Aldosteronism and Peripheral Blood Mononuclear Cell Activation

A Neuroendocrine-Immune Interface


Abstract—Aldosteronism eventuates in a proinflammatory/fibrogenic vascular phenotype of the heart and systemic organs. It remains uncertain whether peripheral blood mononuclear cells (PBMCs) are activated before tissue invasion by monocytes/macrophages and lymphocytes, as is the case for responsible pathogenic mechanisms. Uninephrectomized rats treated for 4 weeks with dietary 1% NaCl and aldosterone (ALDOST, 0.75 μg/h) with or without spironolactone (Spi, 100 mg/kg per daily gavage) were compared with unoperated/untreated and uninephrectomized/salt-treated controls. Before intramural coronary vascular lesions appeared at week 4 of ALDOST, we found (1) a reduction of PBMC cytosolic free [Mg2+]i, together with intracellular Mg2+ and Ca2+ loading, whereas plasma and cardiac tissue Mg2+ were no different from controls; (2) increased H2O2 production by monocytes and lymphocytes together with upregulated PBMC gene expression of oxidative stress–inducible tyrosine phosphatase and Mn2+-superoxide dismutase and the presence of 3-nitrotyrosine in CD4+ and ED-1–positive inflammatory cells that had invaded intramural coronary arteries; (3) B-cell activation, including transcription of immunoglobulins, intracellular adhesion molecule-1, and CC and CXC chemokines and their receptors; (4) expansion of B lymphocyte subset and myosin heavy chain class II–expressing lymphocytes; and (5) autoreactivity with gene expression for antibodies to acetylcholine receptors and a downregulation of RT-6.2, which is in keeping with cell activation and associated with autoimmunity. Spi cotreatment attenuated the rise in intracellular Ca2+, the appearance of oxidative/nitrosative stress in PBMCs and invading inflammatory cells, and alterations in PBMC transcriptome. Thus, aldosteronism is associated with an activation of circulating immune cells induced by iterations in PBMC divalent cations and transduced by oxidative/nitrosative stress. ALDO receptor antagonism modulates this neuroendocrine-immune interface. The full text of this article is available online at http://www.circresaha.org. (Circ Res. 2003;93:e124-e135.)

Key Words: aldosterone ■ peripheral blood mononuclear cells ■ hydrogen peroxide production ■ cytosolic free Mg2+ and Ca2+ ■ transcriptome

Irrespective of its etiologic origins, asymptomatic ventricular systolic dysfunction eventuates in an activation of the circulating renin-angiotensin-aldosterone system (RAAS), whose effector hormones contribute to the appearance of the congestive heart failure (CHF) syndrome. A chronic systemic illness ensues that features oxidative/nitrosative stress in such diverse tissues as skeletal muscle, peripheral blood mononuclear cells (PBMCs) (monocytes and lymphocytes), and heart1–11; elevated circulating levels of proinflammatory cytokines and chemokines12–21; and a wasting syndrome that eventuates in cachexia.22 Pharmacological modulation of RAAS effector hormones has proven clinical benefits in patients with CHF.23–27

A role for angiotensin (Ang) II and aldosterone (ALDO) in the pathogenesis of the systemic illness that accompanies CHF is an area of ongoing research. A rodent model has been used to address the consequences of chronic inappropriate (relative to dietary Na+ intake) elevations in plasma ALDO comparable to those seen in human CHF. Treatment with ALDO and 1% dietary NaCl (ALDOST) rapidly suppresses plasma renin and Ang II.28,29 After 4 weeks of treatment with ALDOST, coronary vascular lesions are first seen in the normotensive, nonhypertrophied right atrium and ventricle and left atrium as well as in the hypertensive, hypertrophied left ventricle.30 Chronic mineralocorticoid excess, in combination with dietary salt excess and independent of blood pressure, is also known to adversely affect the structure of intramural arteries of systemic organs, including kidneys, pancreas, and mesentery, which can be prevented by ALDO-receptor antagonist.31–40

Original received May 20, 2003; resubmission received September 16, 2003; revised resubmission received October 3, 2003; accepted October 9, 2003.

From the Departments of Obstetrics & Gynecology (R.A.A.) and Surgery (S.K.B.) and Divisions of Cardiovascular Diseases (Y.S., L.A.W., L.L., K.T.W.), Connective Tissue Diseases (K.J.W., P.A.H., A.E.P.), and Endocrinology (I.C.G.), Department of Medicine, University of Tennessee Health Science Center, Memphis, Tenn.

Correspondence to Karl T. Weber, MD, University of Tennessee Health Science Center, Division of Cardiovascular Diseases, Room 353 Dobbs Research Institute, 951 Court Ave, Memphis, TN 38163. E-mail kweber@utmem.edu

© 2003 American Heart Association, Inc.

Circulation Research is available at http://www.circresaha.org

DOI: 10.1161/01.RES.0000102404.81461.25
Commonly featured in coronary vascular lesions are inflammatory cells and myofibroblasts. In the monocytes/macrophages and lymphocytes that invade intramural coronary arteries, Sun et al. found an induction of oxidative/nitrosative stress and activation of a redox-sensitive nuclear transcription factor-κB (NF-κB), together with upregulated mRNA expression of a proinflammatory mediator cascade that NF-κB regulates. Cotreatment with either spironolactone (Spi), an ALDO receptor antagonist, or an antioxidant prevented these molecular events. Eplerenone, another ALDO receptor antagonist, is also cardioprotective in this model. This proinflammatory/fibrogenic cardiac phenotype is not seen with ALDO plus a 0.4% NaCl diet or with a 1% NaCl diet alone. Moreover, cardioprotective effects of ALDO receptor antagonism during AldoST are seen with either nondepressor or depressor doses of Spi. The importance of antioxidant defenses, and circulating angiotensin II, is also cardioprotective in this model. A separate group of animals received AldoST together with Spi (100 mg/kg by daily gavage). Animals were observed daily for their physical activity and consumption of food and water. Systolic blood pressure was recorded weekly as previously reported. At the conclusion of weeks 1 to 4 of AldoST and AldoST + Spi, animals were weighed and anesthetized, blood was collected by cardiac puncture, and hearts were harvested.

**Plasma and Cardiac Tissue Mg²⁺ Concentrations**

Plasma was diluted 1:20 with 0.5% lantanum chloride, and Mg²⁺ concentration was quantified in 100 μL specimens using a Varian model 220 FS double-beam fast sequential atomic absorption spectrophotometer (Varian Techtron) using a modification of the method of Bhattacharya. Plasma Mg²⁺ levels are expressed in milligrams per deciliter. Microdetermination for Mg²⁺ concentration in ventricular myocardium was carried out in 12- to 15-mg dehydrated, defatted specimens after complete digestion in 0.75 mol/L Ultrex quality nitric acid (J.T. Baker Chemical Co) at 68°C for 15 hours. This procedure has been shown to extract >99% of Mg²⁺ from dry, defatted tissue. Tissue Mg²⁺ levels are expressed in nEq/mg of fat-free dry tissue.

**Isolation of PBMCs**

Heparinized whole blood (5 to 8 mL) was diluted to 10 mL with PBS (pH 7.4), layered on top of 5 mL Histopaque 1083, and centrifuged for 30 minutes at 400g. PBMCs were aspirated, washed twice, suspended in PBS, and counted with a hemocytometer.

**Quantitation of Total Mg²⁺ and Ca²⁺ Concentration in PBMCs**

Isolated PBMCs were washed three times with 140 mmol/L choline chloride. The PBMCs were then lysed with 2 mL deionized water and subjected to three cycles of 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 0.5% LaCl₃ solution, and the diluent was used to quantitate PBMC Mg²⁺ and Ca²⁺ levels by atomic absorption spectroscopy, as described elsewhere. The protein level in the PBMC suspension was assayed as previously described, and total Mg²⁺ and Ca²⁺ concentrations were expressed in micrograms per milligram of protein.

**Cytosolic Free [Mg²⁺], and [Ca²⁺], in PBMCs**

Separate PBMC aliquots (1 × 10⁶ cells) were loaded with the cell-permeant fluorescent probes mag-fura-2 acetoxymethyl ester and fura-2 acetoxymethyl ester (Molecular Probes) for the radiometric measurement of [Mg²⁺], and [Ca²⁺], respectively, using a Perkin-Elmer LS-50B spectrofluorometer according to the method of Delva et al. After loading cells with mag-fura-2 and washing them, cells were suspended in a buffer containing Mg²⁺ for spectrofluorometric measurement. Some mag-fura-2 leaks back out of the cells and is available to react with extracellular Mg²⁺. EDTA and EGTA are added to the suspension to chelate this extracellular Mg²⁺ so that only intracellular Mg²⁺/mag-fura-2 fluorescence (or resting cytosolic ionized Mg²⁺) is measured. As a result of this chelation of extracellular Mg²⁺, there is a small decrease in fluorescence. It is this latter value, after the addition of EDTA and EGTA, that represents true cytosolic Mg²⁺ and is reported herein. The measurement of Ca²⁺ reported herein is thus made after addition of the chelator EGTA so that only free cytosolic Ca²⁺ is measured. Specific details can be found elsewhere, and an online data supplement is available at http://www.circresaha.org.

**Materials and Methods**

**Animals**

Eight-week-old male Sprague-Dawley rats (Harlan, Indianapolis, Ind) were used. The study was approved by the institution’s Animal Care and Use Committee. Unoperated, untreated age- and gender-matched rats served as one control group (n = 5). Uninephrectomized rats receiving 1% NaCl/0.4% KCl in drinking water and standard laboratory chow served as a second control group (n = 10). Separate groups of uninephrectomized, salt-treated rats received AldoST (0.75 μg/h) by implanted minipump (Alzet) for 1 to 4 weeks (n = 10 at each time point). This dose of AldoST promptly raises the plasma levels in rats to those seen in humans with CHF. AldoST rapidly suppresses plasma renin activity and circulating angiotensin II. A separate group of animals received AldoST together with Spi (100 mg/kg by daily gavage). Animals were observed daily for their physical activity and consumption of food and water. Systolic blood pressure was recorded weekly as previously reported. At the conclusion of weeks 1 to 4 of AldoST and AldoST + Spi, animals were weighed and anesthetized, blood was collected by cardiac puncture, and hearts were harvested.

**Methods**

Several methods were used to fractionate and purify PBMCs. Tissue Mg²⁺ levels are expressed in milligrams per deciliter. Microdetermination for Mg²⁺ concentration in ventricular myocardium was carried out in 12- to 15-mg dehydrated, defatted specimens after complete digestion in 0.75 mol/L Ultrex quality nitric acid (J.T. Baker Chemical Co) at 68°C for 15 hours. This procedure has been shown to extract >99% of Mg²⁺ from dry, defatted tissue. Tissue Mg²⁺ levels are expressed in nEq/mg of fat-free dry tissue.

**Isolation of PBMCs**

Heparinized whole blood (5 to 8 mL) was diluted to 10 mL with PBS (pH 7.4), layered on top of 5 mL Histopaque 1083, and centrifuged for 30 minutes at 400g. PBMCs were aspirated, washed twice, suspended in PBS, and counted with a hemocytometer.
Hydrogen Peroxide Generation by PBMCs
For the measurement of hydrogen peroxide (H₂O₂) production, 100-µL aliquots of whole blood obtained serially from the same animals by cardiac puncture were incubated with 2,7-dichlorofluorescein diacetate (25 µmol/L) for 45 minutes at 37°C. After lysing erythrocytes with FACs lysis solution (Becton Dickinson), leukocytes were washed twice and suspended in PBS (pH 7.4). Lymphocyte and monocyte H₂O₂ production was measured using a FACs Caliber flow cytometer (Becton Dickinson) according to the method of Bass et al.²⁶ For specific details, see the online data supplement.

PBMC Transcriptome
Total RNA was isolated from purified PBMC using a trireagent (Invitrogen). The gene expression analysis was conducted as previously described²⁵,²⁷ using the Affymetrix rat genome U34A chip (Affymetrix), probing ~7000 known genes and 1000 expressed sequence tags. A total of six unoperated/untreated controls, six ALDOST obtained at each time point, and six ALDOST+Spi obtained at each time point went into the characterization of transcriptomes. Each sample analyzed on expression array chips consisted of pooled RNA from three animals. We compared transcriptomes from untreated controls with samples obtained at weeks 1 through 4 of ALDOST to produce a list of genes affected by the treatment. The experiment was repeated, and only genes that showed differential expression (≥2-fold) in response to treatment in both of these independent experiments are reported as differentially expressed genes.

**Multicolor Flow Cytometric Analysis of Lymphocyte Activation**
Heparinized blood was obtained by cardiac puncture, and PBMCs were isolated by density-gradient centrifugation over Histopaque 1077 (Sigma). PBMCs were counted, washed twice, and suspended in Dulbecco’s PBS (Invitrogen Corporation) supplemented with 2% FCS. For cell-surface labeling, PBMCs (9×10⁶/sample) were incubated with a cocktail of FITC-labeled, PE-labeled, PerCP-labeled, and APC-labeled antibodies at 4°C for 20 minutes, washed, and resuspended in PBS-2% FCS. The mouse anti-rat mAbs used for flow cytometry in this study were as follows: FITC-conjugated G4.18 (anti-CD3) and OX-33 (anti-CD45RA), PE-conjugated OX-18 (anti-major histocompatibility [MHC] class I/RT1-A), PerCP-conjugated OX-8 (anti-CD8) and OX-6 (anti-MHC class II/RT1-B), and APC-conjugated IF4 (anti-CD3) and OX-35 (anti-CD4) (all from BD Biosciences, San Jose, Calif). Parallel samples of cells were also incubated with 1g isotopic controls (BD Biosciences). Optimal antibody dilutions were determined in preliminary experiments. All samples were immediately analyzed on a FACS Caliber flow cytometer (Becton Dickinson). Fluorescence data from at least 30 000 cells (from a lymphocyte gate) were collected for each sample. Offline analyses of raw data were performed using WinMDI software (J. Trotter, Scripps Institute).

3-Nitrotyrosine in Invasive Inflammatory Cells
Expression of oxidative/nitrosative stress was studied by immunohistochemical localization of 3-nitrotyrosine. Lymphocytes and macrophages were detected by immunohistochemical assessment using CD4 and ED-1, respectively. 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, Waltham, Mass) or CD4 at a dilution of 1:50 (Becton Dickinson) or ED-1 at a dilution of 1:140 (Harlan Bioproducts) 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, incubated with 0.5 mg/mL diaminobenzidine tetrahydrochloride 2-hydrate plus 0.05% H₂O₂ for 10 minutes, and rewashed in PBS. Negative control sections were incubated with secondary antibody alone, stained with hematoxylin, dehydrated, mounted, and examined by light microscopy.

**Results**

**Animals**
During weeks 1 and 2 of ALDOST treatment, 9- and 10-week-old rats were active, eating, and drinking. They were also gaining weight comparable to age- and gender-matched controls (Figure 1). Rats receiving ALDOST plus Spi cotreatment for weeks 1 and 2 were also healthy, and their body weight was no different from controls (Figure 1). This preclinical stage was followed by the appearance of lethargy and anorexia during weeks 3 and 4 of ALDOST. During this clinical stage, rats failed to gain weight, and their body weight was no longer comparable to controls (Figure 1). On the other hand, animals receiving Spi cotreatment remained healthy, active, and gained weight similar to that observed in both control groups (Figure 1).

Systolic blood pressure at week 1 ALDOST was no different from unoperated/untreated and uninephrectomized/salt-treated control groups but rose gradually thereafter and was significantly greater (P<0.05) than controls at weeks 3 and 4 (Table). Cotreatment with Spi prevented the gradual rise in blood pressure, with animals remaining normotensive throughout the 4-week period of observation (Table).

**Weekly Blood Pressure in Controls and in ALDOST and ALDO+Spi Groups**

<table>
<thead>
<tr>
<th></th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>UD</td>
<td>119±6</td>
<td>122±8</td>
<td>120±7</td>
<td>118±10</td>
</tr>
<tr>
<td>UN</td>
<td>117±5</td>
<td>124±6</td>
<td>118±10</td>
<td>121±9</td>
</tr>
<tr>
<td>ALDOST</td>
<td>121±7</td>
<td>127±7</td>
<td>145±10</td>
<td>187±13*</td>
</tr>
<tr>
<td>ALDO+Spi</td>
<td>123±7</td>
<td>126±9</td>
<td>125±12</td>
<td>131±10</td>
</tr>
</tbody>
</table>

*P<0.05 vs controls; mean±SEM.
Plasma and Cardiac Tissue Mg\textsuperscript{2+} Concentrations
The concentration of plasma Mg\textsuperscript{2+} at 4 weeks of ALDOST treatment (1.51±0.10 mg/dL) was not different from unoperated/untreated or uninephrectomized/salt-treated controls (1.40±0.06 and 1.45±0.15 mg/dL, respectively) and was not altered by cotreatment with Spi (1.74±0.17 mg/dL). The concentration of Mg\textsuperscript{2+} in cardiac tissue in unoperated/untreated and uninephrectomized/salt-treated controls was 79.91±9.61 and 80.17±9.32 nEq/mg FFDT and remained unchanged at 4 weeks ALDOST (75.77±6.34 nEq/mg FFDT).

Total Intracellular Mg\textsuperscript{2+} and Ca\textsuperscript{2+} in PBMCs
Total Mg\textsuperscript{2+} in PBMCs harvested from untreated controls was 1.45±0.05 µg/mg protein. At weeks 1 through 4 of ALDOST treatment, this value was found to be increased to 1.63±0.13, 1.85±0.07, 1.68±0.03, and 1.71±0.11 µg/mg protein, respectively. The total concentration of Ca\textsuperscript{2+} in PBMCs obtained from controls was 0.60±0.05 µg/mg protein. PBMC total Ca\textsuperscript{2+} was increased in response at 1 to 4 weeks of ALDOST treatment to 0.87±0.03, 0.77±0.08, 0.83±0.23, and 1.14±0.11 µg/mg protein, respectively.

Cytosolic Free [Mg\textsuperscript{2+}]i and [Ca\textsuperscript{2+}]i in PBMCs
No difference in PBMC count was observed between controls and ALDOST with or without Spi cotreatment at any weekly time point (data not shown). Compared with controls and as shown in the left panel of Figure 2, PBMC [Mg\textsuperscript{2+}]i was significantly (P<0.05) reduced at week 1 of ALDOST. At week 2, ionized [Mg\textsuperscript{2+}]i levels were again normal and no different from unoperated/untreated or uninephrectomized/salt-treated controls. Therefore, [Mg\textsuperscript{2+}]i was again reduced (P<0.05) at weeks 3 and 4 of ALDOST. Spi cotreatment did not alter these sequential changes in [Mg\textsuperscript{2+}]i observed with 1 to 3 weeks of ALDOST, but [Mg\textsuperscript{2+}]i was at control levels at week 4 (Figure 2, left).

At week 1 of ALDOST, PBMC [Ca\textsuperscript{2+}]i was unchanged from controls but rose progressively thereafter and was greater than both control groups at weeks 2, 3, and 4 (Figure 2, right). Spi cotreatment abrogated intracellular Ca\textsuperscript{2+} loading during weeks 2 through 4 of ALDOST (Figure 2, right).

Hydrogen Peroxide Generation by PBMCs
At week 1 of ALDOST, H\textsubscript{2}O\textsubscript{2} generation was no different from baseline levels before uninephrectomy and to initiating ALDOST (Figure 3). At week 2 of ALDOST, monocytes (left) and lymphocytes (right) demonstrated increased H\textsubscript{2}O\textsubscript{2} production compared with baseline and week 1 values, and this was sustained at weeks 3 and 4 (Figure 3). Spi cotreatment abrogated increased H\textsubscript{2}O\textsubscript{2} generation by both monocytes and lymphocytes at weeks 2, 3, and 4 of ALDOST treatment (Figure 3).

PBMC Transcriptome
For the analysis of PBMC-expressed genes, ie, their transcriptome, three pooled blood samples were obtained from nine controls, whereas those harvested weekly from rats receiving either ALDOST or cotreatment with Spi were harvested from 24 rats per treatment group with six rats at each time point per group. Gene chip array analysis was interrogated and compared for the differential (=2-fold) expression (either upregulated or downregulated) of genes related to shifts in intracellular monovalent and divalent cations, Na\textsuperscript{+}, Mg\textsuperscript{2+}, and Ca\textsuperscript{2+}, the presence of oxidative/nitrosative stress, and PBMC activation and phenotype.

Relevant to the decline in PBMC [Mg\textsuperscript{2+}]i seen during week 1 of the preclinical stage of ALDOST, which was presumably...
accompanied by Na\(^+\) loading (not measured), we found upregulated gene expression of an ATPase inhibitor protein (Figure 4, top left) and an Na\(^+\)-dependent transporter (Figure 4, bottom left), which was sustained over 4 weeks. Spi cotreatment attenuated these responses. Other upregulated PBMC genes seen during this time frame with ALDOST (not shown) included somatostatin receptor and Na\(^+\)/H\(^+\)-dependent serotonin transporter, whereas the \(\alpha_1\) isoform of Na\(^+\)/K\(^+\)-ATPase was downregulated. Spi cotreatment served to attenuate these responses at all time points. We did not find specific genes that only became markedly (>2-fold) upregulated or downregulated at weeks 3 or 4 of ALDOST treatment or that responded in a like manner to Spi cotreatment at these time points.

Intracellular Ca\(^{2+}\) loading, initially of organelles, and subsequently free ionized cytosolic levels appeared during the preclinical stage of ALDOST and were associated with upregulated expression of an ATP-dependent Ca\(^{2+}\) pump (Figure 4, top right) and calmodulin kinase, a Ca\(^{2+}\)-dependent protein kinase C–associated kinase (Figure 4, bottom right). Cotreatment with Spi attenuated these responses. Other Ca\(^{2+}\)-related genes that were upregulated during this time period of ALDOST (not shown) included calgranulin A, a Ca\(^{2+}\)-binding chemokine, and proteins involved in intracellular Ca\(^{2+}\) binding, such as calgranulin B, lipocortin I, and a Ca\(^{2+}\)-binding protein. A downregulation in gene expression (not shown) was seen for Ca\(^{2+}\)-inhibitable adenyl cyclase, whereas FAK-2, a Ca\(^{2+}\)-dependent tyrosine kinase, was unchanged from controls. Spi attenuated the response in Ca\(^{2+}\) binding protein but did not alter these other responses at any time point. We did not find specific genes that were first markedly upregulated or downregulated at weeks 3 or 4 of ALDOST or that were similarly altered by Spi cotreatment.

The presence of oxidative/nitrosative stress in PBMCs throughout 4 weeks of ALDOST treatment was evidenced by responses in their transcriptome. This included an early, upregulated expression of oxidative stress–inducible tyrosine phosphatase (not shown) and such antioxidant defenses as Mn\(^{2+}\)-superoxide dismutase and l-cysteine oxireductase (Figure 5, top and bottom). Inducible NO synthase was also upregulated (not shown) and is integral to NO formation that regulates the mitochondrial electron transport chain, a major source of reactive oxygen species. Glutathione peroxidase...
and reductase, catalase, and NADPH oxireductase were not altered. During weeks 2 through 4 of ALDOST and accompanying the increased H₂O₂ production by PBMCs, we found an activation and iteration in their phenotype. Evidence of early PBMC activation included upregulated expression of intracellular adhesion molecule (ICAM)-1 and integrin α1 (Figure 6, top and bottom), cell adhesion regulator, CC chemokine receptor protein, chemokine receptor CCR2, CXC chemokine receptor, interleukin (IL)-1β and its receptor type 2 and accessory protein, and interferon-γ-inducible GTP cyclohydrolase. Spi attenuated these responses. An iteration in PBMC phenotype was suggested by a downregulation in MHC class I molecule together with upregulated expression of MHC class II Aβ (Figure 6, top right), IgE binding protein, IgG2b rearranged gene, and IgA constant region (not shown). Autoreactivity was evidenced by upregulated gene expression of antibodies to acetylcholine receptors and nerve growth factor and a downregulation to the expression of RT-6.2 (Figure 6, bottom right), each of which was attenuated by Spi cotreatment.

**Lymphocyte Activation**

Our transcriptome data suggested ALDOST treatment is accompanied by B-cell activation (eg, increased immunoglobulin gene transcription). At week 4, we determined the B/T cell ratio in control, ALDOST, and Spi cotreated rats by flow cytometry (Figure 7) and found a relative expansion of the B lymphocyte subset in ALDOST rats compared with UN controls and that was attenuated by Spi. Moreover, MHC class II–expressing lymphocytes were increased in ALDOST
rats at week 4, consistent with the upregulation of MHC II genes. The increase in MHC class II-positive cells is also in keeping with the immune activation induced by ALDOST. Indeed, by multicolor flow cytometry, we confirmed that the B-cell subset is the major class II-expressing population of lymphocytes. Within the CD3+ T-cell population, we did not detect differences in the CD4/CD8 ratio among the various treatment groups (data not shown).

3-Nitrotyrosine in Immune Cells Invading the Coronary Vasculature

As reported previously,51,52 the proinflammatory coronary vascular phenotype first appears at week 4 of ALDOST treatment. Peroxynitrite (ONOO−) is a potent reactive nitrogen species formed by the reaction of NO and superoxide. Its short half-life makes detection of ONOO− difficult. However, its reaction with stable tyrosine residues forms a stable 3-nitrotyrosine derivative and serves as a marker of nitrosative stress. Immunohistochemical evidence of 3-nitrotyrosine was found in inflammatory cells, including CD4+ lymphocytes, that invaded the coronary vasculature of the right and left heart at week 4 of ALDOST (Figures 8A and 8C). ED-1-positive macrophages were also found to have 3-nitrotyrosine labeling (not shown). Cotreatment with Spi prevented the appearance of 3-nitrotyrosine labeling in cells that invaded intramural coronary arteries (Figures 8B) and attenuated the number of inflammatory cells seen at these sites.

Discussion

Herein we hypothesized that chronic treatment with ALDO and 1% dietary NaCl leads to an iteration in PBMC divalent cation composition, which, in turn, accounts for an induction of oxidative/nitrosative stress and activation of these immune cells that subsequently invade the intramural coronary vasculature of the right and left heart. We additionally hypothesized that by abrogating these responses in PBMC divalent cations, Spi would be immunomodulatory. Our study led to several major findings.

Beginning with the preclinical stage of ALDOST, we found a reduction in PBMC [Mg2+], that was significantly lower than levels found in PBMCs obtained from either of our two control groups or historical controls reported by others.59 Furthermore, the fall in this biologically active component of intracellular Mg2+ seen with ALDOST is in keeping with significantly reduced human lymphocyte [Mg2+], found in patients with primary aldosteronism.60 The mechanism responsible for the reduction in [Mg2+], is unknown. It could involve an efflux from the cell, a shift within the cell’s compartments, or both. Delva et al50 reported a Na+-dependent, ALDO-mediated reduction in [Mg2+], in cultured human lymphocytes that involved transcription and protein synthesis; thereby, a putative Na+/Mg2+ exchange site59 was implicated. In chicken erythrocytes, Mg2+ efflux is dependent on extracellular Na+ with a stoichiometry of 1 Mg2+ coupled with the influx of 2 Na+ via a Na+/Mg2+ exchanger.60 Cytosolic free [Mg2+] represents 0.5% to 5% of total cellular Mg2+, and the remainder is bound to ATP and other phosphometabolites sequestered within such organelles as mitochondria and endoplasmic reticulum.61 At week 2 of ALDOST treatment, PBMC [Mg2+] was similar to that seen in our controls. This might reflect homeostatic regulation from these organelles, although the appearance of a new PBMC population cannot be ruled out. A decline in PBMC [Mg2+] was again seen at weeks 3 and 4 of ALDOST. Other explanations accounting for the reduction in [Mg2+] need to be considered. PBMC total Mg2+ concentration was increased during weeks 1 through 4 of ALDOST and likely includes the activation of protein kinase C to promote Mg2+ entry and compartmentalization with the opening of mitochondrial permeability transition pores induced by oxidative/nitrosative stress and Ca2+ loading.62–64 A reduction in organelle Mg2+ stores therefore cannot be implicated in the fall of [Mg2+]. Given that cytosolic free [Mg2+], represents such a small fraction of total Mg2+, it is not likely that the observed increase in total Mg2+ could be attributed to this source. We did not find a reduction in plasma [Mg2+] or a decline in cardiac tissue [Mg2+] with 4 weeks of ALDOST, even though urinary Mg2+ excretion (not measured herein) can be enhanced by ALDOST.65 We cannot implicate dietary Mg2+ deficiency, given that the Mg2+ content of our standard chow (20 to 40 mmol/kg) is in keeping with daily requirements and far greater than that (<2 mmol/kg) needed to induce dietary Mg2+ deficiency.66,67 Future studies are planned to address responsible mechanisms.

Mg2+ is involved in >300 enzymatic reactions, including Mg2+-dependent Na+/K+-ATPase.59,61 A reduction in the activity of this exchanger leads to a rise in intracellular Na+ followed by the stoichiometric exchange of 3 Na+ for 1 Ca2+ via a Na+/Ca2+ exchanger.68,69 At week 1 of ALDOST treatment, analysis of PBMC transcriptome revealed upregu-
lated expression of an ATPase inhibitor protein and Na+-dependent transporter, together with a downregulation in α, isoform of Na+/K+-ATPase and upregulation in ATP-dependent Ca2+ pump, each of which persisted during subsequent weeks of ALDOST treatment. Spi attenuated these responses. Total intracellular Ca2+ was increased throughout the 4-week period of ALDOST and is likely responsible for the early and persistent induction of oxidative/nitrosative stress. PBMC [Ca2+]i, rose at weeks 2 through 4 of ALDOST treatment, in keeping with the saturation of organela stores during week 1. ALDO and extracellular Na+ are each known to upregulate Ca2+ uptake in various cells, including lymphocytes.70,71 Furthermore, ALDO reversibly downregulates the activity of a Na+/Ca2+ exchanger, which would inhibit net Ca2+ efflux from PBMCs.72,73 Collectively, these responses would account for intracellular Ca2+ loading, which we observed for both cytosolic free [Ca2+]i, and total Ca2+ concentration of PBMCs. In both humans and experimental animals, chronic mineralocorticoid excess, derived from either endogenous or exogenous sources and inappropriate for dietary Na+, is associated with a rise in platelet [Ca2+]i, and release of endogenous, circulating ouabain, a Na+/K+-ATPase inhibitor, which normalized after surgical ablation.74-78 Na+-Ca2+ exchange is dependent on cell Na+ and is competitively inhibited by Mg2+.79 A 4-g NaCl diet in a black population with salt-sensitive hypertension is accompanied by increases in erythrocyte Ca2+ and Na+ concentrations and Ca2+-ATPase activity, whereas [Mg2+]i, and Na+/K+-ATPase activity are each reduced.80 Spi cotreatment in our rodent model of ALDOST prevented the rise in [Ca2+]i, that appeared at weeks 2 through 4 of ALDOST treatment, suggesting that it altered Na+ delivery to and Na+/Ca2+ exchange in PBMCs.81 The fall in [Mg2+]i, which was not prevented by Spi, does not therefore seem to be an absolute prerequisite to PBMC Ca2+ loading. However, a reduction in [Mg2+]i, a physiological antagonist to Ca2+ entry, would indirectly elevate [Ca2+]i.82 ALDO also increases the density of Ca2+ current and Ca2+ channel expression, responses blunted by Spi.74 Nifedipine, a dihydropyridine Ca2+ entry blocker, prevents renovascular lesions that accompany chronic mineralocorticoid excess.83 Thus, our findings implicate Na+-dependent intracellular Ca2+ loading as integral to other events that appeared in PBMCs during ALDOST. Increased [Ca2+]i, is an intracellular messenger integral to lymphocyte activation that appears in response to antigen-binding and antigen-presenting cells.84 It has been reported to play a major regulatory role in the generation of reactive oxygen species in chemotactic factor–stimulated neutrophils.85 Concordant with the rise in intracellular Ca2+ was the induction of oxidative/nitrosative stress in PBMCs, as indicated by several lines of evidence. PBMC transcriptome at 1 to 4 weeks of ALDOST treatment revealed overexpression of oxidative stress–inducible tyrosine phosphatase and upregulation of enzymes associated with antioxidant defense systems, such as Mn+-superoxide dismutase (SOD), l-cysteine, and NADPH oxireductases, and inducible NO synthase. In monocytes and lymphocytes obtained from rats receiving ALDOST at 2 to 4 weeks, we found evidence of increased H2O2 production, suggesting antioxidant defenses in these cells were no longer able to neutralize this stress, as presumably had been the case at week 1. Persistent oxidative/nitrosative stress may have contributed to the appearance of the systemic illness and catabolic state seen in these rats at weeks 3 through 4 of ALDOST, which featured lethargy, anorexia, and a failure to gain weight. In preventing oxidative/nitrosative stress, Spi cotreatment prevented this clinical response. At week 4 of ALDOST treatment, we found immunohistochemical localization of 3-nitrotyrosine in inflammatory cells that invaded intramural coronary arteries. Sun et al82 have previously reported the presence of oxidative/nitrosative stress in these immune cells that first invade the coronary circulation at 4 weeks of ALDOST treatment. This included activation of gp91phox, a membrane-bound NADPH oxidase subunit, and the RelA subunit of a redox-sensitive NF-kB, together with a proinflammatory mediator cascade it regulates. This cascade included upregulated mRNA expression of ICAM-1, monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor (TNF)-α. These cellular and molecular events were not seen when ALDOST was combined with either an antioxidant or Spi. Herein, we report the prevention of the rise in PBMC [Ca2+]i by Spi cotreatment, and this negated the induction of oxidative/nitrosative stress in these immune cells that invade the coronary vasculature.

A third finding of our study was the activation of PBMCs before the appearance of the proinflammatory coronary vascular phenotype seen at week 4 of ALDOST treatment. This occurred in the absence of myocardium-derived antigen, given that the heart in our rat model is intact and free of prior injury. This early immunostimulatory state included (1) B-cell activation with increased expression of immunoglobulins; (2) an expansion of the B-cell lymphocyte subset; (3) an increase in MHC class II–expressing lymphocytes; (4) increased expression of ICAM-1, integrin α6, CC chemokine receptor protein and receptor CCR5, CXC chemokine receptor, IL-1β, its receptor type 2, and accessory protein; and (5) inducible interferon-γ. There was also evidence to implicate autoreactivity with increased expression of antibodies to acetylcholine receptors and nerve growth factor and reduced RT-6.2. RT-6, an alloantigenic marker of mature resting T cells, is lost on activation. Cytotoxic T cells do not display RT-6 and are associated with autoimmunity.84-87 RT-6 exists in at least two allelic forms, RT-6.1 and RT-6.2, where RT-6.2 consists of a nonglycosylated 25- and 28-kDa form.88 RT-6.2. RT-6, an alloantigenic marker of mature resting T lymphocytes; (4) infiltrating lymphocytes; expressing lymphocytes; (4) inflammatory cells that invaded intramural coronary arteries. Sun et al82 have previously reported the presence of oxidative/nitrosative stress in these immune cells that first invade the coronary circulation at 4 weeks of ALDOST treatment. This included activation of gp91phox, a membrane-bound NADPH oxidase subunit, and the RelA subunit of a redox-sensitive NF-kB, together with a proinflammatory mediator cascade it regulates. This cascade included upregulated mRNA expression of ICAM-1, monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor (TNF)-α. These cellular and molecular events were not seen when ALDOST was combined with either an antioxidant or Spi. Herein, we report the prevention of the rise in PBMC [Ca2+]i by Spi cotreatment, and this negated the induction of oxidative/nitrosative stress in these immune cells that invade the coronary vasculature.

A third finding of our study was the activation of PBMCs before the appearance of the proinflammatory coronary vascular phenotype seen at week 4 of ALDOST treatment. This occurred in the absence of myocardium-derived antigen, given that the heart in our rat model is intact and free of prior injury. This early immunostimulatory state included (1) B-cell activation with increased expression of immunoglobulins; (2) an expansion of the B-cell lymphocyte subset; (3) an increase in MHC class II–expressing lymphocytes; (4) increased expression of ICAM-1, integrin α6, CC chemokine receptor protein and receptor CCR5, CXC chemokine receptor, IL-1β, its receptor type 2, and accessory protein; and (5) inducible interferon-γ. There was also evidence to implicate autoreactivity with increased expression of antibodies to acetylcholine receptors and nerve growth factor and reduced RT-6.2. RT-6, an alloantigenic marker of mature resting T cells, is lost on activation. Cytotoxic T cells do not display RT-6 and are associated with autoimmunity.84-87 RT-6 exists in at least two allelic forms, RT-6.1 and RT-6.2, where RT-6.2 consists of a nonglycosylated 25- and 28-kDa form.88 RT-6.2. RT-6, an alloantigenic marker of mature resting T lymphocytes; (4) infiltrating lymphocytes; expressing lymphocytes; (4) inflammatory cells that invaded intramural coronary arteries. Sun et al82 have previously reported the presence of oxidative/nitrosative stress in these immune cells that first invade the coronary circulation at 4 weeks of ALDOST treatment. This included activation of gp91phox, a membrane-bound NADPH oxidase subunit, and the RelA subunit of a redox-sensitive NF-kB, together with a proinflammatory mediator cascade it regulates. This cascade included upregulated mRNA expression of ICAM-1, monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor (TNF)-α. These cellular and molecular events were not seen when ALDOST was combined with either an antioxidant or Spi. Herein, we report the prevention of the rise in PBMC [Ca2+]i by Spi cotreatment, and this negated the induction of oxidative/nitrosative stress in these immune cells that invade the coronary vasculature.

A third finding of our study was the activation of PBMCs before the appearance of the proinflammatory coronary vascular phenotype seen at week 4 of ALDOST treatment. This occurred in the absence of myocardium-derived antigen, given that the heart in our rat model is intact and free of prior injury. This early immunostimulatory state included (1) B-cell activation with increased expression of immunoglobulins; (2) an expansion of the B-cell lymphocyte subset; (3) an increase in MHC class II–expressing lymphocytes; (4) increased expression of ICAM-1, integrin α6, CC chemokine receptor protein and receptor CCR5, CXC chemokine receptor, IL-1β, its receptor type 2, and accessory protein; and (5) inducible interferon-γ. There was also evidence to implicate autoreactivity with increased expression of antibodies to acetylcholine receptors and nerve growth factor and reduced RT-6.2. RT-6, an alloantigenic marker of mature resting T cells, is lost on activation. Cytotoxic T cells do not display RT-6 and are associated with autoimmunity.84-87 RT-6 exists in at least two allelic forms, RT-6.1 and RT-6.2, where RT-6.2 consists of a nonglycosylated 25- and 28-kDa form.88 Circulating levels of CC chemokines, including MCP-1, macrophage inflammatory protein (MIP)-1α, and RANTES (Regulated on Activation Normally T Cell Expressed and Secreted), are increased particularly in patients with advanced, symptomatic failure at rest (NYHA class IV) irrespective of its etiologic origins.16 Monocytes and CD3+ lymphocytes (T lymphocytes) obtained from individuals with NYHA class III and IV failure have been studied in culture with respect to their release of CC chemokines.16 In response to stimulation of monocytes with LPS or CD3+ lymphocytes with anti-CD3/anti-CD28 monoclonal antibodies, increased release of MIP-1α and MCP-1 was observed for monocytes and increased release of RANTES by CD3+ lymphocytes (vis-à-vis healthy blood donor controls). The effect of serum
from these patients on superoxide generation by cultured monocytes harvested from healthy blood donors was also examined.16 Spontaneous and provoked generation of superoxide was enhanced and to an extent related to serum MCP-1 levels, which could be inhibited by neutralizing antibodies to this CC chemokine. Serum levels of CXC chemokines, including IL-8, growth-regulated oncogene α, and epithelial neutrophil activating peptide-78, are also elevated in patients with heart failure and to an extent related to the severity of symptomatic heart failure.17 Furthermore, spontaneous and LPS-stimulated monocytes from these patients release elevated amounts of these CXC chemokines.17 Damás et al18 demonstrated upregulated PBMC gene expression (ribonuclease protection assay) for MIP-1α, MIP-1β, IL-8, and their corresponding receptors, which include CCR1 and CCR5, CXC chemokine receptor 1, and CXC chemokine receptor 2 in patients with heart failure of diverse causality. Thus, in humans with symptomatic heart failure, where salt and water retention and elevated plasma levels of ALDO are expected, as well as in our rodent model of ALDOST, there is evidence of sustained monocyte and lymphocyte activation. Such “run-away inflammation” may contribute to the chronic “cytokine storm” seen with the CHF syndrome. An initial immunostimulatory state that begins during week 1 of ALDOST treatment, based on Na+-dependent, ALDO-induced activation of PBMCs, is sustained and begets an immunopathologic state with cardiac lesions at week 4. Dietary-induced Mg2+ deficiency and associated aldosteronism is accompanied by reduced Mg2+ and increased Na+ and Ca2+ in lymphocytes,91 evidence of oxidative/nitrosative stress in plasma, reduced antioxidant reserves in PBMCs, elevated PBMC proinflammatory cytokine production at week 1, and a delayed appearance in cardiac pathology seen at week 3,66,67 together with upregulated expression of stress proteins and glutathione transferase in neutrophils and thymocytes.92,93 The appearance of exaggerated immune cell responses seen with Mg2+ deficiency includes superoxide anion production and enhanced [Ca2+]i, which have been attributed to abnormal Ca2+ handling.94

We believe the antigen-independent activation of cellular immunity seen with ALDOST is related to H2O2 production and its role as second messenger. Reth95 has reviewed the evidence implicating H2O2 as second messenger capable of antigen mimicry. Redox-regulated proteins include transcription factors that can either prevent (p53) or stimulate (p50) their transcriptional activity. Other redox-regulated proteins, which we observed in our analysis of PBMC transcriptome, are the protein tyrosine phosphatases. They are rendered inactive by H2O2. In lymphocytes, H2O2 can be spontaneously generated from superoxide and protons in water or catalyzed by cytosolic SOD. Another source of superoxide is NADPH oxidase/reductase. In response to ALDOST, we found upregulated expression of both SOD and NADPH oxidase/reductase in PBMC transcriptome. Sun et al12 found immunohistochemical evidence that the gp91phox subunit of NADPH oxidase was activated in cells invading the intramural coronary vasculature. NADPH oxidase is pertinent to inducible oxidative/nitrosative stress in lymphocytes during signal transduction.96 Lymphocytes produce H2O2 on stimulation of their antigen receptor, whereas immunoglobulins (or antibodies) do not require a particular antigen binding site to incite H2O2 production.97–99 Receptors can be activated in a ligand-independent manner when immune cells are treated with H2O2. B-cell activation leads to antibody production. In patients with heart failure of diverse origins, circulating antibodies to muscarinic M3-acetylcholine receptors (AChR) have been observed and correlated with the severity of their symptomatic status.100–102 Specific immune responses may be involved in activated PBMCs targeting the intramural coronary vasculature and could explain why cardiac lesions of the right and left heart are not seen until week 4 of ALDOST treatment. Adoptive transfer studies have been planned to address the issues concerning an autoimmune response. However, the need for coronary endothelial cell activation in contributing to this response remains uncertain.

Findings of this study relate to the pathophysiology of chronic cardiac failure in humans, where an activation of the circulating RAAS is accompanied by a progressive systemic illness that features oxidative/nitrosative stress, lethargy, and a catabolic state with wasting. Herein, we found ALDOST in rodents to lead to an early activation of PBMCs before the appearance of lethargy, anorexia, failure to gain weight, and coronary lesions. The prospect of B-cell activation and autoreactivity in the absence of antigen presentation calls into question their potential for autoimmune-mediated injury that targets the intramural vasculature and leads to a progressive structural remodeling of involved vessels. Functional consequences of antibody interference with AchR seen with ALDOST may include a modulation of parasympathetic control of heart rate and conduction,103 reduced baroreceptor discharge,106 impaired vasodilator reserve to Ach,90,107 and nerve growth factor that maintains the integrity of sympathetic innervation.108 Our observations broaden the paradigm embraced by the concept of a neuroendocrine-immune interface.109,110

In summary, findings of this study have addressed several gaps in our knowledge. In aldosteronism, PBMCs are activated before invading the intramural coronary arterial circulation. Activation of these immune cells by intracellular Ca2+ loading leads to the induction of oxidative/nitrosative stress, including H2O2 production, which likely serves as second messenger to mimic antigen-receptor binding and lymphocyte activation. The clinical efficacy of ALDO receptor antagonism in the management of symptomatic heart failure,26,27 where aldosteronism is expected, may include its ability to modulate this neuroendocrine-immune interface.

Acknowledgments

This study was supported in part by NIH grants R01-DK62403, R24-RR-15373, and R21-DK-55263 (to I.C.G.), National Heart, Lung, and Blood Institute grants R01-HL67888 (to Y.S.), and R01-HL62229 (to K.T.W.) and grants from the UTHSC Center of Excellence in Connective Tissue Diseases (to Y.S. and K.T.W.).

References

10 Circulation Research November 14, 2003


57. Deleted in proof.


Aldosteronism and Peripheral Blood Mononuclear Cell Activation: A Neuroendocrine-Immune Interface


Circ Res. 2003;93:e124-e135; originally published online October 23, 2003;
doi: 10.1161/01.RES.0000102404.81461.25

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/93/10/e124

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2003/11/12/93.10.e124.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Online Data Supplement

Expanded Materials and Methods

Cytosolic Free [Mg$^{2+}$], and [Ca$^{2+}$], in PBMC

We measured [Mg$^{2+}$], and [Ca$^{2+}$], in isolated PBMC ratiometrically using the cell permeant fluorescent ion probes mag-fura-2 acetoxymethyl ester and fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR, USA), respectively using a modification of the method of Delva et al. (1). For more details and documentation of the ratiometric measurement of Mg$^{2+}$ and Ca$^{2+}$ see References 2 and 3.

For loading the cells, separate aliquots (1×10$^6$ cells each) were suspended in RPMI-1640 culture media with 0.1% (v/v) bovine albumin. After incubation for 15 min at 37°C to allow equilibration, mag-fura-2 acetoxymethyl ester (10 µmol/L) for the measurement of [Mg$^{2+}$], or fura-2 acetoxymethyl ester (5 µmol/L) for the measurement of [Ca$^{2+}$], was added to the cell suspensions and incubated for an additional 1 hour to allow maximal cell uptake of the fluorescent probes. After centrifugation for 10 min at 1,500×g, the cells loaded with the fluorescent probe were washed twice with RPMI-1640 with 0.1% bovine albumin to remove all the extracellular fluorescent probe, resuspended in the same media and incubated an additional 45 min at room temperature to allow complete hydrolysis of the fluorescent probes by intracellular esterases. The hydrolyzed probe is cell impermeant and interacts with free ionic Mg$^{2+}$ or Ca$^{2+}$ resulting in an emission spectral shift.

For the ratiometric determination of [Mg$^{2+}$], the cells loaded with mag-fura-2 were centrifuged and suspended in 2 mL of a buffer containing (mmol/L) NaCl 140, KCl 5, CaCl$_2$ 1.8, MgSO$_4$ 0.8, HEPES 15, and D-glucose 5 (pH 7.4) and transferred to a quartz cuvette just before
fluorometric measurements were made. Fluorescence emission (510 nm, slit width 5 nm) was measured at alternate excitation at 335 and 370 nm (slit width 10 nm) using a Perkin Elmer LS-50B spectrofluorimeter (Shelton, CT, USA). After the initial emissions were read, 5 mmol/L EDTA and 5 mmol/L EGTA were added to the cuvette to chelate the extracellular Mg$^{2+}$ and Ca$^{2+}$ in the buffer resulting in a rapid-step change in fluorescence at both wavelengths due to a small amount of probe leakage from the cells. The immediate change (<15 seconds) in fluorescence intensities after the addition of EDTA and EGTA was used for calculation of the 335/370 fluorescence ratio for free resting Mg$^{2+}_i$ (R). Triton X-100 was then added to the cuvette to a final concentration of 0.1% (v/v) to lyse the cells. Since the cells were in a medium containing EDTA and EGTA, intracellular Mg$^{2+}$ thus released was chelated, and the resulting fluorescence intensities were used for calculation of the zero Mg$^{2+}$ 335/370 fluorescence ratio ($R_{\text{min}}$). Finally, MgSO$_4$ (100 mmol/L) was added to the cuvette and the resulting fluorescence intensities obtained then were used for calculation of the excess Mg$^{2+}$ 335/370 fluorescence ratio ($R_{\text{max}}$).

Mg$^{2+}_i$ was measured in triplicate and calculated as follows: Mg$^{2+}_i$ (mmol/L) = $K_d \times \frac{[(R-R_{\text{min}})S_f]}{[(R_{\text{max}}-R)S_b]}$ where $K_d$, the dissociation constant for Mg$^{2+}$, is 1.5 mmol/L, and $S_f$ and $S_b$ are the fluorescence intensities at 370 nm for mag-fura-2 with zero Mg$^{2+}$ and excess Mg$^{2+}$, respectively. Ca$^{2+}$ also binds to mag-fura-2 but, given that [Ca$^{2+}$]$_i$ under the present conditions >1000 times lower than the $K_d$ for Ca$^{2+}$ binding to mag-fura-2, only 0.05% of the mag-fura-2 will be complexed with Ca$^{2+}$. Therefore, we did not correct the calculated [Mg$^{2+}$]$_i$ for Ca$^{2+}$.

For the ratiometric determination of [Ca$^{2+}$]$_i$, the cells loaded with fura-2 were centrifuged and suspended in 2 mL of a buffer containing (mmol/L) NaCl 140, KCl 5, MgSO$_4$ 0.8, HEPES 15, and D-glucose 5 (pH 7.4) and transferred to a quartz cuvette just before fluorometric
measurements were made. Fluorescence emission (510 nm, slit width 10 nm) was measured at alternate excitation at 340 and 380 nm (slit width 10 nm). After the initial fluorescence was measured, EGTA (10 mmol/L) was added to chelate the Ca$^{2+}$ in the medium, thus producing a desaturation of fura-2 that leaked from inside the cells and producing a rapid step-change in the fluorescence intensities. These fluorescence intensities obtained immediately (<15 sec) after addition of EGTA were used for calculation of the 340/380 fluorescence ratio for free resting Ca$^{2+}$ (R). Triton X-100 was then added to the cuvette to a final concentration of 0.1% (v/v) to lyse the cells, and the resulting fluorescence intensities were used for calculation of the zero Ca$^{2+}$ 340/380 fluorescence ratio ($R_{\text{min}}$). Finally, CaCl$_2$ (10 mmol/L) was added to the cuvette and the resulting fluorescence intensities obtained then were used for calculation of the excess Ca$^{2+}$ 340/380 fluorescence ratio ($R_{\text{max}}$). Ca$^{2+}$$_i$ was measured in triplicate and [Ca$^{2+}$]$_i$ (nmol/L) was calculated with the same equation as for [Mg$^{2+}$]$_i$ where the Ca$^{2+}$ dissociation constant ($K_d$) was 225 nmol/L.

**PBMC generation of H$_2$O$_2$**

Production of H$_2$O$_2$ by PBMC was measured by a modification of the method of Bass et al. (4) for whole blood using single cell analysis by flow cytometry (5). Whole blood was incubated with 25 µM dichlorofluorescin diacetate (Molecular Probes, Eugene, OR) for 45 minutes at 37°C. During the incubation, the dichlorofluorescin diacetate diffused into the cells and was hydrolyzed to nonfluorescent 2′7′-dichlorofluorescin and thereby trapped inside the cells. 2′7′-dichlorofluorescin is oxidized by H$_2$O$_2$ to fluorescent dichlorofluorescin. The red blood cells were then lysed with FACS lysing solution (Becton Dickinson, San Jose, CA). The remaining leukocytes were then washed three times with phosphate buffered saline (PBS). After
the final wash, the leukocytes were suspended in PBS for flow cytometric analysis using a FACS Caliber flow cytometer (Becton Dickinson, San Jose, CA). Individual lymphocytes and monocytes 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 the use of photomultiplier gain settings such that all intensities of cellular fluorescence would be registered between 10 and 90% of the full scale of the 1000 channel resolution of the instrument.

References


