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Research Article

Nobiletin Protects Endothelial Cell Function via Upregulation of eNOS/ET-1 and Antioxidant Status-Related Genes under Nonstimulated and Inflammatory Conditions

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Nobiletin, a natural polymethoxylated flavonoid compound, has been shown to exert a wide range of biological activities. However, there is limited evidence on the molecular mechanisms by which nobiletin modulates endothelial cell function. Our aim was to investigate the potential of nobiletin to enhance endothelial cell functionality under nonstimulated and inflammatory conditions. To this end, gene expression relevant to nitric oxide (NO) production, endothelin 1 (ET-1), and antioxidant status in human endothelial cells EA.hy926 was determined using real-time PCR. The fluorescence probe 4,5-diaminofluorescein (DAF-2) was used to measure NO production. The results demonstrated significant upregulation of eNOS and antioxidant genes as well as downregulation of ET-1 by nobiletin under nonstimulated and TNF-α-stimulated inflammatory conditions. These findings suggest a promising protective effect of nobiletin with relevance for cardiovascular health, likely through the NO/ET-1 dynamics and Nrf2 signaling pathway.

1. Introduction

Epidemiological studies have suggested that the intake of citrus fruits is inversely associated with the risk of ischemic stroke [1] and coronary heart disease [2]. Citrus fruits, such as oranges, grapefruit, lemons, limes, mandarins, bergamots and pomelos, are abundant in flavonoids, including hesperidin, hesperetin, naringin, naringenin, diosmin, quercetin, rutin, nobiletin, and tangeretin [3]. Polymethoxyflavones (PMFs) such as nobiletin and tangeretin represent the characteristic compounds of the genus [4]. Nobiletin has attracted wide research attention for its various beneficial properties, such as antioxidant [5], anti-inflammatory [6], and anticancer [7] effects. According to epidemiological evidence, the consumption of citrus flavonoids is inversely associated with the risk of cardiovascular mortality [8].

Given the crucial role of endothelial function in the development of cardiovascular disease [9], the effect of citrus flavonoids on endothelial function is of great importance. However, findings of randomized controlled trials (RCTs) investigating how orange juice consumption modulates endothelial function are inconsistent [10]. A recent crossover RCT demonstrated that two-week consumption of blood orange juice improved flow-mediated dilation in healthy overweight and obese participants [11]. Flavanone-rich citrus beverages alleviated the transient decline in postprandial endothelial function in healthy men following a sequential double meal rich in fat [12]. However, orange juice did not acutely affect endothelial function in men at moderate risk of cardiovascular disease [13]. Therefore, the mechanisms by which citrus flavonoids modulate endothelial function remain to be elucidated.

Endothelial dysfunction is defined as a reduction in nitric oxide (NO) production and/or bioavailability, with or without an imbalance between relaxing and contracting factors associated with elevated inflammation [14]. As the major vasodilator produced by endothelial cells, NO plays a pivotal role in maintaining vascular homeostasis. Hence, endothelial nitric oxide synthase (eNOS) function and activity are crucial for endothelial cell functionality [15]. The potent contracting factor endothelin 1 (ET-1) produced by endothelial cells has been reported to reduce eNOS promoter activity and eNOS protein levels, thereby contributing to decreased levels of NO [16]. The imbalance between NO and ET-1 contributes to endothelial dysfunction [17]. Administration of resveratrol reversed a high-cholesterol diet-induced downregulation of NO and upregulation of ET-1 in experimental hypercholesterolemic rabbits [18]. There is, however, a paucity of data regarding how citrus flavonoids regulate ET-1 expression. Of note, excessive oxidative stress contributes to elevated eNOS uncoupling and reduced NO bioavailability [19]. Therefore, redox balance in endothelial cells is of great importance. The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) regulates the expression of antioxidant genes such as heme oxygenase-1 (HO-1), gamma-glutamyl cysteinyl synthetase (GCLC), and superoxide dismutase (SOD) which contribute to the maintenance of cellular redox balance [20]. Notably, endothelial cell dysfunction is a trigger or consequence of oxidative stress and inflammation [14].

Therefore, in this study, we investigated the effect of citrus flavonoids, with a focus on nobiletin, on eNOS, ET-1, and antioxidant gene expression under both nonstimulated and inflammatory conditions.

2. Materials and Methods

2.1. Reagents. Hesperetin, naringenin, and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma-Aldrich (Dorset, UK). Nobiletin, tangeretin, and resveratrol were purchased from Extrasynthese (Genay, France). The purity of all compounds was above 97%. Dulbecco's phosphate-buffered saline (DPBS) without calcium and magnesium and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific (Loughborough, UK). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, penicillin-streptomycin, and trypsin-EDTA were obtained from Lonza (Slough, UK). TRIsure reagent and SensiMix™ were obtained from Bioline (London, UK). The iScript™ cDNA synthesis kit was purchased from Bio-Rad (Hertfordshire, UK). Chloroform, diethylpyrocarbonate (DEPC)-treated (RNase-free) water, and propan-2-ol were obtained from Thermo Fisher Scientific. All other chemicals and reagents were obtained from Sigma-Aldrich unless specified otherwise.

2.2. Cell Culture and Treatments. The experiments were conducted using human endothelial cells EA.hy926 (CRL-2922™) which were obtained from the American Type Culture Collection (ATCC). Cells were cultured in DMEM with 1 g/L of glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 10% fetal bovine serum, and 1% penicillin and streptomycin in a humidified atmosphere with 5% CO_2 at 37°C. EA.hy926 cells were seeded at a density of 1×10^5 cells/mL and left to grow overnight to reach 90% confluence. Stock solutions of flavonoids were prepared in 100% dimethyl sulfoxide (DMSO) at 100 mM in aliquots, with the exception of tangeretin (at 25 mM due to its limited solubility in DMSO). Dilutions were made from stock solutions to specific concentrations immediately prior to treatment. Initially, optimisation of NOS3 induction was conducted by testing effects of different incubation periods (6 h and 18 h), glucose content (1 g/L and 4.5 g/L), and FBS (0% and 10%), with resveratrol (25 and 50 μM) and PMA (10 nM) as positive controls [21]. Following NOS3 optimisation, cells were incubated with naringenin, hesperetin, nobiletin, and tangeretin at different concentrations for 18 h in FBS-free, low-glucose medium (1 g/L).

2.3. Cell Viability. The potential cytotoxicity of test compounds was determined by the neutral red (NR) assay as described previously [22]. Specifically, varying concentrations (0, 25, 50, and 100 μM) of compounds in phenol red-free and serum-free medium were prepared in triplicate and incubated at 37°C for 18 h. After medium removal, cells were incubated with medium containing NR at 40 μg/mL for 2 h. Subsequently, cells were washed with DPBS. The NR dye was extracted using destain solution, and absorbance was read at 540 nm using a plate reader (Tecan SPARK 10M, Männedorf, Switzerland). The viability of the treated cells was calculated as percent relative to control.

2.4. RNA Extraction and Real-Time PCR. RNA was extracted from EA.hy926 cells using TRIsure and quantified using NanoDrop2000 (Thermo Fisher Scientific, with purity assessed by A260/280. Reverse transcription was conducted using the iScript™ cDNA synthesis kit. Real-time PCR was performed using SensiMix from Bioline™ on a StepOne Real-Time PCR system (Thermo Fisher Scientific. Primers were designed using NCBI tools (Table 1) as previously described [23], which were synthesized by Eurofins Genomics (Ebersberg, Germany).

2.5. Measurement of Nitric Oxide. The fluorescence probe 4,5-diaminofluorescein (DAF-2) was used to determine nitric oxide production by EA.hy926 cells following 18 h incubation with different test compounds. Measurements were conducted as previously reported [24]. Following the removal of the cell culture medium and a wash with DPBS, cells were incubated with L-arginine (100 μM) in DPBS for 10 min. Subsequently, cells were incubated with DAF-2 (0.1 μM) in the dark for 5 min. Fluorescence was measured at room temperature with excitation wavelength at 485 nm and emission wavelength at 520 nm with a Tecan plate reader. Autofluorescence of DAF-2 was subtracted from the total fluorescence.

Table 1: Primer sequences of target genes for real-time PCR.

Gene	Accession number	Annealing temp. (°C)	Forward primer	Reverse primer
ACTB	P60709	55	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG
NOS3	P29474	57.3	GCAGCCTCACTCCTGTTTTC	GGTCTTCTTCCTGGTGATGC
HMOX1	P09601	58	CTTCTTCACCTTCCCCAACA	AGCTCCTGCAACTCCTCAAA
GCLC	P48506	58	CAATGGGAAGGAAGGTGTGT	GCGATAAACTCCCTCATCCA
EDN1	P05305	56	GATGCCAATGTGCTAGCCAA	GCTGTTTCTCATGGTCTCCG

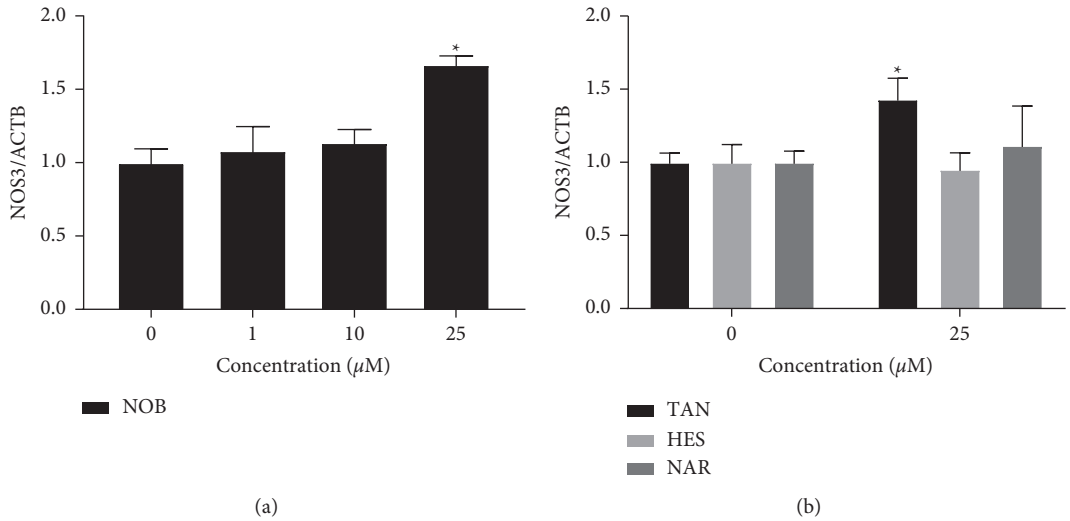

Figure 1: Effect of increasing concentrations of citrus flavonoids: (a) nobiletin (NOB) and (b) tangeretin (TAN), hesperetin (HES), and naringenin (NAR) on NOS3 mRNA levels. Cells were incubated for 18 h with concentrations up to 25 μM. Data are presented as mean ± SD. *indicates significant differences between the treatment group and untreated control group at $p < 0.05$.

2.6. Measurement of Reactive Oxygen Species. The generation of reactive oxygen species (ROS) was determined using the fluorescent probe dihydrodichlorofluorescein (H_2DCF-DA). Confluent endothelial cells in 96-well plates were incubated with 20 μM H_2DCF-DA for 30 min under standard conditions. Cells were then washed with DPBS and incubated with increasing concentrations of nobiletin (0, 1, 5, and 10 μM) for 30 min followed by the addition of 250 μM hydrogen peroxide for 1 h. Fluorescence intensity was measured at 485 nm and 535 nm excitation and emission wavelengths, respectively, using a Tecan plate reader in well-scanning mode. Results were calculated in percent of stimulated control cells.

2.7. Statistical Analysis. Results of three independent passages were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using Statistical Package for the Social Sciences (SPSS, version 24, IBM Corporation, USA). Treatment effects were analyzed by one-way analysis of variance (ANOVA) followed by the post hoc test of Dunnett's (comparing multiple treatments to a single control). $p < 0.05$ was considered as statistically significant.

3. Results and Discussion

3.1. Differential Effects of Citrus Flavonoids on eNOS Expression. The concentrations used in the following experiments were not toxic to cells (Figure S1). Cell viability did not differ between the DMSO-treated group (98.8 ± 3.1%) and untreated group (100 ± 2.1%) ($p = 0.71$); therefore, DMSO used in experiments at 0.4% or lower, was not considered to have a negative effect on cell viability. In order to establish NOS3 induction, EA.hy926 cells were incubated with PMA, a known inducer of NOS3 mRNA and

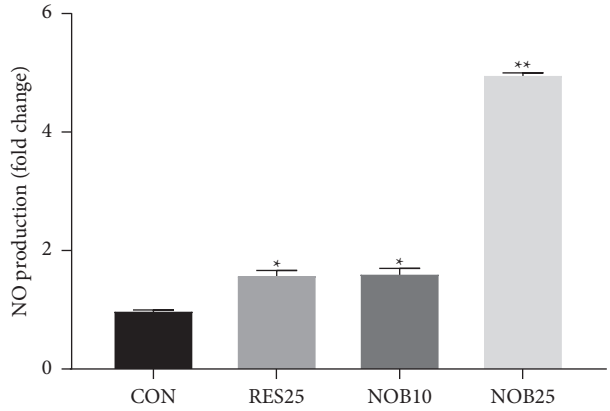

Figure 2: Dose-dependent effect of nobiletin to increase NO production using the DAF-2 assay. Effect of increasing DAF-2 detected NO release from EA.hy926 cells after 18 h incubation with nobiletin (NOB) at 10–25 μM and resveratrol (RES; 25 μM) as a positive control. Data were expressed as a fold change of control. Data are presented as mean ± SD. * and ** indicate significant differences between the treatment group and untreated control group at $p < 0.05$ and $p < 0.01$, respectively. CON: control.

protein levels [25]. As shown in Figure S2, PMA exposure resulted in a dose-dependent induction from 0 to 1000 nM which was significantly higher at 1–1000 nM with maximum induction at 10 nM compared to control. Induction of NOS3 mRNA following incubation with PMA or resveratrol was stronger at 18 h than 6 h (Figure S3). Hence, an incubation period of 18 h was selected to evaluate effect of citrus flavonoids on NOS3 mRNA expression.

In the next step, the potential of nobiletin, tangeretin, naringenin, and hesperetin to induce NOS3 mRNA was evaluated at concentrations ranging from 1 to 25 μM. As shown in Figure 1(a), nobiletin incubation led to a significant increase in NOS3 at 25 μM. Compared to resveratrol which had only resulted in a marked induction at 50 μM (Figure S3), nobiletin was more potent to induce NOS3 mRNA expression at a lower concentration. NOS3 mRNA expression was also significantly upregulated by tangeretin at 25 μM (Figure 1(b)). NOS3 mRNA expression was not significantly changed by hesperetin and naringenin (Figure 1(b)).

NO production was significantly augmented by nobiletin in a concentration-dependent manner, from 1.6-fold upregulation at 10 μM to 5-fold upregulation at 25 μM (Figure 2). Resveratrol was used as a positive control for NO induction resulting in a 1.6-fold NO production at 25 μM. Therefore, nobiletin appeared to be more potent in inducing NO than resveratrol when compared at 25 μM.

Since NO plays a pivotal role in endothelial function, the regulation of its synthesis by citrus flavonoids is crucial to the understanding of their effects on cardiovascular health. Importantly, eNOS expression can be regulated at different levels, such as eNOS promoter activity, eNOS transcription and mRNA stability, and posttranslational regulation [26]. The current study investigated effects on eNOS at the mRNA level and NO release.

Treatment with hesperetin (12.5–100 μM) for 24 h dose-dependently upregulated NO production in human umbilical vein endothelial cells (HUVECs), whereas treatment with naringenin did not result in significant changes in NO production under the same conditions [27]. Additionally, hesperetin (50 μM, 24 h) significantly increased eNOS transcription and protein expression in HUVECs whereas no significant changes were observed following treatment with naringenin [27]. In contrast, incubation with hesperetin and naringenin for 18 h did not change eNOS mRNA levels in EA.hy926 cells in the present study, suggesting cell type- and/or time point-associated differences [28]. Whilst some citrus flavonoids might not modulate eNOS expression, they might contribute to the improvement of cardiovascular health by different mechanisms [29].

Polymethoxylated flavones have been highlighted for their anti-inflammation [30] and anticancer effects [31] in recent years. However, the effects of nobiletin and tangeretin on eNOS expression in human endothelial cells have not been investigated. In comparison, nobiletin and tangeretin were found to be more potent to induce eNOS mRNA levels in our study compared to hesperetin and naringenin. This could be attributed to the presence of the double bond at the 2–3 position in the C ring of nobiletin and tangeretin, which has been associated with an upregulation in eNOS mRNA expression [32], potentially due to the reduction potential of the double bond. Among the investigated citrus flavonoids in the present study, nobiletin was the most effective compound in increasing eNOS mRNA and NO production under the defined conditions. Consistent with these results, nobiletin significantly increased the relaxation of phenylephrine-induced contraction in endothelium-intact rat aorta [33]. In support of this observation, nobiletin has been shown to promote the phosphorylation of eNOS at position Ser-1177 by increasing endothelial $[Ca^{2+}]$i, thereby increasing NO production and vasodilation in phenylephrine-precontracted mesenteric arteries isolated from rats [34]. In keeping with these findings, nobiletin alleviated iron overload damage in vascular endothelium through increasing eNOS phosphorylation and NO production [35]. In addition, nobiletin was found to induce endothelium-independent vasodilation in rat aorta. Specifically, nobiletin inhibited phenylephrine-mediated contraction of endothelium-denuded rat aorta through the activation of Ca^{2+}-activated K^+ (BK) channel and ATP-sensitive K^+ (K_{ATP}) channel, leading to vasodilation via membrane hyperpolarization [33]. Taken together, nobiletin has been shown to exert vasodilator effects by different mechanisms, such as inducing eNOS expression, promoting eNOS phosphorylation as well as activating BK and K_{ATP} channels. Although the phosphorylation level of eNOS at Ser-1177 upon treatment of nobiletin was not measured in this study, a recent study has found a striking discordance between eNOS phosphorylation and NO formation in endothelial cells [36]. The phosphorylation of eNOS as a surrogate marker for activation of the eNOS enzyme can therefore be questioned. The generation of NO was determined as the ultimate outcome to confirm the transcriptional increase in eNOS mRNA levels in the present study, although it cannot be ruled out that other mechanisms are contributing to NO increases.

3.2. Differential Effects of Citrus Flavonoids on Antioxidant Status-Related Genes. Modulation of HMOX1 and GCLC mRNA expression by citrus flavonoids was also evaluated. Nobiletin significantly augmented HMOX1 (Figure 3(a)) and GCLC (Figure 3(b)) mRNA expression at 25 μM, whereas HMOX1 and GCLC mRNA levels were not changed by tangeretin, hesperetin, and naringenin.

Elevated ROS levels lead to a reduction in eNOS cofactor BH_4 and facilitate eNOS uncoupling, thereby reducing NO bioavailability [37]. Hence, antioxidant status plays a crucial role in maintaining endothelial cell function and NO/ET-1 balance. Several proteins/enzymes contribute to maintaining cellular redox balance, including HO-1 and GCLC; however, there is limited research that has considered induction of HO-1 and GCLC by nobiletin and tangeretin in human endothelial cells. The nobiletin-dependent upregulation of HO-1 and GCLC as observed in the current study is suggesting improvement of antioxidant status, thereby supporting findings on the beneficial effects of nobiletin on eNOS and NO production. Present results are in agreement

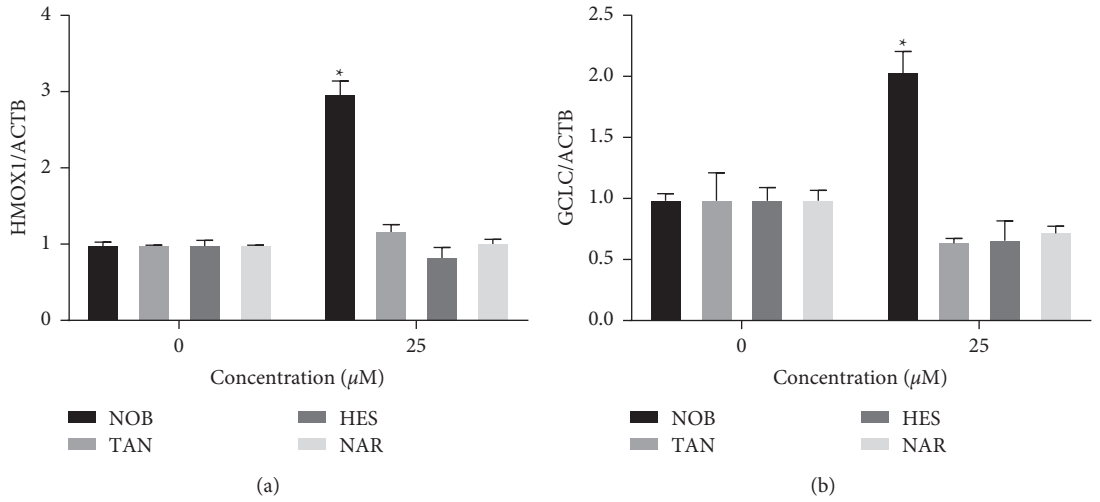

Figure 3: Effect of nobiletin (NOB), tangeretin (TAN), hesperetin (HES), and naringenin (NAR) on HMOX1 (a) and GCLC (b) mRNA levels after 18 h incubation. Data are presented as mean ± SD. *indicates significant differences between the treatment group and untreated control group at $p < 0.05$.

with a previous study that showed upregulation of Nrf-2/HO-1 and MMP pathways following nobiletin administration and amelioration of vascular alterations in L-NAME-induced hypertensive rats [38]. Furthermore, incubation with nobiletin (0–10 μM) resulted in a dose-dependent reduction of hydrogen peroxide-stimulated ROS levels in EA.hy926 cells (Figure 4), confirming the contribution of nobiletin to enhancing and/or stabilizing cellular antioxidant status. The beneficial effect of nobiletin on ROS levels has also been demonstrated *in vivo* in mice fed with a high-fat diet [39]. In addition, the chemical structure of citrus flavonoids indicates their role as radical scavengers and hydrogen-donating antioxidants [40]. Altogether, citrus flavonoids such as nobiletin can not only act as antioxidants to scavenge free radicals but can also upregulate antioxidant status-related gene expression to maintain cellular redox state and limit cell damage. Although the effect on antioxidant enzymes HO-1 and GCLC was not confirmed on protein level in this study, the reduced ROS levels are supportive to the improvement of antioxidant status in endothelial cells, likely through direct and indirect mechanisms.

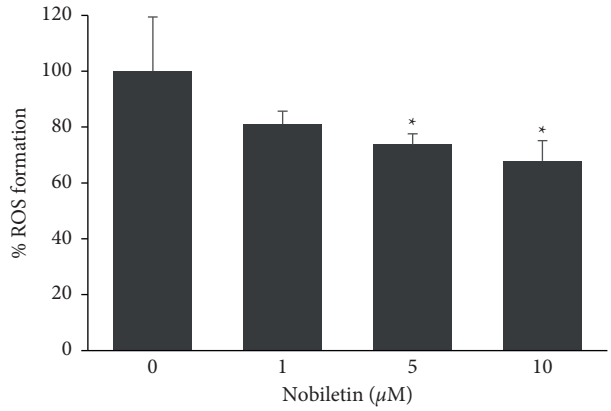

Figure 4: Effect of nobiletin (0–10 μM) on hydrogen peroxide-induced ROS formation. Data are presented as mean ± SD. *indicates significant differences between the treatment group and untreated control group at $p < 0.05$.

3.3. Dose-Dependent Inhibition of ET-1 mRNA Expression by Nobiletin. Given that nobiletin was the most potent compound among the test compounds to induce NOS3, HMOX1, and GCLC mRNA expression, its impact on ET-1 mRNA expression was also investigated. ET-1 mRNA levels were reduced by nobiletin in a concentration-dependent manner, with a 35% decrease at 25 μM ($p < 0.05$) (Figure 5).

Hesperetin and naringenin at 1 μM significantly reduced ET-1 mRNA expression in H_2O_2-treated HUVECs whereas no effect was observed on NOS3 mRNA expression [32]. The present study was the first to demonstrate that nobiletin (1–25 μM) resulted in a concentration-dependent upregulation of NOS3 expression and downregulation of ET-1 in human endothelial cells, providing new insight into how

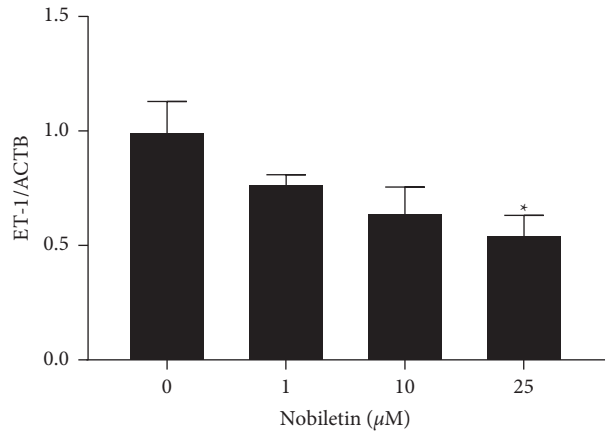

Figure 5: Effect of nobiletin (0–25 μM) on ET-1 mRNA levels after 18 h incubation. Data are presented as mean ± SD. *indicates significant differences between the treatment group and untreated control group at $p < 0.05$.

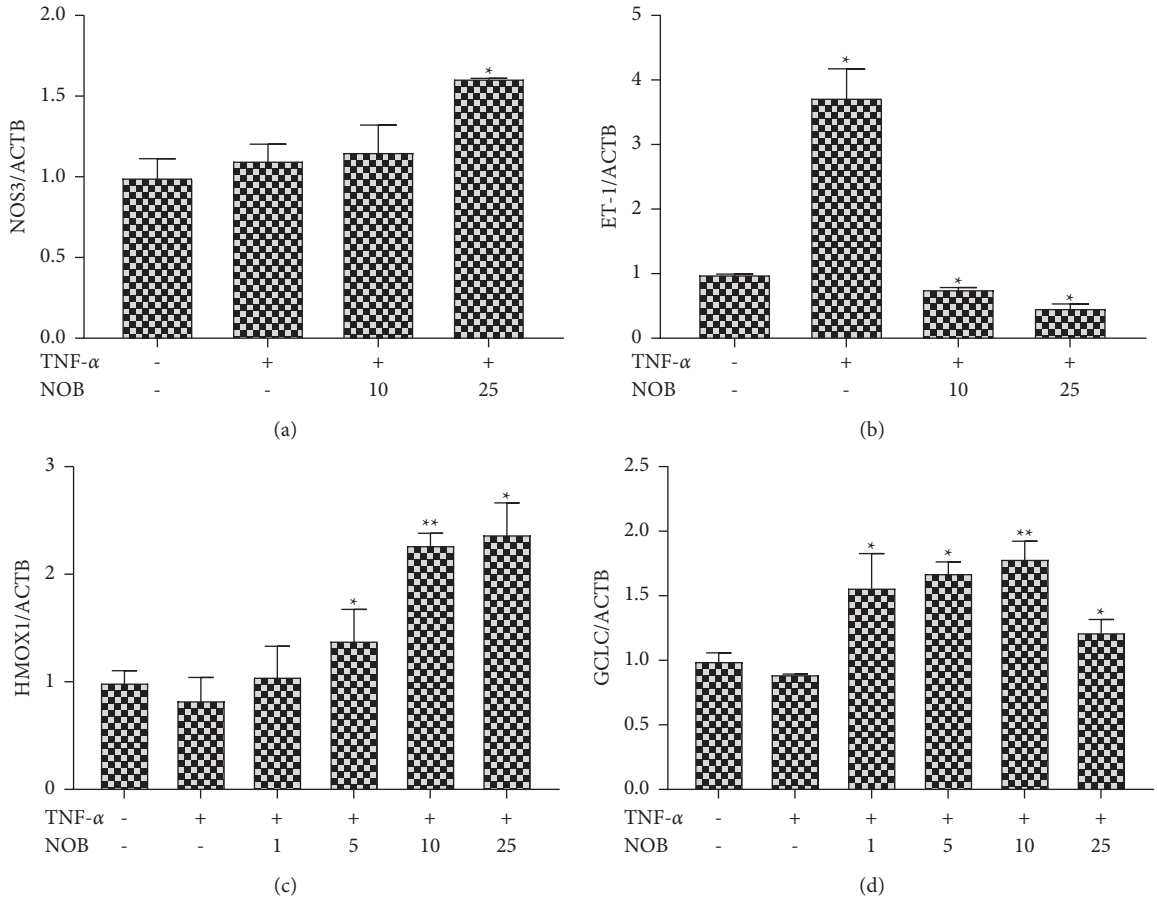

Figure 6: Effect of nobiletin (NOB) on NOS3 (a), ET-1 (b), HMOX1 (c), and GCLC (d) mRNA levels following 1 h pretreatment with nobiletin followed by 18 h incubation with TNF-α (10 ng/mL). Data are presented as mean ± SD. * and ** indicate significant differences between the treatment group and untreated control group at $p < 0.05$ and $p < 0.01$, respectively.

nobiletin may modulate endothelial cell function, since an imbalance between eNOS and ET-1 contributes to endothelial dysfunction [41].

3.4. Effect of Nobiletin on Endothelial Cell Function under TNF-α-Induced Inflammatory Condition. Since nobiletin was the most promising compound in improving endothelial cell function, its effect was further investigated under TNF-α-induced inflammatory conditions. Pretreatment with nobiletin significantly upregulated NOS3 mRNA expression by 1.6-fold ($p < 0.05$) at 25 μM, following incubation of TNF-α (10 ng/mL) for 18 h (Figure 6(a)). Meanwhile, pretreatment with nobiletin at 10–25 μM significantly reduced ET-1 mRNA expression ($p < 0.05$) (Figure 6(b)). In addition, under the same investigated condition, pretreatment with nobiletin significantly increased HMOX1 (Figure 6(c)) and GCLC (Figure 6(d)) mRNA levels.

Inflammation plays a crucial role in contributing to alterations in vessel structure and function, resulting in endothelial dysfunction [42]. Elevated proinflammatory cytokines with a subsequent decrease in anti-inflammatory markers are considered as the link between inflammation and endothelial dysfunction [43]. Proinflammatory cytokine TNF-α is considered to be the major inflammatory factor contributing to the development of endothelial dysfunction. Therefore, the current study aimed at investigating the effect of nobiletin on endothelial cell function under TNF-α-induced inflammatory condition. The present study demonstrated that nobiletin was still effective in upregulating eNOS and antioxidant status-related genes as well as downregulating ET-1 under inflammatory conditions, providing new insight into how nobiletin preserves endothelial function when exposed to proinflammatory cytokines. This is of importance as inflammation contributes to the development and progression of numerous diseases such as cardiovascular disease [44], cancer [45], and neurodegenerative disease [46]. Nobiletin exerted protection against metabolic dysfunction in high-fat-fed mice and prevented obesity, hepatic steatosis, dyslipidemia, and insulin resistance [47]. Although the direct effect of nobiletin on NF-κB pathway activation was not investigated in the present study, nobiletin previously demonstrated protective effects against LPS-induced endotoxic shock in mice, through inhibition of TNF-α, IL-6, and high mobility group box 1 (HMGB1), through the NF-κB signaling pathway [48]. Consistent with these findings, nobiletin was found to significantly inhibit trimethylamine oxide-induced vascular inflammation

through inhibiting NF-κB and MAPK/ERK-related pathways in rats [49]. Taken together, nobiletin has been shown to preserve endothelial function under inflammatory conditions by upregulation of eNOS and the Nrf-2-regulated antioxidant system as well as inhibition of ET-1.

4. Conclusions

In summary, different citrus flavonoids were evaluated for their effects on markers of endothelial cell function. The present data demonstrate the efficacy of nobiletin to upregulate eNOS, downregulate ET-1, and improve antioxidant status through induction of antioxidant enzymes and inhibition of cellular ROS formation, under nonstimulated and inflammatory conditions, thereby providing new evidence on eNOS protection and preservation of endothelial function. Given the potential of nobiletin, *in vivo* human studies, in healthy and patient cohorts, are warranted to establish *in vivo* bioefficacy in a safe manner, which might open new avenues of research in the development of novel natural therapeutic agents.

Data Availability

The data described in the manuscript will be made available upon request.

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

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Supplementary Materials

The data regarding cell viability (Figure S1), effects of PMA (Figure S2), and different incubation periods (Figure S3) on NOS3 mRNA expression are demonstrated in the supplementary file. (*Supplementary Materials*)

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