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Eicosapentaenoic acid inhibits TNF-α-induced Lnk expression in human umbilical vein endothelial cells: involvement of the PI3K/Akt pathway☆

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Abstract

n−3 Polyunsaturated fatty acids (PUFAs) exert anti-inflammatory properties by influencing inflammatory cell activation processes. Lnk is an adaptor protein involving endothelial cell (EC) activation because it is induced by tumor necrosis factor-α (TNF-α). This study was conducted to evaluate the role of eicosapentaenoic acid (EPA), an *n*−3 PUFA, in the regulation of Lnk expression in human umbilical vein endothelial cells (HUVECs). Primary HUVECs were pretreated with EPA for 12 h at various concentrations (0–40 μM) and then exposed for another 12 h in the presence or absence of TNF-α (10 ng/ml). Lnk mRNA and protein were detected using reverse transcriptase polymerase chain reaction, immunoprecipitation and Western blot analysis. Results showed that pretreatment of HUVEC with EPA inhibited TNF-α-induced expression of Lnk in a dose-dependent manner. TNF-α-induced Lnk was also inhibited by a phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002. Thus, we investigated the role of PI3K/Akt signaling pathway in this process. Phosphorylation of Akt was assessed by Western blot analysis. We found that EPA treatment decreased the amount of activated Akt. These results showed that EPA inhibited TNF-α-induced Lnk expression in HUVECs through the PI3K/Akt pathway. This may be a potential mechanism by which EPA protects ECs under inflammatory conditions.
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Keywords: Eicosapentaenoic acid; Lnk; Endothelial cell; TNF; Akt

1. Introduction

The endothelium constitutes the principal barrier of exchange and contact between the blood and surrounding tissues, and therefore plays a critical role in a number of acute and chronic inflammatory diseases [1]. The onset and progression of these disease states involve the activation of endothelial cells (ECs) [2]. Activated ECs express new proteins and secrete chemokines, regulating inflammatory response. In EC activation, the formation of multimolecular complexes, which are initiated by adaptor proteins in many cases, is a crucial step [3].

Lnk, a member of an adaptor protein family that also includes APS and Src homology 2 (SH2) B, is implicated in cytokine receptor signaling [4]. Mice nullizygous for Lnk revealed an essential role for Lnk in B-cell lymphopoiesis and early hematopoiesis [5,6]. Further study showed that Lnk mRNA was upregulated by tumor necrosis factor-α (TNF-α) in primary porcine ECs, indicating a role of Lnk in inflammation [7]. However, the molecular mechanism for the regulation of Lnk expression in ECs has not been reported.

Long-chain *n*−3 polyunsaturated fatty acids (PUFAs) have anti-inflammatory properties and could benefit patients at risk for a variety of inflammatory settings [8]. The detailed mechanism underlying these beneficial effects remains to be elucidated. Cell culture and animal feeding studies have indicated that *n*−3 PUFAs influence inflammatory cell activation processes from signal transduction to protein expression, even involving effects at the genomic level [9,10]. Evidence has shown that exposure of the endothelium to PUFAs directly affected EC metabolism.

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The $n-3$ PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) reduced cytokine-induced expression of pro-inflammatory proteins in the endothelium [11,12].

As part of our investigation of Lnk in ECs, we undertook the present study to evaluate the role of EPA in the regulation of Lnk mRNA and protein in human umbilical vein endothelial cells (HUVECs). To our knowledge, this is the first report to show the regulation of Lnk by EPA.

2. Materials and methods

2.1. Materials and reagents

Human recombinant TNF-α and LY294002 were purchased from Sigma (Saint Louis, MO, USA). *cis*-5,8,11,14,17-EPA was purchased from Sigma as 99% pure free acid. EPA was dissolved in ethanol (100%) and stored at −20°C away from light. Equal amounts of ethanol were used in control incubations. TNF-α was reconstituted in phosphate-buffered saline. Antibodies to Akt and phosphorylated Akt (pAkt) were purchased from Cell Signaling (Beverly, MA, USA). Antibody to Lnk was purchased from Serotec (Oxford, UK). Secondary antibody rabbit antigoat IgG was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.2. Cell culture and activation

Primary cryopreserved pooled HUVECs (no. C-003-5C; first passage) were obtained from Cascade Biologics (Portland, OR, USA). The cells have the characteristics of vascular ECs in terms of cobblestone appearance at confluence and von Willebrand factor expression. After thawing, cells were seeded in 75-cm^2 cell culture flasks and cultured according to the supplier's recommendations in medium 200. Medium 200 was supplemented with low-serum-growth supplement (20 μl/ml) (Cascade Biologics) in the absence of antibiotics. Cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Primary cells were used between passages 3 and 4 in this study. Cells incubated with any treatment in this study had greater than 99% viability based upon the standard trypan blue dye exclusion test. Cells were harvested with 0.25% trypsin and 0.02% EDTA (Sigma). Cultured ECs were first made "quiescent" by withdrawal of growth factor overnight prior to treatment.

2.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from HUVECs using TRIzol reagent and processed according to the manufacturer's instructions. Samples were resuspended in RNase-free water, and concentrations were determined spectrophotometrically. Three micrograms of each sample was used for RT-PCR to generate human Lnk and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR products of 244 and 452 bp, respectively. For semiquantitative analysis, the linearity of amplification of Lnk and GAPDH cDNAs, depending on PCR cycle number, was established in preliminary experiments. Twenty-eight cycles resulted in the best amplification profile to detect differences among samples. We used the following primer pairs: for Lnk, sense 5′-GTG GGG AAT ACG TGC TCA CT-3′ and antisense 5′-AAA GGC AAG AGG ACC GTG TT-3′; for GAPDH, sense 5′-ACC ACA GTC CAT GCC ATC AC-3′ and antisense 5′-TCC ACC ACC CTG TTG CTG TA-3′. Reactions were performed in a volume of 50 μl, under the following conditions: initial denaturation at 95°C for 2 min; followed by 28 cycles of 95°C for 50 s, 58°C for 50 s and 72°C for 40 s; and an additional cycle with extension at 72°C for 7 min. The final PCR products were analyzed by electrophoresis, stained with ethidium bromide and photographed. Digital images were analyzed using Scion Image Software. Relative levels of Lnk mRNA were normalized to GAPDH transcripts from the same reaction.

2.4. Immunoprecipitation

For Lnk detection, cells were harvested and lysed in IP buffer (150 mM NaCl, 1% NP-40, 50 mM Tris–HCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM deoxycholic acid and 1 mM EDTA) for 30 min at 4°C. The lysis product was centrifuged at 4°C and 20,000×*g* for 30 min. The supernatant was collected, and protein content was measured by modified Lowry protein assay kit (Pierce, Rockford, IL, USA). Equal amounts (1 mg) of proteins were incubated overnight with 2.0 μg of goat–anti-Lnk antibodies at 4°C. Protein–antibody complexes were collected on protein A Sepharose beads. The beads were washed thrice with IP buffer and prepared for Western blot analysis.

2.5. Western blot analysis

Proteins were boiled for 5 min in loading buffer [1 M Tris–HCl, 10% β-mercaptoethanol, 10% sodium dodecyl sulfate (SDS) and 20% glycerol] and subjected to electrophoresis on a 10% SDS polyacrylamide gel. The proteins were transferred to a polyvinylidine difluoride (Millipore, Billerica, USA) membrane. The membranes were blocked for 30 min with 5% nonfat milk in Tris-buffered saline with 0.05% Tween-20 (TBST). The samples were incubated overnight with primary antibody goat anti-Lnk (1:400) at 4°C. The membranes were washed thrice in TBST for 5 min each and then blotted for 2 h with horseradish peroxidase-conjugated secondary antibody rabbit antigoat IgG (1:500) at room temperature. The membranes were then washed twice with TBST, followed by an additional wash of TBS. Immunoreactive bands were visualized using enhanced chemoluminescence (ECL) and the Western Blotting Detection System (Pierce). Protein mass was compared after quantifying the intensity of protein bands by Quantity One software (Bio-Rad, California, USA). The experiment was repeated thrice.

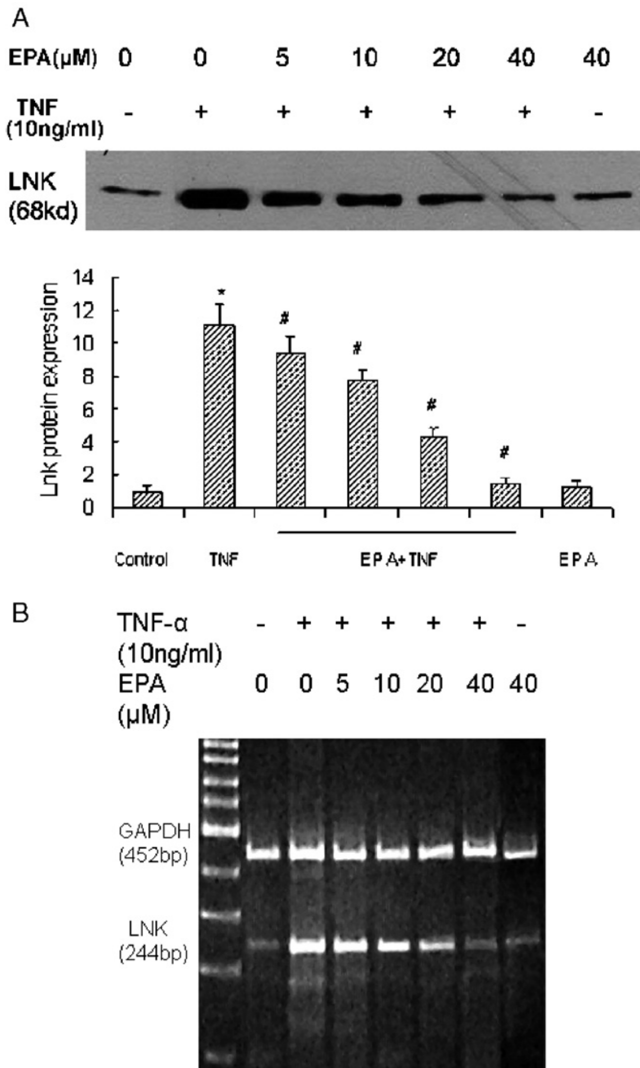

Fig. 1. EPA inhibits TNF-α-induced Lnk expression in HUVECs. HUVECs were treated with the presence or absence of EPA at various concentrations (5, 10, 20 and 40 μM). Twelve hours later, the cells were treated with 10 ng/ml TNF-α for 12 h. Cells were harvested for detection. (A) Lnk protein expression was determined by immunoprecipitation and Western blot analysis. (B) Lnk mRNA expression was determined by RT-PCR. The data are representative of three independent experiments. *$P<.05$ compared with untreated cells; #$P<.05$ compared with cells stimulated with TNF-α in the absence of EPA.

2.6. Akt (Ser^{473}) phosphorylation assay

To assay Akt phosphorylation, treated HUVECs were washed twice with PBS, lysed in sample buffer (50 mM Tris–HCl, 100 mM NaCl, 0.1% SDS, 1% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml pepstatin and 1 μg/ml leupeptin), boiled, separated by SDS polyacrylamide gel electrophoresis and transferred to a polyvinylidine difluoride membrane. The membrane was then incubated at room temperature in a blocking solution of 5% skim milk powder dissolved in 1× TBS [10 mM Tris (pH 8.0) and 140 mM NaCl] for 1 h. The membrane was then incubated overnight with the blocking solution containing phospho-Akt (Ser^{473}) antibody at 4°C. After washing thrice with TBS for 5 min, the blot was then incubated with antirabbit secondary antibody conjugated to horseradish peroxidase in the blocking solution. Detection of the bound antibody by ECL was performed according to the manufacturer's instructions.

2.7. Statistics

Data are expressed as mean±SD. Statistical significance between two groups was tested using unpaired Student's *t* test. Statistical significance between more than two groups was tested using one-way analysis of variance followed by the Student–Newman–Keuls test. Statistical significance was set at $P<.05$.

3. Results

3.1. EPA inhibits TNF-α-induced Lnk expression in HUVECs

To examine the modulating role of EPA on Lnk expression, we pretreated HUVECs with EPA for 12 h at various concentrations (0–40 μM) and then exposed them for another 12 h in the presence or absence of TNF-α (10 ng/ml). As shown in Fig. 1A, immunoblotting analysis revealed that EPA decreased, in a dose-dependent manner, Lnk protein expression induced by TNF-α. EPA did not affect Lnk protein in resting ECs. The inhibitory effects of EPA at high concentrations were not due to their cytotoxic

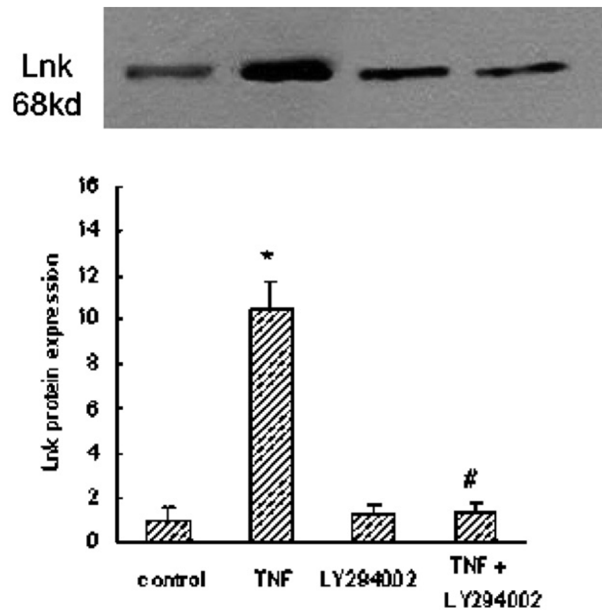

Fig. 2. The stimulation effect of TNF-α on Lnk is blocked by LY294002. HUVECs were incubated for 12 h with 10 ng/ml TNF-α in the presence or absence of LY294002 (10 μM). Immunoprecipitation and Western blot analysis were performed as described in the text. Lnk expression was quantified by densitometric analysis of Western blots from three independent experiments. *$P<.05$ compared with untreated cells; #$P<.05$ compared with cells stimulated with TNF-α only.

effects, as we checked cell viability by trypan blue exclusion test during these assays. Pretreatment with 40 μM EPA almost completely blocked TNF-α-induced Lnk expression.

To assess whether EPA modulates Lnk at the mRNA step, we performed RT-PCR on total HUVEC RNA after cell treatment for 12 h in the presence or absence of EPA followed by TNF-α (10 ng/ml) stimulation for another 12 h. In accordance with immunoblotting results, Lnk mRNA levels were attenuated by EPA in activated ECs (Fig. 1B).

3.2. TNF-α induced Lnk expression through phosphatidylinositol 3-kinase (PI3K)

We then tried to identify the signal transduction pathway that was associated with Lnk regulation. As shown in Fig. 2, HUVECs cultured with TNF-α (10 ng/ml) for 12 h demonstrated markedly (P<.05) enhanced Lnk expression compared with resting cells. Pretreatment for 12 h with 10 μM LY294002, a specific inhibitor of PI3K, significantly (P<.05) reduced Lnk expression in TNF-α-stimulated HUVECs. These results suggest that TNF-α induced Lnk expression through the signaling molecule PI3K.

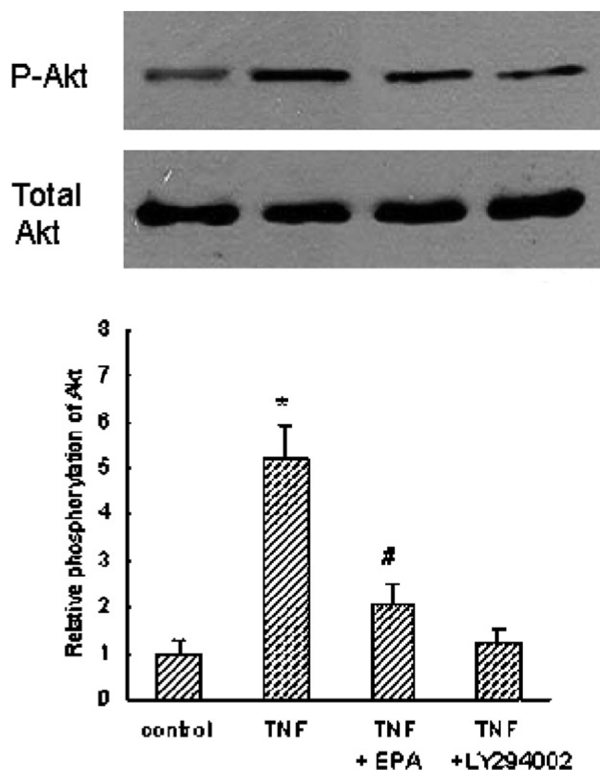

Fig. 3. EPA decreases the amount of activated Akt. HUVECs were pretreated with EPA (40 μM) for 12 h and then exposed to 10 ng/ml TNF-α for 12 h before harvesting. Each lane contains 50 μg of total protein from cell lysates. The blots were probed with anti-phospho-Akt antibody (upper panels) or anti-Akt antibody (lower panels). Results are the combination of three independent experiments and are presented as the level of pAkt compared with that of the control treatment. *P<.05 compared with untreated cells; #P<.05 compared with cells stimulated with TNF-α in the absence of EPA.

3.3. EPA inhibits Lnk expression through the PI3K/Akt pathway

One target of PI3K was the serine/threonine kinase Akt. Because activation of Akt correlates with phosphorylation at Thr^{308} and Ser^{473} residues, we examined the amount of pAkt in HUVECs using an anti-phospho-Akt (Ser^{473}) antibody. pAkt displayed little expression under unstimulated conditions and then increased in cells stimulated by 10 ng/ml TNF-α. Although EPA (40 μM) treatment had no effect on total Akt protein expression, it decreased the amount of the phosphorylated form of Akt in TNF-α-stimulated HUVECs (Fig. 3). These results suggest that EPA suppresses Lnk expression via downregulation of the PI3K/Akt signaling pathway.

4. Discussion

We reported here for the first time that EPA inhibited TNF-α-induced Lnk expression by inhibiting Akt activity in HUVECs. This may be a potential mechanism by which EPA protects ECs under infectious and inflammatory conditions. The precursor of $n-3$ PUFA is linolenic acid, which is metabolized to DHA [13]. EPA is an intermediate fatty acid during the metabolism of DHA [13]. Feeding laboratory animals fish oil reduces acute and chronic inflammatory responses, improves survival to endotoxin and prolongs the survival of grafted organs in models of autoimmunity [14–16]. In general, the optimal effects of $n-3$ PUFA can be explained by the influence of cellular metabolic functions, incorporation into membrane phospholipids, modulation of enzymes and direct impact on gene expression. It is also possible that these fatty acids may modulate cell functions by interfering with signal molecules. EPA increased the expression of genes, including adaptor protein Traf6 in 3T3-Swiss fibroblasts, in the NFκB signaling pathway [17]. DHA inhibits TNF-α-induced phospholipase C activation [18].

Lnk, a recently identified intracellular adaptor protein, negatively regulates B-lymphopoiesis and early hematopoiesis. Lnk-deficient mice show enhanced B-cell production due to the hypersensitivity of B precursors to stem cell factors [19]. Competitive repopulation assays in irradiated host animals have demonstrated that the ability of hematopoietic progenitors to generate various blood cells is greatly enhanced by the absence of Lnk [20]. In the present study, we showed that Lnk expression was induced by TNF-α in primary HUVECs. This is consistent with findings by Boulday et al. [7], who showed that TNF-α-mediated EC activation was associated with increased levels of mRNA for Lnk. This indicates a role for this adaptor protein in the regulation of TNF-α-induced EC activation. Molecular mechanisms underlying Lnk-mediated regulation are not fully understood.

Lnk has been proposed as a negative regulator of cytokine signaling. According to its structure [a proline-rich

amino-terminus, a pleckstrin homology domain, an SH2 domain and a conserved tyrosine near the carboxyl-terminus], Lnk might be involved in diverse signaling pathways [21]. It was reported that tyrosine-phosphorylated Lnk is bound to the SH2 domain of Grb2, phospholipase C-1 and PI3K in activated T cells and functions as a negative mediator of the T-cell receptor signaling pathway [22]. The binding of TNF-α to its membrane receptor initiates a chain of cellular events, including the activation of PI3K [23]. Therefore, we investigated the effect of LY294002, a PI3K inhibitor, on TNF-α-induced Lnk expression. Results showed that TNF-α failed to induce Lnk expression while the PI3K/Akt pathway was blocked by LY294002, suggesting that TNF-α induced Lnk expression through the PI3K pathway. However, downstream targets of PI3K include phospholipase C [24], protein kinase C [25], Rac [26] and the serine/threonine kinase Akt [27]. The target protein involving the effect of EPA on Lnk remains to be elucidated.

$n-3$ Fatty acids have proven to be potent and efficacious broad-spectrum protein kinase inhibitors [28]. EPA inhibited Akt kinase activity in breast cancer cells and may be useful for the treatment of tamoxifen-resistant breast cancer cells with high levels of activated Akt [29]. DHA inhibited the phosphorylation of AKT induced by LPS [30]. EPA has been shown to protect ECs against apoptosis through restoration of cFLIP expression, which is partially mediated through Akt activation [31]. Murata et al. [32] showed that exposure of hepatoma cells to EPA caused upregulation of several insulin-induced activities, including insulin receptor substrate-1-associated PI3K and its downstream target Akt kinase activity. On the other hand, studies have demonstrated that Lnk blocked three major signaling pathways, including Akt in $CD41^+$ megakaryocytes and in primary erythroblasts [33,34]. Thus, we designed an experiment to determine whether EPA modulates TNF-α-induced Lnk expression via their inhibitory effects on Akt. To test this hypothesis, we assessed the effects of EPA on TNF-α-stimulated Akt activity in HUVECs. We observed that EPA significantly decreased the TNF-α-stimulated phosphorylation of Akt in HUVECs. These results suggest that EPA may exert its inhibitory action on Lnk expression by inhibiting Akt activity in TNF-α-stimulated ECs. This illustrates that one mechanism by which EPA affects cellular function is by reducing the activity of Akt, thereby regulating signal transduction pathways in which Akt is involved. This potential mechanism should be kept in mind when considering the therapeutic benefits of $n-3$ fatty acids.

We demonstrated that EPA treatment of HUVEC cells resulted in the decreased induction of Lnk expression in response to TNF-α. Studies are under way to investigate the physiological relevance of the phenomenon. We also showed that TNF-α induced Lnk expression through the PI3K/Akt pathway. The serine/threonine kinase Akt is thought to be an important signaling pathway in cellular proliferation and survival [26]. We speculate that Lnk may play a role in EC proliferation and survival, while further studies are needed to determine the consequence of Lnk induction (or reduction) in ECs.

In summary, the present study showed that EPA inhibited TNF-induced Lnk expression in HUVECs. The PI3K/Akt signaling pathway was involved in this process. This study increased our knowledge concerning Lnk regulation and gave some insights into the mechanisms of the anti-inflammatory effects of EPA.

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