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Lack of inverse agonistic activity of nebivolol, its D- and L-enantiomers and of in vivo metabolized nebivolol in human myocardium

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Abstract

The pharmacological profile of nebivolol may be mediated by its enantiomers and/or its hydroxylated metabolites. Therefore, the cardiac effects of the nebivolol enantiomers as well as of serum specimens containing hydroxylated nebivolol metabolites were studied in human myocardium. For control, the β_1-adrenoceptor selective antagonist metoprolol was used. After pre-stimulation of force of contraction with forskolin (0.3 μM) or isoprenaline (0.01 μM), force developement was decreased only at high concentrations ($\geq$ 300 nM) of nebivolol or its enantiomers in isolated trabeculae. Nebivolol and its enantiomers, in contrast to metoprolol (0.4 μM: − 72% basal force), produced only minor negative intropic effects in isolated trabeculae under basal conditions. Basal force of contraction was not decreased by in vivo metabolized nebivolol in pharmacological concentrations. Neither D- nor L-nebivolol (30 μM) influenced myofibrillar Ca^{2+} responsiveness. Nebivolol and the D-enantiomer, but not the L-enantiomer (all 0.5 μM), improved the frequency-dependent force generation. D-Nebivolol, in contrast to L-nebivolol, was a β_1-adrenoceptor selective compound in membrane preparations from non-failing donor hearts. In conclusion, nebivolol and its enantiomers as well as in vivo metabolized nebivolol produce only minor negative inotropic effects. This and the finding that nebivolol and its D-enantiomer improve the frequency-dependent force generation may be of particular advantage when treating patients with already compromised cardiac function.

Keywords: Nebivolol; Nebivolol, enantiomer; Nebivolol, metabolite; Contractility; Myocardium, human

1. Introduction

Nebivolol is a lipophilic, highly β_1-adrenoceptor selective antagonist without intrinsic sympathomimetic activity (Brixius et al., 2001; Janssens et al., 1989). In recent in vitro studies on the cardiac action, nebivolol has been shown to have minor cardiodepressive effects (Brixius et al., 2001; Bundkirchen et al., 2001), which cannot be explained by lowering peripheral resistance. The minor cardiodepressive effect could not be attributed to an agonistic stimulation of the "putative" β_4-adrenoceptor by nebivolol as it was assumed for bucindolol (Bundkirchen et al., 2002) and was also not attributed to an increase in myofibrillar Ca^{2+} sensitivity (Bundkirchen et al., 2001).

In contrast to other β-adrenoceptor antagonists, nebivolol does not acutely depress cardiac contractility but rather improves ventricular function. This holds true in patients with coronary heart disease (Goldstein et al., 1993), in hypertensive patients (Sanchez, 1991), patients with dilated cardiomyopathy (Wisenbaugh et al., 1993) and healthy volunteers (De Crée et al., 1990, 1992). This beneficial pharmacological profile of nebivolol may partly be attributed to additional vasodilating properties of the β-adrenoceptor antagonist. It has been shown that nebivolol reduces peripheral resistance (Van de Water et al., 1988) and relaxes coronary arteries (Gao et al., 1991) mediated by nitric oxide liberation (Cockcroft et al., 1995).

Nebivolol is a racemic mixture of two enantiomers: D-nebivolol and L-nebivolol. After a single 5 mg oral dose, peak plasma drug concentrations (C_{max}) for unchanged

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nebivolol were 1.48 μg/l in healthy volunteers (Kamali et al., 1997) and those for the active fractions of D-nebivolol and L-nebivolol plus their corresponding hydroxylated metabolites were 7.3 μg/l for D-nebivolol and 13.1 μg/l for L-nebivolol in hypertensive patients (Himmelmann et al., 1996). Thus, the pharmacological profile of nebivolol in cardiac tissue in vivo may differ from its in vitro effects. The aim of the present study was to investigate, whether an enantiomer- or a metabolite-specific inotropic effect may contribute to the unique mode of action of nebivolol. Therefore, the present study investigates the direct cardiac effects and the β_1-adrenoceptor selectivity of D-nebivolol and L-nebivolol as well as their hydroxylated metabolites in human myocardium. Since the hydroxylated metabolites of nebivolol are not available, we studied serum specimens taken from healthy volunteers 2 h after oral administration of nebivolol. For comparison, the negative inotropic effect of metoprolol was examined.

2. Materials and methods

2.1. Human myocardial tissue and serum

2.1.1. Left ventricular myocardium

Left ventricular failing myocardium was obtained from patients suffering from heart failure functionally classified NYHA IV due to dilateted or ischemic cardiomyopathy after cardiectomy during heart transplantation (mean age: 55 ± 4.6 years, $n = 6$, 5 male, 1 female). The preoperative medication consisted of diuretics, angiotensin-converting enzyme inhibitors, cardiac glycosides and nitrates as needed. Patients receiving catecholamines, β-adrenoceptor antagonists or Ca^{2+} channel antagonists were withdrawn from the study. Non-failing human myocardium was obtained from donors with brain death caused by traumatic injury ($n = 3$; 2 males, 1 female; age: 45 ± 4.8 years). The non-failing hearts could not be transplanted for technical reasons. None of the patients had received β-adrenoceptor antagonist treatment before injury.

2.1.2. Right atrial myocardium

Right atrial tissue was taken from patients undergoing aortocoronary bypass operation (mean age: 65 ± 1.77 years, $n = 36$, 6 female, 30 male) without clinical signs of cardiac failure as measured by heart catheterization and by echocardiography. None of the patients had received Ca^{2+} channel antagonists within 7 days of surgery, or β-adrenoceptor agonists 48 h before surgery. Drugs used for general anesthesia were flunitrazepam, fentanyl and rocuronium bromide with propofol.

2.1.3. Serum samples

Serum specimens from 10 healthy volunteers were collected before and 2 h after 5 mg nebivolol was administered orally (T_{max} of nebivolol after a single oral dose is 2.7 ± 1.2 h with a C_{max} of 159 ± 61 ng/ml and a terminal half-time of 22.0 ± 8.2 h; Van Rooy, 1994, unpublished data). The serum specimens was centrifuged (2000 × g, 20 min) and then stored at − 80 °C until further use.

2.2. Measurement of force of contraction

The experiments were performed on isolated, electrically driven (1 Hz, 37 °C, 1.8 mM Ca^{2+}), isometrically contracting muscle preparations. The experiments were performed as previously described (Brixius et al., 2001). The inotropic effect of the β-adrenocepotor antagonists were investigated performing cumulative concentration–response curves (0.01–3 μM) after pre-stimulation with isoprenaline (0.01 μM), forskolin (0.3 μM) or under unstimulated conditions. In addition, the influence of nebivolol, D-NEB or L-NEB was measured on frequency-dependent force generation (0.5 to 3 Hz). A control was obtained from the same muscle preparations before the administration of the respective compound.

Experiments investigating the inotropic effects of serum specimens of nebivolol were performed by adding the serum specimens to the organ bath. By this approach, the organ bath consisted 2/3 serum and 1/3 of a Tyrode's solution. As calculated from the available data on nebivolol's pharmacokinetics (Himmelmann et al., 1996; Kamali et al., 1997), the concentration of metabolized and non-metabolized nebivolol in the organ bath of this respective experiments was approximately 0.25 μM.

2.3. Measurement of myofibrillar Ca^{2+} sensitivity

Triton X-100 skinned fibres were prepared as described previously (Schwinger et al., 1994). From each heart, several skinned-fiber preparations of ventricle were investigated. Concentration–response curves for Ca^{2+} were measured in the presence and in the abscence of D-nebivolol and L-nebivolol (both 30 μM). In all experiments, the final concentration of solvent (dimethyl sulfoxide) was 0.1%. EC_{50} concentration for Ca^{2+} were calculated for each fiber. Experiments were performed as described previously (Schwinger et al., 1994; Brixius et al., 2000).

2.4. β_1- and β_2-adrenoceptor binding studies

2.4.1. Membrane preparation

Due to the described β_1-adrenoceptor downregulation in human failing myocardium (Bristow et al., 1982; Schwinger et al., 1990, 1991), human non-failing myocardium was used for the determination of β_1-adrenoceptor selectivity. Myocardial tissue was taken from the interventricular septum. The myocardial membrane preparation has been described previously (Brixius et al., 2001; Bundkirchen et al., 2003).

2.4.2. Radioligand binding assay

β-Adrenoceptors in cardiac tissue were investigated using [^{3}H]CGP 12.177 ((±)-[^{3}H]4-(3-tertiarybutylamino-

2-hydroxypropoxy)-benzimidazole-2-on HCl) (specific activity 55 Ci/mmol) as the radiolabeled ligand. To obtain a homogenous population of β_1- or β_2-adrenoceptors, competition experiments were performed in the presence of the highly β_1-adrenoceptor selective antagonist CGP 20.712A (2-hydroxy-5-(2-(hydroxy-3-(4((1-methyl-4-trifluoromethyl)-1-*H*-imidazol-2-yl)-phenoxy)-propyl)-aminoethoxyl)-benzamide) (300 nM) or the highly β_2-adrenoceptor selective antagonist ICI 118.551 (*erythro*-(±)-1-(7-methylindan-4-yloyl)-3-isopropylaminobutan-2-ol HCl) (50 nM), respectively. At these concentrations of the highly specific antagonists, only the respective β_1- or β_2-adrenoceptor subpopulation was pharmacologically blocked.

Serum experiments were performed with the radiolabeled ligand [^{125}I]iodocyanopindolol, (specific activity 2000 Ci/mmol). [^{125}I]iodocyanopindolol was used instead of [^{3}H]CGP 12.177 because it reveals a higher specific activity which allows to detect even a small amount of radioligand bound to the membrane preparation. This was necessary because in the serum experiments, specific binding was reduced markedly, most likely due to interactions of the serum proteins with the membrane vesicles. In this respective experiments, the incubation assay contained 90 vol.% serum.

The radioligand binding assay has been described previously (Brixius et al., 2001; Bundkirchen et al., 2003).

2.5. Determination of adenylate cyclase activity

Adenylate cyclase activity was determined in particulate membrane fractions from human non-failing hearts as described previously (Schmidt et al., 1995; Brixius et al., 2001).

2.6. Materials

Nebivolol (±)-[*R**[*S**[*S**-(*S**)]]]-α,α′-[iminobis-(methylene)]bis[6-flouro3,4-dihydro-2*H*-1-benzo-pyran-2-methanol] and the D- and L-enantiomers were provided by Berlin Chemie (Berlin, Germany). Triton X-100 was from Merck (Darmstadt, Germany). [^{3}H]CGP 12.177, [^{125}I]iodocyanopindolol, [^{3}H]cAMP and [^{32}P]α-ATP were obtained from Amersham Pharmacia (Braunschweig, Germany).

2.7. Statistics

All values are means ± S.E.M. Statistical significance was analyzed with Student's *t*-test for paired observations; a *P* value of <0.05 was considered significant. In the radioligand binding studies, K_i values were calculated from IC_{50} values determined by fitting the competition curve with a nonlinear regression analysis assuming only one receptor state (one site fit), regardless if they actually reflect one or two affinity states.

Regression analyses were performed using the computer software GraphPadPrism (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Influence of nebivolol, D-nebivolol and L-nebivolol on force of contraction

3.1.1. Basal force of contraction

To investigate whether nebivolol or its D- and L-enantiomers influence force of contraction in the absence of cAMP-dependent stimulation (indirect determination of inverse agonistic activity), force of contraction was measured in isolated atrial trabeculae (1 Hz, 37 °C, 1.8 mM Ca^{2+}). For comparison, metoprolol was also investigated. Fig. 1A shows original force recordings after one singe concentration of nebivolol or metoprolol (both 0.4 μM), demonstrating the different inotropic responses.

Since the affinities of nebivolol and metoprolol to β-adrenoceptors are not equivalent (the β-adrenoceptor affinity of nebivolol is higher compared to metoprolol (Brixius et al., 2001)), cumulative dose–response curves were obtained to investigate the negative inotropic effects. Fig. 1B shows the negative inotropic responses after cumulative application of nebivolol ($n=6$), D-nebivolol ($n=8$), L-nebivolol ($n=6$) or metoprololol ($n=6$), respectively (0.01–3 μM). Nebivolol as well as D-nebivolol and L-nebivolol decreased only slightly force of contraction in comparison to the control (dimethyl sufoxide, $n=8$), whereas metoprolol decreased force of contraction with high efficacy and potency (Fig. 1B). The maximal decrease in force of contraction (as measured at 3 μM) was (% of maximum; Δ mN/mm^2): metoprolol: −55.7 ± 6.9%; −7.83 ± 1.95 mN/mm^2>D-nebivolol: −32.2 ± 3.4%; −4.38 ± 1.1 mN/mm^2 = L-nebivolol: −33.5 ± 3.3%; −4.89 ± 0.84 mN/mm^2 = nebivolol: −28.1 ± 3.8%; −3.72 ± 1.35 mN/mm^2>solvent: −17.1 ± 2.4%; −2.34 ± 0.72 mN/mm^2.

3.1.2. Pre-stimulated force of contraction

To investigate the influence on isometric force of contraction after cAMP-dependent pre-stimulation, concentration–response curves for the respective compound were obtained in isolated right atrial trabeculae (1 Hz, 37 °C, 1.8 mM Ca^{2+}) after pre-stimulation with isoprenaline (0.01 μM, β-adrenergic stimulation) or forskolin (0.3 μM, determination of intrinsic sympathomimetc activity in a situation of facilitated β-adrenoceptor/G_s protein/adenylate cyclase coupling).

3.1.2.1. Isoprenaline pre-stimulation. Concentration–response curves for nebivolol, D-nebivolol and L-nebivolol (0.01–3 μM) were obtained after pre-stimulation with isoprenaline (10 nM). Fig. 2A gives the percentual changes of force of contraction after cumulative application of D-nebivolol and L-nebivolol. Basal and isoprenaline-induced force of contraction are given in Table 1. The maximal negative inotropic efficacy (measured at 3 μM of the respective compound) was (% of maximum; Δ mN/mm^2): D-nebivolol: −37.1 ± 2.8%; −8.3 ± 1.3 mN/mm^2 ≥ L-

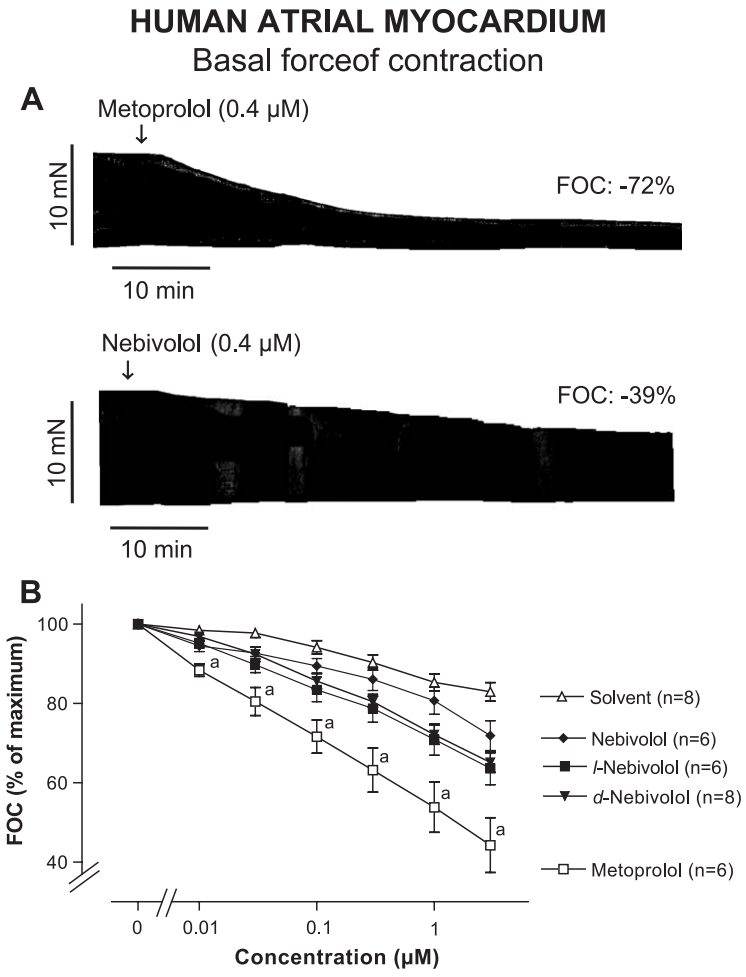

Fig. 1. Inotropic effects of the β-adrenoceptor antagonists metoprolol, nebivolol and its D- and L-enantiomers in human right atrial non-failing myocardium under basal conditions. (A) Original force recordings demonstrating the different inotropic efficacy of nebivolol and metoprolol. (B) Concentration–response curves obtained after cumulative administration of metoprolol, nebivolol, D-nebivolol and L-nebivolol; FOC: force of contraction; $^{a}P<0.05$ vs. nebivolol, D-nebivolol and L-nebivolol.

nebivolol: $-29.1 \pm 3.1\%$; -8.0 ± 1.1 mN/mm^2>solvent: $-12.5 \pm 3.9\%$; -2.3 ± 1.0 mN/mm^2.

To determine whether or not nebivolol and metoprolol, respectively, are able to reverse the isoprenaline-induced increase in adenylate cyclase activity, the influence of the β-adrenoceptor antagonists on cAMP formation was investigated in a membrane preparation. Fig. 3 shows that both nebivolol (0.1 nM–1 mM) and metoprolol (1 nM–1 mM) completely reverse the isoprenaline (1 µM)-induced increase in adenylate cyclase activity.

3.1.2.2. Forskolin pre-stimulation. To investigate whether the minor cardiodepressant effects of nebivolol may be attributed to an intrinsic sympathomimetic activity, cumulative concentration–response curves (0.01–3 µM) of nebivolol, D-nebivolol and L-nebivolol were performed in the presence of forskolin (0.3 µM) which has been shown to facilitate the β-adrenoceptor/G_s protein/adenylate cyclase coupling (Jasper et al., 1988). Fig. 2B gives the percentual changes of force of contraction after cumulative application of nebivolol, D-nebivolol and L-nebivolol. Basal and forskolin-induced force of contraction for each group is given in Table 1. Only in high concentrations nebivolol, D-nebivolol and L-nebivolol induced a significant negative inotropic effect. The negative inotropic efficacy on forskolin pre-stimulated myocardium was (maximum decrease in force of contraction measured at 3 µM) (% of maximum; Δ mN/mm^2): D-nebivolol: $-40.4 \pm 4.9\%$; -12.4 ± 2.5 mN/mm^2 ≥ nebi-nebivolol: $-35.6 \pm 3.9\%$; -8.1 ± 1.2 mN/mm^2 = L-nebivolol: $-33.0 \pm 3.0\%$; -10.8 ± 2.5 mN/mm^2>solvent: $-8.9 \pm 2.5\%$; -2.8 ± 0.8 mN/mm^2).

3.2. Influence of nebivolol, D-nebivolol and L-nebivolol on frequency-dependent force generation

The frequency-dependent force potentiation is a very important physiological mechanism, which enables the heart to increase contractility at higher stimulation frequencies. Fig. 3 shows that in the presence of nebivolol and D-nebivolol, the curve of the frequency-dependent force gen-

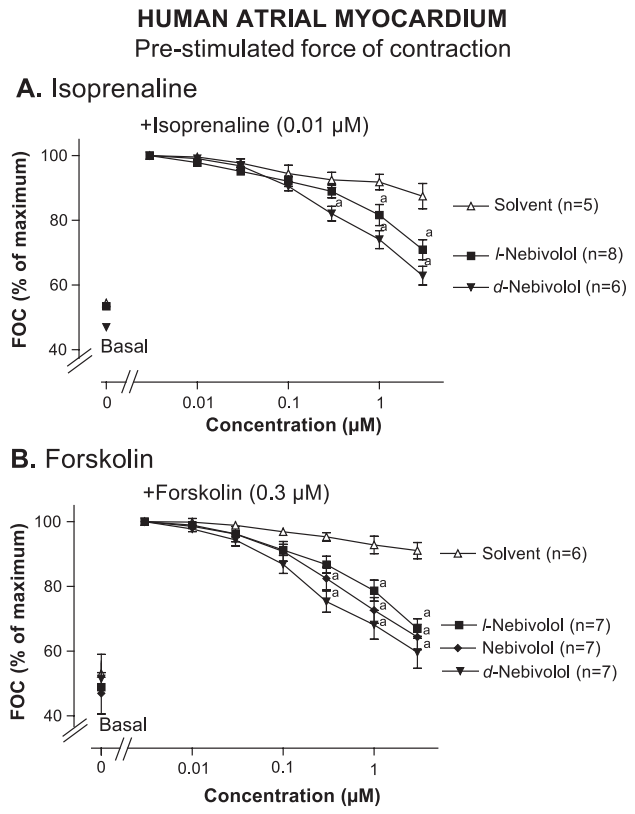

Fig. 2. Concentration–response curves for the cardiac effects of the β-adrenoceptor antagonist nebivolol and its D- and L-enantiomers in human right atrium non-failing myocardium after isoprenaline pre-stimulation (β-adrenergic stimulation, A) and after forskolin pre-stimulation (facilitated β-adrenoceptor/G_s protein/adenylate cylase coupling, B). Solvent: force development in the presence of solvent (dimethyl sulfoxide); $^{a}P<0.05$ vs. + pre-stimulation.

eration was shifted to the right whereas in the presence of L-nebivolol, the curve was not affected (all 0.5 μM). This rightward shift mediated by nebivolol and D-nebivolol was accompanied by a significant increase in the amplitude of frequency-dependent force generation (Table 2). This increase was absent in the presence of L-nebivolol or solvent (data for solvent not shown).

Table 1
Basal force of contraction and force of contraction after cAMP-dependent pre-stimulation

	Forskolin			Isoprenaline	
	FOC basal (mN/mm^2)	FOC + 0.3 μM (mN/mm^2)		FOC basal (mN/mm^2)	FOC + 0.01 μM (mN/mm^2)
Nebivolol ($n=7$)	11.8 ± 2.5	24.0 ± 3.8		–	–
D-Nebivolol ($n=7$)	15.9 ± 3.6	29.0 ± 4.4	D-Nebivolol ($n=6$)	10.2 ± 2.2	21.8 ± 2.9
L-Nebivolol ($n=7$)	17.9 ± 5.2	33.0 ± 6.6	L-Nebivolol ($n=8$)	15.0 ± 2.8	28.0 ± 3.6
Solvent ($n=6$)	14.7 ± 2.5	27.0 ± 3.5	Solvent ($n=5$)	11.3 ± 4.2	20.8 ± 5.5

FOC: force of contraction; solvent: dimethyl sulfoxide.

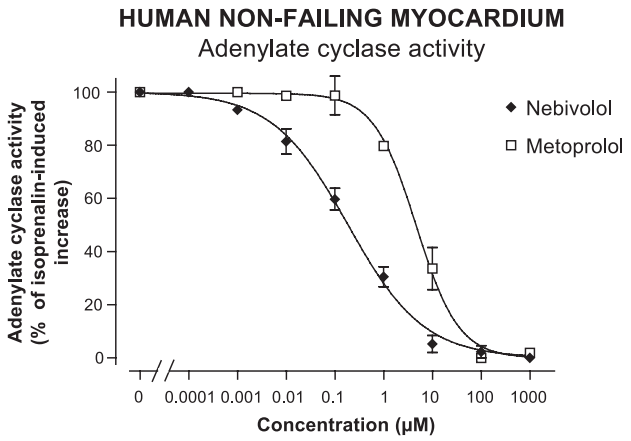

Fig. 3. Influence of nebivolol and metoprolol on the isoprenalin-induced adenylate cyclase activity. Both β-adrenoceptor antagonists completely reverse the isoprenalin-induced increase in cAMP formation.

3.3. Influence of D-nebivolol and L-nebivolol on myofibrillar Ca^{2+} responsiveness

The direct influence of D-nebivolol and L-nebivolol on Ca^{2+} dependent tension development was studied in Triton X-100 skinned fiber preparations of human left ventricular myocardium. D-Nebivolol and L-nebivolol (both 30 μM) did not influence maximal Ca^{2+}-activated tension (control: DT_{max}: 27.2 ± 1.6 mN/mm^2, $n=7$; D-nebivolol: 29.0 ± 1.1 mN/mm^2, $n=7$; L-nebivolol: 29.4 ± 2.0 mN/mm^2, $n=7$). In addition, no shift was observed for the myofibrillar Ca^{2+} sensitivity in the presence of D-nebivolol or L-nebivolol (30 μM). The concentrations needed for a 50% increase of maximal Ca^{2+}-activated tension (EC_{50}) were as follows: control: pCa_{50}: −6.39 ± −7.91; D-nebivolol: −6.33 ± −7.15 and L-nebivolol: −6.27 ± −7.10, respectively. Thus, D-nebivolol and L-nebivolol do not influence cardiac myofibrillar Ca^{2+} responsiveness.

3.4. Binding characteristics of D-nebivolol and L-nebivolol

To study the β_1-adrenoceptor selectivity of D-nebivolol and L-nebivolol, competition experiments to [^{3}H]CGP

Table 2
Frequency-dependent force development before and after 0.5 μM of nebivolol, D-nebivolol, L-nebivolol or solvent in right atrial trabeculae from human myocardium

	FOC_{max} (% of $FOC_{0.5\ Hz}$)		Frequency at FOC_{max} (Hz)	
	Before	After	Before	After
Nebivolol ($n=6$)	170 ± 22.4	215 ± 14.1^a	1.7 ± 0.3	2.3 ± 0.2^a
D-Nebivolol ($n=7$)	175 ± 30.2	240 ± 27.0^a	1.7 ± 0.2	2.2 ± 0.1^a
L-Nebivolol ($n=8$)	172 ± 17.2	176 ± 22.1	1.6 ± 0.1	1.8 ± 0.1
Solvent ($n=5$)	175 ± 19.6	181 ± 30.6	1.9 ± 0.1	1.9 ± 0.2

FOC_{max}: maximal developed force of contraction; solvent: dimethyl sulfoxide; before: before β-adrenoceptor blocker treatment; after: after β-adrenoceptor blocker treatment.

$^a P<0.05$ vs. before.

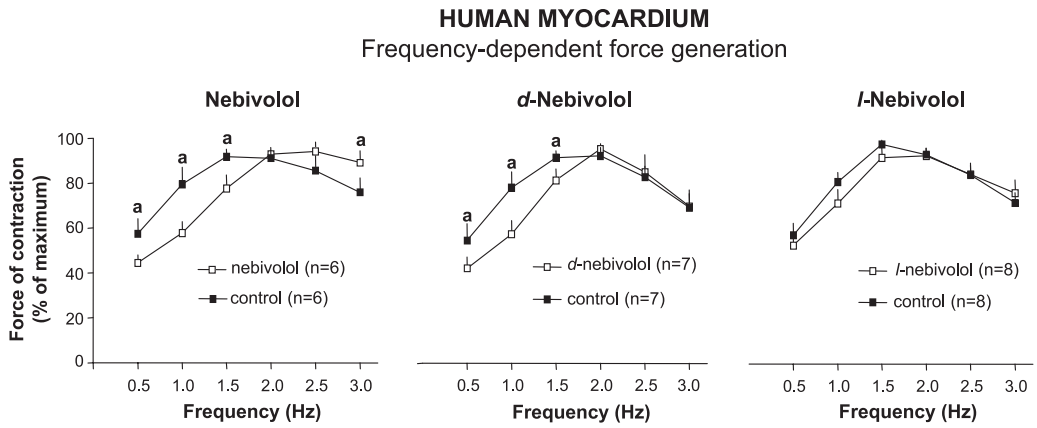

Fig. 4. Frequency-dependent force generation in the absence (controls) or presence (0.5 μM) of nebivolol, D-nebivolol and L-nebivolol, respectively. Note the significant shift in the force frequency curve mediated by nebivolol and D-nebivolol; $^{a}P<0.05$ vs. control.

12.177 binding were performed in the presence of ICI 118.551 (50 nM) in order to obtain a homogeneous population of β_1-adrenoceptors, as well as in the presence of CGP 20.712 A (300 nM) to determine competition of β_2-adrenoceptors. Fig. 4 shows competition curves obtained with D-nebivolol (left panel) and L-nebivolol (right panel) in crude membrane preparations of human non-failing left ventricular myocardium ($n=3$). D-Nebivolol is a β_1-adrenoceptor selective compound with high affinity to β_1-adrenoceptors ($K_i(\beta_1)$: 6.1 nM, 95% confidence interval (CI): 5.39–6.75) and low affinity to β_2-adrenoceptors ($K_i(\beta_2)$: 149.7 nM, CI: 121.9–183.9), $K_i(\beta_2)/K_i(\beta_1)=24.8$. In contrast, L-nebivolol showed only low affinity binding to β_1-adrenoceptors ($K_i(\beta_1)$: 496.0 nM, CI: 386.5–636.0) and also to β_2-adrenoceptors ($K_i(\beta_2)$: 338.5 nM, CI: 294.8–388.1), $K_i(\beta_2)/K_i(\beta_1)=0.68$.

3.5. Investigations using nebivolol serum

3.5.1. Influence of nebivolol serum on myocardial contractility

In order to investigate the potential inotropic effect of nebivolol after in vivo metabolism, the influence of serum samples on force of contraction was measured in isolated trabeculae obtained from right atrial human myocardium. The administration of serum samples to the organ bath led to an initial positive inotropic effect in both groups. 10 min after the serum was administered to the organ bath, force of contraction was increased from 5.54 ± 1.73 to 6.99 ± 2.10 mN/mm^2 in the controls and from 5.32 ± 1.38 to 6.50 ± 1.35 mN/mm^2 in the nebivolol serum group. From 10 min to the end of the registration (1 h), force of contraction was decreased. However, this decrease in force of contraction was similar in both groups. After 1 h, force of contraction

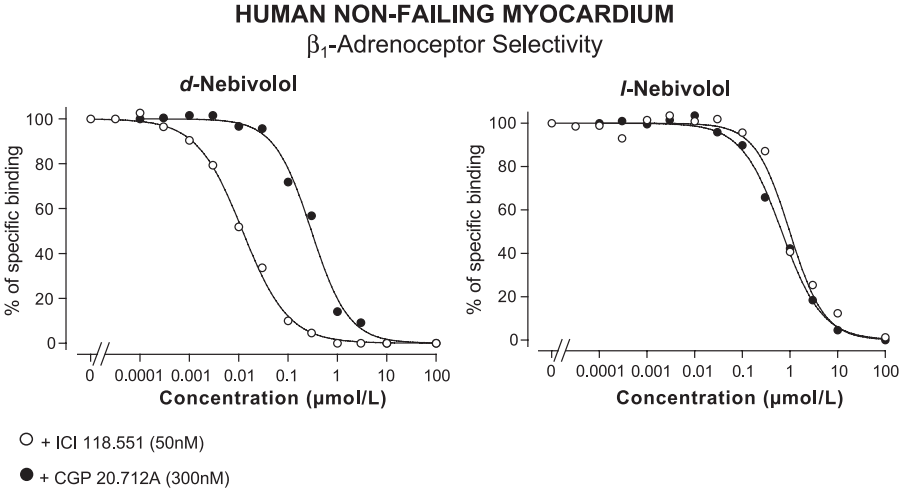

Fig. 5. Inhibition of specific [^{3}H]CGP 12.177 bound to cardiac β_1- and β_2-adrenoceptors by D-nebivolol and L-nebivolol. To obtain a homogenous population of β_1- or β_2-adrenoceptors, competition experiments were performed in the presence of the highly β_1-adrenoceptor selective CGP 20.712A (300 nM) or the highly β_2-adrenoceptor selective ICI 118.551 (50 nM), respectively. Data are expressed as means of three experiments.

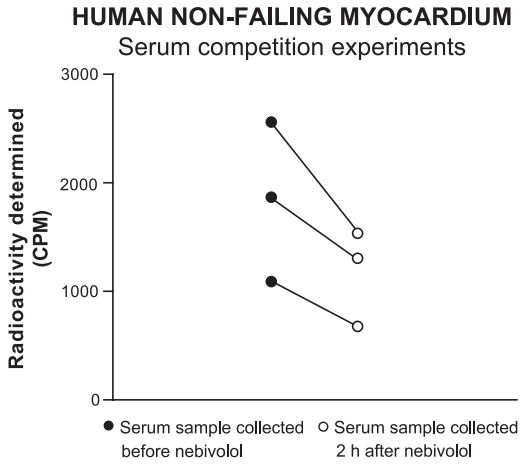

Fig. 6. Inhibition of specific [^{125}I]iodocyanopindolol binding to cardiac β_1- and β_2-adrenoceptors by serum samples ($n = 3$, each experiment performed in triplicate) which were collected before and 2 h after 5 mg nebivolol was administered orally. Note that in all experiments, specific binding of [^{125}I]iodocyanopindolol was lower when serum specimens collected after nebivolol administration were used.

was reduced to 5.32 ± 1.70 mN/mm^2 in the controls ($-26.7 \pm 2.7\%$) and 4.55 ± 1.11 mN/mm^2 ($-32.5 \pm 7.5\%$) in the nebivolol serum group.

3.5.2. Binding experiments

In order to demonstrate that the investigated serum specimens contain nebivolol and/or active metabolites of nebivolol, binding experiments using [^{125}I]iodocyanopindolol were performed. Specific binding of [^{125}I]iodocyanopindolol (at KD) was determined as described above in membrane preparations from non-failing human myocardium. In contrast to the former experiments, serum specimens were used as the competitor instead of D- or L-nebivolol. Serum specimens from three healthy volunteers were collected before and 2 h after 5 mg nebivolol was administered orally. Each experiment was performed in triplicate. As indicated in Fig. 5, specific, nonselective binding of [^{125}I]iodocyanopindolol to β_1- and β_2-adrenoceptors was inhibited more effective by the serum specimens obtained after nebivolol administration in comparison to serum specimens obtained before. Thus, serum samples obtained after administration of one single oral dose of 5 mg nebivolol contain a considerable amount of nebivolol and/or metabolites of nebivolol that bind to β-adrenoceptors (Fig. 6).

4. Discussion

4.1. The high β_1-adrenoceptor selectivity is mediated by D-nebivolol

In the present study, it has been shown that the high β_1-adrenoceptor selectivity of the racemic nebivolol is mediated by the D- but not the L-enantiomer. L-Nebivolol inhibits binding to β_1- and β_2-adrenoceptors only at high concentrations so that this enantiomer might be of minor importance for the β-blocking properties. Consistently, it has been shown that D-nebivolol is a potent selective β_1-adrenoceptor antagonist in rabbit and rat lung membrane preparations, whereas L-nebivolol only blocks β-adrenoceptors at concentrations much higher than the therapeutical plasma concentrations (Pauwels et al., 1988). In a crossover trial including 14 healthy male volunteers, L-nebivolol in contrast to nebivolol and D-nebivolol was not different to placebo in terms of exercise-induced heart rate and systolic blood pressure (Van Neuten and De Crée, 1998).

However, it has been reported that L-nebivolol potentiates the blood pressure lowering effect of D-nebivolol (Xhonneux et al., 1990). This might be attributed to the vasodilative nitric oxide release, which is mainly attributed to the L-enantiomer (Gao et al., 1991). Thus, the role of L-nebivolol for the clinical efficacy of racemic nebivolol remains unclear, although the present study and former investigations suggest that L-nebivolol is devoid of β-adrenoceptor antagonistic properties in pharmacological concentrations.

4.2. Minor impact on force development

Under basal conditions, nebivolol as well as D-nebivolol and L-nebivolol affected force of contraction only slightly in pharmacological relevant concentrations, indicating that these compounds are more or less devoid of inverse agonistic activity. In contrast, metoprolol, a β-adrenoceptor antagonist with a high degree of inverse agonistic activity (Varma et al., 1999), reduced force of contraction under basal conditions with high efficacy and potency. The unique mode of action mediated by nebivolol treatment, i.e. decreasing heart rate and blood pressure effectively in humans, without marked negative inotropic effect, is not understood completely. We have demonstrated in the present study that it is not due to an increased myofibrillar Ca^{2+} responsiveness or due to β-adrenoceptor agonistic properties which have neither been found for nebivolol nor for its enantiomers in the present study.

The present finding that after cAMP-dependent prestimulation using isoprenalin, nebivolol and its enantiomers, in contrast to metoprolol, did not completely antagonize the increase in force of contraction leads to the question whether or not nebivolol is a typical β-adrenoceptor antagonist. Therefore, measurements of adenylate cyclase activity in membrane preparations of non-failing myocardium were performed. In these experiments, nebivolol as well as metoprolol completely reversed the isoprenaline-induced increase in adenylate cyclase activity, providing evidence that nebivolol is a "classic" β-adrenoceptor antagonist.

It might be speculated that nebivolol has a different influence on β-adrenoceptors pending on the region and the environment of their expression. It may be possible that β-blocking efficacy of nebivolol is highest in the sinus node

and the atrioventricular node with smaller efficacy on the contractile myocardium. This explanation appears to be sound with the clinical observation of a "typical" behaviour of nebivolol on heart rate and its "atypical" behaviour on myocardial contractility.

It is also possible that the nitric oxide release of nebivolol (Cockcroft et al., 1995) is important for its attenuated negative inotropic effect since it has been shown that nitric oxide may concentration-dependently inhibit or activate phosphodiesterase III via its effector molecule cGMP (Kojda and Kottenberg, 1999). In addition, nebivolol lacks intrinsic sympathomimetic activity and inverse agonistic activity; in consequence, basal activation of the cellular system will not be affected.

Further studies are needed to answer these questions.

4.3. Nebivolol and D-nebivolol improve the frequency-dependent force generation

In the present study nebivolol, as well as D-nebivolol, are able to improve the frequency-dependent force development by shifting the curve of force frequency dependency to the right and increasing the amplitude of force development.

This is in contrast to metoprolol which did not influence the frequency-dependent force generation in former experiments (Flesh et al., 1999).

The influence of nebivolol and its D-enantiomer on the force frequency relationship might be especially useful in heart failure patients with an reduced capacity of frequency-dependent force potentiation (Schwinger et al., 1993). In a recent study, it has been shown that nebivolol restores the altered force frequency relationship induced by hydroxyl radical treatment (Janssen et al., 1999). The mechanism by which these beneficial effects of nebivolol and its D-enantiomer are mediated remains elusive. The observation that the alterations in the force frequency relationship occur only in the presence of the compounds that display high affinity to β_1-adrenoceptors (i.e. nebivolol and D-nebivolol) suggest that this receptor may be involved in the regulation of the frequency-dependent force generation in human myocardium. One may also speculate that the nitric oxide release induced by nebivolol treatment is responsible for the alterations in the force frequency relationship. There is growing evidence that nitric oxide modulates myocardial contractility, possibly enhancing the force frequency relationship (Shah and MacCarthy, 2000; Paulus et al., 2001). However, it has been demonstrated that the nitric oxide release of nebivolol can be mainly attributed to the L-enantiomer (Gao et al., 1991) which failed to enhance the force frequency relationship in the present study.

4.4. Hydroxylated metabolites of nebivolol

After oral administration, nebivolol displays a peculiar pharmacodynamic profile. It may be speculated that metabolites contribute to the unique mode of action of nebivolol. It has been shown that metabolites of nebivolol are involved in its vasodilating properties in mouse thoracic aorta segments (Broeders et al., 2000). To test the hypotheses that metabolites of nebivolol play an important role for the direct inotropic activity, serum samples from healthy volunteers were investigated. The competition binding experiments demonstrate that the samples that were collected after the administration of nebivolol, were able to inhibit specific binding of a radiolabelled β-adrenoceptor ligand in comparison to the specimens obtained before. However, in contraction experiments with these samples, force of contraction was not affected more by the specimens obtained after nebivolol administration in comparison to that obtained before. Thus, we conclude that in vivo metabolized nebivolol, similar to non-metabolized nebivolol or its enantiomers, does not increase or decrease force of contraction under basal conditions.

4.5. Limitations of the study

The present study was performed under in vitro conditions. Experiments were performed on isolated trabeculae, on skinned fiber preparations and on membrane preparations of human myocardial tissue. It cannot be excluded that in the in vivo effects of the studied compounds may differ from those observed in vitro.

Our results provide evidence that L-nebivolol does not contribute much to the specific β-adrenoceptor blocking effects of nebivolol. However, concentrations used in the in vitro experiments cannot be simple transferred to the measured in vivo plasma concentration. In addition, L-nebivolol might be metabolized in a atypical way in some patients, thus gaining "atypical" metabolites with higher affinity to β-adrenoceptors.

5. Conclusions

In the human myocardium, nebivolol and its D-enantiomer mediate high affinity β_1-adrenoceptor binding. Nebivolol and its enantiomers produce only minor direct negative inotropic effects, which holds also true for in vivo metabolized nebivolol. This and the fact that nebivolol and its D-enantiomer improve the frequency-dependent force generation may be of particular advantage when treating heart failure patients.

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