Hyperparathyroidism and the Calcium Paradox of Aldosteronism

Vikram S. Chhokar, MD; Yao Sun, MD, PhD; Syamal K. Bhattacharya, PhD; Robert A. Ahokas, PhD; Linda K. Myers, MD; Zhiqing Xing, MD, PhD; Richard A. Smith, PhD; Ivan C. Gerling, PhD; Karl T. Weber, MD

Background—Aldosteronism may account for oxi/nitrosative stress, a proinflammatory phenotype, and wasting in congestive heart failure. We hypothesized that aldosterone/1% NaCl treatment (ALDOST) in rats enhances Ca²⁺ and Mg²⁺ excretion and leads to systemic effects, including bone loss.

Methods and Results—At 1, 2, 4, and 6 weeks of ALDOST, we monitored Ca²⁺ and Mg²⁺ excretion, ionized [Ca²⁺]o and [Mg²⁺]o, parathyroid hormone and α₁-antiproteinase activity in plasma, bone mineral density, bone strength, Ca²⁺ and Mg²⁺ content in peripheral blood mononuclear cells (PBMCs) and ventricular tissue, and lymphocyte H₂O₂ production. A separate group received spironolactone (Spiro), an aldosterone receptor antagonist. Age- and gender-matched unoperated and untreated rats served as controls. ALDOST induced a marked (P<0.05) and persistent rise in urinary and fecal Ca²⁺ and Mg²⁺ excretion, a progressive reduction (P<0.05) in [Ca²⁺]o and [Mg²⁺]o, and an elevation in parathyroid hormone (P<0.05) with a fall (P<0.05) in bone mineral density and strength. An early, sustained increase (P<0.05) in PBMC Ca²⁺ and Mg²⁺ was found, together with an increase (P<0.05) in tissue Ca²⁺. Plasma α₁-antiproteinase activity was reduced (P<0.05), whereas lymphocyte H₂O₂ production was increased (P<0.05) at all time points. Spiro cotreatment attenuated (P<0.05) urinary and fecal Ca²⁺ and Mg²⁺ excretion, prevented the fall in [Ca²⁺]o and [Mg²⁺]o, rescued bone mineral density and strength, and prevented Ca²⁺ overloading of PBMCs and cardiomyocytes.

Conclusions—In aldosteronism, Ca²⁺ and Mg²⁺ losses lead to a fall in [Ca²⁺]o and [Mg²⁺]o, with secondary hyperparathyroidism and bone resorption. Ca²⁺ overloading of PBMCs and cardiac tissue leads to oxi/nitrosative stress and a proinflammatory phenotype. (Circulation. 2005;111:871-878.)

Key Words: aldosterone ■ calcium ■ magnesium ■ osteoporosis ■ parathyroid hormone

C hronic ventricular dysfunction is frequently accompanied by congestive heart failure (CHF), a syndrome with signs and symptoms that are rooted in neurohormonal activation. The mineralocorticoid hormone aldosterone (ALDO), acting via its cytosolic receptor in epithelial cells of such classic target tissues as kidneys and colon, contributes to the appearance of CHF. A systemic illness accompanies CHF with features that include oxi/nitrosative stress in such diverse tissues as skin, skeletal muscle, heart, circulating immune cells and blood, as well as a proinflammatory phenotype with wasting of soft tissue and bone. This catabolic state contributes to a progressive downhill clinical course that can eventuate in a wasting syndrome called cardiac cachexia.

See p 830

We have used a rat model to simulate chronic, inappropriate (relative to dietary Na⁺ intake) elevations in plasma ALDO such as those that occur in human CHF. During ALDO/salt treatment (ALDOST), a 2-week preclinical stage gives way to lethargy, anorexia, and a failure to gain weight. Coincident with this clinical presentation is the appearance of a proinflammatory vascular phenotype. As recently reviewed, this vascular remodeling involving ED-1-positive monocytes/macrophages and CD4-positive lymphocytes is found in the normotensive, nonhypertrophied right atrium and ventricle; the left atrium and pulmonary artery; the hypertensive, hypertrophied left ventricle and aorta; and such diverse systemic organs as pancreas, mesentery, and kidneys. A corresponding receptor antagonist, spironolactone (Spiro), administered in either a small nondenpressor dose, which does not prevent hypertension, or a large depressor dose, prevents such remodeling. This contrasts to nonspecific vasoactive agents that are not vasculoprotective despite providing equipotent reductions in arterial pressure. Hence, 3 major lines of evidence link vascular remodeling to ALDOST, not hemodynamic factors. Molecular mechanisms responsible for the appearance of this clinicopathological state during ALDOST are under investigation.
Peripheral blood mononuclear cells (PBMCs; lymphocytes and monocytes) are activated during the preclinical stage of ALDOST. This immunostimulatory state includes upregulated transcription of PBMC genes related to antioxidant defenses, adhesion molecules, and chemokines, together with evidence of autoreactivity. The activation of the PBMC transcriptome appears to be induced by a fall in cytosolic free [Mg\(^{2+}\)] and Ca\(^{2+}\) loading of these cells and is transduced by reactive oxygen species participating in intracellular signal transduction. It could be argued that these responses in PBMC divergent cationic composition are an outcome of Na\(^+-\)dependent ALDO receptor binding and involve Na\(^+-\)Mg\(^{2+}\) and Na\(^+-\)Ca\(^{2+}\) exchangers, a hypothesis supported by ex vivo findings in human lymphocytes exposed to ALDO and the protective role of ALDO receptor antagonism in preventing these cationic responses. However, an indirect mechanism deserves consideration. Studies in rats, dogs, and humans have shown a Na\(^+-\)dependent rise in urinary Ca\(^{2+}\) excretion in response to treatment with an exogenous mineralocorticoid and NaCl. Hypercalcuria and hypermagnesuria are found in patients with primary aldosteronism. Here, we hypothesized that chronic ALDOST in rats enhances urinary and fecal excretion of both Mg\(^{2+}\) and Ca\(^{2+}\) and leads to adverse systemic effects, including secondary hyperparathyroidism (SHPT) with bone loss.

**Methods**

**Animals**

Male 8- to 12-week-old Sprague-Dawley rats (Harlan Sprague Dawley, Inc, Indianapolis, Ind) were used in this study, which was approved by the institution’s Animal Care and Use Committee. Unoperated, untreated age- and gender-matched rats served as controls. As previously reported, ALDOST consists of uninephrectomized rats who received ALDO (0.75 μg/h) by implanted minipump (Alzet), together with 1% NaCl/0.4% KCl in drinking water with 1% NaCl. Food was withheld for the 24-hour period during collection of urine and feces. Urine was kept frozen until determination of Mg\(^{2+}\) and Ca\(^{2+}\) concentrations. Hypercalciuria and hypermagnesuria were found in patients with primary aldosteronism. Here, we hypothesized that chronic ALDOST in rats enhances urinary and fecal excretion of both Mg\(^{2+}\) and Ca\(^{2+}\) and leads to adverse systemic effects, including secondary hyperparathyroidism (SHPT) with bone loss.

**Urinary and Fecal Excretion of Mg\(^{2+}\) and Ca\(^{2+}\)**

Animals were placed in cleaned, mineral-demineralized metabolic cages that were rinsed with distilled, deionized water and allowed to drink water with 1% NaCl. Food was withheld for the 24-hour period during collection of urine and feces. Urine was kept frozen until determination of Mg\(^{2+}\) and Ca\(^{2+}\). After each use, cages were manually cleaned with deionized water, and all nonmetallic parts were washed with diluted (1:100) hydrochloric acid and finally rinsed with distilled, deionized water for future use. Urinary concentrations of these divalent cations were determined as reported elsewhere with an atomic absorption spectrophotometer. Urinary excretion rates were calculated by multiplying the concentration (μg/mL) by the 24-hour urine volume (mL/24 h) and expressed as micrograms per 24 hours. Likewise, Mg\(^{2+}\) and Ca\(^{2+}\) concentrations in feces were determined with some modifications of a protocol reported previously with a Varian FS-220 model atomic absorption spectrophotometer. Briefly, fresh feces collected during the 24-hour period were placed in a cleaned Pyrex glass beaker, heated in an air oven for 48 hours at 102°C, and defatted by extraction with anhydrous ether; weight of the fat-free dry feces was recorded (mg/24 h). Fat-free dry feces (15 to 16 mg) was extracted in 5 mL of 0.75 mol/L Ultrex quality nitric oxide (J.T. Baker Chemical Co) for 24 hours at 68°C, and 1 mL acid extract was used to quantify the Mg\(^{2+}\) and Ca\(^{2+}\) concentrations as described elsewhere. Fecal excretion rates were calculated multiplying the Mg\(^{2+}\) and Ca\(^{2+}\) concentrations (μg/mg) by the fecal mass (mg/24 h) and expressed as milligrams of fat-free dry feces per 24 hours.

**Plasma Ionized Mg\(^{2+}\) and Ca\(^{2+}\) Concentrations**

Concentrations of plasma ionized Mg\(^{2+}\) and Ca\(^{2+}\) were determined via the direct ion-selective electrode techniques with a Nova 8 Analyzer (Nova Biomedical) and expressed in millimoles per liter.

**Bone Mineral Density and Content**

Bone mineral density (BMD) and bone marrow content (BMC) were determined in excised, manually cleaned tibias and femurs by peripheral dual-energy x-ray absorptiometry with GE Lunar PIXI-mus2 (GE Healthcare); quality control and calibration were carried out within 24 hours of each scanning period. This method has been previously validated for rat leg bones.

**Bone Breaking Strength**

An Instron Universal Test System (Instron Corp) was used for mechanical testing. Flexure stress of the femurs was determined by breaking the bones with 3-point bending (gap size, 15 mm). The load was applied at a constant displacement of 15 mm/min to failure. The femurs broke in the mid-diaphyseal region, and data were recorded as flexure stress in megapascals.

**PBMC Total Mg\(^{2+}\) and Ca\(^{2+}\) Concentrations**

Isolated PBMCs were washed 3 times with 140 mmol/L choline chloride. The PBMCs were then lysed with 2 mL deionized water and subjected to 3 cycles of rapid alternate freezing at −70°C and thawing. An aliquot of 1.9 mL of isolated PBMC suspension containing 1 to 5 mg/mL protein was digested with 0.4 mL of 0.75 mol/L Ultrex quality nitric acid (J.T. Baker) for 24 hours at 68°C. The acid-extracted suspension was centrifuged, 1 mL of supernatant was diluted with 3 mL of 0.5% LaCl\(_3\) solution, and the diluted was used to quantify PBMC Mg\(^{2+}\) and Ca\(^{2+}\) levels by atomic absorption spectroscopy as described elsewhere. The protein level in the PBMC suspension was assayed, and total Mg\(^{2+}\) and Ca\(^{2+}\) concentrations were expressed in micrograms per milligram protein.

**Cardiac Tissue Mg\(^{2+}\) and Ca\(^{2+}\) Concentrations**

Microdetermination for Mg\(^{2+}\) and Ca\(^{2+}\) concentrations in ventricular tissue was carried out in 12- to 15-mg demineralized, defatted specimens after complete digestion in 5 mL of 0.75 mol/L Ultrex quality nitric acid (J.T. Baker) for 15 hours at 68°C. This procedure extracts >99% of Mg\(^{2+}\) and Ca\(^{2+}\) from dry, defatted tissue. Tissue Mg\(^{2+}\) and Ca\(^{2+}\) levels are expressed as nanomoles per milligram of fat-free dry tissue.
TABLE 1. Urinary and Fecal Excretion of Mg\(^{2+}\) During ALDOST With or Without Spiro Cotreatment

<table>
<thead>
<tr>
<th></th>
<th>Urinary Mg(^{2+}), (\mu g/24) h</th>
<th>Fecal Mg(^{2+}), mg/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1932.9±970.8 (5)</td>
<td>17.2±2.9 (5)</td>
</tr>
<tr>
<td>ALDOST, wk 1</td>
<td>4358.4±338.3* (5)</td>
<td>37.3±1.6* (5)</td>
</tr>
<tr>
<td>ALDOST, wk 2</td>
<td>5301.4±245.3* (5)</td>
<td>30.2±2.7* (5)</td>
</tr>
<tr>
<td>ALDOST, wk 4</td>
<td>5987.1±679.2* (5)</td>
<td>34.6±5.7* (5)</td>
</tr>
<tr>
<td>ALDOST+Spiro, wk 1</td>
<td>7158.1±779.2* (5)</td>
<td>21.6±1.7* (5)</td>
</tr>
<tr>
<td>ALDOST+Spiro, wk 2</td>
<td>2033.5±831.8* (5)</td>
<td>17.6±1.8* (4)</td>
</tr>
<tr>
<td>ALDOST+Spiro, wk 4</td>
<td>1444.9±808.2† (5)</td>
<td>24.9±7.2† (4)</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n). *P<0.05 vs control; †P<0.05 vs ALDOST.

Plasma \(\alpha_{1}\)-Antiproteinase Activity
As previously reported, plasma \(\alpha_{1}\)-antiproteinase activity (\(\alpha_{1}\)-AP) was assessed with the \(\alpha_{1}\)-AP-410 assay system (Oxis Research) and is expressed in micromoles per liter.

Lymphocyte \(H\_2O\_2\) Production
Lymphocyte \(H\_2O\_2\) production was measured as previously reported. Briefly, 0.1 mL whole blood was incubated with dichlorofluorescein diacetate (25 \(\mu\)mol/L, Molecular Probes) for 45 minutes at 37°C. After red blood cells were lysed with FACS lysing solution (Becton, Dickinson & Co), the leukocytes were suspended in PBS (pH 7.4) for single-cell flow cytometric analysis with an FACS Caliber flow cytometer (Becton, Dickinson & Co). Lymphocytes were discerned by the combination of low-angle forward-scattered and right angle-scattered laser light. Fluorescent emission at 510 to 550 nm was recorded with excitation at 488 nm using a slit width of 10 nm.

Statistical Analysis
Values are presented as mean±SEM. Data were analyzed with ANOVA. Significant differences between individual means were determined by use of Bonferroni multiple comparisons test. Significance was assigned to \(P<0.05\).

Results
Clinical Findings and Urinary Excretion Rate
At weeks 1 and 2 of ALDOST, animals were active and eating. As reported previously, rats appeared healthy and were gaining weight comparable to controls during this preclinical stage, although they exhibited polydipsia and polyuria. At week 4, rats were lethargic and anorectic, and their polydipsia and polyuria continued. With cotreatment with Spiro, animals gained weight comparable to controls, and the appearance of the illness was prevented and polydipsia and polyuria were attenuated.

Average 24-hour urine output for 1-, 2-, and 4-week controls was 19.1±2.5, 17.2±2.3, and 23.4±3.5 mL, respectively. Compared with controls, daily urine output rose to 24.0±4.2, 133.8±13.3 \((P<0.05)\), and 118.2±17.7 \((P<0.05)\) mL during weeks 1, 2, and 4 of ALDOST. Daily urine output at 1, 2, and 4 weeks of Spiro cotreatment was 33.4±6.0, 26.0±6.2, and 14.7±3.0 mL, respectively \((P<0.05\) versus ALDOST).

Urinary and Fecal Excretion of Mg\(^{2+}\) and Ca\(^{2+}\)
Urinary excretion and fecal excretion of Mg\(^{2+}\) are increased during ALDOST compared with unoperated, untreated controls (Tables 1 and 2). These marked elevations in Mg\(^{2+}\) excretion were evident at week 1 and sustained at 2 and 4 weeks. As is the case with the excretion of Mg\(^{2+}\), the urinary excretion and fecal excretion of Ca\(^{2+}\) are also markedly increased during 1, 2, and 4 weeks of ALDOST compared with these controls. Cotreatment with Spiro attenuated urinary and fecal excretion during ALDOST. The reduction in fecal Mg\(^{2+}\) and Ca\(^{2+}\) excretion seen with Spiro was already evident at week 1 and persisted at weeks 2 and 4, the reduction in urinary Mg\(^{2+}\) and Ca\(^{2+}\) excretion was evident at weeks 2 and 4 of ALDOST but not at week 1. This delay in reducing urinary excretion and greater decline in fecal excretion likely represent (1) a corresponding time-dependent elevation in plasma Spiro levels and its potential direct delivery to gastrointestinal tract by twice-daily gavage and (2) the greater ALDO receptor binding density in the kidney (vis-à-vis the colon).31

Plasma Ionized Mg\(^{2+}\) and Ca\(^{2+}\)
Ionized Mg\(^{2+}\) and Ca\(^{2+}\) levels in plasma fell progressively over 4 weeks of ALDOST (Figure 1). At week 4, this...
amounted to a 50% reduction in ionized Mg\(^{2+}\) and 30% reduction in ionized Ca\(^{2+}\). Cotreatment with Spiro prevented the fall in ionized concentrations of these divalent cations seen during ALDOST.

**Parathyroid Hormone**

Compared with unoperated, untreated controls, plasma PTH levels remained significantly elevated at weeks 1, 2, and 4 of ALDOST, although they decreased in magnitude after week 1 (Figure 2).

**BMD and BMC**

BMD and BMC for tibia and femur in age- and gender-matched controls are presented in Table 3; the continued accumulation of minerals in the growth plate of these 9- to 12-week-old rats is evident. BMD and BMC at 1, 2, and 4 weeks ALDOST were therefore compared with each of their age- and gender-matched controls, given the ongoing maturation of bone. A significant decline in BMC for the tibia and femur was observed at the corresponding time points during ALDOST and was most evident at week 4. Spiro cotreatment either attenuated or prevented the decline in BMC and attenuated ALDOST effects on BMC in these bones.

**Bone Strength**

Three-point bending to failure indicated a significant decrease in cortical bone strength at week 4 of ALDOST (Figure 3). Spiro cotreatment prevented this loss in bone strength.

**PBMC Total Mg\(^{2+}\) and Ca\(^{2+}\) Concentrations**

Unlike cytosolic free Mg\(^{2+}\), which fell during weeks 1, 2, and 4 of ALDOST,\(^7\),\(^8\) the total concentration of Mg\(^{2+}\) in PBMC was elevated (\(P<0.05\)) (Figure 4). Total Ca\(^{2+}\) concentration in PBMC was likewise increased (\(P<0.05\)) above control values during 1, 2, and 4 weeks of ALDOST.

**Figure 2.** Plasma PTH levels rose markedly during week 1 of ALDOST and remained significantly (\(P<0.05\)) elevated during weeks 2 through 4.

**Figure 3.** Flexor stress (MPa) at weeks 1, 2, and 4 of ALDOST with or without spironolactone is shown. Three-point bending was done with failure in mid-diaphyseal region of femur. At week 4, ALDOST-treated animals exhibited significant (\(P<0.05\)) decrease in bone strength vs controls. Spiro-cotreated animals did not reveal this decline in bone strength.

**TABLE 3. BMD and BMC of Tibia and Femur During ALDOST With or Without Spiro Cotreatment**

<table>
<thead>
<tr>
<th></th>
<th>Tibia</th>
<th>Femur</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BMD, g/cm(^2)</td>
<td>BMC, g</td>
</tr>
<tr>
<td>Control, wk 1</td>
<td>0.114±0.001</td>
<td>0.290±0.005</td>
</tr>
<tr>
<td>ALDOST, wk 1</td>
<td>0.103±0.001*</td>
<td>0.224±0.005*</td>
</tr>
<tr>
<td>Control, wk 2</td>
<td>0.117±0.002</td>
<td>0.306±0.006</td>
</tr>
<tr>
<td>ALDOST, wk 2</td>
<td>0.113±0.002</td>
<td>0.282±0.007*</td>
</tr>
<tr>
<td>Control, wk 4</td>
<td>0.121±0.001</td>
<td>0.337±0.009</td>
</tr>
<tr>
<td>ALDOST, wk 4</td>
<td>0.115±0.002*</td>
<td>0.285±0.007*</td>
</tr>
<tr>
<td>ALDOST+Spiro, wk 1</td>
<td>0.107±0.002*</td>
<td>0.267±0.007†</td>
</tr>
<tr>
<td>ALDOST+Spiro, wk 2</td>
<td>0.117±0.002*</td>
<td>0.292±0.010</td>
</tr>
<tr>
<td>ALDOST+Spiro, wk 4</td>
<td>0.124±0.002†</td>
<td>0.315±0.006†</td>
</tr>
</tbody>
</table>

Values are mean±SEM (n=5).
*\(P<0.05\) vs control; †\(P<0.05\) vs ALDOST.
**Total Mg²⁺ and Ca²⁺ Concentrations in Ventricular Tissue**

In ventricular tissue obtained from 14-week-old male unoperated, untreated controls, total concentrations for Ca²⁺ and Mg²⁺ were 3.78 ± 0.16 and 46.01 ± 1.05 nEq/mg fat-free dry tissue, respectively. At 4 and 6 weeks of ALDOST, total Ca²⁺ had risen (P < 0.05) to 5.42 ± 0.31 and 6.39 ± 0.65 nEq/mg fat-free dry tissue, respectively, whereas Mg²⁺ remained the same as controls (data not shown). Spiro cotreatment for 4 and 6 weeks significantly attenuated or prevented (P < 0.05) the Ca²⁺ overloading of cardiomyocytes (3.83 ± 0.15 and 3.96 ± 0.28 nEq/mg fat-free dry tissue, respectively), whereas Mg²⁺ levels remained unchanged from controls or that seen with ALDOST.

**Plasma α₁-AP**

Evidence of systemic oxi/nitrosative stress, indicated by reduced plasma α₁-AP, was seen at weeks 1, 2, and 4 of ALDOST compared with controls (Figure 5). Spiro cotreatment attenuated the fall in this inverse correlate of oxi/nitrosative stress that appeared during weeks 1, 2, and 4 of ALDOST that was statistically no different from values found in controls.

**Lymphocyte H₂O₂ Production**

In keeping with the rise in total intracellular Ca²⁺ concentration in PBMCs, lymphocyte H₂O₂ production was significantly increased at weeks 2 to 4 of ALDOST, a delayed response likely corresponding to the exhaustion of antioxidant defenses beyond week 1 (Table 4). Spiro cotreatment prevented the increment of H₂O₂ generation by these cells.

**Discussion**

The first major finding of this study is the early and sustained elevation in urinary and fecal excretion of Mg²⁺ and Ca²⁺ that occurs in rats during ALDOST. The marked loss of Mg²⁺ and Ca²⁺ was evident during preclinical and clinical stages of ALDOST and involved renal and gastrointestinal sites of excretion, both of which are sites of ALDO receptor binding. Hypercalciuria accompanies the short-term treatment of humans or animals with a mineralocorticoid plus dietary Na⁺ loading. The increment in urinary Ca²⁺ excretion, which occurs in the

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**Figure 4.** Total intracellular concentrations for [Mg²⁺] and [Ca²⁺] in PBMCs were increased (*P < 0.05) at weeks 1, 2, and 4 of ALDOST.

**Figure 5.** Systemic evidence of oxi/nitrosative stress during ALDOST was monitored by determining plasma α₁-AP activity. Decline in α₁-AP activity was found at weeks 1, 2, and 4 of ALDOST (*P < 0.05), which was prevented by Spiro cotreatment.

**TABLE 4.** Lymphocyte H₂O₂ Production During ALDOST With or Without Spiro

<table>
<thead>
<tr>
<th>Time</th>
<th>ALDOST</th>
<th>ALDOST + Spiro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALDOST, wk 1</td>
<td>61.17 ± 14.45</td>
<td></td>
</tr>
<tr>
<td>ALDOST, wk 2</td>
<td>129.06 ± 21.31*</td>
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</tr>
<tr>
<td>ALDOST, wk 4</td>
<td>96.94 ± 12.17*</td>
<td></td>
</tr>
<tr>
<td>ALDOST + Spiro, wk 1</td>
<td>57.09 ± 4.95</td>
<td></td>
</tr>
<tr>
<td>ALDOST + Spiro, wk 2</td>
<td>61.51 ± 19.47†</td>
<td></td>
</tr>
<tr>
<td>ALDOST + Spiro, wk 4</td>
<td>41.73 ± 5.56†</td>
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</table>

Values are mean ± SEM (n = 5).

*P < 0.05 vs control; †P < 0.05 vs ALDOST.
distal segment of the nephron, is dietary Na\(^+\) dependent. Urinary Mg\(^{2+}\) excretion also rises with dietary Na\(^+\) loading in rats treated with a mineralocorticoid, whereas the hypermagnesuria found in patients with primary aldosteronism is abrogated by either Spiro or surgical removal of diseased adrenal tissue.26,33,34 Here, we found Spiro cotreatment to attenuate or abrogate the enhanced urinary and fecal excretion of each cation.

The stimulus responsible for the hypermagnesuria and hypercalcemia that accompanies ALDOST is not clearly understood. However, elevations in arterial pressure or the mineralocorticoid hormone itself (in the absence of dietary Na\(^+\)) have been discounted.21,22 A role for such metabolic derangements as polydipsia and metabolic alkalosis that accompany chronic mineralocorticoid/salt treatment have likewise been eliminated.32 In both rats and humans, marked Na\(^+\) loading alone (eg, an 8% NaCl diet) is accompanied by hypercalcemia,25,36 and a proinflammatory vascular phenotype.37,38 The probable mechanism is thought to be related to an expansion of the extravascular space, resulting in decreased proximal tubular resorption and thereby increased distal delivery of Na\(^+\), Mg\(^{2+}\), and Ca\(^{2+}\), with the mineralocorticoid promoting distal tubular Na\(^+\) resorption without retarding Mg\(^{2+}\) or Ca\(^{2+}\) excretion.21,23 This may be further augmented by nitric oxide–mediated increments in medullary blood flow.39,40

The sustained urinary and fecal loss of Mg\(^{2+}\) and Ca\(^{2+}\) that accompanies ALDOST leads to a progressive fall in plasma ionized concentrations of these divalent cations and ultimately to a reduction in BMD. Plasma ionized [Mg\(^{2+}\)], and [Ca\(^{2+}\)], were markedly reduced at week 4, although each cation had already begun to fall during the preclinical stage at weeks 1 and 2 of ALDOST. Bone and its mineral stores are the primary reserve for these cations, with bone resorption facilitated by PTH. Elevations in PTH, secreted in response to reduced plasma ionized [Ca\(^{2+}\)], and [Mg\(^{2+}\)], would be expected in the setting of sustained hypercalcemia and hypermagnesuria. We indeed found elevated plasma PTH levels, consistent with normocalcemic SHPT, during weeks 1 to 4 of ALDOST. Both serum ionized and total plasma Ca\(^{2+}\) is reduced in response to deoxycorticosterone/salt treatment (DOCST),32 together with increased serum PTH and urinary excretion of cAMP, a marker of parathyroid activity. Both primary hyperparathyroidism and SHPT have been reported in patients with primary aldosteronism,41–43 with expected aberrations in serum ionized and total Ca\(^{2+}\), together with elevations in PTH, normalized by adrenal surgery or Spiro.34,43

Our second major finding is the marked reduction in BMD and BMC of tibia and femur that appeared by week 4 of ALDOST. This hitherto-unappreciated fall in BMD and BMC was relatively rapid and was accompanied by a reduction in bone strength, suggesting that the loss of these cations was related to increased PTH secretion. Urinary hydroxyproline, a marker of bone resorption, is increased during ALDOST or DOCST,44 whereas the hypercalcemia seen with 8% NaCl loading alone is likewise accompanied by a loss of bone Mg\(^{2+}\) and Ca\(^{2+}\) and increased urinary excretion of various markers of bone resorption.35,45,46 The cellular basis for bone resorp-

in ALDOST will likely involve both osteoclasts and osteoblasts. PTH provokes bone loss indirectly by stimulating osteoblast production of interleukin-6, which induces osteoclastogenesis and activates osteoclasts.47 Other tissue sources of interleukin-6 may also be contributory. The role of oxi/nitrosative stress in promoting bone resorption in the setting of ALDOST remains to be examined.

In patients with severe heart failure awaiting cardiac transplantation, moderate and marked reductions in BMD, in keeping with osteopenia and osteoporosis, respectively, have been reported.5,6 Elevated circulating levels of intact PTH and its C-terminal PTH-related peptide, representing normocalcemic SHPT, are found in these patients with advanced CHF and in those with moderately severe failure.6,48–50 The confounding effect of furosemide, a potent loop diuretic commonly prescribed to these patients that enhances urinary Mg\(^{2+}\) and Ca\(^{2+}\) excretion, cannot be addressed but would presumably only exaggerate the urinary loss of these cations that accompanies aldosteronism. In reducing both the urinary and fecal loss of these cations, Spiro cotreatment rescued BMD and BMC during 4 weeks of ALDOST.

In addition to bone stores, dietary Ca\(^{2+}\) and Mg\(^{2+}\) provide an important source of these cations, and increments in renal formation of 1,25(OH)\(_2\)D\(_3\), formation, driven by PTH stimulation, would raise their gastrointestinal absorption. Increased duodenal absorption of Ca\(^{2+}\), mediated by 1,25(OH)\(_2\)D\(_3\), accompanies the hypercalcemia associated with DOCST in rats.32 Although not monitored in this study, the active metabolite of this hormone did not appear sufficient to overcome the sustained gastrointestinal losses of Ca\(^{2+}\) that we observed during ALDOST. Future studies are needed to address 1,25(OH)\(_2\)D\(_3\) responses during ALDOST.

Despite the body’s loss of these divalent cations, the reduction in their plasma ionized concentrations, and loss in BMD and BMC, Mg\(^{2+}\) and Ca\(^{2+}\) concentrations in PBMC and Ca\(^{2+}\) in ventricular tissue were increased. This third major finding of our study, intracellular Ca\(^{2+}\) overload, is a phenomenon referred to as a Ca\(^{2+}\) paradox.51 Relative to the Ca\(^{2+}\) overload found in ventricular tissue, we have not identified the cells involved in this response. We would suspect that cardiomyocytes are Ca\(^{2+}\) loaded for several reasons. First, they are an excitable cell and will have a greater density of voltage-gated Ca\(^{2+}\) channels and intracellular Ca\(^{2+}\) release channels than nonexcitable cells. Given their size, two thirds of the structural space of myocardium, and the 40% and 70% increase in tissue Ca\(^{2+}\) concentration seen at 4 and 6 weeks of ALDOST, respectively, it would seem highly probable that they are involved. Aldosterone upregulates the Ca\(^{2+}\) current in cardiomyocytes via T-channel expression. The number of cardiomyocyte Ca\(^{2+}\) channels is increased with mineralocorticoid excess, and intracellular Ca\(^{2+}\) responses to provocation are increased in these cells, as well as cardiac fibroblasts, in this setting.52–55 Finally, cardiomyocytes have PTH receptors, and when incubated with this peptide hormone, a rise in [Ca\(^{2+}\)], occurs.56,57 We found the Ca\(^{2+}\) overload of PBMC to be accompanied by oxi/nitrosative stress, expressed as increased lymphocyte H\(_2\)O\(_2\) production. An activation of NADPH oxidase in inflammatory cells invading the intramu-

ral coronary vasculature has previously been reported.14,17,18
We also have found upregulated transcription for PBMC genes representing antioxidant defenses, chemokines, and cytokines during preclinical and clinical stages of ALDOST.\(^7,8\)

As in human CHF, evidence of oxi/nitrosative stress is present at a systemic level in rats with ALDOST. Here, the early, persistent reduction in plasma \(c\alpha\)-AP activity seen is likely in keeping with \(Ca^{2+}\) overload of PBMCs and ventricular tissue and may include other cells and tissues (eg, platelets, erythrocytes, fibroblasts, endothelial cells, and vascular tissue).\(^31,33-35\)

A direct effect of ALDOST on cultured lymphocyte \([Ca^{2+}]_i\), could not be demonstrated.\(^20\) Widespread \(Ca^{2+}\) loading, which may be a response to elevated plasma PTH levels, could explain the systemic induction of oxi/nitrosative stress. Receptors for PTH are found on PBMCs and cardiomyocytes with receptor-ligand binding capable of raising \([Ca^{2+}]_i\), in these cells.\(^38\)

The role of PTH and other calcitropic hormones—eg, \(1,25(OH)_{2}D_{3}\) and endothelin-1—in regulating PBMC \([Ca^{2+}]_i\), during ALDOST requires further study. As we have shown previously,\(^8\) in preventing this rise in \([Ca^{2+}]_i\), induction of oxi/nitrosative stress, and activation of PBMCs, Spiro cotreatment prevented the proinflammatory vascular phenotype. The demonstrated efficacy of Spiro, together with ACE inhibitor and diuretic, in the management of symptomatic heart failure\(^59\) may have its origins, at least in part, in ameliorating these diverse metabolic responses to chronic, inappetent (relative to dietary Na\(^+\)) elevations in plasma ALDO.

In conclusion, Mg\(^{2+}\) and Ca\(^{2+}\) are known to contribute to the regulation of immune cell function. This is no more evident than during periods of stress such as that which accompanies aldosteronism, leading to the body’s loss of these important divalent cations. Paradoxical Ca\(^{2+}\) overloading and the generation of reactive oxygen intermediates in PBMCs serve to promote immune cell activation. The role of such calcitropic hormones as PTH and \(1,25(OH)_{2}D_{3}\), in promoting this Ca\(^{2+}\) overload remains to be addressed. The prospect that SHPT could be operative during ALDOST in regulating the divalent cation composition of PBMCs, as is the case in chronic renal failure,\(^60\) offers new insights into the pathophysiology of CHF and its proinflammatory phenotype. In the case of the myocardium, Ca\(^{2+}\) overloading of cardiomyocytes may contribute to their electrical remodeling and arrhythmogenic potential.

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