Bradycardia Induces Angiogenesis, Increases Coronary Reserve, and Preserves Function of the Postinfarcted Heart

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Background—We tested the hypothesis that induction of chronic bradycardia would trigger an upregulation of key growth factors and receptors, which would then lead to angiogenesis and improve coronary reserve in the left ventricle after myocardial infarction.

Methods and Results—Bradycardia was induced in rats by administering alinidine via osmotic pumps beginning 1 day after coronary artery ligation. Echocardiographic analysis was conducted before and after treatment. Morphometric analysis was used in perfusion-fixed hearts to document arteriolar and capillary growth. Western blots were used to evaluate growth factor and receptor changes. During the first week of treatment, vascular endothelial growth factor (VEGF), VEGF receptor 1 (Flt-1), and basic fibroblast growth factor proteins were higher in the treated group, whereas VEGF receptor 2 (Flk-1), angiopoietin-1, and angiopoietin-2 were not affected by treatment. After 3 weeks, VEGF protein remained elevated, and bradycardia was associated with a higher capillary length density in the border (40%) and remote (14%) regions and a higher arteriolar length density in the septum (62%), despite a greater increase in left ventricular mass. Although arteriolar length density increased in all size classes, the greatest increase occurred in the smallest (terminal) arterioles. This vascular growth was associated with a 23% greater coronary reserve. Echocardiography revealed a smaller increase in ventricular volume and a greater preservation of ejection fraction in rats treated with bradycardia.

Conclusions—Pharmacologic induction of bradycardia enhances vascularity and coronary reserve, preserves function of surviving myocardium, and therefore, is a noninvasive, therapeutic avenue that provides an alternative to gene therapy.

Key Words: growth substances • ischemia • capillaries • remodeling • echocardiography

Angiogenic therapy for myocardial ischemia has received considerable attention in recent years. Several strategies have emerged, including delivery of growth factor proteins, gene therapy, and stem cell implantation.1,2 Although the proangiogenic effect of these methods is appealing, there are limitations and concerns, including delivery modalities, uncontrolled angiogenesis, limited half-life of growth factors, and effects on other organs.2–4 A potential approach to angiogenic gene therapy for the ischemic and infarcted heart is physiological stimulation of the appropriate angiogenic growth factors and receptors and subsequent facilitation of vascular growth in the surviving myocardium. Angiogenesis in the surviving myocardium is necessary because cardiomyocytes in this region undergo marked compensatory hypertrophy because of the loss of muscle in the infarcted region. Previous studies have shown that angiogenesis in the surviving myocardium is inadequate5,6 and that maximal perfusion and coronary reserve are compromised.7–9 Thus, stimulation of angiogenesis and arteriogenesis offers a therapeutic approach directed at a key factor mechanism of pathological left ventricular (LV) remodeling.

Prolongation of the diastolic interval by bradycardia stimulates myocardial angiogenesis in normal hearts.10–12 Our previous work revealed marked myocardial angiogenesis in rats with chronic bradycardia induced by alinidine and showed that this angiogenic response is dependent on vascular endothelial growth factor (VEGF).13 Subsequently, our in vitro work showed that stretch of myocytes releases growth factors that stimulate angiogenic events in coronary microvascular endothelial cells.14 Thus, one important underlying mechanism involved in bradycardia-induced angiogenesis is the enhanced ventricular diastolic filling resulting in increased stretch of cardiac myocytes and blood vessels, which stimulates expression of angiogenic growth factors and receptors. This type of therapy has advantages over the introduction of single growth factors because it is noninvasive; it
also eliminates the risk of uncontrolled angiogenesis because it activates an intrinsic angiogenic response. The clinical implications for patients with coronary heart disease are of great importance. On the basis of this evidence, we tested the hypotheses (1) that chronic bradycardia in the postinfarcted heart would serve to facilitate angiogenesis by upregulating key growth factors and receptors and (2) that this vascular growth would serve to increase coronary reserve. This study is the first to document the potential therapeutic value of this noninvasive approach and the growth factors and receptors that are affected by chronic bradycardia in the postinfarcted heart. Our data support the concept that pharmacologically induced bradycardia improves coronary reserve through growth factor–induced angiogenesis. In addition, we document preservation of ventricular function by this noninvasive therapy.

Methods

Animal Model

Male Sprague-Dawley rats (Harlan Sprague-Dawley Inc, Indianapolis, Ind) weighing 300 to 350 g were used for all experiments. All procedures were approved by the University of Iowa Animal Care and Use Committee and were in accordance with the regulations of the Animal Welfare Act of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Myocardial infarction (MI) was created by left coronary artery ligation. The rats were anesthetized with a ketamine HCl and xylazine cocktail (100 mg:5 mg/kg body weight IP), a left thoracotomy was performed, the heart was externalized through an incision between the fourth and fifth intercostal space, and the proximal left coronary artery was ligated. The heart was immediately internalized and the chest closed while the lungs were inflated to minimize pneumothorax. Approximately 70% of the rats survived the procedure.

Chronic bradycardia was created by constant infusion of alinidine (Boehringer Ingelheim Pharmaceuticals), a KATP channel antagonist that increases the atrioventricular node refractive period, with an osmotic pump (Alzet). The infusion rate of 0.7 mg·h−1·kg−1 was determined in pilot experiments. Heart rates were recorded by ECG in conscious rats from 3 electrodes attached to dermal pins. This assessment was conducted during the first week of treatment and at the conclusion of the study.

Experimental Design

One day after ligation, the animals were assigned to a group with either alinidine treatment (MI + A) or vehicle only (MI) after determination of ischemic region size, as determined by echocardiography. Analysis of the expression of growth factors and their receptors was based on 3, 7, and 21 days of alinidine treatment periods; analysis of angiogenesis was based on the 21-day alinidine treatment period. Two days after conclusion of the 21-day treatment period, the rats again underwent echocardiographic evaluation and subsequently were anesthetized, hemodynamics were evaluated, and the heart was arrested with lidocaine HCl and preserved by retrograde perfusion-fixation with a glutaraldehyde-parafomaldehyde solution through the aorta on a Langendorff apparatus at a driving pressure of 120 mm Hg.12 The ventricles were cut with a 4-blade guillotine, each of the resulting 2-mm slices was weighed and photographed, and infarct size (expressed as a percentage of LV free wall) was determined by image analysis. One slice was processed and embedded in paraffin, and 6-μm sections were stained with periodic acid-methenamine silver to demarcate cell boundaries. Cardiomyocyte cross-sectional areas at the level of the nucleus in the epimyocardium and endomyocardium were measured by image analysis. Specimens were dissected from (1) a 1-mm region bordering the infarct and (2) the ventricular septum of perfusion-fixed hearts, processed, and then embedded in Spurr’s plastic. One-micron sections were cut perpendicular to the long axes of muscle fibers, placed on glass slides, and stained with Azure II and methylene blue (Richardson’s solution). A second experimental series evaluated myocardial perfusion in a subset of MI and MI + A samples (n = 16).

Stereological Analysis of Angiogenesis

Capillary and arteriole profiles were analyzed by Image Pro software as performed routinely in our laboratory. Vessel length density is based on the following calculation: L (mm/mm3) = (ab/N)1/3, where a and b are the long and short axes, respectively, and N is the number of vessel profiles in the field (numerical density). Arterioles were defined as vessels <50 μm in diameter and having at least 1 layer of smooth muscle; vessels with no smooth muscle and diameters <10 μm were noted as capillaries.

Western Blot Assay

Tissue samples from the border region and septum of 3 to 5 rats per group were quickly rinsed in phosphate-buffered saline, frozen in LN2, and stored at −70°C. Frozen tissues were homogenized in a lysis buffer containing protease inhibitors, and 70 μg protein was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and blocked with 3% nonfat milk. The membranes were blotted with rabbit polyclonal anti–VEGF, Flk-1, Flt-1, basic fibroblast growth factor (bFGF), and Tie-2 antibodies (Santa Cruz Biotechnology, Inc); angiopoietin-1 and angiopoietin-2 antibodies (Alpha Diagnostic International); or mouse monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Chemicon). The antigen-antibody complexes were visualized by the appropriate secondary antibodies and a Supersignal chemiluminescence system (Pierce). GAPDH was used as a loading control.

Ventricular Hemodynamics

Rats were anesthetized with ketamine HCl (50 mg/kg), intubated, and mechanically ventilated. A PE50 catheter filled with heparinized saline (50 U/mL) was then introduced into the right carotid artery and connected to a computerized data-acquisition system (PowerLab/4S, Chart software, AD Instruments; Australia–coupled pressure transducer BP-100, iWorx). After baseline blood pressure was recorded, the catheter was slowly advanced into the LV through the aorta to obtain ventricular pressures.

Echocardiography

The procedures briefly described herein are routinely used in the Echocardiography Laboratory and have been extensively published.16 Rats were evaluated 24 hours after coronary artery ligation and again 3 weeks later. Ketamine HCl (25 mg/kg) was used to induce a semiconscious state. 2D images were acquired in LV short- and long-axis planes with an 8-MHz sector-array probe, yielding 100 frames per second. LV mass, volumes, and ejection fractions were calculated with the area-length method.16 Regions demonstrating akinesia or dyskinesis were visually identified, planimetered, and expressed as percentages of total LV end-diastolic silhouette. One day after infarction, the 2 groups were similar for echocardiographic data. The areas of ischemia (as a percentage of the free wall and septum) were similar, being 45±3% and 48±3% for the MI and MI + A groups, respectively. Ejection fractions were virtually identical for the 2 groups: for MI, 0.40±0.03 and for MI + A, 0.41±0.02.

Myocardial Perfusion

This protocol is similar to that previously used in our laboratory.17 Rats were anesthetized with ketamine/xylazine (75 mg:0.5 mg/kg) and mechanically ventilated. Catheters were introduced into the right common carotid artery (for injection of microspheres), left and right femoral arteries (for hemodynamics and reference blood withdrawal), and the left jugular vein (for infusion of dipyridamole). The carotid artery cannula was advanced into the LV. We injected 7.5×105 microspheres (Biopal) under baseline (rest) conditions and after maximal vasodilation under dipyridamole infusion. Blood was
withdrawn continuously just before, during, and after microsphere infusion, and an equal amount of donor blood was immediately infused (37°C). During these procedures, arterial pressures and heart rates were monitored on a Power Laboratory. After completion of this protocol, the heart was excised and infarct size determined as previously described. Tissue samples from the border region and interventricular septum were excised, weighed, and sent, along with the blood standards, for neutron activation counts at Biopal. Adequate mixing was verified by comparing flows from 2 border regions.

Statistical Analysis
Data are expressed as mean±SEM. Group comparisons are based on Student’s t test or paired t test for unpaired and paired comparisons, respectively. A probability value ≤0.05 was used to denote statistical significance.

Results
Three weeks after coronary artery ligation, the rats were anesthetized with Ketamine HCl and xylazine (100 and 15 mg/kg, respectively) and the heart arrested in diastole and was excised. Infarcts ranged from 50% to 83% of the LV free wall. As shown in Figure 1, alinidine treatment affected a greater degree of compensatory hypertrophy. Compared with the MI group, the MI+A rats’ ventricular weight–to–body weight ratio was 28% higher and cardiomyocytes cross-sectional areas were 35% and 40% higher in the border region and septum, respectively. Ventricular weights were 1.33±0.02 g and 1.18±0.04 g in MI+A and MI groups, respectively (P<0.05). Arterial and ventricular pressures were similar for the 2 groups (data not shown).

Figure 1. Compensatory cardiac hypertrophy 3 weeks after coronary artery ligation. Infarct size is similar in alinidine-treated MI+A and untreated MI groups (upper left). Mass of surviving myocardium increased more in MI+A group, as evidenced by ventricular weight–to–body weight ratios (upper right) and cardiomyocytes cross-sectional areas (lower left). Data are mean±SEM and are based on 9 to 11 rats per group. Statistically significant difference between MI and MI+A groups: *P≤0.05, **P≤0.01. Abbreviations are as defined in text.

Bradycardic Effect of Alinidine
Heart rates in awake rats declined by 20% to 30% after the first day of treatment and remained lower than those of rats in the untreated group. Mean heart rates after 1 week of treatment were 27% lower in the treated group; ie, 390±20 and 285±17.5 bpm (P<0.01), in the MI and MI+A groups, respectively.

Expression of Growth Factors and Their Receptors
To decipher the molecular mechanisms involved in alinidine-induced angiogenesis and arteriogenesis, we investigated 3 major angiogenic growth factor/receptor systems in this study: VEGF/VEGFR receptor 1 and 2 (Flt-1 and Flk-1), bFGF, and angiopoietin 1 and 2/Tie-2. We previously showed that increases in VEGF (3- to 4-fold in the border region and 1.5- to 1.8-fold in the septum) occur during the first week after MI.19 Angiopoietin-2 (Ang-2) is also enhanced 2-fold in both regions 1 week after MI. An increase in bFGF (1.5- to 2.2-fold) occurred in the border region only. In the present study, we document the effect of alinidine treatment on growth factor levels compared with the levels in untreated rats with MI.

Three days after MI, the VEGF protein level had increased by 1.5±0.2-fold in the septum of the MI+A group compared with the untreated MI group (Figure 2). Flt-1 protein level increased by >2-fold in the septum of the MI+A group compared with the untreated MI group (Figure 2). In contrast, Flk-1 was not enhanced over that in the MI group. One week after MI, VEGF protein in the alinidine group remained higher in the septum compared with values in the untreated group (Figure 3A) and also at this time became elevated in the border region. This increase was further enhanced by the third week after MI, but VEGF protein in the septum fell to the level of the untreated group (Figure 3B).

An early (3 days) increase in bFGF protein occurred in the MI+A group (Figure 4) but returned to levels comparable to those of the MI group at 1 and 3 weeks (data not shown). In contrast to VEGF, Flt-1, and bFGF, alinidine induction of bradycardia did not enhance angiopoietin-1 and -2 or Tie-2
above the levels of the MI group at any of the time points (data not shown).

**Angiogenesis**

Despite the greater degree of hypertrophy in the treated group, marked angiogenesis was documented (Figure 5). Compared with the untreated group, capillary length density in the MI+/H11001A group was higher by 42% ($P < 0.01$) and 14% ($P < 0.05$) in the border region and septum, respectively, and arteriolar length density was increased by 62% ($P < 0.01$) in the septum. Figure 5C illustrates arteriolar length densities in the interventricular septum by diameter size class. These data indicate that length densities for the MI+/H11001A group were greater in all size classes than those for the MI group. The largest difference was in the smallest size class, which represents terminal arterioles.

**Myocardial Perfusion**

To document improvement in coronary circulation as a consequence of bradycardia-induced vascular growth, we measured myocardial perfusion in a subset of 16 rats. No significant differences between the 2 groups for mean arterial pressure were noted, and conductance values (flow adjusted for pressure) were also similar at rest (Table). During maximal vasodilation, conductance values were higher in the septum of the MI+/A group compared with the MI group. Coronary reserve, ie, the percentage increase (over resting flow) in conductance during maximal vasodilation, is illustrated in Figure 6. Increases in conductance during maximal vasodilation in the free wall and septum were 52% and 54%, respectively, in the MI+/A group and 38% and 40%, respectively, in the MI group. The increase in the septum of the MI+/A group was 33% higher than that of the MI group ($P < 0.05$).

**Alinidine-Induced Bradycardia Improves Ventricular Function and Remodeling**

The greater increase in ventricular mass (Figure 1) due to alinidine-induced bradycardia was accompanied by a smaller increase in ventricular volume and a smaller decrement in ejection fraction (Figure 7). One day after coronary artery ligation, ejection fractions were virtually identical in the 2 groups. After 3 weeks, ejection fraction fell by 31% in the MI group but by only 9% in the MI+/A group ($P \leq 0.01$). Ventricular volume increased by 103% in the MI rats and by 71% in the MI+/A group ($P \leq 0.03$). Expansion of the ischemic (akinetic) zone increased by 21% in the MI group but was virtually unchanged ($-2.8\%$) in the MI+/A rats ($P \leq 0.01$). Thus, the smaller decline in ejection fraction in the MI+/A group was associated with a greater degree of compensatory hypertrophy and a smaller pathological LV dilatation.
tion. Hemodynamic data, ie, arterial and ventricular pressures, were not significantly different in the 2 groups.

**Discussion**

We provide new evidence that in postinfarcted hearts, chronic bradycardia (1) upregulates VEGF, its Flt-1 receptor, and bFGF; (2) facilitates angiogenesis; (3) increases coronary reserve; and (4) improves ventricular remodeling and preserves function. To our knowledge, this is the first documentation that a bradycardic agent enhances coronary reserve as well as capillary and arteriolar growth in the postinfarcted heart. Analysis of arteriolar densities documented arteriolar growth in all size classes of the arteriolar tree that exceeded those of the untreated rats and provided an anatomic basis for the greater coronary reserve. The most dramatic increase occurred in the terminal arterioles, ie, those with diameters <10 μm, which is indicative of new arterioles. The higher ejection fraction in the rats with bradycardia was associated with a greater degree of compensatory hypertrophy and a smaller increase in ventricular lumen volume.

The predominant effect of alinidine is a decrease in heart rate without significant changes in arterial pressure. Previous studies showed that pretreatment with alinidine reduced infarct size and increased survival rate in rats. In swine, pretreatment with alinidine reduced the size of area at risk, improved regional contractile function during low-flow ischemia, and enhanced myocardial perfusion after acute coronary artery occlusion. The protective effect of alinidine is likely due to a reduced O$_2$ demand and an increased O$_2$ supply attributed to a prolonged diastolic period. In contrast, the present study examined the role of bradycardia on angiogenesis, myocardial perfusion, and remodeling of the ventricle after completed infarction, ie, independent of drug effects on risk area and infarct size.

**Angiogenic Growth Factors and Their Receptors**

As previously noted, MI is associated with upregulation of VEGF, bFGF, hepatocyte growth factor, and VEGF receptors Flt-1 and Flk-1 in the surviving myocardium. These growth factor responses promote some limited degree of angiogenesis in the infarcted heart. Our data on alinidine-treated rats with MI indicate that bradycardia facilitates a further increase in VEGF, Flt-1, and bFGF. The enhancement of VEGF is consistent with our previous work that documented VEGF as a requirement for angiogenesis in a bradycardia nonischemic model and as a stretch-activated growth factor necessary for endothelial cell proliferation.

Mechanical stretch can induce Flt-1 expression in cultured rat cardiac myocytes and may have played a role in the increase observed in this study. On the contrary, although Flk-1 of treated or untreated MI rats increased significantly compared with sham controls, no significant differences were documented between treated and untreated groups. That Flt-1 may have played a key role in the angiogenic response is consistent with a study that showed that marked inhibition of VEGF stimulated angiogenesis in the adult testis that had been administered antisense Flt-1. Moreover, our recent work on embryonic quail hearts showed that neutralization of VEGF-B, a ligand for Flt-1, inhibits tubulogenesis by 65%.
The upregulation of VEGF observed in this study is consistent with our in vitro finding on myocytes subjected to cyclic stretch.13

Angiogenesis and Coronary Reserve
Our finding that capillary and arteriolar growth exceeded the magnitude of compensatory hypertrophy in the infarcted hearts treated with alinidine indicates a substantial angiogenic response. The drug-induced bradycardia affected a greater magnitude of hypertrophy, as indicated by ventricular weight-to-body weight ratios and cardiomyocyte cross-sectional areas. The present study extends the previous findings that documented VEGF-dependent myocardial angiogenesis in normal, uninfarcted rats treated with alinidine.12 Most important, our data document improved coronary reserve as a correlate to the growth of arterioles, the major resistance vessels. Thus, these findings imply that bradycardia may be useful as a postinfarction, noninvasive therapy that enhances coronary reserve by angiogenesis while simultaneously enhancing the magnitude of cardiac hypertrophy and minimizing remodeling and the decline in ventricular function. Studies that used electrical pacing to produce bradycardia demonstrated myocardial capillary growth in normal rabbits35 and those with aortic valve regurgitation.35 Recently, bradycardia was reported to be associated with the development of coronary collateral vessels in humans with obstructive coronary artery disease; however, this retrospective study did not provide either evidence of a cause-and-effect relation between bradycardia and the development of collateral vessels or clues for the underlying molecular mechanisms.36

Ventricular Function and Remodeling
Ventricular dilatation is associated with depressed ventricular function and poor survival.37 Adequate cardiomyocyte hypertrophy is an important adaptation that can minimize or normalize wall stress. Our data indicate that the greater degree of hypertrophy with alinidine-induced bradycardia is associated with better LV function. A beneficial effect of additional cardiac hypertrophy was also found in postinfarcted rats treated with insulin-like growth factor.38 Moreover, restoration of the ejection fraction toward baseline was correlated with the increase in LV mass in dogs with MI.39 These favorable changes in remodeling may or may not be related to the vascular growth stimulated by alinidine-induced bradycardia. The consequence of the more marked cardiac hypertrophy and smaller increase in ventricular volume is normalization of wall stress. Such a response is a key to minimizing decompensated eccentric ventricular hypertrophy, a characteristic of large infarcts, which leads to heart failure.6 Thus, our data indicate that alinidine treatment reversed the natural history of ventricular remodeling.

Conclusions
Alinidine-induced chronic bradycardia in rats with MI was shown to facilitate an improved coronary reserve through angiogenesis and to preserve ventricular function by improving ventricular remodeling. Thus, these novel findings document several adaptations that are of clinical relevance in postinfarction therapy. Most previous studies on therapeutic angiogenesis have focused on delivery of a single growth factor by invasive procedures. The present study used noninvasive, pharmacologically induced bradycardia to upregulate the appropriate growth factors and receptors necessary for angiogenesis and to improve coronary reserve after MI.

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References
4. Schwarz ER, Speckman MT, Patterson M, Hale SS, Isner JM, Kedes LH, Kloner RA. Evaluation of the effects of intramyocardial injection of DNA expressing vascular endothelial growth factor (VEGF) in a myocardial


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