JPP Journal of Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY

JPP Journal of Pharmacy and Pharmacology

JPP 2011, 63: 1587–1594

Received February 24, 2011
Accepted August 30, 2011
DOI
10.1111/j.2042-7158.2011.01364.x
ISSN 0022-3573

Research Paper

Monascus purpureus-fermented rice inhibits tumor necrosis factor-α-induced upregulation of matrix metalloproteinase 2 and 9 in human aortic smooth muscle cells

Chih-Pei Lin[a,c], Po-Hsun Huang[b,d], Hui-Szu Tsai[a], Tao-Cheng Wu[b,d], Hsin-Bang Leu[b,d], Po-Len Liu[e] and Yung-Hsiang Chen[f]

[a]Department of Pathology and Laboratory Medicine, Division of General Laboratory, [b]Division of Cardiology, Taipei Veterans General Hospital, [c]Department of Biotechnology and Laboratory Science in Medicine and Institute of Biotechnology in Medicine, [d]Institute of Clinical Medicine, Cardiovascular Research Center, National Yang-Ming University, Taipei, [e]Department of Respiratory Therapy, College of Medicine, Kaohsiung Medical University, Kaohsiung and [f]Graduate Institute of Integrated Medicine, College of Chinese Medicine, Department of Medical Research, China Medical University and Hospital, Taichung, Taiwan

Abstract

Objectives Inflammation is associated with atherosclerosis. Cholestin (*Monascus purpureus*-fermented rice) contains a naturally occurring statin, which has lipid-modulating, anti-inflammatory and antioxidative effects. This study aimed to investigate the effects of Cholestin extract on the expression of matrix metalloproteinase (MMP)-2 and MMP-9 by tumor necrosis factor (TNF)-α-treated human aortic smooth muscle cells (HASMCs).

Methods Zymography, reverse transcription polymerase chain reaction and immunoblot analyses were used for analysis of MMP expression of TNF-α-stimulated HASMCs. Gel shift assay was used for analysis of transcription factor nuclear factor-κB (NF-κB) activation. Intracellular reactive oxygen species (ROS) generation was also analysed.

Key findings The supplement of HASMCs with Cholestin extract significantly suppresses enzymatic activities of MMP-2 and MMP-9 in TNF-α-stimulated HASMCs. RT-PCR and immunoblot analyses show that Cholestin extract significantly attenuates TNF-α-induced mRNA and protein expressions of MMP-2 and MMP-9. Gel shift assays show that Cholestin treatment reduces TNF-α-activated NF-κB. Furthermore, Cholestin also attenuates intracellular ROS generation in TNF-α-treated HASMCs. The supplement with an ROS scavenger *N*-acetyl-cysteine (glutathione precursor) gives similar results to Cholestin.

Conclusions Cholestin reduces TNF-α-stimulated MMP-2 and MMP-9 expression as well as downregulating NF-κB activation and intracellular ROS formation in HASMCs, supporting the notion that the natural compound Cholestin may have potential application in clinical atherosclerosis disease.

Keywords atherosclerosis; inflammation; nuclear factor-κB; oxidative stress; red yeast rice

Introduction

Red yeast rice, a fermented product of rice and red yeast (*Monascus purpureus*), has been used by the Chinese for many centuries to make rice wine, for maintaining the taste and the color of meat and fish and for its medicinal properties.[1–3] Cholestin™, a dietary supplement related to red yeast rice, has been reported to have lipid-lowering effects and is considered beneficial in subjects with hyperlipidemia.[1] The pharmacological preparation from red yeast rice that is marketed in China, the USA and many other countries is composed of 73.4% starch, 5.8% protein, less than 2% fat and a number of compounds called monacolins (~0.4%), which are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase.[4] It has also been reported that Cholestin contains 2–6% fatty acids including linoleic acid, oleic acid, palmitic acid and stearic acid,[5] some of which exhibit lipid-lowering properties.[6]

Correspondence: Chih-Pei Lin, Department of Biotechnology and Laboratory Science in Medicine and Institute of Biotechnology in Medicine, National Yang-Ming University, Taipei 11221, Taiwan.
E-mail: cplin@vghtpe@gov.tw

Yung-Hsiang Chen, Graduate Institute of Integrated Medicine, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan.
E-mail: yhchen@mail.cmu.edu.tw

Red yeast rice contains a family of naturally occurring statins that have a marked modulating effect on lipids.[1,7,8] The extract has been shown to have free-radical scavenging properties.[9–11] Dhale *et al*. described the isolation and characterization of dihydromonacolin-MV, a new monacolin metabolite, as a potent antioxidant from *M. purpureus*.[12] Recently, a Cholestin extract was found to decrease C-reactive protein and to protect endothelial function through lipid-lowering and anti-inflammatory mechanisms.[13–17] Moreover, a previous study also showed that Cholestin can reduce homocysteine-stimulated endothelial adhesiveness as well as downregulating intracellular reactive oxygen species (ROS) formation, supporting the notion that Cholestin may have potential applications in clinical atherosclerosis.[18]

The proliferation and migration of vascular smooth muscle cells (VSMCs) may play a key role in the development of intimal thickening after arterial-wall injury or in atherosclerosis.[19,20] VSMCs in the tunica media have low mitogenic activity, but during the early stages of arterial-wall injury or atherosclerosis they may undergo a transition from a contractile to a synthetic phenotype and begin proliferating in response to various growth factors, causing intimal hyperplasia of the arterial walls.[19] In addition to growth factor stimulation, the replication and migration of VSMCs may require the degradation or remodelling of the extracellular matrix (ECM) surrounding the cells; an imbalance between the accumulation and degradation of ECM may be crucial in the development of the intimal thickening that forms after vascular-wall interventions.[19] This proteolytic balance within the arterial wall towards matrix breakdown is partly mediated by matrix metalloproteinases (MMPs). MMP-2 and MMP-9 are indispensable for the degradation of type IV collagen, a major component of the basement membrane.[21] Recent experimental data show that inflammatory cytokines, including tumor necrosis factor-α (TNF-α), may induce the expression of the genes encoding MMPs.[22]

Increased oxidative stress may play the main role in the inflammatory mechanisms that cause the progression of atherosclerosis.[23,24] Inflammatory cytokine TNF-α is one well-known cytokine and stimulates the production of MMPs through the activation of intracellular signalling pathways, including redox-sensitive transcription factor nuclear factor-κB (NF-κB). TNF-α has also been shown to promote the expression of MMPs in VSMCs through oxidative-stress-related mechanisms. Since Cholestin may exhibit a 'pleiotropic' effect on vascular protection, the ability of Cholestin to modulate the expression of MMP-2 and MMP-9 activation of redox-sensitive transcription factor NF-κB by TNF-α-treated human aortic smooth muscle cells (HASMCs) was tested in the present study. The antioxidant N-acetyl-cysteine (NAC) was used as a positive control for ROS scavenging. This is the first study to show that Cholestin reduces intracellular ROS production and inhibits NF-κB activation, and consequently decreases MMP-2 and MMP-9 expression in HASMCs. Since the degradation of the ECM and basement membrane by HASMCs and the migration of VSMCs through MMP-2 and MMP-9 overexpression are crucial steps in the pathogenesis of atherosclerosis and restenosis, this study implies that Cholestin may have therapeutic potential in the prevention of cardiovascular disease.

Materials and Methods

Materials

TNF-α and NAC were purchased from Sigma Chemical Co. (MO, USA). Cholestin was kindly provided by Pharmanex (Nu Skin Taiwan Inc., Taiwan Branch) and extracted at room temperature with an 8: 1: 1 (volume) mixture of water, ethanol and dimethyl sulfoxide (DMSO) as previously described.[18] The final concentration of solvents in the testing solution was always < 0.5% to avoid possible interference or cytotoxicity.

Cell culture

HASMCs, purchased as cryopreserved tertiary cultures from Cascade Biologics (OR), were grown in culture flasks in SMC growth medium M231 (Cascade Biologics, Inc.) supplemented with fetal bovine serum (5%), human epidermal growth factor (10 ng/ml), human basic fibroblast growth factor (3 ng/ml), insulin (10 mg/ml), penicillin (100 U/ml), streptomycin (100 pg/ml) and Fungizone (1.25 mg/ml) at 37°C in a humidified 5% CO_2 atmosphere. The growth medium was changed every other day until confluence, when the cells were passaged by division between four culture dishes and again grown to confluence. Cells were used between passages 3 and 8. The purity of the HASMC cultures was verified by immunostaining with a monoclonal antibody against smooth muscle-specific α-actin.

MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, USA) assay was used to measure cell viability. The principle of this assay is that mitochondrial dehydrogenase in viable cells reduces MTT to a blue formazan. Briefly, cells were grown in 96-well plates and incubated with various concentrations of agents. After twice washing HASMCs with PBS, 100 µl of medium containing MTT (0.5 mg/ml) was added to each well and incubation continued at 37°C for an additional 4 h. The medium was then carefully removed so as not to disturb the formazan crystals formed. 100 µl DMSO, which solubilizes the formazan crystals, was added to each well and the absorbance of the solubilized blue formazan read at 540 nm using a microplate reader (Multiskan Ex, ThermoLabsystems) with DMSO as the blank. The reduction in optical density caused by the chemicals was used as a measurement of cell viability, normalized to cells incubated in control medium, which were considered 100% viable.

Gelatin zymography

Conditioned media from control, TNF-α-treated and Cholestin (NAC) + TNF-α-treated cells were collected and concentrated. Equal amounts (20 µg) of total protein were loaded onto 10% SDS polyacrylamide gels containing 1 mg/ml gelatin for the assessment of MMP activity. After gel electrophoresis, the gels were twice washed in 2.5% Triton X-100 for 15 min at room temperature to remove the SDS and permit partial renaturation of the protein. Gels were incubated subsequently at 37°C overnight in a buffer containing 10 mM $CaCl_2$, 150 mM NaCl and 50 mM Tris-HCl, pH 7.5 and then stained with 0.2% Coomassie blue and photographed on a light box. Proteolysis was detected as a white zone in a dark

blue field. For quantitative analysis of MMP-2 and MMP-9 activation, the resultant bands were scanned and then subjected to digital image analysis.[25,26]

Western blot analysis

Protein extracts were prepared as previously described.[27] Briefly, HASMCs were lysed in 100 µl lysis buffer with protease inhibitor (PIERCE), after washing by PBS, and were then centrifuged for 30 min at 4°C and 12 000*g* to harvest the supernatant. The cell total protein was quantified by use of Bio-Rad protein assay reagent. The whole-cell lysates were subjected to SDS-polyacrylamide (10%) gel electrophoresis, followed by electroblotting onto a polyvinylidene fluoride membrane (Amersham Biosciences). Membranes were probed with a goat monoclonal antibody directed to MMP-2 and MMP-9 (Calbiochem, CA, USA) and incubated with horseradish peroxidase-labelled secondary antibody. They were then washed with PBS containing 0.1% Tween 20. Bands were visualized by chemiluminescence detection reagents (PerkinElmer, USA). Anti-α-tubulin antibodies were used as loading control. A digital imaging device was used to perform densitometric quantitation of bands in the laboratory setting. Following acquisition, the image is downloaded by means of a serial interface to a personal computer. There it is analysed to ensure that the series of pixel intensities lie within the linear range and it is then quantitated with local background subtraction. Densitometric analysis was conducted with ImageQuant (Promega) software to semi-quantify the Western blot data.

Isolation of total RNA and reverse transcription polymerase chain reaction analysis

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. Reverse transcription polymerase chain reaction analysis (RT-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 comparison with an external DNA standard that was amplified by the rabbit-specific MMP-2 or MMP-9 primers. The PCR primers used were as follows: MMP2 forward primer, 5′-TTG GAT CCT CCT ACA GCA GCT GCA CCA G-3′, and reverse primer, 5′-AAG AAT TCC CGT AGA GCT CTT GAA TGC-3′; MMP9 forward primer, 5′-AAG GAT CCA GTT TCC GTT 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′-CCT CCG ACG CCT GCT TCA CCA C-3′.

Electrophoretic mobility shift assay

Nuclear protein extracts were prepared as previously described.[28] Briefly, after washing with PBS, the cells were scraped off the plates in 0.6 ml of ice-cold buffer A (HEPES 10 mM, pH 7.9; KCl 10 mM; dithiothreitol (DTT) 1 mM; phenylmethylsulphonylfluoride (PMSF) 1 mM; $MgCl_2$ 1.5 mM; and 2 µg/ml each of aprotinin, pepstatin and leupeptin). After centrifugation at 300*g* for 10 min at 4°C, the cells were resuspended in buffer B (80 µl of 0.1% Triton X-100 in buffer A), left on ice for 10 min, then centrifuged at 12 000*g* for 10 min at 4°C. The nuclear pellets were resuspended in 70 µl of ice-cold buffer C (HEPES 20 mM, pH 7.9; $MgCl_2$ 1.5 mM; NaCl 0.42 M; DTT 1 mM; EDTA 0.2 mM; PMSF 1 mM; 25% glycerol and 2 mg/ml each of aprotinin, pepstatin and leupeptin), then incubated for 30 min at 4°C, followed by centrifugation at 15 000*g* for 30 min at 4°C. The resulting supernatant was stored at −70°C as the nuclear extract. Protein concentrations were determined by the Bio-Rad method.

Electrophoretic mobility shift assay (EMSA) was carried out with the DIG Gel Shift Kit (Roche Diagnostics) following the user's manual. In the first step, single-stranded complementary oligonucleotides containing the binding sequences for transcription factors were annealed and end-labelled with digoxygenin. The NF-κB probe used in the gel shift assay was a 31-mer synthetic double-stranded oligonucleotide (5′-ACA AGG GAC TTT CCG CTG GGG ACT TTC CAG G-3′; 3′-TGT TCC CTG AAA GGC GAC CCC TGA AAG GTC C-5′) containing a direct repeat of the κB site. The labelled probes (48 fmol of double-stranded oligonucleotides) were then incubated for 30 min at 4°C with 10 µg of nuclear extract proteins in 40 mM HEPES buffer, pH 7.9, containing 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 1 mM DTT, 2 µg of poly(dI–dC) and 0.2 µg of poly-(L)-lysine. Then the mixtures were subjected to electrophoresis on a 6% polyacrylamide gel with 0.5× Tris/borate/EDTA running buffer. The DIG-oligonucleotide/protein complexes were transferred to a Hybond-N blotting membrane (Amersham Life Science, Germany) and the shift bands were visualized. Densitometric analysis was conducted with, ImageQuant software (Promega) to semi-quantify the EMSA data. The specificity of the binding reaction was determined by coincubating duplicate samples with 100-fold molar excess of unlabelled oligonucleotide probe and anti-NF-κB (anti-p65) antibody.

Detection of intracellular ROS

The effect of Cholestin on ROS production in HASMCs was determined by a fluorometric assay using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as the probe.[28] This method is based on the oxidation by H_2O_2 of non-fluorescent DCFH-DA to fluorescent DCF. Briefly, 15 µM DCFH-DA was added to the medium in the last 20 min of incubation (37°C, 18 h) and HASMCs were washed by Hank's balanced salt solution (without Ca^{2+}, Mg^{2+}) containing 10% bovine serum albumin. Then 250 µl cell lysis buffer (PBS containing 20% ethanol, 0.1% Tween 20) was added to each well. After centrifuging, the supernatant was transferred to measure the fluorescence intensity (relative fluorescence units) at 485 nm excitation and 530 nm emission using a fluorescence microplate reader (Victor II).

Statistical analyses

Results were expressed as mean ± SEM, and data were analysed using the Kruskal–Wallis test with Dunn's test as the post-hoc test for significant difference. Statistical significance was defined as $P < 0.05$. All statistical procedures were performed with SigmaStat version 3.1 (Jandel, USA).

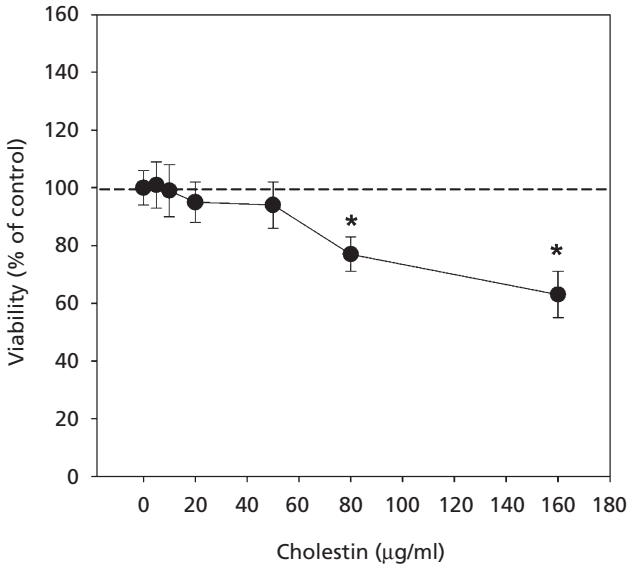

Figure 1 HASMC viability after culture with Cholestin for 24 h as determined by MTT assay. Data are expressed as percentage (mean ± SEM) of survival cells using the untreated group as control (viability = 100%). The results are from five separate experiments, $^{*}P < 0.05$, compared to control.

Results

MTT assay for TNF-α, Cholestin and NSC on HASMCs

Cell viability was assessed using the MTT assay. Treatment of HASMCs with 0–50 μg/ml Cholestin for 24 h did not result in cytotoxicity, whereas high concentrations of Cholestin (≥80 μg/ml) significantly inhibited cell survival (Figure 1). In addition, cell viability did not significantly change with 50 μg/ml Cholestin or 5 mM NAC treatment for 18 h followed by 10 ng/ml TNF-α stimulation for 6 h (data not shown). The results indicate that the significant cytotoxic effects on HASMCs were found in high-dose Cholestin. The non-cytotoxic working concentrations of Cholestin extract (≤50 μg/ml) in the following tests were used to avoid possible interference with cell viability.

Cholestin inhibits TNF-α-induced induction of MMP-2 and MMP-9 enzyme activity and protein expression

A number of studies have demonstrated that MMPs are important for VSMC proliferation and migration into the intima.[19] Recent studies have also reported that TNF-α stimulates the induction of MMPs in VSMCs.[22,26,29–36] Therefore, to determine the efficacy of Cholesin in inhibiting MMP-2 and 9 expression by TNF-α, HASMCs were cultured with TNF-α (10 ng/ml) in the absence or the presence of varying

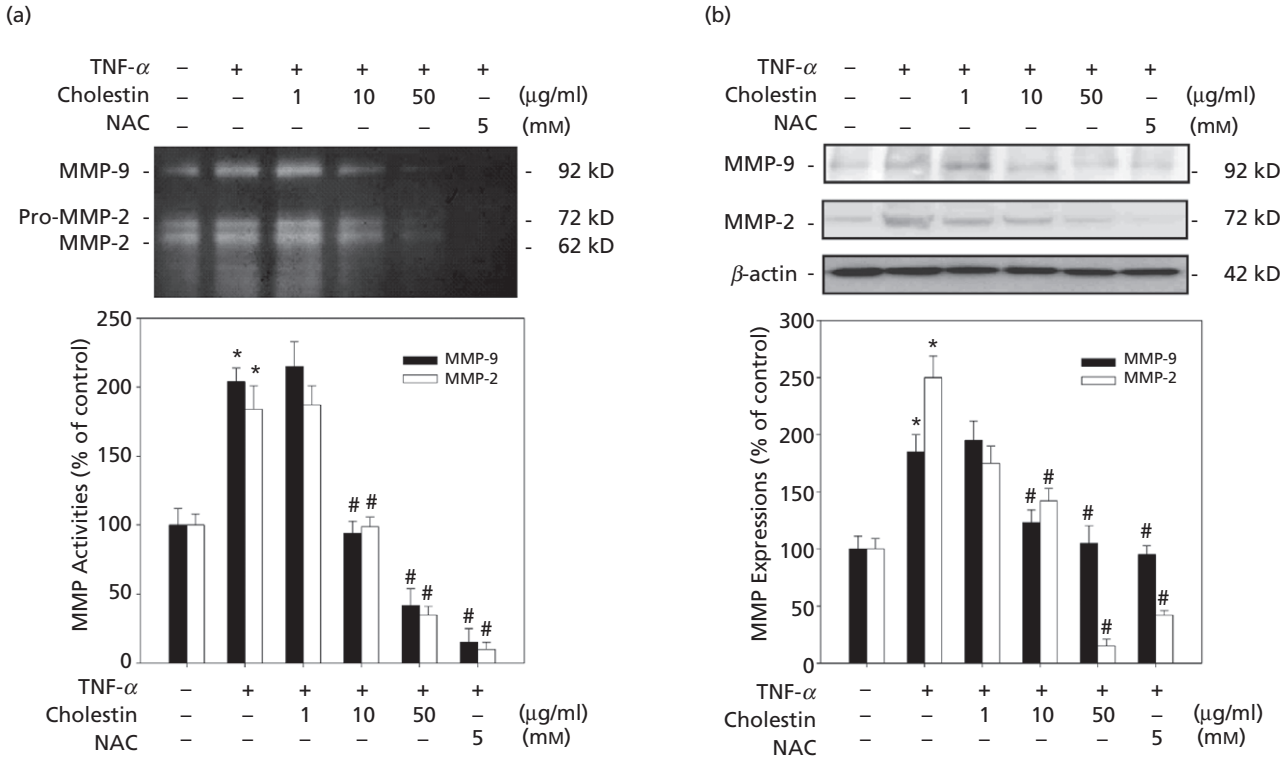

Figure 2 Effects of Cholestin and NAC on TNF-α-stimulated MMP-2 and MMP-9 enzymatic activity and protein expression. (A) Cholestin inhibits the TNF-α (10 ng/ml)-induced increase in MMP-2 and MMP-9 activity as indicated by gelatin zymography. (B) Cholestin inhibits the TNF-α-induced increase in MMP-2 and MMP-9 protein levels in HASMCs as analyzed by Western blotting. Densitometric analysis was conducted with software to semi-quantify zymography and Western blot data. The data are the mean ± SEM for three separate experiments. $^{*}P < 0.05$ compared to control cells. $^{\#}P < 0$ .05 compared to HASMCs treated with TNF-α alone.

concentrations of Cholestin. After 6 h, the conditioned medium and cell lysates were harvested for analysis of MMP-2/MMP-9 enzymatic activity and protein expression by zymography and Western blot, respectively (Figure 2a and b). Media from control HASMCs demonstrated low-level proteolytic activities at 92 kD, 72 kD and 62 kD, corresponding to MMP-9, Pro-MMP-2 and MMP-2, respectively. In contrast, treatment with TNF-α (10 ng/ml) increased the expression of a band of proteolytic MMP-9 and MMP-2 activities. This induction of MMP activity by TNF-α was inhibited in the presence of Cholestin in a dose-dependent manner. Similar results were found in immunoblot results (Figure 2b), although TNF-α has less impact on the enzymatic activities of MMP-2 and MMP-9. These data indicate that Cholestin treatment inhibits TNF-α-stimulated increases in MMP-9/MMP-2 activity and protein expression. The preincubation of cells with the antioxidant NAC (an ROS scavenger control) almost completely abrogates TNF-α-induced upregulation of MMPs.

Cholestin inhibits TNF-α-induced MMP-2 and MMP-9 mRNA expression

RT-PCR assays were performed to investigate whether or not the downregulation of MMP expression by Cholestin occurs at the transcriptional level. As shown in Figure 3, MMP-9/MMP-2 mRNA expression was induced by TNF-α after 3 h.

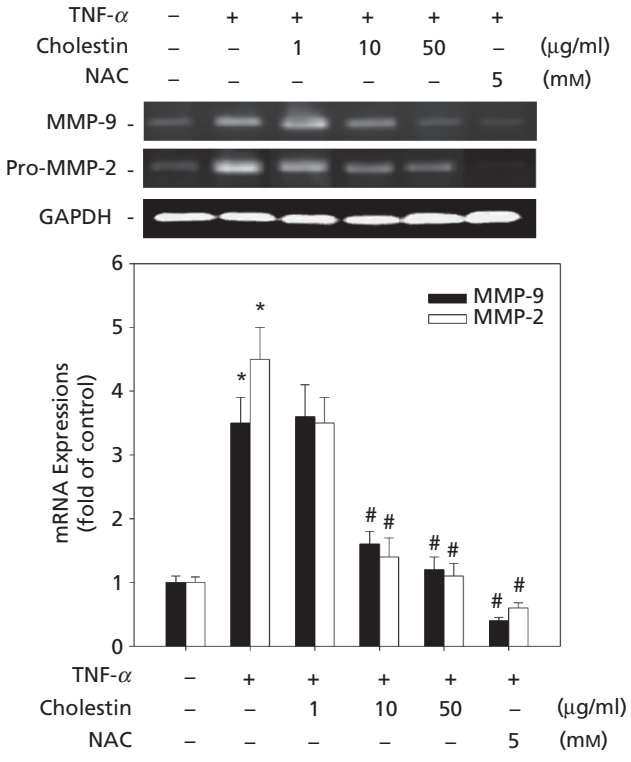

Figure 3 Effects of Cholestin and NAC on TNF-α-stimulated MMP-2 and MMP-9 mRNA expression. RT-PCR analyses of MMP-2 and MMP-9 expressions in cultured HASMCs. The PCR products on the gel were also shown. Three independent experiments gave similar results. The summarized data (mean ± SEM) from three separate experiments are shown in the bar graph. $^{*}P < 0.05$, compared to untreated control group; $^{\#}P < 0.05$, compared to TNF-α-treated group.

A pretreatment with Cholestin strongly inhibited the TNF-α-induced mRNA expression of MMPs in a dose-dependent manner, in accordance with the zymography and immunoblot results. NAC also significantly inhibited MMP mRNA expression from TNF-α exposed to HASMCs. This result suggests that Cholestin inhibits MMP-9 and MMP-2 at the transcriptional level.

Inhibition of TNF-α-induced activation of NF-κB by Cholestin

Transcriptional regulation involving NF-κB activation has been implicated in TNF-α-induced MMP expression in VSMCs.[22,32–34,37] To examine whether Cholestin inhibits NF-κB activation or not, gel-shift assays were performed with the consensus NF-κB binding sequence. Incubation of HASMCs with 10 ng/ml TNF-α caused strong activation of NF-κB at 1 h. The activation of NF-κB induced by TNF-α could be suppressed by the ROS scavenger NAC, as detected by DNA binding activity. The addition of 10 and 50 μg/ml Cholestin showed that TNF-α-derived NF-κB shifted bands were significantly reduced (Figure 4). These results suggest that Cholestin and NAC may downregulate NF-κB activation.

Inhibition of TNF-α-induced intracellular ROS generation by Cholestin

Inflammatory cytokine TNF-α may activate NF-κB in VSMCs via oxidative stress.[34,35] The effect of Cholestin on intracellular ROS generation in HASMCs was studied. Figure 5 shows the effects of 1, 10 and 50 μg/ml Cholestin and 5 mM NAC on intracellular ROS production induced by TNF-α (10 ng/ml for 30 min) in HASMCs. Treatment with Cholestin (10 and 50 μg/ml) or NAC significantly inhibited untreated and TNF-α-induced ROS production in HASMCs.

Discussion

Cholestin (*M. purpureus*-fermented rice) contains a naturally occurring statin that has lipid-modulating, anti-inflammatory and antioxidative effects. In this study, it was found for the first time that Cholestin treatment effectively attenuates TNF-α-induced mRNA and protein expression of MMP-2 and MMP-9. It was also shown that this inhibition of MMPs in HASMCs might come about through ROS and NF-κB signalling pathways.

Initial degradation of the ECM is an inevitable step for vascular cells to hypertrophy, proliferate and migrate. Vascular cells, including VSMCs, secrete MMPs that selectively digest the individual components of the ECM. Among MMPs, MMP-2 and MMP-9 regulate VSMC migration and proliferation by acting specifically on basement membrane components that modulate communication with surrounding activated cells. It has been shown that MMP-2 is constitutively expressed in VSMCs in normal arteries in addition to increased MMP-2 expression; MMP-9 expression is induced in VSMCs and macrophages in atherosclerotic arteries.[38] This study aimed to investigate the anti-atherogenic mechanism of Cholestin in HASMC against TNF-α treatment, as TNF-α functions as a stimulator in the pathogenesis of

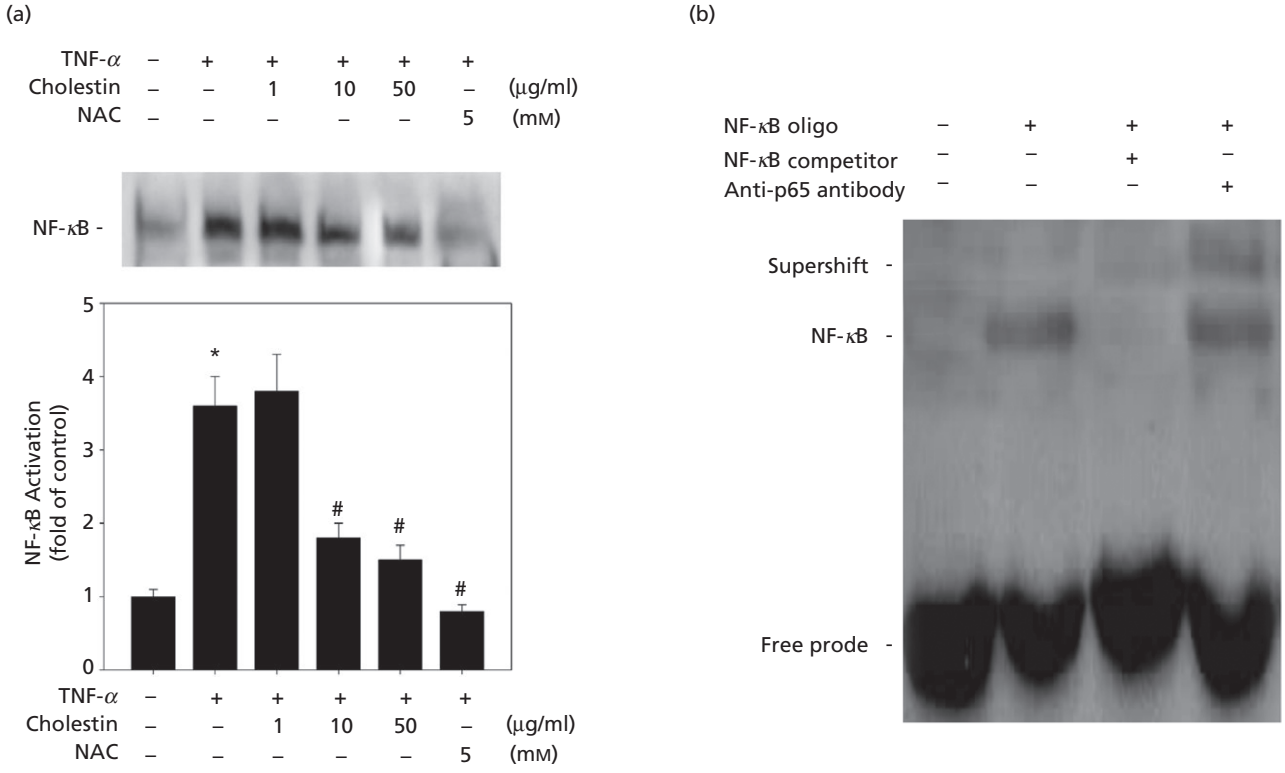

Figure 4 EMSA for NF-κB activation in cultured HASMCs. (A) Nuclear protein extracts were prepared and gel shift assay was performed using DIG-labelled oligonucleotides containing consensus NF-κB. Densitometric analysis was conducted with software to semi-quantify EMSA data. The results are from three separate experiments. $^{*}P < 0.05$, compared to untreated control group; $^{\#}P < 0.05$, compared to TNF-α-treated group. (B) To confirm that the presence of bands is specific to NF-κB, unlabelled oligonucleotide and anti-p65 antibody controls were also performed in separate studies.

vascular lesions such as atherosclerosis.[39] The present study provides new evidence that TNF-α enhances MMP-2 and MMP-9 protein expression and activity in cultured HASMCs. In addition, the study also demonstrated that NAC, an ROS scavenger and glutathione precursor, attenuates MMP-2 and MMP-9 expression. It is possible that TNF-α may contribute to the development of atherosclerotic vascular remodelling at multiple steps mediated by MMP-2 and MMP-9, including the initial activation of the inflammatory cytokine network, recruitment of inflammatory cells to the plaque, plaque destabilization, and finally plaque rupture associated with clot formation. Therefore, the inhibition of MMP-2 and MMP-9 appears to be an appropriate target for the development of anti-atherogenic agents.

In addition, our study examined whether treatment with Cholestin inhibits TNF-α-stimulated MMP-2 and MMP-9 activity in HASMCs. Of considerable interest in this study was the marked reduction by Cholestin of the secretion of MMP-2 and MMP-9 from TNF-α-stimulated VSMC, as determined by zymography and Western blot. These findings suggest that Cholestin may have anti-atherogenic and anti-inflammatory effects on HASMCs through the inhibition of MMP-2 and MMP-9 expression, which have been linked to the progression of plaque rupture and intimal formation in arterial lesions.

The effects of Cholestin were tested because it is derived from a Chinese product, red yeast rice, commonly used

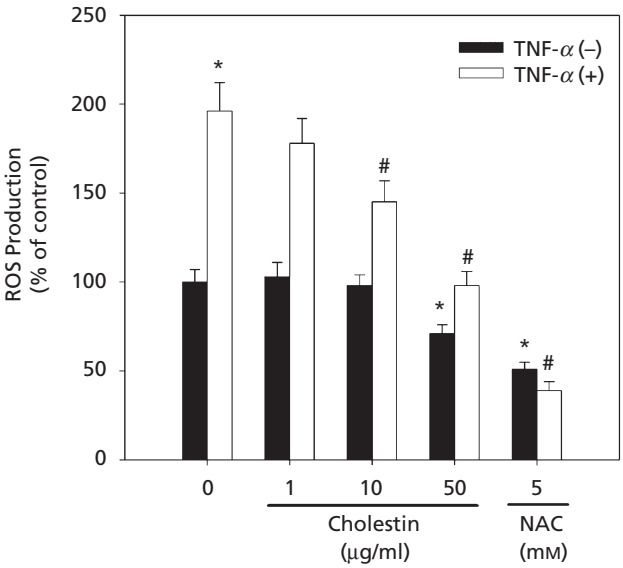

Figure 5 Inhibitory effects of Cholestin and NAC on TNF-α-induced ROS production in HASMCs. HASMCs were labelled with H_2O_2-sensitive fluorescent probe DCFH-DA. Fluorescent intensity of cells was measured with a fluorescence microplate. Data are shown as the mean ± SEM of three independent analyses. $^{*}P < 0.05$, compared to untreated control group; $^{\#}P < 0.05$, compared to TNF-α-treated or control group.

in traditional Chinese medicine for the treatment of blood stasis, a cardiovascular-related disorder.[40] Cholestin is rich in polyphenolic compounds, which inhibit homocysteine-stimulated endothelial adhesiveness as well as downregulating intracellular ROS formation, NF-κB activation and vascular adhesion molecule-1 expression in endothelial cells, supporting the notion that Cholestin may have potential applications in clinical atherosclerosis.[18] In the present study, Cholestin treatment effectively reduced MMP-2 and MMP-9 expression in TNF-α-stimulated HASMCs. Collectively, these data suggest that Cholestin treatment decreases MMP-2 and MMP-9 expression and might therefore assist in the prevention of inflammation and pathogenesis of atherosclerosis.

Transcriptional regulation involving NF-κB activation has been implicated in the TNF-α-induced activation of VSMCs.[41] A key component of MMP expression is the redox-sensitive transcription factor NF-κB.[42] Consistent with previous studies,[21,22,32–34] it was demonstrated that TNF-α activates NF-κB in VSMCs, suggesting that the upregulation of MMP expression in response to TNF-α may be mediated by this transcriptional factor. However, NF-κB is a ubiquitously expressed multiunit transcription factor that is activated by diverse signals, possibly via phosphorylation of the IκB subunit and its dissociation from the inactive cytoplasmic complex, followed by translocation of the active dimers p50 and p65, to the nucleus.[43] In several immortalized cell lines, NF-κB is activated by diverse stimuli, such as TNF-α, interlukin-1β and lipopolysaccharides, and inhibited by the antioxidants pyrrolidine dithiocarbamate and NAC.[44] Since Cholestin has been shown to exhibit antioxidative properties,[9–11] this study demonstrates a similar pattern of Cholestin-sensitive inactivation of MMP expression and NF-κB activity in HASMCs.

The antioxidative components such as sterols, isoflavones,[1] pigments,[45] dimerumic acid[9,11] and dihydromonacolin-MV[12] may possibly contribute to the anti-athergenetic effect of Cholestin. In a previous study of the radical-scavenging abilities of Cholestin using the probe-based ultra-weak chemiluminescence technique, the scavenging activities of Cholestin on $O_2^{-}\cdot$ OH·, and ROO· but not H_2O_2 were demonstrated *in vitro*.[18] In the early stage of atherosclerosis, TNF-α-induced superoxide anion production may play a potential role in NF-κB activation in the vascular wall via activation of IκB kinase.[23] It has been shown that statins have intrinsic antioxidant activity with both antihydroxyl and peroxyl radical activity.[46] Recently, a novel antioxidant mechanism by which statins reduce ROS in endothelial cells has been demonstrated and statin-mediated *S*-nitrosylation of thioredoxin enhanced the enzymatic activity of thioredoxin, resulting in a significant reduction in intracellular ROS.[47] Based on the results of the present study, it is proposed that the inhibitory effect of Cholestin on MMP expression and NF-κB activation may be due to its antioxidant properties and that it acts by directly scavenging ROS. Further study of the direct radical scavenging ability of various components is in progress in order to determine the mechanism of action of Cholestin. Additionally, based on the significant decrease in viability of HASMCs at relatively high concentrations of Cholestin (160 μg/ml), attention should be paid to possible side effects in clinical application.

Conclusion

This study shows that Cholestin treatment effectively reduces MMP-2 and MMP-9 expression in TNF-α-stimulated HASMCs. This is the first study to show that Cholestin reduces intracellular ROS production, NF-κB activation and that it consequently decreases MMP-2 and MMP-9 expression in HASMCs. Since the degradation of the ECM and basement membrane by HASMCs and the migration of VSMCs through MMP-2 and MMP-9 overexpression are crucial steps in the pathogenesis of atherosclerosis and restenosis, this study implies that Cholestin may have therapeutic potential in the prevention of cardiovascular disease.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This study was supported by grants VGH94-289 and VGH V96C1-156 from Taipei Veterans General Hospital, Taipei, Taiwan; grant NSC 100–2320-B-039–008-MY2 from the National Science Council, Taiwan; grant CMU99-COL-03 from China Medical University, Taichung, Taiwan; and grant DOH100-TD-B-111–004 from the Taiwan Department of Health Clinical Trial and Research Center of Excellence.

References

1. Heber D *et al*. Cholesterol-lowering effects of a proprietary Chinese red-yeast-rice dietary supplement. *Am J Clin Nutr* 1999; 69: 231–236.
2. Man RY *et al*. Cholestin inhibits cholesterol synthesis and secretion in hepatic cells (HepG2). *Mol Cell Biochem* 2002; 233: 153–158.
3. Wei W *et al*. Hypolipidemic and anti-atherogenic effects of long-term Cholestin (*Monascus purpureus*-fermented rice, red yeast rice) in cholesterol fed rabbits. *J Nutr Biochem* 2003; 14: 314–318.
4. Ma J *et al*. Constituents of red yeast rice, a traditional Chinese food and medicine. *J Agric Food Chem* 2000; 48: 5220–5225.
5. Zhang W *et al*. Active components of Xuezhikang. *Chin J New Drugs* 1998; 7: 213–214.
6. Katan MB *et al*. Effects of fats and fatty acids on blood lipids in humans: an overview. *Am J Clin Nutr* 1994; 60: 1017S–1022S.
7. Liao CC *et al*. Red yeast rice for a patient with olanzapine-induced dyslipidemia: a test-and-retest case report. *Prog Neuropsychopharmacol Biol Psychiatry* 2008; 32: 1340–1341.
8. Zhao SP *et al*. Effect of xuezhikang, a cholestin extract, on reflecting postprandial triglyceridemia after a high-fat meal in patients with coronary heart disease. *Atherosclerosis* 2003; 168: 375–380.
9. Aniya Y *et al*. Dimerumic acid as an antioxidant of the mold, *Monascus anka*. *Free Radic Biol Med* 2000; 28: 999–1004.
10. Aniya Y *et al*. Screening of antioxidant action of various molds and protection of *Monascus anka* against experimentally induced liver injuries of rats. *Gen Pharmacol* 1999; 32: 225–231.
11. Taira J *et al*. Dimerumic acid as an antioxidant from the mold, *Monascus anka*: the inhibition mechanisms against lipid peroxidation and hemeprotein-mediated oxidation. *Biochem Pharmacol* 2002; 63: 1019–1026.

12. Dhale MA *et al*. Isolation and characterization of dihydromonacolin-MV from *Monascus purpureus* for antioxidant properties. *Appl Microbiol Biotechnol* 2007; 73: 1197–1202.
13. Liu L *et al*. Xuezhikang decreases serum lipoprotein(a) and C-reactive protein concentrations in patients with coronary heart disease. *Clin Chem* 2003; 49: 1347–1352.
14. Zhao SP *et al*. Xuezhikang, an extract of cholestin, protects endothelial function through antiinflammatory and lipid-lowering mechanisms in patients with coronary heart disease. *Circulation* 2004; 110: 915–920.
15. Li JJ *et al*. Effects of xuezhikang, an extract of cholestin, on lipid profile and C-reactive protein: a short-term time course study in patients with stable angina. *Clin Chim Acta* 2005; 352: 217–224.
16. Hu CL *et al*. Effects of withdrawal of Xuezhikang, an extract of cholestin, on lipid profile and C-reactive protein: a short-term time course study in patients with coronary artery disease. *Cardiovasc Drugs Ther* 2006; 20: 185–191.
17. Zhao SP *et al*. Xuezhikang, an extract of cholestin, reduces cardiovascular events in type 2 diabetes patients with coronary heart disease: subgroup analysis of patients with type 2 diabetes from China coronary secondary prevention study (CCSPS). *J Cardiovasc Pharmacol* 2007; 49: 81–84.
18. Lin CP *et al*. Cholestin (Monascus purpureus rice) inhibits homocysteine-induced reactive oxygen species generation, nuclear factor-kappaB activation, and vascular cell adhesion molecule-1 expression in human aortic endothelial cells. *J Biomed Sci* 2008; 15: 183–196.
19. Ross R. Atherosclerosis–an inflammatory disease. *N Engl J Med* 1999; 340: 115–126.
20. Tsai CS *et al*. Urotensin II-induced endothelin-1 expression and cell proliferation via epidermal growth factor receptor transactivation in rat aortic smooth muscle cells. *Atherosclerosis* 2009; 206: 86–94.
21. Kim HS *et al*. Alpha-lipoic acid inhibits matrix metalloproteinase-9 expression by inhibiting NF-kappaB transcriptional activity. *Exp Mol Med* 2007; 39: 106–113.
22. Lee B, Moon SK. Resveratrol inhibits TNF-alpha-induced proliferation and matrix metalloproteinase expression in human vascular smooth muscle cells. *J Nutr* 2005; 135: 2767–2773.
23. Chen YH *et al*. Anti-inflammatory effects of different drugs/agents with antioxidant property on endothelial expression of adhesion molecules. *Cardiovasc Hematol Disord Drug Targets* 2006; 6: 279–304.
24. Lee WJ *et al*. Sesamin mitigates inflammation and oxidative stress in endothelial cells exposed to oxidized low-density lipoprotein. *J Agric Food Chem* 2009; 57: 11406–11417.
25. Lin SJ *et al*. Salvianolic acid B attenuates MMP-2 and MMP-9 expression in vivo in apolipoprotein-E-deficient mouse aorta and in vitro in LPS-treated human aortic smooth muscle cells. *J Cell Biochem* 2007; 100: 372–384.
26. Wu TC *et al*. Carvedilol, a pharmacological antioxidant, inhibits neointimal matrix metalloproteinase-2 and -9 in experimental atherosclerosis. *Free Radic Biol Med* 2007; 43: 1508–1522.
27. Chen JW *et al*. Ginkgo biloba extract inhibits tumor necrosis factor-alpha-induced reactive oxygen species generation, transcription factor activation, and cell adhesion molecule expression in human aortic endothelial cells. *Arterioscler Thromb Vasc Biol* 2003; 23: 1559–1566.
28. Chen YH *et al*. Magnolol attenuates VCAM-1 expression in vitro in TNF-alpha-treated human aortic endothelial cells and in vivo in the aorta of cholesterol-fed rabbits. *Br J Pharmacol* 2002; 135: 37–47.
29. Galis ZS *et al*. Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. *Circ Res* 1994; 75: 181–189.
30. Cho A *et al*. Mitogen-activated protein kinases mediate matrix metalloproteinase-9 expression in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 2000; 20: 2527–2532.
31. Moon SK *et al*. ERK1/2 mediates TNF-alpha-induced matrix metalloproteinase-9 expression in human vascular smooth muscle cells via the regulation of NF-kappaB and AP-1: involvement of the ras dependent pathway. *J Cell Physiol* 2004; 198: 417–427.
32. Kim CH, Moon SK. Epigallocatechin-3-gallate causes the p21/WAF1-mediated G(1)-phase arrest of cell cycle and inhibits matrix metalloproteinase-9 expression in TNF-alpha-induced vascular smooth muscle cells. *Arch Biochem Biophys* 2005; 435: 264–272.
33. Moon SK *et al*. Quercetin exerts multiple inhibitory effects on vascular smooth muscle cells: role of ERK1/2, cell-cycle regulation, and matrix metalloproteinase-9. *Biochem Biophys Res Commun* 2003; 301: 1069–1078.
34. Moon SK *et al*. Reactive oxygen species mediates disialoganglioside GD3-induced inhibition of ERK1/2 and matrix metalloproteinase-9 expression in vascular smooth muscle cells. *FASEB J* 2006; 20: 1387–1395.
35. Zhang HS, Wang SQ. Salvianolic acid B from *Salvia miltiorrhiza* inhibits tumor necrosis factor-alpha (TNF-alpha)-induced MMP-2 upregulation in human aortic smooth muscle cells via suppression of NAD(P)H oxidase-derived reactive oxygen species. *J Mol Cell Cardiol* 2006; 41: 138–148.
36. Jin UH *et al*. Inhibitory effect of *Salvia miltiorrhia* BGE on matrix metalloproteinase-9 activity and migration of TNF-alpha-induced human aortic smooth muscle cells. *Vascul Pharmacol* 2006; 44: 345–353.
37. Tsai CS *et al*. Cilostazol attenuates MCP-1 and MMP-9 expression in vivo in LPS-administrated balloon-injured rabbit aorta and in vitro in LPS-treated monocytic THP-1 cells. *J Cell Biochem* 2008; 103: 54–66.
38. Pasterkamp G *et al*. Atherosclerotic arterial remodeling and the localization of macrophages and matrix metalloproteases 1, 2 and 9 in the human coronary artery. *Atherosclerosis* 2000; 150: 245–253.
39. Recalde D *et al*. Human apolipoprotein A-IV reduces secretion of proinflammatory cytokines and atherosclerotic effects of a chronic infection mimicked by lipopolysaccharide. *Arterioscler Thromb Vasc Biol* 2004; 24: 756–761.
40. Journoud M, Jones PJ. Red yeast rice: a new hypolipidemic drug. *Life Sci* 2004; 74: 2675–2683.
41. McKellar GE *et al*. Role for TNF in atherosclerosis? Lessons from autoimmune disease. *Nat Rev Cardiol* 2009; 6: 410–417.
42. Newby AC. Metalloproteinase expression in monocytes and macrophages and its relationship to atherosclerotic plaque instability. *Arterioscler Thromb Vasc Biol* 2008; 28: 2108–2114.
43. Ghosh S, Baltimore D. Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B. *Nature* 1990; 344: 678–682.
44. Schreck R *et al*. Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. *J Exp Med* 1992; 175: 1181–1194.
45. Jung H *et al*. Color characteristics of monascus pigments derived by fermentation with various amino acids. *J Agric Food Chem* 2003; 51: 1302–1306.
46. Franzoni F *et al*. A comparative study of the in vitro antioxidant activity of statins. *Int J Cardiol* 2003; 90: 317–321.
47. Haendeler J *et al*. Antioxidant effects of statins via S-nitrosylation and activation of thioredoxin in endothelial cells: a novel vasculoprotective function of statins. *Circulation* 2004; 110: 856–861.