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Docosahexaenoic acid inhibits lipopolysaccharide-induced metastatic activities by decreasing inflammation on prostate cancer cell

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Docosahexaenoic acid (DHA) is rich in fish oil with many pharmacological impacts such as anti-inflammation and anti-cancer activities. In the present study, we aimed to investigate the inhibitory effects of DHA on the invasion and inflammation in prostate cancer cells. The cytotoxicity of DHA with or without lipopolysaccharides (LPS) treatment was evaluated by MTT assay. The invasion and wound healing assays were used to determine the roles of DHA in cell migration and invasion after LPS treatment. The expression levels of IL-6 and IL-8 were detected using ELISA assay. The protein expression was investigated by Western blotting. DHA exhibited significant cytotoxicity at the concentration of 100 μM in PC3 cells. Exposure to DHA (6, 12 and 25 μM) dose-dependently inhibited invasion and wound closure potential in PC3 cells after LPS treatment. DHA dose-dependently downregulated LPS-induced expression levels of IL-6 and IL-8. In addition, the LPS-induced protein levels of p-AKT and COX-2 were suppressed by DHA treatment. Our results indicate that low doses of DHA effectively inhibit metastasis by decreasing IL-6, IL-8, p-AKT and COX-2 expression levels after LPS treatment.

1. Introduction

Prostate cancer is one of the leading causes of cancer mortality worldwide. Initially, prostate cancer is highly responsive to androgen-deprivation therapy (Teo et al. 2019). However, the disease ultimately develops castration resistance. Castration-resistant prostate cancer cells are not only unresponsive to androgen-deprivation therapy but also highly metastatic (Gravis 2019) which is a cause of tumor treatment failure (Lombard et al. 2019).

LPS is the component of gram-negative bacterial cell wall. LPS is a strong immune activator and can induce immune response such as cytokine production and inflammation in the tumor microenvironment. Previous studies have indicated that LPS can induce cell migration by inducing the secretion of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in PC3 cells (Michalaki et al. 2004). One study demonstrated that LPS-induced proliferation and invasion through the p38-MAPK and NF-κB signaling pathways (Xu et al. 2015). Therefore, LPS can promote proliferation and invasion in prostate cancer cell.

Omega-3 polyunsaturated fatty acid is beneficial to human health (Nindrea et al. 2019). Alpha-linolenic acid (ALA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) belong to omega-3 polyunsaturated fatty acids. DHA and EPA are present in fish oil and display numerous pharmacological activities including regulating plasma triglyceride concentration, anti-inflammation and platelet aggregation (Preston Mason 2019; Tian et al. 2017). Several studies have reported that DHA and EPA can prevent carcinogenesis and induce cancer cell apoptosis *in vitro* and *in vivo* through multiple pharmacological mechanisms (Jing et al. 2013).

It is well recognized that phytochemicals exhibit different biological functions, such as anti-cancer and anti-inflammatory activities. Although numerous studies have shown that DHA suppresses cell growth and increases apoptosis in various types of cancer cells, no study has examined whether DHA can decrease cancer cell invasion and migration by inhibiting inflammatory mediator release. It is quite a challenge to identify an effective non-toxic compound that can inhibit tumor metastasis for improving survival rates in cancer patients. DHA has less side effects with multiple pharmacological activities. Therefore, this study aimed to determine whether DHA can inhibit cancer prostate cell invasion and migration induced by LPS.

2. Investigations and results

2.1. LPS restrains the growth suppression of PC3 cells by DHA

LPS can promote proliferation in prostate cancer cells. Therefore, we examined the pharmacological mechanism of action of DHA in PC3 cells. The responses of PC3 cells to DHA alone at the concentration of 25, 75 and 100 μM were determined by MTT assay at 24 h time point. The results demonstrated that 75 μM and 100 μM of DHA exhibited significant anti-proliferation activity. Notably, 25, 75 and 100 μM of DHA reduced PC3 cell growth to 13, 20 and 35%, respectively, compared to control group at 24 h (Fig 1). The cytotoxicity of DHA was attenuated when the PC3 cells were co-treated with LPS. Therefore, we believe that LPS may protect against DHA cytotoxicity. DHA at the concentration range of 6–25 μM did not produce cytotoxicity in PC3 cells. Therefore, we selected these DHA concentrations for further analyses.

2.2. DHA suppresses PC3 cell migration and invasion after LPS treatment

To determine the effect of DHA on the metastatic potential of PC3 cells *in vitro* migration and invasion assays were carried out. LPS treatment can significantly induce migration in PC3 cells. DHA treatment (6, 12.5 and 25 μM) dramatically inhibited PC3 cell migration by LPS treatment at 24 h (Fig. 2). We further examined the effects of DHA (25 μM) combined with LPS treatment on PC3 cell invasion by using transwell assay. LPS treatment alone significantly induced PC3 cell invasion at 24 h and 48 h. DHA (25

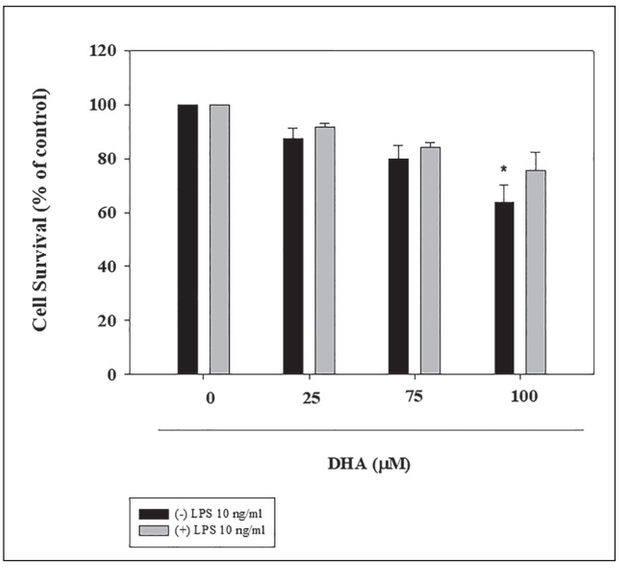

Fig. 1: Effect of DHA with or without LPS stimulation on PC3 proliferation at 24 h by MTT assay. Data are presented as mean ± SEM from three independent experiments. * $p < 0.05$ vs. control group.

μM) did not display significant cytotoxicity in PC3 cell at 48 h (data not shown). However, DHA (25 μM) significantly inhibited LPS-stimulated PC3 cell invasion only at 48 h, while did not exert inhibitory effect at 24 h (Fig. 3).

2.3. DHA downregulates the LPS-induced expression levels of IL-6 and IL-8

LPS can modulate the expression levels of cytokine or pro-inflammatory mediators in different types of cells. In this study, we focused on the expression levels of IL-6 and IL-8. As shown in Fig. 4, 10 ng/mL of LPS treatment increased the secretion of IL-6 and IL-8. Treatment with DHA (6, 12.5 and 25 μM) remarkably downregulated the LPS-induced expression levels of IL-6 in PC3 cells in a concentration-dependent manner.

2.4. DHA inhibits the protein levels of COX-2 and p-AKT after LPS treatment

Previous studies have demonstrated that DHA exerts anti-inflammation activities in different types of tumor cells. Therefore, we examined the roles of DHA in the activation of COX-2 and p-AKT in LPS-treated PC3 cells. The results showed that LPS induced the protein expression levels of COX-2 and p-AKT. After co-treatment with DHA, the levels of COX-2 and p-AKT were significantly reduced in a concentration-dependent manner (Fig. 5). Taken altogether, DHA likely attenuates the expression of inflammation and cell proliferation molecules.

3. Discussion

Epidemiological studies have shown that n-3 polyunsaturated fatty acids are beneficial to human health. DHA exerts potential anti-cancer effects against the invasion, migration and proliferation of different tumor cells. A recent study has reported that DHA can decrease the growth of human prostate cancer cell lines (Oono et al. 2017). Moreover, DHA synergistically enhances the cytotoxicity effect of docetaxel in prostate cancer cells (Shaikh et al. 2008). Base on the previous findings, DHA at a concentration of 100 μM exerted minor effects on the anti-proliferation of PC3 cells. These previous results are consistent with our current findings (Oono et al. 2017; Shaikh et al. 2008). However, a previous study has demonstrated that DHA at a concentration of 30 μM suppressed the viability of PC3 cells by 50 % (Shin et al. 2013).

LPS affects cytokine secretion and immune activation associated with initiation, promotion, progression, invasion, and metastasis of tumor cells (Pei et al. 2010, 2008). Numerous inflammatory media-

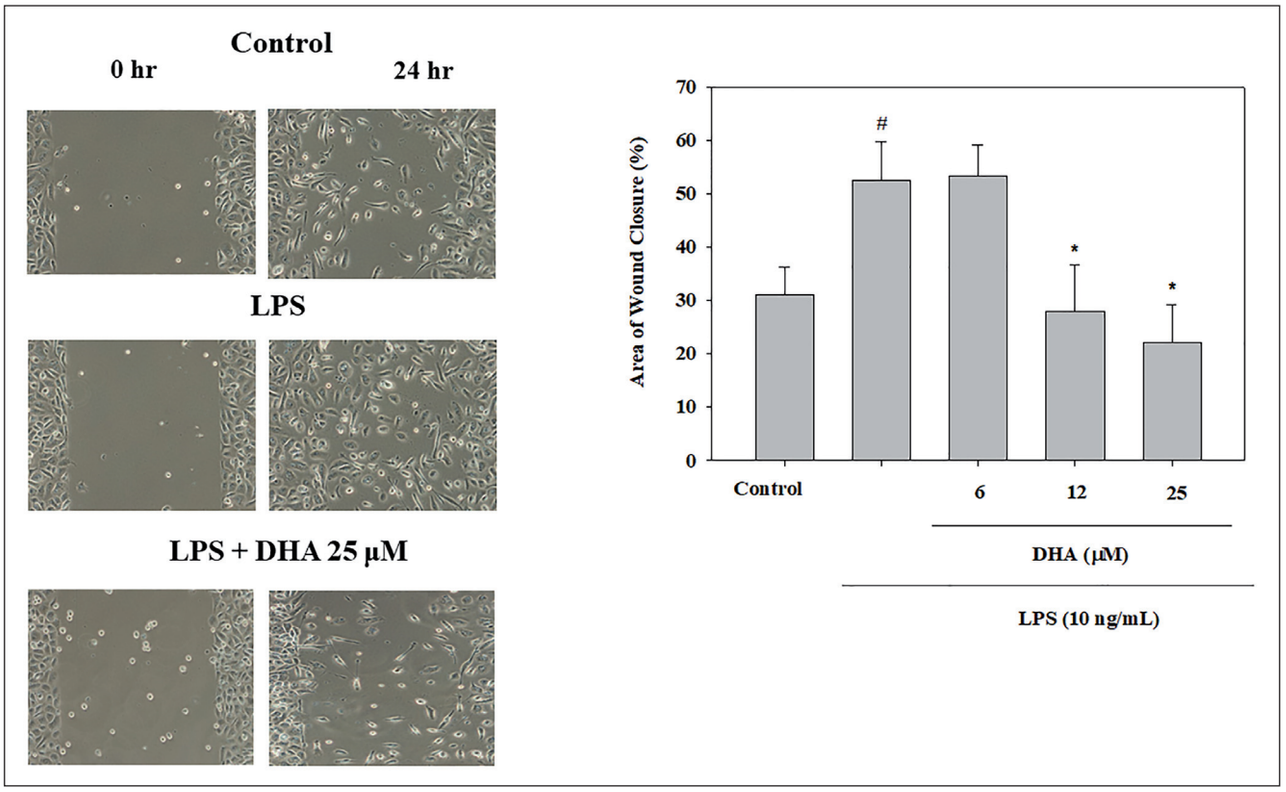

Fig. 2: DHA suppresses wound closure in PC3 cells at 24 hr. For wound healing assay, PC3 cells were seeded in 6 well plate and treatment of DHA with or without LPS stimulation for 24 h. Figures are representative of at least three experiments performed. *indicates $p < 0.05$ (compared with LPS stimulation alone) # indicates $p < 0.05$ (compared with control).

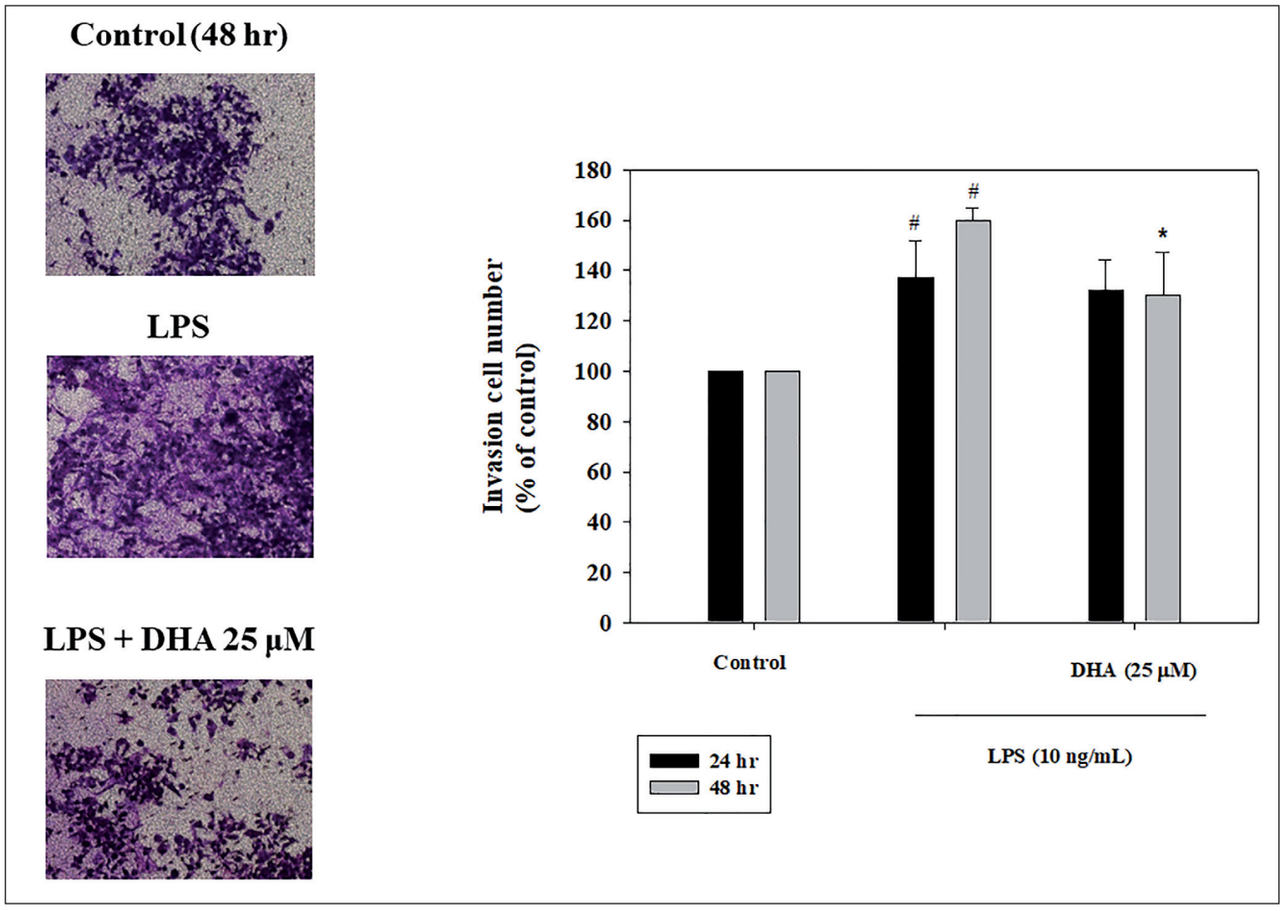

Fig. 3: Invasion assays were conducted to assess the invasion properties of DHA with LPS stimulation for 24 h and 48 h. Figures are representative of at least three experiments performed. *indicates $p < 0.05$ (compared with LPS stimulation alone) # indicates $p < 0.05$ (compared with control).

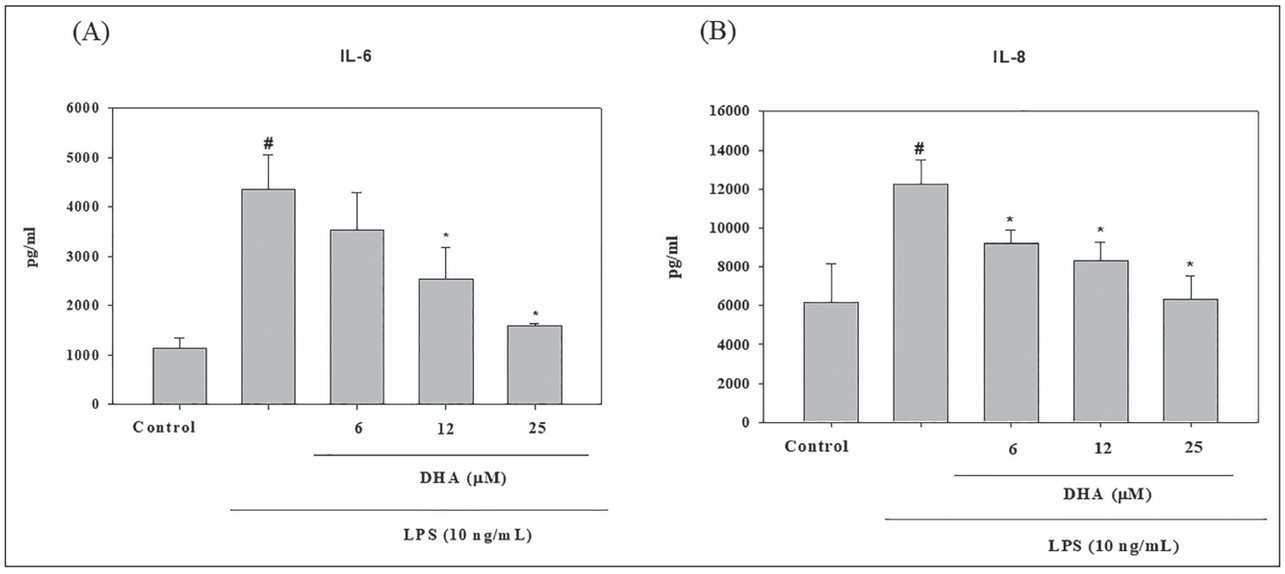

Fig. 4: DHA decreased the production of IL-6 and IL-8 in LPS stimulated PC3 cells for 24 h. IL-6 and IL-8 production in cell supernatants were measured by ELISA. Figures are representative of at least three experiments performed. *indicates $p < 0.05$ (compared with LPS stimulation alone). # indicates $p < 0.05$ (compared with control).

tors are involved in cancer metastasis such as IL-6 and IL-8. Inflammation also becomes a target for cancer prevention and treatment. The cancer cells with metastasis exhibit high levels of IL-8 and IL-6 (Li et al. 2018). High plasma levels of IL-6 and IL-8 are associated with frequent metastasis and poor survival rates. Besides, COX-2 has shown to be overexpressed in many cancer cells including prostate cancer (Yang et al. 2017). Furthermore, LPS promotes cell survival and metastatic activity in cancer cells and significantly activates the expression and activity of COX-2. COX-2 inhibitors can decrease cell proliferation and metastatic activity in cancer cells. A previous study has indicated that DHA has anti-inflammation activity by inhibiting NF-κB and COX-2 in SH-Y5Y cells (Fu et al. 2017). In this study, we found that LPS stimulation increased the expression level of COX-2 as well as the production of IL-6 and

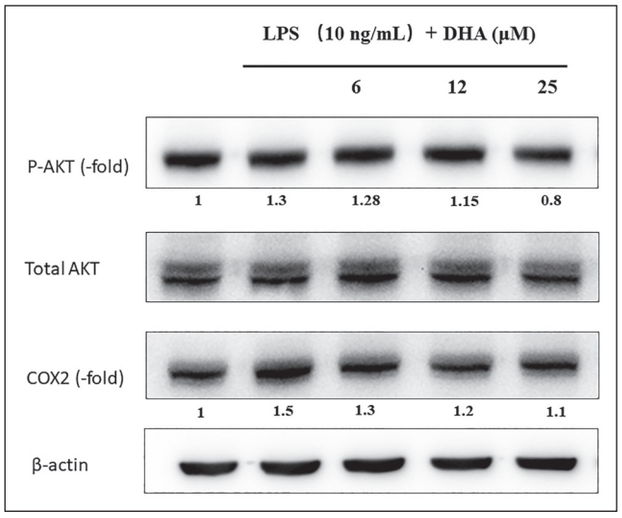

Fig. 5: DHA inhibits the p-AKT and COX-2 protein expression in LPS-stimulated PC3 cells. Human PC3 cells were treated with different concentration of DHA and LPS for 24 hr. Whole cell extracts were prepared and analysed by Western blots for indicated proteins. beta-actin was used as an internal control.

IL-8 in PC3 cells, but DHA suppressed such effects. Furthermore, we used low concentrations of DHA (12.5-25 μM) to inhibit COX-2 protein expression and cytokine production without significantly affecting the cellular toxicity. Collectively, these findings reveal that DHA can modulate immune activity and suppress the development, progression, invasion of prostate cancer cells

Numerous studies indicate that the phosphatidylinositol-3 kinase (PI3K)–AKT pathway is dysregulated in tumor cells (Coutte et al. 2012). There is also cross line between TLR4 and PI3K-AKT pathway during cancer growth (Zhang et al. 2012). High expression of PI3K or AKT is associated with cell survival, metastasis and proliferation. Elevated AKT activity can protect LNCaP cells from TRAIL-induced apoptosis (Chen et al. 2001). In this study, the findings of Western blot analysis indicated that the phosphorylation level of AKT was increased after LPS treatment. However, DHA could suppress the expression level of p-AKT induced by LPS treatment. Taken together, DHA might exhibit anti-metastatic activity by inhibiting p-AKT expression.

In summary, our results indicate that low doses of DHA effectively inhibit the invasion and migration of prostate cancer cells by decreasing IL-6 and IL-8 production, p-AKT and COX-2 protein expression. DHA exhibits anti-invasion, anti-migration and anti-inflammation activities on prostate cancer cells. However, it remains unclear whether DHA has the same effects in other cancer cells. The current study investigates the potential therapeutic effect of DHA on the prevention of prostate cancer metastasis. In the future, the detailed mechanisms of action and animal study should be conducted.

4. Experimental

4.1. Chemicals and reagents

Docosahexaenoic acid (DHA), lipopolysaccharides (LPS; from *E. coli* 026:B6), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) and primary antibodies against β-actin were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Primary antibodies against p-AKT, AKT were obtained from Cell signaling (Danvers, MA, USA), while primary antibodies against COX-2 and horseradish peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Reagents used for cell culture including Ham's F-12K (Kaighn's) Medium, fetal bovine serum (FBS), antibiotic and Trypsin-EDTA (0.25%) were obtained from Gibco Life Technologies (Grand Island, NY, USA). Protein assay kit was supplied by Bio-Rad (Richmond, CA, USA). DHA was dissolved in 99% alcohol and then diluted with media prior to the assay.

4.2. Cell culture

Human prostate carcinoma cells (PC3) were supplied by Food Industry Research and Development Institute (Hsinchu, Taiwan). These cells were cultured in F-12 (Gibco) containing 10% FBS and maintained under standard cell culture conditions.

4.3. Cell proliferation assay

Upon reaching confluence, the cells (1×10^4 cells/well) were seeded in 96-well plates and treated with different concentrations of DHA (25, 75 and 100 μM) with or without LPS (10 ng/ml). After 24 h of treatment, the viability of cells was evaluated by MTT assay with minor modifications. Briefly, the wells were rinsed twice with PBS after discarding the medium. Then, 10 μL of 5 mg/ml MTT solution and 100 μL of fresh medium were added into each well, followed by incubation for 4 h. After centrifugation, the supernatant was discarded, and 100 μL of dimethyl sulfoxide (DMSO) was used to dissolve the obtained MTT-formazan crystals. Finally, the absorbance measurements were performed at 570 nm using a microplate reader. This experiment was repeated for three times.

4.4. Wound healing assay

PC3 cells (1×10^6 cells/well) were cultured a 6-well plate with Ham's F-12K medium consisting of 10% fetal calf serum until reaching confluency. A scratch wound was created on cell monolayers using a sterile 200 μL pipette tip. After rinsing with PBS, the cells were treated with different concentrations of DHA (6, 12 and 25 μM) with or without LPS (10 ng/ml).

4.5. Invasion assay

Matrigel was coated on each 12-well Transwell plate (8 μm pore size). After serum starvation for 24 h, the cells were collected in F-12 medium with 1 % FBS, and re-suspended at a density of 2×10^5 cells/mL. Approximately 500 μL of cell suspension treated with DHA was loaded into the top chamber, while the bottom chamber was loaded with F-12 medium with 10 % FBS and LPS (10 ng/mL) as chemoattractant. Following 24 h and 48 h of incubation, Matrigel and the attached cells in the top chamber were gently scraped off, and the invading cells in the bottom chamber were fixed in 4 % formaldehyde and stained with 0.5 % crystal violet. Finally, the membranes were washed with PBS, and the dye was eluted with acetic acid. The microplate reader was used for absorbance measurement at 595 nm.

4.6. ELISA detection for IL-6 and IL-8

The cells were treated with DHA (6, 12 and 25 μM) and LPS (10 ng/ml) for 24 h. After centrifugation, cell supernatants were collected from treated and control cells. The expression levels of IL-6 and IL-8 were further detected with ELISA assay (Boster, CA, USA) by following the manufacturer's protocol.

4.7. Western blotting

Total protein was isolated from the cells using protein extraction reagents (Pierce Biotechnology, Rockford, IL, USA). Approximately 20 μg of protein sample were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane. The nitrocellulose membrane was incubated with the indicated primary antibody. After that, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody against mouse, goat or rabbit IgG for 1 h. After washing, the blotted membrane was exposed to enhanced chemiluminescent substrate for the visualization of target protein bands.

4.8. Statistical analysis

Data analysis and figure plotting were carried out using Sigma Plot ver. 10.0 statistics software on a compatible hardware. Statistical difference between two variables was compared by unpaired, two-tailed Student's t-test. One-way analysis of variance (ANOVA) was used for comparing control group with more than one treatment group. All data are presented as mean ± standard error of mean (SEM). A P-value of <0.05 was regarded as statistically significant.

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