Vascular Endothelial Growth Factor Blockade Prevents the Beneficial Effects of β-Blocker Therapy on Cardiac Function, Angiogenesis, and Remodeling in Heart Failure

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Background—Impaired angiogenesis in the post-myocardial infarction heart contributes to the progression to heart failure. The inhibition of vascular endothelial growth factor (VEGF) signaling has been shown to be crucial for the transition from compensatory hypertrophy to cardiac failure. Importantly, β-adrenergic receptor blocker therapy has been also shown to improve myocardial perfusion by enhancing neoangiogenesis in the failing heart.

Methods and Results—Eight weeks from surgically induced myocardial infarction, heart failure rats were randomized to receive bisoprolol (B) or vehicle. At the end of a 10-week treatment period, echocardiography revealed reduced cardiac diameters and improved cardiac function in B-treated compared with vehicle-treated rats. Moreover, B treatment was associated with increased cardiac angiogenesis and in vivo coronary perfusion and reduced cardiac fibrosis. Importantly, 2 weeks after B treatment was started, increased cardiac VEGF expression and Akt and endothelial NO synthase activation were observed by comparing B-treated with drug-untreated failing hearts. To test whether the proangiogenic effects of B act via activation of VEGF pathway, rats were intravenously injected with adenoviral vector encoding a decoy VEGF receptor (Ad-Flk) or a control adenovirus (Ad-C), at the start of the treatment with B. After 10 weeks, histological analysis revealed reduced capillary and coronary perfusion in B-treated plus Ad-Flk rats compared with B-treated plus Ad-C rats. Moreover, VEGF inhibition counteracted the positive effects of B on cardiac function and remodeling.

Conclusions—β-Blockade promotes cardiac angiogenesis in heart failure via activation of VEGF signaling pathway. β-Blocker–induced enhancement of cardiac angiogenesis is essential for the favorable effects of this therapy on cardiac function and remodeling. (Circ Heart Fail. 2013;6:1259-1267.)

Key Words: adrenergic β-1 receptor antagonists ■ angiogenesis ■ heart failure ■ vascular endothelial growth factor A

Heart failure (HF) is currently one of the leading causes of morbidity and mortality worldwide, with myocardial infarction (MI) being the most common cause.1,2 The loss of cardiac function after acute MI drives specific cardiac remodeling and hypertrophy processes, aiming at preserving cardiac output.3 However, adequate growth of capillaries and arterioles is necessary to support muscle growth in the surviving myocardium.4 Several lines of evidence have proven that angiogenesis is inadequate in the failing heart, thus contributing to maladaptive left ventricular (LV) remodeling and promoting the transition from adaptive cardiac hypertrophy to LV dilation and dysfunction.5,6 Thus, stimulation of cardiac angiogenesis is considered a promising tool in post-MI therapy.

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During the past decades, large bodies of evidence have shown that β-blocker therapy reduces HF-related morbidity and mortality.7,8 Overall, the success of β-blockers in HF...
treatment is attributed, at least in part, to their ability to block the continuous increased adrenergic overdrive present in the failing human heart.7–9 Moreover, β-adrenergic receptor (βAR) blockade has been proven to exert several additional therapeutic effects, including reduced oxygen consumption, improved cardiac reverse remodeling, blunted apoptosis, inhibited βAR internalization, and reduced risk of arrhythmias.9–12 Importantly, β-blocker therapy has been also shown to improve myocardial perfusion by enhancing neoangiogenesis in the failing heart.13–15 This latter phenomenon seems to be related to β-blocker–dependent heart rate reduction (HRR) that has been demonstrated to enhance coronary reserve as well as capillary and arteriolar growth in normal16 and in the post-MI hearts.17,18 Moreover, it has been demonstrated that HRR is able to activate vascular endothelial growth factor (VEGF)–dependent angiogenic pathway.16

However, to the best of our knowledge, there are no studies investigating the effects of β-blocker therapy on VEGF proangiogenic signaling pathway in HF. Thus, in an experimental model of chronic HF, we demonstrate that bisoprolol (B) treatment induces cardiac VEGF upregulation and that prevention of β-blocker–dependent VEGF induction abrogates the proangiogenic effect of β-blocker. Moreover, we prove that angiogenesis induced by β-blocker plays a crucial role to avoid the transition from compensatory to maladaptive hypertrophy, thus representing an essential mechanism for the therapeutic effects of β-blocker.

Methods

Experimental Groups and Pharmacological Treatment Protocols

Seventy-nine Sprague-Dawley male rats (300 g) entered the study: sham-operated (n=12) and rats with surgically induced MI (n=67) by permanent ligation of the left anterior descending coronary artery as previously described.19 At 8 weeks post-MI, HF rats were randomized to the following treatment groups: (1) placebo (drinking water; n=10); (2) B (10 mg/kg per day in drinking water; n=12); (3) placebo plus intravenous injection of Adenovirus (Ad) vectors encoding Flk1-Fc (AdFlk), a potent angiogenesis inhibitor that acts as a decoy VEGF receptor20 (n=10); (4) placebo plus intravenous injection of Ad encoding for the control Fc fragment (AdCTR)19 (n=10); (5) B plus intravenous injection of AdFlk (n=13); and (6) B plus intravenous injection of Ad-control (n=12). We injected 4×10¹⁰ plaque-forming units of AdFlk or AdCTR into the jugular vein of rats at 8 weeks post-MI (when also B treatment was started). Treatment period was of 10 weeks for all groups. All animal care and experimental protocols were approved by the Ethics Committee for the Use of Animals in Research of our institution.

Echocardiography

Echocardiography was performed 8 weeks after surgery (after randomization to treatments) and repeated at the end of the study (18 weeks after MI) in anesthetized (1.5% isoflurane; v/v) rats with a Vevo770 (VisualSonics) echocardiograph, as previously described.21

Myocardial Perfusion Studies

Myocardial perfusion was determined using 15 μm fluorescent microspheres (Triton Inc.), as previously described.22 Cardiac and blood samples were processed for microspheres determination. Total myocardial blood flow and coronary conductance (coronary blood flow normalized by corresponding perfusion pressure) were measured at basal condition and after maximal coronary dilation by dipyridamole (6 mg/kg per minute IV). Coronary flow reserve was calculated as maximal coronary conductance divided by basal coronary conductance.

Measurement of Infarct Size

Infarct size was examined in all experimental groups at the end of the study period. Briefly, hearts were frozen in liquid nitrogen and sectioned from apex to base into 2-mm slices. To delineate the infarct size, sections were incubated in 1% (wt/vol) triphenyltetrazolium chloride (Sigma) in PBS (pH, 7.4) at room temperature for 15 minutes. For each section, the infarct size of the LV was calculated from enlarged digital photos using SigmaScan version 5.0 software, as described previously.23

RNA Isolation and Real-Time Reverse Transcription Polymerase Chain Reaction

Cardiac total RNA isolations, reverse transcription to cDNA, and quantitative real-time reverse transcription polymerase chain reaction were performed as previously described.24

Histology

Capillary density and arteriolar length density were measured as previously described.23 Briefly, LV specimens were fixed in 4% formaldehyde and embedded in paraffin. After deparaffinization and rehydration, 4-μm-thick sections were prepared and mounted on glass slides. Capillary density and arteriolar length density were evaluated in 5 randomly selected LV sections in either anterior or lateral wall (border zones) at ≈1 mm from the edge of scar tissue and in the lateral wall, far from the infarcted area (remote). Capillaries (5–10 μm thick) were detected by Lectin Bandeiraea simplicifolia I staining. Arterioles (<50 μm thick) were identified by immunofluorescence using anti-smooth muscle α-actin antibody as previously described.25 Arteriolar length density was calculated with the following formula: Length density (mm/mm²)=s(2a/b)/N/N/A, where a and b represent long and short axes, respectively, of individual arterioles, N is total number of arteriolar profiles, and A is the total area in which arterioles were measured.

Cardiac fibrosis has been evaluated by picro-sirius staining. Briefly, after deparaffinization and rehydration, 4-μm-thick sections were prepared, mounted on glass slides and stained with 1% Sirius red in picric acid (Carlo Erba Laboratories, Italy) to detect interstitial fibrosis. All the sections were examined with a microscope (Leitz, DIAPLAN), and images were acquired with a digital camera (Digital JVC, TK-C1380).

Cardiomyocyte Size Measurement

Cardiomyocyte surface area was determined from sections of the LV myocardium from hearts of all study groups. Sections were stained with wheat germ agglutinin coupled to Alexa Flour 488 (1:100; Invitrogen, W11261). Surface areas of cardiomyocytes were measured in 10 randomly selected fields from each individual heart sample (5 hearts per group) using Software ImageJ.

Immunoblotting and VEGF/Akt/Endothelial NO Synthase Measurement

LV samples were lysed in an radio immunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors cocktail (Roche), Measurements of cardiac VEGF, Akt, serin473-phospho-Akt (pAkt), endothelial NO synthase (eNOS), and serin1177-phospho-eNOS (p-eNOS) protein levels were performed using specific primary antibodies (Ab-VEGF, Santacruz; Ab-Akt, Santacruz; Ab-pAkt, Upstate; Ab-eNOS, Upstate; Ab p-eNOS, Upstate). Secondary antibodies were purchased from Immunoreagent Inc. Bands were visualized by enhanced chemiluminescence (Millipore) according to the manufacturer’s instructions and were quantified using densitometry (Chemidoc, Biorad). Each experiment and densitometric quantification was separately repeated 2–3 times.
Results
Effects of B on In Vivo Cardiac Function
Echocardiography performed 8 weeks after MI induction revealed that LV ejection fraction and internal diameter at diastole were not statistically different among HF groups before placebo or B treatment initiation (Figure I in the online-only Data Supplement). Ejection fraction was significantly decreased, and LV diastolic diameter was significantly increased in both HF groups compared with sham, demonstrating a similar degree of HF (Figure I in the online-only Data Supplement). At the end of the study period (18 weeks post-MI), the 2 HF groups still had worse cardiac function compared with sham (Figure 1A and Table 1). As expected, although no differences in infarct size were observed among HF groups (Table 1), 10 weeks of B treatment positively affected both cardiac contractility and LV geometry compared with HF control group (Figure 1B and Table 1). Importantly, HF B-treated rats showed a significant reduction in HR compared with sham and HF control groups (Figure 1C). The heart weight/body weight ratio was significantly increased in control HF group compared with sham rats, consistent with an HF phenotype; importantly, B treatment resulted in a reduction of heart weight/body weight ratio compared with HF control (Table 1).

Table 1. Physical and Echocardiographic Data of Sham-Operated and HF Rats at the End of the Study Period

<table>
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<th>Sham</th>
<th>HF/Control</th>
<th>HF/Bisoprolol</th>
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<td>0.469±0.014</td>
<td>0.454±0.015</td>
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<td>HW, g</td>
<td>1.16±0.04</td>
<td>1.41±0.01*</td>
<td>1.24±0.03*†</td>
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<td>HW/BW, g/kg</td>
<td>2.48±0.08</td>
<td>3.01±0.09*</td>
<td>2.73±0.08*†</td>
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<tr>
<td>Echocardiography</td>
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<td></td>
</tr>
<tr>
<td>HR, beats per minute</td>
<td>321.5±15.9</td>
<td>324.8±7.1</td>
<td>268.8±7.7**</td>
</tr>
<tr>
<td>LVEF, %</td>
<td>64.8±0.7</td>
<td>27.9±1.9*</td>
<td>36.7±1.4†</td>
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<td>LVIDd, mm</td>
<td>8.5±0.2</td>
<td>11.0±0.4*</td>
<td>9.9±0.2†</td>
</tr>
<tr>
<td>LVIDs, mm</td>
<td>5.4±0.1</td>
<td>9.4±0.4*</td>
<td>8.1±0.2†</td>
</tr>
<tr>
<td>LVAWd, mm</td>
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<td>1.54±0.10*</td>
<td>1.46±0.07*</td>
</tr>
<tr>
<td>LVPWd, mm</td>
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<td>2.22±0.08†</td>
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<tr>
<td>Infarct size, %</td>
<td>…</td>
<td>46.3±4.2</td>
<td>44.8±3.1</td>
</tr>
</tbody>
</table>

ANOVA analysis and Bonferroni test were used among all 3 groups. Data are presented as mean±SEM. BW indicates body weight; HF, heart failure; HR, heart rate; HW, heart weight; LVAWd, LV anterior wall diameter at diastole; LVEF, left ventricular ejection fraction; LVIDd, LV internal diameter at diastole; LVIDs, LVID at systole; and LVPWd, LV posterior wall diameter at diastole.

*P<0.05 vs sham; †P<0.05 vs HF/control, **P<0.05 vs sham and HF/control.
Effects of β-Blocker Therapy on Cardiac Remodeling Gene Profile

At the end of the study period, we evaluated cardiac gene expression patterns related to ventricular remodeling in our experimental groups. As a marker of HF, we investigated expression of the mRNA for brain natriuretic peptide in the LV and found this to be significantly increased in the HF control group, whereas in B-treated animals we observed values similar to sham (Figure 1D). We further examined, as markers of remodeling and fibrosis, cardiac mRNA levels of collagen type I and transforming growth factor-β1 (Figure 1E and 1F) and found mRNA levels of both of these markers significantly elevated in HF control rats compared with sham controls; but both were markedly reduced in the B-treated HF rats. Consistently, picro-sirius red staining for cardiac fibrosis performed at the end of the study period showed markedly increased fibrosis in HF control rat hearts compared with B-treated rat hearts. As expected, no fibrosis was detectable in sham-operated rat hearts (Figure 1G). Of note, cardiomyocytes surface area was increased in HF control group compared with sham, as expected. Importantly, B treatment resulted in a slight but significant increase in cardiomyocytes size compared with HF control (Figure IIA in the online-only Data Supplement).

Effects of B on Cardiac Angiogenesis and Perfusion

As expected, HF control rats showed a marked capillary density rarefaction in both LV border and remote zones compared with sham (Figure 2A). Ten weeks of B treatment resulted in a significant increase of capillary density compared with HF controls (Figure 2A). Consistent with capillary density data, arteriolar length density was dramatically reduced in the LV border and remote zones in HF control group compared with sham (Figure 2A, Figure IIIA in the online-only Data Supplement). Importantly, myocardial blood flow and coronary conductance were significantly reduced after maximal vasodilation in HF control group compared with sham rats (Figure 2B and 2C and Figure 2A, Effects of bisoprolol (B) on cardiac capillary and arteriolar network. Left, Representative images of Lectin Bandeiraea simplicifolia I (BS-I) staining of capillaries and of arterioles stained with antibodies against smooth muscle α-actinin in cardiac section obtained from sham, heart failure (HF) rats, and HF rats treated with B (HF/B) at the end of the study period in the lateral wall far from the infarcted area (remote; magnification ×200; bar=50 μm). Right, Bar graphs show data on capillary counts (capillary to myocytes ratio) and arteriolar length density in either left ventricular border or remote zones in all study groups at the end of the study period (n=5 rats per group and 5 sections per animal). Total myocardial blood flow (MBF; B), coronary conductance (C), and coronary flow reserve (CFR; D) in sham, HF, and HF/B rats at the end of the study period (n=10 rats per group). Cardiac protein expression of (E) vascular endothelial growth factor (VEGF), (F) Akt and serin473-phospho(p)-Akt, and (G) endothelial NO synthase (eNOS) and Ser1177-phospho(p)-eNOS in sham, HF, and HF/B hearts at the end of the study period. The expression of glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize VEGF protein levels. pAkt to total-Akt (tAkt) ratio and p-eNOS to eNOS ratio indicated, respectively, the levels of Akt and eNOS phosphorylation in the heart (n=5 hearts per group). Data are presented as mean±SEM. *P<0.05 vs sham; #P<0.05 vs HF. One-way ANOVA analysis with Bonferroni correction in D to G and repeated measures ANOVA with Bonferroni correction in A to C.
Table I in the online-only Data Supplement). B improved myocardial perfusion and reduced coronary vascular resistances in HF/B rats compared with HF control. Accordingly, coronary reserve, which showed a ≈3-fold decrease in HF control rats compared with sham, significantly increased after β-blocker treatment, although final values remained still lower than in sham (Figure 2D and Table I in the online-only Data Supplement).

Effects of B on Cardiac VEGF/Akt/eNOS Pathway

Interestingly, 2 weeks after the start of treatments, in HF control hearts we observed a significant reduction of cardiac VEGF protein levels compared with sham. This finding was associated to a reduced Akt activation and eNOS phosphorylation compared with sham, as reflected by phospho-Akt/total-Akt (pAkt/Akt) and peNOS/teNOS ratios, respectively. Surprisingly, 2 weeks of B treatment induced an increase of cardiac VEGF protein expression, associated to Akt activation and eNOS phosphorylation, even at higher values to those observed in sham hearts (Figure 2E–2G).

Effects of VEGF Inhibition on B-Induced Improvement of Cardiac Function

To test the in vivo pathophysiological relevance of B-dependent cardiac VEGF upregulation and Akt and eNOS activation on cardiac function, angiogenesis, and remodeling, HF rats at 8 weeks post-MI were treated with placebo or B and were also injected intravenously with an adenoviral vector encoding for the ligand-binding domain of VEGF receptor 2 (Flk1) fused to murine IgG2a Fc (Ad-Flk), a potent VEGF inhibitor, or with a control adenovirus that encodes only for the murine IgG2a Fc (Ad-CTR).18 Thus, we obtain 4 study groups: (1) HF rats treated with placebo and injected with Ad-CTR (HF/AdCTR); (2) HF rats treated with placebo and injected with Ad-Flk (HF/AdFlk); (3) HF rats treated with B and injected with Ad-CTR (HF/B/AdCTR); (4) HF rats treated with B and injected with Ad-Flk (HF/B/AdFlk). Importantly, no statistical differences have been found between HF rats treated with placebo and HF/AdCTR rats, as well as between HF rats treated with B and HF/B/AdCTR rats, for all echocardiographic, histological, and molecular parameters. This observation indicates that, as expected, Ad-CTR does not affect cardiac function, remodeling, and angiogenesis. At the end of the study period (10 weeks of treatment), cardiac function has been assessed by echocardiography in all 4 study groups. As expected, in HF/B/AdCTR rats we observed a significant reduction of LV internal diameter and a significant increase of LV ejection fraction percentage compared with HF/AdCTR (Figure 3A). HF/AdFlk showed similar levels of both LV dilation and contractility compared with HF/AdCTR. Surprisingly, the positive effects of B on cardiac function and remodeling were completely abolished when VEGF was inhibited. In fact, HF/B/AdFlk rats showed an increased LV internal diameter and decreased LV ejection fraction when compared with HF/B/AdCTR, at values that were similar to those measured in HF/AdCTR (Figure 3B). Importantly, no differences in infarct size were observed among all 4 HF groups (Table 2). As expected, HF/B/AdCTR and HF/B/AdFlk rats showed similar reduction in HR compared with groups not treated with B (Figure 3C), demonstrating that Ad-Flk administration did not affect B-dependent HRR. Consistently, heart weight/body weight ratio was similar between HF groups, with the only exception of HF/B/AdCTR group in which it was significantly lower (Table 2).

Effects of VEGF Inhibition on B-Dependent Amelioration of Cardiac Remodeling Gene Profile

At the end of the study period, in HF/B/AdCTR hearts we observed a marked reduction of brain natriuretic peptide, collagen type I, and transforming growth factor-β1 mRNAs compared with HF/AdCTR, as assessed by reverse transcription polymerase chain reaction (Figure 3D–3F). In HF/AdFlk rats, cardiac levels of these gene expression patterns were similar to those observed in HF/AdCTR. Importantly, when VEGF was inhibited, B completely failed to reduce the mRNA levels of all the 3 genes investigated, with levels indistinguishable from those observed in rats not treated with β-blocker (Figure 3D–3F). Consistently, we observed a significant reduction of fibrosis in HF/B/AdCTR hearts compared with HF/AdCTR (Figure 3). In HF/AdFlk rats, cardiac fibrosis was similar to that observed in HF/AdCTR rats. More important, when B treatment was associated to VEGF inhibition, the ability of β-blocker to reduce cardiac fibrosis was completely lost, with levels of cardiac fibrosis similar to those observed in rats not treated with B (Figure 3G). Of note, B treatment in HF/B/AdCRT group resulted in an slight but significant increase in cardiomyocytes size compared with HF/AdCTR group (Figure IIB in the online-only Data Supplement). Importantly, when B treatment was associated to VEGF inhibition, the ability of β-blocker to increase cardiomyocytes surface area was completely prevented (Figure IIB in the online-only Data Supplement).

Effects of VEGF Inhibition on B-Induced Cardiac Angiogenesis

After 10 weeks of treatment, capillary density and arteriolar length density were increased in HF/B/AdCTR hearts compared with HF/AdCTR both in the remote and border zones (Figure 4A, Figure IIB in the online-only Data Supplement). Consistently, we observed a parallel increase in myocardial blood flow (after maximal vasodilation), coronary conductance (after maximal vasodilation), and coronary flow reserve in HF/B/AdCTR hearts compared with HF/AdCTR (Figure 4B–4D and Table II in the online-only Data Supplement). Capillary density, arteriolar length density, and all parameters of in vivo myocardial perfusion were similar in HF/AdFlk and HF/AdCTR (Figure 2A–2D, Table II in the online-only Data Supplement), proving that VEGF inhibition is not able to reduce further capillary density and myocardial perfusion in HF rats. Importantly, VEGF inhibition was able to abolish the proangiogenic effect of β-blocker therapy completely; in fact, HF/B/AdFlk hearts showed significant reduction of capillary density, arteriolar length density, and in vivo cardiac perfusion compared with HF/B/AdCTR, at levels similar to HF/AdCTR hearts (Figure 2A–2D, Table II in the online-only Data Supplement).
Effects of VEGF Inhibition on Cardiac VEGF/Akt/eNOS Pathway

To test the effects of VEGF inhibition on β-dependent activation of VEGF/Akt/eNOS pathway that we observed after 2 weeks from treatment initiation, we measured the levels of those proteins at the same time point in all 4 study groups. In HF/B/AdCTR rats, we observed a robust increase of cardiac VEGF protein expression, associated to higher Akt and eNOS phosphorylation levels compared with HF/AdCTR hearts, confirming the ability of β-blocker to increase the activation of this pathway (Figure 4D–4F). In rat hearts treated with placebo plus VEGF inhibition, we observed increased VEGF protein expression comparable with that measured in rats treated only with B, but Akt and eNOS activation levels were similar to those observed in rats treated with placebo (Figure 4E–4G). Of note, in line with histological and in vivo perfusion data, VEGF inhibition completely prevented β-dependent Akt and eNOS activation. In particular, in HF/B/AdFlk hearts, although VEGF protein levels increased similar to HF/B/AdCTR group, pAkt/Akt and peNOS/eNOS ratios were significantly lower to that observed in HF/B/AdCTR (Figure 4E–4G).

Discussion

The most significant finding of the present study is that β-blocker favorably affects performance and remodeling of the failing heart through reactivation of angiogenesis. We show that the effects of β-blocker treatment on cardiac angiogenesis and remodeling in HF are dependent on the activation of VEGF proangiogenic pathway. Indeed, we provide evidence that in HF rats β1AR blockade is able to: (1) prevent HF-related adverse remodeling; (2) improve cardiac angiogenesis and perfusion; and (3) activate the VEGF/Akt/eNOS signaling pathway. More importantly, this is the first report showing that the inhibition of VEGF is able to prevent all the favorable effects induced by β-blocker treatment on cardiac function and structure in HF rats.

It is well established that angiogenesis in the post-MI heart is necessary because cardiomyocytes undergo compensatory hypertrophy to counterbalance for muscle loss in the infarcted region.43 Moreover, disruption of co-ordinated tissue growth and angiogenesis in the heart contributes to the progression from adaptive cardiac hypertrophy to HF.44 Thus, many efforts have been done to potentiate neoangiogenesis in HF.
Table 2. Physical and Echocardiographic Data of HF Rats at the End of the Study Period

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<td>HR, beats per minute</td>
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<td>274.0±4.1†</td>
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<td>LVEF, %</td>
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</table>

ANOVA analysis and Bonferroni test were used among all 4 groups. Data are presented as mean±SEM. BW indicates body weight; HF, heart failure; HF/AdCTR, HF rats treated with placebo and injected with Ad-CTR; HF/AdFlk, HF rats treated with placebo and injected with Ad-Flk; HF/B/AdCTR, HF rats treated with bisoprolol (B) and injected with Ad-CTR; HF/B/AdFlk, HF rats treated with B and injected with Ad-Flk; HR, heart rate; HW, heart weight; LVEF, left ventricular ejection fraction; LVAWd, LV anterior wall diameter at diastole; LVPWd, LV posterior wall diameter at diastole. *P<0.05 vs all other groups; †P<0.05 vs HF/AdCTR and Ad/AdFlk.

Previous studies have demonstrated that bradycardia enhances coronary reserve, capillary density, and arteriolar growth in the postinfarcted heart and that bradycardia-induced cardiac angiogenesis is dependent on VEGF. It has been hypothesized that HRR, prolonging diastole, is able to enhance diastolic filling and stretch myocytes and capillaries, thus increasing cardiac VEGF expression. Here, we report that activation of VEGF proangiogenic pathway underlies B-induced angiogenesis in HF. Of course, β-blockers have negative chronotropic effects, and previous reports have shown that these drugs are able to increase cardiac angiogenesis in post-MI animal models, thus we cannot exclude that in our study increased angiogenesis in B-treated animals is mainly dependent on HRR induced by this drug. Thus, whether bradycardia or other biological effects of β-blockers are detrimental for the induction of VEGF-dependent angiogenesis is still unclear. Future studies will be needed to evaluate separately HR-dependent and HR-independent effects of β-blockers treatment on VEGF-dependent angiogenesis and subsequent restoration of cardiac function and remodeling. However, we offer the first demonstration that B is able to activate the VEGF/Akt/eNOS signaling pathway in HF, and to test the pathophysiological relevance of this mechanism, we administered HF B-treated rats with an adenoviral vector encoding for a VEGF decoy receptor (AdFlk), that is known to act as a potent VEGF inhibitor. Surprisingly, we found that VEGF inhibition prevented not only B-induced cardiac angiogenesis, but, unexpectedly, it also neutralized the favorable effects of β-blocker therapy on cardiac function and remodeling. Indeed, HF rats treated with B and VEGF inhibition showed no significant differences compared with HF/AdCTR rats in terms of cardiac chamber dilation and contractility, as well as for cardiac remodeling genes expression, indicating that B-induced VEGF upregulation is essential in co-ordinating adequate vascular and myocardial growth in post-MI failing hearts. Moreover, our data indicate that 10 weeks of B treatment resulted in a slight but significant increase in cardiomyocytes size compared with HF control group. This finding is in line with the proangiogenic effect of β-blocker treatment, which is known to be essential to support co-ordinated vessel and cardiomyocytes growth. The discrepancy between decreased heart weight/body weight ratio and increased cardiomyocytes size observed in HF rats treated with β-blocker compared with HF control rats could be explainable by the increased cardiac size (LV chamber dilation) and fibrosis observed in HF control compared with HF/B group. Importantly, the effects of B treatment on cardiomyocytes size is in line with the echocardiographic data showing that posterior wall thickness is increased in hearts treated with β-blocker compared with control. Taken together, these results support the notion that the imbalance between myocyte growth and coronary angiogenesis plays a critical role in cardiac function and angiogenesis-dependent activation of cardiac angiogenesis is a crucial part of the mechanism of action of this drug class.

Moreover, our study is consistent with previous observations from our group and others that have identified in VEGF/Akt/eNOS pathway reactivation a potential target to promote co-ordinated angiogenesis in HF. Previously, we have shown that exercise training, independently from HRR, is able to induce VEGF upregulation and Akt activation in the postischemic heart and to improve age-dependent VEGF down-regulation and angiogenesis responses to hindlimb ischemia. Moreover, β2AR overexpression in the failing heart is able to enhance neoangiogenesis, inducing a sustained and co-ordinated Akt and VEGF activation. The involvement of β2AR in the neoangiogenic processes in response to ischemia prompted us to use in this study a selective β1AR blocker, in order to not interfere with β2AR. However, further studies are needed to demonstrate potential differences in proangiogenic properties between unselective β-blockers and selective β1AR blockers. Notably, in the present study, VEGF/Akt/eNOS pathway was strongly suppressed in HF control rats compared with sham animals at 10 weeks post-MI. These molecular changes were associated with maladaptive cardiac remodeling and severe cardiac dysfunction. Importantly, in our model, we demonstrate that B-dependent VEGF/Akt/eNOS pathway activation, which occurs within 2 weeks from treatment initiation, is crucial for the proangiogenic effects of β-blocker treatment. Moreover, we think that the switch on of this pathway promotes growth of new vessels and thus the transition from a maladaptive to an adaptive, angiogenesis-dependent LV remodeling, which attenuates the negative LV remodeling observed in placebo-treated animals late from MI.

In rat hearts treated with placebo plus VEGF inhibition, we observed increased VEGF protein expression comparable with that measured in rats treated with B, but Akt and eNOS activation levels were similar to those observed in rats treated with placebo (Figure 4). This apparent inconsistency is explainable by considering the mechanism of action of Flk that acting as a
decoy for VEGF might induce a rebound endogenous hyper-production of VEGF that is not able to activate VEGF receptors, as also observed by others.18,27

Interestingly, we found that AdFlk-mediated VEGF inhibition at 10 weeks post-MI, a time point when HF is established, is not able to further reduce neither Akt nor eNOS activation compared with HF control rats. This is probably attributable to the fact that the inhibition was induced late from MI, a time point far from the ischemic injury, when VEGF expression and Akt and eNOS activation were almost completely suppressed, at levels even lower than those observed in noninfarcted hearts. Consequently, in our study, injection of AdFlk in placebo-treated rats was not able to worsen cardiac function, angiogenesis, and remodeling when compared with placebo-treated rats injected with AdCTR.

Conclusions
The present study demonstrates that the proangiogenic properties of βAR antagonists are dependent to the reactivation of the VEGF pathway. Prevention of β-blocker–dependent VEGF upregulation in HF results in reduction of cardiac capillary and arteriolar density and avoids all the beneficial effects of this therapy on cardiac function and remodeling. Thus β-blocker–induced enhancement of cardiac angiogenesis is essential for the therapeutic effects of β-blockers in HF.

Sources of Funding
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Disclosures
None.

References

Figure 4. A, Effects of bisoprolol (B) and vascular endothelial growth factor (VEGF) inhibition on cardiac capillary and arteriolar network. Left, Representative images of Lectin Bandeiraea simplicifolia I (BS-I) staining of capillaries and of arterioles stained with antibodies against smooth muscle α-actinin in cardiac section obtained from all 4 heart failure (HF) experimental groups at the end of the study period in the lateral wall far from the infarcted area (remote; magnification ×200; bar=50 μm). Right, Bar graphs show data on capillary counts (capillary to myocytes ratio) and arteriolar length density in either left ventricular border or remote zones in all 4 HF study groups at the end of the study period (n=5 rats per group and 5 sections per animal). Total myocardial blood flow (MBF; B), coronary conductance (C), and coronary flow reserve (CFR; D) in all 4 HF experimental groups at the end of the study period (n=10 rats per group). Cardiac protein expression of (E) VEGF, (F) Akt and serine473-phospho(p)-Akt, and (G) endothelial NO synthase (eNOS) and Ser1177-phospho(p)-eNOS in all 4 groups at the end of the study period. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize VEGF protein levels. pAkt to total-Akt (tAkt) ratio and p-eNOS to eNOS ratio indicated, respectively, the levels of Akt and eNOS phosphorylation in the heart (n=5 hearts per group). Data are presented as mean±SEM. *P<0.05 vs all other HF groups. One-way ANOVA analysis with Bonferroni correction in D to G and repeated measures ANOVA with Bonferroni correction in A to C. HF/AdCTR indicates HF rats treated with placebo and injected with Ad-CTR; HF/AdFlk, HF rats treated with placebo and injected with Ad-Flk; HF/B/AdCTR, HF rats treated with B and injected with Ad-CTR; and HF/B/AdFlk, HF rats treated with B and injected with Ad-Flk.
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Undergoing antiangiogenic therapies. Beta-blocker are reduced in patients with heart failure. Beta-blocker in heart failure. Beta-blocker–induced stimulation of cardiac angiogenesis represents a relevant therapeutic effect of vascular endothelial growth factor inhibition, avoids all the beneficial effects of beta-blocker–dependent angiogenesis, via to support co-ordinated tissue growth and angiogenesis in the heart, thus preventing the progression from adaptive cardiac hypertrophy to mal-adaptive left ventricular remodeling. In this context, the present study demonstrates for the first time that beta-blocker therapy is able to activate the vascular endothelial growth factor/Akt/endothelial NO synthase signaling pathway, that is necessary to support co-ordinated tissue growth and angiogenesis in the heart, thus preventing the progression from adaptive cardiac hypertrophy to left ventricular dilation and dysfunction. Importantly, prevention of beta-blocker–dependent angiogenesis, via vascular endothelial growth factor inhibition, avoids all the beneficial effects of beta-blocker therapy on cardiac function and structure and results in reduction of cardiac vascular network and myocardial perfusion. These results indicate that beta-blocker–induced stimulation of cardiac angiogenesis represents a relevant therapeutic effect of beta-blockers in heart failure and open a new scenario for future studies testing whether the benefits of beta-blocker are reduced in patients with heart failure undergoing antiangiogenic therapies.

**CLINICAL PERSPECTIVE**

Heart failure represents one of the leading causes of mortality worldwide. Beta-blocker therapy represents one of the mainstay of therapeutic armamentarium for its ability to improve survival and to reduce hospitalization in patients with heart failure. This class of drug is known to protect the failing heart from the noxious effects of increased adrenergic drive, but the precise mechanisms by which beta-blocker confers cardioprotection are not completely elucidated. Importantly, beta-blocker therapy has also been shown to improve cardiac angiogenesis and myocardial perfusion, and this latter effect represents a key step to support the adequate growth of vascular network and to permit cardiomyocytes growth in the failing myocardium. In fact, angiogenesis is inadequate in the failing heart, thus contributing to the transition from adaptive cardiac hypertrophy to mal-adaptive left ventricular remodeling. In this context, the present study demonstrates for the first time that beta-blocker therapy is able to activate the vascular endothelial growth factor/Akt/endothelial NO synthase signaling pathway, that is necessary to support co-ordinated tissue growth and angiogenesis in the heart, thus preventing the progression from adaptive cardiac hypertrophy to left ventricular dilation and dysfunction. Importantly, prevention of beta-blocker–dependent angiogenesis, via vascular endothelial growth factor inhibition, avoids all the beneficial effects of beta-blocker therapy on cardiac function and structure and results in reduction of cardiac vascular network and myocardial perfusion. These results indicate that beta-blocker–induced stimulation of cardiac angiogenesis represents a relevant therapeutic effect of beta-blockers in heart failure and open a new scenario for future studies testing whether the benefits of beta-blocker are reduced in patients with heart failure undergoing antiangiogenic therapies.
Vascular Endothelial Growth Factor Blockade Prevents the Beneficial Effects of β-Blocker Therapy on Cardiac Function, Angiogenesis, and Remodeling in Heart Failure

Giuseppe Rengo, Alessandro Cannavo, Daniela Liccardo, Carmela Zincarelli, Claudio de Lucia, Gennaro Pagano, Klara Komici, Valentina Parisi, Oriana Scala, Alessia Agresta, Antonio Rapacciulo, Pasquale Perrone Filardi, Nicola Ferrara, Walter J. Koch, Bruno Trimarco, Grazia Daniela Femminella and Dario Leosco

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### SUPPLEMENTAL MATERIAL

#### Supplemental Tables:

#### Supplemental Table 1

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<th>SHAM at Rest</th>
<th>SHAM after DVP</th>
<th>HF at Rest</th>
<th>HF after DVP</th>
<th>HF BB at Rest</th>
<th>HF BB after DVP</th>
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<td>91±1.58</td>
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Data are expressed as mean±standard error. DVP = Diprydamole; HF = Heart Failure; MBF = Myocardial Blood Flow; MAP = Mean Arterial Pressure; CC = Capillary Conductance; CTR = Coronary Flow Reserve.

* p < 0.05 versus SHAM  sp < 0.05 versus HF

#### Supplemental Table 2

<table>
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<tr>
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<th>HF/AdCTR at Rest</th>
<th>HF/AdCTR after DVP</th>
<th>HF/AdFK at Rest</th>
<th>HF/AdFK after DVP</th>
<th>HF/AdCTR BB at Rest</th>
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<td>85±1.81</td>
<td>73±1.92</td>
<td>83±1.81</td>
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<tr>
<td>CC (MBF/MAP)</td>
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<td>1.3±0.16</td>
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<tr>
<td>CTR (MaxCC/MinCC)</td>
<td>1.16±0.09</td>
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<td>1.18±0.14</td>
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</tbody>
</table>

Data are expressed as mean±standard error. DVP = Diprydamole; HF = Heart Failure; MBF = Myocardial Blood Flow; MAP = Mean Arterial Pressure; CC = Capillary Conductance; CTR = Coronary Flow Reserve.

* p < 0.05 versus Rest
Supplemental Figures:

**Supplemental Figure 1:** (A) Left ventricle ejection fraction (EF, as %), (B) LV internal diameter at diastole (LVIDd, as mm) and (C) heart rate (HR, as bpm) measured by echocardiography at 8 weeks post-MI before bisoprolol or placebo treatments initiation. n = 12 rats per group. Data are presented as mean ± SEM. *P < 0.05 versus sham. One-way ANOVA analysis and Bonferroni test among all groups.
Supplemental Figure 2: Cardiomyocytes Surface Area in (A) Sham, HF control and HF bisoprolol treated rats; (B). (Left panel) HF/Ad-CTR, HF/Ad-FLK, HF/B/Ad-CTR and HF/B/Ad-FLK groups. Representative images of WGA staining and (right panel) Bar graphs showing cardiomyocytes size measured in cardiac section. Magnification 200x. Data are presented as mean±SEM. (A) *P < 0.05 versus sham; #P < 0.05 versus HF. (B) *P < 0.05 versus all other groups. One-way ANOVA analysis with Bonferroni test among all groups.
Supplemental Figure 3: Bar graphs show data on capillary counts (capillary/mm²) in either LV border or remote zones in (A) sham, HF and HF bisoprolol groups; (B) HF/Ad-CTR, HF/Ad-FLK, HF/B/Ad-CTR and HF/B/Ad-FLK groups at the end of the study period (n= 5 rats per group and 5 sections per animal). Data are presented as mean±SEM. (A) *P < 0.05 vs sham; #P < 0.05 vs HF; (B) *P < 0.05 versus all other HF groups. Repeated measures ANOVA with Bonferroni correction.