Stimulation of Platelet Nitric Oxide Production by Nebivolol Prevents Thrombosis

Stefania Momi, Roberta Caracchini, Emanuela Falcinelli, Stefano Evangelista, Paolo Gresele

Objective—dl-Nebivolol, a selective β1-adrenergic receptor antagonist, besides its hypotensive activity exerts vasodilatory and platelet inhibitory effects in vitro by a mechanism involving nitric oxide (NO). Our aim was to evaluate whether nebivolol exerts in vivo antithrombotic effects, to unravel the mechanism of this action and to clarify the relative roles of its 2 enantiomers: d- and l-nebivolol.

Methods and Results—In wild-type mice, dl-nebivolol, l-nebivolol, and d-nebivolol, but not bisoprolol, reduced mortality consequent to platelet pulmonary thromboembolism induced by the intravenous injection of collagen plus epinephrine (−44%, −45%, −29%, respectively; P<0.05), whereas in eNOS−/− mice only dl-nebivolol and d-nebivolol were effective. dl-Nebivolol, l- and d-nebivolol reduced photochemical damage-induced femoral artery thrombosis in wild-type mice, whereas in eNOS−/− mice only dl-nebivolol and d-nebivolol were active. Moreover, dl-nebivolol and l-nebivolol increased plasma, urinary-, and platelet-derived nitrates and nitrites (NOx), NO degradation products, in wild-type but not in eNOS−/− mice. In vivo platelet activation, assessed by platelet P-selectin expression, was reduced by dl-nebivolol and l- and d-nebivolol in wild-type mice but only by dl-nebivolol and d-nebivolol in eNOS−/− mice. In bone marrow–transplanted, chimeric mice with only blood cells, and not the endothelium, producing NO dl-nebivolol and l-nebivolol maintained their antithrombotic activity, whereas they lose it in chimeras with only endothelium, and not blood cells, producing NO. In vitro, with isolated platelets, dl-nebivolol and l-nebivolol, but not d-nebivolol, bisoprolol, and bisoprolol, increased platelet cGMP and NOx formation. Treatment with dl-nebivolol and l-nebivolol increased phosphorylated eNOS in platelets.

Conclusions—Our data show that dl-nebivolol exerts an antithrombotic activity by stimulating the formation of NO by platelets, and that this effect is generated by its l-enantiomer, whereas the d-enantiomer exerts a weak antiplatelet effect because of β-adrenergic receptor–independent stimulation of adenyly cyclase. These results confirm that platelet-derived NO plays a role in thrombosis prevention and it may represent a target of pharmacological intervention. (Arterioscler Thromb Vasc Biol. 2014;34:820-829.)

Key Words: bisoprolol • blood platelets • nebivolol • nitric oxide • thrombosis

Hypertension is associated with an increased risk of arterial thrombotic events, such as stroke and myocardial infarction,1 in which platelets are deeply involved. Previous studies have shown that β-adrenoceptor blockers reduce platelet activation and aggregation,2 but this does not seem to be a class effect because not all β-blockers share this property.3 dl-Nebivolol (a racemic mixture of d- and l-nebivolol) is a third-generation β1 adrenoceptor-selective antagonist that, besides its β-blockade–mediated hypotensive effect, possesses direct vasodilatory properties through a mechanism involving the L-arginine/nitric oxide (NO) pathway.4,5 Of the 2 enantiomers forming the racemic mixture of dl-nebivolol, d-nebivolol, which has an ≈200-fold greater affinity for β1-adrenoceptors than that of l-nebivolol, seems to be mainly responsible of selective β-blockade,6 whereas the l-form is the main effector of the endothelium-dependent vasorelaxant effect.7,8 dl-Nebivolol exerts its vasodilatory action by activating endothelial nitric oxide synthase (eNOS) and by preventing eNOS uncoupling, thus enhancing NO production.9 Endothelium-derived NO is a powerful inhibitor of platelet activation and an antithrombotic agent.10,11 Besides endothelium, eNOS is present in other cells, including platelets,12,13 and several in vitro and in vivo observations in animals and in humans suggest that platelet-derived NO plays a significant anti-inflammatory and antithrombotic role.15-19 dl-Nebivolol was previously reported to inhibit ADP- and collagen-induced aggregation of human platelets in vitro,20 an effect prevented by the previous exposure of platelets to a NOS inhibitor, such as L-NG-nitroarginine methyl ester, and enhanced by preincubation with L-arginine, the substrate of NOS, suggesting that the antiplatelet effect of dl-nebivolol is mediated by an enhanced release of NO from platelets.4

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Despite these premises, no data are available yet on the antithrombotic activity of dl-nebivolol in vivo and on the potential role of platelet-derived NO in it. Therefore, the aim of our study was to assess whether dl-nebivolol exerts an antithrombotic effect, to test which of its 2 enantiomers, d- and l-nebivolol, is responsible for this activity, and to evaluate the role of platelet-versus endothelium-derived NO in its antithrombotic activity.

**Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Antithrombotic Activity**

**Pulmonary Thromboembolism**

The intravenous injection of collagen plus epinephrine led to death 85% of wild-type mice. Pretreatment with dl-nebivolol dose dependently reduced mortality, whereas bisoprolol was ineffective (Figure II in the online-only Data Supplement). dl-Nebivolol (5 mg/kg) and equimolar doses of l-nebivolol (2.5 mg/kg) reduced mortality equally (−44% and −45%, respectively; P<0.05 versus controls), whereas d-nebivolol (2.5 mg/kg) was less effective (−29%; Figure 1A and 1C).

The dose of collagen plus epinephrine required to obtain ≈85% mortality in eNOS−/− mice was lower than that required in wild-type mice (Materials and Methods are available in the online-only Data Supplement). dl-Nebivolol (5 mg/kg) and, slightly, d-nebivolol (2.5 mg/kg) protected also eNOS−/− mice from collagen plus epinephrine-induced mortality, but less than wild-type mice, whereas l-nebivolol (2.5 mg/kg) completely lost its protective activity in this animal strain (Figure 1B and 1C).

dl-Nebivolol (5 mg/kg) and even more l-nebivolol (2.5 mg/kg) protected wild-type mice also from mechanical pulmonary microembolism induced by the intravenous injection of swollen, hardened rat red blood cells (Figure 1D), indicating that a vasodilatory effect on lung microvasculature contributes to the protective action of dl-nebivolol and l-nebivolol against lung embolism-provoked lethality. This effect was not shared by d-nebivolol and bisoprolol.

**Femoral Artery Thrombosis**

Photochemically induced injury to the femoral artery of wild-type mice led to the complete arrest of blood flow in 11.9±0.7 minutes. Pretreatment with dl-nebivolol, l-nebivolol, and

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**Nonstandard Abbreviations and Acronyms**

AR  adrenergic receptor

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**Figure 1.** Collagen plus epinephrine-induced pulmonary thromboembolism. A, Effect of dl-nebivolol, l-nebivolol, d-nebivolol, and bisoprolol in wild type mice (n=20 per group) and (B) in eNOS−/− mice (n=24 per group; except vehicle=38). Effect of l-nebivolol on collagen plus epinephrine-induced thromboembolism in mice. D, Effect of dl-nebivolol, l-nebivolol, d-nebivolol, and bisoprolol in wild-type mice (white column) and in eNOS−/− mice (black column; n=10 per group). Results are expressed as % mortality. *P<0.05 vs vehicle.
D-nebivolol significantly prolonged the time to occlusion, whereas bisoprolol was ineffective (Figure 2A). Thrombus weight was significantly lower in the groups treated with Dl- and L-nebivolol, whereas D-nebivolol and bisoprolol were ineffective (Figure 2B). In eNOS−/− mice, photochemical injury to the femoral artery led to the complete arrest of blood flow in a time significantly shorter than that of wild-type mice (7.7±0.3 minutes; P<0.05 versus wild-type mice). Dl-nebivolol and D-nebivolol still prevented thrombosis in eNOS−/− mice, whereas L-nebivolol lost its protective effect; here too, bisoprolol was inactive (Figure 2C).

Thrombus weight was significantly higher in vehicle-treated, control eNOS−/− mice when compared with that in wild-type mice (0.59±0.02 versus 0.27±0.01; P<0.05). Dl-nebivolol and D-nebivolol reduced thrombus weight, whereas L-nebivolol and bisoprolol were ineffective (Figure 2D).

Venous Thrombosis

After the provocation of inferior vena cava flow restriction, all (n=5) wild-type mice developed a thrombus located just below the ligature (toward the tail) within 48 hours (thrombus weight, 13.4±4.8 mg and length, 4.8±1.5 mm). Pretreatment with Dl-nebivolol and L-nebivolol reduced significantly venous thrombus size (Dl-nebivolol: thrombus weight, 4.5±1.5 mg; thrombus length, 3.7±1.9 and L-nebivolol: thrombus weight, 6.2±2.7 mg; thrombus length, 3.0±1.2; P=0.019 and P=0.04 versus control, respectively). Pretreatment with D-nebivolol did not significantly affect venous thrombosis (thrombus weight, 9.2±3.3 mg; thrombus length, 4.1±2.0; P=NS). These results are in line with the pivotal role played by NO in the regulation of venous endothelial function contributing to prevent venous thrombosis.21

Platelet Inhibition

Platelet Aggregation

Treatment with DL-nebivolol, D-nebivolol, and, even more, L-nebivolol inhibited significantly ex vivo U46619-induced platelet aggregation (Figure 3A). In vitro, U46619-induced platelet aggregation was significantly inhibited by preincubation with DL-nebivolol, L-nebivolol, and D-nebivolol (efficacy: Dl>Dl). To evaluate which β-receptors mediate the inhibitory activity of nebivolol on platelets, we performed in vitro coinoculation experiments with selective β1 (CGP20712A) or β2 (ICI118551) adrenoreceptor antagonists. The inhibitory effect of D-nebivolol was not affected by CGP20712A or ICI118551, whereas the inhibitory effect of Dl- and L-nebivolol was completely abolished by CGP20712A but not by ICI118551 (Figure 3B), indicating that the in vitro inhibitory effects of DL- and L-nebivolol on platelets are mediated by β1-adrenergic receptors (ARs). On the contrary, the inhibitory effect of D-nebivolol on platelet aggregation was abolished by pretreatment with SQ 22536, an

Figure 2. The effect of Dl-nebivolol, L-nebivolol, and bisoprolol on photochemically induced thrombosis in wild-type (A) and eNOS−/− mice (n=10 per treatment; D) and on thrombus weight in wild-type mice (B) and eNOS−/− mice (n=5 per treatment; D). Time to occlusion and thrombus weight in eNOS−/− vehicle-treated mice was significantly shorter than in vehicle-treated wild-type mice (7.7±0.3 vs 10.6±0.4 minutes, respectively; P<0.05). *P<0.05 vs vehicle.
inhibitor of adenylyl cyclase (Figure 3C). Consistently, incubation of platelets with D-l- and D-nebivolol, but not with D- and L-nebivolol, enhanced intraplatelet cAMP (Figure 3D). However, l-nebivolol–induced inhibition of platelet aggregation was abolished by 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one, a soluble guanylyl cyclase inhibitor (Figure 3C).

Platelet P-Selectin Expression

The expression of P-selectin on circulating platelets was significantly enhanced by the intravenous injection of a mixture of collagen plus epinephrine in wild-type mice and even more strikingly in eNOS−/− mice (Table). Pretreatment with DL-nebivolol, L-nebivolol and, to a lesser extent, D-nebivolol significantly reduced platelet P-selectin expression in wild-type mice (Table). The first-generation β-blocker bisoprolol (5 mg/kg PO) also slightly, but not significantly, reduced platelet P-selectin expression in wild-type animals, and not as much as DL-nebivolol and its enantiomers. In eNOS−/− mice, only DL-nebivolol and D-nebivolol reduced slightly, but significantly, platelet P-selectin expression, whereas l-nebivolol and bisoprolol were not effective (Table).

NO Stimulation

Nitrites and Nitrates (NOx)

The treatment of wild-type mice with L-nebivolol (2.5 mg/kg) for 5 days significantly increased plasmatic and urinary NOx; DL-nebivolol slightly, but not significantly, increased NOx, whereas D-nebivolol and bisoprolol did not (Figure 4A and 4B). In eNOS−/− mice, no changes in plasmatic and urinary
nitrites and nitrates were induced by any treatment (not shown).

**Intraplatelet cGMP**

The treatment of mice with either *d*-nebivolol or *l*-nebivolol significantly increased intraplatelet cGMP, whereas *d*-nebivolol and bisoprolol were ineffective (Figure 4C).

**Role of Platelet-Derived NO**

**Femoral Artery Thrombosis in Chimeric Mice**

To unravel the role of platelet- versus endothelium-derived NO in the protective effects of *dL*-nebivolol against femoral artery thrombosis, we studied chimeric mice with circulating blood cells derived from eNOS−/− mice and with blood vessels normally expressing eNOS (wild-type chimeric mice) or vice versa chimeric mice with circulating blood cells normally expressing eNOS and blood vessels devoid of eNOS (eNOS−/− chimeric mice). The time to complete occlusion of the femoral artery in wild-type chimeric mice (in which circulating cells are not able to produce NO) was significantly shorter than that of wild-type mice (8.1±1.85 versus 11.9±0.7 minutes; *P*<0.05), and it was slightly but significantly prolonged by *dL*-nebivolol or *d*-nebivolol, but not by *l*-nebivolol (Figure 5A). In eNOS−/− chimeric mice (in which circulating cells produced NO but the endothelium did not), the time to complete occlusion of the femoral artery was slightly, but significantly, longer than that of eNOS−/− mice (9.66±1.7 versus 7.7±0.33 minutes; *P*<0.001) and not different from that of wild-type mice, and it was significantly prolonged by the

**Table. The Effect of Five-Day Treatment of Wild-Type and eNOS−/− Mice With dl-Nebivolol, Its Enantiomers, and Bisoprolol on the Expression of P-Selectin on Circulating Platelets After Intravenous Challenge With a Mixture of Collagen Plus Epinephrine**

<table>
<thead>
<tr>
<th>Treatments</th>
<th>P-Selectin (% of Positive Platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-Type Mice</td>
</tr>
<tr>
<td>Vehicle</td>
<td>29.1±1.6</td>
</tr>
<tr>
<td><em>d</em>-Nebivolol 5 mg/kg PO</td>
<td>9.8±1.4‡</td>
</tr>
<tr>
<td><em>l</em>-Nebivolol 2.5 mg/kg PO</td>
<td>7.9±1.4‡</td>
</tr>
<tr>
<td><em>d</em>-Nebivolol 2.5 mg/kg PO</td>
<td>14.7±1.5*</td>
</tr>
<tr>
<td>Bisoprolol 5 mg/kg PO</td>
<td>23.9±1.1§</td>
</tr>
</tbody>
</table>

Baseline (mice not challenged with collagen plus epinephrine): wild-type mice=6.4±1.8% of positive platelets; eNOS−/− mice=11±1.6% of positive platelets. n=8 for all groups.

*P*<0.05 vs vehicle group.
†*P*<0.05 vs wild-type mice.
‡*P*<0.001 vs vehicle group.
§*P*<0.054 vs vehicle group. PO indicates per os.

pretreatment with the NO scavenger v2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide significantly reduced *dL*-nebivolol and *l*-nebivolol–induced intraplatelet cGMP increase (Figure 4C).

**Figure 4.** In vivo effect of *dL*-nebivolol, *l*-nebivolol, *d*-nebivolol, and bisoprolol on (A) plasmatic and (B) urinary nitrites and nitrates in wild-type mice; (C) intraplatelet cGMP levels measured in wild-type mice and expressed as pmol/10⁸ platelets, without (black columns) and with (white columns) v2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; (D) platelet-derived nitrites and nitrates in eNOS−/− mice. Platelet-derived NOx were assessed in the supernatant of resting (white columns) and collagen-stimulated (black columns) platelets.

E, In vitro platelet-derived NOx: GF platelets, preincubated with *d*-nebivolol, *l*-nebivolol, and bisoprolol (100 μmol/L), were stimulated with collagen 2 μg/mL, and NOx were measured in the supernatant. Values represent mean±SEM, n=3 to 13 mice per group. *P*<0.05 vs vehicle.

*Inside bars: *P*<0.05 vs corresponding white column.
administration of both dL-nebivolol and l-nebivolol but not by d-nebivolol (Figure 5B).

Control transplanted mice (wild type into wild type and eNOS−/− into eNOS−/−) behaved as the respective not transplanted strains.

To unravel the possible role of the interaction with white cells, thrombosis was induced in wild-type mice depleted of neutrophils and reinfused with neutrophils isolated from eNOS−/− mice. The resultant chimeric mice had normal circulating platelets but neutrophils lacking eNOS. In these conditions, although a slight, not significant, prolongation of the time to thrombotic occlusion of the femoral artery was observed, the antithrombotic effect of dL-nebivolol and l-nebivolol was fully preserved (Figure III in the online-only Data Supplement).

Production of NO From Isolated Platelets

When platelets isolated from mice treated for 5 days with the different drugs were stimulated in vitro with collagen, NOx in the supernatant were significantly higher in mice treated with dL-nebivolol and l-nebivolol but not with d-nebivolol and bisoprolol (Figure 4D). Moreover, incubation in vitro of platelets with dL- and l-nebivolol (100 μmol/L), but not with d-nebivolol, increased the amount of nitrites and nitrates found in the supernatant of collagen-stimulated platelets (Figure 4E). Incubation of GFP in vitro with dL- and l-nebivolol also increased intraplatelet cGMP, an effect blocked by preincubation with ODQ, whereas d-nebivolol was ineffective (Figure IV in the online-only Data Supplement).

eNOS Phosphorylation

Phosphorylated eNOS was measured in platelet pellets of wild-type mice treated for 5 days. dL-Nebivolol and l-nebivolol increased phosphorylation in Ser 1177 of eNOS in platelet extracts (Figure 6; Figure V in the online-only Data Supplement), whereas d-nebivolol and bisoprolol did not affect phosphorylated eNOS expression.

Other effects

Blood Pressure

eNOS-knockout mice had increased blood pressure (130±1.9 mmHg) when compared with wild-type mice (119±3.1 mmHg). In eNOS−/− mice, dL-nebivolol and d-nebivolol significantly reduced blood pressure, whereas l-nebivolol did not (Figure 7A). Bisoprolol was also effective. In wild-type normotensive mice, only D-nebivolol reduced blood pressure (Figure 7B).

Antioxidant Activity

dL-Nebivolol and l-nebivolol significantly reduced ROS formation induced by photochemical injury in femoral arteries, whereas d-nebivolol and bisoprolol did not affect it (Figure VI in the online-only Data Supplement).

Figure 5. The effect of dL-nebivolol, l-nebivolol, d-nebivolol, and bisoprolol on photochemically induced femoral artery thrombosis in (A) wild-type (WT) chimeric mice (eNOS−/− bone marrow donors, WT mice recipients) and (B) eNOS−/−-chimeric mice (WT bone marrow donors and eNOS−/− recipients). n=10 mice in vehicle-treated groups; n=5 mice in all drug-treated groups. *P<0.05 vs control.

Figure 6. The effect of dL-nebivolol, l-nebivolol, p-nebivolol, and bisoprolol on protein expression of total- and phosphorylated (p)-eNOS in platelet extracts assessed by Western blotting using monoclonal-anti-total eNOS and monoclonal-anti-Ser1177 phospho-eNOS antibody. Bottom, Data were quantified using ImageJ software and plotted relative to the controls. Data are expressed and mean±SEM of n=4 experiments. Top, Example of Western blotting for total-eNOS, p-eNOS, and α-tubulin. Lane 1, control; lane 2, dL-nebivolol; lane 3, l-nebivolol; lane 4, d-nebivolol, and lane 5, bisoprolol.)
Our data show that \( dl \)-nebivolol, a third-generation, highly selective \( \beta_1 \)-ARs antagonist, exerts an antithrombotic activity in vivo in mice by enhancing NO release from platelets and that the NO-releasing activity can be ascribed mainly to its \( l \)-enantiomer by \( \beta \)-AR–mediated eNOS activation.

\( dl \)-Nebivolol dose dependently protected wild-type mice from platelet-mediated pulmonary thromboembolism induced by the intravenous injection of collagen plus epinephrine. In this model, \( l \)-nebivolol was as effective as the racemic mixture and more than \( d \)-nebivolol. Moreover, \( dl \)- and \( l \)-nebivolol, but not \( d \)-nebivolol, exerted a partial protective effect also in a model of mechanical pulmonary microembolism induced by the intravenous injection of swollen, hardened rat red blood cells. In this model, vasodilatory agents, such as calcium antagonists or isosorbide mononitrate, but not antiplatelet agents, exert a protective effect by inducing vasodilation of the lung microvasculature. These results are thus consistent with both an antiplatelet and a vasodilatory activity of \( dl \)- and \( l \)-nebivolol, which concur to prevent collagen plus epinephrine-induced pulmonary embolism lethality. However, the antithrombotic effect of \( dl \)- and \( l \)-nebivolol was confirmed in a photochemical injury–induced arterial thrombosis model, known to be platelet mediated and not influenced by vasodilators.

To establish whether also the antithrombotic effects of \( dl \)-nebivolol could be ascribed to an increase of NO production, we performed experiments in eNOS−/− mice, genetically deficient of the endothelial variant of NO synthase. In these mice, \( l \)-nebivolol lost its protective effect: these results show that \( l \)-nebivolol requires a functional eNOS to exert its antithrombotic activity and acts by stimulating NO production. Confirmatory evidence comes from the finding that \( dl \)-nebivolol and, more strikingly, \( l \)-nebivolol induced an in vivo enhancement of plasmatic and urinary nitrites and nitrates, the main end products of NO catabolism. The increased levels of NO attained in vivo were biologically relevant, as documented by the enhanced intraplatelet cGMP detected in mice treated with \( l \)-nebivolol, an effect caused by enhanced NO formation because pretreatment with \( \nu_2 \)-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, a NO scavenger, abolished it. In agreement with the present results, an increase in plasmatic cGMP was previously shown in patients with hypertension treated with \( dl \)-nebivolol (5 mg/d for 4 weeks) but not with hydrochlorothiazide (25 mg/d for 4 weeks).

NO in the circulation is produced both by the endothelium and by blood cells. However, the presence and role of eNOS in platelets have been the object of debate. Since the first report by Radomski et al., showing an increase of cGMP in isolated platelets stimulated by collagen, an effect abrogated by NOS antagonists, numerous studies have confirmed the presence of eNOS in platelets, the ability to synthesize NO, and the inhibitory role on platelet activation. However, other reports have negated the presence of eNOS in platelets and 1 group has even provided evidence that platelet-derived NO and its second messenger, cGMP, have a stimulatory role on platelet activation. Similarly, studies on the role of platelet-derived NO in thrombosis models in vivo have given conflicting results, with data in favor of suggesting a different mechanism of action of the 2 enantiomers on platelets.

Figure 7. The effect of \( dl \)-nebivolol, \( l \)-nebivolol, \( d \)-nebivolol, and bisoprolol on systemic blood pressure in eNOS−/− (A) and wild type (B) mice. Mice were administered with drugs by oral route for 5 days. Blood pressure was measured at baseline (white columns) and 3 hours after the last drug administration on day 5 (black columns). \( n=5 \) mice per group. *\( P<0.05 \) vs baseline.
a significant antithrombotic effect studies suggesting that the absence of platelet eNOS has little consequence on arterial thrombus formation, and even studies showing an impaired ability of eNOS−/− mice to form occlusive thrombi. Differences in experimental conditions and animal models may probably explain the conflicting results. Moreover, the existence of variant forms of eNOS in platelets may explain some of the negative data. Despite controversy, consistent data suggest that platelet-derived NO participates in platelet inhibition and thrombosis prevention and that conditions characterized by defective platelet NO production, such as hypertension or treatment with some antiretroviral drugs, are associated with an enhanced risk of ischemic cardiovascular events.

The pre-eminent role of platelet- instead of endothelium-derived NO in the antithrombotic activity of dl-nebivolol suggests that NO generated by platelets upon activation at an arterial damage site is more effective than that generated by endothelium-derived NO probably because it acts exactly where platelets get activated.

Therefore, to clarify the role of platelet-derived NO in the antithrombotic effects of dl-nebivolol, we used chimeric mice. dl-Nebivolol and l-nebivolol maintained their antithrombotic action in mice in which blood cells, but not the endothelium, produce NO (wild-type chimeras), whereas they lost it in mice in which only endothelial cells produce NO. A confirmation of the ability of dl- and l-nebivolol to induce NO release from platelets comes from the finding that platelets isolated from wild-type mice treated with dl-nebivolol or l-nebivolol, but not with n-nebivolol or bisoprolol, and stimulated in vitro with collagen release increased amounts of NO and that incubation in vitro with l-nebivolol enhances NO release in the supernatant and intraplatelet cGMP, an effect prevented by soluble guanylyl cyclase function. In experiments in wild-type mice depleted of neutrophils and reinfused with neutrophils isolated from eNOS−/− mice, showing that the antithrombotic effect of l-nebivolol is fully preserved, further point to platelets as the main effectors of l-nebivolol-induced NO release in vivo.

dl-Nebivolol was reported to possess an intrinsic β2/β3 agonistic activity, leading to Akt-dependent phosphorylation of eNOS in Ser1177 in endothelial cells; therefore, we assessed phosphorylated eNOS in platelets. dl-Nebivolol and l-nebivolol enhanced eNOS phosphorylation at serine1177, 1 of the phosphorylation sites involved in eNOS activation in platelets.

Tran Quang et al showed that both d- and l-nebivolol stimulate vascular β3-adrenoceptors in rat aortae, and that this may be relevant to nebivolol–induced NO release. However, the presence of β3-adrenoceptors has not been demonstrated in platelets that instead possess β1 and β2 antagonists. The in vitro platelet inhibitory effect of l-nebivolol was prevented by both β1/β2 antagonists, whereas that of d-nebivolol was not. In fact, d-nebivolol exerted a weak antiplatelet effect independent from NO and related to a β-AR–independent stimulation of adenylyl cyclase, as shown by the neutralizing action of the adenylyl cyclase inhibitor SQ 22536. However, this effect seems to contribute marginally to the antithrombotic activity of dl-nebivolol in wild-type mice, in which l-nebivolol was as effective as the racemic mixture.

Nebivolol was recently shown to act as a G-protein-coupled receptor kinase/β-arrestin biased agonist: whether this effect is involved in either adenylyl cyclase or eNOS activation in platelets remains to be established.

In conclusion, our data show for the first time that dl-nebivolol, and in particular its l-enantiomer, exerts an antithrombotic and antiplatelet effect in vivo by stimulating the release of NO from platelets.

The observation that dl-nebivolol reduced significantly all-cause mortality in elderly patients with congestive heart failure of ischemic etiology, whereas first-generation anti-hypertensive β-blockers do not prevent ischemic cardiovascular events when compared with other antihypertensive interventions, suggests that increased platelet-derived NO production in vivo may confer enhanced cardiovascular protection.

However, large, prospective comparative clinical trials with other β-blockers not influencing NO production may confirm whether these additional pleiotropic effects of dl-nebivolol on the cardiovascular system confer a superior profile in terms of prevention of ischemic cardiovascular events.

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Disclosures

Dr Evangelista is an employee of Menarini Group. The other authors report no conflicts.

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**Significance**

Our data show that DL-nebivolol exerts an antithrombotic and antiplatelet activity in vivo in mice by stimulating the release of NO from platelets and that the NO-releasing activity can be ascribed mainly to its L-enantiomer by β-adrenergic receptor-mediated eNOS activation. The pre-eminent role of platelet- over endothelium-derived NO in the antithrombotic activity of DL-nebivolol suggests that NO generated by platelets upon activation at an arterial damage site, is more effective than endothelium-derived NO probably because it acts exactly where platelets get activated. This novel antithrombotic mechanism has implications that go beyond the specific drug tested and shows that the modulation of the production of platelet-derived NO, as well as the sensitization of platelets to the inhibitory effects of NO, may be important targets of future antithrombotic therapies.
Supplemental Figure I - Assessment by RT-PCR of eNOS expression in extracts from (A) blood cells and (B) brain tissue obtained from wild type, eNOS<sup>−/−</sup> and chimeric mice 8 weeks after bone marrow transplantation.

A) Extracts from blood cells:
1=wild type; 2=wild type donor-eNOS<sup>−/−</sup> recipient; 3=eNOS<sup>−/−</sup>; 4=eNOS<sup>−/−</sup> donor-wild type recipient

B) Extracts from brain tissue:
5=wild type; 6=wild type recipient-eNOS<sup>−/−</sup> donor; 7=eNOS<sup>−/−</sup> recipient-wild type donor; 8=eNOS<sup>−/−</sup>
Supplemental Figure II - Collagen+epinephrine-induced pulmonary thromboembolism: dose dependent effect of nebulol in wild type mice (n=20 per group).

Results are expressed as % mortality. *p=0.05 vs vehicle; §p=0.03 vs vehicle.
Supplemental Figure III - Effect of dl-nebivolol, l-nebivolol and d-nebivolol on photochemically-induced femoral artery thrombosis in neutropenic wild type mice reinfused with eNOS⁻/⁻-derived neutrophils. Circulating neutrophil counts were: control=3956±729; control after LY-6G=368±16; control after LY-6G and neutrophils reinfusion=2668±257).
n=5 mice per group. *P<0.05 vs control.
**Supplemental Figure IV** – In vitro effect of preincubation with dl-nebivolol (100μM), l-nebivolol (100μM) and d-nebivolol (100μM) on intraplatelet cGMP levels in U46619-activated murine platelets. Platelets were incubated with drugs in the presence or in the absence of ODQ (10μM); zaprinast (40μM) was added at the end of aggregation. The response of platelets to SNP as a positive control is reported in the inset.

Values represent means ± S.D. of n=8 experiments. *P<0.05 vs baseline.
**Supplemental Figure V** – Effect of dl-nebivolol, l-nebivolol and d-nebivolol on total eNOS and phospho-eNOS detected in lysed platelets.

β-tubulin was used as housekeeping protein. Representative western blotting gels.
**Supplemental Figure VI** - Effect of dl-nebivolol, l-nebivolol, d-nebivolol and bisoprolol on ROS production in femoral artery extracts after photochemically-induced thrombosis. ROS in control of femoral artery not subjected to photochemical injury was 474±69 DCF fluorescence intensity/mg of protein.

n=5 mice per group. *P<0.05 vs control.
Materials and Methods

Animals and Drugs
Male CD-1 (C57BL6, Charles Rivers, Como, Italy) and eNOS−/− mice (JaxMice, Harbour Maine, Maryland, USA), weighing 20-25 g, were used. Mice were caged and fed a regular diet for at least 1 week before use. dl-nebivolol (a racemic mixture containing equal amounts of l- and d-nebivolol), l-nebivolol and d-nebivolol were from Menarini Group (Florence, Italy); bisoprolol from Sequoia Research (Pangbourne, UK). Drugs were dissolved in PEG400 (Sigma-Aldrich Co, Milan, Italy) and 0.5 % DMSO and were administered by oral gavage in a fixed volume of 100 µl/mouse. The study was approved by the Committee on Ethics of Animal Experiments of the University of Perugia and by the Italian Ministry of Public Health (Authorization n° 231/2010-B).

Treatments
dl-nebivolol, its two enantiomers (+SRRR nebivolol=d-nebivolol; -RSSS nebivolol=l-nebivolol), bisoprolol, or their vehicles were administered by oral gavage 3 hrs before thrombogenic challenge. dl-nebivolol was tested in a range of doses (from 0.0625 to 5 mg/kg), while d-nebivolol and l-nebivolol were tested at one single dose (2.5 mg/kg) equimolar to the maximum dose of dl-nebivolol tested. Bisoprolol, that reduces blood pressure and heart rate in humans in the same dose-range of dl-nebivolol (1), was tested at the dose of 5 mg/kg.

For flow cytometry and cGMP measurements, blood was collected from mice 3 hrs after a single oral drug administration. For studies on blood pressure, on plasmatic and urinary nitrites and nitrates, on eNOS expression and phosphorylation and for studies on the role of blood cell-derived NO in the antithrombotic activity of the drug, mice were dosed with the tested drugs for five days and blood and urines were collected 3 hrs after the last drug administration.

In experiments for cGMP measurement, mice were pre-treated daily with 1 mg/kg i.v. of CarboxyPTIO (C-221, Sigma-Aldrich SpA, Milan, Italy), or its vehicle on days -2, -1, and 0 and then administered (on day 0) bisoprolol (5 mg/kg), or dl-nebivolol (5 mg/kg) or its enantiomers (2.5 mg/kg) by oral gavage. Blood was collected 3 hrs after drug administration.

Thrombosis models
Pulmonary thromboembolism
Pulmonary thromboembolism was induced by a method previously described (2). Mice were caged and fed a regular diet for at least one week before use. Thrombotic challenge was generated by the rapid i.v. injection of 100µl of a mixture of collagen (250µg/ml, Mascia Brunelli, Milan, Italy) and epinephrine (1.5 µg/ml, Mascia Brunelli, Milan, Italy) into one of the tail veins. The effect of the intravenous challenge with collagen plus epinephrine was assessed by evaluating, as a cumulative end point, death of the animals or paralysis of the hind limbs for more than 15 min (1, 2). Given that eNOS−/− mice had a higher response to thromboembolic challenge, a dose-response curve to collagen+epinephrine was carried out and a dose of 200 µg/ml collagen and 1.2 µg/ml epinephrine was selected in order to obtain a mortality in untreated animals similar to wild type mice.

A model of non-thrombotic disseminated mechanical pulmonary microembolism, which is sensitive to vasodilators but not to antiplatelet agents, was also used (2). This
model involves the i.v. injection of a suspension of swollen, hardened rat red blood cells (300 µl, hematocrit 12.5 %).

In each session at least five animals per experimental group were tested; controls were run at the beginning and at the end of each experimental session. Surviving animals were sacrificed at the end of each experimental session by an overdose of anesthesia. Mice were accustomed to handling and the injections were carried out by skilled investigators with minimal disturbance to the animals. Data are presented as % of animals dead/total number of animals tested.

**Femoral artery thrombosis**

Photochemically-induced femoral artery thrombosis was generated in anesthetized mice by a method described previously (3). Briefly, mice were anaesthetized with xylazine (5 mg/kg i.p.) and ketamine (50 mg/kg, i.p.) and placed on a heated operating table. A 25G needle venous butterfly was inserted in one of the tail veins for the infusion of rose Bengal. The left femoral artery was carefully exposed and a laser Doppler probe (Transonic System Inc, Ithaca, NY, USA) was positioned onto the branch point of the deep femoral artery, distal to the inguinal ligament, for monitoring blood flow. The exposed artery was irradiated with green light (wavelength 540 nm) of a Xenon lamp (L4887, Hamamatsu Photonics, Hamamatsu, Japan) equipped with a heat-absorbing filter via a 3 mm diameter optic fibre attached to a manipulator. Light irradiation was protracted for 20 minutes; the infusion of rose-Bengal (20 mg/kg) was started 5 min after the beginning of irradiation and lasted for 5 min. The end point was the cessation of blood flow for >30s; in case no occlusion occurred after 30 min, the time was recorded as 30 min (3). One hour after the end of the irradiation period, the femoral artery was excised, opened and the thrombus was removed, dried at 37°C for 24 hours and then weighed.

**Venous thrombosis model**

A flow restriction to the inferior vena cava (IVC) was induced as described (4). Briefly, mice were anesthetized with xylazine (5 mg/kg i.p.) and ketamine (50 mg/kg, i.p.) and placed in a supine position. After laparotomy, intestines were exteriorized and sterile saline was applied during the whole procedure to prevent drying. After gentle separation from aorta, inferior vena cava (IVC) was ligated by a 7.0 polypropylene suture immediately below the renal veins (toward the tail) over a 30-gauge needle and then the needle was removed to obtain a partial flow restriction (stenosis). The needle was placed outside the vessel so that piercing or any other injury to the IVC wall was completely avoided. In stenosis model, all visible side branches (usually 1 or 2) were also ligated. After surgery, peritoneum and skin were closed by monofilament absorbable suture and 6.0 silk, respectively. Mice were euthanized after 48 hours, and thrombi developed in the IVC below the suture (toward the tail) were taken for analysis (4). Thrombus weight and thrombus length were measured. Thrombus size was expressed as the ratio between weight and length.

**Generation of eNOS−/−-wild type chimeras**

Wild-type (C57BL/6) and eNOS−/− (C57BL/6 background) male and female mice, 8-12 weeks-old, were used. Bone marrow transplantation-recipient mice were exposed to a single lethal irradiation dose of 8 Gy from a 18-mV photon beam linear accelerator (Clinac 600/C Varian; Cernusco, Milano, Italy) with a focus-to-skin distance of 63 cm
and a dose-rate of 0.2 Gy/minute. Following irradiation, all mice not receiving bone marrow transplantation died within 14 days.

Bone marrow cells were collected from donor mice into phosphate-buffered saline (PBS) by flushing the shafts of the femur and tibia with PBS. Cells were suspended in PBS, then red blood cells were lysed with ammonium chloride-potassium buffer and the remaining cells were washed twice with PBS. Finally, bone marrow cells were resuspended at a final concentration of 20x10^6 per ml in PBS, and injected (10x10^6 cells/mouse) into recipient mice intravenously, via the lateral tail vein the day after total body irradiation (3).

In some experiments eNOS−/− mice were used as donors and wild-type mice as recipients (eNOS−/− chimeric mice), in others wild-type mice were used as donors and eNOS−/− mice as recipients (wild type chimeric mice). Control experiments were carried out by transplanting bone marrow from wild-type donors into wild-type recipients and from eNOS−/− donors into eNOS−/− recipients.

eNOS expression was assessed by RT-PCR in brain tissue and in blood cells obtained 8 weeks after bone marrow transplantation, to ensure that full engraftment had taken place (Supplemental Figure I) (3). One month after bone marrow transplantation both eNOS−/− and wild type chimeric mice, as well as controls, had almost normal platelet counts (724±44x10^3 platelets/µL and 772±55x10^3 platelets/µL, respectively).

In some experiments selective neutrophil depletion was obtained by the injection of anti-Ly-6G mAb (1A8, BioXCell, West Lebanon, NH) (1mg injected intraperitoneally 36 hours before thrombotic challenge). One hour before the induction of femoral artery thrombosis, neutrophils isolated from eNOS−/− mice were reinfused in neutropenic wild type mice. Each mouse received a total of 7x10^6 cells.

For neutrophil separation, bone marrow cells obtained by flushing femurs and tibiae with ice-cold Hepes Tyrode buffer Mg^2+/Ca^2+ free, containing hirudin (15µg/mL) EDTA (5mM) and EGTA (5mM) to avoid coagulation and cellular agglutination, were filtered through a BD cell strainer and contaminating erythrocytes were eliminated by hypotonic shock. Cells were retrieved, washed and resuspended in ice-cold Mg/Ca free Hepes Tyrode buffer supplemented with EDTA and EGTA. Neutrophils were isolated by hystopaque gradient (45 min 4°C without brake) and resuspended in ice-cold Mg/Ca free Hepes Tyrode. CaCl2 (1mM) and MgCl2 (1mM) were later added immediately before assay. Purity of the neutrophil preparations (consistently > 97%) was verified by flow cytometry and cell viability (>97%) by trypan blue dye exclusion test. Polymorphonuclear morphology was routinely verified by optical microscopy after MayGrunwald-Giemsa staining.

**Blood pressure measurement**

Wild type and eNOS−/− mice were administered dl-nebivolol (5 mg/kg), its enantiomers (2.5 mg/kg each), or bisoprolol (5 mg/kg) for five days, once a day by oral gavage. Systolic blood pressure was measured in conscious mice, after a period of training and acclimation to the procedure, at baseline and 3 hrs after last drug treatment, using a non-invasive computerized tail-cuff system (BP-2000, Visitech System, Apex, USA), as described elsewhere (5).

**Expression of P-selectin on the platelet surface**

Platelet surface P-selectin was measured in whole-blood by flow cytometry (EPICS XL-MCL, Beckman Coulter, Miami, Florida, USA), as previously described (3,6). Whole blood (anticoagulated with sodium citrate 4%, 1:10 v:v) was collected from wild-type
and eNOS\textsuperscript{+/−} mice 2 min after the i.v. injection of a mixture of collagen (150 μg/ml) plus epinephrine (0.25 μM), as described (3); aliquots of 5 μl were stained with a PE-labelled anti-CD41 antibody (Leo.D2, Emfret Analytics, Germany) as a platelet identifier and with a FITC-labelled-anti-P-selectin antibody (CD62P, Emfret Analytics, Germany). The reaction was stopped with PFA 1% (1 ml). A negative control was obtained using an aspecific isotopic antibody. P-selectin expression is reported as percentage of positive cells.

**Platelet aggregation**

Platelet aggregation was assessed by light transmission aggregometry (LTA) *ex vivo* and *in vitro* using U46619, a stable TxA\textsubscript{2} analogue, as a stimulus. For *ex vivo* experiments, mice were treated with dl-nebivolol (5 mg/kg), l- and d-nebivolol (2.5 mg/kg each) for five days, once a day by oral gavage. One hour after the last administration, blood was collected by cardiac puncture in Na-citrate 4% (1/10 vol), and centrifuged at 150g for 15 min to obtain platelet-rich plasma (PRP). Gel filtered platelets (GFP) were obtained by passing PRP through Sepharose 2B (Sigma Chemical, St Louis, USA) columns equilibrated with calcium-free Tyrode’s buffer (3.1 mM HEPES, 4 mM NaH\textsubscript{2}PO\textsubscript{4}·H\textsubscript{2}O, 137 mM NaCl, 2.6 mM KCl, 1 mM MgCl\textsubscript{2}, 5.6 mM dextrose, and 0.1% BSA, pH 7.4) (7) and adjusted to a concentration of 100x10\textsuperscript{3}/μl.

For *in vitro* experiments, GFP were incubated with 1H-[1,2,4]Oxadiazolo[4,3-a] quinoxalin-1-one (ODQ)(Sigma-Aldrich, Milan, Italy) 10μM, a guanylyl cyclase inhibitor, for 5 min before the addition of dl-nebivolol, l-nebivolol and d-nebivolol 100μM, and further incubated for 10 min before stimulation. CGP20712A (300 nM)(Sigma-Aldrich, Milan, Italy), a selective β1-AR antagonist, ICI118551 (100 nM) (Sigma-Aldrich, Milan, Italy), a selective β2-AR antagonist, SQ22,536 (50 μM), an adenylyl cyclase inhibitor, and forskolin (10μM)(Sigma-Aldrich, Milan, Italy), an adenylyl cyclase activator, were incubated for 5 min at 37°C and aggregation was induced by a threshold dose (0.5 μM) of U46619 and followed for 5 min. Results are expressed as maximal amplitude of aggregation (% light transmission).

**Intraplatelet cGMP**

Intraplatelet cGMP, the second messenger generated by NO within platelets, was measured by enzyme immunoassay (Amersham, Amsterdam, The Netherlands). For *in vitro* experiments, immediately after U46619-induced platelet aggregation, zaprinast (40 μM) (Sigma, Milan, Italy), a phosphodiesterase (PDE) inhibitor or its vehicle, was added in order to prevent cGMP degradation, samples were immediately centrifuged at 20,000 x g for 5 min and supernatants stored at −80°C for later determination of intraplatelet cGMP. Sodium nitroprusside (SNP) 10µM, a direct NO donor, was used as a positive control.

For *in vivo* experiments, blood was collected by cardiac puncture from anesthetized mice in sodium citrate 4% containing zaprinast (40 μM)(1/10 vol). PRP was obtained by centrifugation at 150xg for 10 min and then it was centrifuged again at 1000xg for 10 min to obtain a platelet pellet that was extracted with lysis reagent and stored at −80°C. Intraplatelet cGMP was expressed as pmol/10\textsuperscript{8} platelets (8).

Moreover, in selected experiments mice were treated with the NO scavenger CarboxyPTIO (1 mg/kg i.v.)(Sigma, Milan, Italy) once a day for three days before drugs administration.
Intraplatelet cAMP

Intraplatelet cAMP, the second messenger generated within platelets by adenyl cyclase activation, was measured by enzyme immunoassay (Parameter™ Cyclic AMP Assay, R&D System Inc, Minneapolis, USA). GFP (200x10^3/µl) were incubated with the adenyl cyclase inhibitor SQ 22,536 (50 µM) for 15 minutes at 37°C, then dl-nebivolol, l-nebivolol, d-nebivolol (100µM) or vehicle were added for an additional 10 min. Samples were then centrifuged at 12,000 rpm for 7 sec, the supernatant discarded and the platelet pellet was lysed and stored at -80°C for later assay.

Plasmatic and urinary nitrites and nitrates

Nitrites and nitrates (NOx), the degradation end-products of NO, were measured in plasma, urine and platelet supernatants of wild type and eNOS^-/- mice (See online supplemental material for details). Nitrites and nitrates (NOx), the degradation end-products of NO, were measured in plasma and urine and platelet supernatants of wild type and eNOS^-/- mice using the Griess reagent, as previously reported (5). Plasma was obtained by centrifugation of blood at 1,000 x g for 10 min. Urine samples were collected from mice placed in metabolic cages during the 24 hrs preceding the first drug administration (baseline levels) and during the 24 hrs following the last drug administration. Plasmatic and urinary NOx were measured by a colorimetric, non enzymatic method (Molecular Probe, Invitrogen, Milan, Italy), as reported elsewhere (5).

In some selected experiments, NOx were measured in platelet supernatants. Blood was obtained from wild type and eNOS^-/- mice treated with drugs for five days. Platelet rich plasma, adjusted to 250,000 platelets/µl with autologous platelet poor plasma, was incubated with collagen 2µg/ml for 15 min at 37 °C under mild stirring. NOx were assessed in the supernatant plasma, after centrifugation at 12000 rpm for 5 min, by the Griess reagent, at 540 nm using a sodium nitrite calibration curve (6).

eNOS expression by Western blotting

Endothelial nitric oxide synthase (eNOS), total and phosphorylated at Ser(1177) (p-eNOS), was assessed by western blotting in platelet pellets using antibodies from BD Pharmingen (Milan, Italy). Briefly, eighty µg of protein lysate were heated at 90°C for five min in the presence of sample buffer (63 mM Tris HCl pH 6.8, 10% SDS, 50% glycerol, 1% bromophenol blue, 0.25 M DTT) and then analysed by SDS polyacrylamide gel electrophoresis (9% separating) for 90 min at 20 mA. Separated proteins were transferred onto a nitrocellulose membrane at 100 V for 90 min. The membranes were saturated with 5% fat-free dried milk in phosphate-buffered saline and incubated with a rabbit polyclonal antiserum (1:2000 dilution) directed against eNOS and phosphorylated eNOS (Millipore, Milan, Italy) for 2 h at room temperature. The membranes were extensively washed with phosphate buffered saline-Tween 0.05% and then incubated with HP-conjugated anti-rabbit IgG (1:3000 dilution) for 2 h at room temperature. Blots were developed using the enhanced chemiluminescence assay. Individual band densities of immunoblots were integrated by using the Quantiscan software (Biosoft, Cambridge, U.K.). Tissue protein content was measured by the Bio-Rad protein assay. The expression of α-tubulin was used as the internal control.

Antioxidant activity
The formation of reactive oxygen species (ROS) in femoral artery extracts after photochemically-induced thrombosis was measured by OxiSelect ROS assay kit (Cell Biolabs, USA) (sensitivity limit of 10 pM for DCF) according to the manufacturer's instructions (5).

**Statistical analysis**

All values are reported as means ± SEM. Comparisons between groups were made using either the Student's t-test, when two groups were compared, or by the ANOVA followed by the Bonferroni posthoc test, when more than two groups were compared. Differences were deemed significant when p<0.05. Statistical analyses were performed using the GraphPad Prism Software.
References


