# Nebivolol and its 4-Keto Derivative Increase Nitric Oxide in Endothelial Cells by Reducing its Oxidative Inactivation

Luciano Cominacini, MD,* Anna Fratta Pasini, MD,* Ulisse Garbin, MD,* Cristina Nava, MD,*
Anna Davoli, BSc,* Marco Criscuoli, BSc,† Attilio Crea, BSc,‡ Tatsuya Sawamura, MD,§
Vincenzo Lo Cascio, MD*

*Verona, Firenze, and Pomezia, Italy; and Osaka, Japan*

|  |  |
|---|---|
| **OBJECTIVES** | The objective of the present study was to elucidate the vasodilator mechanisms of nebivolol, a high selective $\beta_1$-receptor antagonist with antioxidant properties. |
| **BACKGROUND** | Oxidative inactivation of nitric oxide (NO) is regarded as an important cause of its decreased biological activity. |
| **METHODS** | Oxidative stress was induced through the binding of oxidized (ox)-low-density lipoprotein (LDL) to its specific endothelial receptor, called "lectin-like oxidized LDL receptor-1" (LOX-1), in bovine and human endothelial cells and in Chinese hamster ovary cells stably expressing bovine LOX-1 (BLOX-1-CHO cells). Reactive oxygen species (ROS), superoxide ($O_2^{\cdot-}$), and NO were measured in cells by flow cytometry. |
| **RESULTS** | Nebivolol and its 4-keto derivative prevented in a dose-dependent manner the increase of ROS ($p < 0.001$) and $O_2^{\cdot-}$ ($p < 0.001$) in bovine aortic endothelial cells (BAECs), human umbilical vein endothelial cells (HUVECs), and BLOX-1-CHO cells stimulated with ox-LDL. Atenolol had no effect. The incubation of HUVECs and BAECs with ox-LDL reduced basal and bradykinin-induced NO and nitrite concentration ($p$ from $<0.001$ to $<0.01$). Nebivolol and its 4-keto derivative prevented the reduction of basal and stimulated NO and nitrite concentration ($p$ from $<0.001$ to $<0.01$) while atenolol had no effect. The preincubation of BAECs with blocking anti-LOX-1 monoclonal antibody (LOX-1 mAb) significantly counteracted the effect of ox-LDL on stimulated generation of NO ($p < 0.001$), but the effect was significantly lower than that of nebivolol and its 4-keto derivative alone ($p < 0.01$). |
| **CONCLUSIONS** | In conclusion, the findings of the present study indicate that nebivolol increases NO also by decreasing its oxidative inactivation. (J Am Coll Cardiol 2003;42:1838–44) © 2003 by the American College of Cardiology Foundation |

Endothelium plays an important role in the regulation of vascular tone, the major endothelium-derived relaxing factor being nitric oxide (NO) (1,2). It is recognized that essential hypertension is characterized by impaired endothelium-dependent vasodilation (3), an abnormality that is known to precede atherosclerosis and particularly coronary artery disease (CAD) (4). This is especially relevant because hypertension is a major risk factor for CAD.

The impairment of endothelium-dependent relaxation has been linked to a decreased production and/or biological activity of endothelium-derived NO (5).

Nebivolol, a highly selective $\beta_1$-receptor antagonist with antioxidant properties (6,7), has been shown to cause vasodilation in animal models (8) and humans (9) and to reverse endothelial dysfunction in essential hypertension

(10). It has been hypothesized that nebivolol induces vasodilation through an endothelial $\beta_2$-adrenergic receptor-mediated NO production (11).

Because the vascular release of superoxide ($O_2^{\cdot-}$) is increased in atherosclerotic arteries (12) and $O_2^{\cdot-}$ can inactivate NO in a chemical reaction during which peroxynitrite is formed (13), oxidative inactivation of NO has also been regarded as an important cause of endothelial dysfunction in essential hypertension (14).

The present study was therefore performed to obtain further insight into the mechanism underlying the effect of nebivolol on NO production and, in particular, to explore whether nebivolol reduces oxidative inactivation of NO and, consequently, increases its intracellular availability in endothelial cells exposed to oxidative stress.

## METHODS

**Cell cultures.** Bovine aortic endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described (15,16). Chinese hamster ovary-K1 (CHO-K1) cells and a CHO-K1 cell line stably expressing bovine lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (BLOX-1-CHO)

(17) were cultured as previously described (15). Cell survival was monitored according to the method of Landegren (18).

**Addition and measurement of nebivolol in endothelial cells.** Nebivolol and its 4-keto and 4-OH derivatives (courtesy of Menarini Ricerche, Firenze, Italy) and atenolol (Sigma, St. Louis, Missouri) were dissolved in ethanol and in M-199. Increasing amounts of the drugs (from 1 to 25 $\mu$mol/l) were added to endothelial cell monolayers for 30 min at 37°C. Nebivolol was also incubated at different times (30 to 180 min) with endothelial cells in order to ascertain whether endothelial cells metabolize the drug. The determination of nebivolol and atenolol in cells was performed as previously described (19,20).

**Induction of oxidative stress in endothelial cells.** Oxidative stress in endothelial cells was induced by the addition of oxidized (ox) LDL. The binding of ox-LDL to the endothelial receptor called LOX-1 has already been demonstrated to increase the intracellular concentration of reactive oxygen species (ROS) (15) and $O_2^{\cdot-}$ (21).

**LDL isolation and oxidation.** Whole blood, containing ethylenediamine tetraacetic acid (EDTA) (1 mg/ml), was processed for LDL separation as previously described (15). $Cu^{2+}$ modified LDL was prepared as reported (15), and the extent of LDL oxidation was determined by thiobarbituric acid-reactive substances (TBARS) (15). Protein was measured by the Pierce BCA protein assay reagent (22).

**Translocation of cytosolic nicotinamide adenine dinucleotide phosphate oxidase components.** The translocation of p47phox and Rac1 was evaluated as previously described (23). Briefly, HUVECs ($5 \times 10^7$), incubated with 50 $\mu$g protein/ml of ox-LDL and native LDL (control) for 1 to 10 min at 37°C, were sonicated on ice in 0.5 ml relaxation buffer (10 mM Pipes, pH 7.4, 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl2, 1.25 mM EGTA, 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml pepstatin, 10 $\mu$g/ml aprotinin). Cytosol and membranes were prepared by centrifugation on a discontinuous gradient of 15% and 34% (w/w) sucrose at 120.000 $g$ for 30 min at 4°C. The membrane pellets were transferred to a nitrocellulose membrane and probed with the anti-p47phox (Santa Cruz Biotechnology, Santa Cruz, California) or anti-Rac1 antibody (Santa Cruz Biotechnology).

Before stimulation with ox-LDL, HUVECs were also preincubated with anti-human LOX-1 monoclonal antibody (hLOX-1 mAb, 10 $\mu$g/ml), p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580 (Calbiochem, La Jolla, California) (10 $\mu$mol/l), protein kinase C (PKC) inhibitor bisindolylmaleimide (Calbiochem) (1 $\mu$mol/l), and increasing amounts of nebivolol (from 5 to 25 $\mu$mol/l) for 30 min at 4°C.

**ROS and $O_2^{\cdot-}$ measurement.** Intracellular ROS and $O_2^{\cdot-}$ levels were measured as described (15,21) by following the oxidation of 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and hydroethidine (HE) by flow cytometry (Coulter Corp., Hialeah, Florida). Cells were also tested for the production of $H_2O_2$ (24) and extracellular $O_2^{\cdot-}$ (25) radicals induced by ox-LDL.

For ROS, $H_2O_2$, and $O_2^{\cdot-}$ measurements, endothelial cells were first preincubated with nebivolol or atenolol for 30 min at 37°C, then increasing concentrations (50 to 150 $\mu$g protein/ml) of ox-LDL and native LDL were incubated with the cells for 10 min at 37°C in the presence of 5 mmol/l arginine and 3 $\mu$mol/l tetrahydrobiopterin (TB4). The effect of 4-OH, and 4-keto derivatives of nebivolol on the intracellular ROS and $O_2^{\cdot-}$ concentrations was also evaluated under the previously specified experimental conditions.

To test the response specificity, nebivolol, its 4-keto derivative, and anti-bovine LOX-1 monoclonal antibody (bLOX-1 mAb) (30 $\mu$g/ml) (17) were incubated with BAECs, CHO-K1, and BLOX-1-CHO cells under the previously specified experimental conditions.

**Binding of labeled ox-LDL to BAECs and BLOX-1-CHO cells and inhibition of binding.** Oxidized LDL was labeled using the fluorescent compound N, N-dipentadecyl-aminostyrylpyridinium iodide (di-15-ASP) as described previously (15). The data were fitted with GraFit (Erithacus Software Ltd., Staines, United Kingdom). A 100-fold excess of unlabeled ox-LDL, native LDL (100 $\mu$g protein/ml), bLOX-1 mAb (30 $\mu$g/ml), polyinosinic acid (250 $\mu$g/ml), and nebivolol (25 $\mu$mol/l) were used as inhibitors.

**NO measurement.** Intracellular NO was measured in the presence and absence of endothelial activation with bradykinin (100 nmol/l) (Sigma), as previously described, (21) by utilizing the fluorescent indicator 4, 5 diaminofluorescein diacetate (DAF-2 DA) in flow cytometry (Coulter Corp.). Nitric oxide production was also monitored by following levels of nitrite in the supernatant of stimulated HUVECs and BAECs. Levels of nitrite were determined by a fluorimetric assay (26). For NO measurement, HUVECs and BAECs were first preincubated with nebivolol or atenolol for 30 min at 37°C, then increasing concentrations (50 to 150 $\mu$g protein/ml) of ox-LDL and native LDL were

incubated with endothelial cells for 10 min at 37°C in the presence of bradykinin (100 nmol/l), 5 mmol/l arginine, and 3 $\mu$mol/l TB4. The effect of 4-OH and 4-keto derivatives of nebivolol on the intracellular basal and stimulated NO concentration was also evaluated under the previously specified experimental conditions.

In order to ascertain whether the effect of ox-LDL on intracellular NO concentration was dependent on $O_2^{\cdot-}$ produced by the binding of ox-LDL to LOX-1, bLOX-1 mAb (17) (30 $\mu$g/ml) was used under the previously specified experimental conditions.

Furthermore, to test the contribution of endothelial $\alpha$- or $\beta$-adrenergic receptors and 5-HT serotonergic receptors to NO concentration after preincubation with nebivolol and metabolites, a $\beta_2$-adrenergic antagonist (butoxamine, 0.1 mmol/l; Sigma), an $\alpha$-adrenergic receptor blocker (phentolamine 10 $\mu$mol/l; Sigma), or a 5-HT antagonist (NAN-190; 1 $\mu$mol/l; ICN, Frankurt/Main, Germany) was used. **Endothelial NO synthase activity measurement.** Endothelial NO synthase (eNOS) activity was measured by evaluating the metabolism of 3H arginine to 3H citrulline as described previously (21).

**Statistical analysis.** Statistical analysis was performed by one-way analysis of variance with repeated measures followed by post-hoc Tukey's test for multiple comparisons using the "SYSTAT" program and statistical software manual (SYSTAT Inc., Evanston, Illinois) for Macintosh. Statistical significance was inferred when p < 0.05. All reported p values were derived from Tukey's test.

## RESULTS

The addition of increasing amounts of nebivolol and atenolol in HUVECs and BAECs determined a dose-dependent intracellular increase of the drugs. The intracellular recovery was higher for nebivolol than for atenolol (respectively ranging from 65 ± 6% to 70 ± 8% for nebivolol and metabolites and of 35 ± 4% for atenolol (p < 0.001). The concentration of the nebivolol in endothelial cells was stable for at least 3 h.

In our experimental conditions, the incubation of HUVECs and BAECs with increasing amounts of ox-LDL for 10 min at 37°C induced a sharp and dose-dependent increase in intracellular concentration of ROS and $O_2^{\cdot-}$ (p from <0.001 to <0.01, in experiments performed in triplicate on six different occasions for each concentration of ox-LDL). Similarly, levels of $H_2O_2$ and $O_2^{\cdot-}$ in the supernatant of both HUVECs and BAECs significantly increased after the addition of ox-LDL (p from <0.001 to <0.01).
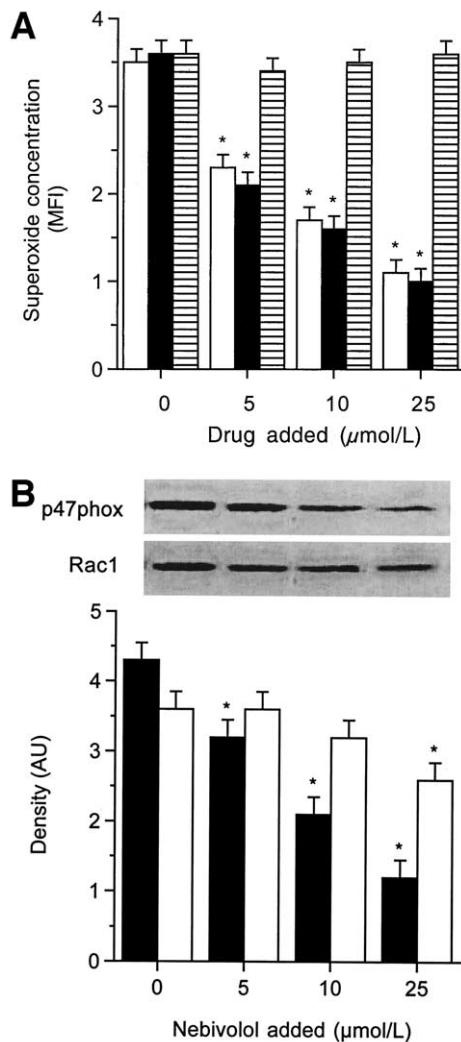
To determine whether the increase of ROS and $O_2^{\cdot-}$ induced by ox-LDL was dependent on NADPH oxidase activation and was, therefore, preceded by the translocation of some cytosolic NADPH oxidase components, we isolated membrane from ox-LDL-stimulated HUVECs and carried out immunoblot analysis of p47phox and Rac1. The trans-
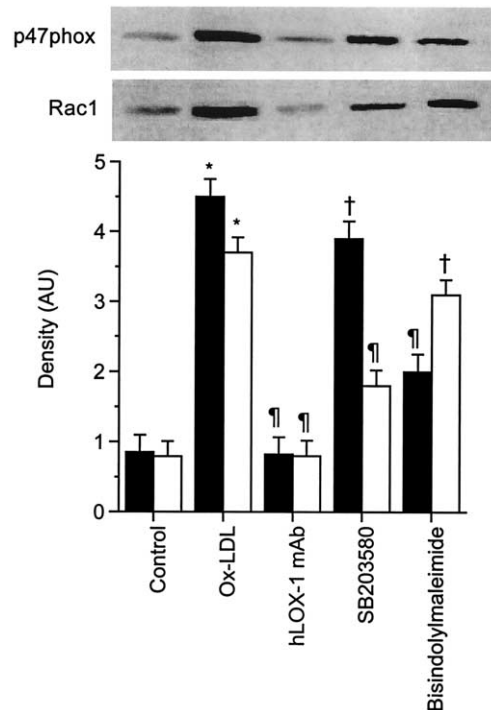


**Figure 1.** Effect of native low-density lipoprotein (LDL), oxidized LDL (ox-LDL), and ox-LDL plus anti-human LOX-1 monoclonal antibody (hLOX-1 mAb), SB203580 and bisindolylmaleimide on the translocation of p47phox and Rac1 to the plasma membrane of human umbilical vein endothelial cells. The cells were preincubated with hLOX-1 mAb, SB203580, and bisindolylmaleimide for 30 min at 37°C and then stimulated with native LDL (control) and ox-LDL (50 $\mu$g protein/ml) for 5 min at 37°C. The figure shows representative blots of six different experiments and the average data obtained by densitometric analysis. Results (means ± SD) are expressed as density in arbitrary units (AU). *p < 0.001 versus control; ¶p < 0.001 versus ox-LDL; †p < 0.05 versus ox-LDL. **Solid bars** = p47phox; **open bars** = Rac1.

location of p47phox and Rac1 was first found at 1 min after stimulation and peaked at 4 min (data not shown). We found that incubation of ox-LDL with HUVECs determined a significant increase in both p47phox and Rac1 translocation. The translocation of p47phox and Rac1 was inhibited by hLOX-1 mAb and respectively by bisindolylmaleimide and SB203580. Figure 1 shows representative immunoblots of p47phox and Rac1 and the summarized data obtained by densitometric analysis. Nebivolol and its 4-keto derivative dose-dependently reduced the intracellular concentration of ROS and $O_2^{\cdot-}$ in HUVECs and BAECs (p from <0.001 to <0.01). Atenolol and 4-OH derivative of nebivolol had no effect. Figure 2A shows the effect of nebivolol, its 4-keto derivative, and atenolol on $O_2^{\cdot-}$-induced by ox-LDL in HUVECs. The effect of nebivolol on intracellular $O_2^{\cdot-}$ concentration was associated to a reduced translocation of both p47phox and Rac1; the effect, however, was much more evident for p47phox (Fig. 2B).

Nebivolol (25 $\mu$mol/l) also reduced $H_2O_2$ from 4.52 ± 0.25 nmol/$10^6$ cells to 2.1 ± 0.21 nmol/$10^6$ cells in HUVECs (p < 0.001) and from 4.92 ± 0.44 nmol/$10^6$ cells to 1.99 ± 0.24 nmol/$10^6$ cells in BAECs (p < 0.001); it also decreased supernatant $O_2^{\cdot-}$ from 10.1 ± 0.8 nmol/$10^6$ cells
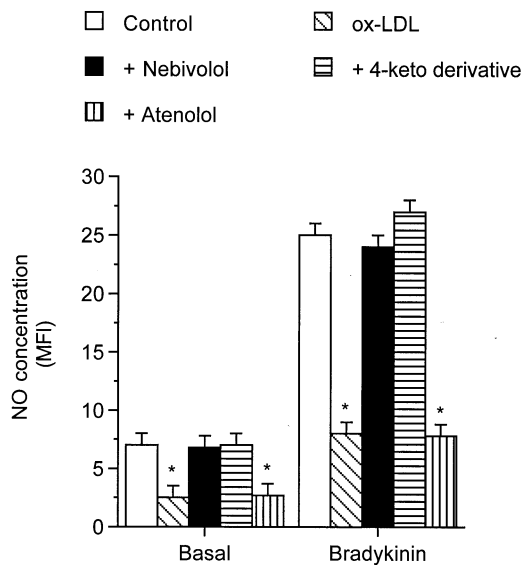
**Figure 3.** Effect of nebivolol, its 4-keto derivative, and anti-bovine LOX-1 monoclonal antibody (bLOX-1 mAb) on oxidized low-density lipoprotein (ox-LDL)-induced variation of superoxide ($O_2^{.-}$) in bovine aortic endothelial cells (BAECs), in Chinese hamster ovary-K1 (CHO) cells, and CHO-K1 cell line stably expressing bovine LOX-1 (BLOX-1-CHO). Nebivolol (25 $\mu$mol/l), its 4-keto derivative (25 $\mu$mol/l), and bLOX-1 mAb (30 $\mu$g/ml) were preincubated with cells for 30 min at 37°C. The cells were then stimulated with ox-LDL (50 $\mu$g protein/ml) for 10 min at 37°C. Results are expressed as mean fluorescence intensity (MFI) and are means ± SD of experiments performed in triplicate in six separate occasions. *p < 0.001 versus ox-LDL.

bLOX-1 mAb, nebivolol, and its 4-keto derivative for 30 min at 37°C. As shown in Figure 3, the $O_2^{.-}$ concentration was markedly reduced in BAECs and BLOX-1-CHO cells preincubated with bLOX-1 mAb (p < 0.001), nebivolol (p < 0.001), and its 4-keto derivative (p < 0.001).

To exclude an interference of nebivolol on ox-LDL binding to LOX-1, binding tests with labeled ox-LDL were performed in BAECs and BLOX-1-CHO cells. The dose-response relationship in di-15-ASP-labeled ox-LDL binding to BAECs and BLOX-1-CHO cells showed saturation kinetics, and a 100-fold excess of unlabeled ox-LDL completely inhibited di-15-ASP-labeled ox-LDL binding to BAECs and BLOX-1-CHO cells (data not shown). Scatchard analysis revealed that di-15-ASP-labeled ox-LDL can bind to BAECs and BLOX-1-CHO cells with a constant dissociation ranging from 31 to 37 $\mu$g/ml. Oxidized LDL, bLOX-1 mAb, and polyinosinic acid significantly reduced the binding of di-15-ASP-labeled ox-LDL both in BAECs and BLOX-1-CHO cells (p from <0.001 to <0.01). Nebivolol, its 4-keto derivative, and native LDL had no effect.

The incubation of HUVECs and BAECs with ox-LDL for 10 min in presence of DAF-2 DA, dose-dependently reduced basal and bradykinin-induced intracellular NO concentration (p from <0.001 to <0.01, n = 12 for each concentration of ox-LDL). Nebivolol and its 4-keto derivative prevented the reduction of basal and stimulated intracellular NO induced by a fixed dose of ox-LDL (50 $\mu$g protein/ml) in HUVECs and BAECs (p from <0.001 to <0.01) while atenolol did not. Figure 4 shows the effect of nebivolol, its 4-keto derivative, and atenolol on the reduction of basal and stimulated intracellular NO concentration induced by ox-LDL in BAECs. Butoxamine, a $\beta_2$-

**Figure 2.** (A) effect of increasing amounts of nebivolol, its 4-keto derivative, and atenolol on intracellular concentration of superoxide ($O_2^{.-}$) induced by oxidized low-density lipoprotein (ox-LDL) in bovine aortic endothelial cells. The cells were stimulated with ox-LDL (50 $\mu$g protein/ml) for 10 min at 37°C. Results are expressed as mean fluorescence intensity (MFI) and are means ± SD of experiments performed in triplicate in six separate occasions. *p < 0.001 versus control. **Open bars** = nebivolol; **solid bars** = 4-keto derivative; **striped bars** = atenolol. (B) Effect of increasing amounts of nebivolol on the translocation of p47phox and Rac1 to the plasma membrane of human umbilical vein endothelial cells. The cells were preincubated with increasing amounts of nebivolol for 30 min at 37°C and then stimulated with ox-LDL (50 $\mu$g protein/ml) for 10 min at 37°C. The figure shows representative blots of six different experiments and the average data obtained by densitometric analysis. Results (means ± SD) are expressed as density in arbitrary units (AU). *p < 0.001 versus control. **Solid bars** = p47phox; **open bars** = Rac1.

to 4.6 ± 0.5 nmol/10$^6$ cells in HUVECs (p < 0.001) and from 9.6 ± 0.9 nmol/10$^6$ cells to 4.2 ± 0.5 nmol/10$^6$ cells in BAECs (p < 0.001). Atenolol had no effect. All the experiments regarding $H_2O_2$ and $O_2^{.-}$ in the supernatant were performed in triplicate on six different occasions.

To test the response specificity of nebivolol and its 4-keto derivative, a fixed amount of ox-LDL (50 $\mu$g protein/ml) was incubated with BAECs, CHO-K1, and BLOX-1-CHO cells for 10 min at 37°C after preincubation with
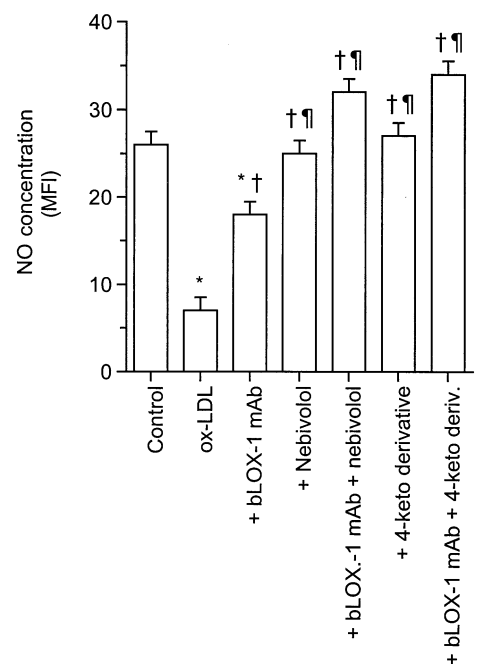
**Figure 4.** Effect of nebivolol, its 4-keto derivative, and atenolol on oxidized low-density lipoprotein (ox-LDL)-induced variations of nitric oxide (NO) in basal and bradykinin-stimulated bovine aortic endothelial cells. The cells were then stimulated with ox-LDL (50 μg protein/ml) for 10 min at 37°C. Results are expressed as mean fluorescence intensity (MFI) and are means ± SD of experiments performed in triplicate in six separate occasions. *p < 0.001 versus control.

adrenergic antagonist; phentolamine, an α-adrenergic receptor blocker; and NAN 190, a 5-HT receptor antagonist had no effect on the counteracting effect of nebivolol.

Similarly, levels of nitrite in the supernatant of bradykinin-stimulated endothelial cells dropped from 635 ± 25 pmol/well to 211 ± 12 pmol/well (p < 0.001, n = 12) in HUVECs and from 696 ± 45 pmol/well to 186 ± 18 pmol/well in BAECs (p < 0.001, n = 12) after the addition of 50 μg protein/ml of ox-LDL. The decrease was prevented by nebivolol (25 μmol/l) (respectively from 642 ± 32 pmol/well to 612 ± 38 pmol/well in HUVECs, and from 711 ± 54 pmol/well to 677 ± 44 pmol/well in BAECs, p = not significant, n = 12) but not by atenolol at the same concentration.

To test if the restoring action of nebivolol on the reduction of intracellular NO concentration induced by ox-LDL was due only to its effect on $O_2^{\cdot-}$, we preincubated BAECs with bLOX-1 mAb and measured bradykinin-stimulated intracellular NO after induction of oxidative stress with ox-LDL. Figure 5 shows that the preincubation of BAECs with bLOX-1 mAb significantly counteracted the effect of ox-LDL on bradykinin-stimulated generation of NO (p < 0.001). The counteracting effect of bLOX-1 mAb on intracellular NO was significantly lower than that induced by nebivolol and its 4-keto derivative alone (p < 0.01) or nebivolol or its 4-keto derivative plus bLOX-1 mAb (p < 0.001).

The results of this study also show that the eNOS activity of BAECs significantly increased by a 5-min contact with 25 μmol/l nebivolol; this effect was almost identical to that induced by 100 nmol/l bradykinin (Fig. 6). Atenolol and ox-LDL had no effect on eNOS activity.



**Figure 5.** Effect of bovine anti-LOX-1 monoclonal antibody (bLOX-1 mAb), nebivolol, and its 4-keto derivative alone or in combination with bLOX-1 mAb on oxidized low-density lipoprotein (ox-LDL)-induced variations of nitric oxide (NO) in bradykinin-stimulated bovine aortic endothelial cells (BAECs). Nebivolol (25 μmol/l), its 4-keto derivative (25 μmol/l), and bLOX-1 mAb (30 μg/ml) were preincubated with BAECs for 30 min at 37°C. The cells were then stimulated with ox-LDL (50 μg protein/ml) for 10 min at 37°C. Results are expressed as mean fluorescence intensity (MFI) and are means ± SD of experiments performed in triplicate on six separate occasions. *p < 0.001 versus control; †p < 0.001 versus ox-LDL; ¶p < 0.01 versus bLOX-1 mAb.

## DISCUSSION

The results of this study show that nebivolol and its 4-keto derivative reduce the concentration of ROS and, in particular, of $O_2^{\cdot-}$ induced by ox-LDL in endothelial cells. The increase in ROS and $O_2^{\cdot-}$ was previously shown to be specifically related to the binding of ox-LDL to LOX-1 and to be almost totally prevented by radical scavengers and by bLOX-1 mAb (15,21). The fact that in this study nebivolol and its 4-keto derivative work not only in endothelial cells, but also in BLOX-1-CHO cells suggests that the effect of the two compounds is specific for the ROS produced by the binding of ox-LDL to LOX-1. This, of course, does not mean that LOX-1 is the only receptor involved in nebivolol-dependent effect because any other receptor producing ROS or $O_2^{\cdot-}$ after stimulation (27,28) could be a potential target of nebivolol activity. Nebivolol has already been demonstrated to possess antioxidant activity (6,7). The effect of nebivolol and, above all, of its 4-keto derivative we found in this study may, therefore, be related to their radical scavenging activity. The possibility that nebivolol can affect the binding of ox-LDL to LOX-1 is excluded by the binding tests with labeled ox-LDL.

Furthermore, it has been previously hypothesized that endothelial NADPH oxidase may be the source of the free
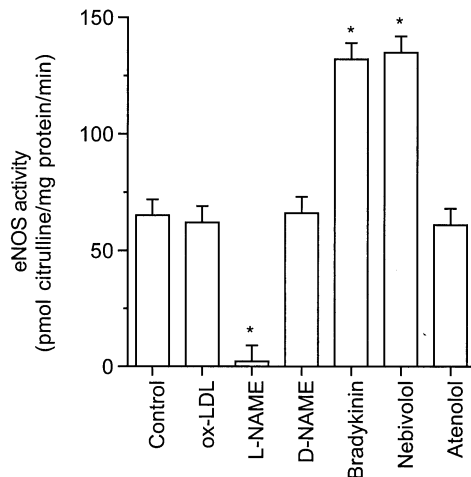
**Figure 6.** Effect of oxidized low-density lipoprotein (ox-LDL), nitro-L-, and D-arginine methyl ester hydrochloride (L- and D-NAME), bradykinin, nebivolol, and atenolol on endothelial nitric oxide synthase (eNOS) activity in bovine aortic endothelial cells (BAECs). Ox-LDL (50 μg protein/ml), L- and D-NAME (2 mmol/l), bradykinin (100 nmol/l), nebivolol (25 μmol/l), and atenolol (25 μmol/l) were preincubated with BAECs for 5 min at 37°C. Results are expressed as pmol citrulline/mg protein/min and are means ± SD of experiments performed in triplicate in six separate occasions. *p < 0.001 versus control.

radicals generated by activated LOX-1 (21,29). NADPH oxidase is a multicomponent enzyme consisting of at least two membrane components (gp91phox and p22phox) and three cytosolic proteins (p47phox, p67phox, and Rac) (30). All the classic NADPH subunits, including p47phox and Rac1 have already been shown to be expressed in human endothelial cells and in particular in HUVECs (31). In resting cells the oxidase is dormant and its components separately exist between the membrane and the cytosol (30). When cells are exposed to appropriate stimuli, NADPH oxidase is activated by association of these components on the plasma membrane (30). Actually, neutrophil and endothelial NADPH oxidase exhibit differences in their activity. In fact, a substantial proportion of NADPH oxidase in unstimulated endothelial cells, in contrast to the neutrophil enzyme, exists as a preassembled intracellular complex associated with the cytoskeleton (31). Nevertheless, a significant translocation of p47phox to the plasma membrane was demonstrated under stimulation with angiotensin II (32), consistent with the oxidase being present in the membrane fraction. Taken together, these findings support the conclusion of our study that the translocation of p47phox and Rac1 really occurred in our experimental conditions and that endothelial NADPH oxidase may be the source of the free radicals generated by activated LOX-1. The fact that the translocation of p47phox and Rac1 was greatly reduced, respectively, by bisindolylmaleimeide and SB203580 may indicate that p38 MAPK is more responsible for the translocation of Rac and PKC for the translocation of p47phox. In this context, it is of interest that these two kinases have already been shown to be associated with LOX-1 activation (33,34). Because in this study the translocation of Rac1 and in particular of p47phox was reduced by nebivolol, a conclusion can be drawn that this compound and its 4-keto derivative may reduce ROS production also by affecting the signaling pathways leading to NADPH oxidase activation.

Previous findings by our group have shown that the binding of ox-LDL (21) and platelets (29) to LOX-1 reduces the intracellular and extracellular concentration of NO in endothelial cells through an increased cellular production of $O_2^{\cdot-}$. In the present study, we demonstrated that nebivolol and its 4-keto derivative prevented the reduction of basal and bradykinin-stimulated NO induced by ox-LDL in BAECs. Because the reduction of NO was counteracted by bLOX-1 mAb, another conclusion of this study is that the effect of nebivolol can be at least partially explained by its effect on $O_2^{\cdot-}$, which can inactivate NO in a chemical reaction during which peroxynitrite is formed (13). From our results it is, however, clear that the effect of nebivolol and its 4-keto derivative on NO cannot be explained only by their effect on $O_2^{\cdot-}$ because the counteracting effect of bLOX-1 mAb was significantly lower than that caused by nebivolol and its 4-keto derivative alone or in combination with the LOX-1 blocking antibody. Taken together, these results suggest that nebivolol and its 4-keto-derivative may increase NO in endothelial cells by working in at least two different ways: they reduce $O_2^{\cdot-}$ concentration and consequently peroxynitrite formation and increase NO production. The conclusion on the effect of nebivolol on NO production is supported by the results we obtained by measuring eNOS activity in endothelial cells. The activity of the enzyme was more than doubled by a 5-min contact with nebivolol; this effect was identical with that induced by bradykinin. It is noteworthy that atenolol and ox-LDL were devoid of any effect on eNOS activity. Our results on NO production are in agreement with a series of previous studies showing that the mechanism of vasodilator action of nebivolol is attributed to activation of eNOS in vascular endothelial cells (9,11,35).

The results of this study and, in particular, the inhibitory effect of nebivolol on $O_2^{\cdot-}$ concentration may have implications in vivo. In fact, oxidative inactivation of NO has been regarded as an important cause of endothelial dysfunction in essential hypertension (14). Furthermore, it has been recognized for a long time that atherosclerotic blood vessels are very susceptible to the development of vasospasm in vivo (36), and the current weight of evidence suggests that impaired endothelium-dependent vasodilation plays a central role in the mechanisms leading to ischemic manifestations (37). In this context, it has been demonstrated that peroxynitrite, generated by a rapid combination of NO and $O_2^{\cdot-}$ (13), inactivates prostacyclin (PGI$_2$) synthase via chemical nitration, leaving PGH$_2$ unmetabolized, which then causes vasospasm and platelet aggregation (38). By reducing $O_2^{\cdot-}$, nebivolol may reduce peroxynitrite and, therefore, reduce vasospasm and platelet aggregation. Finally, ROS have been shown to induce activation of nuclear

factor-kB (15), a transcription factor involved in the first steps of atherosclerotic plaque formation (39) and to play a pivotal role in regulating apoptosis (40). Nebivolol, therefore, besides reducing blood pressure, may help prevent atherosclerotic initiation and complications.

---

**Reprint requests and correspondence:** Dr. Luciano Cominacini, Dipartimento di Scienze Biomediche e Chirurgiche (Medicina D), Università di Verona, Ospedale Policlinico, 37134 Verona, Italy. E-mail: comina@medicinad.univr.it.

## REFERENCES

1. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980;288:373–6.
2. Ignarro LJ, Byrns RE, Buga GM, Wood KS. Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties of nitric oxide radical. Circ Res 1987;61:866–79.
3. Taddei S, Virdis A, Ghiadoni L, Sudano I, Salvetti A. Endothelial dysfunction in hypertension. Fact or fancy? J Cardiovasc Pharmacol 1998;32 Suppl 3:S41–7.
4. Brush JE Jr, Faxon DP, Salmon S, Jacobs AK, Ryan TJ. Abnormal endothelium dependent coronary vasomotion in hypertensive patients. J Am Coll Cardiol 1992;19:809–15.
5. Drexler H, Zeiher AM, Meisner K, Just H. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolemic patients by L-arginine. Lancet 1991;338:1546–50.
6. Van de Water A, Janssens W, Van Nauten J, Xhommeux R, de Cree J. Pharmacological and haemodynamic profile of nebivolol, a chemically novel, potent and selective β-1 adrenergic antagonist. J Cardiovasc Pharmacol 1988;11:552–63.
7. Troost R, Schwedhelm E, Rojczyk S, Tsikas D, Frohlich JC. Nebivolol decreases systemic oxidative stress in healthy volunteers. Br J Clin Pharmacol 2000;50:377–9.
8. Gao YS, Nagao T, Bond RA. Nebivolol induces endothelium dependent relaxations of canine coronary arteries. J Cardiovasc Pharmacol 1991;17:964–9.
9. Cockroft JR, Chowienczyk PJ, Brett SE, et al. Nebivolol vasodilates human forearm vasculature: evidence for an L-arginine/NO-dependent mechanism. J Pharmacol Exp Ther 1995;274:1067–71.
10. Tzemos N, Lim PO, MacDonald TM. Nebivolol reverses endothelial dysfunction in essential hypertension. Circulation 2001;104:511–5.
11. Broeders M, Doevendans P, Bekkers B, et al. Nebivolol: a third-generation β-blocker that augments vascular nitric oxide release. Circulation 2000;102:877–84.
12. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. J Clin Invest 1993;91:2546–51.
13. Ignarro RJ. Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu Rev Pharmacol Toxicol 1990;30:535–60.
14. Taddei S, Virdis A, Ghiadoni L, Magagna A, Salvetti A. Vitamin C improves endothelium dependent vasodilation by restoring nitric oxide activity in essential hypertension. Circulation 1998;97:2222–9.
15. Cominacini L, Fratta Pasini A, Garbin U, et al. Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kB through an increase production of intracellular reactive oxygen species. J Biol Chem 2000;275:12633–8.
16. Cominacini L, Garbin U, Fratta Pasini A, et al. Oxidized low-density lipoprotein increases the production of intracellular reactive oxygen species in endothelial cells: inhibitory effect of lacidipine. J Hypertens 1998;16:1913–9.
17. Sawamura T, Kume N, Aoyama T, et al. An endothelial receptor for oxidized low-density lipoprotein. Nature 1997;386:73–7.
18. Landegren U. Measurement of cell numbers by means of the endogenous enzyme hexosaminidase: application to detection of lymphokines and cell surface antigens. J Immunol Methods 1984;67:379–85.
19. Cheymol G, Poirier JM, Carrupt PA, et al. Pharmacokinetics of beta-adrenoceptor blockers in obese and normal volunteers. Br J Clin Pharmacol 1997;43:563–70.
20. Chiu FC, Zhang JN, Li RC, Raymond K. Efficient assay for the determination of atenolol in human plasma and urine by high-performance liquid chromatography with fluorescence detection. J Chromatogr Biomed Sci Appl 1997;691:473–7.
21. Cominacini L, Rigoni A, Fratta Pasini A, et al. The binding of oxidized low density lipoprotein (ox-LDL) to ox-LDL receptor-1 reduces the intracellular concentration of nitric oxide in endothelial cells through an increased production of superoxide. J Biol Chem 2001;276:13750–5.
22. Smith P, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;105:293–9.
23. Yamamori T, Inanami O, Sunimoto H, Akasaki T, Nagahata H, Kuwabara M. Relationship between p38 mitogen-activated protein kinase and small GTPase Rac for the activation of NADPH oxidase in bovine neutrophils. Biochem Biophys Res Commun 2002;293:1571–8.
24. Zhou M, Diwu Z, Panchuk-Voloshina N, Haugland RP. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. Anal Biochem 1997;253:162–8.
25. McCord JM, Fridovich I. Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969;244:6049–55.
26. Chen L, Salafranca MN, Mehta JL. Cyclooxygenase inhibition decreases nitric oxide synthase activity in human platelets. Am J Physiol 1997;42:1854–9.
27. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griendling KK. Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. Circ Res 2002;91:406–13.
28. Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM, Wautier JL. Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. Am J Physiol Endocrinol Metab 2001;280:685–94.
29. Cominacini L, Fratta Pasini A, Garbin U, et al. The platelet-endothelium interaction mediated by LOX-1 reduces the intracellular concentration of nitric oxide in endothelial cells. J Am Coll Cardiol 2003;41:499–507.
30. Babior BM. NADPH oxidase: an update. Blood 1999;93:1464–76.
31. Li JM, Shah AM. Intracellular localization and preassembly of the NADPH oxidase complex in cultured endothelial cells. J Biol Chem 2002;277:19952–60.
32. Li JM, Shah AM. Mechanism of endothelial cell NADPH oxidase activation by angiotensin II. J Biol Chem 2003;278:12094–100.
33. Li D, William V, Liu L, et al. LOX-1 inhibition in myocardial ischemia-reperfusion injury: modulation of MMP-1 and inflammation. Am J Physiol Heart Circ Physiol 2002;283:1795–801.
34. Li D, Liu L, Chen H, et al. LOX-1 mediates oxidized low-density lipoprotein-induced expression of matrix metalloproteinases in human coronary artery endothelial cells. Circulation 2003;107:612–7.
35. Parenti A, Filippi S, Amerini S, et al. Inositol phosphate metabolism and nitric-oxide synthase activity in endothelial cells are involved in the vasorelaxant activity of nebivolol. J Pharmacol Exp Ther 2000;292:698–703.
36. Shimokawa H, Tomoike H, Nabeyama S, et al. Coronary artery spasm induced in atherosclerotic miniature swine. Science 1983;221:560–2.
37. Shimokawa H, Flavahan NA, Shepherd JT, Vanhoutte PM. Endothelium-dependent inhibition of ergonovine-induced contraction is impaired in porcine coronary arteries with regenerated endothelium. Circulation 1989;80:643–50.
38. Zou MH, Bachschmid M. Hypoxia-reoxygenation triggers coronary vasospasm in isolated bovine coronary arteries via tyrosine nitration of prostacyclin synthase. J Exp Med 1999;190:135–40.
39. Collins T, Cybulsky MI. NF-kappaB: pivotal mediator or innocent bystander in atherosclerosis? J Clin Invest 2001;107:255–64.
40. Curtin JF, Donovan M, Cotter TG. Regulation and measurement of oxidative stress in apoptosis. J Immunol Methods 2002;265:49–72.