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RESEARCH PAPER

Myocardial β_2-adrenoceptor gene delivery promotes coordinated cardiac adaptive remodelling and angiogenesis in heart failure

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BACKGROUND AND PURPOSE

We investigated whether β_2-adrenoceptor overexpression could promote angiogenesis and improve blood perfusion and left ventricular (LV) remodeling of the failing heart.

EXPERIMENTAL APPROACH

We explored the angiogenic effects of β_2-adrenoceptor overexpression in a rat model of post-myocardial infarction (MI) heart failure (HF). Cardiac adenoviral-mediated β_2-adrenoceptor overexpression was obtained via direct intramyocardial injection 4-weeks post-MI. Adenovirus(Ad)-GFP and saline injected rats served as controls. Furthermore, we extended our observation to β_2-adrenoceptor –/– mice undergoing MI.

KEY RESULTS

Transgenes were robustly expressed in the LV at 2 weeks post-gene therapy, whereas their expression was minimal at 4-weeks post-gene delivery. In HF rats, cardiac β_2-adrenoceptor overexpression resulted in enhanced basal and isoprenaline-stimulated cardiac contractility at 2-weeks post-gene delivery. At 4 weeks post-gene transfer, Ad-β_2-adrenoceptor HF rats showed improved LV remodeling and cardiac function. Importantly, β_2-adrenoceptor overexpression was associated with a markedly increased capillary and arteriolar length density and enhanced *in vivo* myocardial blood flow and coronary reserve. At the molecular level, cardiac β_2-adrenoceptor gene transfer induced the activation of the VEGF/PKB/eNOS pro-angiogenic pathway. In β_2-adrenoceptor–/– mice, we found a ~25% reduction in cardiac capillary density compared with β_2-adrenoceptor+/+ mice. The lack of β_2-adrenoceptors was associated with a higher mortality rate at 30 days and LV dilatation, and a worse global cardiac contractility compared with controls.

CONCLUSIONS AND IMPLICATION

β_2-Adrenoceptors play an important role in the regulation of the angiogenic response in HF. The activation of VEGF/PKB/eNOS pathway seems to be strongly involved in this mechanism.

Abbreviations

Ad, adenovirus; ECs, endothelial cells; eNOS, endothelial NOS; FS, LV fractional shortening; GFP, green fluorescent protein; GRK2, G-protein coupled receptor kinase-2; HF, heart failure; ICI118551, erythro-dl-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol; ISO, isoprenaline; KO, knockout; LAD, left anterior descending coronary artery; LV, left ventricular; MI, myocardial infarction; SNS, sympathetic nervous system

Introduction

Heart failure (HF) is a complex clinical syndrome characterized by left ventricular (LV) dysfunction accompanied by generalized hyperactivation of neurohormonal status (Rengo *et al.*, 2004; Mann and Bristow, 2005; Lymperopoulos *et al.*, 2007a). Although the sympathetic nervous system (SNS) overdrive is beneficial immediately after cardiac injury, aiming to preserve cardiac output, over time, it becomes detrimental, facilitating the worsening of cardiac contractility. The prolonged and sustained HF-related SNS activation induces several noxious effects on myocardial and vascular function, and is responsible for the dysregulation of cardiac β-adrenoceptors, including β_1-adrenoceptor down-regulation and β_1- and β_2-adrenoceptor uncoupling from signal-transducing G-proteins (Rockman *et al.*, 2002; Lohse *et al.*, 2003; Feldman *et al.*, 2005; Leosco *et al.*, 2007; Triposkiadis *et al.*, 2009), via increased cardiac G-protein coupled receptor kinase-2 (GRK2) expression/activity (Lefkowitz, 1993; Ungerer *et al.*, 1993; 1994; Rengo *et al.*, 2011). The molecular dysfunction of β-adrenoceptor signalling accounts for the reduced inotropic response to adrenergic stimulation in the failing myocardium.

Over the past two decades, several data have been produced proving different effects between β_1- and β_2-adrenoceptor signalling on cardiac physiology and pathophysiology (Lands *et al.*, 1967; Communal *et al.*, 1999; Zaugg *et al.*, 2000; Xiao *et al.*, 2004). However, the role of β_2-adrenoceptors in HF has not been completely elucidated (Zhu *et al.*, 2005). Previous evidence indicated that cardiac the overexpression of β_2-adrenoceptors improves contractility in the healthy heart (Milano *et al.*, 1994; Dorn *et al.*, 1999; Maurice *et al.*, 1999; Liggett *et al.*, 2000), and potentiates the functional recovery of unloaded rabbit failing myocardium (Tevaearai *et al.*, 2002). Furthermore, other evidence has been obtained indicating that pharmacological stimulation of β_2-adrenoceptors in the post-ischaemic failing heart induces cardioprotection preventing maladaptive remodelling, and curbing HF progression (Ahmet *et al.*, 2004; Xydas *et al.*, 2006).

Recently, β_2-adrenoceptor signalling has been demonstrated to be involved in angiogenesis regulation. In an experimental model of hindlimb ischaemia, it has been demonstrated that β_2-adrenoceptors play a pivotal role in the control of endothelial cells (ECs) function. Adenoviral-mediated β_2-adrenoceptor overexpression in the ischaemic hindlimb leads to enhanced EC proliferation and migration with the final effect of improved ischaemia-induced angiogenesis (Iaccarino *et al.*, 2005). This pro-angiogenic effect seems to be ascribed to increased β_2-adrenoceptor-dependent vascular endothelial growth factor (VEGF) production and release (Iaccarino *et al.*, 2005). Moreover, further findings supporting the important role of β_2-adrenoceptors in angiogenesis have been obtained in β_2-adrenoceptor knockout mice subjected to chronic hindlimb ischaemia (Ciccarelli *et al.*, 2011). Importantly, the impaired angiogenic response to ischaemia, observed in these mice, is restored by intravascular β_2-adrenoceptor gene transfer (Ciccarelli *et al.*, 2011). However, whether β_2-adrenoceptors regulate angiogenesis in the heart has never been previously tested either in physiological or in pathological conditions. With respect to this, previous findings from our group (Leosco *et al.*, 2008) and others (Karam *et al.*, 1990) indicate that angiogenic responses in the failing heart are inadequate and that impairments in the cardiac capillary and arteriolar network during HF plays a key role in post-ischaemic heart dysfunction. At the molecular level, this is associated with a blunted activation of the VEGF/PKB (Akt)/endothelial-NOS (eNOS) pathway. Nevertheless, the angiogenesis impairment can be reverted through interventions, such as exercise training, leading to an improvement in cardiac β-adrenoceptor signalling and reactivation of the PKB pro-angiogenic pathway (Leosco *et al.*, 2008).

We aimed to evaluate for the first time whether the beneficial effects of β_2-adrenoceptor overexpression in the failing myocardium may be correlated to the restoration of HF-related impairment of cardiac angiogenesis. We utilized an adenoviral-mediated intramyocardial gene delivery technique, which allows the robust expression of the transgene in the LV (Rengo *et al.*, 2009; Zincarelli *et al.*, 2010). To further support the notion that the β_2-adrenoceptor plays a crucial role in ischaemia-induced angiogenesis, we also studied the phenotype of post-myocardial infarction (MI) HF β_2-adrenoceptor knockout (KO) mice.

Methods

Experimental procedures

The study protocol was designed in accordance with *The Guide for Care and Use of Laboratory Animals* of the National Institutes of Health (NIH Publication No. 85–23, Revised 1996), and was approved by the Ethics Committee for the Use of Animals in Research of our Institution. An expanded Methods section appears in the online-only Data Supplement. The results of all studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

For experimental MI induction, rats were anaesthetized with 4% isoflurane. Deep anaesthesia was confirmed by the lack of a response to noxious stimuli. Next, rats were intubated and ventilated with a mixture of O_2 and 2% isoflurane with a pressure-controlled ventilator. After thoracotomy, MI was induced by permanent ligation of the left anterior descending coronary artery (LAD). Subsequently, chest and skin were closed in layers. Rats were observed until they awakened, and then returned to the Animal Care Unit. Standard postoperative care, including analgesic (buprenorphine 400 IU·kg^{-1} i.p. 20 min before surgery, ketoprofen 10 mg·kg^{-1} s.c. injection for 2 days after surgery), was provided. MI in mice was induced under general anaesthesia with isoflurane(2%), the heart was exposed and temporarily dislocated via a small left thoracotomy to place a suture ligation of the LAD (Lymperopoulos *et al.*, 2009; 2010). Myocardial gene transfer in rats was achieved by direct intramyocardial injection 4 weeks post-MI (Rengo *et al.*, 2009). Echocardiographic evaluations were performed at 4 weeks post-MI and at the end of the study (Leosco *et al.*, 2008). Basal and isoprenaline-stimulated LV contractility was evaluated by invasive haemodynamic studies at 2 and 4 weeks after gene delivery. At the end of the study period, animals were killed by cervical dislocation under deep anaesthesia.

Myocytes contractility

Myocytes were isolated from the non-infarcted zone of the LV by a standard enzymatic digestion (Leosco et al., 2008; Rengo et al., 2009). Contractility was evaluated under baseline and after agonist-mediated β_1-adrenoceptor stimulation by NA at 10^{-7} M or higher concentrations in the presence of the α_1-adrenoceptor antagonist, prazosin (10^{-6} M).

Myocardial perfusion studies

Myocardial perfusion was determined using 15 μm fluorescent microspheres (Triton Technology, Inc., San Diego, CA, USA). Cardiac and blood samples were processed for microsphere determination. Myocardial blood flow was measured at basal and after maximal vasodilatation.

Histology

Capillary and arteriolar length densities were evaluated in border and remote zones of the infarcted area. Capillaries were detected by Lectin *Bandeiraea simplicifolia*-I staining. Arterioles were identified by immunofluorescence using anti-SM α-actin antibody (Leosco *et al.*, 2008).

β-Adrenoceptor signalling

Receptor binding, adenylyl cyclase activity and GRK2 protein assays were obtained as previously described (Rengo *et al.*, 2010).

VEGF/PKB/eNOS measurement

VEGF, PKB, Ser[473]-phospho-PKB, eNOS and Ser[1177]-phospho-eNOS protein levels were performed by Western blot analysis (Lymperopoulos *et al.*, 2007b; 2008).

Statistical analysis

Data are summarized as mean ± SEM. Comparisons were made with the use of Student's *t*-tests or ANOVA as appropriate. A Bonferroni correction was applied to the probability values whenever multiple comparisons arose. Values of $P < 0.05$ were considered significant.

Results

Study design and transgenes expression

At 1 month after MI, a time point with established HF (Leosco *et al.*, 2008), rats were randomly allocated to three different groups receiving cardiac gene transfer of: adenovirus (Ad)–β_2-adrenoceptor, Ad–green fluorescent protein (GFP), or vehicle (saline). One day before gene delivery, all groups were analysed by echocardiography to confirm the presence of similar levels of LV dysfunction and HF. All groups were then studied over the course of 4 more weeks (Figure 1A), and all assays in the 3 HF groups were compared with a control sham-operated group that received neither MI nor gene transfer (4 experimental

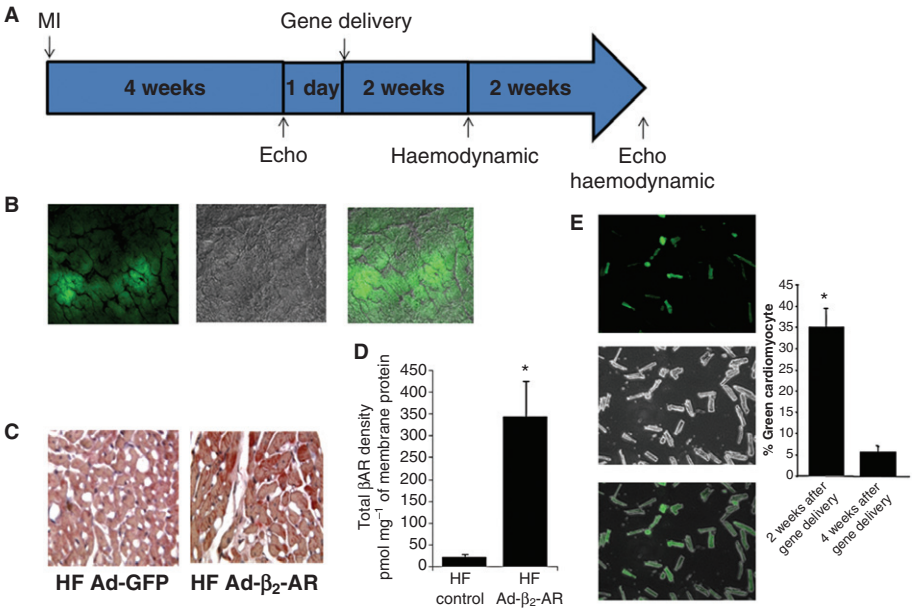

Figure 1

(A)Overall design of the 8-week study. (B) Representative GFP fluorescence microscopy (left), light microscopy (middle) and overlay of both (right) of LV myocardium 2 weeks after intramyocardial Ad-GFP delivery. (C) β_2-Adrenoceptor (AR) immunohistochemistry in LV tissue from Ad-GFP- (left, control) and Ad-β_2-adrenoceptor-treated (right) rats (2 weeks post-gene delivery). Magnification ×40. (D) Total β-adrenoceptor density (B) in cardiac homogenates purified from hearts of HF Ad-β_2-adrenoceptor and HF control (HF-saline and HF Ad-GFP) groups at 2 weeks after gene therapy ($n = 6$ and 8 for each group); *$P < 0.001$ vs. HF control. (E) Right panel; percentage (%) of GFP-stained isolated myocytes assessed 2 and 4 weeks following Ad-GFP *in vivo* gene delivery to HF rats by direct intra-myocardial injection ($n = 5$ for each time point). Green myocytes from each rat heart were counted in five randomly selected fields and expressed as percentage of the total number of myocytes per field. *$P < 0.001$ vs. 4 weeks after gene delivery. Data are presented as means ± SEM. Left panel: representative GFP fluorescence microscopy (upper), light microscopy (middle) and overlay of both (lower) of myocytes 2 weeks after Ad-GFP delivery.

Table 1

Physical and echocardiographic data in sham-operated and HF rats at 4 weeks after myocardial infarction

	Sham	HF saline	HF Ad-GFP	HF Ad-β_2-adrenoceptor
Physical data				
Body wt (kg)	0.408 ± 0.017	0.372 ± 0.012*	0.360 ± 0.018*	0.366 ± 0.010*
Heart wt (g)	1.15 ± 0.09	2.22 ± 0.21*	2.30 ± 0.19*	2.16 ± 0.26*
Heart wt/body wt ($g \cdot kg^{-1}$)	2.86 ± 0.13	5.94 ± 0.63*	6.40 ± 0.56*	5.89 ± 0.60*
Lung wt (g)	1.68 ± 0.18	2.87 ± 0.22*	3.0 ± 0.38*	2.92 ± 0.45*
Lung wt/body wt ($g \cdot kg^{-1}$)	4.06 ± 0.34	7.70 ± 0.85*	8.3 ± 0.92*	7.88 ± 0.67*
Echocardiographic data				
Heart rate (beats min^{-1})	366 ± 22	355 ± 16	348 ± 32	352 ± 28
LV internal diameter (mm)				
Diastolic	5.72 ± 0.45	8.86 ± 0.74*	9.12 ± 0.82*	9.36 ± 1.0*
Systolic	3.05 ± 0.28	6.35 ± 0.62*	6.22 ± 0.74*	6.40 ± 0.82*
LV FS (%)	46.4 ± 6.0	27.8 ± 5.2*	30 ± 4.7*	29.2 ± 5.6*
Interventricular septum (mm)				
Diastolic	1.49 ± 0.9	1.03 ± 0.11*	1.00 ± 0.8*	1.04 ± 0.9*
Systolic	2.64 ± 0.32	1.06 ± 0.12*	1.03 ± 0.6*	1.08 ± 0.10*
LV posterior wall (mm)				
Diastolic	1.53 ± 0.17	1.88 ± 0.18*	1.86 ± 0.13*	1.85 ± 0.17*
Systolic	2.49 ± 0.30	2.57 ± 0.26	2.55 ± 0.15	2.60 ± 0.18

Sham, $n = 10$; HF-saline, $n = 12$; HF Ad-GFP, $n = 11$; HF Ad-β_2-adrenoceptor, $n = 12$.
*$P < 0.05$ vs Sham.
HF, heart failure; LV, left ventricle; FS, fractional shortening.

groups in total). At 2 weeks post-gene delivery, both transgenes (β_2-adrenoceptor and GFP) were robustly expressed in the LV of the respective groups. GFP fluorescence in cardiac sections from Ad-GFP-treated rats confirmed that a large area of the LV free wall was transduced, although the expression was not homogeneous (Figure 1B). β_2-Adrenoceptor immunohistochemistry of cardiac sections from Ad-β_2-adrenoceptor-treated HF rats showed comparable areas of the LV transduced (Figure 1C), and the β-adrenoceptor binding experiment showing a 15-fold increase in membrane receptors compared with HF-saline and HF-GFP hearts (Figure 1D). Moreover, to confirm that our gene delivery technique supported efficient cardiac expression *in vivo*, 2 and 4 weeks after gene delivery of Ad-GFP, GFP expression was evaluated by detection of green fluorescence in cardiomyocytes isolated from the LV as previously described (Rengo *et al.*, 2009). Interestingly, at 2 weeks after Ad-GFP gene delivery we found a transduction efficiency to the LV that was >35% of total isolated LV myocytes, whereas, at 4 weeks post-gene delivery, GFP expression was minimal (less than 5%) (Figure 1E), which is consistent with the duration of *in vivo* expression seen by us and others using Ad (Maurice *et al.*, 1999).

In vivo *effects of cardiac β_2-adrenoceptors overexpression on LV remodelling and cardiac contractility*

At 1 month post-MI and before gene delivery, all HF groups had significantly impaired cardiac function compared with sham rats. As assessed by echocardiography, LV fractional shortening (FS) and internal diameter at diastole were comparable in all the HF groups, indicating a similar degree of cardiac dysfunction (Table 1).

At 2 weeks post-gene delivery, as assessed by *in vivo* invasive haemodynamic analysis, HF control groups (HF-Saline and HF-GFP) showed impaired basal and isoprenaline (ISO)-stimulated cardiac contractility, and increased LV end-diastolic pressure compared with sham, as expected (Figure 2). Two weeks of β_2-adrenoceptor overexpression resulted in enhanced basal cardiac contractility and reduced LV end-diastolic pressure compared with HF-saline and -GFP rats (Figure 2 and Table S1).

At 4 weeks from gene transfer, a time point when adenoviral-mediated β_2-adrenoceptor overexpression was minimal, the beneficial effects of β_2-adrenoceptor gene delivery on basal cardiac contractility disappeared, but β-adrenoceptor mediated contractility induced by ISO was significantly increased compared with HF controls (Table 2). Interestingly, NA was able to increase β_1-adrenoceptor-dependent contractility in cardiomyocytes extracted from AdV-β_2-adrenoceptor infected hearts compared with HF control. This result indirectly shows there is no obliteration of β_1-adrenoceptor responses in isolated cells (Figure S1). At the end of the study-period, HF-saline and HF-GFP rats showed further deterioration of cardiac function and progression of LV maladaptive remodelling (LV dilatation and reduced LVW/BW) compared with the echocardiographic

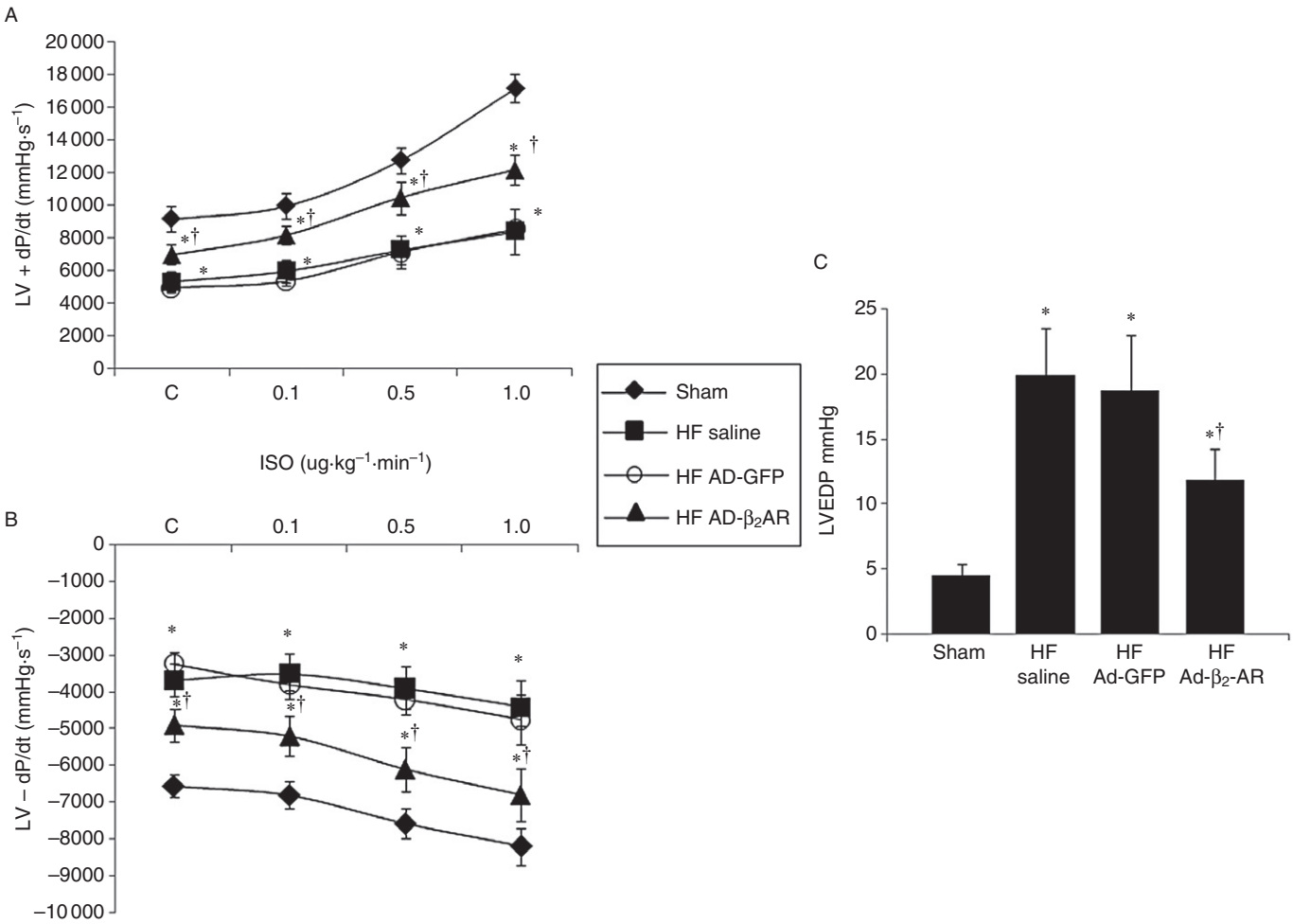

Figure 2

(A) Average LV +dP/dt and LV –dP/dt values (B) at 2 weeks post-gene therapy in the four experimental groups evaluated under basal conditions and after isoprenaline stimulation. (C) Average left ventricle end diastolic pressure (LVEDP) in the four experimental groups. Sham, n = 11; HF-saline, n = 13; HF Ad-GFP, n = 12; HF Ad- β_2-adrenoceptor (AR), n = 12. ANOVA analysis and Bonferroni test were used among all groups. Data are presented as mean ± SEM. *P < 0.05 vs sham at basal or at each respective dose of isoprenaline; †P < 0.05 vs HF-saline and HF Ad-GFP at basal or at each respective dose of isoprenaline.

measurements performed at 1-month post-MI, as expected (Table 1, Table 2 and Figure 3). Importantly, β_2-adrenoceptor overexpression resulted in improved FS, reduced ventricular systolic and diastolic diameters, and increased LVW/BW compared with HF control groups (Table 2 and Figure 3). Finally, at the post-study analysis of LV infarct size, we found similar infarct sizes among all study groups (Table 2). In this regard, the lack of a reduction in infarct size in β_2-adrenoceptor overexpressing HF rats should be ascribed to the fact that gene delivery was performed late after MI when infarct healing was complete.

Taken together, these data indicate that β_2-adrenoceptor overexpression exerts a favourable effect on post-MI LV contractility and remodelling. Interestingly, the positive effects on isoprenaline-stimulated LV contractility and on adaptive hypertrophic remodelling also persisted when the transgene expression was exhausted.

Effects of β_2-adrenoceptors overexpression on cardiac angiogenesis and myocardial blood flow

At 2 weeks after gene delivery, capillary density, but not arteriolar length, was significantly improved in HF-β_2-adrenoceptor rats compared with the HF-saline and HF-GFP groups (Supplemental Table 1).

Figure 4 displays histological and blood flow data in all study groups at 4 weeks after gene delivery. All HF groups showed a significant reduction in both capillary density and arteriolar length compared with sham animals. Interestingly, β_2-adrenoceptor overexpression induced a significant reactivation of the angiogenic mechanims in HF hearts. In fact, capillary and arteriolar density in both border and remote zones of the LV were significantly increased in the HF Ad-β_2-adrenoceptor group compared with HF-saline and HF-GFP

Table 2

Physical, haemodynamic and echocardiographic parameters at 4 weeks after cardiac gene delivery

	Sham-operated	HF Saline	HF Ad-GFP	HF Ad-β_2-adrenoceptor
Physical data				
Body wt (kg)	0.426 ± 0.024	0.388 ± 0.022*	0.390 ± 0.019*	0.382 ± 0.028*
Heart wt (g)	1.17 ± 0.07	2.58 ± 0.28*	2.42 ± 0.19*	2.82 ± 0.23*†
Heart wt/body wt (g·kg^{-1})	2.75 ± 0.14	6.66 ± 0.37*	6.12 ± 0.23*	7.36 ± 0.34*†
Lung wt (g)	1.70 ± 0.09	3.15 ± 0.30*	3.22 ± 0.16*	2.14 ± 0.19*†
Lung wt/body wt (g·kg^{-1})	3.98 ± 0.32	8.22 ± 0.46*	8.27 ± 0.38*	5.60 ± 0.36*†
Hemodynamic and echo data				
Heart rate (beats min^{-1})	355 ± 32	342 ± 28	340 ± 29	365 ± 26
LVSP (mmHg)	136 ± 12	111 ± 10*	109 ± 8*	123 ± 9*†
LVEDP (mmHg)	4.5 ± 0.8	21 ± 5*	20 ± 6**	10 ± 3*†
LV +dP/dt (mmHg·s^{-1})				
Baseline	8842 ± 457	4053 ± 268*	4238 ± 224*	4427 ± 313*
ISO (1 μg·kg^{-1}·min^{-1})	16 724 ± 966	6558 ± 236*	6886 ± 287*	9569 ± 461*†
LV –dP/dt (mmHg·s^{-1})	–6 488 ± 440	–3153 ± 235*	–2923 ± 321*	–3366 ± 357*
Baseline	–8 421 ± 654	–4115 ± 586*	–3934 ± 443*	–5340 ± 523*†
ISO (1 μg·kg^{-1}·min^{-1})				
LV internal diameter (mm)				
Diastolic	5.69 ± 0.37	9.52 ± 0.48*	9.43 ± 0.56*	7.10 ± 0.32*†
Systolic	3.10 ± 0.22	7.22 ± 0.39*	7.29 ± 0.44*	5.10 ± 0.36*†
LV fractional shortening (%)	45.5 ± 8	24.1 ± 4.6*	22.8 ± 5.9*	34.6 ± 7.2*†
Interventricular septum (mm)				
Diastolic	1.47 ± 0.8	1.02 ± 0.9*	0.90 ± 0.8*	1.00 ± 0.7*
Systolic	2.58 ± 0.28	1.03 ± 0.8*	0.97 ± 0.6*	1.03 ± 0.13*
LV posterior wall (mm)				
Diastolic	1.50 ± 0.15	1.94 ± 0.17*	1.97 ± 0.13*	2.32 ± 0.22*†
Systolic	2.51 ± 0.27	2.56 ± 0.28	2.52 ± 0.16	2.88 ± 0.18*†
Infarct size (%)	–	48.8 ± 5.6	51.3 ± 6.7	47.3 ± 7.5

Sham, $n = 11$; HF-saline, $n = 13$; HF Ad-GFP, $n = 14$; HF Ad-β_2-adrenoceptor, $n = 13$.
*$P < 0.01$ vs. sham.
†$P < 0.01$ vs. HF saline and HF GFP.
HF, heart failure; LV, left ventricle; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end diastolic pressure; ISO, isoprenaline.

hearts. Importantly, in HF Ad-β_2-adrenoceptor rats, we observed a further increase in capillary density between 2 and 4 weeks post-gene delivery (Figure 4 and Supplemental Table 1). Moreover, although myocardial blood flow and coronary reserve were significantly reduced in all HF groups compared with sham, β_2-adrenoceptor overexpression resulted in a significant improvement in both perfusion parameters compared with HF-controls (Figure 4).

Myocardial β-adrenoceptor status after in vivo *gene delivery*

Two weeks after gene therapy, cardiac β-adrenoceptor density was significantly reduced in HF controls compared with sham, while the HF Ad-β_2-adrenoceptor group showed a 15-fold increase in total membrane β-adrenoceptors compared with HF controls, proving the efficacy of the viral-mediated gene delivery technique (Figure 1 and Table 3). β_2-Adrenoceptor overexpression was accompanied by higher basal cardiac cAMP levels compared with HF-saline and HF-GFP groups. As expected, in HF control groups, cAMP cardiac levels were lower compared with sham (Table 3). ISO induced roughly 2- to 2.5-fold increases in adenylate cyclase activity in all HF groups. Myocardial GRK2 levels, measured by Western blotting, were significantly up-regulated in HF-saline and GFP groups compared with sham. In β_2-adrenoceptor-infected hearts, cardiac GRK2 expression was decreased almost to the sham levels.

Interestingly, at 4 weeks post-gene delivery, a time point when adenoviral-mediated overexpression of transgenes was minimal, total β-adrenoceptor density and cAMP production remained still higher in Ad-β_2-adrenoceptor-infected hearts compared with HF controls (Table 3). Accordingly, cardiac GRK2 protein expression was still significantly lower in

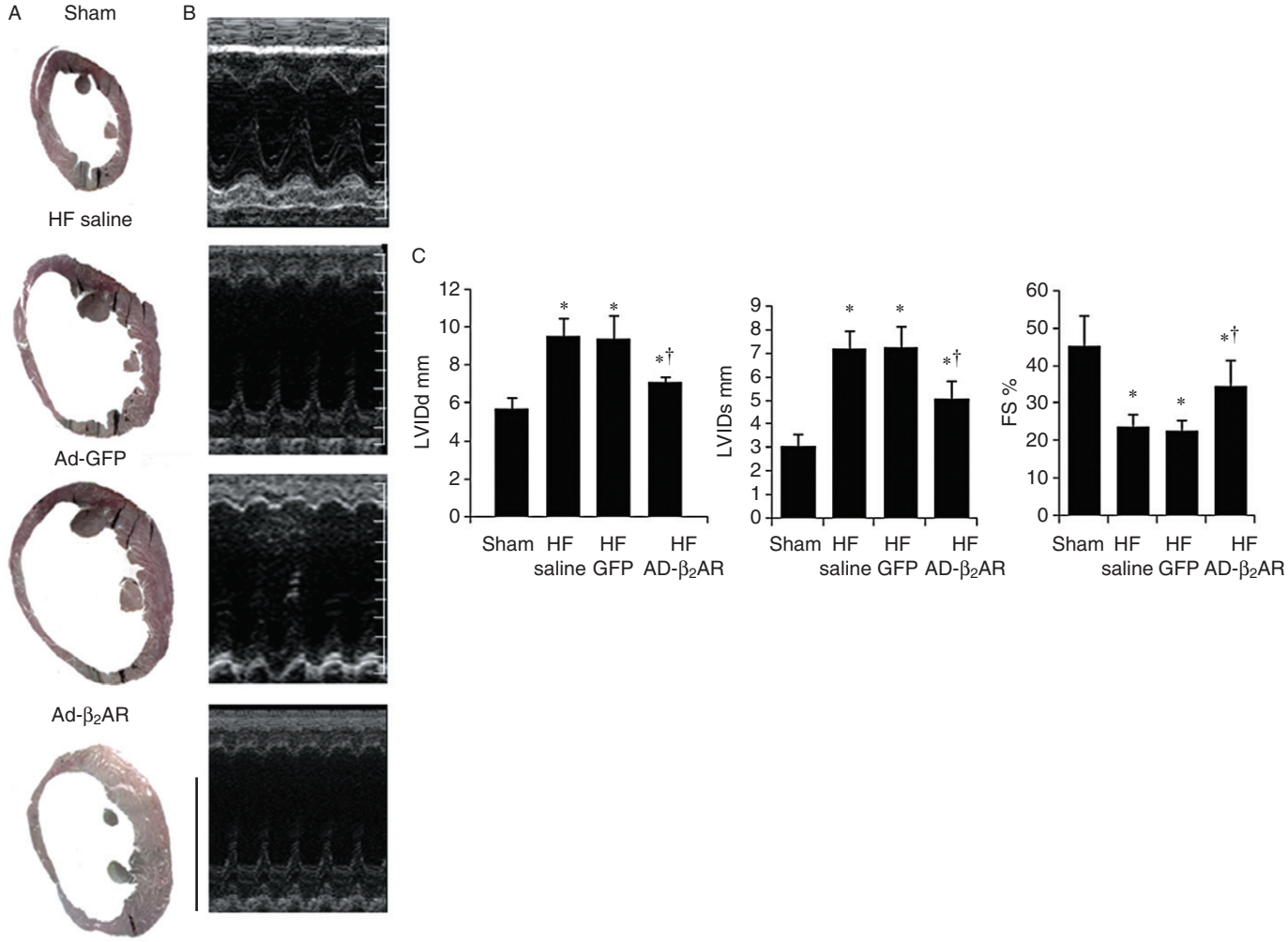

Figure 3

(A) Representative LV cross sections and (B) echocardiographic M-mode recordings from of all study groups at the end of the study period. (C) LV internal diameter at diastole (LVIDd) (left), LVID at systole (LVIDs) (middle) and fractional shortening (FS) (right) as measured by echocardiography 4 weeks after gene delivery. Sham, $n = 11$; HF-saline, $n = 14$; HF Ad-GFP, $n = 11$; HF Ad-β_2-adrenoceptor (AR), $n = 12$. Bar = 10 mm. ANOVA analysis and Bonferroni test among all groups. All data are expressed as mean ± SEM. *$P < 0.05$ vs. sham at basal or at each respective dose of isoprenaline; †$P < 0.05$ vs. HF-saline and HF Ad-GFP at basal or at each respective dose of isoprenaline.

Ad-β_2-adrenoceptor-infected hearts compared with other HF groups.

Cardiac β_2-adrenoceptors overexpression and VEGF/PKB/eNOS pathway

Next, we explored the effects of β_2-adrenoceptor overexpression on the cardiac VEGF/PKB/eNOS pathway, which is known to have a critical role in the control of the angiogenic mechanisms in the heart (Lymperopoulos *et al.*, 2008; 2010).

At 2 weeks after gene transfer, all HF groups showed higher cardiac phospho (p)-PKB/total (t)-PKB ratio compared with sham animals (Figure 5A). Noteworthy, in the HF β_2-adrenoceptor group, PKB activation was associated with enhanced cardiac VEGF and p-eNOS/eNOS protein levels compared with HF control groups. In contrast, HF-saline and HF-GFP groups did not show any increase in VEGF and p-eNOS protein expression, with levels comparable to those observed in sham hearts. These results agree with previous data, indicating that PKB activation is not always associated with enhanced activity of its downstream effectors, and, most importantly, that dissociation between PKB and eNOS/VEGF leads to negative cardiac remodelling (Shiojima *et al.*, 2005; Shiojima and Walsh, 2006). Overall, these data strongly suggest that cardiac β_2-adrenoceptor overexpression is able to restore the integrity of the pro-angiogenic pathway and to promote coordinated cardiac and vascular growth in the post-MI failing myocardium.

Interestingly, 4 weeks post-gene therapy, the activation of the VEGF/PKB/eNOS pathway was blunted in the HF-β_2-adrenoceptor group, whereas, in the HF control groups the p-PKB/t-PKB ratio was still higher compared with both HF-the β_2-adrenoceptor and sham groups (Figure 5B). Importantly,

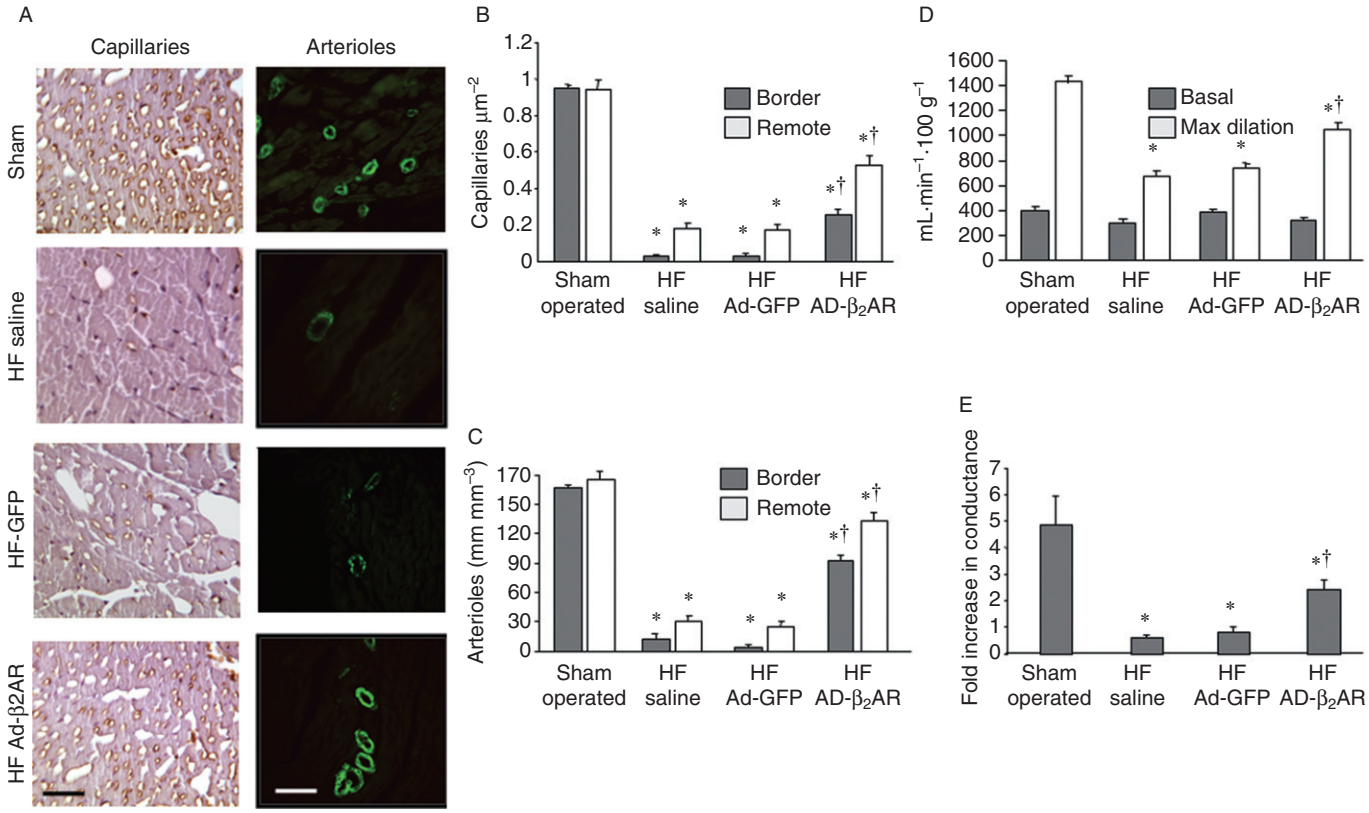

Figure 4

(A) Representative images of (left) Lectin *Bandeiraea simplicifolia* I staining of capillaries in LV sections and (right) of arterioles stained with antibodies against smooth muscle α-actin obtained from all study groups at 4 weeks post-gene therapy in the lateral wall far from the infarcted area (remote). Magnification ×40. Scale bar: 50 μm. (B) Histograms show data on capillary counts, and (C) arteriolar length density in either LV border anterior and lateral, and remote zones in all study groups at 4 weeks after gene therapy ($n = 5$ for each group). (D) Average of myocardial blood flow at basal condition and after maximal coronary dilation by dipyridamole and of coronary reserve measured in all study groups at the end of the study period ($n = 8$ rats for each group). ANOVA analysis and Bonferroni test among all groups. All data are expressed as mean ± SEM. *$P < 0.05$ vs. sham; †$P < 0.05$ vs. HF-saline and HF Ad-GFP.

PKB activation was not associated with enhanced VEGF and eNOS protein levels.

In order to link directly the effects of β_2-adrenoceptor overexpression to the activation of the VEGF/PKB/eNOS pathway, an additional group of Ad–β_2-adrenoceptor HF infected rats was treated with the specific β_2-adrenoceptor antagonist ICI118 551 ($0.2\ \mu g \cdot kg^{-1} day^{-1}$) beginning 5 days before gene delivery. As illustrated in Figure S2, ICI118551 was able to completely prevent β_2-adrenoceptor overexpression-dependent activation of the pro-angiogenic signalling in the hearts. This result suggests a direct effect of β_2-adrenoceptors on the activation of VEGF/PKB signalling.

Interestingly, in β_2-adrenoceptor-infected hearts, the VEGF/PKB/eNOS pathway, evaluated separately in cardiomyocytes and endothelial cells, was activated to a similar extent in both cell subtypes at 2 weeks after gene delivery (see supplemental Figure S3). This indicates that the overall effect on angiogenesis activation in β_2-adrenoceptor-overexpressing hearts could be mediated by pro-angiogenic stimuli originating from both myocytes and EC, thus, confirming previous observations suggesting the existence of reciprocal cross-talk mechanisms between the vasculature and cardiac myocytes that regulate coronary angiogenesis and contractile function (Shiojima *et al.*, 2005; Shiojima and Walsh, 2006).

Impairment of cardiac angiogenesis and LV contractility in post-ischaemic HF β2-adrenoceptor–/– mice

To further support the notion of a crucial role for the β_2-adrenoceptor in the regulation of cardiac function and angiogenesis during HF, homozygous β_2-adrenoceptor–/– (β_2KO) or β_2-adrenoceptor+/+ male mice littermates (control group) underwent MI (to induce HF) or sham operation (Figure 6). One month after surgically induced MI, the mortality rate was threefold higher in β_2-adrenoceptor–/– compared with β_2-adrenoceptor+/+ mice (Figure 6A). Echocardiography performed at this time point showed reduced FS in HF β_2-adrenoceptor+/+ compared with sham mice. The lack of β_2-adrenoceptors was associated with further decrease in FS compared with HF β_2-adrenoceptor+/+ mice (Figure 6B and Supplemental Table 2).

Interestingly, cardiac capillary density was significantly decreased in sham β_2-adrenoceptor–/– compared with sham β_2-adrenoceptor+/+. Moreover, 1 month post-MI, both HF

Table 3

Cardiac β-adrenoceptor receptor signalling at 12 and 30 days from gene delivery

	Sham		HF Saline		HF Ad-GFP		HF Ad-β_2-adrenoceptor	
β-AR density (fmol mg^{-1} membrane protein)								
12 day	67.8 ± 22.1		23.2 ± 6.6*		23.0 ± 6.8*		345.5 ± 82.2*†	
30 day	66.5 ± 11.9		21.0 ± 4.3*		24.1 ± 5.6*		47.8 ± 12.1*†‡	
	Basal	**ISO 10^{-4}**	**Basal**	**ISO 10^{-4}**	**Basal**	**ISO 10^{-4}**	**Basal**	**ISO 10^{-4}**
Adenylyl-cyclase activity (pmol cAMP·mg^{-1}·min^{-1})								
12 days	5.2 ± 1.6	12.1 ± 2.8	2.3 ± 0.8*	5.4 ± 1.0*	2.9 ± 0.9*	6.1 ± 1.2*	4.4 ± 1.0†	10.3 ± 2.5†
30 days	5.6 ± 1.1	13.0 ± 3.1	1.6 ± 0.4*‡	4.2 ± 0.9*‡	1.7 ± 0.7*‡	4.8 ± 0.9*‡	3.6 ± 1.1*†‡	7.2 ± 2.0*†‡
GRK2 protein expression (GRK2/GAPDH D.U.)								
12 days	0.22 ± 0.04		0.78 ± 0.09*		0.80 ± 0.12*		0.40 ± 0.07*†	
30 days	0.18 ± 0.03		1.26 ± 0.24*‡		1.33 ± 0.36*‡		0.75 ± 0.08*†‡	

Sham, $n = 7$; HF-saline, $n = 6$; HF Ad-GFP, $n = 7$; HF Ad-β_2-adrenoceptor, $n = 8$.
*$P < 0.01$ vs. sham operated.
†$P < 0.01$ vs. HF saline and HF-GFP.
‡$P < 0.005$ vs. 12-day values in the same group.
HF = heart failure; GRK2 = G-protein coupled receptor kinase 2.

mouse lines showed a decrease in capillary density compared with the respective sham groups. However, HF β_2-adrenoceptor–/– mice had significantly lower capillary density compared with HF β_2-adrenoceptor+/+ (Figure 6C). At the molecular level, we investigated the VEGF/PKB/eNOS pathway by Western blotting in both β_2-adrenoceptor–/– and β_2-adrenoceptor+/+ mice 1 month post-sham operation or surgically induced MI (Figure 6D). In HF β_2-adrenoceptor+/+ mice, we found an enhanced p-PKB/t-PKB ratio compared with both sham mouse lines. Interestingly, PKB activation was even more pronounced in HF β_2-adrenoceptor–/– compared with HF β_2-adrenoceptor+/+. However, VEGF and p-eNOS/eNOS protein levels in both HF lines were not significantly different from those observed in sham β_2-adrenoceptor–/– and β_2-adrenoceptor+/+ mice.

Discussion

In the present study, the β_2-adrenoceptor has been shown to be involved in the regulation of cardiac angiogenesis in the context of HF. In fact, in a rat model of post-ischaemic HF, we have reported for the first time that adenoviral-mediated cardiac overexpression improves post-MI LV function and remodelling, and that these favourable effects are associated with increased *in vivo* myocardial angiogenesis and blood perfusion, significant activation of the pro-angiogenic VEGF/PKB/eNOS pathway, and a reduction in cardiac β-adrenoceptor down-regulation. These data are consistent with the impaired myocardial capillarization observed in β_2-adrenoceptor–/– mice, that also showed a blunted angiogenic response to ischaemia, more severe post-MI LV dysfunction and higher 30-day post-MI mortality rate compared with control mice.

After MI, adequate growth of new capillaries and arterioles is needed to allow compensatory hypertrophic responses and favourable LV remodelling (Anversa *et al.*, 1986). However, neoangiogenesis is often inadequate in the post-MI heart (Karam *et al.*, 1990; Leosco *et al.*, 2008), and the lack of a valid readaptation of coronary conductance and reserve contributes to infarct expansion and transition from adaptive cardiac hypertrophy to LV dilatation and dysfunction.

Interestingly, several lines of evidence have shown that the β_2-adrenoceptor signals and functions in a substantially different way compared with the β_1-adrenoceptor. In fact, whereas β_1-adrenoceptor activation results in increased cardiomyocyte apoptosis, β_2-adrenoceptor stimulation protects cardiomyocytes against apoptotic stimuli (Communal *et al.*, 1999). Moreover, *in vivo* studies also support the notion that β_2-adrenoceptor activation and signalling in the heart may have positive implications that might be beneficial in HF (Liggett *et al.*, 2000). In particular, the aim of the present investigation was to demonstrate that the replacement of dysfunctioning β_2-adrenoceptors, which is a well-described phenomenon occurring in HF, could improve cardiac angiogenesis in the failing myocardium. In this context, mechanistic studies have shown that β_2-adrenoceptors control EC function (Ahmet *et al.*, 2004) and promote cell survival in other tissues (Morisco *et al.*, 2000; Zhu *et al.*, 2001). Iaccarino *et al.* have shown a selective down-regulation of β_2-adrenoceptors in the skeletal muscle following ischaemia, and neoangiogenesis improvement by β_2-adrenoceptor overexpression in the ischaemic muscle (Iaccarino *et al.*, 2005).

In our study, we demonstrated that the β_2-adrenoceptor controls angiogenesis in the heart; in fact, cardiac adenoviral-mediated β_2-adrenoceptor overexpression was associated with reactivation of this mechanism, which is impaired in the failing myocardium. The recovery of cardiac angiogenesis

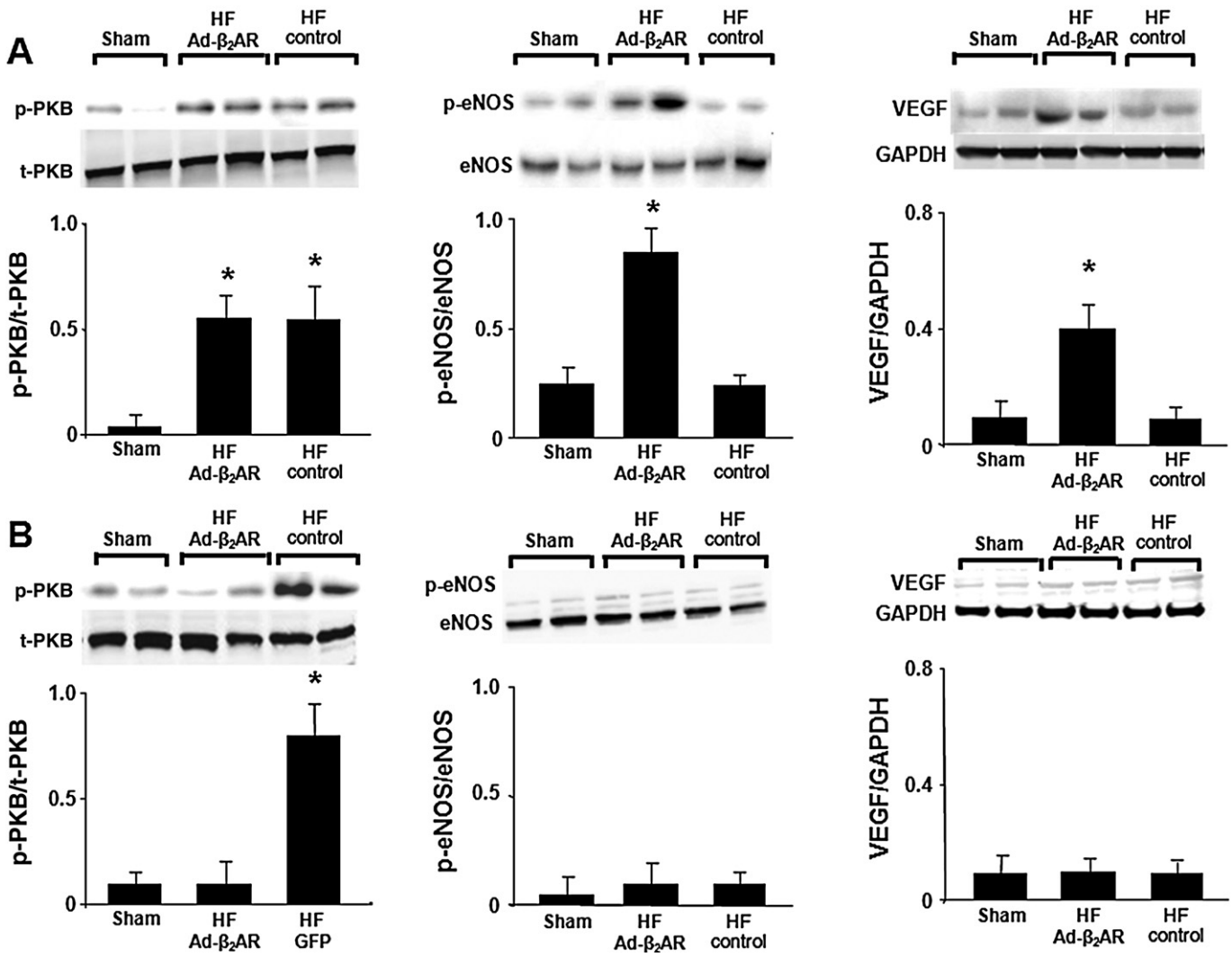

Figure 5

Shown is cardiac protein expression of VEGF, PKB, Ser^{473}-phospho(p)-PKB, eNOS and Ser^{1177}-phospho(p)-eNOS in all study groups at 2 (A) and 4 (B) weeks after gene therapy. Data between HF-saline and Ad-GFP were not statistically different and were pooled together and indicated as HF-control. The expression of GAPDH was used as an internal control to normalize VEGF protein levels. p-PKB/PKB and p-eNOS/eNOS ratio indicated respectively the levels of PKB and eNOS phosphorylation in the heart. Data are expressed as mean ± SEM. *$P < 0.05$ vs. sham ($n = 6$ rats per each group, for each time point).

properties prevents maladaptive LV remodelling and the progression of cardiac dysfunction. Our data, in the context of a clinically relevant model of HF, extend to the failing myocardium what has been reported on the ability of β_2-adrenoceptor overexpression to enhance cardiac contractility *in vivo* (Maurice *et al.*, 1999; Liggett *et al.*, 2000). Using a transgenic mouse model of HF, it has been shown that modest (30-fold) myocardial overexpression of β_2-adrenoceptors can prevent the development of hypertrophy and ventricular dysfunction (Dorn *et al.*, 1999). Furthermore, selective β_2-adrenoceptor stimulation reduced apoptosis and increased contractility in a post-MI HF rat model (Ahmet *et al.*, 2004) and adenoviral-mediated β_2-adrenoceptor gene transfer enhanced cardiac performance in healthy (Maurice *et al.*, 1999) and unloaded rabbit failing hearts after heterotopic cardiac transplantation (Tevaearai *et al.*, 2002). In our study, the overexpression of the receptor was achieved by direct intramyocardial injection of Ad-β_2-adrenoceptors, a technique that allowed us to obtain a ~15-fold increase in membrane β-adrenoceptors. Importantly, these levels lie in the therapeutic range reported by other authors who showed that the effects of β_2-adrenoceptor overexpression on cardiac function are dose-dependent and only a moderate overexpression results in enhanced *in vivo* and *in vitro* signalling and cardiac contractility (Liggett *et al.*, 2000).

Furthermore, we explored the effects of β_2-adrenoceptor overexpression on cardiac β-adrenoceptor signalling. We found improved β-adrenoceptor function at the receptor level 2 weeks post-gene therapy and, importantly, this positive effect was still evident 4 weeks post-gene delivery.

To further support the notion that the β_2-adrenoceptor plays a crucial role in the regulation of the angiogenic mecha-

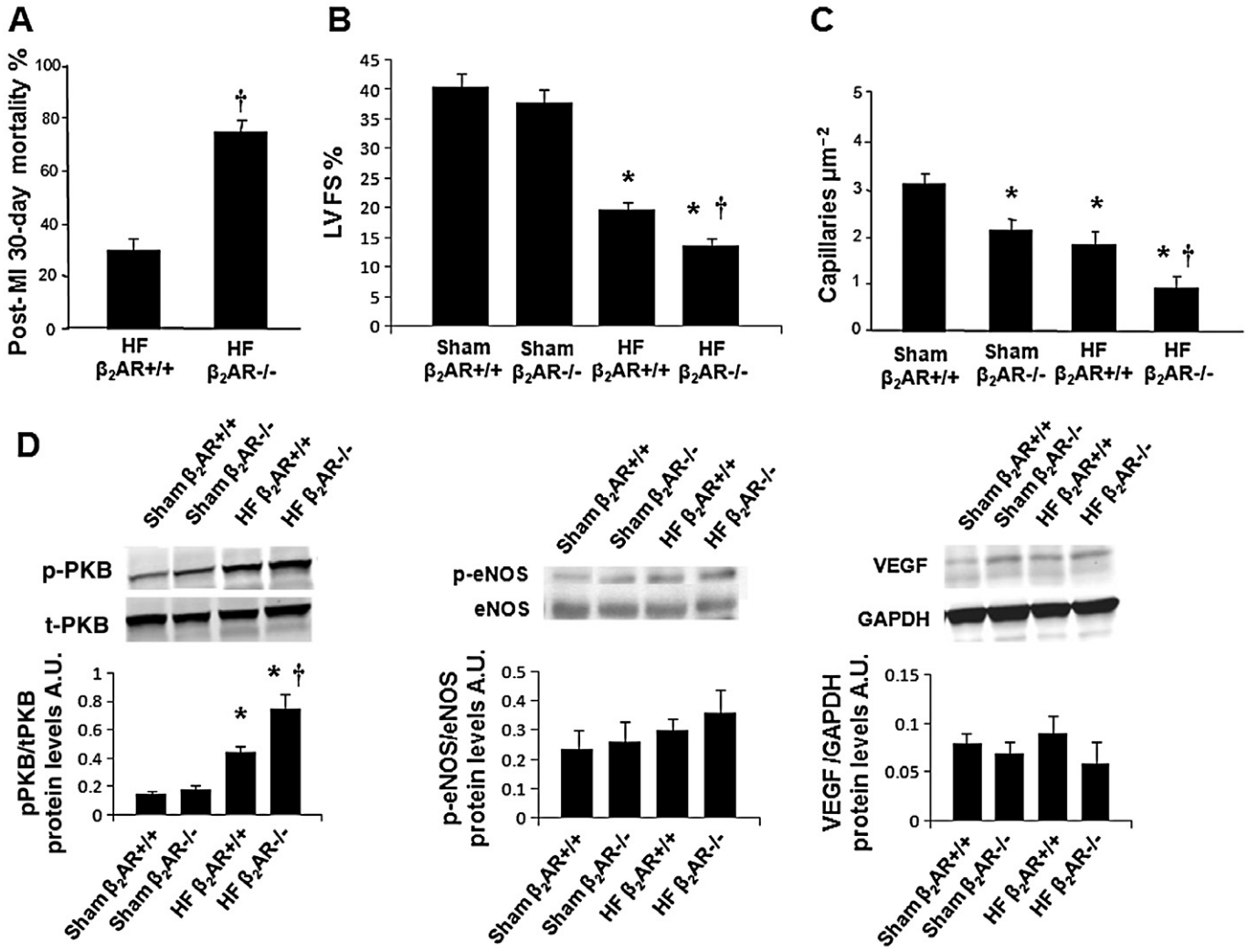

Figure 6

(A) Post-MI 30-day mortality rate in HF β_2-adrenoceptor (AR)+/+ and β_2-adrenoceptor–/– mice (n = 35 mice for each group). (B) LV fractional shortening FS (%) assessed by echocardiography at 4 weeks post-MI or sham operation in β_2-adrenoceptor+/+ and β_2-adrenoceptor–/– mice (n = 15 for each group). (C) Capillary counts (expressed as total capillary density μm^{-2}) in LV remote zone in all study groups (n = 8 for each group). (D) Shown is the cardiac protein expression of Ser^{473}p-PKB/PKB (left), Ser^{1177}-p-eNOS/eNOS and (middle) and VEGF (right) in sham or HF β_2-adrenoceptor+/+ and β_2-adrenoceptor–/– at the end of the study (n = 8 for each group). Data are expressed as mean ± SEM. *$P < 0.05$ vs. sham β_2-adrenoceptor+/+; †$P < 0.05$ vs. HF β_2-adrenoceptor+/+.

nism in the heart, we studied β_2-adrenoceptor–/– mice undergoing MI. Previous observations indicated that total elimination of both cardiac β_1- and β_2-adrenoceptors in mice has little impact on chronotropy/inotropy (mainly controlled by β_1-adrenoceptor) or myocardial basal metabolism (controlled by β_2-adrenoceptor), although functional deficits are clearly revealed by stress conditions, such as stimulation by β-adrenoceptor agonists or maximal exercise (Rohrer *et al.*, 1999). In β_2-adrenoceptor–/– mice, the reduction of anti-apoptotic defenses and the loss of preconditioning mechanisms have been recognized as the main determinants of the overtly exaggerated cardiomyopathy and dramatic mortality in HF models (Bernstein *et al.*, 2005). In the present study, consistent with the results obtained in HF rats, we found for the first time that the lack of β_2-adrenoceptor signalling was associated with a severe impairment of cardiac capillarization, higher 30-day mortality and cardiac dysfunction compared with control mice. Furthermore, we also showed that cardiac capillary density is significantly reduced in the non-infarcted heart of β_2-adrenoceptor–/– mice with respect to controls. The alteration in capillary density observed in β_2-adrenoceptor–/– mice is even more pronounced in the post-MI failing heart lacking β_2-adrenoceptors, also indicating a defect in ischaemia-induced cardiac angiogenesis. Accordingly, the more severe LV dysfunction and the higher mortality after MI observed in KO mice strongly support the protective role of cardiac β_2-adrenoceptors against ischaemia. In the present study, we explored the VEGF/PKB/eNOS pathway, which is strongly implicated in cardiac growth and angiogenesis. The ability of β_2-adrenoceptors to stimulate PKB

has been previously demonstrated in neonatal and adult cardiomyocytes (Morisco *et al.*, 2000; Zhu *et al.*, 2001), as well as in ECs (Iaccarino *et al.*, 2005). However, all these studies focused on the role of β_2-adrenoceptors in promoting cardioprotection through activation of PKB, but none of them examined the pro-angiogenic implications of β_2-adrenoceptor-dependent PKB activation in the heart. Most importantly, PKB activation is able to induce coordinated cardiac growth and angiogenesis only when accompanied by a significant VEGF production and release. In fact, VEGF inhibition early after PKB activation results in impaired coronary angiogenesis and transition from adaptive to maladaptive hypertrophy (Shiojima *et al.*, 2005). Our group has previously demonstrated that PKB is strongly activated immediately after MI with a concurrent VEGF overproduction (Leosco *et al.*, 2008). In the post-acute phase of MI, PKB activation is not accompanied by adequate VEGF production leading to pathological remodelling, characterized by severe LV dilatation and dysfunction (Leosco *et al.*, 2008). Herein, we showed that in HF, the overexpression of β_2-adrenoceptors induces a sustained and coordinated PKB and VEGF activation that is completely inhibited by ICI 118551, a specific β_2-adrenoceptor antagonist. In this regard, *in vitro* studies have demonstrated that overexpression of β_2-adrenoceptors in ECs induces VEGF production and EC proliferation (Iaccarino *et al.*, 2005). All these findings demonstrating the ability of β_2-adrenoceptors to activate PKB (Morisco *et al.*, 2000; Zhu *et al.*, 2001; Iaccarino *et al.*, 2005), as well as to induce *in vitro* and *in vivo* VEGF production (Iaccarino *et al.*, 2005; Leosco *et al.*, 2008), strongly suggest that the activation of the pro-angiogenic pathway in the heart is mechanistically related to increased β_2-adrenoceptor signalling following gene therapy. Interestingly, in β_2-adrenoceptor–/– HF mice at 1 month post-MI, we found low VEGF protein levels, as expected, but more pronounced PKB activation compared with control. We speculate that the impaired angiogenic phenotype observed in β_2-adrenoceptor KO mice requires enhanced PKB activation, although this is not accompanied by VEGF production. As regards the explanation for the reduced PKB and VEGF levels observed 4 weeks post gene therapy, it is important to note that transgene expression is almost exhausted at that time, thus lacking the β_2-adrenoceptor-mediated PKB and VEGF activation. It has been recently proposed that β_2-adrenoceptors can be cardioprotective in acute myocardial injury via PKB/eNOS activation and that GRK2 is a key regulator of this pathway in the heart (Rohrer *et al.*, 1999; Bernstein *et al.*, 2005; Tong *et al.*, 2005). Interestingly, GRK2, which is up-regulated in several animal models of cardiac diseases, has recently been shown to bind and inhibit PKB with consequent reduced activation of the downstream effectors, such as eNOS (Liu *et al.*, 2005). Moreover, GRK2 inhibition in the heart leads to enhanced PKB/eNOS signalling and cardioprotection through activation of NO-dependent anti-apoptotic/survival mechanisms (Liu *et al.*, 2005; Brinks *et al.*, 2010). Of note, in our study, cardiac β_2-adrenoceptor overexpression induced an overall improvement in cardiac β-adrenoceptor signalling and a significant reduction in cardiac GRK2 protein levels. This might represent an additional mechanism to explain cardiac PKB/eNOS activation observed in β_2-adrenoceptor infected hearts.

A potential limitation of the present study could be the lack of a sustained transgenes expression; in fact, β_2-adrenoceptor overexpression was almost exhausted at 1 month from infection, consistent with previous studies utilizing adenovirus. However, only 2 weeks of transgene expression were adequate to activate angiogenesis in HF hearts. Importantly, neovessel formation and maturation also continued after the loss of transgene expression, as demonstrated by the enhanced vascular network at 4 weeks from infection. Importantly, capillary and arteriolar density at the end of the study was even more pronounced than that reported at 2 weeks post-infection, the time of highest transgene expression.

In summary, the present study demonstrates that replacing dysfunctioning β_2-adrenoceptors in the post-ischaemic failing heart results in improved LV remodelling/function and cardiac angiogenesis, which is inadequate in the failing myocardium. This study adds novel additional mechanisms for the beneficial effects of β_2-adrenoceptors in HF and offers potential insights about future therapeutic strategies for HF treatment. In particular, we propose that an intervention aimed at the stimulation of cardiac angiogenesis might have valuable therapeutic consequences in the failing heart.

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Conflict of interest

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Haemodynamic and histological data at 2 weeks after adenoviral-mediated cardiac gene delivery

Table S2 Echocardiographic data from sham-operated or post-MI β_2AR+/+ and β_2AR–/– mice on day 28 post-MI. Analysis of variance with the Bonferroni test was performed among all groups

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