Nebivolol-induced vasodilation of renal afferent arterioles involves β3-adrenergic receptor and nitric oxide synthase activation

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Nebivolol is a novel antihypertensive drug (23, 49) that exhibits a higher degree of selectivity for β1-adrenergic receptors compared with other β-blockers used in clinical practice (5, 34). Nebivolol reduces heart rate, blood pressure, and peripheral and coronary vascular resistance and lowers left ventricular afterload and preload and elicits vasodilation (5, 27, 39). In kidney studies (22), nebivolol at a dose of 1 mg/kg increased renal plasma flow, glomerular filtration rate (GFR), urine flow, and urinary NaCl excretion rate. The effects of nebivolol on GFR were prevented by Nω-monomethyl-L-arginine, a nonselective inhibitor of nitric oxide synthase (NOS). Nebivolol also increased renal nitric oxide (NO) excretion by 71%, which was also prevented by Nω-monomethyl-L-arginine (22). Plasma rennin activity was decreased by nebivolol (13, 22). In the isolated rat kidney preparation, nebivolol caused a dose-dependent reduction in renal perfusion pressure indicating a reduced renal vascular resistance and an increase in NO release that was blocked by N-nitro-l-arginine methyl ester (L-NAME), a NOS inhibitor, and metergoline, a 5-hydroxytryptamine antagonist (25). Studies (16, 17) using myograph and an NO sensor showed that nebivolol dilated mouse renal arteries and increased NO release. Nebivolol also dilated rat renal artery rings that were preconstricted with KCl and phenylephrine; this effect was inhibited by L-NAME (47). With the use of [3H]inulin space determination to estimate contraction and relaxation of renal glomerular vasculature, nebivolol induced relaxation of the renal glomerular microvasculature through ATP efflux with consequent stimulation of P2Y-purinoceptor-mediated NO release from endothelial cells (26). Nebivolol has also shown to increase endothelial nitric oxide synthase (eNOS) expression and activity in various tissues (9, 30, 45, 48). Although it is recognized that NO is involved, the renal vasodilator mechanism of nebivolol has not been delineated. In other tissues, endothelial β3-adrenoreceptors (7, 8, 20, 27, 33, 34, 36, 42, 43), endothelial β2-adrenoreceptors (2, 17, 35), P2Y receptors (26), and Ca2+–activated K+ channels (17) have been implicated in mediating its vasodilating properties. However, the effects of nebivolol on afferent and efferent arteriolar vascular tone have not been established. The present study was performed to determine the magnitude of the juxtamedullary afferent and efferent arteriolar responses to nebivolol and the roles of NOS and sGC in mediating nebivolol-induced vasodilatory responses in afferent arterioles. The detailed studies were focused on afferent arterioles as the model system to evaluate the hypothesis that nebivolol induces vasodilation of renal afferent arterioles of juxtamedullary nephron via an NOS/NOS/sGC/cGMP pathway. We also tested the hypothesis that the afferent arteriolar vasodilation effect may be mediated through the release of NO by activation of NOS via a β3-adrenoreceptor dependent mechanism. While β3-adrenoreceptor mRNA has been shown in the heart and peripheral vasculature (8, 36, 42) and the kidney (31, 50), there is no report on the localization of β3-adrenoreceptors in the renal microvasculature. Accordingly, immunohistochemical studies were performed to localize β3-adrenoreceptors in the kidney.

MATERIALS AND METHODS

The experimental protocols and procedures were approved by the Tulane University Institutional Animal Care and Use Committee.
Videomicroscopic measurements of afferent and efferent arteriolar diameters were performed on the isolated blood-perfused juxtamedullary nephron preparation as previously described (10, 11). Briefly, each experiment used one male Sprague-Dawley rat (Charles River Laboratories, Wilmington, MA), weighing 370–410 g, serving as blood donor and kidney donor. Blood was collected via the carotid arterial cannula and centrifuged and filtered to separate the plasma and cellular fractions. This reconstituted blood was pressurized with a 95% O2-5% CO2 gas mixture. The right kidney was perfused through a cannula inserted in the superior mesenteric artery and advanced to the right renal artery. The perfusate was a Tyrode solution (pH 7.4) containing 5.1% BSA and a mixture of L-amino acids that was pressurized with a 95% O2-5% CO2 gas mixture. The kidney was excised and sectioned longitudinally, retaining the papilla intact with the perfused dorsal two-thirds of the organ. Overlying tissue was removed to expose glomeruli and related vasculature of the juxtamedullary nephrons. The branches of renal artery were ligated to increase the perfusion pressure. After the dissection was completed, the Tyrode perfusate was replaced with the reconstituted blood. Renal perfusion pressure was set at 100 mmHg. The inner cortical surface of the kidney was continuously superfused with a warmed (37°C) Tyrode solution containing 1% BSA. The tissue was transilluminated and video images of the microvessels were evaluated with a microscope (Nikon) equipped with a water-immersion objective (×40) and transferred by a Newvicon camera (model NC-67M; Dage-MTI, Michigan City, IN) through an image enhancer (model MFJ-1452; MFJ Enterprises, Starkville, MS) to a video monitor and a computer. Arteriolar inside diameters were measured at 30-s intervals using a calibrated digital image-shearing monitor (Instrumentation for Physiology and Medicine, San Diego, CA). Treatments were administered by superfusing the tissue with a Tyrode solution containing the agent to be tested or the vehicle solution.

Chemicals. The following agents used in this study: nebivolol HCl was provided by the Forest Research Institute; N,N-diethylphenoxy)-1-[(1S)-1,2,3,4-tetrahydronaphth-1-yl]amino]-(2S)-2-propanol oxide salt (SR 59230A), butoxamine, and (±)-metoprolol (+)-tartrate were purchased from Sigma (St. Louis, MO).

Experimental protocols. For each experiment, a single afferent or efferent arteriole from one rat kidney that showed adequate blood flow was selected for study. The arteriole was visualized and superfused with solutions containing the agents to be tested. Nebivolol was added to the superfusate at concentration varying from 1 nM to 100 μM. In clinical studies, plasma levels of nebivolol in patients treated with a single dose of 10 mg were ~100 nM. While our effective concentrations were slightly higher, higher doses are sometimes needed for in vitro studies. When the superfusate is bathed over the tissues, it mixes with the interstitial fluid and also has to diffuse to its site of action so that the effective concentration at that point may be lower. Nebivolol was superfused alone or after treatment with L-NNA, a NOS inhibitor, after treatment with ODQ, a selective inhibitor of NO-sensitive guanylyl cyclase, after treatment with metoprolol, a selective β1-adrenergic blocker, after treatment with butoxamine, a selective β2-adrenergic blocker, and after treatment with SR 59230A, a selective β3-adrenergic blocker. After a 15- to 20-min equilibration period, an experimental protocol was initiated consisting of consecutive 5- to 15-min treatment period for one dose based on the stabilization of vessel tone. Steady-state diameter determinations were calculated during the final 5 min of each treatment period at a perfusion pressure of 100 mmHg. The first experimental protocol was performed to determine the dose-response relationships for nebivolol. Afferent or efferent arteriolar inside diameters were measured during sequential exposure of the kidney to the following superfusate solutions: (i) control vehicle, (ii) 100 μmol/l L-NNA; (ii) control vehicle, (iii) 100 μmol/l L-NNA; and (iv) control vehicle, (v) 100 μmol/l L-NNA followed by nebivolol at 1, 10, and 100 μmol/l (the effective doses based on dose response experiments); (i) control vehicle, (ii) 1,000 μmol/l L-NNA; and (v) control vehicle, (ii) 1,000 μmol/l L-NNA followed by nebivolol was repeated at 1, 10, and 100 μmol/l. A third series of experiments was performed to determine the effects of soluble guanylate cyclase inhibition by ODQ on the nebivolol mediated vasodilatation of afferent arterioles. Afferent arteriolar inside diameters were measured during sequential exposure of the kidney to the following superfusate solutions: (i) control vehicle; (ii) metoprolol at concentrations of 1.25, 2.5, 5, 10, 20, 40, and 80 μmol/l; (iii) control vehicle, (iv) 20 or 50 μmol/l metoprolol; and (v) control vehicle, (vi) addition of 20 or 50 μmol/l metoprolol followed by nebivolol at 1, 10, and 100 μmol/l. A fifth series of experiments was performed to determine the effects of β3-adrenergic receptor inhibition with SR 59230A and β3-adrenergic receptor inhibition with butoxamine on the nebivolol mediated vasodilatation of afferent arterioles. Afferent arteriolar inside diameters were measured during sequential exposure of the kidney to the following superfusate solutions: (i) control vehicle, (ii) 100 μmol/l nebivolol; (ii) control vehicle, (ii) 100 μmol/l nebivolol plus 2.5 or 5μM SR 59230A; (iii) control vehicle, (iv) 100 μmol/l nebivolol; and (v) control vehicle, (v) 100 μmol/l nebivolol plus 10 or 100 μM butoxamine.

Immunohistochemical studies were performed as previously described (19). Briefly, rat kidneys were perfused and fixed with 4% paraformaldehyde and zinc-saturated formalin. Then, paraffin-embedded kidney sections (3 μm) were processed by immunoperoxidase technique by sequential incubations with a monoclonal rabbit anti-β3-adrenergic receptor primary antibody (cat. no. orb15066; Biobyt, Cambridge, UK) at a 1:200 dilution for 2 h, anti-rabbit secondary antibody, and detection with DAB on methyl green used as counterstain (cat. no. H-3402; Vector Laboratories, Burlingame, CA). For dual color immunostaining, the kidney sections were sequentially incubated at room temperature for additional 30 min with blocking serum followed by 60-min incubation with a mouse smooth muscle actin (SMA) antibody (code no. M0851; DakoCytonation, Carpinteria, CA) using a rabbit/mouse EnVision G2 doublestain system kit (code no. K5361; DakoCytonation). In this case, the detection for β3-adrenergic receptor was performed using DAB-nickel chloride (cat. no. SK-4100; Vector Laboratories), which provides a grey-black chromogen, and SMA was detected with Permanent Red (DakoCytonation), which provides a red chromogen. The specificity of immunostaining was determined by the omission of the primary antibody and substitution with normal horse serum.

Statistical analysis. All data are reported as means ± SE. Data were analyzed by two-way ANOVA or one-way ANOVA, followed by a Bonferroni’s multiple-comparison post hoc test. Values of P < 0.05 were considered statistically significant.

RESULTS

Effects of nebivolol on renal afferent and efferent arterioles. Figure 1 illustrates the effects of nebivolol on afferent and efferent arteriolar diameters. In response to superfusion with nebivolol at concentrations of 0.1, 1, 10, and 100 μmol/l, afferent arteriolar diameters L increased significantly from 16.8 ± 0.2 to 17.5 ± 0.2, 18.5 ± 0.3, 19.4 ± 0.6, and 20.0 ±
arteriolar diameters. Values are means ± SE at renal perfusion pressure of 100 mmHg.

Effects of NOS inhibition with L-NNA on nebivolol-induced vasodilation of afferent arterioles. Figure 2 shows the effects of nebivolol during superfusion with 1-L-arginine (L-NNA). Nebivolol-induced vasodilation was prevented by inhibition of nitric oxide synthase (NOS) with L-NNA given at 1,000 μmol/l (n = 6; P < 0.01), and nebivolol partly restored the diameter of arterioles that were under incomplete NOS blockade with L-NNA given at 100 μmol/l (n = 8; P < 0.01). Values are means ± SE. *P < 0.05, **P < 0.01 vs. control condition; #P < 0.05, ##P < 0.01 vs. L-NNA group at renal perfusion pressure of 100 mmHg.

Effects of sGC inhibition with ODQ on the nebivolol-mediated vasodilation of afferent arterioles. As shown in Fig. 3, pretreatment with ODQ at a concentration of 10 μmol/l decreased afferent arteriolar diameter from 16.9 ± 0.3 to 14.2 ± 0.5 μm (−16.5 ± 1.9%; n = 5; P < 0.01). In the presence of ODQ, superfusion with nebivolol at concentrations up to 100 μmol/l did not elicit perceivable changes in afferent arteriolar diameter (from 14.2 ± 0.5 to 14.5 ± 0.5 μm; 2.4 ± 0.6%; n =

Fig. 2. Afferent arteriolar responses to nebivolol in presence of Nω-nitro-L-arginine (L-NNA). Nebivolol-induced vasodilation was prevented by inhibition of nitric oxide synthase (NOS) with L-NNA given at 1,000 μmol/l (n = 6; P < 0.01), and nebivolol partly restored the diameter of arterioles that were under incomplete NOS blockade with L-NNA given at 100 μmol/l (n = 8; P < 0.01). Values are means ± SE. *P < 0.05, **P < 0.01 vs. control condition; #P < 0.05, ##P < 0.01 vs. L-NNA group at renal perfusion pressure of 100 mmHg.

Fig. 3. Effect of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) on the afferent arteriolar responses to nebivolol. Nebivolol-induced vasodilation was abolished by inhibition of soluble guanylate cyclase (sGC). In the presence of ODQ given at 10 μmol/l, superfusion with nebivolol did not cause significant change in afferent arteriolar diameters. (n = 6). Values are means ± SE at renal perfusion pressure of 100 mmHg.

Fig. 1. Afferent and efferent arteriolar responses to nebivolol. Nebivolol caused dose dependent increases in afferent (A; n = 6) and efferent (B; n = 6) arteriolar diameters. Values are means ± SE. *P < 0.05, **P < 0.01 vs. control condition at renal perfusion pressure of 100 mmHg.

0.7 μm, respectively, with an average maximum increase of 18.9 ± 3.0% at the highest dose (n = 6; P < 0.01). Likewise, efferent arteriolar diameters 2) increased significantly from 17.6 ± 0.2 to 17.9 ± 0.3, 18.7 ± 0.3, 19.9 ± 0.4, and 20.4 ± 0.4 μm (15.8 ± 1.8%; n = 7; P < 0.01 vs. baseline). No significant changes were observed at concentrations of 0.001 and 0.01 μmol/l. Because afferent and efferent arterioles responded similarly to nebivolol, we focused our more detailed studies using juxtamedullary nephron afferent arterioles as our model system.

Effects of NOS inhibition with L-NNA on nebivolol-induced vasodilation of afferent arterioles. Figure 2 shows the effects of nebivolol during superfusion with 1-L-arginine. Pretreatment with L-NNA decreased arteriolar diameter significantly from 17.7 ± 0.3 to 14.9 ± 0.3 μm (−16.1 ± 1.4%; n = 8; P < 0.01) with a concentration of 100 μmol/l and from 17.5 ± 0.2 to 13.7 ± 0.4 μm (−22.0 ± 2.2%; n = 6; P < 0.01) with a concentration of 1,000 μmol/l. In the presence of L-NNA at a concentration of 100 μmol/l, the vasodilator responses to superfusion with nebivolol at concentrations up to 100 μmol/l did not elicit perceivable changes in arteriolar diameter (from 14.2 ± 0.5 to 14.5 ± 0.5 μm; 2.4 ± 0.6%; n =

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Effects of NOS inhibition with L-NNA on nebivolol-induced vasodilation of afferent arterioles. Figure 2 shows the effects of nebivolol during superfusion with 1-L-arginine. Pretreatment with L-NNA decreased arteriolar diameter significantly from 17.7 ± 0.3 to 14.9 ± 0.3 μm (−16.1 ± 1.4%; n = 8; P < 0.01) with a concentration of 100 μmol/l and from 17.5 ± 0.2 to 13.7 ± 0.4 μm (−22.0 ± 2.2%; n = 6; P < 0.01) with a concentration of 1,000 μmol/l. In the presence of L-NNA at a concentration of 100 μmol/l, the vasodilator responses to superfusion with nebivolol at concentrations up to 100 μmol/l did not elicit perceivable changes in arteriolar diameter (from 14.2 ± 0.5 to 14.5 ± 0.5 μm; 2.4 ± 0.6%; n =

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Effects of nebivolol-induced afferent arteriolar vasodilation were prevented by inhibition of sGC. Thus nebivolol-induced afferent arteriolar vasodilation was prevented by inhibition of sGC.

**Effects of nebivolol on metoprolol pretreated renal afferent arterioles.** Figure 4 shows the effects of nebivolol on afferent arterioles treated with metoprolol. 1) We first determined that superfusion with metoprolol up to 80 μmol/l did not caused perceptible changes in afferent arteriolar diameter (1.2 ± 0.2%; n = 4) demonstrating that direct β1-receptor blockade does not elicit afferent arteriolar vasodilation. 2) Furthermore, during β1-receptor blockade with metoprolol (20 μmol/l), superfusion with nebivolol at concentrations of 1, 10, and 100 μmol/l still elicited vasodilation with afferent arteriolar diameters increasing significantly from 17.0 ± 0.1 to 17.5 ± 0.2, 18.8 ± 0.4, and 19.3 ± 0.4 μm (15.0 ± 2.5%; n = 6; P < 0.01 vs baseline). Similar responses occurred in arterioles pretreated with metoprolol at a concentration of 50 μmol/l (n = 2). Thus blockade of β1-adrenergic receptors with metoprolol did not prevent nebivolol-induced dilation of afferent arterioles, indicating that the vasodilation is mediated through a different pathway or mechanism.

**Effects of SR 59230A and butoxamine on nebivolol-induced vasodilation in renal afferent arterioles.** Nebivolol at a concentration of 100 μmol/l alone increased afferent arteriolar diameters significantly from 16.9 ± 0.2 to 19.7 ± 0.3 μm (16.0 ± 1.6%; n = 6; P < 0.01). This effect was markedly attenuated by SR 59230A. When nebivolol (100 μmol/l) and SR 59230A (2.5 or 5.0 μmol/l) were superfused together, the afferent arteriolar vasodilation was attenuated with diameter increasing to 18.0 ± 0.2 and 17.8 ± 0.2 μm, respectively (n = 6; P < 0.05 vs. control condition; P < 0.01 vs nebivolol alone; Fig. 5A). However, butoxamine failed to block the nebivolol-induced vasodilation; superfusion with nebivolol (100 μmol/l) plus butoxamine (100 μmol/l) only slightly blunted the afferent arteriolar vasodilation (−3.0 ± 0.2%; n = 4; P > 0.05 vs. nebivolol alone; P < 0.01 vs. control condition; Fig. 5B). Thus only blockade of β3-adrenergic receptors with SR 59230A markedly reduced the nebivolol-induced dilation of afferent arterioles.

**Immunohistochemical localization of β3-adrenergic receptors.** A monoclonal antibody to the β3-adrenergic receptors was utilized to localize β3-adrenergic receptors in the kidney.
illustrated in Fig. 6, the $\beta_3$-receptor-specific immunoexpression was observed in the endothelial cells (brown chromogen on methyl green counterstain) of renal vasculature (Fig. 6A) and glomerular capillaries (Fig. 6B). Importantly, in glomerular arterioles (Fig. 6, C and D) without the use of counterstain, the colocalization with SMA (chromogen red), a positive marker for smooth muscle cells, indicated that the $\beta_3$-receptor (grey-black) is immunoexpressed by the endothelial cells of the afferent arterioles (Fig. 6C) and efferent arterioles (Fig. 6D).

DISCUSSION

Nebivolol is a recently developed, third-generation $\beta$-adrenoceptor antagonist characterized by combining highly selective $\beta_1$-adrenergic receptor antagonism with NO-dependant vasodilatory activity and reactive oxygen species scavenging effects (15). More recently, increased attention has been focused on its pharmacological roles and cellular mechanisms. In this study, we demonstrate that nebivolol elicits marked dilation of both afferent and efferent arterioles. These findings are consistent with evidence obtained from whole kidney studies showing that nebivolol increased renal plasma flow (22, 25), reduced renal perfusion pressure (22), and dilated renal arteries (16, 17, 47). At a dose of 1 mg/kg, estimated renal plasma flow increased significantly by 19%, while GFR increased 14% at this dose and 22% at 2 mg/kg. The associated increases in urine flow and sodium excretion were substantial and occurred at even lower dose. However, the in vivo effects are likely due to a combination of effects including the actions of $\beta_1$-adrenergic blockade to inhibit sympathetic-mediated stimulation of renin release. This would decrease internal angiotensin II levels and contribute to the overall renal vasodilation (22). These combined actions may also contribute to differential effects on the afferent and efferent arterioles. Vasodilator effects in other tissues include rat and mouse coronary microarteries (8), canine coronary, carotid and pulmonary arteries (14, 24, 41), rat aorta, small mesenteric arteries (1, 7, 38, 47), and human brachial artery (6).

The mechanism of the renal vasodilator action of nebivolol has not been ultimately defined, and the identity of the receptor and pathway mediating nebivolol’s activation of eNOS leading to vasodilation remains uncertain. Several studies have suggested that nebivolol-induced NO release is likely to be mediated by $\beta_3$-adrenergic receptor stimulation (7, 8, 20, 27, 33, 34, 36, 42, 43). Dessy et al. (8) reported that nebivolol failed to dilate aortic rings in $\beta_3$-adrenoceptor-deficient mice and that nadolol, a selective $\beta_1,2$-adrenoceptor blocker, did not attenuate the nebivolol-induced vasodilation in rat and human coronary microvessels indicating that the drug’s vasodilator action is not via $\beta_1$- or $\beta_2$-adrenoceptors. In contrast to the effect of $\beta_1,2$-blockade, the complete $\beta_1,2,3$-blocker bupranolol significantly inhibited the relaxation elicited by nebivolol in rat coronary microvessels supporting a role for $\beta_3$-adrenergic receptors in the NO-mediated vasodilatory effects of nebivolol (8). However, the investigators did not use a highly selective $\beta_3$-blocker in these experiments. In endothelin-1-precontracted rat thoracic aorta rings, nebivolol induced a concentration-dependent relaxation, which was unaffected by nadolol but was significantly reduced by L-748,337, a $\beta_3$-AR antagonist (43). A study (33) using the fluorescent probe diamino-fluorescein showed that nebivolol induces a dose-dependent NO production in mouse heart, but was not able to induce NO production in presence of the $\beta_1$-antagonist SR59230A further supporting a fundamental role for $\beta_3$-adrenergic receptors in cardiac NO production by nebivolol. Nebivolol activates a calcium-independent transduction pathway that implicates an increase in adenylate cyclase and phospholipase

Fig. 6. Immunolocalization of the $\beta_3$-adrenergic receptor in the normal rat kidney. Paraffin-embedded rat perfused kidney sections (3 $\mu$m) were incubated with a monoclonal rabbit anti-$\beta_3$-adrenergic receptor antibody (Biobyrt; Cambridge, UK) at 1:200 dilution for 2 h using an immunoperoxidase technique (A–D). With the use of methyl green as a counterstain (A and B), specific brown stain (DAB) is observed in the endothelial cells of a renal arteriole (Art; A) and glomerular arterioles (Glom; B). C and D: kidney sections without counterstain using costaining with permanent red chromogen (Dako Cytochrome) for smooth muscle actin (red; SMA) and grey-black chromogen with DAB-nickel chloride (Vector Laboratories) for $\beta_3$-adrenergic receptor. C, top right: lower magnification ($\times 40$) field of the selected structures despite in C showing the presence of $\beta_3$-receptor-specific immunostaining at the endothelial cells of the glomerular arterial arteriole (AA). D: presence of $\beta_3$-adrenergic receptor in the endothelium of efferent arterioles (EA). All microphotographs were taken using a digital camera (DS-Fi1) and a $\times 100$ magnification-oil immersion objective (Nikon Eclipse 50i).
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A2 activity. The β₁- or β₂-adrenoceptor antagonists do not inhibit the action of nebivolol. However, its action on cyclic AMP production is inhibited by bupranolol, a β₁,β₃-adrenoceptor antagonist, and S-(−)-cyanopindolol, a selective β₁-adrenoceptor antagonist, suggesting that β₃-adrenoceptor is involved in nebivolol vasodilator properties (20). However, some studies suggest that the cellular mechanisms of the vasodilator effect of nebivolol may also involve activation of the endothelial β₂-adrenoceptors (2, 17, 35), Ca²⁺-activated K⁺ channels (17) and P2Y-purinoceptor (26). It has also been suggested that while the vasodilation induced by nebivolol is mediated by endothelium-derived NO, β₂, β₃-adrenoceptors, 5-hydroxytryptamine receptors (4), and K⁺ channels (47) are not involved in the nebivolol-induced vasodilation. These studies indicate the need for evaluating tissue specific responses thus necessitating our direct studies on the renal microvasculature.

It is well established that NO is formed by NOS. NOS activates the enzyme sGC to produce the second messenger cGMP, which subsequently activates protein kinase G and leads to vasodilation (18, 44). eNOS is the most abundant NOS isoform in the cardiovascular system. eNOS activity is affected by a variety of modifications, such as phosphorylation and acylation, by its cellular localization, and by protein-protein interactions (18, 21). The mechanisms of signal transduction leading to NOS activation are not yet fully delineated. Previous studies (18, 21) suggest that the major factors stimulating nitric oxide synthases are shear stress, serine/threonine protein kinase Akt/PKB, phosphorylated Akt and Ca²⁺-calmodulin. In the present study, we demonstrated that in response to NOS blockade with L-NNA afferent arteriolar diameter decreased significantly as reported previously (12). Nebivolol-induced afferent arteriolar vasodilation was markedly attenuated by pretreatment with L-NNA (100 μM) and prevented by pretreatment with L-NNA (1,000 μM), indicating that nebivolol vasodilates renal afferent arterioles via NOS-NO pathway. These results agree with previous studies showing NO-dependent vasodilator effects of nebivolol in other tissues (17, 25, 35, 36, 47). Nebivolol elicited some vasodilation under condition of incomplete NOS blockade with the lower dose of L-NNA, suggesting that with incomplete NOS suppression nebivolol may stimulate NOS, thereby increasing the availability of NO in the endothelium and smooth muscle to restore endothelial function. While we focused our detailed studies on afferent arterioles because of their greater availability, it is likely that similar mechanisms are responsible for the efferent responses as well. It has already been demonstrated that L-NNA elicits similar vasoconstriction responses in efferent arterioles (12).

In further studies, the vasodilator effects of nebivolol were prevented by ODQ, indicating complete blockade of nebivolol-induced vasodilation by sGC inhibition, which further confirmed that the endothelial localization of the target mechanism activated by nebivolol is NOS and the NOS-NO-sGC-cGMP pathway mediates the nebivolol-induced vasodilation. Nebivolol may activate NOS to produce NO, which leads to activation of sGC and increases in cGMP. These findings are in accordance with evidence obtained from cellular studies on NOS showing that nebivolol induces eNOS activation by a translocation of the enzyme, as well as serine phosphorylation (30) and Akt/protein kinase B (3, 30); by dephosphorylation of Thr, eNOS (8); and through the phosphatidylinositol 3-kinase/Akt pathway (28), inducing phosphorylation of eNOS (32). Nebivolol increases eNOS expression and activity in renal artery (17, 45, 48) and renal NO release in the rat kidney, which was also prevented by NOS inhibition (22, 25).

To determine the role of β₁-adrenoceptor activation in the afferent arteriolar responses to nebivolol and distinguish the vasodilatory action of nebivolol from other β-adrenergic blockers that selectively inhibit β₁-receptors, we tested the effect of nebivolol in the presence of β₁-blockade with metoprolol. The absence of changes with metoprolol and the failure of metoprol to prevent the response to nebivolol indicate that the vasodilation is mediated via a mechanism not directly involving blockade of β₁-adrenergic receptors, suggesting a novel pathway mediating the nebivolol-induced vasodilation. Further studies showed that β₂-adrenoceptors blockade with butoxamine only slightly blunted the vasodilatory effect of nebivolol, suggesting a minor possible role mediated by β₂-adrenoceptors. In contrast, nebivolol-induced afferent arteriolar vasodilation was markedly attenuated after blocking β₃-adrenoceptors with SR 59230A indicating an agonist effect of nebivolol on endothelial β₃-adrenergic receptors, thus suggesting that the vasodilator effect of nebivolol is mediated through the release of NO via a β₃-adrenoceptor-dependent mechanism. These results are supported further by our current immunohistochemical data revealing the presence of β₃-adrenoceptors on endothelial cells of renal arterioles and glomerular capillaries, suggesting their functional role to activate β₃-adrenoceptors and NOS and elicit renal vasodilation. These findings fit well with functional studies in coronary arteries and other tissues showing that the vasodilator effects of nebivolol is mediated via activation of endothelial β₃-adrenergic receptors (7, 8, 20, 27, 43, 33) and molecular studies showing that β₃-adrenoceptors are expressed in the kidney (31, 50) and the endothelial cells of aorta (3) and human coronary resistance arteries (8). These results have important implications for the pathways involved in adrenergic modulation of eNOS pathways in the vascular wall (28). These findings provide mechanistic insights suggesting that β₃-adrenergic receptors may respond to excessive renal sympathetic nerve activation by providing a counteracting renal protective influence to prevent excessive renal vasoconstriction during states of elevated sympathetic activity (8, 34, 36, 42).

In summary, nebivolol markedly vasodilates both renal afferent and efferent arterioles of juxtaglomerular nephrons. The afferent arteriolar responses are due to a mechanism not directly involving blockade of β₁-adrenergic receptors but predominantly mediated through the release of NO via NOS-NO/sGC/cGMP-dependent mechanism involving activation of β₃-adrenergic receptors and NOS, thus revealing the presence and action of β₃-adrenergic receptors as potential regulators of renal hemodynamics during states of high sympathetic activity. The effects of nebivolol to vasodilate renal afferent and efferent arterioles are of potential clinical relevance for conditions related to treatment of hypertension and associated cardiovascular disorders where there is evidence of renal vasoconstriction. The renal microvasculature plays a crucial role in the control of blood pressure. NO-mediated renal vasodilation is impaired in hypertension and diabetes due to a decrease NO bioavailability, NOS activity, and NO-cGMP signaling. The present study provides direct data demonstrating that nebivolol...
elicits vasodilation of the renal microvasculature and suggests that the major pathway is via β3-adrenergic receptor and NOS activation. Nebivolol-induced renal vasodilation could be of benefit in the treatment of hypertension and associated cardiovascular disorders associated with hypertension and endothelial dysfunction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.-G.F. and L.G.N. conception and design of research; M.-G.F. and M.C.P. performed experiments; M.-G.F. and M.C.P. prepared figures; M.-G.F. drafted manuscript; M.-G.F. and L.G.N. edited and revised manuscript; M.-G.F. and L.G.N. approved final version of manuscript.

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