 Effects of nebivolol on proliferation and apoptosis of human coronary artery smooth muscle and endothelial cells


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Abstract

Objective: Secondary failure due to late restenosis continues to occur in 30–50% of individuals after PTCA. β-Blockers play an important role in the treatment of CAD. The aim of this study was to investigate the effects of the new β-blocker nebivolol on cell proliferation of human coronary smooth muscle cells (haCSMCs) and endothelial cells (haECs) in comparison to traditional β-blockers.

Methods: The effect of nebivolol and other β-blockers on proliferation of HaECs and HaCSMCs was analyzed by bromodeoxyuridine incorporation. Apoptosis was measured by determination of hypodiploid DNA in both cell types. Additionally, in HaECs NO formation, endothelin-1 transcription and secretion were determined.

Results: Incubation for 1, 2, 4, 7 or 14 days resulted in a concentration- and time-dependent reduction of proliferation up to 80% in HaECs and HaCSMCs. β-Blockers such as propranolol, metoprolol or bisoprolol did not exert this effect. Nebivolol inhibited accelerated haCSMC proliferation even in the presence of growth factors such as TGFβ1 and PDGF-BB. Nebivolol concentration-dependently induced a moderate apoptosis (10⁻⁹ mol/l: 23%) and a decrease of haCSMCs in the S-phase by 66%. HaECs showed comparable results. During nebivolol incubation NO formation of HaECs increased, while endothelin-1 transcription and secretion were suppressed.

Conclusion: Whereas classical β-blockers do not affect cell growth, only nebivolol inhibits haCSMC or haEC proliferation and induces a moderate rate of apoptosis. Furthermore, in HaECs NO formation increases and endothelin-1 secretion decreases suggesting that nebivolol may represent a β-blocker with great promises in CAD therapy.

Keywords: Endothelial function; Endothelins; Restenosis; Smooth muscle

1. Introduction

Despite significant improvements in the primary success rates of medical and surgical treatments of atherosclerotic disease, secondary failure due to late restenosis continues to occur in 30–50% of individuals [1]. Restenosis after vascular injury probably results from the interdependent actions of the ensuing thrombosis, inflammation, liberation of potent growth-regulatory molecules such as platelet-derived growth factor, basic fibroblast growth factor, and smooth muscle cell accumulation [2]. This involves proliferation, chemotactic cell migration into the intimal layers of the vessel, and secretion of extracellular matrix proteins [3,4].

β-Blockers play an important role in the treatment of ischemic heart disease [5–8]. According to their relative ability to antagonize the actions of sympathomimetic amines, β-adrenergic antagonists may be classified as β1-selective (i.e. metoprolol, bisoprolol) or nonselective (i.e. propranolol) inhibitors [9]. Moreover, certain β-blockers have additional vasodilating properties, such as carvedilol which blocks α-adrenergic receptors [10], or bucindolol

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which exerts direct vasodilating actions [10]. The new β-blocker nebivolol is a highly selective [11] and lipophilic β1-adrenergic receptor antagonist with vasodilating characteristics [12–14]. The drug is devoid of intrinsic sympathomimetic or membrane stabilizing activity [15,16]. Novel therapeutic agents to treat cardiovascular disease should possess some of the following properties: (1) improvement of prognosis in patients, (2) modulation of thrombosis and inflammation, (3) reversal of endothelial cell dysfunction, and (4) inhibition of vascular smooth muscle cell proliferation and migration.

The main aim of this study was to examine whether the new β-blocker nebivolol exerts growth-inhibitory effects on human coronary smooth muscle and endothelial cells. A further objective was to compare the antiproliferative properties of nebivolol with other β-blockers. Furthermore, the effect of nebivolol on NO and endothelin-1 formation in human coronary endothelial cells was analyzed.

2. Methods

2.1. Drugs

Propranolol and metoprolol were from Sigma (Deisenhofen, Germany). Bisoprolol was from Merck (Darmstadt, Germany) and carvedilol from Roche Molecular Biochemicals (Mannheim, Germany). Nebivolol (Berlin-Chemie, Berlin, Germany) is a lipophilic substance that was dissolved in 100% methanol and diluted with three volumes of growth medium to obtain a stock solution of 10⁻³ mol/l. All further dilutions were prepared in growth medium and the final methanol concentration in the experiments was 0.1%. Control cells received the diluent at equal concentrations.

2.2. Cell culture of HaECs and HaCSMCs

Human coronary arteries were obtained from two male patients with end-stage cardiomyopathy (without macroscopic signs of atherosclerosis) undergoing heart transplantation (approved by the local ethics committee). The patients were on a medication with digitonin, furosemide, enalapril and warfarin. Human coronary smooth muscle cells (haCSMCs) and human coronary endothelial cells (haECs) from the left descending coronary artery were passaged as described previously [17,18]. The growth rate of haCSMCs and haECs was identical in the two donors. HaCSMCs were grown in a mixture of Waymouth MB 752/1 medium and nutrient mixture F12 Ham (1:1, v/v) supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Gibco/BRL, Eggenstein, Germany). Smooth muscle origin was confirmed by immunocytochemical stainings using monoclonal antibodies against smooth muscle α-actin (Progen, Heidelberg, Germany).

HaECs were cultured on collagen-coated petri dishes in a special growth medium (EGM-2-2-MV Bullet kit, BioWhitakker, Verviers, Belgium) and fed every third day [19]. Passage numbers from 5 to 8 were used for the experiments. Mycoplasma contamination was excluded by DNA staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Roche Molecular Biochemicals).

2.3. Cellular assays of HaECs and HaCSMCs

Cells were seeded into 96-well plates at a density of 10 000 cells per well. Incubation with nebivolol (10⁻⁷–10⁻⁵ mol/l) was started 24 h after cells were adherent. After 1, 2, 4, 7 and 14 days, final cell numbers were measured by incorporation of bromodeoxyuridine (BrdU) using a cell proliferation ELISA (Roche Molecular Biochemicals) [18]. MTT tests, which measure metabolic activity as an indirect marker for cell proliferation, were performed according to the manufacturer’s instructions (Sigma) [18].

2.4. Measurement of apoptosis of HaECs and HaCSMCs

For determination of cell death haCSMC or HaECs were seeded into 6-well plates and treated with nebivolol (10⁻⁷–10⁻⁵ mol/l) for 24 h. The leakage of fragmented DNA from apoptotic nuclei was measured by the method of Nicoletti et al. [20]. Briefly, apoptotic nuclei were prepared by lysing cells in hypotonic lysis buffer (1% sodium citrate, 0.1% Triton X-100, 50 μg/ml propidium iodide) and analyzed by flow cytometry [18]. Nuclei to the left of the 2 N peak containing hypodiploid DNA were considered as apoptotic [21]. Apoptosis was additionally detected by annexin V staining. Cells were grown on coverslips and incubated with nebivolol for 24 h. Cells were rinsed PBS, pH 7.4, and incubated with FITC-labeled annexin V (Roche Molecular Biochemicals).

2.5. Growth factors

For investigating the effects of growth factors haCSMC were incubated with 20 ng/ml of either platelet-derived growth factor (PDGF)-AA, PDGF-BB or transforming growth factor β₁ (TGFβ₁), or 15 ng/ml basic fibroblast growth factor (bFGF, each from R&D System, Wiesbaden, Germany) in the presence of 10% FCS. Nebivolol was added 24 h after seeding of the cells. The concentrations inducing maximal stimulatory effects on haCSMCs were found in assays in which increasing concentrations (0.1, 10, 15, 20 and 40 ng/ml) of the growth factors were examined [18,22]. The medium containing drug (10⁻⁷–10⁻⁵ mol/l) and growth factors was exchanged every third day and haCSMC were incubated for 7 days.
2.6. Measurement of nitric oxide and endothelin-1 in haECs

Nitric oxide (NO) concentration was determined indirectly by the diazotization method using a calorimetric kit (Roche Molecular Biochemicals) [23]. For the assay 40,000 haECs were seeded into 6-well plates and 24 h later nebivolol was added. After 4 days of incubation growth medium was collected, combined with an equal volume of potassium phosphate, pH 7.5, and centrifuged at 2000 g for 45 min through a nitrater-free ultrafilter. The ultrafiltrate (0.5 ml) was incubated for 10 min with NADPH (0.65 μg/μl), nitrate reductase (0.1 mU/μl), 1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylene diamine dihydrochloride. Stabilized potassium nitrate was used as standard. The detection limit was 0.2 μmol/l nitrate.

Endothelin-1 (ET-1) concentrations were measured after 4 days of incubation using a specific ELISA (Immundiagnostik, Bensheim, Germany). The ELISA had a crossreac-
tion with ET-2, but did not detect ET-3 or Big-ET-1. The detection limit was 0.38 fmol/ml [24,25].

2.7. Prepro ET-1 cDNA cloning and in situ hybridization of HaCECs

Prepro ET-1 RNA probes for in situ hybridization were synthesized from a 541-bp-cDNA fragment. The cDNA was obtained from A10 cells by reverse transcription PCR using the sense primer GGAAACAGATGCCAGTGCT and the antisense primer CCAGACAGCAAGAAGAGGC [26,27]. The prepro ET-1 fragment (bp 503–1043) was cloned into the multiple cloning site of pGEM-T (Promega) flanked by Sp6 and T7 promoters. The insert was used as template for the generation of sense and antisense RNA probes and labeled with digoxigenin-UTP (Roche Molecular Biochemicals). In situ hybridization was performed as described by Ceol et al. [28]. Cells were fixed with 2% formaldehyde and washed in PBS. Prehy-
bridization buffer [50% formamide, 5% dextran sulfate, 500 μg/ml yeast t-RNA, 200 μg/ml herring sperm DNA, 10% DTT, 5×SSC (0.3 mol/l NaCl and 0.3 mol/l sodium citrate)] was applied to the cells for 3 h. The buffer was replaced by hybridization buffer (5 μg/ml DIG-labeled RNA probe in prehybridization buffer) for 12 h at 46°C. Slides were washed in 2×SSC for 15 min at 42°C, then for 15 min in 1×SSC at 37°C, 0.5×SSC at 37°C, 30 min in 0.5×SSC and then in PBS–Tween-20 (PBST). Slices were blocked with 0.5% BSA and sheep serum (1:50) in PBST for 30 min. Then, the gold-labeled anti-DIG antibody (1:30) was applied to the slides in 0.5% BSA–PBST for 30 min. After washing in PBST a silver enhancement reagent (Roche Molecular Biochemicals) was applied for 20 min and the cells were counterstained with eosin.

2.8. Statistical analysis

Statistical analyses were performed using analysis of variance and Student’s–Newman–Keuls test for the assessment of significance. Results in which two or more treatment groups were compared were tested by means of two-way ANOVA [18,22]. P values <0.05 were considered to denote statistically significant differences. The data are presented as the percentage of change compared to control cultures. Each experiment was performed with six replicates. The mean±S.E.M. of these was used for statistical comparison of three to six different sets of experiments. For each experiment cells of only one donor were used. The IC₅₀ is defined as the concentration that resulted in an inhibition of 50% in cases where a maxi-

mum effect could be seen or deduced.

3. Results

3.1. Effect of nebivolol on haCSMCs

haCSMCs were incubated with different concentrations of nebivolol. Even after short incubation periods of 24, 48 h (Fig. 1A) or 96 h (data not shown) the mitotic indices in the BrdU-ELISA were concentration-dependently reduced. After 7 days of incubation, a significant reduction in cell growth was apparent with an IC₅₀ of 6.1 μmol/l (Fig. 1B). The MTT test showed comparable results. Incubation for up to 14 days did not result in any drug resistance (BrdU ELISA: IC₅₀ 5.8 μmol/l, data not shown). Incubation for 7 days in the presence of nebivolol followed by incubation without nebivolol for 7 additional days also showed a concentration-dependent reduction in the growth rate (BrdU-ELISA: IC₅₀ 5.7 μmol/l, MTT test: IC₅₀ 6.0 μmol/ l, Fig. 1C).

3.2. Effect of nebivolol on growth factor-stimulated haCSMC proliferation

In order to evaluate whether nebivolol could inhibit accelerated smooth muscle cell proliferation, haCSMCs were incubated with different growth factors, i.e. PDGFs, bFGF and TGF β1. At the concentrations used all growth factors exerted a mitogenic effect (BrdU-ELISA: PDGF-BB 143±2.5%, P<0.001; bFGF 108±3.0%; TGF β1 139±2.0%, P<0.001; PDGF-AA 110±2.0%, P<0.05; control 100±2.6%, data not shown). An incubation with nebivolol for 7 days (10⁻⁷–10⁻⁵ mol/l) in the presence of the growth factors inhibited proliferation dose-dependently beginning at 10⁻⁷ mol/l (Fig. 2): PDGF-BB: IC₅₀ 6.8 μmol/l, bFGF: IC₅₀ 6.4 μmol/l, TGF β1: IC₅₀ 7.7 μmol/l.
3.3. Comparison of nebivolol with different β-blockers

Permanent incubation of haCSMCs with metoprolol, propranolol, bisoprolol, carvedilol or nebivolol (each $10^{-7}–10^{-5}$ mol/l) showed different growth-inhibitory effects. Continuous incubation with metoprolol or bisoprolol (data not shown) did not influence proliferation (Fig. 3A) or gross metabolic activity (Fig. 3B). Propranolol even induced a discrete increase in cell proliferation (BrdU-ELISA, 118±4.0% vs. control of 100±3.6%, n=6, P<0.01). Continuous incubation with carvedilol concentration-dependently reduced the proliferation (Fig. 3A). In contrast, MTT staining actually increased in the presence of carvedilol, suggesting that metabolic activity increased in a concentration-dependent fashion up to 130±3.6% at $10^{-5}$ mol/l (n=6, P<0.01, Fig. 4B). Nebivolol decreased both MTT staining and proliferation starting at a concentration of $2\times10^{-6}$ mol/l.

3.4. Effect of nebivolol on cell morphology

Microscopic analyses were performed to study morphological alterations induced by nebivolol in haCSMCs. With increasing nebivolol concentrations, haCSMCs became spindle-shaped and reduced their cell volume (data not shown). At a concentration of $4\times10^{-6}$ mol/l nebivolol, DAPI staining revealed disintegration and condensation of the nuclear chromatin structure indicating that nebivolol induced apoptosis (data not shown).
3.5. Effect of nebivolol on apoptosis in haCSMCs

In order to elucidate whether nebivolol induces apoptosis, formation of hypodiploid DNA was measured. haCSMCs were incubated for 24 h with growth medium before nebivolol was added for 48 h. Incorporation of propidium iodide as a marker for membrane damage was also measured. Incubation with nebivolol concentration-dependently induced apoptosis with an increase from 6% in control cells to 23% in cells treated with $10^{-5}$ mol/l nebivolol (Fig. 4). Equivalent data were obtained with annexin V staining (data not shown). Furthermore, there was a decrease in the number of cells in S-phase from 16% (control) to 5% (nebivolol $10^{-7}$ mol/l). The percentage of cells in G1-phase remained unchanged, while the proportion of haCSMCs in the G2-phase decreased from 24% (control) to 17% (nebivolol, data not shown). As a positive control, the apoptotic agent staurosporine ($10^{-5}$ mol/l) was added to the cells, resulting in 50% of cells undergoing apoptosis.

3.6. Effect of nebivolol on proliferation and apoptosis in haECs

Like in haCSMCs, nebivolol produced a concentration-dependent decrease in the mitotic indices of haECs (IC50 6.8 μmol/l BrdU-ELISA, data not shown). The MTT test revealed a statistically significant decrease in metabolic activity at $10^{-6}$ mol/l. Coincubation with 500 μmol/l 1-NAME, an inhibitor of NO synthesis, did not inhibit the decrease in cell proliferation indicating that the antiproliferative effect of nebivolol on haECs was not NO-dependent (data not shown).

Incubation of haECs with different β-blockers showed comparable results as in haCSMCs: metoprolol and bisoprolol did not influence haEC proliferation or gross metabolic activity (data not shown). Propranolol again induced a discrete increase in metabolic activity (BrdU-ELISA $116\pm5$ vs. control of $100\pm4\%$, n=4, $P<0.01$). Incubation with carvedilol reduced concentration-dependently the proliferation ($64\pm5\%$ at $10^{-5}$ mol/l), whereas MTT staining increased ($135\pm3.6\%$ at $10^{-5}$ mol/l, n=6, $P<0.01$, data not shown). Regarding apoptosis in haECs, comparable results were obtained as in haCSMCs with a decrease of cells in the S-phase from 16% to 4%, an increase of apoptotic cells from 7 to 25% (data not shown) and a reduction of the haECs in the G2-phase.

3.7. Effects of nebivolol on nitric oxide production in haECs

In order to determine whether nebivolol increased NO production in vitro, haECs were incubated with increasing concentrations of the drug for 4 days. NO was measured indirectly by analyzing the nitrate concentration in the growth medium. As shown in Table 1, an increase in nitrate formation was evident at $10^{-7}$ mol/l. Incubation...
Fig. 4. Effect of nebivolol on apoptosis and cell cycle progression. Flow cytometry of haCSMCs was performed after incubation with nebivolol for 24 h. The protein kinase inhibitor staurosporine (STS) was used as a positive control. Nebivolol induced significant apoptosis at a concentration of $4 \times 10^{-6}$ mol/l. HaCSMCs in the S-phase decreased concentration-dependently. Data are expressed as means±S.E.M. ($n=4$, Co, control with 0.1% methanol).

with 500 µmol/l L-NAME inhibited nebivolol-induced nitrate synthesis.

3.8. Endothelin-1 secretion in haECs

Since ET-1 has been implicated as an important mediator in the pathogenesis of atherosclerosis, basal ET-1 secretion into the growth medium was measured. In untreated cultures, haECs produced $0.5\pm0.05$ fmol/ml/10 000 cells ET-1 after 4 days (Table 2). In the presence of increasing concentrations of nebivolol, the ET-1 concentration in the growth medium decreased concentration-dependently to $0.13\pm0.01$ fmol/ml/10 000 cells ($10^{-5}$ mol/l, $-86\%$, $P<0.001$, $n=4$). In the presence of 500 µmol/l L-NAME, nebivolol was not able to reduce ET-1 secretion. Since ET-1 is not stored in vesicles but de novo synthesized, additionally in situ hybridization was performed to determine prepro ET-1 mRNA synthesis in haECs. As shown in Fig. 5, control cells produced high amounts of prepro ET-1 mRNA with a cytoplasmatic

Table 1

Influence of nebivolol on nitrate formation ($n=6$)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Nitrates±S.E.M. (µmol/10 000 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control−methanol</td>
<td>0.42±0.04</td>
</tr>
<tr>
<td>Control+methanol</td>
<td>0.38±0.03</td>
</tr>
<tr>
<td>Nebivolol 0.1</td>
<td>0.54±0.02*</td>
</tr>
<tr>
<td>Nebivolol 1</td>
<td>0.76±0.02*</td>
</tr>
<tr>
<td>Nebivolol 5</td>
<td>0.94±0.03</td>
</tr>
<tr>
<td>Nebivolol 10</td>
<td>0.98±0.03*</td>
</tr>
<tr>
<td>Nebivolol 10+L-NAME</td>
<td>0.42±0.03*</td>
</tr>
</tbody>
</table>

Table 2

Effect of nebivolol on ET-1 secretion ($n=4$)

<table>
<thead>
<tr>
<th>Condition</th>
<th>ET-1±S.E.M. (fmol/ml/10 000 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control without methanol</td>
<td>0.50±0.05</td>
</tr>
<tr>
<td>Control with methanol</td>
<td>0.55±0.05</td>
</tr>
<tr>
<td>Nebivolol 0.1</td>
<td>0.55±0.09</td>
</tr>
<tr>
<td>Nebivolol 1</td>
<td>0.54±0.04</td>
</tr>
<tr>
<td>Nebivolol 5</td>
<td>0.26±0.04*</td>
</tr>
<tr>
<td>Nebivolol 10</td>
<td>0.13±0.01*</td>
</tr>
<tr>
<td>Nebivolol 10+L-NAME</td>
<td>0.49±0.04</td>
</tr>
</tbody>
</table>
Fig. 5. In situ hybridization of prepro endothelin-1 mRNA expression. (A) Hybridization of control haECs with antisense prepro ET-1 shows strong cytoplasmic signals. (B) After 12 h of nebivolol (2×10^{-8} mol/l) incubation the cells show only weak prepro-ET-1 mRNA expression. (C) Control cells hybridized with a sense RNA probe reveal only faint nonspecific background staining.
localization. Nebivolol incubation for 12 h at $2 \times 10^{-6}$ mol/l inhibited completely the mRNA production of prepro ET-1.

4. Discussion

The β-blockers nebivolol and carvedilol inhibit the proliferation of human coronary smooth muscle cells very potently and concentration-dependently. An accelerated growth rate induced by different growth factors was significantly inhibited by nebivolol. Application of nebivolol for 7 days followed by incubation for 7 additional days without nebivolol did not reduce this growth-inhibitory effect. Furthermore, haCSMCs did not develop drug resistance after 14 days of continuous drug incubation. Proliferation of haCSMC in vivo is inhibited by increased NO formation [29]. During nebivolol incubation haECs increased NO liberation and reduced prepro ET-1 mRNA production with a subsequent decrease of ET-1 secretion. These effects of nebivolol may inhibit smooth muscle cell proliferation either directly or indirectly through an increased NO production of endothelial cells. These actions on both haECs and haCSMCs make nebivolol as a candidate drug for the prevention of restenosis or even the progression of atherosclerosis. One caveat of this study is that the cells were isolated from patients with end stage cardiomyopathy and not from healthy donors.

Concerning its effects on the cell cycle, nebivolol blocked the entry of cells into the G2 phase in haCSMCs and haECs. Measurement of hypodiploid DNA, annexin V and DAPI staining revealed that nebivolol dose-dependently induced apoptosis in maximally 25% of the cells without detectable signs of necrosis, whereas staurosporine induced apoptosis in more than 50% of the cells. This indicates that nebivolol induces only a moderately increased apoptotic rate in haCSMC and haECs. Such an acceleration of apoptotic death processes may reduce cellular components in atherosclerotic lesions, such that the integrity and stability of the plaques are maintained [30,31].

Since NO is an endogenous anti-atherosclerotic molecule and because it was shown that nebivolol induced relaxation of canine coronary arteries and human forearm arteries in vivo [12,32], we studied the effects of nebivolol on haECs. Incubation of haECs with $10^{-7}$ mol/l nebivolol increased NO formation at low concentrations. On the other side, ET-1 secretion and prepro-ET-1 mRNA expression dose-dependently decreased in the cells.

Such a spectrum of activities on haECs and haCSMCs has not been shown for any other classical β-blocker. Neither metoprolol, bisoprolol nor propranolol reduced proliferation, while only carvedilol reduced the growth rate
of haCSMCs by 40% [33,34]. Celiprolol even stimulated cell growth of rat smooth muscle cells in vitro [33]. These data suggest that the antiproliferative properties of nebivolol are not the result of a β-adrenergic receptor blockade. Growth-inhibitory effects were seen with the voltage-dependent calcium channel blockers nifedipine and verapamil using high concentrations in vitro [35,36]. Carvedilol, however, exerts mild calcium channel-blocking properties at high concentrations [35,36]. No evidence exists for an action of nebivolol on voltage-dependent calcium channels. Since nebivolol can inhibit the augmented proliferation of haCSMCs induced by various growth factors, the site of growth inhibition occurs at some point beyond growth factor receptor signaling at a distal common pathway. Nebivolol also irreversibly inhibited haCSMC proliferation without overt cytotoxicity indicating that it might interact with cell cycle-regulatory proteins. In haECs incubation with L-NAME did not inhibit the antiproliferative action of nebivolol indicating that NO is not responsible for growth inhibition.

Since ET-1 can increase mitogenesis of growth factors by amplifying their effects on DNA synthesis [37,38], the fact that nebivolol decreased ET-1 secretion is an important point. Increased ET-1 secretion has been shown in the early phase of atherogenesis [39,40] as well as in unstable angina [25]. ET-1 promotes the production of extracellular matrix and the formation of fibroproliferative lesions [41,42], whereas blockade of endothelin receptors inhibited neointima formation after balloon angioplasty in a rat model [39,42]. Thus, the inhibition of ET-1 secretion by nebivolol might contribute to its vasodilating action and represent a paracrine mechanism involved in the inhibition of haCSMC proliferation.

ET-1 secretion is inhibited by the release of endothelial NO [43,44]. In our experiments, L-NAME reduced nebivolol-induced NO release and increased ET-1 secretion, demonstrating that NO inhibits ET-1 secretion. This NO-liberating effect and the inhibition of ET-1 secretion may influence endothelial function. The vasoprotective actions of NO have been clearly demonstrated by the fact that gene transfer of endothelial NO synthase reduces proliferation of smooth muscle cells after balloon angioplasty in a rat model [29,45]. In humans, the endothelium of the internal mammary liberates higher concentrations of NO than veins. As a result, graft occlusions were found to occur less frequently during a 10-year follow-up period [46,47]. Since elevated NO levels may be involved in this process [46,47], it might influence restenosis or even inhibit fibromuscular atherosclerotic plaque formation in human coronary arteries. However, for atherosclerotic vessels changes of NO synthase expression and function as well as endothelial cell damage and increased oxidative stress were demonstrated [48,49]. These factors might limit the effect of nebivolol in vivo in atherosclerotic vessels.

It should be mentioned that the IC_{50} concentrations of nebivolol needed to inhibit proliferation of haCSMCs or haECs in vitro were higher than those expected from pharmacokinetic studies in humans. However, serum levels of β-blockers do not accurately reflect the local concentrations present in smooth muscle cells, because for instance propranolol has been demonstrated to accumulate in myocardium [50]. In case of carvedilol growth-inhibitory effects have been described at a dose of 10 μmol/l in vitro [33]. Although this represents a high concentration in vitro, neointima formation was reduced after balloon angioplasty using a dose of only 1 mg/kg in vivo. This indicates that the in vivo levels and the in vitro concentrations needed to produce antimitogenic effects are different. In addition to its growth inhibitory actions, nebivolol reduced ET-1 secretion and increased NO production at concentrations that can be achieved in vivo. Such additional mechanisms might further potentiate the anti-atherogenic effects of nebivolol in vivo by simultaneously acting on endothelial and smooth muscle cells.

In summary, nebivolol possesses several characteristics of a modern pharmacological drug for treating cardiovascular disease. It (1) showed a potent growth-inhibitory effect in the absence but also in the presence of different mitogens and (2) moderately induced apoptosis. Additionally, nebivolol may improve endothelial function by increasing NO formation, thereby decreasing ET-1 secretion.

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