Plasminogen Activator Inhibitor-1 Deficiency Prevents Hypertension and Vascular Fibrosis in Response to Long-term Nitric Oxide Synthase Inhibition

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Background—Long-term inhibition of nitric oxide synthase (NOS) is known to induce hypertension and perivascular fibrosis. Recent evidence also suggests that long-term NOS inhibition induces expression of plasminogen activator inhibitor-1 (PAI-1) in vascular tissues and that PAI-1 may contribute to the development of fibrosis after chemical or ionizing injury. On the basis of these observations, we hypothesized that PAI-1 may influence the vascular response to long-term NOS inhibition by N'-nitro-L-arginine methyl ester (L-NAME).

Methods and Results—We compared the temporal changes in systolic blood pressure and coronary perivascular fibrosis in PAI-1−/− deficient (PAI-1−/−) and wild-type (WT) male mice (N=6 per group). At baseline, there were no significant differences in blood pressure between groups. After initiation of L-NAME, systolic blood pressure increased in both groups at 2 weeks. Over an 8-week study period, systolic blood pressure increased to 141±3 mm Hg in WT animals versus 112±4 mm Hg in PAI-1−/− mice (P<0.0001). The extent of coronary perivascular fibrosis increased significantly in L-NAME-treated WT mice (P<0.01 versus PAI-1−/− mice). Cardiac type I collagen mRNA expression was greater in control (P<0.01) and L-NAME-treated PAI-1−/− (P<0.05) groups than in control WT mice, indicating that PAI-1 deficiency prevents the increase of collagen deposition by promoting matrix degradation.

Conclusions—These findings suggest that PAI-1 deficiency alone is sufficient to protect against the structural vascular changes that accompany hypertension in the setting of long-term NOS inhibition. Direct inhibition of vascular PAI-1 activity may provide a new therapeutic strategy for the prevention of arteriosclerotic cardiovascular disease.

Key Words: nitric oxide synthase ■ plasminogen activators ■ hypertension ■ collagen
L-NAME treatment. Here, we report that PAI-1−/− mice exhibit significant resistance to perivascular fibrosis and collagen deposition after NOS inhibition for 8 weeks. Furthermore, PAI-1−/− mice are protected against L-NAME–induced hypertension.

**Methods**

**Animal Preparation**

PAI-1−/− mice, which were originally generated by P. Carmeliet and colleagues,18,19 and WT mice on the same genetic background (C57BL/6J) were purchased from the Jackson Laboratory (Bar Harbor, Me). PAI-1−/− mice were backcrossed more than 10 times to the control C57BL/6J strain. Male PAI-1−/− mice and WT mice were maintained in a temperature-controlled facility with a fixed light/dark cycle, with 6 animals studied in each experimental group. L-NAME (Sigma Chemical Co), an NOS inhibitor, was administered in the drinking water at 1 mg/mL, 100 to 120 mg · kg−1 · d−1 intake, whereas control animals (PAI-1−/− and WT) received unmodified drinking water. All animals were fed a regular chow diet. Systolic blood pressure was serially determined in conscious, trained mice by a noninvasive tail-cuff device, and body weight was measured every 2 weeks.

**Histopathology and Morphometry**

Histopathology and morphometry were performed by a single investigator who was unaware of the treatment protocol. After 8 weeks, the animals were euthanized for gross and microscopic cardiac analyses. At death, 6 heart tissues were harvested per group, and left and right ventricles were separated from the atria and weighed. The effects of L-NAME on the extent of coronary perivascular fibrosis were also compared. To evaluate coronary perivascular fibrosis, Masson’s trichrome–stained sections were photographed and scanned. The short-axis images of the 10 to 15 arterial images at the lumen and outer border of the tunica media were traced in each section. Perivascular fibrosis, Masson’s trichrome–stained sections were photographed and scanned. The short-axis images of the 10 to 15 arterial images at the lumen and outer border of the tunica media were traced in each section. Perivascular fibrosis (area of fibrosis divided by total vessel area) (collagen deposition stained with aniline blue) immediately surrounding the coronary arterial wall was measured, and the ratio of perivascular fibrosis (area of fibrosis divided by total vessel area) was determined.

Morphometric analysis of left ventricular (LV) myocytes was performed to compare the effects of L-NAME on myocyte cross-sectional area.20,21 The outer borders of myocytes that were cut transversely and that had both a visible nucleus and an unbroken cellular membrane were traced, and the areas were determined. Approximately 100 myocytes were counted per heart section, and the average myocyte size was calculated for each animal.

**Oligonucleotide Primers for Reverse Transcription–Polymerase Chain Reaction Assay for Cardiac PAI-1 and Type I Collagen mRNA**

Relative quantification of mRNA was done by 1-step reverse transcription–polymerase chain reaction (RT-PCR) with real-time amplicon detection with the fluorescent dye SYBR Green I on a LightCycler Instrument (Roche Molecular Biochemicals). RT-PCR was performed in a reaction volume of 20 µL with 500 nM of each primer for a series of 2-fold template dilutions from 1:1 to 1:16, corresponding to 10.0 to 0.625 ng/µL total heart RNA. For reaction buffer and RT-PCR enzymes, the LightCycler RNA Amplification Kit SYBR Green I (Roche Molecular Biochemicals) was used. After reverse transcription at 55°C for 10 minutes and an initial denaturation step at 95°C for 30 seconds, amplification was performed with 50 cycles of denaturation (95°C for 1 second), annealing (63°C for 10 seconds), and extension (72°C for 20 seconds). To monitor amplification in real time, double-strand DNA-dependent SYBR Green I fluorescence was measured at the end of the extension period of each cycle. For each transcript, an inverse correlation was observed between the amount of applied total RNA and the interpolated cycle number (C) at which the magnitude of fluorescence increased with maximum velocity. The slope (m) and y intercept (b) defining this relationship were calculated by linear regression on the equation C=m·log[g total RNA]+b, with excellent goodness of fit ($R^2>0.98$) obtained for all dilution series. The average ratio of PAI-1/GAPDH or type I collagen/GAPDH transcript observed in samples obtained from each mouse was normalized to the average ratio of the transcript observed in samples obtained from control WT mice.

**Statistical Analysis**

Data are expressed as mean±SEM. Paired data were compared by Student’s t tests. Comparisons between multiple groups were performed by 1-way ANOVA followed by Fisher’s protected least significant difference tests. Comparisons of the time-related changes in blood pressure among groups were performed by 2-way ANOVA followed by Bonferroni’s multiple-comparison t tests. Comparisons of the ratios of cardiac PAI-1/GAPDH or cardiac type I collagen/GAPDH transcript were tested by 2-tailed Student’s t test. Results with P<0.05 were considered statistically significant.

**Results**

**Hemodynamic Parameters and Body Weight in Study Groups**

At baseline, there were no significant differences in systolic blood pressure between groups (Table). After initiation of L-NAME, systolic blood pressure increased at 2 weeks in L-NAME–treated WT mice (124±4 mm Hg, mean±SEM; P<0.01 versus baseline, P<0.01 versus untreated WT mice) and in L-NAME–treated PAI-1−/− mice (111±4 mm Hg; P<0.05 versus baseline, P<0.05 versus untreated PAI-1−/−). At this early time point, systolic blood pressure was already significantly higher in the L-NAME–treated WT mice than in similarly treated PAI-1−/− mice (P<0.001). Over the subsequent 6 weeks, systolic blood pressure progressively increased in L-NAME–treated WT mice, whereas L-NAME–treated PAI-1−/− mice failed to exhibit further increases (141±3 mm Hg in L-NAME–treated WT versus 112±4 mm Hg in L-NAME–treated PAI-1−/−, P<0.0001 by ANOVA (Figure 1). Systolic blood pressure did not change in control animals that did not receive L-NAME.
There were no significant differences in heart rate between groups by L-NAME treatment. Body weight was lower in PAI-1^{2/2} groups over 8 weeks compared with WT groups but increased significantly in both groups after 8 weeks of L-NAME treatment (Table).

Effects of L-NAME on LV Hypertrophy
The relative weight of the LV (LV weight/total body weight) was significantly increased in the L-NAME–treated animals compared with untreated controls \((P<0.01)\). The relative increase in LV mass, however, was greater in WT mice than in PAI-1^{1/-} mice \((P<0.01, \text{Figure 2A})\). There were no significant differences in the relative weights of the right ventricle between groups. At the cellular level, LV myocyte hypertrophy was significantly increased in the L-NAME–treated WT mice \((P<0.01, \text{Figure 2B})\). In contrast, there was no cellular hypertrophy in L-NAME–treated PAI-1^{2/2} mice (Figure 2B).

Coronary Perivascular Fibrosis
The ratio of coronary perivascular fibrosis to total vascular area increased significantly after 8 weeks of L-NAME treatment in WT mice \((P<0.01)\), whereas only marginal changes were observed in PAI-1^{1/-} mice treated with L-NAME (Figure 3). Consistent with these quantitative differences in the extent of perivascular fibrosis, there was a visible accumulation of collagen within the media of the coronary arteries in L-NAME–treated WT mice (Figure 4). Collagen accumulation did not differ between the L-NAME–treated PAI-1^{1/-} and control PAI-1^{2/2} groups.

Quantitative RT-PCR for Cardiac PAI-1 and Type I Collagen mRNA
To evaluate whether L-NAME significantly induced PAI-1 expression, we quantified cardiac PAI-1 mRNA levels in L-NAME–treated and control WT mice by 1-step RT-PCR with real-time amplicon detection. RT-PCR yielded sequence-specific amplicons of 351 bp for PAI-1 primers and 351 bp for Type I Collagen primers.

### Systolic Blood Pressure, Heart Rate, and Body Weight in Control and Treated Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Systolic Blood Pressure, mm Hg</th>
<th>Heart Rate, bpm</th>
<th>Body Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline Week 8</td>
<td>Baseline Week 8</td>
<td>Baseline Week 8</td>
</tr>
<tr>
<td>Control WT</td>
<td>102 ± 2 98 ± 2</td>
<td>402 ± 11 439 ± 10</td>
<td>24 ± 0.4 29 ± 0.4</td>
</tr>
<tr>
<td>L-NAME WT</td>
<td>100 ± 2 141 ± 3∗†</td>
<td>385 ± 16 415 ± 9</td>
<td>25 ± 0.7 30 ± 1.0†</td>
</tr>
<tr>
<td>Control PAI-1^{1/-}</td>
<td>102 ± 2 98 ± 2</td>
<td>402 ± 11 439 ± 10</td>
<td>22 ± 1.0 26 ± 0.7§</td>
</tr>
<tr>
<td>L-NAME PAI-1^{1/-}</td>
<td>97 ± 1 112 ± 4§§</td>
<td>390 ± 14 432 ± 17</td>
<td>23 ± 1.0 26 ± 0.6§</td>
</tr>
</tbody>
</table>

Data for systolic blood pressure, heart rate, and body weight in controls and L-NAME–treated groups at baseline and after 8 weeks. Data are mean ± SEM. Statistical significance was determined by Student’s t tests and ANOVA.

∗\(P<0.01\) vs baseline.
†\(P<0.01\) vs other groups.
‡\(P<0.05\) vs baseline.
§\(P<0.05\) vs control PAI-1^{1/-} group.
||\(P<0.01\) vs control PAI-1^{1/-} and L-NAME PAI-1^{1/-} groups.
302 bp for GAPDH primers. The ratio of cardiac PAI-1/GAPDH transcript in L-NAME–treated WT mice was 2.3-fold greater than the corresponding ratio in control WT mice ($P<0.001$, Figure 5).

Furthermore, to examine the mechanism by which PAI-1 deficiency induces matrix degradation and turnover, we performed quantitative RT-PCR for cardiac type I collagen expression. RT-PCR yielded sequence-specific amplicons of 175 bp for type I collagen primers. The ratio of cardiac type I collagen/GAPDH transcript was greater in PAI-1$^{2/-}$ groups than in control WT mice (2.3-fold greater in control PAI-1$^{2/-}$, $P<0.01$, and 2.2-fold greater in L-NAME–treated PAI-1$^{2/-}$ mice, $P<0.05$). The ratio did not increase significantly, however, in L-NAME–treated WT mice (1.4-fold greater than those in the control WT mice, $P=0.12$) (Figure 6).

### Discussion

In the present study, we have demonstrated that genetically modified mice with a deficiency in PAI-1 are resistant to the vascular pathology and hypertension induced by long-term NOS inhibition. This is the first animal model to exhibit resistance to the effects of L-NAME other than eNOS-deficient mice.25,26

The systemic hypertension induced by long-term NOS inhibition is probably explained by both the acute loss of nitric oxide–dependent vasodilation and by the time-dependent development of overt fibrotic structural changes in resistance vessels.1–3 In the present study, after initiation of L-NAME, systolic blood pressure increased significantly in both groups at 2 weeks. After this early increase, however, systolic blood pressure failed to increase further in PAI-1$^{2/-}$ mice, whereas systolic blood pressure progressively increased in WT mice. Furthermore, the extent of coronary perivascular fibrosis increased significantly after 8 weeks of L-NAME treatment in WT mice, whereas only marginal changes were observed in PAI-1$^{2/-}$ mice. These findings indicate that PAI-1 deficiency provides protection against the structural consequences of long-term NOS inhibition. This extends previous observations that PAI-1$^{2/-}$ mice are relatively resistant to fibrosis after chemical injury.17

The corollary of these findings is that vascular PAI-1 overproduction exacerbates the fibrotic process. Interestingly, long-term NOS inhibition has been reported to activate the renin-angiotensin system in the arterial wall,6,8,27,28 which may compound the injurious effects of NO deficiency on the vasculature.29 Our group and others have shown that angiotensin II regulates PAI-1 expression in vitro and in vivo,16,30–33 and NO suppresses PAI-1 expression after stimulation by angiotensin II in aortic smooth muscle cells.9 Together, these factors would be anticipated to increase vascular PAI-1 production during treatment with L-NAME. Indeed, it was recently shown that arterial PAI-1 expression

**Figure 3.** Coronary perivascular fibrosis in controls and L-NAME-treated groups. L-NAME for 8 weeks significantly increased coronary perivascular fibrosis in WT but not in PAI-1$^{2/-}$ mice. Data are mean±SEM. *$P<0.01$ vs the other groups.

**Figure 4.** Micrographs of coronary artery sections stained with Masson’s trichrome stain in control WT (A), L-NAME–treated WT (B), control PAI-1$^{2/-}$ (C), and L-NAME–treated PAI-1$^{2/-}$ (D) mice. In addition to increased perivascular fibrotic lesions, there was a visible accumulation of collagen within media of coronary arteries in L-NAME–treated WT mice (B). Bars=40 μm.

**Figure 5.** Quantitative RT-PCR in cardiac PAI-1 mRNA in control and L-NAME–treated WT mice. Data are mean±SEM. *$P<0.001$ vs control WT.

**Figure 6.** Quantitative RT-PCR in cardiac type I collagen mRNA in controls and L-NAME–treated groups. Data are mean±SEM. *$P<0.01$ and †$P<0.05$ vs control WT.
increases 4-fold in L-NAME–treated rats, which can be prevented by ACE inhibition. The present study confirms and extends this observation that long-term L-NAME treatment increases cardiac PAI-1 mRNA expression and suggests that L-NAME–induced PAI-1 expression may be involved in coronary vascular structural changes. The mechanistic link between PAI-1 and the structural and functional cardiovascular changes induced by long-term NOS inhibition, however, has not previously been appreciated. Furthermore, we confirmed the increased type I collagen mRNA expression in PAI-1+/− groups, although the expression did not increase significantly in L-NAME–treated WT mice. These findings indicate that PAI-1 deficiency may prevent the increase of collagen deposition by promoting accelerated matrix degradation. By directly inhibiting plasminogen activation and by indirectly impairing matrix metalloproteinase activation, PAI-1 may retard matrix turnover and promote pathological tissue remodeling with fibrosis. Thus, long-term treatment with L-NAME not only leads to increased matrix deposition through a variety of mechanisms, including induction of type I collagen production, but also coincidently impairs matrix degradation and turnover by inducing PAI-1. In the present study, L-NAME–induced coronary arterial perivascular fibrosis was abolished in PAI-1−/− mice, indicating that PAI-1 deficiency alone is sufficient to prevent the vascular fibrotic response and to preserve vascular compliance.

Other possibilities can be considered to explain the experimental results described in this study. If eNOS expression or activity is increased in PAI-1−/− mice, this might protect these animals from the effects of L-NAME. There is experimental evidence that NO in fact regulates PAI-1 production, whereas we are not aware of any evidence supporting the converse. Furthermore, basal systolic blood pressure is similar in WT and PAI-1−/− mice, suggesting that a major alteration in vascular NO production is unlikely to exist in the setting of PAI-1 deficiency.

The present study is limited to an exploration of the role of PAI-1 deficiency in protecting against the vascular consequences of long-term NOS inhibition. It is unknown whether PAI-1 deficiency protects against other experimental causes of perivascular fibrosis and hypertension. Furthermore, although it might be reasonable to anticipate that plasminogen activator deficiency might be associated with increased susceptibility to the vascular effects of L-NAME treatment, this hypothesis is currently being tested. In addition, heterozygous PAI-1−deficient mice should also be studied to determine whether or not the antifibrotic effects correlate with gene dose or represent a threshold phenomenon observed only in the complete absence of PAI-1.

At present, a variety of commonly applied medical therapies are known to reduce vascular PAI-1 production. For example, ACE inhibition reduces plasma PAI-1 levels in patients after myocardial infarction and in subjects with an activated renin-angiotensin system. Hormone replacement therapy also effectively lowers PAI-1 levels in postmenopausal women. Both of these therapeutic strategies, however, have multiple other effects that contribute to, and possibly complicate, their effects in terms of vasculoprotection. The present study describes a novel mechanism for protecting the vasculature from the pathological consequences of long-term inhibition of NOS. Direct inhibition of PAI-1 may provide a new strategy for the prevention of hypertensive cardiovascular disease and arteriosclerosis.

Acknowledgments

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References


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