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The cellular mechanisms involved in the vasodilator effect of nebivolol on the renal artery

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Abstract

Nebivolol is known as a highly selective β_1-adrenoceptor antagonist. Based on the reported vasodilator effect of nebivolol, we examined the cellular mechanisms by which the drug induces renal artery vasodilation, an issue of potential relevance for condition associated with high blood pressure. To this purpose, myograph and patch-clamp techniques were used. Small mouse renal arteries were placed in the myograph chamber, and after the optimal concentration for the vasodilator effect of nebivolol was established (50 μM), the arteries were further investigated to assess the potential contribution of nitric oxide (NO) and of Ca^{2+} ions to the nebivolol-induced effect, by exposing the arteries to the specific inhibitors such as N^G-nitro-L-arginine methylester (L-NAME, 100 μM), ethylenglycol-bis-(β-amino-ethylen ester) *N*,*N*′-tetraacetic acid (EGTA, 4 μM) and thapsigargin (1 μM). The expression of NO synthase was evaluated by the Western-blot technique. Using myograph and patch-clamp techniques applied on intact renal artery, we investigated the role of β_2-adrenoceptors, of myoendothelial junctions and of Ca^{2+}-activated K^+ channels in the vasodilatory effects of nebivolol, using 100 μM butoxamine, 40 μM 18 beta-glycyrrhetinic acid, 1 mM tetraethylammonium, and 100 nM iberiotoxin, respectively. The results showed that the cellular mechanisms of the vasodilator effect of nebivolol on the renal artery entail (i) activation of the endothelial β_2-adrenoceptor, (ii) participation of $[Ca^{2+}]_i$, (iii) increase in NO and eNOS, and (iv) activation of Ca^{2+}-activated K^+ channels. The cellular mechanisms underlying vasodilator effect of nebivolol on the artery explain the favorable effect of this drug in hypertension.

Keywords: Renal artery; Nebivolol; Vasodilation; Hyperpolarization

1. Introduction

Owing the importance of the blood vessels for the function and regulation of the cardiovascular system, these are major targets for pharmacotherapy in patients with cardiovascular risk factors or established atherosclerotic disease (Lüscher et al., 2001). Thus, the renal vasculature plays an important role in the control of the blood pressure (Martens and Gelband, 1996): the endothelium-derived hyperpolarizing factor-mediated vasodilation is impaired in the renal microcirculation of hypertensive and diabetic rats (De Vriese et al., 2000). Cardiovascular drugs may exert beneficial effects on the vascular wall both at the level of both the endothelium and smooth muscle cells. Nebivolol is a new selective β_1-adrenoceptor blocker possessing vasodilator properties useful for the management of essential hypertension involving the vascular endothelium (Waeber, 2000; Scheen, 2001; Cosentino et al., 2002; Kubli et al., 2001; Ignarro et al., 2002). This drug is administered as a racemic mixture of D- and L-enantiomers (Scheen, 2001). The D-enantiomer is responsible for the β_1-blocking properties, whereas the L-enantiomer induces vasodilatation via β_2-adrenoceptor binding, with a subsequent rise in endothelial NO synthase-dependent NO production (Waeber,

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2000; Scheen, 2001). In addition, nebivolol is a lipophilic agent: it is metabolized in the liver and transformed into several active metabolites, essentially via the CYP2D6, an isoform of cytochrome *P*450 characterized by a genetic polymorphism (5). Nebivolol is highly specific for β_2-adrenoceptors (Scheen, 2001), and it is recommended for hypertensive patients in a dosage of 5 mg (once a day), for 4 weeks (McNeely and Goa, 1999).

The pharmacological effects of nebivolol have been studied in vitro on segments of mouse thoracic aorta (Broeders et al., 2000; Altwegg et al., 2000), canine coronary and carotid artery (Ritter, 2001), rats and bovine aorta and small mesenteric arteries (Cosentino et al., 2002; De Groot et al., 2003; Parenti et al., 2000), on human aortic smooth muscle cells (SMC) (Andre et al., 2000), as well as on the coronary endothelial and smooth muscle cells (Brehm et al., 2000; Brehm et al., 2001). These studies showed that, nebivolol has a vasodilatory effect (Broeders et al., 2000; Altwegg et al., 2000; Ritter, 2001; De Groot et al., 2003; Parenti et al., 2000), reduces the cell proliferation (Andre et al., 2000; Brehm et al., 2000; Brehm et al., 2001), does not significantly influence glucose or plasma lipid metabolism, and appears to have a protective effect on left ventricular function (Waeber, 2000). The mechanism by which nebivolol induces all these effects, is still unclear. It was observed that nebivolol produced relaxation by endothelial β_2-adrenoceptor-mediated NO production (Waeber, 2000; Broeders et al., 2000; Altwegg et al., 2000; Ritter, 2001; De Groot et al., 2003; Parenti et al., 2000), by activating the production of phospholipases C and A_2 and cyclic adenosine monophosphate (cAMP) (Gosgnach et al., 2001) and by the activating inositol phosphate metabolism (Parenti et al., 2000). In addition, decreased secretion of endothelin-1 in the presence of nebivolol may represent a strategy with great promise for antiproliferative therapy of coronary heart disease (Brehm et al., 2000). The antiproliferative effects of nebivolol were observed on human SMC; the mechanism involves inactivation of Cdk2 (cell-cycle regulatory protein) (Andre et al., 2000). Moreover, it was reported that nebivolol decreased systemic oxidative stress in young healthy volunteers (Troost et al., 2000), and could protect against the hydroxyl radical-induced contractile dysfunction by having a direct effect on the myofilaments, preserving in this way the sarcoplasmic reticulum function (Janssen et al., 2001).

Based on these data, we were interested in the effect of nebivolol on the renal arteries, which plays an important role in the pathogenesis of renal disease, as well as in hypertension. The investigation of the cellular mechanism of nebivolol was another important point of our study. In this study, we tried to determine the involvement of intracellular calcium, NO, myoendothelial junctions, and endothelium-derived hyperpolarizing factor (EDHF) in the response of the smooth muscle cells (SMC) after activation of β_2-adrenoceptor exposed by endothelial cells.

2. Materials and methods

2.1. Animals

Young normal RAP mice (3–4 months old) were used in this study. The experiments on the animals were performed in accordance with "Principles of laboratory animal care" (NH publication no. 83-25, revised 1985).

2.2. Renal arteries preparation for the myograph technique

2.2.1. Experimental procedure for isolation of the intact renal artery

Immediately after death, a laparotomy was performed and both renal arteries were excised together with the kidney and were pinned down on Sylgard resin in a Petri dish containing saline. Segments (1 to 2 mm long) of the renal arteries were dissected out, and two stainless steel wires (∅: 40 μm) were threaded through these segments and mounted in a small-vessel myograph (Model 410A, J.P. Trading, Denmark) as described by Mulvany and Halpern, 1997. The myograph chamber was filled with HEPES salt solution (HPSS) containing (mM): 5 Hepes, 140 NaCl, 4.6 KCl, 1.17 $MgSO_4$, 2.5 $CaCl_2$, and 10 glucose (Chulia et al., 1995; Thurston et al., 1995) maintained at 37 °C and continuously gassed with O_2. After an equilibration period of 20 min, the arteries were set to a normalized internal circumference at which they give a maximum isometric response and which is estimated to be 0.9 times the circumference they would have when relaxed and subjected to a transmural pressure of 100 mm Hg. The mean internal diameter of the renal arteries used (at 100 mm Hg) was in the range of 340±50 μm.

2.2.2. The effect of nebivolol on the renal arteries

To measure the nebivolol-induced relaxation (arteries with intact endothelium), the vessels were contracted in noradrenaline (10^{-8}–10^{-4} M) and then exposed at 2-min intervals to increasing concentrations of nebivolol (10–100 μM) (Parenti et al., 2000).

To test the role of intracelular Ca^{2+} and NO in the vasodilatation of renal arteries, the noradrenaline-precontracted vessels were incubated with 4 μM EGTA calcium-free salt solution (a chelator agent for extracellular calcium) and with 1 μM thapsigargin (an inhibitor of the Ca^{2+}ATP-ase in sarcoplasmic reticulum) (Parenti et al., 2000) for 10 and 15 min, respectively; the activity of nitric oxide synthase (NOS) was blocked by 10-min incubation of the noradrenaline-precontracted vessels with 100 μM L-NAME (Parenti et al., 2000) (an inhibitor of NO synthase) followed by the measurement of the reactivity of the artery to 10–100 μM nebivolol.

Other experiments tested the effect of nebivolol on renal artery reactivity (with intact endothelium) in the presence of 100 μM butoxamine (an antagonist of β_2-adrenoceptor), of 40 μM 18 beta-glycyrrhetinic acid (gap junction blocker),

and 100 nM iberiotoxin (both inhibitors of Ca^{2+}-activated K^+ channels); the vessel was exposed to the inhibitors for 15 min.

2.3. Renal arteries preparation for the patch-clamp technique

For the patch-clamp experiments renal arteries were isolated from mice (as described above) and then mounted in the myograph chamber; an opening cut along the long axis of the vessel was made before the artery fragment was pinned on Sylgard resin with adventitial layer up wards, as described by White and Hiley, 1998. The activity of the smooth muscle cells was recorded as described by Yamamoto et al., 1998. Briefly, the adventitial layer was removed from the smooth muscle layer after a short incubation for 15 min at 35 °C with 0.5 mg/ml collagenase A in nominally Ca^{2+}-free solution containing (mM): 141.5 NaCl, 5.4 KCl, 1 $MgCl_2$, 10 HEPES, 5 glucose with pH adjusted to 7.3 with 1 M NaOH (at room temperature).

2.3.1. Electrophysiological techniques applied on the renal arteries

The patch-clamp whole-cell configuration was used to record the effect of nebivolol on intact renal artery in which the adventitial layer had been removed by enzymatic treatment in order to have access to the SMC layer. Only cells with a resting potential between −30 and −60 mV were used. Changes in membrane potential were recorded in the current-clamp mode. Current clamp recordings were performed using WPC-100 amplifier (ESF electronic, Göttingen, Germany) and borosilicate glass pipettes (GC150T, Harvard Apparatus, Edenbridge, Kent, UK) pulled with a microprocessor-controlled vertical pipette puller PUL-100 (WPI, Sarasota, FL, USA) and heat polished to a resistance of 2–8 MΩ.

Controlled stimuli were delivered, and the digital records were captured with pClamp 8 software (Axon Instruments, Union City, CA, USA) and DigiData 1200 Series Interface (Axon Instruments). The experiments were performed at 37 °C, the bath temperature being controlled with a temperature controller TC-202A (Harvard Apparatus). Membrane potential changes were recorded in the presence of 50 μM nebivolol before and after application of the following inhibitors: 100 μM butoxamine, 40 μM 18 beta-glycyrrhetinic acid, 1 mM tetraethylammonium and 100 nM iberiotoxin, for 200 s.

2.3.2. Solutions for the patch-clamp technique

The composition of standard bath solution for the patch-clamp experiments was (mM): 141.5 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 5 Glucose. The pH was adjusted to 7.3 with 1 M NaOH at room temperature.

The pipette solution contained 120 μM amphotericin B in the solution containing (mM): 10 NaCl, 35 KCl, 60 K_2SO_4, 10 HEPES, 3.44 NaOH, 20 sucrose, 1 EGTA, pH 7.3.

2.4. Western-blotting technique

The isolated renal arteries (mice) were collected in PBS (phosphate-buffered saline), and minced into very small pieces after connective tissue was removed. The pieces were kept on ice and were homogenized in lysis buffer containing: 10 mM Trisma-base, 5 mM EDTA (ethylenediamine tetra-acetic acid), 10% Triton X-100, 25 μM PMSF (phenyl methyl sulfonyl fluoride), 1 μM benzamidine. The membrane proteins were pelleted by centrifugation for 15 min at 9600×g. The protein concentration was measured by the Bradford's method, and proteins were separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In parallel, proteins from renal artery preparations incubated with 50 μM nebivolol (for 40 min at 37 °C) were run on the same gel. High molecular weight markers (6.5 to 205 kDa) were loaded into one lane as a size standard. Equivalent amounts (40 μg) of total protein from renal arteries in the absence and in the presence of nebivolol were added to adjacent lanes, and the samples were run at 120 V for 2 h. Gels were subsequently transferred to a nitrocellulose membrane, at 54 mA, for 45 min. The membranes were washed in 0.05% Tween 20 in PBS, and blocked in 5% BSA (bovine serum albumin) in PBS overnight, at 4 °C. Afterwards, the membranes were incubated for 1 h with the first antibody (rabbit anti-nitric oxide synthase, endothelial, Sigma), and then, washed and incubated for 1 h with goat anti-rabbit IgG (immunoglobulin) coupled with peroxidase. The bound antibody was detected in the presence of 4-chloro-1-naphtol, 1 mg/ml. The density of immunoreactive bands was measured using a microcomputer imaging system.

The beta-actin bands on blots were revealed after interaction with monoclonal anti-β-actin (mouse IgG2a isotype) as a primary antibody, and the same secondary antibody (as above), followed by reaction with 4-chloro-1-naphtol, and densitometric evaluation.

2.5. Viability of endothelial cells in the renal artery

The possible damage of the endothelial cells in renal artery during their preparation for patch-clamp experiments was investigated by fluorescence microscopy. The arteries were incubated in 1 mg/ml FITC-dextran (fluorescein-isothyiocyanate labeled dextran) in PBS, for 20 min and washed out thoroughly.

The viability of endothelial cell was also tested by the patch-clamp technique in current-clamp mode, recording the changes of the membrane potential in the presence of acetylcholine (10 μM; an endothelium-dependent vasodilator), which binds to M_2 muscarinic receptors present only on endothelial cells. The changes in membrane potential are thus markers of endothelial cell function.

2.6. Reagents

Nebivolol was from Berlin-Chemie (Germany). Noradrenaline, acetylcholine, L-NAME, EGTA, thapsigargin, tetraethylammonium, iberiotoxin, butoxamine, 18 beta-glycyrrhetinic acid, FITC-dextran, HEPES, anti-nitric oxide synthase, endothelial (eNOS, 596–609) from rabbit, anti-β-actin (mouse IgG2a isotype), and anti-rabbit IgG (whole molecule)-peroxidase from goat were purchased from Sigma (St. Louis, MO, USA), collagenase A from *Clostridium histolyticum* was from Roche (Switzerland). All others reagents used were of analytical grade.

2.7. Data analysis

The tension developed in noradrenaline-exposed vessels is expressed as active wall tension (mN mm^{-1} artery length) and the induced nebivolol relaxation was given as % of noradrenaline-induced contraction. Half-maximal concentrations (EC50) were calculated from the dose–response curves and were compared by the one-way analysis of variance (ANOVA) test. The data were considered significant when $P<0.05$. The patch clamp data were analyzed using pClamp 8 (Axon Instruments). All values are expressed as mean±S.E.M.

3. Results

3.1. The effect of nebivolol on the vascular response of the renal artery

The arteries relaxed in a dose-dependent manner in response to nebivolol up to 50 μM, and had a maximal response between 50 and 100 μM nebivolol (Fig. 1). Thus, as shown in Fig. 1, nebivolol has a vasodilator effect on renal arteries, and the concentration able to produce a maximal vasodilator effect was 50 μM, where the relaxation was 90.5±3.1% (n=15). EC50 for the nebivolol was 18.1 μM.

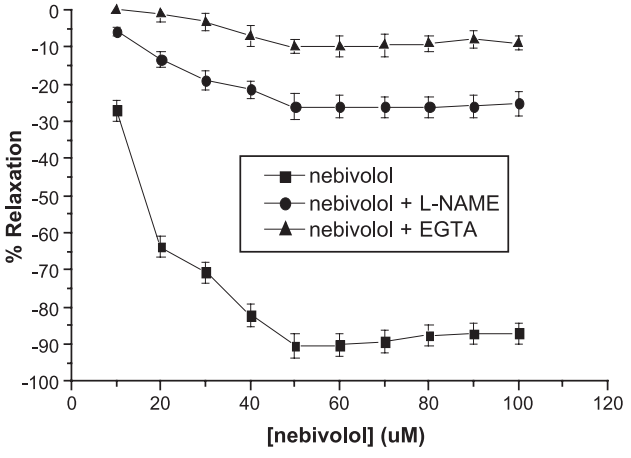

Fig. 1. The vasodilator effect of the various doses of nebivolol (10–100 μM) on the vascular response of the intact renal artery isolated from normal mice (3–4 months old); the inhibition of NO production with 100 μM L-NAME reduces the nebivolol-induced relaxation in the renal artery; reduction of $[Ca^{2+}]_i$ by adding 4 μM EGTA to the organ bath further reduced the nebivolol-induced relaxation ($P<0.05$).

3.2. The involvement of intracellular calcium and NO in the relaxation of the renal arteries to nebivolol

To check whether NO was involved in the vasodilator effect of nebivolol, the activity of NOS was inhibited by adding 100 μM L-NAME to the organ bath of the myograph. The results showed that 100 μM L-NAME diminished the relaxation (at 50 μM) of the renal arteries to 26.2±3.5% (n=7) (Fig. 1). Since it is known that NO production (by eNOS) increases after a rise in free $[Ca^{2+}]_i$ in the endothelial layer, we explored the role of $[Ca^{2+}]_i$ in nebivolol-induced effect. To this intent, external Ca^{2+} was removed by 4 μM EGTA added to the organ bath, and the effect of nebivolol tested as above (Fig. 1). The results indicated very low levels of relaxation, i.e. 10±2% (n=7) in the presence of 50 μM nebivolol (Fig. 1).

The role of $[Ca^{2+}]_i$ was also assessed in the presence of 1 μM thapsigargin, an inhibitor of Ca^{2+} ATP-ase pump in sarcoplasmic reticulum. In this case, the relaxation of arterial wall was reduced to 44.4±11.5% (n=7).

It is known that increases of $[Ca^{2+}]_i$ in endothelial cells generates the release of NO by NOS.

Using the Western blot technique, we compared the eNOS expression from control renal arteries (40 μg/ml protein) to that of arteries incubated in vitro with 50 μM nebivolol (for 40 min). The densitometry of eNOS protein (in six separate experiments) gave mean values of 119.35±0.24 (considering in controls as 100%); thus, nebivolol resulted in an ~20% increase in eNOS (Fig. 2). The Western blotting experiments for β actin showed similar results in both control and nebivolol-treated renal arteries (Fig. 2).

3.3. The involvement of endothelial β_2 adrenoceptors, myoendothelial junctions and Ca^{2+}-activated K^+ channels in the relaxation of the renal arteries to nebivolol

The myograph technique was applied to find out the involvement of endothelial β_2-adrenoceptors, myoendothelial gap junctions, and Ca^{2+}-activated K^+ channels in the vasodilator effect of nebivolol. To this purpose, maximally contracted renal artery, following addition of noradrenaline (10^{-8}–10^{-4} M), was exposed to 50 μM nebivolol in the presence of 100 μM butoxamine (an antagonist of β_2 receptor), 40 μM 18 beta-glycyrrhetinic acid (gap junctions blocker), 1 mM tetraethylammonium (an inhibitor of Ca^{2+}-activated K^+ channels), and 100 nM iberiotoxin (a specific inhibitor of large conductance Ca^{2+}-activated K^+ channels). The nebivolol-induced relaxation decreased to 29.1±12.2% in the presence of butoxamine, 32.7±10.8% in the presence

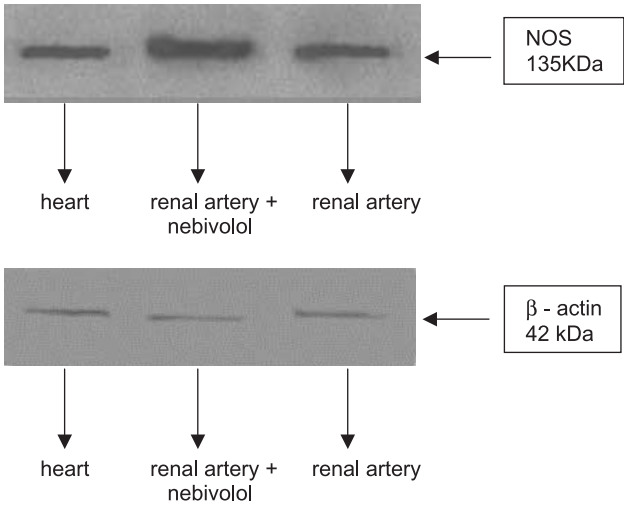

Fig. 2. A representative Western blot assessing augmented expression of eNOS in mice renal arteries incubated 40 min (at 37 °C) with 50 μg/ml nebivolol; heart homogenates (mice) run on the same transfers were used as positive control for eNOS; immunoblotting for β-actin was used as internal control.

of 18 beta-glycyrrhetinic acid, and to 43.3±10.6% and 40±9.7% in the presence of tetraethylammonium and iberiotoxin, respectively (Fig. 3) (n=7).

The patch-clamp technique (in whole cell configuration) was used in parallel to establish the mechanisms involved in vasodilator effects of nebivolol (tested by the myograph technique, as above). The experiments were carried out on SMC from the intact renal arteries, and recordings were made in current-clamp mode.

The recordings showed that 50 μM nebivolol (for 200 s) induced a membrane hyperpolarization of 5±2 mV (n=20) (Fig. 4). The mean membrane resistance of the examined cells was 309±152 MΩ, the access resistance was 52.3±19.6 MΩ, and the capacitance was 17.8±6.2 pF.

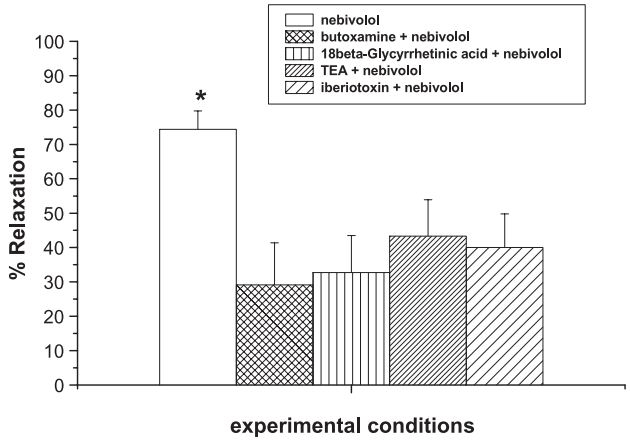

Fig. 3. The involvement of endothelial β_2-adrenoceptors, myoendothelial junctions, and of Ca^{2+}-activated K^+ channels in the relaxation of the contracted renal arteries in response to nebivolol is shown by the reduction of the maximal vasodilator response to 50 μM nebivolol in the presence of 100 μM butoxamine, 40 μM 18 beta-glycyrrhetinic acid, 1 mM tetraethylammonium and 100 nM iberiotoxin (*P<0.05).

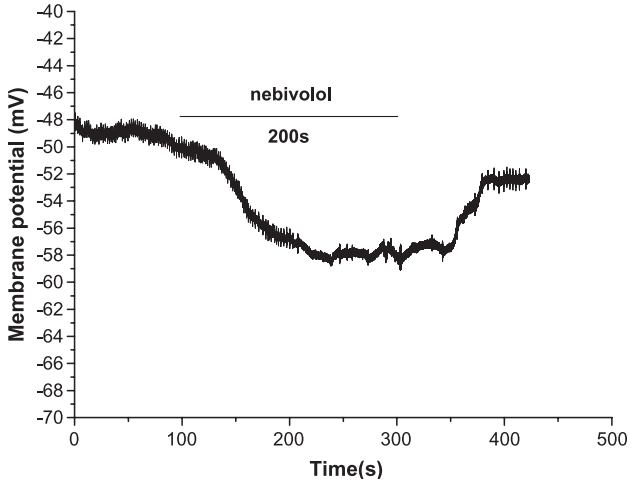

Fig. 4. The hyperpolarization effect of 50 μM nebivolol on the resting membrane potential of smooth muscle cells in intact arteries.

The mean resting membrane potential of the patched cells in the intact vessels segments was −44.3±5.8 mV (n=20).

The effect of butoxamine, 18 beta-glycyrrhetinic acid, tetraethylammonium, iberiotoxin on the SMC response to nebivolol was tested in separate experiments. Nebivolol was applied for 200 s and the preparation was washed (100 s) until the membrane potential reached the steady state value. Then, a blocker was applied, followed (without washing) by the mixture of blocker and nebivolol (200 s each), the preparation was washed again (100 s), and then nebivolol was added again (200 s) to test the reversibility of the hyperpolarization.

As shown in Fig. 5, nebivolol did not induce a substantial change in membrane potential in the presence of butoxamine (applied for 200 s). Similar experiments were done in the presence of butoxamine (applied for 200 s). Similar experiments were done in the presence of 18 beta-glycyrrhetinic acid, tetraethylammonium, and iberiotoxin (these are not shown here). The hyperpolarization induced by nebivolol in the presence of these blockers was far less pronounced then that obtained in the presence of nebivolol only (of 2±1 mV compared to 5±2 mV). This means that the nebivolol-induced hyperpolarization cannot develop plenary in the presence of these blockers. These results indicated the involvement of endothelial β_2-adrenoceptors, myoendothelial junctions, and Ca^{2+}-activated K^+ channels in the relaxation of the renal arteries in response to nebivolol. In addition, we observed that, after washing out blockers, nebivolol maintained its effect on the membrane potential.

When butoxamine, 18 beta-glycyrrhetinic acid, tetraethylammonium or iberiotoxin was applied alone, the resting membrane potential slightly hyperpolarized. The response to nebivolol observed during application of these blockers was lower, which means that every blocker in part contributes to the hyperpolarization effect of nebivolol. Other factors released by endothelial cells, such as NO,

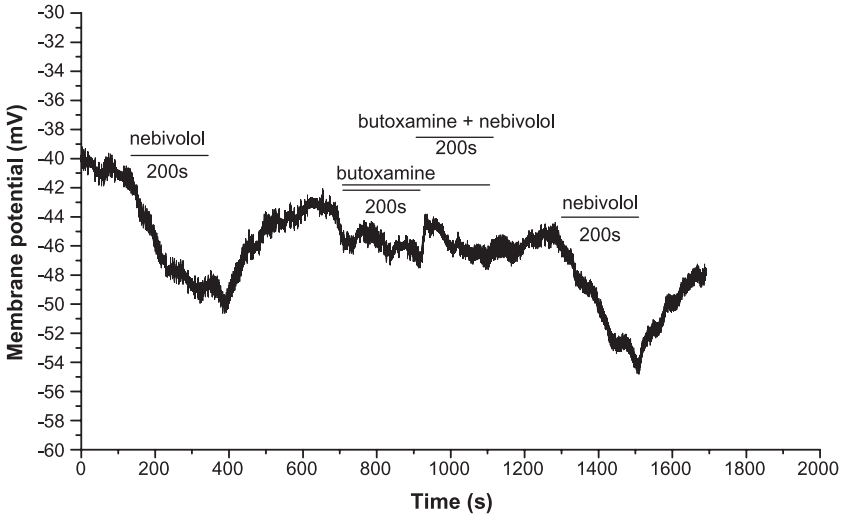

Fig. 5. Reduction of the hyperpolarization effect of 50 μM nebivolol on the resting membrane potential of smooth muscle cells in intact arteries in the presence of 100 μM butoxamine The results show the involvement of endothelial β2-adrenoceptor in the relaxation of the contracted renal arteries induced by nebivolol.

$[Ca^{2+}]_i$, PGI2 (prostacyclin) may participate in the effect of nebivolol.

3.4. The viability of endothelial cells in the renal artery

In order to demonstrate the viability of endothelial cells after preparation of the tissue for patch-clamp studies, the effect of 10 μM acetylcholine on the resting membrane potential was recorded. The membrane potential (in absolute value) increased as a result of the hyperpolarizing effect of acetylcholine on endothelial cells (Fig. 6). Since M_2 muscarinic receptors for binding acetylcholine are present only on endothelial cells, the hyperpolarization effect induced by acetylcholine demonstrates that endothelial cells were not affected during the process of tissue preparation.

In a separate experiment we tested the viability of the cells in the vascular wall using FITC-dextran. The fluorescent FITC-dextran (apparent Mr 4400 D) entered the vascular cells by endocytosis, demonstrating that the endothelial cells were functional.

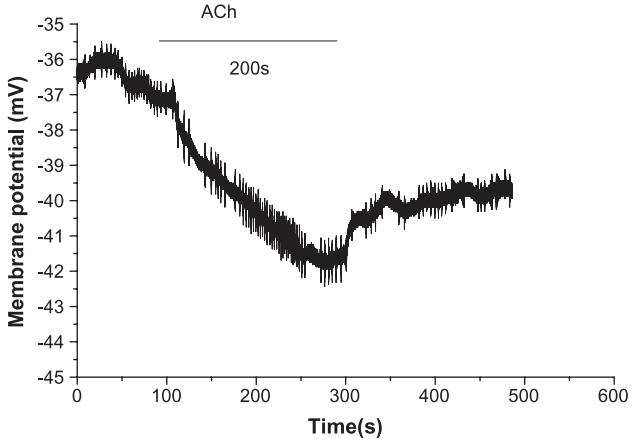

Fig. 6. The hyperpolarization effect of 10 μM acetylcholine, an endothelium-dependent vasodilator, on the resting membrane potential of smooth muscle cells in intact renal arteries, showing that endothelial cells were not affected by the process of tissue preparation.

4. Discussion

The major finding of this study is the vasodilator effect of nebivolol (50 μM) on the mice renal arteries in vitro. This is the first demonstration of the vasorelaxant properties of nebivolol on the renal arteries. It is known that renal arteries are strongly affected in hypertension, and angiotensin II levels are higher in renal interstitial fluid than in plasma (Nishiyama et al., 2002). Thus, we can speculate that nebivolol may have a beneficial effect in restoring the endothelial dysfunction. Other findings are related to the mechanism(s) involved in this process. In order to investigate whether nitric oxide (NO) and intracellular Ca^{2+} are involved in the response of the intact renal arteries to nebivolol, we tested the reactivity to nebivolol in the presence of L-NAME (an inhibitor of NOS), and EGTA (a chelator of extracellular calcium ions), using the miograph technique. The involvement of NO and $[Ca^{2+}]_i$ in the mechanism of nebivolol is supported by our observation which that the vasodilator response to nebivolol was less pronounced under these experimental conditions. Using thapsigargin, an inhibitor of the Ca^{2+}ATP-ase pump in sarcoplasmic reticulum, we demonstrated a possible role of Ca^{2+} in the vasodilator response of renal artery to nebivolol. The increased expression of NOS suggests a role of NO in the vasodilator effect of nebivolol. Interestingly, Broeders et al., 2000, showed that nebivolol induces arterial dilation in humans, and this phenomenon involves endothelial β_2-adrenoceptors binding, with a subsequent rise in endothelial free $[Ca^{2+}]_i$ and endothelial NOS-dependent NO production. Since Chen et al., 1988 reported endothelium-dependent

hyperpolarization of the vascular smooth muscle, this phenomenon is thought to be produced by an unidentified humoral substance, i.e. endothelium-derived hyperpolarizing factor (EDHF) (Chen et al., 1991). In addition, Yamamoto et al., 1999 showed in their results obtained for guinea pig mesenteric arterioles that hyperpolarizing responses detected in smooth muscle cells seem to originate in endothelial cells and are conducted to the muscle layer via myoendothelial gap junctions. Thus, EDHF released from vascular endothelium passes through the myoendothelial gap junction, and afterwards produces hyperpolarization of the SMC by activating the Ca^{2+}-activated K^+ channels. Myoendothelial gap junctions and Ca^{2+}-activated K^+ channels seem to be crucial to the hyperpolarization of SMC. Starting from these data, we presumed that nebivolol may exert a vasodilator effect by activating the β_2-adrenoceptors, myoendothelial gap junctions and Ca^{2+}-activated K^+ channels. In order to check on the above mechanism, both myograph and conventional whole-cell patch-clamp methods were applied to renal arteries preparations. Thus, by myograph technique the response of renal artery to 50 μM nebivolol in the presence of butoxamine (an antagonist of β_2-adrenoceptors), 18 beta-glycyrrhetinic acid (a blocker of gap junctions), tetraethylammonium (an inhibitor of Ca^{2+}-activated K^+ channels), and iberiotoxin (specific inhibitor of Ca^{2+}-activated K^+ channels with large conductance) was investigated. The decreases of the vasodilator response showed that β_2-adrenoceptors (on endothelial cells), myoendothelial gap junctions, and Ca^{2+}-activated K^+ channels are involved in the vasorelaxant effect of nebivolol. Using the patch-clamp technique, we observed that butoxamine, 18 beta-glycyrrhetinic acid, tetraethylammonium, and iberiotoxin reduced nebivolol-induced hyperpolarization of SMC in intact renal arteries. These results show for the first time that the myoendothelial gap junctions and Ca^{2+}-activated K^+ channels are involved in nebivolol-induced hyperpolarization in SMC in isolated renal arteries. In addition, our results demonstrated that, the response of renal arteries to nebivolol is due to the involvement of β_2-adrenoceptors of endothelial cells, conclusion supported by the results which showed that butoxamine prevented the nebivolol-induced hyperpolarization of SMC in intact renal arteries, as observed in current-clamp configuration. These results reflect the reaction of viable cells, as shown by both fluorescence staining with FITC-dextran, and the presence of an hyperpolarizing effect of acetylcholine on endothelial cells, which demonstrate that their viability was maintained after preparation of the tissue for patch-clamp studies.

In conclusion, the results of the present study indicate that nebivolol has a reversible vasodilator effect on renal arteries, and this phenomenon involves endothelial β_2-adrenoceptor binding, with a subsequent rise in $[Ca^{2+}]_i$, endothelial NOS-dependent NO production and EDHF. The latter passes through myoendothelial gap-junctions and activates Ca^{2+}-activated K^+ channels in smooth muscle cells. This may be an important mechanism underlying the nebivolol-induced arterial dilation in humans and thus nebivolol might become an additional therapeutic target in disorders associated with endothelial dysfunction.

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