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Nobiletin Prevents Trimethylamine Oxide-Induced Vascular Inflammation via Inhibition of the NF-κB/MAPK Pathways

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Supporting Information

ABSTRACT: Dietary choline and its containing foods are biotransformed to trimethylamine (TMA) via gut microbial metabolism. Subsequently, as an intermediate molecule, TMA is quickly transported and oxidized in the liver by hepatic flavin monooxygenases to form trimethylamine oxide (TMAO). TMAO was treated as a waste byproduct from choline metabolism, but recent convincing evidence demonstrated the association between the small molecule TMAO and inflammation-related diseases, including blood vessel inflammation and vascular diseases. The scope of this study is to investigate the preventive effect of nobiletin on TMAO-induced blood vessel inflammation. Our results from Western blot showed that the inhibition of TMAO-induced cardiovascular inflammation was correlated with nobiletin-mediated inhibitory effects on NF-κB and MAPK/ERK related pathways. More specifically, nobiletin prevented the oxidative damage of vascular sites (proximal aorta), inhibited the activity of MAPK/ERK, reduced the expression of NF-κB p65 and phospho-NF-κB p65, and consequently decreased the inflammatory response. Flow cytometry analyses showed that nobiletin decreased TMAO-induced apoptosis of HUVEC cells and counteracted TMAO-induced HUVEC cell proliferation. Results from HE staining and immunohistochemical results also showed that nobiletin reduced the degree of inflammation of the proximal aorta in Sprague−Dawley rats. In summary, nobiletin significantly reduced TMAO-induced vascular inflammation via inhibition of the NF-κB/MAPK pathways.

KEYWORDS: *TMAO, nobiletin, vascular inflammation, NF-κB/MAPK pathways, cell proliferation*

INTRODUCTION

Vascular diseases are the most prevalent pathological conditions affecting humans daily life with a high rate of occurrence, disability, and fatality and draw the most frequent medical complication.[1] Referring to a group of disorders occurring in the heart and blood vessels, vascular diseases have been the leading cause of mortality in the world.[2] Risks for vascular diseases include alcohol consumption, tobacco use, less physical activity, unhealthy diet, and obesity. The tremendous burden for quality of life and the medical expenses of vascular disease patients is beyond imagination.[1,2] As is true universally, prevention of vascular diseases is unarguably the best solution. Hence, the findings of reasonable causes of vascular diseases are of profound importance.

A recent investigation from over 25 hundred people revealed that dominant meat eaters showed increased connection between L-carnitine, mainly found in red meat and high-fat dairy products, and increased vascular diseases such as atherosclerosis.[3] L-Carnitine is metabolized by intestinal microbiota to trimethylamine (TMA), which is then rapidly absorbed to portal circulation and transported to the liver, where it is quickly oxidized to trimethylamine oxide (TMAO) by liver enzyme and flavin monooxygenases (FMO), and subsequently released to the blood circulation system. TMAO is an active molecule and the attack of plasma TMAO on vascular cells results in vascular diseases such as atherosclerosis.[4,5] Generating a high expression level of lamins, atherosclerosis induces coronary heart disease, angina, myocardial infarction, and cerebrovascular disease among others.[6,7] Nuclear lamins provide structural support for the nucleus and are also involved in transcriptional regulation, cell cycle control, DNA repair, and signal transduction.[8] A-type lamins, the main component of the nuclear lamina including two major isoforms of lamin A and lamin C, are mainly expressed in tissues such as heart, liver, and kidney.[9] The expression level of lamin A is found to be decreased in

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senescent cells, resulting in increased cell sensitivity to ROS, aging occurrence, and more DNA damage.[10]

To prevent against vascular diseases, increased intake of dietary plants such as fruits and vegetables is strongly recommended in addition to reducing behavioral risk factors pertaining to CVDs incidence. It has been well-documented that phytochemicals from plants have health-promotion benefits for protecting vascular diseases incidence. For example, nobiletin (and its metabolites) is an active ingredient found in the peels of citrus fruits, particularly aged tangerines. It has been found that nobiletin can inhibit cholesterol oxidation and atherosclerosis, thus preventing thrombosis.[11,12] Also, nobiletin was shown to regulate intracellular ROS generation,[13,14] intervene in the NF-κB signaling pathway, and decrease the inflammatory markers of COX-2 and iNOS.[15,16] More recently, it was found that nobiletin enhanced circadian rhythms and prevented metabolic syndrome in a clock-dependent manner by targeting the molecular oscillator.[17]

Moreover, emerging evidence suggests a key role for chronic inflammation in the initiation of vascular diseases.[18,19] As an example, the anti-inflammatory drug canakinumab targeting interleukin-1β has been proved to significantly lower recurrent cardiovascular events.[20] Notably, increasing reports indicated that TMAO-induced vascular events are closely related to inflammation.[21−23] In particular, TMAO-promoted IL-1β release in endothelial cells is closely linked with ROS[22,24,25] and the canonical NF-κB signaling.[21] Knowing the established anti-inflammatory effects of nobiletin, in this study we aimed to investigate whether inflammation is involved in the pathogenesis of TMAO-induced vascular events and whether nobiletin can reduce the vascular pathology induced by TMAO via an anti-inflammation mechanism.

MATERIALS AND METHODS

Materials. Nobiletin with a minimum purity of 98% was isolated in house from aged tangerine peels according to previously described method.[26] TMAO, *N*,*N*,*N*′,*N*′-Tetramethylethane-1,2-diamine, ammonium peroxy sulfate, and hematoxylin & eosin (HE) reagents were purchased from Sigma-Aldrich (St. Louis, USA). Choline chloride of 99% purity was purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Ham's F-12K (Kaighn's) medium, trypsin, fetal calf serum, EZCell Cell Cycle Analysis Kit, RadioImmunoPrecipitation Assay (RIPA) lysis buffer (strong), and Nuclear/Cytosol Fractionation Kit were purchased from Biovision, Inc. (Milpitas, CA, USA). Bicinchoninic acid (BCA) protein Quantitation Kit, goat anti-rabbit IgG, and AnnexinV-FITC/PI apoptosis detection kit were purchased from Bio-Swamp Life Science Lab (Wuhan, Hubei Province, China). Protein marker was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA); MTT, sodium dodecyl sulfate (SDS), poly(vinylidene difluoride) (PVDF) transfer membrane, and chemiluminescence reagents were purchased from Merck Millipore (Billerica, MA, USA). Antibodies IL-1β, TNF-α, MCP-1, IL-6, SOD, CAT-1, NF-κB p65(p65), phospho-NF-κB p65 (p-p65), ERK, phospho-ERK (p-ERK), p38 MAPK (p38), phospho-p38 MAPK (p-p38), iNOS, and COX-2 were purchased from Abcam plc (Cambridge, England). SOD activity colorimetric assay kit was purchased from Nanjing Jiancheng Bioengineering Institute Co., Ltd. (Nanjing, Jiangsu Province, China). Some catalog numbers of reagents and information on cell lines used in this study are listed in the Supporting Information.

Animal Experiments. Forty Sprague−Dawley (SD) rats (4−6 weeks old) were purchased from the Hubei Research Center of Laboratory Animals (Wuhan, Hubei Province, China]. Rats were offered ad libitum access to food and water (more information on animal handling in Supporting Information). The 40 SD rats had a week of adaptive feeding and then were randomly divided into five groups with eight rats in each group (four male and four female rats): normal group (NOR), control group (CON), low concentration of nobiletin treated group (LCN), middle concentration of nobiletin treated group (MCN), and high concentration of nobiletin treated group (HCN). Groups of rats except for NOR were given drinking water with 3% choline chloride. The groups of rats received 100 (LCN), 200 (MCN), or 400 mg/kg of body weight (bw) of nobiletin (HCN) dissolved in corn oil by gavage feeding (1 mL/time, once per day), respectively. The treatments of all groups lasted for 4 weeks after the 1-week adaptive feeding. All rats were euthanized 24 h after the final dose administration by CO_2 asphyxiation and then laparotomy was performed. The samples of proximal aorta and blood from each rat were collected.

Analyses of Histological and Immunohistochemistry. To observe the infiltration of inflammatory cells, the proximal aorta was cut into sections and embedded in paraffin. Then the sections of proximal aorta were stained with HE reagents and visualized under a BH2 optical microscope (Olympus, Tokyo). The samples of paraffin-embedded slices were dewaxed and rehydrated according to routine protocol. According to the manufacturers' guidelines, the samples were incubated first with the antibody of IL-1β, TNF-α, MCP-1, IL-6, SOD, CAT-1, p-p65, p-p38, or iNOS overnight at 4 °C and then with a biotin-conjugated secondary antibody and streptavidin−biotin peroxidase for 30 min. Substrate used was 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and a brown color was detected and regarded as a positive signal under a light microscope. For the immunoreactive score, quantitative analysis of target protein expression levels in three different regions of each histopathological section was performed using Image-Pro Plus 4.5 (Media Cybernetics, Rockville, MD, USA).

Cell Culture. Human umbilical vein endothelial cells (HUVEC) were cultured in Ham's F-12K (Kaighn's) medium, which contained 0.03−0.05 mg/mL of endothelial cell growth supplement and 10% heat-inactivated fetal bovine serum and supplemented with 100 U/mL of penicillin and 100 g/mL of streptomycin. The cells were maintained at 37 °C and with 5% CO_2.

MTT Assay. HUVEC cells were seeded into a 96-well plate at a density of 2×10^4 cells/mL. When cells had reached 70−80% confluence, 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, or 2.0 mM nobiletin (in dimethylsulfoxide) was added to each well. Eight parallel wells were set for each treatment. After the cells were treated for 24 h, MTT assay was applied to detect the cell survival and proliferation using a plate reader (BioTek Synergy 2, Winooski, VT, USA) at a wavelength of 490 nm.

Protein Extraction and Western Blotting. HUVEC cells were cultured in Ham's F-12K (Kaighn's) medium with or without 0.4 mM TMAO and 0.6, 1.0, or 1.4 mM nobiletin of each flask for 24 h. The cells were then harvested and lysed in ice-cold lysis buffer for 30 min, followed by centrifugation at 10 000 rpm for 30 min at 4 °C. To measure the relative expression levels of ERK, p-ERK, p38, p-p38, and COX-2, part of the cultured cells in each group was homogenized in ice-cold RIPA buffer containing 0.1% phenylmethylsulfonyl fluoride. For the detection of relative expression levels of p65, p-p65, and lamin A/C, the protein of the cultured cells in each group was extracted by Nuclear/Cytosol Fractionation Kit according to the protocol. The protein concentrations were measured using the BCA Protein Quantitation Kit. An equal amount of protein for each sample (50 μg) was subjected to SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were blocked with blocking solution containing 5% bovine serum albumin (BSA) and then incubated with the indicated primary antibodies at 4 °C overnight, and subsequently with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The HRP activity was visualized using the Tanon-5200 automatic chemiluminescence analyzer (Tanon Science & Technology Co., Ltd., Shanghai, China), and the density of the protein bands was quantified using ImageJ imaging software (Media Cybernetics, Rockville, MD, USA).

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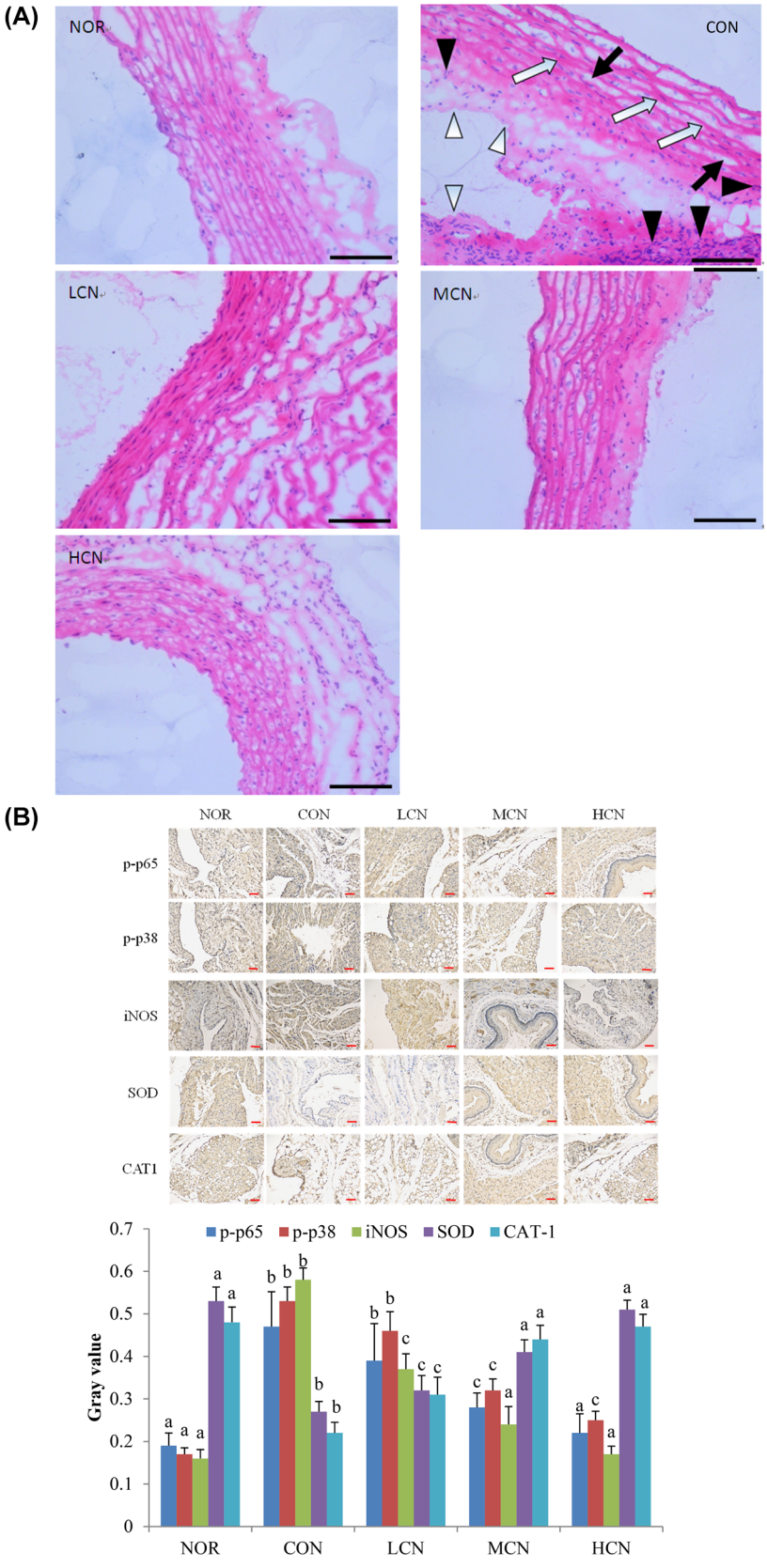

Figure 1. Histopathological changes of rat cardiovascular tissues in different treatment groups. (A) HE staining and immunohistochemistry. White arrow points to intimal thickening, White triangle points to elastic fibers proliferated, black arrow points to vacuolar degeneration, and black triangle points to inflammation in the cardiovascular tissue. Scale bars represent 200 μm. (B) Staining results of proximal aorta of SD rats treated with choline with or without nobiletin. (At the end of the experiment, five rats were randomly selected from each group; the proximal aorta tissues were harvested; and HE and immunohistochemistry staining were performed. Significant differences between treatment groups are expressed by different letters (a–c) when $p < 0.01$.)

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Cell Cycle Analysis. When the cultivated HUVEC cells reached 70−80% confluence, the original medium was discarded and the fresh Ham's F-12K medium containing 0.4 mM TMAO and three different concentrations of nobiletin (0.6, 1.0, or 1.4 mM) were added. The cells were then harvested after being cultured for 24 h. Fluorescence intensity was detected and recorded on a Beckman Coulter FC500 Cytometer in FL-2 channel (Becton Dickinson, San Jose, CA, USA). The analysis of cell cycle distribution was performed with Modfit 4.0 software (Becton Dickinson).

Cell Apoptosis Analysis. When the cells were cultivated for 24 h according to the method of "Protein Extraction and Western Blotting" aforementioned, the cells were then harvested, washed with ice cold PBS buffer three times, resuspended in 200 μL of Binging buffer, fixed in 10 μL of Annexin V-FITC and 10 μL of PI, gently mixed, and incubated at 4 °C for 30 min without light exposure. Binging buffer (300 μL) was added, and the flow cytometry was performed to detect cell apoptosis.

Statistical Analysis. Statistical evaluation was conducted using either one-way Student's t test or one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test. Data were reported as the mean ± SD for the number of independent experiments. Statistically significant differences were reported with a probability value of $p < 0.05$ or $p < 0.01$.

RESULTS AND DISCUSSION

Vascular diseases cause the most fatalities worldwide and are considered a pressing healthcare problem globally. Long-time consumption of red meat and or high-fat foods that are rich in L-carnitine, phosphatidylcholine, and choline among others is closely associated with the development of cardiovascular diseases, such as atherosclerosis.[27,28] Recent research findings of gut microbiota metabolism on TMA-generating foods suggested that TMAO molecule specifically and rapidly stimulates endothelial cell signaling pathway and subsequently results in the pathogenesis of vascular disease. TMAO is also connected to the increased vascular damage via the overproduction of both ROS and inflammatory cytokines.[29] Therefore, on the basis of the reported findings, inhibiting the TMAO generation in the intestinal microbiota could be a valid strategy to efficiently prevent vascular diseases.

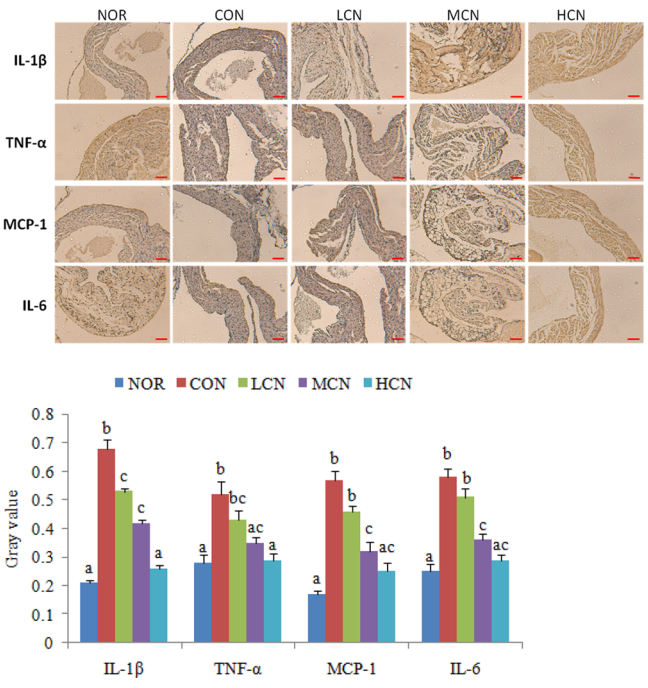

Figure 2. Immunohistochemical results showing the inhibitory effects of nobiletin on the expression of IL-1β, TNF-α, MCP-1, and IL-6 in vivo. The data average in each group is expressed as the mean ± SD. Significant differences between treatment groups are expressed by different letters (a−c) when $p < 0.01$.

For the past several decades, study has suggested that regular consumption of vegetables and fruits can effectively prevent vascular diseases because dietary phytochemicals, particularly rich in plants, can reduce vascular diseases via mechanisms of attenuating oxidative stress and inflammation.[30] As a food and an herb in Asia, particularly China for centuries, aged tangerine peels contain both polyhydroxyflavonoids (PHFs) such as hresperidin and narirutin, and polymethoxyflavones (PMFs), such as nobiletin, 3,5,6,7,8,3′,4′-heptamethoxyflavone, and tangeretin, which exist exclusively in citrus peels. The inhibitory effects of PMFs against inflammation occur via a mechanism of inhibition of NF-κB and ROS production, which are the major pathogenic causes of vascular diseases.[31] In the mechanism study of nobiletin in the prevention of atherosclerosis at vascular walls induced by low-density lipoproteins, it was found that nobiletin targeted macrophage foam-cell formation, reduced plasma cholesterol concentrations, and inhibited the oxidation of low-density lipoproteins.[11]

Histological Assessment. Histological analyses (Figure 1A) showed that mice treated with choline chloride suffered from intimal thickening, endothelial tissue slightly shedding, and locally elevated aortic intima near the heart. Also, the proliferation of elastic fibers in the tunica media of the aorta was observed. Furthermore, the observed vacuolar degeneration in the intima and tunica media marked cardiovascular inflammation induced successfully by choline chloride. The aforementioned histological phenotypes are in accordance with published results that TMAO, metabolically derived from choline,[5] L-carnitine,[1,2] or phosphatidylcholine,[3,7] among others, induces vascular inflammation via MAPK and NF-κB signal pathway.[7,21] After nobiletin treatment, in contrast, aortic intimal thickening (Figure 1A) was dramatically decreased, aortic smooth muscle cells were evenly arranged, and there was no smooth muscle cell hyperplasia observed. Taken together, these results demonstrated that nobiletin can efficiently prevent TMAO-induced cardiovascular inflammation.

Inhibitory Effects on the Expression of p-p65, p-p38, iNOS, SOD, and CAT-1 in the Cardiovascular Tissue by Nobiletin. In evaluating the anti-inflammatory effects of nobiletin in the cardiovascular tissue, we also examined the expression levels of five proteins, namely, p-p65, p-p38, iNOS, SOD, and CAT-1. We found that the expression levels of p-p65, p-p38, and iNOS were low but SOD and CAT-1 were high in the NOR group (Figure 1B). Remarkable upregulation of p-p65, p-p38, and iNOS and much downregulation of SOD and CAT-1 were observed in the CON group (choline treatment, Figure 1B). However, in the cardiovascular tissues of the high dose nobiletin-treated group (HCN), the expression of p-p65, p-p38, and iNOS was downregulated by 53.2, 52.9, and 70.7% compared to the respective expression levels in the CON group, whereas the relative expression levels of SOD and CAT-1 were increased by 189 and 214%, respectively, in the HCN group. Therefore, nobiletin antagonized the inflammatory effect of choline on the expression of inflammation markers of p-p65, p-p38, and iNOS in the cardiovascular tissue; thus, we conclude that nobiletin effectively inhibited TMAO-induced inflammation.

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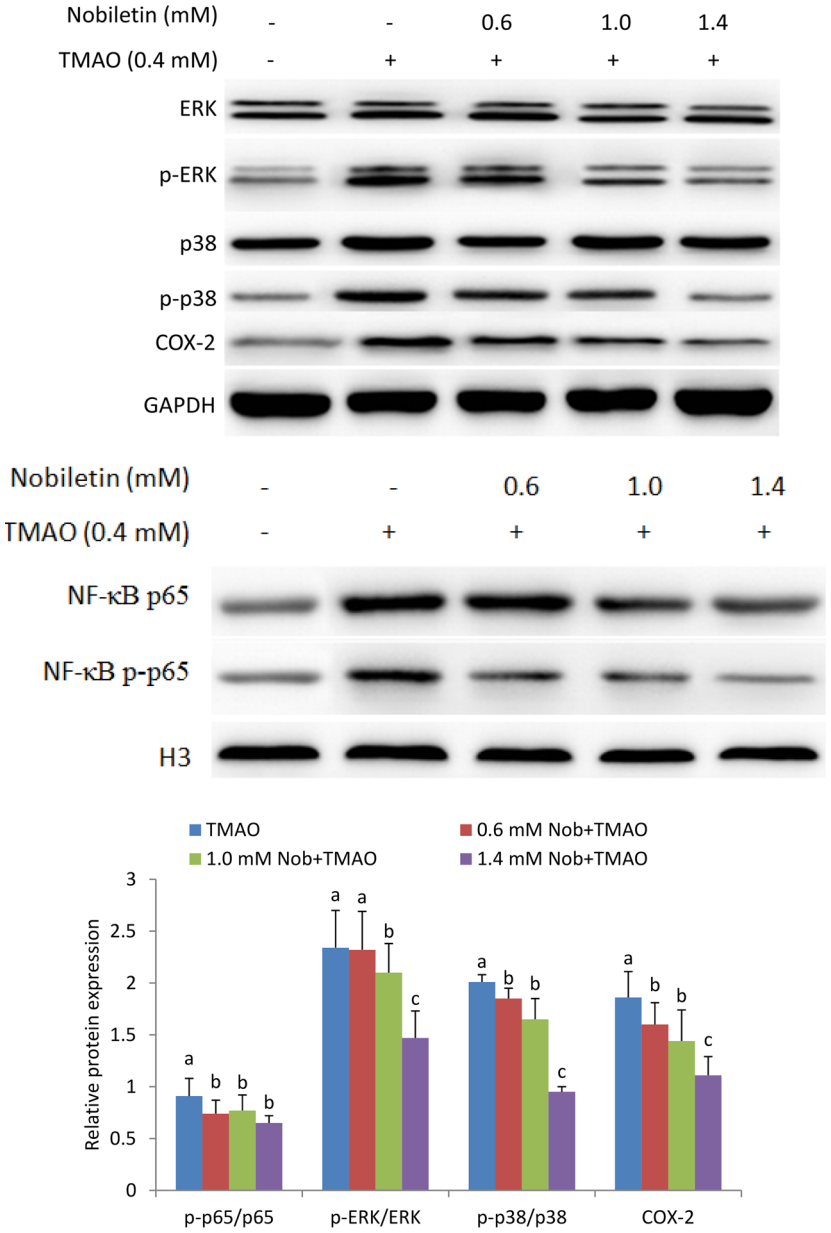

Figure 3. Inhibitory effects of nobiletin on the protein expression of p65, p-p65, lamin A, lamin C, ERK, p-ERK, p38, p-p38, and COX-2 in TMAO-treated HUVEC. The relative protein expression is expressed as the fold increase compared to that of the NOR group after being normalized to H3 or GAPDH in three independent experiments. Data is reported in the mean ± SD (n = 3). Significant differences between treatment groups are expressed by different letters (a–c) when $p < 0.01$.

Effect of Nobiletin on the Expression of IL-1β, IL-6, TNF-α, and MCP-1 in Proximal Aorta. The expression levels of pro-inflammatory cytokines including IL-1β, IL-6, TNF-α, and MCP-1 in cardiovascular tissue were measured by immunohistochemistry (Figure 2). The results demonstrated that the levels of these four cytokines increased significantly in the choline-treated group compared to those in the NOR group. Interestingly, nobiletin significantly reduced the expression level of these pro-inflammatory cytokines induced by choline, indicating that nobiletin counteracted TMAO-induced inflammation. These results further substantiated that nobiletin shows anti-inflammatory activity in the cardiovascular inflammatory tissue.

Cell Viability in HUVEC Cells Treated by Nobiletin. In investigating the anti-inflammation mechanism of nobiletin in the context of cardiovascular system, we first examined the effect of nobiletin on HUVEC cells. We started by examining the cytotoxicity of nobiletin to HUVEC cells using MTT assay. It is found that nobiletin at 1 mM did not show measurable toxicity against HUVEC cells; however, the viability dropped to 66.5% when nobiletin reached 2.0 mM, which is still in the normal survival range (60%) of viability measurement (Supporting Information). The calculated IC_{50} value is 13.7 mM. Therefore, the concentration of 0.6, 1.0, and 1.4 mM nobiletin were selected for subsequent in vitro cell culture experiments.

Inhibitory Effects of Nobiletin on the Activation of NF-κB and the MAPK/ERK Pathway. Increased expression of COX-2 and NF-κB in inflammatory cells is associated with cardiovascular inflammation in patients with cardiovascular diseases.[32] Hence, we further investigated the anti-inflammatory effect of nobiletin on vascular inflammation induced by

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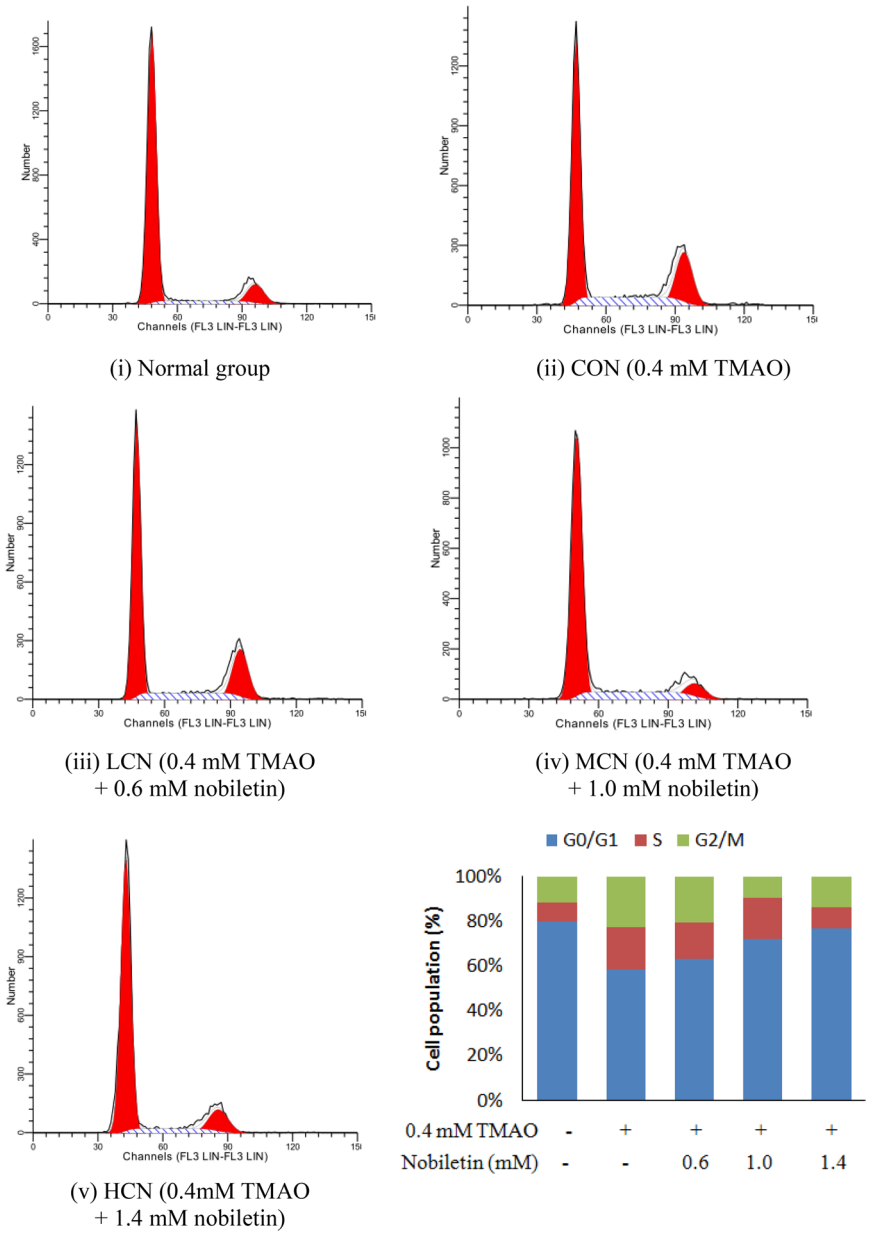

Figure 4. Effects of nobiletin on proliferation and apoptosis of TMAO-treated HUVEC. Cells were treated with 0.4 mM TMAO and 0, 0.6, 1.0, or 1.4 mM nobiletin for 24 h, respectively. Representative images of flow cytometric analysis by PI staining were shown. Data is presented in the mean (n = 3).

TMAO and whether the nobiletin-mediated anti-inflammation is achieved by the inhibition of NF-κB expression and the regulation of MAPK/ERK signaling pathway.

As illustrated in Figure 3, compared to that of TMAO-treated cells, after cotreatment with nobiletin, the phosphorylation l of ERK and p38 proteins decreased in a dose-dependent manner. Relative expression of cellular p65 and p-p65 was increased by 2.64- and 2.32-fold in TMAO-treated HUVEC cells, respectively, compared to that of untreated cells. Notably, nobiletin treatment dose-dependently inhibited the expression of p65 and p-p65 in a manner analogous to its effect in vivo. The above data demonstrated that nobiletin can effectively reduce the phosphorylation level of p38 and p65 in cells and consequently alleviate cardiovascular inflammation.

Effect of Nobiletin on Proliferation of HUVEC Induced by TMAO In Vitro. The effect of nobiletin on TMAO-treated HUVEC cell proliferation was next examined. In drug-untreated HUVEC cells, 80% were distributed at the G_0/G1 phase, only 9% of cells at the S phase and 11% at the G_2/M phase (Figure 4), whereas about 60% cells were in G_0/G1 phase, 19% cells were in S phase, and 21% cells were in G_2/M phases with the treatment of TMAO, indicating that TMAO induced the proliferation of HUVEC cells (Figure 4). More importantly, nobiletin clearly reversed TMAO-induced proliferation in an efficient dose-dependent manner. In particular, nobiletin at 1.4 mM abrogated TMAO-mediated alteration in the cell cycle progression and returned the pattern of cell cycle distribution nearly identical to that in drug-untreated HUVEC cells (Figure 4). Collectively, these data indicated that nobiletin inhibited the proliferation of HUVEC cells induced by TMAO.

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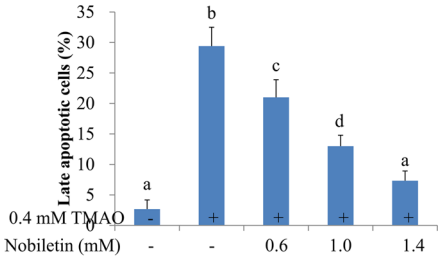

Figure 5. Effects of nobiletin on apoptosis of TMAO-treated HUVEC. Cells were treated with 0.4 mM TMAO and 0, 0.6, 1.0, or 1.4 mM nobiletin for 24 h. Representative images of flow cytometric analysis by Annexin V-FITC/PI staining were shown. The top right quadrant represents Annexin V-FITC-stained cells, or early phase apoptotic cells, whereas the bottom right quadrant represents PI- and FITC-dual-stained cells or late-phase apoptotic or necrotic cells. Values represent the mean ± SD of minimum three independent experiments. Significant differences between treatment groups are expressed by different letters (a–c) when $p < 0.01$.

Effect of Nobiletin on Cell Apoptosis of HUVEC Induced by TMAO In Vitro. We further tested the effect of nobiletin on the apoptosis of TMAO-treated HUVEC cells. As shown in Figure 5, 35.3% of the HUVEC cells underwent apoptosis after TMAO stimulation for 24 h. Notably, the proportion of apoptotic cells was dramatically decreased to 11.1% when cotreated with 1.4 mM nobiletin, suggesting that nobiletin protected HUVEC cells from TMAO-induced apoptosis.

In this study, both in vivo and in vitro experiments indicated the preventive effect of nobiletin on TMAO-stimulated cardiovascular inflammation. Furthermore, we identified that alleviating the activation of the MAPK and NF-κB signaling pathways underlie the preventative effect of nobiletin on vascular endothelial cells.

In summary, acting as a barrier, vascular endothelium usually blocks macromolecules and inflammatory cells from blood to tissue, but TMAO, generated from the metabolism of choline in gut microbiota and subsequent oxidation by liver FMO and then to the blood system, induces vascular inflammation. In our study, we have successfully attested that TMAO induced vascular inflammation by upregulating ERK, p38, p65, and p-p65 in HUVEC cells and treatment with nobiletin effectively inhibited these inflammatory biomarkers dose dependently, which allowed us to conclude that the anti-inflammatory effects of nobiletin on TMAO-stimulated vascular inflammation is achieved by inhibiting the expression of NF-κB and MAPK/ERK signaling pathway. The same phenomenon was observed in our animal study; that is, nobiletin effectively inhibited the expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and MCP-1) in proximal aorta, thus preventing tissue inflammation induced by choline-generated TMAO in cardiovascular tissue.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b01270.

Catalog numbers of reagents and information on cell lines used in this study and additional animal information (PDF)

Graphic showing effects of nobiletin on cell viability (PDF)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ANOVA, analysis of variance; BSA, bovine serum albumin; CON, control group; CVDs, cardiovascular diseases; COX-2, cyclooxygenase 2; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinases; FMO, flavin monooxygenases; GSH-ST, glutathione S-transferase; HCN, high concentration of nobiletin treated group; HE, hematoxylin and eosin; HRP, horse radish peroxidase; HUVEC, human umbilical vein endothelial cell; iNOS, inducible nitric oxide synthase; IL-1β, interleukin 1β; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; LCN, low concentration of nobiletin treated group; MAPK, mitogen activated protein kinase; MCN, middle concentration of nobiletin treated group; MCP-1, monocyte chemotactic protein 1; MDA, malonaldehyde; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor kappa B; NO, nitric oxide; NOR, normal group; OD, optical density; ROS, reactive oxygen species; SD rats, Sprague–Dawley rats; SOD, superoxide dismutase; TMA, trimethylamine; TMAO, trimethylamine oxide; TNF-α, tumor necrosis factor α

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