Journal of the Science of Food and Agriculture

Inhibition of Endothelial Adhesion Molecule Expression by Monascus purpureus-fermented Rice Metabolites, Monacolin K, Ankaflavin, and Monascin

Journal:	*Journal of the Science of Food and Agriculture*
Manuscript ID:	JSFA-10-1026.R1
Wiley - Manuscript type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Lin, Chih-Pei; Taipei Veterans General Hospital, Department of Pathology and Laboratory Medicine Lin, Yun-Lian; National Research Institute of Chinese Medicine, National Research Institute of Chinese Medicine Huang, Po-Hsun; Taipei Veterans General Hospital, Division of Cardiology Tsai, Hui-Szu; Taipei Veterans General Hospital, Department of Pathology and Laboratory Medicine Chen, Yung-Hsiang; China Medical University, Graduate Institute of Integrated Medicine
Key Words:	Cell adhesion molecule, Monascus purpureus rice (red yeast rice), Inflammation, Oxidative stress, Nuclear factor-κB

Journal of the Science of Food and Agriculture

(JSFA-10-1026.R1)

Inhibition of Endothelial Adhesion Molecule Expression by *Monascus purpureus*-fermented Rice Metabolites, Monacolin K, Ankaflavin, and Monascin

Chih-Pei Lin,[1,2,*] Yun-Lian Lin,[3] Po-Hsun Huang,[4,5] Hui-Szu Tsai,[1] Yung-Hsiang Chen[6,*]

[1]*Department of Pathology and Laboratory Medicine, Taipei Veterans General Hospital, Taipei, Taiwan;* [2]*Department of Biotechnology and Laboratory Science in Medicine and Institute of Biotechnology in Medicine, National Yang-Ming University, Taipei, Taiwan;* [3]*National Research Institute of Chinese Medicine, Taipei, Taiwan* [4]*Division of Cardiology, Taipei Veterans General Hospital, Taipei, Taiwan;* [5]*Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan;* [6]*Graduate Institute of Integrated Medicine, China Medical University, Taichung, Taiwan;*

*Address for reprint requests and other correspondence: C.-P. Lin and Y.-H. Chen. Taipei Veterans General Hospital, Taipei 11217, Taiwan; National Yang-Ming University, Taipei 11221, Taiwan. FAX: +886-4-22037690 (e-mail: cplin@vghtpe.gov.tw and yhchen@mail.cmu.edu.tw)

Abstract

BACKGROUND: Inflammation is an independent risk factor of cardiovascular diseases and associated with endothelial dysfunction. *Monascus purpureus*-fermented rice, containing naturally-occurring statins and various pigments, has lipid-modulating, anti-inflammatory, and antioxidative effects.

RESULTS: The effects of monacolin K, ankaflavin, and monascin, as the metabolites from *Monascus*-fermented rice on the expression of cell adhesion molecules (intercellular adhesion molecule-1/ICAM-1, vascular cell adhesion molecular-1/VCAM-1, and E-selectin) by tumor necrosis factor (TNF)-α-treated human aortic endothelial cells (HAECs) were investigated. Supplement of HAECs with these *Monascus*-fermented rice metabolites significantly suppressed cellular binding between the human monocytic cells U937 and TNF-α-stimulated HAECs. Immunoblot analysis showed that *Monascus*-fermented rice metabolites significantly attenuated TNF-α-induced of VCAM-1 and E-selectin but not ICAM-1 protein expression. Gel shift assays showed that *Monascus*-fermented rice metabolites treatment reduced TNF-α-activated transcription factor nuclear factor (NF)-κB. Furthermore, *Monascus*-fermented rice metabolites also attenuated reactive oxygen species (ROS) generation *in vitro* and in TNF-α-treated HAECs. Supplement with an ROS scavenger *N*-acetyl-cysteine gave similar results as compared with

Monascus-fermented rice metabolites.

CONCLUSION: *Monascus*-fermented rice metabolites reduced TNF-α-stimulated endothelial adhesiveness as well as down-regulating intracellular ROS formation, NF-κB activation, and VCAM-1/E-selectin expression in HAECs, supporting the notion that the various metabolites from *Monascus*-fermented rice might have potential implications in clinical atherosclerosis disease.

Keywords: Cell adhesion molecule; *Monascus purpureus* rice (red yeast rice); Inflammation; Nuclear factor-κB; Oxidative stress

Introduction

Red yeast rice, a fermented product of rice and red yeast (*Monascus purpureus*), has been used by Chinese for many centuries to make rice wine, as a food preservative for maintaining the taste and the color in meat and fish, and for its medicinal properties.[1-3] Cholestin™ is a dietary supplement related to red yeast rice that has been reported to have lipid-lowing effects and considered beneficial in subjects with hyperlipidemia.[1] The pharmacological preparation from red yeast rice that has been publicly used in China, United States, and many other countries is composed, in part, of 734 g kg^{-1} starch, 58 g kg^{-1} protein, less than 20 g kg^{-1} fat, and a number of compounds named monacolins (~4 g kg^{-1}), which are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase.[4] It has also been reported that *Monascus*-fermented rice contains 20 – 60 g kg^{-1} fatty acids including linoleic acid, oleic acid, palmitic acid, and stearic,[5] where some of them have lipid lowing properties.[6] *Monascus* species have been proven to produce many functional secondary metabolites. These pigments (yellow pigment: ankaflavin and monascin; orange pigment: monascorubrin and rubropunctanin; red pigment: monascorubramine and rubropuctamine) were investigated and applied to the food colorant in the previous studies.[7, 8] In current study, *Monascus*-fermented product was gradually regarded as the functional food because the monacolins (lipid-lowering agents),[9]

γ-aminobutyric acid (GABA) (hypotensive agent),[10] dimerumic acid,[11-13] and dihydromonacolin-MV (antioxidants)[14] were found.[15] These secondary metabolites have been identified with anti-inflammatory or antioxidative activities.

The salutary effect of HMG-CoA reductase inhibitors (statins) on reducing mortality rate in patients with coronary artery disease (CAD) has been evidenced.[16, 17] The pharmacological benefit of statins is explained by their lipid-modulating effects; but recent experimental and clinical evidence demonstrates that the anti-atherosclerosis activity of statins also includes cholesterol-independent mechanisms.[18, 19] Red yeast rice contains a family of naturally occurring statins that has a marked modulating effect on lipids[1, 20] and the extract of red yeast rice has been shown with free radical scavenging properties,[11, 13, 21] Recently, a *Monascus*-fermented rice extract was found to decrease C-reactive protein and protect endothelial function through lipid-lowing, anti-inflammatory, or antioxidative mechanisms.[22-26]

Elevated endothelial expression of adhesion molecules as mediators of subintimal leukocyte accumulation in atherosclerosis[27, 28] and increased oxidative stress may play the cardinal role in the inflammatory mechanisms for the progression of atherosclerosis.[29] More recently, it was reported that Cholestin extract reduced homocysteine-stimulated endothelial adhesiveness as well as down-regulating

intracellular ROS formation, supporting the notion that the natural compound Cholestin might have potential implications in clinical atherosclerosis disease.[30] Because the concentrations of statins used in the previous study were markedly higher than that in Cholestin-treated group, it was speculated that Cholestin was not only an impure form of statin drug and chemical components other than monacolins might be responsible for the observation. The antioxidative components could possibly contribute to the anti-athergenetic effects of Cholestin. Inflammatory cytokine tumor necrosis factor (TNF)-α has been shown to promote the adhesion of leukocytes to endothelial cells through oxidative stress-related mechanism.[31, 32] Since *Monascus*-fermented rice metabolites, like statins, may also exhibit a "pleiotropic" effect on vascular protection, in the present study, the ability of *Monascus*-fermented rice metabolites, monacolin K (MK) and two yellow pigments – ankaflavin and monascin, was tested in modulating the expression of adhesion molecules and the activation of redox-sensitive transcription factor nuclear factor-κB (NF-κB) by TNF-α-treated human aortic endothelial cells (HAECs).

Materials and methods

Cell culture

Human aortic endothelial cells (HAECs, Cascade Biologics) were grown in Medium 200 (M200) (Cascade Biologics) supplemented with low serum growth supplement (Cascade Biologics) in an atmosphere of 950 ml l^{-1} air and 50 ml l^{-1} CO_2 at 37°C in plastic flasks. The final concentrations of the components in M200 contained 20 ml l^{-1} FBS, 1 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml human fibroblast growth factor, 10 μg/ml heparin, and 10 ml l^{-1} antibiotic-antimycotic mixture (GibcoBRL). At confluence, the cells were subcultured at a 1:3 ratio and used at passage numbers 3 through 8. The human monocytic cell line U937 (American Type Culture Collection) was grown in suspension culture in RPMI-1640 (JRH Bioscience) containing 100 ml l^{-1} FBS, 25 ml l^{-1} [N-(2-hydroxyethyl) piperazine-N'-(2-ethenesulphonic acid)] (HEPES) buffer and 10 ml l^{-1} antibiotic-antimycotic mixture in an atmosphere of 950 ml l^{-1} air and 50 ml l^{-1} CO_2 at 37°C. The cells were routinely subcultured at a 1:4 ratio. TNF-α, *N*-acetyl-cysteine (NAC), and MK were purchased from Sigma Chemical Co. (MO, USA). Ankaflavin and monascin were purchased from reseaLIFE (Switzerland).

MTT assay for cell viability

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, USA) assay was used to measure cell viability [33]. 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 TNF-α, *Monascus*-fermented rice metabolites, or NAC. After washing HAECs by PBS 2 times, 100 µl 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) where DMSO as the blank. The reduction in optical density caused by drugs was used as a measurement of cell viability, normalized to cells incubated in control medium, which were considered 100% viable.

Monocytic cell-endothelial cell adhesion assay

The adherence of monocytic cells U937 to TNF-α-activated HAECs was examined under static conditions. HAECs were grown to sub-confluence in 6-well plates; cells were incubated with *Monascus*-fermented rice metabolites or NAC for 18 h followed by 6-h stimulation with TNF-α. HAECs in 6-well plates were incubated

with U937 (10^6 cells/ml) for 30 min. Finally, 2 ml (20 ml l^{-1}) gluteraldehyde was added to each well to fix the whole cells. Non-adherent cells were removed, and plates were gently rocking washed 5 min twice with HEPES-HBSS (1:50, HEPES 20 mM; HBSS with Ca^{2+} and Mg^{2+}, with out EDTA). The numbers of adherent cells were recognized and determined under inverted microscopy (OLYMPUS) with computer software, ImagePro Plus 4.0 (USA). Under 40X objective lens, twenty randomly chosen fields were counted per well. Experiments were performed in duplicate or triplicate and were repeated at least 3 times.

Western blot analysis

Protein extracts were prepared as previously described.[34] Briefly, HAECs were lysed in 100 µl lysis buffer with protein: protease inhibitor (PIERCE), after washing by PBS, then centrifuge in 4°C, 8,000×*g* for 30 min to harvest the supernatant. The cell total protein was quantified by Bio-Rad protein assay reagent. The whole-cell lysates were subjected to SDS-polyacrylamide (100 g kg^{-1}) gel electrophoresis, followed by electroblotting onto PVDF membrane (Amersham Biosciences). Membranes were probed with a goat monoclonal antibody directed to ICAM-1, VCAM-1, or E-selectin (R&D, USA) and incubated with horseradish peroxidase-labeled secondary antibody, and then washed with PBS containing 1 ml l^{-1}

Tween 20. Bands were visualized by chemiluminescence detection reagents (PerkinElmer, USA). Anti-β-actin antibodies were used as loading control. Densitometic analysis was conducted with software, ImageQuant (Promega), to semiquantify Western blot data.

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts were prepared as previously described.[35] 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 1 ml l^{-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, 250 ml l^{-1} 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.

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-labeled 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 labeled 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, 200 ml l^{-1} glycerol, 1 mM DTT, 2 µg of poly(dI–dC), 0.2 µg of poly-(L)-lysine. Then the mixtures were subjected to electrophoresis on a 60 g kg^{-1} polyacrylamide gel with 0.5× TBE 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. Densitometic analysis was conducted with software, ImageQuant (Promega), to semiquantify EMSA data.

Detection of intracellular ROS production

The effect of *Monascus*-fermented rice and NAC on ROS production in HAECs

was determined by a fluorometric assay using 2',7'-dichlorofluorescein diacetate (DCFH-DA, Molecular Probe) as the probe.[35] This method is based on the oxidation by H_2O_2 of nonfluorescent 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), while the incubation ended up, HAECs were washed by HBSS (with out Ca^{2+}, Mg^{2+}) containing 100 g kg^{-1} BSA. Then 250 μl cell lysis buffer (PBS containing 200 ml l^{-1} EtOH, 1 ml l^{-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 emission using a fluorescence microplate reader (Victor II).

Ultraweak chemiluminescence (uwCL) monitoring of oxygen-derived free radicals

For superoxide anion ($\cdot O_2^-$)-generating system, the following reaction mixture in a total volume of 2.1 ml consisting of 1.0 ml of 2.0 mM lucigenin; 1.0 ml of phosphate-buffered saline, pH 7.4; 0.05 ml of 1.0 M arginine; 0.05 ml of 1.4 μM methylglyoxal was used. After gently mixing the above-mentioned components, the reaction mixture was added to a quartz round-bottom cuvette in the black-box unit of the uwCL analyzer equipped with a high-sensitivity detector [3.3×10^{-15} W/(cm^2·count)] form Jye Horn Co. (Taipei, Taiwan).[36]

For hydroxyl radical (·OH) generating system. The reaction mixture used

consisting the following: 1.0 ml of 3 µM indoxyl-β-glucuronide (IBG), 0.1 ml $FeSO_4$, 1.6 ml of 30 ml l^{-1} H_2O_2, and 0.05 ml of 10 mM EDTA. All the above-mentioned reagents were added to the quartz round-bottom cuvette in the black-box unit of the uwCL analyzer in a sequential order of EDTA, IBG, H_2O_2, and $FeSO_4$.[37]

For hydrogen peroxide (H_2O_2)-generating system, the following reaction mixture were used: 1.0 ml of 2 mM luminol, containg sodium borate, pH 7.3; 1.0 ml of PBS, pH 7.4 and 1.0 ml of 12 ml l^{-1} H_2O_2. The total volume of the reaction mixture was 3.00 ml. All the above-mentioned reagents were then added to the quartz round-bottom cuvette and uwCL was measured using BJL uwCL analyzer.[38] To standardize the system, we use Trolox as the standard; thus, the IC_{50} value of a test compound can be converted.

Statistical analyses

Results were expressed as mean ± SEM, and data were analyzed using ANOVA followed by Dunnett's test or Student's *t*-test for significant difference. Statistical significance was defined as $p<0.05$. All statistical procedures were performed with SigmaStat version 3.1 (Jandel, USA).

Results

MTT assay for MK, ankaflavin, and monascin on HAECs

Cell viability was assessed using the MTT assay. Treatment of HAECs with low dose MK, ankaflavin, and monascin for 24 h did not result in cytotoxicity, whereas high concentration *Monascus*-fermented rice metabolites (≥ 60 μM ankaflavin and ≥ 100 μM MK and monascin) significantly inhibited cell survival (Fig. 1). In addition, cell viability did not significantly change under the conditions of 50 μM MK, ankaflavin, and monascin as well as 5 mM NAC treatment for 18 h followed by 10 ng/ml TNF-α treatment for 6 h (data not shown). The results indicate that the notable cytotoxic effects on HAECs are found in high-dose various *Monascus*-fermented rice metabolites. The non-cytotoxic working concentrations of MK, ankaflavin, and monascin (≤ 50 μM) in the following tests were used to avoid possible interferences on cell viability.

Monascus-fermented rice metabolites inhibits U937 adhesiveness to TNF-α-activated endothelial cells

TNF-α increases early events of the atherosclerotic process by modulating monocyte adhesion and transmigration.[27] Fig. 2 shows that incubation of HAECs with

TNF-α (10 ng/ml for 6 h) significantly increased U937 adhesiveness. The ability of various *Monascus*-fermented rice metabolites was then tested to modulate U937 adhesiveness to TNF-α-activated endothelial cells. As shown in Fig. 2, un-stimulated control HAECs showed minimal binding to U937 cells, but endothelial adhesiveness to U937 was substantially increased (10.4 fold increase, $p<0.05$) when the HAECs were treated with TNF-α. Supplement of HAECs with various *Monascus*-fermented rice metabolites dose-dependently inhibited U937 adhesion to HAECs treated with TNF-α; supplement of HAECs with 5 mM NAC (an ROS scavenger antioxidative control) for 18 h similarly inhibited U937 adhesion to TNF-α-activated HAECs.

Inhibition of TNF-α-induced VCAM-1 and E-selectin expressions by Monascus-fermented rice metabolites

To determine the optimal conditions for TNF-α-induced adhesion molecule expression by HAECs, dose-response studies were performed, in which HAECs were cultured with various concentrations of TNF-α for various time intervals in a pilot study; in accordance with the previous studies,[27] when HAECs treated with TNF-α (10 ng/ml for 6 h), the cell adhesion molecules, ICAM-1, VCAM-1, and E-selectin expressions on HAECs were significantly increased. Next, the effect of various *Monascus*-fermented rice metabolites on TNF-α-induced cell adhesion molecule

expressions on HAECs was investigated. HAECs were pre-treated with 50 μM MK, ankaflavin, or monascin for 18 h and followed by 10 μg/ml TNF-α for 6 h. The results showed that expression of VCAM-1 and E-selectin but not ICAM-1 protein, (Fig. 3; 5 mM of NAC was used as an ROS scavenger control) in TNF-α-stimulated HAECs were significantly suppressed by various *Monascus*-fermented rice metabolites. This result suggests that endothelial VCAM-1 and E-selectin rather than ICAM-1 expression, are more critical to monocyte adhesion in this *in vitro* model.

Inhibition of TNF-α-induced activation of NF-κB by Monascus-fermented rice metabolites

Transcriptional regulation involving NF-κB activation has been implicated in the TNF-α-induced endothelial dysfunction.[27, 39] To examine whether or not *Monascus*-fermented rice metabolites inhibited NF-κB activation, gel-shift assays were performed with the consensus NF-κB binding sequence. This pilot study showed that incubation of HAECs with 10 ng/ml TNF-α caused significant activation of NF-κB at 30 min. The activation of NF-κB induced by TNF-α could be suppressed by ROS scavenger NAC as detected with DNA binding activity. Supplement with 50 μM MK, ankaflavin, and monascin showed that TNF-α-caused NF-κB shifted bands were significantly reduced (Fig. 4). The results suggest that various *Monascus*-fermented

rice metabolites, by down-regulating the NF-κB activation, may inhibit TNF-α-induced VCAM-1 or E-selectin expression in the HAECs, with the result of suppressing monocyte adhesiveness to endothelial cells.

Inhibition of TNF-α-induced intracellular ROS generation by Monascus-fermented rice metabolites

Inflammatory cytokine TNF-α could activate NF-κB in endothelial cells via oxidative stress.[27] The effect of *Monascus*-fermented rice metabolites on intracellular ROS generation in HAECs was studied. Fig. 5 shows the effects of 2 – 50 μM MK, ankaflavin, and monascin on intracellular ROS production induced by TNF-α (10 ng/ml for 6 h) in HAECs. Treatment with NAC or various *Monascus*-fermented rice metabolites dose-dependently inhibited TNF-α-induced ROS production in HAECs.

Ultraweak Chemiluminescence for radical-scavenging abilities of Monascus-fermented rice metabolites

Probe-based uwCL technique was used to measure the production of a panel of three oxygen-derived free radicals.[30] As shown in the Table, *Monascus*-fermented rice metabolites exhibited the major radical-scavenging abilities on $^{\cdot}OH$, whereas less effect was found on $O_2^{-\cdot}$ (with no suppressible activity for MK and ankaflavin), and

there were no obviously suppressible activity for H_2O_2 scavenging (vitamin E analog, Trolox, was used as an experimental standard for uwCL technique).

Discussion

The present study showed that supplement of HAECs with *Monascus*-fermented rice metabolites, including MK, ankaflavin, and monascin, significantly suppressed cellular binding between the human monocytic cells U937 and TNF-α-stimulated HAECs. *Monascus*-fermented rice metabolites significantly attenuated TNF-α-induced VCAM-1 and E-selectin protein expressions. *Monascus*-fermented rice metabolites treatment also reduced TNF-α-activated redox-sensitive transcription factor NF-κB. Furthermore, *Monascus*-fermented rice also attenuated intracellular ROS generation in TNF-α-treated HAECs. Probe-based uwCL technique showed that *Monascus*-fermented rice metabolites exhibited the major radical-scavenging abilities on $^{\cdot}OH$.

The results confirmed that expression of VCAM-1 and E-selectin proteins in HAECs was significantly elevated by TNF-α stimulation; furthermore, this elevation could be suppressed by various *Monascus*-fermented rice metabolites supplement, suggesting that endothelial VCAM-1 and E-selectin, rather than ICAM-1 expression, was more critical to monocyte adhesion in this *in vitro* model. These results also demonstrate that *Monascus*-fermented rice metabolites may decrease TNF-α-induced endothelial adhesiveness to monocytes, at least in part, via VCAM-1 and E-selectin modulation on HAECs. It's known that VCAM-1 and E-selectin expression is focally

elevated in endothelial cells in vascular regions prone to atherogenesis;[28, 40] the data might reflect a link between elevated TNF-α levels and increased leukocyte infiltration in atherosclerosis development; and supplement of *Monascus*-fermented rice may have therapeutic potential attenuating inflammation-related atherogenesis.

Transcriptional regulation involving NF-κB activation has been implicated in the TNF-α-induced endothelial dysfunction.[27] Supplement with *Monascus*-fermented rice metabolites showed that TNF-α-caused NF-κB shifted bands were significantly reduced, suggesting that *Monascus*-fermented rice metabolites, by down-regulating the NF-κB activation, might inhibit TNF-α-induced VCAM-1 and E-selectin expressions in the HAECs, with the result of suppressing monocyte adhesiveness to endothelial cells. Since *Monascus*-fermented rice has shown antioxidative properties,[11, 13, 21] this study demonstrates a similar pattern of *Monascus*-fermented rice-sensitive inactivation of VCAM-1 and E-selectin expressions and NF-κB activity in HAECs.

Inflammatory cytokine TNF-α could activate NF-κB in endothelial cells via oxidative stress.[27] It has been shown that statins have intrinsic antioxidant activity with both anti-hydroxyl and peroxyl radical activity.[41] The Table shows the *in vitro* (cell-free model) RSA of various Cholestin derivates using the uwCL method. By contrast, Figure 5 shows the *ex vivo* (cell culture model) inhibitory effects of

Cholestin metabolites on TNF-α-induced ROS production in HAECs by a fluorescent probe DCFH-DA assay. These data suggest that all MK, ankaflavin, and monascin have the ability to reduce intracellular ROS production. However, *Monascus*-fermented rice pigments further exhibited the major radical-scavenging abilities on ·OH, but MK failed to inhibit ROS directly in vitro. Recently, a novel antioxidant mechanism by which statins reduce ROS in endothelial cells has been demonstrated, and statin-mediated *S*-nitrosylation of thioredoxin has enhanced the enzymatic activity of thioredoxin, resulting in a significant reduction in intracellular ROS.[42] These results suggest that the inhibitory effect of *Monascus*-fermented rice on adhesion molecule expressions and NF-κB activation may be due to its direct or indirect properties on ROS scavenging. Further study for investigating the direct or indirect radical scavenging ability of various *Monascus*-fermented rice metabolites is carried on to distinguish the action mechanism between monacolins and different pigments.

Monascus-fermented rice contains 4 g kg^{-1} HMG-CoA reductase inhibitors belonging to the statin class.[1] The effective dose in the previous study, 50 μg/ml Cholestin, contains approximately 0.2 mg l^{-1} compounds of the statin class and has similar effect on homocysteine-induce endothelial dysfunction as compared to 10 μM simvastatin or pravastatin.[30] The other antioxidative components, such as sterols,

isoflavones,[1] pigments,[43] and dimerumic acid,[11, 13] could possibly contribute to the anti-athergenetic effects of *Monascus*-fermented rice.

In the study, although these three components are from the same *Monascus purpureus*-fermented rice, the levels of TNF-α-stimulated endothelial adhesiveness, intracellular ROS formation, NF-κB activation, and VCAM/E-selectin expression are different among MK, ankaflavin, and monascin. These data suggest that there are still some different mechanisms involved in these metabolites. The MK significantly inhibited the activation of NF-κB and ROS production, but only partially reduced the expression of adhesion molecules. By contrast, ankaflavin and monascin significantly inhibited the activation of NF-κB and ROS production, but seemed to be more effective in reducing TNF-α-induced monocyte adhesion and adhesion molecule expressions. These findings are also compatible with the present understanding that the activation of NF-κB by cytokines, such as TNF-α, could be caused through both redox-dependent and -independent pathways.[44, 45] Other intracellular signaling pathways, such as mitogen-activated protein kinases or activator protein-1, might be involved and warrant further investigation.

In conclusion, the present study demonstrates that TNF-α markedly increases VCAM-1 and E-selectin expressions as well as the adhesiveness of U937 monocytic cells to endothelial cells. Moreover, supplement of *Monascus*-fermented rice

metabolites, MK, ankaflavin, and monascin, or NAC is useful for endothelial dysfunction induced by TNF-α. These data suggest that *Monascus*-fermented rice supplement may be a potential implication to attenuate TNF-α-stimulated activation of the endothelium and may help reduce the risk of vascular disease associated with inflammation.

Acknowledgments

This study was supported by grant VGH 97C1-157 from Taipei Veterans General Hospital, Taipei, Taiwan, grant NSC 97-2320-B-039-022-MY3 from the National Science Counsel, Taiwan.

References

1. Heber D, Yip I, Ashley JM, Elashoff DA, Elashoff RM and Go VL, Cholesterol-lowering effects of a proprietary Chinese red-yeast-rice dietary supplement. *Am J Clin Nutr* **69**:231-236 (1999).
2. Man RY, Lynn EG, Cheung F, Tsang PS and O K, Cholestin inhibits cholesterol synthesis and secretion in hepatic cells (HepG2). *Mol Cell Biochem* **233**:153-158 (2002).
3. Wei W, Li C, Wang Y, Su H, Zhu J and Kritchevsky D, Hypolipidemic and anti-atherogenic effects of long-term Cholestin (Monascus purpureus-fermented rice, red yeast rice) in cholesterol fed rabbits. *J Nutr Biochem* **14**:314-318 (2003).
4. Ma J, Li Y, Ye Q, Li J, Hua Y, Ju D, Zhang D, Cooper R and Chang M, Constituents of red yeast rice, a traditional Chinese food and medicine. *J Agric Food Chem* **48**:5220-5225 (2000).
5. Zhang W, Duan Z and X S, Active components of Xuezhikang. *Chinese J New Drugs* **7**:213-214 (1998).
6. Katan MB, Zock PL and Mensink RP, Effects of fats and fatty acids on blood lipids in humans: an overview. *Am J Clin Nutr* **60**:1017S-1022S (1994).
7. Martinkova L, Patakova-Juzlova P, Krent V, Kucerova Z, Havlicek V, Olsovsky P, Hovorka O, Rihova B, Vesely D, Vesela D, Ulrichova J and Prikrylova V, Biological activities of oligoketide pigments of Monascus purpureus. *Food Addit Contam* **16**:15-24 (1999).
8. Lin YL, Wang TH, Lee MH and Su NW, Biologically active components and nutraceuticals in the Monascus-fermented rice: a review. *Appl Microbiol Biotechnol* **77**:965-973 (2008).
9. Journoud M and Jones PJ, Red yeast rice: a new hypolipidemic drug. *Life Sci* **74**:2675-2683 (2004).
10. Su YC, Wang JJ, Lin TT and Pan TM, Production of the secondary metabolites gamma-aminobutyric acid and monacolin K by Monascus. *J Ind Microbiol Biotechnol* **30**:41-46 (2003).
11. Taira J, Miyagi C and Aniya Y, Dimerumic acid as an antioxidant from the mold, Monascus anka: the inhibition mechanisms against lipid peroxidation and hemeprotein-mediated oxidation. *Biochem Pharmacol* **63**:1019-1026 (2002).
12. Yamashiro J, Shiraishi S, Fuwa T and Horie T, Dimerumic acid protected oxidative stress-induced cytotoxicity in isolated rat hepatocytes. *Cell Biol Toxicol* **24**:283-290 (2008).
13. Aniya Y, Ohtani, II, Higa T, Miyagi C, Gibo H, Shimabukuro M, Nakanishi H

and Taira J, Dimerumic acid as an antioxidant of the mold, Monascus anka. *Free Radic Biol Med* **28**:999-1004 (2000).

14. Dhale MA, Divakar S, Kumar SU and Vijayalakshmi G, Isolation and characterization of dihydromonacolin-MV from Monascus purpureus for antioxidant properties. *Appl Microbiol Biotechnol* **73**:1197-1202 (2007).
15. Lee CL, Wang JJ, Kuo SL and Pan TM, Monascus fermentation of dioscorea for increasing the production of cholesterol-lowering agent--monacolin K and antiinflammation agent--monascin. *Appl Microbiol Biotechnol* **72**:1254-1262 (2006).
16. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). *Lancet* **344**:1383-1389 (1994).
17. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet* **360**:7-22 (2002).
18. Gotto Jr AM, Jr. and Farmer JA, Pleiotropic effects of statins: do they matter? *Curr Opin Lipidol* **12**:391-394 (2001).
19. Takemoto M and Liao JK, Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler Thromb Vasc Biol* **21**:1712-1719 (2001).
20. Zhao SP, Liu L, Cheng YC and Li YL, Effect of xuezhikang, a cholestin extract, on reflecting postprandial triglyceridemia after a high-fat meal in patients with coronary heart disease. *Atherosclerosis* **168**:375-380 (2003).
21. Aniya Y, Yokomakura T, Yonamine M, Shimada K, Nagamine T, Shimabukuro M and Gibo H, Screening of antioxidant action of various molds and protection of Monascus anka against experimentally induced liver injuries of rats. *Gen Pharmacol* **32**:225-231 (1999).
22. Liu L, Zhao SP, Cheng YC and Li YL, Xuezhikang decreases serum lipoprotein(a) and C-reactive protein concentrations in patients with coronary heart disease. *Clin Chem* **49**:1347-1352 (2003).
23. Zhao SP, Liu L, Cheng YC, Shishehbor MH, Liu MH, Peng DQ and Li YL, Xuezhikang, an extract of cholestin, protects endothelial function through antiinflammatory and lipid-lowering mechanisms in patients with coronary heart disease. *Circulation* **110**:915-920 (2004).
24. Li JJ, Hu SS, Fang CH, Hui RT, Miao LF, Yang YJ and Gao RL, 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* **352**:217-224 (2005).

25. Hu CL, Li YB, Tang YH, Chen JB, Liu J, Tang QZ, Zhang QH and Huang CX, 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* **20**:185-191 (2006).
26. Zhao SP, Lu ZL, Du BM, Chen Z, Wu YF, Yu XH, Zhao YC, Liu L, Ye HJ and Wu ZH, 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* **49**:81-84 (2007).
27. Chen YH, Lin SJ, Chen YL, Liu PL and Chen JW, Anti-inflammatory effects of different drugs/agents with antioxidant property on endothelial expression of adhesion molecules. *Cardiovasc Hematol Disord Drug Targets* **6**:279-304 (2006).
28. Ross R, Atherosclerosis--an inflammatory disease. *N Engl J Med* **340**:115-126 (1999).
29. Puddu GM, Cravero E, Arnone G, Muscari A and Puddu P, Molecular aspects of atherogenesis: new insights and unsolved questions. *J Biomed Sci* **12**:839-853 (2005).
30. Lin CP, Chen YH, Chen JW, Leu HB, Liu TZ, Liu PL and Huang SL, 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* **15**:183-196 (2008).
31. Koga T, Claycombe K and Meydani M, Homocysteine increases monocyte and T-cell adhesion to human aortic endothelial cells. *Atherosclerosis* **161**:365-374 (2002).
32. Silverman MD, Tumuluri RJ, Davis M, Lopez G, Rosenbaum JT and Lelkes PI, Homocysteine upregulates vascular cell adhesion molecule-1 expression in cultured human aortic endothelial cells and enhances monocyte adhesion. *Arterioscler Thromb Vasc Biol* **22**:587-592 (2002).
33. Welder AA, A primary culture system of adult rat heart cells for the evaluation of cocaine toxicity. *Toxicology* **72**:175-187 (1992).
34. Chen JW, Chen YH, Lin FY, Chen YL and Lin SJ, 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* **23**:1559-1566 (2003).
35. Chen YH, Lin SJ, Chen JW, Ku HH and Chen YL, 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* **135**:37-47 (2002).

36. Tsai CH, Chang RC, Chiou JF and Liu TZ, Improved superoxide-generating system suitable for the assessment of the superoxide-scavenging ability of aqueous extracts of food constituents using ultraweak chemiluminescence. *J Agric Food Chem* **51**:58-62 (2003).
37. Tsai CH, Stern A, Chiou JF, Chern CL and Liu TZ, Rapid and specific detection of hydroxyl radical using an ultraweak chemiluminescence analyzer and a low-level chemiluminescence emitter: application to hydroxyl radical-scavenging ability of aqueous extracts of Food constituents. *J Agric Food Chem* **49**:2137-2141 (2001).
38. Chen CW, Chiou JF, Tsai CH, Shu CW, Lin MH, Liu TZ and Tsai LY, Development of probe-based ultraweak chemiluminescence technique for the detection of a panel of four oxygen-derived free radicals and their applications in the assessment of radical-scavenging abilities of extracts and purified compounds from food and herbal preparations. *J Agric Food Chem* **54**:9297-9302 (2006).
39. Lin CP, Chen YH, Leu HB, Lin SJ, Chen YL, Huang SL and Chen JW, Anti-inflammatory strategies for homocysteine-related cardiovascular disease. *Front Biosci* **14**:3836-3845 (2009).
40. Hofmann MA, Lalla E, Lu Y, Gleason MR, Wolf BM, Tanji N, Ferran LJ, Jr., Kohl B, Rao V, Kisiel W, Stern DM and Schmidt AM, Hyperhomocysteinemia enhances vascular inflammation and accelerates atherosclerosis in a murine model. *J Clin Invest* **107**:675-683 (2001).
41. Franzoni F, Quinones-Galvan A, Regoli F, Ferrannini E and Galetta F, A comparative study of the in vitro antioxidant activity of statins. *Int J Cardiol* **90**:317-321 (2003).
42. Haendeler J, Hoffmann J, Zeiher AM and Dimmeler S, Antioxidant effects of statins via S-nitrosylation and activation of thioredoxin in endothelial cells: a novel vasculoprotective function of statins. *Circulation* **110**:856-861 (2004).
43. Jung H, Kim C, Kim K and Shin CS, Color characteristics of monascus pigments derived by fermentation with various amino acids. *J Agric Food Chem* **51**:1302-1306 (2003).
44. Krejsa CM, Nadler SG, Esselstyn JM, Kavanagh TJ, Ledbetter JA and Schieven GL, Role of oxidative stress in the action of vanadium phosphotyrosine phosphatase inhibitors. Redox independent activation of NF-kappaB. *J Biol Chem* **272**:11541-11549 (1997).
45. Janssen-Heininger YM, Poynter ME and Baeuerle PA, Recent advances towards

understanding redox mechanisms in the activation of nuclear factor kappaB. *Free Radic Biol Med* **28**:1317-1327 (2000).

Figure legends

Fig. 1. HAEC viability after culture with various Cholestin metabolites, MK, ankaflavin, and monascin, 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 3 separate experiments, *p<0.05, compared to control.

Fig. 2. Effects of Cholestin metabolites, MK, ankaflavin, and monascin, on TNF-α-stimulated adhesiveness of HAECs to U937 monocytic cells. Incubation of HAECs with 10 ng/ml TNF-α increased U937 adhesiveness. HAECs were pre-incubated for 18 h with various Cholestin metabolites or NAC followed by stimulated with TNF-α (10 ng/ml for 6 h) and adhesion assay was performed. The results for 3 separate experiments, each performed in triplicate, are expressed as mean percentage of untreated control ± SEM. *p<0.05, compared to untreated control group; $^{\#}p$<0.05, compared to TNF-α-treated group. Representative photomicrographs show the effects of Cholestin metabolite treatments on the TNF-α-induced adhesion of U937 cells to HAECs. The scale bar length = 100 μm.

Fig. 3. Western blot analysis of ICAM-1, VCAM-1, and E-selectin expressions in

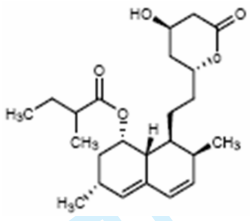
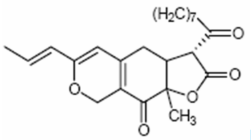
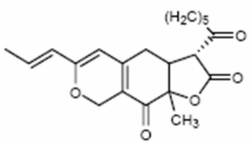
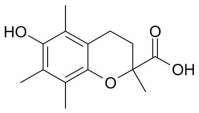
cultured HAECs. Cells were pre-incubated for 18 h with 50 μM MK, ankaflavin, monascin, or 5 mM NAC followed by stimulated with TNF-α (10 ng/ml for 6 h); Western blot analysis was performed as described in "Methods". Densitometric analysis was conducted with software to semiquantify Western blot data. Three independent experiments gave similar results. The summarized data (mean ± SEM) from 3 separate experiments is shown in the bar graph. *p<0.05, compared to untreated control group; $^{\#}p$<0.05, compared to TNF-α-treated group.

Fig. 4. EMSA for NF-κB activation in cultured HAECs. HAECs pre-incubated with for 18 h with 50 μM MK, ankaflavin, monascin, or 5 mM NAC followed by stimulated with TNF-α (10 ng/ml for 30 min) and nuclear protein extracts were prepared and gel shift assay was performed using DIG-labeled oligonucleotides containing consensus NF-κB. Densitometric analysis was conducted with software to semiquantify EMSA data. Three independent experiments gave similar results. The summarized data (mean ± SEM) from 3 separate experiments is shown in the bar graph. *p<0.05, compared to untreated control group; $^{\#}p$ <0.05, compared to TNF-α-treated group.

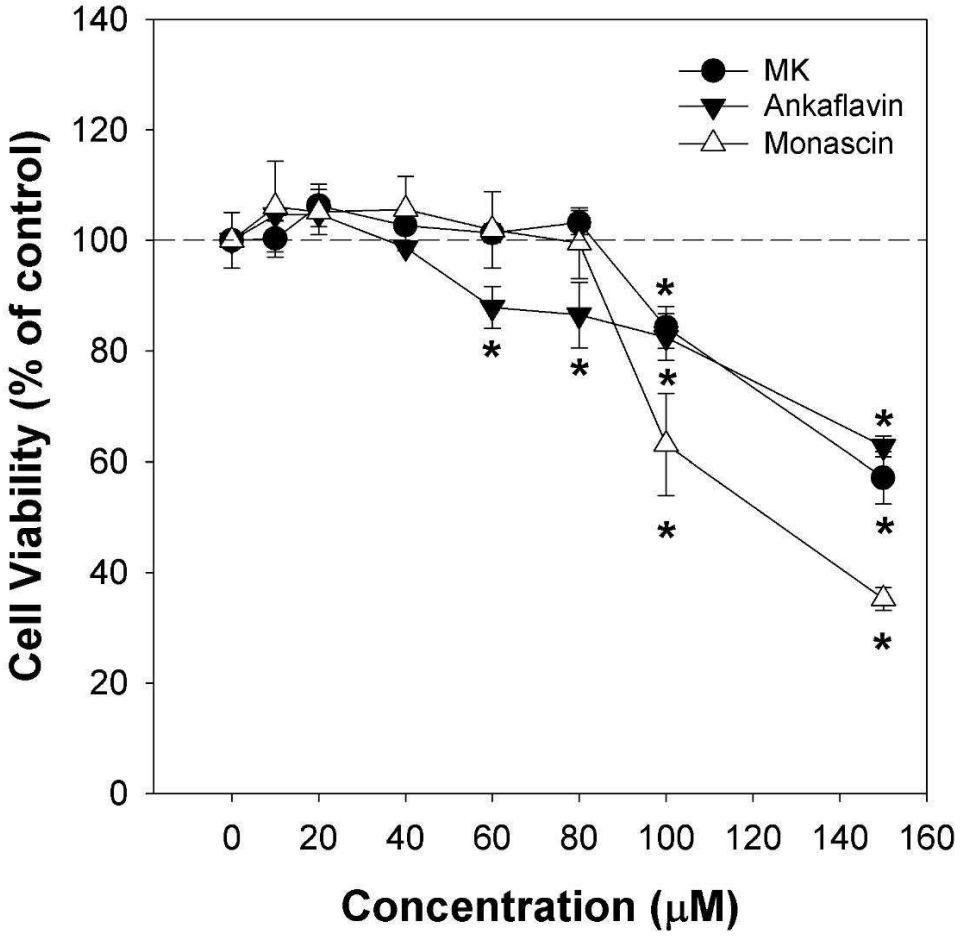
Fig. 5. Inhibitory effects of Cholestin metabolites on TNF-α-induced ROS production

in HAECs. Cells were pre-incubated for 18 h with MK, ankaflavin, monascin, or NAC followed by stimulated with TNF-α (10 ng/ml for 30 min) and intracellular ROS generation (DCF assay) was performed. HAECs were labeled with H_2O_2-sensitive fluorescent probe DCFH-DA (15 μM) for 20 min. Fluorescence intensity of cells was measured with fluorescence microplate. Data are shown as the mean ± SEM of 3 independent analyses. *p<0.05, compared to untreated control group; $^{\#}p$<0.05, compared to TNF-α-treated group.

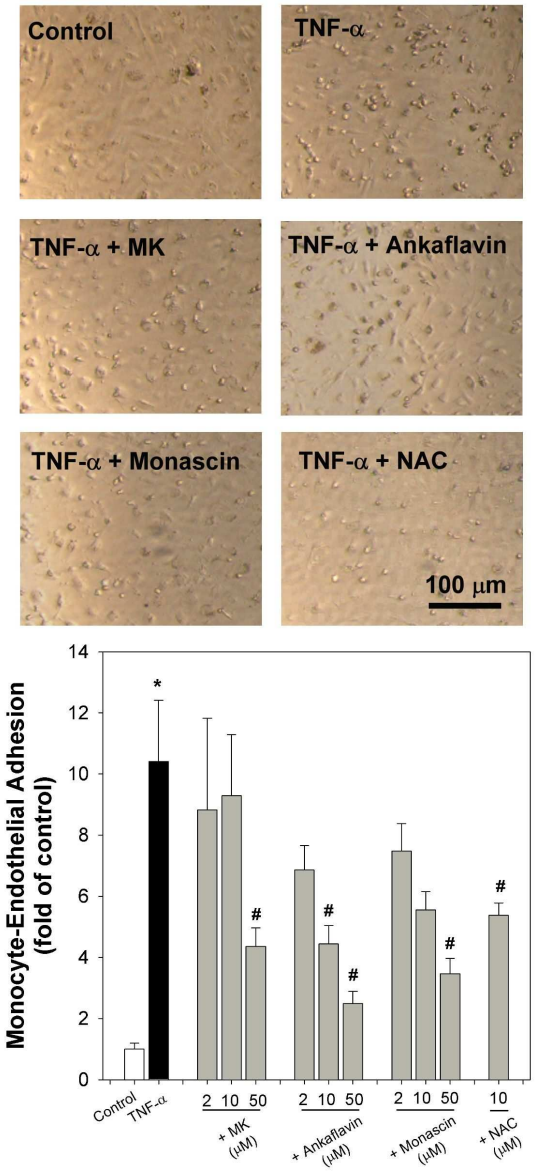
Table. The radical scavenging ability (RSA) of various Cholestin derivates and Trolox (a water soluble vitamin E analog for comparison) using probe-based ultraweak chemiluminescence (uwCL) method

		RSA (IC_{50} value, μM)		
		Superoxide ($O_2^{\cdot-}\cdot$)	Hydroxyl radical ($\cdot OH$)	Hydrogen peroxide (H_2O_2)
Monacolin K		Nil[#]	Nil[#]	Nil[#]
Ankaflavin		Nil[#]	27.84	Nil[#]
Monascin		654.10	41.32	Nil[#]
Trolox		9.51	2.16	395.94

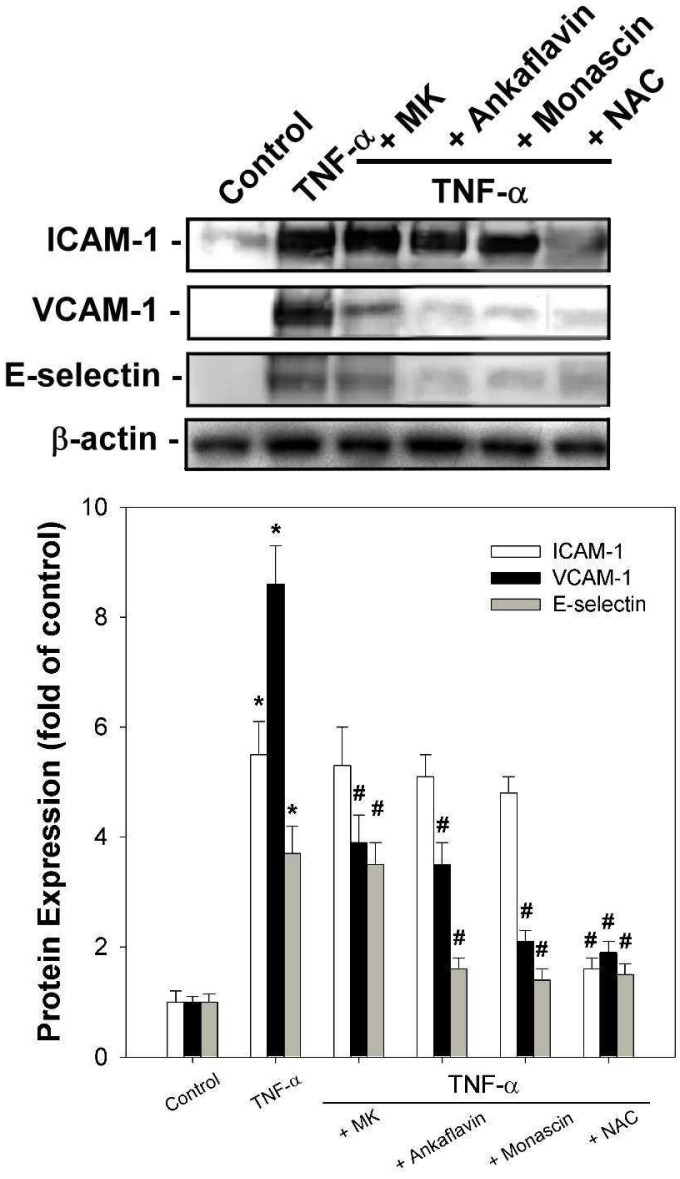
[#] No suppressible activity. The highest concentration used was 700 μM.



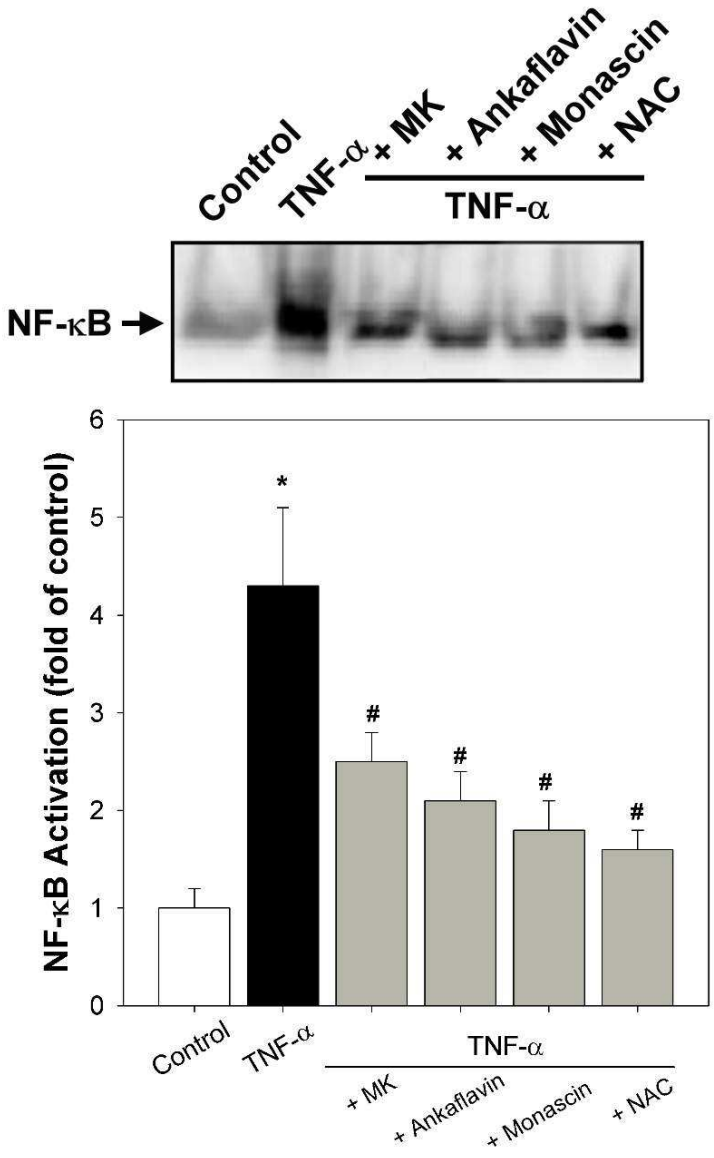
HAEC viability after culture with various Cholestin metabolites, MK, ankaflavin, and monascin, for 24 h as determined by MTT assay
409x406mm (72 x 72 DPI)



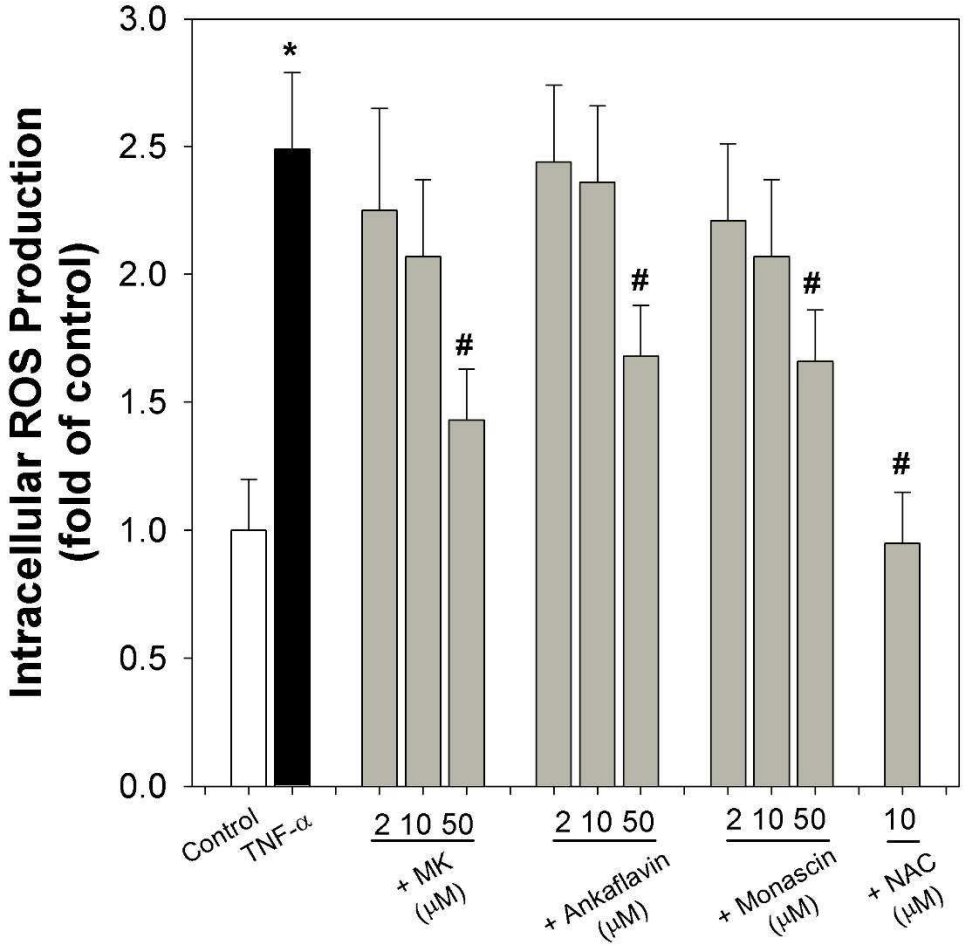
Effects of Cholestin metabolites, MK, ankaflavin, and monascin, on TNF-alpha-stimulated adhesiveness of HAECs to U937 monocytic cells
416x874mm (72 x 72 DPI)



Western blot analysis of ICAM-1, VCAM-1, and E-selectin expressions in cultured HAECs
323x507mm (72 x 72 DPI)



EMSA for NF-κB activation in cultured HAECs
325x466mm (72 x 72 DPI)



Inhibitory effects of Cholestin metabolites on TNF-α-induced ROS production in HAECs
404x398mm (72 x 72 DPI)