Ischemia-Induced Coronary Collateral Growth Is Dependent on Vascular Endothelial Growth Factor and Nitric Oxide

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Background—We hypothesized that ischemia-induced expression of vascular endothelial growth factor (VEGF) and the production of NO stimulate coronary collateral growth.

Methods and Results—To test this hypothesis, we measured coronary collateral blood flow and VEGF expression in myocardial interstitial fluid in a canine model of repetitive myocardial ischemia under control conditions and during antagonism of NO synthase. Collateralization was induced by multiple (1/h; 8/d), brief (2 minutes) occlusions of the left anterior descending coronary artery for 21 days. In controls, collateral blood flow (microspheres) progressively increased to $89\pm9$ mL·min$^{-1}$·100 g$^{-1}$ on day 21, which was equivalent to perfusion in the normal zone. Reactive hyperemic responses (a measure of the severity of ischemia) decreased as collateral blood flow increased. In $N^\delta$-nitro-L-arginine methyl ester (L-NAME)– and L-NAME+nifedipine–treated dogs, to block the production of NO and control hypertension, respectively, collateral blood flow did not increase and reactive hyperemia was robust throughout the occlusion protocol ($P<0.01$ versus control). VEGF expression (Western analyses of VEGF$\text{164}$ in myocardial interstitial fluid) in controls peaked at day 3 of the repetitive occlusions but waned thereafter. In sham-operated dogs (instrumentation but no occlusions), expression of VEGF was low during the entire protocol. In contrast, VEGF expression was elevated throughout the 21 days of repetitive occlusions after L-NAME. Reverse transcriptase–polymerase chain reaction analyses revealed that the predominant splice variant expressed was VEGF$\text{164}$.

Conclusions—NO is an important regulator of coronary collateral growth, and the expression of VEGF is induced by ischemia. Furthermore, the induction of coronary collateralization by VEGF appears to require the production of NO.

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Key Words: nitric oxide ■ collateral circulation ■ hyperemia ■ endothelium-derived factors

The coronary collateral circulation ameliorates adverse outcomes of ischemic heart disease, such as myocardial infarction, angina, and sudden death.1–3 Myocardial viability after acute infarction correlates with the extent of collateral blood flow of the affected vascular segment.4 Expansion of preexisting collaterals involves the addition of new vascular components (endothelium, smooth muscle, and fibroblasts) via mitosis, migration, tissue remodeling, matrix degradation, and differentiation.5,6 Despite the potential for coronary collateralization to abate the consequences of ischemia, there is little consensus about the signals underlying coronary collateral growth. No experiments to date have established a causal role for a particular growth factor in collateralization via receptor blockade or antagonism of downstream signaling.

Nitric oxide (NO) was suggested to stimulate hindlimb collateral growth and mediate vascular endothelial growth factor (VEGF) signaling.7–9 Collateral growth in the ischemic hindlimb was impaired in endothelial NO synthase (eNOS) knockout mice.9 Furthermore, the mitogenic effects of VEGF, but not those of basic fibroblast growth factor, were inhibited by antagonists of NOS.7,8 In the present study, we delineated the role of VEGF in coronary collateralization by assessing its temporal expression during collateral growth and by antagonizing the production of NO to disrupt its signaling.

Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin and conformed to the “Guiding Principles in the Care and Use of Animals” of the American Physiological Society and the NIH Guide for the Care and Use of Laboratory Animals.

Repetitive Coronary Occlusion Model

Mongrel dogs (either sex; 25 to 30 kg) were anesthetized with propofol (50 mg/kg IV) and isoflurane (1.5% to 2.0%) in 100% oxygen. A left thoracotomy was performed under sterile conditions, and the following were implanted as described previously10,11: a heparin-filled catheter in the thoracic aorta for measurement of arterial pressure; a Doppler ultrasonic flow transducer (20 MHz) on the left anterior descending coronary artery (LAD) for measurement of coronary blood flow velocity; a balloon cuff vascular occluder (In

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Vivo Metric) around the LAD for production of brief coronary occlusions; a heparin-filled catheter in the left atrial appendage for administration of drugs and radioactive microspheres; and an intramyocardial catheter (0.8-mm OD, 0.04-mm ID) in the LAD perfusion territory for sampling of interstitial fluid. The catheter had forty 25-gauge holes punched in a 2-cm segment that was situated in the ventricular wall. All catheters and leads were secured, tunneled subcutaneously, and exteriorized between the scapulae; the wounds were repaired, and the dog was treated postoperatively as described previously. Dogs recovered from surgery for 10 days before experimentation, and during this time period, they were trained to stand quietly in a restraining sling. Systemic and coronary hemodynamics and reactive hyperemic responses were monitored immediately before, during, and after each coronary artery occlusion.

Myocardial Interstitial Fluid
Samples of myocardial interstitial fluid (MIF) were collected from the LAD region each morning before subsequent experimentation. Isotonic saline (4 mL) was flushed into the catheter as 4 mL of fluid was withdrawn. The sample was immediately placed on ice, divided into aliquots, frozen, and stored at −80°C until analysis. The sample “window” of the intramyocardial catheter is ~0.4 mL, and the total volume of the catheter is ~2.0 mL. Because there was a 24-hour equilibration between samples, it is likely that a significant portion of the volume within the catheter equilibrated with MIF, so the final dilution of MIF that we analyzed is somewhere between 4- and 6-fold. We had difficulty estimating the recovery of interstitial proteins or the equilibration time between the interstitium and the catheter because of fibrosis. Despite these constraints, we could measure large changes in protein concentration within a 24-hour period, suggesting that the recovery and equilibration times were sufficient to detect differences.

Experimental Protocols
Collateralization was induced by multiple 2-minute occlusions of the LAD with the implanted pneumatic vascular occluder. In 3 groups, occlusions were performed once per hour, 8 times per day, for 21 days: (1) control (n=8); animals subjected to repetitive occlusions; (2) NO inhibition (n=8); Nω-nitro-L-arginine methyl ester (L-NNAME, Sigma, 30 mg/kg BID), an NOS inhibitor, was administered via the atrial catheter 2 days before initiation of occlusions and continued throughout the 21-day protocol; and (3) NO inhibition + nifedipine (n=4); dogs were given L-NNAME according to the same regimen as described in the preceding group in combination with nifedipine (60 mg/d) to offset hypertension produced by inhibition of NOS. In a sham-operated group (n=5), occlusions were not performed, and these animals were used as time controls for analysis of VEGF in MIF. Arterial pressure was monitored daily, and the efficacy of NO blockade was established by monitoring coronary flow and arterial pressure after intravenous injection of acetylcholine (20, 60, and 200 ng/kg).

Regional Myocardial Blood Flow
Carbonized plastic microspheres (15±2 μm diameter, New England Nuclear) labeled with 111In, 103Ru, 51Cr, or 95Nb were used to measure regional myocardial blood flow to the normal and collateral-dependent regions at days 0, 7, 14, and 21 of the occlusion protocol by standard techniques. Measurements of flow were completed in only the control group and the experimental group receiving L-NNAME. We did not measure flow in the sham group, because we have reported previously that the instrumentation does not affect coronary collateralization, and flows to the instrumented region were not different from that in the normal zone.

Western Analysis
VEGF expression in MIF was determined by Western analysis. MIF was collected and diluted in gel loading buffer (containing protease inhibitors, 10 mMol/L dithiothreitol, and 2% SDS). For each sample of MIF, 400 μL initially used for immunoprecipitation was separated in a 10% to 12% polyacrylamide gel. After electrophoretic transfer to nylon membrane (Zeta Probe), the blot was blocked with 5% nonfat milk in TBST (13 mMol/L Tris, pH 7.6; 150 mMol/L NaCl; 0.05% Tween 20). Mouse monoclonal VEGF primary antibody (25 μg, Santa Cruz Biotechnology) was incubated in 1% buffered nonfat milk for 1 hour, followed by washes and an incubation in secondary anti-mouse IgG (Santa Cruz Biotechnology). The bands were detected with a nonradioactive detection system (ECL from Amer sham). Molecular size standards ranging from 200 to 6.5 kDa were run with samples to ensure quantification of the molecular size of the signal. A 50-ng standard of human VEGF (Genentech) was also electrophoresed for comparison to the proteins in the sample. Signals were digitized with a CCD camera-frame digitizer system, analyzed with NIH Image software (density and band area), and expressed as a ratio to the 50-ng standard.

Reverse Transcriptase–Polymerase Chain Reaction
RNA was isolated from cardiac tissue by the acid guanidinium thiocyanate (GTC)phenol method. Transmural samples (~500 mg) of ventricle after 21 days of repetitive occlusions were excised and cleaned of adherent fat in ice-cold PBS. RNA was extracted, precipitated, washed in 70% ethanol, and stored in DEPC-treated H2O at −80°C until analysis.

Total RNA (5 μg) was reverse-transcribed with an oligo dT18 to 24 primer with Ready-To-Go You-Prime First-Strand Beads (Phar macia Biotech) to generate first-strand cDNA (total 33 μL). We added 67 μL sterile water (total volume 100 μL) and used 2 μL of this cDNA solution for polymerase chain reaction (PCR). The resultant cDNA was amplified in 48 μL reaction buffer containing 25 pmol sense and antisense primer, 22 mmol/L Tris-HCl (pH 8.4), 55 mmol/L KCl, 1.65 mmol/L MgCl2, 220 μmol/L dGTP, 220 μmol/L dTTP, 220 μmol/L dCTP, and 2 U recombinant Taq DNA polymerase/mL. The thermal profile used a Gene Amp PCR system 2400 (Perkin-Elmer) consisting of a denaturation step of 94°C for 1 minute, an annealing step of 58°C for 1 minute, and an extension step of 72°C for 1 minute. All samples were initially denatured for a total of 5 minutes (94°C). The sequence of VEGF sense primer was 5′-TTCTGTATC-AGTCTTCTCCTGGTGAAGTTCATGGATG-3′, and that of antisense primer was 5′-CGAAGTTGTTAAGCTTCTGAGT-3′. This primer set amplifies 4 splice variants of VEGF in dogs: VEGF121, VEGF164, VEGF182, and VEGF188.12 The PCR product for VEGF188 was undetectable; therefore, our analysis focused on VEGF121, VEGF164, and VEGF182. We cloned and sequenced PCR products to verify that the products we amplified with our VEGF primers corresponded to the known sequence of VEGF in Genbank. The PCR products of 405, 534, and 606 bp amplified by our VEGF primer corresponded to the mRNA encoding VEGF121, VEGF164, and VEGF182, respectively. The sequence of GAPDH sense primer was 5′-GCCAAAGGGGTGCTATCTCT-3′ and that of antisense primer was 5′-GCCCATCCAGATCCATCTCT-3′, which amplifies 225-bp product.

In preliminary studies, we found that the amount of PCR product increased exponentially from 26 to 36 cycles for VEGF and from 22 to 32 cycles for GAPDH. Saturation was reached after 38 and 34 cycles, respectively. Accordingly, VEGF products were amplified for 30 cycles and GAPDH products for 28 cycles. Each PCR reaction product was electrophoresed through a 2% agarose gel, and the product was visualized by incubation for 20 minutes in a solution containing 10 ng/mL ethidium bromide. Resulting gel bands were imaged with a Fluor imager (Molecular Dynamics). The relative intensities of the bands, expressed as optimal density units, were quantitatively analyzed with ImageQuant software (Molecular Dynamics). VEGF signals (density and band size) were normalized to the GAPDH mRNA signal, of which the latter served as internal standard.

Data Analysis
The percent debt repayment during reactive hyperemia was calculated (excess flow velocity during reactive hyperemia/flow velocity debt)×100. This analysis assumes that flow volume and flow velocities are proportional, which is probably the situation in our experiments, because the Doppler probe was fibrosed securely in
place, and this degree of fibrosis would prevent large changes in vascular caliber. The percent change of peak flow velocity was defined as (peak flow velocity−basal flow velocity)/basal flow velocity×100.

All data are expressed as mean±SEM. The changes in the parameters between the groups and over time were compared by 2-way ANOVA for repeated measurements. If significant differences were observed, then the post hoc Bonferroni-Dunn test was used to detect specific differences between the groups and across time. Differences between the L-NAME and control group reverse transcription (RT)-PCR data were analyzed with Student’s t test for unpaired observations. The level of significance was P<0.05.

Results

Hemodynamic Parameters
Mean arterial pressures in controls and L-NAME+nifedipine–treated animals were similar at all times (Table 1). In dogs treated with L-NAME, mean arterial pressure at day 0 was significantly higher than at other time periods and versus the other groups. Heart rates were similar in the groups (Table 1).

Effects of L-NAME
Acetylcholine (20 to 200 ng/kg) decreased blood pressure and increased coronary blood flow velocity in a dose-dependent manner. L-NAME significantly attenuated the responses to acetylcholine (Table 2).

Reactive Hyperemia
Figure 1 depicts myocardial reactive hyperemic responses. On day 1, the percent increase in peak flow velocity (vasodilator reserve) was similar in all experimental groups. The percent debt repayment was significantly suppressed at day 1 in the L-NAME and L-NAME+nifedipine groups compared with that of controls. Both peak flow velocity and percent debt repayment were decreased in control experiments after progressive repetitive occlusions, but these variables remained unchanged in the L-NAME and L-NAME+nifedipine groups. After 21 days of repetitive occlusions, vasodilator reserve and percent debt repayment were significantly smaller in control than in L-NAME– and L-NAME+nifedipine–treated dogs.

Myocardial Blood Flow
In the control and L-NAME groups, myocardial blood flow to the normal zone was unchanged at the various times, except that at day 21 in the L-NAME group, flow was decreased compared with that at day 0 (Table 1). Coronary collateral blood flow in the control group progressively increased during the repetitive occlusions. In contrast, in L-NAME– and L-NAME+nifedipine–treated dogs, collateral blood flow did not increase during repetitive ischemia (Figure 2).

Expression of VEGF Protein
VEGF protein expression peaked 3 days after initiation of repetitive occlusions and then waned after additional coronary occlusions in control experiments (Figure 3). In L-NAME–treated dogs, VEGF protein was upregulated at day 3 and remained elevated during the entire 21-day occlusion protocol. In the sham group, VEGF levels did not change throughout the protocol. In 2 additional animals that were treated with the combination of L-NAME and nifedipine, VEGF analyses mimicked those observed with L-NAME. In these animals, expression (ratio of signal from MIF/50 ng standard) increased from baseline (0.45) to 0.86 at day 3 and remained elevated until the end of the protocol (day 21: 1.26).

RT-PCR Analysis for VEGF mRNA
Figure 4 depicts RT-PCR for VEGF mRNA in myocardium from ischemic (LAD perfusion area) and nonischemic (left circumflex artery perfusion area) regions after 21 days of repetitive occlusions. The PCR products for VEGF164 and VEGF188 in the ischemic regions of control dogs were at the same levels as those in the nonischemic region. In the L-NAME group, however, the PCR products for VEGF164 and VEGF188 in the ischemic zone were significantly higher than

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MBF indicates myocardial blood flow; Nif, nifedipine.

*P<0.01 vs control, †P<0.05 vs L-NAME, ‡P<0.05 vs day 0.

| TABLE 2. Effects of Acetylcholine Before and After Treatment With L-NAME |
|-----------------------------|-----------------------------|-----------------------------|
|                            | ∆BP, mm Hg                  | % Change Coronary Flow Velocity |
|                            | Before L-NAME | After L-NAME | Before L-NAME | After L-NAME |
| 20 ng/kg                   | 8±1 | 1±1* | 58±10 | 24±11* |
| 60 ng/kg                   | 18±2 | 8±1* | 96±4 | 53±13* |
| 200 ng/kg                  | 25±2 | 12±2* | 135±9 | 80±14* |

BP indicates blood pressure. Data are mean±SEM.

*P<0.01 vs before L-NAME.
those in the nonischemic region. The PCR product for VEGF 121 was barely detectable in these groups.

**Discussion**

We have made 3 new observations: (1) expression of VEGF during coronary collateralization is time-dependent. Maximum production occurs early during repetitive ischemia and then wanes as collateral growth progresses and ischemia lessens, (2) inhibition of NOS prevents coronary collateral growth but augments expression of VEGF after repetitive episodes of myocardial ischemia, and (3) VEGF 164 is the dominant splice variant of VEGF expressed during coronary collateral development. On the basis of these observations, we conclude that ischemia drives the expression of VEGF and that blockade of NO production, which reportedly interferes with the signaling of VEGF, prevents collateral growth.

**Coronary Collateral Growth**

Coronary collateral growth was induced by brief (2 minutes), repetitive episodes of myocardial ischemia in dogs, which is a model that avoids myocardial stunning and tissue necrosis. Repetitive episodes of myocardial ischemia in this model concomitantly induce coronary collateral development and lead to expression of growth factors in MIF. Application of MIF from animals subjected to repetitive episodes of ischemia caused marked proliferation of endothelial and vascular smooth muscle cells in culture.

Previously, we suggested that VEGF was expressed during collateral growth, because MIF-induced proliferation of cultured endothelial cells was blocked by neutralizing antibodies to VEGF at all but the earliest and latest times of collateral growth. There is 1 inconsistency between the present estimates of VEGF in MIF and our previous results using antibody neutralization of VEGF; specifically, we would not have expected anti-VEGF to block endothelial cell proliferation at days 12 to 13 and 19 to 20, because we predict levels of VEGF to be low at these times. We reconcile our cell culture data and the Western analyses by suggesting that during the later stages of collateral development, other growth factors are probably expressed in addition to VEGF. Blockade of 1 factor may reduce the level of all mitogens below a critical threshold value for proliferation.

Figure 1. A, Representative coronary reactive hyperemic responses from a control dog on days 1, 7, 14, and 21 of repetitive occlusions. Reactive hyperemia diminished progressively during repetitive occlusions. B, Representative reactive hyperemic responses from an L-NAME–treated dog on days 1, 7, 14, and 21 of occlusions. Reactive hyperemia diminished over course of experiment. C, Percent peak flow change from baseline during myocardial reactive hyperemia in control, L-NAME–treated, and L-NAME+nifedipine–treated dogs. D, Ratio of flow debt to repayment in control, L-NAME–treated, and L-NAME+nifedipine–treated dogs.

Figure 2. Collateral blood flow in control, L-NAME–treated, and L-NAME+nifedipine–treated dogs at days 0, 7, 14, and 21 of occlusion. Collateral blood flow increased after repetitive coronary occlusions in control dogs. In L-NAME and L-NAME+nifedipine groups, however, collateral flow did not increase. *P<0.05 control vs L-NAME, control vs L-NAME+nifedipine; †P<0.05 vs control; ‡P<0.05 vs day 1.

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tors are expressed in ischemic myocardium.\textsuperscript{20} VEGF possesses unique target cell specificity for vascular endothelial cells,\textsuperscript{15} but collateral growth involves mitosis of both endothelial and vascular smooth muscle cells.\textsuperscript{2,5,6} Other factors must exert activity in concert with VEGF. Nevertheless, our results and those of others\textsuperscript{18,19} support the concept that VEGF is an important mediator of coronary collateral growth.

Under control conditions, expression of VEGF protein in the MIF peaked early during collateral development but waned as collateral growth progressed. VEGF expression appeared to be directly related to the intensity of the ischemic signal, which was severe initially but lessened during the occlusions as collaterals developed. When collateral growth was inhibited by L-NAME and the intensity of the ischemic signal persisted during the entire occlusion protocol, VEGF expression was maintained at high levels. Taken together, these observations suggest that ischemia is the critical factor inducing expression of VEGF during coronary occlusion.

Role of NO on Coronary Collateral Growth

Critical to our conclusions regarding the role of NO in coronary collateralization is validation that L-NAME inhibited eNOS activity. We have 2 observations verifying the efficacy of the blockade. First, dilation to acetylcholine was significantly abrogated by L-NAME. Second, the flow velocity repayment after reactive hyperemia was significantly attenuated by L-NAME, which was previously reported, and our results corroborate this observation.\textsuperscript{21,22} These results attest to adequate antagonism of eNOS.

An implicit assumption we have made in our experiments and conclusions is that the effects of L-NAME are mediated by the inhibition of NO. In addition to NO, NOS also produces superoxide,\textsuperscript{23} and L-NAME is reported to block the production of superoxide by eNOS.\textsuperscript{24} Moreover, L-NAME reportedly has some nonspecific effects, such as blockade of muscarinic receptors\textsuperscript{25} and inhibition of ornithine decarboxylase.\textsuperscript{26} Furthermore, it is possible that the hypertension produced by L-NAME affected collateral development. The increase in arterial pressure was modest, and the effects of hypertension on collateral growth are controversial.\textsuperscript{27,28} Importantly, nifedipine, an L-type calcium channel blocker, prevented hypertension but did not enable collateral growth to occur during L-NAME treatment. An alternative explanation, however, is that L-NAME produces vasoconstriction of...
the collateral vessels, thereby limiting collateral-dependent flow. We believe that this is unlikely, because others have not observed constriction of collateral vessels after inhibition of NOS. Despite these caveats, on the basis of our hemodynamic information and what is known about the signaling for VEGF, we believe that our results are consistent with the actions of L-NAME as an inhibitor of NO production.

The role of NO in angiogenesis and collateralization is controversial. NO inhibits migration and proliferation of vascular smooth muscle cells but promotes endothelial cell migration and tube formation. Our hypothesis that NO is critical for coronary collateral formation is corroborated by the observation that collateral formation in the peripheral circulation was impaired in eNOS-knockout mice. The signaling cascade activated by VEGF includes NO production, which explains why collateral formation is inhibited by NO inhibition or knockout. Our results support the concept that NO is essential for the signaling of VEGF.

**Clinical Implications**

Abaci et al recently reported that coronary collateral development was compromised in patients with diabetes mellitus. NO-mediated vasodilation is impaired in patients with diabetes. Furthermore, Métais et al suggested that NO-mediated VEGF-induced vascular relaxation was reduced in coronary microvessels of patients with coronary artery disease. In our study, inhibition of NO synthesis impaired coronary collateral development, which implies that coronary collateral growth may be impaired in patients with deficits in NO production. Our results are compatible with a paradigm in which collateral growth is dependent on the production of VEGF during the initial ischemic periods and effective endothelial production of NO to facilitate proper signaling.

In conclusion, the expression of VEGF and production of NO are essential for growth and maturation of the coronary collateral circulation in response to myocardial ischemia.

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