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Fucoxanthin suppresses OxLDL-induced inflammation *via* activation of Nrf2 and inhibition of NF-κB signaling

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ABSTRACT

Objective: To explore the impact of fucoxanthin on oxidized low-density lipoprotein (OxLDL)-induced stress and inflammation in human endothelial cells and its underlying mechanisms.

Methods: HUVECs were treated with OxLDL and/or fucoxanthin for a range of time points and concentrations. We evaluated the effects of fucoxanthin on OxLDL-induced HUVECs using the MTT assay, reactive oxygen species accumulation assay, ELISA, RT-PCR, immunofluorescence, and Western blotting.

Results: Fucoxanthin enhanced the cell viability in a dose dependent manner after OxLDL exposure. Furthermore, fucoxanthin pretreatment significantly decreased OxLDL-induced reactive oxygen species production and prevented the activation of the nuclear factor kappa-B pathway, which led to substantial suppression of pro-inflammatory gene expressions. OxLDL-induced upregulation of interleukin-6, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, interleukin-1β, monocyte chemotactic protein-1, cyclooxygenase-1, and tumor necrosis factor-α was significantly reduced by fucoxanthin.

Conclusions: Fucoxanthin can inhibit OxLDL-induced vascular inflammation and oxidative stress in HUVECs by targeting Nrf2 signaling pathways.

KEYWORDS: Oxidized low-density lipoprotein; Fucoxanthin; Atherosclerosis; Inflammation; Oxidative stress; Cell viability; HUVEC; Nrf2 signaling pathway; NF-κB

1. Introduction

Across the globe, atherosclerosis is a major cause of morbidity and mortality[1]. The condition is often accompanied by other comorbidities like metabolic syndrome, hypertension, diabetes, and dyslipidemia[2,3]. Dysregulated lipid metabolism and a maladaptive immune response are the main causes of atherosclerosis. Low-density lipoproteins (LDLs) accumulate under the endothelium after vascular damage[4,5]. During atherosclerosis, excess LDL is converted into oxidized LDL (OxLDL), which initially damages the endothelial barrier, interferes with endothelial healing, and then emerges into the subendothelial space. The initial stages of

Significance

Oxidized low-density lipoprotein plays a central role in atherosclerosis by acting on endothelial cells. The anti-oxidant and anti-inflammatory properties of fucoxanthin contribute to decreasing oxidized low density lipoprotein-induced oxidative stress and pro-inflammatory cytokine levels in human endothelial cells. These findings support the use of fucoxanthin in treatment of the chronic inflammation-associated atherosclerosis and provide a basis for future clinical investigations.

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atherosclerosis are accompanied by the recruitment of monocytes by endothelial cell-derived ligands, mainly vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1[6]. Toll-like receptor (TLR) co-signaling pathways are also activated in the endothelium by OxLDL, leading to pro-inflammatory signaling cascades and inflammasomes[7]. Plaque stability is determined by the balance between pro- and anti-inflammatory signals.

Signals for binding molecules include nuclear factor kappa-B (NF-κB) translocation and intracellularly generated ROS, which play a major role in vascular diseases. In endothelial and non-endothelial cells, OxLDL increases the production of superoxide radicals (O^-_2) and activates NF-κB[8,9]. Inflammation and oxidative stress-related molecules like superoxide dismutase, reactive oxygen species (ROS), and nuclear transcription factor-E2-related factor 2 (Nrf2) are regulated by NF-κB. Heme oxygenase-1 (HO-1) is an enzyme regulated by Nrf2 that protects cells from ROS[10]. The activation of Nrf2 and HO-1 protein expression in HUVECs inhibits apoptosis, inflammation, and pro-inflammatory mediators like interleukin (IL)-6, tumor necrosis factor (TNF)-α, and cyclooxygenase (COX)-2[11]. Additionally, PI3K/AKT provides a cellular defense against oxidative stress and inflammation. Various signal transduction pathways, including PI3K/AKT, regulate Nrf2 and signal to activate antioxidant enzymes[12,13]. In the context of endotoxin-induced endothelial damage, these proteins are cytoprotective, antioxidant, and anti-inflammatory. The expression of these proteins in various cell types can be triggered by a variety of natural compounds and crude herbal extracts. Some Nrf2-mediated genes have antioxidant and anti-inflammatory properties[14]. Therefore, the most effective way to combat OxLDL-induced ROS generation is to supplement antioxidants exogenously.

Diatoms and brown algae produce fucoxanthin (Fux), a carotenoid (Figure 1). This compound possesses multiple biological functions including antidiabetic, anti-obesity, antimicrobial, and anticancer properties[15-18]. In addition, Fux is a powerful antioxidant[19]. Fux has anti-inflammatory properties in lipopolysaccharide-stimulated murine macrophages *in vitro* and improves fat oxidation and decreases lipid levels *in vivo*[20]. However, Fux's effect on inflammation caused by OxLDL remains unexplored. Therefore, determining Fux's ability to reduce OxLDL-induced inflammation in endothelial cells was the aim of this study.

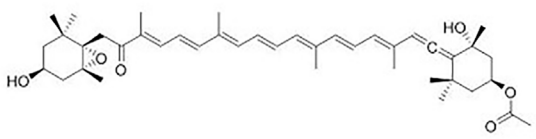

Figure 1. Chemical structure of fucoxanthin (Fux).

2. Materials and methods

2.1. Reagents

Human umbilical vein endothelial cells (HUVECs) were from the Chinese Academy of Sciences in Shanghai. OxLDL from Union-Bio Technology, China. Methyl thiazolyl tetrazolium (MTT) and 2,7-dichlorodi-hydrofluorescein diacetate (DCFH2-DA) from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM) from Gibco BRL/Invitrogen (Carlsbad, CA, USA). Antibodies against IL-6 (#P620), IL-1β (#PBOIL1BI), TNF-α (#PA5-19810), COX-1 (#PA5-16318), ICAM-1 (#MA5407), HO-1 (#PA5-77833), NAD(P)H:quinone oxidoreductase 1 (NQO-1) (#PA5-82294), γ-GCLC (#PA5-44190), p-P65 (#MA5-15160), p-inhibitory kappa B alpha (p-IKBα) (#PA5-36653), and TLR4 (#48-2300) were from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Nrf2 (AB-M-018), β-actin (AB-M-003), secondary antibodies goat anti-rabbit (AB-M-010), and goat anti-mouse (AB-M-009) were from MOLEQULE-ON (New Lynn, Auckland, New Zealand).

2.2. Cell culture and treatment

HUVECs were cultured at 37 ℃ with 10% FBS in DMEM. After 24 h, HUVECs were treated with OxLDL (100 μg/mL) and/or Fux at the indicated concentrations (5, 10, 25 and 50 μmol/mL). The MTT assay (Sigma, MO USA) was used to measure the proliferation of the cells.

2.3. Viability assay

MTT assay was used to assess the viability of the cells. These cells were then maintained in wells of a 12-well plate, at 4×10^5 cells/well, and exposed to various treatments. This was followed by further incubating the cells for 2 h of phosphate buffer saline (PBS) containing 400 μL of MTT (0.5 mg/mL). After removing the medium, the developed formazan product was liquefied in dimethylsulfoxide (400 μL). Finally, a microplate reader (Winooski, VT, USA) was used to determine the absorbance of each well at 570 nm.

2.4. ROS accumulation measurements

DCFH2-DA fluorescence dye was used to determine intracellular ROS accumulation[21]. Cells were seeded in a 6-well plate at 1×10^7 cells/mL, then pretreated with Fux (0-50 μmol/mL for 2 h) followed by OxLDL stimulation (100 μg/mL) for 24 h, at which point intracellular ROS accumulation was quantified. Afterward, DCFH2-DA was supplementary to the culture medium for 30 min

at 37℃. Using Leica D6000 fluorescence microscope (Leica, Germany) the dichlorofluorescein fluorescence intensity inside cells was examined. By comparing the fluorescence intensity of treated cells and vehicle-treated cells, ROS levels were measured.

2.5. Cytokine measurements

HUVECs were cultured in a 12-well plate with approximately 6.5 $\times 10^5$ cells/well in the culture medium. Cells received pretreatment with Fux (0-50 μmol/mL, 2 h) and then OxLDL treatment (100 μg/mL, 72 h). IL-6, monocyte chemotactic protein (MCP)-1, IL-1β, TNF-α, and ICAM-1 were quantified using their respective ELISA kits[14].

2.6. Real–time qPCR

After the treated cells were cleaned with PBS, tRNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was converted to cDNA using a PrimeScript RT reagent kit as per the recommended guidelines of the manufacturer (Takara Bio, Shiga, Japan). Real-time qPCR was performed using SYBR Green system (Applied Biosystems, Foster City, CA, USA) and a ViiA-7 Applied Biosystem (Carlsbad, CA, USA). The mRNA expressions of *IL–6*, *MCP–1*, and *VCAM–1* were standardized to the expression of *β–actin*, the housekeeping gene. The primer sequences are shown in Table 1. The relative gene expression was calculated *via* the $2^{-\triangle\triangle Ct}$[22].

2.7. Production of cytoplasmic and nuclear extracts

The cytoplasmic material was extracted in Buffer Ⅰ [25 mM HEPES pH 7.9, 5 mM KCl, 0.5 mM $MgCl_2$, and 1 mm dithiothreitol (DTT)] for a 5 min cycle. Then, it was mixed with an equivalent amount of Buffer Ⅱ [25 mM HEPES pH 7.9, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM DTT, and 0.4% (*v/v*) NP-40] accompanied with protease and phosphatase inhibitors, and then kept with continuous stirring at 4℃. The cell lysates were put in a centrifuge for 5 min at 4℃ and 500×*g*. The respective upper layers (supernatants) were then moved to fresh 1 mL Eppendorfs. The lower layer (cells pellet) were suspended with fresh Buffer Ⅱ solutions, then it was mixed with the cytoplasmic protein. In order to get rid of any residual nuclei, the pellets were further centrifuged at 10 000×*g* and the upper aqueous phase were separated. Cytoplasmic Nrf2, p-IKBα, total IKBα activation were analyzed by using Western blotting.

For the preparations of nuclear extracts, the cytoplasmic extraction pellets were re-suspended in Buffer Ⅲ [25 mM HEPES pH 7.9, 400 mM NaCl, sucrose (10%), NP-40 (0.05%), and 1 mM DTT] with inhibitors of both phosphate and protease. Then, the tubes were vortexed for 1 h at 4℃ before they were subjected to a centrifugation step of 10 000×*g* for 10 min. The supernatants gathered after this, which contains nuclear proteins, nuclear Nrf2, p-P65 and p65 activation were also analyzed by using Western blotting.

2.8. Western blotting

After treatment, cells were harvested and then washed once with cold PBS, after which cytoplasmic, nuclear, and total extracts were prepared as aforementioned in section 2.7. In each sample, to detect the protein level, a Bio-Rad protein assay was used, and bovine serum albumin was used as the reference standard. SDS-PAGE (8%-15%) was used to resolve equal amounts of protein (50 μg), and the proteins were transferred to nitrocellulose membranes overnight. Five-percent skimmed milk was used for blocking the membranes at 37℃ for 30 min, after which the membranes were readily incubated with the indicated primary antibodies (IL-6, IL-1β, TNF-α, COX-1, ICAM-1, Nrf2, HO-1, NQO-1, γ-GCLC, p-P65, p-IKBα, and TLR4) for 24 h. After this, a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody was incubated with the nitrocellulose membranes for 1 h, and an enhanced chemiluminescence substrate was used to develop the membranes (Pierce Biotechnology, Rockford, IL, USA). An LI-COR chemiluminescence imaging system (3600-00-C-Digit Blot Scanner) was used to examine the samples. Image Studio Lite software (LI-COR Biosciences, Lincoln, NE, USA) was used to generate the graphs of the densitometric band intensities with normalization to the intensity of the untreated control band, which was set to 1[23].

2.9. Immunofluorescence assay

In a four-well glass Tek chamber containing DMEM medium with 10% FBS and Fux for 2 h, OxLDL (100 μg/mL) was added in cells for 24 h. After fixation with 2% paraformaldehyde for 15 min and permeabilization with 0.1% Triton X-100 for 10 min, cells were washed and blocked with 10% FBS in PBS, then incubated with 1.5% FBS for 2 h with an anti-Nrf2 primary antibody. After that, we incubated the cells for 1 h with FITC-conjugated secondary antibodies. The cells were stained with 1 μg/mL DAPI for 5 min. A fluorescence microscope at 200× magnification was used to view the stained cells after they were washed with PBS[24].

Table 1. Primer sequences.

Gene	Sequence (5'-3')
IL–6	Forward: GGTACATCCTCGACGGCATCT
	Reverse: GTGCCTCTTTGCTGCTTTCAC
MCP–1	Forward: TCACCACCATGGAGAAGGC
	Reverse: AGGTGGAAGAATGGGAGTTG
VCAM–1	Forward: AGACAGAGAACAGGGAATTAAATGTGT
	Reverse: AGACAGAGAACAGGGAATTAAATGTGT

2.10. Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 6.0 (GraphPad Software Inc., San Diego, CA, USA). This study analyzed the data using an analysis of variance (one-way analysis of variance) and Tukey's *post-hoc* test. The results were considered significant at $P<0.05$.

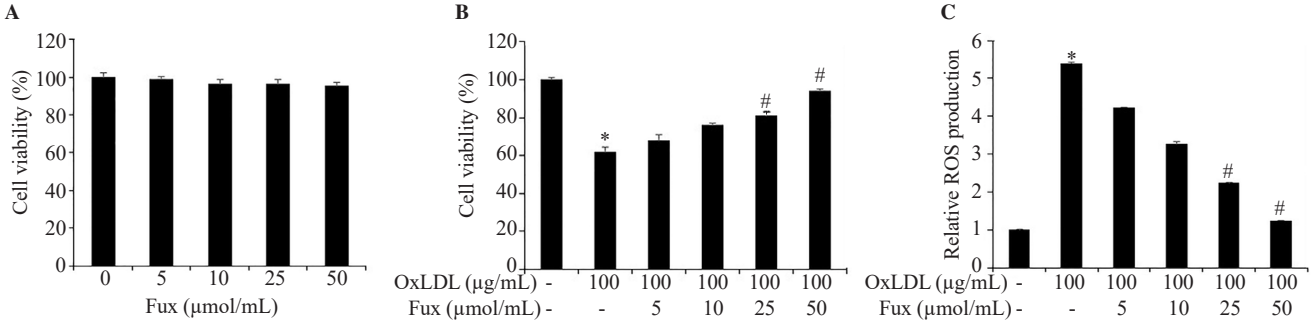

Figure 2. Effect of fucoxanthin (Fux) on reactive oxygen species (ROS) production and cell viability. (A) Human umbilical vein endothelial cells (HUVECs) were treated for 24 h with Fux at 0, 5, 10, 25, and 50 μmol/mL and then analyzed for viability using the MTT assay. (B) HUVECs were exposed to Fux and/or oxidized low-density lipoprotein (OxLDL) for 24 h and then analyzed for viability using the MTT assay. (C) The effect of Fux on OxLDL-induced ROS generation. $^{*}P<0.05$ denotes significant differences in comparison to the control. $^{\#}P<0.05$ denotes significant differences as compared to OxLDL alone treatment group.

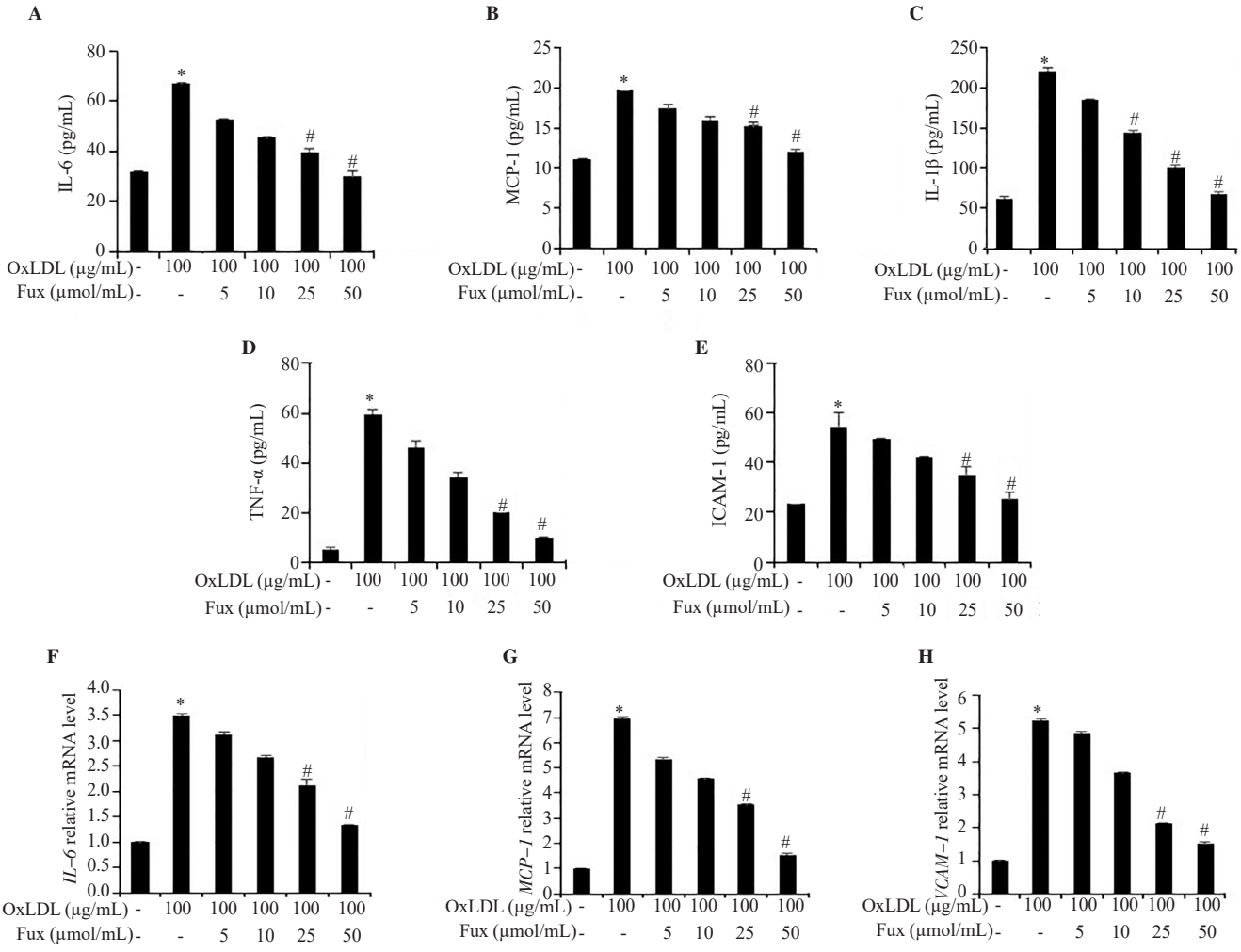

Figure 3. Effect of Fux on interleukin (IL)-6, monocyte chemotactic protein (MCP)-1, IL-1β, tumor necrosis factor (TNF)-α, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in HUVECs. (A-E) IL-6, MCP-1, IL-1β, TNF-α, and ICAM-1 were determined using a commercial ELISA kit; (F-H) Fux reduces the levels of *IL-6*, *MCP-1*, and *VCAM-1* in HUVECs. *IL-6*, *MCP-1*, and *VCAM-1* mRNA expressions were analyzed by RT-PCR. $^{*}P<0.05$ denotes significant differences in comparison to the control. $^{\#}P<0.05$ denotes significant differences as compared to OxLDL alone treatment group.

3. Results

3.1. Fux inhibits OxLDL–induced cytotoxicity in HUVECs

The MTT was used to test cell cytotoxicity. Fux had no cytotoxic effects on HUVECs up to 50 μmol/mL (Figure 2A); therefore, this concentration was used in the following experiments. While OxLDL (100 μg/mL) significantly reduced cell viability by 39.6% (P<0.05), Fux dose-dependently protected the cells from OxLDL-induced cell death (Figure 2B).

3.2. Fux suppresses intracellular ROS dose–dependently

DCFH2-DA fluorescence was used to measure intracellular ROS. Fux pretreatment significantly attenuated OxLDL-induced ROS accumulation in a dose-dependent manner (Figure 2C).

3.3. Fux suppresses OxLDL–induced pro–inflammatory cytokine

Figure 3A-E shows that OxLDL stimulation significantly

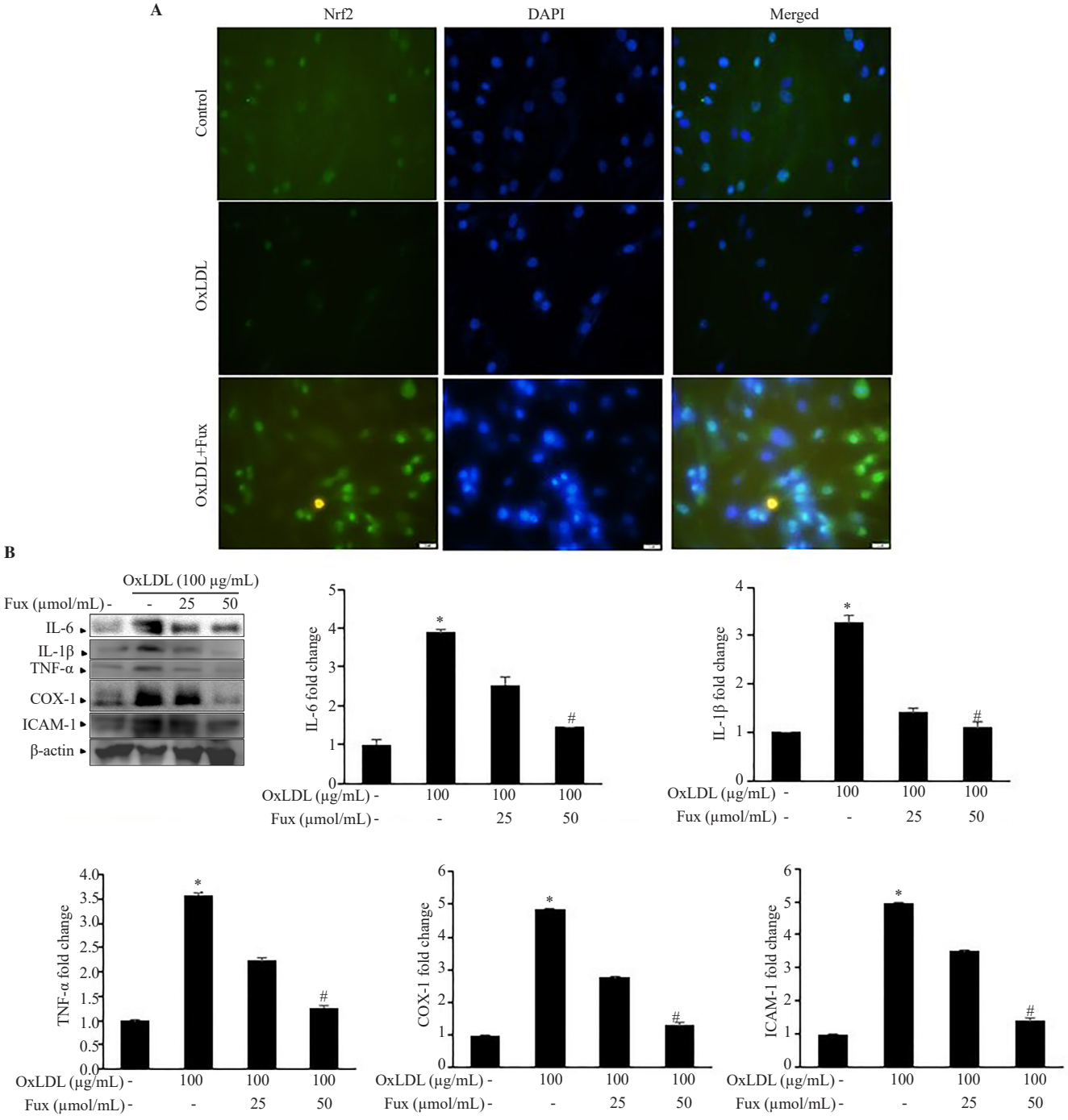

Figure 4. (A) Nrf2 nuclear translocation; (B) Effect of Fux on OxLDL-induced expression of IL-6, COX-1, IL-1β, TNF-α, and ICAM-1. Western blotting was performed. $^{*}P$<0.05 denotes significant differences in comparison to the control. $^{\#}P$<0.05 denotes significant differences as compared to OxLDL alone treatment group.

increased the production of TNF-α, IL-1β, MCP-1, IL-6, and ICAM-1. In contrast, Fux pretreatment significantly suppressed the release of these pro-inflammatory cytokines.

3.4. Fux suppresses IL-6, MCP-1, and VCAM-1 gene expression in OxLDL-treated HUVECs

According to the qRT-PCR results, cells pretreated with Fux had significantly lower levels of these mRNA transcripts than cells treated with OxLDL alone (Figure 3F-H) (P<0.05). Fux suppressed the cytokine production associated with OxLDL-induced inflammation.

3.5. Fux enhances Nrf2 nuclear translocation

We used an immunofluorescence assay to examine the nuclear translocation of Nrf2. In cells treated with OxLDL, nuclear Nrf2 accumulation decreased (Figure 4A). Fux treatment increased Nrf2 nuclear aggregation, as evidenced by the high Nrf2 staining in Fux-treated cells, according to immunofluorescence images.

3.6. Fux suppresses IL-6, IL-1β, TNF-α, COX-1, and ICAM-1 protein expression in OxLDL-treated HUVECs

Treatment with 100 μg/mL of OxLDL for 24 h led to the overexpression of IL-6, IL-1β, TNF-α, COX-1, and ICAM-1 protein while Fux treatment suppressed overexpression significantly (P<0.05) (Figure 4B). These results indicate that Fux could reduce OxLDL-induced inflammation in HUVECs.

3.7. Effect of Fux on Nrf2 activation in HUVECs

OxLDL reduced nuclear Nrf2 levels while co-treating with Fux reversed it dose-dependently (Figure 5A). Furthermore, nuclear Nrf2 localization in HUVECs was also confirmed by immunofluorescence. These data suggested that Fux enriched Nrf2 expression in nuclei.

3.8. Effect of Fux on HO-1, γ-GCLC, and NQO-1 activation

According to Western blotting results, OxLDL reduced HO-1, NQO-1, and γ-GCLC protein expression; while Fux treatment upregulated the expressions dose-dependently (Figure 5B).

3.9. Effect of Fux on OxLDL-associated NF-κB activation

OxLDL stimulation led to an increase in p-P65, p-IKBα, and TLR4 levels (Figure 6). Fux pretreatment significantly attenuated OxLDL-induced increases in p-P65, p-IKBα, and TLR4 levels.

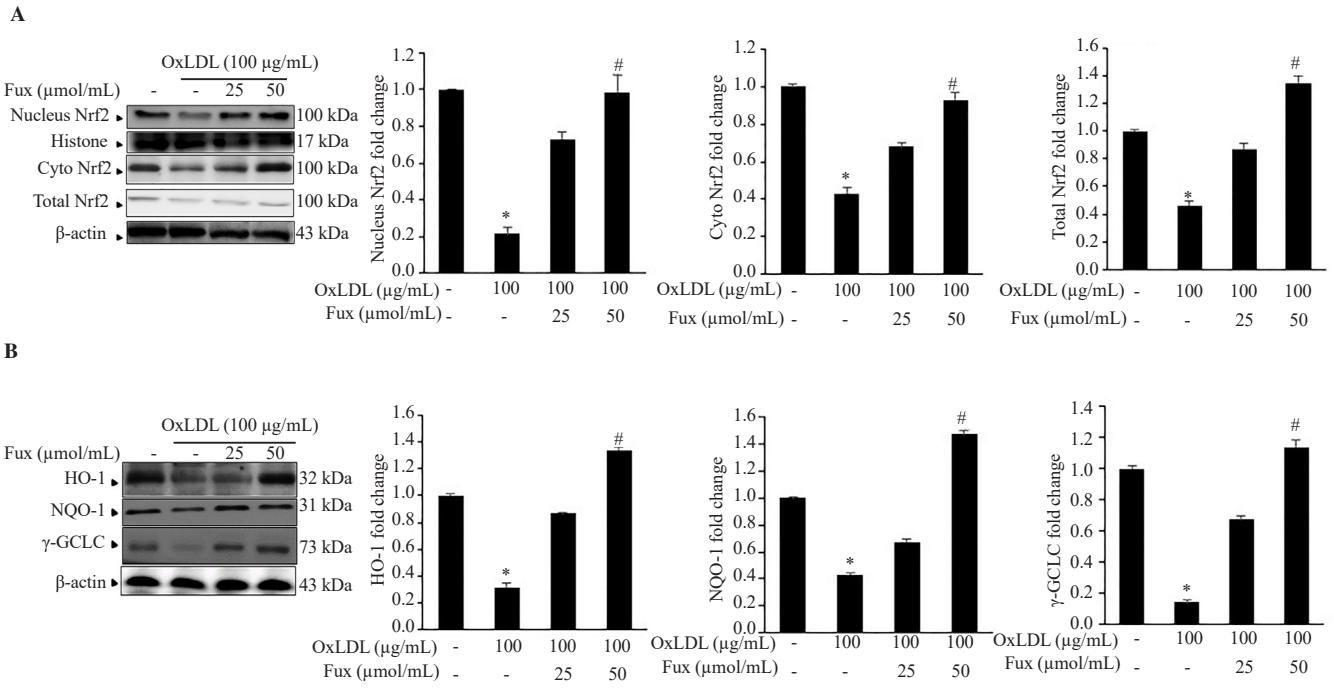

Figure 5. Effect of Fux on Nrf2 (nucleus, cytosolic, and total) (A), HO-1, NQO-1, and γ-GCLC protein expressions (B). *P<0.05 denotes significant differences in comparison to the control. #P<0.05 denotes significant differences as compared to OxLDL alone treatment group.

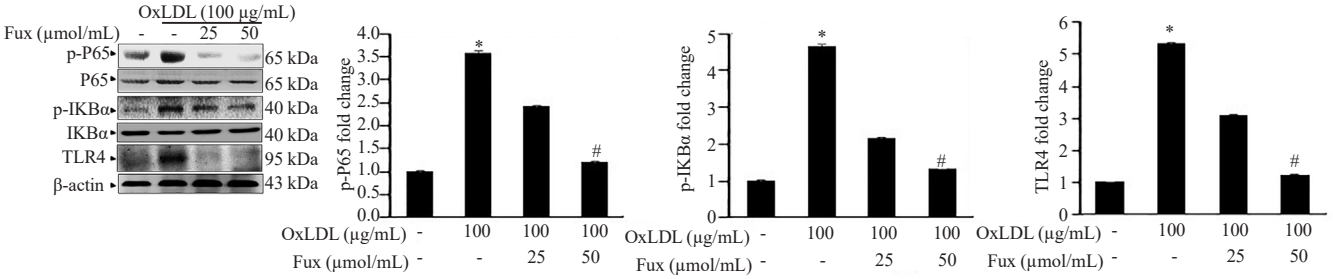

Figure 6. Fux inhibits NF-κB activation in OxLDL-induced HUVECs. Western blot was used to detect p-P65, p-IKBα, and TLR4. $^{*}P<0.05$ denotes significant differences in comparison to the control. $^{\#}P<0.05$ denotes significant differences as compared to OxLDL alone treatment group.

4. Discussion

Atherosclerosis remains one of the leading causes of death in the world, despite huge advances in science[25–28]. This inflammatory condition causes LDL to accumulate in arteries. There is evidence that inflammation plays a significant role in atherosclerosis development. Recent studies have focused on the role of modified lipoproteins, particularly OxLDL. OxLDL may be a key antigen in atherosclerosis, according to several lines of evidence. Several studies have found OxLDL and antibodies against its epitopes in both human and rabbit plasma, as well as in atherosclerosis lesions. However, Holvoet *et al*. reported that OxLDL levels are associated with coronary artery disease in both heart transplant recipients and patients with established coronary artery disease[29,30]. The molecular mechanism underlying Fux's effect on OxLDL-induced damage to HUVECs was investigated in this study, which revealed how pivotal Nrf2 was in this process. Through the Nrf2/TLR4/NF-κB pathways, we showed that Fux regulates HUVEC viability, ROS, and inflammation, clarifying a new role for Fux in protecting against atherosclerosis. OxLDL generates ROS, which plays a major role in the progression of atherosclerosis. Oxidative stress, inflammation, and apoptosis are central to vascular endothelial dysfunction. We used OxLDL to simulate early inflammation in HUVECs. Fux significantly inhibited the inflammatory response in OxLDL-stimulated HUVECs.

In various cell lines, including HUVECs, Fux is antioxidative and cytoprotective[31]. HUVECS stimulated by OxLDL are widely used in anti-inflammatory studies[32,33]. In the current study, HUVECS were stimulated with OxLDL to induce an inflammatory reaction. OxLDL stimulation up-regulated TNF-α, IL-1β, and IL-6 cytokine expression. At both Fux concentrations (25 and 50 μmol/mL), the secretion of TNF-α, IL-1β, and IL-6 was significantly downregulated. These findings suggest that Fux protects the endothelium by acting as a potent anti-inflammatory agent. VCAM-1 and ICAM-1, early markers of atherosclerosis, are increased by NF-κB activation[34]. The promoters of ICAM-1 and VCAM-1 contain from one to three NF-κB binding sites, and NF-κB acts as a "transducer" to activate these genes. OxLDL increased ICAM-1 and VCAM-1 in HUVECs as expected, and Fux pretreatment significantly blocked this effect.

Regarding the molecular basis of Fux's protective effects, we hypothesized that Fux can induce cell-adaptive responses *via* Nrf2. The expression of Nrf2-regulated cytoprotective proteins in endothelial cells may contribute to Fux's anti-inflammatory and atheroprotective effects. Crosstalk exists between Nrf2 and NF-κB, and Nrf2 specifically inhibits the NF-κB transcription machinery. In addition, results show that Nrf2 nuclear translocation is induced by Fux and Fux inhibits NF-κB signaling in HUVECs. In addition, we found that Fux also modulated Nrf2 expression. Due to Nrf2 activation, HO-1, NQO-1, and γ-GCLC expression levels were upregulated following Fux pretreatment. Flavonoids induce HO-1 expression in endothelial cells through activating the p38/Akt pathway, which can inhibit endothelial adhesion and dysfunction[35]. The production of anti-inflammatory cytokines is augmented by HO-1, which attenuates the release of pro-inflammatory cytokines. Fux may work by directly enhancing endothelial HO-1 expression and quenching oxidative stress inside cells.

In conclusion, the protective effect of Fux against OxLDL-induced endothelial dysfunction supports the potential benefits of consuming foods rich in brown algae. Our findings on the intracellular signaling pathways modulating the oxidative insults induced by OxLDL in endothelial cultures may facilitate the discovery of an innovative therapeutic approach to the prevention of atherosclerotic progression.

Conflict of interest statement

The authors declare no conflict of interest.

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Authors' contributions

PR designed the project. PR and AMA performed the experiments and analyzed data. PR drafted the manuscript. PR and AMA performed critical revision of the article. AMA supervised all experiments. All authors have read and approved the final manuscript.

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