RESEARCH PAPER

Blockade of β-adrenoceptors restores the GRK2-mediated adrenal α2-adrenoceptor–catecholamine production axis in heart failure

G Rengo[1,2,3],*, A Lymperopoulos[4],*, C Zincarelli[1,2,3],*, GD Femminella[3], D Liccardo[3], G Pagano[3], C de Lucia[3], A Cannavo[3], P Gargiulo[3], N Ferrara[1,5], P Perrone Filardi[3], WJ Koch[2] and D Leosco[3]

[1]Cardiology Division, Fondazione 'Salvatore Maugeri', IRCCS, Telese Terme (BN), Italy, [2]Center for Translational Medicine and the George Zallie and Family Laboratory for Cardiovascular Gene Therapy, Department of Medicine, Thomas Jefferson University, Philadelphia, PA, USA, [3]Department of Internal Medicine, Cardiovascular and Immunological Sciences, University 'Federico II', Naples, Italy, [4]Dept. of Pharmaceutical Sciences, College of Pharmacy, Nova Southeastern University, Ft. Lauderdale, FL, USA, and [5]Department of Health Sciences, University of Molise, Campobasso, Italy

BACKGROUND AND PURPOSE

Sympathetic nervous system (SNS) hyperactivity is characteristic of chronic heart failure (HF) and significantly worsens prognosis. The success of β-adrenoceptor antagonist (β-blockers) therapy in HF is primarily attributed to protection of the heart from the noxious effects of augmented catecholamine levels. β-Blockers have been shown to reduce SNS hyperactivity in HF, but the underlying molecular mechanisms are not understood. The GPCR kinase-2 (GRK2)–α2adrenoceptor–catecholamine production–axis is up-regulated in the adrenal medulla during HF causing α2-adrenoceptor dysfunction and elevated catecholamine levels. Here, we sought to investigate if β-blocker treatment in HF could lower SNS activation by directly altering adrenal GRK2 levels.

EXPERIMENTAL APPROACH

Four weeks after myocardial infarction-induced HF, adult rats were randomized to 10-week treatment with vehicle (HF/C) or bisoprolol (HF/B). Cardiac function and dimensions were measured. In heart and adrenal gland, GRK2 levels were assessed by RT-PCR and Western blotting and adrenoceptors studied with radioligand binding. Catecholamines and α2adrenoceptors in adrenal medulla chromaffin cell cultures were also measured.

KEY RESULTS

Bisoprolol treatment ameliorated HF-related adverse cardiac remodelling and reduced plasma catecholamine levels, compared with HF/C rats. Bisoprolol also attenuated adrenal GRK2 overexpression as observed in HF/C rats and increased α2adrenoceptor density. In cultures of adrenal medulla chromaffin cells from all study groups, bisoprolol reversed HF-related α2adrenoceptor dysfunction. This effect was reversed by GRK2 overexpression.

CONCLUSION AND IMPLICATIONS

Blockade of β-adrenoceptors normalized the adrenal α2adrenoceptor-catecholamine production axis by reducing GRK2 levels. This effect may contribute significantly to the decrease of HF-related sympathetic overdrive by β–blockers.

Abbreviations

Ad, adenovirus; BNP, brain natriuretic peptide; Col1, collagen type I; GFP, green fluorescent protein; GRK2, GPCR protein-coupled receptor kinase-2; HF, heart failure; HR, heart rate; HW, heart weight; ^{125}I-CYP, [^{125}I]Cyanopindolol; LV, left ventricle; LVIDd, LV internal diameter at diastole; LVPWDd, LV posterior wall diameter at diastole; MI, myocardial infarction; SNS, sympathetic nervous system

Introduction

Heart failure (HF) is a major and growing public health problem in the Western world (Rengo *et al.*, 2004; Hunt *et al.*, 2009). Abnormalities in cardiac β-adrenoceptor signalling and function represent a salient characteristic of chronic HF pathophysiology (Feldman *et al.*, 2005; Tilley and Rockman, 2006). Increased levels and activity of GPCR kinase-2 (GRK2) play a critical role in promoting β_1-adrenoceptor down-regulation and G-protein uncoupling (desensitization) of both β_1- and β_2-adrenoceptor subtypes in the failing heart, resulting in a deterioration of overall cardiac function (Petrofski and Koch, 2003; Rengo *et al.*, 2011; receptor nomenclature follows; receptor nomenclature follows Alexander *et al.*, 2011). Moreover, in the past decade, our group has produced several lines of evidence demonstrating that inhibition of cardiac GRK2 decreases mortality and adverse ventricular remodelling while enhancing cardiac function in models of HF (Iaccarino *et al.*, 2005; Raake *et al.*, 2008; Rengo *et al.*, 2009).

The failing human heart is adrenergically hyperactivated, which helps to maintain cardiac performance in the short term; but, with worsening HF, sympathetic nervous system (SNS) overdrive results in adverse biological effects on cardiomyocytes (Lymperopoulos *et al.*, 2007a; 2009; 2011; Triposkiadis *et al.*, 2009). Adrenergic hyperactivation is accompanied by a generalized neurohormonal activation, finally determining structural end-organ damages such as cardiac dilatation, hypertrophy and fibrosis (Lymperopoulos *et al.*, 2007a; Triposkiadis *et al.*, 2009). Treatment with β–adrenoceptor antagonists (β-blockers) exerts a range of beneficial effects on cardiac function and, importantly, reduces HF-related morbidity and mortality (Bristow, 2000b; Foody *et al.*, 2002). β-blockers can directly prevent catecholamine-mediated toxicity on cardiomyocytes by preventing catecholamines from binding to β-adrenoceptors (Bristow, 2000b; Foody *et al.*, 2002). Importantly, it has also been reported that long-term β-blocker treatment is able to reduce HF-related SNS over-activity in experimental models of HF (Pleger *et al.*, 2007; Rengo *et al.*, 2009). as well as in humans (Nemanich *et al.*, 1990; Andersson *et al.*, 1994; Yoshikawa *et al.*, 1996). However, the underlying molecular mechanism(s) responsible for this effect remains unidentified.

Recently, our group established experimentally that GRK2 up-regulation in the catecholamine-producing adrenal medulla of HF animals leads to enhanced down-regulation and uncoupling of α_2-adrenoceptors (Lymperopoulos *et al.*, 2007a; 2008; 2010). These receptors are physiologically expressed on the chromaffin cell membranes of the adrenal gland and of the SNS nerve terminals where they normally exert negative feedback control on catecholamine production and secretion (Lymperopoulos *et al.*, 2007b). It appears that with progression of HF, adrenal α_2-adrenoceptors become severely dysfunctional due to the actions of GRK2, thus contributing to the chronically elevated catecholamine levels and SNS activity seen with this disease.

In the present study, we investigated the specific adrenal consequences of β-blocker treatment in a rat HF model. We sought to determine if chronic bisoprolol treatment could potentially rescue the dysfunctional α_2-adrenoceptor-catecholamine axis in HF and contribute to lower SNS activity and thus to protect the failing heart. By concentrating on GRK2 levels in our study, we have attempted to find not only the nodal target for adrenal dysfunction in HF but also a potential therapeutic mechanism to produce physiological sympatholysis.

Methods

All animal care and experimental protocols were 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 were approved by the Ethics Committee for the Use of Animals in Research of our Institution. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath et al., 2010). An expanded Methods section appears in the online-only data supplement.

Experimental groups

Sprague–Dawley male rats (~300 g, total = 105) (Charles River Laboratories, Lecco, Italy) entered the study and were randomly assigned to one of the three experimental groups. Sham-operated rats and rats with surgically induced myocardial infarction (MI) were treated with vehicle (drinking water) for 14 weeks (Sham, $n = 20$; HF/C, $n = 40$). At 4 weeks post MI, one group of HF rats was assigned to a 10 week bisoprolol treatment (HF/B, $n = 45$), using 80 mg L^{-1} in drinking water (approximate dose of 10 mg^{-1} kg^{-1} day^{-1}) (Watanabe *et al.*, 2001; Pleger *et al.*, 2007; Rengo *et al.*, 2009). Bisoprolol treatment was started at one-fourth of the full dose, and dosages were doubled every week until administration of the full dose was achieved.

Experimental procedures

MI was induced by surgical ligation of the proximal tract of the left anterior descending coronary artery, as previously described (Leosco *et al.*, 2008). Serial M-mode echocardiographic evaluations were performed at 4 weeks after surgery and at the end of the study protocols in anaesthetized (1.5% isofluorane) rats, using the VisualSONICS VeVo 770 imaging system with a 716 scan head, as described (Pleger *et al.*, 2007; Leosco *et al.*, 2008; Rengo *et al.*, 2009). At the end of the study period, animals were killed by cervical dislocation under deep anaesthesia (ventilated with a mixture of O_2 and 5% isoflurane).

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 (Rengo *et al.*, 2010; Zincarelli *et al.*, 2010).

Western blotting

Western blots to assess protein levels of GRK2 (sc-562; Santa Cruz Biotechnology, Santa Cruz, CA) were performed using protein extracts from homogenized adrenal glands and hearts as described (Leosco *et al.*, 2008; Rengo *et al.*, 2010).

Preparation of tissue fractions

Tissue fractions were obtained as previously mentioned (Leosco et al., 2008).

Saturation ligand-binding

Plasma membranes from excised adrenal gland were prepared and saturation binding was performed using the α_2-adrenoceptor radioligand [^{3}H]RX821002, as reported (Lymperopoulos et al., 2007a).

B-adrenoceptor radioligand binding

Membrane fractions from the left ventricle (LV) were used for β-adrenoceptor radioligand binding studies using the non-selective β-adrenoceptor antagonist ligand [^{125}I]cyanopindolol (^{125}I-CYP) as previously described (Leosco et al., 2008). All assays were performed in triplicate, and receptor density (in fmol) was normalized to mg of membrane protein.

Measurement of infarct size

Infarct size was examined in all experimental groups at the end of the study period, as previously described (Rengo et al., 2009).

Adrenal chromaffin cell isolation and culture

To obtain chromaffin cells, excised adrenal glands from all three study groups were incubated in Locke's solution containing 1 mg·mL^{-1} collagenase (>200 U·mL^{-1}; Biochrom KG, Berlin, Germany), as previously described (Lymperopoulos et al., 2007a; 2008).

In vitro catecholamine secretion measurements

Forty-eight hours post isolation, chromaffin cells were placed in a balanced salts buffer and stimulated with 20 µM nicotine for 30 min, following pretreatment with 10 µM UK14304 (Sigma Aldrich, Milan, Italy) or vehicle for 30 min. At the end of nicotine treatment, the supernatant was collected for determination of its catecholamine content. Adenoviral-transfected cells were infected 48 h post isolation, and cells were subjected to the various treatments 24 h after transfection (72 h post isolation).

Plasma and in vitro catecholamine secretion measurements

Plasma adrenaline and noradrenaline levels were determined by ELISA, performed on rat plasma samples using the BI-CAT EIA kit from ALPCO Diagnostics (Windham, NH), as described previously (Lymperopoulos et al., 2007a; Rengo et al., 2010). In vitro adrenaline and noradrenaline secretion in the supernatant of cultured chromaffin cells were measured by using the same ELISA kit, essentially as described previously (Lymperopoulos et al., 2007a; Rengo et al., 2010).

Adenoviruses

AdGRK2 or AdGFP recombinant adenoviruses were constructed as described and purified using two sequential rounds of CsCl density gradient ultracentrifugation (Lymperopoulos et al., 2008).

Statistics

Data were analysed by one-way ANOVA, followed by a Bonferroni's post hoc analysis, or by unpaired t-testing, as appropri-ate. For all tests, $P < 0.05$ was considered statistically significant after Bonferroni corrections, if needed, and all data are reported as means ± SEM.

Materials

[^{3}H]RX 821002 (specific activity 40–70 Ci mmol^{-1}) was obtained from Perkin Elmer (Milan, Italy) and [^{125}I]CYP (specific activity=2200 Ci mmol^{-1}) from New England Nuclear Corp., Boston, MA, USA. Bisoprolol, and nicotine were from Sigma-Aldrich and isoflurane from Abbott (Latina, Italy).

Results

Effects of β-blocker therapy on cardiac function and dimensions

In order to evaluate the effects of β-blocker treatment in chronic HF, adult male Sprague–Dawley rats were first subjected to MI (coronary arterty ligation) or a sham operation. As shown in Figure 1, MI produced significant cardiac dysfunction and HF after 4 weeks. It was at this time that the rats with MI were randomly assigned to either bisoprolol or placebo treatment. As shown in Figure 1A and D, bisoprolol-treated (HF/B) or control rats (HF/C) had no discernable differences in cardiac function as measured by LV ejection fraction or dimensions (as measured by LV diastolic diameter) upon pretreatment echocardiography, as all rats had LV dysfunction 4 weeks post MI and before starting treatment, compared with Sham-operated control rats. After a 10 week treatment period, both HF groups still had significantly impaired cardiac function compared with sham controls. However, ejection fraction was significantly higher in rats receiving bisoprolol compared with HF controls, although, at the end of the study period, it did not exceed pretreatment values (Figure 1B and C; Table 1). Conversely, control treatment led to further deterioration of cardiac function after 10 weeks (Figure 1B and C; Table 1). Adverse LV remodelling, as measured by ventricular dilatation, also progressed further in control-treated HF rats and this was prevented by bisoprolol treatment (Figure 1E; Table 1). Moreover, β-blocker treatment resulted in significant reduction of heart rate (Figure 1F). Finally, there were no differences in infarct size between the two HF groups (Table 1), which was not surprising as bisoprolol treatment was started at 4 weeks post MI induction, a time by which the infarct scar is completely established. Taken together, these results strongly indicate that bisoprolol treatment is capable of preventing cardiac dysfunction and adverse remodelling in the post-MI failing heart.

Effects of β-blocker therapy on cardiac remodelling gene profile

Following 10 weeks of HF treatment (14 weeks post MI), we evaluated cardiac gene expression patterns related to ventricular remodelling 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/C group, while this was normalized in bisoprolol-treated animals (Figure 2A). We further examined, as markers of remodelling and fibrosis, cardiac mRNA levels of collagen type I (Col1) and TGFβ-1
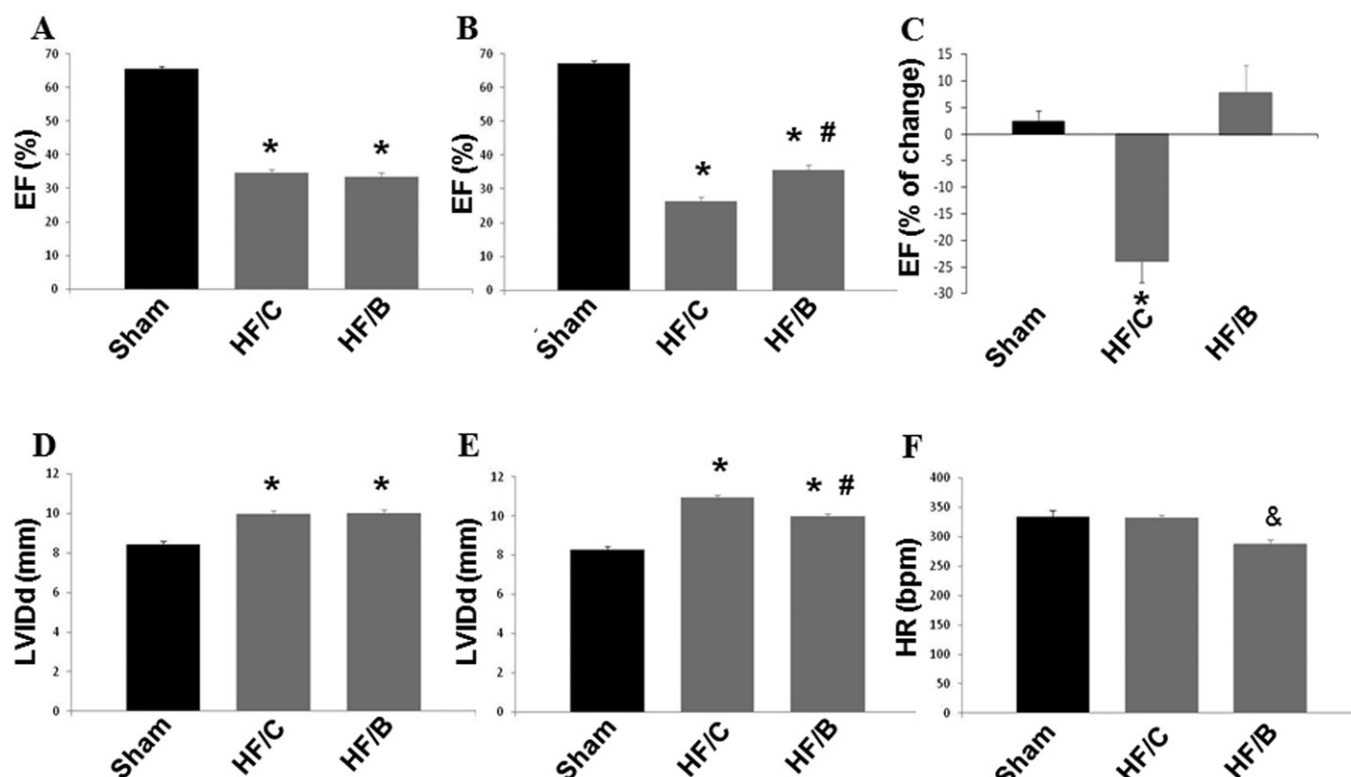
Figure 1

Ejection fraction (EF, as %) measured by echocardiography 4 weeks post MI (before treatments start) (A) and at the end of the study period, 10 weeks after bisoprolol or placebo treatments (B). (C) Percentage change in ejection fraction after the 10 weeks of treatment. LV internal diameter at diastole (LVIDd) measured by echocardiography before (D) and after treatment (E). (F) Heart rate (HR) at 14 weeks after MI. Sham, $n = 12$; HF/C, $n = 18$; HF/B, $n = 20$. Data are presented as mean $\pm$ SEM. *$P < 0.05$ versus sham; #$P < 0.05$ versus HF/C; & $P < 0.05$ versus sham and HF/C. ANOVA analysis and Bonferroni test among all groups.

Table 1

Physical and echocardiographic data in sham-operated and HF rats at the end of the study period

	Sham-operated	HF/control	HF/Bisoprolol
Physical data			
BW (kg)	0.463 ± 0.014	0.446 ± 0.010	0.470 ± 0.013
HW (g)	1.15 ± 0.04	1.40 ± 0.02*	1.28 ± 0.04*†
HW/BW (g·kg⁻¹)	2.50 ± 0.08	3.14 ± 0.12*	2.73 ± 0.09*†
Echocardiography			
HR (bpm)	333.9 ± 11.1	332.4 ± 4.66	287.5 ± 7.28&
LV EF (%)	67.1 ± 0.9	26.3 ± 1.2*	35.5 ± 1.7*†
LVIDd (mm)	8.27 ± 0.17	10.95 ± 0.13*	9.97 ± 0.15*†
LVIDs (mm)	5.14 ± 0.16	9.51 ± 0.16*	8.18 ± 0.20*†
LVAWDd (mm)	1.72 ± 0.03	1.46 ± 0.05*	1.45 ± 0.06*
LVPWDd (mm)	1.68 ± 0.06	2.02 ± 0.06*	2.17 ± 0.08*†
Infarct size (%)	–	44.8 ± 3.7	45.2 ± 4.3

ANOVA analysis and Bonferroni test were used among all three groups. Data are presented as mean $\pm$ 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.
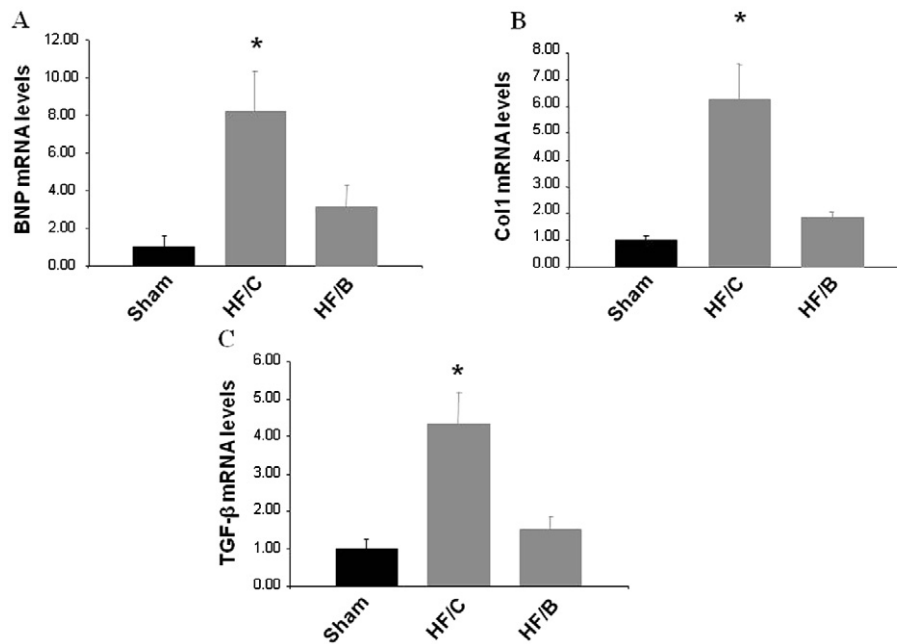
Figure 2

mRNA levels for (A) BNP, (B) collagen type I (Col1), (C) TGFβ1 in hearts from all experimental groups at the end of the study period (Sham, n = 10; HF/C, n = 12; HF/B, n = 12). All values were standardized to amplified 28S rRNA. *P < 0.05 versus Sham or HF/B groups. ANOVA analysis and Bonferroni test among all groups. Data are presented as mean ± SEM and plotted as fold over sham values.

(Figure 2B and C) and found mRNA levels of both of these markers significantly elevated in HF/C rats compared with sham controls; but both were markedly reduced in the bisoprolol-treated post-MI HF rats. These results demonstrate the positive effects of bisoprolol treatment on cardiac adverse remodelling also at the molecular level.

Effects of β-blocker therapy on plasma catecholamines in HF

We next investigated plasma catecholamine levels in our experimental groups at the end of the study period. Both adrenaline and noradrenaline were markedly elevated in HF rats compared with sham controls as expected (Figure 3). Ten weeks of bisoprolol treatment resulted in a significant reduction of circulating plasma levels of both noradrenaline and adrenaline compared with placebo group (Figure 3). However, our data show that bisoprolol treatment in HF did not induce a complete restoration of circulating catecholamine levels, as they remain significantly higher than those in sham control rats.

## Adrenal GRK2 levels in HF after β-blocker therapy

Because we recently reported that GRK2, a critical regulator of adrenal catecholamine production, is up-regulated in the adrenal medulla of HF animals (Lymperopoulos et al., 2007a), the finding that bisoprolol administration significantly reduces circulating catecholamine levels in HF prompted us to evaluate how this treatment could affect GRK2 expression levels in the adrenal glands of HF animals. Consistent with our previous studies, adrenal GRK2 protein levels in the HF/C
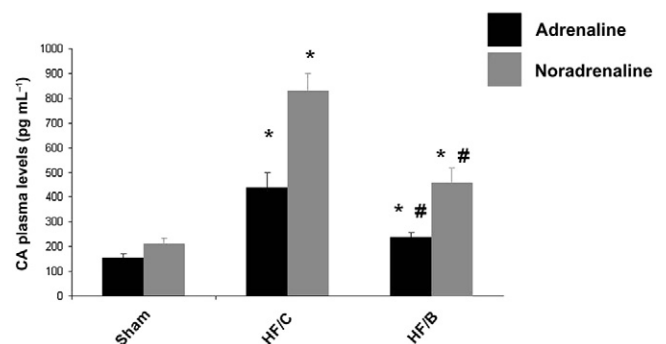
Figure 3

Plasma catecholamine (CA) levels in the three experimental groups at the end of the study. Data for noradrenaline and adrenaline are presented separately as means ± SEM. *P < 0.05 versus Sham or HF/B groups (n = 10 for each group); #P < 0.05 versus Sham or HF/C. ANOVA analysis and Bonferroni test among all groups.

group were significantly elevated compared with sham control rats (Figure 4A). Interestingly, 10 weeks of bisoprolol treatment resulted in normal adrenal GRK2 levels indistinguishable from sham rats and in line with reduced circulating catecholamine levels (Figure 4A). Adrenal GRK2 mRNA levels were also increased in HF/C rats compared with sham; bisoprolol treatment was able to normalize GRK2 mRNA levels in the adrenals (Supplemental Figure S1). Of importance, we found similar increases of GRK2 levels in the myocardium of post-MI HF rats that was associated with significant β-adrenoceptor down-regulation compared with sham rats
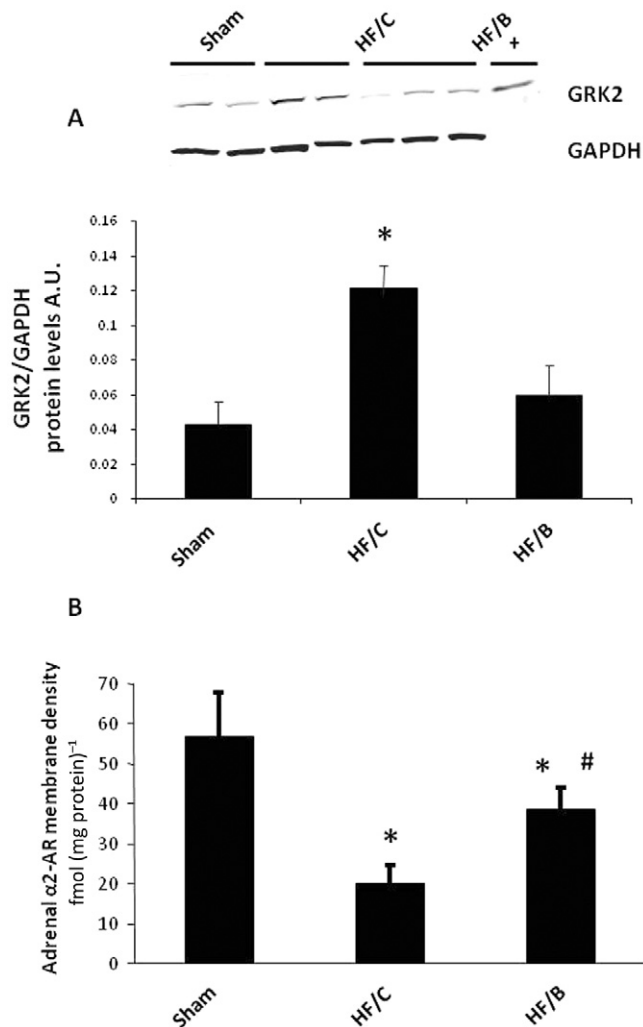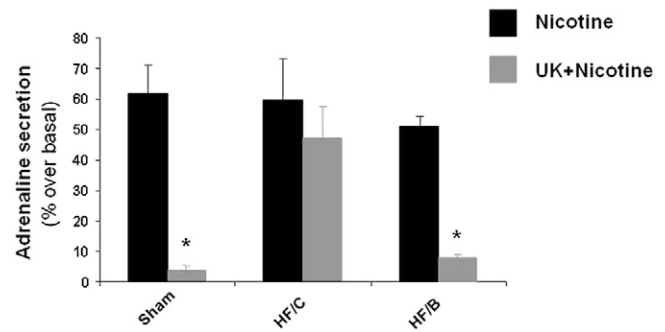
Figure 4

(A) GRK2 expression in adrenal homogenates purified from all three experimental groups at the end of the study period ($n = 7$ for each group). Representative Western blots (upper panel) and average densitometric quantitative analysis from blots showing the ratio of GRK2 to GAPDH (lower panel). + = positive control. (B) Total α_2-adrenoceptor (α_2AR) density in plasma membranes purified from the adrenal glands of all three experimental groups at the end of the study ($n = 6$ for each group). *$P < 0.05$ versus Sham or HF/B; #$P < 0.05$ versus HF/C. Data are presented as mean ± SEM. ANOVA analysis and Bonferroni test among all groups.

(Supplemental Figures S2 and S3). These results are consistent with our previous observations (Leosco *et al.*, 2008; Rengo *et al.*, 2009; 2010), and bisoprolol treatment resulted in a normalization of cardiac GRK2 protein levels and significantly increased β-adrenoceptor density in the heart compared with HF/C rats, and these levels were restored to normal (Sham) levels (Supplemental Figures S2 and S3).

Adrenal α2-adrenoceptor density in HF after β-blocker therapy

Previous data from our group have demonstrated that during HF the increased expression and activity of GRK2 triggers a
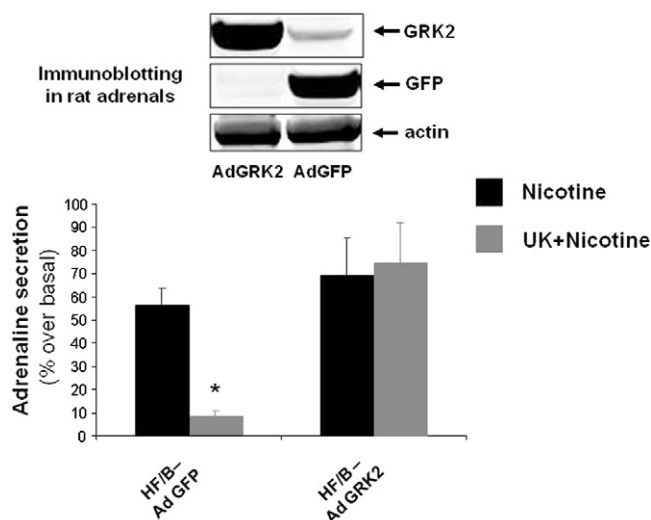

Figure 5

Secretion of adrenaline from cultures of chromaffin cells, isolated from the adrenals of sham, HF/C and HF/B rats, in response to nicotine (20 μmol·L^{-1}), following pretreatment with vehicle (Nicotine) or with UK14304 (10 μmol·L^{-1}; UK + Nicotine). *$P < 0.05$, versus HF/C-UK + Nicotine, $n = 6$. Data are presented as mean ± SEM. ANOVA analysis and Bonferroni test among all groups.

substantial α_2-adrenoceptor dysregulation in the adrenal gland, resulting in loss of receptor inhibitory function and increased catecholamine secretion (Lymperopoulos *et al.*, 2007a). This prompted us to investigate the effects of β-blocker therapy on adrenal α_2-adrenoceptor density in our HF rats. As shown in Figure 4B, the HF/C group displayed marked adrenal α_2-adrenoceptor down-regulation compared with healthy sham animals; however, bisoprolol treatment induced a significant improvement of adrenal α_2-adrenoceptor density. These data strengthen the notion that β-blocker treatment reduces plasma catecholamine levels in HF by restoring the adrenal GRK2-α_2-adrenoceptor-catecholamine secretion axis.

In vitro *adrenaline release from isolated chromaffin cells*

To better evaluate the effect of β-blocker treatment on α_2-adrenoceptor dysfunction in HF, we examined catecholamine release from chromaffin cells isolated from the three experimental groups and treated *in vitro* with nicotine, a pharmacological stimulator of catecholamine release, and pretreated with or without the α_2-adrenoceptor-specific agonist UK14304. In cultured chromaffin cells from all the three groups, nicotine induced similar levels of adrenaline release. In sham-derived cells, UK14304 blunted adrenaline release in response to the stimulus as expected; whereas in chromaffin cells derived from the HF/C group, the α_2-adrenoceptor agonist was not able to inhibit adrenaline secretion in response to nicotine stimulation, indicating α_2-adrenoceptor desensitization. Notably, when chromaffin cells from HF/B animals were stimulated with UK14304, nicotine-induced adrenaline release was significantly reduced, similar to that in cells from sham rats (Figure 5). Furthermore, *in vivo* β-blocker treatment was also able to restore the function of α_2-adrenoceptors towards inhibition of nicotine-induced noradrenaline secretion in chromaffin cells (Supplemental Figure S4). These data support the hypothesis that β-blocker treatment is able to reverse GRK2-mediated α_2-adrenoceptor desensitization through reduction of GRK2

Figure 6

Representative Western blot showing expression of transgenes in cultures of chromaffin cells (upper panel). Secretion of adrenaline from chromaffin cells isolated from the adrenals of HF/B rats infected *in vivo* with AdGFP or AdGRK2 in response to nicotine treatment with or without pretreatment with UK14304 (UK + Nicotine) (lower panel). *$P < 0.05$, versus AdGRK2-UK + Nicotine, $n = 6$. Data are presented as mean ± SEM. ANOVA analysis and Bonferroni test among all groups.

expression levels and thus can restore the inhibitory function of α_2-adrenoceptors on catecholamine release from chromaffin cells during HF.

Effects of GRK2 overexpression on adrenaline release from chromaffin cells isolated from HF bisoprolol at 14 weeks post MI

To test whether normalization of adrenal GRK2 protein levels is critical for the ability of bisoprolol to prevent HF-related α_2-adrenoceptor down-regulation *in vivo*, we extracted the adrenal glands of bisoprolol-treated HF rats and cultured them for 24 h. Then the adrenal cells were infected either with a GFP adenovirus (Ad-GFP) as a control or Ad-GRK2. Induced expression of the transgenes (GRK2 and GFP) was confirmed by Western blotting in cellular protein extracts (Figure 6, upper panel), and 24 h after infection, catecholamine secretion from isolated chromaffin cells was tested using nicotine and the α_2-adrenoceptor-specific agonist UK14304, as above. As shown in Figure 6 (lower panel), chromaffin cells extracted from bisoprolol-treated rats and infected *in vitro* with Ad-GFP were shown to have normalized negative feedback as UK14304 blocked nicotine-induced adrenaline release into the media. Importantly, when GRK2 was overexpressed in these cells, the inhibition of nicotine-induced catecholamine release by UK14304 was lost (Figure 6). Similar results have been obtained for noradrenaline secretion in the same setting of experiments (Supplemental Figure S5). These results strongly suggest that the effects of bisoprolol on restoring α_2-adrenoceptor inhibitory function

in HF are dependent on GRK2 levels/activity in the adrenal glands.

Discussion

In this study, we report a novel molecular mechanism that explains, at least in part, the ability of long-term β-blocker treatment in chronic HF to reduce the enhanced circulating catecholamine levels that significantly worsen the prognosis of patients affected by this condition (Lymperopoulos *et al.*, 2007a; Triposkiadis *et al.*, 2009). Ten weeks of bisoprolol treatment in rats with post-MI HF lowered adrenal GRK2 to normal levels and consequently markedly reduced adrenal catecholamine production via reversal of the dysfunction of adrenal α_2-adrenoceptors, which are desensitized and down-regulated in HF. This finding is of particular physiological and clinical relevance since several experimental studies have reported that β-blocker treatment counteracts the catecholaminergic hyperactivation of chronic HF in animal models (Pleger *et al.*, 2007; Rengo *et al.*, 2009), not only by preventing adrenoceptor activation by agonist but also, chronically, by reducing circulating catecholamine levels. However, no evidence had been provided regarding the molecular mechanisms underlying this beneficial effect of β-blockade in HF.

Circulating catecholamines are derived from two main sources: the peripheral SNS nerve terminals, which release noradrenaline and the adrenal medulla, which produces mainly adrenaline and to a lesser extent, noradrenaline. In the adrenal gland, catecholamine production is tightly regulated by the adrenoceptors, in particular α_2-adrenoceptors expressed in the chromaffin cells of the medullary region, which exerts tonic inhibition of catecholamine release, and also β_2-adrenoceptors, which can facilitate catecholamine secretion (Lymperopoulos *et al.*, 2007b). Like most other GPCRs, the α_2-adrenoceptors can be desensitized by phosphorylation via GRKs, and chronic action by these kinases such as GRK2 can promote receptor down-regulation (i.e. receptor degradation). During HF, GRK2 is up-regulated in the adrenal medulla, causing α_2-adrenoceptor dysfunction and ultimately leading to catecholamine hypersecretion from the chromaffin cells (Lymperopoulos *et al.*, 2007a). By decreasing GRK2 levels in the adrenal gland, β-blocker treatment appears to restore adrenal α_2-adrenoceptor density and signalling at the plasma membrane and catecholamine feedback inhibition, and this represents a mechanism whereby drugs such as bisoprolol (used in this study) can reduce SNS overdrive in chronic HF.

Overall, the success of β-blockers in HF treatment is attributed to their ability to block the constantly increased sympathetic overdrive present in the failing heart, thus reducing mortality and morbidity in HF patients (Bristow, 2000a; Foody *et al.*, 2002). Moreover, in experimental models and in human HF, β-adrenoceptor blockade exerts several additional therapeutic effects, including improved cardiac reverse remodelling, blunted apoptosis, inhibited β-adrenoceptor internalization, reduced oxygen consumption and reduced risk of arrhythmias (Foody *et al.*, 2002). In addition to these functional and clinical effects, β-blockade also appears to reduce the autonomic derangement and neurohumoral excitation in HF (Nemanich *et al.*, 1990; Andersson *et al.*, 1994;

Yoshikawa *et al*., 1996). In particular, we have recently demonstrated that long-term metoprolol treatment in HF rats induces a significant reduction of both circulating noradrenaline and adrenaline levels (Rengo *et al*., 2009). This latter property of β-blocker treatment is particularly important for, at least, two main reasons. First, catecholamines exert negative effects on the heart and peripheral vasculature also through α-adrenoceptors, which are not antagonized by β-blockers (Lamba and Abraham, 2000). Second, SNS outflow can enhance and perpetuate the hyperactivation of other neurohormonal axes, such as the renin–angiotensin and endothelin systems (Goldsmith, 2004; Lee and Tkacs, 2008). Thus, β-blocker treatment, by reducing HF-related SNS overdrive, is also able to prevent the noxious effects of catecholamines on α_2-adrenoceptors and to diminish activity of other cardiotoxic neurohormonal systems.

However, it is important to note that there are also studies showing no effects of β-blocker on circulating catecholamine levels (Santostasi *et al*., 1998; The RESOLVD Investigators, 2000; Blanchet *et al*., 2003; Foucart *et al*., 1991; Gilbert *et al*., 1996; Al-Hesayen *et al*., 2005; Lowes *et al*., 2002) and thus, the ability of β-blocker to reduce SNS hyperactivity in HF patients is still a matter of debate. Such discrepancies among studies might be attributed to several factors: (1) the particular β-blocker agent used (e.g. non-subtype-selective vs. β_1-adrenoceptor-selective drugs); (2) duration of treatment (long-term vs. short-term); (3) HF severity (NYHA class and ejection fraction); (4) catecholamine levels before starting treatment (high vs. low levels). In our model of severe HF, due to the induction of a large MI (infarct size ~45%), documented by the dramatically impaired ejection fraction, altered LV remodelling and high circulating catecholamine levels, we found almost a normalization of circulating catecholamine levels in HF/B compared with HF/C group.

As mentioned above, in the SNS nerve endings and in the adrenal glands, catecholamine secretion is tightly regulated by adrenoceptors acting as 'presynaptic autoreceptors' in this regard (Lymperopoulos *et al*., 2007b). Thus, the possibility of reducing catecholamine levels using β-blockers has already been suggested and is mechanistically linked to the blockade of the facilitatory β_2-adrenoceptors presynaptic autoreceptor subtype (Foucart *et al*., 1988; 1991). In our study we chose bisoprolol, a β_1-adrenoceptor-selective blocker, in order to preclude any involvement of the β_2-adrenoceptors subtype, which is purported to be a facilitatory presynaptic autoreceptor in the adrenals and SNS nerve terminals (Foucart *et al*., 1988; 1991; Lymperopoulos *et al*., 2007a). Of note, the bisoprolol-blocked β_1-adrenoceptors, contrary to the β_2-adrenoceptors, appear not involved at all in neuronal or adrenal catecholamine release and hence in the modulation of catecholamine levels (Foucart *et al*., 1988; 1991; Lymperopoulos *et al*., 2007a). Moreover, several lines of evidence have shown that the β_2-adrenoceptor signals and functions in a substantially different manner from the β_1-adrenoceptor in cardiomyocytes. Importantly, the detrimental effects of sympathetic overdrive in the failing heart seem to be conferred mainly by cardiac β_1- rather than β_2-adrenoceptors (Zhu *et al*., 2005).

It should be mentioned here that whether the observed effects of bisoprolol on the adrenal GRK2–α_2-adrenoceptor-catecholamine axis secretion axis are receptor-dependent still remains an open question that we plan to address in future experiments. For example, it is not clear from our present results that GRK2 lowering in the adrenal gland (and myocardium) is specifically due to β_1-adrenoceptor blockade *per se* or whether there could be a receptor-independent event such as a more general beneficial actions of the drug on the heart, which indirectly feeds back to the adrenal medulla to suppress catecholamine secretion. However, we also investigated the effects of bisoprolol treatment on adrenal GRK2–α_2-adrenoceptor-catecholamine production axis at an earlier time point: 2 weeks after initiation of bisoprolol treatment (6 weeks post MI). At this time point and, although there was no difference between the ejection fraction and left ventricular end-diastolic diameter between bisoprolol-treated and HF control groups, we still observed a significant reduction in the mRNA levels of HF-related remodelling/fibrosis genes in the bisoprolol-treated group (Supplemental Figures S6 and S7). However, we did not find any differences in GRK2 protein levels and α_2-adrenoceptor function between bisoprolol and HF control groups (Supplemental Figures S8 and S9). Taken together, these latter results, although not conclusive, suggest that bisoprolol-dependent reverse remodelling preceded GRK2 and normalization of α_2-adrenoceptors. Another important question that warrants further investigation in the future is whether these beneficial adrenal effects of bisoprolol are shared by other agents in this drug class (β-blockers) or at least by the other β_1-adrenoceptor-selective blockers that have been shown to be beneficial in HF, such as metoprolol.

An interesting therapeutic issue may be explained by our data on adrenal GRK2- α_2-adrenoceptor signalling following chronic β_1-adrenoceptor blockade. The poor sympatho-inhibitory efficacy of the α_2-adrenoceptor agonist moxonidine in HF patients might be ascribable to GRK2-dependent α_2-adrenoceptor dysfunction. In fact, the MOXSE and MOXCON trials (Swedberg *et al*., 2000; 2002), two recent clinical trials of moxonidine for the treatment of HF, were discontinued because of excess mortality in the treatment group. A possible reason for the failure of these trials could have been the dysfunction of peripheral α_2-adrenoceptors. Thus, cardiac sympathetic nerve terminals and the adrenal glands might not adequately respond to α_2-adrenoceptor agonists because of the impaired receptor function. Importantly, in both studies, treatment with β-blockers. within 2 months before recruitment was an exclusion criterion. The present study demonstrates that β-blocker treatment was able to restore α_2-adrenoceptor function in HF, and this might result not only in the observed reduction of circulating catecholamine levels but also, perhaps, in potentially increased efficacy of moxonidine in HF treatment. Therefore, β-blocker treatment coupled with α_2-adrenoceptor agonists should be reconsidered.

Of note, these favourable molecular/physiological alterations in the adrenal gland post MI are associated with beneficial effects on the heart in terms of LV contractility and remodelling. In particular, bisoprolol treatment seems to prevent further progression of cardiac dysfunction. In fact, in HF/C rats we observed a progressive decrease in ejection fraction and increase in LV diameters by comparing the echocardiograpic results obtained at 4 and 10 weeks post MI. On the other hand, bisoprolol halted the progression of HF, as shown by the similar values in ejection fraction and LV diameters

obtained by echocardiography at 4 and 10 weeks post MI. These findings are in complete accordance with previous reports indicating that β-blocker treatment affects ejection fraction and reduces LV diameters in the failing heart (Bristow, 2000b; Sabbah, 2004; Tevaearai and Koch, 2004). Moreover, the positive effects of bisoprolol treatment in the heart are evident also at the molecular level. GRK2 is normally up-regulated in the failing heart and significantly impairs cardiac β-adrenoceptor signalling and function (Petrofski and Koch, 2003; Rengo et al., 2011). Indeed, we found a marked up-regulation of GRK2 in hearts of untreated post-MI rats, whereas bisoprolol-treated rats showed a restoration of cardiac GRK2 protein levels. Normalization of cardiac GRK2 levels represents further additional molecular evidence for cardiac improvement by β-blockers in HF (Petrofski and Koch, 2003; Rengo et al., 2009). Of course, we cannot discriminate between the beneficial effect of bisoprolol obtained through direct β-adrenoceptor blockade in the heart and the indirect effect related to the reduction of the circulating catecholamine levels. These findings are in line with previous observations by our group in the failing myocardium (Pleger et al., 2007; Rengo et al., 2009) and in the aged heart (Leosco et al., 2007) and by others in hypertensive animals (Eckhart et al., 2002). Finally, markers of adverse cardiac remodelling were normalized after bisoprolol treatment such as BNP, TGF-β1, and Col-1 in HF rats.

In conclusion, the present study suggests a novel molecular mechanism for the beneficial effects of β-blocker treatment by curbing the autonomic derangements that confound and aggravate chronic HF. In addition, this study supports our previous findings that adrenal GRK2 up-regulation is an important pathogenic mechanism for the sympathetic overdrive in HF, and its lowering or inhibition represents a novel potential sympatholytic strategy for HF therapy.

Acknowledgements

Conflict of interest

None declared.

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Supporting information

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

Figure S1 Adrenal GRK2 mRNA levels in all experimental groups at the end of the study period (Sham, $n = 7$; HF/C, $n = 8$; HF/B, $n = 8$). All values were standardized to amplified 28S rRNA. *$P < 0.05$ versus Sham or HF/B groups. ANOVA analysis and Bonferroni test among all groups. Data are presented as mean $\pm$ SEM and plotted as fold over sham values.

Figure S2 GRK2 expression in heart homogenates purified from all three experimental groups at the end of the study period ($n = 7$ for each group). Representative Western blots (upper panel) and average densitometric quantitative analysis from blots showing the ratio of GRK2 to GAPDH (lower panel). *$P < 0.05$ versus Sham or HF/B. Data are presented as mean $\pm$ SEM. ANOVA analysis and Bonferroni test among all groups.

Figure S3 Total β-adrenoceptor density in plasma membranes purified from the hearts of all three experimental groups at the end of the study ($n = 6$ for each group). *$P < 0.05$ versus Sham or HF/B. Data are presented as mean $\pm$ SEM. ANOVA analysis and Bonferroni test among all groups.

Figure S4 *In vitro* noradrenaline secretion from chromaffin cells isolated from the adrenals of sham, HF/C and HF/B rats in response to 20 μmol·L^{-1} nicotine treatment, following pretreatment with vehicle (Nicotine) or with 10 μmol·L^{-1} UK14304 (UK + Nicotine). *$P < 0.05$, compared with HF/C-UK + Nicotine, $n = 6$. Data are presented as mean $\pm$ SEM. ANOVA analysis and Bonferroni test among all groups.

Figure S5 *In vitro* noradrenaline secretion from chromaffin cells isolated from the adrenals of HF/B rats infected *in vivo* with AdGFP or AdGRK2 in response to nicotine treatment with or without pretreatment with UK14304 (UK + Nicotine). *$P < 0.05$, compared with AdGRK2-UK+Nicotine, $n = 6$. Data are presented as mean $\pm$ SEM. ANOVA analysis and Bonferroni test among all groups.

Figure S6 Ejection fraction (EF) as measured by echocardiography 6 weeks post MI (2 weeks after treatments start; upper panel); LV internal diameter at diastole (LVIDd) measured by echocardiography 2 weeks after treatments start (lower panel). HF control, $n = 6$; HF Bisoprolol, $n = 6$. Data are presented as mean $\pm$ SEM. ANOVA analysis and Bonferroni test among all groups.

Figure S7 Heart mRNA levels of TGFβ1 (upper panel) collagen type I (Col1) (lower panel); in all experimental groups at 2 weeks after treatment start (Sham, $n = 6$; HF control, $n = 6$; HF bisoprolol, $n = 6$). All values were standardized to amplified 28S rRNA. *$P < 0.05$ versus Sham; #$P < 0.05$ versus HF control. ANOVA analysis and Bonferroni test among all groups. Data are presented as mean $\pm$ SEM and plotted as fold over sham values.

Figure S8 GRK2 expression in adrenal homogenates purified from all three experimental groups at 2 weeks post-treatment start ($n = 5$ for each group). Average densitometric quantitative analysis from blots showing the ratio of GRK2 to GAPDH. *$P < 0.05$ versus Sham. Data are presented as mean $\pm$ SEM. ANOVA analysis and Bonferroni test among all groups.

Figure S9 *In vitro* adrenaline secretion from chromaffin cells isolated from the adrenals of HF control and HF bisoprolol rats (2 weeks after treatment start) in response to 20 μmol·L^{-1} nicotine treatment, following pretreatment with vehicle (Nicotine) or with 10 μmol·L^{-1} UK14304 (UK + Nicotine); $n = 5$ for each group. Data are presented as mean $\pm$ SEM. ANOVA analysis and Bonferroni test among all groups.

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