Third-Generation β-Blockers Stimulate Nitric Oxide Release From Endothelial Cells Through ATP Efflux
A Novel Mechanism for Antihypertensive Action

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Background—Nebivolol and carvedilol are third-generation β-adrenoceptor antagonists, which unlike classic β-blockers, have additional endothelium-dependent vasodilating properties specifically related to microcirculation by a molecular mechanism that still remains unclear. We hypothesized that nebivolol and carvedilol stimulate NO release from microvascular endothelial cells by extracellular ATP, which is a well-established potent autocrine and paracrine signaling factor modulating a variety of cellular functions through the activation of P2-purinoceptors.

Methods and Results—Contraction and relaxation of renal glomerular vasculature were measured by determination of intracapillary volume with [3H]-inulin. Biologically active NO was measured with highly sensitive porphyrinic NO microsensors in a single glomerular endothelial cell (GEC). Extracellular ATP was measured by a luciferin-luciferase assay. Enzymatic degradation of extracellular ATP by apyrase and blockade of P2Y-purinoceptors by suramin or reactive blue 2 inhibited both β-blocker–induced glomerular vasorelaxations and β-blocker–stimulated NO release from GECs. Both β-blocker–induced vasorelaxations were in the micromolar concentration range identical to that required for the β-blocker stimulation of ATP and NO release from GECs. The maximum of NO release for nebivolol and carvedilol was very similar (188 ± 14 and 226 ± 17, respectively). Blockade of ATP release by a mechanosensitive ion channel blocker, Gd3+, inhibited the β-blocker–dependent release of ATP and NO from GECs.

Conclusions—These results demonstrate for the first time that nebivolol and carvedilol induce relaxation of renal glomerular microvasculature through ATP efflux with consequent stimulation of P2Y-purinoceptor–mediated NO release from GECs. (Circulation. 2003;107:2747-2752.)

Key Words: nitric oxide ■ endothelium ■ receptors, adrenergic, beta

Although β-blockers have been highly recommended medications for the treatment of hypertension,1 their use may result in an undesirable increase in peripheral vascular resistance (PVR) and a reduction in cardiac output. Nebivolol is a recently developed selective β1-adrenergic receptor antagonist that lowers blood pressure acutely and reduces PVR without depressing left ventricular function.2 Similar benefits in hemodynamic profile have been shown for carvedilol, another recently developed third-generation unselective β-adrenoceptor antagonist (blocker of α1- and β1/β2-adrenoceptors).3–5 These effects of β-blocking activity contrast those caused by traditional β-blockers. The blood pressure–decreasing effect of nebivolol and carvedilol seems to result from a direct vasorelaxant effect of the drugs not mediated by adrenoceptors but possibly by NO generation from endothelial cells. This suggestion comes from studies that relied on the determination of the secondary effects of NO, eg, vasorelaxation after inhibition of NO synthesis or endothelium removal2,4,6,7 or the measurements of biologically inactive products of NO, eg, nitrites,5,8 but not based on direct measurement of NO. Therefore, it is still unclear whether β-blockers themselves augment NO production, and the presence of an endogenous mediator of the vasodilating effect of the drugs is suspected. If so, the transduction pathway that leads to the β-blocker–induced NO release remains to be defined.

It has been shown that nebivolol and carvedilol specifically caused relaxation in rat small arteries,5,6,7 which may be effective in protecting the microcirculation in various cardiovascular disease states. Vascular tone in arterial microvasculature is controlled by both perivascular nerves and endothelial cells. The role of extracellular adenosine nucleotides in this dual control mechanism is prominent.9 ATP, released as a neurotransmitter by sympathetic nerves, acts on ligand-gated P2X-purinoceptors located on vascular smooth muscle cells to produce vasoconstriction. ATP released from endo-

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glomerular microvasculature by activating the P2Y-purinoceptors/endothelial NO synthase (eNOS)/cytosolic guanylate cyclase pathway. Our preliminary studies using a porphyrinic microsensor for direct electrochemical measurements of biologically active NO have indicated that both nebulin and carvedilol and exogenously-added ATP are capable of liberating NO from endothelial cells with very similar kinetics. In the present study, the attempt has been made to elucidate the hypothesis that ATP release from endothelial cells might be the mediator of the vasodilating effect of both β-blockers in arterial microvasculature. We have analyzed whether the vasodilating effect of the β-blockers in renal rat glomerular microvasculature was modulated by the purinergic cell-signaling blockade. We have also investigated the possibility of a concomitant release of endogenous ATP and biologically active NO in response to the exposure of β-blockers to cultured microvascular endothelial cells.

Methods

Isolation of Renal Glomeruli
Glomeruli were isolated from the renal cortex of adult male Wistar rats (200 to 250 g). Rats were decapitated under the ether anesthesia, and the kidneys were removed and placed in ice-cold Dulbecco’s PBS containing (in mmol/L) NaCl 137, KCl 2.7, Na2HPO4 8.1, KH2PO4 1.5, CaCl2 0.9, MgCl2 0.49, and glucose 5.6 at pH 7.4. The minced cortex was mashed through a gradual nylon sieve (the pore size in sequence: 250, 120, and 70 μm). Rat glomerular endothelial cells (GECs) were cultured as described previously. Briefly, isolated glomeruli were treated with 0.1% collagenase for 20 minutes (type IV) and washed twice in phenol red-free DMEM (VEC Technologies) with 450 mg/dL glucose, 10% FBS, 2 mmol/L supplemental glutamine, 100 μmol/L penicillin, and 100 μg/mL streptomycin; 200 μL of the cell suspension was then plated into tissue culture flasks previously coated with fibronectin (Coming). The culture was incubated in 95% air and 5% CO2 at 37 °C and passaged by an enzymatic (trypsin) procedure. GECs were characterized by their morphology, positive staining for factor VIII, and the possession of angiotensin-converting enzyme activity.

Determination of Glomerular Inulin Space
Glomerular inulin space (GIS) was measured according to previously described methods. Immediately after isolation, ~2000 glomeruli were suspended in 200 μL PBS containing 1% BSA and 0.5 μCi [3H]-inulin. Samples were preincubated for 30 minutes at 37°C in a shaking water bath (1.7 Hz). Incubation was continued with 1 μmol/L angiotensin II (ang II) for 5 minutes and then with nebulin (racemate, Berlin-Chemie) or carvedilol (SmithKline Beecham) for the indicated time. Some experiments were done in the presence of purinergic cell-signaling modulators or NOS inhibitors. Reactions were terminated by centrifugation (5 seconds at 5000g) of 200 μL of the suspension in the microtube containing 100 μL of ice-cold silicone oil (Wecker Silicone). The tip of the microtube with the glomerular pellet was cut off, and the content was resuspended in 500 μL of 0.3% Triton X-100. The supernatant (20 μL) was treated in an identical manner. After solubilization, the radioactivity of the samples (triplicate) was measured in a liquid scintillation counter. The inulin space of a single glomerulus was computed as follows:

\[ \text{Glomerular inulin space (GIS)} = \frac{\text{counts per minute of inulin}}{\text{counts per minute of standard}} \times \frac{\text{volume of inulin}}{\text{volume of standard}} \]

The results are expressed as picoliter per glomerulus or as a percentage of the basal GIS value (621±17 pl/gglomerulus).

NO Measurement
NO microsensors were prepared and applied according to the procedures published previously. The active carbon-fiber tip (working electrode) was gently placed close to the surface (5±2 μm) of the cell membrane of GECs by means of a computer-controlled micromanipulator. Ten microliters of the test substances were added to reach a final concentration in the cell medium as follows: 1 μmol/L calcium ionophore (Cal) A23187, 1 μmol/L ATP, and 0.01 to 10 μmol/L nebulin and carvedilol. In some experiments, GECs were pretreated for 5 minutes with the purinergic cell-signaling modulators or the NOS inhibitors.

ATP Measurement
The extracellular ATP concentration was quantitated by luminometry using a luciferin-luciferase assay (Abofax). Briefly, samples of medium (50 μL) were boiled for 1 minute and centrifuged to eliminate possible cell contaminants. The plate was in a luminometer (Berthold GmbH) and processed automatically by injection of 100 μL of luciferin-luciferase reagent into each well and measured for 10 seconds. Standard curves were performed using molecular biology grade ATP, which was diluted in the same buffer and processed parallel to the samples. When drug effects were measured, a small volume of the drug (β-blocker alone or in combination with other agents used) was gently applied immediately before mechanical stimulation of cells. A medium displacement method was used as a mechanical stimulus of the cells. Half of the volume of the bathing medium was gently pipetted up and down twice with a micropipette. ATP was measured 5 minutes after medium displacement. Bioluminescence controls were performed with each drug solution to eliminate drug effect on luciferase activity as well as to control ATP contamination. All chemicals were purchased from Sigma, unless otherwise noted.

Statistics
Statistical analyses were performed by one-way ANOVA followed by Dunnett’s t test to determine significance. Values are given as mean±SEM. P<0.05 was considered to be significant.

Results

Effect of β-Blockers on Ang II–Precontracted Glomeruli
We have analyzed the relaxation and contraction of glomeruli by using [3H]-inulin, because this agent only penetrates extracellular space. Most of the extracellular space of isolated glomeruli exists as intracapillary space, and, therefore, an increase or decrease in GIS reflects a relaxation or contraction of glomerular microvasculature, respectively. The isolated renal glomeruli in the buffer free of vasoactive agents have a globular structure and maximal volume, which is reflected by basal GIS. Thus, to investigate relaxation of totally relaxed glomerular vasculature after isolation, similar to other models for studying blood vessels relaxing responses, we preincubated the glomeruli with a vasoconstrictor agent (1 μmol/L ang II). Consistent with our previous observations, ang II rapidly decreased GIS, which peaked at 5 minutes (83±1% of basal GIS) and remained unchanged for up to 30 minutes. As shown in Figure 1A, nebulinol and carvedilol induced relaxation of ang II–precontracted glomeruli in a concentration-dependent manner with the total relaxing effect at concentrations ≥1 μmol/L (Figure 1A). The vasorelaxing effect of both β-blockers peaked at 3 minutes, and it was maintained also at 10 minutes of incubation (Figure 1B).
Figure 1. Concentration-dependent (A) and time-dependent (B) relaxing effect of nebivolol (--) and carvedilol (--) on ang II–precontracted glomeruli. Glomeruli were preincubated with 1 μmol/L ang II for 5 minutes. To evaluate concentration-dependent effect of the drug, glomeruli incubation was continued with various concentrations of β-blocker for 3 minutes. For estimation of time-dependent effect of the drug, glomeruli incubation was continued with or without 1 μmol/L β-blocker for the indicated time. n=6 to 8; *P<0.01 β-blocker+ang II vs ang II alone.

Neither nebivolol nor carvedilol alone affected the basal GIS value. To investigate the possible role of ATP in β-blocker–induced glomerular vasorelaxation, the response to the drugs was evaluated in the presence of apyrase to eliminate ATP from the extracellular solution. Apyrase (10 U/mL) completely inhibited the drug-induced relaxation of ang II–precontracted glomeruli (Figure 2). To determine whether the requirement of extracellular ATP for β-blocker–induced relaxation involves signaling through purinergic receptors, similar studies were performed in the presence of the P2-purinoceptor antagonists suramin and reactive blue 2. Both suramin (10 μmol/L), a broad spectrum P2-purinoceptor inhibitor, and reactive blue 2 (50 μmol/L), a P2Y-purinoceptor inhibitor, prevented the relaxing effect of the drugs. To investigate the involvement of the endothelial isoforms of NOS in glomerular vasorelaxation, the changes in GIS values were determined in the presence of N\textsuperscript{G}-nitro-L-arginine (L-NNA), a potent eNOS inhibitor, and N-[3-(aminomethyl)benzyl]acetamidine (1400W), a selective iNOS inhibitor. L-NNA (100 μmol/L), but not 1400W (100 μmol/L), completely prevented the relaxing effect of the drugs. To know whether the β-blockers induce relaxation of the glomeruli in a cGMP-dependent manner, the changes in GIS were additionally explored in the presence of H\textsuperscript{11}oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), a selective inhibitor of NO-sensitive guanylyl cyclase. ODQ 10 μmol/L completely inhibited the glomerular vasorelaxing response to the β-blockers.

Effect of β-Blockers on NO and ATP Release

Typical amperograms of NO release from a single GEC are shown in Figure 3. The patterns of NO release stimulated with nebivolol and carvedilol (1 μmol/L each) in a GEC were similar to those after exogenously added 1 μmol/L ATP. The maximum NO concentration was observed between 8 to 10 seconds and was reached 1 second earlier for ATP than the β-blockers. The kinetics of NO release was appreciably slower for the β-blockers and ATP compared with a receptor-independent NO agonist, Cal, during the entire course of NO release. A linear increase of peak NO concentration with an increasing concentration of the drugs from 0.05 to 5 μmol/L was observed (Figure 4). The maximal NO release in GECs was very similar for nebivolol (188±14 nmol/L) and carvedilol (226±17 nmol/L) and represented 55% and 65% of the maximum concentration that can be achieved in these cells (after stimulation with Cal), respectively.

To define the molecular mechanism underlying the β-blocker–induced NO release in GECs, we assessed whether this response results from the ATP elevation in the extracellular medium and the coupling of a P2Y-purinoceptors by ATP. Similar to the studies with the isolated glomeruli, NO release from GECs stimulated by the drugs (1 μmol/L each) was inhibited in the presence of apyrase and the P2-
purinoceptors inhibitors, suramin and reactive blue 2 (Figure 5). β-blocker–stimulated NO release was also inhibited by L-NAME, but it was unaffected by 1400W. The changes in NO concentrations induced by different β-blocker concentrations were in excellent agreement with the respective changes in ATP content in the extracellular medium in response to mechanical stimulation (Figure 4). Mechanical stimuli, in different experimental models, evoked a release of ATP from a variety of cells, including vascular endothelial cells. We used a medium displacement method, which was successfully applied to elicit nucleotide release from different cell types. ATP release stimulated by medium displacement increased 12.8 ± 0.9-fold in the presence of 1 μmol/L nebivolol and 15.7 ± 1.1-fold in the presence of 1 μmol/L carvedilol compared with cells mechanically stimulated without the β-blockers (9.8 ± 0.5 nmol/L). The drugs, up to 10 μmol/L, did not significantly change the ATP concentration in the medium when the cells were unexposed to mechanical stimulation (2.1 ± 0.4 versus 3.2 ± 0.6 nmol/L for nebivolol and 3.3 ± 0.6 nmol/L for carvedilol, respectively). However, β-blocker–dependent increase in ATP concentration in the medium was observed when the mechanically nonstimulated cells were coincubated with 100 μmol/L ARL67156, an ecto-ATPase inhibitor (6-N,N-diethyl-D-β,γ-dibromo-methyleneATP) (3.8 ± 0.5-fold for 1 μmol/L nebivolol and 4.8 ± 0.6-fold for 1 μmol/L carvedilol versus ARL 67156 alone; P > 0.01). ARL67156 also markedly potentiated the β-blocker–dependent (1 μmol/L) increase in ATP release after mechanical stimulation of the cells (Figure 6). These data suggest that the drugs increased ATP release from the cells in response to mechanical stimulus but did not decrease the breakdown of ATP in the extracellular fluid. Administration of both β-blockers to the cells at concentrations up to 10 μmol/L neither alone nor together with mechanical stimulation altered lactate dehydrogenase activity in the extracellular medium and trypan blue exclusion. This confirms that ATP release did not occur as a result of cytotoxicity induced by the drug or the medium displacement procedure.

Figure 4. Concentration-dependent effect of nebivolol and carvedilol on NO and ATP release from GECs. The maximal concentrations of NO release after addition of various concentrations of the β-blocker were quantified in real time by a NO porphyrinic microsensor on the surface of cell membranes. The amount of ATP released into the extracellular medium was measured with a luciferin-luciferase bioluminescent assay 5 minutes after medium displacement in the presence of various concentrations of the drug. Mechanical basal (9.8 ± 0.5 nmol/L) represents the amount of ATP measured in the cell medium exposed to mechanical stimulation without any agents (n = 7 to 8 for both NO and ATP).

Figure 5. Effect of purinergic cell-signaling modulators and NOS inhibitors on β-blocker–stimulated NO release from GECs. Cells were preincubated with 10 μmol/L suramin, 50 μmol/L reactive blue 2, 10 U/mL apyrase, 200 μmol/L Gd³⁺, 100 μmol/L L-NNA, and 100 μmol/L 1400W for 30 minutes, followed by addition of 1 μmol/L nebivolol or carvedilol. n = 7 to 8; *P < 0.01 control (β-blocker alone).

Figure 6. Effect of ARL67156 and Gd³⁺ on β-blocker–stimulated ATP release from GECs. Nebivolol or carvedilol 1 μmol/L was administered to the cells in the presence of 100 μmol/L ARL67156 or 200 μmol/L Gd³⁺ and immediately subjected to a medium displacement. Mechanical basal (9.8 ± 0.5 nmol/L) represents the amount of ATP measured in the medium of the cells exposed to mechanical stimulation without any agents. ATP was measured in the extracellular medium 5 minutes after a medium displacement. n = 7 to 8; *P < 0.01 vs β-blocker alone; +P < 0.01 vs ARL67156 alone.
In an attempt to clarify whether the β-blockers lead to ATP release through the mechanism transducing the mechanical stimulus in the GECs, we evaluated the potential effect of lanthanide Gd
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 (GdCl₃), which inhibits the opening of mechanosensitive nonselective cation channels in epithelial cells. Exposure to 200 μmol/L Gd
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 markedly decreased both NO (Figure 5) and ATP (Figure 6) release from GECs after stimulation with the β-blockers.

Discussion

The present study represents the first report showing that ATP is a putative signaling agent for direct NO-dependent vasodilating action of nebivolol and carvedilol in renal rat glomerular microvasculature. The relaxant effect of both β-blockers was clearly dependent on the ability of endothelium to generate biologically active (diffusible) NO, because it was blocked by pretreatment with the eNOS inhibitor L-NNA at a concentration that also effectively blunted β-blocker–stimulated NO release from GECs. By contrast, the presence of the iNOS inhibitor did not affect β-blocker–dependent vasorelaxation of glomeruli or NO release from GECs, additionally confirming the concept that the drugs’ vasodilating action is attributable to activation of eNOS. The evidence for involvement of ATP and purinergic receptors in β-blocker–stimulated NO-dependent glomerular vasorelaxation was obtained from experiments in which glomeruli and GECs were preincubated with apyrase for enzymatic degradation of extracellular ATP and suramin and reactive blue 2 for blocking P2Y-purinoceptors. Furthermore, the vasorelaxing action of the β-blockers was in a range of micromolar concentrations identical with that required for stimulation of both ATP and NO release from GECs. The immediate effect of the drugs on NO release monitored by using a porphyric NO microsensor that allows NO detection in real time (response time ≅0.1 ms) virtually excludes the possibility that the drugs could have been metabolized in our experiments. To obtain more information about the mechanism involved in the β-blocker–stimulated ATP release in GECs, we tested the influence of a mechanosensitive ion channel blocker, Gd
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, on this effect. Addition of Gd
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 inhibited β-blocker–dependent ATP release, suggesting that the drugs facilitate ATP release through the same effector mechanism as mechanical stimuli. It has been previously suggested that nebivolol, depending on the blood vessel, might exert its NO-mediated vasodilatory action through endothelial β₂-adrenoceptor or 5-hydroxytryptamine (5-HT) receptor ligations.⁸,¹⁷ In our experiments, the effect of both β-blockers on ATP and NO release in GECs was not shared with propranolol, a well-established β₁-/β₂-adrenoceptor and 5-hydroxytryptamine (5-HT)-receptor blocker¹⁷ (data not shown). Propranolol neither stimulated ATP and NO release from GECs itself nor inhibited these effects of nebivolol and carvedilol. These data suggested that ATP release with subsequent NO release, both in response to nebivolol or carvedilol, was not associated with an agonistic effect of the drugs on β₁-/β₂-adrenoceptors or 5-HT-receptors in GECs.

The final effect of ATP action on vascular beds depended on the type of activated purinoreceptors and their location. In the glomerular vasculature, the existence of P₂-purinoceptors, mainly P₂Y, has been confirmed in cultured endothelial cells and smooth muscle–like glomerular mesangial cells.¹⁸ It is commonly documented that activation of P₂Y-purinoceptors by ATP leads to an intracellular increase in [Ca²⁺] through the phospholipase-C/inositol-1,4,5-triphosphate (IP₃) pathway.⁹,¹⁸ The eNOS activity can be increased by a Ca²⁺/calmodulin-dependent or kinase-dependent mechanism. It has been previously demonstrated that nebivolol itself or its putative endogenous mediator could induce an IP₃/Ca²⁺-dependent activation of eNOS in endothelial cells.⁷,⁸ These results disagree with the other observations suggesting that nebivolol did not change intracellular [Ca²⁺] and phospholipase-C activity but is rather attributable to Ca²⁺-independent mechanism, implicating an increase in phospholipase-A₂ activity and prostacyclin production.¹⁹ This would be consistent with the fact that ATP is also a potent stimulator of prostacyclin production in endothelial cells.⁹ Thus, it may be speculated that the discrepancies between the aforementioned results in the vascular beds and species studied could be explained by differences in intracellular transduction signaling by which ATP released by the drug leads to vasodilation.

We have previously shown that exogenous ATP induces relaxation of glomerular vasculature in a cGMP-dependent manner.¹¹ It seems that ATP-mediated NO release after stimulation with the β-blockers may finally activate NO-dependent cytoplasmic guanylyl cyclase in smooth muscle–like mesangial cells, because the drugs’ relaxing effect was completely blocked by the enzyme inhibitor ODQ. On the other hand, mechanisms other than endothelium/cGMP-dependent vasorelaxation should be considered when analyzing the direct action of ATP on smooth muscle cells while endothelium is damaged. In glomerular vasculature, the activation of P₂Y-purinoceptors located on smooth muscle–like mesangial cells has been shown to increase intracellular cAMP concentration that, in turn, may inhibit contraction induced by vasoactive agents.²⁰ Similar vasorelaxing effects of ATP are observed in coronary arterial vessels. Direct application of ATP, for example, relaxed both guinea pig and rabbit coronary artery segments in the presence and absence of the endothelium, suggesting the presence of P₂Y-purinoceptors on coronary smooth muscles.²¹ However, it is worthwhile to emphasize that a common finding in numerous blood vessels lacking endothelium is contraction via action of ATP at a P₂X-purinoceptor, whereas some vessels, once contracted, can relax with addition of ATP, suggesting the presence of both P₂X- and P₂Y-purinoceptors on the vascular smooth muscle.⁹,¹⁰ It has been consistently shown that the vasorelaxant effect of nebivolol changed to a concentration-dependent contractile response in rat mesenteric arteries after either endothelium denudation or blocking NO and prostacyclin production.² In addition to a role for luminal ATP or agents acting via luminal ATP in regulating hemostasis, ATP released into the bloodstream is thought to serve an anti-thrombogenic role, because it is an antagonist of ADP at the platelet P₂t-purinoceptors.⁹ Furthermore, metabolism of purine nucleotides to adenosine from the site of ATP release by well-characterized ecto-nucleotidases on the endothelial cells
would also be expected to inhibit additional platelet activity and promote dilatation of arterioles.

In conclusion, the results of this study present evidence for the first time that the vasodilator effect of nebivolol and carvedilol in renal glomerular microvasculature is associated with the activation of ATP efflux with consequent stimulation of P2Y-purinoceptor-mediated liberation of NO from glomerular microvascular endothelial cells (Figure 7). This mechanism of the β-blockers’ vasodilating action, if proven beneficial in different vascular beds, suggests a novel approach to design pharmacological interventions in cardiovascular disorders, especially relevant to microcirculation protection.

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