Nicorandil Promotes Myocardial Capillary and Arteriolar Growth in the Failing Heart of Dahl Salt-Sensitive Hypertensive Rats

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Abstract—Long-term administration of vasodilators increases shear stress, which is thought to be important for vascular growth in the heart. Nicorandil, an activator of ATP-sensitive potassium channels with a nitrate-like action, is a potent vasodilator. We have now investigated the effects of nicorandil on vascular growth and gene expression in the failing heart of Dahl salt-sensitive (DS) hypertensive rats. DS rats fed a high-salt diet from 6 weeks of age develop concentric cardiac hypertrophy secondary to hypertension at 11 weeks, followed by heart failure at 18 weeks. DS rats on such a diet were treated with a nonanthypertensive oral dose of nicorandil (6 mg/kg per day) or vehicle from 11 to 18 weeks of age. Treatment of DS rats with nicorandil improved cardiac function and attenuated the development of heart failure. Myocardial capillary and arteriolar densities did not differ between vehicle-treated DS rats and age-matched controls. The abundance of mRNAs for endothelial NO synthase (eNOS), vascular endothelial growth factor (VEGF), the VEGF receptor Flt-1, and basic fibroblast growth factor (bFGF) in the myocardium was markedly reduced in vehicle-treated DS rats compared with controls. Treatment of DS rats with nicorandil greatly increased capillary and arteriolar densities and inhibited the downregulation of eNOS, VEGF, fms-like tyrosin kinase-1, and bFGF gene expression. This, nicorandil stimulates coronary capillary and arteriolar growth and thereby likely suppresses the development of heart failure in DS rats. Nicorandil may prove beneficial for the treatment of hypertensive heart failure as well as of ischemic heart disease. (Hypertension. 2005;46:719-724.)

Key Words: endothelial growth factors ■ fibroblast growth factor ■ potassium channels ■ heart failure

Nicorandil is a nicotinamide ester with 2 distinct mechanisms of pharmacological action: it induces the opening of ATP-sensitive potassium (K$_{ATP}$) channels, thereby triggering the dilation of peripheral and coronary resistance arterioles, and, through effects of its nitrate moiety, it elicits the dilation of systemic veins and epicardial coronary arteries. Thus, nicorandil increases coronary blood flow, reduces preload and afterload, and exerts an antianginal action.1-3 The Impact of Nicorandil in Angina (IONA) study, which included subjects with left ventricular (LV) dysfunction (ejection fraction of <45%), demonstrated a significant improvement in clinical outcome in patients with stable angina treated with nicorandil, suggesting a possible efficacy of this drug in the treatment of heart failure.3

Acute administration of nicorandil has indeed been shown to be effective in the treatment of heart failure, predominantly as a result of its vasodilator effects.4,5 A single oral dose of nicorandil increased coronary blood flow and ameliorated exercise-induced LV dysfunction in patients with previous myocardial infarction.6 Intravenous administration of nicorandil also attenuated exercise-induced LV diastolic dysfunction in individuals with hypertrophic cardiomyopathy, probably as a result of its beneficial effect on abnormal coronary microcirculation.7 These findings support the notion that nicorandil may ameliorate heart failure associated with defective coronary microcirculation.

Abnormalities of the microvasculature, such as a reduction in vascular density, thickening of the arteriolar wall, and inadequate angiogenesis, contribute to the pathogenesis of various forms of heart disease.8 Thus, stimulation of angiogenesis in the heart might be expected to benefit individuals with heart failure. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) promote angiogenesis by inducing the migration and proliferation of endothelial and smooth muscle cells, as well as the formation of vascular tubes and networks.9-10 Arteriolar growth is dependent on bFGF; as revealed by the observation that coronary arteriolar growth in neonatal rats was inhibited by treatment...
with neutralizing antibodies to this growth factor.14 Mice that lack VEGF exhibit impaired angiogenesis and the consequent development of ischemic cardiomyopathy,13 suggesting that VEGF is an important regulator of the balance between cardiac oxygen consumption and vascular growth.

Long-term administration of vasodilators increases shear stress, which is an important stimulus for the release of bFGF and VEGF, and these growth factors may then function as mediators of shear stress–induced angiogenesis.13–16 We hypothesized that long-term administration of nicorandil, a potent vasodilator,1 might induce myocardial angiogenesis through stimulation of the production of angiogenic growth factors such as bFGF and VEGF, and that such an effect might contribute to the beneficial action of this drug in heart failure.

Methods

Animals and Experimental Protocols
Male inbred Dahl salt-sensitive (DS) rats were obtained from Eisai (Tokyo, Japan) and handled in accordance with the guidelines of Nagoya University Graduate School of Medicine as well as with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). Weaning rats were fed laboratory chow containing 0.3% NaCl until 6 weeks of age. DS rats fed an 8% NaCl diet after 6 weeks manifest compensated concentric LV hypertrophy attributable to hypertension at 11 weeks and a distinct stage of fatal LV failure with chamber dilation at 18 weeks. DS rats were therefore fed an 8% NaCl diet from 6 weeks of age and received either a nonanthipertensive dose (6 mg/kg of body mass per day) of nicorandil (Chugai) or vehicle (DS-ChF+V, respectively; n = 8 per group) via a gastric tube from 11 to 18 weeks of age. The dose of nicorandil was determined from our preliminary observations and the results of a previous study.17 DS rats maintained on the 0.3% NaCl diet after 6 weeks of age served as age-matched controls (DS-C; n = 8). The diet and tap water were provided ad libitum throughout the experiment. Systolic blood pressure was measured weekly by the indirect tail-cuff method. At 18 weeks of age, rats were killed with an overdose of sodium pentobarbital (50 mg/kg), and the heart was removed for analysis.

Echocardiographic and Hemodynamic Analyses
At 18 weeks of age, rats were subjected to transthoracic echocardiography as described.18 In brief, M-mode echocardiography was performed with a SONOS 7500 ultrasound system and an ultraband high-fidelity manometer-tipped catheter (SPR-407; Millar Instruments) that had been calibrated relative to atmospheric pressure was introduced through the right carotid artery into the left ventricle. Tracings of LV pressure and the ECG were digitized to perfusion. The ventricle. Tracings of LV pressure and the ECG were digitized to

Immunohistochemistry
The left ventricle was fixed with ice-cold 4% paraformaldehyde for 16 to 24 hours, embedded in paraffin, sectioned transversely (thickness 3 μm), and processed for immunohistochemistry to determine the extents of coronary capillary formation and vascular density. Antibodies to tissue transglutaminase (CUB 742; Dako) and to von Willebrand factor (vWF; F5320; Dako) were used to visualize endothelial cells, and smooth muscle cells were detected with antibodies to α-smooth muscle actin (M0851; Dako). After removal of paraffin with xylene and dehydration with a series of ethanol solutions, the tissue sections were subjected to microwave irradiation (750 W) for 15 minutes in 0.01 mol/L citrate buffer (pH 6.0). Sections were then placed in an automated immunostainer (Ventana Medical Systems) as described.19 For negative controls, primary antibodies were replaced with mouse IgG. Capillaries positive for tissue transglutaminase or vWF were counted in ≥5 different microscopic fields (×400) of each section, and capillary density was expressed as the average number of capillaries per field. Absolute density was assessed from the number of α-smooth muscle actin–positive microvessels in the LV wall at a magnification of ×100; 8 nonoverlapping fields were examined to avoid double sampling of vessels. Arterioles were defined as vessels with an internal diameter of <50 μm that had ≥1 layer of smooth muscle cells; vessels with no smooth muscle and a diameter of <10 μm were considered capillaries.20

Reverse Transcription and Quantitative Polymerase Chain Reaction
Total RNA was extracted from LV tissue and treated with DNase with the use of a spin-vacuum total RNA isolation kit (Promega). cDNA was synthesized from 2 μg of total RNA with the use of an oligo(dT) primer and SuperScript II reverse transcriptase (Gibco BRL). Quantitative polymerase chain reaction (PCR) analysis was performed with a Prism 7700 Sequence Detector (Perkin-Elmer) as described.21 with primers and TaqMan probes specific for cDNAs encoding atrial natriuretic peptide (ANF), brain natriuretic peptide (BNP), β-myosin heavy chain (β-MHC), VEGF, or the VEGF receptor fetal liver kinase-1 (KDR).18,22 PCR was also performed with oligonucleotides specific for endothelial NO synthase (eNOS) cDNA (5′-CCGGAAGATGAGAAGGGTTT-3′, 5′-CTGCTGTGTTAAGTGCTC TTC-3′, and 5′-CTGAAGAGCAGAGAGGTTACAAAATTCCGA-3′ as the forward primer, reverse primer, and TaqMan probe, respectively; GenBank accession No. AJ011115), for Flt-1 cDNA (5′-GAGGAAAGCCTCAGGTACCTCA-3′, 5′- GTGTTGCCTGCGACACTCTTTG-3′, and 5′-GTCGCAAGAACCCT CAGACAAGTCCAAACC-3′, respectively; GenBank accession No. NM019306) or for bFGF cDNA (5′-AGCGACCCACACATGCA ACTAC-3′, 5′-ACCTGCTGTAGAAGAGAGGTTG-3′, and 5′- TCCAAACAGAGAAGAGAAGTTGTTGC-3′, respectively; GenBank accession No. x61697). TaqMan rodent GAPDH control reagents (Perkin-Elmer) were used for detection of GAPDH mRNA as an internal standard. The PCR products of each target gene were subcloned by TA cloning (pGEM-T Easy; Promega) and verified by sequencing. Serial dilutions of cloned plasmid DNA were analyzed for each target gene to determine standard curves for quantitative analysis. A positive control with the cDNA template used to generate the standard curve and a negative control (water control) that lacked any cDNA were also performed.

Immunoblot Analysis
Tissue samples (80 μg of protein) were subjected to SDS-PAGE on a 15% gel, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was incubated at room temperature first for 1 hour with 5% nonfat milk and 0.1% Tween-20 in Tris-buffered saline, and then overnight with rabbit polyclonal antibodies to VEGF or to β-MHC (Santa Cruz Biotechnology) at a 1:1000 dilution in the same solution. After washing, the membrane was incubated for 1 hour with a 1:10000 dilution of horseradish peroxidase–conjugated goat antibodies to rabbit IgG (Medical and Biological Laboratories), and immune complexes were then detected with ECL (enhanced chemiluminescence) reagents (Amer sham). The intensity of the VEGF and β-MHC bands was quantified by densitometry with ATTO CS Analyzer (version 1.01) software.18 We used 10 ng of recombinant human VEGF or FGF1 (Sigma) as positive controls for immunoblot analysis.

Statistics
Data are expressed as means±SEM. Differences among groups were assessed by 1-way factorial ANOVA. Within-group comparisons were performed by 2-way repeated-measures ANOVA. When a significant difference was detected, intergroup comparisons were
Effects of Nicorandil on Echocardiographic, Hemodynamic, and Other Parameters in 18-Week-Old DS Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DS-C</th>
<th>DS-CHF+V</th>
<th>DS-CHF+NCR</th>
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</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>399±10</td>
<td>289±9</td>
<td>320±25</td>
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<td>Systolic blood pressure (mm Hg)</td>
<td>156±2</td>
<td>255±4*</td>
<td>267±6*</td>
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<tr>
<td>Heart rate (bpm)</td>
<td>411±23</td>
<td>438±17</td>
<td>425±15</td>
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<tr>
<td>LV weight (mg)/tibial length (mm)</td>
<td>20.1±0.4</td>
<td>29.9±2.4*</td>
<td>30.5±1.6*</td>
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<tr>
<td>Lung weight (mg)/tibial length (mm)</td>
<td>40±4</td>
<td>110±11*</td>
<td>64±11†</td>
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<tr>
<td>LVPWT (mm)</td>
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<td>1.9±0.1*</td>
<td>1.8±0.1*</td>
</tr>
<tr>
<td>LVd (mm)</td>
<td>7.5±0.1</td>
<td>8.5±0.3*</td>
<td>7.9±0.2†</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>42±1</td>
<td>24±3*</td>
<td>32±2†</td>
</tr>
<tr>
<td>LVEDP (mm Hg)</td>
<td>5±1</td>
<td>19±2*</td>
<td>10±1†</td>
</tr>
</tbody>
</table>

Data are means±SEM. *P<0.05 vs DS-C rats; †P<0.05 vs DS-CHF+V rats.

Results

Effects of Nicorandil on LV Failure and Remodeling

Systolic blood pressure was significantly higher in DS-CHF+V rats than in DS-C rats at 7 weeks of age and thereafter. Systolic blood pressure did not differ between DS-CHF+V and DS-CHF+NCR rats from 6 to 18 weeks (Table; Figure 1). Heart rate was similar among all 3 groups (Table). The ratio of LV weight to tibial length, an index of LV hypertrophy, was 49% greater in DS-CHF+V rats than in DS-C rats at 18 weeks, and the overload-induced increase in this parameter was not affected by nicorandil. The ratio of lung weight to tibial length, an index of pulmonary congestion, was increased by 175% in DS-CHF+V rats compared with that in age-matched DS-C rats, indicative of the development of CHF; treatment with nicorandil significantly reduced the load-induced increase in this parameter.

Echocardiography revealed that LVPWT and LVd were significantly greater in DS-CHF+V rats than in DS-C rats. LV fractional shortening (LVFS) was decreased in DS-CHF+V rats relative to that in DS-C rats. Treatment with nicorandil reduced the increase in LVd and the decrease in LVFS in DS rats on the high-salt diet, whereas LVPWT was not affected by this drug. Hemodynamic analysis revealed that LVEDP was greater in DS-CHF+V rats than in DS-C rats, and that treatment with nicorandil ameliorated this increase in LVEDP (Table).

Effects of Nicorandil on Coronary Capillary and Arteriolar Growth

Immunostaining of the myocardium with antibodies to tissue transglutaminase or to vWF to detect capillary endothelial cells revealed that capillary density did not differ significantly between DS-CHF+V and DS-C rats (Figure 2). Treatment with nicorandil significantly increased myocardial capillary density. Immunostaining for α-smooth muscle actin revealed that whereas the density of arterioles with an internal diameter of >25 μm was similar in DS-CHF+V and DS-CHF+NCR rats, that of arterioles with an internal diameter of between 10 and 25 μm was significantly increased in DS-CHF+NCR rats compared with DS-CHF+V rats (Figure 3).

Effects of Nicorandil on Load-Induced Reprogramming of Cardiac Gene Expression

Hemodynamic overload resulted in marked upregulation of the expression of fetal-type cardiac genes, including those for ANP, BNP, and β-MHC in the left ventricle of DS rats (Figures 4 and 5). Treatment with nicorandil inhibited the increase in the expression of these genes.

Effects of Nicorandil on Cardiac Expression of VEGF, VEGF Receptors, bFGF, and eNOS

The abundance of VEGF and bFGF mRNAs in the LV myocardium was markedly reduced in DS-CHF+V rats.

Figure 1. Time course of systolic blood pressure in DS rats fed a high-salt diet and treated either with vehicle (DS-CHF+V) or with nicorandil (DS-CHF+NCR), as well as in age-matched controls fed a low-salt diet (DS-C). Data are means±SEM. *P<0.05 vs DS-C rats.

Figure 2. Capillary density in the left ventricle of DS-C, DS-CHF+V, and DS-CHF+NCR rats at 18 weeks of age. Data are means±SEM. †P<0.05 vs DS-CHF+V rats.
compared with that in DS-C rats (Figures 4 and 6A). Nicorandil inhibited the downregulation of VEGF and bFGF gene expression. The amount of VEGF or bFGF protein in the LV myocardium was also markedly smaller in DS-CHF+/V rats than in DS-C rats (Figure 6). Again, treatment with nicorandil inhibited this decrease in VEGF and bFGF expression. The abundance of mRNAs for the VEGF receptor Flt-1 and for eNOS in the LV myocardium was significantly decreased in DS-CHF+/V rats compared with DS-C rats (Figures 4 and 7). Nicorandil inhibited the downregulation of Flt-1 and eNOS gene expression. The amount of the mRNA for the VEGF receptor Flk-1 (KDR) did not differ significantly among the 3 groups of rats.

**Discussion**

We have shown that chronic administration of a nonantihypertensive dose of nicorandil increased VEGF and bFGF gene expression in the LV myocardium, as well as promoted coronary capillary and arteriolar growth, and attenuated the development of decompensated heart failure in DS rats.

Long-term treatment of rabbits with vasodilators such as adenosine and a xanthine derivative in doses that markedly increase coronary and muscle blood flow results in an increase in capillary density in heart and skeletal muscle. An increase in blood flow may enhance vascular growth through increases in shear stress, wall tension, or stretch.13,24

**Figure 3.** Myocardial density of arterioles (indicated by arrows), according to internal diameter, in the left ventricle of DS-C, DS-CHF+/V, and DS-CHF+/NCR rats at 18 weeks of age. Data are means±SEM. *P<0.05 vs DS-C rats; †P<0.05 vs DS-CHF+/V rats.

**Figure 4.** Representative polyacrylamide gel electrophoresis of reverse transcription and PCR products of the indicated mRNAs in the left ventricle of DS-C, DS-CHF+/V, and DS-CHF+/NCR rats at 18 weeks of age. Positive and negative controls are also shown.

**Figure 5.** Expression of fetal-type cardiac genes in the left ventricle of DS-C, DS-CHF+/V, and DS-CHF+/NCR rats at 18 weeks of age. The abundance of each mRNA was normalized by that of GAPDH mRNA and then expressed relative to the mean value for DS-C rats. Data are means±SEM. *P<0.05 vs DS-C rats; †P<0.05 vs DS-CHF+/V rats.

**Figure 6.** VEGF and bFGF mRNA and protein abundance in the left ventricle of DS-C, DS-CHF+/V, and DS-CHF+/NCR rats at 18 weeks of age. A, The amounts of VEGF and bFGF mRNAs were corrected for the amount of GAPDH mRNA and then expressed relative to the mean value for DS-C rats. Data are means±SEM. *P<0.05 vs DS-C rats; †P<0.05 vs DS-CHF+/V rats. B, Representative immunoblots for VEGF and bFGF in LV tissue. Recombinant VEGF and bFGF were used as positive controls, with GAPDH as a loading control.
Nicorandil induces vasodilation by opening $K_{\text{ATP}}$ channels and through its nitrate-like action.\(^1,2\) We therefore hypothesized that chronic treatment with nicorandil might increase blood flow and stimulate coronary angiogenesis. DS-CHF+NCR rats showed a marked increase in the myocardial density of capillaries and arterioles compared with DS-CHF+V rats, suggesting that chronic administration of nicorandil promotes the formation of functional coronary microvessels in the failing heart. Although the mechanism of this effect of nicorandil remains to be elucidated, nicorandil did not induce tube formation by cultured human umbilical vein endothelial cells under normoxic or hypoxic conditions (data not shown), suggesting that the angiogenic action of the drug in vivo is not direct.

VEGF and bFGF stimulate angiogenesis under physiological and pathological conditions.\(^9,10,16\) In the present study, nicorandil markedly increased VEGF and bFGF expression at the mRNA and protein levels in the failing heart of DS rats. VEGF is a circulating glycoprotein that promotes blood vessel growth in response to ischemia and other stimuli.\(^25\) Nicorandil did not increase the abundance of VEGF and bFGF mRNAs in cultured cardiomycocytes (data not shown). Although the mechanism by which nicorandil induces myocardial VEGF expression remains unclear, several mechanisms appear possible. First, nicorandil increases coronary blood flow and shear stress by dilating coronary resistance arterioles, and shear stress seems to activate VEGF expression and the angiogenic cascade.\(^6,26\) Second, nicorandil, which activates $K_{\text{ATP}}$ channels and induces nitrate-like effects, might increase the production of interstitial adenosine through NO- or $K_{\text{ATP}}$ channel-mediated activation of ecto-5'-nucleotidase.\(^27,28\) Adenosine is thought to function as an angiogenic factor and upregulates VEGF expression in cultured myocardial vascular smooth muscle cells.\(^29\) Nicorandil thus likely induces VEGF expression, at least in part, by an adenosine-dependent pathway. Finally, enhancement of eNOS expression by nicorandil might contribute to upregulation of VEGF expression. Deficiency of eNOS resulted in marked impairment of myocardial capillary development and an associated reduction in VEGF expression in the neonatal mouse myocardium.\(^30\)

VEGF signaling in endothelial cells is mediated by 2 receptor tyrosine kinases: Flt-1 and Flk-1.\(^31,32\) Both receptors are necessary for normal mouse development, and differences in their signaling properties have been identified. The amount of Flt-1 mRNA was decreased in the heart of DS-CHF+V rats compared with DS-C rats, whereas the amount of Flk-1 mRNA was similar in the 2 groups, indicating differential regulation of the expression of these 2 receptors. Nicorandil inhibited the downregulation of Flt-1 gene expression apparent in DS-CHF+V rats, whereas it did not affect Flk-1 gene expression. These results suggest that signaling by VEGF and Flt-1 is an important determinant of the difference in cardiac pathophysiology between DS-CHF+V and DS-CHF+NCR rats. Selective downregulation of certain VEGF isoforms and Flt-1 as well as a reduced capillary density have been detected in patients with dilated cardiomyopathy.\(^33\) Although the relationship between capillary density and this condition remains unclear, these observations implicate VEGF and Flt-1 in the pathophysiology of dilated cardiomyopathy. In the present study, capillary density did not differ between DS-CHF+V and DS-C rats, despite the significant decrease in VEGF expression in the former animals. This apparent discrepancy might reflect the complex balance between the actions of angiogenic and angiostatic factors in the myocardium.\(^34\)

A role for bFGF in arteriolar growth is well documented. A chronic increase in blood flow in rabbits was found to result in a sustained increase in the amounts of bFGF mRNA and protein in vascular smooth muscle cells.\(^35\) We have now shown that nicorandil increased the amounts of bFGF mRNA and protein in the myocardium of DS rats. Administration of neutralizing antibodies to bFGF inhibited coronary arteriolar growth in neonatal rats,\(^11\) whereas administration of bFGF to dogs with ameroid occlusion of a coronary artery enhanced collateral development and collateral perfusion.\(^36\) Furthermore, arterial enlargement in response to high flow is preceded by an increase in bFGF levels in arterial smooth muscle cells.\(^37,38\) Our present demonstration of an increase in myocardial arteriolar density in response to chronic nicorandil treatment is thus consistent with the nicorandil-induced increase in bFGF gene expression.

**Perspectives**

We have shown that a nonantihypertensive dose of nicorandil stimulated coronary capillary and arteriolar growth and likely thereby suppressed the development of heart failure in DS rats. Our results suggest that chronic administration of nicorandil may prove effective for the treatment of hypertensive heart failure as well as for that of ischemic heart disease. Further studies are needed to examine the effect of nicorandil on survival in this animal model as well as to determine whether chronic treatment with a higher dose of this drug exhibits a greater beneficial effect on heart failure.

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References
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Figure 1

Transglutaminase and α-smooth muscle actin

Negative controls for immunostaining

Figure 2

Tube formation of HUVEC under normoxia and hypoxia conditions.