Original Contribution

Carvedilol, a pharmacological antioxidant, inhibits neointimal matrix metalloproteinase-2 and -9 in experimental atherosclerosis

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Abstract

Matrix metalloproteinase (MMP) is critical to the progression of atherosclerosis and neointima hyperplasia after vascular injury. We investigated the effects of carvedilol, a pharmacological antioxidant with α- and β-adrenergic blocking activity, on MMP-2 and MMP-9 expression. Vascular injury was induced with the balloon catheters on abdominal aortas of high-cholesterol-fed rabbits. On Day 21, there was significant aortic neointima formation with increased oxidative DNA damage by immunostaining with 8-hydroxy-2′-deoxyguanosine and enhanced MMP-2 and MMP-9 expressions by Western blotting, which were significantly reduced by oral administration of carvedilol (20 mg/kg/day) or probucol (100 mg/kg/day). Vascular expression (by Western blot), activity (by gelatin zymography), and mRNA levels of MMP-2 and MMP-9 were also reduced by carvedilol or probucol. Besides, pretreatment with carvedilol or probucol but not propranolol, a β-blocker, or prazocin, an α-blocker, inhibited tumor necrosis factor-α-stimulated expressions and activities of MMP-2 and MMP-9 in human aortic smooth muscle cells. On electrophoretic mobility-shift assay, carvedilol inhibited the binding activities of activator protein-1 and specific protein-1, two major transcription factors for MMP promoter regions. Accordingly, carvedilol, a pharmacological antioxidant, inhibited in vivo and in vitro expression of MMP-2 and MMP-9 properly by modulating the redox-related pathways, suggesting its potential clinical implications.

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Keywords: Antioxidant; β-Blocker; Carvedilol; Matrix metalloproteinase; Neointimal hyperplasia; Oxidative injury

Introduction

Migration of vascular smooth muscle cells (VSMCs) plays an important role in the pathogenesis of atherosclerosis, restenosis, and vascular graft stenosis [1]. Matrix metalloproteinase (MMP), a family of enzymes which selectively digest individual components of extracellular matrix, may play an important role in the development of atherosclerotic plaque and vascular remodeling by allowing VSMCs to migrate from media into the intima [2–5]. Among them, MMP-2 and MMP-9 (also named gelatinase A and B, respectively) that could be produced by both VSMCs and inflammatory cells are the major MMPs associated with VSMC migration [6–8] and neointima formation after vascular injury [9,10]. Recent evidence further showed that plasma MMP-2 and -9 levels are not only increased in patients with coronary artery disease [11] but also upregulated and activated in coronary circulation after coronary angioplasty [12], suggesting their potential role in both experimental [13] and clinical atherosclerosis [14].
Increased oxidative stress, a cardinal feature of vascular inflammation, has been implicated as important in clinical atherosclerosis and vascular remodeling [14]. It was proposed that in vitro activation and expression of MMPs [15,16] and the in vivo activation of MMP-2 and -9 [17] could be affected by increasing MMP transcription and proenzyme activation and limiting endogenous inhibition of MMP activity [18]. However, whether the pharmacological intervention on vascular oxidative stress could modify the expression of MMPs and related vascular remodeling had not been clarified.

Carvedilol, a pharmacological antioxidant with both α1- and β-adrenoceptor antagonist activity, has been widely used for clinical hypertension and heart failure [19–21]. However, little is known about its role in clinical atherosclerosis. Similar to other types of antioxidants [22–24], carvedilol was shown to inhibit the proliferation and migration of VSMCs and neointima formation in balloon-injured rats [25] and reduce in-stent restenosis in a porcine model [26]. It was also demonstrated that carvedilol, similar to probucol, another pharmacological antioxidant, could inhibit in vitro atherogenesis through its antioxidative effect on human aortic endothelial cells [27]. Recently, carvedilol was shown to reduce the oxidative DNA damage indicated by 8-hydroxy-2′-deoxyguanosine (8-OHdG) in hypertensive patients, suggesting the clinical relevance of its antioxidative effect [28]. To test the hypothesis that pharmacological antioxidants such as carvedilol and probucol could inhibit vascular MMPs expression in experimental atherosclerosis, the present study was conducted with an in vivo atherosclerosis model with balloon injury in hypercholesterolemic rabbits and with the in vitro culture system of human aortic smooth muscle cells (HASMCs) stimulated by tumor necrosis factor-α (TNF-α), an inflammatory cytokine critical to atherogenesis. It was revealed that both carvedilol and probucol reduced oxidative damage and the expression and activity of MMP-2 and MMP-9 in vascular neointima. Carvedilol further inhibits TNF-α-stimulated MMP expression in HASMCs, suggesting its potential implication in clinical atherosclerosis.

Material and methods

In vivo study

Balloon-injury experiments in rabbits

A total of 24 adult male New Zealand White rabbits (2.5 to 3 kg) were provided 60 g/kg/day of commercial normal chow diet and water ad libitum for 2 weeks. They were then randomly divided into four groups with 6 in each group. Animals in group 1 were continuously fed with normal chow diet (ND) and served as the control. Animals in group 2 were fed a 2% high-cholesterol (HC) diet (Purina Mills Inc., LLS, MS) for 6 weeks. Animals in group 3 (HC + probucol) and group 4 (HC + carvedilol) were given probucol (Sigma, St. Louis, MO) (100 mg/kg/day) and carvedilol (Roche, NJ) (20 mg/kg/day), respectively, by oral administration in addition to 2% high-cholesterol diet for total 6 weeks.

At the end of the third week, balloon injury of abdominal artery was performed for these four groups as described previously [29]. Briefly, a 3F Fogarty embolectomy catheter (Biosensor, USA) was inserted through the femoral artery of anesthetized rabbits and passed to the abdominal aorta (16 cm), inflated with normal saline, and withdrawn four times. Heparin (100 units/kg) was administered immediately after the balloon-injury process. The animals were sacrificed at the end of the third week after balloon injury; the abdominal aortas were cut into 5 segments. A small part of each arterial segment was taken, immersion-fixed with 4% buffered paraformaldehyde, paraffin-embedded, and then cross-sectioned for morphometry and immunohistochemistry. The remaining large portion of each arterial segment was immediately frozen in liquid nitrogen for protein and RNA isolation. In each animal, arterial blood was collected again from the ear artery.

All the animals were treated under protocols approved by the Institutional Animal Care Committee of the National Yang- Ming University (Taipei, Taiwan, ROC). The experimental procedures and animals’ care conformed to the Guide for the Care and Use of Laboratory Animals, published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996).

Biochemical measurements

Arterial blood was collected from the ear artery of rabbits into tubes containing sodium citrate. Blood samples were collected before and 6 weeks after the high-cholesterol diet was fed. The plasma cholesterol and triglyceride were measured using automatic biochemical analyzers (Spotchem TM SP 4410 Kyoto Daiichi Kagaku Co. Ltd.).

Immunohistochemical staining and morphometry

Immunohistochemical staining was performed on serial 5-μm-thick paraffin-embedded sections from rabbit abdominal aortas using anti-MMP-2 (Calbiochem, SD), anti-MMP-9 (Calbiochem), and anti-8-OHdG (JaICA, Fukuroi, Shizuoka, Japan) antibodies. Morphometric analyses were performed on hematoxylin-eosin-stained cross sections for each artery by the use of Image-Pro Plus (Media Cybernetics Inc., USA). The areas of intima hyperplasia were quantified by computer-assisted planimetry, and the extent of the lesions was expressed as a proportion of the total surface area (surface area of lesions/total surface area of the thoracic aorta) and a ratio of the intima and media. For double-label immunostaining of MMP-2 and α-actin (Dakocytoinforma- tin, Glostrup, Denmark) or MMP-9 and α-actin, commercial kits were used according to the manufacturers’ instruction (Vectastain Elite ABC Kit, Vector). In double-label immuno- nstaining, MMP-2 and -9 were stained purple, while α-actin was stained brown. The double-label cells were stained deep brown. In double-label immunofluorescence microscopy, 8-OHdG was stained with green fluorescence and MMP-2 or MMP-9 was stained with red fluorescence. The double-label cells were stained with orange to yellow fluorescence.
Quantitative polymerase chain reaction (Q-PCR)

Total RNA was isolated from arterial segments using a TRIZOL reagent kit according to the manufacturer’s instructions. cDNA was synthesized from total RNA using Superscript II reverse transcriptase. Quantitative real-time PCR was performed using a LightCycler and the FastStart DNA Master SYBR Green I kit (Roche). The levels of MMP-2 and MMP-9 mRNA expression were determined in arbitrary units by a comparison with an external DNA standard that was amplified by the rabbit-specific MMP-2 or MMP-9 primers. PCR primers used were as follows. MMP2 forward primer, 5′-TTG GAT CCT CCT ACA GCA GCT GCA CCA C-3′, and reverse primer, 5′-AAG AAT TCC CGT AGA GCT TTT GAA TGC-3′; MMP9 forward primer, 5′-AAG GAT CCA GCT TCC CAT CTT CCA G-3′, and reverse primer, 5′-AAG AAT TCG GCG CCG GTA GGG CTG GTA-3′; GAPDH forward primer, 5′-TGC CCC CTC TGC TGA TGC C-3′, and reverse primer, 5′-CTG CAC CCT GCT TCA CCA C-3′.

Western blot analysis

Rabbit arterial total tissue fractions were prepared and modified as described previously [29], and Western blot analysis was conducted to determine the levels of MMP-2 and MMP-9 in arterial segments. Equal amounts of protein extraction were subjected to SDS-PAGE and electrophoretically transferred to a PVDF membrane; the membrane was probed with goat anti-MMP-2 or mouse anti-MMP-9 antibody and then incubated with horseradish peroxidase-conjugated secondary antibody and developed using the enzyme-linked chemiluminescence detection reagents. Mouse anti-β-actin (Labvision/NeoMarkers, CA) antibodies were used as loading controls.

Gelatin zymography analysis

MMP-2 and MMP-9 activities were determined by gelatin zymography as described previously [30]. The rabbit arterial total tissue fractions were mixed with an equal volume of 2X Tris–glycine–SDS sample buffer, and then applied to gelatin zymography gels. For this set of study, a total of 20 μg of protein extracted from the vascular tissue was loaded for the gelatinolytic activity of MMPs in each group of the animals. Protein concentration was measured by the Bio-Rad protein assay. After electrophoresis, proteins were renatured in renaturing buffer and placed at 37 °C for overnight developing in developing buffer. Gelatinase activity was revealed by negative staining with Coomassie brilliant blue R-250 (0.1% Coomassie brilliant blue R-250, 45.5% methanol, 9% acetic acid) and quantified by densitometry.

In vitro study

Cell culture

HASMCs (Cascade Biologics, OR) were grown and passaged as described previously [31]. Cells were used at passages 3–8. Before treatment or stimulation with reagents, the cells were serum-starved overnight.

Western blot and gelatin zymography analysis

HASMCs were pretreated with 5 μmol/L of probucol, carvedilol (α,β-blocker and antioxidant activity), or 10 μmol/L of propranolol (a β-blocker, Sigma) or prazocin (an α-blocker, Sigma) for 18 h followed by TNF-α (20 ng/mL, PeproTech, Inc, Rocky Hill, NJ) stimulation for 24 h. The conditioned media were collected and Western blot and gelatin zymography analysis were performed for MMP-2 and MMP-9.

Statistical analysis

All data were expressed as means±SE. The difference in mean values among different groups was analyzed by one-way ANOVA and subsequent post hoc Dunnett’s test. A value of P<0.05 was considered statistically significant.

Results

Effects of carvedilol and probucol on plasma lipids

There was no significant difference in body weight among groups at baseline and at 3 and 6 weeks after feeding with a 2% high-cholesterol diet (data not shown). Plasma lipid levels, especially plasma cholesterol levels, were rapidly increased after high-cholesterol feeding. There was no significant difference in plasma total cholesterol and triglyceride levels among HC+carvedilol, HC+probucol, and HC animals either before or after being fed a high-cholesterol diet for 6 weeks (Table 1).

Carvedilol and probucol reduced neointima hyperplasia

The effects of carvedilol or probucol on the neointima hyperplasia were quantified by histomorphometric analysis of the abdominal aortic cross sections in the animals at Day 21 after the balloon injury. The intima/media area ratio and the intima/media thickness ratio of the aortas were significantly increased in the HC than in control animals. Compared with that in the HC group, both the intima/media area ratio (0.40±0.03 in HC+carvedilol group versus 0.45±0.04 in HC+probucol group versus 0.69±0.07 in HC group, P<0.05) and the thickness ratio (0.48±0.04 in HC+carvedilol group versus 0.52±0.04 in HC+probucol group versus 0.83±0.09 in HC group, P<0.05) were significantly reduced in carvedilol-(HC+carvedilol) or in probucol-(HC+probucol) treated animals (Fig. 1).

Table 1

<table>
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<tr>
<th></th>
<th>Total cholesterol (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
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<tr>
<td></td>
<td>Baseline 6 weeks</td>
<td>Baseline 6 weeks</td>
</tr>
<tr>
<td><strong>HC</strong></td>
<td>1345.00±147.15</td>
<td>31.33±6.64</td>
</tr>
<tr>
<td><strong>HC+probucol</strong></td>
<td>1221.67±195.55</td>
<td>46.00±8.89</td>
</tr>
<tr>
<td><strong>HC+carvedilol</strong></td>
<td>1220.00±88.62</td>
<td>38.00±5.13</td>
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HC: high-cholesterol diet alone group; HC+probucol: high cholesterol-diet with probucol treatment group; HC+carvedilol: high-cholesterol diet with carvedilol treatment group.
Carvedilol and probucol inhibited MMP-2 and -9 expressions and reduced oxidative DNA damage in neointima of the abdominal aortas

Compared to the HC animals, animals treated with carvedilol (HC + carvedilol) or probucol (HC + probucol) had significantly reduced neointima formation and the expressions of MMP-2 and MMP-9 in neointima (Fig. 2). To evaluate vascular oxidative stress in these animals, immunostaining of 8-OHdG for oxidative DNA damage was performed. There were 8-OHdG-positive endothelial cells in the aortas of animals in every group. The expression of 8-OHdG was observed in the HC animals, but it was only limited to a few smooth muscle cells in the probucol-(HC + probucol) or probucol-(HC + probucol) treated animals (Fig. 3A). The double immunofluorescence microscopic studies of 8-OHdG and MMPs showed that only small foci of 8-OHdG labeling were colocalized with MMPs in the neointima (Figs. 3B and 3C).

Carvedilol and probucol inhibited the enzymatic activities of MMP-2 and MMP-9 in the abdominal aorta

Gelatin zymographic analysis revealed that the major gelatinolytic activities were observed at molecular masses of 62 and 72 kDa, which most likely correspond to the active and latent forms of MMP-2, respectively. A 92-kDa band was also detected in the rabbit vessel tissue extracts, which was assigned as MMP-9. Gelatin zymography showed that the activities of vascular pro-MMP-2, MMP-2, and MMP-9 were significantly increased in the HC animals; treatment with carvedilol (HC + carvedilol) or probucol (HC + probucol) significantly decreased their activities (Fig. 4).
Fig. 2. The immunohistochemical analysis for the matrix metalloproteinase (MMP) expression in the abdominal aortas of rabbits in different groups. All the rabbits received endothelial denudation over abdominal aorta. Compared to those with normal diet (control), hypercholesterolemic (HC) rabbits had markedly thickened neointima with increased MMP-2 and MMP-9 staining. (A and B) The weaker immunopositive staining of MMP-2 and MMP-9 of neointima in the probucol-(HC+probucol) or carvedilol-treated (HC+carvedilol) rabbits than HC subjects. Thus the neointima and MMPs expressions were significantly reduced in the treatment group. The internal elastic lamina is indicated by the arrows. The scale bar represents 25 μm.
Carvedilol and probucol inhibited the protein and mRNA expression of MMP-2 and MMP-9 in the abdominal aortas.

Western blot analysis showed that vascular expressions of MMP-2 and -9 in abdominal aortas were significantly increased in the HC animals compared to control animals. Carvedilol and probucol treatments significantly decreased the protein expressions of MMP-2 and -9 (Figs. 5A and 5B).

To explore whether the changes of MMP-2 and MMP-9 were modulated at the transcriptional level, the mRNA expressions of MMP-2 and MMP-9 on the abdominal aortas of high-cholesterol-fed rabbits were assessed by quantitative real-time...
PCR. MMP-2 and MMP-9 mRNA expressions were significantly elevated in the HC animals compared to control animals. Carvedilol or probucol treatment significantly inhibited the increase of mRNA expression of MMP-2 and MMP-9 (Figs. 5C and 5D).

Carvedilol and probucol inhibited the expressions and activities of MMP-2 and -9 in TNF-α-stimulated HASMCs

It was shown in our in vivo study that in hypercholesterolemic rabbits, the VSMCs might be activated and synthetic with the expression of MMPs in the neointima after balloon injury. Then, in the in vitro study, inflammatory cytokine TNF-α was used to stimulate the expression of MMPs in HASMCs. To further investigate the direct pharmacological effects on MMP expression in HASMCs, carvedilol (an α,β-blocker with antioxidant activity), probucol (an antioxidant), propranolol (a β-blocker), and prazocin (an α-blocker) were then used. The cultured conditioned media for HASMC, which was pretreated with different drugs for 18 h followed by TNF-α (20 ng/ml) stimulation for 24 h, were subjected to Western blot and gelatin zymography assays. Results showed the amounts (Figs. 6A and 6B) and activities (Fig. 6C) of MMP-2 and -9 that were increased by TNF-α stimulation were significantly suppressed by pretreatment with carvedilol (5 μmol/L) or probucol (5 μmol/L) for 18 h, indicating the direct inhibitory effects of carvedilol or probucol on MMPs in HASMCs. By contrast, pretreatment with propranolol (5–20 μmol/L) or prazocin (5–20 μmol/L) failed to reduce the activities of MMP-2 or -9 in TNF-α-stimulated HASMCs (data not shown). Taken together, it was suggested that the inhibition on the amounts and activities of MMPs by carvedilol could potentially depend on its antioxidant but not α- or β-adrenergic blocker activities.

Discussion

The principal findings of this study included that (1) in hypercholesterolemic rabbits, oxidative DNA damage and the
amounts of MMP-2, active MMP-2, and MMP-9 were significantly increased in the neointimal layer of the aorta after balloon injury; (2) treatment with carvedilol or probucol significantly reduced neointima hyperplasia, oxidative DNA damage, and the amounts of MMP-2, active MMP-2, and MMP-9 in the neointimal layer of these animals; (3) pretreatment with carvedilol or probucol but not propranolol, a β-adrenergic blocker, or prazocin, an α-adrenergic blocker, could directly inhibit the amounts and presence of MMP-2 and MMP-9 in TNF-α-stimulated HASMCs. Taken together, it was indicated that in experimental atherosclerosis, pharmacological antioxidants such as carvedilol could modulate the intracellular oxidative stress and inhibit the presence of MMP-2, active MMP-2, and MMP-9 in neointima after vascular injury.

Increased neointimal expression of MMPs after vascular injury

It has been shown that balloon injury comprises cell migration, cell proliferation, and matrix deposition and apoptosis, resulting in constrictive arterial remodeling and neointima formation where MMPs may play a significant role [32]. However, the detailed role of MMPs in atherosclerosis is largely not known. In the present study, all the animals received endothelial denudation induced by balloon injury over abdominal aorta. Though also seen in the neo-endothelial cells, the expressions of MMP-2 and -9 were mainly confined to the VSMCs in neointima rather than to macrophages or to VSMCs in the medial layer, suggesting the cell-type-specific expression of MMP-2 and -9 in the activated, synthetic VSMCs in the neointima. However, as seen in our in vitro study, VSMCs could produce and secrete MMPs on stimulation. Thus, the in vivo expression of MMPs may contain both intracellular and extracellular MMPs that could be suggested by the presence of MMPs in both intracellular and extracellular areas in the neointima. This is compatible with a previous suggestion that the synthesis of MMPs is a prolonged process and VSMCs are a major source of MMP production in arterial neointima [33]. The above could be also
supported by the in vitro expression of MMP-2 and -9 that was significantly upregulated by TNF-α stimulation in HASMCs in the present study.

It was recently shown that a broad-spectrum MMP inhibitor did not have a beneficial effect on atherosclerosis in the apolipoprotein E knockout mouse model, suggesting that more selective compounds targeting some specific types of MMPs would be preferable [34]. However, short-term statin treatment, though reducing MMP-2 expression, did not modify neointimal morphology in stented arteries of normal cholesterolemic rabbits [35]. In the present study, both MMP-2 and MMP-9 expression, together with neointimal hyperplasia, were reduced by carvedilol treatment in a balloon-injury hypercholesterolemic rabbit model. It was compatible with the current suggestion that some particular types of MMPs such as MMP-9 might be more critical to intimal thickening or neointimal hyperplasia after vascular injury [36,37]. Indeed, serum MMP-9 level that could be elevated after coronary intervention was suggested as a major prognostic predictor in patients with coronary artery disease [38,39]. MMP-9 was also associated with vulnerable carotid atherosclerotic lesions [40].

Cell-type-specific inhibition on oxidative injury and MMP expression in neointimal vascular smooth muscle cells

It was proposed that increased oxidative stress could be critical to the expression of MMPs both in vitro and in vivo [15–17,36]. Currently, 8-OHdG is known as the DNA oxidative products that may indicate the DNA damage caused by reactive oxygen species [24]. Thus, the intracellular oxidative stress/injury indicated by the presence of 8-OHdG could theoretically induce and prior to the intracellular production and extracellular secretion of MMPs on stimulation.

In the present study, similar to the expression of MMPs, 8-OHdG-positive cells were observed in the newly generated endothelium and neointima in injured aorta of rabbits. However, while MMPs may be present in both intracellular and extracellular areas, the 8-OHdG-positive staining was mainly seen in the nuclei and cytoplasma of numerous VSMCs in the neointima rather than in the medial layer. Treatment with carvedilol or probucol in a relatively clinically relevant dose, though minimally altering the endothelial expression of MMPs, could significantly reduce the 8-
OHdG staining in the cytoplasm of neointimal VSMCs, providing novel evidence of cell-type-specific inhibitory effects of pharmacological antioxidants. It has been suggested that the antiatherosclerosis effects of carvedilol or probucol were correlated well with their antioxidative properties in the hypercholesterolemic rabbits without balloon injury [41]. Thus, the in vivo findings of present study showed that they could inhibit intracellular oxidative injury and the intracellular as well as extracellular expression of MMP-2 and MMP-9 along with neointima hyperplasia in a similar trend. The direct inhibitory effects of carvedilol on MMP-related AP-1 transcriptional pathways in TNF-α-stimulated HASMCs further provided a novel mechanism to its in vivo effects.

Fig. 5. Carvedilol and probucol inhibited protein and mRNA expression of MMP-2 and -9 in the abdominal aortas of high-cholesterol-fed, endothelial-denuded rabbits. The protein and mRNA expressions of MMP-2 (A and C) and MMP-9 (B and D) in abdominal aorta extracts were determined by Western blot and real-time PCR in different groups. Three independent experiments gave similar results. Summarized data (means±SE) are shown as a bar graph from three separate experiments by densitometry (*P<0.05 versus control group; †P<0.05 versus HC group).

In vitro effects of carvedilol on TNF-α-stimulated MMP expression

Inflammatory cytokines such as interleukins and TNF-α are the major participants in the development and progression of atherosclerosis in clinical coronary artery disease [42]. TNF-α may increase intracellular ROS production and activate redox-sensitive transcription pathways and alter the expression and activity of MMPs in various vascular cells including endothelial cells, smooth muscle cells, and macrophages [43–46]. These cellular events may participate both in neointima hyperplasia and in the formation of atherosclerotic plaques [47,48]. In the present study, TNF-α may express along with the expression of MMP-2 and -9 in the whole neointimal layer of the injured
Fig. 6. Carvedilol and probucol inhibit the amounts and activities of MMP-2 and -9 in the TNF-α-stimulated human aortic smooth muscle cells (HASMCs). HASMCs were pretreated with carvedilol or probucol (5 μmol/L for 18 h), followed by TNF-α-stimulation (20 ng/mL for 24 h). The amounts of MMP-2 (A) and MMP-9 (B) were determined by Western blot, and the activities of proMMP-2, MMP-2, and MMP-9 in conditional medium were determined by gelatinase zymography (C), respectively. Three independent experiments gave similar results. Summarized data (mean±SE) are shown as a bar graph from three separate experiments by densitometry (*P<0.05 versus control group; †P<0.05 versus TNF-α-treated group).
vessels (data not shown). It was compatible with the previous suggestion that VSMC-derived TNF-α could serve as a marker of modulated VSMC phenotype after acute vascular injury and might contribute to local cellular activation and proliferation of VSMCs at sites of arterial injury [49].

Recent evidence suggested that the activation of MMPs by TNF-α may be through different signal transduction pathways leading to various regulation mechanisms of the gene activation in different cells [50,51]. It was found that TNF-α-induced MMP-9 promoter stimulation leads to a proportional increase of enzyme expression and secretion [52]. Though the regulatory elements of the promoter region of human MMP-9 gene may include NF-κB, AP-1, Sp-1, and poliovirus enhancer activator 3 [53], a decreased transcriptional activity of AP-1 sites is sufficient to reduce MMP-9 promoter activity in different cancer cells in vitro [54]. On the other hand, there are two Sp-1 transcription factor binding sites in the MMP-2 promoter region, which may explain the potential decrease in MMP-2 levels by decreased binding of Sp-1 transcription factor [55]. However, previous findings suggested that AP-1 and Sp-1 rather than NF-κB could contribute to the expression of MMP-2 and -9 in cancer cells [54,55]. We have previously shown that carvedilol could directly inhibit the activation of redox-sensitive transcription factor AP-1 and NF-κB in TNF-α-stimulated human aortic endothelial cells [27]. Future studies are indicated to verify the direct effects of carvedilol on intracellular transcription pathways in activated vascular endothelial and smooth muscle cells in vitro and to further clarify the individual mechanisms of the activation of MMP-2 and -9 in the in vivo model of atherosclerosis.

**Potential role of pharmacological antioxidants in clinical atherosclerosis**

Though the direct antiatherosclerosis effects of pharmacological antioxidants such as carvedilol and probucol have been shown in this study, their complex pharmacological mechanisms in addition to antioxidant capacity might also contribute to the inhibitory effects on experimental atherosclerosis. However, identical to that shown in the previous studies, plasma cholesterol levels were similarly increased by high-cholesterol diet with or without treatment of probucol, suggesting that the antiatherosclerotic mechanisms of this low-dose probucol may be independent of its cholesterol-lowering effects in the present study. It was also concerned that whether the blood pressure-lowering effect of carvedilol may have an impact on its inhibitory effect on MMP expression and neointimal hyperplasia. It was previously shown that angiotensin-converting enzyme inhibitor but not other different antihypertensive agents including verapamil, hydralazine, and minoxidil could inhibit neointima hyperplasia in a rat balloon-injury model, suggesting that direct vascular protection rather than a blood pressure-lowering effect may be required for the inhibition on VSMCs migration and proliferation after balloon injury [56]. In the present study, carvedilol or probucol but not propranolol, a β-blocker, or prazocin, an α-blocker, could inhibit TNF-α-induced MMP expressions in HASMCs, suggesting the direct, antioxidant-related inhibition of carvedilol on activated VSMCs. This may give a rationale, at least in part, to its in vivo effects on MMPs expression and neointima formation after balloon injury. Besides, carvedilol was shown to inhibit the oxidation of low-density lipoprotein by VSMCs in vitro [57], suggesting that it may potentially exert vascular protection by multiple antioxidant-related mechanisms [56,57].

Interestingly, recent large clinical studies failed to show the beneficial effects of antioxidant vitamins including vitamins C and E on long-term outcomes in patients at risk of atherosclerosis [58]. However, pretreatment with pharmacological antioxidants such as probucol, though could be associated with significant side effects, has successfully reduced restenosis after coronary intervention in patients with coronary artery disease [59]. An alternative strategy using pharmacological antioxidants with multiple cardiovascular effects and proven clinical safety such as carvedilol may be mandatory for clinical atherosclerosis [27,28].

**Study limitations**

There are some issues that need to be addressed further. In the present study, our in vivo data indicated the general presence of both MMP-2 and MMP-9 in most of the cells and extracellular areas in the neointima of the aorta of hypercholesterolemic rabbits. This could be due to the presence of both intracellular and extracellular MMPs produced and secreted by VSMCs in the neointima after balloon injury. In fact, we have shown in the in vitro part of this study that VSMCs could produce and secrete MMP-2 and -9 on stimulation such as TNF-α. It is then difficult to evaluate the in vivo presence of MMPs based on each single cell in the neointima in this balloon-injury model. On the other hand, it has been shown by Sata et al. that in the balloon-injury model, circulating progenitor cells from bone marrows could also contribute to up to 10–30% of the intimal smooth muscle cells [60,61]. Theoretically, they may also produce and secrete MMPs. Thus, we cannot exclude the possibility that the cells in the neointima after balloon injury may come from different origins and have different contributions to the presence of intracellular and extracellular MMPs. They may also have different responses to the treatment of carvedilol or probucol. However, it is difficult to differentiate the neointimal cells of different origins in vivo. Accordingly, similar to that used in the study of Waksman and co-workers [62,63], we used immuno-positive area rather than immuno-positive cells to evaluate the presence of both intracellular and extracellular MMPs in the neointima after balloon injury. The data did indicate the significant reduction of intimal expression of MMPs by carvedilol or probucol treatment that is consistent with the data of gelatinase zymography and Western blot for MMPs.

**Conclusions**

The current study provided additional evidence that pharmacological antioxidants such as carvedilol may inhibit oxidative DNA damage and the presence as well as the activities
of MMP-2 and MMP-9, together with neointimal hyperplasia after balloon injury in hypercholesterolemic rabbits. Carvedilol could also directly reduce the activity of MMP-2 and MMP-9 in TNF-α-stimulated HASMCs in vitro. These findings not only demonstrated the novel protective mechanisms of carvedilol in experimental atherosclerosis but also suggest its potential role as an adjunctive medical treatment for clinical atherosclerosis disease.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2007.08.010.

References


