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RESEARCH PAPER

N-3 polyunsaturated fatty acids block the trimethylamine-N-oxide- ACE2-TMPRSS2 cascade to inhibit the infection of human endothelial progenitor cells by SARS-CoV-2

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Abstract

Severe acute respiratory syndrome coronavirus 2(SARS-CoV-2) is a novel coronavirus that infects many types of cells and causes cytokine storms, excessive inflammation, acute respiratory distress to induce failure of respiratory system and other critical organs. In this study, our results showed that trimethylamine-N-oxide (TMAO), a metabolite generated by gut microbiota, acts as a regulatory mediator to enhance the inerleukin-6 (IL-6) cytokine production and the infection of human endothelial progenitor cells (hEPCs) by SARS-CoV-2. Treatment of N-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) could effectively block the entry of SARS-CoV-2 in hEPCs. The anti-infection effects of N-3 PUFAs were associated with the inactivation of NF-κB signaling pathway, a decreased expression of the entry receptor angiotensin-converting enzyme 2 (ACE2) and downstream transmembrane serine protease 2 in hEPCs upon the stimulation of TMAO. Treatment of DHA and EPA further effectively inhibited TMAO-mediated expression of IL-6 protein, probably through an inactivation of MAPK/p38/JNK signaling cascades and a downregulation of microRNA (miR)-221 in hEPCs. In conclusion, N-3 PUFAs such as DHA and EPA could effectively act as preventive agents to block the infection of SARS-CoV-2 and IL-6 cytokine production in hEPCs upon the stimulation of TMAO.

Keywords: Trimethylamine-N-oxide; SARS-CoV-2; interleukin-6; microRNA-221; human endothelial progenitor cells.

1. Introduction

The coronavirus disease 2019 (COVID-19) arises from the infection of a novel coronavirus, severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), and induces the pandemic of the century through spreading around the world since 2020 [1]. Around 410 million cases and more than 5.8 million deaths worldwide as of Feb 2022. Unlike its predecessor, SARS-CoV, the majority of SARS-CoV-2 infections are believed to be the result of exposure to individuals who were pre-symptomatic, asymptomatic or mildly symptomatic for COVID-19 [2]. Another important difference with SARS-CoV-2 is its preference for vulnerable populations with pre-existing conditions such as hypertension (HTN), cardiovascular disease (CVD), Type 2 diabetic mellitus (DM) and chronic respiratory disease (CRD) [3–5]. SARS-CoV-2 infection is induced by binding to the entry receptor angiotensin-converting enzyme-2 (ACE2) and triggering the activation of downstream transmembrane ser-

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ine protease 2 (TMPRSS2) protein, a serine protease, locating inside of cellular membrane [6]. Both ACE2 and TMPRSS2 proteins are highly expressed in many types of cells and tissues including the nasopharynx/ lung epithelial cells, the cardiovascular system and gastrointestinal tracts [7]. It is plausible that SARS-CoV-2 might infect several types of cells including endothelial cells [8] and causes acute respiratory distress, cytokine storm and excessive inflammation leading to respiratory failure, organ failure and mortality [6].

Human endothelial progenitor cells (hEPCs) are derived from bone marrow (BM) and mobilize to damaged tissues to form new blood vessels after an ischemic injury [9]. Several studies indicate that hEPCs express cell surface markers such as CD31, CD105 (endoglin), CD 144 and CD309 (Flk-1/KDR) [10,11]. These BM-derived hEPCs differentiate into endothelial colony forming cells and human vascular endothelial cells for the neovascularization [12]. Many studies suggest that hEPCs are implicated in the tissue repairing of blood vessels damaged by various pathological conditions [13–15]. Although respiratory deterioration represents the major clinical symptom, SARS-CoV-2 infection may also involve in the cardiovascular dysfunction [16]. The entry of SARS-CoV-2 into hEPCs, endothelial colony forming cells and human vascular endothelial cells also requires the entry receptor/serine protease complexes, ACE2-TMPRSS2 complexes, which are found in cells of the lungs, blood vessels, heart, and gastrointestinal system [17]. The spike (S) protein on the exterior of SARS-CoV-2 binds to the ACE2 receptors and prime to the host cell serine protease TMPRSS2 to trigger the infection [6]. A recent study indicated that patients with SARS-CoV-2 infection have life-threatening complication of cardiovascular system and worse outcomes than the healthy ones [18].

According to the clinical findings in Wuhan China, many of these patients with chronic diseases including HTN, DM and CVD use ACE inhibitors and angiotensin-receptor blocker which upregulate the level of ACE-2 receptor [19–21]. Therefore, it's plausible that an augmented expression of ACE-2 and TMPRSS2 would facilitate the entry of SARS-CoV-2 into many types of mammalian cells [22]. A recent study showed that NF-κB and AP-1 transcription factors were involved in the expression of ACE2 in vascular smooth muscle cells [23]. Several studies indicated that NF-κB and MAPK signaling pathways were involved in inflammatory response [24,25]. Inactivation of inflammatory cascades such as NF-κB and MAPK/JNK signaling pathways and decreased expression of interleukin-6 (IL-6) played important roles in anti-inflammation strategies [26]. Our previous studies indicated that microRNA (miR)-221 played an important role in hEPCs [27,28]. Therefore, it's plausible that targeting to the expression of IL-6 production and multiple key regulatory pathways could prevent the scenario of SARS-CoV-2 infection and inflammation.

Recent studies suggest that chronic diseases such as HTN, DM, and CVD are correlated with the overconsumption of dietary saturated fat [29]. In the human intestine, dietary phosphatidylcholine, choline and carnitine present in meat, are metabolized by gut microbes, converted into trimethylamine (TMA) and further converted into trimethylamine-N-oxide (TMAO) by hepatic flavin monooxygenase-3 (FMO3) in the liver [30]. A recent study showed that TMAO could induce platelet hyperactivation and increase thrombosis risk [31]. Thus, TMAO has been shown to contribute to various aspects of CVD, such as heart failure, atherosclerosis, stroke and HTN [32–38] making TMAO an antinutrient and potentially detrimental to human health. Other studies also revealed a significant association between CVD and gut-flora-derived metabolites [39].

Recent studies indicated that high-fat-diet or red-meat consumption increased post-prandial plasma levels of TMAO in healthy non-obese adults [40,41]. Others studies also showed that a high-saturated-fat diet increases circulating ACE2 concentrations in healthy non-obese adults [42]. These findings suggested that an increment of plasma TMAO level maybe correlate with the increased ACE2 protein expression. It is noting that diabetic animals had significantly increased TMAO concentrations in comparison with normal controls in an experimental animal study [43].

Epidemiological studies indicated that fish oil consumption is inversely correlated with the incidence of CVD [44]. Interestingly, a previous study showed a strong correlation between fish intake and an increased urinary excretion of TMAO [41]. The biological functions of fish oil are contributed by certain N-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). DHA and EPA are reported to have strong influence on intestinal microbiota and the regulation of immune function [45]. Our previous studies already demonstrated that N-3 PUFAs could enhance the physiological function of hEPCs and prevent ischemic injuries especially in diabetic animals [27,46]. However, the roles of gut microbiota-dependent metabolite, TMAO, and the dietary factors such as N-3 PUFAs in the infection of hEPCs by SARS-CoV-2 have yet to be well-documented. Therefore, we investigated whether N-3 PUFAs such as DHA and EPA could modulate SARS-CoV-2 infection and suppress cytokine storm in hEPCs upon TMAO stimulation.

2. Materials and methods

2.1. Reagents and antibodies

Free types of EPA (# 90110) and DHA (# 90310) were purchased from Cayman Chemical Inc. (Ann Arbor, MI, USA). The following antibodies were acquired from the Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA): anti-ACE2 (#F2520), anti-TMPRSS2 (#D2420), anti- phosphorylation-p38 (Y182) (#C0705), anti-β-actin (#L3004), and anti-Lamin A (L1919) antibodies. Anti-phosphorylation IκBα (p-IκBα; S32/36) (#16), anti- phosphorylation p65 (p-p65/RelA; S536) (#17), and anti-phosphorylation-JNK (p-JNK; T183/Y185) (#11) antibodies were purchased from Cell signaling Technology Inc. (Danvers, MA, USA). Anti- phosphorylation -c-Jun (p-c-Jun/S63) (#42926) and anti-c-fos (#42256) antibodies were purchased from GeneTex Inc. (Irvine, CA, USA). Antibodies against human CD31 conjugated to fluorescein isothiocyanate , human CD 105 conjugated to APC, human CD144 conjugated to PerCP-Cy5.5 were purchased from BD PharMingen Inc. (San Diego, CA, USA). Antibody against human CD309 conjugated to PE was purchased from eBioscience Inc. (San Diego, CA, USA). MCDB-131 medium, SB203580 (a specific inhibitor of MAPK/p38), Bay-11-7082 (a specific inhibitor of NF-κB) and SP600125 (a specific inhibitor of MAPK/JNK) were acquired from Sigma (St Louis, MO, USA). A commercial protein extraction kit, Nuclear Protein Extract Reagent (NE-PER), was purchased from Pierce Biotechnology Inc. (Lackford, IL, USA). Trizol reagent and Lipofectamine LTX with plus reagent were purchased from Invitrogen Inc. (Carlsbad, CA, USA). The two-step real-time polymerase chain reaction (RT-PCR) kit was purchased from Promega Inc. (Madison, WI, USA). The specific Taqman MicroRNA assays, including the primers for has-miR-221 and U6 snRNA, were purchased from Applied Biosystems (Carlsbad, CA, USA). The control vector and anti-miR-221 plasmids were purchased from System Biosciences Inc. (Mountain View, CA, USA). Fetal bovine serum (FBS) was obtained from the Thermo Fisher Scientific Inc. (Pittsburgh, PA, USA). EGM-2 growth kit was purchased from Lonza, Inc. (Allendale, NJ, USA).

2.2. Preparation of hEPCs

Human EPCs were generated from fresh human umbilical cord blood mononuclear cells (MNCs) and prepared according to the method as described in previous studies [11,47]. Protocols conformed to the ethical guidelines in a prior approval by the institutional review board of the China Medical University Hospital. In brief, MNCs were isolated by using Ficoll-Paque procedure [11,47]. Colonies of hEPCs were selected and cultured in 10% FBS MCDB-131 culture media with a EGM-2 growth kit in a gelatin-coated tissue culture dish.

Immunophenotyping of hEPCs was analyzed by a fluoresence-activated cell sorting method and flow cytometry analysis as described in previous studies (FACs; Becton Dickinson, San Diego, CA) [47]. Briefly, the characterization of hEPCs -specific surface markers ($CD31^+$/ $CD105^+$/ $CD144^+$/ $CD309^+$) was performed and identified by using flow cytometry analysis according to previous protocol [47]. We used primary monoclonal antibodies against human CD31 conjugated to fluorescein isothiocyanate , human CD 105 conjugated to APC, human CD144 conjugated to PerCP-Cy5.5 and human CD309 conjugated to PE. hEPCs between passage six and nine were used in the current study.

2.3. Cell culture of hEPCs

hEPCs were seeded onto a gelatin (50 μg/mL) -coated tissue culture dish and cultured in antibiotic-free 10% (FBS) MCDB-131 medium with EGM-2 growth kit. TMAO was dissolved in dimethyl sulfoxide (DMSO) at a stock solution of 600 mM.

2.4. Entry of SARS-CoV-2 pseudotyped lentivirus

hEPCs (1×10^5 cells) were seeded in 96-well plates with MCDB-131 medium containing TMAO (0 and 300 μM) in the presence of DHA or EPA for 24 h. To detect entry of SARS-CoV-2 pseudotyped Lentivirus into hEPCs, aliquots of SARS-CoV-2 plasmid (nCoV-S-Luc) 5 μL were transferred to each well of a 96-well plate and incubated in an incubator at 37°C for 48 h. Photomicrographs of cell morphology were also documented by using inverted phase-contrast microscope (300X) at 48 h time point. At the end of experimental period, Bright-Glo Reagent was added to each well for the luciferase activity analysis. The luciferase assay was performed in a multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

2.5. Neovasculogenesis (vascular tube formation) assay

hEPCs were added to Matrigel-free culture plates and cultured in MCDB-131 medium containing TMAO (0 and 300 μM) in the presence of EPA or DHA (0, 25, 50, 125 μM) for 24 h. Aliquots of SARS-CoV-2 plasmid (nCoV-S-Luc) 5 μL were then transferred to each culture plate for 48 h. To prepare Matrigel-coated 96 well plates, aliquots of 50 -μL Matrigel (at a concentration of 4 mg/mL) were used as a supporting matrix for vascular tube formation, added to 96-well culture plates and incubated at 37°C until gelatinization. To perform neovasculogenesis assay, hEPCs were cultured in MCDB-131 medium containing 10% FBS. At the end of SARS-CoV-2 plasmid transfection, hEPCs were collected from culture plates and seeded to the Matrigel-coated 96-well culture plates (1×10^5 cells/well) for 6 h. At the end of neovasculogenesis experiment, hEPCs were fixed with 0.5 mL of glutaraldehyde/paraformaldehyde solution (2.5%) and stained with modified calcein-AM solution. Tubular structure formations on the 3-D Matrigels were visualized under Olympus IX-71 inverted phase – contrast microscopy (40X), and photomicrographs were documented and analyzed with Olympus DP-71 digital camera and imaging system (Olympus, Tokyo, Japan).

2.6. Extraction of cellular proteins

Protein extractions were executed by NE-PER kit with inhibitors of phosphatase and protease. To remove the cell debris, cellular proteins were centrifuged for 10 minutes at 12,000 x g. The remaining supernatants were obtained as a cytoplasmic fraction.

2.7. Western Blotting analysis

Cellular proteins (70 μg) were fractioned by using 10% sodium-dodecyl sulfate polyacrylamide gel electrophoresis . The resulting SDS-PAGE gel was electroblotted to polyvinylidene difluoride membrane and detected with anti-ACE2 monoclonal antibody. The blots were stripped and reprobed with β-actin antibody as an internal control. Detection of other proteins including TMPRSS2, p-IκB-α, p-p38, p-JNK, p-p65/RelA, c-fos, p-c-Jun, and lamin A in cell lysates were performed by using similar procedure described above.

2.8. Quantitative real-time PCR (qPCR)

Total RNA samples from hEPCs were prepared from each subgroup with Trizol reagent and converted into cDNA for analysis using a two-step RT-PCR kit. Briefly, cDNA samples were used in a PCR mix containing the has-miR-221 specific primers, as described above. U6 snRNA was used as the internal control. Quantitative PCR experiments were performed using the real-time PCR detection system (Applied Biosystems, Carlsbad, CA, USA). The expression levels of miR-221 adjusted by the internal control U6 snRNA in hEPCs are presented as a fold of the corresponding untreated control subgroup. Transfections of anti-sense anti-miR-221 plasmids and internal control vector were conducted by using Lipofectamine LTX with plus transfection reagent.

2.9. ELISA analysis

hEPCs (1×10^5 cells) were seeded in 24-well plates with MCDB-131 medium containing TMAO (0 and 300 μM) in the presence of DHA or EPA for 24 h. At the end of experimental period, cell supernatant was collected for the measurement of IL-6 production according to the manufacturer's protocol.

2.10. Statistical analysis

Biostatistics analyses were performed to determine the difference between treatment and control subgroups of hEPCs by using SAS software (Cary, NC, USA). Confirmation of difference was performed by using the one-way ANOVA model and Tukey's post hoc test at the P=.05 level. Confirmation of difference in the relative entry efficiency of SARS-CoV-2 was examined by using student t-test at the P=.05 level.

3. Results

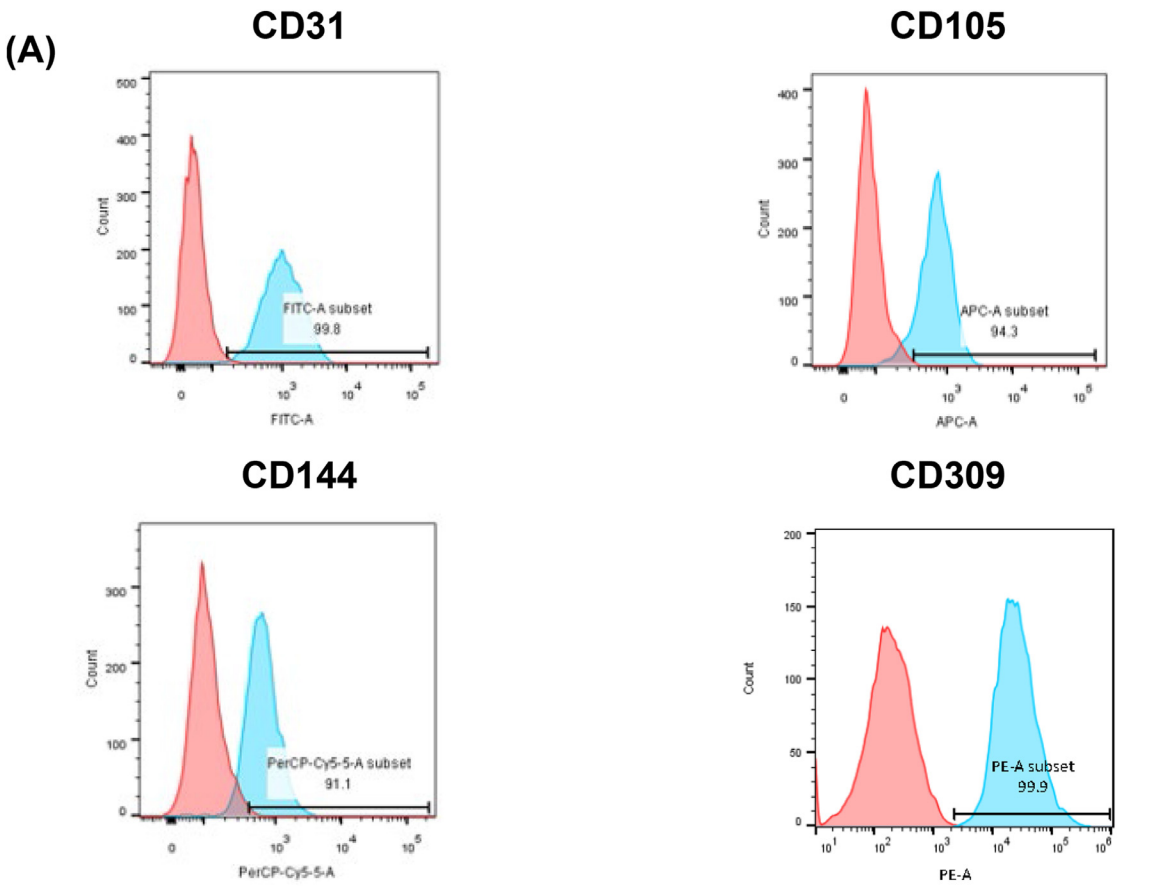
3.1. N-3 PUFAs effectively block the entry of SARS-CoV-2 and the expression of ACE2 and TMPRSS2 proteins in hEPCs upon TMAO stimulation

In this study, we investigated whether TMAO, a metabolite of gut microbiota, could augment the infection of hEPCs by SARS-CoV-2. We characterized specific surface markers in hEPCs (Fig. 1A). As shown in Figure 1A, hEPCs bear cell surface markers such as CD31, CD105, CD144, and CD309. All positive markers were above 90% of gated cells. Moreover, TMAO (at a concentration of 300 μM) could significantly enhance the entry of SARS-CoV-2 into hEPCs (P<.05) (Fig. 1B). These findings suggested that TMAO, a well-known inflammation agent, could increase the infection of hEPCs by SARS-CoV-2. N-3 PUFAs have been known to their anti-inflammatory effects. Therefore, we further examined whether N-3 PUFAs such as DHA and EPA could interfere the action of TMAO in hEPCs. As shown in Figure 1B, DHA and EPA significantly inhibit the infection of hEPCs by SARS-CoV-2 (P<.05). Cell morphology of hEPCs, infected by SARS-CoV-2, was shown in Figure 1C. Our previous studies indicated that N-3 PUFAs such as DHA and EPA could prevent ischemic injuries through an induction of neovasculogenesis in hEPCs [46]. Therefore, we further examined whether DHA and EPA could exert any effects on increment of neovascularization in hEPCs. As shown in Figure 1D, treatment of TMAO alone didn't alter neovascularization levels in SARS-CoV-2 -infected hEPCs in comparison with TMAO-untreated control subgroup. These results suggested that TMAO-mediated entry of SARS-CoV-2 could not affect neovascularization levels in hEPCs. However, treatment of DHA or EPA could significantly enhance neovascularization levels in hEPCs, respectively (P<.05). In conclusion, N-3 PUFAs such as DHA and EPA could effectively alleviate TMAO-mediated entry of SARS-CoV-2 into hEPCs.

To study the action of mechanisms, we further examine whether TMAO could modulate the expression of key regulatory proteins such as ACE2 and TMPRSS2 proteins in hEPCs. As shown in Figure 1E, TMAO significantly induce the expression of ACE2 and TMPRSS2 proteins in hEPCs. Thus, we further investigated the inhibitory effects of N-3 PUFAs on the expression of ACE2 and TMPRSS2 proteins in TMAO-treated hEPCs. Our results demonstrated that treatment of DHA and EPA could effectively inhibit TMAO-mediated expression of ACE2 and TMPRSS2 proteins for the infection of hEPCs by SARS-CoV-2 (Fig. 1E). Among all of them, DHA seemed to be more effective than EPA on blocking the expression of TMPRSS2 protein. In contrast, EPA seemed to be more effective than DHA on inhibiting the expression of ACE2 protein in hEPCs upon TMAO stimulation. These results suggested that DHA and EPA differentially inhibited the expression of ACE2 and TMPRSS2 proteins for the infection of hEPCs by SARS-CoV-2.

3.2. TMAO mediated the infection of hEPCs by SARS-CoV-2 and the expression of IL-6 protein through modulation of cellular signaling pathways and microRNA-221 level

We further examined the mechanisms of action during TMAO-mediated infection of hEPCs by SARS-CoV-2 and the expression



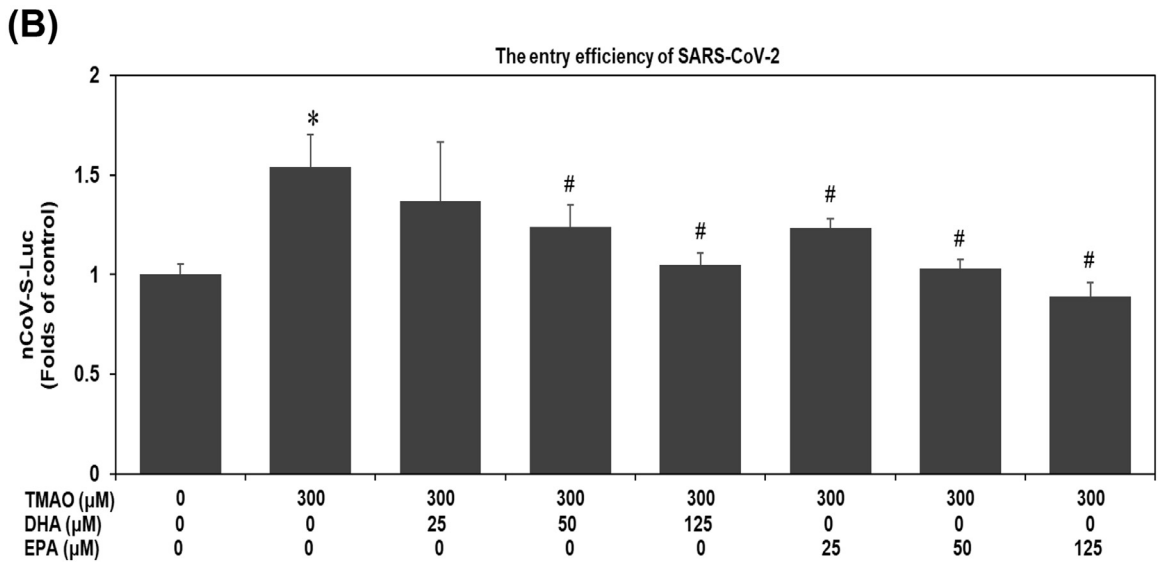
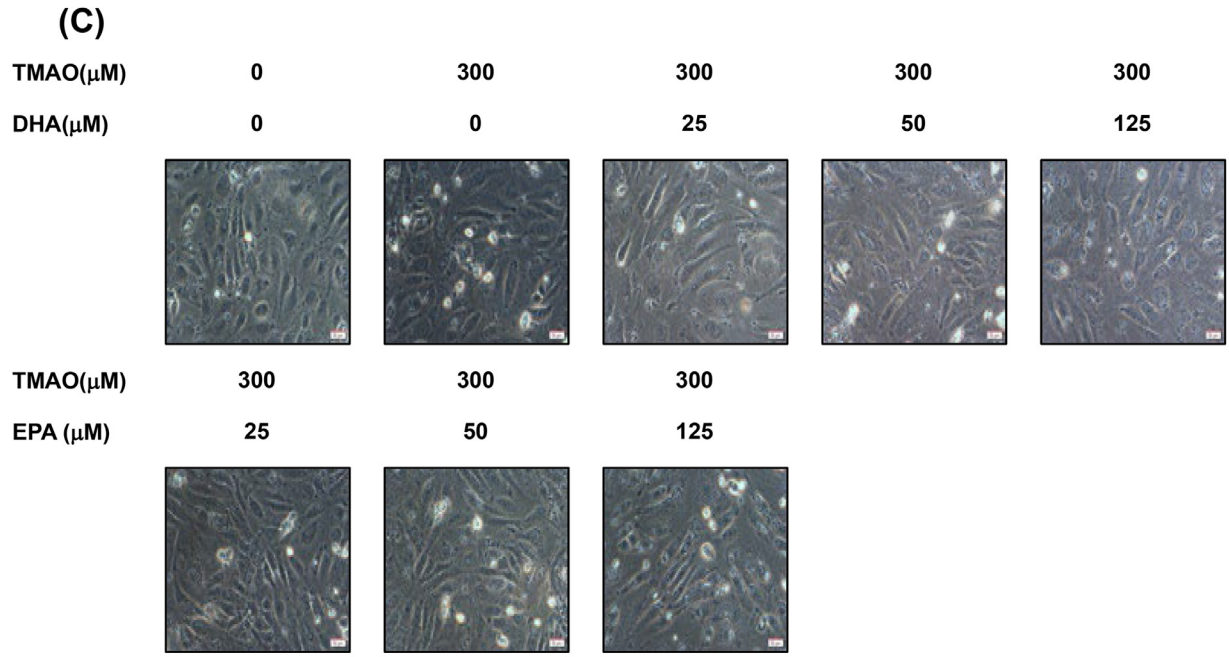
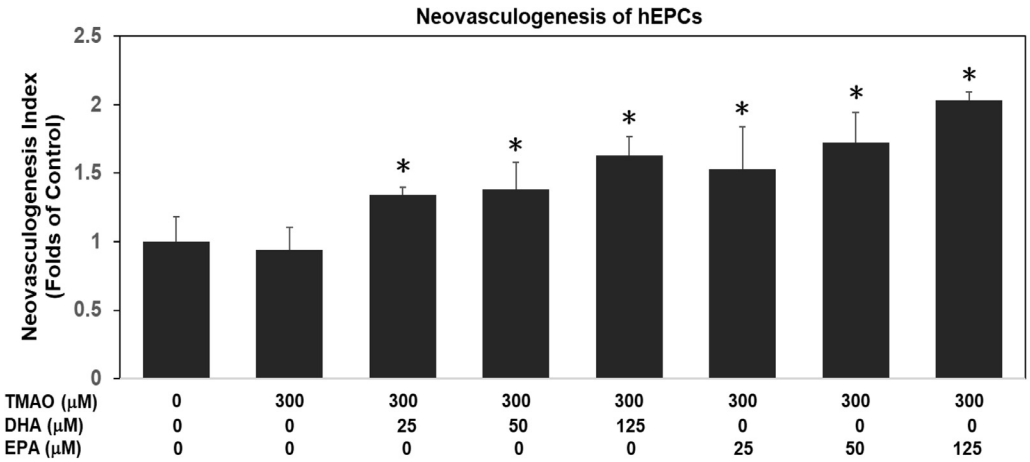
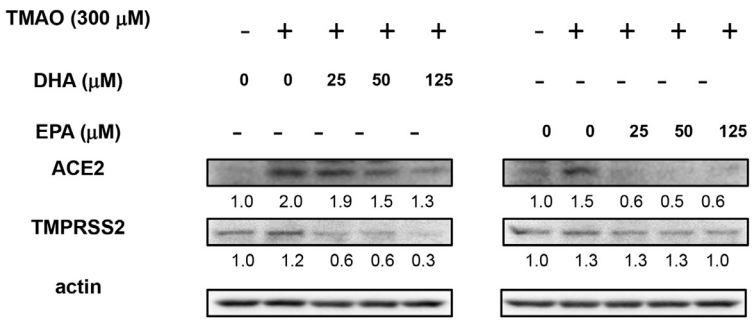


Fig. 1. N-3 PUFAs effectively block the entry of SARS-CoV-2 and the expression of ACE2 and TMPRSS2 proteins in hEPCs upon TMAO stimulation (A)Characterization of specific cell surface markers in hEPCs by flow cytometry analysis. hEPCs were cultured with TMAO (0 and 300 μM) in the presence of DHA and EPA (at concentrations of 0, 25, 50, and 125 μM, respectively) for 24 h. (B) Measurement of entry efficacy was performed by using SARS-CoV-2 plasmid (nCoV-S-Luc) infection as described in Materials and Methods. Experiments were carried out in triplicate. The values are presented as mean ± standard deviation (SD) in each subgroup. An asterisk represented a statistical difference in comparison with untreated control subgroup ($P<.05$). A pound sign represented a statistical difference in comparison with TMAO (300 μM) -treated subgroup ($P<.05$). (C) At the end of SARS-CoV-2 plasmid transfection, photomicrographs of cell morphology were also documented by using inverted phase-contrast microscope (300X). In the lower right corner of each images, a scale bar represented 30 μM (D) Neovasculogenesis levels of SARS-CoV-2 plasmid infected hEPCs were measured at 6 h time point. A detailed neovasculogenesis procedure was described in Materials and Methods Section. An asterisk represented a statistical difference in comparison with TMAO-treated alone subgroup ($P<.05$). (E) Measurement of cytoplasmic proteins including ACE2, TMPRSS2 and actin was performed by using Western Blotting analysis as described in Materials and Methods. The integrated densities of each protein (ACE2 and TMPRSS2) were adjusted with the corresponding internal control protein (actin) and shown in the bottom row.







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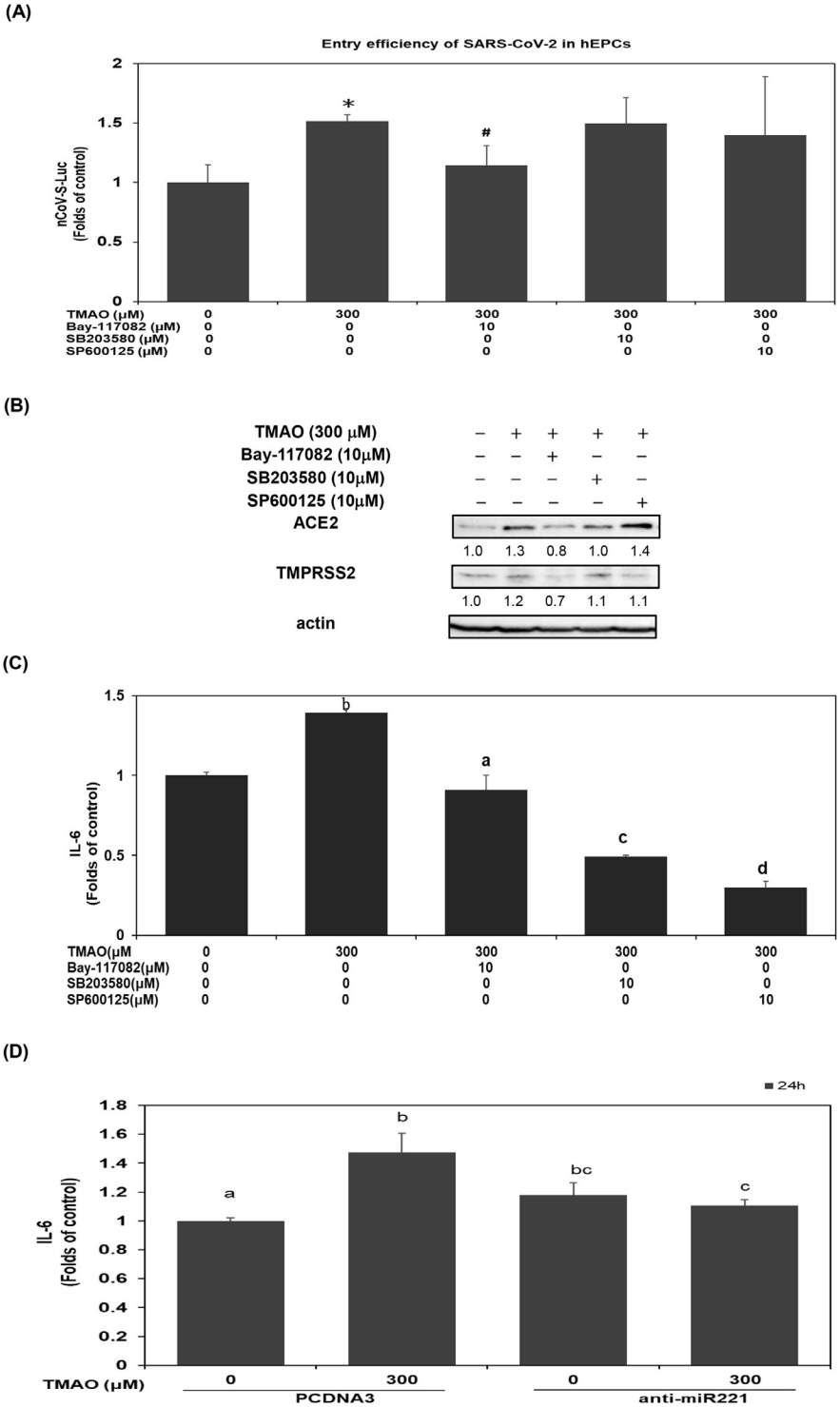

Fig. 2. TMAO mediated the infection of hEPCs by SARS-CoV-2 and the expression of IL-6 protein through modulation of cellular signaling pathways and microRNA-221 level. hEPCs were cultured with TMAO (0 and 300 μM) in the presence or absence of Bay-117082, SB203580, or SP600125 (at a concentration of 10 μM) for 24 h. (A) Measurement of entry efficacy was performed by using SARS-CoV-2 plasmid (nCoV-S-Luc) infection as described in Materials and Methods. Experiments were carried out in triplicate. The values are presented as mean ± SD in each subgroup. An asterisk represented a statistical difference in comparison with untreated control subgroup ($P<.05$). A pound sign represented a statistical difference in comparison with TMAO (300 μM)-treated subgroup ($P<.05$). (B) Measurement of cytoplasmic proteins including ACE2, TMPRSS2, and actin was performed by using Western Blotting analysis as described in Materials and Methods. The integrated densities of each protein (ACE2 and TMPRSS2) were adjusted with the corresponding internal control proteins (actin) and shown in the bottom row. Measurement of IL-6 protein (C) and miR-221 (F) and was performed by using ELISA assay and qPCR analysis, respectively, as described in Materials and Methods. (D, E) hEPCs were cultured with TMAO (0 and 300 μM) in the presence or absence of anti-sense plasmid against miR-221 (anti-miR-221) for 24 h. Measurement of IL-6 protein (D) and miR-221 (E) was performed as described in Materials and Methods. Different letters represent statistical differences among different subgroups ($P<.05$).

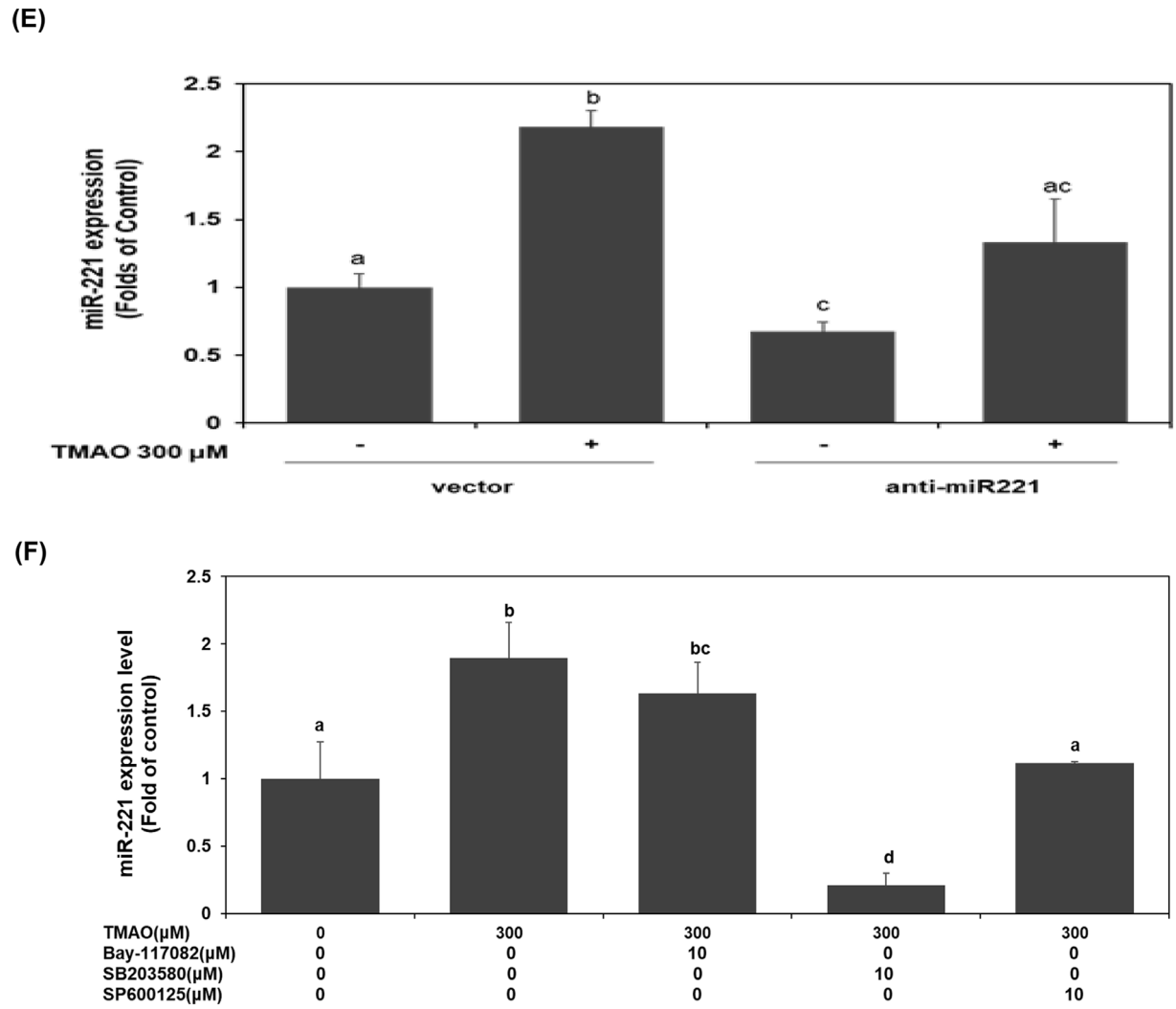

Fig. 2. Continued

of key regulatory proteins. As shown in Figure 2A, treatment of Bay-117082 (a specific inhibitor of NF-κB) significantly blocked the entry SARS-CoV-2 into hEPCs upon TMAO stimulation. However, treatment of SB203580 (a specific inhibitor of MAPK/p38) and SP600125 (a specific inhibitor of MAPK/JNK) couldn't inhibit TMAO-mediated entry of SARS-CoV-2 into hEPCs. These findings suggested that NF-κB signaling pathway plays a crucial role in determining TMAO-mediated entry of SARS-CoV-2 into hEPCs. Therefore, we further examined the effects of these inhibitors on the expression of key regulatory proteins in hEPCs upon TMAO stimulation. As shown in Figure 2B, treatment of Bay-117082 effectively inhibited the expression of ACE2 and TMPRSS2 proteins in hEPCs upon TMAO stimulation. These results suggested that NF-κB signaling pathway might play important roles in TMAO-mediated entry of SARS-CoV-2 into hEPCs and the expression of key regulatory proteins including ACE2 and TMPRSS2.

It's well-known that TMAO is one of factors that induce inflammatory response. Therefore, we further examined whether these signaling pathways (NF-κB, MAPK/p38 and JNK) play important roles in TMAO-mediated expression of IL-6 protein in hEPCs. As shown in Figure 2C, treatment of SB203580 and SP600125 could significantly inhibit the expression of IL-6 in hEPCs upon TMAO stimulation ($P<.05$). Our results also showed that treatment of Bay-117082 moderately reduced TMAO-mediated expression of IL-6 in hEPCs. These results suggested that TMAO might induce the expression of IL-6 protein mainly through the activation of MAPK/p38 and JNK signaling cascades in hEPCs (Fig. 2C).

Our previous study indicated that miRNA-221 (miR-221) played an important role in hEPCs [27]. Thus, we further examined the role of miR-221 in the regulation of IL-6 production in hEPCs. As shown in Figure 2D, treatment of anti-sense plasmid against miR-221 (anti-miR-221) effectively reduced TMAO-mediated expression IL-6 in hEPCs ($P<.05$). These results suggested that miR-221 played an important role in TMAO-mediated expression IL-6 in hEPCs.

To study the additional roles and actions, we further examined whether TMAO could modulate the expression of miR-221 in hEPCs. As shown in Figure 2E, our results suggested that treatment of TMAO significantly induced the expression of miR-221 in hEPCs ($P<.05$). Treatment of anti-sense plasmid against miR-221 (anti-miR-221) effectively reduced TMAO-mediated expression miR-221 in hEPCs ($P<.05$).

To study the underlying mechanisms, we further investigated that whether TMAO-mediated expression of miR-221 was through the modulation of MAPK/p38 and JNK signaling pathways in hEPCs. As shown in Figure 2F, treatment of SB203580 and SP600125 could significantly inhibit the expression of miR-221 in hEPCs upon

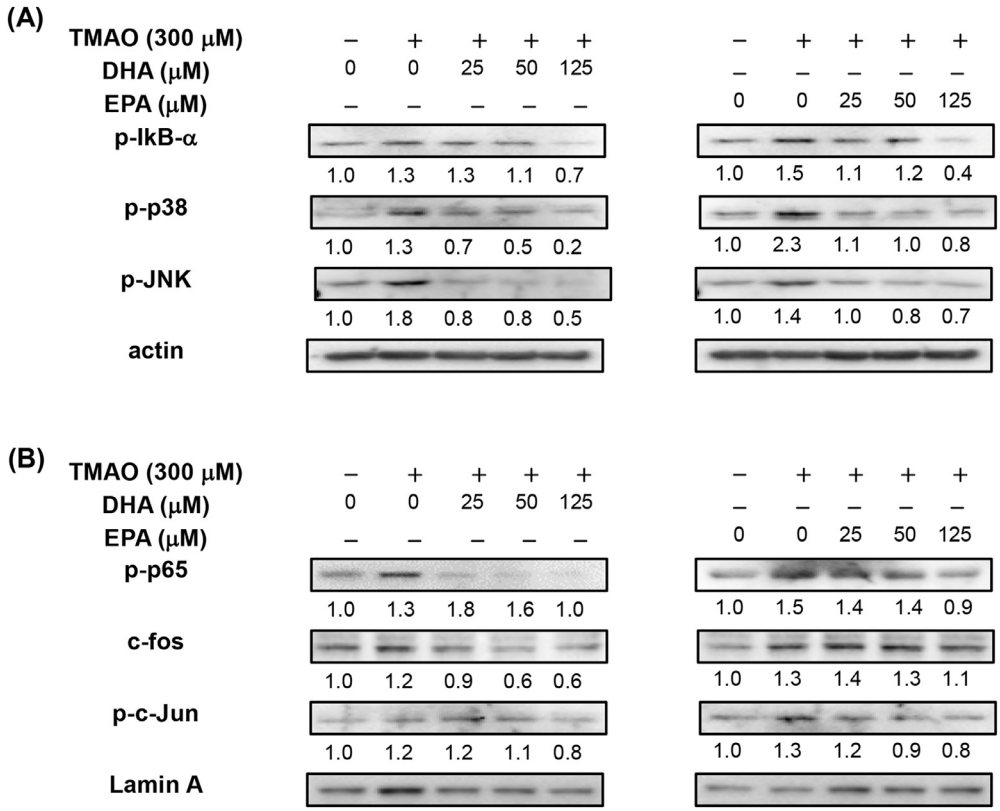

Fig. 3. N-3 PUFAs effectively blocked TMAO-mediated activation of signaling pathways in hEPCs hEPCs were cultured with TMAO (0 and 300 μM) in the presence or absence of DHA and EPA (at concentrations of 0, 25, 50, and 125 μM) for 24 h. Measurement of (A) cytoplasmic proteins (p-IκB-α, p-p38, p-JNK, and actin) and (B) nuclear proteins (p-p65, c-fos, p-c-Jun, and lamin A) was performed by using Western Blotting analysis as described in Materials and Methods. The integrated densities of each cytoplasmic proteins (p-IκB-α, p-p38, p-JNK) and nuclear proteins (p-p65, c-fos, p-c-Jun) were adjusted with the corresponding control proteins actin and lamin A, respectively. The quantification values were shown in the bottom row.

TMAO stimulation ($P<.05$). However, treatment of Bay-117082 couldn't inhibit TMAO-mediated expression of miR-221 in hEPCs. These results indicated that MAPK/p38 and JNK signaling pathways play pivotal roles in TMAO-mediated expression of miR-221 in hEPCs (Fig. 2F).

In conclusion, these findings indicated that MAPK/p38 and JNK signaling pathways played central roles and might regulate TMAO-mediated expression of IL-6 through epigenetic regulation of miR-221 level in hEPCs. However, NF-κB signaling pathway was involved in TMAO-mediated expression of IL-6 through a miR-221-independent pattern in hEPCs.

3.3. N-3 PUFAs effectively blocked TMAO-mediated activation of signaling pathways in hEPCs

Our findings already demonstrated the molecular mechanisms of action (Fig. 2). Therefore, we further investigated whether TMAO could modulate the activation of these related signaling pathways in hEPCs. As shown in Figure 3A, TMAO effectively induced the phosphorylation of I-κB, p38, and JNK proteins in hEPCs. Moreover, TMAO effectively increased nuclear levels of NF-κB/p-p65 (Rel-A), c-fos and phosphorylated c-Jun (p-c-Jun) proteins (Fig. 3B). These results suggested that TMAO could activate NF-κB, MAPK/p38, and MAPK/JNK signaling pathways in hEPCs.

Our results (Fig. 1) already demonstrated that N-3 PUFAs could block TMAO-mediated entry of SARS-CoV-2 into hEPCs and the expression of ACE2 and TMPRSS2. Therefore, we further investigated whether DHA and EPA could modulate the activation of these key signaling pathways in hEPCs. As shown in Figure 3A and B, N-3 PUFAs such as DHA and EPA could significantly inactivate the NF-κB signaling pathway by reducing the phosphorylation level of IκB-α protein and nuclear translocalization of NF-κB/p-p65 transcription factor in hEPCs. Interestingly, DHA and EPA could also significantly inhibit the phosphorylation levels of p38 and JNK signaling proteins (Fig. 3A) as well as the nuclear levels of c-fos and p-c-Jun proteins in hEPCs upon TMAO stimulation (Fig. 3B). These results indicated that N-3 PUFAs such as DHA and EPA could significantly block TMAO-mediated entry of SARS-CoV-2 into hEPCs and the expression of ACE2 and TMPRSS2, in part, through an inactivation of NF-κB signaling pathway. Moreover, DHA and EPA could inhibit the activation of MAPK/p38 and JNK signaling cascades in hEPCs.

3.4. N-3 PUFAs significantly inhibit TMAO-mediated expression of IL-6 protein through downregulation of miR-221 in hEPCs

Our results already demonstrated that N-3 PUFAs such as DHA and EPA could inhibit TMAO-mediated activation of NF-κB, MAPK/p38 and JNK signaling pathways in hEPCs (Fig. 3). Our results also indicated that the activation of MAPK/p38 and JNK signaling pathways played crucial roles in the TMAO-mediated expression of miR-221 and IL-6 in hEPCs (Fig. 2C and F). Therefore, we further investigated whether N-3 PUFAs DHA and EPA could modulate the expression of miR-221 and IL-6 in hEPCs upon TMAO stimulation. As shown in Figure 4A, TMAO significantly induced the expression of miR-221 in hEPCs ($P<.05$). Treatment of N-3 PUFAs such as DHA and EPA could significantly inhibit TMAO-mediated expression of miR-221 in hEPCs ($P<.05$). DHA and EPA (at a dosage of 125 μM) inhibited the expression of miR-221 up to 85% and 75%

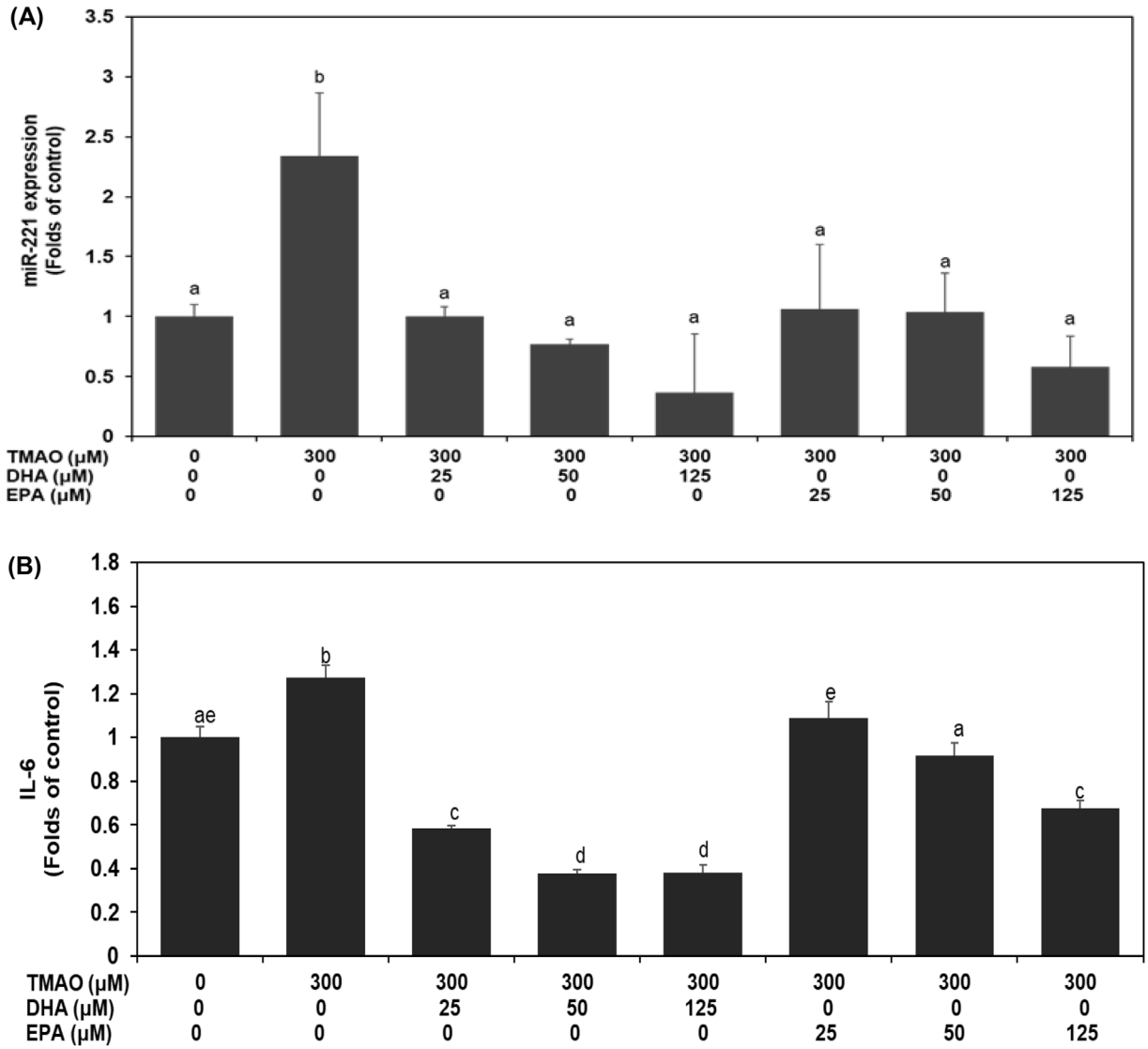

Fig. 4. N-3 PUFAs significantly inhibit TMAO-mediated expression of IL-6 protein through downregulation of miR-221 in hEPCs hEPCs were cultured with TMAO (0 and 300 μM) in the presence or absence of DHA and EPA (at concentrations of 0, 25, 50, and 125 μM) for 24 h. Measurement of miR-221 (A) and IL-6 protein (B) was performed by using qPCR analysis and ELISA assay, respectively, as described in Materials and Methods. Different letters represent statistical differences among different subgroups ($P<.05$).

in comparison with TMAO-treated subgroup ($P<.05$). These results suggested that DHA and EPA might inhibit TMAO-mediated expression of miR-221 through the inactivation of MAPK/p38 and JNK signaling pathways in hEPCs. Therefore, we further examined whether DHA and EPA could modulate TMAO-mediated expression of IL-6 in hEPCs. As shown in Figure 4B, our results further demonstrated that DHA and EPA significantly inhibited TMAO-mediated expression of IL-6 in hEPCs ($P<.05$) (Fig. 4B). These findings suggested that DHA and EPA could inhibit TMAO-mediated expression of IL-6 through an inactivation of the MAPK/p38/JNK signaling pathways and epigenetic downregulation of miR-221 in hEPCs.

4. Discussion

Many recent COVID-19 studies focus on the entry of SARS-CoV-2 and its interaction with the ACE2/TMPRSS2 complex [6]. Infection of SARS-CoV-2 causes cytokine storm, inflammation and dysfunction of biological systems in human [48,49]. TMAO, an important gut-microbe dependent metabolite, is one of factors induce inflammation and involves in inflammatory response and inflammation-related immunity [50,51]. It is well known that intake of healthy diets such as Mediterranean diet could benefit from a reduced risk of CVD [52]. Mediterranean diet is featured with high intake of plant foods and moderate consumption of fish and N-3 PUFAs such as DHA and EPA [52]. Many studies demonstrated that DHA and EPA obtain anti-inflammation effects [53,54]. A recent study also indicated that Mediterranean diet might have strong impact on gut microbiota and associated metabolome [55].

In this study, our results demonstrated that TMAO could enhance the infection of hEPCs by SARS-CoV-2 through an increased expression of key regulatory proteins including ACE2 and TMPRSS2 (Fig. 1). N-3 PUFAs such as DHA and EPA have been well-known as their anti-inflammatory functions in several studies [54,56]. Therefore, we further examined whether DHA and EPA could also act as anti-infection agents against SARS-CoV-2 in hEPCs. Our results further demonstrated that DHA and EPA could interfere the entry of SARS-CoV-2 into hEPCs, in part, through a downregulation of ACE2 and TMPRSS2 proteins (Fig. 1). Previous studies showed anti-inflammatory functions of DHA, EPA and their derived mediators by reducing inflammatory biomarkers *in vitro* and *in vivo* [57,58].

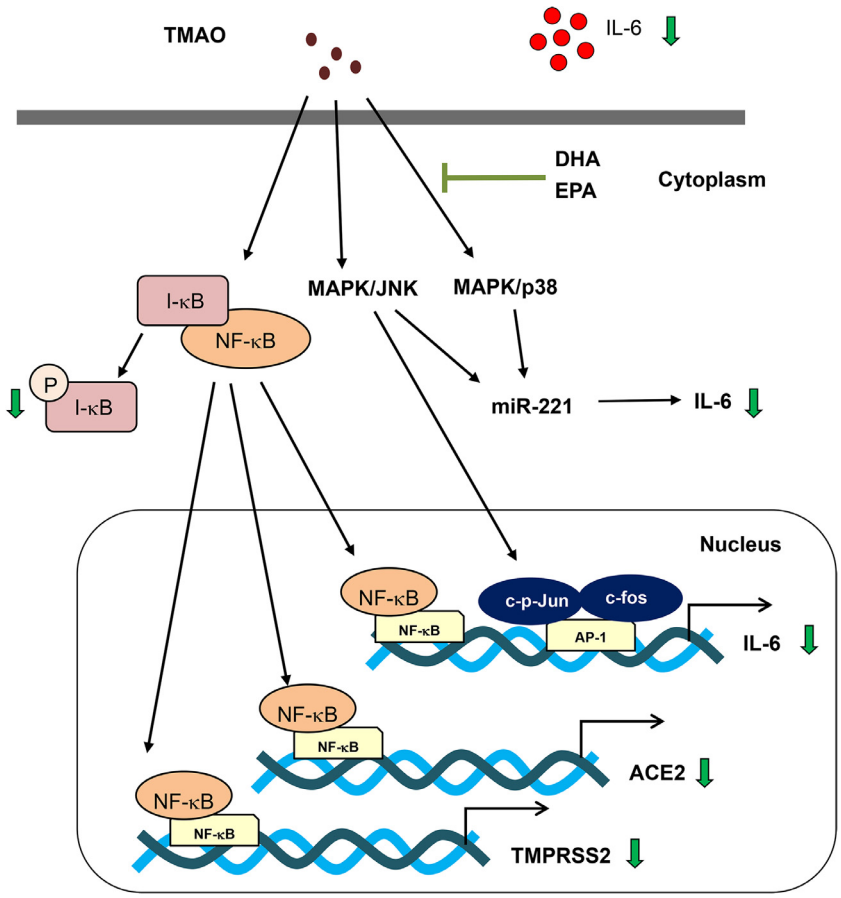

Fig. 5. Proposed mechanisms of signaling pathways regulated by N-3 PUFAs in hEPCs upon TMAO stimulation. Green arrows indicate decreases in expression level.

Our results demonstrated that a novel role of DHA and EPA in the interference of SARS-CoV-2 infection in hEPCs. DHA and EPA could act as effective mediators to block the action of SARS-CoV-2 by suppression of ACE2/ TMPRSS2 axis.

We also demonstrated that the molecular mechanisms of action of TMAO -mediated increment of SARS-CoV-2 infection in hEPCs and the increased expression of ACE2 and TMPRSS2 proteins were through an activation of NF-κB signaling pathway (Fig. 2A and B). Our results further indicated specific inhibitors of MAPK/p38/ JNK signaling pathways couldn't suppress the ACE2 and TMPRSS2 proteins. These findings suggested a crucial role of NF-κB signaling pathway in determining the expression of key regulators during SARS-CoV-2 infection in hEPCs. Our results were also consistent with previous findings and demonstrated a putative binding site of NF-κB in the promoter region of *ACE2* [23].

Interestingly, TMAO could induce the expression of IL-6 cytokine production and the upregulation of miR-221 through the activation of MAPK/p38 /JNK signaling pathways in hEPCs (Fig. 2C andF). These results indicated that MAPK/p38 and MAPK/JNK signaling pathways played important roles in determining IL-6 and miR-221 expression in hEPCs although a mild association between the activation of NF-κB signaling pathway and IL-6 production was found in this study (Fig. 2C). Our results further demonstrated that treatment of anti-miR-221 plasmid could suppress the expression of IL-6 protein in hEPCs upon TMAO stimulation (Fig. 2D). These results demonstrated that TMAO might modulate the expression of IL-6 protein through an epigenetic regulation of miR-221 level.

Our previous studies indicated that N-3 PUFAs such as DHA and EPA might act as anti-inflammatory agents and reduce the expression of miR-221 in hEPCs [27]. To study the molecular mechanisms of action, we investigated the effects of DHA and EPA on the activation of these signaling cascades in hEPCs. As shown in Figure 3A-B, DHA and EPA effectively inhibited the activation of NF-κB, MAPK/p38 and MAPK/JNK signaling cascades through decreased levels of phosphorylated I-κB, p38, and JNK proteins. Moreover, DHA and EPA effectively suppressed nuclear levels of NF-κB /p-p65 (RelA), c-fos, and p-c-Jun transcription factors in hEPCs. These findings could verify the actions of DHA and EPA on the prevention of SARS-CoV-2 infection by an inactivation of NF-κB signaling pathway in hEPCs.

We further examined whether DHA and EPA could modulate the expression of miR-221 and IL-6 protein in hEPCs upon TMAO stimulation in this study. As shown in Figure 4, DHA and EPA could significantly inhibit the expression of miR-221 and IL-6 protein in hEPCs. These findings suggested that N-3 PUFAs such as DHA and EPA could act as preventive agents to block TMAO-mediated expression of ACE2/TMPRSS2 complexes through the inactivation of NF-κB signaling pathway and reduce the infection of hEPCs by SARS-CoV-2. Moreover, DHA and EPA act as anti-inflammatory agents to suppress the expression of IL-6 production through the inactivation of MAPK/p38 and MAPK/JNK signaling pathways and downregulation of miR-221 in hEPCs. The proposed mechanism is described in Figure 5.

In conclusion, our results demonstrated that TMAO, a gut metabolite of carnitine, might enhance the infection of hEPCs by SARS-CoV-2 and the expression of IL-6 through an activation of NF-κB, MAPK/p38, and JNK signaling pathway and an epigenetic upregulation of miR-221. N-3 PUFAs such as DHA and EPA could

significantly inhibit the infection of hEPCs by SARS-CoV-2 and inflammation through a downregulation of IL-6 cytokine production upon TMAO stimulation.

Author contributions

The authors have no financial conflicts of interest in this work. This manuscript has not been simultaneously submitted to other journals. All authors meet criteria for authorship and that the authors will sign a statement attesting authorship, disclosing all potential conflicts of interest, and releasing the copyright should the manuscript be acceptable for publication.

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Declaration of competing interests

The authors have no financial conflicts of interest in this work.

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