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Nebivolol prevents desensitization of β-adrenoceptor signaling and induction of cardiac hypertrophy in response to isoprenaline beyond β_1-adrenoceptor blockage

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Ozakca I, Arioglu-Inan E, Esfahani H, Altan VM, Balligand JL, Kayki-Mutlu G, Ozcelikay AT. Nebivolol prevents desensitization of β-adrenoceptor signaling and induction of cardiac hypertrophy in response to isoprenaline beyond β_1-adrenoceptor blockage. *Am J Physiol Heart Circ Physiol* 304: H1267–H1276, 2013. First published March 1, 2013; doi:10.1152/ajpheart.00352.2012.—The importance of chronic stimulation of β-adrenoceptors in the development of cardiac dysfunction is the rationale for the use of β-blockers in the treatment of heart failure. Nebivolol is a third-generation β-blocker, which has further properties including stimulation of endothelial nitric oxide synthase and/or β_3-adrenoceptors. The aim of this study was to investigate whether nebivolol has additional effects on β-adrenoceptor-mediated functional responses along with morphologic and molecular determinants of cardiac hypertrophy compared with those of metoprolol, a selective β_1-adrenoceptor blocker. Rats infused by isoprenaline (100 $\mu g \cdot kg^{-1} \cdot day^{-1}$, 14 days) were randomized into three groups according to the treatment with metoprolol (30 $mg \cdot kg^{-1} \cdot day^{-1}$), nebivolol (10 $mg \cdot kg^{-1} \cdot day^{-1}$), or placebo for 13 days starting on *day 1* after implantation of minipump. Both metoprolol and nebivolol caused a similar reduction on heart rate. Nebivolol mediated a significant improvement on cardiac mass, coronary flow, mRNA expression levels of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) and atrial natriuretic peptide and phospholamban (PLN)/SERCA2a and phospho-PLN/PLN ratio compared with metoprolol and placebo. Nebivolol prevented the detrimental effects of isoprenaline infusion on isoprenaline (68% of control at 30 μM), BRL37344 (63% of control at 0.1 μM), and forskolin (64% of control at 1 μM) responses compared with metoprolol (isoprenaline, 34% of control; BRL37344, no response; forskolin, 26% of control) and placebo (isoprenaline, 33% of control; BRL37344, 28% of control; forskolin, 12% of control). Both β-blockers improved the changes in mRNA expressions of β_1- and β_3-adrenoceptors. Our results suggest that nebivolol partially protects the responsiveness of β-adrenoceptor signaling and the development of cardiac hypertrophy independent of its β_1-adrenoceptor blocking effect.

nebivolol; inotropy; adrenergic stimulation; β-adrenoceptors

PLASMA CATECHOLAMINE LEVELS play a pivotal role in the short- and long-term regulation of cardiac function. In acute term, as seen in the fight-or-flight response, the activation of sympathetic drive induces positive effects on inotropy, chronotropy, and lusitropy, which are mediated especially by cardiac β_1-adrenoceptors. However, sustained activation of β-adrenoceptors by circulating catecholamines can cause detrimental effects on cardiac muscle. This phenomenon can be observed in the progress of the heart failure; in the early stages of the pathology, the increase of the plasma catecholamines compensates the dysfunction of the cardiac muscle, but in the long term the sympathetic overactivation contributes to dysfunction of the heart (19). The experiments made in cultured cardiac myocytes and in transgenic mice showed that β_1-adrenoceptor signaling produces greater adverse biological effects compared with β_2-adrenoceptors (3, 10, 31). As a consequence, the β-blocking agents are now considered as a first-line therapy for chronic heart failure (16). The common pharmacological property of the clinically effective β-blockers in the treatment of heart failure is to antagonize β_1-adrenoceptor activation. These agents are referred as cardioselective β-blockers (e.g., metoprolol, atenolol). Recently, a new β-blocker class, which has additional properties including vasodilation, has been assessed to get better clinical benefit in the failing heart. As a member of third-class β-blockers, nebivolol is a vasodilating β-blocker, which has been approved for the treatment of hypertension. Cardioprotective effect of nebivolol related to β_1-adrenoceptor blocking and vasodilating activity has been demonstrated in the Study of Effects of Nebivolol Intervention on Outcomes and Rehospitalization in Seniors With Heart Failure (SENIORS) trial in heart failure (13, 33). Nebivolol-induced vasodilation is mediated by nitric oxide (NO), which resulted from the β_3-adrenoceptor stimulation (1, 8, 9, 28).

In heart failure, contrary to β_1- and β_2-adrenoceptors, β_3-adrenoceptor expression is increased and no desensitization occurs to this subtype (6, 7, 24). These properties make β_3-adrenoceptors an interesting partner of theraupetic interventions in failing heart. Besides their negative inotropic effect on cardiac contractility, β_3-adrenoceptor-mediated vasodilation and NO release in the myocardium and also stimulation of cardiac Na-K ATPase activity especially in failing conditions (4) suggested the possible benefits of β_3-adrenoceptor stimulation in hyperadrenergic state. Nebivolol, combining the β_1-adrenoceptor blocking and β_3-adrenoceptor stimulating effects, could be evaluated as a unique pharmacologic agent in heart failure (1). The dual action mechanisms of nebivolol can elicit a different perspective in the regulation or interaction of β-adrenoceptor subtypes in hyperadrenergic state.

In the present study, we examined the effects of nebivolol, compared with metoprolol, on the morphologic, molecular, and functional changes of β-adrenoceptor subtypes in isoprenaline (Iso)-induced cardiac hypertrophy.

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MATERIALS AND METHODS

Animals and treatments. All animal procedures were approved by Ankara University Animal Care and Use Committees and adhered to the Directive 2010/63/EU. Animals were housed in individual cages at 22 ± 1°C and 12-h:12-h light/darkness cycle. Eight-week-old male Sprague-Dawley rats were anesthetized by ketamine-xylazine combination and treated with isoprenaline via subcutaneous osmotic minipump (Alzet, 2002; Durect, Cupertino, CA). Isoprenaline infusion rate was averaged 100 $\mu g \cdot kg^{-1} \cdot day^{-1}$ for 14 days, as determined by preliminary experiments. On the first day, 24 h after operation, rats were randomized into three group ($n = 54$): *1*) treated with placebo (inert vehicle: DMSO 30%, distilled water 70%; Iso); *2*) metoprolol tartrate (30 $mg \cdot kg^{-1} \cdot day^{-1}$; M); or *3*) nebivolol hydrochloride (10 $mg \cdot kg^{-1} \cdot day^{-1}$; N). All agents were given orally via gastric gavage for 13 days starting on *day 1*. Sham-operated rats ($n = 18$) served as controls (C). The doses of metoprolol and nebivolol were assessed in preliminary experiments according to their ability to reduce heart rate to similar levels. Starting from *day 2*, heart rate measurements were performed in all groups at the same conditions for 13 days.

Isolated rat heart preparation and experimental design. Rats were anesthetized by ether inhalation. The heart was quickly removed and placed in oxygenated Krebs solution (95% O_2-5% CO_2, pH 7.35 to 7.40) containing (in mmol/l) 120 NaCl, 25 $NaHCO_3$, 4.8 KCl, 1.2 KH_2PO_4, 1.25 $MgSO_4$, 1.25 $CaCl_2$, and 10 glucose. The aorta was cannulated and perfused with oxygenated Krebs solution at constant pressure (60 mmHg) by using the Langendorff method. A latex water-filled balloon was inserted into the left venticular chamber and connected to a pressure transducer for continous measurement of heart contractility. For each heart, the experiment was started with a progressive increase of the latex baloon inserted inside the left ventricle to generate a ventricular volume-developed pressure relationship. When the maximal pressure was reached, the left ventricular (LV) end-diastolic pressure (LVEDP) was adjusted properly. Ten minutes of equilibration in isovolumic working conditions were imposed. In spontaneously beating conditions, baseline data were recorded for at least 10 min more. After equilibration, the hearts were instantly paced at 8.3 Hz to the end of the experiment. Heart rate, LV developed pressure (LVDP), and maximum rate of positive and negative change in left ventricle pressure ($+dP/dt_{max}$ and $-dP/dt_{max}$) were measured online using a dedicated software (MP100 Data Acquisition System; Biopac-System, Goleta, CA). Flow rate values were monitored by flowmeter (Transonic Systems, Ithaca, NY) inserted to the perfusion line.

Experiments on isolated papillary muscle. In a second experimental group of rats, after anesthetization by ether inhalation, the heart was immediately removed and placed in Krebs solution. The experiments were performed as previously described (14). Papillary muscles were dissected from left ventricle, placed in an experimental chamber, and superfused at a flow rate 5 ml/min with oxygenated Krebs solution (95%O_2−5%CO_2, pH 7.35 to 7.40) and warmed (30°C). The tissues were stabilized for 60 min and stimulated at a pacing cycle length of 1,700 ms, and stimulus pulse width was 1 to 2 ms and amplitude was twice the diastolic threshold. Tension was recorded by using a mechanoelectric force transducer (HSE F30; Harvard Apparatus GmbH, March-Hugstetten, Germany). Ventricular tissues were stretched stepwise (10 μm increments) to a length at which contraction force was maximal. The experiments then were performed at 90% of maximal tension. After equilibration, cumulative concentration-response curves of isoprenaline, a nonselective β-adrenoceptor agonist, noradrenaline, a selective β_1-adrenoceptor agonist in the presence of prazosin and desipramine, fenoterol, a selective β_2-adrenoceptor agonist, BRL37344, a selective β_3-adrenoceptor agonist, and forskolin, a membrane-permeable cyclic AMP (cAMP) analog, were determined by superfusion with successive increasing concentrations of the agonists. For all concentrations, tension was measured online using an acquisition unit (MP35 Data Acquisition System, Biopac-System). At the end of the preparation, pieces of left ventricle tissue were immediately frozen in liquid nitrogen and stored at −80°C for subsequent PCR experiments.

Total RNA isolation, RT-PCR, and real-time PCR experiments. Hearts were powdered with liquid nitrogen and homogenized with an ultrasonic homogenizer (Bandelin Electronics, Berlin, Germany) before RNA extraction. Total RNA was extracted with the TRIzol reagent (Sigma-Aldrich Chemical, St. Louis, MO) according to the manufacturer's protocol. The optical density values and amounts of RNA were determined spectrophotometrically using Nanodrop (NanoDrop) at wavelength 260 nm (λ_{260}) and 280 nm (λ_{280}). In addition, 18S/28S bands were used to evaluate RNA integrity (data not shown). After DNase I treatment (Roche Diagnostics GmBH, Mannheim, Germany), 1 μg of RNA from each sample was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Quantitative real-time PCR was performed using SYBR Green 1 Master (Roche Diagnostics) on Lightcycler 480 (Roche Applied Science, Indianapolis, IN). All reactions were run in triplicates. Relative gene expression was normalized to TBP (TATA-box binding protein). Primer sequences and accession numbers used for real-time PCR to detect the expression of each target gene are shown in Table 1. The real-time PCR efficiency rate (E) in the exponential phase and gene expression ratio were calculated according to the following formulas (27): $E = 10^{[-1/slope]}$ and ratio = $(E_{Target})^{\Delta CP_{Target}(control-sample)}/(E_{Reference})^{\Delta CP_{Reference}(control-sample)}$.

SDS-gel electrophoresis, immunoblotting, and Western blot analysis. Frozen left ventricle tissues were powdered with liquid nitrogen and homogenized in ice-cold radioimmunoprecipitation assay buffer (Sigma-Aldrich Chemical) including protease inhibitor cocktail (100×; Sigma-Aldrich Chemical) and sodium orthovanadate (1 mM; Sigma-Aldrich Chemical). Homogenates were centrifuged at 1,300 *g* for 5 min at 4°C and the supernatants were centrifuged at 16,000 *g* for 30 min at 4°C, and the 16,000 *g*-supernatants were used for immunoblotting. The protein concentrations of lysates were measured with the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein from each heart lysate (20 μg/lane) were loaded

Table 1. *Nucleotide sequences for the primers, size of PCR products, and PCR amplification efficiency rate of each primer set*

Gene	GenBank ID	Primers		Product Size, bp	Efficiency Rate, E
		Forward	Reverse		
Adrenoceptor					
β_1	NM_012701.1	GCTCACCAACCTCTTCATCA	CCGTCACACATAGCACGTCT	158	2.09 (0.9974)
β_2	NM_012492	GGAATGACAGCGACTTCTTG	GATAACCGACATGAGGATGG	116	2.56 (0.9227)
β_3	NM_013108	GCAGAACTCACCGCTCAAC	CAGGCTCCTTGCTAGATCTC	90	2 (0.9905)
Sarcoplasmic reticulum Ca^{2+} ATPase	NM_001110823.2	CTCTGAGAGTTGACCAGTCGAT	AGTATTGACTCCAGTCGCCA	176	1.81 (0.9958)
Atrial natriuretic peptide	NM_012612	GGTAGGATTGACAGGATTGG	AGATGAAGACAGGAAGCTGC	192	1.83 (0.9918)
TATA-box binding protein	NM_001004198	GCAATCAACATCTCAGCAGC	TGGTGTGGCAGGAGTGATAG	154	1.78 (0.9840)

Given in parentheses are the r^2 of the standard curves. All the genes are from rat origin.

Table 2. *Effects of metoprolol and nebivolol treatments on heart rate during in vivo procedure*

Day	C	Iso	M	N	CM	CN
0	386.3 ± 8.4	372.9 ± 5.7	373.7 ± 2.5	375.4 ± 4.6	369.0 ± 3.0	359.8 ± 4.0
7	386.5 ± 3.7	527.6 ± 11.2***	462.7 ± 16.9***††	484.9 ± 14.5***†	333.8 ± 3.3**	329.4 ± 2.3***
14	374.1 ± 4.9	531.3 ± 10.2***	481.5 ± 4.4***††	470.8 ± 6.6***†††	334.4 ± 1.0*	334.0 ± 2.1**

Values are means ± SE; $n = 10$ for control (C), isoprenaline (Iso), metoprolol tartrate (M), and nebivolol hydrochloride (N) and $n = 5$ for metoprolol-treated controls (CM) and nebivolol-treated controls (CN). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. C; †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$ vs. Iso.

onto 8% SDS-polyacrylamide gels for sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERCA2a) and GAPDH and 10% tricine-SDS-polyacrylamide gels for phpspho-phospholamban and phospholamban, separated in a minigel apparatus (Mini-PROTEAN-III; Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with 2% BSA (Equitech-Bio, Kerrville, TX) in TBS-Tween and incubated overnight at 4°C with anti-SERCA2a antibody (1:5,000), anti-phospholamban (PLN) antibody (1:2,000), anti-phospho-phospholamban (Ser16/Thr17) (p-PLN) antibody (1:2,000) from Cell Signaling (Beverly, MA), and anti-GAPDH (1:10,000) from Santa Cruz Biotechnology (Santa Cruz, CA). The antigens were detected by enhanced chemiluminescence method (ECL Western Blotting Substrate; Pierce, Rockford, IL) with horseradish peroxidase-linked anti-mouse IgG (1:10,000) from Santa Cruz Biotechnology or horseradish peroxidase-linked anti-rabbit IgG (1:2,000) from Cell Signaling. Relative band densities were analyzed using ImageJ (http://rsbweb.nih.gov/ij/) and were normalized to GAPDH as loading controls.

Histomorphometric analysis. Mid-LV specimens were embedded in optimal cutting temperature (Tissue-Tek, Sakura Finetek Europe, Germany), and multiple (5 μm) transversal sections were cut with cryostat. For myocytes area and capillary density, cryo-sections were defrosted slowly in acetone and then washed in PBS for 3 × 5 min. Immunohistochemical staining of endothelial cells was preformed with Isolectin B4 (Vector Laboratories, Burlingame, CA; 1/50) 1 h at room temperature. After a wash in PBS for 3 × 5 min, Avidin-FITC (Vector Laboratories; 1/150) was applied for 2 h in the dark. After a wash in PBS for 3 × 5 min, wheat germ agglutinin (Vector Laboratories; 1/150) was applied for 2 h in the dark. After a wash in PBS for 3 × 5 min, tissues were mounted with Vectashield (Vector Laboratories) and observed with Axioimager Z1 Apotome system with MRm Rev3 AxioVision-Deconvolution 3D camera (Zeiss, NY). The data analysis was performed with Axiovison software (Zeiss, NY). Cells (50–120) from 20 sections per heart were measured.

Heart rate measurements. Heart rate was measured in triplicate by using the tail-cuff method (MAY 9610 Indirect Blood Pressure Recorder System; Commat, Ankara, Turkey) before initiation of treatments and during in vivo treatment daily.

Drugs. Isoproterenol, noradrenaline, fenoterol, BRL37344 [(±)-(R*,R*)-[4-[2-[[2-(3-Chlorophenyl)-2-hydroxyethyl]amino]propyl] phenoxy]acetic acid sodium hydrate), forskolin, prazosin, desipramine, metoprolol, and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich Chemical. Nebivolol racemate was a generous gift from Abdi Ibrahim (Istanbul, Turkey). All drugs were prepared as stock solutions in distilled water, with the exception of nebivolol, which was dissolved in DMSO.

Statistical analysis. All results are expressed as means ± SE. Stastical analysis was performed using one-way ANOVA followed by the Newman-Keuls multiple comparison test. P value <0.05 was considered statistically significant. Data were analyzed by using GraphPad Prism 5 (GraphPad Software, San Diego, CA).

RESULTS

Effects of metoprolol and nebivolol treatments on heart rate and cardiac morphology after isoprenaline infusion. During 14-day isoprenaline infusion, heart rates of rats increased ~30% compared with controls (Table 2). In β-blocker treatment protocol, the equi-effective doses were determined by assessing negative chronotropic effects of metoprolol and nebivolol on isoprenaline-induced tachycardia. Selected doses of both metoprolol and nebivolol resulted in similar reductions (9.3% for M; 11.3% for N) on isoprenaline-induced tachycardia (Table 2). It is known that chronotrophy of the heart is controlled by β_1-adrenoceptor activation. Thus it can be assumed that metoprolol and nebivolol-mediated negative chronotrophy is an indicator of their β_1-adrenoceptor blocking efficacy. After 14-day isoprenaline-infusion, cardiac hypertrophy was observed as indicated by an increased LV weight-to-body weight ratio (LV/BW) and increased cardiomyocyte size. Although no difference was observed in body weight between four groups (Fig. 1*A*), chronic infusion of isoprenaline caused an increase in LV/BW compared with controls (Fig. 1*B*). Nebivolol, but not metoprolol, treatment significantly decreased LV/BW and cardiomyocyte size after 14-day isopren-

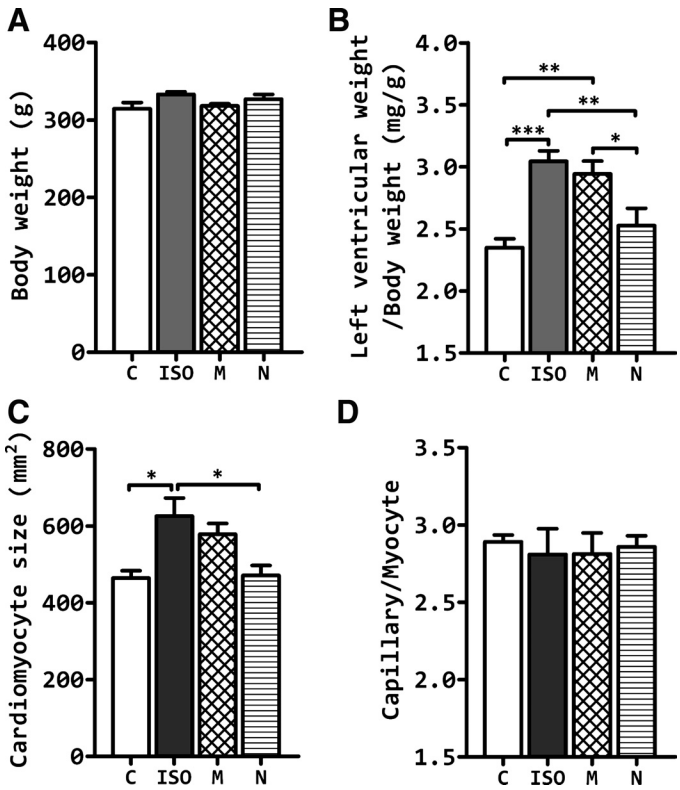

Fig. 1. Morphologic assessment of cardiac hypertrophy. *A*: evaluation of body weights of all groups at the end of 14-day isoprenaline (Iso) infusion and/or β-blocker treatment. *B*: left ventricular (LV) weight-to-body weight ratio of all experimental groups ($n = 9$–11 per group). *C*: cardiomyocyte size. *D*: capillaries per myocyte. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by post hoc analysis. C, control; M, metoprolol tartrate; N, nebivolol hydrochloride.

aline infusion (Fig. 1, *B* and *C*). The number of capillary per myocyte did not change among four groups (Fig. 1*D*).

Effects of metoprolol and nebivolol treatments after isoprenaline infusion on isolated heart. To further characterize the hypertrophy model and to evaluate the possible effect(s) of β-blocker treatments on cardiac hemodynamics, myocardial function was evaluated using an isolated Langendorff heart preparation. The performance of isolated heart preparations in control and isoprenaline-treated groups was observed in the absence (−) and presence (+) of pacing. Spontaneous heart rate was similar among all groups [C vs. Iso vs. M vs. N (beats/min): 190 ± 15 vs. 213 ± 14 vs. 242 ± 16 vs. 227 ± 15; $P > 0.05$ in all groups]. Without pacing, LVDP was found to be significantly decreased in Iso, M, and N groups compared with control; despite LVEDP, +dP/d*t* and −dP/d*t* were not different among all groups (Fig. 2). In the present model, all hearts were perfused at constant pressure (60 mmHg). This model allowed evaluation of differences in coronary flow as an indicator of coronary vascular resistance and elimination of flow demand variances of each heart due to the increase in cardiac mass in response to isoprenaline infusion. Pacing at 8.3 Hz was selected because it closely resembled the in vivo heart rate of isoprenaline-treated rats. With pacing, LVDP fell in all groups; the reduction in Iso, M, and N groups was not found to be significant compared with controls with pacing (Fig. 2*A*). Pacing significantly increased the LVEDP in all groups compared with that in the absence of pacing (Fig. 2*B*). In a detailed analysis of LVEDP values in the presence of pacing, only nebivolol treatment induced less increase in end-diastolic pressure, suggesting a better diastolic filling was performed in response to nebivolol (Fig. 2*B*). The coronary flow was normalized to the LV weight (flow/LV). In the absence of pacing, coronary flow (flow/LV) slightly reduced in response to isoprenaline infusion (Fig. 2*C*). Although metoprolol treatment did not improve the basal coronary flow, nebivolol treatment significantly restored coronary flow compared with Iso and M group (Fig. 2*C*). With pacing, coronary flow tended to increase in control and β-blocker-treated groups but not in Iso group; however, in N group the coronary flow was found significantly different compared with the M group (Fig. 2*C*). In the presence of pacing, +dP/d*t* and −dP/d*t* were not different among all groups (Fig. 2, *D* and *E*).

Effects of metoprolol and nebivolol treatments after isoprenaline infusion on functional responses on papillary muscle. Fourteen-day isoprenaline infusion resulted in ~67%, 57%, and 72% reduction of the positive inotropic responses to isoprenaline, noradrenaline, and fenoterol, respectively, with a significant shift to rightforward in concentration-response curves compared with controls (Table 3 and Fig. 3). In addition to isoprenaline infusion, metoprolol treatment affected neither E_{max} nor pD_2 values compared with Iso group in concentration-response curves of all three agonists (Table 3). On the other hand, nebivolol treatment increased the E_{max} of isoprenaline response ~68% of control group and also shifted the isoprenaline concentration-response curve rightforward compared with the Iso group (Table 3 and Fig. 3*A*). Nebivolol produced a slight increase in the maximum response of noradrenaline and also shifted the noradrenaline concentration-response curve to rightforward compared with the Iso group (Table 3 and Fig. 3*B*). Like metoprolol, nebivolol had no effect on the E_{max} value of fenoterol concentration-response curve. Although metoprolol treatment had no effect on pD_2 value, nebivolol shifted the fenoterol concentration-response curve to rightforward compared with the Iso group (Table 3 and Fig. 3*C*).

BRL37344 mediated negative inotropy at concentrations between 0.01 and 100 nM and positive inotropy at 300 and 1,000 nM concentrations on isolated papillary muscles of controls (Fig. 4*A*). The concentration-dependent opposite inotropic effects of BRL37344 have been explained as becoming nonselective on β-adrenoceptors at high concentrations and mediating positive inotropic effects through β_1- and/or β_2-adrenoceptors. In Iso and M groups, not only the negative inotropic effect of BRL37344 but also positive inotropic effect observed at high concentrations were abolished. On the contrary, nebivolol treatment improved BRL37344-induced negative inotropic effect at 100 nM (C vs. N: 100.0 ± 14.8% vs. 62.91 ± 11.51%; $P < 0.05$) (Fig. 4*B*). At the concentrations higher than 100 nM, unlike controls, BRL37344-mediated positive inotropy has been completely abolished in the N group.

The inotropic responses of forskolin were obtained at three different concentrations (1, 3, and 10 μM) cumulatively. The percentage of the inotropic response to forskolin at 1 μM was reduced significantly in all groups compared with controls (Fig. 5, *A* and *B*). At 3 μM, however, only the inotropic

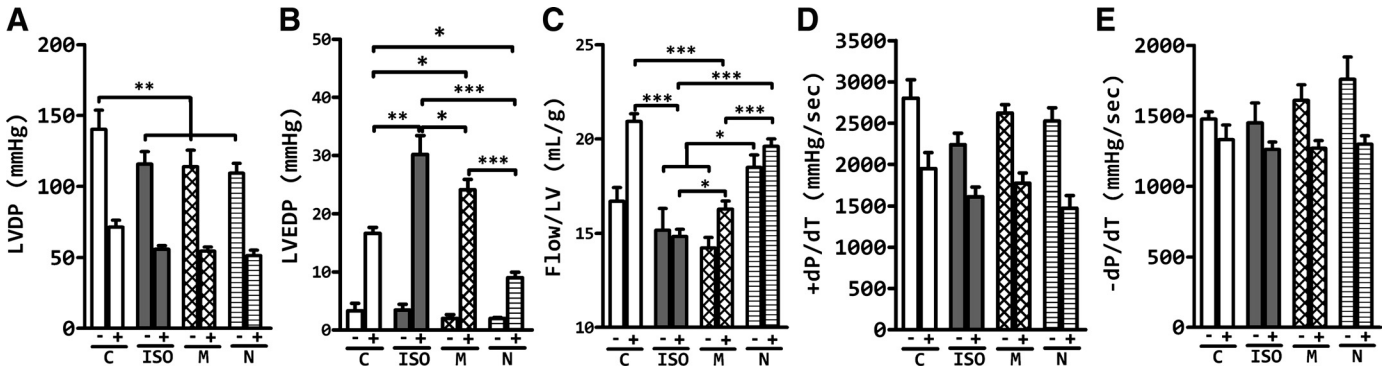

Fig. 2. Baseline Langendorff isolated heart data. LV developed pressure (LVDP; *A*), LV end-diastolic pressure (LVEDP; *B*), flow/LV (*C*), positive change in LV pressure (+dP/dt; *D*), and negative change in LV pressure (−dP/dt; *E*) values were assessed in the absence (−) and presence (+) of pacing (n = 5–7 per group). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ by post hoc analysis.

Table 3. *The pD_2 and E_{max} values of β-adrenoceptor agonists with different selectivities on isolated papillary muscle*

	C	Iso	M	N
Iso				
pD_2	7.60 ± 0.06	6.91 ± 0.08***	6.91 ± 0.20***	6.32 ± 0.01***†††‡‡
E_{max}, %	150.08 ± 15.76	50.47 ± 7.44***	51.86 ± 4.97***	101.56 ± 8.30*†‡
Noradrenaline				
pD_2	6.70 ± 0.06	6.17 ± 0.07**	6.06 ± 0.19***	5.53 ± 0.10***†††‡
E_{max}, %	123.56 ± 9.98	53.51 ± 5.35***	45.32 ± 8.43***	72.33 ± 12.94*
Fenoterol				
pD_2	6.77 ± 0.10	6.11 ± 0.06**	6.06 ± 0.19**	5.39 ± 0.24***†
E_{max}, %	235.33 ± 24.63	66.39 ± 8.18***	45.70 ± 8.64***	75.63 ± 15.02***

Values are means ± SE; $n = 6–10$ per group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. C; †$P < 0.05$; ††$P < 0.01$; †††$P < 0.001$ vs. Iso; ‡$P < 0.05$; ‡‡$P < 0.01$.

responses of isoprenaline- and metoprolol-treated groups were found to be significantly different compared with controls (Fig. 5*A*). There was no significant difference among all groups at 10 μM (Fig. 5*A*).

Effect of metoprolol and nebivolol treatments after isoprenaline infusion on the mRNA expression levels of cardiac β-adrenoceptor subtypes and cardiac hypertrophy markers. Chronic isoprenaline infusion caused a significant decrease in β_1-adrenoceptor mRNA levels (Fig. 6*A*). Both metoprolol or nebivolol treatments increased the β_1-adrenoceptor mRNA levels compared with C and Iso group (Fig. 6*A*). Nebivolol treatment was found to be more effective to increase β_1-adrenoceptor expression levels compared with metoprolol. Both isoprenaline infusion and β-blocker treatments caused an increase in β_2-adrenoceptor mRNA levels. However, only nebivolol treatment was found to be significantly different compared with control group (Fig. 6*B*). Chronic infusion of isoprenaline resulted in a significant upregulation of β_3-adrenoceptor mRNA levels compared with controls (Fig. 6*C*). Both metoprolol and nebivolol treatments caused significant reductions in upregulated β_3-adrenoceptor mRNA levels compared with C and Iso group (Fig. 6*C*).

Downregulation of SERCA2a mRNA levels, which is known as a marker of cardiac hypertrophy, was observed on our experimental cardiac hypertrophy model (Fig. 6*D*). Although metoprolol treatment failed to improve the downregulated SERCA2a mRNA levels, nebivolol therapy increased the mRNA levels of SERCA2a to the control levels (Fig. 6*D*). The mRNA levels of atrial natriuretic peptide (ANP), another gene evaluated as a marker of cardiac hypertrophy in the present study, were increased significantly in response to isoprenalin infusion as referred in literature (17, 32) (Fig. 6*E*). Metoprolol treatment had no effect on the upregulated ANP mRNA levels (Fig. 6*E*); on the other hand, nebivolol decreased the mRNA levels of ANP to the control values (Fig. 6*E*).

Effect of metoprolol and nebivolol treatments after isoprenaline infusion on the excitation-contraction coupling protein levels. Western blot analysis indicated that SERCA2a expression was decreased in response to isoprenaline infusion (C vs. Iso: 100.0 ± 5.2% vs. 82.3 ± 2.8%; $P < 0.05$) (Fig. 7*A*). The treatment with metoprolol did not change the reduction, but nebivolol treatment induced a slight amelioration on SERCA2a protein level (M vs. N: 83.2 ± 1.8% vs. 87.8 ± 4.8%, $P > 0.05$) (Fig. 7*A*). Total PLN was markedly decreased (C vs. Iso vs. M vs. N: 100.0 ± 3.8% vs. 70.3 ± 13.1% vs. 56.6 ± 5.0% vs. 102.2 ± 17.9%; C vs. M, $P < 0.05$; M vs. N, $P < 0.05$), so the ratio of PLN to SERCA2a was found to be reduced in Iso and M groups (Fig. 7*B*), but nebivolol treatment improved the PLN-to-SERCA2a ratio to control levels (Fig. 7*B*). The ratio of PLN Ser16/Thr17 phosphorylation (C vs. Iso vs. M vs. N: 100.0 ± 14.6% vs. 99.6 ± 10.4% vs. 65.5 ± 11.1% vs. 70.3 ± 25.8%; $P > 0.05$ in all groups) to total PLN was increased in

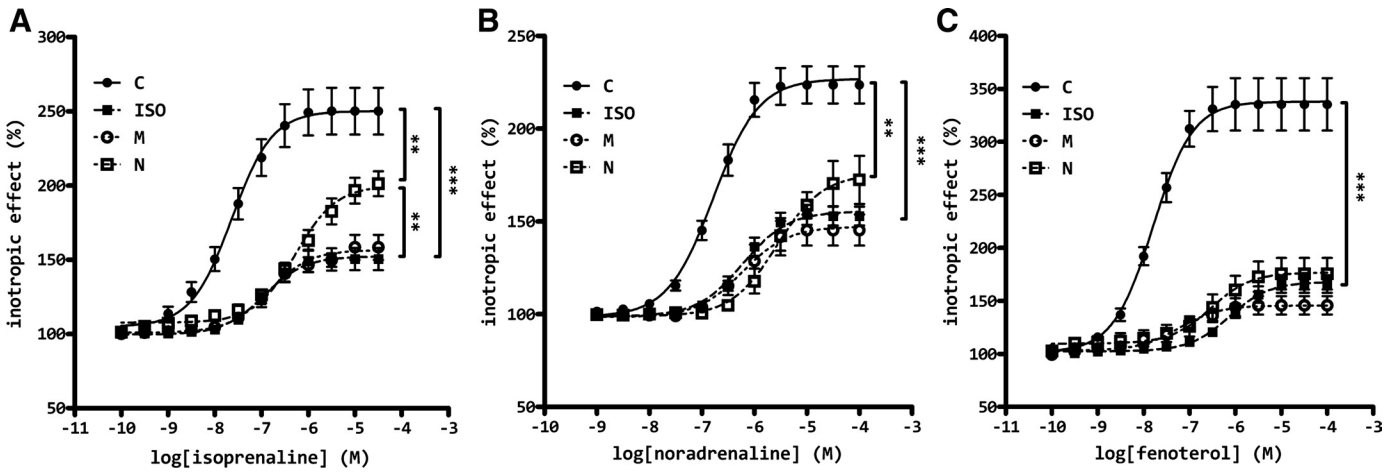

Fig. 3. Inotropic responses of several β-adrenoceptor agonists. Concentration-response curves of isoprenaline (*A*; $n = 8–10$ per group), noradrenaline (*B*; $n = 6–8$ per group), and fenoterol (*C*; $n = 6–8$ per group) on isolated papillary muscle preparations at the end of 14-day experimental procedures are shown. For noradrenaline, experiments were performed in the presence of an α_1-adrenoceptor antagonist, prazosin (1 μM), and uptake2 inhibitor, desipramine (10 μM). **$P < 0.01$; ***$P < 0.001$ by post hoc analysis.

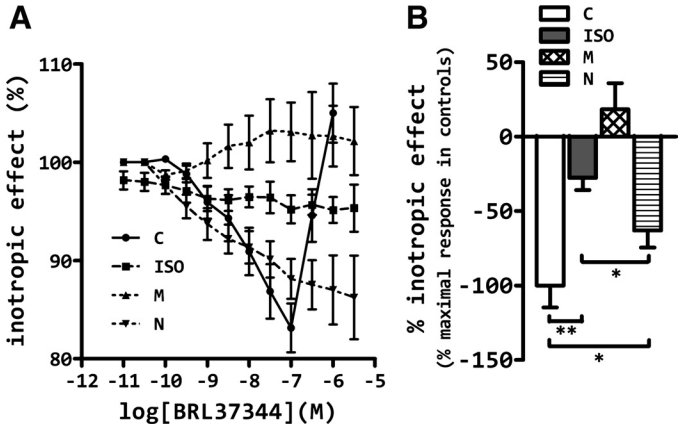

Fig. 4. Inotropic responses of BRL37344. Concentration-response curves of BRL37344 (*A*) and percent changes of 100 nM BRL37344-mediated inotropic responses (*B*) on isolated papillary muscle preparations at the end of 14-day experimental procedures. For *B*, the responses obtained with 100 nM BRL37344 in controls were expressed as 100%. Statistical significance of 100 nM of BRL37344 was performed by 1-way ANOVA among C, Iso, and N followed by Newman-Keuls multiple comparison test ($n = 6$ per group). $^{*}P < 0.05$; $^{**}P < 0.01$.

Iso and M groups, but nebivolol treatment reduced the p-PLN/PLN significantly (Fig. 7*C*). Collectively, these results indicate that in hyperadrenergic state the reduction in SERCA2a protein level could be compensated with release of inhibitory effect of PLN on SERCA2a activation. The opposite effect of nebivolol on excitation-contraction coupling proteins is paralleled with its reversal effects on cardiac hypertrophy.

DISCUSSION

The present study demonstrated that nebivolol partially prevented the changes in β-adrenoceptor-mediated inotropic responses along with morphologic and molecular determinants of cardiac hypertrophy induced by isoprenaline infusion. Metoprolol treatment, on the other hand, was found to be ineffective on the same parameters, suggesting that nebivolol has some additional properties, which would be important in its action mechanism. The superior characteristic of nebivolol over cardioselective β-blockers is to produce NO- and/or β_3-adrenoceptor-mediated vasodilation (8, 9, 28).

Consistent with the previous studies (21), isoprenaline infusion successfully induced a cardiac hypertrophy model in the present study. The properties of the experimental model are *1*) increased heart rate during isoprenaline infusion, *2*) increased left ventricle weight-to-body weight ratio and cardiomyocyte size, and *3*) changes in the mRNA expressions of SERCA2a and ANP, which are known as hypertrophy markers in left ventricular tissues of the rats. As a result of isoprenaline infusion, decreased LVDP values can also be evaluated as a marker of cardiac dysfunction (Fig. 3, *A* and *B*). In this hypertrophy model, the functional responses were evaluated in the isolated papillary muscle preparations in the presence of nonselective (isoprenaline), selective β_1- (noradrenaline in the presence of prazosin and desipramine), selective β_2- (fenoterol), selective β_3- (BRL37344) adrenoceptor agonists, and forskolin, membrane-permeable cAMP activator.

Several groups have shown the vasodilating effect of nebivolol in different vascular beds. Among them, coronary microvessels, a major site for the regulation of coronary resistance and perfusion reserve, could have remarkable therapeutic importance. Our results showed that nebivolol treatment induced a better perfusion in the isolated heart, which is observed as increased coronary flow in a constant-pressure perfused Langendorff system. The size of cardiomyocytes was reduced in response to nebivolol, although the number of capillaries per myocyte did not change. Thus the effect of nebivolol on coronary flow seems to be associated with vasodilation. The effect of pacing on LVEDP also showed that nebivolol treatment caused a better relaxation during diastole compared with all other groups.

Our experiments with forskolin allow us to test the target at which nebivolol acts to increase β-adrenoceptor signaling. As a direct activation of adenylate cyclase, forskolin-induced contractile performance was also improved by nebivolol treatment. These results led us to focus on the possible action site of nebivolol. The improvement on isoprenaline-, BRL37344-, and forskolin-mediated responses induced by nebivolol treatment suggested that nebivolol may affect a common pathway, which probably involves downstream signaling of β_1- and

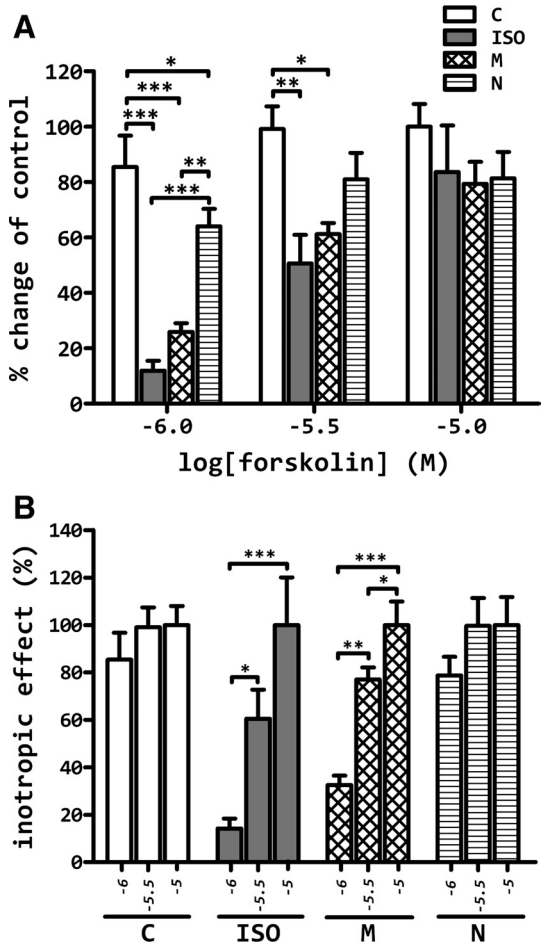

Fig. 5. Inotropic responses of forskolin. *A*: inotropic responses obtained at different forskolin concentrations (1, 3, and 10 μM). Values are shown as percent change of maximum inotropic response of controls. *B*: inotropic responses of each group obtained at different forskolin concentrations (1, 3, and 10 μM). Values are shown as percent change of maximum inotropic response of each group, so for each group maximum inotropic response was accepted as 100% ($n = 6$ per group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by post hoc analysis.

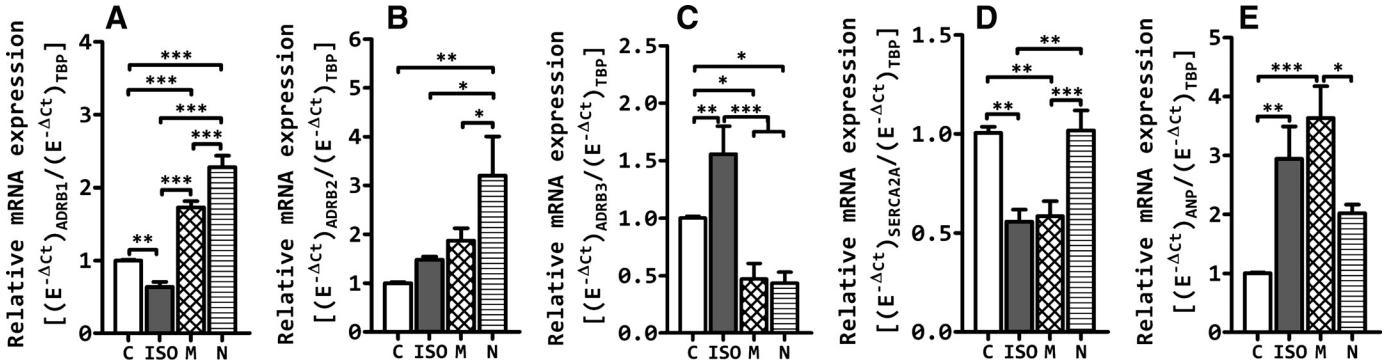

Fig. 6. Changes in expressions of β-adrenoceptor subtypes and cardiac hypertrophy markers. β_1-Adrenoceptor (*A*), β_2-adrenoceptor (*B*), β_3-adrenoceptor (*C*), sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a; *D*), and atrial natriuretic peptide (ANP; *E*) mRNA levels are shown. Values were normalized to TATA-box binding protein (TBP) and expressed as percent control values ($n = 6–8$ per group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by post hoc analysis.

β_3-adrenoceptors and also cAMP signaling. Although the possible changes in protein expressions of β-adrenoceptors have not been investigated in the current study, the comparison of receptor-mediated (isoprenaline) and cAMP-mediated (forskolin) responses among all groups support this conclusion.

The second major outcome is the fact that in contrast with metoprolol, nebivolol induced a decrease on cardiac mass and an improvement on mRNA expressions of cardiac hypertrophy markers. As shown in the current study, re-expression of fetal genes such as ANP and alteration in the expression of Ca^{2+} handling proteins such as SERCA2a are observed in cardiac hypertrophy (20). As is well known, the downregulation of SERCA2a could be associated with the sustained cytosolic Ca^{2+} elevation, which results in the activation of hypertrophic signal in the myocyte (20). In the light of demonstrating both reduction of cardiac mass and alterations in the expressions of cardiac hypertrophy markers in nebivolol-treated group, it could be speculated that nebivolol acts through a mechanism that overcomes cytosolic Ca^{2+} overload, independent from its β_1-adrenoceptor blocking effect.

Other evidence of the surmounted effect of nebivolol on Ca^{2+} overload is the change of protein expressions of SERCA2a and PLN. In the heart, intracellular Ca^{2+} homeostasis is under control of SERCA2a activity, which controls both the rate of cytosolic Ca^{2+} removal and the degree of SR Ca^{2+} load. The rate of Ca^{2+} removal by SR is regulated mainly

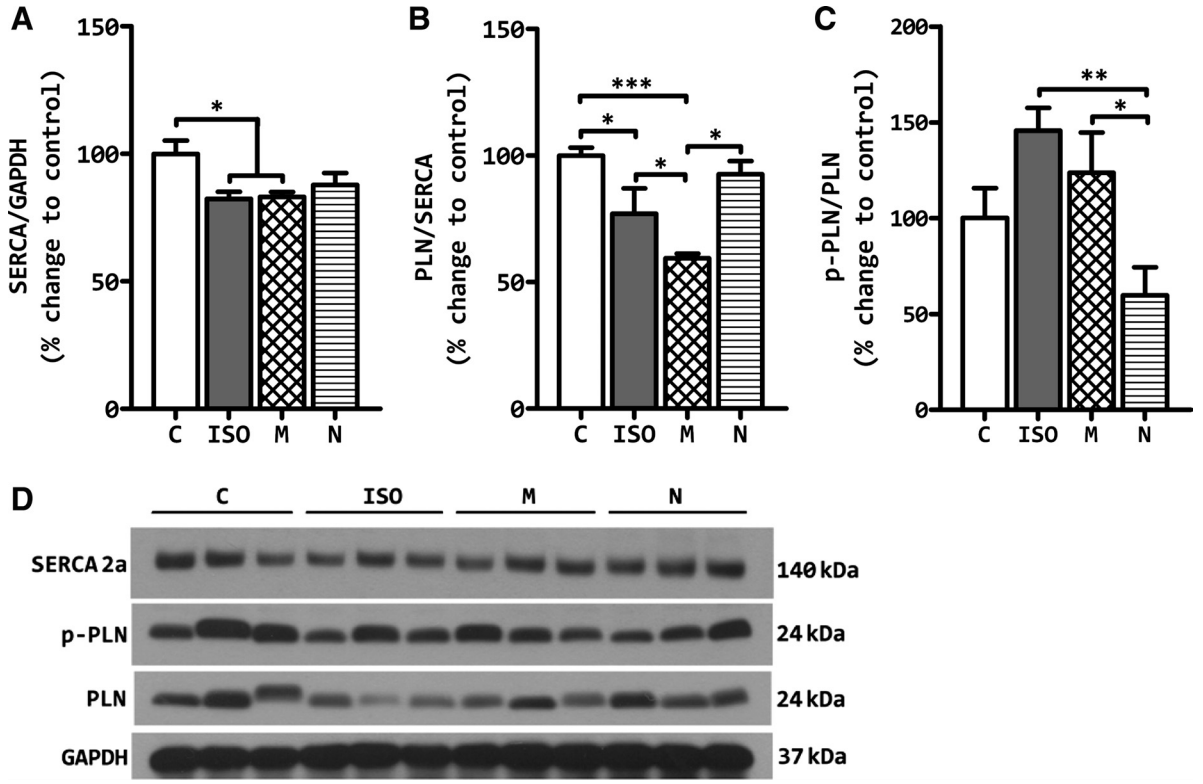

Fig. 7. Analysis of excitation-contraction coupling proteins. SERCA2a (*A*), SERCA2a/phospholamban (PLN; *B*), phospho-PLN/PLN (*C*), and representative Western blotting images ($n = 3$ for each group) (*D*) are shown. Values were normalized to GAPDH as a lane loading control and expressed as percent control values ($n = 5$ to 6 per group). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by post hoc analysis.

by the expression level of SERCA2a, PLN, and its phosphorylated form (18). β-adrenoceptor-mediated PLN phosphorylation increases the rate of Ca^{2+} transport from cytosolic compartment into SR lumen. The increased phosphorylation of PLN and increased Ca^{2+} levels accumulated in the SR accelerates relaxation and increases contractility, which contribute to the increased cardiac output after sympathetic activation (15). As in the present study, the reduction in SERCA2a expression protein level in response to isoprenaline infusion was shown in previous studies (29). However, both reduced PLN-to-SERCA2a ratio and increased phosphorylation of PLN indicate the removal of restriction on SERCA2a activity in Iso and M groups. The altered regulations of PLN on SERCA2a activity can point out a compensatory mechanism for decreased SERCA2a expression to maintain the cardiac function in isoprenaline-induced cardiac hypertrophy. It is possible that such a compensation mechanism that is observed in the early stages of cardiac hypertrophy may prevent the progression to decompensated stage of heart failure. In nebivolol-treated group, abolition of compensatory regulation of PLN can indicate the reversal effect of nebivolol on impaired Ca^{2+} handling. The effects of nebivolol treatment on p-PLN/PLN can be observed as a reduction in LVEDP in the presence of pacing (Fig. 2*B*) and restoration of inotropic responses to ligands, which have different affinities (Fig. 3*A*, Fig. 4*B*, and Fig. 5*B*). The antibody used in the present study recognizes both phosphorylation sites for PLN [Ser16 for protein kinase A (PKA) and Thr17 for Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)], so we could not detect the respective contribution of both kinases to phosphorylation of PLN.

The conventional β-adrenoceptor/cAMP/PKA downstream elements have been known as a well-described signaling cascade of β-adrenoceptor stimulation. In addition to this cascade, the activation of CaMKII by enhanced intracellular Ca^{2+} from β-adrenoceptor stimulation has been demonstrated (34, 38). It is important to note that CaMKII activation also plays a pivotal role in certain pathological conditions like chronic β-adrenoceptor stimulation-induced cardiac hypertrophy (22, 31) and heart failure (40). The results of two recent studies have pointed out a possible aspect about the synergistic effects of CaMKII and NADPH oxidase activation and reactive oxygen species production (11, 26) in diseased heart. It could be possible to point out a relationship between increased cardiac oxidative stress by stimulation of NADPH oxidase activity (36, 37) and enhanced CaMKII activity (22, 31), both induced by chronic isoprenaline infusion. Although nebivolol-induced inhibition of NADPH oxidase activity is not shown in the present study, several groups observed the mentioned effect of nebivolol in different studies (12, 25, 30, 35, 39). The effects of nebivolol treatment seem not to be selective for a receptor subtype or mediator, because nebivolol restores both isoprenaline- and BRL37344-induced inotropic responses together with receptor-independent pathways of β-adrenoceptor signaling (forskolin). Based on the hypothesis discussed above, Na-K ATPase is an attractive cellular regulatory pump that may affect Ca^{2+} handling in cardiomyocytes. In failing conditions, β_3-adrenoceptor stimulation reduces the glutathionylation of Na-K pump through NO synthase-dependent mechanism and by attenuating Na^+ levels decreases Ca^{2+} overload (4). This mechanism can also explain the counteracting effect of nebivolol on Ca^{2+}-regulated hypertrophic responses.

In the current study, isoprenaline infusion induced a downregulation in the mRNA expression levels of β_1-adrenoceptors together with an upregulation in the mRNA expression levels of β_2- and β_3-adrenoceptors. Both metoprolol and nebivolol treatments induced upregulation of decreased β_1-adrenoceptor mRNA levels and downregulation of increased β_3-adrenoceptor mRNA levels. It can be suggested that only β_1-adrenoceptor blockage can be enough to improve the changes on mRNA levels of β-adrenoceptors induced by isoprenaline infusion. Although the mRNA levels of β_1- and β_3-adrenoceptors were ameolirated in response to both β-blockers, only nebivolol treatment resulted in improvement of the functional responses.

One of the important characteristics of the β_3-adrenoceptors is being activated at plasma catecholamine levels higher than required for β_1-/β_2-adrenoceptors. In this point of view, in addition to the downregulation and desensitization mechanisms of β_1-/β_2-adrenoceptors in sympathetic overstimulation, β_3-adrenoceptor activation is thought to act as a counter mechanism in the hyperadrenergic conditions. The state of cardiac β_3-adrenoceptors in the presence of high plasma catecholamine levels is still an unanswered question. In hyperadrenergic conditions although there is no consensus regarding the functions of the β_3-adrenoceptors, the molecular regulation of these receptors has been detected by many groups (6, 7, 24). The idea that the role of β_3-adrenoceptor stimulation could be more than negative inotropism in sympathetic overactivation has been supported by transgenic models. The studies that were performed in genetically modified mice showed that β_3-adrenoceptors protect the heart from detrimental effects of sustained hyperadrenergic state (23). In failing conditions although β_3-adrenoceptor-mediated negative inotropic reponse is abolished, the deletion of β_3-adrenoceptors worsened cardiac remodelling and cardiac hypertrophy partially caused by absence of coupled state of endothelial nitric oxide synthase (23). In addition, β_3-adrenoceptor knockout mice are not protected from myocardial ischemia/reperfusion injury following exercise (5). The protective role of β_3-adrenoceptor overexpression in isoprenaline-induced hypertrophic model was also observed in mice (2).

A potential limitation of the present study is the lack of changes in protein expression of β-adrenoceptor subtypes. Especially the changes in β_3-adrenoceptor mRNA levels could not easily be correlated with the improvement in BRL37344-mediated inotropic responses in β-blocker-treated groups. According to the present data, β_1-adrenoceptor blockage (with both metoprolol and nebivolol) seems to be effective in the regulation of β_1- and β_3-adrenoceptor mRNA levels, but the inotropic responses improved only in nebivolol-treated group.

In conclusion, our results suggest that nebivolol treatment ameliorates the β-adrenoceptor signaling pathway via β_3-adrenoceptor stimulation in the presence of adrenergic overstimulation. The involvement of β_3-adrenoceptor activation as a mechanism for nebivolol action has been supported by the lack of improvement in response to metoprolol therapy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: I.O., V.M.A., and A.T.O. completed conception and design of research; I.O., E.A.I., H.E., and G.K.M. performed experiments; I.O. and H.E. analyzed data; I.O., H.E., V.M.A., J.-L.B., and A.T.O. interpreted results of experiments; I.O. prepared figures; I.O. drafted the manuscript; I.O., E.A.I., V.M.A., J.-L.B., and A.T.O. edited and revised manuscript; I.O., V.M.A., J.-L.B., and A.T.O. approved final version of manuscript.

REFERENCES

1. **Balligand JL.** β_3-Adrenoceptor stimulation on top of β_1-adrenoceptor blockade "Stop or Encore?" *J Am Coll Cardiol* 53: 1539–1542, 2009.
2. **Belge C, Sekkali B, Tavernier G, Poulier AC, Bertrand L, Vanoverschelde JL, Hilfiker-Kleiner D, Langin D, Balligand JL.** Cardiomyocyte-specific overexpression of β_3-adrenoceptors attenuates the hypertrophic response to catecholamines in vivo (Abstract). *Circulation* 116: II_148, 2007.
3. **Bisognano JD, Weinberger HD, Bohlmeyer TJ, Pende A, Raynolds MV, Sastravaha A, Roden R, Asano K, Blaxall BC, Wu SC, Communal C, Singh K, Colucci W, Bristow MR, Port DJ.** Myocardial-directed overexpression of the human β_1-adrenergic receptor in transgenic mice. *J Mol Cell Cardiol* 32: 817–830, 2000.
4. **Bundgaard H, Liu CC, Garcia A, Hamilton EJ, Huang Y, Chia KK, Hunyor SN, Figtree GA, Rasmussen HH.** β_3-Adrenergic stimulation of the cardiac Na+-K+ pump by reversal of an inhibitory oxidative modification. *Circulation* 122: 2699–2708, 2010.
5. **Calvert JW, Condit ME, Aragón JP, Nicholson CK, Moody BF, Hood RL, Sindler AL, Gundewar S, Seals DR, Barouch LA, Lefer DJ.** Exercise protects against myocardial ischemia-reperfusion injury via stimulation of β_3-adrenergic receptors and increased nitric oxide signaling: role of nitrite and nitrosothiols. *Circ Res* 108: 1448–1458, 2011.
6. **Cheng HJ, Zhang ZS, Onishi K, Ukai T, Sane DC, Cheng CP.** Upregulation of functional β_3-adrenergic receptor in the failing canine myocardium. *Circ Res* 89: 599–606, 2001.
7. **Dincer UD, Bidasee KR, Güner S, Tay A, Ozcelikay AT, Altan VM.** The effect of diabetes on expression of β_1-, β_2-, and β_3-adrenoreceptors in rat hearts. *Diabetes* 50: 455–461, 2001.
8. **de Groot AA, Mathy MJ, van Zwieten PA, Peters SL.** Involvement of the β_3 adrenoceptor in nebivolol-induced vasorelaxation in the rat aorta. *J Cardiovasc Pharmacol* 42: 232–236, 2003.
9. **Dessy C, Saliez J, Ghisdal P, Daneau G, Lobysheva II, Frérart F, Belge C, Jnaoui K, Noirhomme P, Feron O, Balligand JL.** Endothelial β_3-adrenoreceptors mediate nitric oxide-dependent vasorelaxation of coronary microvessels in response to the third-generation β-blocker nebivolol. *Circulation* 112: 1198–1205, 2005.
10. **Engelhardt S, Hein L, Wiesmann F, Lohse MJ.** Progressive hypertrophy and heart failure in β_1-adrenergic receptor transgenic mice. *Proc Natl Acad Sci USA* 96: 7059–7064, 1999.
11. **Erickson JR, Joiner ML, Guan X, Kutschke W, Yang J, Oddis CV, Bartlett RK, Lowe JS, O'Donnell SE, Aykin-Burns N, Zimmerman MC, Zimmerman K, Ham AJ, Weiss RM, Spitz DR, Shea MA, Colbran RJ, Mohler PJ, Anderson ME.** A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell* 133: 462–474, 2008.
12. **Fang Y, Nicol L, Harouki N, Monteil C, Wecker D, Debunne M, Bauer F, Lallemand F, Richard V, Thuillez C, Mulder P.** Improvement of left ventricular diastolic function induced by β-blockade: a comparison between nebivolol and metoprolol. *J Mol Cell Cardiol* 51: 168–176, 2011.
13. **Flather MD, Shibata MC, Coats AJ, Van Veldhuisen DJ, Parkhomenko A, Borbola J, Cohen-Solal A, Dumitrascu D, Ferrari R, Lechat P, Soler-Soler J, Tavazzi L, Spinarova L, Toman J, Böhm M, Anker SD, Thompson SG, Poole-Wilson PA; Investigators SENIORS.** Randomized trial to determine the effect of nebivolol on mortality and cardiovascular hospital admission in elderly patients with heart failure (SENIORS). *Eur Heart J* 26: 215–225, 2005.
14. **Gauthier C, Laurent K, Charpentier F, Drouin E, Chevallier JC, Le Marec H.** Endomyocardial biopsies: a new approach for studying the electrical and mechanical properties of human ventricular myocardium. *J Mol Cell Cardiol* 26: 1267–1271, 1994.
15. **Hagemann D, Xiao RP.** Dual site phospholamban phosphorylation and its physiological relevance in the heart. *Trends Cardiovasc Med* 12: 51–56, 2002.
16. **Heart Failure Society of America, Lindenfeld J, Albert NM, Boehmer JP, Collins SP, Ezekowitz JA, Givertz MM, Katz SD, Klapholz M, Moser DK, Rogers JG, Starling RC, Stevenson WG, Tang WH, Teerlink JR, Walsh MN.** HFSA 2010 Comprehensive Heart Failure Practice Guideline. *J Card Fail* 16: e1–e194, 2010.
17. **Izumo S, Nadal-Ginard B, Mahdavi V.** Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. *Proc Natl Acad Sci USA* 85: 339–343, 1988.
18. **Kranias EG, Hajjar RJ.** Modulation of cardiac contractility by the phospholamban/SERCA2a regulatome. *Circ Res* 110: 1646–1660, 2012.
19. **Lohse MJ, Engelhardt S, Eschenhagen T.** What is the role of β-adrenergic signaling in heart failure? *Circ Res* 93: 896–906, 2003.
20. **Lorell BH, Carabello BA.** Left ventricular hypertrophy: pathogenesis, detection, and prognosis. *Circulation* 102: 470–479, 2000.
21. **McMartin L, Summers RJ.** Functional analysis of desensitization of the β-adrenoceptor signalling pathway in rat cardiac tissues following chronic isoprenaline infusion. *Br J Pharmacol* 127: 1012–1020, 1999.
22. **Métrich M, Lucas A, Gastineau M, Samuel JL, Heymes C, Morel E, Lezoualc'h F.** Epac mediates β-adrenergic receptor-induced cardiomyocyte hypertrophy. *Circ Res* 102: 959–965, 2008.
23. **Moens AL, Leyton-Mange JS, Niu X, Yang R, Cingolani O, Arkenbout EK, Champion HC, Bedja D, Gabrielson KL, Chen J, Xia Y, Hale AB, Channon KM, Halushka MK, Barker N, Wuyts FL, Kaminski PM, Wolin MS, Kass DA, Barouch LA.** Adverse ventricular remodeling and exacerbated NOS uncoupling from pressure-overload in mice lacking the β_3-adrenoreceptor. *J Mol Cell Cardiol* 47: 576–585, 2009.
24. **Moniotte S, Kobzik L, Feron O, Trochu JN, Gauthier C, Balligand JL.** Upregulation of β_3-adrenoceptors and altered contractile response to inotropic amines in human failing myocardium. *Circulation* 103: 1649–1655, 2001.
25. **Oelze M, Daiber A, Brandes RP, Hortmann M, Wenzel P, Hink U, Schulz E, Mollnau H, von Sandersleben A, Kleschyov AL, Mülsch A, Li H, Förstermann U, Münzel T.** Nebivolol inhibits superoxide formation by NADPH oxidase and endothelial dysfunction in angiotensin II-treated rats. *Hypertension* 48: 677–684, 2006.
26. **Pandey D, Gratton JP, Rafikov R, Black SM, Fulton DJ.** Calcium/calmodulin-dependent kinase II mediates the phosphorylation and activation of NADPH oxidase 5. *Mol Pharmacol* 80: 407–415, 2011.
27. **Pfaffl MW.** A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45, 2001.
28. **Rozec B, Quang TT, Noireaud J, Gauthier C.** Mixed β_3-adrenoceptor agonist and alpha$_1$-adrenoceptor antagonist properties of nebivolol in rat thoracic aorta. *Br J Pharmacol* 147: 699–706, 2006.
29. **Shibata M, Takeshita D, Obata K, Mitsuyama S, Ito H, Zhang GX, Takaki M.** NHE-1 participates in isoproterenol-induced downregulation of SERCA2a and development of cardiac remodeling in rat hearts. *Am J Physiol Heart Circ Physiol* 301: H2154–H2160, 2011.
30. **Sorrentino SA, Doerries C, Manes C, Speer T, Dessy C, Lobysheva I, Mohmand W, Akbar R, Bahlmann F, Besler C, Schaefer A, Hilfiker-Kleiner D, Lüscher TF, Balligand JL, Drexler H, Landmesser U.** Nebivolol exerts beneficial effects on endothelial function, early endothelial progenitor cells, myocardial neovascularization, and left ventricular dysfunction early after myocardial infarction beyond conventional β_1-blockade. *J Am Coll Cardiol* 57: 601–611, 2011.
31. **Sucharov CC, Mariner PD, Nunley KR, Long C, Leinwand L, Bristow MR.** A β_1-adrenergic receptor CaM kinase II-dependent pathway mediates cardiac myocyte fetal gene induction. *Am J Physiol Heart Circ Physiol* 291: H1299–H1308, 2006.
32. **van den Bosch BJ, Lindsey PJ, van den Burg CM, van der Vlies SA, Lips DJ, van der Vusse GJ, Ayoubi TA, Doevendans PA, Smeets HJ.** Early and transient gene expression changes in pressure overload-induced cardiac hypertrophy in mice. *Genomics* 88: 480–488, 2006.
33. **van Veldhuisen DJ, Cohen-Solal A, Böhm M, Anker SD, Babalis D, Roughton M, Coats AJ, Poole-Wilson PA, Flather MD, Investigators SENIORS.** B-blockade with nebivolol in elderly heart failure patients with impaired and preserved left ventricular ejection fraction: data From

SENIORS (Study of Effects of Nebivolol Intervention on Outcomes and Rehospitalization in Seniors With Heart Failure). *J Am Coll Cardiol* 53: 2150–2158, 2009.

34. **Wang W, Zhu W, Wang S, Yang D, Crow MT, Xiao RP, Cheng H.** Sustained β_1-adrenergic stimulation modulates cardiac contractility by Ca^{2+}/calmodulin kinase signaling pathway. *Circ Res* 95: 798–806, 2004.
35. **Whaley-Connell A, Habibi J, Johnson M, Tilmon R, Rehmer N, Rehmer J, Wiedmeyer C, Ferrario CM, Sowers JR.** Nebivolol reduces proteinuria and renal NADPH oxidase-generated reactive oxygen species in the transgenic Ren2 rat. *Am J Nephrol* 30: 354–360, 2009.
36. **Zhang GX, Kimura S, Nishiyama A, Shokoji T, Rahman M, Yao L, Nagai Y, Fujisawa Y, Miyatake A, Abe Y.** Cardiac oxidative stress in acute and chronic isoproterenol-infused rats. *Cardiovasc Res* 65: 230–238, 2005.
37. **Zhang GX, Ohmori K, Nagai Y, Fujisawa Y, Nishiyama A, Abe Y, Kimura S.** Role of AT_1 receptor in isoproterenol-induced cardiac hypertrophy and oxidative stress in mice. *J Mol Cell Cardiol* 42: 804–811, 2007.
38. **Zhang R, Khoo MS, Wu Y, Yang Y, Grueter CE, Ni G, Price EE Jr, Thiel W, Guatimosim S, Song LS, Madu EC, Shah AN, Vishnivetskaya TA, Atkinson JB, Gurevich VV, Salama G, Lederer WJ, Colbran RJ, Anderson ME.** Calmodulin kinase II inhibition protects against structural heart disease. *Nat Med* 11: 409–417, 2005.
39. **Zhou X, Ma L, Habibi J, Whaley-Connell A, Hayden MR, Tilmon RD, Brown AN, Kim JA, Demarco VG, Sowers JR.** Nebivolol improves diastolic dysfunction and myocardial remodeling through reductions in oxidative stress in the Zucker obese rat. *Hypertension* 55: 880–888, 2010.
40. **Zhu WZ, Wang SQ, Chakir K, Yang DM, Zhang T, Brown JH, Devic E, Kobilka BK, Cheng H, Xiao RP.** Linkage of β_1-adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca^{2+}/calmodulin kinase II. *J Clin Invest* 111: 617–625, 2003.