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

In both humans and rats, inappropriate (relative to dietary Na$^+$) elevations in circulating aldosterone are accompanied by a proinflammatory/fibrogenic vascular phenotype that involves intramural arteries of the heart and systemic organs. In a rat model of aldosterone/salt treatment (ALDOST), the appearance of such vascular remodeling is Na$^+$- and time-dependent and is prevented by cotreatment with spironolactone, an aldosterone receptor antagonist, in either nondepressor or depressor dosage. Lesions include ED1-positive monocytes/macrophages and CD4-positive lymphocytes that first invade the perivascular space of involved vessels at week 4 of ALDOST. An 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.)

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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$_{2+}$]i and to determine lymphocyte H$_2$O$_2$ production in PBMCs. The intramural coronary artery vasculature found in PBMCs harvested from rats receiving 1 to 4 weeks ALDOST and rise in [Ca$_{2+}$]i, 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 nitrotyrosine at a dilution of 1:100 (Upstate Biotech) in PBS, washed in PBS. Negative control sections were incubated with 20 to 40 mmol/kg Mg$_{2+}$, and [Ca$_{2+}$]i, and to determine lymphocyte H$_2$O$_2$ production in PBMCs. The intramural coronary artery vasculature found in coronal sections of right and left ventricular tissue was examined for evidence of invading inflammatory cells and oxidative/nitrosative stress as reflected by 3-nitrotyrosine labeling.

**Methods**

**Animals**

Eight-week-old male Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, Ind) were used in this study. Five groups were studied: (1) untreated, unoperated age-matched rats, which served as controls; (2) uninephrectomized rats given standard chow (20 to 40 mg/kg Mg$_{2+}$) together with 1% NaCl/0.4% KCl in drinking water and aldosterone (0.75 μg/h) administered by implanted minipump (ALDOST); (3) ALDOST plus Mg$_{2+}$-supplemented diet (Mg$_{2+}$ content 40 to 60 mg/kg); (4) ALDOST plus cotreatment with an L-type CCB, (amilodipine, 10 mg·kg$^{-1}$·d$^{-1}$ by gavage); and (5) ALDOST plus NAC (200 mg/kg QD IP). Each group was studied at 1 to 4 weeks of age.

**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, 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).
Body Weights (g) During ALDOST and With Various Interventions

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>253±11</td>
<td>282±14</td>
<td>309±12</td>
<td>342±15</td>
</tr>
<tr>
<td>ALDOST</td>
<td>250±12</td>
<td>279±18</td>
<td>266±17*</td>
<td>252±13*</td>
</tr>
<tr>
<td>ALDOST + Mg2+ diet</td>
<td>255±10</td>
<td>279±14</td>
<td>294±15</td>
<td>283±16†</td>
</tr>
<tr>
<td>ALDOST + CCB</td>
<td>247±9</td>
<td>276±13</td>
<td>288±11</td>
<td>279±11‡</td>
</tr>
<tr>
<td>ALDOST + NAC</td>
<td>250±11</td>
<td>283±12</td>
<td>290±13</td>
<td>289±14‡</td>
</tr>
</tbody>
</table>

*P<0.05 vs controls; †P<0.05 vs ALDOST.

[Mg2+]i, was either attenuated by the Mg2+-supplemented diet or NAC but was not affected by CCB.

PBMC [Ca2+]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 [Ca2+]i was prevented with the Mg2+-supplemented diet, amlodipine, or NAC.

Lymphocyte Hydrogen Peroxide Production

H2O2 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 Mg2+-supplemented diet, lymphocyte H2O2 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 H2O2 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 cardiac myocytes (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 Mg2+-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 [Mg2+]i that accompanies ALDOST was attenuated by a Mg2+-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 [Mg2+]i. Herein, we confirmed this response and documented its presence throughout 4 weeks of ALDOST. Mechanisms responsible for the early and sustained fall in [Mg2+]i, may be several fold. Hypermagnesuria accompanies aldosteronism and likely contributes to the fall in [Mg2+].18,19 Additionally, lymphocyte [Mg2+]i, is reduced in patients with primary aldosteronism, and in ex vivo studies of human lymphocyte, [Mg2+]i fell in response to incubation with physiologically relevant concentrations of aldosterone.17 Delva et al15 suggested this Na+-dependent, aldosterone receptor–mediated response involves the transcription and synthesis of a putative Na+/Mg2+ exchanger; however, Mg2+ efflux was not measured in that study. In preventing the decline in [Mg2+]i, 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. 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 \( [\text{Mg}^{2+}]_i \)-deficient diet. PBMC activation, which appears during week 1 of such a diet, precedes inflammatory cell invasion of cardiac tissue by several days.

Figure 2. Cytosolic free \( \text{Ca}^{2+} \) concentration (\( [\text{Ca}^{2+}]_i \)) 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: \( [\text{Mg}^{2+}]_i \)-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 (\( \text{H}_2\text{O}_2 \)) 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: \( [\text{Mg}^{2+}]_i \)-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 \([\text{Mg}^{2+}]\) that appears with ALDOST by a \([\text{Mg}^{2+}]\)-supplemented diet. A reduced activity of \([\text{Mg}^{2+}]\)-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. Mibebradil, 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 H2O2 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 H2O2, 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 H2O2 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 \([\text{Mg}^{2+}]\), 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 Mg\(^{2+}\) deficiency.\(^{14,22,28}\) The proinflammatory phenotype seen with dietary Mg\(^{2+}\) deficiency is prevented when such a diet is simultaneously made Ca\(^{2+}\)-deficient.\(^{5,2}\) A Mg\(^{2+}\)-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 Ca\(^{2+}\). \(^{43}\) Herein, we found cotreatment with a CCB to attenuate PBMC Ca\(^{2+}\) loading during ALDOST. In blocking Ca\(^{2+}\) entry, amiodipine attenuated H\(_2\)O\(_2\) production by these cells. Further evidence supporting the importance of Ca\(^{2+}\) loading in leading to an altered PBMC redox state is derived from our previous findings with spironolactone cotreatment, which prevented the rise in [Ca\(^{2+}\)]\(_i\), and induction of oxidative stress, including H\(_2\)O\(_2\) production, and attenuated the upregulation of transcriptome of the proinflammatory PBMC phenotype.\(^1\) Our in vivo findings underscore the importance of an equilibrium between intracellular Mg\(^{2+}\) and Ca\(^{2+}\) 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 phenotype and the presence of 3-nitrotyrosine in invading inflammatory cells at 4 weeks of ALDOST. Sun et al\(^7\) previously reported that cotreatment with either an antioxidant, pyrroldine diethiocarbamate, 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 in a wasting syndrome termed cardiac cachexia.\(^51\) We believe inappropriate chronic elevations in plasma aldosterone (relative to dietary Na\(^+\)) contribute to this illness through an immunostimulatory state mediated by iterations in PBMC dialytically oxidative/nitrosative stress, a contention supported by findings of the present study.

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