Nebivolol Induces Nitric Oxide Release in the Heart Through Inducible Nitric Oxide Synthase Activation

Angelo Maffei, Alba Di Pardo, Rosa Carangi, Pierluigi Carullo, Roberta Poulet, Maria T. Gentile, Carmine Vecchione, Giuseppe Lembo

Abstract—Nebivolol is a β1-adrenergic receptor antagonist that also reduces blood pressure by evoking endothelial NO production and vasodilation. We aimed at assessing whether nebivolol induces NO production also in the heart and delineating the molecular mechanisms involved. Using the fluorescent probe diaminofluorescein, we found that nebivolol induces a dose-dependent NO production in the heart, statistically significant already at $10^{-7}$ mol/L. It is not an effect because of the blockade of β1-adrenergic receptor, because this effect is not shared by another drug of the same class, atenolol. Because nebivolol has been reported to act as an agonist on other β-adrenergic receptors, we tested NO production in the presence of receptor antagonists. Nebivolol was not able to induce NO production in presence of the β3-antagonist SR59230A, indicating a fundamental role for β3-adrenergic receptors in cardiac NO production by nebivolol. Moreover, inducible NO synthase inhibition abolishes NO release in the heart, indicating that nebivolol induces NO production by acting on the inducible isofrom of the enzyme. The action of nebivolol on inducible NO synthase was confirmed by real-time PCR experiments, showing cardiac overexpression of inducible NO synthase but not neuronal NO synthase or endothelial NO synthase, after 5 hours of treatment with nebivolol. In conclusion, our study demonstrates that nebivolol also stimulates NO production in the heart. This action of nebivolol is exerted via a signaling pathway starting from the activation of β3-adrenergic receptors and leading to overexpression of inducible NO synthase. Cardiac NO production by nebivolol could participate in the cardiovascular effects of nebivolol treatment in patients affected by hypertension and heart failure. (Hypertension. 2007;50:652-656.)

Key Words: adrenergic agents ■ adrenergic beta receptors ■ heart ■ NO ■ NO synthase

β-Blockers are among the antihypertensive drugs most indicated against heart failure.1 β-Blockers act principally to inhibit the adverse effects of the sympathetic nervous system in patients with heart failure.2-5 In fact, whereas cardiac adrenergic drive initially supports the performance of the failing heart, long-term activation of the sympathetic nervous system exerts deleterious effects that can be antagonized by the use of β-blockers.

In addition to the inhibitory effects on β1-adrenergic receptors, the novel β-blocker nebivolol has additional hemodynamic properties.4 In particular, nebivolol is able to stimulate endogenous production of NO by inducing phosphorylation of the endothelial enzyme endothelial NO synthase (NOS; eNOS).5 The particular properties of nebivolol also determine favorable effects on cardiac function in patients with heart failure when compared with classical β-blockers,6 but the mechanisms underlying these beneficial differences have not been well characterized. In this regard, whereas nebivolol action on NO can result in favorable outcomes at vascular levels, the effects of NO on the heart are not so straightforwardly beneficial.

In the heart, NO is produced not only by the endothelium but also by the cardiomyocyte.7 The modulatory effects of NO on cardiac function are complex, because of both the expression of all 3 NOS isoforms in the heart and the multiplicity of NO intracellular targets.8 More important, NO production in cardiac pathologies is profoundly altered. Actually, in heart failure there is an increased release of NO, particularly from the inducible isofrom inducible NO synthase (iNOS),9 which seems to be a counterregulatory mechanism trying to protect the heart from functional deterioration.10 Also, neuronal NO synthase (nNOS)–derived NO production is increased in human failing hearts,11 whereas vascular eNOS activity is depressed.7 However, the precise role of all of the NOS isoforms in this and other cardiac pathologies is still debated. In this study we aimed to investigate whether nebivolol is able to influence NO release also in the heart, as already observed in vessels, and to characterize the mechanisms activated and the NOS isoforms involved in this nebivolol action.

Methods

Studies were conducted on male C57BL/6N mice (Charles River Laboratories, Calco, Italy; n=60), aged 10 to 12 weeks. Mice were...
housed 4 per cage, kept in a temperature controlled room (23°C to 25°C) with a 12-hour light/dark cycle, and fed standard rodent chow and water ad libitum. The experiments were performed after the mice were acclimatized to our housing conditions for ≥1 week. On the day of experiments, mice were sedated by CO2 and the heart was excised. All of the experimental procedures were in accordance with national guidelines for research in animals and were approved by the review committee of our institution.

Evaluation of NO Production

Isolated heart was mounted on a Langendorff apparatus and perfused at a constant hydrostatic pressure of 75 mm Hg with an oxygenated Krebs buffer containing diaminofluorescein diacetate (DAF-2DA; 10 μmol/L; Alexis), a fluorescent probe for detection of NO production in tissues, for 5 hours. After the first 30 minutes of incubation, the heart was stimulated with nebivolol (10⁻⁴ to 10⁻² mol/L) or atenolol (10⁻⁴ to 10⁻³ mol/L). Some experiments were performed in the presence of N⁶-nitro-L-arginine methyl ester (L-NAME; 300 μmol/L), a pan-NOS inhibitor; of L-N⁶-(1-iminoethyl)-lysine (L-NIL; 200 μmol/L), an iNOS inhibitor; of 7-nitroindazole (200 μmol/L), an nNOS inhibitor; of IC1118 551 (100 nmol/L), a β₂-adrenergic receptor antagonist; or in the presence of SR59230A (100 nmol/L), a β₁-adrenergic receptor antagonist. Finally, some experiments were performed in eNOS knockout mice (B6.129P2-Nos3tm1Unc/J) and in their relative control strain (C57BL/6J; n = 4 per strain; Jackson Laboratories).

Hearts were removed, fixed in formalin, and then dehydrated, diaphanized, and included in paraffin. They were cut into 5-μm-thick sections in a microtome. Specimens were observed under an Axioskop2 fluorescence microscope (Zeiss) with a ×200 magnification. Twenty-four-bit color pictures were taken equipped with a fluorescein isothiocyanate filter (excitation: 450 to 490 nm; emission: 515 to 560 nm). Twenty-four-bit color pictures were taken using a digital camera system coupled to imaging software (Spot, Diagnostic Instruments) under constant exposure time, gain, and offset. To account for fluorescence decay, all of the images were taken in the first 30 seconds of light exposure. Fluorescence intensity was quantified using the imaging software and expressed in fluorescence arbitrary units. This procedure was validated in a first experimental series, during which different times and DAF concentrations were used to evaluate NO production after increasing stimulations with acetylcholine in the presence or absence of L-NAME to reach the best sensitivity and accuracy.

Evaluation of NOS Expression

Heart total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA (100 ng) from each sample was transcribed into cDNA using the RT-PCR Expand Reverse Transcriptase kit (Roche) according to the supplier’s instructions. Two microtubes (10% of reverse transcription reaction) of each cDNA preparation were subsequently used as template for 25 μL of PCR containing 1 μmol/L of eNOS, nNOS, and iNOS primers, as described previously, and 7.875 μL of SYBR green PCR master mix (Applied Biosystems).

Real-time PCR was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems) under the following conditions: 50°C for 2 minutes; 95°C for 10 minutes; 40 cycles 95°C for 40 seconds; and 63°C for 1 minute. Before real-time PCR analysis, each primer pair was confirmed to yield a single band at the appropriate size using conventional PCR at the above-listed cycling conditions. Relative eNOS, iNOS, and nNOS gene expression levels were determined using the Relative Quantification (ddCt) Study of 7500 System SDS Software (Applied Biosystems).

Statistical Analysis

Results are presented as mean±SE. Differences between groups were compared using 1-way or 2-way ANOVA followed by the Bonferroni post-hoc test, as appropriate. A value of P<0.05 was considered significant.

Nebivolol Induces NO Production in the Heart

We have evaluated NO production in the heart after exposure to nebivolol. Nebivolol induces a clear increase in DAF-2DA fluorescence, an index of NO production, in the mouse heart (Figure 1). The amount of NO depends on the dose of nebivolol used. In particular, nebivolol induces a statistically significant NO production already at a concentration of 10⁻⁷ mol/L, and this production increases progressively with increasing drug dosages. ED₅₀, ie, the dose at which nebivolol exerts half its effect on NO production, has been calculated as 0.56 μmol/L.

To evaluate whether the increase in NO production exerted by nebivolol should be attributed to its action as a β₂-adrenergic receptor antagonist, we investigated the effect of another drug of the same class, atenolol. Atenolol was not able to induce an increase in cardiac NO production (data not shown).

Nebivolol Induces iNOS Overexpression in the Heart

to determine which NOS isoform is activated by the stimulation with nebivolol, we tested NO production in the heart after exposure to nebivolol in the presence of different strategies that inactivated single NOS isoforms. As expected, the pan-NOS inhibitor L-NAME totally abolished NO production induced by nebivolol. The increase in NO production exerted by nebivolol was completely abolished also by the preincubation with the iNOS pharmacological inhibitor L-NIL (Figure 2). In contrast, the nNOS inhibitor 7-nitroindazole had no effects on nebivolol-induced NO production. In the absence of selective pharmacological inhibitors of eNOS, the contribution of this isoform to the
NO production induced by nebivolol in the heart was evaluated in genetically modified mice. There was no difference in nebivolol-induced NO production between eNOS knockout mice and relative controls (argon fluorescence: 28.7 ± 4.7 versus 31.6 ± 3.9 fluorescence arbitrary units; \( P \) value was not significant).

Because the inducible isoform of NOS is regulated through its expression, we evaluated whether nebivolol determined changes in the expression of the NOS isoforms. Nebivolol induced a significant increase in iNOS mRNA expression while not modifying the expression of the other NOS isoforms (Figure 3), thus indicating an effect of the drug on the nuclear promotion of iNOS expression.

**Nebivolol Induces NO Production in the Heart by Activating \( \beta_1 \)-Adrenergic Receptors**

Finally, we investigated the receptor activated by nebivolol to induce NO production by the use of pharmacological antagonists of the adrenergic receptors that have shown activation by nebivolol in previous studies. Through this strategy, we observed that nebivolol-induced NO production was completely abolished in the presence of the specific \( \beta_1 \)-adrenergic receptor antagonist SR59230. In contrast, the \( \beta_2 \)-adrenergic receptor antagonist ICI118 551 had no effect on nebivolol-induced NO release (Figure 4).

**Discussion**

In this study, we have demonstrated for the first time that nebivolol induces NO production in the heart. Nebivolol is known to have hemodynamic properties that go beyond its action as an antagonist of \( \beta \)-adrenergic receptors. These properties consistently differentiate nebivolol from other \( \beta \)-blockers, leading to a slightly better therapeutic profile. Focusing on cardiac physiology, nebivolol treatment in hypertensive patients is associated with preservation of cardiac output and reduced peripheral resistance, in contrast to what was observed with atenolol. In accordance, nebivolol reduces mortality and cardiovascular hospital admissions in patients with heart failure. The improved cardiac hemodynamic profile of nebivolol may be because of the induction of NO. Actually, NO improves coronary blood flow, reduces peripheral resistance, improves cardiac output, attenuates cardiac hypertrophy, and mediates the protective effects of ischemic preconditioning. Thus, induction of cardiac NO by nebivolol but not by other \( \beta \)-adrenergic receptor antagonists, such as atenolol, may account for the improved cardiac profile of the drug. In fact, the dose of nebivolol needed to induce a significant increase in NO release is equivalent to the plasma concentration, which is usually achieved in nebivolol-treated patients. This strongly suggested that greater cardiac NO availability is normally achieved during pharmacological intervention and participates in the pharmacological profile of nebivolol. However, the physiological significance of NO production in the heart is still debated. Further investigations are needed to understand the real contribution of nebivolol-induced NO production to heart physiology. On this issue, another \( \beta \)-receptor antagonist with NO-inducing properties, celiprolol, has been shown to prevent the transition toward heart failure in mice. This effect can be attributable to NO production, because L-NAME was able to blunt celiprolol beneficial action. Because nebivolol has been demonstrated to be an effective treatment for heart failure in a powered randomized, controlled study, it can be hypoth-
rescence arbitrary units) in the heart after exposure to 10−5 mol/L of nebulol in presence of ICI118 551, a β3-adrenergic receptor antagonist, or SR59230A, a β3-adrenergic receptor antagonist. B, Quantification of NO production (DAF-2DA–induced fluorescence, expressed in fluorescence arbitrary units) in the heart after exposure to 10−5 mol/L of nebulol alone or in the presence of the different β-adrenergic inhibitors (P<0.05 vs control).

esized that a similar mechanism may contribute to nebulolol action.

Some further clues could arise from the investigation of the molecular mechanisms through which nebulolol induces NO production in the heart. Two main targets should be identified: upstream, the receptor to which nebulolol should link to exert its effect, and downstream, the NOS isoform that is activated by the intracellular signaling pathway initiated by the interaction between nebulol and this receptor. We have explored these issues through both an analytical procedure, such as real-time PCR, and the use of pharmacological inhibitors. To explore the role of eNOS, for which a selective pharmacological inhibitor is lacking, genetically engineered mice were used. Although genetically engineered mice are usually considered more selective than inhibitors, the pharmacological inhibitors that we used have been largely recognized as selective and effective tools to study NOS isoforms and β-adrenergic receptors. Thus, we are confident that the signaling we identified (β-adrenergic/iNOS) is the intracellular pathway through which nebulolol induces cardiac NO synthesis.

With regard to the first question, nebulolol action on cardiac NO production does not depend on its main pharmacological effect, ie, the inhibition of β1-adrenergic receptor, because cardiac NO is not induced by other drugs of the same functional class. Instead, our results show that nebulolol is not able to evoke NO production in the heart when β3-adrenergic receptor is blocked by a selective pharmacological inhibitor, indicating that nebulolol exerts its action via stimulation of this adrenergic receptor subclass. This result is in agreement with previous observations showing that nebulolol is able to induce vascular NO production by activating β3-adrenergic receptors.23 In ventricular myocytes, β3-adrenergic–induced NO release has been involved in the modulation of cardiac sympathetic drive, counteracting the deleterious effects exerted by excessive catecholaminergic β3-adrenoceptor stimulation.26 According to this hypothesis, nebulolol could offer a double protection in failing hearts by both antagonizing β3-adrenoceptors and stimulating β3-adrenergic–mediated NO release.

With regard to the second question, our study suggests that nebulolol stimulates the activity of the inducible form of NOS. iNOS expression is increased in human heart failure.27 Such overexpression was once considered deleterious, but recent evidence have indicated instead that it can be a compensatory mechanism by which the heart tries to reduce injury. In fact, iNOS-produced NO limits β3-adrenergic responsiveness in myocytes from both normal and failing human hearts.28,29 This observation strengthen our hypothesis that the release of NO stimulated by nebulolol can counteract the β3-adrenergic effects in heart failure. Moreover, iNOS has been shown to mediate cardioprotection induced by other factors, such as endotoxin derivatives,30 adenosine agonists,31 or late ischemic preconditioning.32 More important, targeted gene therapy with iNOS has been demonstrated to protect against myocardial infarction, both short-term and long-term, without adverse functional consequences.33 It seems plausible that nebulolol, by inducing β3-adrenergic/iNOS-mediated NO production in the heart, may exert a similar protection, thus emerging as a pharmacological tool for the prevention of cardiac disease. However, this hypothesis still needs to be tested in future research evaluating the effects of nebulolol against experimental heart failure and the role of NO in these effects.

**Perspectives**

Our study has indicated a novel molecular effect of nebulolol on the heart, showing that it induces NO production via a β3-adrenergic/iNOS intracellular pathway. Our observations add new insights into the effects of nebulolol treatment. In fact, it is known that nebulolol induces NO production in vessels and that this mechanism contributes to the favorable hemodynamic effects observed in patients taking the drug. The finding that nebulolol can induce NO production also in the heart suggests further therapeutic advantages in cardiac patients. Studies in animal models of disease and in patients should be planned and realized in the near future to investigate the actual clinical relevance of this novel mechanism of action for nebulolol.
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None.

REFERENCES
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