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

Rengo et al: β-Blocker Induces Angiogenesis via VEGF/Akt/eNOS Pathway

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Abstract

Background—Impaired angiogenesis in the post-myocardial infarction (MI) heart contributes to the progression to heart failure (HF). 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—8 weeks from surgically-induced MI, HF 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 to 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 eNOS activation were observed by comparing B-treated to drug-untreated failing hearts. To test whether the pro-angiogenic 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 bisoprolol. After 10 weeks, histological analysis revealed reduced capillary and coronary perfusion in B-treated+Ad-Flk compared to B-treated+Ad-C. Moreover, VEGF inhibition counteracted the positive effects of B on cardiac function and remodeling.

Conclusions—β-blockade promotes cardiac angiogenesis in HF via activation of VEGF signaling pathway. β-blocker-induced enhancement of cardiac angiogenesis is essential for the favourable effects of this therapy on cardiac function and remodelling.

Key Words: heart failure, β-adrenergic receptor blocker, angiogenesis, vascular endothelial growth factor
Nowadays, heart failure (HF) remains one of the leading causes of morbidity and mortality worldwide, with myocardial infarction (MI) as the most common etiology (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) remodelling 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.

Over the last 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 β-AR 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 normal and (16) 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 pro-angiogenic signalling pathway in HF. Thus, in an experimental model of chronic HF, we demonstrate that bisoprolol (B) treatment induces cardiac VEGF
up-regulation 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

Ninety-seven Sprague-Dawley male rats (300g) entered the study. Sham-operated (n=12) and rats with surgically induced myocardial infarction (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/day in drinking water; n=12); 3) placebo plus intravenous (i.v.) injection of Adenovirus (Ad) vectors encoding Flk1-Fc (AdFlk), a potent angiogenesis inhibitor that acts as a decoy VEGF receptor (20) (n=10); 4) placebo plus i.v. injection of Ad encoding for the control Fc fragment (AdCTR) (18) (n=10) 5) B plus intravenous (i.v.) injection of AdFlk (n=13), and 6) B plus i.v. injection of Ad-control (n=12) . We injected 4x10^{10} 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% isofluorane; 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⁻¹ min⁻¹ 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 min. For each section, the infarct size of the LV was calculated from enlarged digital photos using SigmaScan 5.0 software, as described previously (23).

RNA isolation and real-time RT-PCR

Cardiac total RNA isolations, reverse transcription to cDNA and quantitative real-time RT-PCR were carried out as previously described (24).
Histology

Capillary density and arteriolar length density were measured as previously described (22). Briefly, left ventricular specimens were fixed in 4% formaldehyde and embedded in paraffin. After de-paraffinization and re-hydratation, 4 μm-thick sections were prepared, mounted on glass slides. Capillary density and arteriolar length density were evaluated in five 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 (BS-I) staining. Arterioles (<50 μm thick) were identified by immunofluorescence using anti-SM α-actin antibody as previously described (22). Arteriolar length density was calculated with the following formula: \( \text{Lv} = (\Sigma a/b)/N \cdot N/A \), 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 de-paraffinization and re-hydratation, 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 left ventricular myocardium from hearts of all study groups. Sections were stained with wheat germ agglutinin (WGA) 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/eNOS measurement**

Left ventricular (LV) samples were lysed in a RIPA buffer with protease and phosphatase inhibitors cocktail (Roche). Measurements of cardiac VEGF, Akt, serin473-phospho-Akt (pAkt), endothelial nitric oxide 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, USA). Each experiment and densitometric quantification was separately repeated at least three times.

**Statistics**

Normally distributed, continuous variables were compared by one-way analysis of variance (ANOVA), followed by a Bonferroni’s post hoc analysis or by repeated measures ANOVA, as appropriate. Data are reported as means±SEM. Statistical significance was accepted at p <0.05.

**Results**

**Effects of bisoprolol on in vivo cardiac function**

Echocardiography performed 8 weeks after MI induction revealed that LV ejection fraction (EF) and internal diameter at diastole were not statistically different among HF groups before placebo or B treatment initiation (Suppl. Fig.1). EF was significantly decreased and LV diastolic diameter was significantly increased in both HF groups compared with sham, demonstrating a similar degree of HF (Suppl. Fig. 1). At the end of the study period (18 weeks post-MI) the 2 HF groups still have worse cardiac function compared to sham (Fig. 1A
Effects of β-blocker therapy on cardiac remodelling 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 (BNP) in the LV and found this to be significantly increased in the HF control group, while in B-treated animals we observed values similar to sham (Fig. 1D). We further examined, as markers of remodeling and fibrosis, cardiac mRNA levels of collagen type I (Col1) and TGFβ-1 (Fig. 1E-F) 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 (Fig. 1G). Of note, cardiomyocytes surface area was increased in HF control group compared to sham, as expected. Importantly, bisoprolol treatment resulted in a slight but significant increase in cardiomyocytes size compared to HF control (Suppl. Fig. 2A).
Effects of bisoprolol on cardiac angiogenesis and perfusion

As expected, HF control rats showed a marked capillary density rarefaction in both LV border and remote zones compared to sham (Fig. 2A). Ten weeks of B treatment resulted in a significant increase of capillary density compared to HF controls (Fig. 2A). Consistently with capillary density data, arteriolar length density was dramatically reduced in the LV border and remote zones in HF control group compared to sham (Fig. 2A, Suppl. Fig. 3A). Interestingly, a significant growth of arterioles was found in LV the border and remote zones after 10 weeks of bisoprolol treatment (Fig. 2A, Suppl. Fig. 3A). Importantly, myocardial blood flow and coronary conductance were significantly reduced after maximal vasodilation in HF control group compared with sham rats (Figure 2B, 2C and Suppl. Table 1). Bisoprolol 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 Suppl. Table 1).

Effects of bisoprolol 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 to sham. These finding was associated to a reduced Akt activation and eNOS phosphorylation compared to sham, as reflected by phospho-Akt/total-Akt (pAkt/tAkt) 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 (Fig. 2E-F-G).
Effects of VEGF inhibition on bisoprolol-induced improvement of cardiac function

To test the in vivo patho-physiological relevance of B-dependent cardiac VEGF up-regulation, and Akt and eNOS activation on cardiac function, angiogenesis and remodelling, 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 with B and injected with Ad-CTR (HF/B/AdCTR); 4) HF rats treated with B and injection 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 EF% compared to HF/AdCTR (Fig. 3A). HF/AdFlk showed similar levels of both LV dilation and contractility compared to 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 EF when compared to HF/B/AdCTR, at values that were similar to those measured in HF/AdCTR (Fig. 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 to groups not treated with B (Fig. 3C), demonstrating that
Ad-Flk administration did not affect B-dependent HRR. Consistently, HW/BW ratio was similar between HF groups, with the only exception of HF/B/AdCTR group where it was significantly lower (Table 2).

Effects of VEGF inhibition on bisoprolol-dependent amelioration of cardiac remodelling gene profile

At the end of the study period, in HF/B/AdCTR hearts we observed a marked reduction of BNP, Col1 and TGFβ-1 mRNAs compared to HF/AdCTR, as assessed by RT-PCR (Fig. 3D-E-F). 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 3 genes investigated, with levels indistinguishable from those observed in rats not treated with β-blocker (Fig. 3D-E-F). Consistently, we observed a significant reduction of fibrosis in HF/B/AdCTR hearts compared to HF/AdCTR (Fig. 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 (Fig. 3G). Of note, bisoprolol treatment in HF/B/AdCRT group resulted in a slight but significant increase in cardiomyocytes size compared to HF/AdCTR group (Suppl. Fig. 2B). Importantly, when bisoprolol treatment was associated to VEGF inhibition, the ability of β-blocker to increase cardiomyocytes surface area was completely prevented (Suppl. Fig. 2B).

Effects of VEGF inhibition on bisoprolol-induced cardiac angiogenesis

After 10 weeks of treatments, capillary density and arteriolar length density were increased in HF/B/AdCTR hearts compared to HF/AdCTR both in the remote and border zones (Fig. 4A,
Suppl. Fig. 3B). 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 to HF/AdCTR (Fig. 4 B-C-D and Suppl. Table 2). Capillary density, arteriolar length density, all parameters of in vivo myocardial perfusion were similar in HF/AdFlk and HF/AdCTR (Fig. 2A-B-C-D, Suppl. Table 2), proving that VEGF inhibition is not able to further reduce capillary density and myocardial perfusion in HF rats. Importantly, VEGF inhibition was able to completely abolish the proangiogenic effect of β-blocker therapy; in fact, HF/B/AdFlk hearts showed significant reduction of capillary density, arteriolar length density and in vivo cardiac perfusion compared to HF/B/AdCTR, at levels similar to HF/AdCTR hearts (Fig. 2A-B-C-D, Suppl. Table 2).

Effects of VEGF inhibition on cardiac VEGF/Akt/eNOS pathway

To test the effects of VEGF inhibition on B-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 to HF/AdCTR hearts, confirming the ability of β-blocker to increase the activation of this pathway (Fig. 4D-E-F). In rat hearts treated with placebo plus VEGF inhibition, we observed increased VEGF protein expression comparable to that measured in rats treated only with B, but Akt and eNOS activation levels were similar to those observed in rats treated with placebo (Fig. 4E-F-G). Of note, in line with histological and in vivo perfusion data, VEGF inhibition completely prevented B-dependent Akt and eNOS activation. In particular, in HF/B/AdFlk hearts, although VEGF protein levels resulted increased similar to HF/B/AdCTR group, pAkt/tAkt and peNOS/teNOS ratios were significantly lower to that observed in HF/B/AdCTR (Fig. 4E-F-G).
Discussion

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

It is well established that angiogenesis in the post-MI heart is necessary because cardiomyocytes undergo compensatory hypertrophy in order to counterbalance for muscle loss in the infarcted region (4,5). Moreover, disruption of coordinated tissue growth and angiogenesis in the heart contributes to the progression from adaptive cardiac hypertrophy to HF (5). Thus, many efforts have been done in order to potentiate neoangiogenesis in HF.

Previous studies have demonstrated that bradycardia enhances coronary reserve, capillary density and arteriolar growth in the post-infarcted heart (17,18), and that bradycardia-induced cardiac angiogenesis is dependent on VEGF (16). It has been hypothesized that HRR, prolonging diastole, is able to enhance diastolic filling and stretch myocytes and capillaries, thus increasing cardiac VEGF expression (16). Here we report that activation of VEGF proangiogenic pathway underlies bisoprolol-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 (13,14), 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 heart rate dependent and 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 signalling 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 (20). Surprisingly, we found that VEGF inhibition prevented not only B-induced cardiac angiogenesis, but, unexpectedly, it also neutralized the favourable effects of β-blocker therapy on cardiac function and remodelling. Indeed, HF rats treated with B and VEGF inhibition showed no significant differences compared to HF/AdCTR rats in terms of cardiac chamber dilation and contractility, as well as for cardiac remodelling genes expression, indicating that B-induced VEGF upregulation is essential in coordinating 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 to HF control group. This finding is in line with the proangiogenic effect of β-blocker treatment, which is known to be essential to support coordinated vessel and cardiomyocytes growth. The discrepancy between decreased HW/BW ratio and increased cardiomyocytes size observed in HF rats treated with β-blocker compared to HF control rats could be explainable by the increased cardiac size (LV chamber dilation) and fibrosis observed in HF control compared to 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 to control. Taken together these results support the notion that the imbalance between myocyte growth and coronary angiogenesis plays a critical role in cardiac function (20,22) and demonstrates
that β-blocker-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 coordinated angiogenesis in HF (20,22,25). Previously, we have shown that exercise training, independently from HRR, is able to induce VEGF upregulation and Akt activation in the post-ischemic heart (22) and to improve age-dependent VEGF downregulation and angiogenesis responses to hindlimb ischemia (26). Moreover, β2AR overexpression in the failing heart is able to enhance neoangiogenesis inducing a sustained and coordinated Akt and VEGF activation (21). 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 to sham animals at 10 weeks post-MI. These molecular changes were associated with maladaptive cardiac remodelling and severe cardiac dysfunction. Importantly, in our model we demonstrate that B-dependent VEGF/Akt/eNOS pathway activation, which occurs within two weeks from treatment initiation, is crucial for the proangiogenic effects of β-blocker treatment. Moreover, we believe that the switch on of this pathway promotes new vessels growth and thus the transition from a maladaptive to an adaptive, angiogenesis-dependent LV remodelling, which attenuates the negative LV remodelling observed in placebo-treated animals late from MI.

In rat hearts treated with placebo plus VEGF inhibition, we observed increased VEGF protein expression comparable to 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 also 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 to HF control rats. This is probably due 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 non-infarcted hearts. Consequently, in our study, injection of AdFlk in placebo treated rats was not able to worse cardiac function, angiogenesis and remodeling when compared to placebo treated rats injected with AdCTR.

In conclusion 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 remodelling. Thus β-blocker-induced enhancement of cardiac angiogenesis is essential for the therapeutic effects of β-blockers in HF.

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Disclosures

None.

References


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Table 1. Physical and echocardiographic data in 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>BW (kg)</td>
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<td>0.469±0.014</td>
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<td>HW (g)</td>
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<td>HW/BW (gxKg⁻¹)</td>
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<td>2.73±0.08*†</td>
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<td>Echocardiography</td>
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</tr>
<tr>
<td>HR (bpm)</td>
<td>321.5±15.9</td>
<td>324.8±7.1</td>
<td>268.8±7.7**</td>
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<td>LV EF (%)</td>
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<td>LVIDd (mm)</td>
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<td>LVIDs (mm)</td>
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<td>Infarct size (%)</td>
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<td>44.8±3.1</td>
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ANOVA analysis and Bonferroni test were used among all three groups. Data are presented as mean±SEM. *P < 0.05 versus Sham; †P < 0.05 versus HF/control, **p< 0.05 versus Sham and HF/control. BW = body weight, HW = heart weight, HR = heart rate, LV EF = LV ejection fraction, LVIDd = LV internal diameter at diastole, LVIDs = LVID at systole, LVAWDd = LV anterior wall diameter at diastole, LVPWDd = LV posterior wall diameter at diastole.
Table 2. Physical and echocardiographic data HF rats at the end of the study period

<table>
<thead>
<tr>
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<th>HF/AdCTR</th>
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<td>HW/BW (gxKg⁻¹)</td>
<td>3.29±0.15</td>
<td>3.06±0.11</td>
<td>2.76±0.09*</td>
<td>3.05±0.12</td>
</tr>
<tr>
<td><strong>Echocardiography</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HR (bpm)</td>
<td>334.4±13.2</td>
<td>317.0±13.2</td>
<td>274.0±4.1†</td>
<td>276.5±11.3†</td>
</tr>
<tr>
<td>LV EF (%)</td>
<td>27.3±0.9</td>
<td>24.3±1.8</td>
<td>36.1±0.7*</td>
<td>26.0±1.6</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>10.7±0.3</td>
<td>11.0±0.2</td>
<td>9.8±0.2*</td>
<td>10.9±0.2</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>9.3±0.3</td>
<td>9.7±0.3</td>
<td>7.9±0.2*</td>
<td>9.7±0.1</td>
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<tr>
<td>LVAWDd (mm)</td>
<td>1.37±0.11</td>
<td>1.42±0.08</td>
<td>1.51±0.08</td>
<td>1.40±0.15</td>
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<tr>
<td>LVPWDd (mm)</td>
<td>1.87±0.21</td>
<td>2.05±0.08</td>
<td>2.35±0.10*</td>
<td>2.07±0.12</td>
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<tr>
<td>Infarct size (%)</td>
<td>47.6±5.2</td>
<td>45.3±3.8</td>
<td>48.1±4.6</td>
<td>44.1±4.2</td>
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ANOVA analysis and Bonferroni test were used among all four groups. Data are presented as mean±SEM. *P < 0.05 versus all other groups; †P < 0.05 versus HF/AdCTR and Ad/AdFlk.

BW = body weight, HW = heart weight, HR = heart rate, LV EF = LV ejection fraction, LVIDd = LV internal diameter at diastole, LVIDs = LVID at systole, LVAWDd = LV anterior wall diameter at diastole, LVPWDd = LV posterior wall diameter at diastole.
Figures Legends

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 the end of the study period, 10 weeks after bisoprolol or placebo treatments. n = 12 rats per group. mRNA levels for (D) BNP, (E) collagen type I (Col1), (F) TGFβ1 in hearts from sham, HF and HF/B groups at the end of the study period (n = 10 per group). All values were standardized to amplified 28S rRNA. (F) Fibrosis in cardiac section from all three experimental groups at the end of the study period (n= 5 for each group). Representative panels of picro-sirius red staining (magnification x200) (left panel) and average quantitative analysis (right panel); Scale bar: 50 μm. Data are presented as mean±SEM. *P < 0.05 versus sham; #P < 0.05 versus HF; ^ P < 0.05 versus sham and HF. One-way ANOVA analysis with Bonferroni test among all groups.

Figure 2. (A) Effects of bisoprolol on cardiac capillary and arteriolar network. (Left panel) 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, HF and HF/B rats at the end of the study period in the lateral wall far from the infarcted area (remote). Magnification x200. Scale bar: 50 μm. (Right panel) Bar graphs show data on capillary counts (capillary to myocytes ratio) and arteriolar length density in either LV 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).

(B) Total myocardial blood flow (MBF), (C) Coronary Conductance and (D) Coronary Flow Reserve (CFR) in sham, HF and HF/B rats at the end of the study period (n=10 rats per group). Cardiac protein expression of (E) VEGF, (F) Akt, Serin473-phospho(p)-Akt, (G)
eNOS, and Ser1177-phospho(p)-eNOS in sham, HF and HF/B hearts 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. p-Akt to total-Akt 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), (E), (F), (G) and repeated measures ANOVA with Bonferroni correction in (A), (B) and (C).

**Figure 3.** (A) Left ventricle ejection fraction (EF), (B) LV internal diameter at diastole (LVIDd) and (C) heart rate (HR) measured by echocardiography at the end of the study period, 10 weeks after bisoprolol or placebo treatments, with or without VEGF inhibition. n=10 rats per group, mRNA levels for (D) BNP, (E) collagen type I (Col1), (F) TGFβ1 in hearts from all four HF experimental groups at the end of the study period (n = 10 per group). All values were standardized to amplified 28S rRNA. (F) Fibrosis in cardiac section from all four HF experimental groups at the end of the study period (n= 5 for each group). Representative picro-sirius red staining (magnification x200) (left panel) and average quantitative analysis (right panel); Scale bar: 50 μm. Data are presented as mean±SEM. *P < 0.05 versus all other HF groups; ^P < 0.05 versus HF groups not treated with bisoprolol. One-way ANOVA analysis and Bonferroni test among all groups.

**Figure 4.** (A) Effects of bisoprolol and VEGF inhibition on cardiac capillary and arteriolar network. (Left panel) 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 four HF experimental groups at the end of the study period in the lateral wall far from the infarcted area (remote). Magnification x200.
Scale bar: 50 μm. (Right panel) Bar graphs show data on capillary counts (capillary to myocytes ratio) and arteriolar length density in either LV border or remote zones in all four HF study groups at the end of the study period (n= 5 rats per group and 5 sections per animal). (B) Total myocardial blood flow (MBF), (C) Coronary Conductance and (C) Coronary Flow Reserve (CFR) in all four HF experimental groups at the end of the study period (n=10 rats per group). Cardiac protein expression of (E) VEGF, (F) Akt, Serin473-phospho(p)-Akt, (G) eNOS, and Ser1177-phospho(p)-eNOS in all four 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. p-Akt to total-Akt ratio, and p-eNOS to eNOS ratio indicated respectively the levels of Akt and eNOS phosphorilation in the heart (n=5 hearts per group). Data are presented as mean±SEM. *P < 0.05 versus all other HF groups. One-way ANOVA analysis with Bonferroni correction in (D), (E), (F), (G) and repeated measures ANOVA with Bonferroni correction in (A), (B) and (C).
Figure 3

A. EF (%)

B. LV end-diastolic diameter (mm)

C. Heart rate (bpm)

D. BNP mRNA levels

E. Galectin-3 mRNA levels

F. Fibrosis (%)

G. Representative images of fibrosis
Figure 4

A. Capillaries Arterioles

B. MBF (mL/min/g)

C. Coronary conductance (mL/mmHg x min x g)

D. 

E. VEGF

F. p-Akt

G. p-eNOS

VEGF/CARDH protein levels (Fold over HF/CTR)

p-Akt/Akt protein levels (Fold over HF/CTR)

p-eNOS/eNOS protein levels (Fold over HF/CTR)
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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**SUPPLEMENTAL MATERIAL**

**Supplemental Tables:**

### Supplemental Table 1

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<tr>
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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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<tbody>
<tr>
<td>MBF (ml/min/g)</td>
<td>1.56±0.07</td>
<td>9.64±0.09</td>
<td>1.15±0.13</td>
<td>1.25±0.14*</td>
<td>1.45±0.14</td>
<td>3.05±0.16*</td>
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<td>MAP (ml/mmHg x min x g)</td>
<td>104±1.82</td>
<td>91±1.58</td>
<td>83±1.99*</td>
<td>72±1.17*</td>
<td>86±1.81*</td>
<td>77±1.92*</td>
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<td>CC (MBF/MAP)</td>
<td>1.5±0.07</td>
<td>10.6±0.74</td>
<td>1.4±0.17</td>
<td>1.7±0.21*</td>
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<td>5.1±0.25*</td>
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<td>CTR (MaxCC/MinCC)∥</td>
<td>7.0±0.40</td>
<td>1.25±0.73*</td>
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Data are expressed as mean±standard error. DVP = Dipyridamole; HF = Heart Failure; MBF = Myocardial Blood Flow; MAP = Mean Arterial Pressure; CC = Capillary Conductance; CTR = Coronary Flow Reserve

* p < 0.05 versus SHAM  †p < 0.05 versus HF

### Supplemental Table 2

<table>
<thead>
<tr>
<th></th>
<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>
<th>HF/AdCTR BB after DVP</th>
<th>HF/AdFK BB at Rest</th>
<th>HF/AdFK BB after DVP</th>
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<tr>
<td>MBF (ml/min/g)</td>
<td>0.99±0.07</td>
<td>1.02±0.09</td>
<td>0.94±0.13</td>
<td>0.91±0.14</td>
<td>1.76±0.14</td>
<td>4.99±0.16*</td>
<td>1.08±0.14</td>
<td>1.12±0.16</td>
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<tr>
<td>MAP (ml/mmHg x min x g)</td>
<td>82.9±1.82</td>
<td>75±1.38</td>
<td>78±0.97</td>
<td>71±1.17</td>
<td>85±1.81</td>
<td>73±1.92</td>
<td>83±1.81</td>
<td>75±1.92</td>
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<tr>
<td>CC (MBF/MAP)</td>
<td>1.15±0.09</td>
<td>1.39±0.14</td>
<td>1.16±0.18</td>
<td>1.31±0.21</td>
<td>2.01±0.15</td>
<td>6.76±0.26*</td>
<td>1.31±0.16</td>
<td>1.51±0.22</td>
</tr>
<tr>
<td>CTR (MaxCC/MinCC)∥</td>
<td>1.16±0.09</td>
<td>1.12±0.07</td>
<td>3.50±0.21*</td>
<td>1.18±0.14</td>
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</tbody>
</table>

Data are expressed as mean±standard error. DVP = Dipyridamole; 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.