Bone Loss in Rats with Aldosteronism

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ABSTRACT: *Objective:* We hypothesized that aldosteronism is accompanied by hypercalciuria and hypermagnesuria that lead to bone loss, which could be rescued by hydrochlorothiazide and spironolactone. *Methods:* We monitored 24-hour urinary Ca^{2+} and Mg^{2+} excretion; plasma ionized $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ and plasma K^+; and bone mineral density of the femur. The following groups (n = 5 in each group) were studied: age- and gender-matched, untreated controls; controls + 4 weeks hydrochlorothiazide; 4 weeks aldosterone/salt treatment (ALDOST, 0.75 μg/h and dietary 1% NaCl/0.4% KCl); 4 weeks ALDOST + hydrochlorothiazide (50 mg/kg in prepared food); and 4 weeks ALDOST + hydrochlorothiazide + spironolactone (200 mg/kg day in divided doses by twice-daily gavage). *Results:* ALDOST increased ($P < 0.05$) urinary Ca^{2+} and Mg^{2+} excretion four- and twofold, respectively; hydrochlorothiazide cotreatment attenuated ($P < 0.05$) Ca^{2+} excretion in controls and during ALDOST without affecting augmented Mg^{2+} excretion whereas hydrochlorothiazide + spironolactone normalized Ca^{2+} and reduced Mg^{2+} excretion ($P < 0.05$). Compared with controls, plasma $[Ca^{2+}]_o$ at 4 weeks of ALDOST was reduced (0.89 ± 0.02 versus 0.83 ± 0.03 mmol/L; $P < 0.05$) but remained no different from levels in controls with hydrochlorothiazide and hydrochlorothiazide + spironolactone (0.88 ± 0.04 and 0.97 ± 0.03 mmol/L, respectively). Plasma $[Mg^{2+}]_o$ fell ($P < 0.05$) with ALDOST + hydrochlorothiazide (0.23 ± 0.01 versus 0.34 ± 0.01 mmol/L) and was prevented with spironolactone co-treatment (0.33 ± 0.01 mmol/dL). Hypokalemia (2.9 ± 0.2 mmol/L) occurred in rats with ALDOST + hydrochlorothiazide but not with spironolactone co-treatment. At 4 weeks of ALDOST, plasma parathyroid hormone was increased (30 ± 4 versus 11 ± 3 pg/mL; $P < 0.05$) and bone mineral density was reduced (0.153 ± 0.006 versus 0.170 ± 0.002 g/cm^2; $P < 0.05$). Co-treatments with either hydrochlorothiazide or hydrochlorothiazide + spironolactone each prevented bone loss. *Conclusions:* Hypercalciuria and hypermagnesuria accompany aldosteronism and account for a decline in their plasma ionized concentrations and secondary hyperparathyroidism with bone resorption. Attenuation of bone loss in aldosteronism can be achieved with hydrochlorothiazide; however, mono- and divalent cation homeostasis, together with bone integrity, are each preserved with the combination hydrochlorothiazide + spironolactone. **KEY INDEXING TERMS:** Aldosterone; Calcium; Magnesium; Parathyroid hormone; Bone loss. **[Am J Med Sci 2005;330(1):1–7.]**

Congestive heart failure (CHF), a clinical syndrome whose origins are rooted in neurohormonal activation that includes effector hormones of the circulating renin-angiotensin-aldosterone system, is accompanied by a systemic illness. Pathophysiologic features include oxi/nitrosative stress in blood and such diverse tissues as skin, skeletal muscle, immune cells, and heart[1-5] and a proinflammatory phenotype with elevated plasma levels of chemokines and cytokines and a catabolic state with loss of soft tissues and bone that eventuate in a wasting syndrome termed *cardiac cachexia*.[6-12] Pathogenic mechanisms involved in the appearance of this illness are under investigation.

An animal model of aldosteronism has been used to represent one aspect of the CHF neurohormonal profile. Uninephrectomized rats receive 1% NaCl in drinking water and aldosterone (ALDO) by minipump to inappropriately (relative to dietary Na^+ intake) raise its plasma levels to those seen in cases of human CHF. Referred to as *aldosterone/salt treatment (ALDOST)*, this regimen is accompanied by a proinflammatory phenotype induced by the marked and persistent excretion of Ca^{2+} and Mg^{2+} in both urine and feces and which adversely influences extra- and intracellular concentrations of these divalent cations.[13-18] Pathophysiologic responses accompany the negative balance of Ca^{2+} and Mg^{2+}. They include parathyroid

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hormone–mediated loss of bone mineral density (and bone strength) and intracellular Ca^{2+} loading with induction of oxi/nitrosative stress in diverse cells and an immunostimulatory state with activation of peripheral blood mononuclear cells that invade the coronary and systemic circulations to create a proinflammatory vascular phenotype.[13-22]

Herein we focused on the bone loss seen with aldosteronism,[13] which far exceeds that seen solely with the hypercalciuria accompanying a high-NaCl diet alone.[23] We hypothesized that pharmacologic intervention could attenuate the urinary loss of these divalent cations seen with ALDOST and thereby would prevent bone loss. To uncouple hypercalciuria and hypermagnesuria during ALDOST, we selected hydrochlorothiazide, to promote urinary Ca^{2+} resorption and compared this intervention to the combination of hydrochlorothiazide and spironolactone, an ALDO receptor antagonist, to reduce both Ca^{2+} and Mg^{2+} excretion. Age- and gender-matched, unoperated, untreated rats served as controls. Accordingly, we monitored 24-hour urinary Ca^{2+} and Mg^{2+} excretion, plasma ionized $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$, plasma K^+, and femur mineral density.

Methods

Animal Model

Male, 8- to 12-week-old Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were used in this study, approved by the institution's Animal Care and Use Committee. There were five groups with five rats in each group, unless otherwise specified. Age- and gender-matched, unoperated, untreated rats served as controls. As previously reported, ALDOST consists of uninephrectomized rats who receive ALDO (0.75 μg/h) by implanted minipump (Alzet, Cupertino, CA) together with a 1% NaCl/0.4% KCl drinking water and standard laboratory chow (Harlan Tekland 2215 Rodent Diet) containing 1.13% Ca^{2+}.[24] Separate groups of animals received ALDOST plus hydrochlorothiazide or hydrochlorothiazide alone (treated controls). Hydrochlorothiazide was mixed into powdered standard chow (50 mg/kg body weight) and water was added. The mixture was placed in a pan and cut into small squares and air dried. We did not monitor daily food intake. Yet another group received ALDOST and hydrochlorothiazide plus spironolactone (200 mg/kg/day) in divided doses by twice-daily gavage. At 4 weeks of treatment, the animals were anesthetized and killed, and blood and femur samples were harvested.

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 "bathed" in 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 hours 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), rinsed three times with deionized water, and finally rinsed twice with distilled-deionized water for future use as previously reported.[13,14]

Urinary Ca^{2+} and Mg^{2+} concentrations were determined as reported elsewhere[13,14] 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-hour urine volume (mL/24 hours) and expressed as μg/24 hours.

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 K^+ were determined by the direct ion-selective electrode technique using a Nova 8 Analyzer (Nova Biomedical, Waltham, MA) and expressed in mmol/L.

Parathyroid Hormone

Plasma parathyroid hormone (PTH) was measured by the intact PTH immunoassay (IRMA) using a commercial kit (Nichols Institute Diagnostics, San Clemente, CA). IRMA is a two-site immunoradiometric assay for the measurement of the biologically intact 84 amino acid chain of PTH molecule. Blood (2 mL) was collected from the rat heart into a chilled EDTA tube and immediately centrifuged ($1600\times g$) for 15 minutes. 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 hours. Beads were then washed twice with washing solution and each test tube was counted with a gamma counter for 1 minute. A standard curve was generated using prepared intact PTH standards and plasma PTH values and expressed as pg/mL plasma.

Bone Mineral Density

Bone mineral density was determined for excised, manually cleaned femurs by peripheral dual-energy x-ray absorptiometry using GE Lunar PIXImus2 (GE Healthcare, Fairfield, CT). Quality control and calibration were carried out within 24 hours of each scanning period. This method has been validated for rat long bones.[25] We have previously reported on the equivalence of this noninvasive assessment of tibia and femur bone mineral density with their total concentrations of Mg^{2+} and Ca^{2+} determined by atomic absorption spectrophotometry.[13]

Statistical Analysis

Values are presented as mean ± SEM. Data were analyzed using analysis of variance. Significant differences between individual means were determined using the post hoc Bonferroni multiple comparisons test. Significance was assigned to $P < 0.05$.

Results

Urinary Ca^{2+} and Mg^{2+} Excretion

We ascertained that the 24-hour urinary Ca^{2+} excretion rate in unoperated controls was 895.7 ± 138.2 μg/24 hours. Treatment of control rats with hydrochlorothiazide led to an expected reduction in urinary Ca^{2+} excretion (Table 1). Four weeks of ALDOST raised urinary Ca^{2+} excretion more than fourfold. Co-treatment with hydrochlorothiazide reduced the 24-hour urinary Ca^{2+} excretion seen with ALDOST, whereas the addition of spironolactone to the hydrochlorothiazide regimen further reduced ($P < 0.05$) urinary Ca^{2+} excretion to levels seen in untreated controls.

Urinary Mg^{2+} excretion in unoperated, untreated controls was 969.6 ± 137.2 μg/24 hours. This was significantly increased by hydrochlorothiazide treatment (see Table 1). At 4 weeks of ALDOST, 24-hour urinary Mg^{2+} excretion was markedly increased ($P < 0.05$) above control values, as was the case with ALDOST + hydrochlorothiazide. The addition of spironolactone to the ALDOST + hydrochlorothiazide regimen significantly reduced urinary Mg^{2+} excretion compared with ALDOST alone, whereas it did not normalize urinary Mg^{2+} excretion to levels seen in controls.

Body Weight

Body weights for 12-week-old male controls treated with hydrochlorothiazide for 4 weeks were not significantly different from one another (310 ± 6 versus 286 ± 6 mg, respectively). At 4 weeks of ALDOST, however, body weight was significantly less than either of these controls (224 ± 11), in keeping with our previous findings, in which ALDOST is accompanied by reduced weight gain.[17] Co-treatment with either hydrochlorothiazide or hydrochlorothiazide + spironolactone prevented this failure to gain weight (288 ± 9 and 276 ± 4 mg, respectively), which we also previously reported for spironolactone co-treatment.[17]

Plasma Ionized $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$

At week 4 of ALDOST, plasma $[Ca^{2+}]_o$ was reduced ($P < 0.05$) compared with control values (0.83 ± 0.03 versus 0.89 ± 0.02 mmol/L, respectively). However, ALDOST + hydrochlorothiazide prevented this fall in ionized Ca^{2+} (0.88 ± 0.04 mmol/L) and co-treatment with hydrochlorothiazide + spironolactone was accompanied by a rise in $[Ca^{2+}]_o$ to 0.97 ± 0.03 mmol/L that was greater ($P < 0.05$) than ALDOST values, but not greater than the values in the five control rats in this study or the 19 historical controls in this laboratory (0.97 ± 0.01; 0.88–1.09 mmol/L).

Plasma ionized $[Mg^{2+}]_o$ was reduced at 4 weeks of ALDOST, but did not reach statistical significance (0.31 ± 0.02 mmol/L) versus control values (0.34 ± 0.01 mmol/L). At 4 weeks of ALDOST + hydrochlorothiazide, plasma $[Mg^{2+}]_o$ was significantly less than with ALDOST alone (0.23 ± 0.01 mmol/L), and this was totally rescued by ALDOST + hydrochlorothiazide + spironolactone (0.33 ± 0.01 mmol/L).

Plasma K^+

In separate studies, plasma K^+ concentrations were determined in 9-, 10- and 12-week-old untreated control rats. These values were found to be 4.4 ± 0.1, 3.8 ± 0.1, and 4.3 ± 0.2 mmol/L, respectively, and ranged between 3.4 and 5.1 mmol/L. A value of less than 3.4 mmol/L was therefore chosen to define hypokalemia. At week 4 of hydrochlorothiazide treatment, none of the control rats developed hypokalemia (3.6 ± 0.1 mmol/L), as was also the case for those receiving ALDOST alone (4.0 ± 0.3 mmol/L). Four of five rats with ALDOST + hydrochlorothiazide had hypokalemia. Plasma K^+ fell below 3.0 mmol/L in 2 animals receiving ALDOST + hydrochlorothiazide. However, none of the rats receiving ALDOST + hydrochlorothiazide + spironolactone had hypokalemia. Plasma K^+ did not exceed 5.1 mmol/L in any animal in any of the treatment groups, including those with spironolactone co-treatment. At week 4 of ALDOST + hydrochlorothiazide

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

	Ca^{2+}g/24 h (n)	Mg^{2+}g/24 h (n)
Control	895.7 ± 138.2 (5)	969.6 ± 137.2 (5)
Control + hydrochlorothiazide	152.4 ± 14.0[a] (5)	2245.8 ± 556.1[a] (5)
ALDOST	4968.5 ± 1077.8[a] (4)	3856.3 ± 439.6[a] (5)
ALDOST + hydrochlorothiazide	2066.5 ± 846.8 (5)	3181.7 ± 1065.7 (5)
ALDOST + hydrochlorothiazide + spironolactone	854.0 ± 152.4[b] (5)	1887.4 ± 707.6[b] (5)

Values mean ± SEM.
[a] *$P < 0.05$ vs. control.*
[b] *$P < 0.05$ vs. ALDOST.*

treatment, plasma K^+ level was 2.9 ± 0.2 and was significantly ($P < 0.001$) less than the 4.3 ± 0.2 mmol/L seen with ALDOST + hydrochlorothiazide + spironolactone.

Parathyroid Hormone

At 4 weeks of ALDOST, plasma PTH levels were increased ($P < 0.05$) compared with control values (30 ± 4 versus 11 ± 3 pg/mL).

Bone Mineral Density

Bone mineral density for the femur of 12-week-old, male, unoperated, untreated control rats was 0.170 ± 0.005 g/cm^2 (Figure 1). At 4 weeks of ALDOST, bone mineral density was significantly ($P < 0.05$) reduced to 0.153 ± 0.014 g/cm^2. This reduction in bone mineral density was rescued by co-treating rats with ALDOST using either hydrochlorothiazide alone or hydrochlorothiazide plus spironolactone. Four weeks' treatment of controls with hydrochlorothiazide did not result in a fall in bone mineral density.

Discussion

This study led to several major findings. First, and confirmatory to recent reports from our laboratory,[13,14] is the hypercalciuria and hypermagnesuria induced by long-term ALDOST. Short-term studies in rodents[26-29] have identified the hypercalciuria seen with mineralocorticoid excess using either ALDO or deoxycorticosterone (DOC), together with dietary NaCl. An elevation in urinary Ca^{2+} excretion is also seen in humans during short-term administration of a mineralocorticoid, which is inappropriate relative to dietary sodium intake.[30,31] In primary aldosteronism, basal hypercalciuria is accentuated by dietary Na^+ loading[31] and is normalized by spironolactone or surgical removal of diseased adrenal tissue.[32] Hypermagnesuria is also seen in patients with primary aldosteronism and resolves after adrenal surgery or in response to spironolactone treatment.[33]

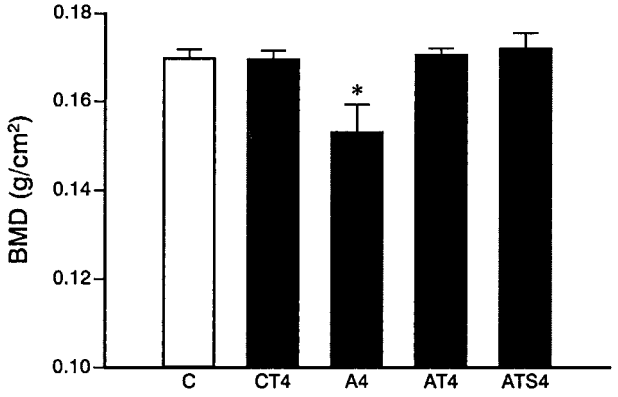

Figure 1. Bone mineral density (BMD) was monitored in rat femur for the various treatment groups and in age- and gender-matched controls (C). CT4, control + 4 weeks hydrochlorothiazide; A4, 4 weeks ALDOST; AT4, 4 weeks ALDOST + hydrochlorothiazide; ATS4, 4 weeks ALDOST + hydrochlorothiazide + spironolactone.

The stimulus for the hypercalciuria and hypermagnesuria that accompany ALDOST is not completely understood. Elevations in arterial pressure, which occur in response to chronic mineralocorticoid excess, and the mineralocorticoid hormone per se, in the absence of inappropriate dietary Na^+ intake, have long been discounted.[26,27] Additionally, polydipsia and metabolic alkalosis, which accompany chronic mineralocorticoid/salt treatment, have likewise been eliminated.[29] Dietary Na^+ loading alone (e.g., an 8% NaCl diet) leads to a suppression of plasma ALDO and is accompanied by hypercalciuria.[34-38] Prevailing opinion suggests the mechanism responsible for the hypercalciuria seen during ALDOST is likely related to an expansion of extravascular fluid volume including intravascular and interstitial compartments. This results in decreased proximal tubular Na^+ and Ca^{2+} resorption and their increased distal delivery. The mineralocorticoid hormone promotes distal Na^+ resorption without an effect on Ca^{2+}, whereby Ca^{2+} excretion is increased.[26-28,31] Furthermore, nitric oxide-mediated enhancement in medullary blood flow may be facilitative.[39-41]

The sustained increment in urinary Ca^{2+} and Mg^{2+} excretion that accompanies 4 weeks of ALDOST led to a fall in plasma ionized $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$, which was statistically significant for $[Ca^{2+}]_o$. Co-treatment with hydrochlorothiazide or hydrochlorothiazide + spironolactone each prevented the fall in $[Ca^{2+}]_o$. However, the fall in $[Mg^{2+}]_o$ was accentuated by ALDOST + hydrochlorothiazide but could be rescued with spironolactone co-treatment. The fall in $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ provide well-known stimuli to the parathyroid glands to release PTH. Plasma levels of PTH were significantly increased at week 4 of ALDOST. Increased plasma PTH levels would be expected to increase Ca^{2+} and Mg^{2+} resorption from bone and Ca^{2+} absorption from kidneys. PTH levels are increased in rats treated with DOC/salt.[29,34,42] Secondary hyperparathyroidism has been observed in patients with primary hyperaldosteronism,[43] in whom reduced serum ionized Ca^{2+} and elevated PTH levels are normalized by spironolactone or adrenal surgery.[32,43] In addition to bone stores, gastrointestinal Ca^{2+} absorption would be augmented by $1,25(OH)_2D_3$ formed in kidneys and driven by PTH. Increased duodenal absorption of Ca^{2+} by this active vitamin D metabolite accompanies the hypercalciuria associated with DOC/salt treatment.[29]

Our second major finding is the loss of bone mineral density that accompanies 4 weeks of ALDOST. The compensatory responses provided by PTH to preserve plasma ionized Ca^{2+} and Mg^{2+} were likely responsible for the fall in femur bone mineral den-

sity at week 4 of ALDOST. Bone loss in the rat model of mineralocorticoid/salt excess has been suggested by others[42,44,45] based on increased urinary hydroxyproline, a marker of bone resorption. The secondary aldosteronism that appears in patients with CHF is accompanied by reductions in bone mineral density of moderate to marked severity, together with elevated levels of intact PTH or its C-terminal PTH-related peptide.[7,8,10-12,46-49] The influence of furosemide, a potent loop diuretic commonly used in these patients that promotes urinary Ca^{2+} and Mg^{2+} excretion, may exacerbate bone loss and secondary hyperparathyroidism.

An expected reduction in urinary Ca^{2+} excretion was found with hydrochlorothiazide alone in control rats. This was also the case when hydrochlorothiazide was given during ALDOST and in combination with spironolactone during ALDOST. This represents our third major finding and one not previously reported. In rescuing urinary Ca^{2+} excretion, each regimen prevented bone loss. Hydrochlorothiazide has demonstrated pharmacologic actions within the distal tubule that include enhanced Ca^{2+} resorption while raising urinary Mg^{2+} excretion.[50-52] It is this property that reverses secondary hyperparathyroidism and the hypercalciuria associated with vitamin D administration.[53,54] We found hydrochlorothiazide to reduce ALDOST-induced hypercalciuria on average by 70%, which, over the course of 4 weeks of ALDOST, prevented the reduction in bone mineral density.

Aldosterone increases thiazide receptor density principally in the renal cortex, and this, in turn, has led to a greater natriuresis than that observed in thiazide-treated controls and in situations in which the magnitude of the natriuresis correlated with receptor density.[55-58] We did not measure urinary Na^{+} excretion in this study. However, relative to its influence on urinary Ca^{2+} and Mg^{2+} excretion, we found hydrochlorothiazide responses in control rats and in ALDOST rats to be comparable. The use of spironolactone in combination with hydrochlorothiazide provided an additive effect in further reducing urinary Ca^{2+} excretion and would be in keeping with its antagonism of ALDO receptors present within more distal segments of the nephron. The combination of hydrochlorothiazide + spironolactone in the management of CHF has not been systematically examined in recent times and may prove as effective a diuretic regimen as furosemide alone without the marked hypercalciuria and hypermagnesuria that accompany this loop diuretic.

The reduction in urinary Ca^{2+} excretion provided by hydrochlorothiazide, however, occurred at the expense of increased urinary Mg^{2+} and K^{+} excretion, which can adversely influence their intracellular levels in diverse tissues.[59-64] Hypokalemia appeared in a substantial number of rats receiving ALDOST + hydrochlorothiazide despite a diet supplemented with 0.4% KCl and in some cases was marked (<3.0 mmol/L). The addition of spironolactone to hydrochlorothiazide prevented the appearance of hypokalemia in rats with ALDOST, as is the case in diuretic-treated individuals.[65,66] An elevation in plasma K^{+} levels above 5.1 mmol/L was not observed in any of the experimental groups, each of whom had normal renal function.

Our findings suggested that the hypercalciuria and hypermagnesuria of aldosteronism can be attenuated by hydrochlorothiazide and hydrochlorothiazide + spironolactone, and this will prevent the loss of bone mineral density. This conclusion would imply that bone strength would also be preserved.[13,14] In preventing osteopenia, these interventions may prevent bone fractures, an important health care problem, particularly among elderly populations, in whom heart failure is so prevalent. In the setting of aldosteronism, such as appears with CHF, the appearance of hydrochlorothiazide-induced hypokalemia can be offset by spironolactone co-administration. Our study suggested that the combination of hydrochlorothiazide + spironolactone will better preserve both mono- and divalent cation homeostasis and bone integrity in aldosteronism. The addition of spironolactone to an angiotensin-converting enzyme inhibitor and loop diuretic in the overall management of CHF has proven effective in reducing mortality and morbidity events, including ventricular arrhythmias and the risk of sudden cardiac death.[67-69] The renal and gastrointestinal losses of Ca^{2+} and Mg^{2+} that accompany aldosteronism are considerable and are measured in milligram quantities in feces and microgram levels in urine. A marked reduction in bone mineral density is found at and beyond week 4 of ALDOST.[13] A high-NaCl diet alone and a loop diuretic will each induce hypercalciuria and hypermagnesuria. In the setting of aldosteronism, these interventions could lead to an exaggerated loss of these divalent cations and to adverse responses, including parathyroid hormone-mediated bone loss and Ca^{2+} loading of diverse tissues (e.g., myocardium) and cells (e.g., platelets and lymphocytes).[70] The judicious use of a thiazide diuretic, in combination with spironolactone, may protect against such adverse outcomes.

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