RESEARCH

Cardiovascular Therapeutics

Different Pharmacological Properties of Two Enantiomers in a Unique β-Blocker, Nebivolol

Louis J. Ignarro

Department of Medical and Molecular Pharmacology, University of California Los Angeles School of Medicine, Center for Health Sciences, Los Angeles, California, USA

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Correspondence
Professor Louis J. Ignarro, PhD, Department of Medical and Molecular Pharmacology, University of California Los Angeles (UCLA) School of Medicine, 23–305A Center for Health Sciences, Box 951735 Los Angeles, CA 90095-1735, USA.
Tel.: +1-310-825-5159 or +1-310-825-7137;
Fax: +1-310-206-0589;
E-mail: lignarro@mednet.ucla.edu

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Nebivolol is a racemic combination of d-nebivolol (+SRRR nebivolol) and l-nebivolol (–RSSS nebivolol) that differs chemically from other β-blockers, with an absolutely symmetrical configuration developing from a central nitrogen atom. D-nebivolol and l-nebivolol divaricate pharmacologically and therapeutically, with a noticeably different profile from that of conventional β-blockers; for instance, the selective blocking of β_1-adrenoceptors is determined almost exclusively by d-nebivolol. Both enantiomers act synergistically with respect to blood pressure reduction: the effect of nebivolol on heart rate is exclusively exerted by d-nebivolol, with these hypotensive effects enhanced by the addition of the l-enantiomer, which in itself does not influence systolic and diastolic blood pressure. Furthermore, this pronounced and lasting blood pressure reduction is roughly equal to the effect of conventional β-blockers in high doses. In certain vascular districts, nebivolol stimulates endothelial nitric oxide (NO) synthesis, thereby increasing the availability of NO in the endothelium, smooth muscle, and platelets and, consequently, producing a sustained vasodilation, with decreases in peripheral resistance and blood pressure. These effects are not shared by other β-adrenoceptor blockers used as references and mainly rely on the l-enantiomer. L-nebivolol also increases NO availability under conditions of oxidative stress by the inhibition of endothelial NO synthase (eNOS) uncoupling, thereby reducing NO inactivation. Furthermore, neither nebivolol nor its enantiomers show any intrinsic sympathomimetic activity and undesirable β-blocker effects, such as a decrease in cardiac output, which do not occur or are less pronounced with the combination of d-nebivolol and l-nebivolol. In conclusion, the independent pharmacologic and clinical effects of d-nebivolol and l-nebivolol act synergistically to produce a cardiovascular profile that differs noticeably from that of conventional β-blockers.

ATP = adenosine5-triphosphate
BAECs = bovine aortic endothelial cells
CVECs = cultured bovine coronary postcapillary venular endothelial cells
ECs = endothelial cells
EDHF = endothelium-derived hyperpolarizing factor
EDRF = endothelium-derived relaxing factor
eNOS = endothelial nitric oxide synthase
ET = endothelin
GTN = glycerol trinitrate
HUVECs = human umbilical vein endothelial cells
i.v. = intravenous
LDL = low density lipoprotein
L-NAME = N-nitro-L-argine methyl ester
NADPH = nicotinamide adenine dinucleotide phosphate
NO = nitric oxide
NOS = nitric oxide synthase
L-NMMA = N^6-monomethyl-L-arginine
L-NNA = N-nitro-L-arginine
ODQ = 1H-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-l-one
PLA2 = phospholipase A2
PKA = protein kinase A
PLD = phospholipase D
SPC = summary of product characteristics
SHRs = spontaneously hypertensive rats

Introduction

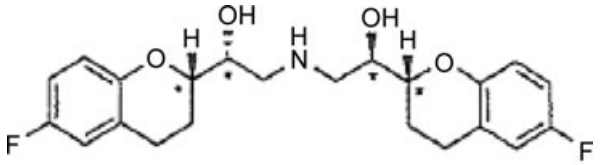
Nebivolol is an innovative drug that differs chemically, pharmacologically, and therapeutically from all other β-blockers. The compound is a racemic combination of d-nebivolol (+SRRR nebivolol) and l-nebivolol (–RSSS nebivolol) (Fig. 1).

Chemical Characteristics

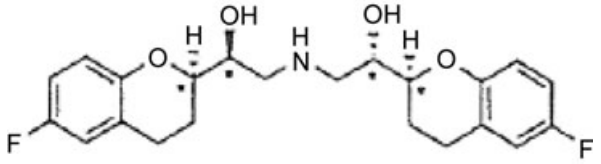
Differences in the Basic Chemical Structure *Versus* Other β-Blockers

It is immediately obvious that the chemical structure of nebivolol differs from that of other β-blockers (Siebert et al. 2007). Conventional β-blockers are derivatives of an oxypropanolamine structure that can still be seen in carvedilol, whereas sotalol, which is authorized as an antiarrhythmic drug and not for the treatment of essential hypertension, and nebivolol both deviate from this basic structure (Labrid et al. 1989). Atenolol, bisoprolol, and metoprolol are all isopropylamine derivatives, while nebivolol is known as a bis-iminodiethanol derivative (Siebert et al. 2007).

Contrary to all other β-blockers, nebivolol displays an evident symmetrical configuration, with a complex structure developing from a central nitrogen atom (Janssen 1991; Siebert et al. 2007). A common feature of conventional β-blockers is an aromatic or heteroaromatic ring structure (commonly $OCH_2CH(OH)CH_2NHR$, where R varies among β-blockers) attached to the oxypropanolamine structure through the oxygen atom via an ether bridge. However, nebivolol has a distinct structure in which the oxygen atom is incorporated into a fluorochroman structure that directly connects to the central bis-iminodiethanol structure (Labrid et al. 1989; Siebert et al. 2007).



D-Nebivolol (*SRRR*-Isomer)



L-Nebivolol (*SRRR*-Isomer)

Figure 1 Structural formulae of the two enantiomers of nebivolol.

Number of Chiral Centers and Spatial Configuration

Owing to its particular chemical structure, nebivolol bears four chiral centers compared to all other conventional β-blockers that have only one chiral center (Siebert et al. 2007). The number of chiral centers has a significant impact on the spatial configuration of the active substances. Conventional β-blockers can form only two mirror-image configurations (enantiomers), referred to as D- and S-enantiomers (Siebert et al. 2007). On the other hand, owing to its four symmetrically arranged chiral centers, nebivolol can assume 10 different spatial configurations, each of which has only one mirror-image partner and cannot be spatially superimposed on the other configurations (Siebert et al. 2007).

Spatial Configuration and Pharmacological Properties

A molecule's spatial configuration has serious implications for its receptor-binding properties. If the active substance has an unfavorable configuration, it will have little or no effect on the receptor (Labrid et al. 1989). In the case of conventional β-blockers, the β-blocking effect of the S-enantiomer is approximately 100- to 200-fold stronger than that of the corresponding D-enantiomer, (Mutschler et al. 2001). With nebivolol, on the other hand, only 2 of the 10 possible configurations are pharmacologically active at clinical dosages. These are referred to as d-nebivolol (+SRRR) and l-nebivolol (–RSSS) (Pauwels et al. 1988). As a result of a selective synthesis and production process, the medicinal product (Nebilet®) contains only these two eutomers and, contrary to conventional β-blockers, no inactive distomers.

Pharmacological Characteristics

While conventional β-blockers exclusively inhibit the effects of catecholamines at the β-adrenergic receptors, d-nebivolol and l-nebivolol each develops specific pharmacologic effects through two different mechanisms (Ignarro 2004; Janssens et al. 1991).

Selective Blocking of β_1-Receptors by d-Nebivolol

Binding Studies

At clinical doses, selective blocking of β_1-adrenoceptors is determined almost exclusively by d-nebivolol (+SRRR nebivolol), whose affinity for the β_1-adrenoceptors is approximately 175-fold greater than that of l-nebivolol (Pauwels et al. 1988; Van der Water et al. 1988b). This binding behavior is contrary to all other β-blockers, where the l-enantiomer is the more effective antagonist and in which the OH group of the oxypropanolamine structure must be present in the S-configuration in order to achieve high β_1-adrenoceptor affinity (Barrett and Cullum 1968; Kober et al. 1982). However, in d-nebivolol, both OH groups are arranged exactly inversely in a configuration that suggests a mechanism of binding to the β-adrenoceptor site in a manner different to that of other β-blockers (Siebert et al. 2007).

In an attempt to confirm and extend the available data on the binding selectivity of the two nebivolol enantiomers, their ability to interact with 46 different receptors or channels and 6 enzymes, mostly of human origin (recombinant or from human tissues), was tested (Criscuoli and Evangelista, unpublished data). Affinity values are in good general agreement with those obtained in the past, using receptors from animal tissues (Pauwels et al. 1988). In particular, d-nebivolol showed comparable affinity for β_1- and 5-HT_{1A} human receptors (Ki = 1.7 and 2.8 nM, respectively) and its affinity for β_2-adrenoceptors was much lower (125 nM). L-nebivolol showed a much lower affinity than d-nebivolol for the β_1, β_2, and β_3 adrenoceptors (90 nM, 217 nM, and >1500 nM, respectively), but maintained a good affinity for the 5-HT_{1A} receptor (15 nM). Interestingly, both enantiomers showed a much lower affinity for the rat-type of 5-HT_{1A} receptor. This different selectivity was confirmed in human myocardium, where d-nebivolol had an affinity for β_1-and β_2-adrenoceptors of 6 and 150 nM, respectively, compared to 496 and 338 nM, respectively, for the l-enantiomer (Bundkirchen et al. 2003a).

It should be noted that dl-nebivolol, when challenged with cell membranes of the left ventricular myocardium of a nonfailing human heart in binding competition experiments to the radioligand ^{3}H-CGP 12.1777 in the presence of β_1- and β_2-selective antagonists, was found to be the most selective β_1 antagonist among the most commonly used β-blockers, a finding well in line with those obtained in vascular tissue and cultured cell preparations (Brixius et al. 2001). Furthermore, this selectivity was retained by the metabolized drug, as assessed by serum samples of patients treated with nebivolol (Bundkirchen et al. 2003b).

A recent study examining the selectivity of a wide range of β-blockers has confirmed the high selectivity of dl-nebivolol in Chinese hamster ovary cells stably expressing human β_1- or β_2-adrenoceptors (Baker 2005). As shown in Table 1, the β_1 selectivity of most clinically used β-blockers is poor in intact cells, and some compounds that are traditionally classed as "β_1-selective" have also higher affinity for the β_2-adrenoceptor. Among the compounds tested, only bisoprolol and nebivolol were endowed with sharp selectivity for the β_1-adrenoceptor.

Effect on Cardiac Contractility In Vitro

In the studies of Brixius et al. (2001) and Bundkirchen et al. (2003b), nebivolol and its enantiomers did not show any intrinsic sympathomimetic activity (as measured by increases in forskolin-stimulated force of contraction in isolated ventricular (Brixius et al. 2001) and atrial (Bundkirchen et al. 2003b) trabeculae from human nonfailing hearts). Consequently, nebivolol did not modify the enhancing effect of forskolin on adenylate cyclase. Neither d-nebivolol nor l-nebivolol $\leq$30 μM influenced myofibrillar Ca^{2+} responsiveness. Furthermore, dl-nebivolol and d-nebivolol, but not l-nebivolol, all at 0.5 μM, improved the frequency-dependent and maximum-force generation that is linked to the activation of β-adrenoceptors (Table 2).

The antagonist effect of dl-nebivolol on isoproteronol-induced increases in the heart rate of guinea pig-isolated atria is mimicked by its d-enantiomer; however, the l-enantiomer is 100-fold less potent than the d-enantiomer (Janssens et al. 1991). The presence of the l-enantiomer did not significantly affect the β_1-adrenoceptor antagonism afforded by d-nebivolol.

Cardiovascular Effects In Vivo

Hemodynamic effects elicited by intravenous (i.v.) dl-nebivolol were mainly ascribed to d-nebivolol in normotensive rats, as indicated by the almost complete inhibition of adrenergic stimulation with isoproterenol elicited by this enantiomer (Sacco et al. 2005). In addition, only d-nebivolol produced a hypotensive effect comparable to that of the racemate (Fig. 2). L-nebivolol did not exert any effect against β-adrenergic stimulation, confirming that the β_1 selectivity of the drug can be ascribed to its d-enantiomer.

While reduction in heart rate induced by nebivolol was exclusively exerted by the d-enantiomer in spontaneously hypertensive rats (SHRs), the hypotensive effects

Table 1 Log K_D values of β-blockers and β-agonists for binding to the human β_1- and β_2-adrenoceptors in Chinese hamster ovary cells and their selectivity-ratios values (modified from Baker, 2005, with permission).

β-ligand	Log K_D for β_1	Log K_D for β_2	Selectivity: β_1 vs. β_2	Selectivity: β_2 vs. β_1
CGP 20712A	-8.81 ± 0.03	-6.11 ± 0.05	502.2	
ICI 89406	-8.91 ± 0.09	-7.07 ± 0.06	69.2	
Practolol	-6.14 ± 0.05	-4.99 ± 0.07	>14.1	
Nebivolol	-9.04 ± 0.04	-7.89 ± 0.08	14.1	
Xamoterol	-7.22 ± 0.04	-6.07 ± 0.08	14.1	
Bisoprolol	-7.83 ± 0.04	-6.70 ± 0.05	13.5	
Betaxolol	-8.21 ± 0.07	-7.38 ± 0.06	6.8	
Atenolol	-6.66 ± 0.05	-5.99 ± 0.14	4.7	
ICI 215001	-6.37 ± 0.05	-5.86 ± 0.04	3.2	
Acebutolol	-6.46 ± 0.03	-6.08 ± 0.07	2.4	
Metoprolol	-7.26 ± 0.07	-6.89 ± 0.09	2.3	
CGP 12177	-9.21 ± 0.04	-9.39 ± 0.07		1.5
Labetolol	-7.63 ± 0.05	-8.03 ± 0.07		2.5
Carvedilol	-8.75 ± 0.09	-9.40 ± 0.08		4.5
Pronethanol	-6.44 ± 0.07	-7.36 ± 0.07		8.3
Propranolol	-8.16 ± 0.08	-9.08 ± 0.06		8.3
Sotalol	-5.77 ± 0.11	-6.85 ± 0.09		12.0
CL 316243	>-3	-4.10 ± 0.19		>12.6
Alprenolol	-7.83 ± 0.06	-9.04 ± 0.07		16.2
Bupranolol	-8.51 ± 0.04	-9.85 ± 0.05		21.9
Nadolol	-7.23 ± 0.04	-8.60 ± 0.07		23.4
Timolol	-8.27 ± 0.08	-9.68 ± 0.02		25.7
ICI 118551	-6.52 ± 0.02	-9.26 ± 0.03		549.5
Clinically used β-agonists				
Salbutamol	-4.66 ± 0.07	-6.12 ± 0.07		28.8
Terbutaline	-3.82 ± 0.07	-5.62 ± 0.06		63.1
Salmeterol	-5.38 ± 0.01	-8.83 ± 0.07		2818.4

Values represents mean ± standard error of measurement of 4–10 separate experiments.
Mammalian (Chinese hamster ovary) cells stably expressing either the human β_1-adrenoceptor or the human β_2-adrenoceptor were cultured for cell-binding studies using appropriate ligands for each receptor subtype (Baker 2005).

Table 2 Frequency-dependent force development before and after 0.5 μM of dl-nebivolol, d-nebivolol, l-nebivolol, or vehicle in right atrial tabeculae from human myocardium (modified from Bundkirchen et a., 2003b).

	FOC_{max} (% of $FOC_{0.5Hz}$)		Frequency at FOC_{max} (Hz)	
	Before	After	Before	After
dl-nebivolol (n = 6)	170.0 ± 22.4	215.0 ± 14.1*	1.7 ± 0.3	2.3 ± 0.2*
d-nebivolol (n = 7)	175.0 ± 30.2	240.0 ± 27.0*	1.7 ± 0.2	2.2 ± 0.1*
l-nebivolol (n = 8)	172.0 ± 17.2	176.0 ± 22.1	1.6 ± 0.1	1.8 ± 0.1
Vehicle (n = 5)	175.0 ± 19.6	181.0 ± 30.6	1.9 ± 0.1	1.9 ± 0.2

FOC_{max}, maximal developed force of contraction.
*$P < 0.05$ vs. respective value before the exposure to drug.

of the d-enantiomer were enhanced by the addition of the l-enantiomer, which in itself did not influence systolic and diastolic blood pressure (Xhonneux et al. 1990). In pithed rats, a model used to study the effects of hypotensive drugs that avoid the baroreceptor reflexes, dl-nebivolol and d-nebivolol at low doses were both found to be potent antagonists of β_1-adrenoceptors, reducing the heart rate induced by (–)epinephrine (Schneider et al. 1990). Furthermore, the l-enantiomer was 1000-fold less potent than the d-enantiomer as β_1-adrenoceptor antagonist in this test (Schneider et al. 1990). In this experimental model, the selectivity of the racemate and its d-enantiomer was confirmed by their lack of interaction with α-, β_2-, 5-HT_2-, and angiotensin II receptors

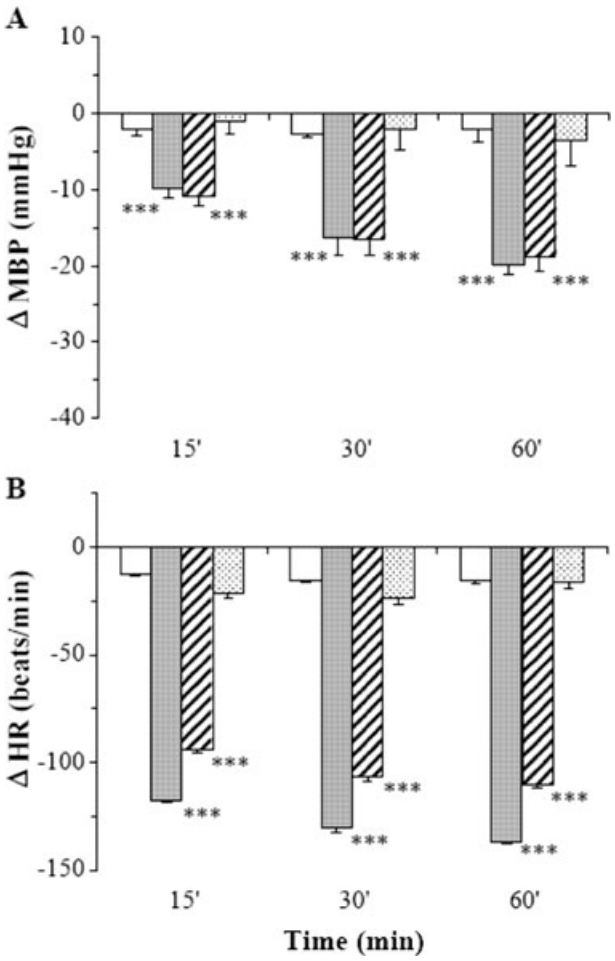


Figure 2 Effects of intravenous administration of dl-, d-, and l-nebivolol on (**A**) mean blood pressure (MBP) and (**B**) heart rate (HR) in normotensive rats. Groups were: vehicle (open bar), dl-nebivol at 1 mg/kg (gray bar), and d-nevivolol (hatched bar) or l-nebivolol (dotted bar), both at 0.5 mg/kg. Data reported are mean differences *versus* basal values of 5–8 rats (***$P <$ 0.001 vs. control) (reproduced from Sacco et al. 2005, with permission).

and by lack of interaction with sympathetic neurotransmission. Likewise, the cardiovascular effects of d-nebivolol in close-chest anesthetized dogs were due to the antagonism of the β_1-adrenoceptor and differed from those observed with the l-enantiomer, which reduced peripheral resistances (Van der Water et al. 1988b).

In humans, the effects of racemic nebivolol 2.5, 5.0, and 10.0 mg, d-nebivolol 2.5 mg, l-nebivolol 2.5 mg, and placebo, each given once daily for 7 days, were tested (Van Nueten and De Crée 1998). Both dl-nebivolol 5.0 mg and d-nebivolol 2.5 mg significantly reduced exercise-induced increases in heart rate and systolic blood pressure to a similar extent, while there was no significant effect with l-nebivolol 2.5 mg or placebo. These data confirm that the β-blocking effects of nebivolol reside in the d-enantiomer. Further studies reviewed by De Cree et al. (1991) showed that the racemic enantiomer mixture produced unique combined effects in both forms: d-nebivolol, a highly selective β_1-adrenoceptor antagonist, lowers heart rate and blood pressure and l-nebivolol improves left ventricular performance at rest.

Stimulation of Endothelial Nitric Oxide (NO) Release by l-Nebivolol

The early pharmacologic and clinical studies have already shown that the combination of d-nebivolol and l-nebivolol has properties that cannot be explained by the blocking of adrenergic receptors (De Cree et al. 1991; Himmelmann et al. 1996; Stoleru et al. 1993). Further research proved the existence of a second mechanism of action, which was described as an interaction with the L-arginine/NO pathway (Gao et al. 1991; Ignarro 2004). By now, it has been confirmed a number of times that the activity of endothelial NO synthase endothelial nitric oxide synthase (eNOS) increases in the presence of l-nebivolol (Evangelista et al. 2007; Ladage et al. 2006; Mason et al. 2006). Because of this, the availability of NO in the endothelium increases and NO moves to the smooth muscle cells. As a consequence, a sustained vasodilation occurs and peripheral resistance and blood pressure decrease. The nebivolol-induced vasodilating effect is less pronounced than that obtained with NO donors and phosphodiesterase-5 inhibitors and is not subject to tachyphylaxis (Nodari et al. 2003).

Nebivolol is the first and, so far, the only drug whose summary of product characteristics (SPC) mentions an interaction with the L-arginine/NO pathway (nebivolol 5 mg tablets, European SPC) (eMC 2007). The unique effects of l-nebivolol are mediated via a target site at the endothelium and can thus be detected even in the arterioles and capillaries where conventional β-blockers have neither target sites nor effects (Arosio et al. 2002; Dessy et al. 2005). Furthermore, l-nebivolol increases NO availability under conditions of oxidative stress by the inhibition of eNOS uncoupling and, therefore, by reducing NO inactivation (Evangelista et al. 2007; Mason et al. 2006). Nebivolol racemate and/or its l-enantiomer has been found to be protective in experimental models of thrombosis and atherosclerosis (Fratta Pasini et al. 2005; Mollnau et al. 2003; Troost et al. 2000), probably through the stimulation of NO release from the endothelium and platelets (Falciani et al. 2001; Ignarro 2004). The nebivolol-induced release of NO from platelets seems to be another important mechanism involved in the vasoprotective effects of this drug.

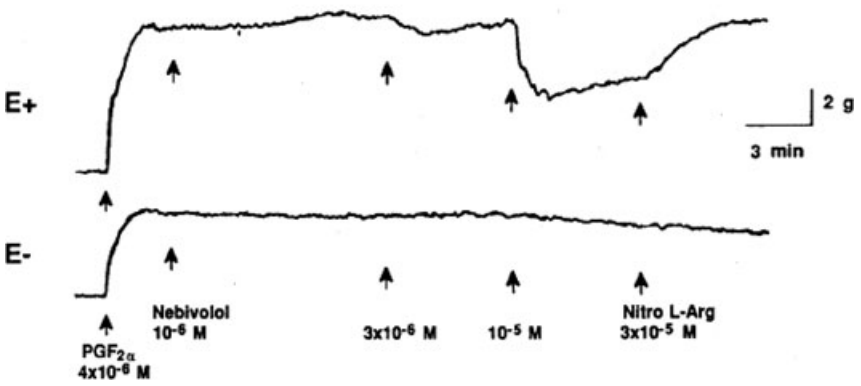


Figure 3 Changes in tension invoked by cumulatively added nebivolol in canine left anterior descending coronary arteries with and without endothelium during contractions from prostaglandin $F_{2\alpha}$ (4×10^{-6}) (E+, with endothelium; E–, without endothelium) (reproduced from Gao et al. 1991, with permission).

Nitrogen Monoxide-Dependent Vasodilation

The vasodilatory properties of nebivolol were firstly described by Gao et al. (1991) in dog coronary arteries. This tissue, contracted by the use of prostaglandin F2-alpha ($PGF_{2\alpha}$), was relaxed in a concentration-dependent manner by nebivolol only in the presence of an intact endothelium (Fig. 3). The l-enantiomer induced similar relaxation, while d-nebivolol was less potent in vasodilating the coronaries (Gao et al. 1991). Adenosine 5′-diphosphate (ADP)-induced relaxation was similarly potentiated by dl-nebivolol and l-nebivolol. The vasodilation was counteracted by nitro-L-arginine or methylene blue and unaffected by indomethacin, phentolamine, propranolol, or methysergide, thus ruling out the role of α and β_{1-2} adrenoceptors and 5-HT receptors in this effect (Gao et al. 1991). At that time, the authors hypothesized that the release of endothelium-derived relaxing factor (EDRF) (later recognized as NO) played a pivotal role in vasodilation.

Most studies were then devoted to confirming the NO-dependent vasodilatory properties of nebivolol, originally discovered by Gao et al. (1991) and subsequently confirmed in pig coronary arteries by Hashimoto et al. (1996). In fact, this vasorelaxant effect has been further studied, in various experimental conditions, on vessels differing by vascular district, diameter, and function, and in different species. The involvement of NO has been generally verified by evaluating the effect of L-arginine/NO pathway inhibitors, but, sometimes, a measurement of NO production has also been performed with different assays. Moreover, in some studies, the NO release from cultured endothelial cells (ECs) has been evaluated.

In the dog, we confirmed the early evidence of the NO-mediated vasorelaxant action of nebivolol in the coronary artery (Gao et al. 1991) and extended it to a structurally and functionally different vessel, the pulmonary artery (Ignarro et al. 2002a). However, in both vessels, this nebivolol activity appeared to be only partially endothelium-dependent, especially at concentrations >10 μM. At these concentrations, nebivolol-induced vasorelaxation was also only partially antagonized by inhibitors of the L-arginine/NO pathway, namely N^{ω}-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ), and methylene blue. These nebivolol-induced effects, definitely weaker than those seen with vasodilators such as acetylcholine and sodium nitroprusside, were not related to β_1-adrenoceptor antagonism. In fact, atenolol was completely inactive in these models.

In the rat, the existence of an endothelium/NO-dependent vasodilatory response to nebivolol has been confirmed in peripheral resistance vessels and in cardiac microvasculature (Altwegg et al. 2000; Dessy et al. 2005; Georgescu et al. 2005; Kakoki et al. 1999; Maffei et al. 2006; Parenti et al. 2000). This effect was observed at nebivolol concentrations ≥300 nM, an effect that was not shared by atenolol ≤30 μM and was antagonized by L-NAME, the inhibitor of the L-arginine/NO pathway.

In a preconstricted rat mesenteric vascular bed, the decrease in perfusion pressure induced by the racemate in a concentration-dependent manner was mimicked by the l-enantiomer but not by d-nebivolol (Parenti et al. 2000; Fig. 4), showing that in these vessels, the vasodilation of the drug can be ascribed to the l-enantiomer.

The effects of nebivolol on the rat renal microvasculature were studied by Kalinowski et al. (2003). In this study, renal glomeruli were isolated and contracted by angiotensin II, with the effect of added nebivolol measured as changes in glomerular inulin space. Nebivolol (≥1 μM) produced a fast (within 3 min) and complete reversal of the angiotensin II-induced contraction, an effect that could be prevented by pretreatment with N^{ω}-nitro-L-arginine (L-NNA), a potent eNOS inhibitor. The studies performed with an elastic artery, such as rat aorta, produced heterogeneous results. In one case, a

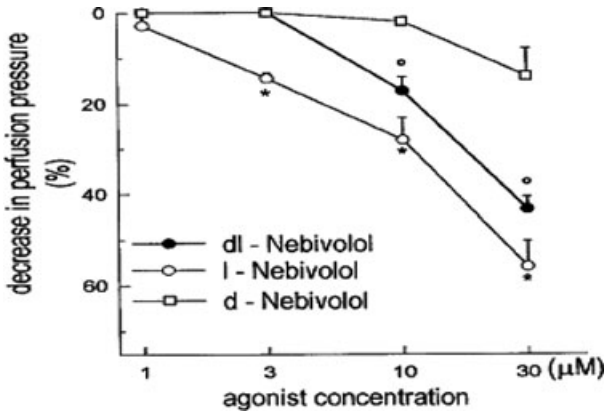


Figure 4 Percentage decrease in perfusion pressure induced by increasing concentrations of dl-nebivolol (n = 8), l-nebivolol (n = 8), and d-nebivolol (n = 4) in preparations of rat isolated mesenteric vascular beds preconstricted by the thromboxane analog U46619 0.3 μM. Points represent the mean values; vertical bars indicate standard error of measurement values. (*$P < 0.05$ vs. d-nebivolol; °$P < 0.05$ vs. d-nebivolol) (reproduced from Parenti et al. 2000, with permission).

potent NO-dependent vasodilation was observed (Kakoki 1999); however, no effect was measured in another study (Altwegg et al. 2000) for concentrations $\leq$30 μM. Conversely, we demonstrated in our laboratory that nebivolol does relax rat aorta rings, when used at concentrations $\geq$10 μM, with a mechanism that is partially endothelium-, eNOS-, and guanylate cyclase-dependent and mostly NO-dependent, as estimated from the complete antagonism exerted by oxyhemoglobin, both in intact and endothelium-denuded arteries (Ignarro et al. 2002a). Not only the reference β-blocker atenolol, but also prototypes of the other major classes of antihypertensive drugs, the calcium channel blocker amlodipine, the angiotensin II receptor 1 antagonist ZD 7155, and the ACE inhibitor enalaprilat, were completely inactive as dilators of the rat aorta at comparable concentrations in this model (Buga and Ignarro 2000).

These data were basically confirmed by de Groot et al. (2003) and Rozec et al. (2006), who verified that nebivolol concentration dependently (starting from 1 to 3 μM) relaxed phenylephrine- or endothelin-precontracted rat aorta rings. This effect was blocked by incubation with L-NNA or by the removal of the endothelium. Metoprolol was completely inactive (de Groot et al. 2003; Rozec et al. 2006). The potential interaction between the vasodilating effect of nebivolol and that of sildenafil was studied by Rosenkranz et al. (2006). They found that nebivolol had an EC_{50} of 3.5 μM in relaxing phenyleprine-induced rat aorta contractions and that, unlike glycerol trinitrate (GTN), sildenafil did not potentiate the effect of nebivolol (Rosenkranz et al. 2006). The observation that different mechanisms of action are recruited by sildenafil and nebivolol might suggest that patients treated with nebivolol can be safely treated with sildenafil.

The effects of nebivolol on the coronary circulation were studied by Gryglewski et al. (2001) in the guinea pig-isolated heart by the Langendorff method. Bolus injections of 10 or 30 μM of nebivolol acutely increased the basal coronary flow by 2- to 3-fold. These effects were partially NO-dependent as the preinfusion of L-NAME reduced them by approximately 70% (Gryglewski et al. 2001). Both d- and l-nebivolol were similarly effective in increasing coronary flow (Chlopicki et al. 2002). Conversely, in the ischemic-reperfused myocardium of the isolated working rabbit heart, the protective effect of nebivolol was attributed to the l-enantiomer (Lu et al. 1990).

In most *in vitro* studies on human macrovessel preparations performed up to now, nebivolol did not produce measurable vasodilation or NO release (on internal mammary artery and saphenous vein) (d'Uscio et al. 1998; van der Zee et al. 1997). However, nebivolol was able to relax human microcoronary arteries precontracted with endothelin-1 or potassium chloride, an effect that was fully endothelium-dependent and only partially inhibited by L-nitroarginine (Dessy et al. 2005). The vasodilating effect of nebivolol in this site (microvessels from 70 to 170 μm in diameter) indicates the capability of the drug in the regulation of coronary resistance and perfusion reserve.

Finally, the clinical pharmacology studies previously carried out in healthy volunteers (Bowman et al. 1994; Cockcroft et al. 1995), with the plethysmographic measure of forearm blood flow (a model similar to an isolated organ system), were repeated in moderately hypertensive patients. Contrasting results were obtained: NO-dependent vasodilation was observed in one case (Dawes et al. 1999), but not in another study (Ghiadoni et al. 2003). However, even in the latter, a vasodilatory effect became evident if vitamin C was coinfused with nebivolol (Ghiadoni et al. 2003). It was hypothesized that, in this case, the hypertension-related endothelial dysfunction was greater than in the Dawes et al. (1999) study and that the scavenging of excess oxygen radicals was needed to protect released NO from fast inactivation.

Tzemos et al. (2001), using a similar method of assessment, showed that nebivolol (5 mg/day), but not atenolol (50 mg/day), was able to revert endothelial dysfunction in hypertensive patients after chronic (8 weeks) treatment with therapeutic oral doses of the drugs (Fig. 5). In the study, nebivolol, but not atenolol,

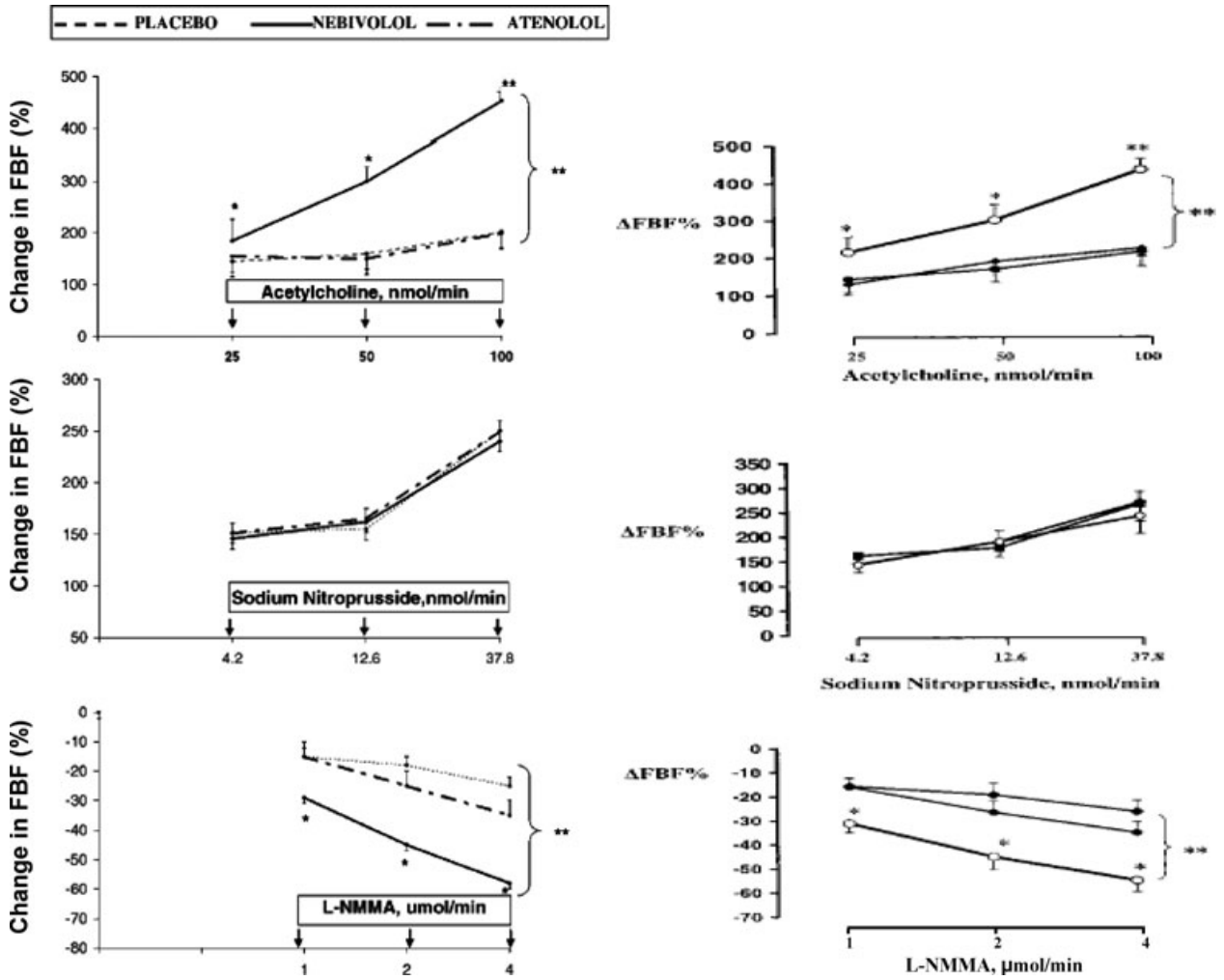


Figure 5 Percentages changes in vasodilation as measured by forearm blood flow (FBF) from baseline preceding each drug perfusion for three dose levels of acetylcholine, sodium nitroprusside, and N^6-monomethyl-L-arginine (L-NMMA) after placebo (◇), nebivolol (○), and atenolol (●) therapy. Values are mean ± standard error of measurement (*$P < 0.05$, **$P < 0.001$ for differences between the treatments) (reproduced from Tzemos et al. 2001, with permission).

produced a significant increase in acetylcholine-mediated (NO-dependent) forearm vasodilation. Only nebivolol-based treatment improved the vasoconstrictive response to N^6-monomethyl-L-arginine (L-NMMA) compared to baseline, suggesting an additional improvement of basal (tonic) NO release.

Soon after, Arosio et al. (2002) reported that even a single oral dose of nebivolol 5 mg (but not atenolol 100 mg) was able to markedly enhance the vasodilator response to iontophoretically applied acetylcholine (measured as blood flow in the third finger tip of the left hand by means of laser Doppler technique) in hypertensive patients. Recently, Lekakis et al. (2005) showed that in patients with coronary artery disease, the 4-week administration of nebivolol (5 mg/kg/day), but not atenolol (50 mg/kg/day), significantly increased the flow-mediated dilatation of the brachial artery. In addition, Kubli et al. (2001) reported that the same oral dose of nebivolol enhanced acetylcholine-stimulated cutaneous vasodilation in healthy volunteers, as measured 3 h after both single and repeated (8 days) administration by laser Doppler technique. Again, atenolol was inactive (Kubli et al. 2001).

The described series of *in vitro* studies confirmed that, at least in certain vascular districts, nebivolol could stimulate an increase in endothelial NO that becomes available at the smooth muscle layers and induces vasorelaxation. These effects are not shared by other β-adrenoreceptor blockers used as references and mainly rely on the l-enantiomer. The variability of the response is possibly linked to species, vascular bed or vessel dimension specificity, or to a compound of these parameters.

The relative weakness of this *in vitro* vasorelaxant action of nebivolol, mostly observed at concentrations >1 μM, may lead some to doubt its relevance to the antihypertensive effects of nebivolol in *in vivo* models and, even more, in its therapeutic use. In particular, it has been speculated that it is hardly tenable that the marked acute vasodilating effects observed with nebivolol, at doses used in clinical practice, can simply rely on the stimulation of endothelial NO pathway, at least as is presently assessed.

On the other hand, there is a common feeling that the observed effects are a hallmark of nebivolol's capacity to exert a vasoprotective, antiendothelial stress action that could be of utmost importance in preventing vascular complications of hypertension. As pointed out in the most recent studies with therapeutic dosage in human vasodilation models, such an action could be particularly evident after chronic treatment. Furthermore, the evidence that a number of nebivolol metabolites are endowed with potent NO-releasing activity (Evangelista et al. 2007; Himmelmann et al. 1996; Maffei et al. 2006) could allow one to speculate that they are, at least in part, responsible for the observed *in vivo* activity.

NO Release from Tissues and Cells

A recent study has clearly shown that nebivolol was able to release NO from mesenteric (resistance) arteries and, at a lesser extent, from large (aorta) rat vessels by means of the diaminofluorescein method (Maffei et al. 2006). Nebivolol (1–10 μM), but not atenolol ($\leq$100 μM), induced NO release from ECs and adventitia layers (Fig. 6) in a similar fashion to that produced by acetylcholine (1 μM) (Maffei et al. 2006). This effect was, at least in part, mimicked by the d-enantiomer and the A4-OH, A4-OH′, and A6 metabolites (Maffei et al. 2006). In SHRs, the capability of nebivolol to induce NO release was maintained as compared to normotensive rats, whereas that of acetylcholine was markedly reduced (Fig. 6). Nebivolol-induced NO release from the mesenteric arteries was confirmed by Mason et al. (2006) using nanosensors placed inside the vessels. The study found that 10 μM of nebivolol inhibited eNOS uncoupling and endothelial dysfunction in hypertensive animals. The ratio of NO/$ONOO^-$, indicators of endothelial function, was significantly increased by exposing the vessels to nebivolol, indicating that the drug allows NO to become available for vasodilation (Mason et al. 2006). In the same experimental conditions, atenolol was inactive and the l-enantiomer was more active than the d-enantiomer (Fig. 7). The basis for nebivolol activity is attributed to its peculiar membrane interactions and the antioxidant activity exerted at nanomolar to micromolar levels.

Direct measurement of NO release from the endothelium or cultured ECs of animal origin resulted in heterogeneous findings. Nebivolol, already known to release NO from cultured pig coronary artery cells (Hashimoto et al. 1996), was found to be able to elicit NO release out of cultured bovine ECs obtained from either the coronary venules (at 10 μM) (Parenti et al. 2000) or the aorta (at $\geq$0.1 μM) (Gryglewski et al. 2001).

Bovine aortic ECs bovine aortic endothelial cells (BAECs) were also used by Dessy et al. (2005) to show

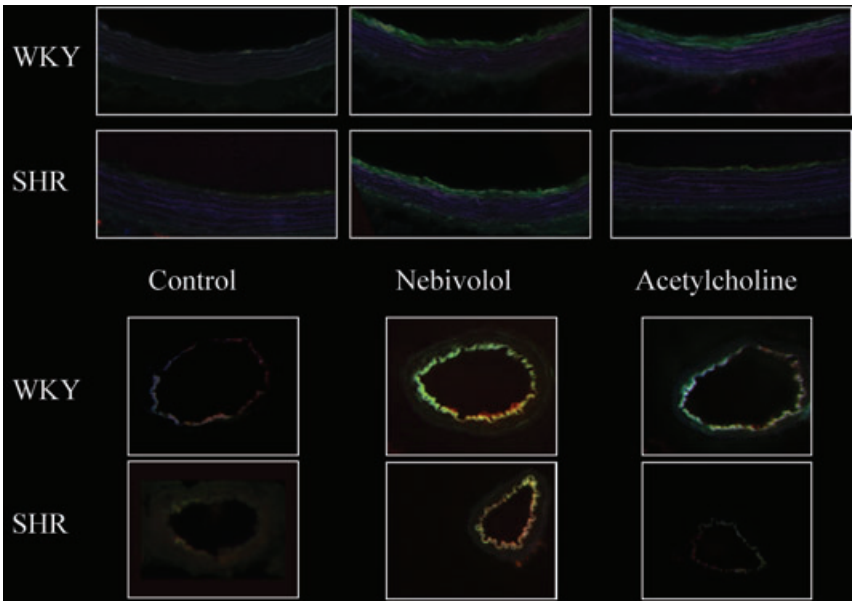


Figure 6 Nitric oxide (NO) release from aortic (upper panels) and mesenteric (lower panels) arteries induced by nebivolol and acetylcholine in WKY (normotensive) and SHR (spontaneously hypertensive) rats, recorded as immunofluorescence by means of the diaminofluorescein method. (Lembo 2003).

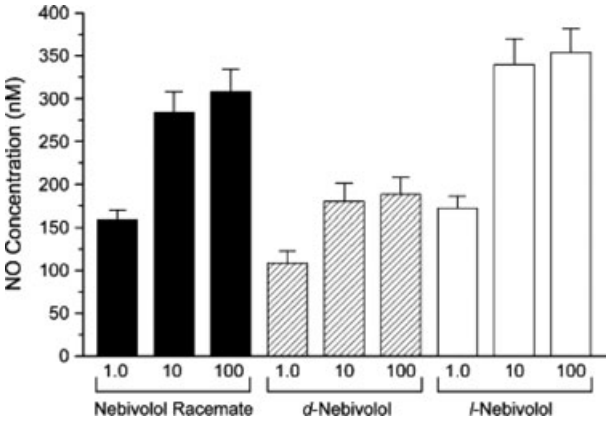


Figure 7 Maximal nitric oxide (NO) concentration released from the endothelium of the mesenteric arteries of WKY rats. NO release was stimulated with nebivolol racemate, l-nebivolol, or d-nebivolol (concentration 1–100 μM/L; n = 6) (reproduced from Mason et al. 2006, with permission).

that nebivolol is able to release NO. In their experimental setup, with a commercially available amperometric system and electron spin resonance spectroscopy, the NO-releasing effect was evident at nebivolol concentrations of >1 μM (Dessy et al. 2005). Nebivolol appeared to exert a very potent effect on the rat renal vascular bed, where it elicited NO release starting from 10 nM concentrations (Kakoki 1999). Moreover, nebivolol stimulated NO release from a single isolated glomerulus, as measured with the Malinski method, with relatively slow kinetics, mostly similar to that of adenosine 5′-triphosphate (ATP)-induced release. The concentration *versus* release curve was alike to that found by Gryglewski in BAECs (Kalinowski et al. 2003). In mouse aorta where dl-nebivolol itself was not active, plasma from a nebivolol-treated mouse (either semipurified or as such) was able to induce a marked release of NO (Broeders et al. 2000).

Data from studies with cultured human ECs seem to indicate that nebivolol is able to increase NO release, though with variable potency depending on the cell origin. In fact, this effect was observed only with a $\geq$10 μM concentration by Gosgnach et al. (2001) in human umbilical vein ECs (HUVECs), but it was evident even with a 0.1 μM concentration in a couple of previous studies on human coronary artery ECs measured as nitrate concentration in the cell growth medium (Brehm et al. 2001). Figure 8 shows that nebivolol 10 μM, but not metoprolol or carvedilol, caused a time-dependent increase in NO release from HUVECs (Ladage et al. 2006).

On the other hand, HUVECs exposed to plasma of hypertensive patients treated for 1 month with 5 mg/day of nebivolol, but not with 100 mg/day of atenolol, showed reduced production of O_2^- and reactive oxygen species if challenged with oxidative factors, along with preserved NO levels and stimulated eNOS activity (Fratta Pasini et al. 2005).

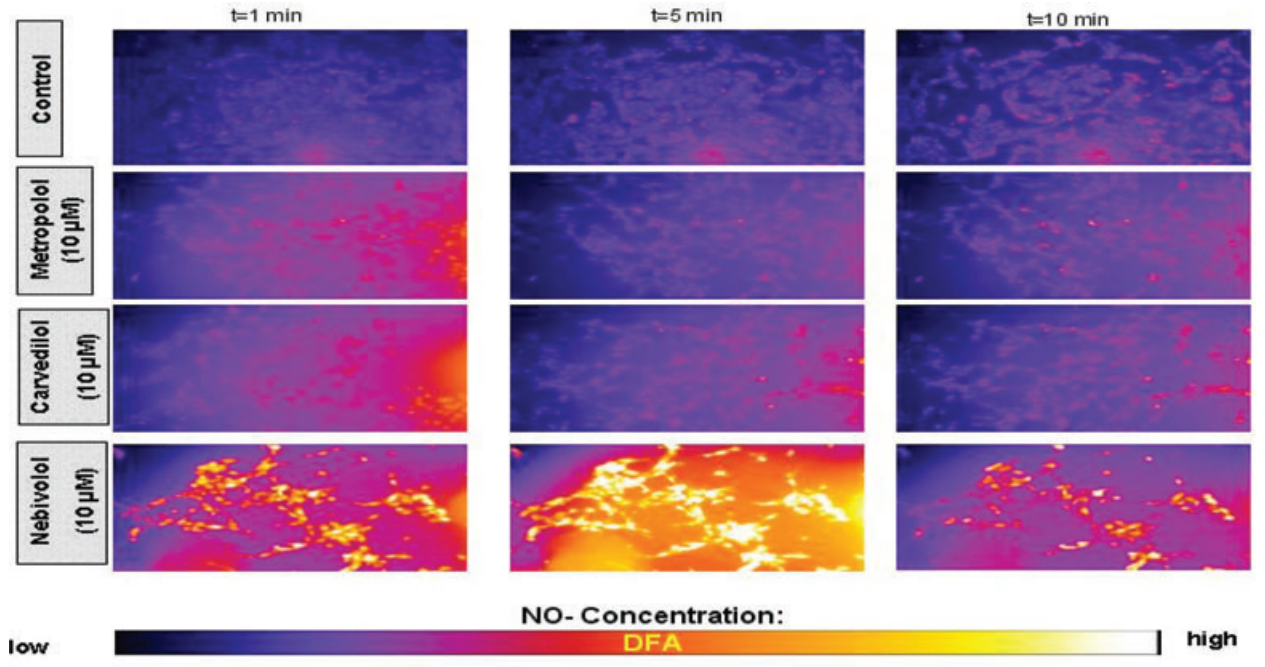


Figure 8 Bioimaging of changes induced by 10 μM nebivolol, 10 μM metoprolol, and 10 μM carvedilol in nitric oxide (NO) release by human umbilical vein endothelial cells via diaminofluorescein fluorimetry (modified from Ladage et al. 2006, with permission).

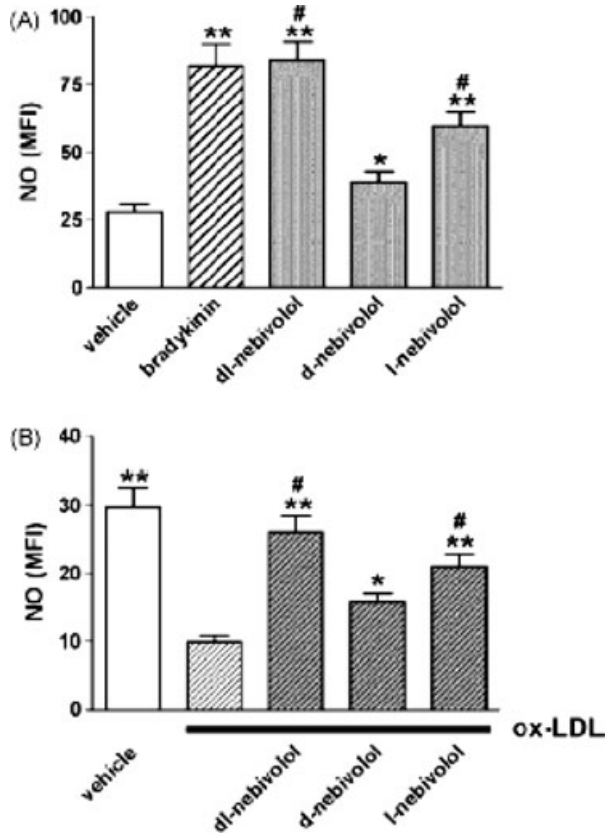


Figure 9 Effect of bradykinin, dl-nebivolol, d-nebivolol, and l-nebivolol on (**A**) basal intracellular nitric oxide (NO) availability and (**B**) oxidized low density lipoprotein (ox-LDL)-induced decreases in NO, expressed as mean fluorescence intensity (MFI), in human umbilical vein endothelial cells (*$P < 0.05$, **$P < 0.01$ vs. vehicle [control; A] or ox-LDL [B]; #$P < 0.01$ vs. d-nebivolol) (modified from Evangelista et al. 2007, with permission).

Mason et al. (2005) showed that in HUVECs and iliac artery ECs isolated from age-matched black and white donors, the rate of NO release was almost 5-fold slower in blacks than in whites. Nebivolol 1–5 μM decreased O_2^- and $ONOO^-$ concentrations and restored NO bioavailability in blacks to the level recorded in cells isolated from white patients, with this restoration independent of the β_1-adrenoceptor blockade (atenolol was inactive) (Mason et al. 2005).

A recent study evaluated not the release but the intracellular formation of NO in oxidized low-density lipoprotein (LDL)-treated HUVECs, and nebivolol was found to significantly increase NO levels, as did the l-enantiomer and some of its metabolites (Evangelista et al. 2007). The exposure of HUVECs to bradykinin, dl-nebivolol, d-nebivolol, and l-nebivolol significantly increased the NO concentration, but the effects of the racemic and l-enantiomer were significantly higher than that of d-nebivolol (Fig. 9A). Exposure to oxidized LDL (ox-LDL) induced a marked decrease in the availability of intracellular NO, which was restored by prior incubation with dl-nebivolol, d-nebivolol, and l-nebivolol (Fig. 9B). The effect of d-nebivolol on this parameter was again significantly lower as compared to l- and dl-nebivolol.

Vasodilation and EC Receptors

Depending on the studies, evidences for the involvement of different receptors have been obtained. These data have always been based on indirect proofs (i.e., on the interference by relatively specific agonist or antagonists for the studied receptor) and none can be considered conclusive.

Nebivolol vasodilating effects on a rat renal vascular bed and aorta were antagonized by NAN 190, a selective blocker of 5-HT_{1A} serotonin receptors (Kakoki 1999). The apparent role of this receptor subtype (or, maybe, of a peripheral 5-HT_{1A}-like subtype) in determining the vasodilating and NO-releasing effects of nebivolol could be related to the compound's very high affinity for these receptors, as shown by binding experiments. However, previous results, besides negating any agonist activity of nebivolol at central 5-HT_{1A} receptors (Janssens et al. 1991), had also ruled out the possibility of an interaction with peripheral receptors in the guinea pig ileum *in vitro* (Janssens and Cools 1994). Moreover, de Groot et al. (2003) were unable to reproduce the data of Kakoki (1999) in an almost identical experimental setup: active nebivolol concentrations were two orders of magnitude higher, and aorta relaxation was completely insensitive to both specific 5-HT_{1A} (NAN 190) and unselective (methysergide) serotonin receptors antagonists. Furthermore, the nebivolol-induced relaxation of dog coronary arteries was not inhibited by methysergide (Gao et al. 1991). These heterogeneous data might once again indicate that the quality of the vasodilating response to nebivolol depends on species, tissue/organ, and vessel types.

A second receptor type that has been indicated by certain research groups to be involved in the nebivolol-induced NO release and vasodilation is β_3-adrenoceptor. In fact, bupranolol (a mixed β_{1-3} antagonist), but not nadolol (a mixed β_{1-2} antagonist), inhibited nebivolol's effects on rat and human coronary microarteries (Dessy et al. 2005), BAECs (Dessy et al. 2005), and HUVECs (Evangelista et al. 2007; Gosgnach et al. 2001). In HUVECs, the release of NO by nebivolol was partially antagonized by the selective β_3 antagonist SR 59230A (Evangelista et al. 2007; Ladage et al. 2006), but further inhibition was obtained with the simultaneous blockade of β_2-adrenoceptors (Evangelista et al. 2007) or estrogen receptors (Ladage et al. 2006). Moreover, de Groot

et al. (2003) showed that nebivolol-induced relaxation of rat aorta was inhibited by the relatively selective β_3 antagonist cyanopindolol and mimicked by the β_3 agonist BRL 37344. Similar results were obtained by Rozec et al. (2006): relaxation induced by nebivolol was unaffected by nadolol and significantly reduced by the selective β_3 antagonist L-748337. However, a previous study by Janssens and Cools (1994) clearly showed that nebivolol was not able to act as either an agonist or an antagonist at β_3-adrenoceptors in guinea pig ileum, based on the lack of interference of alprenolol on nebivolol's effects and nebivolol on BRL 37344's effects (Janssens and Cools 1994). Furthermore, recent binding studies, performed at the human recombinant receptor (Meini et al. 2005), confirmed that the racemate and its enantiomers have only a μM affinity for this receptor subtype.

The NO-releasing activity of the presumed nebivolol mouse metabolite (Broeders et al. 2000) was antagonized by butoxamine (a selective β_2 antagonist, with no effects on serotonin receptors) and mimicked by salbutamol. Accordingly, it appeared to depend on the activation of endothelial β_2-adrenoceptors, and it was hypothesized that metabolic modifications can produce major changes in nebivolol's pharmacologic properties. However, the extension of mouse data to other species/systems appears to be difficult because of the failure of plasma from nebivolol-treated rats (high oral doses for 5 days) and hypertensive patients (repeated therapeutic doses) to elicit NO release from cultured ECs (Balligand 1998, and van der Zee's personal communication 2001,, respectively). Nebivolol is known to be a weak ligand to β_2-adrenoceptors, devoid of any intrinsic sympathomimetic activity in several *in vitro* and in *in vivo* models (Janssens et al. 1989), but, in apparent contrast with previous results, we recently found that nebivolol-induced relaxation of endothelium-intact rat aorta was inhibited by butoxamine 100 μM in a similar fashion as salbutamol- and isoproterenol-induced relaxations, implying a direct activation of β_2-adrenoceptors in the vessel wall (Ignarro et al. 2002a). The same concentration of butoxamine inhibited, at least in part, the vasodilating effect of nebivolol, obtained at $\geq$50 μM, in mouse renal artery (Georgescu et al. 2005).

In the rat mesenteric vascular bed (Ledda 1999, personal communication), nebivolol-induced vasodilation was blocked by prazosin and involvement of endothelial α_1-adrenoceptors, known to be capable of mediating NO release in other systems (Zschauer et al. 1997), was hypothesized. Recently, Rozec et al. (2006) found that nebivolol shifted the concentration–response curve to phenylephrine to the right with a pA_2 of 6.5. Although nebivolol is a very weak ligand for α_1-adrenoceptors (with an affinity approximately 1000-fold lower than for β_1-adrenoceptors), some binding to these receptors cannot be ruled out at the tested concentrations (>1 μM).

Similarly, Garban and coworkers (2004) put forward the hypothesis that the vasorelaxant response of nebivolol in rat aorta was partially due to its interaction with estrogen receptors, particularly if nebivolol stimulated the dissociation of estradiol binding at a very high concentration (500 μM).

In the hypothesis that nebivolol as such is responsible for NO-dependent vasodilation, there are presently four receptor types that are candidates for mediating its effects on the EC: β_3-adrenoceptors, 5-HT_{1A}-like serotonin receptors, β_2-adrenoceptors, and estrogen receptors. However, based on *in vitro* affinity data, the last three receptor types are unlikely to exert any major *in vivo* role (and older studies appear to confirm this).

The recent functional data on rat and human microarteries (Dessy et al. 2005) would suggest that β_3-adrenoceptors are likely to be involved. Yet, it remains to be demonstrated whether nebivolol binds as an agonist to these receptors. After this evidence is obtained, fine-tuning of signal transduction or minor molecular differences among subtypes in different species or tissues could then be advocated to explain the previously shown inactivity at β_3-adrenoceptors in different models.

The very high affinity of nebivolol for the 5-HT_{1A} receptors also renders this receptor type a plausible candidate as the site for nebivolol interaction with the EC, especially if a more refined evaluation of the subtypes actually involved in vascular actions helps to explain the observed contradictions. The molecular interaction of β antagonists with these receptors is a common finding, but its translation into a pharmacologic effect appears to depend on a single antagonist: cyanopindolol was found to be a potent antagonist at the central receptors (Hoyer et al. 1994), tertatolol showed agonist activities at peripheral receptors (Verbeuren 1993), and bucindolol was devoid of any effect whatsoever (Watts et al. 2000). The hypothesis that one or more nebivolol metabolites are responsible for the actions attributed to the parent compound would lead one to further consider β_2-adrenoceptors as candidate receptors. Identification of the mouse metabolite(s) and its (their) isolation from the plasma of other experimental species and from human plasma are necessary steps toward the clarification of this issue.

Vasodilation and Intracellular Mechanisms

We have searched to elucidate the mechanisms operating in the nebivolol-induced relaxation of the rat aorta

(Ignarro et al. 2002a, 2002b). As indomethacin significantly enhanced the effect of 1–30 μM of nebivolol, it was apparent in this model that (1) vasoconstricting prostanoids are involved in the phenylephrine-induced contraction of the isolated vessel, and (2) nebivolol does not act through vasodilatory prostanoids (Ignarro et al. 2002a). Endothelin receptor (either endothelin ET_A or ET_B) blockade had only minor consequences on nebivolol-induced relaxation, but the inhibition of calcium-activated potassium channels of the BK_{Ca} type with charybdotoxin significantly reduced nebivolol's effect on endothelium-intact aorta (Ignarro et al. 2002a). This inhibition was additive to that produced by eNOS blockade with L-NMA, but combined inhibitors were not sufficient to completely abolish nebivolol's effect, thus indicating the presence of additional mechanisms to EDRF and endothelium-derived hyperpolarizing factor (EDHF) (Ignarro et al. 2002a). Acetylcholine-induced relaxation was similarly inhibited, but GTN effects were less affected by the same inhibitors (Ignarro et al. 2002a). The potential activation of the Ca^{2+}-induced potassium channel by nebivolol in mouse renal artery was confirmed by the results of Georgescu et al. (2005), which showed that the nebivolol vasodilation was antagonized by tetraethylammonium.

In the study of Parenti et al. (2000), tapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase, was able to inhibit nebivolol-induced dilatation of the rat mesenteric vascular bed. This suggests that intracellular calcium movements are involved in modulating the vessel's response. By integrating these data with the finding that IP_1, an inositol triphosphate (IP_3) metabolite, accumulated in cultured bovine coronary postcapillary venular ECs cultured bovine coronary postcapillary venular endothelial cells (CVECs) after nebivolol challenge (Parenti et al. 2000), it can be speculated that EC receptor stimulation by nebivolol activates membrane phospholipase C (PLC) and induces IP_3 release. The latter frees Ca^{2+} ions from the endoplasmic reticulum, which, in turn, activates EC NO synthase. Increased formation of tritiated citrulline from labeled L-arginine was reported in CVECs (Parenti et al. 2000) as an index of an increased eNOS activity induced by nebivolol.

Gryglewski et al. (2001) showed that nebivolol-induced NO release from BAECs was accompanied by a concentration-dependent increase in intracellular calcium. L-NNA inhibited by about 70% the NO release produced by either nebivolol or the calcium ionophore A 23187, but removal of extracellular calcium reduced the nebivolol-induced NO release much less than that induced by ionophore, indicating that calcium influx from the extracellular space is only partially involved in nebivolol-activated release (Gryglewski et al. 2001). Also Dessy et al. (2005) found that nebivolol induced a L-NAME-inhibitable increase in intracellular calcium in BAECs. Maffei et al. (2006) in rat aorta and Georgescu et al. (2005) in mouse renal artery showed similar results. This appears to agree with the above data in the rat mesenteric bed.

In contrast to the above, a study with HUVECs (Gosgnach et al. 2001) did not show any modification of PLC activity by nebivolol, while significant increases in phospholipase D (PLD) and, mostly, in phospholipase A_2 phospholipase A2 (PLA_2) and adenylate cyclase were observed. The increase in cAMP formation was correlated to the increase in NO content of the culture medium (Gosgnach et al. 2001). It was speculated that, in this system, eNOS was activated by protein kinase A (PKA)-mediated phosphorylation. This process appeared to be independent of intracellular calcium levels (which were not increased following cell stimulation with nebivolol) (Gosgnach et al. 2001). The increase in PLA_2 seems primarily related to the increased production of prostacyclin (PGI_2). The apparent contrast with the original findings of Gao et al. (1991) and Cominacini et al. (2003) may again suggest that the coupling of nebivolol-activated receptors with intracellular events is dependent on the examined tissue/cellular system.

Kalinowski et al. (2003) showed that ATP is an essential mediator of nebivolol-induced relaxation of the renal microvasculature in the rat as both the P_2-antagonist suramin and the ATP-degrading enzyme apyrase block nebivolol activity. This view is reinforced by the observation that ATP releases NO from the glomerulus in the same way as nebivolol, with kinetics distinct from that of calcium ionophore. P_2-receptor activation is supposed to lead to an intracellular rise in Ca^{2+} and to the consequent activation of NO synthase. A recent study (Kozlovski et al. 2006) has shown that this mechanism involving extracellular ATP seems to be not operating in the nebivolol-induced release of NO from guinea pig heart.

A number of studies examined the possibility that nebivolol could produce its NO-releasing effect by modulating the expression of endothelial NO synthase. No increased eNOS expression was found in human end-stage heart failure samples (Brixius et al. 2006) or in biopsies of myocardial tissue and mammary arteries taken from patients undergoing cardiovascular surgery after 30-day therapy with nebivolol or atenolol (Mory 2001). However, isolated rat aortas exposed to nebivolol 1–10 μM (Maffei et al. 2006) and mouse renal arteries exposed to nebivolol 50 μM (Georgescu et al. 2005) showed a marked eNOS activation. Furthermore, the activation of eNOS in BAECs was reported by Dessy et al. (2005). This

study did not detect changes in the phosphorylation of eNOS on serine 1177, suggesting that in these cells and experimental conditions, nebivolol-evoked eNOS activation does not involve a phosphorylation signal of this particular site; however, nebivolol induced a dephosphorylation of threonin (Thr) 495, known as an inhibitory site. A recent study in HUVECs (Ladage et al. 2006) has shown that the application of nebivolol significantly increased eNOS translocation and serine 1177 phosphorylation of the enzyme; however, in these cells, nebivolol did not alter eNOS phoshorylation at Thr 495 and at serine 114. Mollnau et al. (2003) have shown that chronic nebivolol treatment prevented NO synthase uncoupling and improved cGMP-dependent protein kinase (cGK-1) activity, as assessed by the phosphorylation of P-VASP (vasodilator-stimulated phosphoprotein) in hyperlipemic rabbits.

In another *in vivo* study, by Cosentino et al. (2002), oral nebivolol (10 mg/kg) and atenolol (100 mg/kg), administered daily for 8 weeks, were compared for their chronic effects on endothelial injury in salt-induced hypertension in Dahl rats. Both drugs completely prevented the induction of hypertension, but only nebivolol was able to significantly reduce the salt-induced impairment of endothelium-dependent relaxations of both the aorta and mesenteric artery preparations initiated by acetylcholine and to restore decreased eNOS activity in salt-treated rat aortas (Cosentino et al. 2002).

Oelze et al. (2006) addressed their study to assess whether nebivolol improved NO bioavailability by stimulating eNOS or whether this phenomenon is secondary to the antioxidant properties of nebivolol. Wistar rats were orally treated with nebivolol (10 mg/kg daily for 5 days) while being constantly infused with angiotensin II. The angiotensin II infusion caused hypertension and endothelial dysfunction in the aortas in spite of an increase in eNOS above the control values: superoxide anion levels increased and soluble guanylate cyclase levels decreased; cGK-1 expression did not change, but its activity was decreased (Oelze et al. 2006). NOS inhibition in hypertensive animals decreased the oxygen concentration, suggesting that NOS is deranged to produce superoxide following angiotensin II treatment (uncoupling) (Oelze et al. 2006). The treatment of angiotensin II-infused rats with nebivolol normalized endothelial function, restoring all the altered parameters (Oelze et al. 2006). It exerted a potent antioxidative action and, in particular, prevented eNOS uncoupling (Oelze et al. 2006). In the heart membranes of angiotensin II-treated animals, nebivolol, but not atenolol and metoprolol, prevented eNOS uncoupling interfering with the assembly of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Oelze et al. 2006).

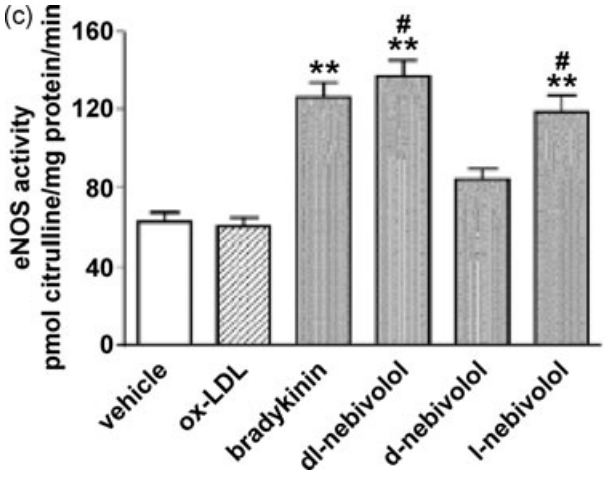


Figure 10 Effect of oxidized low-density lipoprotein (ox-LDL), bradykinin, dl-nebivolol, d-nebivolol, and l-nebivolol on endothelial nitric oxide synthase (eNOS) in human umbilical vein endothelial cells (**$P < 0.01$ vs vehicle group; #$P < 0.01$ vs. d-nebivolol) (modified from Evangelista et al. 2007, with permission).

Table 3 Effect of dl-nebivolol, d-nebivolol, and l-nebivolol on intracellular Ca^{2+} in human umbilical vein endothelial cells (modified from Evangelista et al. 2007).

	Ca^{2+} (nM)
Control	83 ± 7
dl-nebivolol 10 μM	327 ± 12*
d-nebivolol 10 μM	94 ± 12
l-nebivolol 10 μM	625 ± 17*

*$P < 0.001$ vs control (vehicle).

The impact of the enantiomers on the eNOS activation in HUVECs was studied by Evangelista et al. (2007). Figure 10 shows that eNOS activity significantly increased after a 5-min contact with dl-nebivolol and l-nebivolol: this effect was of a similar extent to that produced by bradykinin, while d-nebivolol and ox-LDL did not affect eNOS activity. As a further confirmation of these data, the exposure to dl-nebivolol or l-nebivolol induced changes in intracellular Ca^{2+}, while the exposure to d-nebivolol did not (Table 3).

The possibility that nebivolol could affect inducible NOS (iNOS) was addressed in the study of Gryglewski et al. (2001) by evaluating the effects in cultured mouse macrophages challenged with *Escherichia coli* lipopolysaccharide. Neither expression nor activity of iNOS was modified by nebivolol. Reported results would indicate

that the cellular mechanisms mediating the increase in endothelial NO release were highly dependent on the tissue/cell culture examined, being possibly related to the different receptor types and/or coupled transduction system activated by nebivolol in each experimental setting (Gryglewski et al. 2001). However, these findings strongly confirm that eNOS activation and uncoupling both seem to play a role in nebivolol-induced NO release from vessels, with the l-enantiomer being the major actor of this effect. Nebivolol appears to interact with the endothelial NO pathway in two complementary ways: it increases NOS activity and reduces the NO-scavenging radical superoxide anion, by redirecting deranged NOS activity, from superoxide to NO production.

Stimulation of NO from Platelets by Nebivolol

Noticeable concentrations of NO are present in platelets, and the L-arginine/NO pathway is known to play a role in the modulation of platelet aggregation (Hogan et al. 1988). An *in vitro* study evaluated the effects of nebivolol on human platelet aggregation (Falciani et al. 2001) and found that ADP- and collagen-induced aggregation of human platelets was inhibited by nebivolol (0.1–100.0 μM) in a concentration-dependent manner. Moreover, L-arginine enhanced and L-NAME reduced the effect of nebivolol (Fig. 11) (Falciani et al. 2001).

A piece of evidence supporting an *in vivo* antiaggregating effect of nebivolol was produced by Sala et al. (2001), who showed that chronic nebivolol (5 mg/day for 4 weeks), but not hydrochlothiazide (25 mg/day for 4 weeks), induced a significant increase in platelet cyclic GMP (from 4.8 $\pm$ 0.7 to 7.1 $\pm$ 0.6 pmol/10^9 platelets) in a small group of essential hypertensives (n = 5).

In addition, the NO-releasing effect of nebivolol in platelets seems to be a property mainly due to the l-enantiomer. In an acute thrombosis model in mice, the prior administration of dl-nebivolol and l-nebivolol was protective toward the mortality induced by collagen + epinephrine (Gresele 2007 unpublished data). Data from Gryglewski et al. (2001) showed that i.v. administration of the racemate was able to decrease the extracorporeal formation of a thrombus in anaesthetized rats similar to known NO donors.

These studies provide the first evidence of an NO-mediated action of nebivolol outside the vessel wall. Owing to the importance of platelet reactivity in cardiovascular homeostasis, this effect deserves to be studied in *in vivo* models, especially after prolonged treatment, and in patients undergoing clinical trials.

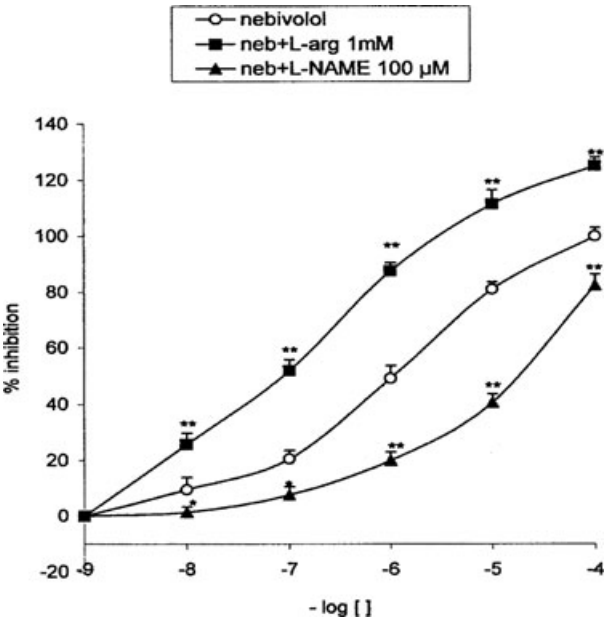


Figure 11 Concentration-dependent inhibitory effect of nebivolol on ADP-induced human platelet aggregation. L-NAME (100 μM) reduces and L-arginine (1 mM) enhances nebivolol effect. Mean values and standard error of measurement (n = 4–6) are reported (*$P < 0.05$, **$P < 0.01$ vs. nebivolol alone) (modified from Falciani et al. 2001, with permission).

Nebivolol: A Unique Cardiovascular Combination Drug

D-nebivolol and l-nebivolol each has its own independent pharmacologic and clinical effects. The combination of the two substances has beneficial cardiovascular actions for the following reasons:

(1) Both molecules act synergistically with respect to blood pressure reduction. The combination achieves a pronounced and lasting blood pressure reduction that is roughly equal to the effect of conventional β-blockers in high doses (Van der Water et al. 1988a, 1998b; Xhonneux et al. 1990)
(2) Undesirable β-blocker effects, such as a decrease in cardiac output, do not occur or are less pronounced with the combination of d-nebivolol and l-nebivolol (De Cree et al. 1991; Himmelmann et al. 1996; Moen and Wagstaff 2006; Stoleru et al. 1993; Van der Water et al. 1988a; Zanchetti 2004).
(3) The pharmacologic and clinical profile of the combination exceeds that of a conventional β-blocker. Numerous clinical and preclinical studies have shown that contrary to conventional β-blockers, nebivolol normalizes endothelial dysfunction and has pronounced antiinflammatory, antioxidative,

and antiproliferative—in short, antiatherosclerotic—effects. By stimulating the eNOS and, in general, the NO availability at the endothelial, smooth muscle, and platelet levels, nebivolol acts against a central and prognostically relevant pathomechanism for the development and progression of hypertension (Moen and Wagstaff 2006; Zanchetti 2004) and atherosclerosis (Mollnau et al. 2003; Wolf et al. 2007).

Conclusions

In conclusion, nebivolol is a unique, fixed-combination cardiovascular drug. The active ingredients, d-nebivolol and l-nebivolol, each have different and independent pharmacologic effects that synergistically concur to beneficial clinical effects with a profile that differs noticeably from that of conventional β-blockers.

Addendum

Chemical Names for Agents Listed for Code Number in the Text

A 23187: 5-(methylamino)-2-[[(2R,3R,8S,9R,11R)-3,9,11-trimethyl-8-[(1S)-1-methyl-2-oxo-2-(1H-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-
BRL 37344: 4-[2-[[2-(3-chlorophenyl)-2-hydroxy-ethyl]amino]propyl]-phenoxy]acetic acid sodium
CGP 12177: 2H-Benzimidazol-2-one, 4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-
CGP 20712A: 2-hydroxy-5-[2-[[2-hydroxy-3-[4-[1-methyl-4-(trifluoromethyl)-1H-imidazol-2-yl]phenoxy]propyl]amino]ethoxy]-, methanesulfonate
CL 316243: 1,3-Benzodioxole-2,2-dicarboxylic acid, 5-[(2R)-2-[[(2R)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-, sodium salt; ICI 89406: N-[2-[[3-(2-cyanophenoxy)-2-hydroxypropyl]amino]ethyl]-N′-phenyl-
ICI 215001: 2-[4-[2-[[(2S)-2-hydroxy-3-phenoxypropyl]amino]ethoxy]phenoxy]-
L 748337: N-[[3-[(2S)-2-hydroxy-3-[[2-[4-[(phenylsulfonyl)amino]phenyl]ethyl]amino]propoxy]phenyl]methyl]-
NAN 190: 1H-Isoindole-1,3(2H)-dione, 2-[4-[4-(2-methoxyphenyl)-1-piperazinyl]butyl]-, hydrobromide
ZD 7155: 1,6-Naphthyridin-2(1H)-one, 5,7-diethyl-3,4-dihydro-1-[[2′-(2H-tetrazol-5-yl)[1,1′-biphenyl]-4-yl]methyl]-, hydrochloride

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