Bradycardia Stimulates Vascular Growth During Gradual Coronary Occlusion

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Objective—In cultured endothelium, stretch induces release of growth factors that contribute to angiogenesis. We tested the hypothesis that bradycardia, which prolongs ventricular diastolic filling time and volume, promotes collateral vessel growth.

Methods and Results—An ameroid occluder was placed on coronary arteries of dogs with normal heart rates (AM) or bradycardia (55 bpm; AM+BC). A third group had normal heart rates and no ameroid (control [CON]). Four weeks after occluder placement, myocardial blood flow at rest and maximal vasodilation (adenosine) at equivalent heart rates and vascular morphometry of hearts were measured. In AM dogs, conductance (myocardial flow/diastolic pressure) of collateral-dependent myocardium was similar to collateral-independent myocardium during rest but increased to only one third of CON during maximal vasodilation. In contrast, in AM+BC dogs, conductance was similar in collateral-dependent and -independent regions during maximal vasodilation. Arteriolar length density in collateral-dependent myocardium was 80% greater in AM+BC than AM dogs. Capillary length density in collateral-dependent region of AM dogs was lower than CON but normal in AM+BC dogs. The angiotropin receptor Tie-2 increased in collateral-dependent regions of AM and AM+BC groups, whereas vascular endothelial growth factor increased in collateral-dependent and -independent regions only in AM+BC dogs.

Conclusion—Chronic bradycardia during gradual coronary artery occlusion facilitates angiogenesis/arteriogenesis in collateral-dependent myocardium and preserves maximal perfusion. (Arterioscler Thromb Vasc Biol. 2005;25:2122-2127.)

Key Words: bradycardia ■ collateral circulation ■ angiogenesis ■ arteriogenesis ■ remodeling ■ capillaries

Interest in angiogenic therapy as an alternative to surgical approaches for amelioration of myocardial ischemia has grown in recent years. Precapillary vessel growth (arteriogenesis) is necessary to maintain myocardial perfusion to tissue distal to an occluded artery. Although collateral growth from adjacent vessels may provide adequate perfusion during resting conditions, it usually is not able to maintain sufficient perfusion during times of increased oxygen demand. Thus, there is considerable interest in developing therapies that accelerate vessel growth and consequently rescue the myocardium before cell necrosis occurs. Approaches currently under development include administration of growth factors or stem cells or alterations in gene expression. However, each approach is associated with limitations that preclude their widespread use.

Our previous studies and those of others demonstrated that stretch of isolated cardiomyocytes and endothelial cells upregulated key growth factors and receptors. Moreover, stretch induced by inflation of an intraventricular balloon in an ex vivo model produced a nearly 6-fold increase in vascular endothelial growth factor (VEGF) message. Although increasing stretch in vivo is more difficult, an approach used in our laboratory and others is prolongation of the diastolic interval during bradycardia. In rats, alrinidine-induced chronic bradycardia stimulated myocardial angiogenesis and upregulation of VEGF. Neutralizing antibodies for VEGF suppressed the alrinidine-stimulated angiogenesis. Subsequently, we documented bradycardia-induced angiogenesis in the surviving myocardium after infarction. Increases in vascularity and coronary reserve induced by bradycardia were sufficient to preserve contractile function in rats after myocardial infarction.

The mechanism for the bradycardia-induced angiogenesis may be related to an increased ventricular diastolic filling time and volume, which stretches myocytes and blood vessels and increases release of growth factors and their receptors.
This physiological approach to induce angiogenesis is advantageous because it activates an intrinsic system within the heart to prevent myocardial infarction. Although we demonstrated previously that bradycardia-induced angiogenesis increases coronary reserve and preserves contractile function in a model of myocardial infarction with an abrupt occlusion, effects of bradycardia on development of the collateral circulation in the heart during gradual arterial stenosis insufficient to produce infarction are unknown. In the present study, we tested the hypothesis that bradycardia, which prolongs diastolic filling time and left ventricular volume, accelerates development of the coronary collateral circulation during a gradual coronary occlusion by upregulation of growth factors and their receptors.

Methods

Expanded methods can be found in the online supplement at http://atvb.ahajournals.org.

Induction of Bradycardia and Stimulation of Collateral Growth

All surgical procedures and protocols conform to the Guiding Principles for the Care and Use of Animals, were approved by the Council of the American Physiological Society, and were reviewed and approved by the VA Medical Center and the University of Iowa animal care and use committees. Anesthetized, ventilated dogs underwent a thoracotomy; a proximal coronary artery was isolated and an ameroid constrictor placed on the vessel. A subset of dogs underwent atrioventricular (AV) nodal ablation using radiofrequency ablation before ameroid implantation. Dogs with AV nodal ablation were implanted with a pacemaker and heart rates maintained at 55 bpm. Heart rates in normal conscious dogs are 70 to 80 bpm. The incisions were closed, the chests evacuated, and the animals allowed to recover.

General Preparation

Three groups of dogs were studied. Ameroid dogs (AM; n = 8) had an occluder placed on the coronary artery and normal heart rates. Ameroid plus bradycardia dogs (AM + BC; n = 7) had an occluder placed on the coronary artery and AV nodal ablation with pacing at 55 bpm during ameroid closure. Control dogs (CON; n = 5) had no occluder and normal heart rates. Four weeks after occluder implantation, the dogs were anesthetized to obtain hearts for assessment of vascularity. In a subset of dogs, systemic hemodynamics and myocardial blood flow were measured.

Measurement of Myocardial Perfusion

Although throughout the period of ameroid closure, the heart rates of AM + BC dogs were maintained at 55 bpm, to compare myocardial perfusion between groups at similar levels of myocardial oxygen consumption, we increased heart rates in AM + BC dogs to a rate similar to the AM group (125 to 150 bpm) during measurement of myocardial perfusion. Myocardial perfusion was measured before (resting) and during maximal vasodilation (adenosine; 1 mg/kg per minute IV) using neutron-activated microspheres. Mean arterial pressure was maintained at resting levels (±10 mm Hg) during infusion of adenosine using a snare on the aorta. At the completion of the study, the heart was arrested with lidocaine and fixation perfused. Tissue samples were excised from the left anterior descending (LAD) and left circumflex (LCx) regions and equally divided into epimyocardium and endomyocardium for measurement of myocardial perfusion. Conductances during diastole were calculated by dividing myocardial perfusion in each layer (mL·min⁻¹·100 g⁻¹) by diastolic arterial pressure (mm Hg).

Analysis of Capillaries, Collateral Arteries, and Noncollateral Arteries

After perfusion fixation, a gelatin–dye mixture was injected into the left main coronary artery to verify closure of the ameroid and to allow direct visualization of collateral arteries between the LAD and LCx (ie, arterial–arterial connections). Collaterals as well as arteries from collateral-dependent (CD) and collateral-independent (CI) regions were excised and prepared for analysis. Images of vessels were digitized and lumen diameters, wall thickness, and wall-to-lumen ratios determined using an Image Pro image analysis program. We also prepared sections for electron microscopy to estimate the relative volumes of cells and extracellular components of the intima and media tunics. Tissue samples for capillary and arteriolar analyses were dissected from midmyocardium in: (1) the region distal to the ameroid occluder (CD region) and (2) normal myocardium (the CI region). Images of several fields were digitized to measure length density, capillary or arteriolar numerical density, and volume density.

Western Immunoblot

Myocardial samples from the CD and CI regions were obtained for Western blot analysis of VEGF, hypoxia-inducible factor (HIF), Tie-1, Tie-2, angiopeptin-1, or angiopeptin-2 after arrest of the heart but before fixation of the heart. Protein levels were normalized to GAPDH.

Statistical Analysis

Data are presented as mean±SEM. Data were compared using a repeated-measures or 2-way ANOVA. Post hoc comparisons were performed using Student-Newman–Keuls test or Bonferroni correction for multiple comparisons with P < 0.05 for statistical significance.

Results

Systemic Hemodynamics

There were no differences in the body weights (kg) of CON dogs (19±2; n = 5), AM (20±1; n = 7), or AM + BC dogs (22±2; n = 8). For comparisons of myocardial perfusion among groups, it was essential to closely approximate myocardial oxygen consumption by pacing the AM + BC dogs to 125 to 150 bpm similar to levels in anesthetized CON and AM dogs. There were no significant differences in mean blood pressure among the groups under resting conditions (CON 94±12 mm Hg; AM 95±8 mm Hg; AM + BC 88±10 mm Hg). During adenosine, diastolic and mean pressure tended to decrease more in the AM + BC group compared with CON and AM dogs (mean blood pressure CON 98±9 mm Hg; AM 90±6 mm Hg; AM + BC 74±12 mm Hg). Because AM + BC dogs were paced during measurement of myocardial perfusion, heart rates were not different among the groups (CON 147±10 bpm; AM 153±16 bpm; AM + BC 126±24 bpm).

Bradycardia Increases Coronary Conductance

Coronary blood flow in the epimyocardium and endomyocardium was measured at rest and during maximal vasodilation with adenosine. At rest, epimyocardial conductance was lower in the CI myocardium in AM dogs, whereas there were no differences in conductance of either layer in the AM + BC compared with CON group (Figure 1A and 1B). In CD myocardium, the conductance was greater in endomyocardium of AM + BC dogs under resting conditions compared with AM.

Maximal vasodilation with adenosine increased epimyocardial and endomyocardial conductances ~5-fold in CI myocardium in CON dogs. Conductance in the CI region...
compared with AM dogs. Bars represent mean ± SE; *P < 0.05 vs AM. BC increased at rest and during maximal vasodilation in AM dogs. Endomyocardial conductance in CD region and AM BC dogs during maximal vasodilation compared with BC dogs was similar to CON dogs. Endomyocardial conductance decreased in CI region in AM and AM BC dogs during maximal vasodilation compared with CON dogs. Endomyocardial conductance in CD region increased at rest and during maximal vasodilation in AM + BC compared with AM dogs. Bars represent mean ± SE; #P < 0.05 vs AM.

during adenosine in the AM group was less in epimyocardium and endomyocardium compared with CON dogs. In contrast, maximal conductance in AM + BC dogs was similar to the CON group in epimyocardium but lower in endomyocardium. In CD myocardium, conductance was greater in both layers during maximal vasodilation in the AM + BC group compared with AM. These data suggest that bradycardia attenuated the decrease in maximal flow attributable to coronary artery stenosis in CD and CI myocardium.

**Bradycardia Increases Arteriolar Length Density**

Mean lumen diameters of arterioles in CD myocardium from CON (12±2 μm), AM (11±2 μm), and AM + BC dogs (10±1 μm) were similar. Thus, gradual occlusion alone had no effect on mean arteriolar size. Arteriolar length density was also similar in CI myocardium among all 3 groups. In contrast, in CD myocardium, arteriolar length density was significantly higher in the AM + BC group compared with AM (i.e., nearly twice that of AM dogs; Figure 2).

Mean capillary diameters in CD and CI myocardium were similar in the 3 groups (CI region CON 4.0±0.2 μm, AM 4.5±0.2 μm, AM + BC 4.0±0.2 μm; CD region AM 4.5±0.3 μm, AM + BC 3.8±0.1 μm). Although capillary length density was similar in CI myocardium in all 3 groups (CON 7563±367 mm/mm²; AM 7098±318 mm/mm²; AM + BC 7276±520 mm/mm²), it was only lower in CD myocardium of AM compared with the CI region (AM 5793±432 mm/mm²; #P < 0.05 versus CI region). Capillary length density in CD region of AM + BC was comparable to CI region (AM + BC 6696±377 mm/mm²). These data suggest that bradycardia stimulates arteriolar growth and prevents loss of capillaries in ischemic myocardium.

Because increases in arteriolar and capillary length density in ischemic myocardium could be related to myocyte cell size and not growth of vasculature, we measured cardiomyocyte cross-sectional areas. Mean cardiomyocyte cross-sectional area in CD myocardium of AM dogs was not significantly different from CON hearts (Figure 2B). In contrast, cardiomyocyte cross-sectional area in CD area of AM + BC dogs was significantly higher than CON and AM dogs (Figure 2B). Thus, the increase in arteriolar length density in AM + BC dogs represents true arteriolar growth because arteriolar growth exceeded the increase in cardiomyocyte hypertrophy.

**Bradycardia Stimulates Collateral Vessel Remodeling**

Collateral vessels between the LAD and LCx and noncollateral vessels of similar size range (>100 μm in diameter) from CD and CI regions were analyzed. Mean diameters of vessels from all groups were similar (data not provided).

Wall-to-lumen ratios in AM dogs were significantly lower in collaterals and noncollaterals compared with CON or AM + BC groups (Figure 3). In the CD region, bradycardia increased wall-to-lumen ratios nearly 2-fold. The remodeling process of collateral vessels was revealed by image analysis of electron micrographs in both of the ameroid groups (Figure 4A through 4C). Expansion of the extracellular compartment of the media affected a decline in the percent of the media occupied by smooth muscle (i.e., only 67±5% and 75±6% in the AM and the AM + BC dogs, respectively, with 93±3% in CON dogs). The major difference between the AM and AM + BC collaterals is that the latter contained more smooth muscle cells. The percent of tunica media occupied by vascular smooth muscle was similar in all groups in noncollateral vessels in CD and CI regions. These data suggest that gradual coronary occlusion stimulated remodeling of collateral vessels, and that imposition of bradycardia
increased the thickness of the tunica media. The greater wall-to-lumen ratio associated with bradycardia was observed in collateral and noncollateral vessels in CD myocardium because the wall-to-lumen ratio of noncollateral vessels remained similar to CON dogs.

Upregulation of VEGF and Tie-2

In biopsies from the CD and CI regions, the 2-fold greater HIF protein in the CD compared with the CI samples suggested that the 2 regions differed with respect to hypoxia (data not shown). However, stretch has also been shown to upregulate HIF-1α in the nonischemic heart. Although a trend toward elevated VEGF protein was seen in the AM group, it was significantly elevated only in the AM + BC dogs in dependent and independent regions (Figure 5A). The angiopoietin receptor Tie-2 increased 2-fold in the CD regions of both ameredoid groups (Figure 5B). Bradycardia was also effective in upregulating this endothelial cell receptor in CD and CI regions in dogs paced at a reduced heart rate. Angiopoietins 1 and 2 and Tie-1 were not altered by gradual coronary stenosis or bradycardia (data not shown).

Discussion

The goal of these studies was to determine the effects of bradycardia on growth of the collateral circulation, resistance vessels, and capillary bed in a model of gradual coronary artery occlusion. Our data support 3 major findings. First, bradycardia increased maximal myocardial conductance in CD myocardium of dogs with ameredoid occlusion. The greater increase in conductance with adenosine in bradycardic dogs indicates that maximal perfusion was elevated, suggesting that during times of increased metabolic demand a greater O₂ supply may be delivered. Second, consistent with the perfusion data, bradycardia increased arteriolar length density in CD myocardium. Capillary length density was maintained at normal levels in CD myocardium in bradycardic dogs despite increases in cardiomyocyte cross-sectional area. Thus, bradycardia stimulated arteriolar and capillary growth in the CD region. Third, the drop in wall-to-lumen ratio of collateral and noncollateral vessels in the ameredoid group did not occur in bradycardic dogs. This finding indicates that bradycardia stimulated growth of the tunica media during remodeling of collateral channels. Finally, bradycardia increased 2 key proteins that facilitate angiogenesis, namely VEGF and the angiopoietin receptor Tie-2.

Bradycardia Stimulates Arteriolar and Collateral Growth and Improves Myocardial Perfusion

A number of studies have reported growth of the myocardial capillary bed in animals with bradycardia associated with either electrical pacing or the negative chronotropic drug alinidine. Most recently we documented arteriolar growth and preservation of coronary reserve in surviving myocardium in alinidine-treated rats with myocardial infarction. The current study extends these findings by documenting, for the first time, that bradycardia stimulates collateral as well as arteriolar growth in ischemic myocardium. These anatomic adaptations are the basis for the attenuation of the decline in coronary reserve associated with stenosis of a coronary artery.

Bradycardia has several effects that may stimulate vessel growth. First, during bradycardia, all cardiomyocytes and vessels are in a relative state of longitudinal stretch for a prolonged period of time because of prolongation of diastole. In our in vitro studies, stretch of cardiomyocytes increased VEGF, whereas...
stretch of endothelial cells upregulated key tyrosine kinase receptors Flk-1, Tie-1, and Tie-2. Thus, the period of relative stretch of these hearts was prolonged and increased the time period of this stimulus. Second, intramural vessels remained in a relatively open, noncompressed state for a longer interval of time. Our documentation of capillary and arteriolar growth in dogs with bradycardia during a coronary artery occlusion is consistent with our previous studies in bradycardic rats with or without infarction. Third, bradycardia alone, independent of an occlusion, may stimulate angiogenesis. Together, these studies indicate that lowering heart rate either during a gradual coronary occlusion or after myocardial infarction facilitates capillary and arteriolar growth.

Bradycardia Stimulates Collateral Growth
Enhanced shear stress is a major stimulus for collateral growth. Collateral flow is increased when a coronary artery is narrowed, and the accompanying elevated shear stress in collateral vessels triggers activation of endothelial cells and the attraction of monocytes and macrophages that play key roles in collateral growth. Consistent with our study, shear stress triggers Flk-1 and Tie-2 activation. These events promote endothelial proliferation necessary for an increase in collateral vessel diameter. Flt-1 may be a key player in collateral growth because placental growth factor (PIGF), a ligand for Flt-1, enhances collateral growth in ischemic hindlimbs. The effectiveness of P1GF was dependent on monocytes that contain the Flt-1 receptor. Fluid shear stress also markedly increases fibroblast growth factor-2 (FGF-2). This suggests that this growth factor may contribute to smooth muscle growth in developing collaterals.

Our electron micrographs document medial reorganization in collateral vessels in both ameroid groups. This and other structural changes (eg, migration, proliferation, and apoptosis of several cell types) have been described in coronary and hindlimb collateral remodeling. The increase in extracellular matrix, smooth muscle disorientation, and disappearance of the internal elastic membrane were typical of AM and AM+BC collaterals. The significant morphological difference between the 2 groups was the greater number of smooth muscle cells, which underlies the 70% greater wall-to-lumen ratio in the AM+BC group. The higher coronary reserve in AM+BC than AM dogs indicates greater collateral flow because the LAD, which supplies blood flow to the region, was totally occluded. However, it is not clear whether the greater coronary conductance was attributable to enhanced numbers or greater vasodilatory capacity of collaterals. In this regard, it is important to note that VEGF restores impaired vasomotor responses in collateral vessels.

Model of Collateral Growth
The model used in these studies resulted in collateral growth in the virtual absence or limited degree of infarction as noted in our dissections and histological slides. It has the advantage of allowing myocardial and vascular adaptations to occur in the setting of a gradual development of vessel occlusion. Although bradycardia is a stimulus for vascular growth in this model, the sequence of events that enables the vascular growth is yet to be addressed. Our growth factor and receptor protein data are limited to 1 time point (ie, 4 weeks after placement of the ameroid occluder). Nevertheless, the increase in VEGF and Tie-2 are consistent with our previous work in the postinfarction bradycardia model and by others in models of collateral vessel development.

The increase in Tie-2 is important because it is a receptor for angiopoietin-1, which has been shown to interact with VEGF in the induction of functional, mature blood vessels in hindlimb ischemia. These experiments showed that the density of smooth muscle α-actin–positive vessels was enhanced by preadministration or coadministration of angiopoietin-1 with VEGF. Moreover, angiopoietin facilitated a greater recovery of blood flow to the ischemic limb. Although our study did not explore all potential angiogenic or arteriogenic growth factors, previous work demonstrated improved CD flow after growth factor administration. Administration of FGF-2 enhanced collateral flow and vascul arity to ischemic myocardium. However, most of our knowledge concerning collateral growth is based on the hindlimb ischemia model. These studies have shown that FGF-2 accelerates arteriogenesis, and that gene delivery of VEGF combined with FGF-2 has additive and synergistic effects on collateral development. Moreover, FGF receptor-1 and syndecan-4 protein and mRNA are increased during the early phase of collateral development. Inhibition of FGF in this hindlimb ischemia model inhibited monocyte chemoattractant protein–1–stimulated arteriogenesis. FGF-2, in combination with platelet-derived growth factor-BB, promoted functional and stable vascular networks and collateral growth. Development of the collateral circulation is most
likely dependent on several growth factors rather than a single growth factor. With the use of a physiological stimulus to induce growth of the collateral circulation, the heart can provide the necessary factors in the appropriate sequence to stimulate adequate growth to maintain perfusion of myocardial tissue at risk for ischemia.

Summary and Clinical Implications
This study documented a therapeutic role for bradycardia in development of the collateral circulation during a progressive coronary occlusion. Our data are the first to show that bradycardia increases conductance to CD myocardium during coronary artery occlusion. The bradycardia-stimulated increase in conductance was consistent with an elevation in growth factors, receptors, and vascular growth. Although in the short term (4 weeks) bradycardia was beneficial, the long-term effects and mechanisms responsible for the dramatic reduction in vascular resistance need further investigation. However, it is important to note that low heart rate has benefits in addition to vascular growth. Previous studies have shown that bradycardia by sinoatrial node ablation in monkeys decreases plaque formation, and that high heart rate in mice is predictive for intima-media thickening.35

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