Carvedilol Inhibits Tumor Necrosis Factor-α–Induced Endothelial Transcription Factor Activation, Adhesion Molecule Expression, and Adhesiveness to Human Mononuclear Cells


Objective—We tested the hypothesis that carvedilol, a β-adrenoceptor and α-adrenoceptor antagonist with potent antioxidant property, could inhibit tumor necrosis factor-α (TNF-α)–induced endothelial adhesiveness to human mononuclear cells (MNCs), an early sign of atherogenesis.

Methods and Results—Circulating MNCs were isolated from the peripheral blood of healthy subjects. Compared with control condition, pretreatment of carvedilol (10 μmol/L for 18 hours) or probucol (5 μmol/L for 18 hours), but not propranolol, prazosin, or both propranolol and prazosin significantly decreased TNF-α–stimulated adhesiveness of cultured human aortic endothelial cells (HAECs) to MNCs. Carvedilol inhibited TNF-α–stimulated endothelial vascular cell adhesion molecule-1 (VCAM-1) and E-selectin (66.0±2.0% and 55.60±1.0% of control, P<0.05, respectively) expression, whereas probucol inhibited only VCAM-1 expression (79.0±5.0% of control, P<0.05). Propranolol, prazosin, or both did not alter the expression of adhesion molecules. Further, pretreatment with carvedilol significantly inhibited TNF-α–stimulated intracellular reactive oxygen species (ROS) production and the activation of redox sensitive nuclear factor kappa B and activator protein-1 transcription pathways.

Conclusions—Carvedilol reduced TNF-α–stimulated endothelial adhesiveness to human MNCs by inhibiting intracellular ROS production, transcription factor activation, and VCAM-1 as well as E-selectin expression, suggesting its potential role in clinical atherosclerosis disease. (Arterioscler Thromb Vasc Biol. 2004;24:2075-2081.)

Key Words: antioxidant ■ atherosclerosis ■ carvedilol ■ cell adhesion molecules ■ endothelium

In the early phase of atherogenesis, cell surface adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), and endothelial cell selectin (E-selectin) could express on endothelial cells to recruit circulating mononuclear cells (MNCs), mainly monocytes, and facilitate their binding to endothelium and migrating to subendothelial space.1–3 Reactive oxygen species (ROS) may serve as a common intracellular messenger for various redox-sensitive transcription pathways that lead to adhesion molecule expression in vascular endothelial cells.4,5 Accumulating in vitro and in vivo evidence showed that substances with antioxidant activity such as probucol and ginkgo biloba extract could scavenge intracellular ROS and inhibit endothelial adhesiveness to monocytes by reducing the expression of various adhesion molecules.4,6 However, because of the presence of significant side effects or lack of clinical evidence, these drugs are rarely used for human atherosclerosis disease.8 Carvedilol, 1-[(carbazol-4-yl)-3-[(2-methoxyphenoxymethyl)-amino]-propanol-2, a nonselective β-adrenoceptor antagonist with α1 adrenoceptor blocking and potent antioxidant activity, is currently used for treatment of hypertension or symptomatic heart failure.9,10 Previous in vitro evidence showed that carvedilol, as an oxidant scavenger, could inhibit cardiomyocyte membrane lipid peroxidation and protect cells from oxidant injury.11 It is further suggested that the clinical benefits of carvedilol in cardiac protection might be largely attributed to its antioxidant activity.9,12 Recently, carvedilol was shown to reduce low-density lipoprotein oxidation in vitro13,14 and inhibit smooth muscle cell migration, proliferation, or neointimal formation after vascular injury in vivo.15 However, the direct mechanism of carvedilol on endothelial protection has not been fully clarified. We then tested the hypothesis that carvedilol may reduce tumor necrosis factor-α (TNF-α)–induced intracellular ROS production, redox-sensitive transcription pathway activation, and adhesion molecules expression, resulting in the inhibition of endothelial adhesiveness to human MNCs in vitro—an early sign of atherogenesis. These findings may provide a novel rationale to the possible role of carvedilol in clinical atherosclerosis disease.
Methods

Reagents
Carvedilol, obtained from Roche, was dissolved in dimethyl sulfoxide as a stock solution. 1-(Isopropylamino)-3-(1-naphthoxy)-2-propanol hydrochloride (abbreviated as propranolol), a synthetic β-adrenergic receptor blocking agent, prazosin, a synthetic α-adrenergic receptor blocking agent, and probucol, an antioxidant that can inhibit in vivo atherosclerosis and in vitro atherogenesis,16 were used as contrast groups. Propranolol, prazosin, and probucol were purchased from Sigma and dissolved in phosphate-buffered saline or alcohol, respectively. Before experiments, the stock reagents solutions were diluted as selected concentrated working solution with medium 200 (Cascade Biologics).

Endothelial Cells Culture
Human aortic endothelial cells (HAECs) were isolated from aortic portion of donors by Cascade Biologics. Cells were identified positive for the von Willebrand factor and CD31 and negative for α-actin by immunohistochemistry. HAECs were cultured in endothelial cell-supplemented medium 200 containing 2% fetal bovine serum, 10 ng/mL human epidermal growth factor, 10 μg/mL heparin, 100 U/mL penicillin, 100 pg/mL streptomycin, and 1.25 μg/mL fungizone. In this experiment, HAECs with 2 to 8 passages were used and cells were grown in a 5% CO2 humidified atmosphere at 37°C. Fresh medium was changed every third day.

Measurement of Reagents Cytotoxicity
The cell cytotoxicity of experimental drugs were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (please see http://atvb.ahajournals.org).

Evaluation for the Viability of Endothelial Cells
The viability of HAECs was evaluated by examining the cellular morphology counterstained with hematoxylin and by measuring the lactate dehydrogenase (LDH) concentration in culture medium in the absence or presence of TNF-α and/or carvedilol.

Intracellular ROS Production Assay
2',7'-dihydrodichlorofluorescein diacetate (Molecular Probes) was used to measure intracellular H₂O₂ production in HAECs. When the studies were performed, the cells were loaded with 20 μmol/L of 2',7'-dihydrodichlorofluorescein diacetate for 45 minutes after being treated with 2 or 10 μmol/L of carvedilol, 5 μmol/L of probucol, or 10 μmol/L of propranolol for 18 hours in 48-well plates. HAECs were washed with warm HBSS 3 times and subsequently stimulated with 2 ng/mL TNF-α. The intracellular ROS production of HAECs was counted by CytoFluor 2300 (Millipore) at emission of 530 nm and absorption of 485 nm after TNF-α was stimulated for 10, 20, 40, or 60 minutes.

Cell Enzyme-Linked Immunosorbent Assay
The detailed procedure of cell enzyme-linked immunosorbent assay has been previously described.9 In brief, to measure the expression of cell surface adhesion molecules, the cell enzyme-linked immunosorbent assay was used. Confluent HAECs in 96-well plates were pretreated with probucol, carvedilol, propranolol, prazosin, or both propranolol and prazosin in the selected concentration for 18 hours before being stimulated with 2 ng/mL TNF-α for the indicated time. The expressions of VCAM-1 and ICAM-1 were evaluated after TNF-α stimulation for 6 hours and the expression of E-selectin was evaluated after 3 hours of stimulation (Please see http://atvb.ahajournals.org).

Western Blot
Western blot analysis was performed for the detection of VCAM-1, ICAM-1, or E-selectin expression by using goat IgG. Confluent HAECs were pretreated with carvedilol (10 μmol/L), probucol (5 μmol/L), propranolol (10 μmol/L), prazosin (10 μmol/L), or both propranolol (10 μmol/L) and prazosin (10 μmol/L) for 18 hours in 100-mm culture dishes, then stimulated with 2 ng/mL TNF-α for 6 hours or 3 hours, respectively. The activated cells were scraped and the cytoplasm total protein was extracted. The protein extracts were applied to 10% SDS-PAGE and blotted to polyvinylidine difluoride membrane. The blotted polyvinylidine difluoride membrane was incubated with phosphate-buffered saline/Tween-20 (0.05%) solution containing 2% skim milk for blocking nonspecific antigen and incubated with goat anti-human VCAM-1, ICAM-1, or E-selectin antibodies (1 μg/mL), respectively, at room temperature for 1 hour. After primary antibody reaction, the membranes were incubated with horseradish peroxidase-conjugated rabbit anti-goat monoclonal antibodies (0.25 μg/mL), detected by chemiluminescence reagent (NEN), and exposed to film (BioMax MR; Kodak).

Electrophoretic Morbility Shift Assay
HAECs were pretreated with carvedilol (10 μmol/L) or propranolol (10 μmol/L) for 18 hours and then stimulated with TNF-α (2 ng/mL) for 30 minutes at 37°C before nuclear protein was extracted. The NF-kB oligonucleotide (5'-AGT TGA GGG GAC TTT CCC AGG C-3’ and 3’-trichloroacetic acid [TCA] ACT CCC CTG AAA GGG TCC G-T-5’) or AP-1 oligonucleotide (5’-CCG GAA-3’ and 3’-GGG AAC TCT GCA GTC GCC GGT T-5’) (Promega) were end-labeled with [γ-32P]ATP (3000 Ci/mmol at 10 mCi/ mL). The labeled oligonucleotide could be separated from unincorporated nucleotides by chromatography through a Sephadex G-25 spin column (Pharmacia) equilibrated in TE buffer. Nuclear protein extracts (20 μg) were incubated with 32P-labeled NF-κB or AP-1 consensus oligonucleotide for 20 minutes before loading to 6% nondenaturing polyacrylamide gel and running the gel at room temperature in 0.25X TBE buffer at 250 volts until the bromophenol blue dye was below the gel. When the electrophoresis was complete, the gel was exposed to X-ray film (BioMax MS; Kodak).

MNC Isolation
Human MNCs were isolated from the peripheral venous blood in the forearms of healthy adults. Immediately after drawing blood, 30 mL whole blood was transferred to cell preparation tubes containing 0.1 mol/L sodium citrate solution. The tubes were inverted 10 times gently. Then, the blood was centrifuged and washed with EDTA/HBSS solution as per operation instructions. The isolated MNCs were resuspended with RPMI 1640 medium, which was serum-free for labeling. When the labeling procedure was completed, the RPMI1640 medium was removed by centrifugation and the cells were washed with EDTA/HBSS, which contained 2 mmol/L Ca²⁺, 2 mmol/L Mg²⁺, and 20 mmol/L HEPES. The viability of the isolated MNCs was assessed using trypan blue exclusion. There was 95% viability, at least in all samples used in these experiments. The isolation procedures were performed at room temperature within 2 hours after the blood was drawn.

MNC and Endothelial Cell Adhesion Assay
Before the experiments of adhesion assay, MNCs were labeled with 10 μmol/L 2',7'-bis(2-carboxyethyl)-5'-carboxyfluorescein acetoxyethyl ester (BCECF/AM) (Boehringer-Mannheim) in serum-free RPMI1640 medium for 45 minutes at 37°C in darkness, then the surplus 2',7'-bis(2-carboxyethyl)-5'-carboxyfluorescein acetoxyethyl ester was washed with HBSS. Labeled MNCs were added to control or treated HAECs that were confluent in 6-well plates, and 3×10⁵ cells of leukocyte were added for each well. After incubation at 37°C for 1 hour, nonadherent cells were removed with gentle HBSS washing 3 times. The degree of MNC adhesion to HAECs was counted by Multilabel Counter Victor® II (Wallace) at emission of 530 nm and absorption of 435 nm after cells were lysed with DMSO.

Statistical Analysis
The data were expressed as the mean±SEM and analyzed by ANOVA program, followed by Dunnett test. P<0.05 was considered statistically significant.
Results

Cell Cytotoxicity of Experimental Drugs
The cell cytotoxicity of carvedilol, prazosin, propranolol, and probucol were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. It was shown that the dosage used of each drug in the following experiments did not affect the cell viability of HAECs (Please see http://atvb.ahajournals.org). Furthermore, TNF-α or carvedilol or both together did not impair the viability of HAECs determined by the morphology of HAECs and the LDH concentration in the culture medium (Figure I, available online at http://atvb.ahajournals.org).

Carvedilol Inhibited the Adhesiveness of MNCs to TNF-α–Stimulated HAECs
HAECs were pretreated with 10 μmol/L carvedilol, 10 μmol/L prazosin, 10 and 50 μmol/L propranolol, and 5 μmol/L probucol for 18 hours before being stimulated with 2 ng/mL TNF-α, then interacted with 2’7’-bis(2-carboxyethyl)-5’-carboxyfluorescein acetoxymethyl ester–labeled MNCs. The MNC binding to TNF-α–stimulated HAECs was significantly increased as compared with control. Pretreatment with 5 μmol/L probucol or 10 μmol/L carvedilol, but not prazosin or propranolol, significantly inhibited MNC binding to TNF-α–stimulated HAECs. Coincubated with prazosin and 10 or 50 μmol/L of propranolol did not inhibit MNC adhesiveness to HAECs (Figure 1a to 1i). The cells were then lysed with DMSO and their relative fluorescence units were counted by fluorometer (Multilabel Counter Victor). As shown in Figure 1j, probucol and carvedilol significantly inhibited MNC binding to TNF-α–stimulated HAECs by 52.1±6.2% and 51.0±4.0% (P<0.05), respectively.

Carvedilol Reduced TNF-α–Induced Expression of VCAM-1 and E-Selectin in HAECs
The effects of carvedilol on VCAM-1, ICAM-1, and E-selectin expression on TNF-α–stimulated HAECs were determine by cell enzyme-linked immunosorbent assay (Figure 2). High-dosage (10 μmol/L) but not low-dosage (2 μmol/L) carvedilol significantly inhibited TNF-α–induced VCAM-1 and E-selectin (66±2% and 55±1%) but not ICAM-1 expression (106±22%) in HAECs. Pretreatment with 5 μmol/L probucol significantly downregulated VCAM-1 but not ICAM-1 or E-selectin expression (79±5%, 104±2%, and 109±4%, respectively). Pretreatment with prazosin or propranolol or both had no effects on any cell adhesion molecule expression. The findings of cell enzyme-linked immunosorbent assay were confirmed by Western blot analysis (Figure 3). Pretreatment with 5 μmol/L probucol or 10 μmol/L carvedilol significantly inhibited VCAM-1 expression, but only the latter significantly inhibited E-selectin expression. TNF-α–induced ICAM-1 expression was not altered by the aforementioned treatments.

Carvedilol Attenuated TNF-α–Induced NF-κB and AP-1 Activation in HAECs
TNF-α induced the nuclear expression of NF-κB and AP-1 in HAECs, which was not seen in control condition. Pretreatment with carvedilol significantly reduced the densities of the NF-κB and AP-1 shifted bands induced by TNF-α (Figure 4). Propranolol had no such effects. Supershift bands were also performed with anti-p65 or anti-c-jun antibodies to confirm the specific presence of bands to NF-κB or AP-1 (Figure II, available online at http://atvb.ahajournals.org).

Carvedilol Reduced Intracellular ROS Formation in TNF-α–Stimulated HAECs
Stimulation with 2 ng/mL TNF-α significantly increased intracellular H₂O₂ production in HAECs in a time-dependent manner, beginning at 20 minutes of exposure (Figure 5A and 5B). Pretreatment with 2 μmol/L or 10 μmol/L carvedilol for 18 hours could reduce TNF-α–induced H₂O₂ formation in HAECs. The effect of 5 μmol/L probucol was similar to that of 10 μmol/L carvedilol. Pretreated with 10 μmol/L propranolol did not alter the H₂O₂ production in TNF-α–stimulated HAECs (Figure 5B).
Discussion

During early atherosclerosis, circulating monocytes and lymphocytes may interact with VCAM-1, whereas neutrophils interact with ICAM-1 on endothelial cells to establish the firm adhesion, a critical step of atherogenesis. In the present study, MNCs were isolated from the peripheral blood of healthy adults for the in vitro adhesiveness of HAECs, which is similar to the firm adhesion during in vivo atherogenesis.

Carvedilol was shown to decrease TNF-α-stimulated endothelial VCAM-1 and E-selectin expression, whereas probucol reduced VCAM-1 only. However, both could inhibit endothelial adhesiveness to human MNCs in a similar degree. Taken together, VCAM-1 may play a crucial role in this in vitro model of atherogenesis, which is compatible with our recent findings that serum level of soluble VCAM-1, rather than soluble ICAM-1, was correlated to the angiographic severity of coronary artery stenosis.

During early atherogenesis, cytokines such as TNF-α may activate membrane-bound NADPH oxidase and increase intracellular ROS production, which could then activate redox-sensitive transcriptional pathway such as NF-κB and induce the expression of VCAM-1 in vascular endothelial cells. Antioxidants such as probucol and pyrrolidine dithiocarbamate could inhibit in vitro endothelial VCAM-1 expression by downregulating NF-κB activation and prevent in vivo atherogenesis. Recent evidence also sug-

Figure 2. The expression of VCAM-1, ICAM-1, and E-selectin in TNF-α-stimulated HAECs determined by cell enzyme-linked immunosorbent assay. HAECs were pretreated with 10 μmol/L carvedilol, 10 μmol/L prazosin, 10 μmol/L propranolol, or 5 μmol/L probucol for 18 hours, followed by TNF-α stimulation (2 ng/mL for 6 hours). The expression of VCAM-1 was significantly inhibited by carvedilol or probucol. The expression of ICAM-1 was not changed by either one of these drugs. The expression of E-selectin was significantly inhibited by carvedilol only. Data are expressed as the mean±SEM of 3 experiments in triplicate. *P<0.05 as compared with TNF-α-stimulated HAECs.

Figure 3. The expression of VCAM-1, ICAM-1, and E-selectin in TNF-α-stimulated HAECs determined by Western blot. HAECs were pretreated with 10 μmol/L carvedilol, 10 μmol/L prazosin, 10 μmol/L propranolol, and 5 μmol/L probucol for 18 hours, followed by TNF-α stimulation (2 ng/mL for 6 hours). The expression of VCAM-1 was reduced by probucol and by carvedilol but not by prazosin or propranolol or combination treatment. The expression of ICAM-1 was not changed by either one of these drugs. The expression of E-selectin was significantly reduced by carvedilol only. Three independent experiments gave similar results. Summarized data (mean±SEM) are shown as a bar graph from 3 separate experiments by densitometry.
suggested that carvedilol might exert endothelial protection via multiple mechanisms such as inhibiting low-density lipoprotein oxidation by scavenging ROS,7,14 preserving intracellular mitochondrial function by reducing excessive oxidative stress,25 inhibiting xanthine–xanthine oxidase-induced LDH release,26 enhancing vasodilatation by inhibition of endothelin-1 biosynthesis,27 stimulating endothelial nitric oxide release through ATP efflux,28 and preventing endothelial apoptosis through modulation of Fas/Fas ligand and caspase-3 pathway.29,30 Our findings further indicated that carvedilol could inhibit redox-sensitive transcription factor NF-κB and AP-1 activation in HAECs. Interestingly, carvedilol or probucol, but not propranolol, a synthetic nonselective β-adrenergic receptor blocker, or prazosin, a synthetic α1-adrenergic receptor blocker, or both together, could significantly inhibit TNF-α-stimulated adhesion molecules expression as well as endothelial adhesiveness to human MNCs. Accordingly, antioxidant activity other than α- or β-adrenoceptor antagonist activity may contribute to the antiatherogenesis effect of carvedilol.

In the present study, pretreatment with carvedilol reduced endothelial expression of VCAM-1 and E-selectin, whereas probucol only inhibited the former. Thus, the redox-sensitive NF-κB activation, which could be inhibited by either carvedilol or probucol, is critical to TNF-α-stimulated VCAM-1 expression.10,31,32 However, the inhibition of both NF-κB and AP-1 activation seems required for the inhibition of TNF-α-stimulated E-selectin expression. Different from the significant increase by strong (10 ng/mL) TNF-α stimulation,33,34 E-selectin was mildly increased by 2 ng/mL of TNF-α.35,36 Recent evidence suggested that although superoxide is crucial to TNF-α-induced VCAM-1 expression, both superoxide and H2O2 contribute to E-selectin expression in endothelial cells.37,38 Furthermore, endothelial E-selectin gene may present DNA-binding sequences for both NF-κB and AP-1,79 and multiple NF-κB binding sites in the human E-selectin gene are required for maximal TNF-α-induced expression.80

then speculated that the inhibitory effect of carvedilol on TNF-α–induced E-selectin expression might be related to its broad antioxidant capacity with the inhibition of both NF-κB and AP-1 activation.10,11,41 Interestingly, although both NFκB and AP-1 action were significantly inhibited, ICAM-1 expression was not suppressed by carvedilol. In fact, both redox-sensitive NF-κB and AP-1 activation42 and redox-independent NF-κB activation43,44 may contribute to TNF-α–induced endothelial ICAM-1 expression. The limited effect of carvedilol shown in the present study is compatible with the notion that redox-independent NF-κB activation is critical to ICAM-1 expression in TNF-α–stimulated HAECs.42,45 (Please see http://atvb.ahajournals.org).

In the present study, the concentration of carvedilol used in vitro was compatible with that usually used for clinical hypertension and heart failure.9 The in vitro protection by

**Figure 4.** The gel shift assay for activation of NF-κB and AP-1 in HAECs. Compared with untreated control (lane a), TNF-α (lane b; 2 ng/mL for 30 minutes) increased the activity of NF-κB and AP-1 in HAECs. Pretreatment with 10 μmol/L of carvedilol for 18 hours (lane c) inhibited the activation of NF-κB and AP-1 stimulated by TNF-α. Pretreatment with 10 μmol/L of propranolol for 18 hours (lane d) had no such effects. Three separate experiments gave similar results.

**Figure 5.** A, Representative fluorescent photomicrographs show the effects of carvedilol on H2O2 generation in TNF-α–stimulated HAECs. Untreated control HAECs (a). HAECs stimulated by 2 ng/mL TNF-α for 20 minutes (b). HAECs stimulated by 2 ng/mL TNF-α for 60 minutes (c). HAECs pretreated with 5 μmol/L probucol for 18 hours followed by TNF-α stimulation (2 ng/mL for 60 minutes) (d). HAECs pretreated with 10 μmol/L carvedilol for 18 hours followed by TNF-α stimulation (2 ng/mL for 60 minutes) (e). HAECs pretreated with 10 μmol/L propranolol for 18 hours followed by TNF-α stimulation (2 ng/mL for 60 minutes) (f). Three independent experiments gave similar results. B, The inhibitory effects of carvedilol on H2O2 production in HAECs. Compared with control groups (○), TNF-α treatment (2 ng/mL for 10, 20, 40, or 60 minutes) significantly increased H2O2 production in a time-dependent fashion in HAECs (□) (P<0.05). Pretreatment with 2 μmol/L (□) or 10 μmol/L (●) carvedilol or 5 μmol/L probucol (▲) significantly decreased TNF-α–stimulated H2O2 production in HAECs. Pretreatment with 10 μmol/L propranolol (●) did not affect TNF-α–stimulated H2O2 production in HAECs. The data were presented with 3 independent experiments.
carvedilol from oxygen free-radical–induced damage could be attained at concentrations that are consistent with the plasma levels of the drug attained in patients on a dose of 25 to 50 mg per day (ie, 100 to 300 nmol/L). However, it was recently shown that carvedilol treatment failed to reduce restenosis after directional coronary artherectomy in patients with coronary artery disease. In that study, carvedilol was given orally 24 hours before the procedure. Such duration might not be enough for a full vascular protection of carvedilol in vivo. In the present study, carvedilol was given 18 hours before TNF-α stimulation, which could significantly reduce in vitro atherogenesis, suggesting that a sufficient duration may be required for the preventive effect of carvedilol. This is compatible with the evidence that sufficient pretreatment duration of antioxidant such as probucol is critical to preventing restenosis after coronary intervention.

In conclusion, carvedilol, by inhibiting the redox-sensitive NF-κB and AP-1 activation, may inhibit TNF-α–stimulated VCAM-1 and E-selectin expression and the adhesiveness of endothelial cells to human MNCs. The mechanism could be related to its broad antioxidant activity and direct endothelial protection, rather than α- and β-adrenergic block effects. The findings may provide not only a novel mechanism of vascular protection by carvedilol but also the novel rationale of its potential implication to the prevention and treatment of atherosclerosis diseases in humans.

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