Diuretics and Bone Loss in Rats With Aldosteronism

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OBJECTIVES	We hypothesized that the increased urinary Ca^{2+} and Mg^{2+} excretion and bone loss that accompanies aldosteronism is aggravated with furosemide and is attenuated by spironolactone.
BACKGROUND	Furosemide, a loop diuretic, is commonly used in patients with congestive heart failure (CHF), in which chronic, inappropriate (dietary Na^+) elevations in plasma aldosterone (ALDO) and a catabolic state that includes bone wasting are expected.
METHODS	In age- and gender-matched, untreated controls, four weeks of aldosterone/salt treatment (ALDO/salt, 0.75 μg/h + 1% NaCl/0.4% KCl in drinking water), four weeks of ALDO/salt + furosemide (40 mg/kg in prepared food), and four weeks of ALDO/salt + furosemide + spironolactone (200 mg/kg/day in divided doses by twice-daily gavage), we monitored: 24-h urinary Ca^{2+} and Mg^{2+} excretion; plasma-ionized $[Ca^{2+}]o$ and $[Mg^{2+}]o$, K^+, and parathyroid hormone (PTH); and bone mineral density (BMD) in the femur.
RESULTS	The ALDO/salt increased ($p < 0.05$) urinary Ca^{2+} and Mg^{2+} excretion ($4,969 \pm 1,078$ and $3,856 \pm 440$ μg/24 h, respectively) compared with controls (896 ± 138 and 970 ± 137 μg/24 h, respectively); furosemide co-treatment further increased ($p < 0.05$) urinary Ca^{2+} and Mg^{2+} excretion ($6,976 \pm 648$ and $6,199 \pm 759$ μg/24 h, respectively), whereas spironolactone co-treatment attenuated ($p < 0.05$) these incremental losses ($4,003 \pm 515$ and $3,915 \pm 972$ μg/24 h). Plasma $[Ca^{2+}]o$ was reduced ($p < 0.05$) at week 4 ALDO/salt + furosemide and was accompanied by hypokalemia (<3.4 mmol/l) that were rescued by spironolactone. Plasma PTH was increased ($p < 0.05$) compared with controls (30 ± 4 vs. 11 ± 3 pg/ml, respectively), whereas BMD was decreased ($p < 0.05$) with ALDO/salt and ALDO/salt + furosemide, but not with spironolactone co-treatment.
CONCLUSIONS	In aldosteronism, hypercalciuria and hypermagnesuria and accompanying decrease in plasma-ionized $[Ca^{2+}]o$ and $[Mg^{2+}]o$ lead to hyperparathyroidism that accounts for bone wasting. Furosemide exaggerates these losses, whereas its combination with spironolactone attenuates these responses to prevent bone loss. (J Am Coll Cardiol 2005;46:142–6) © 2005 by the American College of Cardiology Foundation

The origins of congestive heart failure (CHF), a clinical syndrome with characteristic signs and symptoms, are rooted in neurohormonal activation that includes the renin-angiotensin-aldosterone system, sympathetic nervous system, arginine vasopressin, and a family of natriuretic peptides. Congestive heart failure is accompanied by a systemic illness that includes bone wasting. Bone mineral density (BMD) is reduced in patients with CHF of moderate to marked severity, in which a potent loop diuretic, furosemide, is commonly used for prolonged periods of time (1–5). Pathogenic mechanisms involved in the appearance of bone wasting in CHF are under investigation.

This laboratory has focused on one aspect of the complex neurohormonal profile seen in CHF: aldosteronism, defined as inappropriate (relative to dietary Na^+ intake), chronic elevation in plasma aldosterone (ALDO). A rat model of aldosteronism is used in which uni-nephrectomized rats receive a 1% NaCl diet together with an infusion of ALDO (by mini-pump) to increase its plasma levels to those found in human CHF (6). In this ALDO/salt treatment model,

we have observed an early and persistent increase in urinary and fecal Ca^{2+} and Mg^{2+} excretion, which ultimately leads to a reduction in BMD and bone strength (7,8). The long-term use of furosemide in the setting of aldosteronism could prove detrimental, but has not been examined. Herein, we hypothesize furosemide would augment the hypercalciuria and hypermagnesuria seen with aldosteronism and lead to a loss of bone minerals, which could be rescued by co-treatment with spironolactone, an ALDO receptor antagonist that reduces both urinary and fecal losses of these divalent cations (8). Accordingly, at four weeks of ALDO/salt we monitored: 24-h urinary Ca^{2+} and Mg^{2+} excretion; plasma-ionized $[Ca^{2+}]o$ and $[Mg^{2+}]o$ concentrations, K^+ and parathyroid hormone (PTH); and BMD of femur. Separate animals received ALDO/salt together with furosemide in combination with spironolactone. Age- and gender-matched, unoperated, untreated rats served as controls.

METHODS

Animal model. Male Sprague-Dawley rats (Harlan, Indianapolis, Indiana) were used in this study approved by the institution's Animal Care and Use Committee. There were four groups with five rats in each group unless otherwise specified. Age- and gender-matched, unoperated, untreated

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Abbreviations and Acronyms
ALDO = aldosterone
BMD = bone mineral density
CHF = congestive heart failure
DOC = deoxycorticosterone
PTH = parathyroid hormone
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rats served as controls. As previously reported (7), ALDO/salt consists of uni-nephrectomized rats receiving ALDO (0.75 μg/h) by implanted mini-pump (Alzet, Cupertino, California) together with 1% NaCl/0.4% KCl in drinking water and standard laboratory chow (Harlan Teklad 2215 Rodent Diet, Madison, Wisconsin) containing 1.13% Ca^{2+}. A separate group of animals received ALDO/salt plus daily furosemide. Furosemide (40 mg/kg) was mixed into powdered standard chow, and water was added. The mixture was placed in a pan and cut into small squares and air dried. During the 24-h collection of urine in a metabolic cage (see the following text), animals continued to receive furosemide provided by twice-daily gavage. Another group of rats received furosemide plus spironolactone (200 mg/kg/day) in divided doses by twice-daily gavage. Finally, animals were anesthetized and killed, and blood and femur were harvested at four weeks of treatment.

Urinary Ca^{2+} and Mg^{2+} excretion. On the day of the metabolic study, food was withheld, but water with 1% NaCl was provided. Animals were gently bathed using distilled water to remove any feces or food that could contaminate collected urine. Animals were then placed in a cleaned, minerally decontaminated and distilled-deionized, water-rinsed metabolic cage. Urine was collected over 24 h and kept frozen for Ca^{2+} and Mg^{2+} assay. After each use, cages were manually cleaned with deionized water, all nonmetallic parts were washed with diluted hydrochloric acid (3N), and cages were rinsed three times with deionized water and finally rinsed twice with distilled-deionized water for future use as previously reported (7,8).

Urinary Ca^{2+} and Mg^{2+} concentrations were determined as reported elsewhere (7,8) using an atomic absorption spectrophotometer. Urinary Ca^{2+} and Mg^{2+} excretion rates were calculated from the product of their concentration (μg/ml) by the 24-h urine volume (ml/24 h) and expressed as μg/24 h.

Plasma-ionized $[Ca^{2+}]o$ and $[Mg^{2+}]o$ and plasma K^+. The concentrations of plasma-ionized $[Ca^{2+}]o$ and $[Mg^{2+}]o$ and plasma K^+ were determined by the direct ion-selective electrode technique using a Nova 8 Analyzer (Nova Biomedical, Waltham, Massachusetts) and expressed in μmol/l.

Parathyroid hormone. Plasma PTH was measured by the intact PTH immunoassay (IRMA) using a commercial kit (Nichols Institute Diagnostics, San Clemente, California). The IRMA is a two-site immunoradiometric assay for the measurement of the biologically intact 84-amino-acid chain of the PTH molecule. Blood (two ml) was collected from

the rat heart into a chilled ethylenediaminetetraacetic acid tube and immediately centrifuged (1,600 g) for 15 min. Plasma was then separated and kept at -80 °C. For IRMA, each plasma sample (200 μl) was added to the tube containing 100 μl of the ^{125}I-PTH antibody solution and PTH antibody-coated beads and incubated for 24 h. Beads were then washed twice with washing solution, and each test tube was counted with gamma counter for 1 min. A standard curve was generated using prepared intact PTH standards, and plasma PTH values were expressed as pg/ml.

BMD. The BMD was determined for excised, manually cleaned femurs by peripheral dual-energy X-ray absorptiometry using GE Lunar PIXImus2 (GE Healthcare, Fairfield, Connecticut). Quality control and calibration were carried out within 24 h of each scanning period. This method has been validated for rat long bones (9). We have previously reported on the equivalency of this noninvasive assessment of tibia and femur BMD with their total concentrations of Ca^{2+} and Mg^{2+} determined by atomic absorption spectrophotometry (7).

Statistical analysis. Values are presented as mean $\pm$ standard error of the mean (SEM). Analysis was on square root-transformed data to minimize variability. Significant differences between individual means were determined using the Bonferroni multiple comparisons test. Significance was assigned as $p < 0.05$.

RESULTS

Urinary Ca^{2+} and Mg^{2+} excretion. The Ca^{2+} and Mg^{2+} excretion in unoperated, untreated controls were 896 ± 138 and 970 ± 137 μg/24 h, respectively (Table 1). Four weeks of ALDO/salt was accompanied by hypercalciuria and hypermagnesuria. In co-treating rats receiving ALDO/salt with furosemide, the 24-h urinary Ca^{2+} and Mg^{2+} excretion increased further. The addition of spironolactone to the ALDO/salt + furosemide regimen significantly reduced this level of divalent cation loss.

Plasma-ionized $[Ca^{2+}]o$ and $[Mg^{2+}]o$. Plasma $[Ca^{2+}]o$ in unoperated, untreated controls was 0.89 ± 0.02 mmol/l. At four weeks of ALDO/salt and with furosemide co-treatment, $[Ca^{2+}]o$ was reduced (0.83 ± 0.03 and 0.77 ± 0.09 mmol/l, respectively, $p < 0.05$). A decrease in plasma-ionized $[Ca^{2+}]o$ was not seen with the ALDO/salt + furosemide + spironolactone regimen (0.87 ± 0.05 mmol/l).

Plasma-ionized $[Mg^{2+}]o$ in untreated controls was 0.34 ± 0.01 mmol/l. At four weeks of ALDO/salt and with ALDO/salt + furosemide, plasma $[Mg^{2+}]o$ was reduced but did not reach statistical significance (0.31 ± 0.02 and 0.30 ± 0.01 mmol/l, respectively). Plasma $[Mg^{2+}]o$ was 0.32 ± 0.02 mmol/l in the group receiving ALDO/salt + furosemide + spironolactone.

Plasma K^+. Plasma K^+ levels in unoperated untreated controls was 4.3 ± 0.2 mmol/l and ranged between 3.4 and 5.1 mmol/l. At four weeks of ALDO/salt plus 1% NaCl and

Table 1. Urinary Ca^{2+} and Mg^{2+} Excretion at Week 4 Treatment

	Ca^{2+} (μg/24 h)	Mg^{2+} (μg/24 h)
C	895 ± 138 (5)	970 ± 137 (5)
A	4,969 ± 1,078 (4)*	3,856 ± 440 (5)*
AF	6,976 ± 648 (4)*†	6,199 ± 759 (5)*†
AFS	4,003 ± 515 (4)*‡	3,915 ± 972 (5)‡

Mean ± SEM (n). *p < 0.05 vs. C; †p < 0.05 vs. A; ‡p < 0.05 vs. AF.
A = 4 wks ALDO/salt; AF = 4 wks ALDO/salt + furosemide; AFS = 4 wks ALDO/salt + furosemide+spironolactone; C = untreated controls. Other abbreviations as in text.

0.4% KCl in drinking water, plasma K^+ was 4.0 ± 0.03 mmol/l. In rats receiving ALDO/salt + furosemide, plasma K^+ decreased (p < 0.05) to 3.0 ± 0.1 mmol/l, whereas those on the regimen of ALDO/salt + furosemide + spironolactone, plasma K^+ was 4.7 ± 0.02 mmol/l.

A value below 3.4 mmol/l was defined as hypokalemia. All five rats receiving ALDO/salt + furosemide developed hypokalemia, with two of five rats having a plasma K^+ level below 3.0 mmol/l. None of the rats receiving ALDO/salt + furosemide + spironolactone developed hypokalemia, and in none of these rats did plasma K^+ exceed 5.1 mmol/l.

Parathyroid hormone. Plasma levels of PTH in unoperated and untreated controls was 11 ± 3 pg/ml. At four weeks of ALDO/salt, circulating levels of PTH had increased (p < 0.05) to 30 ± 4 pg/ml.

BMD. The BMD for the femurs of 12-week-old, male-matched, untreated controls was 0.169 ± 0.002 g/cm^2. In keeping with the marked and sustained hypercalciuria and hypermagnesuria seen with ALDO/salt, BMD was significantly (p < 0.05) reduced at four weeks of ALDO/salt alone and with ALDO/salt + furosemide (0.153 ± 0.006 and 0.151 ± 0.006 g/cm^2, respectively). Co-treatment with spironolactone, which attenuated the excretion of Ca^{2+} and Mg^{2+} seen with ALDO/salt plus loop diuretic, prevented this decrease in BMD (0.165 ± 0.003 g/cm^2).

DISCUSSION

Our study shows the importance of well-known properties of furosemide and spironolactone as they apply to aldosteronism. It further addresses the impact of urinary Ca^{2+} and Mg^{2+} excretion on bone health in this setting. There were several major findings. First, the marked hypercalciuria and hypermagnesuria that accompanies chronic ALDO/salt is exaggerated by furosemide [7,8]. Short-term studies in rats have previously identified the hypercalciuria that accompanies mineralocorticoid excess and that was induced by either ALDO or deoxycorticosterone (DOC) treatment, together with inappropriate dietary NaCl [10–13]. In humans, short-term treatment with a mineralocorticoid (inappropriate for dietary Na^+) likewise leads to hypercalciuria [14,15]. The elevated baseline urinary Ca^{2+} excretion seen in patients with primary aldosteronism is augmented further by dietary Na^+ loading [15] and can be normalized by surgical removal of diseased adrenal tissue or spironolactone treatment [16]. Urinary Mg^{2+} excretion is also increased in

patients with primary aldosteronism and is abrogated by either spironolactone or adrenal surgery [17].

The hypercalciuria associated with aldosteronism is thought to be related to an expansion of extravascular fluid volume with resultant reductions in proximal tubular Na^+ and Ca^{2+} resorption. This leads to their increased delivery to distal segments of the nephron, where the mineralocorticoid promotes Na^+ resorption without affecting Ca^{2+} that results in hypercalciuria. A subsequent decrease in plasma $[Ca^{2+}]$o stimulates the secretion of PTH [18]. The mineralocorticoid hormone itself and the accompanying elevation in arterial pressure have each been eliminated as causative of heightened Ca^{2+} excretion [10–12,15], as have accompanying polydipsia and metabolic alkalosis secondary to renal acidification [13,19].

As expected, plasma-ionized $[Ca^{2+}]$o decreased with ALDO/salt and was markedly reduced with ALDO/salt + furosemide; this was rescued by spironolactone co-treatment. A decrease in $[Mg^{2+}]$o was also seen with ALDO/salt and ALDO/salt + furosemide. Parathyroid hormone is released in response to these iterations in plasma $[Ca^{2+}]$o and $[Mg^{2+}]$o [8,18]. Elevations in plasma PTH and PTH-driven renal formation of $1,25(OH)_2D_3$ respectively promote Ca^{2+} resorption from bone and Ca^{2+} absorption from the gastrointestinal tract. Serum levels of PTH and duodenal absorption of Ca^{2+} are each increased in rats treated with DOC/salt [13,20,21]. Secondary hyperparathyroidism has been reported in patients with primary aldosteronism [22], in which reduced $[Ca^{2+}]$o and increased PTH concentrations have been normalized by spironolactone or adrenal surgery [16,22].

Furosemide has well-known nephrogenic effects that promote the urinary excretion of Ca^{2+} and Mg^{2+}. Herein, we found furosemide to exaggerate urinary Ca^{2+} excretion that accompanies ALDO/salt. Of additional importance and despite a diet containing 0.4% supplemental KCl, which prevents hypokalemia in ALDO/salt, hypokalemia developed in many of the rats with aldosteronism that were receiving furosemide. Spironolactone co-treatment prevented hypokalemia. Hypokalemia and hypomagnesemia, together with reduced concentrations of these cations in skeletal muscle, accompany chronic diuretic therapy in patients with cardiovascular disease and can be rescued by spironolactone co-treatment [23–26]. Additionally, it is now recognized that furosemide inhibits renal tubular 11-beta-hydroxysteroid dehydrogenase-2, a guardian enzyme that preserves the specificity of the promiscuous ALDO receptor for mineralocorticoids. In so doing, furosemide may permit more plentiful glucocorticoids to act as a mineralocorticoid and further enhance urinary K^+ excretion [27,28]. Potassium-sparing diuretics, such as spironolactone, are associated with a reduced risk of death and hospitalization for progressive heart failure and all-cause mortality in patients with heart failure [29].

The loss of BMD during furosemide treatment in rats with ALDO/salt is our second major finding. This occurs as a

compensatory response to elevations in circulating PTH evoked to preserve extracellular Ca^{2+} homeostasis. The long-term usage of furosemide may have deleterious effects on Ca^{2+} and Mg^{2+} balance and lead to secondary hyperparathyroidism despite increased gastrointestinal Ca^{2+} absorption (30–33). In our study, in which hypercalciuria and hypermagnesuria appear in response to ALDO/salt, additional losses of Ca^{2+} and Mg^{2+} induced by furosemide lead to a rapid decline in BMD. Others have inferred that bone loss occurs with rat models of chronic mineralocorticoid excess. For example, urinary hydroxyproline, an indirect measure of bone resorption, is increased in this setting (21,34,35).

Reductions in BMD and elevations in plasma PTH are found in patients with advanced heart failure awaiting cardiac transplantation as well as in patients with moderately severe CHF (1–5,36–39). The chronic use of furosemide in these symptomatic patients, in whom secondary aldosteronism is expected, may be contributory to the observed osteopenia and secondary hyperparathyroidism. The importance of preserving bone homeostasis and the prevention of bone loss with its increased potential for fracture is clear, particularly among elderly patients. Thiazide diuretics are known to reduce bone loss and the risk of bone fracture in elderly men and women and in postmenopausal women in general (40–44). The selection of a diuretic in any given patient should take into account their state of Ca^{2+} balance. For example, in elderly patients, a thiazide diuretic or ALDO receptor antagonist seems preferable to a loop diuretic. A thiazide will rescue urinary Ca^{2+} but not urinary Mg^{2+} losses that accompany aldosteronism, whereas the combination of hydrochlorothiazide and spironolactone normalizes urinary Ca^{2+} and Mg^{2+} excretion in this setting (45). Unless the reduction in plasma-ionized $[Ca^{2+}]o$ and $[Mg^{2+}]o$ that accompany hypercalciuria and hypermagnesuria are each rescued, an elevation in plasma PTH with bone resorption can be expected (46–48). Among nursing home residents, the daily dose of furosemide is an important predictor of hyperparathyroidism (49).

Third, we found that spironolactone co-treatment serves to prevent the reduction in BMD seen at four weeks of ALDO/salt + furosemide. Spironolactone is protective against bone loss by reducing both urinary and fecal Ca^{2+} and Mg^{2+} excretion (8). Albeit a salutary response in this rat model, it remains to be seen whether long-term spironolactone use in humans would prevent osteopenia and even osteoporosis in heart failure patients receiving furosemide. Additional measures, such as dietary Ca^{2+} supplements, could theoretically prevent bone loss by attenuating PTH secretion. In rats with chronic mineralocorticoid/salt excess, however, a Ca^{2+}-supplemented diet alone did not lower plasma PTH (50). A diet with combined Ca^{2+} and Mg^{2+} supplements would likely provide a more complete suppression of PTH release as noted earlier, particularly when furosemide is used in the setting of aldosteronism.

Although it was introduced into clinical practice more than 30 years ago, the safety of furosemide in the long-term management of CHF has never been systematically evaluated (51). This notwithstanding, the combination of a small dose of spironolactone, together with furosemide and an angiotensin-converting enzyme inhibitor, has proven effective in the overall management of CHF, including the reduction in risk for sudden cardiac death (52). The efficacy of spironolactone as co-treatment with furosemide in CHF may include its ability to rescue the renal and gastrointestinal losses of Ca^{2+} and Mg^{2+} that occur with aldosteronism and hence better preserve the extracellular and intracellular homeostasis of these divalent cations (8). In so doing, spironolactone could prevent the hyperparathyroidism and PTH-mediated Ca^{2+} loading of myocardium and the accompanying increased propensity for arrhythmias.

Study limitations. Our study has several limitations. First, we did not monitor daily food consumption in each animal and therefore cannot account for the exact dose of administered furosemide. In preparing food supplemented with furosemide, we selected a dose previously reported as effective in rodents (31,53). Second, we monitored plasma PTH only in the ALDO/salt treatment group, in which it was elevated, as is the case in rats receiving DOC/salt (21,34) and in patients with primary aldosteronism (16,22). We therefore cannot address the appearance of hyperparathyroidism in furosemide-treated animals with aldosteronism. Serum PTH and PTH activity (i.e., urinary cyclic adenosine monophosphate) are each increased in normal rats, human infants, and adult volunteers receiving furosemide (30–32,54,55). A calcimimetic agent, which acts as an agonist at the calcium-sensing receptor in parathyroid glands, serves to prevent hyperparathyroidism in furosemide-treated rats (55). Finally, we cannot address the potential of spironolactone to prevent the occurrence of hyperparathyroidism in rats with aldosteronism receiving furosemide. However, spironolactone prevents a decrease in plasma-ionized $[Ca^{2+}]o$ and $[Mg^{2+}]o$ (8). It therefore would be expected to be effective in this regard.

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