However, other studies did not find any alternation in calcitropic hormones including vitamin D (4, 13). We believe that the upregulation of these calcium transporters is more likely due to the increased solute delivery to the DCT. Similar upregulation of DCT calcium transport molecules induced by hypercalcemia was observed in gentamicin-treated mice in the present study, as well as in streptozotocin-induced diabetic rats (21). The upregulation of downstream transporters has also been observed in sodium and water transporters after furosemide administration (18). In response to the increased sodium load, structural and function adaptation have been observed in the DCT and collecting duct with increased transport capacity (1, 17). Previous studies have revealed conflicting results that calbindin-D28k was unchanged (13) or upregulated (28) by loop diuretics. It is likely that the variations in the degree of calciuria and different methods may explain these varied results.

To evaluate the effect of volume depletion and sodium deficiency, mice treated with furosemide were given salt supplementation in the drinking water. Our results demonstrate that salt supplement corrects volume deficit but does not affect furosemide-induced hypercalcuria. These findings are consistent with previous studies that showed that salt supplement in furosemide-treated young rats did not affect calciuria and nephrocalcinosis (10). The upregulation of calcium transport molecules are also similar between these two groups of mice, as shown in the present study. In fact, salt supplement alone induces an increase of urinary calcium content, although the change is smaller than that in furosemide-treated mice. With increased salt intake, the calcium reabsorption in the proximal tubule is diminished (22). It appears that increased distal delivery of calcium induced by reduced reabsorption in either proximal tubule or TALH upregulates calcium transport molecules in the distal tubules. These findings support the compensatory mechanism to reduce calcium loss by increasing DCT calcium reabsorption machinery.

Clinically, prolonged use of furosemide is associated with persistent hypercalcemia and nephrocalcinosis especially in infants (7). These patients do not develop hypocalcemia because the serum calcium level is highly regulated by the parathyroid hormone, vitamin D, and calcitonin. However, diminished bone mineral content was observed in longer treatment, indicating a negative calcium balance (8). To maintain calcium homeostasis, compensatory mechanisms must be activated to prevent calcium loss. It has been shown that intestinal calcium absorption is enhanced during chronic furosemide treatment without alteration in serum vitamin D level (4). The upregulation of calcium channels and calcium binding proteins in the DCT is likely to be another compensatory mechanism to reduce calcium loss.

Thiazides have been used to diminish furosemide-induced nephrocalcinosis in infants. Hufnagle et al. (16) reported that furosemide (2 mg·kg\(^{-1}\)·day\(^{-1}\)) increased urine calcium 10- to 20-fold compared with that shown in untreated infants. The addition of chlorothiazide (20 mg·kg\(^{-1}\)·day\(^{-1}\)) resulted in a 4- to 15-fold decrease in calcium excretion (16). In the present study, we demonstrate similar calcium conservation effects in mice: chlorothiazide reduces furosemide-induced renal calcium wasting by 40%. Clearly, these two drugs are used synergistically to enhance the diuretic effect, but they affect renal calcium handling in opposite directions.

The mechanisms of hypocalciuric action of thiazides have been controversial. Bindels and colleagues (24) indicated that thiazides increase calcium reabsorption only in the proximal tubule because of volume depletion. They reported the same hypocalciuric response to thiazide in the TRPV5 knockout mice, which do not reabsorb calcium in the DCT. We have shown previously that, when the proximal tubule effect is blocked by salt supplementation, thiazides still reduce urine calcium excretion in wild-type mice (20). Because furosemide causes hypercalcemia in the presence of volume depletion, it provides an opportunity for testing whether the thiazide effect is limited to the proximal tubule. The coadministration of chlorothiazide and furosemide did not result in a higher hematocrit or more weight loss than furosemide-treated mice (Table 2). These data indicate that the degree of volume depletion is similar between the two groups. Under these conditions, chlorothiazide still reduces furosemide-induced calcium wasting, suggesting that thiazides may increase calcium reabsorption in the DCT. The enhancement of DCT calcium reabsorption by inhibition of sodium chloride cotransporter NCC is further supported by a recent study reported by Cheng et al. (5) in patients with Gitelman syndrome due to loss-of-function mutations in the NCC. They demonstrated that hypocalciuria in these patients is minimally corrected with volume repletion by saline infusion. These findings indicate that hypocalciuria in Gitelman syndrome, a condition similar to NCC inhibition by thiazides, is in part due to increased calcium reabsorption in the DCT. We should point out that our data only provide indirect evidence of thiazide effects on DCT calcium reabsorption. Further studies are needed to prove these effects with direct measurement of DCT calcium reabsorption, particularly in conditions with a high distal calcium load as described in our studies.

In conclusion, our studies demonstrate that furosemide treatment induces an upregulation of calcium transport molecules in the DCT. These changes may be stimulated by a load-dependent effect and represent a compensatory adaptation in the DCT. Our finding that addition of chlorothiazide reduces furosemide-induced hypercalcemia without affecting volume status suggests that thiazides may increase calcium reabsorption in the DCT. Further studies are needed to confirm the direct effects of thiazides on calcium transport in the DCT.

ACKNOWLEDGMENTS

Part of this work has been presented at the 2005 World Congress of Nephrology (Singapore).
REFERENCES