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Eicosapentaenoic acid inhibits TNF-α-induced matrix metalloproteinase-9 expression in human keratinocytes, HaCaT cells

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Abstract

Eicosapentaenoic acid (EPA) is an omega-3 (ω-3) polyunsaturated fatty acid (PUFA), which has anti-inflammatory and anti-cancer properties. Some reports have demonstrated that EPA inhibits NF-κB activation induced by tumor necrosis factor (TNF)-α or lipopolysaccharide (LPS) in various cells. However, its detailed mode of action is unclear. In this report, we investigated whether EPA inhibits the expression of TNF-α-induced matrix metalloproteinases (MMP)-9 in human immortalized keratinocytes (HaCaT). TNF-α induced MMP-9 expression by NF-κB-dependent pathway. Pretreatment of EPA inhibited TNF-α-induced MMP-9 expression and p65 phosphorylation. However, EPA could not affect IκB-α phosphorylation, nuclear translocation of p65, and DNA binding activity of NF-κB. EPA inhibited TNF-α-induced p65 phosphorylation through p38 and Akt inhibition and this inhibition was IKKα-dependent event. Taken together, we demonstrate that EPA inhibits TNF-α-induced MMP-9 expression through inhibition of p38 and Akt activation.

Keywords: Eicosapentaenoic acid; Tumor necrosis factor-α; Matrix metalloproteinase-9; NF-κB

Ninety-two kilodalton gelatinase (matrix metalloproteinases-9, MMP-9) is a zinc-dependent endopeptidase and is related to tumor cell invasion and metastasis [1,2]. MMP-9 is involved in the proteolytic degradation of extracellular matrix (ECM) proteins such as type III and IV collagens, and elastin and in development, tissue remodeling, and inflammation [3]. Several transcriptional factors such as activator protein-1 (AP-1), Sp-1, and nuclear factor kappa B (NF-κB) are implicated in the activation of MMP-9 expression induced by various growth factors and cytokines [4,5].

Tumor necrosis factor-α (TNF-α) is known to activate some transcriptional factors including NF-κB [6]. Phosphorylation of IκB-α by IκB-α kinase (IKK) triggers IκB-α ubiquitinylation and degradation, which liberates NF-κB, allowing it to translocate to the nucleus [7]. In addition to the degradation of IκB-α, phosphorylation of NF-κB (RelA/p65) is also critical for NF-κB transcriptional activation. Moreover, proinflammatory cytokines, such as, TNF-α and IL-1β phosphorylate RelA/p65 and subsequently stimulate NF-κB transactivation through pathways distinct from its translocation to the nucleus [8–10]. TNF-α can activate various protein kinases including mitogen activated protein kinases. TNF-α also activates phosphatidylinositol-3-OH kinase (PI3K) and its downstream kinase Akt (protein kinase B). Akt is required for NF-κB activation through multiple mechanisms [11,12].

Abbreviations: EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid; TNF-α, tumor necrosis factor-alpha; MMP-9, matrix metalloproteinases-9; NF-κB, nuclear factor kappa B; IκB-α, inhibitory subunit of NF-κB; IKK, IκB-α kinase.

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TNF-α activates Akt, which phosphorylates and activates IKKα, thus promoting NF-κB function [13].

Eicosapentaenoic acid (EPA), an omega-3 (ω-3) polyunsaturated fatty acid (PUFA), is abundant in fish oil and is therapeutically useful in various diseases such as inflammatory disease and in prostate and colon cancers. EPA competes with arachidonic acid (AA) for incorporation into cell membrane phospholipids [14] and as a substrate of cyclooxygenase-2 (COX-2) [15]. Prostaglandins (PGs) are derived from membrane PUFAs and play important roles in inflammation, immune response, and wound healing [16]. Whereas ω-6 PUFAs such as AA generate 2-series prostaglandins (PGE_2), and ω-3 PUFAs, such as EPA, generated 3-series PGs (PGE_3) [15]. PGE_2 is more mitogenic and inflammatory than PGE_3. Moreover, ω-3 and ω-6 PUFAs distinctively act on NF-κB activity. NF-κB is activated by AA but not by EPA [17]. Some reports have demonstrated that EPA suppresses the NF-κB activation induced by various stimuli [18–22]. However, the detailed action mechanism of EPA has not been elucidated. In this report, we investigated effects and mechanism of EPA in TNF-α-induced MMP-9 expression in HaCaT, human immortalized keratinocytes.

Materials and methods

Materials. EPA was purchased from Sigma (St. Louis, MO). All inhibitors (U0126, MEK1-specific inhibitor; SP600125, JNK-specific inhibitor; SB202190, active p38-specific inhibitor; SB202474, inactive p38 inhibitor; wortmannin, PI3K/Akt inhibitor; BAY 11-7082, NF-κB inhibitor) were purchased from Calbiochem (San Diego, CA), and all antibodies were purchased from Cell Signaling Technology (Beverly, MA).

Cell culture and EPA treatment. HaCaT cells were cultured in monolayers at 37 °C in 5% CO_2 using Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Human prostate cancer cells (PC-3) and human umbilical vein endothelial cells (HUVEC) were grown in DMEM and endothelial growth media (EGM, Clonetics, Inc.) under the above condition, respectively. For all experiments, cells were cultured to 80% confluence and starved using FBS-free media for 24 h. EPA was dissolved in DMSO (20 mM), diluted with culture media to the indicated concentrations (final DMSO concentration, 0.1%), and pretreated to cells for 24 h before TNF-α treatment.

Detection of MMP-9 expression by zymography. Briefly, media were lyophilized and resuspended in distilled water without denaturation. Resuspended samples were incubated with zymography sample buffer for 20 min at room temperature and then run on 10% polyacrylamide gels containing 10% gelatin (Invitrogen, Carlsbad, CA). After electrophoresis, gels were renatured by incubation in renaturing buffer (50 mM Tris–HCl, pH 7.4, 2% (v/v) Triton X-100) for 30 min at room temperature. They were then incubated in developing buffer (50 mM Tris–HCl, pH 8.0, 2.5 mM $CaCl_2$, and 0.02% (w/v) sodium azide) for 24 h at 37 °C. Proteolytic bands were visualized by staining gels with 0.5% (w/v) Coomassie brilliant blue. MMP-9 band was confirmed by comparing with band using culture media of HT1080 known to constitutively express MMP-9.

Western blotting. Western blot analysis was performed as previously described [23]. Cells were lysed with lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM DTT) containing 1% Triton X-100. Equal amounts of protein were resolved in gradient (8–16%) SDS–PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were subsequently blocked with 5% skim milk and then incubated with the indicated antibody. The blotting proteins were visualized by the enhanced chemiluminescence.

Preparation of nuclear extracts and NF-κB DNA binding assays. Briefly, cells were lysed with lysis buffer (25 mM Hepes, pH 7.8, 50 mM KCl, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 5 mM PMSF) containing 0.5% nonidet P-40 (NP-40). Cytosolic fractions were removed by centrifugation and nuclear pellets were solubilized using extraction buffer containing 1% NP-40. Equal amounts of nuclear proteins were subjected to Western blotting or gel shift assays. Electrophoretic mobility shift assays (EMSAs) were performed according to the manufacturer's instructions (Gel Shift Assay System; Promega, Madison, WI).

NF-κB reporter assay. HaCaT cells were transfected using FuGENE 6 transfection reagent (Roche, Switzerland) according to the manufacturer's instructions. After 6 h post-transfection, cells were replenished with fresh serum-free medium and incubated for 12 h. The cells were then pretreated with the indicated concentrations of EPA for 24 h and then treated with TNF-α. After 24 h of incubation, cells were washed once with ice-cold PBS and lysed in Reporter Lysis Buffer (Promega, Madison, WI) for 20 min at room temperature. As an internal control, cells were co-transfected with β-galactosidase.

Results

EPA inhibited TNF-α-induced MMP-9 expression and p65 phosphorylation

To assess whether TNF-α induces MMP-9 expression, HaCaT cells were treated with various concentrations of TNF-α (Fig. 1A). After 72 h post-incubation, MMP-9 protein levels were determined by zymography and MMP-9 bands were confirmed by comparing the bands obtained using HT1080 culture media. Treatment of HaCaT cells with TNF-α induced MMP-9 expression dose-dependently. Therefore, we decided to use 5 ng/ml TNF-α in this study. HaCaT cells were pretreated with EPA for 24 h and then treated with TNF-α (Fig. 1B). EPA was found to inhibit TNF-α-induced MMP-9 expression in a dose-dependent manner. As reported, treatment of TNF-α induced phosphorylation of p65 and IκB-α (Fig. 1C) and EPA inhibited TNF-α-induced p65 phosphorylation but not IκB-α phosphorylation (Fig. 1D). Disruption of NF-κB pathway by BAY 11-7082 abolished TNF-α-induced MMP-9 expression (Fig. 1E), suggesting that NF-κB activation is required for TNF-α-induced MMP-9 expression in HaCaT cells.

EPA suppressed TNF-α-induced NF-κB transactivation without inhibiting p65 nuclear translocation

HaCaT cells were pretreated with EPA and then treated with TNF-α (Fig. 2A and B). One hour after TNF-α treatment, nuclear fractions were prepared and the levels of nuclear p65 and the DNA binding activity of NF-κB were determined by Western blotting and by EMSA, respectively. TNF-α induced the nuclear translocation of p65 and activated the DNA binding activity of NF-κB. However, EPA did inhibit neither nuclear translocation of p65 nor DNA binding activity of NF-κB (Fig. 2A and B). To investigate the effect of EPA on TNF-α-induced NF-κB transactivation, HaCaT cells were transiently transfected with NF-κB/luciferase reporter genes (Fig. 2C). TNF-α