Nebivolol prevents desensitization of β-adrenoceptor signaling and induction of cardiac hypertrophy in response to isoprenaline beyond β₁-adrenoceptor blockage

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Submitted 2 May 2012; accepted in final form 25 February 2013

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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 sympathethetic drive induces positive effects on inotropy, chronotropy, and lusitropy, which are mediated especially by cardiac β₁-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 β₁-adrenoceptor signaling produces greater adverse biological effects compared with β₂-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 β₁-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 β₁-adrenoceptor blocking and vasodilative 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 β₃-adrenoceptor stimulation (1, 8, 9, 28).

In heart failure, contrary to β₁- and β₂-adrenoceptors, β₃-adrenoceptor expression is increased and no desensitization occurs to this subtype (6, 7, 24). These properties make β₃-adrenoceptors an interesting partner of therapeuitic interventions in failing heart. Besides their negative inotropic effect on cardiac contractility, β₃-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 β₃-adrenoceptor stimulation in hyperadrenergic state. Nebivolol, combining the β₁-adrenoceptor blocking and β₃-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.
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 isopenalin via subcutaneous osmotic mini-pump (Alzet, 2002; Durect, Cupertino, CA). Isopenalin infusion rate was averaged 100 μg·kg⁻¹·day⁻¹ for 14 days, as determined by preliminary experiments. On the first day, 24 h after operation, rats were randomized into three groups (n = 54): (i) treated with placebo (inert vehicle: DMSO 30%, distilled water 70%; Iso); (ii) metoprolol tartrate (30 mg·kg⁻¹·day⁻¹; M); or (iii) nebivolol hydrochloride (10 mg·kg⁻¹·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% O₂-5% CO₂, pH 7.35 to 7.40) containing (in mmol/l) 120 NaCl, 25 NaHCO₃, 4.8 KCl, 1.2 KH₂PO₄, 1.25 MgSO₄, 1.25 CaCl₂, 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 ventricular chamber and connected to a pressure transducer for continuous measurement of heart contractility. For each heart, the experiment was started with a progressive increase of the latex balloon 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 8.5 Hz to the end of the experiment. Heart rate, LV developed pressure (LVPD), 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%O₂-5%CO₂, 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 mechanicoelectric 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 isopenalin, a nonselective β-adrenoceptor agonist, noradrenaline, a selective β₁-adrenoceptor agonist in the presence of prazosin and desipramine, fenoterol, a selective β₂-adrenoceptor agonist, BRL37344, a selective β₂-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 (λ₂₆₀) and 280 nm (λ₂₈₀). 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 Transcripter 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 TB (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): $E = 10^{-\frac{1}{\text{slope}}}$. $\Delta_{\text{C(T) Target}}$ = $\Delta_{\text{C(T) Standard}}$ and ratio = $(E_{\text{Target (sample)}} E_{\text{Target (control)}}) / (E_{\text{Reference (sample)}} E_{\text{Reference (control)}})$.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank ID</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product Size, bp</th>
<th>Efficiency Rate, E</th>
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<tr>
<td>Adrenocceptor</td>
<td></td>
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<tr>
<td>β₁</td>
<td>NM_012701.1</td>
<td>GCTGACCACAACCTCTTCATGA</td>
<td>CGTGCCACCATAGACGCTTCT</td>
<td>158</td>
<td>2.09 (0.9974)</td>
</tr>
<tr>
<td>β₂</td>
<td>NM_012492</td>
<td>GGATAGGGAGGACATTCTTG</td>
<td>GATAACCGACATGGAGTGG</td>
<td>116</td>
<td>2.56 (0.9227)</td>
</tr>
<tr>
<td>β₃</td>
<td>NM_013108</td>
<td>CGGAAACTCAAGGGCCTAAA</td>
<td>CAGGCTTCTCTGACTATCTC</td>
<td>90</td>
<td>2.0 (0.9905)</td>
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<td>Sarcoplasmic reticulum Ca²⁺ ATPase</td>
<td>NM_001110823.2</td>
<td>CTCCTGGAATTCGGACGCCTGAT</td>
<td>AGATTTGACTGGACGGCA</td>
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<td>1.81 (0.9958)</td>
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<tr>
<td>Atrial natriuretic peptide</td>
<td>NM_012612</td>
<td>GGTAGGTTGAGCAGGATTGG</td>
<td>AGATGAGACAGGAGAATGAC</td>
<td>192</td>
<td>1.83 (0.9918)</td>
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<tr>
<td>TATA-box binding protein</td>
<td>NM_001004198</td>
<td>GGAATCAGCAGCTACAGACG</td>
<td>TGTTGGCAGGAGAATGAC</td>
<td>154</td>
<td>1.78 (0.9840)</td>
</tr>
</tbody>
</table>

Given in parentheses are the r² of the standard curves. All the genes are from rat origin.
onto 8% SDS-polyacrylamide gels for sarcoplasmic reticulum (SR) Ca\(^{2+}\) ATPase (SERCA2a) and GAPDH and 10% tricine-SDS-polyacrylamide gels for phospho-phospholamban and phospholamban, separated in a minigel apparatus (Mini-PROTENAE-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) 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 5 min. Immunohistochemical staining of endothelial cells was performed 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 Axiosimager Z1 Aporeme system with MRm Rev3 AxioVision-Deconvolution 3D camera (Zeiss, NY). The data analysis was performed with Axiovision 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-[(3-Chlorophenyl)-2-hydroxyethyl]amino]propyl] phenoxylacetic 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. Statistical 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 β1-adrenoceptor activation. Thus it can be assumed that metoprolol and nebivolol-mediated negative chronotropy is an indicator of their β1-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. 1A), chronic infusion of isoprenaline caused an increase in LV/BW compared with controls (Fig. 1B). Nebivolol, but not metoprolol, treatment significantly decreased LV/BW and cardiomyocyte size after 14-day isoprenaline-infusion.

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

<table>
<thead>
<tr>
<th>Day</th>
<th>C</th>
<th>Iso</th>
<th>M</th>
<th>N</th>
<th>CM</th>
<th>CN</th>
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<tr>
<td>0</td>
<td>386.3 ± 8.4</td>
<td>372.9 ± 5.7</td>
<td>373.7 ± 2.5</td>
<td>375.4 ± 4.6</td>
<td>369.0 ± 3.0</td>
<td>359.8 ± 4.0</td>
</tr>
<tr>
<td>7</td>
<td>386.5 ± 5.7</td>
<td>527.6 ± 11.2***</td>
<td>462.7 ± 16.9***↑↑</td>
<td>484.9 ± 14.5***↑↑</td>
<td>333.8 ± 3.3*</td>
<td>329.4 ± 2.3***</td>
</tr>
<tr>
<td>14</td>
<td>374.1 ± 4.9</td>
<td>531.3 ± 10.2***</td>
<td>481.5 ± 4.4***↑↑</td>
<td>470.8 ± 6.6***↑↑</td>
<td>354.4 ± 1.0*</td>
<td>334.0 ± 2.1**</td>
</tr>
</tbody>
</table>

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.
Effects of metoprolol and nebivolol treatments after isoprorenaline 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 isoprorenaline-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/dt and −dP/dt 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 isoprorenaline infusion. Pacing at 8.3 Hz was selected because it closely resembled the in vivo heart rate of isoprorenaline-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. 2A). Pacing significantly increased the LVEDP in all groups compared with that in the absence of pacing (Fig. 2B). 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. 2B). 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 isoprorenaline infusion (Fig. 2C). Although metoprolol treatment did not improve the basal coronary flow, nebivolol treatment significantly restored coronary flow compared with Iso and M group (Fig. 2C). 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. 2C). In the presence of pacing, +dP/dt and −dP/dt were not different among all groups (Fig. 2, D and E).

Effects of metoprolol and nebivolol treatments after isoprorenaline infusion on functional responses on papillary muscle. Fourteen-day isoprorenaline infusion resulted in ~67%, 57%, and 72% reduction of the positive inotropic responses to isoprorenaline, noradrenaline, and fenoterol, respectively, with a significant shift to rightward in concentration-response curves compared with controls (Table 3 and Fig. 3). In addition to isoprorenaline infusion, metoprolol treatment affected neither E\textsubscript{max} nor PD\textsubscript{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\textsubscript{max} of isoprorenaline response ~68% of control group and also shifted the isoprorenaline concentration-response curve rightward compared with the Iso group (Table 3 and Fig. 3A). Nebivolol produced a slight increase in the maximum response of noradrenaline and also shifted the noradrenaline concentration-response curve to rightward compared with the Iso group (Table 3 and Fig. 3B). Like metoprolol, nebivolol had no effect on the E\textsubscript{max} value of fenotrol concentration-response curve. Although metoprolol treatment had no effect on PD\textsubscript{2} value, nebivolol shifted the fenotrol concentration-response curve to rightward compared with the Iso group (Table 3 and Fig. 3C).

BRL37344 mediated negative inotropy at concentrations between 0.01 and 100 nM and positive inotropy at 300 and 1000 nM concentrations on isolated papillary muscles of controls (Fig. 4A). 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\textsubscript{2}- and/or β\textsubscript{3}-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. 4B). 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

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

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 β1-adrenoceptor mRNA levels (Fig. 6A). Both metoprolol or nebivolol treatments increased the β1-adrenoceptor mRNA levels compared with C and Iso group (Fig. 6A). Nebivolol treatment was found to be more effective to increase β1-adrenoceptor expression levels compared with metoprolol. Both isoprenaline infusion and β-blocker treatments caused an increase in β2-adrenoceptor mRNA levels. However, only nebivolol treatment was found to be significantly different compared with control group (Fig. 6B). Chronic infusion of isoprenaline resulted in a significant upregulation of β3-adrenoceptor mRNA levels compared with controls (Fig. 6C). Both metoprolol and nebivolol treatments caused significant reductions in upregulated β3-adrenoceptor mRNA levels compared with C and Iso group (Fig. 6C).

Downregulation of SERCA2a mRNA levels, which is known as a marker of cardiac hypertrophy, was observed on our experimental cardiac hypertrophy model (Fig. 6D). Although metoprolol treatment failed to improve the downregulated SERCA2a mRNA levels, nebivolol therapy increased the mRNA levels of SERCA2a to the control levels (Fig. 6D). 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 isoprenaline infusion as referred in literature (17, 32) (Fig. 6E). Metoprolol treatment had no effect on the upregulated ANP mRNA levels (Fig. 6E); on the other hand, nebivolol decreased the mRNA levels of ANP to the control values (Fig. 6E).

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. 7A). 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. 7A). 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. 7B), but nebivolol treatment improved the PLN-to-SERCA2a ratio to control levels (Fig. 7B). 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

### Table 3. The pD2 and E_max values of β-adrenoceptor agonists with different selectivities on isolated papillary muscle

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>Iso</th>
<th>M</th>
<th>N</th>
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<tbody>
<tr>
<td>Iso</td>
<td>pD2</td>
<td>7.60 ± 0.06</td>
<td>6.91 ± 0.08***</td>
<td>6.91 ± 0.20***</td>
</tr>
<tr>
<td></td>
<td>E_max, %</td>
<td>150.08 ± 15.76</td>
<td>50.47 ± 7.44***</td>
<td>51.86 ± 4.97***</td>
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<tr>
<td>Noradrenaline</td>
<td>pD2</td>
<td>6.70 ± 0.06</td>
<td>6.17 ± 0.07**</td>
<td>6.06 ± 0.19***</td>
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<tr>
<td></td>
<td>E_max, %</td>
<td>123.56 ± 9.98</td>
<td>53.51 ± 5.35***</td>
<td>45.32 ± 8.43***</td>
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<tr>
<td>Fenoterol</td>
<td>pD2</td>
<td>6.77 ± 0.10</td>
<td>6.11 ± 0.06**</td>
<td>6.06 ± 0.19***</td>
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<tr>
<td></td>
<td>E_max, %</td>
<td>235.33 ± 24.63</td>
<td>66.39 ± 8.18***</td>
<td>45.70 ± 8.64***</td>
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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.
Iso and M groups, but nebivolol treatment reduced the p-PLN/PLN significantly (Fig. 7C). 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 β1-blockers is to produce NO- and/or β3-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 β1- (noradrenaline in the presence of prazosin and desipramine), selective β2- (fenoterol), selective β3- (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 β1- and β3-adrenoceptor pathways.
β-1-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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ overload, independent from its β-1-adrenoceptor blocking effect.

Other evidence of the surmounted effect of nebivolol on Ca²⁺ overload is the change of protein expressions of SERCA2a and PLN. In the heart, intracellular Ca²⁺ homeostasis is under control of SERCA2a activity, which controls both the rate of cytosolic Ca²⁺ removal and the degree of SR Ca²⁺ load. The rate of Ca²⁺ removal by SR is regulated mainly

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**Fig. 6.** Changes in expressions of β-adrenoceptor subtypes and cardiac hypertrophy markers. β₁-adrenoceptor (A), β₂-adrenoceptor (B), β₃-adrenoceptor (C), sarcoplasmic reticulum Ca²⁺ 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.

**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). β3-adrenoceptor-mediated PLN phosphorylation increases the rate of Ca2+ transport from cytosolic compartment into SR lumen. The increased phosphorylation of PLN and increased Ca2+ 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 Ca2+ handling. The effects of nebivolol treatment on p-PLN/PLN can be observed as a reduction in LVEDP in the presence of pacing (Fig. 2B) and restoration of inotropic responses to ligands, which have different affinities (Fig. 3A, Fig. 4B, and Fig. 5B). The antibody used in the present study recognizes both phosphorylation sites for PLN [Ser16 for protein kinase A (PKA) and Thr17 for Ca2+/calmodulin-dependent protein kinase II (CaMKII)], so we could not detect the respective contribution of both kinases to phosphorylation of PLN.

The current study, isoprenaline infusion induced a down-regulation in the mRNA expression levels of β1-adrenoceptors together with an upregulation in the mRNA expression levels of β2- and β3-adrenoceptors. Both metoprolol and nebivolol treatments induced upregulation of decreased β1-adrenoceptor mRNA levels and downregulation of increased β3-adrenoceptor mRNA levels. It can be suggested that only β1-adrenoceptor blockage can be enough to improve the changes on mRNA levels of β-adrenoceptors induced by isoprenaline infusion. Although the mRNA levels of β1- and β3-adrenoceptors were ameliorated in response to both β-blockers, only nebivolol treatment resulted in improvement of the functional responses.

One of the important characteristics of the β3-adrenoceptors is being activated at plasma catecholamine levels higher than required for β1-/β2-adrenoceptors. In this point of view, in addition to the downregulation and desensitization mechanisms of β1-β2-adrenoceptors in sympathetic overstimulation, β3-adrenoceptor activation is thought to act as a counter mechanism in the hyperadrenergic conditions. The state of cardiac β3-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 β3-adrenoceptors, the molecular regulation of these receptors has been detected by many groups (6, 7, 24). The idea that the role of β3-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 β3-adrenoceptors protect the heart from detrimental effects of sustained hyperadrenergic state (23). In failing conditions although β3-adrenoceptor-mediated negative inotropic response is abolished, the deletion of β3-adrenoceptors worsened cardiac remodelling and cardiac hypertrophy partially caused by absence of coupled state of endothelial nitric oxide synthase (23). In addition, β3-adrenoceptor knockout mice are not protected from myocardial ischemia/reperfusion injury following exercise (5). The protective role of β3-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 β3-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, β1-adrenoceptor blockage (with both metoprolol and nebivolol) seems to be effective in the regulation of β1- and β3-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 β3-adrenoceptor stimulation in the presence of adrenergic overstimulation. The involvement of β3-adrenoceptor activation as a mechanism for nebivolol action has been supported by the lack of improvement in response to metoprolol therapy.

ACKNOWLEDGMENTS

We thank Hilal Ozdag from Ankara University Biotechnology Institute for support to perform real-time PCR experiments and for allowing the author access to laboratory facilities.
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