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Docosahexaenoic Acid Inhibits Cytokine Expression by Reducing Reactive Oxygen Species in Pancreatic Stellate Cells

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Pancreatic stellate cells (PSCs) are activated by inflammatory stimuli, such as TNF-α or viral infection. Activated PSCs play a crucial role in the development of chronic pancreatitis. Polyinosinic-polycytidylic acid (poly (I:C)) is structurally similar to double-stranded RNA and mimics viral infection. Docosahexaenoic acid (DHA) exhibits anti-inflammatory activity. It inhibited fibrotic mediators and reduced NF-κB activity in the pancreas of mice with chronic pancreatitis. The present study aimed to investigate whether DHA could suppress cytokine expression in PSCs isolated from rats. Cells were pre-treated with DHA or the antioxidant N-acetylcysteine (NAC) and stimulated with TNF-α or poly (I:C). Treatment with TNF-α or poly (I:C) increased the expression of monocyte chemoattractant protein 1 (MCP-1) and chemokine C-X3-C motif ligand 1 (CX3CL1), which are known chemoattractants, and enhanced intracellular and mitochondrial reactive oxygen species (ROS) production and NF-κB activity, but reduced mitochondrial membrane potential (MMP). Increased intracellular and mitochondrial ROS accumulation, cytokine expression, MMP disruption, and NF-κB activation were all prevented by DHA in TNF-α- or poly (I:C)-treated PSCs. NAC suppressed TNF-α- or poly (I:C)-induced expression of MCP-1 and CX3CL1. In conclusion, DHA inhibits poly (I:C)- or TNF-α-induced cytokine expression and NF-κB activation by reducing intracellular and mitochondrial ROS in PSCs. Consumption of DHA-rich foods may be beneficial in preventing chronic pancreatitis by inhibiting cytokine expression in PSCs.

Key Words Cytokines, Docosahexaenoic acid, Pancreatic stellate cells, Reactive oxygen species

INTRODUCTION

Chronic pancreatitis is a multifactorial inflammatory syndrome in which repetitive inflammation leads to fibrotic tissue replacement. It results in chronic pain, exocrine and endocrine pancreatic insufficiency, reduced quality of life, and a shorter life expectancy [1]. This disease cannot be cured and is the most lethal disease of the pancreas [1,2]. Since prolonged inflammation in the pancreas is a critical factor for the development of chronic pancreatitis, it is essential to control pancreatic inflammation at an early stage to prevent chronic pancreatitis.

Pancreatic stellate cells (PSCs) exhibit quiescent and activated phenotypes. When quiescent PSCs are exposed to stimuli, such as TNF-α, they become activated myofibroblast-like cells. Chronic activation of PSCs leads to the development of chronic pancreatitis [3]. During chronic pancreatitis, the expression of monocyte chemoattractant protein 1 (MCP-1) and chemokine C-X3-C motif ligand 1 (CX3CL1), also known as fractalkine, is upregulated [4-6]. MCP-1 is an inflammatory chemokine that regulates monocytes and T lymphocytes and contributes to the pathogenesis of mononuclear infiltration [4]. CX3CL1 is a large cytokine composed of 373 amino acids. Soluble CX3CL1 potently attracts T cells and monocytes, whereas the membrane-bound chemokine promotes strong adhesion of leukocytes to activated endothelial cells. The unique functional and structural characteristics of CX3CL1 facilitate its participation in inflammation-related diseases, including atherosclerosis, rheumatoid arthritis, human immunodeficiency virus infection, and several other disorders [7]. Therefore, better understanding the roles of these chemokines in the pathogenesis of chronic pancreatitis may lead to new therapies directed towards MCP-1 and CX3CL1 regulation.

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Persistent activation of PSCs by cytokines, such as TNF-α, during acute pancreatitis may be involved in the progression from acute pancreatitis to chronic pancreatic injury and fibrosis [8]. TNF-α induces the generation of reactive oxygen species (ROS) and NF-κB activation, leading to the expression of adhesion molecules in lung epithelial A549 cells [9]. In endothelial cells, binding of TNF-α to TNF receptor (TNFR) induces NF-κB activation [10]. TNF-α induces CX3CL1 expression in human umbilical vein endothelial cells through the regulation of the NF-κB pathway [11]. TNF-α increases MCP-1 expression in PSCs, which is mediated by activator protein-1 and mitogen-activated protein kinases [12].

There are various causes of chronic pancreatitis, including viral infection [13]. Polyinosinic-polycytidilic acid (poly (I:C)) is structurally similar to double-stranded RNA, which is present in some viruses. Therefore, it has been used to mimic viral infection [14]. Poly (I:C) recognized by Toll-like receptor 3 (TLR3) induces NF-κB activation and MCP-1 expression in PSCs [15]. Poly (I:C)/TLR3 triggers NF-κB activation and interleukin-6 (IL-6) production in astrocytes [16] and ROS-mediated NF-κB activation in macrophages [17]. Poly (I:C) induces CX3CL1 expression via activation of TLR3/NF-κB in glomerular endothelial cells [18]. These studies show the relationship between ROS and NF-κB activation or cytokine expression in cells exposed to TNF-α or poly (I:C).

Docosahexaenoic acid (DHA) is an omega-3 fatty acid. It can be found in cold oceanic fish oil and is synthesized from α-linolenic acid. The anti-inflammatory effects of DHA have been reported in various inflammatory diseases, including atherosclerosis and rheumatoid arthritis [19-21]. DHA downregulates the production of inflammatory cytokines by suppressing NF-κB nuclear translocation [22,23]. Previously, we have reported that DHA inhibits the expression of fibrotic mediators (α-smooth muscle actin and fibronectin) by reducing NF-κB expression in mouse pancreatic tissues with chronic pancreatitis [24]. DHA inhibits cerulein-induced acute pancreatitis in rats by reducing pancreatic edema, myeloperoxidase activity, levels of lipid peroxide and IL-6, and activation of NF-κB [25]. These studies show that the antioxidant activity of DHA may contribute to its inhibitory effect on pancreatic inflammation.

Since active PSCs play a critical role in the development of chronic pancreatitis by inducing inflammatory cytokines, it is essential to investigate whether DHA inhibits inflammatory cytokine expression in activated PSCs by reducing ROS and NF-κB activity in these cells.

The aim of the present study was to investigate the mechanism underlying the anti-inflammatory effect of DHA on TNF-α- or double-stranded RNA-induced inflammatory cytokine expression in PSCs to assess its potential as a preventive agent for inflammatory stimuli- and virus-induced chronic pancreatitis.

MATERIALS AND METHODS

Animals and reagents

Sprague- Dawley male rats (150 to 250 g) at 6 weeks of age were obtained from Orient Bio (Orient Bio Inc., Seongnam, Korea). Three animals per cage were housed under a 12/12 hours light/dark cycle and provided *ad libitum* access to food and water. After 1 week of acclimation period, the rats were anesthetized and used as pancreas donors. All experimental procedures were approved by the Institutional Review Board (IRB) at Institutional Animal Care and Use Committee of Yonsei University (No. IACUC-A-201508-405-02).

Gey's balanced salt solution (GBSS with NaCl 7 g/L), protease, penicillin-streptomycin antibiotics, FBS, TNF-α (SRP3177), poly (I:C), (P1530), DHA (D2534), Lipofectamine 2000, 5,6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-DCF-DA) (D6883), and mounting solution (HC08) were purchased from Sigma-Aldrich (St Louis, MO, USA). The density gradient medium, Nycodenz, was purchased from Nycomed Pharmas AS (Oslo, Norway). Collagenase P and deoxyribonuclease were purchased from Roche Diagnostics GmbH (Mannheim, Germany). Dulbecco's Modified Eagle Medium nutrient mix F-12 and T4 polynucleotide kinase were purchased from Gibco (Grand Island, NY, USA). MitoSOX (M36008) and MCP-1 enzyme-linked immunosorbent assay (ELISA) kit were obtained from Invitrogen (Carlsbad, CA, USA). CX3CL1 ELISA kit was purchased from Abcam (Cambridge, UK).

Isolation and culture of PSCs

PSCs were isolated from 6-week-old male Sprague Dawley rats (150 to 250 g). Briefly, the animals were sacrificed by carbon dioxide inhalation. The pancreas was dissected, removed, placed in a 50 mL glass beaker containing an ice-cold solution of 0.9% NaCl, trimmed of adipose and connective tissue as well as large blood vessels and moved into a biological safety cabinet. It was then transferred to a 60 mm plastic petri dish. Using an insulin syringe, the pancreas was injected with GBSS containing collagenase P (0.05%), protease (0.02%), and deoxyribonuclease (0.1%). The tissue was incubated at 37°C for 4 minutes in a high-speed shaking water bath (240 cycles/ minutes) and for 3 minutes in a low-speed shaking water bath (120 cycles/ minutes). The partially digested pancreatic tissue was finely minced using scissors and incubated again at 37°C for 7 minutes in a shaking water bath at 120 cycles/minutes. The digested tissue was passed through a 100 μm mesh and then centrifuged at 2,000 ×*g* for 5 minutes at 4°C. After the supernatant was carefully aspirated, the cell pellet was washed with a solution containing GBSS and NaCl along with 0.3% bovine serum albumin (BSA), and then centrifuged at 2,000 ×*g* for 5 minutes at 4°C. The supernatant was carefully removed via pipetting and the cell pellet was thoroughly resuspended in 9.5 mL of GBSS-NaCl solution containing 0.3% BSA and 8

mL of 28.7% Nycodenz solution. Six milliliters of GBSS-NaCl solution containing 0.3% BSA was taken in another centrifuge tube and the cell suspension was layered beneath using a 20 mL syringe, taking care not to disrupt the interface. The solution was then centrifuged at 1,400 ×*g* for 20 minutes at 4°C. The thin white band just above the interface was collected using a 5-mL transfer pipette without disturbing the density gradient layers. Cells were washed with GBSS-NaCl solution containing 0.3% BSA and centrifuged at 2,000 ×*g* for 5 minutes at 4°C [14,15]. The cells were counted (approximately 3 million/g pancreas) and cultured in Dulbecco's Modified Eagle Medium nutrient mix F-12 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37°C. PSCs were harvested between passages 2 and 4 after isolation for downstream analysis.

Cell culture and treatment

The cells were incubated in serum-free medium for 24 hours before treatment with TNF-α or poly (I:C). For poly (I:C) preparation, 10 μg poly (I:C) was mixed with 5 μL of Lipofectamine 2000 and 985 μL of serum-free medium and incubated for 15 minutes at 21°C to 23°C. TNF-α was dissolved in distilled water. DHA was dissolved in 0.5 M ethanol.

Experimental protocol

For the time-course experiments, to examine the expression of MCP-1 and CX3CL1, cells (1.25×10^4 cells/well) were plated in a 6-well plate and were stimulated with TNF-α (10 ng/mL) or poly (I:C) (1 mg/mL) for 3 hours (for determination of mRNA expression) and 24 hours (for determination of protein levels).

To determine the effect of DHA on the expression of MCP-1 and CX3CL1, cells (1.25×10^4 cells/well) were plated in a 6-well plate and pre-treated with DHA (10 or 50 μM) for 1 hour and stimulated with TNF-α (10 ng/mL) or poly (I:C) (1 mg/mL) for 1 hour (for determination of intracellular and mitochondrial ROS levels, mitochondrial membrane potential [MMP], and NF-κB activity), 3 hours (for determination of mRNA expression), and 24 hours (for determination of protein levels). The 1 hour-culture time for evaluating ROS levels and NF-κB activation was adapted from previous studies that used cells stimulated with TNF-α or poly (I:C) [9,10,16,17].

Real-time PCR (RT-PCR)

The gene expression levels of MCP-1 and CX3CL1 were assessed by real-time PCR. Total RNA was isolated using the TRI reagent (RNA/DNA/protein isolation reagent, Molecular Research Center, Cincinnati, OH, USA) and reverse-transcribed into cDNA using a random hexamer and M-MLV reverse transcriptase (Promega, Madison, WI, USA) at 23°C for 10 minutes, 42°C for 60 minutes, and 95°C for 5 minutes. The cDNA was incubated with SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan) containing 10 pg/mL of forward and reverse primers, and amplified using the Light Cycler PCR system (Roche Applied Sciences, Indianapolis, IN, USA). RT-PCR was performed using the following rat-specific primers for MCP-1: 5'-ACGTGCTGTCTCAGCCAGAT-3' (forward) and 5'-GTTCTCCAGCCGACTCAT TG-3' (reverse), CX3CL1: 5'-CACAAGATGACCTCGCCAAT-3' (forward) and 5'-GCTGTCTCGTCTCCAGGATG-3' (reverse) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-GCTCTCTGCTCCTCCCTGTT-3' (forward) and 5'-CACACCGACCTTCACCATCT-3' (reverse). For PCR amplification, the cDNA was amplified by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 45 seconds. During the first cycle, the 95°C step was extended to 3 minutes. GAPDH was amplified in the same reaction as the reference gene.

Measurement of intracellular ROS levels

Intracellular ROS levels were assessed using carboxy-DCF-DA. The cells were incubated with 5 μM carboxy-DCF-DA for 1 hour. Cells were washed twice and harvested with PBS and plated into black 96-well plates. The change in fluorescence was measured using a fluorometer (VICTOR 5 Wallac 1420 multi-label counter, Perkin Elmer Life and Analytical Sciences, Waltham, MA, USA) at excitation and emission wavelengths of 485 and 535 nm, respectively.

Measurement of mitochondrial ROS levels

MitoSOX was used to assess mitochondrial ROS levels. The cells were incubated with 2 μM MitoSOX for 1 hour, washed twice, harvested with PBS, and plated into black 96-well plates. The change in fluorescence was measured using a fluorometer (VICTOR 5 Wallac 1420 multi-label counter, Perkin Elmer Life and Analytical Sciences at excitation and emission wavelengths of 514 and 585 nm, respectively.

Fluorescence detection of MMP

Tetraethyl benzimidazolyl carbocyanine iodide (JC-1) is a novel cationic carbocyanine dye that accumulates in the mitochondria and can be used to measure MMP. For laser-scanning confocal microscopy, the cells (1.25×10^4 cells) were seeded on a glass slide and then treated with DHA (50 μM) for 1 hour prior to treatment with TNF-α (10 ng/mL) or poly (I:C) (1 μg/mL) for 1 hour. The cells were stained with JC-1 fluorescent dye, washed with PBS, and mounted on microscope slides with mounting solution. Images were obtained by confocal microscopy (Zeiss LSM 510; Carl Zeiss Inc., Thornwood, NY, USA) at an excitation wavelength of 514 nm and emission wavelength of 529 nm and overlaid using the LSM software.

Electrophoretic mobility shift assay

PSCs were rinsed with ice-cold PBS, harvested by trypsinization, and pelleted by centrifugation at 5,000 ×*g* for 5 minutes. Cells were then lysed in a buffer containing 10 mM HEPES,

10 mM KCl, 1.5 mM $MgCl_2$, 0.05% Nonidet P-40, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride, and centrifuged at 13,000 ×*g* for 10 minutes. The pellets were resuspended in nuclear extraction buffer containing 10 mM HEPES, 420 mM NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol, 0.2 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride, and clarified by centrifugation at 13,000 ×*g* for 10 minutes. The supernatants were collected and used as nuclear extracts. Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). An NF-κB gel shift oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3'; Promega, Madison, WI, USA) was labeled with [^{32}P] dATP (Amersham Biosciences, Piscataway, NJ, USA) using T4 polynucleotide kinase. The end-labeled probe was purified from unincorporated [^{32}P] dATP using a Bio-Rad purification column (Bio-Rad Laboratories) and recovered in Tris-EDTA (TE) buffer. Nuclear extracts (0.5 μg) were preincubated with the buffer containing 12% glycerol, 12 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM DTT, 25 mM KCl, 5 mM $MgCl_2$, and 0.04 μg/mL poly-deoxy-inosinic-deoxy-cytidylic acid for 30 minutes at 21°C to 23°C. The samples were subjected to electrophoretic separation at 4°C on a non-denaturing 5% acrylamide gel. The gel was dried at 80°C for 2 hours and exposed to a radiography film for 5 to 8 days at –80°C with intensifying screens.

ELISA

The levels of MCP-1 or CX3CL1 in the cell culture medium were determined using the MCP-1 ELISA kit or CX3CL1 ELISA kit, according to the manufacturer's instructions.

Statistical analysis

All data are expressed as the mean ± SE. All experiments were repeated three times. Number of each group was four. The statistical differences among groups were evaluated by one-way ANOVA followed by Tukey's test. Statistical anal-

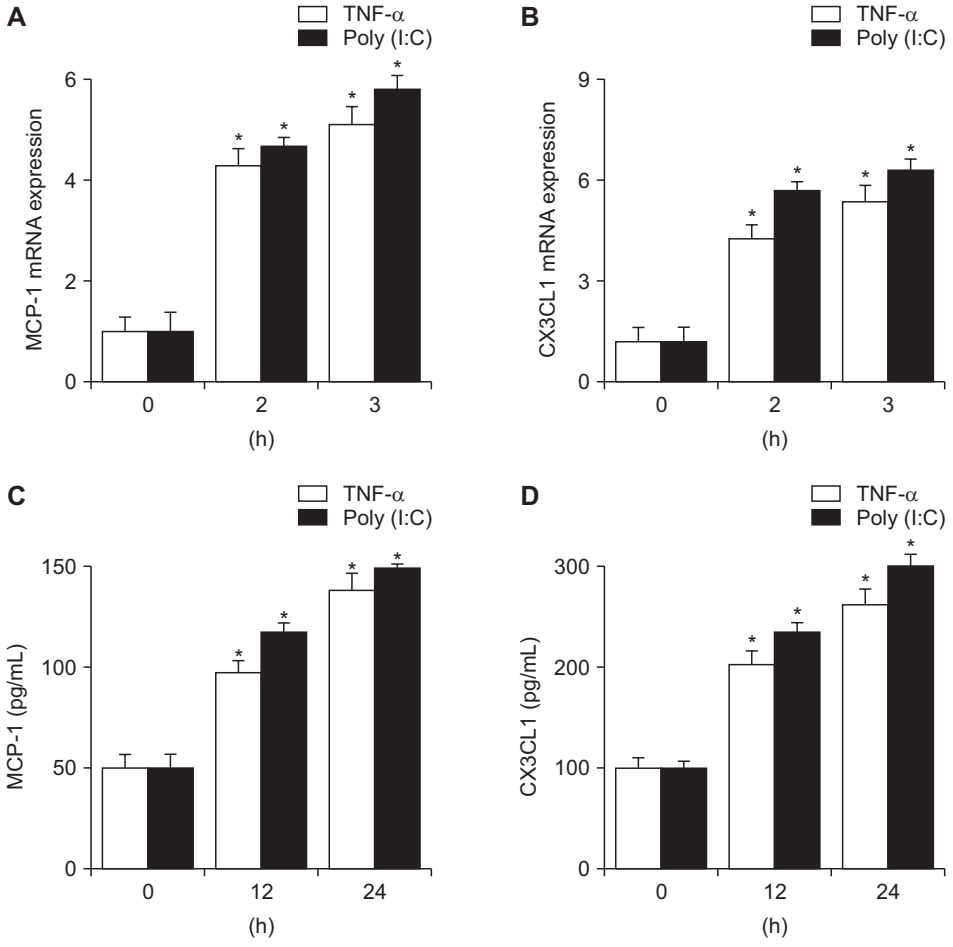

Figure 1. TNF-α and poly (I:C) induce the expression of MCP-1 and CX3CL1 in pancreatic stellate cells (PSCs). Cells were treated with TNF-α at 10 ng/mL or poly (I:C) at 1 μg/mL for the indicated time periods. (A, B) mRNA expression was determined by real-time PCR analysis and normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (C, D) Protein levels in the medium were determined by enzyme-linked immunosorbent assay (ELISA). Data are expressed as the mean ± SE. MCP-1, monocyte chemoattractant protein 1; poly (I:C), polyinosinic-polycytidylic acid; NAC, N-acetylcysteine; CX3CL1, chemokine C-X3-C motif ligand 1. **P* < 0.05 vs. 0 hour.

ysis was performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

TNF-α and poly (I:C) induce the expression of MCP-1 and CX3CL1 in PSCs

RT-PCR and ELISA were performed to establish the effect of TNF-α (10 ng/mL) or poly (I:C) (1 μg/mL) on the mRNA and protein expression of MCP-1 and CX3CL1 (Fig. 1). Both TNF-α and poly (I:C) treatment increased the relative mRNA and protein levels of MCP-1 and CX3CL1 in a time-dependent manner. Maximal MCP-1 and CX3CL1 mRNA expression was observed after 3 hours of incubation (Fig. 1A and 1B). Protein levels of MCP-1 and CX3CL1 were highest at 24 hours of culture (Fig. 1C and 1D). These results indicated that TNF-α and poly (I:C) induced the expression of MCP-1 and CX3CL1 in a time-dependent manner. Therefore, to investigate the effect of DHA, cells were stimulated with TNF-α (10 ng/mL) or poly (I:C) (1 μg/mL) for 3 hours (for measuring mRNA expression) and 24 hours (for determination of protein levels in the medium) in the following experiments.

DHA suppresses TNF-α- or poly (I:C)-induced expression of MCP-1 and CX3CL1 in PSCs

RT-PCR was performed to investigate the inhibitory effect of DHA on the expression of MCP-1 and CX3CL1 mRNA in PSCs. The cells were pre-treated with DHA (10, 50 μM) for 1 hour and then incubated with TNF-α (10 ng/mL) or poly (I:C) (1 μg/mL) for 3 hours (Fig. 2A and 2B). TNF-α or poly (I:C) increased the mRNA expression of MCP-1 and CX3CL1, and

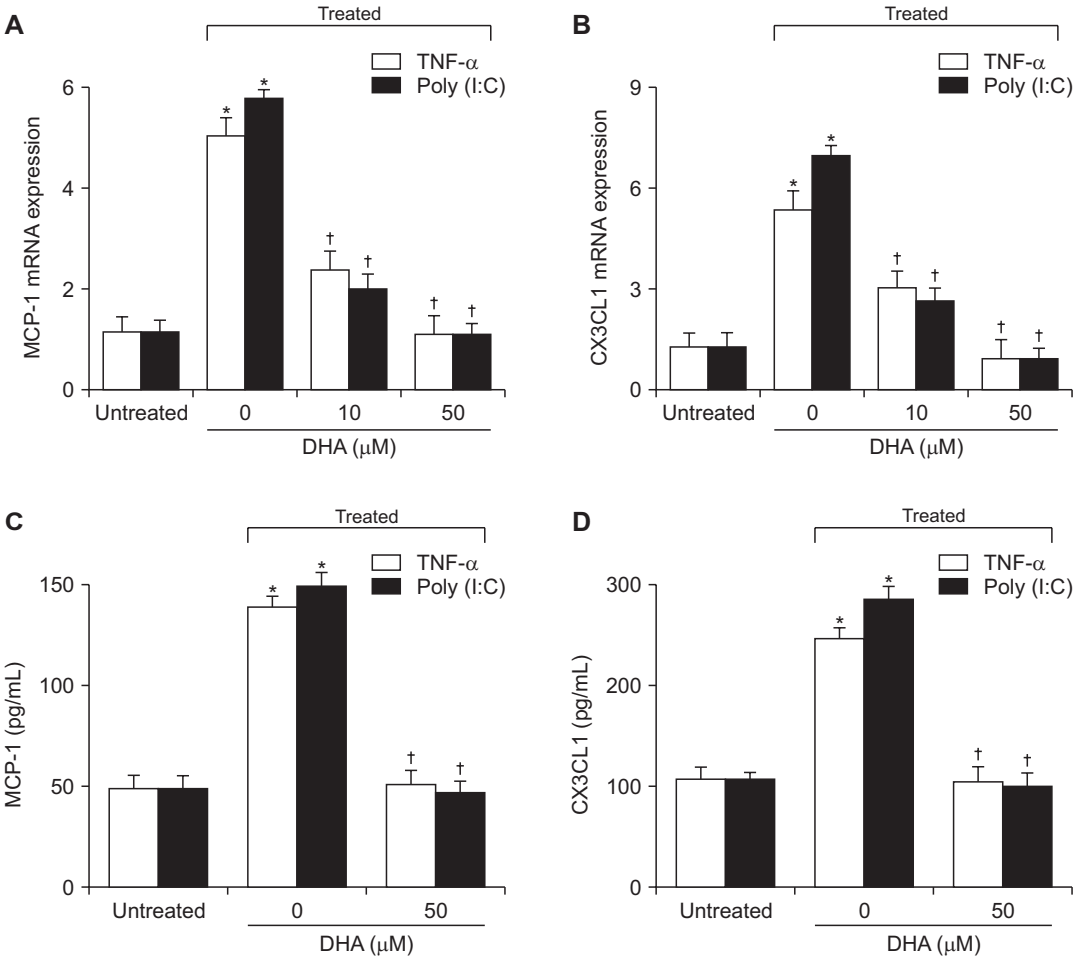

Figure 2. DHA inhibits TNF-α- and poly (I:C)-induced expression of MCP-1 and CX3CL1 in pancreatic stellate cells (PSCs). (A, B) The cells were pre-treated with the indicated concentrations of DHA and stimulated with TNF-α (10 ng/mL) or poly (I:C) (1 μg/mL) for 3 hours. mRNA expression was determined by real-time PCR analysis and normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (C, D) The cells were pre-treated with DHA (50 μM) and stimulated with TNF-α (10 ng/mL) or poly (I:C) (1 μg/mL) for 24 hours. Protein levels in the medium were determined by enzyme-linked immunosorbent assay (ELISA). Data are expressed as the mean ± SE. MCP-1, monocyte chemoattractant protein 1; poly (I:C), polyinosinic-polycytidylic acid; DHA, docosahexaenoic acid; CX3CL1, chemokine C-X3-C motif ligand 1. *$P < 0.05$ vs. untreated cells; †$P < 0.05$ vs. cells treated with TNF-α or poly (I:C) alone.

this increase was significantly reduced by DHA in a concentration-dependent manner. ELISA was performed to evaluate whether DHA could suppress TNF-α- or poly (I:C)-induced increase of MCP-1 and CX3CL1 protein levels. The cells were pre-treated with DHA (50 μM) for 1 hour and then incubated with TNF-α or poly (I:C) for 24 hours. TNF-α- or poly (I:C)-induced increases of the protein levels of MCP-1 and CX3CL1 were significantly reduced by DHA (Fig. 2C and 2D). These results indicated that DHA inhibited TNF-α- and poly (I:C)-induced expression of MCP-1 and CX3CL1 at both mRNA and protein levels.

DHA attenuates TNF-α- or poly (I:C)-induced increase of intracellular and mitochondrial ROS levels and decrease of mitochondrial membrane potential (MMP) in PSCs

We measured intracellular and mitochondrial ROS levels in cells stimulated with TNF-α or poly (I:C) for 1 hour (Fig. 3). Intracellular ROS levels in the cells treated with TNF-α or poly (I:C) (control) were 100% to 120% higher than those of untreated cells (none), whereas ROS levels in the mitochondria increased by 100% to 150% in cells treated with TNF-α or poly (I:C). Pre-treatment with DHA (50 μM) prevented these reductions in intracellular ROS (Fig. 3A) and in mitochondrial ROS (Fig. 3B).

To further examine whether TNF-α or poly (I:C) treatment causes impairment of MMP, the cells were treated with 5,5',6,6'-tetrachloro-1,1',3,3'-JC-1 and then observed under a confocal microscope. As shown in Figure 4A, the red fluorescence observed in the cells indicates mitochondria with high MMP, whereas the green fluorescence indicates mitochondria with low MMP. The ratio of the intensities of the red and green fluorescence shown in Figure 4B indicates that the MMP was decreased by 40% in TNF-α-treated cells and by 30% in poly (I:C)-treated cells. DHA pre-treatment suppressed this decrease. These results indicated that DHA reduced the levels of intracellular and mitochondrial ROS as well as attenuated mitochondrial dysfunction in TNF-α- or poly (I:C)-treated PSCs.

DHA inhibits TNF-α or poly (I:C)-induced activation of NF-κB in PSCs

Next, we determined the active nuclear NF-κB formed in cells pre-treated with DHA for 1 hour and stimulated with TNF-α or poly (I:C) for 1 hour. As shown in Figure 5, NF-κB-DNA binding activity was increased by treatment with TNF-α or poly (I:C). Pre-treatment of cells with DHA suppressed TNF-α- or poly (I:C)-induced NF-κB activation.

NAC suppresses TNF-α- or poly (I:C)-induced expression of MCP-1 and CX3CL1 in PSCs

To assess the role of ROS on cytokine expression, we determined whether the antioxidant N-acetylcysteine (NAC) suppresses the expression of MCP-1 and CX3CL1 in cells stimulated with TNF-α or poly (I:C). The cells were pre-treated with NAC for 1 hour and treated with TNF-α or poly (I:C) for 3 hours (Fig. 6A and 6B) and 24 hours, respectively (Fig. 6C and 6D). NAC suppressed both mRNA and protein expression of MCP-1 and CX3CL-1 in cells stimulated by TNF-α or poly (I:C). These results suggested that TNF-α- or poly (I:C)-induced expression of MCP-1 and CX3CL1 may be mediated by ROS.

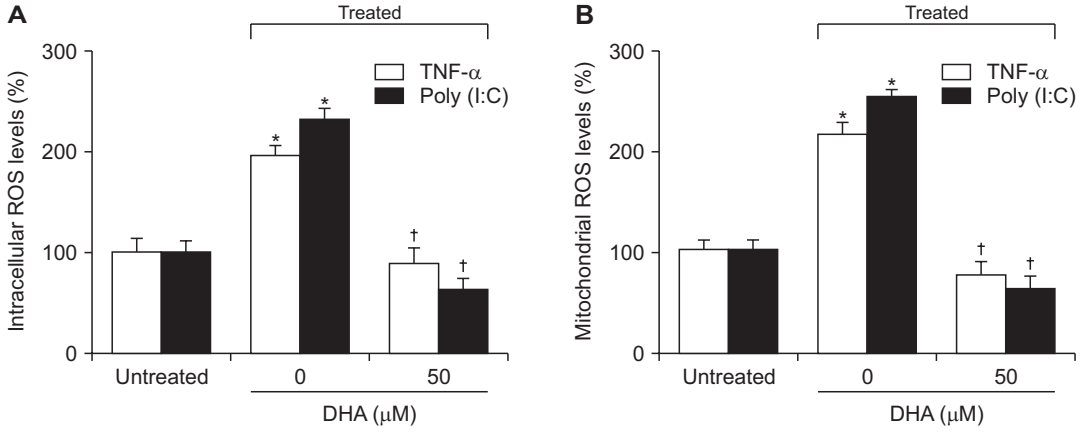

Figure 3. Effect of DHA on TNF-α- or poly (I:C)-induced increase in intracellular and mitochondrial ROS levels in pancreatic stellate cells (PSCs). PSCs were pre-treated with 50 μM DHA for 1 hour, and then stimulated with TNF-α (10 ng/mL) or poly (I:C) (1 μg/mL) for 1 hour. (A) Intracellular ROS levels were determined by 5,6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) assay. (B) Mitochondrial ROS levels were measured using MitoSOX reagent (Invitrogen, Carlsbad, CA, USA). Data are expressed as the mean ± SE. ROS, reactive oxygen species; poly (I:C), polyinosinic-polycytidylic acid; DHA, docosahexaenoic acid. **P* < 0.05 vs. untreated cells; †*P* < 0.05 vs. cells treated with TNF-α or poly (I:C) alone.

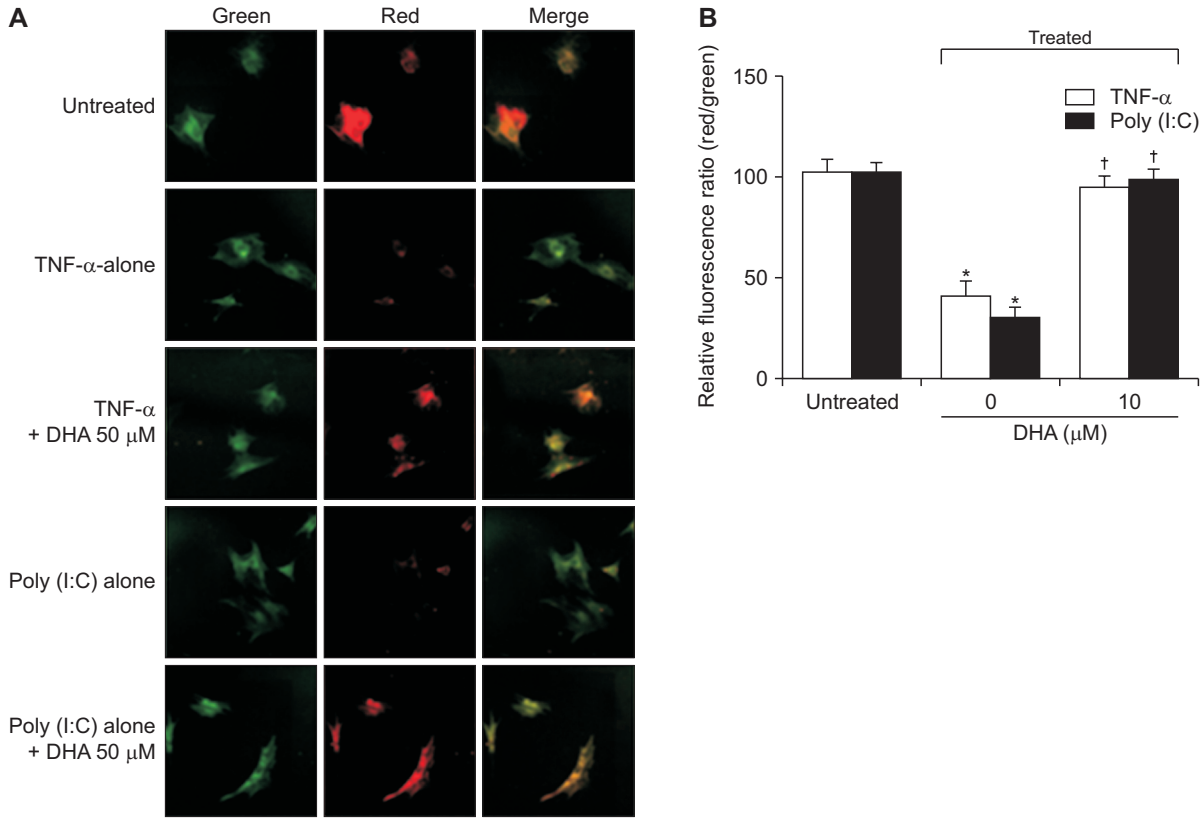

Figure 4. Effect of DHA on TNF-α- or poly (I:C)-induced disruption of mitochondrial membrane potential (MMP) in pancreatic stellate cells (PSCs). MMP was determined by confocal microscopy of cells pre-treated with DHA (50 μM) for 1 hour and stimulated with TNF-α (10 ng/mL) or poly (I:C) (1 μg/mL) for 1 hour prior to staining with JC-1 fluorescent dye (×200 magnification). (A) High MMP is indicated by red fluorescence and low MMP by green fluorescence. (B) The ratio of the intensities of red to green fluorescence was determined for the images shown in the (A). Values are expressed as the mean ± SE. DHA, docosahexaenoic acid; poly (I:C), polyinosinic-polycytidylic acid. *P < 0.05 vs. untreated cells; †P < 0.05 vs. cells treated with TNF-α or poly (I:C) alone.

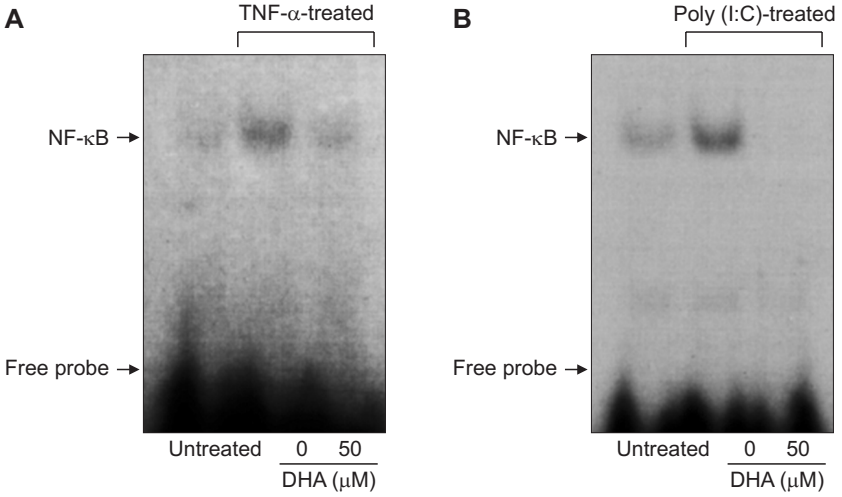

Figure 5. Effect of DHA on TNF-α- or poly (I:C)-induced activation of NF-κB in pancreatic stellate cells (PSCs). The cells were pre-treated with 50 μM DHA for 1 hour and then stimulated with TNF-α (10 ng/mL) (A) or poly (I:C) (1 μg/mL) (B) for 1 hour. NF-κB-DNA binding activity in the nuclear extracts was determined by electrophoretic mobility shift assay. DHA, docosahexaenoic acid; poly (I:C), polyinosinic-polycytidylic acid.

DISCUSSION

In the present study, we showed that TNF-α and poly (I:C) induced the expression of MCP-1 and CX3CL1 and that DHA inhibited this upregulation in PSCs. MCP-1 and CX3CL1 levels are increased in chronic pancreatitis [26,27]. These two cytokines regulate monocytes and T lymphocytes and contribute to the pathogenesis of pancreatitis [4,7]. TNF-α

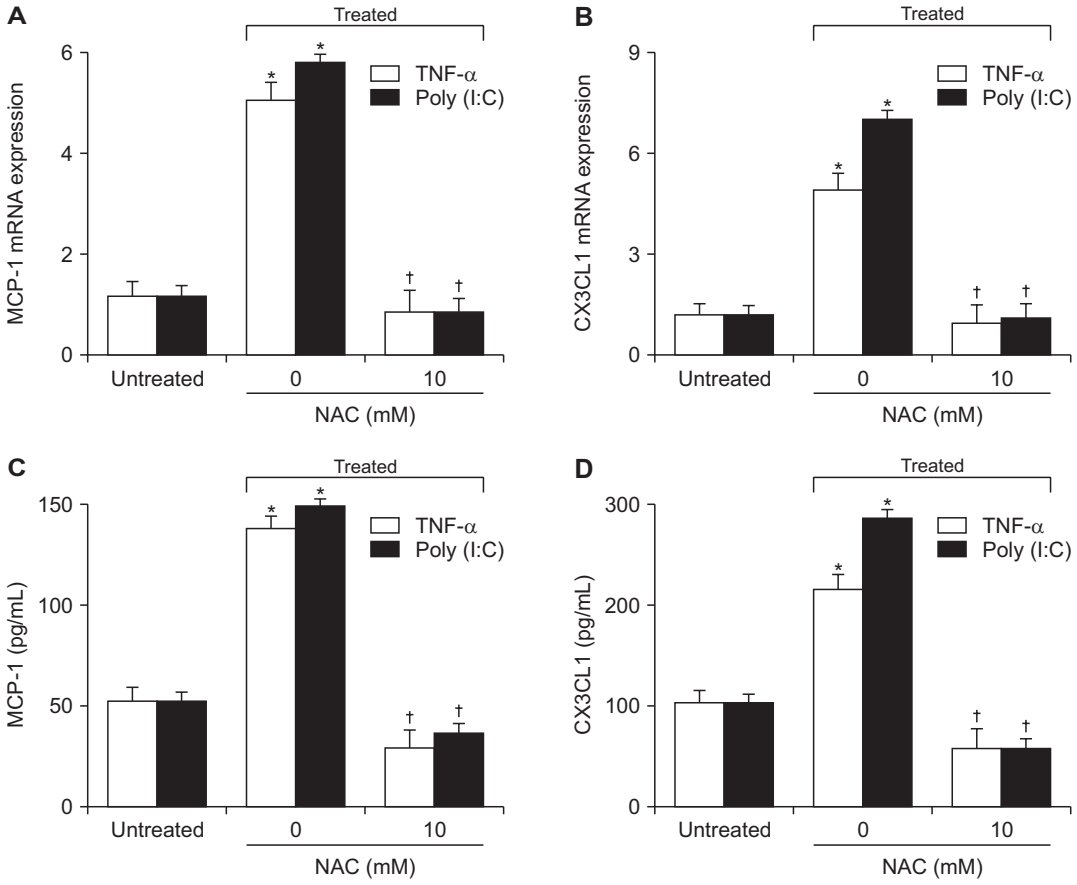

Figure 6. Effect of the antioxidant N-acetylcysteine (NAC) on TNF-α- or poly (I:C)-induced expression of MCP-1 and CX3CL1 in pancreatic stellate cells (PSCs). (A, B) The cells were pre-treated with NAC (10 mM) and stimulated with TNF-α (10 ng/mL) or poly (I:C) (1 μg/mL) for 3 hours. mRNA expression was determined by real-time PCR analysis and normalized to that of GAPDH. (C, D) The cells were pre-treated with NAC (10 mM) and stimulated with TNF-α (10 ng/mL) or poly (I:C) (1 μg/mL) for 24 hours. Protein levels in the medium were determined by enzyme-linked immunosorbent assay (ELISA). Data are expressed as the mean ± SE. MCP-1, monocyte chemoattractant protein 1; poly (I:C), polyinosinic-polycytidylic acid, CX3CL1, chemokine C-X3-C motif ligand 1. **P* < 0.05 vs. untreated cells; †*P* < 0.05 vs. cells treated with TNF-α or poly (I:C) alone.

and poly (I:C) induced an inflammatory response and DHA suppressed this effect, suggesting that DHA might prevent the development of chronic pancreatitis. However, the mechanism by which TNF-α and poly (I:C) induce cytokine expression in PSCs and the process by which DHA reduces inflammation are not fully understood.

In the present study, we demonstrated that treatment with TNF-α or poly (I:C) increased intracellular and mitochondrial ROS levels. Excessive production of ROS is associated with inflammatory diseases, and mitochondrial ROS impacts the expression of proinflammatory cytokines [28-33]. Mitochondria are also known to be targets of ROS. Uncontrolled overproduction of ROS can impair the mitochondria [34]. A recent study showed that increased intracellular ROS cause mitochondrial dysfunction and a subsequent increase in the production of mitochondrial ROS [35]. Mitochondrial ROS activate inflammatory signaling pathways to induce proinflammatory cytokines and inflammasome formation [36-38]. Here, we found that TNF-α and poly (I:C) treatment resulted in dysfunctional MMP and increased mitochondrial ROS, which were reduced by DHA treatment. NAC, a known antioxidant, prevented TNF-α- and poly (I:C)-induced cytokine expression in PSCs. This indicated that increased production of ROS induced the upregulation of inflammatory cytokine expression and that DHA reduced the expression of inflammatory cytokines by reducing ROS levels.

NF-κB is a transcription factor that influences inflammation by regulating the production of proinflammatory cytokines [39]. ROS also influence inflammation and activate NF-κB signaling [40]. Huang et al. [41] have reported that prolonged activation of NF-κB correlates with the development and severity of chronic pancreatitis.

In the present study, we found that TNF-α and poly (I:C) induced NF-κB activation, and DHA inhibited this activation. Our results suggest that TNF-α and poly (I:C) increase inflammatory cytokine expression by activating NF-κB, which is induced by the production of intracellular and mitochondrial ROS, and that DHA reduces this inflammatory response by

reducing ROS levels and preventing mitochondrial dysfunction. Since high amounts of intracellular ROS can damage mitochondria and thus increase mitochondrial ROS [34,35], reducing intracellular ROS might prevent the disruption of mitochondria and thus, decrease mitochondrial ROS in PSCs.

Signals mediating TNF-α-induced NF-κB activation are initiated by the engagement of TNFR at the plasma membrane and then relayed through specific TNFR-associated proteins. TNF-α-associated death domain-containing protein is an adaptor protein that interacts with TNFR and is required for TNF-α-mediated induction of NF-κB [42]. TNF-α (10 ng/mL) induced the expression of adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) via ROS-mediated NF-κB activation in lung epithelial cells [9] and endothelial cells [10]. TNF-α induced NF-κB activation in pancreatic acinar cells [43]. PSCs express ICAM-1 mainly through the activation of NF-κB, thus playing a role in the pathogenesis of pancreatic inflammation [44].

Regarding poly (I:C) signaling, the viral mimic poly (I:C), a synthetic analog of dsRNA, activates the TLR3 pathway [45]. Poly (I:C)-mediated expression of cytokines, such as IL-6 and MCP-1, is mediated by NF-κB in corneal fibroblasts [46]. In the pancreas, viral infections have been suggested as potential mediators of β-cell death in early type 1 diabetes mellitus. Poly (I:C) activates NF-κB, which induces the expression of inducible nitric oxide synthase and cell apoptosis [47]. These studies demonstrated the ROS-NF-κB-cytokine axis in TNF-α- or poly (I:C)-stimulated cells, which supports the present finding that increased intracellular and mitochondrial ROS induce NF-κB activation and cytokine expression in PSCs. In addition, we found that mitochondrial ROS are related to mitochondrial dysfunction, as determined by the reduction of MMP, in PSCs exposed to TNF-α or poly (I:C).

MCP-1 is a major chemokine expressed during pancreatitis [48] and reflects the severity of acute and chronic pancreatitis [49,50]. CX3CL1 activates PSCs and the levels of CX3CL1 increase during acute and chronic pancreatitis [51]. In the present study, we found that TNF-α or poly (I:C) treatment increased MCP-1 and CX3CL1 expression. In addition, TNF-α or poly (I:C) treatment increased intracellular and mitochondrial ROS and NF-κB activity, but decreased MMP in PSCs. These results show that inflammatory stimuli- or viral infection- induced expression of MCP-1 and CX3CL1 is dependent on ROS-mediated NF-κB activation and mitochondrial dysfunction in PSCs.

Regarding the antioxidant and anti-inflammatory effects of DHA, we previously showed that in cerulein-stimulated AR42J cells, DHA reduces ROS levels and induces catalase expression via activation of PPAR-γ [52]. Activation of PPAR-γ results in the inhibition of MCP-1 expression in PSCs [53]. The activation of PPAR-γ by rosiglitazone in macrophages repressed the transcription of CX3CL1 gene and prevented plasma membrane translocation, suggesting that PPAR-γ activation may suppress CX3CL1 signaling [54]. Thus, DHA may decrease ROS levels by activating PPAR-γ in activated PSCs. Further studies are required to determine whether DHA activates PPAR-γ and induces its target gene catalase to reduce ROS levels in activated PSCs.

Intracellular ROS levels can be detected using carboxy-DCF-DA. The change in fluorescence can be measured using a fluorometer or a confocal microscope as a fluorescence photograph. In the present study, intracellular ROS levels were determined using fluorometer since this method determines the changes of ROS quantitatively. Determining a fluorescence photograph may be useful as a supportive way for determining intracellular ROS for the further study.

Several studies demonstrated that TNF-α activated radical production in mitochondria principally at the ubiquinone site and TNF-α damages the mitochondrial chain at complex III, which consequently results in the increased production of mitochondrial ROS inside the mitochondrion in several cell lines [55-57]. Unger et al. [58] reported that poly (I:C) induced production of mitochondrial ROS in bronchial epithelial cells. The data suggest that TNF-α and poly (I:C) may induce production of mitochondrial ROS by damaging the mitochondrial chain at complex III in PSCs. These studies support the present findings that show the increase mitochondrial ROS in PSCs stimulated with TNF-α or poly (I:C).

In the present study, the ROS induction rate in mitochondria is slightly higher than intracellular ROS. It may be explained by the method to detect ROS. Intracellular and mitochondrial ROS levels were measured using carboxy-DCF-DA and MitoSOX, respectively. Carboxy-DCF-DA can determine ROS in the cytosol and penetrate the outer mitochondrial membrane. MitoSOX is able to enter the mitochondrial matrix [59]. Therefore, there can be differences between induction rates of intracellular and mitochondrial ROS. Several studies reported that the ROS induction rate in mitochondria was higher than intracellular ROS in the cells exposed to oxidative stress [60,61].

PSC can be evaluated by immunocytochemistry using antibodies specific for PSC markers such as α-smooth muscle actin (α-SMA) [62,63]. Activated PSCs have increased α-SMA. In the present study, we used the cells that present α-SMA using immunocytochemistry, which was demonstrated in our previous study [64]. In addition, in the present study, NF-κB bands were confirmed using a cold probe or competition reaction as described in our previous study [65].

Regarding the effect of DHA on ROS levels, the low concentration of DHA (≤ 60 μM) had ROS-scavenging activity [66,67] and antioxidant activity [68] whereas the high concentration of DHA (≥ 100 μM) has prooxidant activity [69]. For cytotoxicity of DHA, DHA (50 μM) has cytotoxic effects in cancer cell lines, whereas high concentrations of DHA (> 100 μM) did not affect the viability of normal cells [70,71]. The data suggest that DHA (50 μM) may not be cytotoxic to PSCs isolated form rat pancreas. In the present study, viable cell numbers were not changed by DHA (50 μM) for 24 hour-cul-

ture.

In conclusion, DHA reduces the magnitude of the inflammatory response in PSCs induced by TNF-α or poly (I:C) treatment, which causes increased levels of intracellular and mitochondrial ROS, NF-κB activation, and MCP-1 and CX-3CL1 expression. These findings suggest that consumption of DHA-rich foods and DHA supplementation may prevent inflammation during chronic pancreatic disease.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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