Effects of Chronic Heparin Administration on Coronary Vascular Adaptation to Hypertension and Ventricular Hypertrophy in Sheep

Michael F. Flanagan, MD; Teruhiko Aoyagi, MD; Lucy W. Arnold, MD; Christine Maute, PhD; Alan M. Fujii, MD; Joseph Currier, MD; Dennis Bergau, MA; Henry B. Warren, VMD; Karel Rakusan, MD, PhD

Background—Hypertension decreases myocardial perfusion capacity in adults for several reasons, including insufficient coronary angiogenesis with left ventricular (LV) hypertrophy, arteriolar hypertrophy, and altered vasomotion. Heparin influences growth factors that promote angiogenesis and vasodilation and inhibit arteriolar wall thickening.

Methods and Results—Adult sheep were given heparin 200 U/kg body wt SC twice daily throughout 6 weeks of LV and coronary hypertension from a progressively constricted ascending aortic band (n=14). They were compared with untreated sheep with (n=13) and without (n=13) aortic stenosis. After 6 weeks, maximum myocardial perfusion was measured during adenosine infusion in the conscious state by the microsphere method. Sheep with aortic stenosis had less maximum coronary flow per gram, less conductance reserve, and thicker arteriolar walls in the LV and nonhypertrophied right ventricle. Capillary density decreased in the LV endomyocardium and remained unchanged in the right ventricle. Heparin-treated sheep had significant partial normalization of coronary conductance reserve and maximum perfusion in both ventricles and capillary density in the LV endomyocardium. Arteriolar wall thickness was unchanged. Compared with untreated sheep with aortic stenosis, in heparin-treated sheep LV FGF-2 protein increased 2-fold, whereas FGF-2 mRNA remained unchanged. VEGF mRNA and protein increased 3-fold and 1.4-fold, respectively, whereas TGF-β1 mRNA declined 3-fold.

Conclusions—Heparin administration during LV hypertension increases heparin-binding angiogenic factors FGF-2 and VEGF in the LV and ameliorates decreases in LV perfusion capacity and capillary density. (Circulation. 1999;100:981-987.)

Key Words: heparin ■ angiogenesis ■ growth substances ■ hypertrophy ■ hypertension ■ microcirculation

Abnormal myocardial perfusion capacity, intermittent ischemia, and secondary fibrosis occur with hypertension in adults and may contribute to its adverse consequences, including heart failure, arrhythmias, and sudden death. The limitation in coronary perfusion capacity in adults is due to left ventricular (LV) hypertrophy without proportionate microvascular growth and effects of hypertension on coronary structure and vasomotion. In contrast to adults, at a young age, hypertension results in coronary angiogenesis that is proportionate to LV hypertrophy and less impairment of coronary conductance and myocardial function. Developmentally regulated changes in heparin-binding and modifiable growth factors (eg, fibroblast growth factor [FGF]-2 and vascular endothelial growth factor [VEGF]) mediate perinatal physiological coronary angiogenesis and arterial adaptations to increasing pressure and may be responsible for the advantageous coronary adaptations exhibited with hypertension by the young.

Methods to promote coronary angiogenesis into ischemic myocardium by administration of FGF-2 or VEGF are receiving much attention. Methods to modulate coronary arterial wall thickening by inhibition of vascular smooth muscle transforming growth factor [TGF]-β, and FGF-2 are also being investigated.

Heparins may be useful to modify endogenous growth factors to favorably affect angiogenesis, vasodilation, and arterial smooth muscle proliferation processes that contribute to myocardial perfusion abnormalities with hypertension and ventricular hypertrophy. Heparin interacts with multiple heparin-binding growth factors and receptors that modulate angiogenesis, including FGF-2, VEGF, TGF-β1, and others in ways that promote angiogenesis.
TABLE 1. Hemodynamic Data

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart Rate, bpm</th>
<th>Cardiac Index, ml·min⁻¹·kg⁻¹</th>
<th>LV Peak Pressure, mm Hg</th>
<th>LV End-Diastolic Pressure, mm Hg</th>
<th>Aortic Peak Pressure, mm Hg</th>
<th>Aortic Diastolic Pressure, mm Hg</th>
<th>Pressure Gradient, mm Hg</th>
<th>Coronary Mean Flow Pressure, mm Hg</th>
<th>Diastolic Perfusion Pressure, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>100±9</td>
<td>130±21</td>
<td>121±5</td>
<td>8±1</td>
<td>121±6</td>
<td>91±4</td>
<td>0±1</td>
<td>104±5</td>
<td>80±4</td>
</tr>
<tr>
<td>LWH</td>
<td>101±8</td>
<td>119±19</td>
<td>188±9*</td>
<td>18±3*</td>
<td>121±8</td>
<td>92±4</td>
<td>65±5*</td>
<td>126±8</td>
<td>72±6</td>
</tr>
<tr>
<td>LWH+Heparin</td>
<td>92±5</td>
<td>114±21</td>
<td>189±7*</td>
<td>15±2*</td>
<td>110±4</td>
<td>84±4</td>
<td>73±6*</td>
<td>117±5</td>
<td>71±4</td>
</tr>
<tr>
<td>Adenosine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>139±10</td>
<td>191±26</td>
<td>98±4</td>
<td>9±2</td>
<td>92±4</td>
<td>40±5</td>
<td>5±4</td>
<td>60±4</td>
<td>27±4</td>
</tr>
<tr>
<td>LWH</td>
<td>150±9</td>
<td>212±25</td>
<td>193±11*</td>
<td>19±4</td>
<td>79±4</td>
<td>46±3</td>
<td>114±8*</td>
<td>100±6*</td>
<td>28±5</td>
</tr>
<tr>
<td>LWH+Heparin</td>
<td>149±11</td>
<td>281±42</td>
<td>200±12*</td>
<td>11±4</td>
<td>78±6</td>
<td>46±3</td>
<td>122±10*</td>
<td>103±6*</td>
<td>36±5</td>
</tr>
</tbody>
</table>

Abbreviations as in Figure 1.
*P<0.05 aortic stenosis.

Heparin-binding growth factors were purified from frozen LV with standard protein isolation techniques and heparin affinity chromatography. Total protein concentration in the elution was determined by Pierce Coomassie assay. ELISA was used to measure LV FGF-2 protein by use of a kit (R&D Systems). Samples in triplicate from each individual were measured together on multiwell plates. The assay was repeated. Similar ELISA assays for human VEGF and TGF-β had no cross-reactivity with sheep.

Western immunoblotting was used to measure VEGF protein. LV protein, recombinant VEGF (Santa Cruz Biotechnology), and molecular-weight protein standards (Bio Rad) were size-fractionated with SDS-PAGE (Bio-Rad Laboratories) according to the manufacturer’s recommendation. The proteins were electrophoretically trans-
ferred to PVDF membrane (Millipore) in a wet-transfer device (Bio Rad) according to the manufacturer’s instructions. Western blotting was done with primary antibody to VEGF (rabbit anti-human VEGF, Genentech) and Vectastain ABC-AP Kit (Vector Laboratories) according to the manufacturer’s instructions.

**RNA Extraction and Analysis**

Total RNA was extracted from the LV by the acid guanidinium thiocyanate–phenol-chloroform method, and Northern blot analysis was performed as previously described. The blots were hybridized with the following cDNAs labeled with \[^{32}P\]dCTP by the random priming method: (1) a 500-bp fragment of human FGF-2 (R&D Systems), (2) a 930-bp fragment of human VEGF (Genentech), (3) a 1.4-kb fragment encoding human TGF-\(\beta_1\) (Drs Bradley Arrick and Rik Derynck, Dartmouth Medical School, Hanover, NH), and (4) a 0.8-kb fragment of human GAPDH (Dr Constance Brinckerhoff, Dartmouth Medical School, Hanover, NH). Autoradiograms in the linear range were quantified by densitometry (Adobe Photoshop and Silverscreen or NIH Image). The data for each individual were normalized to the density of the corresponding signal for GAPDH.

**Statistical Analysis**

Results are expressed as mean±SEM. Statistical analysis of the effects of heparin on the responses to hypertension was done by multivariate ANOVA. The relationships of coronary conductance and capillary density to LV FGF-2, VEGF, and TGF-\(\beta_1\) were assessed by simple regression analysis. It is recognized that these relationships are biologically complex and interrelated and may not be linear.

**Results**

**LV Hypertrophy and Hemodynamics**

Aortic stenosis increased the ratio of LV to body weight by 42% in both untreated and heparin-treated sheep over sham sheep (2.7±0.1 versus 1.9±0.1, \(P<0.01\)). No RV hypertrophy occurred.

**Hemodynamic data** are shown in Table 1. The severity of LV hypertension was similar in heparin-treated and untreated sheep with aortic stenosis. Heparin-treated sheep had a statistically insignificant decrease in aortic pressure. Hemodynamic factors that may influence coronary flow were similar in heparin-treated and untreated aortic stenosis sheep.

**Myocardial Perfusion**

At rest, LV perfusion was similar in all groups (data not shown). Maximum coronary flow and conductance data are shown in Figures 1 and 2, respectively. LV coronary conductance reserve decreased with aortic stenosis. Heparin administration increased LV coronary conductance reserve 72% and maximum myocardial perfusion 38% over untreated sheep with aortic stenosis, but conductance remained less than in sham sheep.

**RV coronary conductance reserve** also decreased with aortic stenosis but was 59% greater in heparin-treated than untreated sheep. In heparin-treated sheep, maximum perfusion of both ventricles was similar to that in sham sheep.

**Arteriolar Wall Thickness and Lumen Size**

Morphometric data of arteriolar wall thickness and size in the LV are shown in Table 2 and Figure 3. Similar findings were present in the RV. Arteriolar relative wall thickness increased and mean lumen diameter decreased with aortic stenosis and were unaffected by heparin. The frequency distribution of arterioles of different sizes was similar in all groups (data not shown).

**Microvascular Density**

Capillary density data from 2 independent techniques are shown in Table 2. Capillary density decreased in the LV.
endothelium with aortic stenosis. Heparin ameliorated these effects; LV endothelial capillary density with the alkaline phosphate and silver stain techniques was 17% ($P=0.06$) and 26% higher ($P=0.05$), respectively, in heparin-treated sheep than in untreated aortic stenosis sheep. In the LV midwall and RV, no changes in capillary or arteriolar density were evident.

**LV FGF-2**

Data of LV FGF-2 protein levels are shown in Figure 4. The effects of aortic stenosis and heparin were significant and opposing. Untreated sheep with aortic stenosis had 71% less LV FGF-2 protein than sham sheep. Heparin-treated sheep had 2-fold greater LV FGF-2 protein than untreated sheep with aortic stenosis, but it remained 31% less than in sham sheep. The increase in LV FGF-2 protein in heparin-treated sheep was not associated with a detectable change in FGF-2 mRNA (Figure 5). In individual untreated and treated sheep with aortic stenosis, LV FGF-2 protein level correlated with LV endothelial capillary density (alkaline phosphatase method, $P<0.001$, $R^2=0.88$), maximum perfusion ($P<0.001$, $R^2=0.91$), and coronary reserve ($P<0.001$, $R^2=0.85$).

**LV VEGF**

Data on LV VEGF mRNA and protein levels are shown in Figures 5 and 6, respectively. Levels of VEGF mRNAs in the LV were similar in untreated aortic stenosis sheep and sham sheep. Heparin-treated sheep had 3-fold more VEGF mRNA than untreated aortic stenosis sheep. VEGF$_{165}$ protein was 51% greater in heparin-treated than untreated sheep with aortic stenosis and 213% greater than in sham sheep (effect of heparin, $P=0.06$). In sheep with aortic stenosis, LV VEGF$_{165}$ RNA correlated with LV endothelial capillary density ($P=0.006$, $R^2=0.74$), maximum perfusion ($P=0.004$, $R^2=0.78$), and coronary reserve ($P=0.001$, $R^2=0.84$).

**LV TGF-$$\beta_1$$**

Untreated sheep with aortic stenosis had a trend toward a mild increase in LV TGF-$\beta_1$ mRNA compared with sham sheep (Figure 5). In contrast, TGF-$\beta_1$ mRNA decreased 66% in heparin-treated sheep from the level in untreated aortic stenosis sheep. The level of LV TGF-$\beta_1$ RNA has a negative relationship with LV endothelial capillary density ($P=0.007$, $R^2=0.6$), maximum perfusion ($P=0.008$, $R^2=0.66$), and coronary reserve ($P=0.037$, $R^2=0.54$) in sheep with stenosis.

**Discussion**

This study demonstrates that administration of heparin to young adult sheep during development of LV hypertension and hypertrophy partially ameliorates the decrement in LV capillary density. These findings were seen with 2 independent methods in 2 sets of animals. The increase in capillary density in heparin-treated sheep most likely was from promotion of angiogenesis, although decreased rarefaction is

---

**TABLE 2. LV Microvascular Morphometrics**

<table>
<thead>
<tr>
<th>Group</th>
<th>Cap Density, Endo, AP, cap/mm$^2$</th>
<th>Cap Density, Endo, ST, cap/mm$^2$</th>
<th>Cap Density, Midwall, AP, cap/mm$^2$</th>
<th>Cap Density, Midwall, ST, cap/mm$^2$</th>
<th>Arteriolar Midwall X-Sec Density, arterioles/mm$^2$</th>
<th>Arteriolar Midwall Length Density, $\mu$m/mm$^2$</th>
<th>Arteriolar ID, $\mu$m</th>
<th>Arteriolar Wall Thickness, $\mu$m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1744±106</td>
<td>2438±316</td>
<td>1865±121</td>
<td>2059±194</td>
<td>3.1±0.7</td>
<td>8.8±2.0</td>
<td>14.1±1.8</td>
<td>3.8±0.6</td>
</tr>
<tr>
<td>LVH</td>
<td>1489±40</td>
<td>1646±101</td>
<td>1700±121</td>
<td>2094±178</td>
<td>3.1±0.7</td>
<td>7.7±0.9</td>
<td>9.9±1.1</td>
<td>4.1±0.3</td>
</tr>
<tr>
<td>LVH+Heparin</td>
<td>1735±95</td>
<td>2082±114</td>
<td>1923±96</td>
<td>2113±120</td>
<td>2.5±0.4</td>
<td>7.3±0.6</td>
<td>9.0±1.4</td>
<td>4.5±0.1</td>
</tr>
</tbody>
</table>

$P$-value, effect

- Hypertension 0.044 0.004 NS NS NS NS NS NS
- Heparin 0.063 0.05 NS NS NS NS NS NS

Cap indicates capillary; Endo, endomyocardium; AP, alkaline phosphatase stain for postarteriole capillaries; ST, silver stain technique for all capillaries; x-sec, cross-sectional; and ID, internal diameter. Other abbreviations as in Figure 1.
possible. This is consistent with previous studies demonstrating angiogenic effects of heparin that can accelerate coronary collateral growth. The changes occurred in the LV endomyocardium, where hypertrophy with hypertension occurs most intensely, and capillary density is most greatly influenced by alterations in angiogenesis. The increase in maximal flow and improved flow distribution with less myocardium per capillary can increase maximal myocardial oxygenation.

A second result shown here is that the increase in LV capillaries with heparin correlates with increases in LV FGF-2 and VEGF and decreased TGF-β1 mRNA. Previous studies have demonstrated that heparin increases the amount and angiogenic effect of FGF-2 and VEGF. Heparin increases FGF-2 by impeding its degradation and by releasing FGF from extracellular heparan sulfate–binding sites. In vitro, heparin increases coronary FGF-2 and FGF receptor-1 content and FGF-2 release. In addition, heparin can substitute for heparan sulfate as a required factor for FGF-2 receptor binding and may separately bind FGF to cells and facilitate internalization. VEGF also requires heparins for binding to cell receptors, and addition of exogenous heparin releases VEGF from extracellular heparin-like binding sites and increases its half-life and binding to endothelial receptors. Secondary interactions may contribute; FGF-2 upregulates VEGF mRNA. Therefore, a heparin-mediated increase in FGF-2 protein may increase VEGF mRNA. Together, FGF and VEGF synergistically accelerate angiogenesis. Both FGF and VEGF promote coronary angiogenesis in several species, including humans. In sheep, age-associated changes in cardiac FGF-2 and VEGF levels correlate with coronary angiogenesis with pressure-overload hypertrophy. In lambs, administration of protamine, which binds and reduces the amount of vascular heparins and inhibits FGF-2 and VEGF receptor binding, impedes age-associated coronary angiogenesis.

TGF-β has complex interactions with growth factors, angiogenesis, and vascular smooth muscle. Vascular hypertrophy and fibrosis with hypertension are mediated in part by TGF-β. Heparin modifies TGF-β metabolism. In addition, down-regulation of TGF-β with heparin could result from changes in FGF-2 (or other factors), modifying TGF-β expression.

These studies suggest that in this model, heparin may promote LV coronary capillary angiogenesis, at least in part, through actions that increase the amount of LV FGF-2 and VEGF and reducing TGF-β. To the best of our knowledge, the effects observed here of heparin on cardiac FGF-2 and on VEGF and TGF-β mRNA in vivo are novel.

The coronary angiogenic effects of heparin seen in this study may involve effects of heparin on angiogenic processes not examined here. These include heparin facilitation of...
FGF and VEGF receptor binding,\textsuperscript{11,12} upregulation of nitric oxide,\textsuperscript{8,15} and interference with inhibition of angiogenesis. Heparin blocks inactivation of FGF-2, VEGF, and TGF-\(\beta\) by \(\alpha\)-macroglobulin,\textsuperscript{8} interferes with endothelin and thrombospordin (which inhibit angiogenesis and promote vascular smooth muscle proliferation),\textsuperscript{8,14,15} and strongly binds angiostatin and endostatin, powerful inhibitors of angiogenesis.

A third observation of this study is that heparin appears to ameliorate the adverse effects of hypertension on coronary conductance and maximum perfusion. Coronary arteriolar wall thickness increased and lumen diameter and coronary conductance decreased in both ventricles with ascending aortic stenosis. This is consistent with well-described changes with hypertension of arterial wall thickening, lumen narrowing, abnormal endothelium, and abnormal vasomotion.\textsuperscript{1,4} In heparin-treated sheep, the decrement in coronary conductance with hypertension was less in both ventricles, implying an effect of heparin treatment on direct hypertension-induced changes in coronary vascular structure or vasomotion, separate from ventricular hypertrophy.

Heparin partially reverses pulmonary arterial thickening with hypertension in rats,\textsuperscript{18} perhaps through effects on FGF-2, thrombospordin, platelet-derived growth factor, TGF-\(\beta\), endothelin, and nitric oxide.\textsuperscript{8,9,14,15,19} Morphometric analysis in this study does not demonstrate changes in arteriolar structure that provide a basis for the ameliorating effect of heparin treatment on coronary conductance. The magnitude of heparin effects on coronary conductance was moderate and anticipated associated changes in coronary lumen size exponentially smaller and within the SDs of the data. However, no tendency for a decrease in coronary wall thickness in heparin-treated animals was observed.

Alternatively, heparin effects on coronary conductance could be from effects on hypertension-induced abnormalities in the coronary endothelium and vasomotion. Many heparin-modifiable factors, including FGF and VEGF, influence vasomotion.\textsuperscript{8,14,15,19,20} Heparin administration lowers systemic\textsuperscript{14} and pulmonary\textsuperscript{15} hypertension by regulating vascular nitric oxide, endothelin,\textsuperscript{14,15} and possibly other substances. Supportive effects of heparin on endothelial integrity,\textsuperscript{2,22} mediated by heparin-induced changes in vascular FGF,\textsuperscript{17,20,28} and VEGF,\textsuperscript{29} may ameliorate the adverse effects of barotrauma and improve arterial conductance.\textsuperscript{20,27,28} These effects were not directly examined in this study.

Important effects of the heparin type, dose schedule, amount, route, duration, and timing of onset of treatment were not addressed in this study. Although heparin administration after development of pulmonary hypertension does partially reverse vascular changes,\textsuperscript{18} it is unknown whether heparin would reverse changes in coronary conductance or induce coronary angiogenesis if given after hypertension is established. The persistence of effects is not clear; over longer time periods, the effects of heparin could lessen. Alternative routes of administration and types of heparin may circumvent anticoagulant effects and other disadvantages with subcutaneous heparin administration.

In conclusion, in this model, administration of heparin during LV hypertension appears to modulate the heparin-binding angiogenic factors FGF-2, VEGF, and TGF-\(\beta\) in the LV and ameliorate decreases in LV perfusion capacity and capillary density. To the best of our knowledge, this represents a novel in vivo model for therapeutic coronary angiogenesis through pharmacological manipulation of endogenous regulators of angiogenesis.

Acknowledgments
This study was supported by a Clinician Scientist Award (89-411) from the national chapter and a Grant-in-Aid from the national (93014180) and New Hampshire chapters (9407773S) of the American Heart Association; R&D Cardiovascular Kabi-Pharmacia, Stockholm, Sweden; and by the Victoria Cubberley Memorial Fund, South Acworth, NH. We thank Dr James E. Lock for support and guidance of this project, Yuri Aoyagi and Ervin Meluleni for technical assistance, and Nancy Peacock for secretarial assistance.

References


Effects of Chronic Heparin Administration on Coronary Vascular Adaptation to Hypertension and Ventricular Hypertrophy in Sheep

Circulation. 1999;100:981-987
doi: 10.1161/01.CIR.100.9.981
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/100/9/981

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/