Increased Activity of Nuclear Factor-κB Participates in Cardiovascular Remodeling Induced by Chronic Inhibition of Nitric Oxide Synthesis in Rats

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Background—Chronic inhibition of endothelial nitric oxide (NO) synthesis by the administration of N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME) to rats induces early vascular inflammatory changes [monocyte infiltration into coronary vessels, nuclear factor-κB (NF-κB) activation, and monocyte chemoattractant protein-1 expression] as well as subsequent arteriosclerosis (medial thickening and perivascular fibrosis) and cardiac fibrosis. However, no direct evidence for the importance of NF-κB in this process is known.

Methods and Results—We examined the effect of a cis element decoy strategy to address the functional importance of NF-κB in the pathogenesis of cardiovascular remodeling. We found here that in vivo transfection of cis element decoy oligodeoxynucleotides against NF-κB to hearts prevented the L-NAME–induced early inflammation and subsequent coronary vascular medial thickening. In contrast, NF-κB decoy oligodeoxynucleotide transfection did not decrease the development of fibrosis, the expression of transforming growth factor-β1 mRNA, or systolic pressure overload induced by L-NAME administration.

Conclusions—The NF-κB system participates importantly in the development of early vascular inflammation and subsequent medial thickening but not in fibrogenesis in this model. The present study may provide a new aspect of how endothelium-derived NO contributes to anti-inflammatory and/or antiarteriosclerotic properties of the vascular endothelium in vivo. (Circulation. 2000;102:806-812.)

Key Words: endothelium-derived factors ■ inflammation ■ proteins ■ cells ■ nitric oxide ■ nuclear factor-κB

The vascular endothelium becomes dysfunctional in the early stages of vascular diseases.\textsuperscript{1-3} Such endothelial abnormalities are associated with reduced activity of nitric oxide (NO) and increased expression of inflammation-promoting genes.\textsuperscript{4-8} Recently, endothelium-derived NO has been recognized to be an anti-inflammatory and antiarteriosclerotic molecule. Mice lacking endothelial-type NO synthase exhibit hypertension and an enhanced vascular remodeling in response to injury.\textsuperscript{9,10} We recently reported that chronic inhibition of NO synthesis by the administration of N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME) induces early inflammation [monocyte infiltration, monocyte chemoattractant protein-1 (MCP-1) expression, and nuclear factor-κB (NF-κB) activation] and late cardiovascular remodeling in rats (Figure 1).\textsuperscript{11-15} We also demonstrated that normalization of arterial hypertension by hydralazine treatment did not attenuate such pathological changes,\textsuperscript{11,13,16,17} suggesting a minor role of systemic arterial hypertension in the development of such pathological changes in this model.

NF-κB is presumed to be an important redox-sensitive transcriptional factor that regulates transcription of genes encoding inflammatory cytokines, adhesion molecules, and chemokines.\textsuperscript{18} NF-κB is activated by a battery of stimuli leading to atherogenic events, including oxidative stress, tumor necrosis factor, and angiotensin II.\textsuperscript{19-21} MCP-1 is a member of the C-C chemokines and a potent chemotactic factor for monocytes.\textsuperscript{22} Increased activity of NF-κB\textsuperscript{23} or MCP-1\textsuperscript{24} has been observed in arteriosclerotic and/or atherosclerotic lesions. We recently demonstrated that increased superoxide anion production contributes to the pathogenesis of ACE activation and that ACE inhibition, angiotensin II type 1 receptor blockade, or antioxidant therapy with N-acetylcysteine prevents vascular inflammation, MCP-1 expression, NF-κB activation, and subsequent remodeling in the rat model (Figure 1).\textsuperscript{25} These observations suggest that the increase in local angiotensin II activity plays a primary role in the development of such early and late cardiovascular pathological changes (Figure 1). It is also suggested that increased
activity of NF-κB would participate in the inflammatory changes through transcription of MCP-1 in the rat model of chronic inhibition of NO synthesis (Figure 1). However, no direct evidence for the functional importance of NF-κB in the formation of such cardiovascular inflammation and remodeling has been addressed.

To achieve effective blockade of NF-κB activity in vivo, transfection of a cis element decoy against the NF-κB binding site seems to be a useful strategy. Recently, Morishita et al elegantly innovated the cis element decoy strategy as an effective and feasible in vivo gene therapy. They reported that transfection of NF-κB decoy oligodeoxynucleotides (ODNs) to rat hearts by injection of hemagglutinating virus of Japan (HVJ)-liposome complex in the ascending aorta markedly reduced myocardial injury due to myocardial infarction. Thus, we used this novel strategy and investigated the role of NF-κB in the development of early inflammatory changes and late cardiovascular remodeling in a rat model of chronic inhibition of NO synthesis.

Methods

Decoy ODN Sequences and Preparation of HVJ-Liposome Complexes

The sequences of NF-κB decoy ODNs and scramble decoy ODNs used in this study were as follows (consensus sequences of binding site for NF-κB are italicized): NF-κB decoy ODN: 5'-CCTTGAAGGGATTTCCCTCC-3'; 3'-GGAACCTCCCTAAAGGG-GAGG-5'. Scramble decoy ODN: 5'-TTGCCTGACCTGAC-TAGCC-3'; 3'-AAGGCCATGGACATCGG-5'.

The NF-κB decoy ODNs, but not scramble decoy ODNs, have been shown to bind the NF-κB transcriptional factor. HVJ-liposome complexes were prepared as described. The final concentration of decoy ODNs was 15 μmol/L.

In Vivo Transfection of ODNs Into Hearts

After a rat was anesthetized with intraperitoneal pentobarbital, the right common carotid artery was surgically exposed. A balloon catheter 1.5 mm in diameter was introduced into the common carotid artery and advanced to the ascending aorta. After the catheter tip was positioned at the aortic sinus of Valsalva, the balloon was inflated, and HVJ-liposome complex containing FITC-labeled or unlabeled ODNs (1 mL at 4°C) was infused selectively into the coronary arteries. Then the catheter was removed, the wound was closed, and the animals were allowed to recover from the surgery.

Animal Model of Chronic Inhibition of NO Synthesis

The present study protocol was reviewed and approved by the Committee on Ethics on Animal Experiments, Kyushu University Faculty of Medicine, and the experiments were conducted according to the Guidelines for Animal Experiments of Kyushu University Faculty of Medicine.

Four groups of male Wistar-Kyoto rats were studied. The control group received normal water and chow. The L-NAME group received L-NAME in drinking water (1 mg/mL). The L-1NF group received L-NAME 2 days after NF-κB decoy transfection. The L-1SD group received L-NAME 2 days after scramble decoy transfection.

On day 3 after L-NAME administration was begun, systolic blood pressures (by the tail-cuff method) were measured. Then the rats were euthanized for morphometric, immunohistochemical, and biochemical analyses. Furthermore, some rats in each group received L-NAME for 7 days and untreated water during the following 21 days. On day 28, they were euthanized for morphometric analysis.

Electrophoretic Mobility Shift Assays

To determine the increase in NF-κB binding to the nucleus, electrophoretic mobility shift assays were performed. Five rats in each group were used. Nuclear extracts were prepared from the whole heart homogenates as described. The NF-κB oligonucleotides corresponding to putative consensus sequences (NF-κB: 5'-AGTGGAGGGACCTTCCAGGC-3') (Promega Biotechnology Inc) were labeled with [γ-32P]ATP and T4 polynucleotide kinase and purified by Sephadex G-50 column (Pharmacia Biotechnology Inc). Nuclear extract (10 μg) was incubated with 1 × 10^5 cpm of labeled probe and 2 μg of poly(dI-dC) in a buffer containing 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 4% glycerol, 100 mmol/L NaCl, 5 mmol/L DTT, and 100 g/L BSA for 30 minutes at room temperature (25°C). Then the samples were electrophoresed on 5% acrylamide/0.5×TBE gel (1×TBE: 90 mmol/L Tris borate, 2 mmol/L EDTA). After electrophoresis, gels were dried and subjected to autoradiography. Autoradiographs were later subjected to laser densitometry. Specificity was determined by the addition of excess cold oligonucleotide to the nuclear extracts 10 minutes before addition of radiolabeled probe.

Northern Blot Analysis

Total RNA was extracted from each sample, poly(A)+RNA was purified, and then Northern blot hybridization was performed as described. The cDNA probes used were as follows: a rat MCP-1, a rat transforming growth factor (TGF-β), cDNA (a gift from Dr T. Nakamura, Department of Biochemistry, Kyushu University), and a mouse GAPDH (American Type Culture Collection). Relative amounts of MCP-1 and TGF-β mRNA were normalized against the amounts of GAPDH mRNA.

Histopathology and Immunohistochemistry

Tissue sections were prepared as described. After fixation with methylacrylamid solution, the left ventricle was separated from the atria, right ventricle, and great vessels. The left ventricle was cut into 5 pieces perpendicular to the long axis. All tissues were dehydrated, embedded in paraffin, cut into slices 5 μm thick, and mounted on slides. The sections were stained with either hematoxylin-eosin, Masson’s trichrome, or immunostaining with antibodies against macrophage/monocyte (ED1, Serotec), proliferating cell nuclear antigen (PCNA) (Dako), nonimmune mouse IgG (Zymed), or p50/NF-κB (Santa Cruz). The slides were washed and incubated with biotinylated, affinity-purified goat anti-mouse IgG. After avidin-biotin amplification, the slides were incubated with 3'3'-diaminobenzidine and counterstained with hematoxylin.

Morphometry and cell counting were performed by a single observer who was blind to the treatment protocols as described.
Systolic Blood Pressure and Cardiac Tissue ACE Activity

<table>
<thead>
<tr>
<th>Systolic Blood Pressure, mm Hg</th>
<th>ACE Activity, nmol · mg⁻¹ · h⁻¹</th>
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<tbody>
<tr>
<td><strong>Control group (n=8)</strong></td>
<td></td>
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<tr>
<td>Day 0</td>
<td>131±6</td>
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<tr>
<td>Day 3</td>
<td>135±6</td>
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<tr>
<td>Day 28</td>
<td>126±4</td>
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<tr>
<td><strong>L-NAME group (n=8)</strong></td>
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<tr>
<td>Day 0</td>
<td>132±7</td>
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<tr>
<td>Day 3</td>
<td>168±4†</td>
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<tr>
<td>Day 28</td>
<td>148±4†</td>
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<tr>
<td><strong>L+NF group (n=8)</strong></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>142±6</td>
</tr>
<tr>
<td>Day 3</td>
<td>162±7*</td>
</tr>
<tr>
<td>Day 28</td>
<td>147±8†</td>
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<tr>
<td><strong>L+SD group (n=8)</strong></td>
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<tr>
<td>Day 0</td>
<td>141±6</td>
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<tr>
<td>Day 3</td>
<td>173±7*</td>
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<tr>
<td>Day 28</td>
<td>151±10†</td>
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</table>

Data are mean±SEM.

*P<0.01 vs control group; †P<0.05 vs control group.

In Vivo Transfection of Decoy ODNs Into Heart
Electrophoretic mobility shift assay showed that the NF-κB binding affinity was markedly increased in the hearts from the L-NAME group on day 3 of treatment (Figure 2A). The increased NF-κB activity was suppressed by NF-κB decoy ODN transfection but not by scramble decoy ODN transfection in vivo (Figure 2A). One day after intracoronary infusion of FITC-labeled ODNs, we examined the location of delivered decoy ODNs with the fluorescence microscope. The fluorescence was localized intensely in the nuclei (yellow) as well as cytoplasm (green) of vascular endothelial cells. Weak to moderate fluorescence (orange) was noted in the nuclei in vascular smooth muscle cells, cardiomyocytes, and some interstitial cells (Figure 2B).

Effects of the Transfection of NF-κB Decoy ODNs on the Inflammatory and Proliferative Changes and the mRNA Levels of MCP-1 and TGF-β₁ on Day 3
We observed no evidence of inflammation in the control group (Figure 3A). Attachment of mononuclear leukocytes to the endothelium of coronary vessels was seen in the L-NAME (data not shown) and L+SD groups (Figure 3A). A marked infiltration of mononuclear leukocytes in the perivascular areas immediately surrounding the coronary arteries and veins and the myocardial interstitial spaces was observed in those 2 groups (Figure 3A). The majority of leukocytes that had infiltrated into the lesions were found to be ED1-positive monocytes (Figure 3A). Nuclear staining for PCNA antibody was observed in some endothelial cells, vascular smooth muscle cells in the media, monocytes, or myofibroblast-like cells (Figure 3A). We also examined localization of NF-κB activation by immunohistochemistry (Figure 3B). Compared with the control group, the translocation of p50/NF-κB immunoreactivity to the nucleus, from faint cytoplasmic staining to a prominent nuclear pattern, was observed in the endothelial cells, some smooth muscle cells in the media, and infiltrated inflammatory cells (monocytes and myofibroblasts) in the L-NAME group. This change suggests that redistribution of p50/NF-κB from the cytoplasm to the nucleus has occurred. Such translocation of NF-κB immunoreactivity was observed in some areas in the myocardial myocyte to a similar extent in the control and L-NAME groups. These results suggest that NF-κB activation may occur predominantly in coronary vessels.

When ED1-positive monocytes or PCNA-positive cells were counted by use of immunohistochemistry, the number of immunopositive cells per section was significantly greater in the L-NAME group than in the control group (Figure 4A and 4B). These inflammatory and proliferative changes were markedly reduced by NF-κB decoy ODN transfection but not by scramble decoy ODN transfection (Figure 4A and 4B).

We examined the expression of MCP-1 and TGF-β₁ mRNA in the heart (Figure 4C and 4D). The cardiac MCP-1 and TGF-β₁ mRNA levels were significantly increased in the L-NAME group. The increased expression of MCP-1 mRNA was significantly reduced in the L+NF group but not in the L+SD group. In contrast, the increased expression of TGF-β₁...
mRNA was not reduced by NF-κB decoy or scramble decoy ODN transfection (Figure 4C and 4D).

Effects of the Transfection of NF-κB Decoy on Cardiovascular Remodeling on Day 28
The increase in the medial thickening (the wall-to-lumen ratio) of coronary arteries seen in the L-NAME group was prevented in the L+NF group but not in the L+SD group (Figure 5). In contrast, the increases in perivascular and cardiac fibrosis were not affected by NF-κB decoy or scramble decoy transfection (Figure 5). We did not examine left ventricular hypertrophy or function, because no such changes are evident within 28 days of treatment.11

Tissue ACE Activity
Cardiac tissue ACE activity was increased in the L-NAME group (Table). This increased ACE activity was not influenced by NF-κB decoy ODN transfection or scramble decoy ODN transfection (Table).

Discussion
The novel findings that have emerged from the present study are that in vivo transfection of cis element decoy against NF-κB binding site to the heart markedly suppressed NF-κB activity and prevented early inflammatory changes. Furthermore, transfection of NF-κB decoy ODNs inhibited the late development of coronary vascular medial thickening after inhibition of NO synthesis. The observed effect of NF-κB
decoy transfection was independent of arterial hypertension induced by L-NAME administration. Our present findings suggest that the NF-κB system may be essential in the development of early inflammation and subsequent coronary vascular medial thickening in our model.

In the present study, we could transfect the decoy ODNs superselectively into coronary arteries and demonstrate that the strategy achieved NF-κB blockade in rat hearts (Figure 2). Thus, our present observations suggest that increased activity of NF-κB participates essentially in the L-NAME–induced increases in MCP-1 expression and subsequent monocyte recruitment. This claim is supported by in vitro evidence that cis-acting elements for the NF-κB binding site in the MCP-1 gene promoter region are responsible for increased transcription of the MCP-1 gene and that inhibition of NO synthesis increases the activity of NF-κB in vitro. We previously demonstrated the increased production of MCP-1 protein in endothelial cells, medial smooth muscle cells, and infiltrating inflammatory cells. Because the NF-κB activation in immunohistochemistry was also observed in these cells (Figure 3B), it is likely that increased NF-κB–mediated transcription and protein production of MCP-1 participate essentially in the development of inflammatory changes in our model.

Transfection of NF-κB decoy ODNs also inhibited the increase in PCNA (a marker of cell proliferation)-positive cells. Nuclear staining for PCNA antibody was observed in endothelial cells, vascular smooth muscle cells in the media, infiltrating monocytes, and myofibroblasts. These results suggest that increased NF-κB–mediated transcription of inflammation-promoting genes, including MCP-1, activated those cells and thus induced the vascular proliferative changes. These proliferating cells can secrete growth-promoting factors such as platelet-derived growth factor, fibroblast growth factor, and reactive oxygen species. Therefore, we hypothesize that NF-κB–mediated transcription of MCP-1 induced the recruitment of monocytes and activated vascular smooth muscle cells and monocytes during the early phase, which in turn caused proliferation of vascular smooth muscles by producing those growth-promoting factors. Transfection of NF-κB decoy ODNs thereby inhibited the development of vascular medial thickening during the late phase in the present study. A recent study demonstrated that

![Figure 3. A, Histopathological and immunohistochemical pictures of coronary arteries. Coronary artery sections stained with hematoxylin-eosin (HE) and those immunohistochemically stained for monocyte/macrophage (ED1), proliferating cells (PCNA), and nonimmune IgG (negative control) on day 3 after L-NAME administration was begun. B, Coronary artery sections immunohistochemically stained for p50/NF-κB from control rat and rat that received L-NAME for 3 days. Right, Expanded view of area outlined with black lines in middle panel. Arrowheads denote endothelial cells with nuclear staining with an anti-p50/NF-κB antibody. Bar=50 μm.](http://circ.ahajournals.org/)


MCP-1 may directly stimulate proliferation and migration of cultured vascular smooth muscle cells. Hence, it is reasonable to assume that the NF-κB system plays a key role in early vascular inflammation and subsequent vascular medial thickening after blockade of NO synthesis in vivo.

Despite a marked inhibition of monocyte infiltration, transfection of NF-κB decoy ODNs could not reduce gene expression of TGF-β1 as well as perivascular and cardiac fibrosis. TGF-β1 may induce transformation of fibroblasts to myofibroblasts, stimulate production of extracellular matrix proteins, and thus play an central role in tissue fibrosis. There are no NF-κB binding sites in the promoter region of the TGF-β1 gene. Thus, the NF-κB system may not be involved in cardiac fibrogenesis in our model.

We previously demonstrated that local activity of ACE plays an important role in early cardiovascular inflammation and late remodeling in our model. In this study, there was no significant difference in the enzyme activity between hearts from the L-NAME, L+NF, and L+SD group. Thus, it is likely that the observed effects of NF-κB transfection were independent of local ACE activity.

We previously demonstrated that pharmacological inhibition of endothelial NO synthesis by L-NAME administration caused early cardiovascular inflammation and subsequent remodeling in rats. This conclusion is supported by our previous reports demonstrating that (1) endothelial NO production was blunted in the aorta from rats that received L-NAME but not from those that received D-NAME, (2) treatment with l-arginine prevented the L-NAME–induced inflammation and remodeling, and (3) administration of D-NAME did not induce such cardiovascular pathological changes. However, such pathological changes as seen in the rat model have not been described in genetically mutant mice lacking the endothelial NO synthase gene. These mice exhibit greater inflammatory and proliferative vascular responses to injury. The mechanisms of the difference between the rat and mouse models is probably multifactorial. One plausible explanation is that a defective endothelial NO synthase gene since birth might be compensated by other genes, so that no inflammatory or proliferative vascular changes have been observed at rest in mice lacking...
the endothelial NO synthase gene. In contrast, postnatal blockade of NO synthesis by administration of L-NAME to adult rats caused early inflammatory vascular responses and subsequent remodeling, as seen in the present study. Thus, these observations in the L-NAME–treated rats as well as in the mice lacking endothelial NO synthase may have clinically relevant implications, in that endothelium-derived NO is recognized to be an endogenous anti-inflammatory and/or anti-atherosclerotic factor.

In conclusion, the present study has provided direct in vivo evidence of the role of the NF-κB system in the development of vascular medial thickening at least by inducing MCP-1 in the rat model of chronic inhibition of NO synthesis. Although we did not examine whether other cis elements of the promoter region, such as AP-1 and SP-1, are involved in the regulation of MCP-1 expression in our model, the present study may provide a new aspect of how endothelium-derived NO contributes to anti-inflammatory and/or anti-atherosclerotic properties of the vascular endothelium in vivo. It appears that NO decreases monocyte recruitment to the arterial wall at least by suppressing NF-κB–mediated transcription of MCP-1 in vivo. Thus, preservation of normal endothelial NO activity may be an effective therapeutic strategy to reduce cardiovascular ischemic events in patients with vascular diseases. It might be of clinical interest to know whether an NF-κB decoy strategy can reduce restenosis after coronary angioplasty or the vulnerability of an atherosclerotic plaque prone to rupture.

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