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Altered NF-κB gene expression and collagen formation induced by polyunsaturated fatty acids☆

Yi Jia, John J. Turek*

Department of Basic Medical Sciences, Purdue University, West Lafayette, IN 47907-2026, USA

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Abstract

Inability to control collagen formation in vital organs (e.g., fibrosis) or stimulate healthy collagen production (CP) in connective tissues (e.g., ligaments) is a major cause of death and disability. This study tested the hypothesis that arachidonic acid (AA) and eicosapentaenoic acid (EPA) influenced CP in 3T3-Swiss fibroblasts by altering gene expression in the nuclear factor-kappa B (NF-κB) pathway. 3T3-Swiss fibroblasts were grown in medium containing either AA or EPA. Lipopolysaccharide (LPS) was used to activate NF-κB, and parthenolide was used to block it. Cells treated with EPA had increased expression of genes in the NF-κB pathway when exposed to LPS and also produced more collagen. Parthenolide blocked NF-κB activation to a greater extent in EPA-treated cells and also decreased CP induced by NF-κB activation. Genes in the NF-κB signaling pathway that had increased expression in EPA-treated cells included the toll-like receptor 4 (Tlr4), adaptor proteins [TNF receptor-associated factor 6 (Traf6), myeloid differentiation primary response gene 88], signal transduction kinases (NF-κB-inducing kinase, inhibitors of kappa light polypeptide gene enhancer isoforms), inhibitor protein (I-κB alpha chain), transcription factors (nuclear factor of kappa light chain gene enhancer, p105 and NF-κB subunit p100), DNA binding proteins (cAMP response element binding protein) and response genes known to affect CP [interleukin 6 (IL-6), inducible nitric oxide synthase (iNOS), monocyte chemotactic protein-1]. This study raises the possibility that fatty acids may be used as adjuvants in combination with other therapies (e.g., selective targeting of the NF-κB pathway) to control collagen formation.

Keywords: Polyunsaturated fatty acids; Collagen; Gene expression; Nuclear factor kappa B; Arachidonic acid; Eicosapentaenoic acid; IL-6; MCP-1; iNOS

1. Introduction

Appropriate collagen formation is essential for the healing of various tissue and organs. Skin and dense connective tissues (e.g., tendons and ligaments) require enhanced production of healthy collagen for healing but without scarring [1], whereas vital organs need to repair without fibrosis [2]. Multiple cellular and extracellular factors can influence collagen formation, such as interleukin 6 (IL-6) [3], PGE_2 [4], growth factors [5,6], as well as matrix metalloproteinases [7] and integrins [8]. Essential n-3 and n-6 polyunsaturated fatty acids (PUFAs) have also been shown to influence collagen formation. Linoleic acid (18:2 n-6) suppressed collagen production (CP) in avian chondrocytes [9]. Porcine medial collateral ligament fibroblasts treated with eicosapentaenoic acid (EPA, 20:5 n-3) produced more of their total cell protein as collagen compared to fibroblasts treated with arachidonic acid (AA, 20:4 n-6). The increased collagen formation was correlated with increased IL-6 production [3]. We have observed that CP in murine 3T3-Swiss fibroblasts could be regulated by exposure to different n-6:n-3 PUFA ratios and these effects were mediated, in part, by PGE_2 and changes in the signaling via the different PGE receptor subtypes [10]. Collagen production in AA-treated fibroblasts could be reduced by EP1, EP2 and EP4 PGE receptor agonists, but EPA-treated cells only responded to EP2 and EP4 agonists.

As nontoxic nutrients, PUFA potentially may be used as adjuvants in combinations with drugs or other therapies to control collagen formation. However, an understanding of the mechanism of lipid influence on CP is necessary in order to make rational therapeutic use of these nutrients. Our previous research implicated the nuclear factor-kappa B (NF-κB) pathway as a principal mediator of collagen formation as influenced by PUFA. The NF-κB pathway

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* Corresponding author. Tel.: +1 765 494 5854; fax: +1 765 494 0781.
E-mail address: turekj@purdue.edu (J.J. Turek).

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has also been shown to be activated by PUFA in vascular endothelial cells [11,12].

Nuclear factor-κB (NF-κB), including NF-κB1 (p50/p105), NF-κB2 (p52/p100), Rel A (p65), Rel B and c-Rel, is a family of inducible transcription factors that have the binding sites for promoters or enhancers of many collagen formation associated genes such as IL-6, inducible nitric oxide synthase (iNOS) and monocyte chemoattractant protein-1 (MCP-1) [13–15]. We hypothesized that treatment of fibroblasts with different PUFA would alter expression of genes in the NF-κB pathway and also the collagen formation. In the present study, we used gene expression profiling to compare the different response of NF-κB-related genes to AA and EPA treatments in 3T3-Swiss fibroblasts and correlated the gene expression profile with CP.

2. Materials and methods

2.1. Reagents

Reagents were purchased from Sigma, St. Louis, MO, unless specified otherwise.

2.2. Cell culture and fatty acid enrichment

Mouse fibroblast (3T3-Swiss albino; American Type Culture Collection CCL-92, Rockville, MD) were maintained as subconfluent monolayers in six-well plates (Corning Costar, Cambridge, MA) with Dulbecco's modified Eagle's medium (DMEM), 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 10% bovine calf serum (Hyclone, Logan, UT). Subconfluent cultures grown for 24 h in maintenance medium were washed twice and changed to fresh medium minus calf serum. In place of serum, the control medium was supplemented with 5 mg/ml fatty acid-free bovine serum albumin (BSA), and the test media were supplemented with the soap form of either EPA or AA (NuChek Prep, Elysian, MN) at a concentration of 25 μM. After 48 h, the medium was replaced (EPA or AA enriched) and contained either 10 μg/ml of O55:B5 *Escherichia coli* lipopolysaccharide (LPS) to stimulate induction of NF-κB or LPS plus parthenolide (25 μM), which is an inhibitor of NF-κB. Cells incubated with any of the above treatments had greater than 99% viability based upon the standard trypan blue dye exclusion test.

To test the effect of fatty acids on gene expression, incubation was continued for an additional 24 h and the cells harvested for total RNA isolation. Controls included cells from normal growth medium and also cells enriched with fatty acids but untreated with LPS. After 24 h of incubation, the cells were washed with Hank's balanced salt solution and total RNA extracted from the cells using a commercially available kit (RNAqueous, Ambion, Austin, TX).

2.3. Gene expression profiling

RNA was reverse transcribed using MMVL reverse transcriptase (Promega, Madison, WI) according to the methods for the Superarray pathway specific gene expression profiling system (mGEA1013020 Mouse NF-κB Pathway GEArray and MM-016-12 Mouse NF-κB signaling pathway Q, Superarray, Bethesda, MD) and labeled using ^{33}P-dCTP. The resulting probe was heat denatured and hybridized for 24 h at 68°C for GEArray and 60°C for GEArray Q series membranes. After rinsing, the hybridization membranes were sealed in plastic membranes, exposed to a phosphoimaging screen for 4–12 h and results recorded using a Packard Cyclone Phosphoimager (Packard Bioscience, Downers Grove, IL). The array was analyzed using image analysis software (Optimas 6.1, Media Cybernetics, Silver Spring, MD) and the results expressed as a percentage relative to the various housekeeping genes (β-actin, GAPDH, peptidyl-prolyl isomerase A, ribosomal protein L13a) and PUC18 plasmid DNA as a negative control.

2.4. Collagen formation

After the initial 48 h of PUFA enrichment, the media were replaced with fresh fatty acid-enriched medium containing 50 μM ascorbic acid and 5 μCi of ^{3}H-proline (Amersham, Arlington Heights, IL) with or without treatments for 24 h, and cells were harvested and assayed for collagen, total protein and DNA.

2.5. Collagen assay

Collagen formation was determined as we have previously described [3]. The medium was collected and the cells washed twice with cold phosphate-buffered saline (PBS). The cells were pelleted by centrifugation and the PBS wash combined with the medium fraction. The cell pellet was suspended in 1.0 ml of ammonium hydroxide–Triton X-100 cell lysing solution (AT solution). Following a 15-min incubation at 37°C, 750 μl of the lysate is combined with the medium fraction. The two fractions, cell and medium, then underwent trichloroacetic acid (TCA) precipitation (equal volume of 20% TCA added to the cell plus media fraction). The acid insoluble precipitate was then rinsed several times with 10% TCA to remove free ^{3}H-proline. The precipitate was redissolved in 0.05 N NaOH in 0.05 M TES buffer plus 0.005 M $CaCl_2$ and half of the solution incubated for 6 h at 37°C with protease-free collagenase (100 U/ml) in TES (Sigma, type VII collagenase) and half of the solution serves as a control. Following the digestion, TCA was again added to precipitate the acid insoluble proteins; however, the collagen fragments generated by collagenase treatment remain in solution. The supernatant and precipitate were counted in a scintillation counter, and collagen, noncollagenous and total protein production reported as DPM/μg DNA.

2.6. DNA assay

The remaining 250 μl of the AT solution cellular-lysate from the collagen synthesis assay was used for total DNA determination. Picogreen (50 μl) (Molecular Probes, Eugene, OR) was added to 50 μl of lysate or to 50 μl of

known DNA standards, and fluorescence of the dye binding to double-stranded DNA was measured in a spectrofluorometer. A DNA standard curve was generated by linear regression, and sample DNA values was used to obtain the unknown values.

2.7. Statistical analysis

Data were presented as means±standard error and analyzed by both one-way and two-way ANOVA procedures of SAS (SAS Institute, Cary, NC). A Tukey test was used to analyze significant main and interaction effects. The correlation analysis was performed by Prism software (GraphPad, San Diego, CA). A P value < .05 was considered statistically significant.

3. Results

3.1. Effect of polyunsaturated fatty acids on NF-κB activation

Table 1 contains only those genes having a significant response to either PUFA or LPS treatment. 3T3-Swiss fibroblasts treated with EPA (20:5 n-3) but not stimulated by LPS had increased expression levels for the transmembrane toll-like receptor 4 (Tlr4, 2.8-fold increase) and the adaptor protein [TNF receptor-associated factor (Traf6), 2.4-fold increase] compared to AA-treated cells (Table 1). Other genes (ikBα, Ikkα, Ikkγ, iNOS and MCP-1) showed only slight increases between the EPA- and AA-treated cells [the fold increase expression values of EPA compared to AA (EPA/AA) were between 1.3 and 1.6].

Stimulation of 3T3 fibroblasts with LPS significantly increased the expression of numerous NF-κB pathway specific genes in EPA-treated cells compared to AA-treated cells. The increased expression of genes in the NF-κB pathway induced by LPS was significantly decreased or reversed by treatment with the NF-κB inhibitor parthenolide (Table 1). In EPA-treated cells stimulated with LPS, parthenolide decreased the expression for some genes (IkBα, Myd88, NF-κB2, Tlr4 and Traf6) to the levels of non-LPS stimulated cells. However, for a number of genes (Creb, Ikkβ, Ikkγ, IL-6, iNOS, MCP-1, NF-κB1 and NIK) parthenolide decreased expression to such an extent that AA-treated cells exhibited greater levels of expression for

Table 1
Effect of EPA or AA on NF-κB pathway gene expression in 3T3 fibroblasts

Genes in NF-κB pathway	EPA/control	AA/control	EPA/AA		
	LPS	LPS	No LPS	LPS	LPS + parthenolide
Cell membrane signaling					
Tlr4	3.4±1.4	−1.2±0.2	2.8±1.6	4.1±2.0	1.2±0.4
Traf6	3.2±1.8	−0.9±0.3	2.4±0.5	3.1±3.1	1.2±0.4
Myd88	4.3±0.8	−1.3±0.6	1.2±0.4	5.5±3.7	1.0±0.3
Signal transduction kinases					
NIK	3.5±2.0	−1.4±0.2	1.1±0.1	4.9±3.6	−10.8±0.1
Ikkα	1.9±0.2	−1.1±0.1	1.3±0.1	2.1±0.5	−1.6±0.1
Ikkβ	2.6±1.6	−1.3±0.2	1.2±0.1	3.3±1.9	−1.6±0.1
Ikkγ	1.5±0.3	−5.7±2.7	1.3±0.3	8.2±5.8	−4.5±0.1
IKK-I	2.2±1.1	−1.0±0.6	1.2±0.1	2.2±2.4	−1.8±0.1
Inhibitor protein					
IkBα	2.6±0.8	−4.3±0.9	1.4±0.5	11.2±1.8	3.1±0.5
Transcription factors					
NFκB1	2.5±1.5	−1.2±0.6	1.1±0.1	3.0±2.2	−1.9±0.2
NFκB2	4.2±3.4	−0.9±0.2	1.1±0.1	3.6±4.7	1.4±0.4
DNA binding proteins					
Creb	2.3±0.9	−1.5±0.2	1.1±0.1	3.4±1.7	−1.5±0.1
Response genes					
IL-6	3.4±1.9	−2.7±0.8	1.0±0.1	9.4±3.0	−1.9±0.1
iNOS	–	–	1.5±0.1	7.2±1.5	−2.4±0.1
MCP-1	–	–	1.6±0.5	4.4±1.8	−12.1±0.1

Treatments include 10 μg/ml LPS, LPS+NF-κB inhibitor (parthenolide, 25 μM) and no LPS. As a control, 3T3 fibroblasts were grown in culture medium with 5 mg/ml BSA. The data are expressed as the ratio of the expression of the individual genes relative to β-actin for EPA-treated cells compared to AA-treated cells. A positive number indicates that the expression level in EPA-treated cells was greater than AA-treated cells. A negative number indicates that expression in AA-enriched cells was greater than EPA-enriched cells. These data are a result of three different experiments where gene expression was assayed in duplicate. Myd88, myeloid differentiation primary response gene 88; Ikkα, conserved helix-loop-helix ubiquitous kinase; Ikkβ, inhibitor of kappa light polypeptide gene enhancer, kinase beta; Ikkγ, inhibitor of kappa light polypeptide gene enhancer, kinase gamma; IKK-i, IKK-i mRNA for inducible IκB kinase; IkBα, IκB alpha chain; NFκB1, nuclear factor of kappa light chain gene enhancer, p105; NFκB2, nuclear factor kappa B subunit p100; Creb, cAMP response element binding protein.

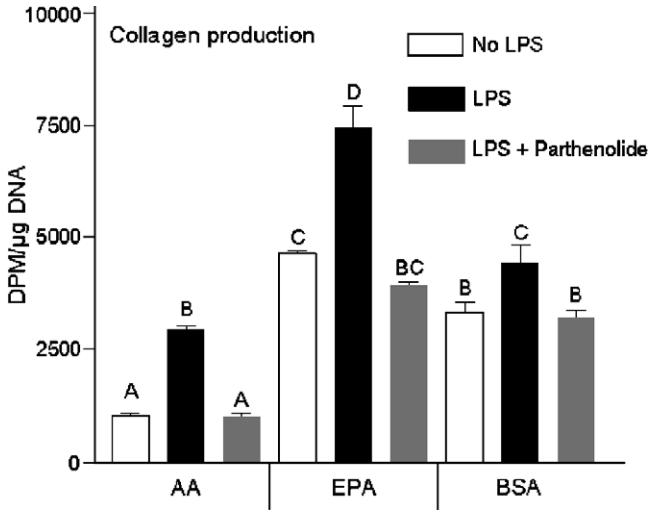

Fig. 1. The effect of different fatty acid on CP (CP) by 3T3-Swiss fibroblast ($n=3$, ±S.E.). These data are a result of triplicate experiments. Cells were treated with fatty acids (25 μmol/L) for 72 and 24 h with either 10 μg/ml of O55:B5 *E. coli* LPS to stimulate induction of NF-κB or LPS plus parthenolide (25 μM), an inhibitor of NF-κB. Bars with different letters (A, B, C, D) are significantly different ($P<.05$).

these genes than EPA-treated cells. Therefore, EPA-treated 3T3 fibroblasts were more responsive to both activation and inhibition of the NF-κB pathway.

3.2. Effect of LPS and parthenolide on CP in EPA- and AA-treated cells

We previously reported that 3T3-Swiss fibroblasts treated with EPA had increased CP and collagen as a percentage of total protein (C-PTP) compared to AA-treated cells [10]. These findings were verified by the present experiments, and we also determined the effects of LPS and NF-κB inhibition on CP in PUFA-treated cells.

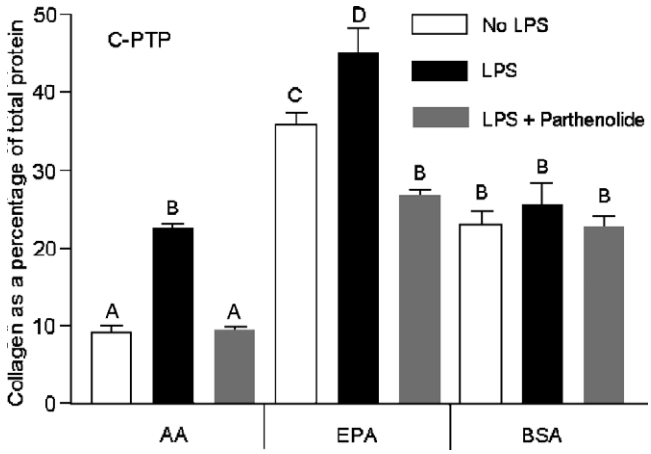

Fig. 2. The effect of different fatty acid on the CP as a percentage of total protein (C-PTP) CP by 3T3-Swiss fibroblast ($n=3$, ±S.E.). Cells were treated with fatty acids (25 μM) for 72 and 24 h with either 10 μg/ml of O55:B5 *E. coli* LPS to stimulate induction of NF-κB or LPS plus parthenolide (25 μM), an inhibitor of NF-κB. Bars with different letters (A, B, C, D) are significantly different ($P<.05$).

LPS treatment significantly ($P<.05$) increased CP for all groups, and this effect was blocked by the addition of the NF-κB inhibitor parthenolide (Fig. 1). Collagen production among the three treatments was significantly different with EPA>BSA>AA. Collagen as a percentage of total cell protein was significantly increased by LPS compared to non-LPS treatment for EPA and AA cells but not the BSA control. Collagen as a percentage of total protein was significantly greater in EPA-treated cells compared to AA and BSA treatments. Treatment with parthenolide decreased C-PTP in AA-treated cells to the same level as non-LPS-stimulated cells. However, for EPA-treated cells, parthenolide decreased C-PTP below non-LPS levels (Fig. 2).

4. Discussion

Controlling collagen formation is essential for attenuation of fibroproliferative diseases and proper healing of wounds and dense connective tissues such as ligaments.

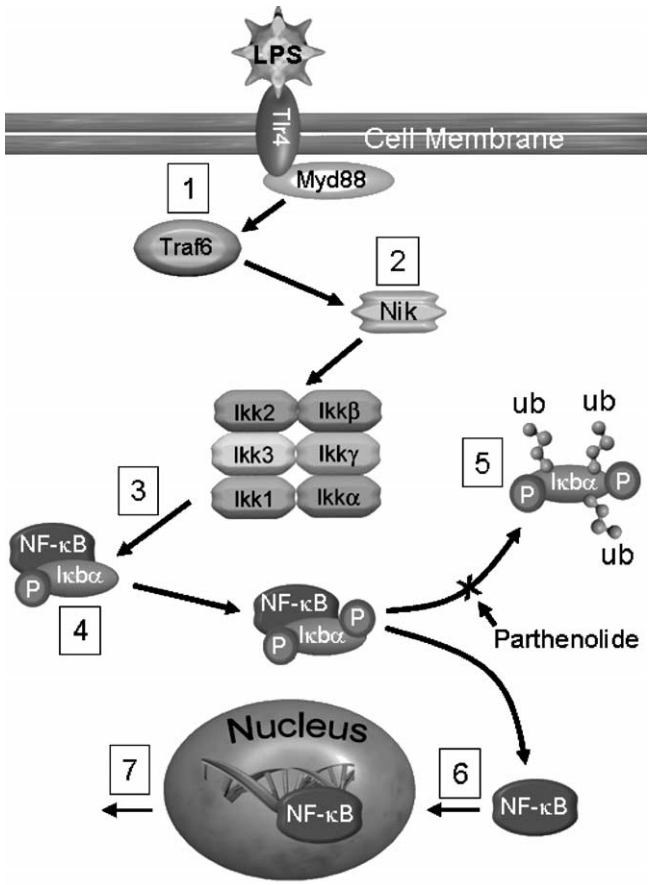

Fig. 3. Activation of NF-κB pathway by LPS [25,48,49]. (1) Lipopolysaccharide signaling and adaptor proteins. (2) MAP kinase family. (3) IκB kinase complexes. (4) Interaction of NF-κB family members with IκB family members. (5) Polyubiquitination and degradation that is blocked by parthenolide. (6) Translocation to nucleus. (7) Production by responsive genes (i.e., IL-6, iNOS, MCP-1). Myd88, myeloid differentiation primary response gene 88; Ikk, IκB kinase complex; IκB, inhibitor of kappa light polypeptide gene enhancer in B-cell. NF-κB family components (e.g., p50, p65, p100 and p105 subunits).

Fibroproliferative disease in the kidney due to diabetes [16], in the lung due to acute respiratory distress syndrome [17,18], in the skin due to scleroderma [2] and in the liver due to viral hepatitis [19] occurs when excess collagen (primarily type I) is synthesized and secreted. Ligament injuries present a different problem in CP. Some ligaments, such as the anterior cruciate ligament, have little ability to heal [1], while others, such as the medial collateral ligament, have an effective repair response [20,21]. Even though the medial collateral ligament heals, it may require weeks or months to remodel, and scar tissue formation can result in a weaker and functionally impaired ligament [20,22].

Since many collagen formation-associated genes, such as IL-6, iNOS and MCP-1, have promoter or enhancer elements for the transcription factor, NF-κB [14,23], we investigated the differential expression of NF-κB-related genes in EPA- and AA-treated 3T3-Swiss fibroblasts. Nuclear factor κB is a transcription factor that is activated in response to oxidative stress [11], cytokines such as TNF-α [24] and IL-1 [13], and also LPS [25]. For our experiments, we chose LPS to activate NF-κB since this factor does not affect the growth of the fibroblasts in culture or trigger an apoptotic pathway like TNF-α. Lipopolysaccharide signaling from the membrane is initiated via a transmembrane receptor (Tlr4), and the phosphorylation of IκB kinase complexes (IKK, including Ikkα, Ikkβ, Ikkγ and IKK-i) is triggered by signaling through adaptor proteins and MAP kinase pathways (Fig. 3). Polyubiquitination and subsequent degradation of the inhibitory IκB proteins allows the translocation of the NF-κB dimers into the nucleus. In the nucleus, the dimers bind to targeted DNA elements and regulate numerous responsive genes involved in the immune response, cell apoptosis, inflammation and oncogenesis [26].

Our experiment showed that genes in the NF-κB pathway that responded to LPS stimulation had increased expression in EPA-treated 3T3-Swiss fibroblasts compared to those treated with AA. Parthenolide is an antiinflammatory sesquiterpene that inhibits NF-κB by blocking the degradation of IκB proteins [27], and it also inhibits MAP kinases [28] and the activation of STAT proteins by blocking tyrosine phosphorylation [29]. The differential gene expression response to LPS stimulation between EPA- and AA-treated cells was significantly reduced or even reversed by treatment with parthenolide. Treatment with PUFA also affected genes at multiple points of the NF-κB signaling cascade. The NF-κB signaling pathway functional gene groups that were affected included the transmembrane receptor (Tlr4), adaptor proteins (Myd88 and Traf6), signal transduction kinases (NIK, Ikkα, Ikkβ, Ikkγ and IKK-i), NF-κB family (NF-κB1 and NF-κB2), IκB family (IkBα), DNA binding proteins (Creb) and responsive genes (IL-6, iNOS and MCP-1). The fact that the LPS receptor (Tlr4) and one of the adapter proteins (Traf6) had increased levels of expression with EPA treatment alone could account for some of the increased activity throughout the NF-κB signaling cascade (Fig. 3). However, the change in differential expression in response to parthenolide suggests that PUFA affects on NF-κB activation are mediated by factors in addition to signaling from the membrane. For example, expression of the NF-κB-inducing kinase (NIK) was increased with LPS stimulation in EPA-treated cells (EPA-treated cells had a 4.6-fold increase in expression compared to AA-treated cells), but decreased to such an extent with parthenolide that AA-treated cells had higher levels of expression (EPA-treated cells had a level of expression that was 10.8-fold less than AA-treated cells).

The NF-κB responsive genes IL-6, iNOS and MCP-1 that had their expression altered by PUFA treatment have all been associated with regulation of collagen formation. Activation of the NF-κB signaling pathway results in increased levels of IL-6 that in turn lead to increased collagen gene transcription [30] and enhanced production of the tissue inhibitors of metalloproteinases (TIMP) [31]. Rats treated with IL-6 had increased collagen synthesis in the liver tissues and in the culture supernatants of hepatic lipocytes [32]. IL-6 gene deletion delayed wound healing with attenuated reepithelialization, angiogenesis and collagen accumulation in BALB/c mice [33]. Eicosapentaenoic acid treatment of porcine medial collateral ligament fibroblasts was correlated with IL-6 production [3]. In human studies, an increased IL-6 amount was found in the fibroblasts from patients with diffuse cutaneous systemic sclerosis, and there was a positive correlation between IL-6 gene expression and CP [34].

Inducible nitric oxide synthase is formed when the activated NF-κB genes bind the 5′-flanking region of the iNOS promoter and induce iNOS formation [23]. The endogenous NO formed by iNOS from the amino acid L-arginine plays an important role in collagen formation during wound healing and fibrosis [35,36]. Various wound models had been studied to show the relationship of NO, iNOS and collagen formation. Wound irradiation significantly reduced iNOS expression, NO synthesis and CP in the fibroblasts from normal rats [37]. Inhibition of NO synthase in wound fibroblasts from polyvinyl alcohol sponges implanted in rats significantly decreased collagen synthesis [38]. Dermal fibroblasts from iNOS knock-out mice proliferated more slowly and synthesized less collagen, and NO donors restored the collagen synthesis to normal level [39]. In vivo studies in rats, however, demonstrated that long-term treatment with an iNOS inhibitor led to fibrosis of the heart and kidney. It was hypothesized that NO scavenged reactive oxygen species by formation of peroxynitrite, and blocking the formation of NO by iNOS inhibition contributed to the development of fibrosis [35]. Therefore, appropriate induction of iNOS from the NF-κB signaling pathway may also be a critical mechanism controlling collagen formation in both fibrosis and proper wound healing.

Monocyte chemoattractant protein-1 has an important role in the regional exudation of monocytes and macrophages in chronic inflammatory diseases and may be

induced by the binding of NF-κB to its enhancer region [40]. Enrichment of MCP-1 increased α1(I) collagen mRNA expression in normal dermal fibroblasts and an anti-MCP-1 neutralizing antibody decreased the skin collagen content and type I collagen mRNA expression in a dermal sclerosis murine model [41]. The expression of α1 (I) collagen mRNA was induced by MCP-1 in both rat lung fibroblasts [42] and normal human skin fibroblasts in a dose-dependent manner [43]. Experiments also showed that MCP-1 was involved in the fibrosis of lung [44], kidney [45] and skin wound healing process [46].

The incorporation of ^{3}H-proline by fibroblasts was measured to indicate the collagen formation with same treatments as those used for gene profiling. Our results demonstrate that LPS stimulated collagen formation for all treatments (EPA, AA, BSA control). Lipopolysaccharide treatment significantly increased both the CP and C-PTP compared to the non-LPS-treated fibroblasts. Parthenolide decreased CP to control levels for all groups and C-PTP to control levels in EPA- and AA-treated cells. With similar treatments, EPA-enriched fibroblasts produced more CP and C-PTP than AA-treated cells. Our previous study showed that EPA-treated 3T3 fibroblasts nonstimulated with LPS produced more collagen than AA-treated cells, and that this was due to regulation of collagen formation via PGE_2 receptor subtype (EP) signaling. Eicosapentaenoic acid-treated cells responded to PGE_2 via EP2 and EP4 receptors but AA-treated cells through EP1, EP2 and EP4 receptors. EP1 signaling increases protein kinase C (PKC) activity, whereas EP2 and EP4 signaling is mediated by an increase in cAMP [10].

Stimulation by LPS activates the NF-κB pathway, which can in turn induce collagen formation through responsive genes such as IL-6, iNOS and MCP-1. Moreover, our results showed significant ($P<.05$) correlations between the CP or the C-PTP and the expression in some NF-κB-related genes, such as the LPS transmembrane receptors (Tlr4) (CP, $R=.702$; C-PTP, $R=.654$) and adaptor proteins (Myd88 and Traf6) (Myd88 with CP, $R=.618$; Traf6 with CP, $R=.618$; Traf6 with C-PTP, $R=.694$). These changes in CP and gene expression were induced by treatment with EPA and AA. It is unknown if treatment with the 18 carbon n-3 and n-6 precursors (linolenic acid, 18:3 n-3; and linoleic acid, 18:2 n-6) or other combinations of PUFA would have similar results. In our previous study, fatty acid analysis of 3T3 fibroblasts treated with AA or EPA indicated significantly different amount of AA and EPA in the cells, as expected. However, cells treated with EPA also had significantly more docosapentaenoic acid (DPA; 22:5 n-3) than cells treated with AA. There were also no significant differences in the amounts of 16:1 n-7, 18:0, 18:1 n-9, 18:2 n-6 or 22:6 n-3 among the treatments [10]. Therefore, the changes in CP and gene expression are likely to be directly related to the amount of AA and EPA enrichment, and possibly to DPA, 22:5 n-3. Future experiments using treatments with DPA alone would determine if this fatty acid is contributing to the effect on CP by altering signaling from the LPS receptor or the expression of other genes in the signaling pathway.

Collectively, these data indicate that AA and EPA have the ability to directly or indirectly modify collagen formation by altering the expression of numerous NF-κB pathway-specific genes. These in vitro results may not be representative of what would occur in vivo or in other cell culture systems. The effect of fatty acids on gene expression may be individualized to different cell types. Linoleic acid (18:2 n-6) treatment of human microvascular endothelial cells led to an activation of NF-κB through induction of oxidative stress [12]. In human myotubes, the fatty acid palmitate led to activation of NF-κB through a proteosome dependent mechanism, but unsaturated fatty acids did not have this effect [47]. Therefore, the effects of PUFA on NF-κB activation and CP may be dependent upon the target tissue or organ or the type of stimulus. In summary, this study provides evidence that PUFA can alter CP in fibroblasts through an NF-κB-dependent pathway. In vivo experiments will be necessary to determine the efficacy of PUFA as adjuvants for control of collagen formation.

Acknowledgments

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References

[1] Hefti FL, Annetrudi K, Fasel J, Morscher EW. Healing of the transected anterior cruciate ligament in the rabbit. J Bone Joint Surg 1995;73A:373–83.
[2] Ghosh AK. Factors involved in the regulation of Type I collagen gene expression: Implication in fibrosis. Exp Biol Med 2002;227: 301–14.
[3] Hankenson KD, Watkins BA, Schoenlein IA, Allen KGD, Turek JJ. Omega-3 fatty acids enhance ligament fibroblast collagen formation in association with changes in interleukin-6 production. Exp Biol Med 2000;223:88–95.
[4] Varga J, Diaz-Perez A, Rosenbloom J, Jimenez SA. PGE2 causes a coordinate decrease in the steady state levels of fibronectin and types I and III procollagen mRNAs in normal human dermal fibroblasts. Biochem Biophys Res Commun 1987;147:1282–8.
[5] Wang X, Smith P, Pu LL, Kim YJ, Ko F, Robson MC. Exogenous transforming growth factor beta(2) modulates collagen I and collagen III synthesis in proliferative scar xenografts in nude rats. J Surg Res 1999;87:194–200.
[6] Wang JF, Olson ME, Ball DK, Brigstock DR, Hart DA. Recombinant connective tissue growth factor modulates porcine skin fibroblast gene expression. Wound Repair Regen 2003;11:220–9.
[7] Oshiro W, Lou J, Xing X, Tu Y, Manske P. Flexor tendon healing in the rat: a histologic and gene expression study. J Hand Surg 2003; 28:814–23.
[8] Li S, Van Den Diepstraten C, D'Souza SJ, Chan BMC, Pickering JG. Vascular smooth muscle cells orchestrate the assembly of type I collagen via {alpha}2{beta}1 integrin, rhoA, and fibronectin polymerization. Am J Pathol 2003;163:1045–56.
[9] Watkins BA, Xu H, Turek JJ. Linoleate impairs collagen synthesis in primary cultures of avian chondrocytes. Proc Soc Exp Biol Med 1996;212:153–9.

[10] Jia Y, Turek JJ. Polyenoic fatty acid ratios alter fibroblast collagen production via PGE_2 and PGE receptor subtype response. Exp Biol Med 2004;229:676–83.
[11] Hennig B, Toborek M, Joshi-Barve S, Barger SW, Barve S, Mattson MP, et al. Linoleic acid activates nuclear transcription factor-kappaB (NF-kappaB) and induces NF-kappaB-dependent transcription in cultured endothelial cells. Am J Clin Nutr 1996;63:322–8.
[12] Park HJ, Lee YW, Hennig B, Toborek M. Linoleic acid-induced VCAM-1 expression in human microvascular endothelial cells is mediated by the NF-kappa B-dependent pathway. Nutr Cancer 2001; 41:126–34.
[13] Zhang YH, Lin JX, Vilcek J. Interleukin-6 induction by tumor necrosis factor and interleukin-1 in human fibroblasts involves activation of a nuclear factor binding to a kappa B-like sequence. Mol Cell Biol 1990;10:3818–23.
[14] Shimizu H, Mitomo K, Watanabe T, Okamoto S, Yamamoto K. Involvement of a NF-kappa B-like transcription factor in the activation of the interleukin-6 gene by inflammatory lymphokines. Mol Cell Biol 1990;10:561–8.
[15] Liou HC. Regulation of the immune system by NF-kappaB and IkappaB. J Biochem Mol Biol 2002;35:537–46.
[16] Hill C, Flyvbjerg A, Rasch R, Bak M, Logan A. Transforming growth factor-beta2 antibody attenuates fibrosis in the experimental diabetic rat kidney. J Endocrinol 2001;170:647–51.
[17] Khadaroo RG, Marshall JC. ARDS and the multiple organ dysfunction syndrome. Common mechanisms of a common systemic process. Crit Care Clin 2002;18:127–41.
[18] Tomashefski Jr JF. Pulmonary pathology of acute respiratory distress syndrome. Clin Chest Med 2000;21(3):435–66.
[19] Kanzler S, Baumann M, Schirmacher P, Dries V, Bayer E, Gerken G, et al. Prediction of progressive liver fibrosis in hepatitis C infection by serum and tissue levels of transforming growth factor-beta. J Viral Hepat 2001;8:430–7.
[20] Frank C, Amiel D, Woo S-Y, Akeson W. Normal ligament properties and ligament healing. Clin Orthop Relat Res 1985;196:15–25.
[21] Clayton ML, Miles JS, Abdulla M. Experimental investigations of ligamentous healing. Clin Orthop Relat Res 1968;61:146–53.
[22] Akeson WH, Garfin S, Amiel D, Woo SL-Y. Para-articular connective tissue in osteoarthritis. Semin Arthritis Rheum 1989;18:41–50.
[23] Iwai-Kanai E, Hasegawa K, Fujita M, Araki M, Yanazume T, Adachi S, et al. Basic fibroblast growth factor protects cardiac myocytes from iNOS-mediated apoptosis. J Cell Physiol 2002;190:54–62.
[24] Kouba DJ, Chung KY, Nishiyama T, Vindevoghel L, Kon A, Klement JF, et al. Nuclear factor-kappa B mediates TNF-alpha inhibitory effect on alpha 2(I) collagen (COL1A2) gene transcription in human dermal fibroblasts. J Immunol 1999;162:4226–34.
[25] Zhang G, Ghosh S. Molecular mechanisms of NF-kappaB activation induced by bacterial lipopolysaccharide through Toll-like receptors. J Endotoxin Res 2000;6:453–7.
[26] Albert SB. The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol 1996;14:649–81.
[27] Hehner SP, Hofmann TG, Droge W, Schmitz ML. The antiinflammatory sesquiterpene lactone parthenolide inhibits NF-kappa B by targeting the I kappa B kinase complex. J Immunol 1999;163:5617–23.
[28] Hwang D, Fischer NH, Jang BC, Tak H, Kim JK, Lee W. Inhibition of the expression of inducible cyclooxygenase and proinflammatory cytokines by sesquiterpene lactones in macrophages correlates with the inhibition of MAP kinases. Biochem Biophys Res Commun 1996; 226:810–8.
[29] Sobota R, Szwed M, Kasza A, Bugno M, Kordula T. Parthenolide inhibits activation of signal transducers and activators of transcription (STATs) induced by cytokines of the IL-6 family. Biochem Biophys Res Commun 2000;267:329–33.
[30] Duncan MR, Berman B. Stimulation of collagen and glycosaminoglycan production in cultured human adult dermal fibroblasts by recombinant human interleukin 6. J Invest Dermatol 1991;97:686–92.
[31] Ito A, Itoh Y, Sasaguri Y, Morimatsu M, Mori Y. Effects of interleukin-6 on the metabolism of connective tissue components in rheumatoid synovial fibroblasts. Arthritis Rheum 1992;35:1197–201.
[32] Choi I, Kang HS, Yang Y, Pyun KH. IL-6 induces hepatic inflammation and collagen synthesis in vivo. Clin Exp Immunol 1994;95: 530–5.
[33] Lin ZQ, Kondo T, Ishida Y, Takayasu T, Mukaida N. Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. J Leukoc Biol 2003; 73:713–21.
[34] Zurita-Salinas CS, Richaud-Patin Y, Krotzsch-Gomez E, Llorente L, Alcocer-Varela J, Diaz-de-Leon L, et al. Spontaneous cytokine gene expression by cultured skin fibroblasts of systemic sclerosis. Correlation with collagen synthesis. Rev Invest Clin 1998; 50:97–104.
[35] Ferrini MG, Vernet D, Magee TR, Shahed A, Qian A, Rajfer J, et al. Antifibrotic role of inducible nitric oxide synthase. Nitric Oxide 2002;6:283–94.
[36] Norrby K. Constitutively synthesized nitric oxide is a physiological negative regulator of mammalian angiogenesis mediated by basic fibroblast growth factor. Int J Exp Pathol 2000;81:423–7.
[37] Schaffer M, Weimer W, Wider S, Stulten C, Bongartz M, Budach W, et al. Differential expression of inflammatory mediators in radiation-impaired wound healing. J Surg Res 2002;107:93–100.
[38] Schaffer MR, Efron PA, Thornton FJ, Klingel K, Gross SS, Barbul A. Nitric oxide, an autocrine regulator of wound fibroblast synthetic function. J Immunol 1997;158:2375–81.
[39] Shi HP, Efron DT, Most D, Barbul A. The role of iNOS in wound healing. Surgery 2001;130:225–9.
[40] Ueda A, Ishigatsubo Y, Okubo T, Oshimura T. Transcriptional regulation of the human monocyte chemoattractant protein-1 gene. Cooperation of two NF-kappa B sites and NF-kappa B/Rel subunit specificity. J Biol Chem 1997;272:31092–9.
[41] Yamamoto T, Ishioka K. Role of monocyte chemoattractant protein-1 and its receptor, CCR-2, in the pathogenesis of bleomycin-induced scleroderma. J Invest Dermatol 2003;121:510–6.
[42] Gharaee-Kermani M, Denholm EM, Phan SH. Costimulation of fibroblast collagen and transforming growth factor beta 1 gene expression by monocyte chemoattractant protein-1 via specific receptors. J Biol Chem 1996;271:17779–84.
[43] Takeuchi A, Isobe K, Miyaishi O, Sawada M, Fan ZH, Nakashima I, et al. Microglial NO induces delayed neuronal death following acute injury in the striatum. Eur J Neurosci 1998;10:1613–20.
[44] Gharaee-Kermani M, McCullumsmith RE, Charo IF, Kunkel SL, Phan SH. CC-chemokine receptor 2 required for bleomycin-induced pulmonary fibrosis. Cytokine 2003;24:266–76.
[45] Lloyd CM, Minto AW, Dorf ME, Proudfoot A, Wells TNC, Salant DJ, et al. RANTES and monocyte chemoattractant protein-1 (MCP-1) play an important role in the inflammatory phase of crescentic nephritis, but only MCP-1 Is involved in crescent formation and interstitial fibrosis. J Exp Med 1997;185:1371–80.
[46] Habibipour S, Oswald TM, Zhang F, Joshi P, Zhou XC, Dorsett-Martin W, et al. Effect of sodium diphenylhydantoin on skin wound healing in rats. Plast Reconstr Surg 2003;112:1620–7.
[47] Weigert C, Brodbeck K, Staiger H, Kausch C, Machicao F, Haring HU, et al. Palmitate, but not unsaturated fatty acids, induces the expression of interleukin-6 in human myotubes through proteasome-dependent activation of nuclear factor-kappaB. J Biol Chem 2004; 279:23942–52.
[48] Palsson-McDermott EM, O'Neill LA. Signal transduction by the lipopolysaccharide receptor. Toll-like receptor-4. Immunology 2004; 113:153–62.
[49] Liu S, Gallo DJ, Green AM, Williams DL, Gong X, Shapiro RA, et al. Role of toll-like receptors in changes in gene expression and NF-kappa B activation in mouse hepatocytes stimulated with lipopolysaccharide. Infect Immun 2002;70:3433–42.