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 (\(\text{O}_2^+\)) generation. SARA normalized \(\text{O}_2^+\) 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; \(P=\text{NS}\)) 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 \(\text{O}_2^+\) 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
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 O2− Production in Intact Segments and NADH/NADPH Oxidase Activity in Vessel Homogenates**

O2− 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 diphenylene iodinium (DPI) (10 μmol/L), and the effect on O2− 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 O2−**

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 Transcriptase–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′-GGGATGGAACCCAGGCTACC-3 (starts at translation start codon) and 5′-GTACCTTGGCCACCTACGCA-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 herring DNA and 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′-CTGTGCCCAGGCTTTGATGTT-3′, reverse primer 5′-CTCCCATCATCCGGTTCTTG-3′, and probe 6FAM-CTGTGAAAGCCAGGGATTCCCCA-TAMRA. The following primers and fluorescent-tagged probes were used to quantify GADPH: 5′-GATGGTGAAGGTCGATTGA-3′, reverse primer
5’-AGTTAAAAGCAGCCCTGGTGAC-3’, and probe 6FAM-CAGGCGCCCAATGCGGCCA-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...
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respectively). In additional experiments, we determined the and NADPH-dependent oxidases (0.27 \( \pm \) 0.04 to 0.16 \( \pm \) 0.05 nmol/mg per protein/min in HL and HL-SARA animals, respectively) and NADPH-dependent oxidases (0.27 \( \pm \) 0.02 to 0.07 \( \pm \) 0.02 nmol/mg per protein/min in HL and HL-SARA animals, respectively). In additional experiments, we determined the role of various sources of \( \mathrm{O}_2^- \) in homogenates. Homogenates alone and in response to xanthine (100 \( \mu \)mol/L) did not produce any signal beyond background, ruling out contributions by xanthine oxidases. \( \mathrm{O}_2^- \) in response to NADH and NADPH was inhibitable by >90% with DPI but not by L-NMMA (10 \( \mu \)mol/L), suggesting a flavin containing oxidoreductase other than NOS (data not shown). We then evaluated in situ production of \( \mathrm{O}_2^- \) using H&E staining (hydroethidium) to provide an idea of the spatial distribution of \( \mathrm{O}_2^- \) in the vessel wall (Figure 4). Sections of aorta derived from HL animals (n=3) demonstrated intense staining in the intima, media, and adventitia (Figure 4C). In contrast, animals that were rendered HL but received SARA (n=3) had markedly reduced levels of staining in the media and adventitia (Figure 4D). Rabbits fed NC and rabbits fed SARA with NC did not demonstrate changes in spatial localization of \( \mathrm{O}_2^- \) signal.

Vasomotor Function With SARA
SARA administration for 6 weeks improved responses to acetylcholine in the HL-SARA group (Figure 5). Peak relaxations markedly improved in the HL-SARA group compared with the HL group (87 \( \pm \) 3\% versus 59 \( \pm \) 2\%, \( P<0.01 \) by ANOVA), whereas there was no significant change in the ED\(_{50}\) dose (Table 1). Relaxations to the endothelium-independent agonist nitroglycerin were unaffected by both lipid feeding and treatment with the drug (Figure 5 and Table 1). Vasoconstrictor responses to phenylephrine remained unchanged (peak responses or EC\(_{50}\)) in response to lipid feeding and SARA administration (Figure 6 and Table 2). Responses to angiotensin II were heightened in the lipid-fed animals (58 \( \pm \) 3 and 49 \( \pm \) 3\% in HL-SARA and HL, respectively, \( P<0.05 \) for peak constriction versus NC and NC-SARA). Interestingly, SARA administration did not alter the heightened sensitivity to angiotensin II in the HL-SARA animals (Figure 6 and Table 2).

Evidence for Vascular Expression of MR
Real-time RT-PCR analysis of aorta for MR transcript revealed expression in control samples (n=4, 147 625 \( \pm \) 34 940 copies of MR transcript normalized to GAPDH) and from samples from HL animals n=4, 59 878 \( \pm \) 37 444).

Discussion
These results are the first to demonstrate a role for SARA in ameliorating endothelial function and alleviating free radical stress in early atherosclerosis and supports prior studies that blockade of MR may be beneficial.

Vascular Responses in Atherosclerosis and the Role of the Mineralocorticoid Pathway
Treatment with SARA improved endothelial function and reduced NADH/NADPH oxidase activity, suggesting an important and previously unreported interaction of MR with \( \mathrm{O}_2^- \)-generating oxidases in the vasculature. A recent study in humans demonstrated that short-term MR blockade in patients with congestive heart failure who were already taking an ACE inhibitor results in marked improvements in forearm resistance vessel function, suggesting that MR blockade improves endothelial function independent of and in addition to ACE inhibition. As shown previously, we demonstrated an increase in the sensitivity to angiotensin II with atherosclerosis. This is believed to be secondary to an increase in angiotensin II type 1 receptor density in the vessel wall. Treatment with SARA did not alter this heightened responsiveness, implying that at least in the HL rabbit, MR antagonism does not alter angiotensin II type 1 receptor-dependent contractile responses.

Potential Mechanisms of Improvement in Vascular Function With SARA
There are a number of potential explanations for the observed findings. The first and foremost is that MR antagonism modifies the influence of aldosterone on \( \mathrm{O}_2^- \)-generating

**TABLE 1. Effects of SARA on Responses to Acetylcholine and Nitroglycerin**

<table>
<thead>
<tr>
<th>Groups</th>
<th>% Ach</th>
<th>ED(_{50}) Ach</th>
<th>% NTG</th>
<th>ED(_{50}) NTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>102 ( \pm ) 1</td>
<td>2.4 ( \pm ) 0.1 ( \times ) 10(^{-8})</td>
<td>104 ( \pm ) 2</td>
<td>7.1 ( \pm ) 0.2 ( \times ) 10(^{-8})</td>
</tr>
<tr>
<td>NC-SARA</td>
<td>100 ( \pm ) 3</td>
<td>3.2 ( \pm ) 0.2 ( \times ) 10(^{-8})</td>
<td>107 ( \pm ) 2</td>
<td>5.7 ( \pm ) 0.1 ( \times ) 10(^{-8})</td>
</tr>
<tr>
<td>HL</td>
<td>58 ( \pm ) 2\†</td>
<td>1.2 ( \pm ) 0.1 ( \times ) 10(^{-7})\†</td>
<td>104 ( \pm ) 3</td>
<td>1.3 ( \pm ) 0.1 ( \times ) 10(^{-8})</td>
</tr>
<tr>
<td>HL-SARA</td>
<td>87 ( \pm ) 3**</td>
<td>6.0 ( \pm ) 0.1 ( \times ) 10(^{-7})\††</td>
<td>112 ( \pm ) 4</td>
<td>1.0 ( \pm ) 0.2 ( \times ) 10(^{-8})</td>
</tr>
</tbody>
</table>

Ach indicates acetylcholine; NTG, nitroglycerin; and ED\(_{50}\), half-maximal dose. Values for peak relaxations are reported as mean \( \pm \) SEM, and ED\(_{50}\) is reported as the mean dose.

\( \* \) \( P<0.01 \) vs HL; \( \dagger \) \( P<0.05 \) vs NC and NC-SARA.

Figure 6. Concentration-response curves to phenylephrine (left) and angiotensin II (right). All values are mean \( \pm \) SEM. \( \* \) \( P<0.05 \) versus NC groups. \( \** \) \( P<0.01 \) versus NC groups.
like effect. 3 We did not perform responses to angiotensin I in possible that MR antagonism resulted in an ACE inhibitor–

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 EC50 PE</th>
<th>% All EC50 All</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>NC-SARA</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>HL</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>HL-SARA</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Peak constriction is expressed as mean±SEM, and EC50 is expressed as mean.

*P<0.05 vs NC and NC-SARA.
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