Effects of L-Arginine on Fibroblast Growth Factor 2–Induced Angiogenesis in a Model of Endothelial Dysfunction

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Background—Nitric oxide availability, which is decreased in advanced coronary artery disease associated with endothelial dysfunction, is an important mediator of fibroblast growth factor-2 (FGF-2)–induced angiogenesis. This could explain the disappointing results of FGF-2 therapy in clinical trials despite promising preclinical studies. We examined the influence of L-arginine supplementation to FGF-2 therapy on myocardial microvascular reactivity and perfusion in a porcine model of endothelial dysfunction.

Methods and Results—Eighteen pigs were fed either a normal (NORM, n=6) or high cholesterol diet, with (HICHOL-ARG, n=6) or without (HICHOL, n=6) L-arginine. All pigs underwent ameroid placement on the circumflex artery and 3 weeks later received surgical FGF-2 treatment. Four weeks after treatment, endothelial-dependent coronary microvascular responses and lateral myocardial perfusion were assessed. Endothelial cell density was determined by immunohistochemistry. FGF-2, fibroblast growth receptor-1, endothelial-derived nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), and syndecan-4 levels were determined by immunoblotting. Pigs from the HICHOL group showed endothelial dysfunction in the circumflex territory, which was normalized by L-arginine supplementation. FGF-2 treatment was ineffective in the HICHOL group (circumflex/left anterior descending blood flow ratios: 1.01 (rest) and 1.01 (pace), after and before treatment). Addition of L-arginine improved myocardial perfusion in response to FGF-2 at rest (ratio 1.13, \(P<0.02\) versus HICHOL) but not during pacing (ratio 0.94, \(P=\text{NS}\)), and was associated with increased protein levels of iNOS and eNOS.

Conclusion—L-arginine supplementation can partially restore the normal response to endothelium-dependent vasorelaxants and myocardial perfusion in response to FGF-2 treatment in a swine model of hypercholesterolemia-induced endothelial dysfunction. These findings suggest a role for L-arginine in combination with FGF-2 therapy for end-stage coronary artery disease. (Circulation. 2005;112[Suppl I]:I-202–I-207.)

Key Words: angiogenesis | growth substances | endothelium | nitric oxide

Therapeutic angiogenesis constitutes a potential alternative approach for patients with advanced coronary artery disease (CAD) who are not candidates for conventional revascularization techniques. Despite promising preclinical results with the use of growth factors, however, clinical trials testing their efficacy in humans have thus far not met the expectations generated by animal studies.\(^1\)\(^,\)\(^2\) Perhaps the most encouraging results have come from a phase I study using a surgically implanted perivascular sustained-delivery system for fibroblast growth factor-2 (FGF-2) in territories that cannot be revascularized at the time of coronary artery bypass grafting.\(^3\) Although that study showed dose-dependent improvements in angina class and perfusion defect size on stress sestamibi imaging that persisted at a mean of 32 months of follow-up,\(^4\) the benefits were still modest and the population small. The reasons explaining this relative lack of success remain unclear, but there is a significant body of evidence suggesting that endothelial dysfunction, which affects virtually all patients who are targeted for angiogenic therapy, may play a pivotal role in the resistance to the effects of exogenous growth factors in that population. FGF-2 and other angiogenic growth factors operate in large part through the release of endothelial-derived nitric oxide (NO) via the activation of tyrosine kinase receptors.\(^5\) Proliferative endothelial cells express significantly more endothelial NO synthase (eNOS) mRNA than confluent cells,\(^6\) and NO synthase inhibitors and decreased NO availability can impair angiogenesis.\(^7\)\(^–\)\(^9\) Moreover, we have recently shown that the myocardial angiogenic response to FGF-2 was inhibited by induction of endothelial dysfunction secondary to chronic administration of a choles-

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terol-enriched diet in a porcine model of ischemia.\textsuperscript{10} Interestingly, in a related study where hypercholesterolemic mice exhibited attenuated FGF-induced collateral vessel formation in association with derangement of the NO synthase pathway, oral administration of L-arginine (the substrate for eNOS) fully restored the angiogenic response to FGF.\textsuperscript{11} L-arginine is a particularly attractive candidate for combination therapy with growth factors in humans, where its administration has been associated with improved epicardial and microvascular responses to acetylcholine in patients with endothelial dysfunction.\textsuperscript{12} In addition, a double-blind, randomized study investigating the effects of chronic dietary supplementation with L-arginine in humans with nonobstructive CAD, an increase in coronary blood flow in response to acetylcholine (associated with a decrease in plasma endothelin concentrations and an improvement in patients’ symptoms) was observed.\textsuperscript{13} On the basis of these results, we sought to evaluate the potentially beneficial effects of L-arginine supplementation on the inhibited angiogenic response to exogenous FGF-2 in a porcine model of chronic myocardial ischemia with hypercholesterolemia-induced endothelial dysfunction.

**Methods**

**Study Design**

Twenty-four Yucatan miniswine (Sinclair Research, Columbia, Mo) were either fed a regular chow (NORM group, n=8) or a hypercholesterolemic diet (n=16) with (HICHL-ARG group, n=8) or without (HICHL, n=8) oral supplementation of L-arginine (50 mg/kg twice daily by mouth). Eighteen pigs (6 per group) completed the experimental protocol. The high-cholesterol diet (consisting of 4% cholesterol, 17.2% coconut oil, 2.3% corn oil, 1.5% sodium cholate, and 75% regular chow) and L-arginine administration were started at 7 weeks of age and continued throughout the entire study period (total period of 20 weeks). All animals received humane care in compliance with the Harvard Medical Area Institutional Animal Care and Use Committee and the National Research Council’s Guide for the Care and Use of Laboratory Animals, prepared by the Institute of Laboratory Animals and published by the National Institutes of Health. For all surgical procedures, anesthesia was induced with ketamine (10 mg/kg intramuscularly), thiopental (5 to 10 mg/kg intravenously), and thiopental 2.5% to effect. The animals were intubated with a cuffed endotracheal tube, mechanically ventilated at 12 to 20 breaths per minute, and maintained under deep general anesthesia with a gas mixture of oxygen at 1.5 to 2 L/min and isoflurane at 0.75% to 3.0% concentration. Thirty minutes before the end of each survival procedure, a dose of buprenorphine (0.03 mg/kg intramuscularly) was administered and a fentanyl patch (4 mg/kg) placed on the animal and left for the first 48 hours after surgery and subsequently as needed.

At 20 weeks of age, all pigs underwent placement of a 1.75-mm ameroid constrictor on the proximal circumflex artery through a left mini-thoracotomy. Eight million isotope-labeled red-gold microspheres (BioPhysics Assay Laboratory) were injected in the left anterior descending and circumflex arteries (distal to the ameroid implantation site) were dissected from the surrounding tissue with a ×40 dissecting microscope and examined in isolated organ chambers as described previously.\textsuperscript{14} The responses to sodium nitroprusside (SNP) (1 nM to 100 μmol/L), an endothelium-independent cyclic guanine monophosphate-mediated vasodilator, as well as to adenosine 5’-diphosphate (ADF; 1 nM to 10 μmol/L), vascular endothelial growth factor (VEGF; 1 μM to 1 nM), and FGF (1 μM to 1 nM), as endothelium-dependent receptor-mediated vasodilators that act via bioavailable NO, were studied.\textsuperscript{15}

**Perfusion Analyses**

Myocardial blood flows were determined during each procedure with isotope-labeled microspheres (ILMs; BioPAL) using methods previously reported.\textsuperscript{16} ILMs of different isotopic mass were used at each experimental stage. Red (gold-labeled) microspheres were injected during temporary circumflex occlusion at the time of ameroid placement to identify myocardial samples that originated from the circumflex coronary distribution (those with the lowest count of gold-labeled microspheres). Black (samarium-labeled) and blue (lutetium-labeled) ILMs were used during the second procedure respectively at rest and under pacing at 150 bpm to determine baseline blood flow in the lateral territory 3 weeks after ameroid placement, at the time of FGF treatment administration. Pink (lutetium-labeled) and green (ytterbium-labeled) ILMs were injected at rest and during pacing at the final procedure, 4 weeks after instauation of FGF therapy. After euthanasia, 10 circumferential transmural left ventricular sections were collected for ILM assays in each animal, weighed, and dried. Each sample was exposed to neutron beams and microsphere densities were measured in a gamma counter. Adjusted myocardial blood flows, reflecting changes in lateral myocardial perfusion during FGF treatment, were determined from the myocardial samples that showed the highest (left anterior descending [LAD] distribution) and the lowest (circumflex distribution) count of red microspheres, calculating the ratios of blood flow in the collateral-dependent territory compared with the control anterior wall, and then comparing the posttreatment versus pretreatment phase using the following equation: Blood flow post-treatment / blood flow pre-treatment = pink / black (at rest) or = green / blue (under pacing).

**Western Blotting**

Total tissue lysates were obtained and the supernatant protein concentration was measured spectrophotometrically at a 595-nm wavelength with a micro bichinchnic acid protein assay (Pierce).
Forty μg of total protein were fractionated by 4% to 20% gradient SDS-polyacrylamide gel electrophoresis (Invitrogen) and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were stained with Ponceau S and then incubated with 5% nonfat dry milk in 50 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, and 0.1% Tween 20 (TBST) buffer for 1 hour at room temperature to block nonspecific binding. Membranes were then incubated with specific antibodies against the following antigens: FGFRI diluted to 1:3000, Syndecan-4 diluted 1:250 (both from Zymed), FGF2 diluted 1:1000 (US Biological), and eNOS and inducible nitric oxide synthase (iNOS), both diluted 1:2500 (both from BD Pharmagen). All first antibodies were incubated for 1 hour at room temperature. After being washed with TBST (3×5 minutes), the membranes were incubated for 1 hour in 2.5% nonfat dry milk in TBST containing the appropriate secondary antibody, either a sheep anti-rabbit or sheep anti-mouse immunoglobulin G (Jackson Immunolabs), conjugated to horseradish peroxidase. Peroxidase activity was visualized by means of an enhanced chemiluminescence and exposed to x-ray films (Amersham).

**Immunohistochemistry**

Staining with CD31 antibody (BD Biosciences Pharmingen) was done according to methods previously reported.17

**Data Analysis**

Data are reported as mean±SEM. Comparisons between groups were analyzed by 1-way ANOVA with the Bonferroni multiple comparison test, followed by a 2-tailed Student’s t test using GraphPad Prism (GraphPad Software Inc). Microvessel responses are expressed as percent relaxation of the preconstricted diameter and were analyzed by 2-way ANOVA examining the relationship between vessel relaxation, log concentration of the vasoactive agent of interest, and the experimental group or myocardial territory of origin (SAS Version 8 and GraphPad Software Inc). Immunoblotting were analyzed after digitalization of x-ray films using a flat-bed scanner (ScanJet 4c, Hewlett Packard) and NIH Image 1.62 software (National Institute of Health). Ponceau S staining was used to determine proper fractionation and equivalent loading of proteins. Only samples with similar fractionation and loading values with less than a 20% difference were analyzed further. The optical density ratio of the bands to that of Ponceau S were used to correct for small uneven loading (<20%).

**Results**

**Animal Model**

Among the 24 pigs that initially entered the study, 2 pigs from the HICHOL group died before completion of the experimental protocol. The first pig died in the hours after ameroid placement, probably from arrhythmia. A second pig died at the time of contrast injection into the right coronary artery immediately preceding the treatment procedure (FGF treatment), going into ventricular fibrillation that was unresponsive to pharmacological intervention and defibrillation. There was 1 death in the HICHOL-ARG group 4 days after FGF treatment that was caused by acute aortic thrombosis at the iliac bifurcation level, and another related to acute heart failure after pacing at the time of treatment. Two pigs from the NORM group died within 24 hours after ameroid placement. Serum cholesterol levels were not significantly different in the HICHOL and HICHOL-ARG groups, but were significantly higher than the NORM pigs at all time points (Figure 1). Total serum cholesterol levels for these 3 groups were 9.2±2.3, 8.6±2.1, and 1.5±0.1 mmol/L, respectively, at the time of ameroid placement, 8.5±1.6, 7.1±1.8, and 1.0±0.1 mmol/L at the time of FGF treatment, and 7.3±10.6, 7.8±1.1, and 0.9±0.1 mmol/L at the time of harvest (P<0.01).

**Myocardial Perfusion**

The results of isotope-labeled microsphere assays in determining circumflex myocardial perfusion are depicted in Figure 3. Three weeks after ameroid placement, at the time of FGF treatment initiation, baseline circumflex coronary flow was similar among the 3 groups at rest (NORM, 0.50±0.04 mL·min⁻¹·g min⁻¹; HICHOL, 0.64±0.06 mL·min⁻¹·g min⁻¹; HICHOL-ARG 0.53±0.03 mL·min⁻¹·g min⁻¹; P=NS) but significantly lower in the HICHOL-ARG group (0.38±0.02 mL·min⁻¹·g min⁻¹) than in the 2 other groups under pacing (NORM, 0.54±0.06 mL·min⁻¹·g min⁻¹; HICHOL, 0.53±0.06 mL·min⁻¹·g min⁻¹; P<0.05 versus each). After 4 weeks of FGF therapy, at the time of the final procedure, circumflex myocardial blood flow both at rest and under pacing was significantly higher in the normal diet group than in its high cholesterol counterparts. Respective ratios of blood flow in the circumflex to the LAD territories in the posttreatment versus pretreatment settings were 1.16±0.05 (normal diet) versus 1.01±0.03 (high cholesterol diet) at rest (P<0.05), and 1.43±0.14 (normal diet) versus 1.01±0.05 (high cholesterol diet) under pacing (P<0.05).

**Microvessel Reactivity**

Figure 2 shows the relaxation curves to increasing concentrations of vasodilators after preconstriction with the thromboxane/prostaglandin endoperoxide analogue U46619. Endothelial dysfunction in the high cholesterol diet group was evidenced by the significantly impaired vasorelaxation to ADP, VEGF, and FGF, 3 endothelium-dependent vasodilators, in the circumflex distribution (P=0.008, P=0.03, and P<0.0001 versus the LAD territory, respectively; Figure 2B). Contrastingly, there was no difference in the curve responses from the circumflex and LAD territories in the NORM group (Figure 2A). L-arginine administration normalized the relaxation responses to both VEGF and FGF, but not to ADP (P=0.01 for the difference between curves from the ischemic and non-ischemic area; Figure 2C). There was no significant difference in relaxation to the endothelium-independent agent SNP between the ischemic and non-ischemic area in any of the groups.

**Coronary angiograms confirmed the complete closure of the ameroid constrictors in all animals, with impaired distal filling of the circumflex artery (TIMI 2 flow). There was no significant difference in the visible collateral vessel development between the 3 groups.**

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**Figure 1.** Serum total cholesterol levels (in mmol/L) for the 3 treatment groups as measured at different time points.

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Coronary angiograms confirmed the complete closure of the ameroid constrictors in all animals, with impaired distal filling of the circumflex artery (TIMI 2 flow). There was no significant difference in the visible collateral vessel development between the 3 groups.
thus corresponding in increases from baseline perfusion of 16% and 43% for the normal diet group at rest and under pacing, respectively, whereas there were no changes in both conditions for the high cholesterol groups. L-arginine administration resulted in improvements in perfusion at rest that were similar to those observed in the normal diet group (ratio of 1.15 ± 0.03; \( P < 0.05 \) versus HICHOL and \( P = \text{NS} \) versus NORM). There was no significant improvement observed under pacing, however (ratio of 0.94 ± 0.04; \( P = \text{NS} \) versus HICHOL and \( P < 0.05 \) versus NORM).

Endothelial Cell Density
The density of CD 31-positive capillary endothelial cells in the left lateral wall of pigs from the normal diet group 4 weeks after initiating FGF treatment was significantly higher than the density found in the high cholesterol diet group (91 ± 12 versus 50 ± 9, respectively; \( P < 0.05 \)). Despite a slight increase after L-arginine supplementation (62 ± 11) compared with the HICHOL group, the difference did not reach statistical significance (Figure 4).

Western Blot Analyses
Myocardial eNOS and iNOS protein expression were significantly higher in the group receiving L-arginine than the other
2 groups. Densitometry analyses of protein levels in the ischemic territory are presented in the Table, and Figure 5 shows representative images of the gels. There was no significant difference in the levels of expression of FGF-2, FGF receptor-1, and syndecan-4.

**Discussion**

In this study, significant coronary microvessel endothelial dysfunction was induced in a porcine model of chronic myocardial ischemia by administration of a cholesterol-rich diet. This was associated with a markedly decreased functional response to the angiogenic effects of exogenous FGF in comparison with pigs undergoing the same FGF treatment but kept on a regular chow. Chronic oral supplementation of L-arginine to pigs receiving the same high-cholesterol regimen could partially restore the normal response of their coronary microvasculature to endothelium-dependent vasorelaxing agents, as well as myocardial perfusion at rest in response to exogenous FGF treatment.

In similar experimental designs comparing groups of pigs under the same diet regimen, we have previously shown that hypercholesterolemia induces endothelial dysfunction, with resulting significant impairments of both VEGF- and FGF-2–mediated myocardial angiogenesis. The mechanisms responsible for the induction of endothelial dysfunction by hypercholesterolemia are not completely understood. Several hypotheses, such as an increase in oxidative stress resulting from the induction of xanthine oxidase, NADH/NADPH oxidase, and uncoupling of eNOS with inactivation from reactive oxygen species, have been proposed, as well as increased levels of asymmetric dimethylarginine, an arginine analogue that competes with L-arginine for eNOS.

In our study, the normalization of the microvascular response to VEGF and FGF resulting from L-arginine administration was associated with significant increases in levels of eNOS and iNOS, a finding that is in agreement with increased enzyme activity by competitive reinforcement of the presence of its functional substrate, especially in view of previous experiments that have shown that the competitive inhibition of eNOS by asymmetric dimethylarginine can be reversed by supplemental L-arginine. Although still unclear, the relationship between FGF-2 and iNOS induction has already been suggested in a model of healing gastric ulcers in rats.

Despite completely normalized vasorelaxing responses to VEGF and FGF in pigs receiving L-arginine, however, the response to ADP remained abnormal even if there was a trend toward an improvement, with a smaller difference compared with the response in the control anterior territory than in the group receiving the high cholesterol diet only. The reason for this is unclear, but it is known that the onset of tissue ischemia is associated with significant changes in the expression of heparan sulfate (such as syndecan-4)–carrying core proteins, leading to alterations in the extracellular matrix. A previous study in mice expressing the transgene for syndecan-4 has also shown improved responses to FGF-2 vasorelaxation associated with a blunted response to ADP, demonstrating the heterogeneity in endothelial receptor–dependent vasodilator response patterns.

In contrast to 2 recent studies in apolipoprotein E–hypercholesterolemic mice where the attenuated collateral vessel formation in response to a FGF-2 disk angiogenesis system and hind-limb ischemia could be fully restored by the oral administration of L-arginine, we found only partial recovery of myocardial perfusion after FGF treatment in our group receiving L-arginine. Contrary to observations made in parallel experiments testing the combination of L-arginine and VEGF, where perfusion was significantly increased in both resting...
and pacing conditions, the association of L-arginine with FGF resulted in perfusion improvements at rest only in the present study. Importantly, immunohistochemical studies with CD31 revealed significant increases in endothelial cell density in the ischemic territory of pigs treated with VEGF and L-arginine, but not in those receiving the FGF-L-arginine combination. Consequently, it is plausible that the improvement in perfusion observed with FGF and L-arginine at rest may be attributable at least in part to improvements in maximal microvascular dilation responses. Although sufficient at rest, this effect may not have fulfilled the important increases observed with FGF and L-arginine supplementation.

Our model cannot reproduce the multiple comorbid conditions observed in CAD patients and the complex interactions by which these conditions can impair endothelial function, but endothelial dysfunction was the common denominator of growth-factor mediated angiogenesis inhibition when both VEGF and FGF were tested with it.\textsuperscript{10,17} The apparent selective improvement of VEGF-induced compared with FGF-induced therapeutic angiogenesis by L-arginine supplementation, however, better orients the application of future approaches in the treatment of patients with advanced CAD.

Our results show that endothelial integrity plays a crucial role in protein growth factor-based therapeutic angiogenesis, which may explain in part the disappointing results of clinical trials compared with animal studies so far. Refinements in our understanding of the molecular interactions governing endothelial function and angiogenesis will help design newer strategies for therapeutic angiogenesis using regulators of endothelial function, synthetic NO donors, eNOS activators, or antioxidants in combination with the most mechanistically suited and effective growth factors.

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