Inhibition of NO Synthesis Induces Inflammatory Changes and Monocyte Chemoattractant Protein-1 Expression in Rat Hearts and Vessels

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Abstract—We recently showed that chronic inhibition of NO synthesis by Nω-nitro-L-arginine methyl ester (L-NAME) causes coronary vascular remodeling (ie, vascular fibrosis and medial thickening) in rats. To test the hypothesis that the inhibition of NO synthesis induces inflammatory changes in the heart, we characterized the inflammatory lesions that occurred during L-NAME administration and determined whether inflammation involved the induction of monocyte chemoattractant protein-1 (MCP-1) in vivo. During the first week of L-NAME administration to Wistar-Kyoto rats, we observed a marked infiltration of mononuclear leukocytes (ED1-positive macrophages) and fibroblast-like cells (α-smooth muscle actin–positive myofibroblasts) into the coronary vessels and myocardial interstitial areas. These inflammatory changes were associated with the expression of proliferating cell nuclear antigen and MCP-1 (both mRNA and protein). The areas affected by inflammatory changes, as well as the expression of MCP-1 mRNA, declined after longer (28 days) treatment with L-NAME and were replaced by vascular and myocardial remodeling. Our results support the hypothesis that the inhibition of NO synthesis induces inflammatory changes in coronary vascular and myocardial tissues and involves MCP-1 expression. Results also suggest that the early stages of inflammatory changes are important in the development of later-stage structural changes observed in rat hearts. (Arterioscler Thromb Vasc Biol. 1998;18:1456-1464.)

Key Words: endothelium-derived factors ■ nitric oxide ■ remodeling ■ monocyte chemoattractant protein-1 ■ macrophages

The vascular endothelium becomes dysfunctional in the early stages of arteriosclerosis and atherosclerosis and remains dysfunctional throughout the course of the disease.1,2 Hypertension, hyperlipidemia, and aging are also associated with endothelial dysfunction.1–6 Such dysfunction has been shown to lead to the abnormal synthesis or release of NO, the principal mediator through which the endothelium-derived relaxing factor functions.2–4 NO has been demonstrated to regulate vascular tone and to inhibit platelet aggregation, thrombus formation, leukocyte adhesion, and vascular proliferation,4,7,8 suggesting that endothelium-derived NO regulates the development of structural changes (remodeling) of the blood vessels.

Chronic vascular diseases such as arteriosclerosis and atherosclerosis exhibit many features of inflammation associated with a reduction in NO synthesis in the endothelium.9–15 For example, recent experimental evidence suggests that the inhibition of NO synthesis increases vascular oxidative stress,11 activates the transcription of regulatory proteins,12,13 and induces the expression of various genes, including those encoding adhesion molecules and inflammatory cytokines.14 In addition, it has been shown that in endothelial cells in culture, inhibition of NO synthesis increases the expression of the gene coding for monocyte chemoattractant protein-1 (MCP-1) and that MCP-1 expression is associated with the activation of a transcription protein, nuclear factor-κB (NF-κB).15 MCP-1 is a potent chemokine for monocytes16–18; its expression is induced by inflammatory cytokines and peptide growth factors in monocytes, endothelial cells, and vascular smooth muscle cells in vitro19 as well as in atherosclerotic and arteriosclerotic lesions in vivo.20,21

We22–25 and others26–28 have recently shown that long-term (4 to 8 weeks) inhibition of NO synthesis with Nω-nitro-L-arginine methyl ester (L-NAME) induces vascular remodeling in rats and pigs. This remodeling was produced by an increase in activity of angiotensin-converting enzyme (ACE) in the heart and vessels and was prevented by treatment with the ACE inhibitor temocapril.23,24 ACE has also been shown to be activated in vascular and myocardial inflammatory lesions.29,30

These data obtained with our model of L-NAME–induced inhibition of NO synthesis have led us to hypothesize that a
reduction in NO synthesis induces inflammatory changes, including the activation of localized ACE, that leads to the development of vascular and myocardial remodeling. There is no direct evidence to link the inhibition of NO synthesis with inflammation in the heart. We therefore determined whether the inhibition of NO synthesis with L-NAME induces the infiltration of inflammatory cells into hearts and vessels. We characterized the temporal and spatial changes in inflammatory lesions occurring during the course of L-NAME administration and examined whether these inflammatory changes involve MCP-1.

Methods

Animal Model of Chronic Inhibition of NO Synthesis

The present experiments were reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and conducted according to the Guidelines for Animal Experiments of Kyushu University Faculty of Medicine. Male Wistar-Kyoto rats, obtained from an established colony at the Animal Research Institution of Kyushu University Faculty of Medicine, housed singly in a pyrogen-free facility and fed untreated drinking water. The second (L) group received untreated drinking water. The third group (L-NAME) (Sigma Chemical Co) in their drinking water was 100 mg/kg body weight. The third group (L-NAME plus L-arginine (70 mg/mL, Sigma Chemical Co) in its drinking water was 100 mg/kg body weight. The third group (L-NAME) received L-NAME (Sigma Chemical Co) in their drinking water. The fourth group (D-NAME-group) received L-arginine. Each group was euthanized on the third, seventh, and 28th days of treatment. Five rats in each of the L-NAME, L-arginine, and D-NAME groups were euthanized on the 3rd day of treatment.

Each animal was anesthetized with intraperitoneal pentobarbital, its abdomen was opened, and the abdominal aorta was cannulated. The chest was opened and an incision made in the right atrium. The heart was perfused via the aorta with oxygenated Krebs-Henseleit solution at a pressure of 90 mm Hg, and the coronary vasculature was fixed for 60 minutes with methacarn solution. The heart was cut into 5 pieces perpendicular to the long axis. In addition, the thoracic aorta, the proximal segment of mesenteric artery, the left renal artery, and the left kidney were isolated from each animal. All tissue samples were fixed in methacarn solution for a few days, dehydrated, embedded in paraffin, and cut into slices 5 μm thick. Sections were mounted on slides and stained with hematoxylin-eosin solution for estimation of inflammatory cell infiltration.

For immunohistochemistry, paraffin slices 5 μm thick were preincubated with 3% skim milk to decrease nonspecific binding. Sections were incubated overnight at 4°C with the mouse anti-rat macrophage/monocyte antibody (ED1, Serotec Inc); rabbit anti–l-lymphocyte antibody (CD3, Dako Co), mouse anti-human smooth muscle actin (α-SM actin) antibody (Dako Co), mouse anti-human proliferating cell nuclear antigen (PCNA) antibody (Dako Co), or nonimmune mouse or rabbit IgG (Zymed Laboratory Inc). The slides were washed and incubated with biotinylated, affinity-purified goat anti-rabbit IgG (Nitirei) as the secondary antibody. After avidin-biotin amplification, the slides were incubated with 3,3′-diaminobenzidine and counterstained with hematoxylin. To detect MCP-1, the mouse monoclonal antibody clone B4, specific for rat MCP-1,31 was used, along with the immunohistochemical methods described.

Morphometry and cell enumeration were performed by a single observer who was blinded to all treatment protocols. To quantify the areas affected by inflammatory changes, the hematoyxlin-eosin–stained whole heart sections (5 per heart) were scanned at ×40 magnification by use of a light microscope equipped with a high-resolution video camera (Microphoto-FXA, Nikon). The areas of clustered inflammatory cell infiltration were determined by using a personal computer (Apple Computer). The sum of the total areas of clustered inflammatory cell infiltration of the entire field and the sum of the total heart areas of the visual field of the section were calculated. Areas of large arterial and venular lumen were excluded from this measurement. Then the percentage of areas affected by inflammatory cell infiltration in each heart (100 multiplied by the area affected by inflammatory change divided by the total heart area of the section) was reported. This quantification method does not include scattered areas of inflammatory cell infiltration but does include clustered inflammatory areas. Thus, this method may underestimate the true inflammatory areas seen in this model.

To quantify the number of immunopositive cells in inflammatory areas in hearts, 3 heart sections per heart were stained immunohistochemically by antibodies against ED1, CD3, α-SM actin, or PCNA and scanned at ×100 magnification. Five to 6 clusters of vascular and myocardial inflammatory lesions (500 to 1000 nuclei per lesion) were selected at random in each heart. The number of cells positive for ED1, CD3, α-SM actin, or PCNA was counted; the sum of the total cells in the inflammatory lesion was reported; and the percentage of immunopositive cells per total counted cells was reported for each animal.

To quantify monocyte infiltration into other blood vessels (aorta, mesenteric artery, and renal artery), at least 4 cross sections of each vessel immunohistochemically stained with an antibody against ED1 were examined, and cells positive for monocyte antigen per section were counted. The average number of monocytes in the intima per section was calculated. In addition, longitudinal sections of the kidney stained by an antibody against ED1 were also examined.

Northern Blot Analysis

Five rats each in the control and L groups were euthanized on the third, seventh, and 28th days of treatment. Five rats in the L-arginine group received L-arginine (70 mg/mL, Sigma Chemical Co) in their drinking water.
group were euthanized on the third day of treatment. After this procedure, rat hearts were removed, the atria and great vessels were trimmed away, and the hearts were snap-frozen in LN2 and stored at −80°C. The right kidney was also isolated, frozen, and stored at −80°C.

Total RNA was extracted from each sample by the acid guanidinium–thiocyanate-phenol-chloroform method (ISOGEN, Nippon Gene), and poly(A)+ RNA was purified on an oligo(dT) cellulose column (Takara Shuzo). Five-microgram aliquots of poly(A)+ RNA were denatured with formaldehyde and formamide, fractionated by electrophoresis on formaldehyde-agarose gels, transferred to nylon membranes (Hybond N+, Amersham), and immobilized by UV irradiation. The membranes were hybridized overnight with rat MCP-1 cDNA31 or mouse GAPDH cDNA (American Type Culture Collection, Rockville, Md) and labeled with [32P]dCTP by random priming (Takara Shuzo). The filters were exposed by autoradiography to Kodak XAR5 film for 24 hours at −70°C with intensifying screens. Relative amounts of MCP-1 mRNA were normalized against the amounts of GAPDH mRNA.

Measurement of NO Production
On the seventh day of treatment, the thoracic aorta was removed en bloc from the rats of each group and placed in cold Krebs-Henseleit solution. The extravascular tissue was removed rapidly, and the vessel lumen was flushed with the solution. Then the aorta was cut into 5-mm-ring segments.

The 5-mm-ring segments of the aortas were incubated with 2 mL HBSS medium containing calcium ionophore A23187 (1 μmol/L) and L-arginine (100 μmol/L). At selected time points (0, 60, and 120 minutes), samples of the medium (100 μL) were collected for measurement of NO2 plus NO3 (NOx), a stable oxidation product of NO. NOx was measured with a chemiluminescence NOx detector (270B, Sievers Co). Signals from the detector were analyzed by a computerized integrator and recorded as areas under the curve. Specific NO-generating capacity was expressed as nanomoles per hour per gram dry weight.

Statistical Analysis
Data are expressed as mean±SE. Changes in parameters of a group over time were compared by 1-way ANOVA and Bonferroni’s multiple comparison test. Differences between groups were determined by 2-way ANOVA and a multiple comparison test. A level of P<0.05 was considered statistically significant.

Results

Systolic Arterial Pressure and Heart Rate
Compared with the control group, the L and L+L-arg groups showed a progressive rise in systolic arterial pressure throughout the study. In addition, the L and L+L-arg groups showed a transient reduction in heart rate on the third and seventh days of treatment, whereas heart rate remained constant in the control group. Rats that received D-NAME (D group) showed no significant changes in systolic arterial pressure and heart rate during the study (Table 1).
Histopathology and Immunohistochemistry of the Heart

On the third and seventh days of treatment, tissue sections from all of the L group rats exhibited marked infiltration of mononuclear cells into the perivascular areas that immediately surrounded the coronary vessels (Figure 1). Attachment of mononuclear leukocytes to the coronary arterial and venous lumens was also observed. Infiltration of inflammatory cells into myocardial interstitial spaces was associated with myocardial myocyte necrosis (Figure 1). In contrast, there was rare infiltration by polymorphonuclear cells. Vascular and myocardial inflammatory lesions were unevenly but equally distributed in the left and right ventricles (Figure 2A).

The areas affected by inflammatory changes declined from day 3 to day 28 (Figure 2A).

We observed no evidence of inflammation in the control rats (Figure 1) or the D groups of rats (data not shown). On day 3, the areas of inflammation in the L + L-arg group (0.3 ± 0.1%) were significantly lower than in the L group (7.0 ± 0.9%).

Cells reacting with antibodies directed against ED1, CD3, PCNA, and MCP-1 were rarely observed in the control group, and cells stained with α-SM actin were limited to the vascular media (data not shown).

We examined the inflammatory lesions in the L group by immunohistochemistry. On day 3, we found that a considerable proportion (~60%) of inflammatory cells that had infiltrated into the lesions were ED1-positive monocytes (Figures 3A and 2B). Spindle-shaped, fibroblast-like cells positive for α-SM actin (myofibroblasts) constituted 10% of the inflammatory cells (Figures 3C and 2B), and there was a small number of CD3-positive T cells (Figures 3B and 2B).

The percentage of ED1-positive monocytes declined, whereas the percentage of α-SM actin–positive myofibroblast cell increased during the course of L-NAME treatment. The cells in the inflammatory areas that were not derived from monocytes, T cells, or myofibroblasts (~25% on the third day, 40% on the seventh day, and 50% on the 28th day) appeared to be spindle-shaped fibroblasts. These changes in cell populations were not affected by L-arginine treatment (not shown). We used α-SM actin as a marker for myofibroblasts. However, the cellular origin of these immunopositive cells is uncertain. They may have been derived from either fibroblasts or vascular smooth muscle cells that migrated into the inflammatory lesions.

We detected PCNA-positive cells in the inflammatory lesions of the vascular intima and media and in perivascular and myocardial interstitial areas of the L group (Figure 3D) on the third (33 ± 16%), seventh (21 ± 8%, P < 0.01 versus the third day), and 28th (14 ± 4%, P < 0.01 versus the third day) day of treatment. The percentage of PCNA-positive cells on the third day was significantly lower (P < 0.01) in the L + L-arg group (10 ± 4%) than in the L group.

Expression of MCP-1 in the Heart

Because the leukocytes that had infiltrated into the inflammatory lesions were predominantly monocytes, we examined the expression of MCP-1 in rat hearts by immunohistochemistry and Northern blot analysis. We found that most of the coronary arteries and veins in the visual field of the section were positively stained with an MCP-1 antibody in the L group of rats on the third and seventh days of treatment. We detected MCP-1–positive cells in the media (vascular smooth muscle cells) of coronary arteries as well as veins (Figure 4). MCP-1 immunoreactivity was also observed in some mononuclear leukocytes (possibly monocytes) that had infiltrated into the intima and adventitia. MCP-1–positive cells in the vascular inflammatory lesions became less prominent from day 3 to day 28 (data not shown). In the myocardial inflammatory areas, we detected MCP-1–positive cells on the third (~50% to 60%), seventh (20% to 40%), and 28th (10% or less) day of treatment.

Figure 2. A, Time course of inflammatory cell infiltration into vascular and myocardial areas in L group. Black bars indicate areas of clustered inflammatory cell infiltration in the left plus right ventricles; shaded bars, areas of clustered inflammatory cell infiltration in left ventricle; and stippled bars, areas of clustered inflammatory cell infiltration in right ventricle. †P < 0.01 versus values on day 3. B, Time course of inflammatory cells with antibodies directed against ED1, CD3, and α-SM actin in hearts of L group. *P < 0.01 versus values on day 3.
In concert with mononuclear cell infiltration, the cardiac MCP-1 mRNA level in the L group was much higher after 3 days but declined with further treatment; at all time points, however, expression of MCP-1 message was significantly higher in the L group than in the control group (Figure 5). The increased expression of MCP-1 mRNA observed on day 3 of L-NAME treatment was not observed in rats of the L group (Figure 6).

Inflammatory Changes in the Aorta, Mesenteric Artery, and Kidney
We examined monocyte infiltration into the aorta, mesenteric artery, and renal artery by immunohistochemistry on the third day of treatment (Table 2). The number of ED1-positive monocytes that had infiltrated into the intima of these vessels was significantly increased in the L group. The increase in monocyte infiltration was not significant in the D or L group.

We also examined the expression of MCP-1 in the kidney by immunohistochemistry and by Northern blot analysis on the third and seventh day of treatment. In contrast to the increased monocyte infiltration into the heart and blood vessels, there was no detectable increase in monocyte infiltration into the kidney (data not shown). The transcript levels of MCP-1 in the kidney of the L group rats were not affected (Figure 7).

NO$_x$-Generating Capacity
NO$_x$ production from the aortic segments with and without endothelium was measured in the control group (Figure 8). Removal of the endothelium markedly decreased aortic NO$_x$ production in the control group to the level of that in the L group segments with endothelium, indicating that NO$_x$ measured in the current study was produced and released from the endothelium.

NO$_x$ production from the aorta with endothelium was compared among all 4 groups (Figure 8). NO$_x$ production in the L group aortas was markedly less than that in the control group aortas. NO$_x$ production in the D group aortas was not affected. Treatment with L-arginine normalized the L-NAME–induced decrease in NO$_x$ production.
Discussion

This in vivo study demonstrates for the first time that inhibition of NO synthesis induces the infiltration of mononuclear leukocytes and myofibroblasts into the coronary vessels as well as into myocardial interstitial areas. In addition, we found that infiltration of these cells is accompanied by cardiomyocyte necrosis and that MCP-1 expression is associated temporally and spatially with the inflammatory lesions. These data support the hypothesis that chronic inhibition of NO synthesis modulates inflammatory changes in coronary vessels and myocardial tissues and involves MCP-1 in rat hearts.

We confirmed that endothelial NO synthesis is inhibited by L-NAME administration by measuring NO-generating capacity in the aorta. We regarded it unlikely that the vascular and cardiac inflammatory changes we observed resulted from nonspecific activity of L-NAME, because L-arginine inhibited the L-NAME-induced inflammatory changes almost completely. Furthermore, we found that the L-NAME-induced inflammatory changes in both right and left ventricles and administration of D-NAME did not induce the inflammatory changes. These results suggest that the inflammatory changes we detected in this model are most likely due to a reduction in NO synthesis or release.

Previously, we had shown that coronary vascular remodeling (medial thickening and perivascular fibrosis) and myocardial remodeling (fibrosis) developed after 28 to 56 days of L-NAME treatment.22–25 We have demonstrated here that this remodeling was preceded by inflammatory lesions, suggesting that these inflammatory changes are the primary initial events responsible for remodeling. The reason for the reduction in inflammatory lesions that we observed at day 28, despite the continuous administration of L-NAME, is not clear. We also found that most of the inflammatory cells that had infiltrated into the lesions were monocytes and myofibroblasts. In addition, a moderate number of these inflammatory cells were shown to express PCNA, a marker of cell

Figure 4. Immunohistochemical localization of MCP-1. Sections immunohistochemically stained with MCP-1 antibody (A, B, and D through F) and nonimmune IgG (C). A, Large coronary artery section in control group. Bar=100 μm. B, Large coronary artery section in L group after 3 days of treatment. Bar=100 μm. C, Large coronary artery section in L group after 3 days of treatment. Bar=100 μm. D, Small coronary artery section in L group after 3 days of treatment. Bar=50 μm. E, Small coronary vein section in L group after 3 days of treatment. Bar=50 μm. F, Section in myocardial inflammatory lesion after 3 days of treatment. Bar=30 μm.
proliferation, suggesting that these infiltrating inflammatory cells were activated. Other investigators had shown that chronic administration of L-NAME for 28 days or more causes structural changes in the aorta, mesenteric arteries, and renal arteries.\textsuperscript{26–28} We have observed here early monocyte infiltration to those vessels by day 3 of L-NAME administration. Therefore, our present results suggest that early inflammatory and proliferative changes play a key role in the development of subsequent structural changes observed during late phases in this model. Although a short-term reduction in NO synthesis has been shown to increase neutrophil rolling and adherence to the endothelium,\textsuperscript{32,33} no such polymorphonuclear cells was observed in the lesions.

We have also extended the in vitro finding of Zeiher et al\textsuperscript{15} and Tsao et al\textsuperscript{34} by demonstrating that in vivo inhibition of NO synthesis increases MCP-1 expression in the heart. MCP-1 is a protein that has been shown to possess proinflammatory activity and to mediate trafficking of monocytes to inflammatory sites.\textsuperscript{16–18} We observed increased MCP-1 protein production in coronary vessels and monocytes that had infiltrated into the inflammatory lesions by immunohistochemistry. However, although we demonstrated increased MCP-1 mRNA and protein in cardiac tissues, we did not detect infiltration of monocytes or altered MCP-1 expression in other organs such as the kidney, suggesting that upregulation of MCP-1 in our model may be localized.

The mechanism by which the inhibition of NO synthesis upregulates MCP-1 in our model is unclear and probably multifactorial. One possibility is that increased expression of MCP-1 may be mediated, at least in part, by activation of the renin-angiotensin system. This concept is supported by find-

| Table 2: Monocyte Infiltration Into the Intima of Aortas, Mesenteric Arteries, and Renal Arteries, as Determined by Immunohistochemistry |
|-----------------|-----------------|-----------------|-----------------|
|                  | Control Group   | L Group         | L+L-Arg Group   | D Group         |
|                  | (n=8)           | (n=8)           | (n=8)           | (n=8)           |
| Aortas           | 0.2±0.2         | 2.2±0.4*        | 0.6±0.3         | 0.2±0.2         |
| Mesenteric arteries | 0.4±0.4        | 4.2±0.9*        | 0.8±0.3         | 0.4±0.2         |
| Renal arteries   | 0.4±0.3         | 3.3±0.6*        | 0.6±0.3         | 0.6±0.3         |

Data are mean±SEM.
*P<0.05 versus control group by 1-way ANOVA and Bonferroni’s multiple comparison test.
no effect on hypertension induced by L-NAME administration. In preliminary studies, we found that the plasma l-arginine levels were >100× higher in the L+L-arg group than in the L group, indicating that l-arginine had been absorbed. One possible interpretation is that in addition to improvement of NO activity, l-arginine supplementation might have reduced inflammatory changes via currently unrecognized mechanisms.

The current results may have clinical implications. The adhesion of mononuclear cells to and their infiltration into the blood vessel wall have been assumed to be early crucial events in vascular disease.9,10 We have shown here that reduced NO synthesis may produce inflammatory and proliferative changes in vivo. Thus, endogenous NO synthesis may decrease MCP-1 in endothelial cells and monocytes and may contribute to the antiarteriosclerotic and antiatherosclerotic effects of endothelium-derived NO. Because l-arginine, a precursor of NO, has been shown to attenuate endothelial adhesion to monocytes and to inhibit the extent of atherosclerotic lesions in cholesterol-fed animals,42–45 it is suggested that NO may interfere with the chemotactic activity of the endothelial layer itself by both autocrine and paracrine mechanisms.

A second implication is related to recent findings that the vulnerability of atherosclerotic plaques to rupture is related to their macrophage content.46,47 In addition, in human atherosclerotic lesions, ACE has been found to be activated in regions with macrophage infiltration.29 The finding that transfer of the endothelial NO synthase gene inhibits neointimal formation after balloon injury48 suggest that therapeutic treatments (eg, lowering of cholesterol, inhibition of ACE, and antioxidants) that improve NO-generating capacity in diseased blood vessels may have beneficial effects by protecting the unstable atherosclerotic plaque from rupture, thereby protecting the patients from the ensuing acute myocardial infarction.

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