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Mixed β_3-adrenoceptor agonist and α_1-adrenoceptor antagonist properties of nebivolol in rat thoracic aorta

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1 Nebivolol, a selective β-adrenoceptor (β_1-AR) antagonist, induces vasodilatation by an endothelium- and NO-cGMP-dependent pathway. However, the mechanisms involved in the vascular effect of nebivolol have not been established. Thus, we evaluated the role of α_1 and β_3-ARs in nebivolol-induced vasodilatation.

2 The responses to nebivolol were investigated *in vitro* in thoracic aortic rings isolated from male Sprague–Dawley rats.

3 Nebivolol (0.1–10 μM) significantly shifted the concentration–response curve to phenylephrine, an α_1-AR agonist, to the right in a concentration-dependent manner ($pA_2 = 6.5$). Conversely, the concentration–response curve to endothelin 1 (ET1) was unaffected by nebivolol.

4 In ET1-precontracted rings, nebivolol induced a concentration-dependent relaxation, which was unaffected by nadolol (a β_1/β_2-AR antagonist) but was significantly reduced by L-748,337 (a β_3-AR antagonist), endothelium removal or pretreatment with L-NMMA (an NOS inhibitor). Similar results were obtained with a β_3-AR agonist, SR 58611A.

5 It was concluded that, in rat aorta, nebivolol-induced relaxation results from both inhibition of α_1-ARs and activation of β_3-ARs. In addition, we confirmed that the endothelium and the NO pathway are involved in the vascular effect of nebivolol. The identification of these vascular targets of nebivolol indicate that it has therapeutic potential for the treatment of pathological conditions associated with an elevation of sympathetic tone, such as heart failure and hypertension.
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Abbreviations: Ach, acetylcholine; α-ARs, α-adrenoceptors; β-ARs, β-adrenoceptors; cGMP, cyclic guanosine monophosphate; E_{max}, maximal effect; ET1, endothelin 1; L-748,337, [(*S*)-*N*-[4-[2-[[3-[3-(acetamidomethyl)phenoxy]-2-hydroxypropyl]amino]ethyl]phenyl]benzenesulfonamide]; L-NMMA, N^G-monomethyl-L-arginine monoacetate; NO, nitric oxide; PE, phenylephrine; SR 58611A, [(*RS*)-*N*-[(25)-7-ethoxycarbonylmethoxy-1,2,3,4-tetrahydronapht-2-yl]-(2)-2-(3-chlorophenyl)-2 hydroethanamide hydrochloride]

Introduction

In the last century, the discovery of β-adrenoceptors (β-ARs) and their antagonists, β-AR blockers, represented a very important development in cardiovascular pharmacotherapy. In the early 1960s, propanolol, a noncardioselective β-blocker, was introduced for therapy. Later, the second generation of β_1-AR selective blockers and finally the third generation β-blockers appeared on the market. The third generation of β-blockers represent a new therapeutic class of drug with high β_1 selectivity and a haemodynamic profile different from that of classical β-blockers, due to their ability to modulate vascular reactions (Toda, 2003). Thus, while previous generations of β-blockers showed contrasting or negligible effects on endothelial function, some of the third generation β-blockers have specific vasodilatory effects. Of all these compounds (carvedilol, nebivolol, celiprolol, bucindolol) with their various pharmacological and haemodynamic effects, nebivolol, a racemic mixture of SR3 (d-) and RS3 (*l*-) eniantomers, is the most selective β_1-AR antagonist currently available for clinical use (Waeber, 2000; Scheen, 2001). The pharmacological effects of nebivolol have been studied *in vitro* in rings of various animals and human blood vessels (Georgescu *et al*., 2005) and it has been shown to induce differential effects in various vascular beds. Taken together, the data indicate that nebivolol has a vasodilatory effect (Altwegg *et al*., 2000; Broeders *et al*., 2000; Ritter, 2001; De Groot *et al*., 2003), inhibits cell proliferation (Andre *et al*., 2000; Brehm *et al*., 2001), and has a protective effect on left ventricular function (Waeber, 2000). This latter effect of nebivolol probably results from its ability to induce vasodilatation, which would improve acute tolerance by unloading the failing left ventricle (Mangrella *et al*., 1998), in addition to a direct effect on the heart (Nodari *et al*., 2003). The exact mechanism and the receptor that mediates all these effects, and particularly its vasodilatory

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action, are still debatable. Endothelial nitric oxide (NO)-synthase-dependent NO release, cyclic guanosine monophosphate (cGMP) and β_2-ARs (Broeders *et al*., 2000; Ignarro *et al*., 2002; Ignarro, 2004; Zanchetti, 2004), P2Y-purinoceptors (Kalinowski *et al*., 2003) and Ca^{2+}-activated K^+ channels (Georgescu *et al*., 2005) have all been implicated in the vasodilator action of nebivolol. It has also been suggested that nebivolol increases NO as a result of its antioxidant properties (Fratta Pasini *et al*., 2005), stimulates inositol phosphate metabolism (Parenti *et al*., 2000), 5-HT_{1A} receptors (Kakoki *et al*., 1999) and β_3-ARs (Gosgnach *et al*., 2001; De Groot *et al*., 2003; Dessy *et al*., 2005).

With regard to the possibility that β_3-ARs are involved in the vasodilator effects of nebivolol, it should be noted that in several *in vivo* and *in vitro* studies with specific agonists and antagonists, β_3-ARs have been shown to be involved in the relaxation of various vascular beds (Tavernier *et al*., 1992; Shen *et al*., 1996; Trochu *et al*., 1999). In humans, β_3-ARs localized in the endothelium and activation of an NO signalling pathway have been implicated in the vasodilatation induced by β_3-AR agonists in the coronary and internal mammary arteries (Dessy *et al*., 2004; Rozec *et al*., 2005). In rat aorta, stimulation of endothelial β_3-ARs activates the NO pathway leading to an increase in intracellular cGMP (Trochu *et al*., 1999; Rautureau *et al*., 2002). Moreover, data from molecular and biochemical experiments confirm that β_3-ARs are predominantly located in the endothelium in rat thoracic aorta, mouse coronary microarteries, human internal mammary artery and coronary microarteries (Rautureau *et al*., 2002; Dessy *et al*., 2004; Rozec *et al*., 2005). Brahmadevara *et al*. (2003) reported that the relaxant effects of β_3-AR agonists such as CGP 12177A and BRL 37344, in phenylephrine-constricted but not PGF2α-constricted aortic rings appeared to be related to an α_1-AR blockade and not to β_3-AR or atypical β-AR activation. Nevertheless, those authors showed that another β_3-AR agonist, SR 58611A ([(*RS*)-*N*-[(25)-7-ethoxycarbonylmethoxy-1,2,3,4-tetrahydronapht-2-yl]-(2)-2-(3-chlorophenyl)-2 hydroethanamide hydrochloride]), in contrast to BRL 37344, produced relaxation of PGF2α-constricted rings, which suggests that SR 58611A produces relaxation by a mechanism different from α_1-AR blockade (Brahmadevara *et al*., 2003). In contrast, to our previous work (Trochu *et al*., 1999), the effect of SR 58611A was not blocked by the β_3-AR antagonist, SR 59230A. It is well known that the affinity and potency of a β_3-AR agonist or antagonist can differ greatly between species (Granneman & Lahners, 1994; Gauthier *et al*., 1999) and tissues (Arch, 2002). Moreover, SR 59230A has been shown to display agonist and antagonist effects on mouse β_3-ARs (Hutchinson *et al*., 2005). The specificity of SR 59230A in rat tissues is unclear (Candelore *et al*., 1999) and the selective β_3-AR antagonist, L-748,337 ([(*S*)-*N*-[4-[2-[[3-[3-(acetamidomethyl)phenoxy]-2-hydroxypropyl]amino]ethyl]phenyl]benzenesulfonamide]), should be used instead in these tissues.

Based on these findings, the present *in vitro* study was performed in the rat aorta to clarify whether the effect of nebivolol on NO could be related to activation of β_3-ARs in our experimental model. Although, from results of binding studies performed in noncardiovascular tissue (Pauwels *et al*., 1988), nebivolol is not thought to be an α-AR antagonist (Ritter, 2001), we also investigated the possible involvement of α-ARs in the nebivolol-induced relaxation.

Methods

Animals

The experiments were performed on 7-weeks-old (250–300 g) male Sprague–Dawley rats (Charles River Laboratories, L'Arbresle, France). The rats were housed in groups of five 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 0700 hours. Food and water were available *ad libitum*. One week was allowed after arrival of the rats before the experiments were performed. The experiments were carried out in compliance with the guidelines of Nantes University.

Tissue preparation and tension studies in rat aortic rings

Rats were anaesthetized with pentobarbital (30 mg kg^{-1} i.p.). Descending thoracic aortae were isolated, 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 (mM): NaCl, 118.3; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; 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).

The presence of a functional endothelium was confirmed by the demonstration of at least 70% relaxation in response to 1 μM acetylcholine (Ach) in rings precontracted with 0.3 μM phenylephrine (PE). In denuded vascular rings, endothelium removal was confirmed by the absence of Ach-induced relaxation. In other experiments, aortic rings were contracted with ET1 and the concentration of ET1 (3–5 nM) 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 either nebivolol or SR 58611A, a β_3-AR agonist, was then constructed. Relaxation produced by each concentration of agonist was measured after a steady state had been reached. Values are expressed as the percentage change in the maximal tension of vessel rings after addition of ET1. As nebivolol and SR 58611A induced long-lasting relaxations, spontaneous time-dependent relaxation was concomitantly evaluated in control rings precontracted with ET1 and subtracted from the relaxation produced by the agonist. The spontaneous relaxation was evaluated concomitantly with each steady-state relaxation in coupled treated rings throughout the experiment. At the last relaxant agonist concentration, the final spontaneous relaxation was evaluated. Some rings were equilibrated in Krebs containing nadolol (a β_1 and β_2-AR antagonist), L-748,337 (a specific β_3-AR antagonist) (Candelore *et al*., 1999) or N^G-monomethyl-L-arginine monoacetate (L-NMMA, an NO synthase inhibitor) for 30 min.

Drugs

L-PE hydrochloride, Ach chloride, ET1, nadolol and L-NMMA were obtained from Sigma (St Louis, MO, U.S.A.). SR 58611A and L-748,337 were generous gifts from Sanofi-Synthélabo (Montpellier, France) and Merck (Rahway, U.S.A.) respectively. Nebivolol racemate was a generous gift from Menarini Research (Firenze, Italy). All drugs were prepared as stock solutions in distilled water, with the exception of (i) nadolol which was dissolved in hydrochloric acid before being neutralized to pH 7.4 with NaOH 1 N, (ii) L-748,337 which was dissolved in dimethyl sulphoxide (DMSO; Sigma). The final concentration of the solvent in the organ bath was less than 0.1% v v^{-1} and was used as a control for the effect of the active drug. ET1 was prepared as a 0.01 mM stock solution in distilled water containing 0.1% bovine serum albumin and kept at −20°C. All other dilutions were prepared daily.

Data and statistical analysis

Results are expressed as the mean ± s.e.m. of n experiments. 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 nebivolol (GraphPad, Prism). For some concentration–response curves, the determination of agonist potencies corresponding to concentrations producing 50% of maximum effect (EC_{50}) were calculated by fitting curves with the Boltzmann equation. pD_2 values were then determined according to the equation p$D_2 = -\log$ (molar EC_{50}) and compared using Student's t-test for unpaired data. A P-value <0.05 was considered statistically significant.

Results

Antagonism of phenylephrine-induced contraction by nebivolol

In rat aortic rings, PE produced a concentration-dependent increase in contraction (Figure 1a). Pretreatment of the ring with nebivolol (0.3–10 μM) shifted the concentration–response curve to PE to the right in a concentration-dependent manner (Figure 1a; Table 1). The shift was accompanied by an attenuation of the maximal response induced by PE. The pA_2 value determined from the Schild plot was 6.5 and the slope was 1.44. By contrast, in the same range of concentrations, nebivolol had no significant effect on the ET1-induced contraction of rat aorta (Figure 1b; Table 1). Thus, in the following study, ET1 was used to precontract rat aorta in order to investigate the vasorelaxant effects of nebivolol.

Comparison of relaxant effects induced by nebivolol and SR 58611A, a β_3-AR agonist

The integrity of the endothelium or the efficiency of endothelial removal was assessed by the application of 1 μM Ach on aortic rings precontracted with PE. In intact aortic rings, the Ach- induced relaxation was 90.6 ± 0.8% ($n = 55$) and 91.4 ± 1.2% ($n = 64$) for the nebivolol and SR 58611A experiments, respectively. In contrast, removal of the endothelium abolished the Ach-induced relaxation in the nebivolol (−1.3 ± 0.3%, $n = 24$) and SR 58611A (−1.9 ± 0.3%, $n = 12$) groups. Rings were then precontracted with ET1 so that the level of contraction was similar in the different experimental conditions (Table 2). In precontracted rings, the application of

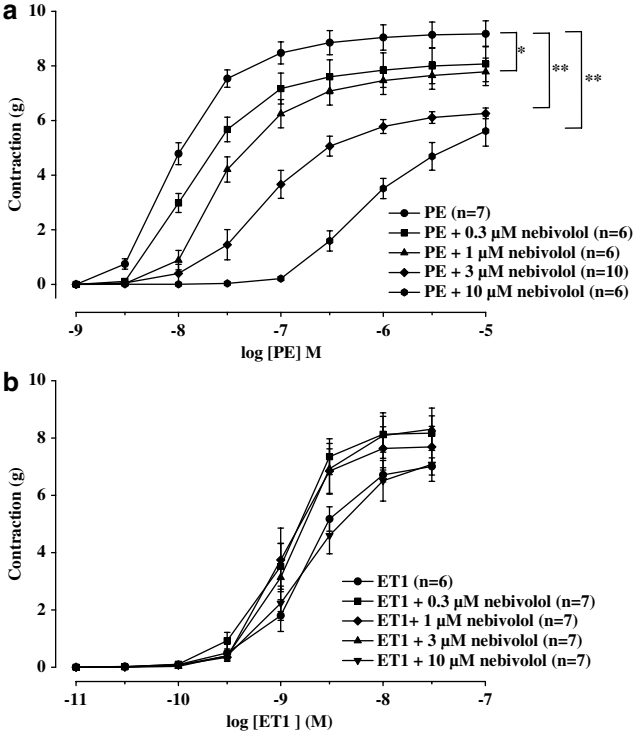

Figure 1 Concentration–response curves to phenylephrine (PE; a) and endothelin (ET1; b) in the absence and the presence of increasing concentrations of nebivolol constructed in rat thoracic aortic rings. Each point is the mean of n experiments and vertical lines show the s.e.m. When no error bar is shown, the error is smaller than the symbol. *$P<0.05$ and **$P<0.01$ indicate significant differences from PE or ET1 alone.

Table 1 pD_2 values of PE and ET1 in the presence of increasing nebivolol concentrations (mean ± s.e.m.)

PE	*8.05 ± 0.04 (n = 7)*	*ET1*	*8.78 ± 0.07 (n = 6)*
PE + nebivolol (0.3 μM)	7.86 ± 0.02 ($n = 6$)*	ET1 + nebivolol (0.3 μM)	8.99 ± 0.08 ($n = 7$)
PE + nebivolol (1 μM)	7.60 ± 0.06 ($n = 6$)*	ET1 + nebivolol (1 μM)	8.95 ± 0.07 ($n = 7$)
PE + nebivolol (3 μM)	7.13 ± 0.12 ($n = 10$)*	ET1 + nebivolol (3 μM)	8.86 ± 0.09 ($n = 7$)
PE + nebivolol (10 μM)	6.14 ± 0.08 ($n = 6$)*	ET1 + nebivolol (10 μM)	8.68 ± 0.07 ($n = 7$)

*$P<0.01$ *versus* agonist alone.

Table 2 Maximum tension induced by ET1 in different experimental conditions (mean ± s.e.m.)

Experimental conditions	*Maximum tension* (g)
Control	6.01 ± 0.33 ($n = 28$)
L-748,337 (7 μM)	6.17 ± 0.25 ($n = 26$)
L-NMMA (100 μM)	6.88 ± 0.21 ($n = 26$)
Nadolol (10 μM)	5.98 ± 0.22 ($n = 26$)
Without endothelium	6.19 ± 0.30 ($n = 24$)

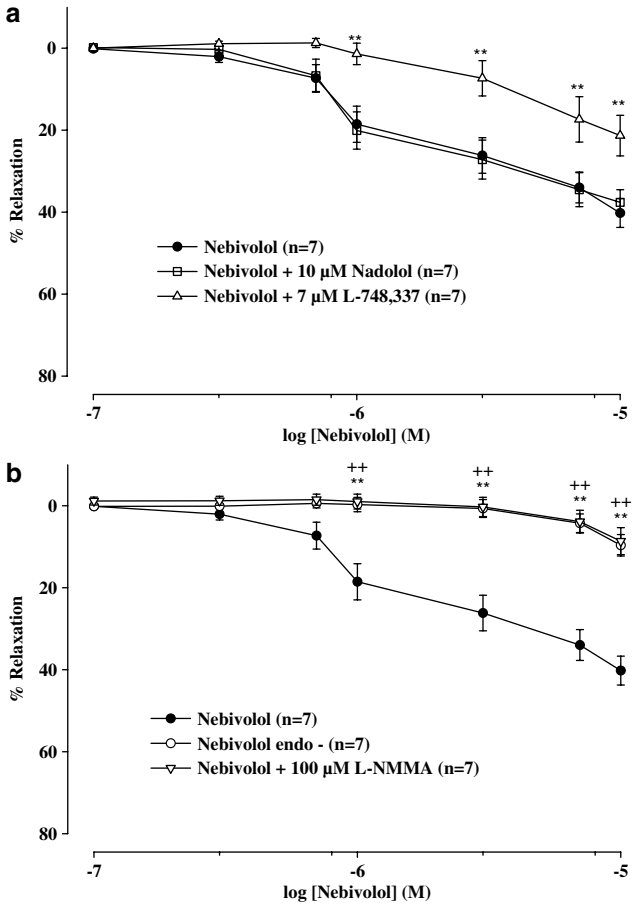

Figure 2 Concentration–relaxation response curves for nebivolol in rat thoracic aortic rings precontracted with ET1 in different experimental conditions. The mean curves are shown resulting from subtraction of the spontaneous relaxation of control vessels pretreated or not with nadolol or L-748,337 (a), L-NMMA (b) and after endothelium removal (b). Results are expressed as the percentage of relaxation from the maximal contraction level induced by ET1. Each point is the mean of n experiments, and vertical lines show the s.e.m. When no error bar is shown, the error is smaller than the symbol ($^*P<0.05$ and $^{**}P<0.01$ indicate significant differences of arterial rings pretreated with L-NMMA from control; $^{+}P<0.05$ and $^{++}P<0.01$ indicate significant differences of arterial rings without endothelium from control).

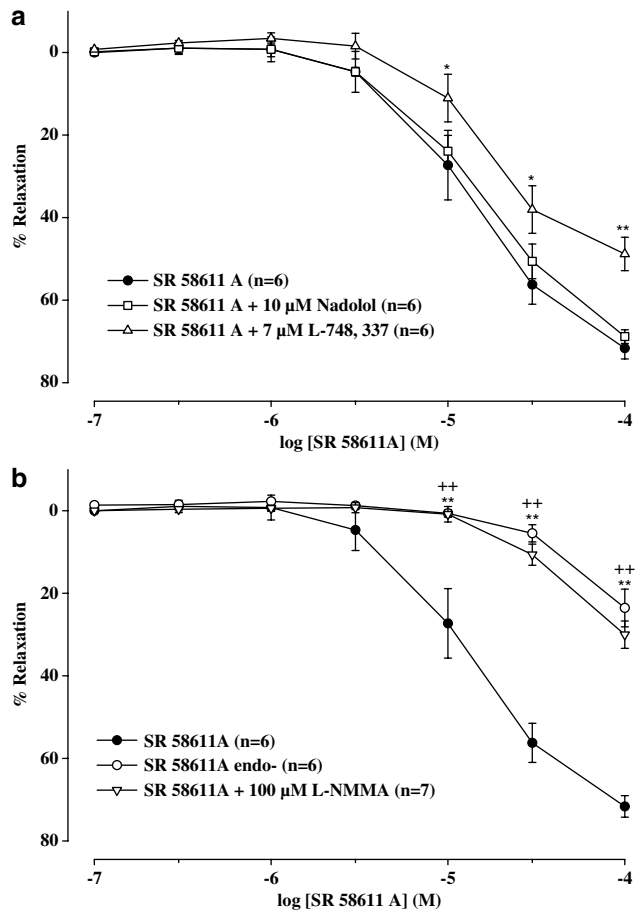

Figure 3 Concentration–relaxation curves for SR 58611A in rat thoracic aortic rings precontracted with ET1 in different experimental conditions. The mean curves are shown resulting from subtraction of the spontaneous relaxation of control vessels pretreated or not with nadolol or L-748,337 (a) and L-NMMA (b) and after endothelium removal (b). Results are expressed as the percentage of relaxation from the maximal contraction level induced by ET1. Each point is the mean of n experiments, and vertical lines show the s.e.m. When no error bar is shown, the error is smaller than the symbol ($^*P<0.05$ and $^{**}P<0.01$ indicate significant differences of arterial rings pretreated with L-NMMA from control; $^{+}P<0.05$ and $^{++}P<0.01$ indicate significant differences of arterial rings without endothelium from control).

cumulative concentrations of nebivolol (0.1–10 μM; Figure 2a) and SR 58611A (0.1–100 μM; Figure 3a) induced a concentration-dependent relaxation. For a given concentration, the maximal response was achieved within 10–15 min and 10–12 min for nebivolol and SR 58611A, respectively. The variation in agonist-induced relaxation observed during the experiment was not caused by a gradual reduction in the precontracted state of the tissue because the contraction produced by ET1 was maintained at a relatively constant level for the entire experiment. In fact, the spontaneous time-dependent relaxation of vessels in control rings was found to be responsible for 15.1 ± 2.8% ($n = 7$) and 6.1 ± 4.7% ($n = 6$) of the relaxant effects of nebivolol and SR 58611A, respectively, at the end of the experiments. To take into account this low spontaneous time-dependent relaxation, the corresponding spontaneous relaxation of control rings was then subtracted from that exhibited by nebivolol or SR 58611A. In these conditions, the maximal effect (E_{max}) values were 40.2 ± 3.5% ($n = 7$) and 71.6 ± 2.6% ($n = 6$) for 10 μM nebivolol and 100 μM SR 58611A, respectively.

Involvement of β_3-ARs in the relaxant effect of nebivolol

To investigate whether nebivolol produces relaxant effects only through activation of β_3-ARs, concentration–response curves

for nebivolol were also determined in the presence of two β-AR antagonists, nadolol (a β_1, β_2-AR antagonist) and L-748,337 (a selective β_3-AR antagonist; Candelore *et al*., 1999). Neither nadolol (10 μM) nor L-748,337 (7 μM) alone modified the ET1-induced contraction of rat aortic rings (Table 2). The concentration–response curve for nebivolol was unaffected by 30 min pretreatment with 10 μM nadolol ($E_{max} = 40.2 \pm 3.5\%$; $n = 7$), whereas it was significantly reduced in the presence of 7 μM L-748,337 ($E_{max} = 21.3 \pm 4.9\%$; $n = 7$; $P < 0.05$ *versus* nebivolol alone; Figure 2b). Similarly, pretreatment with nadolol of ET1-precontracted rings had no effect on the relaxation responses to SR 58611A ($E_{max} = 68.8 \pm 1.6\%$; $n = 6$), but pretreatment with L-748,337 altered the relaxant effect to SR 58611A ($E_{max} = 48 \pm 4.0\%$; $n = 6$; $P < 0.05$ in the presence of 7 μM L-748,337 *versus* SR 58611A alone; Figure 3a).

Involvement of the endothelium and NO pathway in the nebivolol effect

The relaxant effect of nebivolol was almost abolished after removal of the endothelium ($E_{max} = 8.6 \pm 3.3\%$; $n = 6$; $P < 0.05$ *versus* nebivolol in intact rings) and markedly attenuated by 30 min pretreatment with 100 μM L-NMMA ($E_{max} = 9.6 \pm 2.6\%$; $n = 6$; $P < 0.05$ *versus* nebivolol alone; Figure 2b). Similarly, the relaxant effect to SR 58611A was reduced by endothelium removal ($E_{max} = 23.37 \pm 4.25\%$; $n = 6$; $P < 0.05$ *versus* SR 58611A in intact rings) and L-NMMA pretreatment ($E_{max} = 30.0 \pm 3.3\%$; $n = 6$; $P < 0.05$ *versus* SR 58611A alone; Figure 3b).

Discussion

The results from this study demonstrate that nebivolol, a β-blocker of the third generation, induces relaxation of rat aorta by stimulation of endothelial β_3-ARs which then activates the NO pathway. In addition, we have shown, for the first time, that an antagonist effect of nebivolol on α_1-ARs contributes to this vasorelaxation.

Nebivolol produced a concentration-dependent shift to the right of concentration–response curves to PE, an α_1-AR agonist, and decreased the maximal response to PE, whereas it had no effect on the concentration–response curve to another contractile agent, ET1. This suggests that antagonism of α_1-ARs by nebivolol contributes to its vasorelaxant effect in the rat aorta. The subtype(s) of α_1-ARs antagonized by nebivolol cannot be ascertained from the present experiment. However, in the rat aorta, the contraction to PE is mediated in part *via* the α_{1D}-AR subtype (Hussain & Marshall, 1997). Also, the antagonistic affinity estimated for nebivolol in the rat aorta ($pA_2 = 6.5$) was well correlated with previously published pA_2 values for other α_{1D}-AR antagonists (Hussain & Marshall, 1997). The reduced efficiency of PE obtained with increasing concentrations of nebivolol, as well as the finding that the slope of the Schild plot differs from unity (slope = 1.44) suggest that either nebivolol is a noncompetitive antagonist of α_1-ARs, or it has other properties at the concentrations used. The results from the present study do not accord with data obtained previously suggesting that nebivolol has a very low affinity for α_1-ARs and is devoid of α_1-AR antagonistic activity (Broeders *et al*., 2000; Ritter, 2001). However, previously the effects of nebivolol on α_1-ARs were only investigated *in vivo* in humans and rats (Loots & De Clerck, 1990; Schneider *et al*., 1990; Bowman *et al*., 1994; Van Bortel *et al*., 1997) and thus, the results were difficult to interpret due to the influence of numerous regulatory neurohormones on the cardiovascular system.

Our findings further explain the beneficial properties of nebivolol for the treatment of heart failure. Heart failure results in increased sympathetic nervous activity (Shimizu & Mc Grath, 1993) ensuring adequate tissue perfusion; α_1-ARs mediate this vasoconstriction that results in an increased workload of the heart. Nebivolol, like carvedilol, another β-blocker, reduces the afterload of the heart, possibly by its effects on α_1-ARs, thus improving the survival of a patient with chronic heart failure (Louis *et al*., 2001).

The other important finding of the present work was the demonstration that the NO-mediated vasodilatory effect of nebivolol is definitely dependent on the activation of endothelial β_3-ARs in the rat aorta. Thus, the nebivolol-induced relaxation was unaffected by pretreatment with nadolol indicating that it is not mediated by β_1- or β_2-ARs. By contrast, L-748,337, a specific β_3-AR antagonist (Candelore *et al*., 1999), reduced the relaxant effect of nebivolol. Furthermore, nebivolol-induced relaxation was reduced by almost 90% after endothelium removal or inhibition of NOS by L-NMMA, confirming the endothelial localisation of the target mechanism activated by nebivolol. This finding is in accordance with previous data suggesting that nebivolol possesses β_3-AR agonistic properties in rat aorta (De Groot *et al*., 2003), in human umbilical vein endothelial cells (Gosgnach *et al*., 2001) and in human and rodent coronary vessels (Dessy *et al*., 2005). Nevertheless, it was found that nebivolol in the presence of the selective β_3-AR antagonist still induced a small relaxation response. This could firstly be explained by the fact that L-748,337 is a competitive antagonist. Secondly, other receptors could also be involved in the vasodilatation induced by nebivolol, such as 5-HT_{1A} receptors as suggested by Kakoki *et al*. (1999) in the same vascular bed. To exclude the involvement of other endothelial receptors, further studies need to be carried out using antagonists of, for instance, 5-HT or purine receptors.

The present findings are further corroborated by results from our previous studies demonstrating the endothelial localisation of β_3-ARs in rat aorta (Rautureau *et al*., 2002). Stimulation of these receptors produced a relaxation which was antagonized by a β_3-AR antagonist (SR 59230A or L-748,337) and abolished after endothelium removal or inhibition of NOS in rings precontracted with either PE (Trochu *et al*., 1999) or ET1 (present study). It is important to note that the present results obtained in rat aortic rings precontracted with ET1 confirm the functional presence of β_3-ARs and their involvement in the relaxation response to SR 58611A previously observed in rat aortic rings precontracted with PE (Trochu *et al*., 1999; Rautureau *et al*., 2002). In contrast to the findings of Brahmadevara *et al*. (2003; 2004), our results demonstrate that SR 58611A-induced relaxation is antagonised by a selective β_3-AR antagonist, L-748,337, confirming those obtained previously in rat aortic rings precontracted with PE when the effect of SR 58611A was abolished by 10 μM SR 59230A, another β_3-AR antagonist. In addition, the present data obtained from rat aortic rings precontracted with ET1 eliminate the possibility that the relaxant effects of several β_3-ARs agonists, such as SR 58611A or BRL 37344, are due to

the inhibition of α_1-ARs. Nevertheless, L-748,337 failed to antagonize the SR 58611A- and nebivolol-induced relaxation, completely. In this regard, Chlopicki *et al*. (2002) evaluated the vasodilatory potency of nebivolol, using an ultrasonic flowmeter, in guinea-pig heart and concluded that in this vascular bed, nebivolol-induced vasodilatation was not affected by L-748,337. However, in the guinea-pig, a weak or no response to β_3-AR agonist stimulation have been reported in at least two different tissues: myocardium and adipose tissue (Carpene *et al*., 1994; Gauthier *et al*., 1999). Thus, tissue and interspecies differences in the binding and activation of β_3-ARs ligands should be taken into account when comparing results of different studies. L-748,337 is indeed a selective competitive antagonist of β_3-ARs but seems to have a higher affinity *in vitro* for human compared to rat β_3-ARs. In the same recombinant cell preparations, SR 59230A, another β_3-AR antagonist, showed similar affinity for human and rat β_3-ARs, but also exhibited an agonist effect that was dependent on the level of receptor expression (Candelore *et al*., 1999). In our previous studies, it was shown that L-748,337 acted as a selective competitive antagonist of β_3-ARs in both rat (Mallem *et al*., 2004) and human (Rozec *et al*., 2005) blood vessels. As suggested for β_3-AR agonists, the potency/efficacy of antagonists for this receptor could vary according to the functional assays used (Arch, 2002). The demonstration that the endothelium and NO are involved in the nebivolol-induced vasodilatation accords with findings from previous studies performed in different vascular beds of various species: canine coronary and carotid arteries (Gao *et al*., 1991; Ritter, 2001); rat and mice thoracic aorta (Kakoki *et al*., 1999; Broeders *et al*., 2000; Cosentino *et al*., 2002); rat small mesenteric arteries (Altwegg *et al*., 2000) and human and rodent coronaries (Chlopicki *et al*., 2002; Dessy *et al*., 2005). In most of these studies, NO was shown to be involved in the vasorelaxation (Gao *et al*., 1991; Cockcroft *et al*., 1995; Mangrella *et al*., 1998). However, in others different signalling pathways were suggested to be activated, such as an endothelial-independent pathway involving cGMP (Ignarro *et al*., 2002) or EDHF (Dessy *et al*., 2005).

Although, more and more clinical trials have highlighted the possiblity that nebivolol could be an effective treatment for heart failure and improve ventricular function, the beneficial haemodynamic effects of nebivolol are only partly explained by its vascular effects in coronary microarteries, but this might also constitute the mechanism for myocardial function improvement (Dessy *et al*., 2005). Thus, the effect of nebivolol on endothelial β_3-ARs opens up new fields of clinical investigation and, particularly, the evaluation of compounds possessing both β_1-blocker and β_3-AR agonistic properties as likely drugs for treating heart failure. β_1-blockers with a vasodilator effect would induce arteriolar and venous dilatation which would reduce afterload and preload, respectively, to the failing heart. The use of such drugs might be particularly relevant for the treatment of elderly patients with heart failure, when endothelial vasodilator reserve is limited (Flather *et al*., 2005). In hypertensive patients, nebivolol enhances or restores NO-mediated vasodilatation. However, the mechanism involved in this effect has not been clearly identified, although the stimulation of β_3-ARs has been shown to have a beneficial effect in a canine model of perinephritic hypertension (Donckier *et al*., 2001). Also, an upregulation of β_3-ARs has been recently described in aorta of spontaneous hypertensive rats (Mallem *et al*., 2004).

In conclusion, this study has further defined the pharmacological properties of nebivolol, a β-blocker of the third generation, by demonstrating that it has an affinity for α_1- and β_3-ARs in the rat aorta. In addition, the endothelium- and NO-dependent vasorelaxant effects of this drug suggest that it is likely to be of therapeutic interest for the treatment of elderly patients with heart failure or hypertension, as it may improve tolerability and haemodynamic conditions.

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References

ALTWEGG, L.A., D'USCIO, L.V., BARANDIER, C., COSENTINO, F., YANG, Z. & LUSCHER, T.F. (2000). Nebivolol induces NO-mediated relaxations of rat mesenteric but not large elastic arteries. *J. Cardiovasc. Pharmacol.,* **36,** 316–320.

ANDRE, D.E., ARNET, U., YANG, Z. & LUSCHER, T.F. (2000). Nebivolol inhibits human aortic smooth muscle cell growth: effects of cell cycle regulatory proteins. *J. Cardiovasc. Pharmacol.,* **35,** 845–848.

ARCH, J.R. (2002). beta(3)-Adrenoceptor agonists: potential, pitfalls and progress. *Eur. J. Pharmacol.,* **440,** 99–107.

BOWMAN, A.J., CHEN, C.P. & FORD, G.A. (1994). Nitric oxide mediated venodilator effects of nebivolol. *Br. J. Clin. Pharmacol.,* **38,** 199–204.

BRAHMADEVARA, N., SHAW, A.M. & MACDONALD, A. (2003). Evidence against beta 3-adrenoceptors or low affinity state of beta 1-adrenoceptors mediating relaxation in rat isolated aorta. *Br. J. Pharmacol.,* **138,** 99–106.

BRAHMADEVARA, N., SHAW, A.M. & MACDONALD, A. (2004). Alpha1-adrenoceptor antagonist properties of CGP 12177A and other beta-adrenoceptor ligands: evidence against beta(3)- or atypical beta-adrenoceptors in rat aorta. *Br. J. Pharmacol.,* **142,** 781–787.

BREHM, B.R., WOLF, S.C., BERTSCH, D., KLAUSSNER, M., WESSELBORG, S. & SCHULZE-OSTHOFF, K. (2001). Effects of nebivolol on proliferation and apoptosis of human coronary artery smooth muscle and endothelial cells. *Cardiovasc. Res.,* **49,** 430–439.

BROEDERS, M.A., DOEVENDANS, P.A., BEKKERS, B.C., BRONSAER, R., VAN GORSEL, E., HEEMSKERK, J.W., EGBRINK, M.G., VAN BREDA, E., RENEMAN, R.S. & VAN DER ZEE, R. (2000). Nebivolol: a third-generation beta-blocker that augments vascular nitric oxide release: endothelial beta(2)-adrenergic receptor-mediated nitric oxide production. *Circulation,* **102,** 677–684.

CANDELORE, M.R., DENG, L., TOTA, L., GUAN, X.M., AMEND, A., LIU, Y., NEWBOLD, R., CASCIERI, M.A. & WEBER, A.E. (1999). Potent and selective human beta(3)-adrenergic receptor antagonists. *J. Pharmacol. Exp. Ther.,* **290,** 649–655.

CARPENE, C., CASTAN, I., COLLON, P., GALITZKY, J., MORATINOS, J. & LAFONTAN, M. (1994). Adrenergic lipolysis in guinea pig is not a beta 3-adrenergic response: comparison with human adipocytes. *Am. J. Physiol.,* **266,** R905–R913.

CHLOPICKI, S., KOZLOVSKI, V.I. & GRYGLEWSKI, R.J. (2002). NO-dependent vasodilation induced by nebivolol in coronary circulation is not mediated by β-adrenoceptors or by 5 HT_{1A} -receptors. *J. Physiol. Pharmacol.,* **53,** 615–624.

COCKCROFT, J.R., CHOWIENCZYK, P.J., BRETT, S.E., CHEN, C.P., DUPONT, A.G., VAN NUETEN, L., WOODING, S.J. & RITTER, J.M. (1995). Nebivolol vasodilates human forearm vasculature: evidence for an L-arginine/NO-dependent mechanism. *J. Pharmacol. Exp. Ther.,* **274,** 1067–1071.

COSENTINO, F., BONETTI, S., REHORIK, R., ETO, M., WERNER-FELMAYER, G., VOLPE, M. & LUSCHER, T.F. (2002). Nitric-oxide-mediated relaxations in salt-induced hypertension: effect of chronic beta1-selective receptor blockade. *J. Hypertens.,* **20,** 421–428.

DE GROOT, A.A., MATHY, M.J., VAN ZWIETEN, P.A. & PETERS, S.L. (2003). Involvement of the beta3 adrenoceptor in nebivolol-induced vasorelaxation in the rat aorta. *J. Cardiovasc. Pharmacol.,* **42,** 232–236.

DESSY, C., MONIOTTE, S., GHISDAL, P., HAVAUX, X., NOIRHOMME, P. & BALLIGAND, J.L. (2004). Endothelial beta3-adrenoceptors mediate vasorelaxation of human coronary microarteries through nitric oxide and endothelium-dependent hyperpolarization. *Circulation,* **110,** 948–954.

DESSY, C., SALIEZ, J., GHISDAL, P., DANEAU, G., LOBYSHEVA, I., FRERART, F., BELGE, C., JNAOUI, K., NOIRHOMME, P., FERRON, O. & BALLIGAND, J.L. (2005). Endothelial β_3-adrenoreceptors mediate nitric oxide-dependent vasorelaxation of coronary microvessels in response to the third generation β-blocker nebivolol. *Circulation,* **112,** 1198–1205.

DONCKIER, J.E., MASSART, P.E., VAN MECHELEN, H., HEYNDRICKX, G.R., GAUTHIER, C. & BALLIGAND, J.L. (2001). Cardiovascular effects of beta 3-adrenoceptor stimulation in perinephritic hypertension. *Eur. J. Clin. Invest.,* **31,** 681–689.

FLATHER, M.D., SHIBATA, M.C., COATS, A.J., VAN VELDHUISEN, D.J., PARKHOMENKO, A., BORBOLA, J., COHEN-SOLAL, A., DUMITRASCU, D., FERRARI, R., LECHAT, P., SOLER-SOLER, J., TAVAZZI, L., SPINAROVA, L., TOMAN, J., BOHM, M., ANKER, S.D., THOMPSON, S.G., POOLE-WILSON, P.A. & Seniors Investigators. (2005). Randomized trial to determine the effect of nebivolol on mortality and cardiovascular hospital admission in elderly patients with heart failure (SENIORS). *Eur. Heart J.,* **26,** 215–225.

FRATTA PASINI, A., GARBIN, U., NAVA, M.C., STRANIERI, C., DAVOLI, A., SAWAMURA, T., LO CASCIO, V. & COMINACINI, L. (2005). Nebivolol decreases oxidative stress in essential hypertensive patients and increases nitric oxide by reducing its oxidative inactivation. *J. Hypertens.,* **23,** 589–596.

GAO, Y.S., NAGAO, T., BOND, R.A., JANSSENS, W.J. & VANHOUTTE, P.M. (1991). Nebivolol induces endothelium-dependent relaxations of canine coronary arteries. *J. Cardiovasc. Pharmacol.,* **17,** 964–969.

GAUTHIER, C., TAVERNIER, G., TROCHU, J.N., LEBLAIS, V., LAURENT, K., LANGIN, D., ESCANDE, D. & LE MAREC, H. (1999). Interspecies differences in the cardiac negative inotropic effects of beta(3)-adrenoceptor agonists. *J. Pharmacol. Exp. Ther.,* **290,** 687–693.

GEORGESCU, A., PLUTEANU, F., FLONTA, M.L., BADILA, E., DOROBANTU, M. & POPOV, D. (2005). The cellular mechanisms involved in the vasodilator effect of nebivolol on the renal artery. *Eur. J. Pharmacol.,* **508,** 159–166.

GOSGNACH, W., BOIXEL, C., NEVO, N., POIRAUD, T. & MICHEL, J.B. (2001). Nebivolol induces calcium-independent signaling in endothelial cells by a possible beta-adrenergic pathway. *J. Cardiovasc. Pharmacol.,* **38,** 191–199.

GRANNEMAN, J.G. & LAHNERS, K.N. (1994). Analysis of human and rodent beta 3-adrenergic receptor messenger ribonucleic acids. *Endocrinology,* **135,** 1025–1031.

HUSSAIN, M.B. & MARSHALL, I. (1997). Characterization of alpha1-adrenoceptor subtypes mediating contractions to phenylephrine in rat thoracic aorta, mesenteric artery and pulmonary artery. *Br. J. Pharmacol.,* **122,** 849–858.

HUTCHINSON, D.S., SATO, M., EVANS, B.A., CHRISTOPOULOS, A. & SUMMERS, R.J. (2005). Evidence for pleiotropic signaling at the mouse beta3-adrenoceptor revealed by SR59230A [3-(2-Ethylphenoxy)-1-[(1,*S*)-1,2,3,4-tetrahydronapth-1-ylamino]-2*S*-2-propanol oxalate]. *J. Pharmacol. Exp. Ther.,* **312,** 1064–1074.

IGNARRO, L.J. (2004). Experimental evidences of nitric oxide-dependent vasodilatory activity of nebivolol, a third generation beta-blocker. *Blood Press.,* **1,** 2–16.

IGNARRO, L.J., BYRNS, R.E., TRINH, K., SISODIA, M. & BUGA, G.M. (2002). Nebivolol: a selective β(1)-adrenergic receptor antagonist that relaxes vascular smooth muscle by nitric oxide- and cyclic GMP-dependent mechanisms. *Nitric Oxide,* **7,** 75–82.

KAKOKI, M., HIRATA, Y., HAYAKAWA, H., NISHIMATSU, H., SUZUKI, Y., NAGATA, D., SUZUKI, F., KIKUCHI, K., NAGANO, T. & OMATA, M. (1999). Effects of vasodilatory beta-adrenoceptor antagonists on endothelium-derived nitric oxide release in rat kidney. *Hypertension,* **33,** 467–471.

KALINOWSKI, L., DOBRUCKI, L.W., SZCZEPANSKA-KONKEL, M., JANKOWSKI, M., MARTYNIEC, L., ANGIELSKI, S. & MALINSKI, T. (2003). Third-generation beta-blockers stimulate nitric oxide release from endothelial cells through ATP efflux: a novel mechanism for antihypertensive actions. *Circulation,* **107,** 2747–2752.

LOOTS, W. & DE CLERCK, F. (1990). Differential effects of nebivolol on adrenoceptors in the heart and in resistance arterioles in the rat. Quantitative intravital microscopic analysis. *Eur. J. Pharmacol.,* **179,** 177–186.

LOUIS, A., CLELAND, J.G., CRABBE, S., FORD, S., THACKRAY, S., HOUGHTON, T. & CLARK, A. (2001). Clinical trials update: CAPRICORN, COPERNICUS, MIRACLE, STAF, RITZ-2, RECOVER and RENAISSANCE and cachexia and cholesterol in heart failure. Highlights of the Scientific Sessions of the American College of Cardiology. *Eur. J. Heart. Fail,* **3,** 381–387.

MALLEM, M.Y., TOUMANIANTZ, G., SERPILLON, S., GAUTIER, F., GOGNY, M., DESFONTIS, J.C. & GAUTHIER, C. (2004). Impairment of the low-affinity state beta1-adrenoceptor-induced relaxation in spontaneously hypertensive rats. *Br. J. Pharmacol.,* **143,** 599–605.

MANGRELLA, M., FICI, F. & ROSSI, F. (1998). Pharmacology of nebivolol. *Pharmacol. Res.,* **38,** 419–431.

NODARI, S., METRA, M. & DEI CAS, L. (2003). β-Blocker treatment of patients with diastolic heart failure and arterial hypertension. A prospective, randomized, comparison of the long-term effects of atenolol vs Nebivolol. *Eur. J. Heart Failure,* **5,** 621–627.

PARENTI, A., FILIPPI, S., AMERINI, S., GRANGER, H.J., FAZZINI, A. & LEDDA, F. (2000). Inositol phosphate metabolism and nitric-oxide synthase activity in endothelial cells are involved in the vasorelaxant activity of nebivolol. *J. Pharmacol. Exp. Ther.,* **292,** 698–703.

PAUWELS, P.J., GOMMEREN, W., VAN LOMMEN, G., JANSSEN, P.A. & LEYSEN, J.E. (1988). The receptor binding profile of the new antihypertensive agent nebivolol and its stereoisomers compared with various beta-adrenergic blockers. *Mol. Pharmacol.,* **34,** 843–851.

RAUTUREAU, Y., TOUMANIANZ, G., SERPILLON, S., JOURDON, P., TROCHU, J.N. & GAUTHIER, C. (2002). β_3-Adrenoceptor in rat aorta: molecular and biochemical characterization and signalling pathway. *Br. J. Pharmacol.,* **137,** 153–161.

RITTER, J.M. (2001). Nebivolol: endothelium-mediated vasodilating effect. *J. Cardiovasc. Pharmacol.,* **38,** S13–S16.

ROZEC, B., SERPILLON, S., TOUMANIANTZ, G., SEZE, C., RAUTUREAU, Y., BARON, O., NOIREAUD, J. & GAUTHIER, C. (2005). Characterization of beta3-adrenoceptors in human internal mammary artery and putative involvement in coronary artery bypass management. *J. Am. Coll. Cardiol.,* **46,** 351–359.

SCHEEN, A.J. (2001). Pharma-clinics medication of the month, Nebivolol (Nobiten). *Rev. Med. Liege,* **56,** 788–791.

SCHNEIDER, J., FRUH, C., WILFFERT, B. & PETERS, T. (1990). Effects of the selective beta 1-adrenoceptor antagonist, nebivolol, on cardiovascular parameters in the pithed normotensive rat. *Pharmacology,* **40,** 33–41.

SHEN, Y.T., CERVONI, P., CLAUS, T. & VATNER, S.F. (1996). Differences in beta 3-adrenergic receptor cardiovascular regulation in conscious primates, rats and dogs. *J. Pharmacol. Exp. Ther.,* **278,** 1435–1443.

SHIMIZU, K. & MC GRATH, BP. (1993). Sympathetic dysfunction in heart failure. *Bailleres Clin. Endocrinol. Metab.,* **7,** 439–463.

TAVERNIER, G., GALITZKY, J., BOUSQUET-MELOU, A., MONTASTRUC, J.L. & BERLAN, M. (1992). The positive chronotropic effect induced by BRL 37344 and CGP 12177, two beta-3 adrenergic agonists, does not involve cardiac beta adrenoceptors but baroreflex mechanisms. *J. Pharmacol. Exp. Ther.,* **263,** 1083–1090.

TODA, N. (2003). Vasodilating beta-adrenoceptor blockers as cardiovascular therapeutics. *Pharmacol. Ther.,* **100,** 215–234.

TROCHU, J.N., LEBLAIS, V., RAUTUREAU, Y., BÉVÉRELLI, F., LE MAREC, H., BERDEAUX, A. & GAUTHIER, C. (1999). Beta 3-adrenoceptor stimulation induces vasorelaxation mediated essentially by endothelium-derived nitric oxide in rat thoracic aorta. *Br. J. Pharmacol.,* **128,** 69–76.

VAN BORTEL, L.M., DE HOON, J.N., KOOL, M.J., WIJNEN, J.A., VERTOMMEN, C.I. & VAN NUETEN, L.G. (1997). Pharmacological properties of nebivolol in man. *Eur. J. Clin. Pharmacol.,* **51,** 379–384.

WAEBER, B. (2000). Nebivolol: a beta blocker with vasodilator properties. *Schweiz Rundsch. Med. Prax.,* **89,** 631–633.

ZANCHETTI, A. (2004). Clinical pharmacodynamics of nebivolol: new evidence of nitric oxide-mediated vasodilating activity and peculiar haemodynamic properties in hypertensive patients. *Blood Press,* **1,** 17–32.

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