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RESEARCH PAPER

Investigation of the different adrenoceptor targets of nebivolol enantiomers in rat thoracic aorta

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Background and purpose: Nebivolol is a highly selective β_1-adrenoceptor antagonist with β_3-adrenoceptor agonist properties and is a racemate mixture of D- and L-enantiomers. However, the cellular mechanisms of the effects of each enantiomer are not yet clear and are a matter for debate. The aim of the present experiments was to determine the adrenoceptors involved in the vascular effects of D- and L-enantiomers of nebivolol in rat thoracic aorta.
Experimental approach: Responses to nebivolol enantiomers were evaluated in rings of thoracic aorta from male Sprague-Dawley rats.
Key results: D-nebivolol (0.1–10 $\mu mol \cdot L^{-1}$), but not L-nebivolol, significantly shifted to the right the concentration-response curve to phenylephrine, an α_1-adrenoceptor agonist, in a concentration-dependent manner. For the following experiments, aortic rings were constricted with endothelin 1 and now both enantiomers produced an endothelium-dependent relaxation of the rings involving the nitric oxide pathway. This relaxation was not modified by 1 $\mu mol \cdot L^{-1}$ CGP 20,712A (β_1-adrenoceptor antagonist), but significantly blunted by 7 $\mu mol \cdot L^{-1}$ L-74,8337 (β_3-adrenoceptor antagonist). However, only the vasorelaxation induced by D-nebivolol was significantly reduced by 1 $\mu mol \cdot L^{-1}$ ICI 118,551 (β_2-adrenoceptor antagonist).
Conclusions and implications: Our results suggest that the nebivolol enantiomers act on different targets. D-nebivolol induced vasorelaxation by activating β_2- and β_3-adrenoceptors and antagonizing α_1-adrenoceptors. L-nebivolol induced vasorelaxation by activating only β_3-adrenoceptors in our model. Our results emphasize that nebivolol is a β_1-adrenoceptor antagonist with several important pharmacological differences from other β_1-adrenoceptor antagonists.
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Keywords: nebivolol; β-adrenoceptor; α_1-adrenoceptor; nitric oxide; vasodilation; rat aorta

Abbreviations: E_{max}, maximal effect; ET1, endothelin 1; HUVEC, human umbilical vein endothelial cells; pD_2, –log (molar EC_{50})

Introduction

Nebivolol is a lipophilic, third generation β-blocker, devoid of intrinsic sympathomimetic activity (Janssens *et al.*, 1989; Brixius *et al.*, 2001). More precisely, nebivolol is a highly selective β_1-adrenoceptor antagonist that also possesses vasodilator properties, attributed largely to nitric oxide (NO) (Van de Water *et al.*, 1988; Bundkirchen *et al.*, 2003). It is used as a racemate mixture of two enantiomers, D-nebivolol (+SRRR nebivolol) and L-nebivolol (–RSSS nebivolol). The antihypertensive activity of nebivolol is mainly ascribed to D-nebivolol, which presents a 100-fold greater affinity for β_1-adrenoceptor than L-nebivolol (Pauwels *et al.*, 1991). Few studies report that the endothelium-dependent vasorelaxation could be produced by both enantiomers (Cockcroft *et al.*, 1995), but more particularly by L-nebivolol (Gao *et al.*, 1991; Mason *et al.*, 2006).

Although nebivolol involves NO in its vasodilatory actions, the precise mechanisms remain unclear. Several mechanisms have been described as underlying the endothelium-dependent effects of nebivolol. Among them, the antioxidant properties of nebivolol can increase the amount of NO by reducing its oxidative inactivation (Cominacini *et al.*, 2003; Fratta Pasini *et al.*, 2005; Evangelista *et al.*, 2007). In addition, there are presently four receptors that are candidates for nebivolol's effects or those of its metabolites on endothelial cells: β_3-adrenoceptors (Gosgnach *et al.*, 2001; de Groot *et al.*, 2003; Dessy *et al.*, 2005; Rozec *et al.*, 2006), β_2-adrenoceptors (Broeders *et al.*, 2000; Georgescu *et al.*, 2005), 5-HT_{1A} receptors (Kakoki *et al.*, 1999) and the oestrogen receptors of the plasma membrane (Kakoki *et al.*, 1999). However, based on *in vitro* affinity data, the last three receptors are unlikely to exert any major *in vivo* role; older studies appear to confirm this (see Ignarro, 2008). In addition, we have demonstrated earlier that the racemate of nebivolol was able to inhibit the

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vasoconstriction induced by activation of α_1-adrenoceptors (Rozec *et al.*, 2006). This result contrasts with the absence of effects of nebivolol on endothelin (Rozec *et al.*, 2006) and prostaglandin-induced vasoconstriction (see Mangrella *et al.*, 1998). Concerning the effects of each of the nebivolol enantiomers on different adrenoceptors, there are only few studies reported in the literature.

In this context, the aim of the present study was to investigate the role of different adrenoceptors, α_1-, β_1-, β_2- and β_3-adrenoceptors (nomenclature follows Alexander *et al.*, 2008), in the vasorelaxation induced by D-nebivolol and L-nebivolol in rat thoracic aorta. We demonstrate that D-nebivolol produced a vasorelaxation by activation of β_2- and β_3-adrenoceptors and by inhibition of α_1-adrenoceptors, whereas L-nebivolol produced vasorelaxation only by activation of β_3-adrenoceptors.

Methods

Animals

All animal procedures and these experiments were carried out in compliance with the guidelines of Nantes University. The experiments were performed on 10-week-old (350–450 g) male Sprague-Dawley rats (Elevage Janvier, Le Genest St Isle, France). The rats were housed in groups of three per Plexiglas cage under standard conditions of temperature (21–24°C), humidity (40–60%) and 12 h light/dark cycle with light period starting at 07:00. Food and water were freely available. After arrival of the rats and before the experiments, 1 week was allowed.

Tissue preparation and tension studies in rat aortic rings

Rats were anaesthetized with pentobarbital (30 $mg \cdot kg^{-1}$ i.p.). The descending thoracic aorta was excised, cleared of fat and connective tissue and cut into 3 mm rings. In some rings, the endothelium was removed by gentle rubbing of the intimal surface with a fine pair of small forceps. Rings were suspended on stainless-steel wires in a 10 ml organ bath containing Krebs solution composed as follows ($mmol \cdot L^{-1}$): NaCl, 118.3; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 15; EDTA (ethylenediaminetetraacetic acid), 0.016; glucose, 11.1; and $CaCl_2$, 2.5 (pH 7.4). Bath temperature was maintained at 37 ± 0.5°C, and the Krebs solution was continuously oxygenated with a 95% O_2, 5% CO_2 gas mixture. Rings were progressively stretched to a resting tension of 2 g. Isometric tension was recorded by a force displacement transducer (IT2, EMKA Technologies, Paris, France) and displayed on a computer (IOX1.5.7 software, EMKA Technologies). Data were analysed using Datanalyst software (EMKA Technologies).

Functional endothelium was checked by the presence of at least 70% relaxation in response to 1 $\mu mol \cdot L^{-1}$ acetylcholine (Ach) in rings pre-contracted with 1 $\mu mol \cdot L^{-1}$ phenylephrine. In denuded vascular rings, endothelium removal was confirmed by the absence of Ach-induced relaxation. In other experiments, aortic rings were contracted with endothelin 1 (ET1) and the concentration of ET1 (3–5 $nmol \cdot L^{-1}$) was adjusted to produce a similar level of tone (around 80% of the maximal response) for each experimental condition. A cumulative concentration-response curve to nebivolol (racemate, D-nebivolol, L-nebivolol) was then constructed. Relaxation produced by each concentration of nebivolol was measured after a steady-state was reached. Values are expressed as the percentage change in the maximal tension of vessel rings after addition of ET1. As nebivolol induced long-lasting relaxations, spontaneous time-dependent relaxation was concomitantly evaluated in control rings pre-contracted with ET1 and subtracted from the relaxation produced by nebivolol. The spontaneous relaxation was evaluated concomitantly to each steady-state relaxation in coupled treated rings, as long as it was necessary. Some rings were equilibrated in Krebs containing CGP 20,712A (a β_1-adrenoceptor antagonist), ICI 118,551 (a β_2-adrenoceptor antagonist), L-748,337 (a specific β_3-adrenoceptor antagonist) (Candelore *et al.*, 1999) or N^G-monomethyl-L-arginine monoacetate (L-NMMA, an NO synthase inhibitor) for 30 min. Only one protocol was performed on each ring.

Data and statistical analysis

Results are expressed as the mean ± SEM of n experiments. The statistical significance of a drug effect was assessed using one-way analysis of variance (ANOVA) followed by a Dunnett's test. Comparison of the different concentration-response curves was performed by two-way ANOVA (concentration, treatment) with repeated measures completed when appropriate by a Bonferroni *t*-test. A P value < 0.05 was considered statistically significant. The pA_2 value was estimated from Schild plots made by plotting the log (dose ratio – 1) against the log of the molar concentration of D- and L-nebivolol (GraphPad Prism version 5.01, GraphPad Software, San Diego, CA, USA). For some concentration-response curves, the determination of agonist potencies corresponding to concentrations producing 50% of maximum effect (EC_{50}) was calculated by fitting curves with the Boltzmann equation. pD_2 values were then determined according to the equation $pD_2 = -\log$ (molar EC_{50}) and compared using Student's *t*-test for unpaired data. A P value < 0.05 was considered statistically significant. ANOVA analysis were performed with SigmaStat® 3.0 software (SSPS Science Software, Erkrath, Germany) for Windows®.

Drugs

L-phenylephrine hydrochloride, acetylcholine chloride, ET1, CGP 20712A [(±)-2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl]amino] ethoxy]-benzamide, ICI 118,551 [(±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol were obtained from Sigma (St. Louis, MO, USA). L-NMMA (N^G-monomethyl-L-arginine monoacetate) was obtained from Calbiochem (Gibbstown, NJ, USA). L-748, 337 [(S)-N-[4-[2-[[3-[3-(acetamidomethyl)phenoxy]-2-hydroxypropyl] amino]ethyl] phenyl]benzenesulphonamide] was a generous gift from Merck (Rahway, NJ, USA). Nebivolol racemate, D-nebivolol and L-nebivolol were generous gifts from Menarini Research (Firenze, Italy). All drugs were prepared as stock solutions in distilled water, with the exception of nebivolol racemate, L-nebivolol, D-nebivolol and L-748,337 which was dissolved in dimethyl sulphoxide (Sigma). The final

concentration of the solvent in the organ bath was less than 0.1% v.v^{-1} and was used as controls for the effect of the active drug. ET1 was prepared as a 0.01 mmol·L^{-1} stock solution in distilled water containing 0.1% bovine serum albumin and kept at –20°C. All other dilutions were prepared daily.

Results

α_1-adrenoceptor antagonist property of D-nebivolol

In rat aortic ring, phenylephrine produced concentration-dependent contractions (Figure 1). Pre-treatment of the ring with D-nebivolol (0.3–10 µmol·L^{-1}) shifted the concentration-response curve to phenylephrine to the right in a concentration-dependent manner (Figure 1A, Table 1). The pA_2 value determined from the Schild plot was 6.90 and the slope was 1.08 ± 0.14. This Schild plot slope value did not differ from unity and was thus consistent with a competitive antagonism. By contrast, L-nebivolol did not modify in a relevant way the concentration-response curve to phenylephrine (Figure 1B, Table 1). Indeed, phenylephrine-induced contraction was not significantly modified by L-nebivolol (0.3–1 µmol·L^{-1}) pre-treatment although at the highest concentration (10 µmol·L^{-1}), L-nebivolol reduced dramatically the contractile effects of phenylephrine.

Comparison of the relaxant effect induced by nebivolol racemate and its enantiomers

The integrity and function of the endothelium was assessed by the application of 1 µmol·L^{-1} Ach on aortic rings pre-contracted with phenylephrine. In intact aortic rings, the Ach-induced relaxation was 89.8 ± 1.8% (n = 14), 88.9 ± 0.8% (n = 68) and 88.3 ± 0.9% (n = 66) in nebivolol racemate, D-nebivolol and L-nebivolol protocols respectively.

In a previous study (Rozec *et al.*, 2006), we demonstrated that nebivolol racemate pre-treatment on rat thoracic aorta did not affect the concentration-response curve to ET1. Therefore, we chose this vasocontractor agent to study the relaxant effect induced by enantiomers of nebivolol, thus avoiding the effects of the racemate and D-enantiomer of nebivolol on α_1-adrenoceptors, described above.

Rings were pre-contracted with ET1 to a similar level in all experimental conditions (Table 2). In pre-contracted rings, the application of cumulative concentrations of nebivolol racemate and nebivolol enantiomers (0.1–10 µmol·L^{-1}) induced a concentration-dependent relaxation (Figure 2). The relaxant effect of nebivolol and its enantiomers was initially slow and for a given concentration, the maximal response was achieved within 10–12 min. This long-lasting effect led us to evaluate spontaneous time-dependent relaxation of vessels in parallel control rings. The maximal spontaneous time-dependent relaxation of vessels in control rings was found to be responsible for 15.1 ± 2.8% (n = 7), 20.1 ± 4.3% (n = 6) and 19.3 ± 4.7% (n = 6) of relaxant effect at the end of nebivolol racemate, D-nebivolol and L-nebivolol experiments respectively. To take into account this low spontaneous relaxant effect, the corresponding spontaneous relaxation of

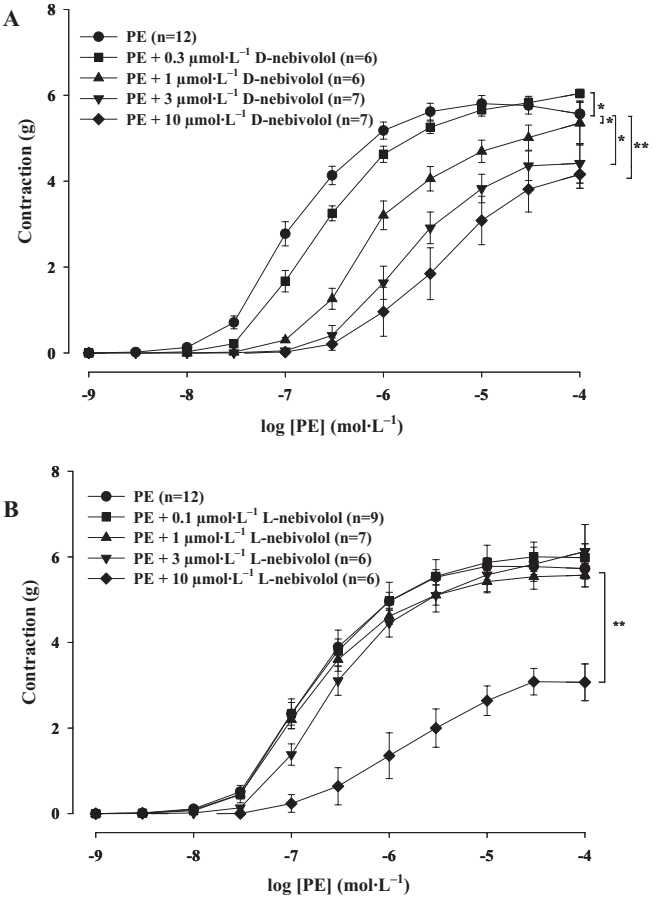

Figure 1 Concentration-response curves to phenylephrine (PE) in the absence and the presence of increasing concentration of D-nebivolol (A) and L-nebivolol (B) constructed in rat thoracic aortic rings. Each point is the mean of n experiments obtained from n rats and vertical lines show the SEM. When no error bar is shown, the error is smaller than the symbol. *P < 0.05 and **P < 0.01 indicate significant differences from PE alone.

Table 1 pD_2 values of phenylephrine (PE) in the presence of increasing nebivolol enantiomer concentrations (mean ± SEM)

D-nebivolol		*L-nebivolol*	
PE	6.78 ± 0.07 (n = 12)	PE	6.78 ± 0.07 (n = 12)
PE + D-nebivolol (0.3 µmol·L^{-1})	6.60 ± 0.06 (n = 6)	PE + L-nebivolol (0.3 µmol·L^{-1})	6.75 ± 0.11 (n = 9)
PE + D-nebivolol (1 µmol·L^{-1})	6.13 ± 0.08 (n = 6)*	PE + L-nebivolol (1 µmol·L^{-1})	6.82 ± 0.09 (n = 7)
PE + D-nebivolol (3 µmol·L^{-1})	5.74 ± 0.10 (n = 7)*	PE + L-nebivolol (3 µmol·L^{-1})	6.51 ± 0.13 (n = 6)
PE + D-nebivolol (10 µmol·L^{-1})	5.30 ± 0.17 (n = 7)*	PE + L-nebivolol (10 µmol·L^{-1})	5.38 ± 0.25 (n = 6)*

pD_2, –log (molar EC_{50}).
*P < 0.05 versus PE alone.

Table 2 Maximum tension induced by endothelin 1 in different experimental conditions (mean ± SEM)

Experimental conditions	*Maximum tension (g)*
Control	5.5 ± 0.2 (n = 38)
Without endothelium	5.7 ± 0.2 (n = 26)
L-NMMA	5.2 ± 0.2 (n = 26)
CGP 20,712A (1 μmol·L^{-1})	5.7 ± 0.2 (n = 30)
ICI 118,551 (7 μmol·L^{-1})	5.4 ± 0.2 (n = 30)
L-748,337 (7 μmol·L^{-1})	5.8 ± 0.3 (n = 24)

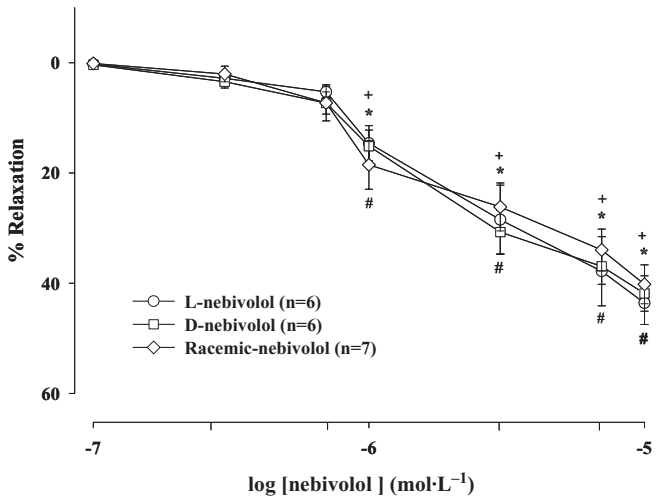

Figure 2 Concentration-relaxation response curves to nebivolol racemate, D-nebivolol and L-nebivolol in rat thoracic aortic rings precontracted with ET1. The mean curves are shown resulting from substraction of the spontaneous relaxation of control rings. Results are expressed as the percentage of relaxation from the maximal contraction induced by ET1. Each point is the mean of n experiments and vertical lines show the SEM. When no error bar is shown, the error is smaller than the symbol. #, +, *$P < 0.05$ indicate significant differences of nebivolol racemate, D-nebivolol and L-nebivolol respectively from basal condition (in the absence of nebivolol racemate or enantiomers). ET1, endothelin 1.

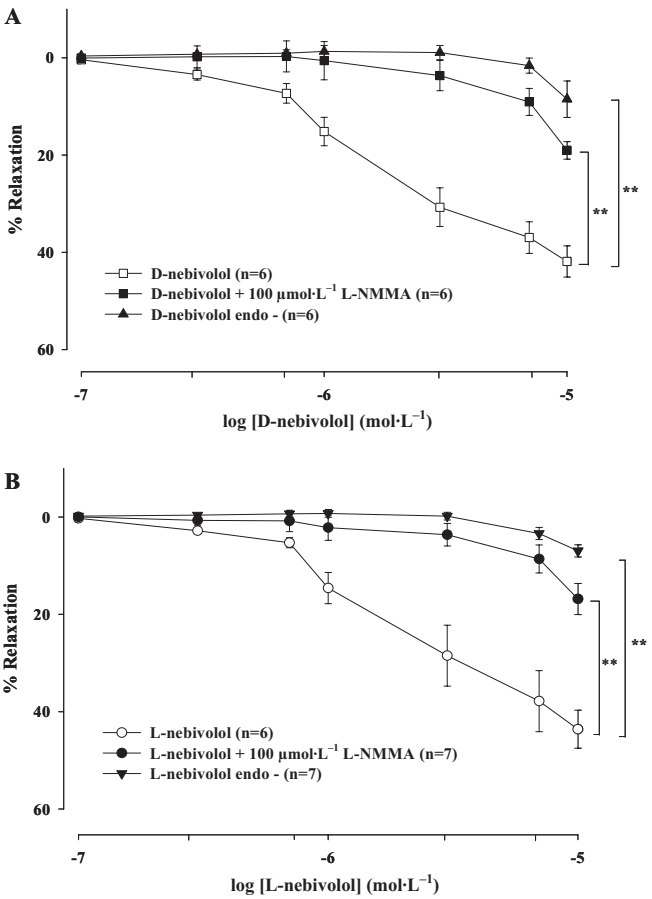

Figure 3 Involvement of the endothelium and the NO pathway in the relaxant effect of D-nebivolol (A) and L-nebivolol (B). The mean curves are shown resulting from subtraction of the spontaneous relaxation of control rings after endothelium removal (endo -) or after pre-treatment with 100 μmol·L^{-1} of L-NMMA. Results are expressed as the percentage of relaxation from the maximal contraction induced by ET1. Each point is the mean of n experiments obtained from n rats and vertical lines show the SEM. When no error bar is shown, the error is smaller than the symbol. **$P < 0.01$ indicates significant differences of arterial rings pre-treated with L-NMMA or without endothelium, from control. ET1, endothelin 1; NO, nitric oxide.

control rings was then subtracted from that exhibited by nebivolol racemate and enantiomers. In these conditions, the maximal effect (E_{max}) values were 38.6 ± 3.7% (n = 7), 40.2 ± 2.4% (n = 6) and 42.2 ± 4.5% (n = 6) for 10 μmol·L^{-1} nebivolol racemate, D- and L-enantiomers respectively.

Involvement of endothelium and the NO pathway in the vasorelaxing effects of nebivolol enantiomers

The efficiency of endothelial removal was assessed by the application of 1 μmol·L^{-1} Ach on aortic rings pre-contracted with phenylephrine. Endothelial removal abolished the relaxation to Ach in D-nebivolol (0.8 ± 1%; n = 6) and L-nebivolol (1.8 ± 1.2%; n = 6) groups.

The relaxation of both nebivolol enantiomers (D- and L-nebivolol) were almost abolished after endothelium removal (D-nebivolol: E_{max} = 9.0 ± 3.1%, n = 6, $P < 0.05$ versus D-nebivolol in intact rings; Figure 3A) (L-nebivolol: E_{max} = 6.9 ± 1.2%, n = 7, $P < 0.05$ vs. L-nebivolol in intact rings; Figure 3B) and markedly attenuated by 30 min pre-treatment with 100 μmol·L^{-1} L-NMMA (D-nebivolol, E_{max} = 19.0 ± 1.8%, n = 6, $P < 0.05$ versus D-nebivolol alone; Figure 3A) (L-nebivolol, E_{max} = 16.9 ± 3.2%, n = 6, $P < 0.05$ vs. L-nebivolol alone; Figure 3B).

Involvement of the three β-adrenoceptors in the relaxation of rat thoracic aorta induced by nebivolol enantiomers

To identify the β-adrenoceptors involved in the relaxation of rat thoracic aorta, concentration-response curves for L- and D-nebivolol were also performed in the presence of selective β-adrenoceptor antagonists: CGP 20712A (β_1-adrenoceptor antagonist), ICI 118,511 (β_2-adrenoceptor antagonist) and L-748,337 (β_3-adrenoceptor antagonist).

The relaxation induced by either enantiomer was not modified by 1 μmol·L^{-1} CGP 20712A (Table 3, Figure 4). However, D-nebivolol-induced relaxation was significantly ($P < 0.05$) blunted by 30 min. pre-treatment with 7 μmol·L^{-1}

Table 3 pD_2 values of nebivolol enantiomers in the absence or the presence of β-adrenoceptor antagonists selective for each subtype (mean ± SEM)

	D-nebivolol	*L-nebivolol*
Control	5.75 ± 0.07 (*n* = 6)	5.67 ± 0.12 (*n* = 6)
+CGP 20,712A (1 μmol·L^{-1})	5.55 ± 0.11 (*n* = 8)	5.52 ± 0.12 (*n* = 7)
+ICI 118,551 (7 μmol·L^{-1})	5.49 ± 0.07 (*n* = 8)*	5.49 ± 0.11 (*n* = 7)
+L-748,337 (7 μmol·L^{-1})	5.23 ± 0.05 (*n* = 6)*	5.17 ± 0.04 (*n* = 7)*

pD_2, –log (molar EC_{50}).
*P < 0.05 versus control.

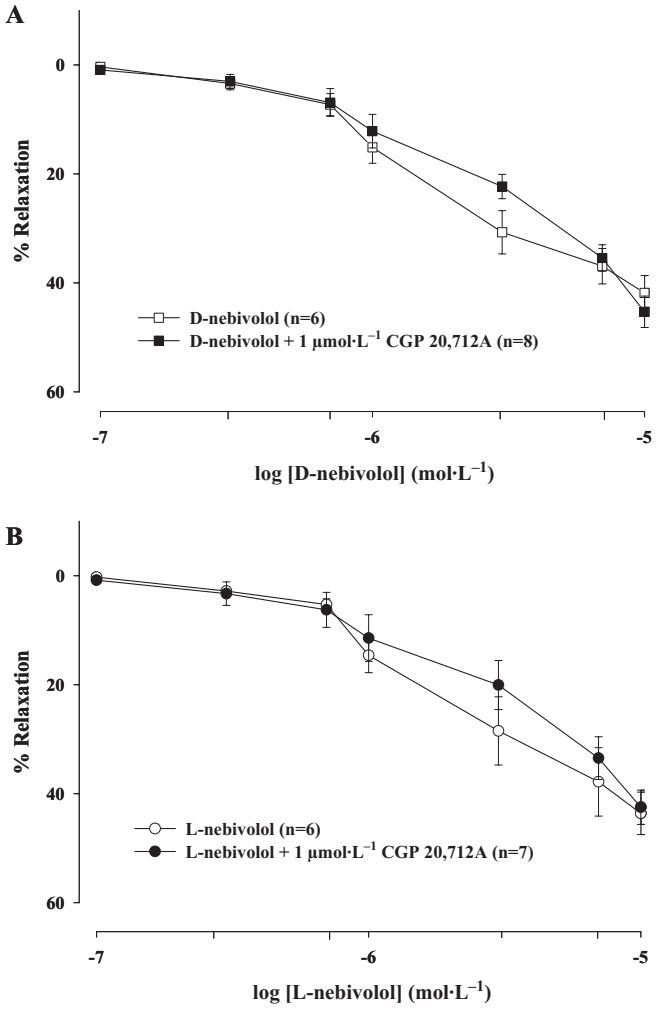

Figure 4 Involvement of β_1-adrenoceptors in the relaxant effect of D-nebivolol (A) and L-nebivolol (B). The mean curves are shown resulting from subtraction of the spontaneous relaxation of control rings after pre-treatment with 1 μmol·L^{-1} of CGP 20,712A (β_1-adrenoceptor antagonist). Results are expressed as the percentage of relaxation from the maximal contraction induced by ET1. Each point is the mean of n experiments obtained from n rats and vertical lines show the SEM. When no error bar is shown, the error is smaller than the symbol. ET1, endothelin 1.

ICI 118,551 (E_{max} = 24.9 ± 2.9%; n = 6), compared with the control condition (E_{max} = 40.2 ± 2.4%; n = 6) whereas the same pre-treatment did not affect L-nebivolol-induced relaxation (Table 3, Figure 5).

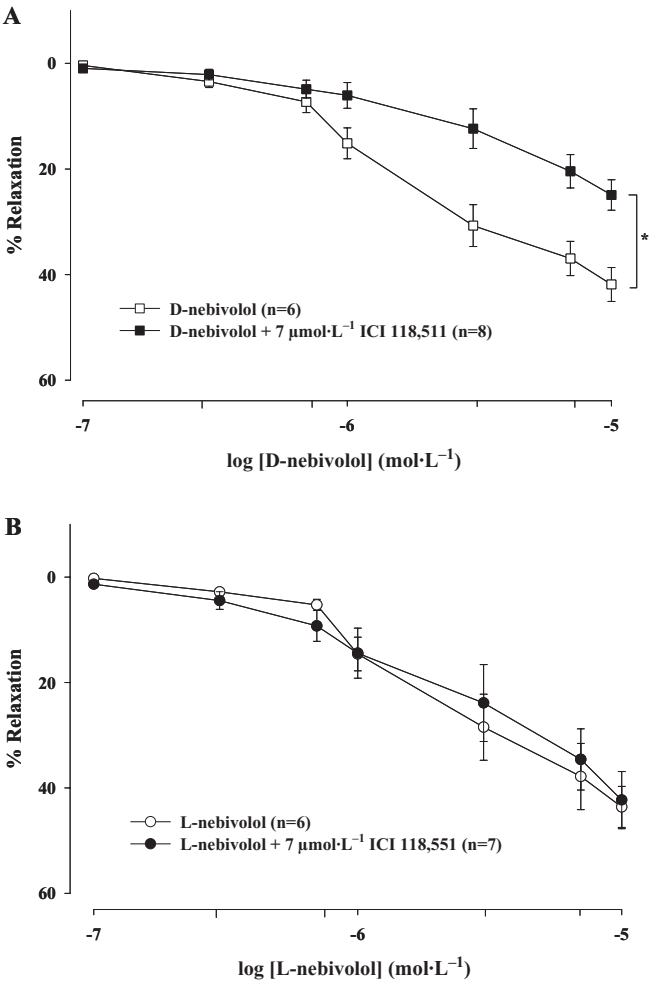

Figure 5 Involvement of β_2-adrenoceptors in the relaxant effect of D-nebivolol (A) and L-nebivolol (B). The mean curves are shown resulting from subtraction of the spontaneous relaxation of control rings after pre-treatment with 7 μmol·L^{-1} of ICI 118,511 (β_2-adrenoceptor antagonist). Results are expressed as the percentage of relaxation from the maximal contraction induced by ET1. Each point is the mean of n experiments obtained from n rats and vertical lines show the SEM. When no error bar is shown, the error is smaller than the symbol. *P < 0.05 indicates significant differences of arterial rings pre-treated with ICI 118,511 from control. ET1, endothelin 1.

We demonstrated the involvement of β_3-adrenoceptors in the relaxant effect of nebivolol racemate on the same vascular bed (Rozec *et al.*, 2006) by using one of the most selective β_3-adrenoceptor antagonist developed, L-748,337 (Candelore *et al.*, 1999). In the present study, pre-treatment of aortic rings by L-748,337 greatly reduced the relaxant effect of D-nebivolol (E_{max} = 24.1 ± 2.2%; pD_2 = 5.23 ± 0.05; n = 6) and L-nebivolol (E_{max} = 18.9 ± 2.5%; pD_2 = 5.17 ± 0.04; n = 6) (Table 3, Figure 6).

Discussion

In the present study, we showed that D-nebivolol presented α_1-adrenoceptor antagonist effects in rat thoracic aorta, whereas L-nebivolol was devoid of this property. We demonstrated that both enantiomers induced an endothelial

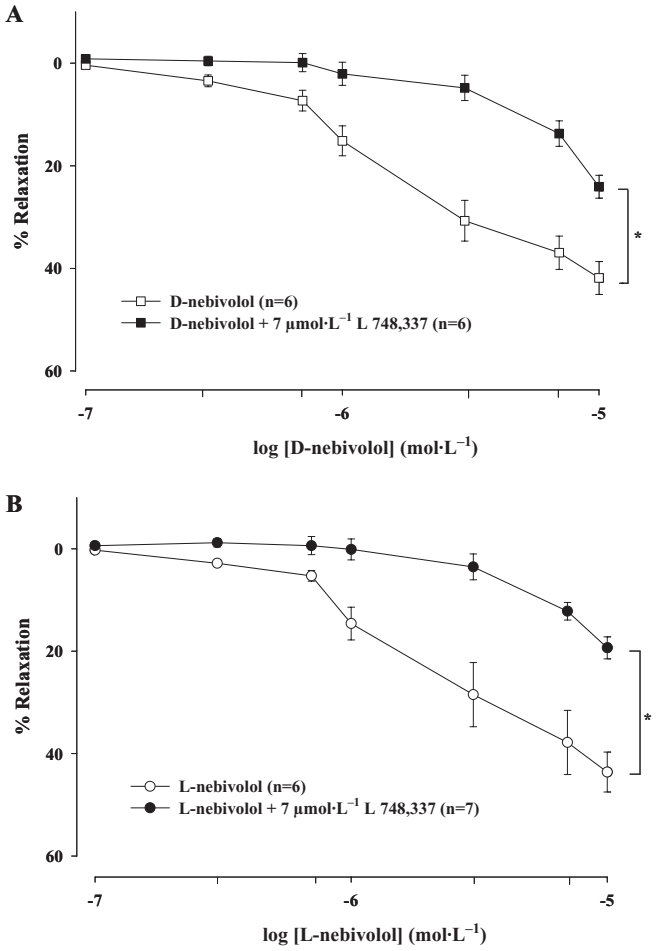

Figure 6 Involvement of β_3-adrenoceptors in the relaxant effect of D-nebivolol (A) and L-nebivolol (B). The mean curves are shown resulting from subtraction of the spontaneous relaxation of control rings after pre-treatment with 7 µmol·L^{-1} of L-748,337 (β_3-adrenoceptor antagonist). Results are expressed as the percentage of relaxation from the maximal contraction induced by ET1. Each point is the mean of n experiments obtained from n rats and vertical lines show the SEM. When no error bar is shown, the error is smaller than the symbol. *$P < 0.05$ indicates significant differences of arterial rings pre-treated with L-748,337 from control. ET1, endothelin 1.

and NO-dependent vasorelaxation and that several β-adrenoceptors could be involved in this response. The vasorelaxation induced by both enantiomers did not result from an action on β_1-adrenoceptors but, at least in part, by activation of β_3-adrenoceptors. In addition, D-nebivolol was able to stimulate β_2-adrenoceptors.

We showed that D-nebivolol shifted to the right the concentration-contraction curve to phenylephrine, an α_1-adrenoceptor agonist, in a concentration-dependent manner. This result is in agreement with our previous study performed with nebivolol racemate in the same model (Rozec *et al.*, 2006). Our present experiments cannot distinguish between the α_1-adrenoceptor subtypes antagonized by D-nebivolol. The antagonist affinity estimated for D-nebivolol in the rat aorta ($pA_2 = 6.9$) was comparable to previously published pA_2 values for other α_{1D}-adrenoceptor antagonists (Hussain and Marshall, 1997) and close to the pA_2 value ($pA_2 = 6.5$) obtained with the racemate in rat thoracic aorta (Rozec *et al.*, 2006). In the present study, the Schild plot slope (1.08) suggested a competitive antagonism whereas in our previous study, the Schild plot slope of 1,4 obtained for the racemate suggested a non-competitive antagonism. This discrepancy could be explained by a competitive antagonism of a heterogeneous α_1-adrenoceptor population by nebivolol racemate. In addition, a slope different from unity for the racemate could signify that several drug properties were expressed in the concentration range used to make the measurements. Consistent with our results, nebivolol-induced vasodilation was blocked by prazosin (an α_1-adrenoceptor antagonist) in rat mesenteric vascular bed (see Ignarro, 2008). These results are not in agreement with many others suggesting that nebivolol is devoid of α_1-adrenoceptor antagonist properties (Schneider *et al.*, 1990; Van Bortel *et al.*, 1997; Ritter, 2001). However, many of those results were obtained *in vivo* where there would be many cardiovascular regulatory systems influencing the final outcome. Furthermore, binding assays suggesting the absence of α_1-adrenoceptor antagonist properties of nebivolol were performed on rat lung membranes (Pauwels *et al.*, 1988), whereas the distribution of α_1-adrenoceptor subtypes differs between tissues. The use of new techniques such as tissue segment binding methods (Muramatsu *et al.*, 2005), which preserve the receptor environment in native tissues, may provide an explanation for such discrepancies. As it has been shown that blockade of endothelin receptors (either ET_A or ET_B) had only minor consequences on nebivolol-induced relaxation (Ignarro *et al.*, 2002) and nebivolol had no significant effect on the ET1-induced contraction of rat aorta (Rozec *et al.*, 2006), we chose to use ET1 to contract rat thoracic aorta rings, in our experiments.

In the present study, D- and L-nebivolol induced an endothelium-dependent vasodilation involving the NO pathway of rat thoracic aorta, comparable to the relaxation induced by the racemate. The present work is the first to evaluate the vasorelaxation induced by nebivolol enantiomers in this vascular bed. In canine coronary arterial rings, L-nebivolol produced a significantly greater endothelium-dependent relaxation than D-nebivolol. Furthermore, L-nebivolol, but not D-nebivolol potentiated the endothelium-dependent relaxation induced by adenosine (Gao *et al.*, 1991). Other *in vitro* studies suggest that the NO production induced by the nebivolol racemate is mainly due to the L-enantiomer, in both human umbilical vein endothelial cells (HUVEC) (Evangelista *et al.*, 2007) and rat microvessels (Mason *et al.*, 2006). Surprisingly, D-nebivolol was mainly involved in the effects on arterial blood pressure in rats (Sacco *et al.*, 2005), by increasing the NO production in both conductance and resistance arteries (Maffei *et al.*, 2006). In addition, L-nebivolol was able to potentiate the hypotensive effects of D-nebivolol (Xhonneux *et al.*, 1990) in the rat. In this later study, the authors consider that the racemate mixture of 50% D-nebivolol and 50% L-nebivolol seems to provide the optimal proportions for anti-hypertensive effects. It is important to note that in humans, after the standard dose of 5 mg, nebivolol has a maximum plasma concentration (C_{max}) of 1.48 ng·mL^{-1}, equivalent to 3.3 nmol·L^{-1} (Kamali *et al.*, 1997). This low concentration still produces a significant decrease in systemic vascular resistance, playing a puta-

tive role in the improvement of heart function by decreasing left ventricular afterload (Goldstein *et al.*, 1993; Dawes *et al.*, 1999).

In order to investigate which β-adrenoceptors mediated the relaxation of rat aorta induced by nebivolol enantiomers, we used a panel of β-adrenoceptor antagonists, selective for each subtype. As expected, the relaxations induced by both enantiomers were not modified by a β_1-adrenoceptor antagonist, CGP 20,712A. D-nebivolol-induced relaxation was antagonized by ICI 118,551 indicating a participation of β_2-adrenoceptors in this effect. Nebivolol has been described to be a weak ligand for β_2-adrenoceptors, devoid of any intrinsic sympathomimetic activity in several *in vivo* and *in vitro* models (Janssens *et al.*, 1989). More recently, few studies have evaluated the putative effect of nebivolol on β_2-adrenoceptors but their conclusions are discordant. Indeed, Evangelista *et al.* (2007) in a cellular model (HUVEC), reported a β_2-adrenoceptor agonist effect of nebivolol on NO production, but in this study, the authors did not use a selective β_2-adrenoceptor antagonist but nadolol, a β_1-/β_2-adrenoceptor antagonist. In addition, they only investigated the effect of the racemate. In the same way, Georgescu *et al.* (2005) reported the involvement of β_2-adrenoceptors in the effects of nebivolol racemate on the vasorelaxation of mouse renal artery. In another study perfomed in mice, Broeders *et al.* (2000), demonstrated a β_2-adrenoceptor agonist effect of nebivolol metabolites on NO production, but in this study the racemate seemed to have no effect on β_2-adrenoceptors. Thus, our study is the first to report a differential effect of D- and L-nebivolol enantiomers on β_2-adrenoceptors in a physiological model.

Surprisingly, we demonstrated that both nebivolol enantiomers produced a vasorelaxation through activation of β_3-adrenoceptors. Indeed, the relaxation of rat thoracic aorta induced by D- and L-nebivolol was strongly reduced by L-748,337, the most selective β_3-adrenoceptor antagonist available at the present time. We have obtained similar results in a previous study with the racemate, where its relaxant effects were not affected by nadolol, a mixed $\beta_{1,2}$-adrenoceptor antagonist, but significantly reduced by L-748,337 (Rozec *et al.*, 2006). This finding is in accordance with earlier work suggesting that nebivolol possessed β_3-adrenoceptor agonist properties. Nebivolol dilates human and rodent coronary resistance microarteries through an agonist effect on endothelial β_3-adrenoceptors to release NO and promote neoangiogenesis (Dessy *et al.*, 2005). In rat aorta, de Groot *et al.* (2003) showed that nebivolol-induced relaxation was inhibited by cyanopindolol, a compound known to block β_3-adrenoceptors, and mimicked by BRL 37344, a preferential β_3-adrenoceptor agonist. In HUVECs, nebivolol effects on cAMP production and nitrite formation were inhibited by cyanopindolol and bupranolol, a mixed $\beta_{1,2,3}$-adrenoceptor antagonist (Gosgnach *et al.*, 2001). In the same model, the release of NO by nebivolol was partially inhibited by a β_3-adrenoceptor antagonist, SR 59230A (Ladage *et al.*, 2006; Evangelista *et al.*, 2007). However, in guinea pig ileum, nebivolol was not able to act as either an agonist or an antagonist at β_3-adrenoceptors (Ignarro, 2008).

Our study demonstrates the discrepancies that could arise from *in vitro* studies of drugs with 'well-established targets'. Indeed, there is a gap between results obtained in ligand-receptor binding studies and *ex vivo* whole tissue approaches. In this latter approach, the cellular environment of the receptors can clearly influence the pharmacological effect of a drug. Considering (i) those discrepancies; (ii) the action of nebivolol on β_3-adrenoceptors (cloned 20 years ago); (iii) the recent indication of nebivolol in heart failure; and (iv) results in recent clinical trials (highlighting the good tolerance of this drug even in elderly patients), it is important to identify more precisely the adrenoceptor targeted by nebivolol in the cardiovascular system. Moreover, pharmacological studies of β-blockers which are now considered as 'gold standards' for the treatment of heart failure although their precise mechanisms are still unclear, will lead to new therapeutic approaches.

Furthermore, our work opens new perspectives in the clinical evaluation of nebivolol and its enantiomers, in so far as each of them presents specific properties. We have mentioned the low incidence of side effects of nebivolol. For instance, the β_2-adrenoceptor agonist properties of D-nebivolol and probably of nebivolol metabolites (Broeders *et al.*, 2000) could limit the contraindication in respiratory disease. The association of cardiovascular disease and chronic obstructive pulmonary disease (COPD) is far from unusual. Despite clear evidence of the effectiveness of β-adrenoceptor antagonists in cardiovascular diseases, clinicians often hesitate to administer them in the presence of COPD because lung function can be reduced even by a selective β_1-blocker. A preliminary study shown that it is possible to suggest the use of nebivolol in hypertensive patients with COPD (Cazzola *et al.*, 2004).

In conclusion, our results demonstrate that nebivolol enantiomers could act on different targets. D-nebivolol produced a vasorelaxation by activation of β_2- and β_3-adrenoceptors and antagonism of α_1-adrenoceptors. L-nebivolol produced a vasorelaxation only by activation of β_3-adrenoceptors in our model. These results emphasize that nebivolol is a β-blocker with several important pharmacological properties that distinguish it from other classical β-blockers.

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Conflict of interests

The nebivolol enantiomers were a gift from Menarini.

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