Aldosterone and Not Plasminogen Activator Inhibitor-1 Is a Critical Mediator of Early Angiotensin II/N\textsuperscript{G}-Nitro-L-Arginine Methyl Ester–Induced Myocardial Injury

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Background—Angiotensin II (Ang II) increases levels of aldosterone and plasminogen activator inhibitor-1 (PAI-1). Both aldosterone and PAI-1 seem to promote cardiovascular (CV) injury. Our objective was to determine the roles of PAI-1 and aldosterone in the development of myocardial and renal damage in a model with high Ang II and low nitric oxide (NO) availability, a pattern seen in patients with heart failure, diabetes mellitus, and arteriosclerosis.

Methods and Results—Mice on a moderately high sodium diet were treated with the NO synthase inhibitor \textsuperscript{N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME)} for 14 days plus Ang II during days 8 through 14. The roles of aldosterone and PAI-1 in the development of CV injury were assessed using the mineralocorticoid receptor antagonist spironolactone (0, 1.5, 15, and 50 mg \textcdot 100 g\textsuperscript{-1} \textcdot day\textsuperscript{-1}) and PAI-1–deficient mice (PAI-1\textsuperscript{−/−}). Ang II/L-NAME–treated mice showed glomerular ischemia, proteinuria, and necrosis of myocytes and vascular smooth muscle cells with an associated mixed inflammatory response, deposition of loose collagen, and neovascularization. Compared with saline-drinking mice, Ang II/L-NAME–treated mice had significantly increased heart to body weight (HW/BW) ratios, cardiac and renal damage assessed by histological examination, PAI-1 immunoreactivity, and proteinuria. Spironolactone treatment decreased PAI-1 immunoreactivity and reduced in a dose-dependent fashion cardiac and renal damage. PAI-1\textsuperscript{−/−} animals had a similar degree of CV injury as PAI-1\textsuperscript{+/+} animals.

Conclusions—Mineralocorticoid receptor antagonism, but not PAI-1 deficiency, protected mice from developing Ang II/L-NAME–mediated myocardial and vascular injury and proteinuria, suggesting that aldosterone, but not PAI-1, plays a key role in the development of early Ang II/L-NAME–induced cardiovascular injury. (Circulation. 2003;108:2517-2523.)

Key Words: plasminogen activator inhibitor ◆ angiotensin ◆ myocardium ◆ kidney

An activated renin-angiotensin-aldosterone system has long been linked to increased cardiovascular (CV) risk.\textsuperscript{4} There is an extensive literature documenting the adverse CV effects of angiotensin II (Ang II)\textsuperscript{2,3} and, more recently, aldosterone.\textsuperscript{4,5} The proximate mediators of these effects are uncertain. However, one proposed mediator is plasminogen activator inhibitor-1 (PAI-1), because it promotes CV injury and is stimulated by aldosterone.\textsuperscript{6}

Several recent studies indicate that PAI-1 is associated with adverse CV effects. Increased circulating levels of PAI-1 are independently associated with primary cardiovascular events\textsuperscript{7} and reinfarction.\textsuperscript{8} Transgenic mice that express active human PAI-1 in endothelial cells develop coronary artery perivascular fibrosis, coronary artery thrombosis, and myocardial infarction.\textsuperscript{9} PAI-1–deficient mice develop less-extensive vascular fibrosis than do wild-type mice when administered an inhibitor of NO synthase.\textsuperscript{10} Furthermore, increased PAI-1 immunoreactivity has been detected in the endothelium and media of the aorta and coronary arteries of rats treated with \textsuperscript{N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME)}. The induction of PAI-1 preceded the development of vascular structural changes and was prevented by coadministration of an ACE inhibitor, suggesting that PAI-1 may influence the development of vascular lesions under the influence of Ang II.\textsuperscript{11}

The potential role of aldosterone in promoting CV injury is highlighted by the RALES study, which showed that in patients with severe heart failure undergoing optimal medical therapy including ACE inhibitors, addition of the mineralocorticoid receptor (MR) antagonist spironolactone reduces cardiac morbidity and mortality by 30%.\textsuperscript{12} The specific mechanisms involved in aldosterone-mediated CV damage are not well defined. In heart failure patients, spironolactone...
increases NO bioactivity, improves endothelial dysfunction, and decreases left ventricular volume and mass, suggesting that aldosterone affects endothelial function and cardiac remodeling.\textsuperscript{13,14}

Animal studies suggest a role for aldosterone in the development of vascular inflammation, myocyte death, and cardiac fibrosis.\textsuperscript{15-18} Our laboratory has studied a rat model of aldosterone-mediated CV damage that is characterized by low NO availability and high levels of Ang II,\textsuperscript{18} a pattern of NO and Ang II seen in patients with diabetes mellitus.\textsuperscript{19}

The goal of the present studies was to examine the roles of PAI-1 and aldosterone in this model of CV injury with high Ang II and low NO availability.

**Methods**

**Animals**

Eight-week-old male, PAI-1–deficient strain B6.129S2-serpine \textsuperscript{1} (PAI-1\textsuperscript{-/-}) and wild-type (WT) mice on the same genetic background (C57BL/6J) were purchased from Jackson Laboratory (Bar Harbor, Me). After 1 week of acclimatization, mice were transferred to metabolic cages for study protocols. Mice had free access to drinking fluid and Purina Laboratory Rodent 5001 chow. The Harvard Medical School Animal Care and Use Committee approved all studies.

**Ang II/L-NAME Model**

Animals received L-NAME (0.1 mg · mL\textsuperscript{-1} · drinking fluid) and Ang II (0.7 mg · kg\textsuperscript{-1} · day\textsuperscript{-1}) unless otherwise stated. L-NAME (Sigma) was administered in drinking water from days 1 to 14. Vehicle or Ang II (American Peptide) was administered on days 8 through 14 via Alzet osmotic subcutaneous minipumps (Model 2001, DURECT). Ang II (American Peptide) was used for M-20 and DAKO EnVision+ (DAKO Cytomation) for H-135 PAI-1 antibody detection. Slides were counterstained with hematoxylin. Heart sections probed with M-20 and H-135 antibodies showed similar patterns of immunoreactivity; no PAI-1 M-20 immunoreactivity was observed in slides pretreated with PAI-1 M-20 blocking peptide (sc-6644P, Santa Cruz Biotech) (data not shown).

The myocardial damage score (MDS) was determined in sections containing right and left ventricles (2 to 3 sections per animal) using a scale from 0 to 4, as described,\textsuperscript{18} where 0 indicated no damage; 1, isolated myocyte damage; 2, focal area of damage; 3, 2 or more areas of damage; and 4, diffuse areas of damage compromising more than 50% of the myocardium.

For determination of myocyte immunohistochemical staining, 10 to 12 fields were analyzed for each section and scored on a scale of 0 to 4, where 0 indicated no foci of immunoreactive cells; 1, a single focus with <10 immunoreactive cells; 2, a single focus with more than 10 immunoreactive cells; 3, 2 foci with >10 immunoreactive cells; and 4, 3 or more foci with >10 immunoreactive cells. Coronary arteries were scored on a scale of 0 to 4 for marker expression in or around the vessel wall, where 0 indicated no significant immunoreactivity; 1, <5 immunoreactive cells in or around an artery; 2, 5 to 20 immunoreactive cells in or around an artery; 3, >20 immunoreactive cells in or around an artery; and 4, >20 immunoreactive cells in or around 2 or more arteries.

Coronal kidney sections (2 to 3 \textmu m) stained with H&E or periodic acid-Schiff reagent were examined by light microscopy. Glomerular damage was characterized by the presence of ischemic or thrombotic changes, and renal vascular damage was characterized by the presence of fibrinoid necrosis of arterial or arteriolar walls. The number of injured glomerular tufts and injured vessels are expressed as number of injuries per 100 glomeruli.

**Statistical Analysis**

Data are expressed as mean±SEM. Paired data were compared by Student’s \(t\) tests. Comparisons between multiple groups were made with one-way ANOVA followed by Bonferroni’s multiple comparison test. Data were analyzed using Graphpad Prism version 3.0 statistical software package. In the Table, day 10 urine values were used in lieu of day 14 values in 1 animal each in the Ang II 0.2/L-NAME 0.1 and the Ang II 0.7/L-NAME 0.3 groups because of oliguria.

**Results**

**Development of the Ang II/L-NAME Model in Mice**

The Ang II/L-NAME regimen previously shown to cause CV injury in rats\textsuperscript{18} did not induce significant cardiac or renal damage in mice (data not shown). Therefore, the duration of Ang II treatment was increased from 3 to 7 days, and 2 doses of Ang II (0.2 and 0.7 mg · kg\textsuperscript{-1} · day\textsuperscript{-1}) and L-NAME (0.1 and 0.3 mg · mL\textsuperscript{-1}) were tested.

There were no significant differences between treatment groups in body weight or the day-14 urinary aldosterone-to-creatinine ratio (Table).
Cardiac Damage

Treatment with L-NAME (0.1 mg · mL⁻¹) and Ang II (0.7 mg · kg⁻¹ · day⁻¹) caused a significant increase (P<0.01) in the MDS compared with saline treatment (Figure 1). Damaged hearts showed organizing myocardial necrosis, with a mixed inflammatory infiltrate, loose collagen deposition, and neovascularization (granulation tissue). Vascular damage consisted of similar granulation tissue deposited around vessels with intimal thickening and often frank vascular wall necrosis. There was no significant perivascular or interstitial fibrosis.

In Ang II/L-NAME–treated mice, areas of myocardial and vascular damage showed prominent PAI-1 immunoreactivity in vascular endothelial cells, vascular smooth muscle cells, macrophages and monocytes, and connective tissue within areas of intimal proliferation (Figures 2C and 3E). In control mice, only faint PAI-1 reactivity was seen in some endothelial cells (Figures 2F and 3F).

Renal Damage

A significant increase in proteinuria was observed in all Ang II/L-NAME–treated groups (P<0.05, paired t test day 0 versus day 14) (Table). Histological evaluation of kidneys from the Ang II/L-NAME group showed glomerular damage and fibrinoid necrosis of small vessels with a significant increase in the extent of vascular injury in Ang II 0.7 mg · kg⁻¹ · day⁻¹ dose compared with the other groups (Figures 1 and 2G through 2J). On the basis of these results, Ang II 0.7 mg · kg⁻¹ · day⁻¹ for 7 days and L-NAME 0.1 mg · mL⁻¹ for 14 days were used in subsequent studies.

Effect of Aldosterone Receptor Blockade on Ang II/L-NAME–Induced Cardiovascular Injury in Mice

The effect of an MR antagonist on CV injury was tested in mice receiving Ang II/L-NAME plus spironolactone (0, 1.5, 15, and 50 mg · 100 g⁻¹, n=7 per group) or placebo (n=6). In the 1.5- and 15-mg · 100 g⁻¹ spironolactone groups, 1 animal in each group died spontaneously; tissues and urine from these mice were not analyzed. Damage mediated by Ang II (0.7 mg · kg⁻¹ · day⁻¹) and L-NAME (0.1 mg · mL⁻¹) was similar in degree and type to that observed in the previous study in which dietary sodium was administered via drinking water (data not shown).

Renal Damage

Treatment with Ang II/L-NAME increased proteinuria compared with that in the control group (P<0.05). With spironolactone treatment, there was a dose-dependent decrease in proteinuria from 9.1±3.9 to 1.6±0.3 µg · mL⁻¹/mg · dL⁻¹. P<0.05 (Figure 4A).

Cardiac Damage

Treatment with Ang II/L-NAME significantly increased HW/BW and MDS compared with that in controls. HW/BW was similar in groups receiving Ang II/L-NAME irrespective of the spironolactone dose (Figure 4C). MDS decreased from 2.7±0.4 to 1.5±0.3 with increasing spironolactone doses, P=0.002 (Figure 4B).
Blood Pressure and PAI-1 Immunostaining

MAP and MDS were measured on day 14 in 3 additional groups of animals on a high-sodium diet receiving Ang II/L-NAME, Ang II/L-NAME/spironolactone 50 mg · 100 g⁻¹ · day⁻¹/L-NAME 0.1 mg · mL⁻¹) and (D through F) control mice. Ang II/L-NAME-treated mice show areas of organizing myocardial necrosis with granulation tissue and increased PAI-1 immunostaining (brown). Control mice show no myocardial damage and sparse PAI-1 immunostaining. Kidney sections from (G and H) Ang II/L-NAME–treated mice showing microvascular lesions with fibrinoid necrosis of small arterioles with extension of necrosis into glomeruli or glomerular microthrombi and control mice (I and J) with no evidence of vascular or tubular damage. A and D, H&E; B and E, Masson’s trichrome; C and F, PAI-1 H135 immunostaining; and G through J, PAS. A through F, Magnification ×72, bars=100 μm; G and I, magnification ×144, bars=50 μm; H and J, magnification ×560, bars=25 μm.

Effect of PAI-1 Deficiency on Ang II/L-NAME–Induced Cardiovascular Injury in Mice

WT and PAI-1⁻/⁻ mice were randomized to 1% NaCl (control) or 1% NaCl/Ang II/L-NAME, n=8 per group. No PAI-1 PCR product was detectable in DNA from PAI-1⁻/⁻ mice, whereas the anticipated 836-bp product was detectable in all WT mice. The control 203-bp GAPDH-PCR product was detected in all mice (data not shown).

Day-14 urinary protein to creatinine ratio was elevated to similar levels in mice receiving Ang II/L-NAME (WT, 46.2±15.7 μg · mL⁻¹/mg · dL⁻¹; PAI-1⁻/⁻, 38.9±10.2 μg · mL⁻¹/mg · dL⁻¹) compared with control groups (WT, 9.7±3.6 μg · mL⁻¹/mg · dL⁻¹ and PAI-1⁻/⁻, 5.3±0.5 μg · mL⁻¹/mg · dL⁻¹), P<0.05 (Figure 6A).

WT and PAI-1⁻/⁻ mice treated with Ang II/L-NAME had a significant increase in HW/BW ratio, MDS, and vascular damage score compared with their control groups (Figures 6B through 6D). Histologically, the myocardial and vascular injury in PAI-1⁻/⁻ mice was similar to that in WT mice. The extent of damage (MDS) in PAI-1⁻/⁻ mice was similar to that seen in WT mice, and the vascular injury score was increased compared with that in WT mice (P<0.05).

Discussion

Administration of Ang II/L-NAME caused cardiac and renal injury in mice that was reduced in a dose-dependent manner by aldosterone blockade with spironolactone. Although
PAI-1 expression was increased in areas of Ang II/L-NAME–induced cardiac damage. PAI-1 deficiency was not protective, suggesting that PAI-1 is not a causative agent in the acute development of Ang II/L-NAME–mediated myocardial injury. Rather, it is likely that PAI-1 is generated in response to damage and may play a role in the resolution of damage.

Our observation that PAI-1 expression is increased with Ang II/L-NAME treatment is consistent with previous reports showing an increase in PAI-1 with Ang II, aldosterone, and NO synthase inhibition. However, our finding that PAI-1 deficiency does not reduce Ang II/L-NAME–induced renal and cardiac injury differs from that of 2 studies showing that PAI-1 deficiency reduces cardiovascular fibrosis caused by chronic (8- to 16-week) administration of L-NAME. This difference in results may be related to the different types of damage observed in the 2 models. In the Ang II/L-NAME model, the primary process seems to be necrosis of myocytes and vascular smooth muscle cells with a mixed inflammatory infiltrate, loose collagen deposition, and neovascularization (granulation tissue). No significant fibrosis was seen at the end of the 14-day Ang II/L-NAME treatment. A similar histological picture was present across all of our studies. In contrast, the chronic L-NAME model caused perivascular or interstitial fibrosis. PAI-1 is thought to retard matrix turnover and promote pathological remodeling and fibrosis through inhibition of plasminogen activation and through indirect effects on matrix metalloproteases. In the Ang II/L-NAME model, PAI-1 may play a role in the repair, fibrosis, and remodeling that would follow the 14-day damage.

The reduction in Ang II/L-NAME–mediated vascular damage with spironolactone is consistent with a growing body of literature, suggesting that aldosterone promotes vascular injury or dysfunction. The MR is present in vascular tissue, suggesting a morphological basis for its action. In patients with heart failure, administration of spironolactone improves endothelium-dependent vasodilatation, and in rodents, min-

Figure 3. Micrographs of coronary artery. Consecutive sections of representative coronary artery from mice treated with Ang II/L-NAME (A, C, and E) and control mice (B, D, and F). Coronary arteries from Ang II/L-NAME–treated mouse show fibrinoid necrosis of the vessel wall with intimal thickening, a mixed inflammatory response, and increased PAI-1 immunostaining. A and B, H&E; C and D, Masson’s trichrome; E and F, and PAI-1 H135 antibody staining. Magnification ×225, bars = 50 μm.

Figure 4. Effect of increasing doses of spironolactone on Ang II/L-NAME–mediated proteinuria and myocardial injury. A, Twenty-four urinary protein (μg · mL⁻¹)/creatinine (mg · dL⁻¹) on day 14; B, MDS; C, HW/BW for control group (white bars) or Ang II/L-NAME/spironolactone groups (dark bars) (0, 1.5, 15, and 50 mg · 100 g⁻¹ · day⁻¹), P<0.05 (proteinuria) and P=0.002 (MDS) for linear trend across spironolactone-treated groups.
eralocorticoids affect vascular tone and contractility. Aldosterone infusion increases vascular expression of proinflammatory molecules. Aldosterone blockade reduces vascular damage and inflammation in rats. 

Figure 5. Effect of spironolactone on blood pressure, myocardial and vascular injury, and PAI-1 expression in Ang II/L-NAME–treated mice. A, MAP (mm Hg); B, MDS; C, vascular injury score; D, myocardial PAI-1 immunostaining score; and E, vascular PAI-1 immunostaining score in groups treated with Ang II/L-NAME (dark bars), Ang II/L-NAME/spironolactone (50 mg \( \cdot 100 g^{-1} \cdot \text{day}^{-1} \)) (striped bar), or control group (white bars), n=8 to 9 mice per group, except n=4 to 5 per group for MAP. Bars represent mean±SEM. *P<0.05, **P<0.005 vs control.

Figure 6. Myocardial and renal injury in WT and PAI-1−/− mice treated with Ang II/L-NAME. A, Twenty-four-hour urinary protein (mg/mL) /creatinine (mg/dL) on day 14; B, MDS; C, vascular injury score, and D, HW/BW. Filled symbols and dark bars represent mice treated with Ang II/L-NAME; open symbols and white bars correspond to control mice. *P<0.01 vs control mice of same genotype. Bars represent mean±SEM in A and D. In B and C, symbols represent individual scores; horizontal bar represents median for the group.
rats treated with Ang II/L-NAME, stroke-prone hypertensive rats, and uninephrectomized rats infused with Ang II or aldosterone.\textsuperscript{18}\textsuperscript{,}\textsuperscript{31} In many of these models, as in the present study, CV protection by MR antagonists is not dependent on blood pressure reductions.\textsuperscript{32}\textsuperscript{,}\textsuperscript{33}

The present study has some limitations. Blood pressure was not measured in the PAI-1\textsuperscript{−/−} mice. Furthermore, although the histological character of the myocardial and vascular injury appeared similar in the wild-type and PAI-1\textsuperscript{−/−} mice, some compensation for the PAI-1 deficiency may have influenced the induction of or mechanisms involved in Ang II/L-NAME-mediated injury. If so, this provides a possible explanation for the increased perivascular inflammation observed in PAI-1\textsuperscript{−/−} mice. The perivascular and myocardial mixed inflammatory infiltrates are most likely forming in response to vascular smooth muscle cell and myocyte cell injury. Although less likely, a primary inflammatory process cannot be ruled out.

In conclusion, the present study documents that, in mice, Ang II/L-NAME causes damage to coronary and renal arteries, myocyte necrosis, and myocardial inflammation via processes that are not dependent on PAI-1. Damage is reduced by MR blockade, suggesting that aldosterone is an important mediator of Ang II–induced CV injury. These results raise the possibility that aldosterone is involved in the development of CV injury in clinical situations characterized by high Ang II and low NO availability, such as diabetes mellitus.

Acknowledgments

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References

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