Aldosteronism and a Proinflammatory Vascular Phenotype
Role of Mg\(^{2+}\), Ca\(^{2+}\), and H\(_2\)O\(_2\) in Peripheral Blood Mononuclear Cells

Robert A. Ahokas, PhD; Yao Sun, MD, PhD; Syamal K. Bhattacharya, PhD; Ivan C. Gerling, PhD; Karl T. Weber, MD

Background—Chronic, inappropriate (relative to dietary Na\(^+\)) elevations in circulating aldosterone, such as occur in congestive heart failure, are accompanied by a proinflammatory vascular phenotype involving the coronary and systemic vasculature. An immunostimulatory state with activated peripheral blood mononuclear cells (PBMCs) precedes this phenotype and is induced by a fall in cytosolic free [Mg\(^{2+}\)], and subsequent Ca\(^{2+}\) loading of these cells and transduced by oxidative/nitrosative stress.

Methods and Results—We sought to further validate this hypothesis in rats with aldosterone/1%NaCl treatment (ALDOST) by using several interventions as cotreatment: a Mg\(^{2+}\)-supplemented diet; amlodipine, a CCB; and N-acetylcysteine, an antioxidant. Blood samples were obtained at weeks 1 to 4 of ALDOST to monitor [Mg\(^{2+}\)], [Ca\(^{2+}\)], and H\(_2\)O\(_2\) production in PBMCs. Coronal ventricular sections were examined for invading inflammatory cells and 3-nitrotyrosine labeling, a marker of oxidative/nitrosative stress. In response to ALDOST and compared with untreated controls, we found an early and persistent reduction in [Mg\(^{2+}\)], with a subsequent rise in [Ca\(^{2+}\)], and H\(_2\)O\(_2\) production, each of which was either attenuated or abrogated by the Mg\(^{2+}\)-supplemented diet and by N-acetylcysteine, whereas amlodipine prevented Ca\(^{2+}\) loading and an altered redox state. Cotreatment with these interventions either markedly attenuated or prevented the appearance of the proinflammatory coronary vascular phenotype and the presence of 3-nitrotyrosine in invading inflammatory cells.

Conclusions—We suggest that the immunostimulatory state that appears during aldosteronism and leads to a proinflammatory coronary vascular phenotype is induced by a fall in [Mg\(^{2+}\)], with Ca\(^{2+}\) loading of PBMCs and is transduced by H\(_2\)O\(_2\) production in these cells. (Circulation. 2005;111:51-57.)

Key Words: calcium ■ stress ■ pathology ■ aldosterone ■ magnesium
Our current working hypothesis suggests PBMC activation in ALDOST is induced by a reduction in biologically active, cytosolic free [Mg\(^{2+}\)], and subsequent Ca\(^{2+}\) loading of these cells and that it is transduced by oxidative/nitrosative stress. Further evidence in support of this contention, albeit circumstantial, is the response to cotreatment with spironolactone, which attenuates Ca\(^{2+}\) loading and the induction of oxidative/nitrosative stress in these cells and their invasion of the coronary vasculature. The present study was undertaken in PBMCs harvested from rats receiving 1 to 4 weeks ALDOST to monitor [Mg\(^{2+}\)] and [Ca\(^{2+}\)] in altering their redox state. The following interventions were examined as cotreatment with ALDOST: a Mg\(^{2+}\)-supplemented diet to prevent the fall in PBMC [Mg\(^{2+}\)], and rise in [Ca\(^{2+}\)]; an L-type Ca\(^{2+}\) channel blocker (CCB; amlopidine) to attenuate the rise in intracellular Ca\(^{2+}\) in these immune cells and which has been shown to ameliorate the appearance of proinflammatory coronary lesions in the setting of chronic mineralocorticoid excess; and an antioxidant (N-acetylcysteine [NAC]) to attenuate oxidative/nitrosative stress in these inflammatory cells. Blood samples were obtained at weeks 1 to 4 of ALDOST to further address the role of iterations in [Mg\(^{2+}\)] and [Ca\(^{2+}\)], in altering their redox state. PBMCs harvested from rats receiving 1 to 4 weeks ALDOST were incubated with 0.1% Triton X-100 (Rmin), and after addition of 10 mmol/L CaCl\(_2\) (Rmax). [Ca\(^{2+}\)] \(_i\) was calculated as follows: 

\[
\frac{[\text{Mg}^{2+}]_i \times K_d}{[\text{Mg}^{2+}]_i - [\text{Mg}^{2+}]_i + S_f \times S_f} 
\]

where \(K_d\), the dissociation constant for Mg\(^{2+}\), is 1.5 mmol/L, and \(S_f\) and \(S_i\) are the fluorescence intensities at 370 nm with 0 Mg\(^{2+}\) and excess Mg\(^{2+}\), respectively. For measurement of [Ca\(^{2+}\)] \(_i\), in the fura-2-loaded cells, fluorescence emission at 510 nm (slit width 7 nm) was recorded with excitation at 340 and 380 nm (slit width 10 nm), respectively, after addition of 10 mmol/L EGTA (R), after addition of 0.1% Triton X-100 (Rmin), and after addition of 10 mmol/L CaCl\(_2\) (Rmax). [Ca\(^{2+}\)] \(_i\) was calculated with the same equation as for [Mg\(^{2+}\)], using the Ca\(^{2+}\) dissociation constant (\(K_d\)) of 225 mmol/L, and \(S_f\) and \(S_i\) were the fluorescence intensities at 380 nm with 0 Ca\(^{2+}\) and excess Ca\(^{2+}\), respectively.

### PBMC Hydrogen Peroxide Production

Lymphocyte H\(_2\)O\(_2\) production was measured as reported previously. Briefly, 0.1 mL of whole blood was incubated with 2,7-dichlorofluorescin diacetate (25 μmol/L, Molecular Probes) for 45 minutes at 37°C. After red blood cells were lysed with FACS lysis solution (Becton Dickinson), leukocytes were suspended in PBS (pH 7.4) for single-cell flow cytometric analysis with a FACS Caliber flow cytometer (Becton Dickinson). 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.

### Immunohistochemistry (3-Nitrotyrosine)

Coronal cryostat sections (6 μm) were prepared, air-dried, fixed in 10% buffered formalin for 5 minutes, and washed in PBS for 10 minutes. Sections were then incubated with primary antibody against 3-nitrotyrosine at a dilution of 1:100 (Upstate Biotech) in PBS containing 1% BSA for 60 minutes. Sections were then washed in PBS for 10 minutes and incubated with IgG-peroxidase-conjugated secondary antibody (Sigma) with a dilution of 1:150, washed in PBS for 10 minutes and incubated with 0.05% H\(_2\)O\(_2\) for 10 minutes, and again washed in PBS. Negative control sections were incubated with secondary antibody alone, stained with hematoxylin, dehydrated, mounted, and examined by light microscopy.

### Statistical Analysis

Values presented are mean±SEM. Data were statistically analyzed by 1-way ANOVA. Significant differences between individual means were determined with the post hoc Bonferroni multiple comparison test, where significance was assigned to \(P<0.05\).

### Results

#### Clinical Observations

At weeks 1 and 2 ALDOST, animals were active, eating, and gaining weight at levels comparable to controls. Anorexia, lethargy, and failure to gain weight were seen at weeks 3 and 4. Rats in the 3 intervention groups receiving ALDOST plus either Mg\(^{2+}\)-supplemented diet, CCB, or NAC did not appear as ill as those receiving ALDOST alone. This is reflected in their body weights, which were less than controls at weeks 3 and 4 but greater than in those with ALDOST alone (Table). [Mg\(^{2+}\)] and [Ca\(^{2+}\)] Concentrations in PBMCs

In untreated, untreated controls receiving standard chow with 20 to 40 mmol/kg Mg\(^{2+}\), the mean concentration of [Mg\(^{2+}\)] in PBMCs was 349±17 μmol/L (Figure 1). This cytosolic free component of intracellular Mg\(^{2+}\) was significantly reduced throughout weeks 1 to 4 ALDOST. This fall in

#### PBMC Cytosolic Free [Mg\(^{2+}\)] and [Ca\(^{2+}\)]

Cytosolic free [Mg\(^{2+}\)] and [Ca\(^{2+}\)] were measured as reported previously with a modification of the ratiometric method and the fluorescent molecular probes mag-fura-2 and fura-2 (Molecular Probes), respectively. Briefly, PBMCs were washed and suspended in RPMI 1640 media containing 0.1% bovine serum albumin (pH 7.4). For loading the probes, the cells were incubated with mag-fura-2/AM (10 μmol/L) for Mg\(^{2+}\) or fura-2/AM (5 μmol/L) for Ca\(^{2+}\) for 1 hour at 37°C. After being washed 3 times with fresh RPMI 1640 media, the cells were suspended in analysis buffer consisting of (in mmol/L) NaCl 140, KCl 5, CaCl\(_2\) 1.8, MgSO\(_4\) 0.8, HEPES 15, and D-glucose 5 (pH 7.4) for the measurement of fluorescence with a Perkin-Elmer LS-50B spectrofluorometer. Fluorescence emission at 510 nm (slit width 10 nm) was recorded with excitation at 335 and 370 nm (slit width 10 nm) for the measurement of [Mg\(^{2+}\)] in the cells loaded with mag-fura-2 after addition of 5 mmol/L EDTA and 5 mmol/L EGTA (R), after addition of 0.1% Triton X-100 (Rmin), and after addition of 100 mmol/L MgSO\(_4\) (Rmax). [Mg\(^{2+}\)] was calculated as follows: 

\[
[Mg^{2+}]_i = K_d \times (R - R_{min}) / [(R_{max} - R_{min})] 
\]

where \(K_d\), the dissociation constant for Mg\(^{2+}\), is 1.5 mmol/L, and \(S_f\) and \(S_i\) are the fluorescence intensities at 370 nm with 0 Mg\(^{2+}\) and excess Mg\(^{2+}\), respectively. For measurement of [Ca\(^{2+}\)] \(_i\) in the fura-2-loaded cells, fluorescence emission at 510 nm (slit width 7 nm) was recorded with excitation at 340 and 380 nm (slit width 10 nm), respectively, after addition of 10 mmol/L EGTA (R), after addition of 0.1% Triton X-100 (Rmin), and after addition of 10 mmol/L CaCl\(_2\) (Rmax). [Ca\(^{2+}\)] \(_i\) was calculated with the same equation as for [Mg\(^{2+}\)], using the Ca\(^{2+}\) dissociation constant (\(K_d\)) of 225 mmol/L, and \(S_f\) and \(S_i\) were the fluorescence intensities at 380 nm with 0 Ca\(^{2+}\) and excess Ca\(^{2+}\), respectively.
[Mg\(^{2+}\)] \(_i\) was either attenuated by the Mg\(^{2+}\)-supplemented diet or NAC but was not affected by CCB. PBMC [Ca\(^{2+}\)] \(_i\) began to rise during week 2 and was statistically greater than in controls at weeks 3 and 4 ALDOST (Figure 2). This gradual rise in PBMC [Ca\(^{2+}\)] \(_i\) was prevented with the Mg\(^{2+}\)-supplemented diet, amlodipine, or NAC.

**Lymphocyte Hydrogen Peroxide Production**

H\(_2\)O\(_2\) production rose progressively in PBMCs harvested from rats during weeks 1 to 4 ALDOST and was significantly greater than controls at weeks 2 to 4 (Figure 3). In response to the Mg\(^{2+}\)-supplemented diet, lymphocyte H\(_2\)O\(_2\) production was increased at week 1 ALDOST but did not differ from controls at weeks 2 to 4. During cotreatment with amlodipine or NAC, lymphocyte production of H\(_2\)O\(_2\) was attenuated compared with ALDOST and was not different from controls.

**Immunohistochemistry**

Superoxide anions react with nitric oxide to form short-lived peroxynitrite and ultimately stable nitrotyrosine protein moieties. The expression of 3-nitrotyrosine, as detected by immunohistochemistry, was used as an in situ marker of oxidative/nitrosative stress in the heart (Figure 4A). In controls, 3-nitrotyrosine is expressed by coronary vascular endothelial and smooth muscle cells; it is not found in the perivascular space or in cardiomyocytes (panel A). In ALDOST rats, perivascular swelling and infiltration by inflammatory cells is seen surrounding intramural coronary arteries together with 3-nitrotyrosine labeling (Figure 4B). The Mg\(^{2+}\)-supplemented diet or CCB attenuated the expansion of the perivascular space and cellular infiltration and the appearance of 3-nitrotyrosine staining (Figure 4C and 4D). NAC treatment completely prevented such vascular remodeling and appearance of 3-nitrotyrosine with ALDOST (Figure 4E).

**Discussion**

The present study yielded several major findings. First, the early and persistent reduction in PBMC [Mg\(^{2+}\)] \(_i\) that accompanies ALDOST was attenuated by a Mg\(^{2+}\)-supplemented diet and NAC but not by CCB. As we and others have previously reported,\(^{10,11,13,17}\) chronic elevations in plasma aldosterone or deoxycorticosterone (DOC), another mineralocorticoid, inappropriate relative to dietary Na\(^{+}\), are associated with reduced lymphocyte [Mg\(^{2+}\)] \(_i\). Herein, we confirmed this response and documented its presence throughout 4 weeks of ALDOST. Mechanisms responsible for the early and sustained fall in [Mg\(^{2+}\)] \(_i\), may be several fold. Hypermagnesuria accompanies aldosteronism and likely contributes to the fall in [Mg\(^{2+}\)].\(^{18,19}\) Additionally, lymphocyte [Mg\(^{2+}\)] \(_i\) is reduced in patients with primary aldosteronism, and in ex vivo studies of human lymphocyte, [Mg\(^{2+}\)] \(_i\), fell in response to incubation with physiologically relevant concentrations of aldosterone.\(^{17}\) Delva et al\(^{15}\) suggested this Na\(^{+}\)-dependent, aldosterone receptor–mediated response involves the transcription and synthesis of a putative Na\(^{+}\)/Mg\(^{2+}\) exchanger; however, Mg\(^{2+}\) efflux was not measured in that study. In preventing the decline in [Mg\(^{2+}\)], with NAC, findings of the present study suggest reactive oxygen species contribute to this response.
Further studies are needed to elucidate the mechanisms responsible for the decline in \([\text{Mg}^{2+}]_i\) during ALDOST in rats are planned.

The fall in PBMC \([\text{Mg}^{2+}]_i\) that appears with ALDOST is accompanied by a subsequent rise in cytosolic free \([\text{Ca}^{2+}]_i\) in these cells. Increased monocyte and lymphocyte \([\text{Ca}^{2+}]_i\), controls numerous cell functions, including adhesion, motility, gene expression, and cell proliferation. \([\text{Ca}^{2+}]_i\) loading of lymphocytes, as well as platelets and vascular smooth muscle cells, occurs in response to chronic DOC/salt treatment in rats and with mineralocorticoid administration in humans. The importance of reduced \([\text{Mg}^{2+}]_i\) in initiation of increased \([\text{Ca}^{2+}]_i\), and the induction of oxidative/nitrosative stress in PBMCs, together with an exaggerated immune cell response, has also been observed in rats fed a \([\text{Mg}^{2+}]^-\)-deficient diet, precedes inflammatory cell invasion of cardiac tissue by several

**Figure 2.** Cytosolic free \([\text{Ca}^{2+}]_i\) concentration ([\(\text{Ca}^{2+}\)]) in PBMCs obtained at weeks 1 to 4 ALDOST are presented as open columns and compared with values for controls (dashed line). Each panel represents separate intervention as solid column: \([\text{Mg}^{2+}]^-\) supplemented (Suppl) diet; amlodipine (CCB); and NAC. Mean ± SEM (n = 5 at all time points). *P < 0.05 vs controls. †P < 0.05 vs ALDOST. See text for details.

**Figure 3.** Hydrogen peroxide (H\(_2\)O\(_2\)) production by PBMCs, expressed as mean channel brightness (MCB), during weeks 1 to 4 of ALDOST are presented as open columns and compared with values for controls (dashed line). Each panel represents separate intervention as solid column: \([\text{Mg}^{2+}]^-\) supplemented (Suppl) diet; amlodipine (CCB); and NAC. Mean ± SEM (n = 5 at all time points). *P < 0.05 vs controls. †P < 0.05 vs ALDOST. See text for details.
weeks. Herein, we were able to prevent the fall in [Mg^{2+}] that appears with ALDOST by a Mg^{2+}-supplemented diet. A reduced activity of Mg^{2+}-dependent Na^+/K^+-ATPase may be contributory to the observed rise in PBMC [Ca^{2+}], DOC/salt treatment augments the number of Ca^{2+} channels and raises [Ca^{2+}], in various cell populations. An aldosterone-induced expression and increased activity of T-type Ca^{2+} channels has been reported in adrenal cells, cardiomyocytes, and prostate epithelial cells and is blocked by spironolactone. Whether this occurs in PBMCs during ALDOST is unknown, but if so, it would enhance the transfer of extracellular Ca^{2+} directly to mitochondria via the endoplasmic reticulum and would contribute to the induction of oxidative/nitrosative stress in these cells. Mibefradil, a dual T- and L-type CCB, inhibits the proliferation of stimulated PBMCs, reduces leukocyte adhesion by interfering with integrin signaling, and is organ protective in rat models of chronic mineralocorticoid/salt excess. In the present study, we found amlo-dipine, an L-type channel blocker, attenuates PBMC Ca^{2+} loading and the appearance of coronary vascular lesions. Other dihydropyridine CCBs have proved to be organ protective in rats with chronic mineralocorticoid excess. Lymphocytes are otherwise nonexcitable cells and are where the membrane potential of T cells is primarily determined by both voltage- and Ca^{2+}-dependent K^+ channels, whose behavior resembles voltage-dependent Ca^{2+} channels. Dihydropyridine CCBs inhibit Ca^{2+} entry in lymphocytes via their influence on Ca^{2+}-activated K^+ channels. Nonetheless, enhanced PBMC Ca^{2+} entry occurs during ALDOST. We cannot discount a release of this cation from intracellular organelles. It has been reported that once present, oxygen and nitrogen metabolites can augment Ca^{2+} entry via voltage-dependent channels, which is supported by our findings with NAC that abrogated PBMC Ca^{2+} loading during ALDOST.

Our second major finding is the induction of oxidative/nitrosative stress in PBMCs that accompanies increased [Ca^{2+}], and which was evidenced by increased H_2O_2 production in circulating lymphocytes. Additional evidence of oxidative/nitrosative stress is the immunohistochemical detection of 3-nitrotyrosine we found in monocytes and lymphocytes invading the perivascular space of intramural coronary arteries. Reactive oxygen species, such as H_2O_2, serve as intracellular messengers in activating transcription of inflammatory cells, thus promoting their proinflammatory behavior. We have previously reported on the early activation of PBMC transcriptome during ALDOST. This includes a differential expression of genes that encode an oxidative stress–inducible tyrosine phosphatase and such antioxidant defenses as superoxide dismutase and that are coupled to upregulated expression of proinflammatory genes and those in keeping with lymphocyte activation and autoreactivity. The rise in lymphocyte H_2O_2 production during ALDOST reflects an imbalance between the generation of reactive oxygen and nitrogen species and their inadequate neutralization by likely depleted antioxidant defenses. The fall in [Mg^{2+}], and accompanying responses in intracellular Ca^{2+} and redox state observed in PBMCs during ALDOST were prevented or attenuated by dietary Mg^{2+} supplementation. Similar responses to Mg^{2+} supplementation...
have been reported in rodent and human platelets during chronic mineralocorticoid excess (inappropriate for dietary Na⁺ intake) and in rodent neutrophils in response to dietary Mg²⁺ deficiency.14,22,28 The proinflammatory phenotype seen with dietary Mg²⁺ deficiency is prevented when such a diet is simultaneously made Ca²⁺-deficient.2,5 A Mg²⁺-supplemented diet has been reported to attenuate endothelin-1 production by heart, aorta, and kidneys in rats receiving DOC/salt treatment.41,42 This calcitropic hormone produced by endothelial cells is also released by parathyroid glands in response to reduced serum ionized Ca²⁺.43 Herein, we found cotreatment with a CCB to attenuate PBMC Ca²⁺ loading during ALDOST. In blocking Ca²⁺ entry, amloidipine attenuated H₂O₂ production by these cells. Further evidence supporting the importance of Ca²⁺ loading in leading to an altered PBMC redox state is derived from our previous findings with spironolactone cotreatment, which prevented the rise in [Ca²⁺]i, and induction of oxidative stress, including H₂O₂ production, and attenuated the upregulation of transcriptome of the proinflammatory PBMC phenotype.11 Our in vivo findings underscore the importance of an equilibrium between intracellular Mg²⁺ and Ca²⁺ in the generation of reactive oxygen and nitrogen species, which in physiologically relevant concentrations may represent a final common pathway in the activation of lymphocytes and monocytes. Left unbridled, marked increments in reactive oxygen and nitrogen intermediates would adversely affect cell survival.

Finally, interventions used in the present study either attenuated or abrogated the proinflammatory coronary vascular phenotypic alteration and the presence of 3-nitrotyrosine in invading inflammatory cells at 4 weeks of ALDOST. Sun et al.14 previously reported that cotreatment with either an antioxidant, pyrrolidine dithiocarbamate, or spironolactone abrogates the appearance of oxidative/nitrosative stress and the number of inflammatory cells that invade the coronary vasculature at week 4 of ALDOST. Several clinical trials support the efficacy of aldosterone receptor antagonism, together with treatment with an ACE inhibitor and diuretic, in the overall management of symptomatic heart failure,44,45 in which aldosteronism is accompanied by evidence of oxidative/nitrosative stress in plasma, PBMCs, and such diverse tissues as skeletal muscle and heart.46–50 An integral feature of this systemic disorder is a catabolic state with simultaneous loss of lean tissue, fat, and bone and which eventuates of this systemic disorder is a catabolic state with simultaneous loss of lean tissue, fat, and bone and which eventuates of this systemic disorder is a catabolic state with simultaneous loss of lean tissue, fat, and bone and which eventuates.

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