Mineralocorticoid Receptor Antagonism in Experimental Atherosclerosis

Sanjay Rajagopalan, MD; Damon Duquaine, BS; Steven King, PhD; Bertram Pitt, MD; Paresh Patel, MD

Background—Aldosterone has been implicated in the effects of angiotensin II in the vasculature. We hypothesized that there is local expression of the mineralocorticoid receptor (MR) in the vasculature and that the use of a selective aldosterone receptor antagonist (SARA) improves endothelial function in early atherosclerosis.

Methods and Results—New Zealand rabbits were placed on normal chow or 1% cholesterol diets, randomized to placebo or SARA (eplerenone, 50 mg/kg twice daily), and killed at the end of 6 weeks for various studies. In the hyperlipidemic (HL) chow group, there was a 2.3-fold increase in superoxide (O$_{2}^{-}$) generation. SARA normalized O$_{2}^{-}$ generation in intact aortas and reduced NADH and NADPH oxidase activity to basal levels (0.31±0.04 and 0.27±0.02 in HL versus 0.16±0.05 and 0.07±0.02 in HL-SARA, respectively; P<0.01 by ANOVA). This was associated with improvements in peak relaxations to the endothelial-dependent agonist acetylcholine (82±6% in HL-SARA versus 61±4 in HL; P<0.01 by ANOVA; ED$_{50}$ 6.8×10$^{-8}$ mol/L in HL-SARA and 1.2×10$^{-7}$ mol/L in HL; P=NS) to near-normal levels. Vessels from the HL group demonstrated hyperreactivity to angiotensin II that could not be corrected with SARA. Plasma aldosterone levels by radioimmunoassay demonstrated a 4- to 5-fold increase in response to SARA but no differences with lipid feeding. Real-time reverse transcriptase–polymerase chain reaction studies revealed expression of MR in the aorta of HL rabbits and those of controls.

Conclusions—MR antagonism improves endothelial function and reduces O$_{2}^{-}$ generation in diet-induced atherosclerosis. Targeting aldosterone by blocking its receptor has potential antiatherosclerotic effects. (Circulation. 2002;105:2212-2216.)

Key Words: atherosclerosis • nitric oxide • superoxide • endothelium

The renin-angiotensin system (RAS) is upregulated in the vasculature of atherosclerotic vessels. It has been hypothesized that locally generated angiotensin II by modulation of NADH/NADPH-dependent oxidases may result in inactivation of nitric oxide (NO), setting the stage for progression of atherosclerosis. Indeed, blockade of RAS using an angiotensin II type 1 receptor blocker has been shown to ameliorate endothelium-dependent vasomotion by decreasing the activity of NADH oxidases in the vessel wall and to reduce experimental atherosclerosis. Recently, there have been several lines of data to suggest that some of the effects of angiotensin II may be mediated by aldosterone. Furthermore, mineralocorticoid receptor (MR) antagonism in patients with an upregulated RAS is associated with improvements in forearm blood flow, suggesting an effect on the nitric oxide synthase (NOS) pathway. We therefore sought to determine if pharmacological manipulation of the aldosterone pathway using a selective aldosterone receptor antagonist (SARA) improved NO availability and endothelial function in experimental atherosclerosis.

Animal Model

Thirty-two male New Zealand white rabbits (Kuiper Animal Farms, Ind) were used in the study. Sixteen rabbits were fed a standard diet of rabbit chow, and 16 were fed an atherogenic diet consisting of standard rabbit chow supplemented with 1.0% cholesterol (Purina chow). After allowing the rabbits to acclimatize to the chow and environment for 2 weeks, the rabbits were gavage fed with a selective mineralocorticoid receptor antagonist (eplerenone; n=16, 100 mg/kg) or normal saline (n=16) 2 times daily for 6 weeks, after which they were euthanized.

The University of Michigan Committee on Use and Care of Animals approved the protocol. Rabbits were cared for according to standards in the Guide for the Care and Use of Laboratory Animals.

Organ Chamber Studies

Segments of the thoracic aorta were suspended in individual organ chambers filled with Krebs buffer (25 mL) of the following composition (in mmol/L): NaCl 118.3, KCl 4.69, CaCl$_2$ 1.87, MgSO$_4$ 1.20, K$_2$HPO$_4$ 1.03, NaHCO$_3$ 25, and glucose 11.1 (pH 7.40), as previously described. The solution was aerated continuously with a 95% O$_2$ and 5% CO$_2$ mixture and maintained at 37°C. Care was taken not to injure the endothelium during preparation of the rings. Tension was...
recorded with a linear-force transducer. Over a period of 1 hour, the resting tension gradually was increased and the ring segment frequently exposed to 80 mmol/L KCl until the optimal tension for generating force during isometric contraction was reached. In preliminary experiments, this proved to be 3 g in all subsets of animals. The vessels were left at this resting tension throughout the remainder of the study. To prevent synthesis of prostaglandins, all experiments were performed in the presence of 10 μmol/L indomethacin. The vessels were then precontracted with gradual doses of L-phenylephrine (0.15 μmol/L).

Vasodilator Responses

After a stable contraction plateau was reached with phenylephrine, which was 40% to 50% of peak tension generated with maximal-dose KCl, the rings were exposed to the endothelium-dependent agonist acetylcholine (1 nmol/L to 10 μmol/L) or the endothelium-independent agent nitroglycerin (1 nmol/L to 10 μmol/L). The vessels were then washed thoroughly and allowed to equilibrate for another hour before being subjected to vasoconstrictors.

Vasoconstrictor Responses

Vessels were allowed to equilibrate for at least 2 hours at the resting tension of 3 g before being subjected to graded doses of phenylephrine (1 nmol/L to 10 μmol/L) or angiotensin II (1 nmol/L to 0.1 μmol/L). Responses were then expressed as a percentage of the peak response to 80 mmol/L KCl.

Estimation of Aortic \( \text{O}_2^- \) Production in Intact Segments and NADH/NADPH Oxidase Activity in Vessel Homogenates

\( \text{O}_2^- \) anion production was measured in aortic segments using lucigenin (5 μmol/L) chemiluminescence as described previously. A 10% vessel homogenate was prepared in 50 mmol/L phosphate buffer by homogenizing aortic segments in a glass-to-glass motorized homogenizer. The chemiluminescence that occurred over the ensuing 5 minutes in response to the addition of either NADH or NADPH (both 100 μmol/L) to 20 μL homogenate was recorded as previously described. In some experiments, homogenates were incubated with diphenyle iodonium (DPI) (10 μmol/L), and the effect on \( \text{O}_2^- \) was evaluated. Values were standardized to the amount of protein present. Protein content was measured using a commercially available kit (Dc protein assay, BioRad Laboratories).

In Situ Detection of \( \text{O}_2^- \)

Unfixed frozen ring segments were cut into 30-μm sections and placed on a glass slide. The oxidatively active fluorescent dye hydroethidium (2×10^-6 mol/L) was applied to the surface of each section and incubated for a period of 30 minutes at room temperature in a dark humidified chamber. The sections were then scanned with a laser confocal microscope using excitation, and emission wavelengths were 488 and 610 nm, respectively, as described. Atherosclerotic and control tissues were processed and imaged in parallel. Laser settings were identical for acquisition of images from atherosclerotic and control specimens.

Quantitative Real-Time Reverse Transcripase–Polymerase Chain Reaction for MR

MR primers were arrived at from a consensus sequence of human, rat, mouse, and squirrel monkey MRs. Primers were 5'-GGGATGGAGACCAAAGGCTACC-3' (starts at translation start codon) and 5'-GTACCTTTGGCCACCTCTCGA-3' (in steroid-binding domain). Polymerase chain reaction (PCR) amplification was performed for 35 cycles (95°C for 30 seconds, 55°C for 30 seconds, and 68°C for 3 minutes). DNA was removed from preparations of total RNA using DNase I and DNase removal reagents (Ambion). First-strand cDNA synthesis was performed with random primers and 5 μL of RNA using SuperScript II reverse transcriptase (RT) (Life Technologies). After treatment with RNaseH, samples were diluted into 50 μg/mL, and the DNA was used as templates for PCR. PCR amplifications and analyses were performed by TaqMan using an ABI 7700 sequence detection system (Perkin Elmer). The following primers and fluorescent-tagged probes were used to quantify MR: forward primer 5'-CTGTGCCAGGCTTTGGATGTT-3', reverse primer 5'-CTCCCATCATTGGGATCTGT-3', and probe 6FAM-CTGTGCAAGGCGGATTCCCA-TAMRA. The following primers and fluorescent-tagged probes were used to quantify GADPH: 5'-GATGTGTAAGGCGGATGAA-3', reverse primer

Figure 1. Plasma aldosterone levels in the animal groups. Values are mean±SEM. **P<0.001 vs NC and HL. P=NS for HL-SARA vs NC-SARA by ANOVA.

Figure 2. The effect of SARA on \( \text{O}_2^- \) production. **P<0.01 vs HL-SARA and NC groups.

Figure 3. NADH and NADPH oxidase activity in aortic homogenates. All values are mean±SEM and represent 6 to 8 animals in each group. **P<0.01 vs HL-SARA and NC groups. ***P<0.001 vs HL-SARA and NC groups.
5'-AGTTAAAAGCAGCTTGGTAC-3', and probe 6FAM-CAGGCGCCCAATGCGCCA-TAMRA.

Standard curves were generated using dilutions of plasmid DNA containing the target sequence into 50 μg/mL herring DNA. Calculations were based on the linear response of the threshold cycle when PCR products were first detected versus the log of the input template copy number. This response was linear over a wide range of copy numbers (10^6 to 10^9) achieving correlation coefficients of >0.98.

Statistics
All of the data in the present study are expressed as mean±SEM. Comparisons among groups were made by one-way ANOVA. For differences between paired observations, a t test was used when appropriate. When significance was detected, a post hoc Newman-Keuls multiple-comparison test was performed. All statistical analysis was performed using Graph Pad software (version 3.02).

Results
At the end of 8 weeks of 1% cholesterol administration, average total cholesterol levels in the animals randomized to saline and the SARA group were 1465±333 and 1471±50 mg/dL, respectively (P=NS by paired t test). Total cholesterol levels in the normal chow (NC) and NC-SARA groups were 38±9 and 52±17 mg/dL, respectively. Figure 1 demonstrates plasma aldosterone levels in the experimental groups. Aldosterone receptor antagonism increased circulating levels of aldosterone by 4-fold in the control group (NC-SARA, 1499±243 pg/mL) compared with the saline group (NC, 360±13 pg/mL). Levels increased 5-fold in the hyperlipidemic (HL) group (HL-SARA, 1235±140 pg/mL) compared with the saline group (HL, 230±36 pg/mL). The differences between HL-SARA and NC-SARA were not significant (P=NS by ANOVA).

O_2^- Generation and NADH/NADPH Oxidase Activity
Figure 2 reveals a 2.3-fold increase in O_2^- production in intact aortic segments at the end of 8 weeks of lipid feeding in HL animals compared with controls (3445±863 counts/mg per minute in HL versus 1295±341 counts/mg per minute in NC animals, P<0.01 by ANOVA). In contrast, SARA resulted in normalization of these counts to baseline levels (HL-SARA, 1071±442 counts/mg). Figure 3 depicts oxidase activity in response to NADH and NADPH in the various animal groups. SARA treatment over 6 weeks normalized

![Figure 4. Confocal microscopy images demonstrating dihydro-ethidium (DHE) staining in aorta of rabbits. A and B, Sections from control animals (NC and NC-SARA, respectively). C and D, Sections from HL and HL-SARA animals, respectively. A total of n=3 rabbits in each group were examined, and the representative segment from each group is shown. I indicates intima; M, media; and A, adventitia.](image)

![Figure 5. Dose-response curves to acetylcholine (Ach) (left) and nitroglycerin (right). Values represent responses to graded doses of drug expressed as a percent of preconstricted tension in response to phenylephrine. All values represent mean±SEM. **P<0.01 vs HL; P=NS for HL-SARA vs NC and NC-SARA by ANOVA.](image)
Concentration-response curves to phenylephrine (left) and angiotensin II (right). All values are mean ± SEM. **P<0.01 versus NC and NC-SARA. 

Figure 6. Concentration-response curves to phenylephrine (left) and angiotensin II (right). All values are mean ± SEM. *P<0.05 versus NC groups. **P<0.01 versus NC groups.
oxidases. Such an effect would be consistent with previous observations that blockade of the MR results in reduced free radical injury in conditions associated with excess aldosterone.\(^7\) We have provided evidence for the presence of MR transcript in the vasculature consistent with the notion that the vasculature has the potential to respond to aldosterone. The numbers were too small to make definitive statements about differences between animal groups, and, moreover, we have not reported measures of enzyme/receptor activity. Additional studies will be needed to understand the regulation of the local mineralocorticoid axis in atherosclerosis.

There is the possibility that elevation in potassium levels contributed to the beneficial effects noted in this study.\(^10,11\) We think that this is unlikely, because human studies of MR antagonism with eplerenone at comparable doses have not revealed significant increases in serum potassium.\(^12\) It is possible that MR antagonism resulted in an ACE inhibitor–like effect.\(^3\) We did not perform responses to angiotensin I in our study; however, the fact that SARA administration did not restore the hyperresponsiveness seen with angiotensin II in HL animals is indirect evidence that this is unlikely to be secondary to an ACE inhibitor–like effect. Finally, it is possible that the effects could represent a free radical–scavenging effect of eplerenone. The structure of eplerenone does not predict such an effect.\(^12\)

In conclusion, MR antagonism ameliorates endothelial function through an NADH/NADPH oxidase–dependent mechanism. These findings provide a novel role for SARA in the treatment of atherosclerosis.

**Acknowledgments**

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**References**


**TABLE 2. Effects of SARA on Vasoconstrictor Responses**

<table>
<thead>
<tr>
<th>Groups</th>
<th>% PE</th>
<th>EC(50) PE</th>
<th>% All</th>
<th>EC(50) All</th>
</tr>
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<td>NC</td>
<td>124±1</td>
<td>2.3±0.1×10(^{-7})</td>
<td>29±3</td>
<td>2.6±0.2×10(^{-8})</td>
</tr>
<tr>
<td>NC-SARA</td>
<td>121±2</td>
<td>3.4±0.1×10(^{-7})</td>
<td>33±2</td>
<td>3.9±0.1×10(^{-9})</td>
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<tr>
<td>HL</td>
<td>130±2</td>
<td>4.4±0.1×10(^{-7})</td>
<td>49±3*</td>
<td>3.6±0.2×10(^{-9})</td>
</tr>
<tr>
<td>HL-SARA</td>
<td>129±1</td>
<td>6.3±0.1×10(^{-7})</td>
<td>58±3*</td>
<td>7.3±0.2×10(^{-9})</td>
</tr>
</tbody>
</table>

Peak constriction is expressed as mean±SEM, and EC\(50\) is expressed as mean.

\(^*P<0.05\) vs NC and NC-SARA.
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