Myocardial Angiogenesis and Coronary Perfusion in Left Ventricular Pressure-Overload Hypertrophy in the Young Lamb

Evidence for Inhibition With Chronic Protamine Administration

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Rene G. Flanagan, and James E. Lock

In contrast to young growing animals, pressure-overload hypertrophy in adults is frequently associated with diminished myocardial capillary density and maximal coronary flow per gram. To determine the role of angiogenesis in maintaining perfusion capacity in the hypertrophying heart, the angiogenesis inhibitor protamine sulfate was administered to young lambs during the development of left ventricular (LV) pressure-overload hypertrophy. Baseline and maximum (adenosine) myocardial perfusion was measured in four groups of chronically instrumented 10-week-old lambs subjected to 1) ascending aortic bands since the age of 4 weeks (LVH group, \( n = 10 \)), 2) sham operation at the age of 4 weeks (SHAM group, \( n = 8 \)), 3) aortic bands and twice daily injections of protamine since the age of 4 weeks (LVH+P group, \( n = 9 \)), and 4) sham operation and injection of protamine (SHAM+P group, \( n = 8 \)). Capillary density was measured postmortem. Peak LV pressure and the LV/body weight ratio were similarly increased in LVH and LVH+P compared with sham-operated lambs (\( p < 0.001 \)). In LVH lambs, LV capillary number increased by 32% compared with sham-operated lambs (\( p < 0.05 \)), and capillary density, coronary flow reserve, and minimal coronary resistance remained normal. In contrast, LVH+P lambs had no significant increase over SHAM lambs in LV capillaries and total maximum coronary flow. The LVH+P lambs had lower LV subendomyocardial capillary density and higher minimal coronary resistance per gram (\( p < 0.05 \) versus LVH lambs). Right ventricular capillary density and minimal resistance were similar in all groups. These findings support the hypotheses that myocardial angiogenesis with pressure-overload hypertrophy is important in maintaining maximal LV coronary flow in the young and that impairment of angiogenesis results in diminished coronary flow capacity. (Circulation Research 1991;68:1458–1470)

Pressure-overload hypertrophy of the left ventricle (LV) is associated with dysrhythmias,\(^1\) pump dysfunction,\(^2,3\) and death.\(^1\) How hypertrophy is linked to these abnormalities is unknown, although inadequate coronary perfusion may play an etiologic role.\(^2,4\) Clinical\(^4\) and experimental studies in animals\(^2,12\) have demonstrated abnormal coronary perfusion in adults with pressure-overload hypertrophy. Four potential mechanisms have been proposed for the observed limitations in coronary vascular reserve: 1) increase in ventricular mass without concomitant microvascular growth,\(^4,5,11–17\) 2) generalized arteriolar “drop out” or “rarefaction”,\(^4,17,18\) 3) abnormal thickening or vasomotion of the coronary resistance vessels,\(^5,6,19,20\) and 4) extravascular compressive effects.\(^4,5\) Several of these factors may operate simultaneously, and their relative importance may change.

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during the time course of the hypertrophy. 4 Due to limitations in manipulating these factors independently, it is unclear which are actually important in mediating the observed diminution in coronary reserve with hypertrophy. 4–6,12

This investigation focuses on the role of microvascular growth in maintaining coronary vascular flow capacity in the hypertrophied LV. In adult animals with pressure-overload hypertrophy, there is increased myocyte diameter and ventricular thickness without significant microvascular hyperplasia13,15,16,21,22 (for review see Reference 16), which results in increased intercapillary diffusion distance16,23 and diminished arteriolar density.11,23 In contrast, in young growing animals there is concurrent physiological growth of the myocardium and coronary microvasculature.12,14,15,25 With pressure-overload hypertrophy in the immature animal, there is increased cardiac myocyte and nonmuscle cell proliferation,22 normal capillary density,12,13,15,16,21,22 and normal coronary vascular flow reserve.10,12,26–30 Hence, investigation of the coronary circulation of the immature hypertrophying heart could provide information concerning the mechanisms influencing myocardial perfusion with pressure overload.

Recently, a number of factors that modulate angiogenesis have been found in myocardium, including acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), and heparinlike polysaccharides.31–35 Agents that inhibit angiogenesis have also been described.36 Protamine sulfate reversibly inhibits endothelial binding of bFGF37 as well as endothelial cell mitogenesis32,37 and migration38 induced by bFGF in vitro. When administered systemically, protamine also reversibly inhibits angiogenesis in vivo in a number of different systems,36,39 including capillary growth in the coronary vasculature.40 Rakan and Turek40 demonstrated that myocardial capillary growth during early postnatal development is impeded by protamine. In the current investigation, protamine was used to determine whether coronary angiogenesis could be inhibited in young growing lambs with pressure overload and to study the role of coronary microvascular growth in maintaining perfusion in the hypertrophying heart. By altering microvascular growth independent of other factors that may contribute to diminished coronary perfusion, we hoped to corroborate the hypothesis that a limitation in coronary vascular growth leads to diminished coronary perfusion capacity. We show that in the hypertrophying young heart, protamine treatment both diminishes capillary density and maximal coronary perfusion. Because the effect of protamine on coronary flow reserve does not appear secondary to altered coronary vascular reactivity, it suggests that limited coronary vascular growth with hypertrophy is an important factor that could lead to diminished perfusion reserve with hypertrophy.

Materials and Methods

Supracoronary Aortic Stenosis

Studies were performed using Suffolk and Hampshire lambs of either sex (Earle Parsons and Sons Inc., Hadley, Mass.). Lambs were chosen because they have been used extensively in previous studies of perinatal cardiovascular physiology26,29,30,41 and because the ovine coronary vasculature may be similar to humans in its collateral growth characteristics in response to ischemia.42 The lambs were housed and treated in accordance with the guidelines of the Children’s Hospital Animal Use Committee and the guidelines for the care and use of laboratory animals of the Institute of Laboratory Animals Resources, National Council (DHHS Publication No. [NIH] 85-23, revised 1985).

Four groups of lambs received a thoracotomy at 4 weeks of age and were subsequently studied 6 weeks later. The thoracotomy was performed in the third right intercostal space using sterile surgical technique and general anesthesia with halothane and intravenous ketamine (10 mg/kg/hr, Quad Pharmaceuticals, Inc., Indianapolis, Ind.). Arterial pressure was monitored using a pigtail catheter (UMI Corp., Ballston Spa, N.Y.) or vascular sheath (Cordis Corp., Miami, Fla.) placed percutaneously in the femoral artery.

The SHAM group (n=8) underwent dissection of the aortic root and encirclement of the aorta with umbilical tape, which was then removed. In all experimental groups, the thorax was closed in layers and evacuated with a chest tube. All catheters and tubes were removed after surgery. The lambs were monitored and given 2 ml i.m. penicillin-streptomycin (Combiotic, Pfizer Inc., New York), twice daily for 5 days.

The LVH group (n=10) received an ascending aortic band to induce LV pressure overload. Simultaneous arterial and LV pressures were monitored using the side arm of a 6F sheath in the femoral artery and a 5F pigtail catheter advanced through the sheath into the LV. Umbilical tape was encircled twice around the ascending aorta and tightened until LV systolic pressure was 25–35 mm Hg greater than peak femoral pressure. The systolic pressure gradient increased as the lambs grew (see “Results”).

The SHAM+P group (n=8) underwent a surgical procedure similar to that of the SHAM group. In addition, they received 10 mg/kg body wt s.c. protamine sulfate (Sigma Chemical Co., St. Louis) twice daily for 6 weeks starting the day of the procedure at 4 weeks of age. Protamine was administered in a 50 mg/ml solution of normal saline buffered with HEPES (Sigma). The dose per body surface area was similar to that previously reported to inhibit murine angiogenesis in growing myocardium40 and tumors.36,39 Preliminary studies showed that larger doses caused inhibition of growth or weight loss. To examine the influence of protamine in sheep without coexisting physiological coronary growth, 2-year-old adult sheep (n=4) underwent thoracotomy and the same protocol of protamine administration. The data from the protamine-treated adult sheep were compared with untreated adult sham-operated controls (n=6) and analyzed separately from SHAM+P lambs.
The LVH+P group (n=9) underwent the same banding procedure as did the LVH lambs and received protamine sulfate chronically for 6 weeks, as described for the SHAM+P group. Two additional lambs in the LVH+P group and two in the LVH group died suddenly with no apparent cause found at autopsy and were excluded from further analysis. None of the SHAM or SHAM+P lambs died before instrumentation.

To determine baseline capillary morphometrics at entry into the study, an additional BASE group (n=9) of lambs did not undergo thoracotomy but were killed at 4 weeks of age for histological studies (see below).

**Instrumentation**

At 9 weeks of age, after anesthesia and ventilation, the left femoral artery was entered percutaneously, and a Tygon catheter (Norton Plastics, Synthetic Division, Akron, Ohio) was passed retrograde into the thoracic aorta, secured in the groin, and tunneled subcutaneously to exit in the flank. Via a sterile left thoracotomy, Tygon catheters were inserted into the left atrium and hemiazygos vein and tunneled to exit in the infrascapular region. The chest was closed, and the lambs were allowed to recover from the thoracotomy for 3–14 days (median, 5 days), during which time they received Combicort intramuscularly. Protamine sulfate administration was continued in the SHAM+P and LVH+P groups until the day before study.

On the day of study, the lambs were weighed and sedated with 10 mg/kg i.m. ketamine, and a 7F sheath (Cordis) was placed percutaneously in the right femoral artery. A transducer-tipped catheter (model SPC-350, Millar Instruments, Houston) was advanced through the sheath into the thoracic aorta. The aortic and left atrial fluid-filled catheters were connected to strain-gauge pressure transducers (model P23ID, Gould Inc., Oxnard, Calif.). The strain-gauge pressure transducers and the transducer-tipped catheter were calibrated in vitro with a mercury manometer before each study. The zero offset of the transducer-tipped catheter was cross-calibrated in vivo using the aortic fluid-filled catheter. The transducer-tipped catheter was then advanced into the LV using fluoroscopy.

**Data Collection**

At least 60 minutes after catheter placement, LV and aortic pressure were amplified, displayed through a multichannel recorder (Gould model 2800 V or model E/M AR 6V, Honeywell Inc., Pleasantville, N.Y.), and recorded on an eight-channel analog tape recorder (model 8868A, Hewlett-Packard Co., Palo Alto, Calif.). Cardiac output and regional myocardial blood flow were measured with radiolabeled (51Cr, 85Sr, 141Ce, or 125I) 15±2-µm microspheres suspended in 10% dextran (3M, St. Paul, Minn.). The microspheres were dispersed by vortex agitation, ultrasonically separated for at least 60 minutes, and again vigorously agitated for 1 minute immediately before use. A reference arterial sample was collected over 120 seconds from the aortic catheter into a preweighed heparinized syringe using a withdrawal pump (model 600-910/920, Harvard Apparatus, South Natick, Mass.) at 7 ml/min, starting 30 seconds before injection of 1–5×10⁶ microspheres into the left atrium. The reference sample flow rate was calculated as the weight of the blood collected per minute divided by the specific gravity. Data were rejected and measurements were repeated if LV pressure and heart rate varied by more than 20% during administration of microspheres. Blood pressure and flow were measured at baseline and during intravenous infusion of adenosine (Sigma) at 4 µM/kg for a duration of 20–25 minutes. The dose and duration of adenosine administered has previously been demonstrated to result in maximal myocardial blood flow and minimal coronary vascular resistance. In preliminary studies in sheep, increasing doses of adenosine from 2 to 8 µM/kg/min resulted in no greater decrease in coronary vascular resistance. In this study, myocardial blood flow measured during infusion of adenosine is termed “maximum” and coronary resistance with adenosine is termed “minimum.” Coronary flow reserve is the increase in flow from baseline to maximum flow.

At the end of each experiment, the lambs were sedated with ketamine, and the heart was arrested in diastole with potassium chloride. The heart was immediately excised, drained, trimmed of epicardial fat, and weighed. Growth of the ventricles from the age of 4 weeks (at aortic banding or sham surgery) to study at age 10 weeks was estimated by comparison with the mean LV and right ventricular (RV) weight of the 4-week-old BASE lambs. The heart was divided into eight regions (LV apex, LV anterior papillary muscle, LV posterior free wall, LV posterior papillary muscle, LV anterior free wall, mid septum, RV papillary muscle, and RV free wall). Samples were excised for histology. The remainder of each region was divided into three transmural layers, 0.4–2.0 g in weight, of approximately equal thickness for assessment of blood flow. The radioactivity in isotope standards, reference arterial blood samples, and myocardial tissue samples were counted in duplicate for 10 minutes in a gamma well counter (LKB 1282 Compugamma, LKB Diagnostics, Inc., Gaithersburg, Md.) at appropriate energy windows and corrected for background and crossover activity using an on-line computer program (ULTRA TERM, LKB Diagnostics). Regional layer flows were calculated using the formula Qt=Qr(Ct/Cr), where Qt is tissue sample flow rate, Qr is reference sample flow rate (in milliliters per minute), Ct is tissue sample count rate, and Cr is reference sample count rate; the result was then divided by the sample weight and expressed as flow in milliliters per minute per gram tissue. Transmural regional flow was calculated as the sum of flow in the three transmural layers divided by the weight of the tissues from the three layers and expressed in milliliters per minute per gram. LV blood flow per
gram was calculated as the sum of flow in all six LV regions divided by the combined tissue weight. Total LV coronary flow was calculated as LV flow per gram times LV weight (in grams). Total RV coronary flow was calculated in a similar manner from the two regions analyzed. Cardiac output was calculated as \( Q_r = \frac{C_s}{C_r} \), where \( C_s \) is count rate per milliliter aliquot from microsphere vial, and indexed to body weight by dividing by body weight in kilograms.

The analog tape recording was converted into a digital format by transfer to a microcomputer-based workstation with an eight-channel analog-to-digital conversion system sampling at a rate of 250 samples/channel/sec. A composite average beat was calculated from 17.5 seconds of data. The averaged waveform was used to calculate mean and diastolic coronary perfusion pressure. Since ascending aortic pressure proximal to the aortic band was difficult to measure repeatedly, the coronary mean pressure was calculated, using the method described by Bache et al., as the integrated LV pressure from the onset of the aortic pressure upstream to the dicrotic notch plus the integrated distal aortic diastolic pressure from the dicrotic notch to the onset of systolic upstroke, divided by the cycle length. The calculated mean coronary pressure and diastolic pressures were accepted as valid since they were always similar to directly measured pressures in the ascending aorta when obtained. Diastolic perfusion pressure was calculated as the mean difference between the aortic and ventricular pressures during the time period from the aortic dicrotic notch to the systolic upstroke. Coronary vascular resistance for each region of myocardium was calculated as the ratio of coronary mean pressure and the measured transmural flow to all layers of that myocardial region. The hemodynamic severity of the aortic stenosis was assessed by using the cardiac output and the gradient between the LV and aortic peak systolic pressure.

**Histological Evaluation**

Capillary density in the myocardium was assessed at the age of entry into the study, at 4 weeks of age in BASE lambs and at 10 weeks of age in the other lamb groups. Longitudinal and transverse sections of LV anterior papillary, posterior LV free wall, and RV free wall myocardium were excised, and the epicardium was marked with India ink. Specimens were frozen in liquid \( N_2 \) within 1 hour postmortem and later stored at \(-70^\circ C\). Ten-micrometer sections were cut in a cryostat, fixed in acetone (\( 4^\circ C \) for 5 minutes), and stained by the indoxyl tetrazolium method for alkaline phosphatase, an enzyme specific for the endothelium. Sections were stained, as previously described, with 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt, and nitro blue tetrazolium (Sigma) in a buffer of MgSO\(_4\) and sodium metaborate (pH 9.2–9.4) for 16 hours. No counterstain was used.

Coronary cross-sectional capillary density was analyzed in the inner one third of the LV papillary muscle (\( CD_{LV_{endo}} \)), in the middle third of the LV free wall myocardium (\( CD_{LV_{mid}} \)), and in the midwall of the RV. Capillary density was assessed by counting the mean number of capillaries in a 141 x 141-\( \mu \)m graticule grid in 10–20 randomly chosen cross-sectional fields at \( \times 320 \) magnification (model ICM, Zeiss, FRG). Since many hearts had a capillary “blush” in the immediate subendomycocardial region, the area within 50 \( \mu \)m of the endocardial surface was excluded from analysis. In each myocardial region, 400–800 capillaries were counted. The observer was unaware of the group identity of the slide during histological analysis of the capillaries. Posterior LV wall thickness (W) was measured at \( \times 10 \) from a transverse cross-sectional slide of the LV taken between the heads of the anterior and posterior papillary muscles. The number of capillaries (N) in a transverse linear array across the ventricular wall was calculated from the cross-sectional capillary density by assuming a distribution in a close hexagonal array\(^2\) using the formula

\[
N = 1.0243W \sqrt{\frac{1}{3} CD_{LV_{endo}} + \frac{2}{3} CD_{LV_{mid}}}
\]

**Data Analysis**

Results are reported as mean±SEM. Statistical analysis of the differences between the four primary experimental groups was performed using a multifactorial analysis of variance with a 2 x 2 design examining the effects of hypertrophy and protamine. Differences in parameters compared simultaneously with additional groups were analyzed using a one-way analysis of variance. When the computed \( F \) value indicated \( p<0.05 \), intergroup mean values were compared by using the Student-Newman-Keuls test. Student’s \( t \) test was used for comparison of paired data within lamb groups and between adult sheep groups.

Linear and nonlinear regression analysis was used to examine the relation of coronary vascular resistance to capillary density. Since the exact relation between coronary vascular resistance and vascular growth is unknown, several possible relations were explored. Nonlinear experimental fits were calculated using a power function of the form \( Y=aX^b+c \) and a nonlinear least-squares method. Third-order polynomial curve fits \( Y=aX^3+bX^2+cX+d \) were calculated using direct least-squares methods. For the two hypertrophy groups and for the pooled data, the nonlinear models did not provide a significant reduction in unexplained variance over simple linear regression, and the results of the latter are therefore presented.

**Results**

**Somatic and Myocardial Growth**

Body weight and heart weight data are shown in Table 1. Body weight increased 28% in lambs with and in lambs without aortic bands, from 4 weeks of age at the time of the initial thoracotomy to 10 weeks of age at the time of the study. Lambs receiving protamine had an 18% growth in body weight, which
was significantly less than that in untreated lambs \((p<0.03)\), similar to previous reports.\(^{40,46}\)

The LV weight in 10-week-old SHAM and SHAM+P lambs was increased by 29% and 30%, respectively, compared with LV weight in 4-week-old BASE lambs \((p<0.01)\). The increase in LV weight from 4 to 10 weeks of age was significantly greater in aortic-banded lambs than in sham-operated controls \((p<0.01)\). The LV weight in LVH and LVH+P lambs increased similarly, 50% and 61%, respectively, compared with 4-week-old BASE lambs. The LV/body mass ratio was commensurately 48% greater in LVH and LVH+P lambs compared with SHAM lambs \((p<0.001)\). Thus, while protamine did impair somatic growth, it did not impair physiological LV growth nor the LV hypertrophic response to pressure overload. The RV/body mass ratio in LVH and LVH+P lambs was also increased but to a smaller degree (25%) compared with unbanded lambs \((p<0.02)\).

### Hemodynamic Data

Systemic hemodynamics are shown in Table 2. Six weeks after aortic banding, baseline peak LV pressure in the LVH and LVH+P groups was elevated to \(168\pm10\) and 168±5 mm Hg, respectively \((p<0.001\) compared with SHAM and SHAM+P groups). The severity of aortic stenosis was hemodynamically similar in LVH and LVH+P groups, since the systolic peak-to-peak pressure gradient between the LV and aorta was similar \((74\pm12\) and 79±12 mm Hg, respectively) and the cardiac index and heart rate were similar in all groups. End-diastolic LV pressures were slightly but not significantly higher in lambs with aortic bands. Calculated coronary mean pressure and diastolic perfusion pressure were similar in LVH and LVH+P groups.

Adenosine infusion decreased systemic vascular resistance and aortic pressures and increased cardiac index similarly in all four groups (Table 2). LV peak pressure, systolic peak-to-peak pressure gradient, coronary mean pressure, and diastolic perfusion pressure were the same in LVH and LVH+P during adenosine infusion. Although the relative change in systemic vascular resistance in response to adenosine was similar in all groups, the absolute level was higher in SHAM+P lambs \((p=0.034\) nontreated versus protamine-treated).

### Myocardial Perfusion

Total LV blood flow (in milliliters per minute) in sham-operated lambs was unaffected by protamine at baseline and increased similarly in response to adenosine-induced vasodilation by 313% and 311% in SHAM and SHAM+P lambs, respectively (Figure 1A). In the LVH and LVH+P lambs, baseline total LV blood flow was similarly greater than in the groups without hypertrophy \((p<0.05)\). Although baseline LV coronary flow in LVH lambs was elevated, it still increased threefold with adenosine, similar to the SHAM and SHAM+P lambs. Thus, LV hypertrophy in young lambs did not impair coronary flow reserve. In contrast, while the LVH+P lambs had a LV baseline blood flow 69% greater than that of sham-operated lambs \((p<0.01)\), maximal total LV blood flow was only 24% more than that of sham-operated lambs and was less than that in LVH lambs \((p=0.06)\).

Myocardial oxygenation during periods of stress and increased myocardial oxygen consumption is dependent on the degree to which coronary blood flow per gram of myocardium can increase.\(^{44}\) Maximal and baseline myocardial perfusion expressed as coronary flow per gram LV are demonstrated in Figure 1B. The elevated LV blood flow in lambs with hypertrophy was not entirely due to the higher LV weight since baseline LV perfusion per gram was 54% higher than that of SHAM and SHAM+P lambs \((p<0.01)\). Since coronary flow reserve was similar in LVH and unbanded lambs, maximal transmural LV perfusion per gram in LVH lambs was similarly 53% greater than in the sham-operated groups \((7.51\pm0.6\) versus \(4.81\pm0.5\) ml/min/g, \(p<0.03)\). However, the higher LV perfusion in LVH lambs was present only in the midmyocardium and subepimyocardium \((p<0.03)\), whereas subendomycardial flow during adenosine infusion was equivalent to that of non-banded lambs \((4.6\pm0.7\) versus \(4.4\pm0.6\) ml/min/g, respectively).

In contrast to lambs with hypertrophy alone, lambs with both hypertrophy and protamine treatment had significantly lower maximal LV myocardial perfusion per gram \((p<0.02)\) in all regions. As in LVH lambs, in

<table>
<thead>
<tr>
<th>Table 1. Heart and Body Weight Data in Four Groups of Lambs</th>
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<tr>
<td><strong>Body wt (kg)</strong></td>
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<tr>
<td><strong>SHAM</strong></td>
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<td><strong>LVH</strong></td>
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<td><strong>LVH+P</strong></td>
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| **Values are mean±SEM.** | **LVH and LVH+P lambs** an increased by 29% and 30%, respectively, compared with LV weight in 4-week-old BASE lambs \((p<0.01)\). The increase in LV weight from 4 to 10 weeks of age was significantly greater in aortic-banded lambs than in sham-operated controls \((p<0.01)\). The LV weight in LVH and LVH+P lambs increased similarly, 50% and 61%, respectively, compared with 4-week-old BASE lambs. The LV/body mass ratio was commensurately 48% greater in LVH and LVH+P lambs compared with SHAM lambs \((p<0.001)\). Thus, while protamine did impair somatic growth, it did not impair physiological LV growth nor the LV hypertrophic response to pressure overload. The RV/body mass ratio in LVH and LVH+P lambs was also increased but to a smaller degree (25%) compared with unbanded lambs \((p<0.02)\).**

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LVH+P lambs the subendomyocardial perfusion during adenosine was significantly lower than subepimyocardial perfusion (59%, p<0.001). Thus, administration of protamine, an angiogenesis inhibitor, to young animals during development of pressure-overload LV hypertrophy reduced the coronary perfusion capacity. As is observed in adult animals with LV pressure-overload hypertrophy, the diminished perfusion predominated in the subendocardium.

Perfusion per gram in the RV was 53% greater than LV perfusion in the LVH+P lambs (p<0.02). The RV coronary flow reserve in LVH+P lambs was 491±73%, similar to that of the other groups. Thus, the effect of protamine in LVH+P lambs predominated in the LV, the ventricle that had the greatest increase in mass.

Table 2: Systemic Hemodynamics Measured at Baseline and After Adenosine Infusion in Four Groups of Lambs

<table>
<thead>
<tr>
<th></th>
<th>SHAM</th>
<th>SHAM+P</th>
<th>LVH</th>
<th>LVH+P</th>
<th>Hypertrophy</th>
<th>Protamine</th>
<th>Interaction</th>
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<tr>
<td>Heart rate (beats/min)</td>
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<tr>
<td>Baseline</td>
<td>123±12</td>
<td>137±15</td>
<td>146±7</td>
<td>145±6</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Adenosine</td>
<td>139±12</td>
<td>156±10</td>
<td>165±8</td>
<td>161±15</td>
<td>NS</td>
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<td>Cardiac index (ml/min/kg)</td>
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<tr>
<td>Baseline</td>
<td>168±48</td>
<td>157±22</td>
<td>159±11</td>
<td>142±14</td>
<td>NS</td>
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<tr>
<td>Adenosine</td>
<td>266±23</td>
<td>223±27</td>
<td>259±17</td>
<td>249±21</td>
<td>NS</td>
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<td>Systemic resistance</td>
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<td>(mm Hg/ml/min/kg)</td>
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<tr>
<td>Baseline</td>
<td>0.77±0.28</td>
<td>0.70±0.08</td>
<td>0.56±0.06</td>
<td>0.65±0.06</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Adenosine</td>
<td>0.21±0.01</td>
<td>0.33±0.05</td>
<td>0.20±0.02</td>
<td>0.23±0.03</td>
<td>NS</td>
<td>0.034 NS</td>
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<td>LV peak pressure (mm Hg)</td>
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<tr>
<td>Baseline</td>
<td>101±5</td>
<td>112±5</td>
<td>168±10</td>
<td>168±5</td>
<td>&lt;0.001</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Adenosine</td>
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Values are mean±SEM. Values of p were determined by two-way analysis of variance. SHAM, lambs subjected to sham operation; SHAM+P, lambs subjected to sham operation and protamine sulfate injections; LVH, lambs with left ventricular hypertrophy induced by an aortic band; LVH+P, lambs with left ventricular hypertrophy induced by an aortic band receiving protamine sulfate injections; Hypertrophy, LVH and LVH+P vs. SHAM and SHAM+P; Protamine, SHAM+P and LVH+P vs. SHAM and LVH; Interaction, hypertrophy and protamine injections; Adenosine, values measured with adenosine infusion; LVEDP, left ventricular end-diastolic pressure; PPSG, peak to peak systolic gradient; diastolic perfusion pressure, aortic mean diastolic pressure minus left ventricular mean diastolic pressure.

Minimum LV Coronary Vascular Resistance With Pressure Overload

With infusion of adenosine, autoregulation of coronary blood flow is abolished, and flow becomes linearly dependent on perfusion pressure.44 Minimum coronary resistance (Figure 2) was calculated to account for the effect of the higher coronary perfusion pressure in lambs with ascending aortic bands (LVH and LVH+P) than in normotensive control lambs, to allow a more meaningful comparison. Minimum coronary vascular resistance may provide an index proportional to the cross-sectional area of the coronary vascular resistance bed.5 Minimum LV coronary vascular resistance in LVH (12.6±1.04 mm Hg/ml/min/g) and SHAM (12.4±2.1 mm Hg/ml/min/g) lambs was similar. Thus, young animals with
pressure-overload hypertrophy maintain normal minimum LV coronary vascular resistance.\textsuperscript{2,5,6,17}

**Minimum LV Coronary Vascular Resistance: Effect of Protamine Administration During LV Growth and Development of Hypertrophy**

In response to adenosine, LV resistance per gram decreased 79±2% and RV resistance decreased 86±2% in SHAM lambs. In SHAM+P lambs, LV and RV resistance per gram decreased similarly (74±4% and 83±4%, respectively). Thus, immature lambs receiving protamine did not appear to have an altered coronary vascular reactivity to adenosine.

Minimum LV coronary vascular resistance per gram (Figure 2) was higher in protamine-treated lambs than in untreated lambs (\(p<0.02\)), roughly in proportion to the estimated increase in LV mass during treatment. Thus, LVH+P lambs with a 61% increase in LV mass compared with 4-week-old BASE lambs had a 65% increase in LV minimal coronary vascular resistance per gram (compared with untreated lambs). Chronic protamine treatment resulted in a small (21%) but significant increase in minimum LV resistance in SHAM+P lambs compared with untreated lambs, roughly proportional to the 25% increase in LV mass that occurred during the 6 weeks of protamine treatment. Minimum LV resistance in LVH+P lambs was 43% higher than in SHAM+P lambs, although the interaction was not statistically significant.

**No Effect of Protamine on LV Minimum Coronary Vascular Resistance Without LV Growth**

To examine the influence of protamine treatment without associated myocardial growth, minimum coronary vascular resistance during adenosine infusion (at the same dose of 4 μg/kg/min) was determined in 2-year-old adult sham-operated sheep who were treated with the same protamine protocol (\(n=4\)) and in untreated adult sham-operated sheep (\(n=6\)). Protamine-treated adult sheep had similar LV weight and minimum coronary vascular resistance when compared with untreated adult sheep (resistance, 15.7±1.2 versus 15.5±1.8 mm Hg/ml/min/g, respectively; \(p=NS\)). Thus, chronic protamine exposure had no apparent effect on coronary vascular resistance in ventricles that were not increasing in mass.

**Minimum RV Coronary Vascular Resistance**

To further determine whether the change in vascular resistance was specific for the hypertrophied ventricle or whether it was due to a generalized vascular effect of protamine independent of angio-

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**Figure 1.** Bar graphs showing left ventricular (LV) blood flow. SHAM, sham-operated lambs; LVH, aortic-banded lambs with LV hypertrophy; SHAM+P, sham-operated lambs receiving protamine sulfate; LVH+P, aortic-banded lambs with LV hypertrophy receiving protamine sulfate. Total LV blood flow in ml/min (panel A) and LV perfusion in ml/min/g (panel B) in the four experimental groups at baseline (hatched bars) and during adenosine infusion (solid bars) are shown. Values of \(p\) were determined by two-way analysis of variance.\(^{*}p<0.01\) vs. sham-operated (nonhypertrophic) lambs. \(\dagger p<0.03\) vs. sham-operated (nonhypertrophic) lambs. \(\ddagger p<0.02\) interaction of protamine treatment and hypertrophy on perfusion during adenosine infusion.

**Figure 2.** Bar graph showing minimum coronary vascular resistance during adenosine infusion in the left ventricle (LV; aortic-banded outflow) and in the right ventricle (RV; non-banded outflow) in four experimental groups. SHAM, sham-operated lambs; LVH, aortic-banded lambs with LV hypertrophy; SHAM+P, sham-operated lambs receiving protamine sulfate; LVH+P, aortic-banded lambs with LV hypertrophy receiving protamine sulfate. \(^{*}p<0.02\) vs. non-protamine-treated lambs.
genesis (e.g., alteration of coronary vascular reactivity or vessel drop out), RV vascular resistance (Figure 2) was measured in the same lambs. Minimum coronary vascular resistance in the RV was similar in all groups of lambs. Protamine-treated lambs had a slight, but not significant, elevation of RV minimum resistance per gram compared with SHAM and LVH, roughly proportional to the increase in RV mass associated with growth. In SHAM+P lambs, the minimum coronary vascular resistance per gram was similar in both ventricles, whereas in LVH+P lambs the resistance per gram was less in the RV than in the hypertrophied LV (p<0.055). The influence of protamine on vascular resistance was greater in the ventricle undergoing the largest increase in mass in response to aortic banding and growth. This is inconsistent with a generalized effect of protamine on vascular resistance independent of angiogenesis.

**LV Wall and Capillary Morphometry**

Samples for capillary density were judged inadequate for accurate cross-sectional capillary counts in two lambs (one LVH, one LVH+P) and were not included in the histological analysis. There were no significant differences in capillary density detected between the LV subendomycardium (anterior papillary muscle) and the midmyocardium (free wall) within any of the groups (Table 3).

LVH lambs had a significant increase in LV wall thickness compared with the BASE group (27±7%, p<0.001) (Table 3) and yet inner and midwall capillary densities were similar to SHAM lambs due to an increase in LV cross-sectional capillary number concordant with the increase in LV mass (Figure 3). The number of capillaries across the LV wall was 27±6% greater in LVH lambs than in age-matched SHAM lambs (p<0.05) (Figure 3A).

In LVH+P lambs, the number of capillaries across the LV wall (Figure 3A) was not significantly increased over 4-week-old BASE or 10-week-old SHAM+P lambs. The blunted increase in LV capillaries in LVH+P lambs, coupled with an increase in LV wall thickness similar to LVH (22±2% over SHAM, p<0.001), resulted in a significantly lower LV midmyo-

![Figure 3](http://circres.ahajournals.org/)

**Figure 3.** Bar graphs showing left ventricular (LV) capillary number and density. BASE, unoperated 4-week-old lambs used for baseline studies; SHAM, sham-operated 10-week-old lambs; SHAM+P, sham-operated lambs receiving protamine sulfate; LVH, aortic-banded 10-week-old lambs with LV hypertrophy; LVH+P, aortic-banded lambs with LV hypertrophy receiving protamine sulfate. Panel A: Calculated number of capillaries in a linear section across the LV wall. Panel B: Number of capillaries per square millimeter in the LV subendomycardium.

*p<0.01 vs. BASE. tp<0.05 vs. SHAM and SHAM+P. tp<0.05 vs. LVH.

### Table 3. Morphometric Data From Four Groups of Lambs

<table>
<thead>
<tr>
<th>Group</th>
<th>LV post midwall thickness (mm)</th>
<th>Capillary density (capillaries/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LV post midwall thickness</td>
<td>Capillary density</td>
</tr>
<tr>
<td></td>
<td>subendo</td>
<td>RV midwall</td>
</tr>
<tr>
<td>SHAM (n=8)</td>
<td>10.4±0.7</td>
<td>2,003±106</td>
</tr>
<tr>
<td>SHAM+P (n=8)</td>
<td>10.4±0.5</td>
<td>2,133±137</td>
</tr>
<tr>
<td>LVH (n=9)</td>
<td>12.7±0.7</td>
<td>2,197±151</td>
</tr>
<tr>
<td>LVH+P (n=9)</td>
<td>12.6±0.2</td>
<td>1,651±134</td>
</tr>
</tbody>
</table>

*p by ANOVA

| Hypertrophy       | NS                             | NS                                | NS                               |
|                   | Protamine                      | NS                                | NS                               |
|                   | Interaction                    | 0.036                             | 0.017                            |

Values are mean±SEM. LV, left ventricular; post, posterior; AP, anterior papillary muscle; subendo, subendomyocardium; RV, right ventricular; SHAM, lambs subjected to sham operation; SHAM+P, lambs subjected to sham operation and protamine sulfate injections; LVH, lambs with LV hypertrophy (aortic-banded); LVH+P, lambs with LV hypertrophy (aortic-banded) receiving protamine sulfate injections; ANOVA, two-way analysis of variance; Hypertrophy, LVH and LVH+P vs. SHAM and SHAM+P; Protamine, SHAM+P and LVH+P vs. SHAM and LVH; Interaction, hypertrophy and protamine injections; NS, not significant.
cardial and subendomyocardial capillary density in LVH+P lambs ($p<0.04$) (Table 3, Figure 3A). In contrast to LVH+P lambs, chronic protamine administration did not demonstrably alter capillary density in either young SHAM+P or in adult sheep ($n=4$) compared with untreated age-matched shams (adult protamine-treated sheep, 105–110% of adult control sheep, $p=NS$). Capillary density in the less hypertrophied RV was not different in LVH+P compared with the other groups. Thus, the changes in capillary density qualitatively correlated with the changes in minimal coronary vascular resistance and were limited to the hypertrophied LV in LVH+P lambs. The diminished LV capillary density in LVH+P lambs is similar to findings of diminished subendomyocardial capillary density in adult animals with pressure-overload hypertrophy of similar duration.12–17

Relation of Minimal Coronary Resistance and Capillary Density

Linear regression analysis was used to correlate changes in coronary capillary density and minimum vascular resistance observed in LVH and LVH+P lambs (Figure 4). Capillary density was used as a general index of tissue vascularity. A direct physiological linkage between density and total coronary vascular resistance is limited since the majority of the coronary vascular resistance occurs proximal to the capillaries.4,17–19 However, protamine inhibits growth of larger resistance vessels as well as capillaries.36 In hypertrophied adult myocardium, changes in the density of arteriolar resistance vessels are paralleled by similar changes in capillary density.11 In this study, a significant inverse relation exists between LV minimum coronary vascular resistance and LV midwall capillary density in lambs with pressure-overload hypertrophy ($r=0.66, p=0.004$). This correlation between vascular density and vascular resistance observed with inhibition of angiogenesis provides support for the hypothesis linking coronary microvascular growth and coronary vascular resistance during development of pressure-overload hypertrophy.

Discussion

The development of LV pressure-overload hypertrophy in adults is accompanied by a decrease in microvascular density11–17 and abnormal coronary vascular resistance,2,5,6,9,11,12 which may be due to inadequate growth of the coronary microvasculature.2,4,6–8,17 However, it has been difficult to separate the role of blunted coronary angiogenesis from other factors thought to influence microvascular density4,17,18 and minimum coronary vascular resistance5,6,12,17,19 in hypertensive vascular beds. These previous studies in mature animals of the role of angiogenesis in maintaining perfusion capacity in the hypertrophied LV have thus far been limited because of the lack of a system in which microvascular growth might be independently altered. In contrast to mature animals, young animals have ongoing coronary growth accompanying physiological myocardial growth.13–16,25,40 The coronary circulation of the young may therefore provide insight into the mechanisms in adults leading to diminished coronary flow reserve with LV hypertrophy. In this study, protamine sulfate, an angiogenesis inhibitor, was used to modulate coronary vascular growth in immature lambs during development of LV pressure-overload hypertrophy and to elucidate the impact of coronary vascular growth, or lack of growth, on the maintenance of coronary perfusion capacity in pressure-overload LV hypertrophy.

A principal finding of this study was that young lambs with LV pressure-overload hypertrophy have an increase in LV capillary number concordant with the increase in LV mass and maintenance of normal LV capillary density, coronary vascular resistance, and perfusion reserve. Therefore, at least in the young lamb with pressure-overload hypertrophy, the chronic increase in coronary systolic pressure did not appear to result in a change in coronary resistance in either the RV or LV. Similarly, LV myocardial compressive effects associated with the higher LV systolic pressure did not influence LV coronary resistance, although transmural flow distribution to the subendomyocardium may have been altered in the presence of adenosine-induced vasodilation. Administration of protamine sulfate during development of LV pressure-overload hypertrophy inhibited a concordant increase in LV capillaries and was associated with a diminished LV capillary density and maximal perfusion per gram. The LV minimum coronary
resistance per gram, which may be an index of microvascular cross-sectional area,\textsuperscript{5} correlated inversely with LV capillary density and was elevated in hypertrophied lambs who received protamine. Thus, both physiological and morphometric assessments suggest that the cross-sectional area of the coronary vessels did not increase in proportion to the hypertrophy in protamine-treated lambs.

The magnitude of the changes in capillary density,\textsuperscript{11–17} coronary perfusion reserve,\textsuperscript{2,4–7,9,11} and vascular resistance\textsuperscript{2,4,5,6,12,17} induced by LV pressure-overload hypertrophy in previous investigations in adult animals are similar to what we observed in young lambs with protamine inhibition of angiogenesis and LV pressure-overload hypertrophy of similar duration and severity. These findings support the concept that microvascular angiogenesis may be an important adaptive component of the myocardial hypertrophic response to pressure overload. In the young, coronary angiogenesis allows LV myocardial perfusion capacity to increase proportionally with LV mass. When coronary angiogenesis is insufficient for the increase in myocardial mass, as seen in adults with pressure-overload ventricular hypertrophy,\textsuperscript{11–17,23,24} diminished myocardial perfusion capacity may result.\textsuperscript{4,6,7,9–12}

Rakusan and Turek\textsuperscript{40} found that protamine inhibits its coronary capillary angiogenesis accompanying physiological postnatal cardiac growth. In that investigation, the animals (rats) had much greater somatic and cardiac growth than the more mature lambs in this study. It is possible that protamine did not result in a measured decrease in capillary density in this study because the cardiac growth in the SHAM+P lambs was insufficient to induce a change in capillary density measurable with the histological and morphometric methods used. An effect of protamine on coronary capillary growth was only evident when the growth rate of the capillaries and LV mass was accelerated by pressure overload. We did observe a trend for increased minimal LV and RV resistance per gram in SHAM+P lambs. This may be a more sensitive index of vascular growth, since small changes in microvascular density and cross-sectional area may cause exponentially larger changes in vascular resistance.\textsuperscript{18}

A potential limitation of the use of protamine sulfate in the study of the hemodynamic impact of myocardial angiogenesis is the possibility of cardiovascular effects of protamine other than those due to inhibition of angiogenesis (e.g., alteration of coronary vascular reactivity, arterial thickening, generalized vascular toxic effects leading to vessel drop out, and inhibition of myocardial growth or function). Protamine may influence systems (e.g., DNA histone, vascular wall heparin–smooth muscle interactions,\textsuperscript{47} nonvascular receptors for bFGF, platelet-derived growth factor,\textsuperscript{48} and insulin\textsuperscript{49}) that potentially could alter coronary vessels or affect myocardial growth and lead to confounding effects. However, in a previous study,\textsuperscript{50} no acute systemic hemodynamic effects were observed in sheep after protamine administration. Chronic high dose protamine administration to humans for treatment of malignancy resulted in no reported cardiovascular side effects.\textsuperscript{46} In previous studies,\textsuperscript{36,37} protamine inhibition of capillary proliferation appeared to be specific and reversible. Protamine did not inhibit proliferation of a number of nonendothelial cell lines.\textsuperscript{36} Protamine treatment of young, rapidly growing mice\textsuperscript{40} resulted in only a slight reduction in body and heart weight and no apparent cardiac pathology apart from inhibition of angiogenesis.

In the present study, physiological cardiac growth and the LV hypertrophic response to pressure overload did not appear to be impaired in lambs given protamine. The specificity of protamine actions on coronary growth, blood flow, and vascular reactivity was examined using coronary flow to the RV as an internal control. Unlike the LV, RV capillarity and minimal coronary vascular resistance were not affected by protamine. The unchanged RV minimum resistance indicates that protamine does not cause a change in hypertensive coronary resistance vessels independent of ventricular hypertrophy. The specificity of protamine action was further investigated using age-matched growing lambs that received protamine but did not have pressure-overload hypertrophy and adult sheep that received protamine but had neither hypertrophy nor physiological growth. Unlike lambs with hypertrophy, protamine administration to sham-operated lambs and to nongrowing adult sheep had no significant effect on coronary perfusion or capillary density. The relative specificity of the effect of protamine suggests that the inhibitory effect of protamine on coronary perfusion was specific to hypertrophying ventricles with growing vasculature. The histological data demonstrated that changes in vascular resistance were paralleled by a decrease in capillary density, supporting the contention that the changes in coronary vascular resistance associated with protamine were a result of impaired neovascularization.

Protamine inhibits the growth of larger vessels as well as capillaries.\textsuperscript{36} Since most of the coronary vascular resistance is proximal to the capillaries,\textsuperscript{4,17–19} protamine presumably also inhibited the growth of coronary resistance vessels. The growth of small arteries and arterioles may involve changes in several morphometric parameters that directly influence vascular resistance, including vessel density, diameter, length, and branching pattern.\textsuperscript{18} The relative changes in these coronary vascular parameters with myocardial hypertrophy and with protamine treatment are not yet clear. The quantification of these changes in myocardium is difficult\textsuperscript{4} and beyond the scope of the present investigation. A previous study by Breisch et al\textsuperscript{11} of LV pressure-overload hypertrophy of similar duration in adult pigs demonstrated a qualitatively similar decrease in capillary density (15–25\%) and a similar although somewhat greater decrease in arteriolar density (34\%). In the present study, capillary number and density were used...
as general indexes of coronary microvascular growth. The significant correlation of capillary density and minimum coronary vascular resistance seen with protamine administration supports the hypothesis that coronary vascular growth is an important determinant of coronary vascular resistance during development of pressure-overload hypertrophy.

The present study is limited to immature animals with one model of pressure overload at a single point during the time course of ventricular hypertrophy. In adults with pressure-overload hypertrophy, the time course of angiogenesis may differ, and other mechanisms may operate to limit myocardial perfusion. For example, an appropriate degree of coronary angiogenesis may be achieved late during the development of some, but not all, forms of pressure-overload hypertrophy in adults. Myocardial perfusion may also be impeded in adults by changes in small coronary artery structure or reactivity that do not appear to be present in younger animals. Therefore, extrapolation of the relation of vascular growth and myocardial perfusion in young lambs to adults with pressure-overload hypertrophy should be placed within the context of these limitations.

Protamine inhibition of angiogenesis may be mediated directly and/or indirectly through interference with heparin-binding growth factors. Protamine sulfate directly reversibly inhibits endothelial aFGF and bFGF receptor binding as well as endothelial cell mitogenesis and migration induced by bFGF in vitro. Additionally, protamine may inhibit angiogenesis by interfering with heparin-binding growth factors through indirect mechanisms by binding endogenous heparin-like molecules. Heparin released from mast cells that accumulate at sites of angiogenesis, including immature and hypertrophied myocardium, may promote angiogenesis via mechanisms involving heparin-binding growth factors, pericytes, and endothelial cell migration. Heparin-like molecules in the extracellular matrix bind and modulate the extracellular storage and degradation of several heparin-binding growth factors including bFGF. Therefore, protamine could possibly inhibit angiogenesis by binding and interfering with endogenous heparin. The demonstration of aFGF and bFGF in myocardium and inhibition of coronary angiogenesis by protamine suggests that cardiac heparin-binding growth factors may play a role in microvascular growth accompanying pressure-overload hypertrophy in the young. The role of heparin and heparin-binding growth factors in coronary neovascularization in the young and in the adult deserves further study. The ability to downregulate coronary angiogenesis with protamine and to upregulate it with thyroxine demonstrates that the process can be pharmacologically modulated, suggesting a potential for the use of angiogenesis promoters in adult hypertrophic and ischemic heart disease.

In conclusion, the immature lamb increases LV capillary number and maintains minimum coronary vascular resistance and maximum coronary perfusion during development of pressure-overload hypertrophy. The angiogenesis inhibitor protamine impedes coronary microvascular growth accompanying LV hypertrophy in the young lamb, resulting in diminished capillary density and maximum myocardial perfusion and elevated LV minimum coronary vascular resistance. These findings provide evidence that coronary angiogenesis is important in maintaining perfusion reserve during LV pressure-overload hypertrophy in the immature LV. Inhibition of myocardial angiogenesis with protamine sulfate suggests that heparin and heparin-binding growth factors may promote coronary neovascularization with pressure-overload hypertrophy in the young. Angiogenesis inhibitors may provide a means of clarifying the role of coronary vascular growth and perfusion, as well as the impact of limited microvascular growth, on alterations in myocardial function reserve and electrophysiological function in pressure-overload hypertrophy.

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References


**KEY WORDS** • angiogenesis • fibroblast growth factor • coronary circulation • hypertrophy • protamine
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M F Flanagan, A M Fujii, S D Colan, R G Flanagan and J E Lock

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