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Ankaflavin and Monascin Regulate Endothelial Adhesion Molecules and Endothelial NO Synthase (eNOS) Expression Induced by Tumor Necrosis Factor-α (TNF-α) in Human Umbilical Vein Endothelial Cells (HUVECs)

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ABSTRACT: Previous studies have established that red mold rice can regulate blood pressure in spontaneously hypertensive rats (SHR) and that *Monascus*-fermented products, including monacolin K, ankaflavin (AF), and monascin (MS), can inhibit expression of adhesion factors such as E-selectin and endothelin-1 to prevent human acute monocytic leukemia cell line THP-1 monocytes from adhering to human aortic endothelial cells. However, it remains unknown whether AF and MS act directly on human umbilical endothelial cells (HUVECs) to enhance nitric oxide (NO) synthesis through the stimulation of endothelial NO synthase (eNOS) expression. To address this knowledge gap, this study investigated whether AF and MS directly regulate NO synthesis and attenuate adhesion factor expression induced by treatment with tumor necrosis factor-α (TNF-α) in HUVECs. The results revealed that both AF and MS (20 μM) treatments promoted increases in eNOS expression and decreases in vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and endothelin-1 mRNA and protein expression resulting from 12 h of TNF-α treatment. These effects are attributed to the ability of AF and MS to inhibit extracellular signal-regulated protein kinase (ERK) phosphorylation and nuclear factor κB (NF-κB) translocation from the cytoplasm into the nucleus, thereby exerting antihypertensive activity.

KEYWORDS: *Monascus-fermented products, human umbilical endothelial cells (HUVECs), adhesion factors, ankaflavin, monascin, antihypertensive activity*

INTRODUCTION

In Europe and the United States, >51% of total mortality is caused by cardiovascular diseases, including stroke, hypertension, coronary heart disease, etc., as a result of high blood cholesterol.[1] Similarly, an average of 107 people die of cardiovascular or related diseases every day in Taiwan, and this trend is worsening.[1] Hypertension is one critical factor of metabolic syndrome, associated with dyslipidemia and hyperglycemia and cardiovascular prevention.[2] Endothelial dysfunction is an important manifestation of hypertension. It is characterized by the expression of adhesion molecules, decreased release of nitric oxide (NO) from endothelial nitric oxide synthase (eNOS), release of constrictor molecules such as endothelin-1 and thromboxane A2, and increased vascular permeability.[3] Elevated endothelial expression of adhesion molecules, levels of oxidative stress, and inflammatory factors have been reported to result in atherosclerosis and hypertension.[4−6]

Red mold rice (RMR) is produced through the fermentation of ordinary rice with *Monascus* species. In East Asian countries, RMR has traditionally functioned as a flavoring, coloring, and preservative in food and as a medicinal herb for improving digestion and revitalizing the blood.[7] Many studies have shown that some secondary metabolites of *Monascus* species improved hyperlipidemia,[8,9] hypertension,[10,11] and hyperglycemia,[12,13] slowed tumor promotion[14] Alzheimer's disease development,[15,16] and reduced oxidative stress[17,18] and fatigue during exercise.[19]

We have found that *Monascus*-fermented products contain angiotensin-converting enzyme inhibitor (ACEI) (IC_{50} = 12.2 mg/mL), thereby effectively improving hypertension in spontaneously hypertensive rats.[17] *Monascus* spp. can produce several bioactive metabolites such as γ-aminobutyric acid (GABA), ankaflavin (AF), and monascin (MS). We have found that red mold dioscorea (RMD) showed a greater antihypertensive effect than RMR. In comparison to RMR, RMD contained higher amounts of GABA, MS, and AF. Interestingly, the RMD group (containing 0.2445 mg of GABA) had greater antihypertensive effect than the GABA group (0.2445 mg kg^{-1} day^{-1}),[17] indicating that other metabolites from RMD may also play a role in the antihypertentive ability. Therefore, this study investigated the effects of AF and MS on adhesion molecule production attenuation and eNOS expression regulation in human umbilical endothelial cells (HUVECs).

MATERIALS AND METHODS

Materials and Chemicals. Crystal violet, propidium iodide (PI), sodium dodecyl sulfate (SDS), Triton X-100, tumor necrosis factor-α (TNF-α), trypsin, and trypan blue were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was purchased from Life Technologies (Auckland, New Zealand). Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemical Industries (Saitama, Japan). Endothelial mitogen, M-199 medium, sodium bicarbonate, amphotericin, heparin, hydrocortisone, penicillin, and

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streptomycin were purchased from HyClone Laboratories (Logan, UT). Endothelin-1 antibody, eNOS antibody, VCAM-1 antibody, E-selectin antibody, GAPDH antibody, p-ERK antibody, ERK antibody, and NF-κB p-65 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of Red Mold Rice. The *Monascus purpureus* NTU 568 culture strain was maintained on potato dextrose agar (PDA, Difco Co., Detroit, MI) slanted at 10 °C and transferred monthly. Long-grain rice (*Oryza sativa*) was used to produce RMR. Briefly, 500 g of substrate was soaked in deionized water for 8 h, and excess water was removed with a sieve. The substrate was autoclaved (HL-341 model, Gemmy Corp., Taipei, Taiwan) for 20 min at 121 °C in a "koji dish", which was made of wood with the dimensions of 30 × 20 × 5 cm. After cooling, the substrate was inoculated with a 5% (v/w) spore suspension and cultivated at 30 °C for 7−10 days. During the culturing stage, 100 mL of water was added daily to the substrate from the second day to the fifth day. At the end of cultivation, the crushed and dried product with the mold was extracted by 95% ethanol at 50 °C for 3 days. The crude extracts were obtained after filtering and concentrating under reduced pressure.

Monascin Isolation and Purification. Crude extract of RMR was coated on silica gel and subjected to dry flash chromatography. Sufficient *n*-hexane was passed through the column to remove the oily hydrophobic materials. Extensive gradient elution was then employed using different ethyl acetate in *n*-hexane ratios to yield numerous fractions. Similar fractions were combined according to thin layer chromatography (TLC), and the solvent was removed under reduced pressure. These fractions were further analyzed by high-performance liquid chromatography (HPLC), and then fractions with a similar single peak profile were combined, respectively. HPLC separations were performed on a Shimadzu LC-6AD series apparatus with an SPD-6AV UV detector, equipped with a 250 × 20 mm i.d. preparative Cosmosil AR-II column (Nacalai Tesque, Inc., Kyoto, Japan). Finally, the fraction with the desired compound was concentrated to dryness. The effective compounds AF and MS were identified by nuclear magnetic resonance (NMR, Varian Gemini, 200 MHz, FT-NMR, Varian Inc., Palo Alto, CA) and electrospray ionization−mass spectrometry (ESI-MS, FinniganMAT LCQ, Thermo Electron Co., Waltham, MA) analysis. All analyses were performed using the ESI interface with the following settings: positive ionization mode; temperature of the capillary, 25 °C; spray voltage, 4.5 kV; capillary voltage, 6 V; sheath gas (N_2) flow, 30 AU; and auxiliary gas (N_2) flow, 10 AU. The ESI interface and mass spectrometry parameters were optimized to obtain maximum sensitivity unit resolution.

Cell Culture. HUVECs were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). HUVECs were grown under standard cell culture conditions (humidified atmosphere, 5% CO_2, 37 °C) in an M-199 medium containing 20% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin, 5 units/mL heparin, and 25 μg/mL endothelial mitogen.

Assay for Cell Viability. Cells were seeded on 24-well plates (3×10^4 cells/well) and treated with various concentrations of AF and monascin MS for 24 h. The medium was then removed, washed with phosphate-buffered saline (PBS), and stained with 2 g/L crystal violet in 100 mL/L phosphate-buffered formaldehyde for 20 min before being washed with water. The crystal violet bound to the cells was dissolved in 20 g/L SDS solution, and its absorbance at 600 nm was measured.[20]

Assay for Cell Cycle. After 24 h of exposure to AF and MS, respectively, the medium was aspirated and adherent cells were harvested and centrifuged at 300*g* for 5 min. Cells were washed with PBS, fixed with 700 mL/L ice-cold ethanol at −20 °C overnight, and then stained with PI at room temperature for 30 min. The cell cycle distribution was analyzed by flow cytometry using a FACScan-LSR flow cytometer equipped with CellQuest software (BD Biosciences, San Jose, CA).[20]

Induction of Adhesion Factors by TNF-α (10 ng/mL) Treatment for 12 h. HUVECs were plated at 90−95% confluence in all experiments. AF and MS (10 μM) were added to HUVEC cells, respectively, and then HUVECs were treated with TNF-α (10 ng/mL) for 12 h to induce vascular cell adhesion molecule (VCAM)-1 and E-selectin expression by modifying the method of Kwon et al.[21]

Measurement of NO Level. The production and release of NO were determined by commercial kit (Cayman, Ann Arbor, MI).

Western Blot. Proteins separated by SDS−polyacrylamide gel electrophoresis were electrophoretically transferred to polyvinylidene difluoride membranes. Blots were first incubated in PBS containing 50 g/L nonfat dry milk for 2 h to block nonspecific binding sites and then in a 1:1000 dilution of primary antibodies at 4 °C overnight, and, finally, after washing, the membrane was washed three times each for 5 min in phosphate-buffered saline Tween-20 (PBST), shaken in a solution of horseradish peroxidase (HRP)-linked anti-rabbit IgG secondary antibody, and washed three more times each for 5 min in PBST. The expressions of proteins were detected by enhanced chemiluminescent (ECL) reagent (Millipore, Billerica, MA).

Assay for Reverse-Transcription PCR.[20] Total RNA was isolated using Trizol (Life Technologies) according to the manufacturer's instructions. A 5 μg aliquot of purified total RNA was employed for reverse transcription using Super-Script III (Life Technologies). The reaction mixture was incubated at 42 °C for 1 h, and the reaction was terminated by heating at 70 °C for 10 min. Amplification of the reverse transcription product by PCR was performed using Promega Taq DNA Polymerase (Promega Co., Madison, WI). All reactions were carried out in a thermal cycler (model 2400, Perkin-Elmer, Norwalk, CT) with the following primers:[22] VCAM-1 (forward primer, 5′-ATG-CCTGGGAAGATGGTCGTGA-3′; reverse primer, 5′-TGGAGCT-GGTAGACCCTCGCTG-3′), E-selectin (forward primer, 5′-ATCAT-CCTGCAACTTCACC-3′; reverse primer, 5′-ACACCTCACCAAAC-CCTTC-3′), and β-actin (forward primer, 5′-GACTACCTCATGAA-GATC-3′; reverse primer, 5′-GATCCACATCTGCTGGAA-3′) (PREMIER Biosoft Int., Palo Alto, CA). PCR was performed in 25 μL of 10 mM Tris-HCl (pH 9.0), 25 mM $MgCl_2$, 10 mM dNTP, 5 units of Taq DNA polymerase, and 10 μM of each primer and terminated by heating at 94 °C for 10 min. After thermocycling and electrophoresis of the PCR products (25 μL) on 1% agarose gel, which was stained with 1 μg/mL ethidium bromide and visualized using a UVPGDS-7900 digital imaging system (UVP AutoChemi System, Cambridge, U.K.), the absence of contaminants was routinely checked by the RT-PCR assay of negative control samples without a primer addition.

Cell Morphological Stain.[23] Cells were collected and stained with Liu's stain dye. Cell morphology was observed under an inverted microscopy.

Statistical Analysis. Data were expressed as the mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance (ANOVA) using the general linear model procedure of SPSS version 17.0 (SPSS Institute, Inc., Chicago, IL), followed by ANOVA with Duncan's test.

RESULTS

Cytotoxicity and Cell Cycle Distribution. Although 24 h of treatment with AF or MS (ranging from 1 to 20 μM) was not observed to exert a cytotoxic effect on HUVECs, the cell viability levels of both the AF and MS groups were found to be 80% lower than that of the control group (Figure 1). When the effects of 24 h treatment with 20 μM of AF or MS on HUVEC morphology and the cell cycle were evaluated, neither treatment was found to change the HUVEC morphology or cell cycle distribution (Figure 2). However, no cytotoxic effect was found in AF and MS treatments for 12 h (data not shown).

NO Production by AF- and MS-Treated HUVECs. When the effect of NO production by HUVECs after 12 h of AF or MS treatment was examined, both AF and MS treatments were found to have increased NO production by HUVECs in a dose-dependent manner, with significant differences in production observed between treatment with 15 or 20 μM of AF or MS ($p < 0.05$) (Figure 3A,B). When a decrease in NO production

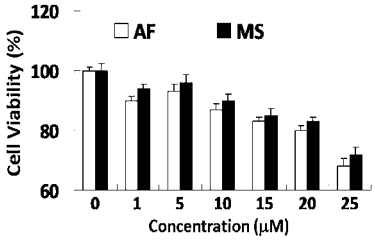

Figure 1. Cell viability of HUVECs treated by AF or MS for 24 h, respectively. Data are presented as the mean ± SD (n = 3).

was subsequently induced by 12 h of treatment with 10 ng/mL of TNF-α and the results of the AF and MS groups were compared with those of the control group, both AF and MS treatments were found to have promoted recovery of NO production by HUVECs (Figure 3C), suggesting that AF and MS treatments may prevent inflammatory factor-induced hypertension via regulation of the NO level.

Inhibition of eNOS Expression in TNF-α-Treated HUVECs. In accordance with the results thus far, the effect of TNF-α on the eNOS expression of HUVECs for 12 h was investigated. Comparison of the results of the AF and MS groups with those of the control group indicates that AF and MS treatments significantly attenuated the inhibitory effect of TNF-α on eNOS expression and promoted recovery of eNOS expression to normal levels in the AF and MS groups ($p < 0.05$) (Figure 4).

Induction of Inflammatory Signaling in TNF-α-Treated HUVECs. Although 12 h of TNF-α treatment was observed to have increased the extracellular signal-regulated kinase (ERK) phosphorylation (p-ERK) in HUVECs (Figure 5A),

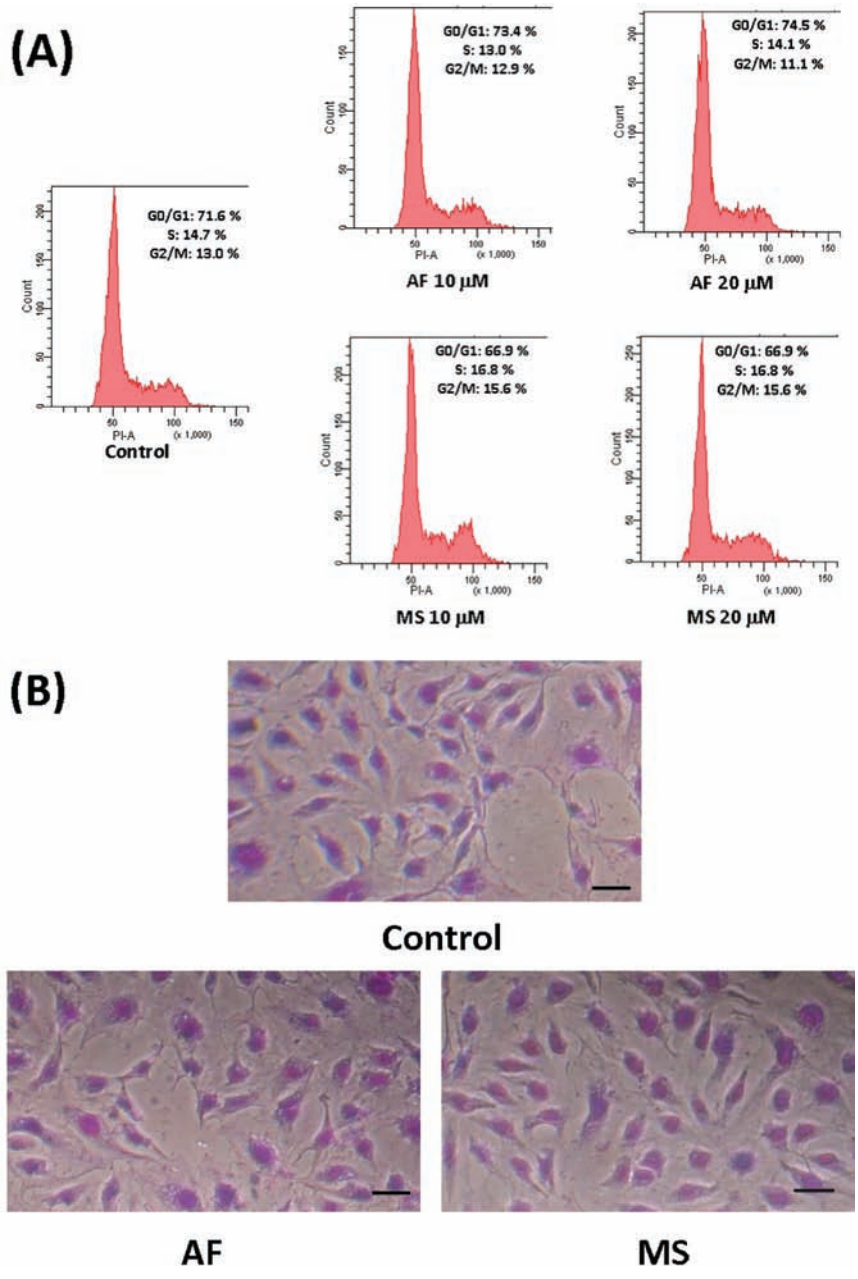

Figure 2. Cell cycle distribution (A) and cell morphology (B) of HUVECs treated by AF or MS for 24 h, respectively. Data are presented as the mean ± SD (n = 3).

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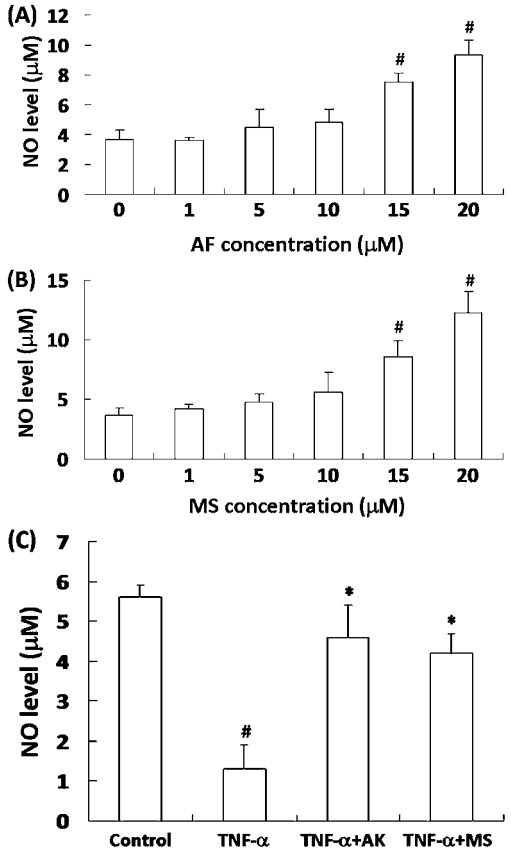

Figure 3. NO production of HUVECs treated by 20 μM AF (A) or MS (B) for 12 h, respectively; inhibitory effect of NO level in TNF-α-treated HUVECs with AF or MS for 12 h (C). Data are presented as the mean ± SD (n = 3). (#) Significantly different from control group at $p < 0.05$.; (*) significantly different from TNF-α-treated group at $p < 0.05$.

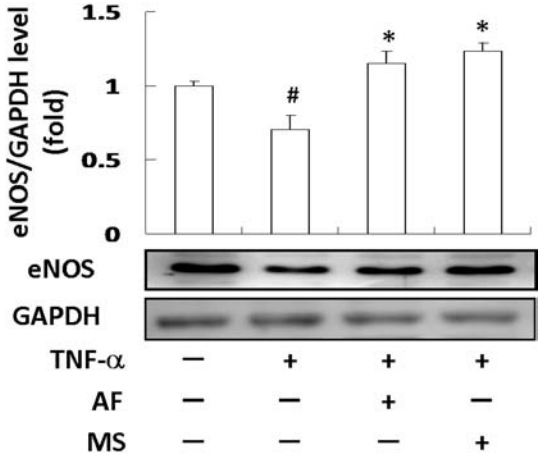

Figure 4. Preventive effect of AF or MS (20 μM) on 10 ng/mL TNF-α induced a decrease in eNOS expression of HUVECs for 12 h of treatment. Data are presented as the mean ± SD (n = 3). (#) Significantly different from control group at $p < 0.05$; (*) significantly different from TNF-α-treated group at $p < 0.05$.

subsequent treatment with AF or MS treatment for 12 h was observed to have decreased p-ERK levels. Twelve hours of TNF-α treatment was also found to have decreased cytosolic NF-κB levels and increased nuclear NF-κB levels, suggesting that TNF-α treatment may promote inflammatory signaling

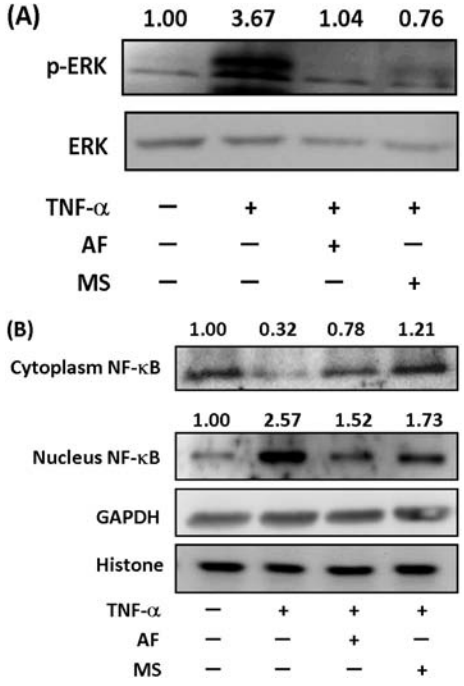

Figure 5. Inhibitory effects of AF and MS (20 μM) on ERK phosphorylation (A) and NF-κB activation (B) in HUVECs treated with 10 ng/mL TNF-α for 12 h.

through NF-κB activation. However, both AF and MS treatments were observed to have attenuated NF-κB transport from the cytosol into the nucleus (Figure 5B).

Expression of Adhesion Factors in TNF-α-Treated HUVECs. NF-κB transcription promotes adhesion factor expression, including that of VCAM-1 and E-selectin. The results of RT-PCR analysis indicate that 12 h of both AF and MS treatments inhibited increases in the VCAM-1 and E-selectin mRNA expression levels of HUVECs that had been induced by 12 h of TNF-α treatment (Figure 6A). When the initial values obtained for the VCAM-1 and E-selectin mRNA levels of HUVECs were assessed by performing real-time PCR, the results confirmed that AF and MS treatments significantly inhibited elevation in VCAM-1 and E-selectin expression levels ($p < 0.05$) (Figure 6B,C). Western blot data also revealed that both AF and MS treatments inhibited increases in VCAM-1 and E-selectin protein expression in HUVECs induced by 12 h of TNF-α treatment (Figure 7).

Effects of AF and MS Treatment on Endothelin-1 Expression of TNF-α-Treated HUVECs. The results indicate that although TNF-α treatment significantly elevates the endothelin-1 expression of HUVECs ($p < 0.05$), which has been associated with hypertension, both AF and MS treatments can attenuate this expression, indicating that both AF and MS may exert antihypertensive activity (Figure 8).

DISCUSSION

Monascus spp. can produce several bioactive metabolites. These encompass not only a variety of pigments, including red (monascorubramine and rubropunctanin), orange (monascorubrin and rubropunctanin), and yellow pigments,[24] but also isoflavones, polyketide monacolins, dimerumic acid, and GABA.[25,26] Kohama et al.[27] found that feeding RMR to spontaneously hypertensive rats (SHR) can effectively inhibit a rise in blood pressure. Examining this finding, Tsuji et al.[28] identified

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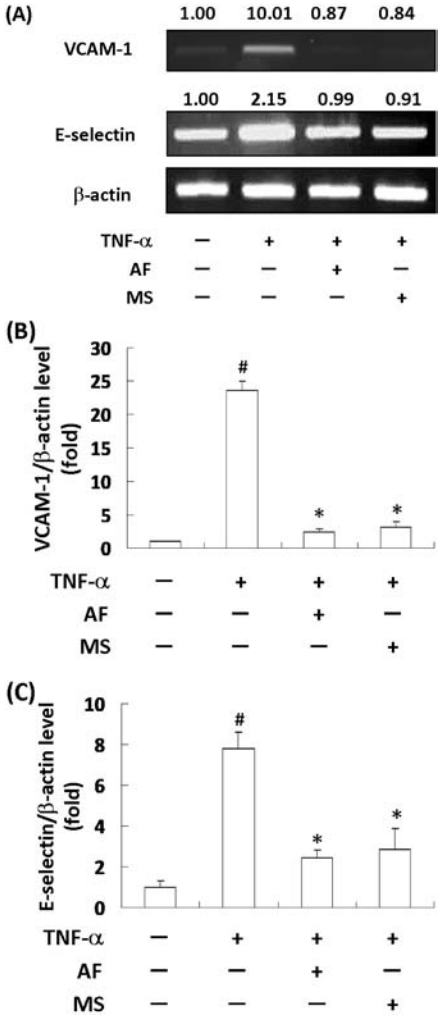

Figure 6. Inhibitory effects of AF and MS (20 μM) on VCAM-1 and E-selectin in HUVECs treated with 10 ng/mL TNF-α for 12 h as measured by RT-PCR (A); levels of VCAM-1 (B) and E-seletin (C) mRNA expression in HUVECs measured by real-time PCR. Data are presented as the mean ± SD (n = 3). (#) Significantly different from control group at $p < 0.05$; (∗) significantly different from TNF-α-treated group at $p < 0.05$.

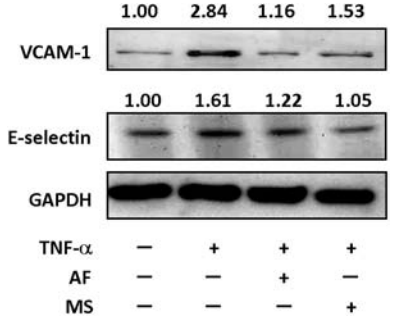

Figure 7. Effects of AF and MS (20 μM) on VCAM-1 and E-selectin protein expression in 10 ng/mL TNF-α treated HUVECs for 12 h by Western blot assay.

GABA, the primary inhibitory neurotransmitter in the central nervous system, as the substance that lowers blood pressure. GABA is known as one of the major inhibitory neurotransmitters in the sympathetic nervous system and plays an important role in cardiovascular function. It has been reported

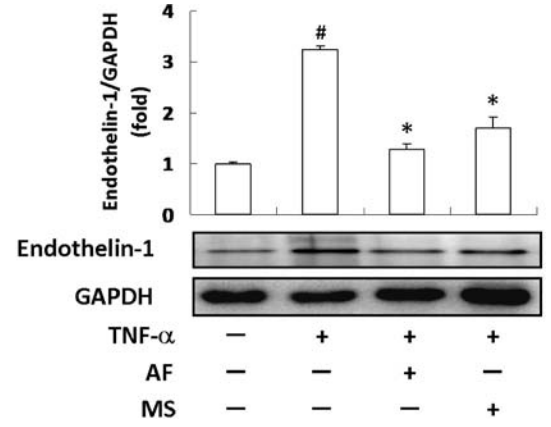

Figure 8. Inhibitory effects of AF and MS (20 μM) on endothelin-1 protein expression in 10 ng/mL TNF-α treated HUVECs for 12 h by Western blot assay. Data are presented as the mean ± SD (n = 3). (#) Significantly different from control group at $p < 0.05$; (∗) significantly different from TNF-α-treated group at $p < 0.05$.

to decrease blood pressure in experimental animals and humans after oral as well as systemic administration.[29]

Adding 0.2−0.3% of *Monascus*-fermented product containing GABA and glucosamine to feed has been found to reduce the blood pressure of rats with congenital hypertension from >200 to <180 mmHg.[30] In a previous study of an SHR model using a noninvasive blood pressure machine to measure tail pulse, rats were fed RMR and RMD (fermented by *M. purpureus* NTU 568) to assess the short-acting (24 h) and long-acting (8 week) blood-pressure-lowering effect.[11] In the short-acting study of RMD, one-time administration of 150 mg/kg of bw was found to have reduced systolic blood pressure by 13 mmHg and diastolic blood pressure by 19 mmHg. In the long-acting study, 8 weeks of RMD administration was found to have reduced systolic and diastolic blood pressures by 27 and 22 mmHg, respectively. These results indicate that both short- and long-term administration of RMD can significantly lower blood pressure ($p < 0.05$).[11]

The vascular remodeling in the aorta caused by hypertension results in vascular lesions observed in vascular disease. The elastin fibers in the aorta of the *Monascus*-treated SHRs were significantly straighter than those of the control rats in our previous study ($p < 0.05$), suggesting that administration of *Monascus*-fermented products has beneficial effects on hypertension.[11] With regard to the differences between the *Monascus*-fermented products, administration of RMD (150 mg/kg of bw; containing 24.46 mg of GABA, 5.17 mg of MS, and 1.06 mg of AF) was found to exert a greater antihypertensive effect than administration of RMR (150 mg/kg of bw; containing 19.68 mg of GABA, 2.53 mg of MS, and 0.78 mg of AF) or GABA (0.245 mg/kg bw).[11] In comparison to RMR, RMD contained higher amounts of GABA, MS, and AF. Interestingly, the RMD group (containing 0.2445 mg of GABA) had greater antihypertensive effect than the GABA group (0.2445 mg kg^{-1} day^{-1}),[11] which means that other metabolites from RMD may also play a role in antihypertensive ability.

Numerous risk factors, such as physical inactivity, smoking, overnutrition, and aging, appear to contribute to the development of vascular dysfunction.[31] Accumulating evidence suggests that the inflammatory cytokine TNF-α plays a pivotal role in the disruption of macrovascular and microvascular circulation both in vivo and in vitro. TNF-α regulates NOS expression

and/or activity, which exerts direct effects on NO production; for example, human aortic endothelial cells treated with TNF-α for 8 h had induced iNOS mRNA expression, but down-regulated eNOS expression.[32] Other studies have also shown that TNF-α significantly decreased eNOS expression in endothelial cells ($p < 0.05$).[33,34] TNF-α also induces the gene expression of various inflammatory cytokines and chemokines, either dependent on or independent of the activation of transcriptional factors, such as NF-κB and AP-1.[35,36] This TNF-α-mediated signaling initiates and accelerates atherogenesis, thrombosis, vascular remodeling, vascular inflammation, endothelium apoptosis, vascular oxidative stress, and impaired NO bioavailability, which contribute to the blunted vascular function. Transcriptional regulation involving NF-κB activation has been implicated in TNF-α-induced endothelial dysfunction.[4,36] A previous study reported that AF and MS can inhibit the binding activity of NF-κB and DNA,[37] thereby suppressing VCAM-1, intercellular adhesion molecule (ICAM)-1, and oxidative stress levels, whereas this study found that AF and MS can inhibit ERK phosphorylation to attenuate NF-κB activation. It is known that VCAM-1 and E-selectin expressions are focally elevated in endothelial cells in vascular regions prone to atherogenesis.[38] In addition, AF and MS have both been found to decrease the TNF-α-induced ICAM-1, VCAM-1, and E-selectin expression of human aortic endothelial cells (HAECs) and attenuate adhesion of THP-1 monocytes to HAECs.[37] Furthermore, one study has indicated that inflammatory factors could result in oxidative stress and NF-κB activation,[39] and several other studies have found that administration of RMR can exert antioxidative properties to attenuate inflammation.[40,41]

Several studies have investigated the inhibition of phenol on TNF-α-induced inflammation. Among these studies, one found that polyphenols derived from apples could significantly attenuate TNF-α-induced NF-κB activation in HUVECs to improve hypertension ($p < 0.05$).[42] Another found that genistein, a soy phytoestrogen, may improve vascular function and hypertension by activating eNOS and increasing NO production in HUVECs, as well as improve blood pressure, in SHRs.[43] Given the importance of NO in modulating vascular homeostasis, it is tempting to propose that AF and MS exert vasculoprotective effects by regulating NO levels. Previous studies have established a role for estrogen in vascular endothelial cells in enhancing NO synthesis by genomic stimulation of eNOS expression[44] and by ER-mediated and nongenomic eNOS activation.[45] Indeed, endothelium-derived NO is not only a potent vasodilator but also possesses anti-inflammatory, antiatherogenic, antithrombotic, and antiapoptotic properties.[46] As neither AF nor MS treatment was found to directly promote heart artery dilatation ex vivo in this study (data not shown), it is hypothesized that AF and MS regulate blood pressure by improving inflammation and up-regulating the eNOS expression in HUVECs.

In conclusion, the present study demonstrated that TNF-α treatment markedly increases VCAM-1, E-selectin, and endothelin-1 expression and decreases eNOS generation to reduce NO level in HUVECs but that supplementation with either of the *Monascus*-fermented metabolites AF or MS can attenuate the endothelial dysfunction induced by TNF-α treatment. These results suggest that both AF and MS treatments are potential means of attenuating TNF-α-stimulated endothelin-1 activation, inhibiting eNOS expression, and reducing NO generation, and thus may help to abate the risk of vascular disease associated with inflammation, thereby preventing hypertension.

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Notes

The authors declare no competing financial interest.

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