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Docosahexaenoic acid prevented tumor necrosis factor alpha-induced endothelial dysfunction and senescence ☆

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ABSTRACT

We investigated how docosahexaenoic acid (DHA) regulated tumor necrosis factor-alpha (TNF-α)-induced senescence and dysfunction in endothelial cells (EC). We used RT-PCR to examine the expression of several genes related to senescence and dysfunction in EC. TNF-α-induced p21 protein levels were investigated by Western blot (WB) and fluorescence antibody techniques. TNF-α induced the senescence marker β-galactosidase and the expression of several senescence and endothelial dysfunction-related genes, e.g., *CDKN1A*, *SHC1* and *GLB1*. DHA attenuated TNF-α-induced senescence-related gene expression and p21 protein expression. DHA attenuated TNF-α-induced gene expression related to dysfunction of EC, such as plasminogen activator inhibitor 1 (*SERPINE1*), lectin-like oxidized low-density lipoprotein receptor-1 (*OLR1*), thromboxane A2 receptor (*TXA2R*) and p38 MAPK (*MAPK14*). DHA reversed the TNF-α-mediated reduction of endothelial nitric oxide synthase (*NOS3*) gene expression. TNF-α-mediated upregulation of these genes was inhibited by allopurinol and apocynin. These results indicated that DHA regulated the expression of several genes that are associated with senescence and dysfunction of EC.
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1. Introduction

Aging is an important risk factor for cardiovascular disease [1]. Specifically, vascular senescence may lead to endothelial dysfunction and subsequently the onset of atherosclerosis and thrombosis [2]. Reports have demonstrated that the circulating level of tumor necrosis factor-alpha (TNF-α) is increased in the elderly [3]. Furthermore, TNF-α production is enhanced in the aortic wall [4] and coronary arteries of aged rodents [5]. Nitric oxide (NO), which is produced by endothelial nitric oxide synthase (eNOS), has been implicated as the major mediator of endothelium-dependent relaxation. However, TNF-α significantly reduces eNOS expression in endothelial cells [6,7]. Therefore, dysregulation of TNF-α expression may be associated with vascular aging [8]. Furthermore, TNF-α induces the generation of reactive oxygen species (ROS) by activation of NADPH oxidase in endothelial cells. Excessive generation of ROS may be a fundamental factor in endothelial senescence [9].

In vascular endothelial cells, lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1) was identified as the receptor for oxidized LDL [10]. *In vivo* studies indicated that endothelial cells, including early atherosclerotic lesions that accumulated in advanced atherosclerotic plaques, express LOX-1 [11,12]. Stimulation of LOX-1 mediated by oxidized LDL strongly induces production of vascular endothelial growth factor (VEGF). In addition, activation of LOX-1 promotes cell adhesion molecules in endothelial cells, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) [13]. In the elderly, it is possible that cell adhesion could be mediated by LOX-1 [14]. Moreover, a previous study demonstrated that stimulation with a thromboxane A2 (TXA2) receptor (TXA2R) agonist induced the expression of adhesion molecules by human umbilical vein endothelial cells [15]. Enhanced production of TXA2 and the associated change in redox regulation precede the aggravation of endothelial cells injury [16]. Moreover, TNF-α induces expression of plasminogen activator inhibitor 1 (PAI-1) in cultured human endothelial cells [17]. These reports suggest that TNF-α induces LOX-1, PAI-1 and TXA2R expression and may lead to endothelial cell dysfunction.

Abbreviations: DHA, docosahexaenoic acid; ICAM-1, intercellular adhesion molecule-1; LOX1, lectin-like oxidized low-density lipoprotein receptor-1; TXA2R, thromboxane A2 receptor; TNF-α, tumor necrosis factor-alpha; PAI-1, plasminogen activator inhibitor-1; eNOS, endothelial nitric oxide synthase; ROS, reactive oxygen species; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor

☆ Gene approved symbol: eNOS (*NOS3*), LOX-1 (*OLR1*), PAI-1 (*SERPINE1*), p21 (*CDKN1A*), p66 (*SHC1*), p 38 MAPK (*MAPK14*).

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With regard to endothelial cell viability, cell cycle regulatory proteins p16, p21 and p53 are associated with senescence. p21 protein is a cyclin-dependent kinase (CDK) inhibitor, and it regulates p53 transcription and induces senescence [18]. Moreover, p16 similarly inhibits the cell cycle in human senescent cells [19]. Likewise, p66 reduces lifespan [20] and regulates intracellular redox balance by upregulating ROS concentration [21]. In fact, the lifespan of p66 (-/-) mice was prolonged and this was attributed to an increased resistance to oxidative stress [22]. In addition, senescence-associated β-galactosidase (GLB1) is widely recognized as an enzymatic biomarker of senescent cells [23]. Fish oil is the best source of docosahexaenoic acid (DHA), and it can prevent cardiovascular disease [24,25]. It is believed that DHA inhibits TNF-α-induced VCAM-1 expression with abrogation of the NF-κB signaling pathway and AP-1 activation [26,27]. In fact, we demonstrated that DHA reduces TNF-α-induced LOX-1 expression, and DHA attenuated several events related to redox regulation in THP-1 cells [28].

Aging-related endothelial dysfunction might contribute to various pathologies. Importantly, several reports have indicated that DHA has a protective effect against endothelial dysfunction. However, the mechanism by which DHA reduces cell senescence is not known. The purpose of this study was to investigate the protective effects of DHA for TNF-α-stimulated senescence in human endothelial cells. Here, we examined TNF-α-induced senescence-related gene expression and endothelial dysfunction as well as involvement of ROS, with an emphasis on senescence-related gene expression and endothelial dysfunction.

2. Materials and methods

2.1. Materials

TRIzol reagent, DNase, fetal bovine serum (FBS), and Superscript III were purchased from Life Technologies Japan, Inc. (Tokyo, Japan). Bradford reagent was purchased from Bio-Rad (Richmond, CA, USA). Anti-p21 antibodies were purchased from Cell Signaling Technology (Tokyo, Japan) and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). DHA, allopurinol and apocynin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Human TNFα was purchased from Roche Applied Science (Mannheim, Germany). All other reagents were purchased from Sigma-Aldrich unless otherwise indicated.

2.2. Cell culture

The human endothelial cell line ISO-HAS (Tohoku University, Sendai, Japan) was cultured in 50% Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS, Life Technologies, Tokyo Japan) and 50% conditioned medium from the angiosarcoma cell line ISO-1 [29]. The ISO-HAS cell line was established from tumor tissue from a human hemangiosarcoma. ISO-HAS cells were shown to constitutively express von Willebrand factor (vWF), CD31, KDR, Flt-1 and VEGF [30,31]. ISO-HAS cells were seeded at a ratio of 1:1 in culture flasks (75 cm^2, Becton Dickinson, Bedford, MA) and grown in DMEM/ISO-1 at 37 °C under 5% CO_2 and in a humidified atmosphere. The culture medium was periodically renewed until the cells reached 80-90% confluence, at which point they were treated with 0.25% trypsin (Sigma-Aldrich, Inc. Tokyo, Japan).

2.3. Treatment of cultures

ISO-HAS cells were plated on 100-mm culture dishes, 24-well or 96-well plates (Nunc, Thermo Fisher Scientific, Osaka Japan) at an initial density of 20×10^4 cells per cm^2 and were grown in DMEM/ISO-1 containing 10% FBS until confluence was reached. One day later, the ISO-HAS cells were exposed to TNF-α (1–10 ng/mL) and/or DHA (10–50 μM); 10% DMEM/ISO-1 lacking TNF-α, DHA and resveratrol served as a control.

2.4. Immunofluorescent analysis

ISO-HAS cells (20×10^4 cells per cm^2) were plated on culture slides (Nunc, Thermo Fisher Scientific) and were rinsed twice with PBS before being fixed in 4% paraformaldehyde at room temperature for 15 min. The fixed cells were washed three times with PBS. The Alexa Fluor 488-conjugated antibody against p21 (Cell Signaling, #5487, Tokyo, Japan, 1:50) was applied for 16 h at 4 °C. After being washed an additional three times with PBS, images were captured using a fluorescent microscope (BZ-8000) and analysis was carried out with the BZ-8100 Dynamic Cell Count image analysis program (Keyence, Osaka, Japan). Specifically, for quantification of results, fluorescence intensity was measured under the same exposure conditions in the same area with the BZ-8000 fluorescent microscope. Experiments shown are representative of those that were independently repeated at least five times.

2.5. Senescence-associated β-galactosidase staining

Senescence-associated (SA) β-galactosidase (gal) staining was performed using a cellular senescence cell histochemical stain kit (SA β-gal staining kit, Sigma, CS0030) according to the manufacturer's protocol [23]. Briefly, ISO-HAS cells were incubated with 10 ng/mL TNF-α for 18 h at 37 °C. ISO-HAS cells were then washed twice with PBS, fixed with 4% paraformaldehyde and SA-β-gal staining was examined using a microscope (BZ-8000). Experiments shown are representative of those that were independently repeated at least five times.

2.6. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extraction was performed with the Trizol reagent (Life Technologies) according to the manufacturer's instructions. RNA samples were treated with DNase I (Life Technologies) at room temperature for 15 min to remove genomic DNA. One microgram of total RNA was reverse transcribed by use of Superscript III (Life Technologies). RT-PCR was performed for p21 (*CDKN1A*), p66 (*SHC1*), *GLB1*, eNOS (*NOS3*), LOX-1 (*OLR1*), PAI-1 (*SERPINE1*), *TXA2R and p38 MAPK (MAPK14).* PCR reactions were incubated initially at 94 °C for 10 min, 55 °C for 2 min and 72 °C for 3 min for 35 cycles using the GoTaq-Green Master Mix (Promega, Tokyo, Japan) with a PCR System 9700 (Life Technology). The primer sequences were obtained from GenBank and designed using the primer design software Primer Express (Applied Biosystems) (Table 1). PCR gene products were examined by electrophoresis on a 2% NuSieve agarose (3:1) gel (FMC Products, Rockland, ME) and visualized with UV illumination after ethidium bromide staining. The amount of mRNA expressed was calculated relative to 18S ribosomal RNA. The PCR product was analyzed by electrophoresis using the same gel. Both quantitation and reproducibility were maintained at high levels.

2.7. Western blot

ISO-HAS cells were lysed in RIPA buffer (Takara Bio Inc., Tokyo Japan) containing a complete protease inhibitor cocktail (Roche Diagnostics). Protein concentrations were measured using a Bradford reagent protein assay kit (Bio-Rad, Richmond, CA) with bovine serum albumin (BSA) as a standard. Cell lysates (10 to

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