Influence of Nebivolol and Metoprolol on Inflammatory Mediators in Human Coronary Endothelial or Smooth Muscle Cells. Effects on Neointima Formation After Balloon Denudation in Carotid Arteries of Rats Treated with Nebivolol

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Key Words

Atherosclerosis, carotid arteries • Muscle, smooth • Endothelial cell, β -blocker

Abstract

Objective and Background: Inflammation plays a critical role in all stages of atherogenesis. Proliferating vascular smooth muscle cells (SMC) and endothelial cells (EC) enhancing the inflammatory response, both contribute to the progression of atherosclerosis. Anti-proliferative, anti-inflammatory and anti-oxidative therapy seems to be a promising therapeutic strategy. The aim of this study was to assess the anti-proliferative and anti-inflammatory effect of the β -blocker nebivolol in comparison to metoprolol *in vitro* and to find out whether nebivolol inhibits neointima formation *in vivo*. **Methods and Results:** Real-time-RT-PCR revealed a decrease in VCAM-1, ICAM-1,

PDGF-B, E-selectin and P-selectin mRNA expression in human coronary artery EC and SMC incubated with nebivolol for 72 hours while metoprolol did not have this effect. Nebivolol reduced MCP-1 and PDGF-BB protein in the culture supernatant of SMC and EC, respectively. Sprague-Dawley rats were treated with nebivolol for 0 or 35 days before and 28 days after carotid balloon injury. Immunohistological analyses showed that pre-treatment with nebivolol was associated with a decreased number of SMC layers and macrophages and an increased lumen area at the site of the arterial injury. The intima area was reduced by 43% after pre-treatment. **Conclusion:** We found that nebivolol reduced the expression of proinflammatory genes in endothelial cells and vascular smooth muscle cells *in vitro* whereas metoprolol did not. *In vivo*, nebivolol inhibited neointima formation by reducing SMC proliferation and macrophage accumulation.

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Introduction

Oxidative stress, characterized by lipid and protein oxidation in the vascular wall, has been shown to play a central role in the development of endothelial dysfunction leading to inflammation and atherosclerosis [1].

Endothelial dysfunction triggers a cascade of events that contribute to the pathogenesis of atherosclerosis [2]. Apart from SMC proliferation, inflammation plays a central role in all stages of atherosclerosis. Leukocyte adhesion to endothelial cells results in the recruitment of macrophages and T-lymphocytes to the developing lesion, where they produce growth-promoting factors. The inhibition of inflammatory mediators and growth factors thus has to be an additional strategy to fight atherosclerosis and restenosis.

In several studies β -adrenergic receptor blockers have been shown to improve survival after myocardial infarction [3] and some evidence suggests that they may have antiatherosclerotic effects [4]. The activation of β -adrenergic receptors causes endothelial injury [5, 6] and promotes the development of atherosclerosis [7].

Since the connection between endothelial dysfunction and coronary artery disease is well established, to reverse endothelial dysfunction is an attractive pharmacological aim. One candidate drug to achieve this is nebivolol, a selective β_1 -receptor blocker with vasodilating properties [8, 9].

The aim of this study was to characterize the effects of the third generation β_1 -receptor blocker nebivolol in comparison to metoprolol on the expression of inflammatory genes and growth factors in human coronary artery EC and SMC *in vitro*. Moreover, we tested whether nebivolol reduces fibromuscular plaque formation after endothelial injury of the rat carotid artery *in vivo*.

Materials and Methods

Materials

Materials were from Sigma (Deideshofen, Germany) except for ketamine hydrochloride and xylazine which were from Parke-Davis (Leverkusen, Germany). Metoprololtartrate was a gift of Merckle (Ulm, Germany). Nebivolol was a gift of Berlin-Chemie (Berlin, Germany). Human coronary artery smooth muscle and endothelial cells (SMC, EC) and all cell culture media were cultured as described previously [10]. Smooth muscle origin was confirmed immunocytochemically using a monoclonal antibody against smooth muscle α -actin (Progen, Heidelberg, Germany). EC were grown in EGM-2-MV Bulletkit medium supplemented with 5% FCS. Passages 4 to 8 were used for the experiments. Mycoplasma contamination was excluded.

All ELISAs were from R&D Systems (Wiesbaden, Germany) and performed according to the manufacturer's instructions.

Methods

RNA isolation and real-time-RT-PCR. Total RNA was isolated from cells incubated with the indicated β -blockers (10^{-5} mol/l) for 72 hours using RNeasy (Tel-Test, Friendswood, USA). Aliquots of total RNA (1 μ g/50 μ l) were reverse-transcribed with TaqMan[®] Reverse Transcription Reagents (2.5 μ mol/l random hexamers, 62.5 U Multi Scribe[™] reverse transcriptase, 20 U RNase inhibitor, 5.5 mmol/l MgCl₂, 2 mmol/l dNTP mixture in a volume of 50 μ l Applied Biosystems) at 25°C for 10 min., 48°C for 30 min and 95°C for 5 min in a Peltier thermal cycler (MJ Research, USA). Subsequently, cDNA samples (2 μ l) were amplified in 20 μ l of 1x SYBR[®]-Green-PCR-Master Mix (Applied Biosystems, Branchburg, New Jersey, USA) and 300 nmol/l of the respective forward and reverse primers in an ABI Prism[™] 7000 sequence detection system (Applied Biosystems, Foster City, USA) (2 min. at 50°C, 10 min. at 95°C, 15 sec. at 95°C and 1 min. at 60°C for a total of 40 cycles). mRNA expression of treated (sample) cells relative to untreated (control) cells was determined using the $2^{-\Delta\Delta CT}$ method [11].

The primers designed using Primer Express[®] Primer Design Software v2.0 and purchased from Invitrogen (Karlsruhe, Germany) were as follows:

human P-selectin forward - CAA GTG CCC AGA ACT CTT TGC; human P-Selectin reverse - TCC ACG AGT GTC AGA ACA ATC C; human M-CSF-1 forward - GTG ACC AAG CCT GAT TGC AA; human M-CSF-1 reverse - GAG GCC GGG TCA CTG CTA; human E-selectin forward - TGT GCA AGT TCG CCT GTC C; human E-selectin-E reverse - CAT GTC CGA GCT GCA GAG C; human TLR4 forward - GTG GTG TCC CAG CAC TTC ATC; human TLR4 reverse - CTG CCA GGT CTG AGC AAT CTC; human IL-6 forward - GCA TGG GCA CCT CAG ATT G; human IL-6 reverse - TGC CCA GTG GAC AGG TTT CT; human bFGF forward - TTG TGT GCT GTT GCC GAA TAC; human bFGF reverse - GCG GTT CGA GAA GGT TTT GA; human PDGF-A forward - CGG AGG AGG AAG AGA AGC ATC GA; human PDGF-A reverse - TGA CCG TCC TGG TCT TGC A; human PDGF-B forward - CCA TTC CCG AGG AGC TTT ATG; human PDGF-B reverse - CAG CAG GCG TTG GAG ATC AT; human VCAM-1 forward - ACA AAG GCA GAG TAC GCA AAC A; human VCAM-1 reverse - GGC TGA CCA AGA CGG TTG TAT C; human GAPDH forward - CCA CCC ATC GCA AAT TCC; human GAPDH reverse - TGG GAT TTC CAT TGA TGA CAA G; human MCP-1 forward - GCT GAC CCC AAG CAG AAG TG; human MCP-1 reverse - TCT TCG GAG TTT GGG TTT GC; human IL-1 α forward - TCA AGG AGA GCA TGG TGG TAG TAG; human IL-1 α reverse - TGG CTT AAA CTC AAC CGT CTC TT; human GM-CSF forward - TTC TGC TTG TCA TCC CCT TTG; human GM-CSF reverse - CTC ATC TGG CCG GTC TCA CT.

Animals

Adult male Sprague-Dawley rats (Charles River, Kisslegg) underwent balloon-induced injury of the common carotid ar-

Fig. 1. Influence of nebivolol and metoprolol on the expression of PDGF-B mRNA in EC. In-situ hybridization of PDGF-B mRNA of representative cells is shown. Hybridization of control EC with antisense PDGF-B shows strong cytoplasmic signals (A). After 12 h of nebivolol incubation (10^{-5} mol/l) this signal was dramatically reduced showing only small amounts of PDGF-B mRNA (B). Incubation with metoprolol (10^{-5} mol/l) did not reduce the signal for PDGF-B mRNA (C). Control cells hybridized with sense-RNA-probe reveal only faint nonspecific background staining (D).

tery [12]. All surgical procedures were performed according to the guidelines of the ethics committee of the University of Tübingen. Rats weighing 420 to 530 g were anesthetized by intraperitoneal injection of ketamine (100 mg/kg bodyweight) and xylazine (5 mg/kg bodyweight). A cervical incision was made, and the left carotid artery was isolated. After dissection of the internal and external carotid bifurcation, the distal external carotid segment was ligated with a 4-0 silk suture. Through a small arteriotomy made in the external carotid, a 2F Fogarty balloon catheter (Baxter Edwards LIS, USA) was inserted and advanced 2 cm below the carotid bifurcation. The balloon was distended with saline to 2 atm using an ACS Inflator Plus (Advanced Cardiovascular Systems) and gently withdrawn to the level of the bifurcation. This was repeated for a total of three times. Finally, the balloon catheter was withdrawn and some

backbleeding was allowed through the arteriotomy to eliminate any air bubbles or blood clots. Then the external carotid artery was ligated proximal to the arteriotomy using 4-0 silk sutures. Food and water intake was identical in controls and nebivolol treated animals.

Drug administration

Nebivolol (10 mg/kg bodyweight) was administered with the normal drinking water in the presence of methylcellulose as solvent for 35 days before and 28 days after balloon angioplasty. One group of animals was treated for 28 days after balloon angioplasty without pre-treatment. To ensure drug resorption and β -blockade, ECGs were recorded. Under nebivolol therapy, heart rate decreased from 421 ± 12 bpm to 362 ± 7 bpm, respectively.

Fig. 2. Effect of nebivolol on neointima areas of rat carotid arteries. Animals were pre-treated for 35 days with nebivolol (n=6). Then balloon denudation was performed and animals were treated for an additional 28 days. Neointima area was measured in 3 representative sections. Nebivolol reduced neointima area significantly in treated groups. Intima area was calculated by quantitative morphometry.

Fig. 3. Reduction of neointima area of rats treated with nebivolol (10 mg/kg bodyweight). Representative carotid artery sections (5µm) are shown of control and nebivolol 35 days pre-treatment. Animals were treated with nebivolol. (A) shows control animals after 28 days without treatment after balloon denudation and (B) a representative section of a nebivolol treated animal.

Morphometry

The cross-sectional area of the intima and media of the lamina elastica interna was measured on Elastica van Gieson stained paraffin sections using a computerised image analysis system (KS 300, Zeiss, Germany). Smooth muscle cells were stained using a mouse monoclonal antibody against smooth muscle cell α -actin (clone: AC-74, Sigma-Chemie). Macrophages were detected using mouse anti-rat ED1 monoclonal antibody (Linaris, Wertheim, Germany) and counted in representative sections.

Statistical analysis

Values are expressed as mean $\pm$ SEM. Having checked homogeneity of variance using the F ratio test, Dunnett's test was used to compare different treatments. T-tests were done to compare the two groups control (no treatment) and nebivolol in the in vivo experiments. Differences were considered significant where $p < 0.05$.

Results

Effect of metoprolol and nebivolol on the expression of adhesion molecules in EC

VCAM-1 mRNA expression in EC was decreased from $100 \pm 0.75\%$ to $33.4 \pm 2.3\%$ (n=3, $p < 0.05$) after 3 days of incubation with nebivolol (10^{-5} mol/l). Metoprolol (10^{-5} mol/l) did not change VCAM-1 mRNA transcripts ($128.9 \pm 21.2\%$, n=3, n.s.). P-selectin mRNA also was reduced after nebivolol but not after metoprolol (control 100 ± 16 , n=3, nebivolol 38.7 ± 10.9 , n=4, $p < 0.05$, metoprolol $88.7 \pm 18.6\%$, n=3, n.s.). E-selectin mRNA reduction was seen after nebivolol ($20.9 \pm 2.1\%$, n=4, $p < 0.01$, control $100 \pm 17.9\%$) but not after metoprolol treatment (146.8 ± 17.9 , n=3, n.s.).

	Control	Treatment
Mean	0.1423	0.09621
SEM	0.006128	0.006627
n	9	6
p<		0.01

Table 1. Media area after nebivolol treatment (10 mg/kg bw). Areas are presented in mm² mean $\pm$ SEM.

	Control	Treatment
Mean	85.33	46.17
SEM	8.358	5.974
n	9	6
p<		0.01

Table 2. Number of intimal macrophages after nebivolol treatment (10 mg/kg bw).

Influence of nebivolol and metoprolol on growth factor expression in EC

After three days of incubation, nebivolol reduced PDGF-B mRNA transcripts in EC from 100 $\pm$ 10.7% to 60 $\pm$ 9.5% (n=4, p<0.05) whereas only a tendency towards a reduced expression was seen after incubation with metoprolol (76.68 $\pm$ 17.4%; n=4, n.s.). PDGF-A mRNA expression was not altered by both β -blockers (data not shown). In-situ hybridization revealed that mRNA of PDGF-B was reduced by nebivolol (Fig. 1A, 1B) but not by metoprolol (Fig. 1C). The concentration of the protein PDGF-BB in the cell culture supernatant was decreased from 2.7 $\pm$ 0.79 pg/10.000 cells (n=5) to 0.544 $\pm$ 0.350 pg/10.000 cells after three days of incubation with nebivolol (n=5, p<0.01) but not after incubation with metoprolol (1.38 $\pm$ 0.497 pg/10.000 cells, n=5, n.s.). bFGF mRNA (control 100 $\pm$ 7.6%, nebivolol 36.8 $\pm$ 12.1%, p<0.02, metoprolol 77.8 $\pm$ 27.7%, n=3, n.s.) and protein were reduced by nebivolol to 2.2 $\pm$ 0.255 pg/10.000 cells (p<0.01, control 4.6 $\pm$ 0.61 pg/10.000 cells, metoprolol 3.27 $\pm$ 0.25 pg/10.000 cells, n=5, n.s.). This is in line with the observation that nebivolol but not metoprolol (10⁻⁵ mol/l) had antiproliferative effects.

Impact of nebivolol and metoprolol on the expression of inflammatory mediators in EC

Toll-like receptor 4 mRNA expression in EC was reduced after incubation with nebivolol (10⁻⁵ mol/l) but not after metoprolol (10⁻⁵ mol/l) application (control 100 $\pm$ 8.5%, nebivolol 50.2 $\pm$ 9.3%, p<0.05, metoprolol 99.4 $\pm$ 2.9%, n=3, n.s.). MCP-1 transcripts were reduced after three days of incubation with nebivolol but unchanged after metoprolol (control 100 $\pm$ 0.24%, nebivolol

70 $\pm$ 10.43%, p<0.01, metoprolol 105 $\pm$ 7.7%, n=3, n.s.). The measurement of MCP-1 in the cell culture supernatant using an ELISA confirmed the downregulation under nebivolol (control 1517 $\pm$ 191, nebivolol 958 $\pm$ 138 (p<0.05), metoprolol 1302 $\pm$ 165 pg/ml/10 000 cells, n=3, n.s.). GM-CSF in the supernatant of the ECs was unchanged after three days of metoprolol incubation (3.153 $\pm$ 0.1096 pg/10⁶ cells, n=6, control 3.67 $\pm$ 0.27 pg/10⁶ cells, n=6, n.s.) but after nebivolol reduced to 0.6315 $\pm$ 0.129 pg/10⁶ cells, n=6, p<0.01). IL-6 mRNA was reduced by nebivolol (control 100 $\pm$ 4.4%, nebivolol 37 $\pm$ 7.0%, p<0.01, metoprolol 77 $\pm$ 15.6, n=3, n.s.).

Influence of nebivolol and metoprolol on the expression of MCP-1 in SMC

In SMC incubated with nebivolol for three days, MCP-1 mRNA expression was significantly reduced (control 100 $\pm$ 2.6%, nebivolol 59.7 $\pm$ 6.9% (n=3; p<0.01), metoprolol 84.6 $\pm$ 3.9% (n=3; n.s.)). After nine days of incubation with nebivolol, the concentration of MCP-1 in the cell culture supernatant was dramatically reduced (control 824.3 $\pm$ 113.9 pg/10.000 cells (n=5), nebivolol 44.7 $\pm$ 26.9 pg/10.000 cells (n=5, p<0.01), metoprolol 863.1 $\pm$ 66.4 pg/10.000 cells (n=5; n.s.)).

Effect of nebivolol and metoprolol on the expression of adhesion molecules and growth factors in SMC

Three days after incubation with nebivolol, the expression of PDGF-B mRNA was reduced in SMC (p<0.05, control 100 $\pm$ 18.7%, nebivolol 71.6 $\pm$ 15.8%, metoprolol 87.1 $\pm$ 20.2%, n=3, n.s.). PDGF-A mRNA was downregulated after nebivolol treatment but not after

metoprolol (data not shown). P-selectin mRNA expression was reduced by both nebivolol and metoprolol (control $100 \pm 8.0\%$, nebivolol $4.6 \pm 1.2\%$, metoprolol $5.2 \pm 2.3\%$, $p < 0.05$, $n = 3$). VCAM-1 mRNA was reduced after three days incubation with nebivolol ($33.4 \pm 2.4\%$, control $100 \pm 1.1\%$, $n = 3$, $p < 0.01$) but not with metoprolol ($128.9 \pm 30.0\%$, $n = 3$, n.s.).

Effects of nebivolol on plaque formation in rat carotid arteries after balloon angioplasty

In order to find out whether these changes in the expression of various pro-atherogenic factors induced by nebivolol might influence the induction of fibromuscular plaques *in vivo*, carotid artery denudation experiments were performed. Since metoprolol showed nearly no effects on gene expression in vascular cells, we only used nebivolol in our animal experiments.

Reduction of neointima formation under nebivolol

In rats treated with nebivolol for 35 days before and another 28 days after balloon-induced injury of the carotid artery, neointima formation was reduced by 42.6% (Fig. 2, control: $0.22 \pm 0.02 \text{ mm}^2$, $n = 9$, nebivolol $0.128 \pm 0.014 \text{ mm}^2$, $n = 6$, $p < 0.01$).

Animals that had received no pre-treatment, did not show a reduction in neointima formation after 28 days of nebivolol treatment after arterial denudation (data not shown).

Effect of nebivolol on intimal smooth muscle cells

In order to find out whether the number of SMC in the intima was reduced, α -actin positive cell layers were counted in representative sections. Pre-treatment for 35 days reduced the number of α -actin positive cell layers significantly in the nebivolol treated group (nebivolol 22 ± 3 cell layers, control 36 ± 2 layers, $n = 5$, $p < 0.005$, Fig. 3).

Effects of nebivolol on media

Media area was also reduced significantly in animals treated for 35 days (nebivolol 10 mg/kg bodyweight) before and 28 days after injury (Table 1). Animals that were not pre-treated showed no significant reduction in media area (data not shown).

Effects of nebivolol on intimal macrophages

Since macrophages play an important role in atherogenesis, the number of intimal macrophages was determined. Pre-treatment for 35 days before balloon-induced injury of the carotid artery with nebivolol reduced the

number of macrophages significantly (Table 2).

Discussion

A critical event in the initiation of atherosclerosis is the adherence of mononuclear cells to the endothelium and their extravasation. These processes require the expression of adhesion molecules by endothelial cells, including P- and E-selectin and VCAM-1 [13]. In cholesterol-induced lesions, VCAM-1 expression rises before the infiltration of leukocytes into the vessel wall [14]. Hypomorphic variants of VCAM-1 introduced in mice sensitive for atherosclerotic plaque formation by apoE gene deficiency showed reduced lesion formation [13].

We found that nebivolol decreased VCAM-1 mRNA expression in ECs whereas metoprolol did not show any effect. Nebivolol also reduced P- and E-selectin mRNA expression in ECs. These members of the selectin family mediate leukocyte "rolling" which stands at the very beginning of the cascade of events leading to plaque formation. P- and E-selectin also seem to contribute to leukocyte recruitment in atherosclerosis susceptible mice [15, 16]. In LDL-receptor and E-/P-selectin double-deficient mice the plaque size was reduced by about 40% after 8, 22 and 37 weeks indicating that both selectins play an important role [16]. Even a single injection of anti-P-selectin antibodies limits plaque formation after carotid denudation in apolipoprotein deficient $E^{-/-}$ mice with a significantly smaller number of macrophages [17].

In mice susceptible to atherosclerosis a decrease of vascular monocyte accumulation was found when they lacked the MCP-1 gene or its receptor [18, 19]. The blockade of MCP-1 limited the progression of the atherosclerotic lesion and makes the plaque more stable [20]. Nebivolol reduced MCP-1 mRNA and protein expression in EC. After nine days of incubation with nebivolol, MCP-1 protein was almost undetectable in the cell culture supernatant. Incubation of EC and SMC with nebivolol reduced GM-CSF gene expression and protein concentration.

Accordingly, nebivolol also reduced the number of macrophages in the atherosclerotic site after balloon-induced injury of the rat carotid artery. Macrophages accumulated at the site of a developing atherosclerotic lesion contribute to plaque formation [21]. In our cell culture experiments, nebivolol reduced the expression of the proatherogenic growth factor PDGF-BB in EC. Metoprolol did not have this effect. The combination of genes downregulated by nebivolol led us to the hypoth-

esis that some central mediators might be responsible for these biochemical effects. One possible candidate is nuclear factor- κ B (NF- κ B). This transcription factor controls the gene expression of cytokines, chemokines, growth factors, and cell adhesion molecules as well as some acute phase proteins [22, 23]. NF- κ B itself is activated by oxidative stress e.g. OH $\cdot$. For nebivolol itself antioxidative actions were described [24, 25].

In rats pre-treated with nebivolol for 35 days, we observed a reduced neointima formation as well as a reduction in the number of SMC layers in the intima. The media area was reduced in animals pre-treated before balloon injury. Without pre-treatment, there was no reduction. We found that macrophage invasion in the intima and SMC proliferation was reduced in rats treated with nebivolol.

Nebivolol might also improve endothelial function caused by an improved NO liberation and reduced oxidative stress [9, 10, 26]. Moreover, reactive oxygen radicals were reduced in a model with hypercholesteremic rabbits by reducing O $_2^{\cdot-}$ [25, 27] in the vessel wall. Nebivolol possesses several antioxidative characteristics, it directly interacts with reactive oxygen radicals [24, 25]. In a rat model of angiotensin II-mediated hypertension it could be shown that nebivolol induced a dissociation of p67 (phox) and Rac1, and additionally an inhibition of NADPH oxidase activity in rat heart membranes. These effects were not demonstrated in rats treated with metoprolol [28]. Nebivolol reduces O $_2^{\cdot-}$ and ONOO $\cdot$ concentrations and restores NO bioavailability in blacks, independently of beta1-selective blockade [29]. In hypertensive patients nebivolol was able to reverse endothelial

dysfunction [9]. On the other side platelets play an important role in the initiation of atherosclerosis. Nebivolol inhibits platelet activation in hypertensive patients [30, 31]. All these characteristics make nebivolol a candidate drug that might inhibit the progression of atherosclerosis. However, the fact that nebivolol inhibited neointima formation only if administered before balloon catheter injury represents a limitation for patients who need urgent angioplasty.

In conclusion, these data show that nebivolol reduces neointima formation after endothelial injury. However, a pre-treatment phase is required to induce these vasculo-protective effects. *In vitro*, a number of NF- κ B dependent genes were downregulated (e.g. VCAM-1, E-selectin, MCP-1). This might explain the effects of nebivolol on plaque formation observed in our *in vivo* experiments. Metoprolol on the other hand seems to be a pure β -adren-ergic receptor antagonist without any of the effects on the expression of pro-atherogenic mediators. These results suggest that nebivolol may be clinically useful in preventing and treating progression of atherosclerosis. However, this has to be shown in large clinical studies.

Abbreviations

SMC (smooth muscle cells); EC (endothelial cells); VCAM-1 (vascular cell adhesion molecule 1); ICAM-1 (intercellular adhesion molecule 1); PDGF-B (platelet-derived growth factor B); MCP-1 (monocyte chemotactic protein-1); NO (nitric oxide); bFGF (basic fibroblast growth factor); IL-6 (interleukin-6).

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