Polyenoic Fatty Acid Ratios Alter Fibroblast Collagen Production *Via* PGE_2 and PGE Receptor Subtype Response

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Previous experiments have shown that dietary *n*-6 and *n*-3 polyenoic fatty acids (PFA) have different effects on collagen production, a process that may be related to the formation of prostaglandins (PG). This study tested the hypothesis that fibroblast collagen production could be regulated by different *n*-6:*n*-3 PFA ratios and that the effects were mediated by PGE_2 and altered signaling via the different PGE receptor subtypes. Compared to a bovine serum albumin control, eicosapentaenoic acid (EPA; 20:5 *n*-3) treated cells significantly ($P < 0.05$) increased both collagen production and collagen as a percentage of total cellular protein (C-PTP), but arachidonic acid (AA; 20:4 *n*-6) reduced collagen production and C-PTP. As the amount of AA decreased and that of EPA increased, collagen production and C-PTP increased, especially when ratio of *n*-6:*n*-3 PFA was less than 1:1. C-PTP was significantly correlated with the amount of PGE_2 in the medium. AA- or EPA-treated cells produced similar C-PTP when incubated with 10^{-6} *M* indomethacin, a cyclooxygenase inhibitor. Addition of exogenous PGE_2 to cell cultures treated with 10^{-6} *M* indomethacin for 48 hrs decreased C-PTP in both AA and EPA groups. Decreased C-PTP was observed in AA-treated cells exposed to EP1, EP2, and EP4 PGE receptor agonists and in EPA-treated cells exposed to EP2 and EP4 agonists. AA-treated cell responded to activators of cyclic adenosine monophosphate and protein kinase C by decreasing C-PTP, but EPA-treated cells were unresponsive. In conclusion, collagen production in 3T3-Swiss fibroblasts induced by different *n*-6:*n*-3 PFA ratios was correlated with PGE_2 production and altered responsiveness and signaling *via* the different PGE receptor subtypes. Exp Biol Med 229:676–683, 2004

Key words: collagen; PGE_2; omega-3 fatty acids; omega-6 fatty acids; fibroblast

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It is essential to control collagen formation in the therapies of fibroproliferative diseases (1) and for proper healing of injuries (2). Although some mechanisms for the regulation of type I collagen gene expression have been identified (3), multiple factors such as transforming growth factor beta (4), connective tissue growth factor (5), matrix metalloproteinases (6), and integrins (7) influence collagen production.

The different effects of dietary *n*-3 and *n*-6 polyenoic fatty acids (PFAs) on collagen formation provide a promising approach to study these mechanisms. Previous studies identified that linoleic acid (18:2 *n*-6) suppressed collagen formation in avian chondocytes (8). In porcine medial collateral ligament fibroblasts, eicosapentaenoic acid (EPA; 20:5 *n*-3) increased collagen production (CP), and the amount of collagen as a percentage of total cell protein (C-PTP) and arachidonic acid (AA; 20:4 *n*-6) decreased them. The prostaglandin E_2 (PGE_2) production was increased in AA-treated cells and decreased in EPA-treated cells (9). Other studies showed that PGs can suppress type I collagen gene expression in fibroblasts at the transcriptional level (10). These results provide evidence that the effects of *n*-3 and *n*-6 PFAs on collagen production may be partly associated with PGE_2.

The various biological effects of PGE_2 are mediated primarily through an autocrine G-protein PGE receptor that exists as four subtypes, EP1, EP2, EP3, and EP4. These receptors are associated with different signal transduction pathways including different messenger molecules, such as cyclic adenosine monophosphate (cAMP), protein kinase C (PKC), and calcium (11). The fatty acid–induced changes in collagen production that are mediated by PGE_2 may be linked to different EP receptor activities and production of corresponding messengers.

The purpose of this study was to test the hypothesis that collagen production in murine 3T3-Swiss fibroblasts could be regulated by exposure to different *n*-6:*n*-3 PFA ratios and that these effects were mediated, in part, by PGE_2 and changes in the signaling *via* the different PGE receptor subtypes.

Material and Methods

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

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, 4 m*M* L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, and 10% bovine calf serum (Hyclone, Logan, UT). To test the effects of fatty acids on collagen formation, subconfluent cultures grown for 24 hrs 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 fatty acid–enriched test media were supplemented with different ratios of *n*-6 and *n*-3 BSA-loaded fatty acid soaps with a final concentration of 25 μ*M*. The test media were supplemented with different ratios of AA to EPA (1:25, 1:10, 1:5, 1:1, 5:1, 10:1, and 25:1) or AA and EPA alone. Cells were grown for 48 hrs in the test media before the addition of treatments, described below.

Collagen Formation. After the initial 48-hr PFA enrichment, the media was replaced with fresh fatty acid–enriched medium containing 50 μ*M* ascorbic acid and 5 μCi of ^{3}H-proline (Amersham, Arlington Heights, IL), with or without treatments for 24 hrs. Cells were harvested and assayed for collagen, total protein, and DNA.

Treatment with PGE_2, PGE_2 Agonists and Activators, and Inhibitors of cAMP, PKC, and Calcium. These treatments were performed to determine whether fatty acids altered the response of cells to PGE_2 *via* the PGE receptors (EP receptors) and to confirm whether there were differences in the signaling from the EP receptors. Cells for this experiment were treated with 10^{-6} *M* indomethacin (INDO) during the 48-hr fatty acid enrichment to suppress endogenous PGE_2 production. After the 48-hr fatty acid enrichment, cells were washed and then incubated with fresh fatty acid–enriched medium containing 50 μ*M* ascorbic acid and 5 μCi of ^{3}H-proline plus the following treatments for 24 hrs before the cells were harvested. For EP receptor agonist treatments, all cells were incubated with the following reagents at 10^{-6}, 10^{-8}, and 10^{-10} *M*: INDO as a baseline control, exogenous PGE_2 (Cayman Chemical, Ann Arbor, MI) and different EP receptor agonists (EP1, 17-phenyl-trinor-PGE_2; EP2, butaprost; EP3, sulprostone; EP2-4, misoprostol; Cayman Chemical, Ann Arbor, MI). To confirm that the signaling from the EP receptors was altered by fatty acid enrichment, cells were treated with cAMP activator, forskolin 250 μ*M*; cAMP inhibitor, SQ22536 1 m*M* (Signal Transduction Products, San Clemente, CA); PKC activator, phorbol 12-myristate 13-acetate (PMA, 10 μ*M*); PKC inhibitor H-7 (A.G. Scientific, San Diego, CA), 10 μ*M*; calcium activator, calcium ionophore (A23187, 10 μ*M*); and calcium chelator/inhibitor, ethylene glycol bis(2-aminoethyl ether)-N,N,N′ N′-tetraacetic acid (EGTA, 1 m*M*). At the end of the experiment, cells were harvested to determine collagen production, total protein, and DNA.

Fatty Acid Analysis. To verify the incorporation of the fatty acids into the cell membranes, fatty acid compositions were analyzed as previously described (8). Briefly, cells were harvested using a cell-scraper and were pelleted and suspended in 100% methanol and quenched with liquid N_2 before freezing at −70°C. Lipids were extracted with chloroform/methanol (2:1, vol./vol.), and polar lipids were isolated by solid-phase extraction. Lipids were saponified and fatty acid methyl esters prepared using 14% boron trifluoride in methanol. Fatty acid methyl esters were analyzed using a gas chromatography and were identified by comparison of their retention times with those of standards (GLC 422; Nu-Chek-Prep, Elysian, MN). Fatty acid compositions were expressed as area percentages.

Collagen Assay. Collagen was assayed as described previously (9). The media were collected and the cells washed twice with cold phosphate buffered saline. The cells were pelleted by centrifugation, and the phosphate-buffered saline wash was combined with the media 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 media fraction. The two fractions, cell and media, 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 was incubated for 6 hrs at 37°C with protease-free type VII collagenase in TES; the other half of the solution served as a control. Following the digestion, TCA was again added to precipitate the acid-insoluble proteins; however, the collagen fragments generated by collagenase treatment remained in solution. The supernatant and precipitate were counted in a scintillation counter, and collagen production, noncollagenous production, and total protein production were reported as decays per min (DPM)/μg DNA.

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 lysate or 50 μl 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 were used to obtain the unknown values.

PGE_2 Assay. Quantitation of total PGE_2 produced by 3T3-Swiss fibroblast cells was by an ELISA kit (Monoclonal PGE_2 EIA Kit-514010; Cayman Chemical, Ann

Table 1. Fatty Acid Composition (Mean ± SEM) of 3T3-Swiss Fibroblasts (Area%)[a]

	BSA	EPA	AA
20:4 *n*-6	14.0 ± 3.2^{ab}	3.7 ± 0.5^{a}	38.7 ± 0.9^{c}
20:5 *n*-3	1.1 ± 0.6^{a}	26.0 ± 1.9^{c}	ND
22:4 *n*-6	0.9 ± 0.2^{a}	0.24 ± 0.2^{a}	4.9 ± 1.1^{b}
22:5 *n*-3	1.6 ± 1.3^{ab}	6.7 ± 0.4^{c}	0.08 ± 0.14^{a}
n-6 polyenoic fatty acid	28.0 ± 2.7^{a}	8.0 ± 0.6^{b}	47.5 ± 0.4^{c}
n-3 polyenoic fatty acid	4.5 ± 1.4^{ab}	34.1 ± 2.8^{c}	1.04 ± 0.1^{a}
n-6:*n*-3	6.7 ± 2.5^{c}	0.2 ± 0.01^{b}	46.2 ± 5.4^{a}

[a] Cells were treated with bovine serum albumin-soap loaded fatty acids (25 μ*M*) for 72 hrs and then harvested for fatty acid analysis. Mean values (n = 3) within rows having different superscripts are significantly different ($P < 0.05$) by one-way ANOVA and Tukey test. BSA, bovine serum albumin; EPA, eicosapentaenoic acid; AA, arachidonic acid; ND, not detected; PFA, polyunsaturated fatty acids. The fatty acid analysis from cells treated with different ratios of *n*-6 and *n*-3 fatty acids is not shown.

Arbor, MI), according to the manufacturer's recommended protocol.

Statistical Analysis. Data were presented as means ± SEM 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). $P < 0.05$ was considered statistically significant.

Results

Fatty Acid Analysis of 3T3-Swiss Fibroblasts. The cellular fatty acid compositions were significantly altered in the lipid content analysis of 3T3-Swiss fibroblasts when they were exposed to different enrichments (Table 1). Cells that were treated with an increased ratio of *n*-6 and *n*-3 fatty acid had significantly increased levels of 20:4, 22:4, and total *n*-6 fatty acids and decreased levels of 20:5, 22:5, and total *n*-3 fatty acids compared with the BSA control ($P < 0.05$). The amount of total *n*-6 and *n*-3 fatty acids in the cells was significantly correlated ($P < 0.05$) with the amount of AA ($r = 0.741$ for *n*-6 and –0.736 for *n*-3) and EPA ($r = -0.817$ for *n*-6 and 0.926 for *n*-3) in the medium.

Effect of *n*-6:*n*-3 Fatty Acid Ratio on Collagen Production. EPA-treated cells had increased CP and collagen as a percentage of total protein (C-PTP) compared with AA-treated cells (Fig. 1). EPA-treated cells produced significantly more collagen than the BSA control (EPA; 5448 ± 652 DPM/μg DNA and BSA; 3374 ± 198 DPM/μg DNA, $P = 0.046$). The amount of collagen produced by AA-treated cells (969 ± 32 DPM/μg) was significantly lower ($P = 0.014$) than the BSA control and the EPA-treated cells ($P < 0.001$). We also analyzed the ^{3}H-proline incorporation into the amount of total acid-insoluble protein (TP) and the interaction effects for C-PTP. For TP, BSA-control cells produced significantly more protein than AA-treated cells (BSA; 14,769 ± 557 DPM/μg DNA and AA; 11,155 ± 399 DPM/μg DNA, $P < 0.001$). EPA-treated cells were not significantly different from the control but were significantly greater than AA-treated cells (13,750 ± 208 DPM/μg DNA; $P < 0.001$). EPA-enriched cells produced significantly more ($P < 0.001$) C-PTP than the BSA-control (EPA; 39.5% ± 4.1% and BSA; 22.9% ± 1.7%, $P = 0.008$; Fig. 2). The amount of collagen as a percentage of total protein in AA-treated cells (8.7% ± 0.2%) was significantly lower than the BSA-control ($P = 0.043$) and the EPA-treated cells ($P < 0.001$).

Different ratios of *n*-6 and *n*-3 fatty acid were used to determine the effect of PFAs on collagen formation. As the amount of EPA increased and AA decreased, the TP, CP, and collagen as a percentage of total protein increased, especially when ratio of *n*-6:*n*-3 PFAs was less than 1:1. The correlation analysis compared AA and EPA amounts with CP and collagen as a percentage of total protein (C-PTP). Both AA and EPA treatments had significant ($P < 0.001$) correlation with CP ($r = -0.838$ for AA and $r = 0.735$ for EPA) and C-PTP ($r = -0.834$ for AA and $r = 0.797$ for EPA), and there was a significant correlation ($r = 0.992$) between CP and C-PTP.

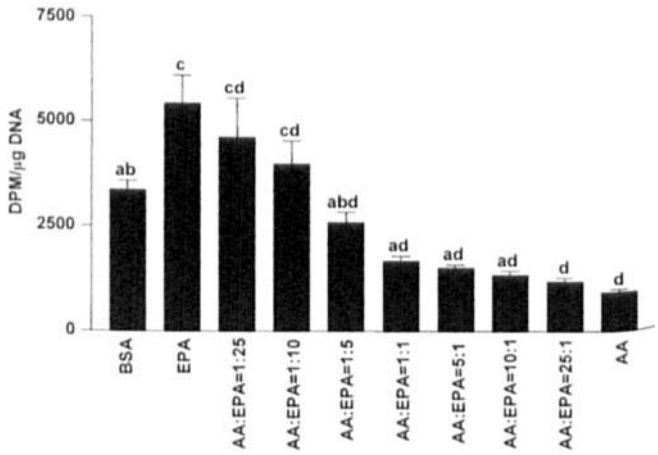

Figure 1. Effect of different fatty acid ratios on collagen production by 3T3-Swiss fibroblast (n = 3, ± SEM). Cells were treated with bovine serum albumin-soap loaded fatty acids (25 μ*M*) for 72 hrs. The amount of collagen is expressed as decays per min (DPM) from ^{3}H-proline per microgram of DNA. Bars with different letters are significantly different ($P < 0.05$). BSA, bovine serum albumin; EPA, eicosapentaenoic acid; AA, arachidonic acid.

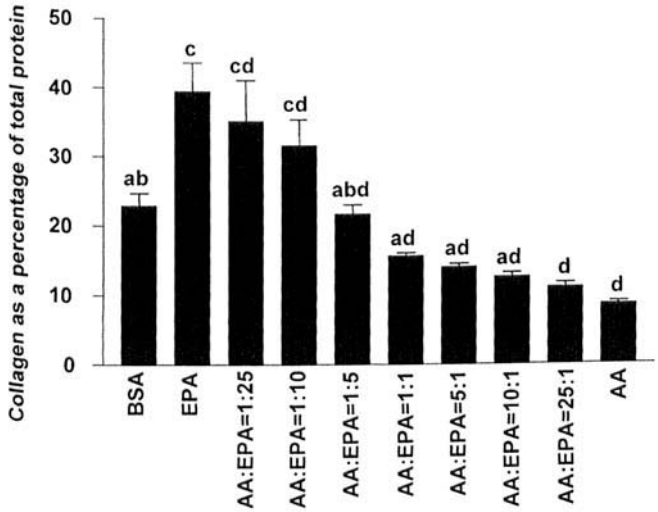

Figure 2. Effect of different fatty acid ratios on collagen production as a percentage of total protein by 3T3-Swiss fibroblast (n = 3, ± SEM). Cells were treated with bovine serum albumin–soap-loaded fatty acids (25 μM) for 72 hrs. The amount of collagen and total protein were originally determined as decays per min (DPM) from ^{3}H-proline per microgram of DNA. Bars with different letters are significantly different ($P < 0.05$). BSA, bovine serum albumin; EPA, eicosapentaenoic acid; AA, arachidonic acid.

Effect of INDO and Exogenous PGE_2. To determine the interaction of cyclooxygenase (COX) and PGE_2 in collagen formation, we exposed the AA-, EPA-, and BSA-treated cells to different concentrations (10^{-6}, 10^{-8}, and 10^{-10} M) of INDO, a COX inhibitor, for 72 hrs (Fig. 3). When treated with a concentration of INDO greater than

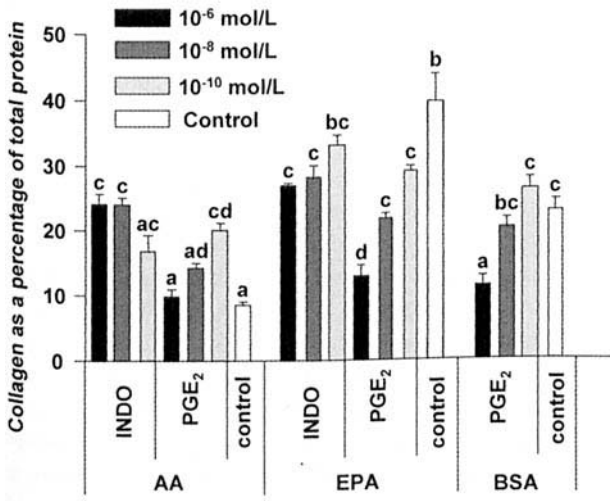

Figure 3. The effect of indomethacin (INDO) and exogenous prostaglandin E_2 (PGE_2) on collagen as a percentage of total protein in 3T3-Swiss fibroblasts (n = 3, ± SEM). Cells incubated with INDO alone were treated for 72 hrs with bovine serum albumin–soap-loaded fatty acids (25 μM) and INDO at 10^{-6}, 10^{-8}, and 10^{-10} M. Cells treated with PGE_2 were incubated with fatty acids and 10^{-6} M INDO for 48 hrs, and then fresh fatty acid–enriched medium added containing PGE_2 at 10^{-6}, 10^{-8}, and 10^{-10} M for an additional 24 hrs. Bars with different letters are significantly different ($P < 0.05$). BSA, bovine serum albumin; EPA, eicosapentaenoic acid; AA, arachidonic acid. The control was cells treated with AA or EPA alone.

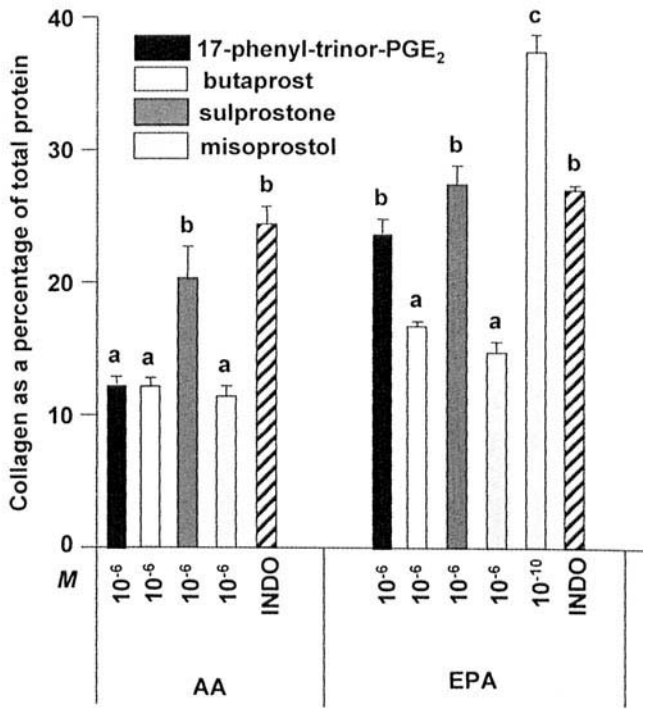

Figure 4. Effect of prostaglandin E receptor (EP) agonists on collagen as a percentage of total cellular protein by 3T3-Swiss fibroblasts (n = 3, ± SEM). Cells were treated for 48 hrs with bovine serum albumin–soap-loaded fatty acids (25 μM) and indomethacin (INDO, 10^{-6} M). The media were then replaced with fresh fatty acid–enriched medium containing the EP agonists 17-phenyl-trinor-prostaglandin E_2 (EP1 agonist), butaprost (EP2 agonist), sulprostone (EP3 agonist), and misoprostol (EP4 agonist) each at 10^{-6}, 10^{-8}, and 10^{-10} M. Bars with different letters are significantly different ($P < 0.05$). EPA, eicosapentaenoic acid; AA, arachidonic acid; INDO, indomethacin. Only significant results are shown.

10^{-8} M, AA-treated cells significantly increased and EPA-treated cells significantly decreased collagen as a percentage of C-PTP ($P < 0.001$) compared with cells without INDO. There was no significant change in BSA-control cells. Treatment with 10^{-6} M INDO blocked most fatty acid–induced changes in C-PTP and approached the BSA control level in EPA- and AA-enriched cells (AA: 24.3% ± 1.3%; EPA: 27.1% ± 0.3%; and BSA: 26.5% ± 1.6%).

Furthermore, to determine the interaction of PGE_2 with collagen formation, exogenous PGE_2 (10^{-6}, 10^{-8}, and 10^{-10} M) was added to AA-, EPA-, and BSA-treated cells for 24 hrs after preincubation with 10^{-6} M INDO for 48 hrs to retard the production of endogenous PGE_2. All groups treated with 10^{-6} M PGE_2 produced significantly less ($P < 0.05$) C-PTP than cells treated with 10^{-6} M INDO alone. AA-treated cells also had significantly ($P = 0.011$) reduced C-PTP when treated with 10^{-8} M PGE_2. The AA-treated cells had significantly less C-PTP than EPA-treated cells in 10^{-10} M PGE_2 (AA: 20.1% ± 0.9%; EPA: 29.2% ± 0.6%, $P = 0.038$), but not in 10^{-6} M and 10^{-8} M PGE_2.

Effect of Different EP Receptor Agonists. AA-treated cells had decreased collagen as a percentage of total protein (C-PTP) when treated with EP1, EP2, and EP4

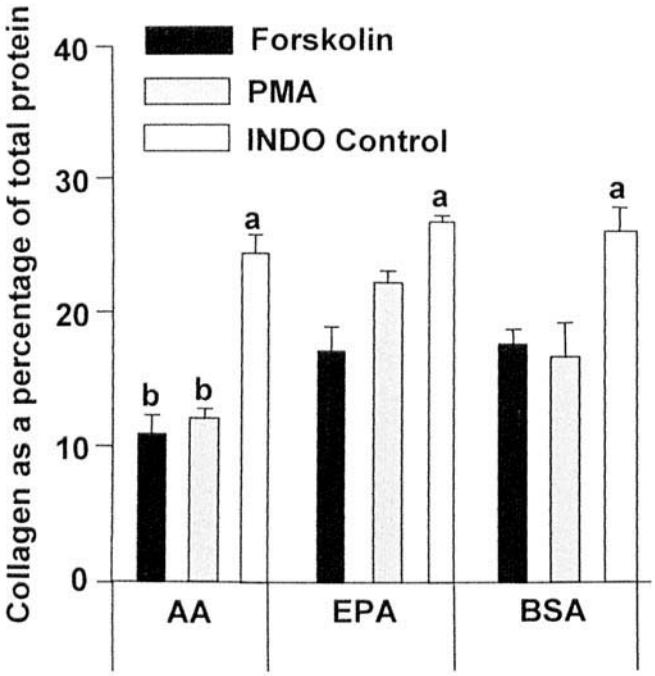

Figure 5. Effect of activators and inhibitors of cyclic adenosine monophosphate, protein kinase C, and calcium on collagen production as a percentage of total cellular protein by 3T3-Swiss fibroblasts (n = 3, ± SEM). Cells were treated for 48 hrs with bovine serum albumin–soap-loaded fatty acids (25 μ*M*) and indomethacin (10^{-6} *M*). Cells were then incubated with fresh fatty acid–enriched medium for an additional 24 hrs with the addition of cyclic adenosine monophosphate activator, forskolin 250 μ*M*; cyclic adenosine monophosphate inhibitor, SQ22536 1 m*M*; protein kinase C activator, phorbol 12-myristate 13-acetate 10 μ*M*; protein kinase C inhibitor H-7 10 μ*M*; calcium activator, calcium ionophore (A23187) 10 μ*M*; Calcium chelator/inhibitor, EGTA,) 1 m*M*. BSA, bovine serum albumin; EPA, eicosapentaenoic acid; AA, arachidonic acid. The controls were cells treated with AA or EPA plus INDO (10^{-6} *M*). Bars with different letters are significantly different ($P < 0.05$). Only significant results are shown.

agonists. However, EPA-treated cells had decreased C-PTP only when treated with EP2 and EP4 agonists (Fig. 4). Compared with the baseline control (cells treated for 72 hours with 10^{-6} *M* INDO only), AA-treated cells had significantly decreased C-PTP when exposed to 10^{-6} *M* 17-phenyl-trinor-PGE_2 (EP1 agonist, 12.3% ± 0.6%, P = 0.036), butaprost (EP2 agonist, 12.1% ± 0.6%, P = 0.029), or misoprostol (EP4 agonist, 11.4% ± 0.8%, P = 0.015). EPA-treated cells had significantly decreased C-PTP when exposed to 10^{-6} *M* butaprost (17.0% ± 0.4%, P = 0.041) or misoprostol (14.7% ± 0.8%, P = 0.004). Interestingly, EPA-treated cells increased C-PTP when exposed to 10^{-10} *M* misoprostol (37.4 ± 1.3%, P = 0.040). The EP3 agonist sulprostone did not significantly change C-PTP for either AA or EPA treatments.

Effect of Activators and Inhibitors of cAMP, PKC, and Calcium. Activation of the EP receptor subtypes induces different second-message molecules, such as cAMP in EP2, EP3, and EP4 and PKC and calcium in EP1. Only AA-treated cells exposed to forskolin, a cAMP activator, and phorbol 12-myristate

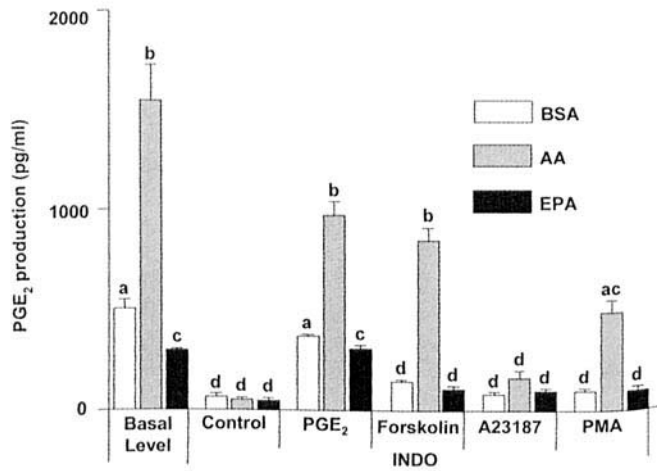

Figure 6. Effect of activators of cyclic adenosine monophosphate, protein kinase C, and calcium on prostaglandin E_2 production by 3T3-Swiss fibroblasts (n = 3, ± SEM). For the basal levels, cells were incubated with bovine serum albumin–soap-loaded fatty acids (25 μ*M*) or bovine serum albumin (BSA) alone. Cells for all other treatments were incubated for 48 hrs with BSA or the fatty acids plus 10^{-6} *M* indomethacin (INDO). The medium was then replaced with fresh fatty acid–enriched medium with INDO (control) or with the addition of prostaglandin E_2 (10^{-10} *M*); cyclic adenosine monophosphate activator, forskolin 250 μ*M*; calcium activator, calcium ionophore (A23187) 10 μ*M*; and protein kinase C activator, phorbol 12-myristate 13-acetate 10 μM for 24 hrs. Bars with different letters are significantly different ($P < 0.05$). EPA, eicosapentaenoic acid; AA, arachidonic acid; BSA, bovine serum albumin; INDO, indomethacin.

13-acetate (PMA), a PKC activator, produced significantly more C-PTP (forskolin: 10.9% ± 1.3%, P = 0.003; PMA: 12.2% ± 0.6%, P = 0.016) than cells treated with 10^{-6} *M* INDO alone (Fig. 5).

PGE_2 Assay. In all groups, 48 hrs pretreatment with 10^{-6} *M* INDO inhibited endogenous PGE_2 production (Fig. 6). AA-treated cells produced more PGE_2 compared with either BSA- or EPA-treated cells ($P < 0.001$; AA: 1552.9% ± 352.1 pg/ml; BSA: 509.5 ± 87.8 pg/ml; and EPA: 299.8 ± 17.1 pg/ml), and EPA-treated cells produced less than the BSA control ($P < 0.05$). When pretreated with INDO and changed to fresh medium with exogenous PGE_2 (10^{-10} *M*) or different pathway activators for 24 hrs, only AA-treated cells exposed to forskolin (250 μ*M*) or PMA (10 μ*M*) restored their PGE_2 production (850.3 ± 122.9 pg/ml in forskolin and 491.3 ± 121.1 pg/ml in PMA). This was a significant increase ($P < 0.001$) compared with those treated with INDO alone (61.5 ± 198.8 pg/ml). Moreover, the results from forskolin treatment were comparable to those using exogenous PGE_2 (977.5 ± 141.7 pg/ml). Correlation of the C-PTP with PGE_2 amount was significant (r = –0.505, $P < 0.001$).

Discussion

During the healing process, the requirement for collagen formation varies with the type of injury. Connective tissues require enhanced production of healthy

collagen without scarring, whereas vital organs need repair without fibrosis. In addition to PGE_2, collagen formation may be influenced by growth factors such as transforming growth factor beta (4) and connective tissue growth factor (5), as well as matrix metalloproteinases (6) and integrins (7). Therefore, potential therapies to control collagen formation may require an approach targeting multiple molecules or mechanisms.

We have observed that *n*-3 and *n*-6 PFA influence the gene expression of numerous growth factors, matrix metalloproteases, tissue inhibitors of matrix metalloproteases, integrins, and various transcription factors (12). For that reason, PFA-induced changes in collagen formation could potentially be used as an adjuvant for therapies designed to enhance healthy collagen production or reduce fibrosis. In our experiments, the *n*-3 PFA, eicosapentaenoic acid (EPA) significantly increased CP and collagen as a percentage of total protein (C-PTP) in 3T3-Swiss fibroblasts, although the total protein amount was less than the BSA control. AA, a typical *n*-6 PFA, decreased both the collagen production and collagen as a percentage of total cellular protein. Previous studies in our laboratory reported similar results with porcine ligament fibroblasts (9). Moreover, we also investigated the effects of different *n*-6:*n*-3 PFA ratios on collagen formation and found that collagen formation had a high positive correlation with the amount of EPA and a negative correlation with AA. When the *n*-6:*n*-3 PFA ratio was less than 1:1, collagen formation increased in a dose-dependent manner.

The changes in collagen formation by PFAs are regulated in part by COX and PGs as well as different cytokines (3). Increased PGE_2 can inhibit type I and III collagen formation (13), and the suppression of type I collagen gene expression is mediated at the transcriptional level (10). Our studies also showed significant negative correlation between collagen percentage and the amount of PGE_2. Because AA and EPA are progenitors of two-series and three-series PGs, respectively, we hypothesized that these PGs are involved in the different changes of collagen formation with PFA enrichments. As PG precursors, AA and EPA compete for COX, which can be inhibited effectively by INDO (14). Our experiments verified the hypothesis by the inhibition of endogenous PGE_2 by INDO and the inhibition of collagen formation by exogenous PGE_2. AA- and EPA-treated cells incubated with 10^{-6} *M* INDO produced amounts of C-PTP similar to the BSA control. Also, addition of exogenous PGE_2 significantly decreased C-PTP in both AA- and EPA-treated cells compared with INDO alone, but at 10^{-10} *M* PGE_2, EPA-treated cells produced more C-PTP than AA-treated cells. The COX product of EPA is PGE_3, and EPA competes with AA for this enzyme and reduces PGE_2 (15). The results of our PGE_2 assay indicated more PGE_2 production from AA-treated cells compared with EPA. *In vivo* studies in mice also showed similar effects of *n*-3 and *n*-6 PFAs on PGE_2 production (16).

The PGs derived from AA and EPA bind to four different PGE receptors (EP receptors) that act through different pathways. Activation of the EP1 receptor induces an influx of calcium and activates PKC through an unclear G protein–mediated pathway (17). The EP2 and EP4 receptors activate cAMP, whereas the EP3 receptor acts by inhibiting cAMP activation (11, 17, 18). In this study, there was a difference between *n*-6 and *n*-3 PFA-treated cells in their response when exposed to different EP agonists. Significant changes to collagen formation were found in AA-treated cells exposed to EP1, EP2, and EP4 agonists and in EPA-treated cells exposed to EP2 and EP4 agonists. Incubation with cAMP and PKC activators induced a collagen percentage decrease in AA groups but not in EPA groups. The 3T3-Swiss fibroblasts were unresponsive to EP3 receptor agonists, and neither AA- nor EPA-treated cells changed collagen formation when exposed to sulprostone (EP3 agonist). Fibroblasts from other sources have also been shown to be primarily responsive to EP1, EP2, and EP4 agonists. In human gingival fibroblasts, interleukin 1β–induced interleukin 6 production was regulated by EP1, EP2, and EP4 agonists (19), and in human embryo lung fibroblasts, expression of type I collagen α1 genes was primarily through the EP2 receptor (18). PGE_2 stimulated basic fibroblast growth factor mRNA expression is mainly controlled through EP1 or EP2 and EP4 receptors in normal human fibroblasts (20). Experiments about the effects of PFAs on gene expression also observed differences related to EP receptors. Sellmayer *et al.* (21) demonstrated that the effect of AA on early growth–related genes (*c-fos* and *Egr-1*) in 3T3-Swiss fibroblasts was mediated by PGE_2 and PKC via the EP1 receptor. Studies of AA-induced *c-fos* in human prostate cancer cells showed that the effect of PGE_2 was mediated via EP2 and EP4 receptors (22). The differences noted in the responsiveness of cells to activation of the EP receptors may be dependent on cell type.

Our studies indicated that EP1, EP2, and EP4 agonists similarly reduced collagen as a percentage of total protein (C-PTP) in AA-treated cells, but only EP2 and EP4 agonists reduced C-PTP in EPA-treated cells. Moreover, our experiments with activators and inhibitors of signaling molecules confirmed that AA- and EPA-treated cells differed in their response to EP agonists. The contribution of cAMP and PKC to PGE_2 production have also been seen in rat chondrocytes (23), human lung fibroblasts (24), and murine BALBc/3T3 fibroblasts (10). In our experiments, forskolin (cAMP activator) and PMA (PKC activator) decreased C-PTP compared with the INDO control in AA-treated cells. In EPA-treated cells, forskolin decreased C-PTP, but the effect was not significant ($P = 0.17$). In addition, the amount of PGE_2 in AA-treated cells increased a similar amount following the addition of forskolin as it did following the addition of exogenous PGE_2.

The effects of PGE_3 on collagen formation have not been studied. However, it is known that in NIH-3T3

fibroblasts, both PGE_3 and PGE_2 induced COX-2 mRNA *via* similar mechanisms, but PGE_3 may decrease COX-2 induction and PGE_2 synthesis through a negative-feedback loop whereas PGE_2 increases COX-2 expression *via* a positive feedback loop (15). *In vivo* studies of knockout mice for intestinal polyposis showed that AA stimulated more PGE_2 production by boosting COX-2 expression through the EP2 receptor via a positive feedback loop (25). The difference between PGE_3 and PGE_2 on COX-2 mRNA could be partially responsible for the interesting result that EPA-treated cells increased C-PTP in 10^{-10} *M* misoprostol (EP4 agonist). The EPA-induced collagen increase may result from PGE_3 binding to EP2 and EP4 receptors and down-regulating COX-2, resulting in decreased PGE_2. Alternatively, PGE_3 could compete with and inhibit the binding of PGE_2 to EP2 and EP4 receptors, or low levels of PGE_3 or EP4 agonists, and block PGE_2 without triggering a secondary messenger response from the receptors (acting as antagonists).

In addition to altered signaling from the EP receptors or differential responsiveness to prostanoid type, the activation of alternative pathways for collagen formation could also be a factor in our results. We have previously shown in porcine medial collateral ligament fibroblasts that increased collagen production in EPA-treated cells was positively correlated with increased interleukin 6 production, which has promoter elements for the transcription factor, NF-κB (9, 26). Activation of this transcription factor or other alternative pathways (27) *via* transforming growth factor beta signaling may result in the formation of some procollagen elements that can act as profibrotic agents and that are insensitive to EP1/EP3 activation or to treatment with forskolin and PMA in EPA-treated cells.

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1. Eickelberg O, Kohler E, Reichenberger F, Bertschin S, Woodtli T, Erne P, Roth M. Extracellular matrix deposition by primary human lung fibroblasts in response to TGF-1 and TGF-3. Am J Physiol Lung Cell Mol Physiol 276:L814–L824, 1999.
2. Dowdy PA, Miniaci A, Arnoczky SP, Fowler PJ, Boughner DR. The effect of cast immobilization on meniscal healing. An experimental study in the dog. Am J Sports Med 23:721–728, 1995.
3. Ghosh AK. Factors involved in the regulation of type I collagen gene expression: implication in fibrosis. Exp Biol Med 227:301–314, 2002.
4. Cutroneo KR. How is type I procollagen synthesis regulated at the gene level during tissue fibrosis. J Cell Biochem 90:1–5, 2003.
5. Wang JF, Olson ME, Ball DK, Brigstock DR, Hart DA. Recombinant connective tissue growth factor modulates porcine skin fibroblast gene expression. Wound Repair Regen 11:220–229, 2003.
6. 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 28:814–823, 2003.
7. 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 alpha2beta1 integrin, RhoA, and fibronectin polymerization. Am J Pathol 163:1045–1056, 2003.
8. Watkins BA, Xu H, Turek JJ. Linoleate impairs collagen synthesis in primary cultures of avian chondrocytes. Proc Soc Exp Biol Med 212:153–159, 1996.
9. Hankenson KD, Watkins BA, Schoenlein IA, Allen KG, Turek JJ. Omega-3 fatty acids enhance ligament fibroblast collagen formation in association with changes in interleukin-6 production. Exp Biol Med 223:88–95, 2000.
10. Riquet FB, Lai WF, Birkhead JR, Suen LF, Karsenty G, Goldring MB. Suppression of type I collagen gene expression by prostaglandins in fibroblasts is mediated at the transcriptional level. Mol Med 6:705–719, 2000.
11. Hwang D. Fatty acids and immune responses—a new perspective in searching for clues to mechanism. Ann Rev Nutr 20:431–456, 2000.
12. Turek JJ, Schoenlein IA. Altered gene expression in 3T3 fibroblasts enriched with different polyunsaturated fatty acids. Faseb J 16:A263, 2002.
13. 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 147:1282–1288, 1987.
14. Barnett J, Chow J, Ives D, Chiou M, Mackenzie R, Osen E, Nguyen B, Tsing S, Bach C, Freire J, Chan H, Sigal E, Ramesha C. Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. Biochim Biophys Acta 1209:130–139, 1994.
15. Bagga D, Wang L, Eisner RF, Glaspy JA, Reddy ST. Differential effects of prostaglandin derived from ω-6 and ω-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion. Proc Natl Acad Sci U S A 100:1751–1756, 2003.
16. Broughton KS, Wade JW. Total fat and (n-3):(n-6) fat ratios influence eicosanoid production in mice. J Nutr 132:88–94, 2002.
17. Coleman RA, Smith WL, Narumiya S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 46:205–229, 1994.
18. Choung J, Taylor L, Thomas K, Zhou X, Kagan H, Yang X, Polgar P. Role of EP2 receptors and cAMP in prostaglandin E2 regulated expression of type I collagen alpha1, lysyl oxidase, and cyclo-oxygenase-1 genes in human embryo lung fibroblasts. J Cell Biochem 71:254–263, 1998.
19. Noguchi K, Shitashige M, Endo H, Kondo H, Ishikawa I. Binary regulation of interleukin(IL)6 production by EP1 and EP2/EP4 subtypes of PGE2 receptors in IL1stimulated human gingival fibroblasts. J Periodont Res 37:29–36, 2002.
20. Sakai Y, Fujita K, Sakai H, Mizuno K. Prostaglandin E2 regulates the expression of basic fibroblast growth factor messenger RNA in normal human fibroblasts. Kobe J Med Sci 47:35–45, 2003.
21. Sellmayer A, Danesch U, Weber PC. Effects of different polyunsaturated fatty acids on growth-related early gene expression and cell growth. Lipids 31(Suppl):S37–S40, 1996.
22. Chen Y, Hughes FM. Prostaglandin E2 and the protein kinase A pathway mediate arachidonic acid induction of c-fos in human prostate cancer cells. Br J Cancer 82:2000–2006, 2000.
23. Schwartz Z, Gilley RM, Sylvia VL, Dean DD, Boyan BD. The effect of prostaglandin E2 on costochondral chondrocyte differentiation is mediated by cyclic adenosine 3',5'-monophosphate and protein kinase C. Endocrinology 139:1825–1834, 1998.
24. Zhu YK, Liu XD, Skold MC, Umino T, Wang H, Romberger DJ, Spurzem JR, Kohyama T, Wen FQ, Rennard SI. Cytokine inhibition of fibroblast-induced gel contraction is mediated by PGE2 and NO acting through separate parallel pathways. Am J Respir Cell Mol Biol 25:245–253, 2001.

25. Sonoshita M, Takaku K, Sasaki N, Sugimoto Y, Ushikubi F, Narumiya S, Oshima M, Taketo MM. Acceleration of intestinal polyposis through prostaglandin receptor EP2 in Apc716 knockout mice. Nature Med 7:1048–1051, 2001.
26. 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 10:3818–3823, 1990.
27. Cutroneo KR, Phan SH. TGF-beta1-induced Smad 3 binding to the Smad 7 gene: knockout of Smad 7 gene transcription by sense phosphorothioate oligos, autoregulation, and effect on TGF-beta1 secretion: bleomycin acts through TGF-beta1. J Cell Biochem 89:474–483, 2003.