Different effects of n-6 and n-3 polyunsaturated fatty acids on the activation of rat smooth muscle cells by interleukin-1β

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Abstract **There is good evidence that the n-3 polyunsaturated fatty acids (PUFAs) in fish oil have antiinflammatory effects and reduce the pathogenesis of atherosclerosis. However, the mechanisms underlying these actions are largely unknown. This study was designed to investigate the effects of membrane incorporation of two major components of fish oil [eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)], on rat smooth muscle cells (SMCs) activation induced by interleukin-1β (IL1β). We compared their effects with those of n-6 arachidonic acid (AA). Expression of vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1 adhesion molecules involved in SMCs migration was enhanced by AA, whereas EPA and DHA had no similar effects. We established that AA potentiates IL1β-induced expression of the type IIA secreted phospholipase A2 (sPLA2) gene, whereas EPA and DHA reduce this stimulation. EPA and DHA also abolished proinflammatory prostaglandin PGE2 production by inhibiting the IL1β-induced production of cyclooxygenase-2 (COX-2) mRNA. Much interest was then focused on three transcriptional factors implicated in inflammation control and especially in modulating rat sPLA2 and COX-2 gene transcription: nuclear factor-κB, CCAAT/enhancer binding protein β, and E26 transformation-specific-1. electrophoretic mobility shift assay revealed that the binding activity of all three factors was increased by AA and reduced (or not affected) by n-3 PUFA. These results indicate that EPA and DHA act in opposition to AA by modulating various steps of the inflammatory process induced by IL1β, probably by reducing mitogen-activated protein kinase p42/p44 activity.**—Bousserouel, S., A. Brouillet, G. Béréziat, M. Raymondjean, and M. Andréani. **Different effects of n-6 and n-3 polyunsaturated fatty acids on the activation of rat smooth muscle cells by interleukin-1β.** ***J. Lipid Res.*** **2003.** 44: **601–611.**

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Early atherosclerosic lesions have many features of an inflammatory process. Macrophages and endothelial cells cooperate in the release of massive amounts of proinflammatory cytokines, including interleukin-1β (IL1β) (1). IL1β participates in the activation of vascular smooth muscle cells (SMCs), which contribute to the development of neointimal lesions (2). The inflammatory response to vascular injury may involve the migration of medial SMCs and their proliferation in the intima. SMCs change from a contractile to a secretory phenotype during this process and overproduce vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) in response to proinflammatory stimuli, which are involved in SMC migration (3, 4). IL1β also stimulates the production of arachidonic acid (AA)-derived lipid mediators that are involved in inflammation and in the maintenance of many vascular functions, as well as in the production of IL1β itself (1). The fatty acid precursor of eicosanoids is released from the cell membrane by phospholipase A2, which hydrolyzes phospholipids at the *sn*-2 position to generate unesterified free fatty acids, very often AA (20:4n-6) (5). Secreted type IIA phospholipase A2 (sPLA2) and cyclooxygenase-2 (COX-2) cooperate in the synthesis of eicosanoids (6), and their expression in SMCs is stimulated by IL1β (7, 8). Human atherosclerotic arteries also contain large amounts of sPLA2 (9) and COX-2 (10), suggesting that these enzymes are important in the process of atherogenesis.

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Abbreviations: AA, arachidonic acid; C/EBP, CCAAT/enhancer binding protein; COX-2, cyclooxygenase-2; DHA, docosahexaenoic acid; EMSA, electrophoretic mobility shift assay; EPA, eicosapentaenoic acid; ERK, extracellular signal-regulated kinase; IL1β, interleukin-1β; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MEK, MAPK kinase; SMC, smooth muscle cell; sPLA2, secreted phospholipase A2; VCAM-1, vascular cell adhesion molecule-1; ZAL, Z-IE(o-t-butyl)A-leucinal.

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There is now considerable evidence that diets rich in fish oil attenuate the progression of a variety of chronic diseases and can protect against cardiovascular lesions, including atherosclerosis (11). Epidemiological, animal, and clinical studies all indicate that eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), two major n-3 polyunsaturated fatty acids (PUFAs) from marine lipids, are potent antiinflammatory and antiatherogenic agents. Further analyses suggest that balance between n-3 and n-6 PUFAs may also be critical in regulating inflammation (11, 12). EPA and DHA may help protect against atherosclerosis by inhibiting the proliferation of SMCs (13, 14) or by causing apoptosis (15).

These n-3 PUFAs are competitive inhibitors of AA metabolism, particularly the COX pathway (16), and decrease the formation of prostaglandins and leukotrienes. EPA is also metabolized to give a series of eicosanoids that are less active or have other antiinflammatory properties (12). In contrast, DHA is not metabolized by the COX or lipoxygenase pathways (17), but can be converted to EPA. Interestingly, both EPA and DHA have been described as more potent inhibitors of COX-2 than the selective reference compound NS-398 (16).

Many recent studies have confirmed that PUFAs modulate the inflammatory response. In various systems, n-3 PUFAs seem to act in opposition to n-6 AA supplementation that potentiates the inflammatory reaction by increasing cell responsiveness to cytokines (18). Several lines of evidence suggest that the change of membrane characteristics following the incorporation of EPA or DHA alters the signal transduction elicited by IL1β (19, 20). The present study was done to evaluate how EPA, DHA, and AA incorporated into cell phospholipids influence the IL1β-induced expression of proatherogenic and pro-inflammatory genes in SMCs (VCAM-1, MCP-1, type IIA sPLA2, and COX-2). We find that AA potentiates SMC inflammation induced by IL1β, whereas the incorporation of EPA or DHA is without effect. We also determined the different impact of these PUFAs on the binding activities of nuclear factor-κB (NF-κB), CCAAT/enhancer binding protein (C/EBP), and E26 transformation-specific-1 (Ets-1), which mediate the expression of sPLA2 (8, 21) and COX-2 (22, 23). This study provides new insight into the mechanism by which certain essential PUFAs promote (AA), or not (EPA and DHA), the activation of SMCs. These findings support epidemiological reports suggesting that diets rich in n-3 PUFA and low in n-6 help protect against atherosclerosis.

METHODS

Reagents

Type I collagen from calf skin, glutamine, penicillin, streptomycin, fatty acid-free BSA, indomethacin, nordihydroguaiaretic acid, leupeptine, pepstatin, PMSF, and *Naja mossambica* type II sPLA2 were purchased from Sigma Chemical Co. Fetal calf serum was from Dominique Dutscher distribution. Murine-mammary lentivirus reverse transcriptase, lipofectAMINE, and random primers were from Life Technologies, Inc. Oligonucleotides were from Oligo Express. Hybond N+ nylon membranes, ECL direct nucleic acid labeling system, and ECL reagent kit for horseradish peroxidase were from Amersham Pharmacia Biotech. Fluorescent substrate 1-hexadecanoyl-2-(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphoglycerol was from Interchim. IL1β was purchased from Immugenex Corp. AA, DHA, and EPA were from Cayman Chemical and are protected from oxidation by BHT in a 0.1% ethanol solution. Luciferase reporter assay kit and pSV-βgal plasmid were from Promega, Inc. The antibodies against the NF-κB p65 subunit, I-κB isoform α, the C/EBP isoform β, and monoclonal phosphospecific mitogen-activated protein kinase (MAPK) antibody, which detects only the catalytically active forms of phosphorylated p42/44, were provided by Santa Cruz Biotechnology. The antibody against extracellular signal-regulated kinase 1/2 (ERK1/2), MAPK, was from New England Biolabs, Inc. Arachidonyltrifluoromethyl ketone (AACOCF3) and PD98059 were from CalBiochem.

Isolation and culture of rat aortic SMCs

Vascular SMCs were isolated by enzymatic digestion of thoracic aortic media from male Wistar rats (weight 300 g, Elevage Janvier) as previously described (8). The cells were grown to confluence and made quiescent by incubation for 24 h in serum-free medium containing 0.2% fatty acid-free BSA. These SMCs were then incubated or not with EPA, DHA, or AA for 24 h. Concentrations of PUFA from 10 μM to 100 μM were tested and complexed with fatty acid-free BSA at ratios of 4 to 1 (AA) or 3 to 1 (DHA or EPA) before adding them to the cells. After incorporation of PUFAs, the medium was changed and cells were incubated with IL1β for 24 h; appropriate inhibitors were added in some experiments (see figure legends).

Incorporation of PUFA into membrane phospholipids

The amount of PUFA incorporated into phospholipids was estimated by extracting unesterified fatty acids from cells before and after 1 day of PUFA incubation. The fatty acids were separated by gas chromatography and detected by mass spectrometry as previously reported (8). They were quantified with reference to heptadecanoic methyl ester (internal standard), and the amount of each PUFA is expressed as a percentage of the sum of all fatty acids (C16:0, C16:1, C18:0, C18:1, C18:2, C20:4, C20:5, C22:4, C22:5, and C22:6) used to estimate the total lipid phase of the membrane.

RT-PCR

Total RNA was extracted and 1.5 μg was used as a template for RT. First-strand cDNA synthesis and semiquantitative PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA amplification as an internal control were previously reported (8).

The primers used for type II sPLA2 were 5′-GTG GCA GAG GAT CCC CCA AGG-3′ (forward) and 5′-GCA ACT GGG GGT GTT CCC TCT GCA-3′ (reverse), and for GAPDH were 5′-CCA TGG AGA AGG CTG GGG-3′ (forward) and 5′-CAA AGT TGT CAT GGA TGA CC-3′ (reverse). The COX-2 primer was designed from published sequences of rat COX (24): 5′-CTG TAT CCC GCC CTG CTG GTG-3′ (forward) and 5′-ACT TGC CGT TGA TGG TGG CTG TCT T-3′ (reverse). The VCAM-1 and MCP-1 primers were designed from the sequence of the rat gene (25): VCAM-1 5′-GGA GAC ACT GTC ATT ATC TCC TG-3′ (forward) and 5′-TCC TTT CAT GTT GGC TTT TCT TGC-3′ (reverse); MCP-1 5′-ATG CAG GTC TCT CTG TCA CG (forward) and TTC TGG ACC CAT TCC TTA GG-3′ (reverse). PCR was performed using the following conditions: denaturation at 95°C for 1 min, oligonucleotide annealing for 1 min at 64°C for sPLA2 or at 50°C for VCAM-1 and MCP-1, and primary extension at 72°C for 1 min. Annealing and extension steps for COX-2 PCR were pro-

cessed for the same time at 72°C for 5 min. We used 22 amplification cycles for sPLA2 and GAPDH primers and 30 cycles for COX-2, VCAM-1, and MCP-1 primers.

The PCR products of sPLA2 (284 bp), COX-2 (279 bp), VCAM-1 (336 bp) or MCP-1 (256 bp), and GAPDH (amplified from the same cDNA; 195 bp) were electrophoresed in 2% agarose gels and transferred to hybond N+ nylon membranes. The identities of the amplified cDNA products were confirmed by hybridization with 5′-CAA CCG TGT GCA GAA AGG TGG ATG TGG CAC-3′ for sPLA2, 5′-GTG AAC CAC GAG AAA TAT GAC AAC TCC CTC-3′ for GAPDH, 5′-GGG GAG ACC ATG GTA GAA CTT GGA G-3′ for COX-2, 5′-GTC TGT TAA TGG CTC GTA CAC CAT C-3′ for VCAM-1, and 5′-CTC AGC CAG ATG CAG TTA ATG CCC-3′ for MCP-1. The oligonucleotide probes were labeled and detected using the ECL direct nucleic acid labeling detection kit (Amersham Pharmacia Biotech). The bands on the autoradiography films were scanned and quantified by densitometry with Quantity One software (BioRad).

sPLA2 assay

sPLA2 activity was measured using a selective fluorometric assay (8).

Electrophoretic mobility shift assay

Nuclear extracts were prepared from SMCs as described previously (8). The double-stranded oligonucleotides were 5′ end-labeled using T4 polynucleotide kinase. Binding reactions were carried out in a 20 μl binding-reaction mixture (10 mM Hepes, pH 7.9, 50 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 0.2% Nonidet P-40, 0.5 mM EDTA) containing 7 μg nuclear protein and 50,000 cpm of labeled probe. Samples were incubated at room temperature for 15 min and fractionated on 5% polyacrylamide gel electrophoresis in 0.25 × TBE (45 mM Tris borate, 1 mM EDTA) at 150 V for 3 h and then transferred to 3MM paper, dried in a gel dryer under vacuum at 80°C, and then exposed to Hyperfilm MP (Amersham Pharmacia Biotech). The NF-κB oligonucleotides 5′-GTT ACA AAG GGA AAT TAC CAT TTG ATC-3′ corresponded to the region (−140/−131) of the rat type II sPLA2 promoter (26). The C/EBP oligonucleotides 5′-GGG ATG AAC TTT CGA AAT CAG CT-3′ corresponded to the sequence from −227 to −240 of the rat type II sPLA2 promoter (21). The nuclear factor Y (NFY) oligonucleotides 5′-GGG GTA GGA ACC AAT GAA ATG AAA CGT TA-3′ corresponded to the binding site of the rat albumin promoter (27). The Ets-1 oligonucleotides 5′-GTT CGC CAA CCG GAA GTT AGG ATC-3′ corresponded to the PEA3 binding site (28). The specificity of the DNA-protein complexes was determined using competition assays with a 100-fold molar excess of each unlabeled double-stranded probe. Components of NF-κB and C/EBP proteins were identified by supershift assay using antibodies against the p65 subunit or C/EBPβ (Santa Cruz Biotechnology, Inc.), as previously described (8).

Transfection and luciferase assays

Cultured rat SMCs were placed in 12-well dishes 24 h before transfection at a concentration that gave 70% confluence. The cells were transfected using 1.5 μl of LipofectAMINE Plus (Life Technologies, Inc.), 300 ng of a plasmid construct containing the region (−486/+46) of the rat sPLA2 promoter (29) fused to luciferase reporter gene pGL3 basic vector (Promega), and 100 ng of pSV-βgal plasmid (Promega) for 3 h, as recommended by the manufacturer. Transfected cells were cultured for 24 h in serum-free medium and incubated for 24 h in the same medium containing 50 μM EPA, DHA, or AA. The cells were then washed twice with phosphate-buffered saline and incubated or not with IL1β (10 ng/ml, 24 h) in serum-free medium. The luciferase activity was determined with a luciferase reporter assay kit (Promega) with signal detection for 12 s by a luminometer (Lumat LB9507, Berthold, Inc.). β-galactosidase activities were measured to normalize variations in transfection efficiency.

Western blotting

Whole-cell lysates were prepared using a lysis buffer containing 0.5% nonidet P-40 in 20 mM Tris (pH 7.5) and the following proteinase inhibitors: 1 μg/ml leupeptine, 1 μg/ml pepstatin, 1 mM PMSF, and 1 mM EDTA. Nuclear extracts were prepared as described in the electrophoretic mobility shift assay (EMSA) section. Equal amounts of protein (20 μg) were fractionated by SDS-PAGE and transferred to PVDF membranes. Free binding sites were blocked with 5% nonfat milk in PBS/0.1% Tween 20 overnight at 4°C, and the membranes washed in PBS-Tween. Appropriate primary antibodies were then added, followed by secondary horseradish peroxidase-coupled antibodies and bands identified using the ECL Western blotting kit (Amersham Pharmacia Biotech).

Measurement of PGE2 by commercial enzyme immunoassay

PGE2 synthesis was evaluated using a commercial enzyme immunoassay kit (Cayman Chemical Co.).

Statistical analysis

The measured values are expressed as means ± SD. Multiple comparisons were performed by one-way ANOVA followed by the Bonferroni multiple comparison procedure. $P < 0.05$ was considered to be significant.

RESULTS

Incorporation of PUFAs into membrane phospholipids

EPA and DHA might modulate signal transduction elicited with AA by displacing AA from the cell membrane or making less AA available for metabolism into eicosanoids (20). We investigated the mechanism by which n-3 PUFAs reduce proatherogenic processes by first exploring the effect of incorporating PUFAs into membrane phospholipids. Incubating rat aorta SMCs with AA, EPA, or DHA for 24 h increased the amount of the respective PUFA incorporated into the cell phospholipids (**Table 1**). The amount of AA in SMCs was increased 4-fold, EPA was increased 18-fold, and DHA 9-fold. These results indicate that SMC membranes were highly enriched under our experimental conditions. Incubation with PUFA for 48 h did not further enhance incorporation (data not shown). Incorporation of PUFA did not modify significantly the pro-

TABLE 1. Incorporation of PUFAs into membrane phospholipids

Fatty Acid	Untreated	AA	EPA	DHA
C20:4 (n-6)	1.61 ± 0.66	6.62 ± 1.2[a]	2.46 ± 0.68	1.94 ± 0.65
C20:5 (n-3)	0.28 ± 0.1	0.07 ± 0.02	5.05 ± 1.79[a]	0.93 ± 0.37
C22:6 (n-3)	0.26 ± 0.1	0.16 ± 0.05	0.38 ± 0.15	2.36 ± 0.98[a]

AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SMC, smooth muscle cell. Serum-starved SMCs were incubated or not (untreated) for 24 h with 50 μM AA, EPA, or DHA-BSA complexes (AA, EPA, DHA). The fatty acid composition of phospholipids was quantified by GC-MS as described in Methods. Fatty acid levels are expressed as mol%age of total fatty acid peak. Values are means ± SD for six determinations.

[a] $P < 0.01$ versus untreated.

tein content of the treated cells, and the amounts of phospholipids were not changed by incubating with AA (577 ± 41 nmol/mg protein) or EPA (593 ± 51 nmol/mg protein), but cells treated with DHA (514 ± 49 nmol/mg protein) had slightly less phospholipid than untreated cells (585 ± 39 nmol/mg protein).

Effect of PUFA on VCAM-1 and MCP-1 expression

IL1β is a multipotent inflammatory mediator that plays a central role in vascular disease. We mimicked the activation of SMCs associated with inflammatory responses by incubating the cells with IL1β (control). Published reports indicate that IL1β stimulates the activation and proliferation of aorta SMCs (1), and that the inflammatory process is potentiated by n-6 AA (18, 30). As proliferating SMCs overproduce adhesion molecules (3), we tested the IL1β-induced expression of VCAM-1 and MCP-1 in cells treated with PUFA. VCAM-1 mRNA in SMCs was detected by semiquantitative RT-PCR (**Fig. 1A**). In our culture conditions, we noticed a basal level of VCAM-1 mRNA in untreated cells that was not stimulated with IL1β (control). In AA-enriched cells, IL1β induced three times more VCAM-1 accumulation than control cells. The n-3 PUFAs had much less effect, with EPA reducing VCAM-1 mRNA by 56% compared with AA and DHA by 43%. Treatment with IL1β alone slightly increased the MCP-1 mRNA in cultured SMCs (Fig. 1B), while incorporation of n-6 AA up-regulated IL1β-induced MCP-1 accumulation by 68%. In comparison with AA-enriched cells, EPA incorporation reduced IL1β-induced MCP-1 mRNA accumulation by 40%, at the same level as control cells, whereas DHA pretreatment did not.

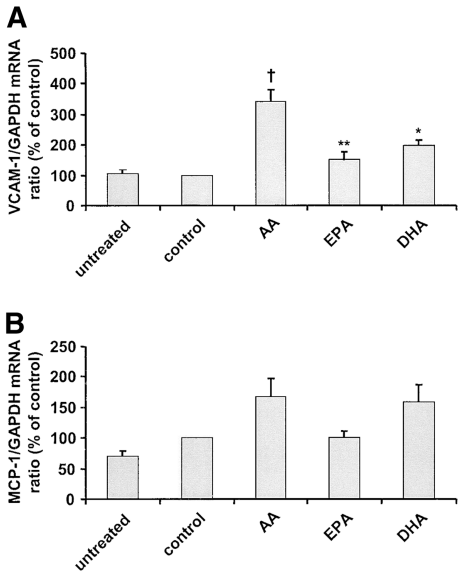

Fig. 1. Effect of PUFA supplementation on adhesion molecules mRNA in smooth muscle cells (SMCs). A: Semiquantitative RT-PCR analysis of vascular cell adhesion molecule-1 (VCAM-1) or (B) monocyte chemoattractant protein-1 (MCP-1) mRNA were normalized against GAPDH amplification. Adhesion molecule-GAPDH mRNA ratios were calculated by densitometry scanning as described in Methods. Serum-starved SMCs (untreated) were incubated or not (control) with 50 μM PUFA [arachidonic acid (AA), eicosapentaenoic acid (EPA), or docosahexaenoic acid (DHA)] for 24 h before stimulation by interleukin-1β (IL1β) (10 ng/ml, 24 h). The results are expressed as the percentage of stimulation over the value obtained with cells treated by IL1β alone (control). Each value represents the means ± SD (bars) of scanning analyses from three independent experiments. † $P < 0.001$ versus control; * $P < 0.05$; and ** $P < 0.01$ versus AA.

Effect of PUFA on IL1β-induced sPLA2 activity and gene expression

As reported previously (8), sPLA2 gene expression detected by RT-PCR, which is silent in rat SMCs under resting conditions, is markedly induced by IL1β (**Fig. 2C**), and sPLA2 enzymatic activity in the cell supernatant was 7-fold ($P < 0.01$) greater than in untreated cells (Fig. 2A). Pretreatment of SMCs with AA increased the stimulation of sPLA2 activity in response to IL1β from 2.6 (control) to 5.9 nmol/min/ml (AA), together with a slight increase in IL1β-induced sPLA2 mRNA. The incorporation of EPA or DHA did not enhance IL1β-induced sPLA2 activity (−64% and −68% of the activity in AA-treated cells) or mRNA (−34% and −57%). In addition, n-3 PUFA supplementation decreased sPLA2 mRNA to significantly below that of IL1β-stimulated cells (control). DHA produced greater inhibition than EPA in both experiments. Incorporation of either EPA or DHA without IL1β treatment did not affect the basal expression of sPLA2, and adding these n-3 PUFAs and IL1β together did not alter the sPLA2 gene expression induced by IL1β (data not shown).

We identified the signaling pathways leading to the stimulation of sPLA2 gene expression by IL1β in AA-enriched cells (Fig. 2B) using pharmalogical inhibitors of COX and lipoxygenase activities [indomethacin and nordihydroguaiaretic acid (NDGA)] and two inhibitors of cytosolic PLA2 activity acting via the MAPK ERK1/2 pathway (PD98059) or the enzyme itself (AACOCF3). Two other inhibitors likely acting on the activity of transcription factors were also assessed: the inhibitor of p38 MAPK (SB203580), and the specific proteasome inhibitor Z-IE(o-t-butyl)A-leucinal (ZAL) that blocks the NF-κB pathway. In AA-enriched cells, IL1β-induced sPLA2 gene expression was abrogated by AACOCF3, PD98059, and ZAL, as expected from our previous study (8). The sPLA2 activity was inhibited by indomethacin, whereas the lipoxygenase inhibitor did not affect the IL1β-induced response.

Effect of PUFA on IL1β-induced sPLA2 gene transcription

The increased sPLA2 gene expression by IL1β and its inhibition by PUFA could be due to the accumulation of endogenous mRNA or to increased gene transcription. We therefore examined the activation of the rat gene sPLA2 promoter (−496 + 20) fused to the luciferase gene reporter in transient transfection experiments. IL1β alone (10 ng/ml, 24 h) increased the transcriptional activity of the sPLA2 promoter (Fig. 2D), but by very little. On the other hand, when AA was incorporated into SMCs, stimulation with IL1β was significant (+42%) compared

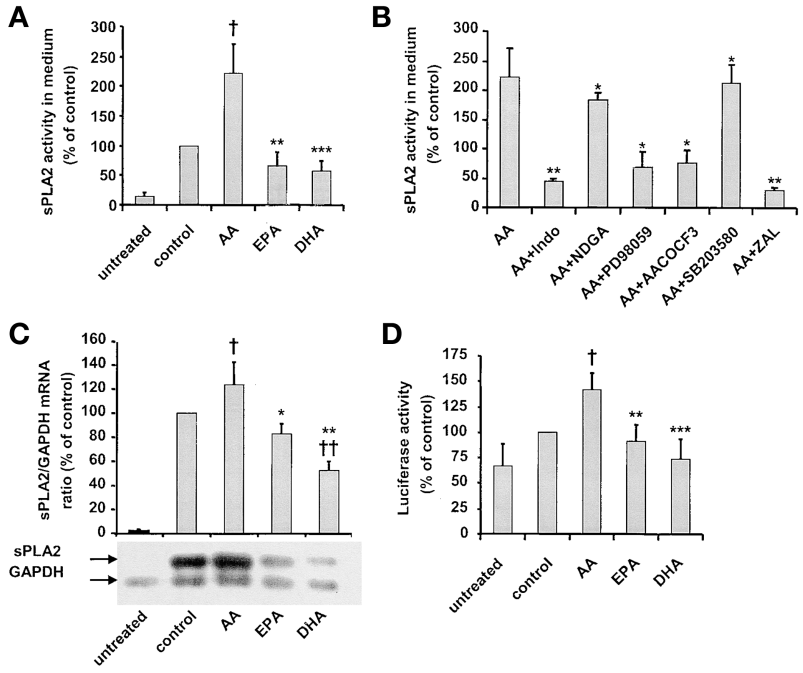

Fig. 2. Effect of PUFA supplementation on IL1β-induced secreted phospholipase A2 (sPLA2) expression in SMCs. Serum-starved SMCs (untreated) were incubated or not (control) with 50 μM PUFA (AA, EPA, or DHA) for 24 h before stimulation by IL1β (10 ng/ml, 24 h). The results are expressed as the percentage of stimulation over the value obtained with IL1β alone (control). A: sPLA2 enzymatic activity was measured in medium by spectrofluorimetric assay and (B) in AA enriched cells, indomethacin (10 μM), nordihydroguaiaretic acid (NDGA) (10 μM), AACOCF3 (20 μM), PD98059 (25 μM), SB203580 (10 μM), or Z-IE(o-t-butyl) A-leucinal (ZAL) (1 μM) were incubated 30 min before IL1β stimulation. Data are means ± SD from three independent experiments performed in duplicate. C: sPLA2 mRNA expression was detected by semiquantitative RT-PCR and a representative autoradiogram of a typical experiment performed in triplicate was shown (lower part), and graph values represent sPLA2-GAPDH mRNA ratios calculated by densitometer scanning relative to the ratio obtained by IL1β stimulation alone (upper part). D: sPLA2 promoter activity was determined by luciferase reporter assay in transiently transfected SMCs as indicated in Methods. All luciferase activity values were reported to β-galactosidase activity and represent the means ± SD (bars) from three independent experiments in which different conditions were tested in duplicate. † $P < 0.05$ and †† $P < 0.001$ versus control; * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$ versus AA.

with cells treated with the cytokine alone (control). In contrast, the incorporation of neither EPA nor DHA increased sPLA2 transcription elicited by IL1β. Moreover, DHA produced greater inhibition than EPA, and the transcriptional activity of the sPLA2 promoter was 26% lower than in cells treated with IL1β alone (control), reaching approximately the value of untreated cells.

Effect of PUFA on IL1β-induced PGE2 synthesis and COX-2 mRNA

We next looked at the effect of n-3 PUFA on the synthesis of PGE2 in cultured SMCs using an enzyme immunoassay. PGE2 is one of the primary compounds generated by COX from AA, and this prostaglandin has many effects on SMC functions (7). The basal release of PGE2 was extremely low (12 pg/ml), while IL1β (10 ng/ml, 24 h) increased (525 pg/ml, $P < 0.001$) the production of PGE2 (**Fig. 3A**). The incorporation of AA greatly augmented the IL1β-induced PGE2 release (9.5-fold) and as expected, this effect was completely blocked by the COX-inhibitor (10 μM indomethacin) but not by the 12/15-lipoxygenase-inhibitor NDGA. IL1β-stimulated PGE2 production by cells enriched in EPA or DHA was lower than that of IL1β-stimulated cells (control). Incorporation of EPA or DHA alone, without IL1β stimulation, did not affect basal PGE2 synthesis (data not shown). In contrast, PGE2 production was clearly increased after AA incorporation alone with an amount that did not differ significantly from IL1β treatment (data not shown).

We also measured the COX-2 mRNA in cultured SMCs by RT-PCR. The basal level of COX-2 mRNA in untreated cells was very low, almost undetectable by this technique (Fig. 3B). IL1β strongly stimulated COX-2 mRNA production ($P < 0.001$). Pretreatment of SMCs with AA increased IL1β-induced COX-2 mRNA by +87%. The COX-inhibitor indomethacin (10 μM) completely blocked the effects of AA and IL1β (data not shown). In contrast, the incorporation of EPA or DHA into phospholipids decreased IL1β-induced accumulation of COX-2 mRNA to below that of control cells (−36% for EPA and −63% for DHA). These finding are in agreement with the effects of AA, EPA, and DHA on PGE2 synthesis reported herein.

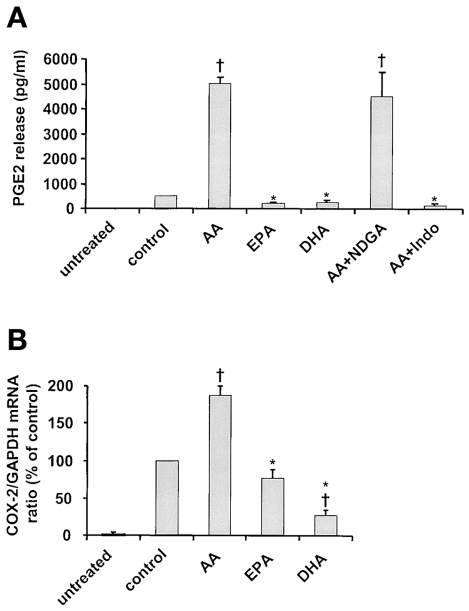

Fig. 3. Effect of PUFA supplementation on IL1β-induced PGE2 release and cyclooxygenase-2 (COX-2) expression in SMCs. A: PGE2 release quantification in cell-free medium was analyzed by using a commercial enzyme immunoassay. SMCs were treated as previously described except the inhibitor addition in AA-enriched cells of 10 μM indomethacin (AA+Indo) or 10 μM NDGA (AA+NDGA) 30 min before IL1β stimulation. Data represent the means ± SD (bars) of three independent experiments performed in duplicate. B: COX-2 expression was detected by semiquantitative RT-PCR analysis of COX-2 mRNA. COX-2-GAPDH mRNA ratios are expressed as the percentage of the stimulatory effect obtained by IL1β alone (control) Data represent the means ± SD (bars) of two independent experiments performed in duplicate. † $P < 0.001$ versus control; * $P < 0.001$ versus AA.

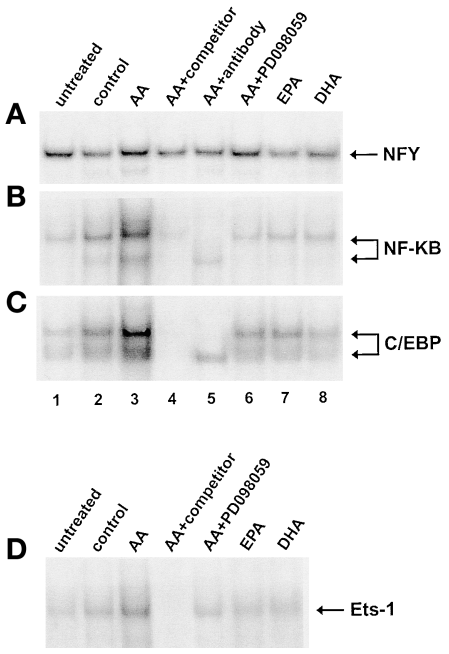

Fig. 4. Effect of PUFA on IL1β-induced DNA binding activity of nuclear factor-κB (NF-κB), CCAAT/enhancer binding protein (C/EBP), and E26 transformation-specific-1 (Ets-1) in SMCs. Electrophoretic mobility shift assay from nuclear extracts prepared from cells treated as previously and incubated with labeled oligonucleotide bearing (A) a nuclear factor Y (NFY) binding site, (B) the NF-κB-, or (C) C/EBP binding site of sPLA2 promoter, and (D) a Ets-1 binding site as described in Methods. A representative autoradiogram of three independent experiments is shown. Arrows denote oligonucleotide-transcription factor complexes. A large excess (×100) of the respective unlabeled probe (competitor, lane 4) and supershift assay with antibodies against p65 subunits (B, lane 5) or C/EBPβ isoform (C, lane 5) were used to validate the specificity of binding.

Effect of PUFA on IL1β-induced transcription factor activation

We have previously shown that the NF-κB and C/EBP transcription factors participate in the regulation of rat sPLA2 gene transcription in rat SMCs (8, 21). We performed EMSA studies using as an internal control the ubiquitously expressed NFY factor (**Fig. 4A**). Untreated cells (Fig. 4B, lane 1) contained a small amount of complex detected with double-stranded labeled oligonucleotide bearing the NF-κB recognition site of the rat sPLA2 promoter (−140–131). IL1β alone increased the NF-κB DNA binding activity (Fig. 4B, lane 2). Cells enriched in AA showed greatly enhanced IL1β-induced NF-κB binding on their responsive elements (Fig. 4B, lane 3). In contrast, cells treated with EPA or DHA had clearly less binding (Fig. 4B, lane 7 or 8 respectively), similar to that of untreated cells. Nuclear extract from AA-enriched cells that had not been stimulated with IL1β also had NF-κB binding activity, while cells enriched with EPA or DHA alone produced no NF-κB complexes (data not shown). The NF-κB binding activity, visualized as two major complexes, was specifically competed out by the excess of the corresponding unlabeled oligonucleotide (Fig. 4B, lane 4). The strong upper band that was completely abolished by the anti-NF-κB antibody p65 (Fig. 4B, lane 5) probably corresponded to the p50/p65 heterodimer complex, whereas the lower band was slightly displaced (probably the p50 homodimer complex). A time-dependent increase in NF-κB binding activity was detected up to 24 h in IL1β-stimulated SMCs as previously shown (8), and also in AA-treated cells (data not shown). This is in accordance with results indicating that the cellular enrichment with PUFA elicited an intracellular oxidative stress resulting in the prolonged NF-κB activation (31).

Similar results were obtained with a double-stranded oligonucleotide bearing the C/EBP site of the rat sPLA2 promoter (−227–240) (Fig. 4C). IL1β slightly increased C/EBP binding, and cells treated with AA produced more of these IL1β-induced complexes. Once again, cells enriched with EPA or DHA showed no induction. An antibody against the C/EBPβ isoform removed most of the induced complexes (Fig. 4C, lane 5). Our previous experiments showed that the C/EBPβ isoforms are the main ones involved in complex formation, with a little implication of C/EBPδ in nuclear extracts from stimulated SMCs (21). The incorporation of EPA or DHA into phospholipids also prevented IL1β-stimulated C/EBPβ binding activity, as with the NF-κB transcription factor.

This could perhaps be because cells supplemented with EPA or DHA contain lesser amounts of these transcription factors. We therefore analyzed nuclear extracts by Western

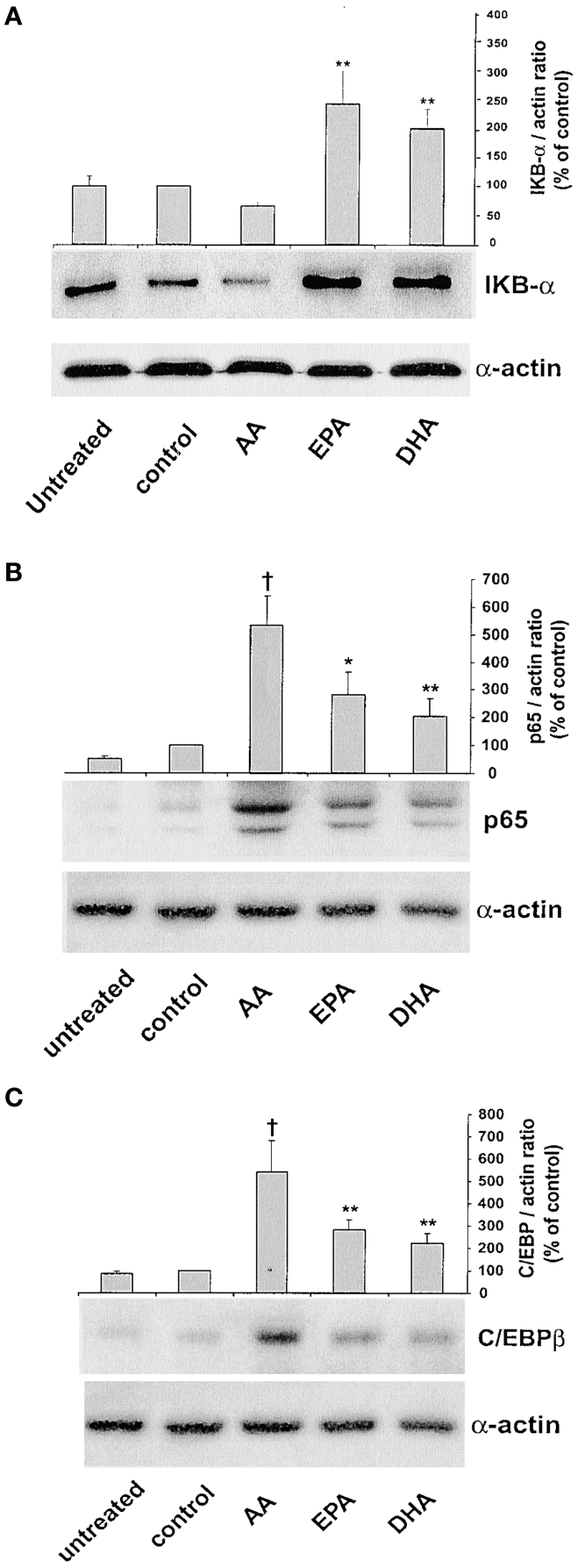

Fig. 5. Effect of PUFA supplementation on I-κBα, NF-κB, and C/EBPβ protein. Nuclear extracts were prepared from cells treated as previously, and 20 μg of protein were loaded on a 10% SDS-PAGE gel, blotted, and incubated with specific antibodies, (A) anti-I-κBα, (B) anti-p65 NF-κB subunits, or (C) anti-C/EBPβ. A representative blot of three separate experiments that gave essentially identical results is shown (lower part). Semiquantitative measurements of protein levels were normalized with α-actin detection and were expressed relative to the ratio from IL1β-treated cells (control), which was taken as 100% (upper part). The quality of the preparation and the amount of protein loaded were evaluated with anti-α-actin antibody. † $P < 0.001$ versus control; * $P < 0.05$ and ** $P < 0.01$ versus AA.

blotting and found that the reduction of NF-κB and C/EBP binding activity in EMSA was correlated with less p65 NF-κB (**Fig. 5B**) and C/EBPβ (Fig. 5C) proteins than in AA-treated cells. However, the amounts of p65 and C/EBPβ proteins in EPA- and DHA-treated cells did not correlate well with results obtained in EMSA when compared with cells treated only with IL1β. Since the activation of NF-κB involves I-κB degradation in the cytosol, we assessed whether the suppressive effects on the NF-κB binding activity result from the nondegradation of I-κBα, using Western blotting analyses. In contrast with n-3 PUFA, AA reduced cytosolic I-κBα expression (Fig. 5A). This effect was closely correlated with the time course of IL1β-induced NF-κB activation in AA-treated cells and with the translocation of p65 to the nucleus (Fig. 5B).

Another transcription factor, Ets-1, has recently been implicated in IL1β-induced sPLA2 gene transcription (V. Antonio, personal communication). Nuclear extracts from control cells provided one more intense complex than untreated cells with labeled double-stranded oligonucleotide bearing the Ets-1 consensus site (Fig. 4D, lane 2). There was a great deal of this complex in AA-enriched cells, but not in EPA- or DHA-enriched cells, where the IL1β-induced Ets-1 binding activity was the same as in untreated cells.

Effect of PUFA on IL1β-induced MAPK pathway

IL1β activates MAPK pathways, including p38 MAPK and ERK signaling cascades (1). These kinases are thought to phosphorylate and activate various transcription factors, which in turn modulate the expression of target genes (28, 32, 33). The specific MAPK kinase (MEK) inhibitor PD98059 inhibited the IL1β-induced activation of NF-κB, C/EBP (Fig. 4B, C, lane 6), and Ets-1 (Fig. 4D, lane 5) in AA-enriched cells. These results are in agreement with our previous studies demonstrating that PD98059 prevents the IL1β-stimulated production of sPLA2 mRNA (8) or sPLA2 activity (Fig. 2B). In contrast, the specific p38 MAPK inhibitor SB203580 had no effect. These data suggest that expression of the sPLA2 gene is mediated through NF-κB, C/EBP, and Ets-1 transcription factors involving the p42/44 MAPK pathway.

We tested the effects of AA on IL1β-induced p42/44 MAPK activity in this signaling pathway by Western blotting experiments with the phosphospecific ERK1/2 antibody (**Fig. 6A**). IL1β-stimulated phosphorylation of ERK1/2 was detected as early as 15 min (control) and was increased by the same induction procedure in AA-enriched cells. In contrast, n-3 PUFA incorporation does not enhance IL1β-induced ERK1/2 phosphorylation. Moreover, EPA pretreatment clearly reduced ERK1/2 phosphorylation to that of untreated cells. However, the amount of total ERK protein remained unchanged, even after DHA and EPA supplementation (Fig. 6B).

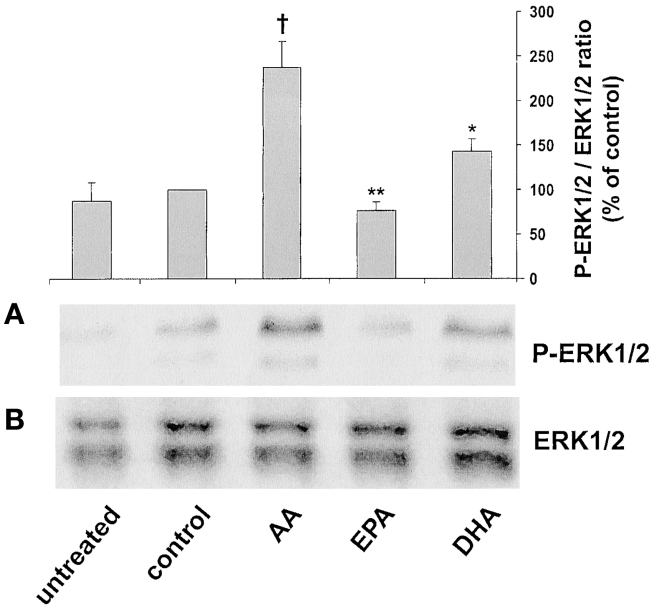

Fig. 6. Effect of PUFA supplementation on IL1β-induced extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in SMCs. Whole lysates of cells were treated as previously, except that the stimulation by IL1β (10 ng/ml) was only for 15 min, and were loaded on 8% SDS-PAGE gels. A: Representative blot of Western blotting analysis with antibody specific for the active phosphorylated ERK1/2 is shown (lower part). Relative phosphorylated protein was normalized by densitometric scanning against total ERK protein detection (upper part), and the results are expressed as described previously. B: The amount of proteins loaded was controlled by incubating a duplicate membrane with an anti-ERK1/2 antibody. † $P < 0.01$ versus control; * $P < 0.05$ and ** $P < 0.01$ versus AA.

DISCUSSION

Many recent studies have confirmed that PUFAs alter the inflammatory response (11, 12). Epidemiological studies have provided considerable evidence for a direct correlation between dietary n-3 PUFA intake and protection against cardiovascular disease. The antiatherogenic properties of fish oils may be due to their capacity to modulate the inflammatory responses of macrophages and endothelial cells (19, 20, 34). Several investigators have reported that EPA and DHA, the active components of fish oils, reduce the proliferation of vascular SMCs and hyperplasia of the arterial wall intima (13, 14). Besides this effect on proliferation, we propose that the change in membrane characteristics caused by the incorporation of EPA or DHA may also modulate atherogenesis by reducing the expression of proatherogenic and proinflammatory proteins.

The inflammatory response of SMCs was activated by incubation with IL1β, the cytokine that plays a central role in vascular physiopathology (1). Several lines of evidence suggested that the incorporation of EPA or DHA alters the signal transduction triggered by IL1β (19, 20, 34). Other studies have shown that n-6 AA seems to act in the opposite manner to the n-3 PUFA, activating inflammation (35) and increasing cell responsiveness to cytokines (18, 30). We have confirmed that IL1β stimulated two genes that are normally not expressed in primary culture of SMCs, sPLA2 and COX-2, and that this in turn increased PGE2 production. The VCAM-1 and MCP-1 genes are expressed

in untreated cells under our culture conditions, and their expression is not greatly augmented by IL1β.

Incubation of rat aorta SMCs with AA, EPA, or DHA for 24 h (see Methods) resulted in significant incorporations with PUFA concentrations of 50 μM and albumin-PUFA ratios of of 3 for EPA and DHA and 4 for AA. These parameters have been extensively used in various in vitro experiments (13, 14, 18, 19, 36–38). Free forms of PUFA such as AA and EPA are susceptible to oxidation to produce large amounts of lipid peroxides. AA and EPA used in these studies were peroxide-free products, so that any PUFA action was not influenced by the formation of lipid radicals. AA and EPA are also protected from oxidation by BHT, which completely suppressed the formation of lipid peroxides in the medium after PUFA supplementation (38). Analysis of the phospholipid fatty acid composition revealed important levels of n-3 PUFA incorporation, conversely with no significant augmentation of the percentage of AA in EPA and DHA supplementation. Such results were confirmed by in vitro and in vivo studies (36–38). We observed that the triglyceride content did not significantly change after supplementation with any PUFA under our culture conditions and SMCs did not contain any increase in lipid droplets detected by oil red staining (data not shown). These results differ from previous data with SMCs isolated from rabbit or guinea pig incubated with higher PUFA concentrations in the presence of serum (39, 40). Thus, the differential accumulation of PUFA may be due to these different lipid delivery parameters or species differences, and often vary from one type of cell or tissue to another (41).

We find that the incorporation of AA into phospholipids potentiates activation of SMCs by IL1β by enhancing the expression of the genes encoding VCAM-1, MCP-1, sPLA2, and COX-2. In contrast to AA, EPA, and DHA had much less effect with production of these adhesion molecules and proinflammatory proteins. Moreover, incorporation of n-3 PUFA decreased the activation of SMCs by IL1β to below that of control cells in most experiments. Our results partly corroborate the finding of others (19, 34), who observed that EPA and DHA attenuate VCAM-1 and MCP-1 production in IL1β-activated primary human umbilical vein endothelial cells. By limiting the expression of VCAM-1 and MCP-1 to below that of AA-enriched cells, EPA and DHA may actively prevent the early phase of atherogenesis. This is the first report, to our knowledge, showing that the antiinflammatory action of EPA and DHA may be due to a decrease in sPLA2 gene expression. Our studies with the inhibitor AACOCF3 confirm that activation of the cPLA2 gene contributes to the release of sPLA2 stimulated by IL1β from AA-enriched cells. This is in agreement with previous results showing that AA released by cPLA2 early in cytokine stimulation is crucial for the subsequent type IIA sPLA2 induction responsible for delayed AA release (6, 8, 18, 42). Current evidence suggests that cPLA2 and sPLA2 are functionally coupled to the COX pathway for stimulus-initiated production of bioactive prostaglandins (6, 43). Many studies have demonstrated that fish oil acts as a PGE2 antagonist (12). We have demonstrated that n-6 AA incorporation markedly increases PGE2 synthesis in response to IL1β, while EPA and DHA completely reduce this production. Our studies on the impact of EPA and DHA on IL1β-induced COX-2 activity suggest that the inhibition of PGE2 production is partly due to a reduction of COX-2 mRNA, as in endothelial cells or macrophages (44, 45), in addition to the decrease of sPLA2 enzyme activity.

The nuclear impact by which fish oils exert their antiinflammatory properties is not fully understood. Because most of the inflammatory molecules are not produced under basal conditions, cytokine activation requires the initiation of gene transcription. This leads to the hypothesis that activation of one or several transcription factors contributes to the concerted activation of these inflammatory genes (34). The effect of PUFAs and their derivatives on transcription was thought to be mediated by peroxisome proliferator-activated receptor (PPAR). EPA and DHA, which are PPAR ligands, may have antiatherogenic properties by modulating sPLA2 (8) and COX-2 (46) gene expression. However, treatment of SMCs with various PUFAs and then with IL1β had no influence on the activation of PPAR detected by EMSA and Western blotting (data not shown). The transcription factors NF-κB and C/EBPβ have received considerable attention over the past several years, as they may be involved in the regulation of several proinflammatory genes (32). Our group has shown that NF-κB and C/EBPβ play a central role in sPLA2 (8, 21) or COX-2 transcription (23). Finally, Ets-1 is another important transcription factor in cytokine-mediated inflammation (28). It is implicated in COX-2 gene expression (47), and recent studies in our laboratory indicate that it is also involved in IL1β-induced sPLA2 gene transcription (V. Antonio, personal communication).

Our second important finding is that the binding activities of these three transcription factors are upregulated by AA pretreatment, whereas the n-3 PUFAs have no effect or inhibit IL1β-induced binding. These changes in the NF-κB system have been described in the promonocytic cell lines U937 (48) or Raw 264.7 (45) and in endothelial cells (34). We have also shown that the coordinated IL1β-induced binding of NF-κB, C/EBPβ, and Ets-1 in AA-enriched cells is abolished by the MEK inhibitor PD98059. The substrates of MAPK include nuclear transcription factors such as NF-κB (33), C/EBPβ (32), and Ets-1 (28), which we find are also down-regulated by n-3 PUFA. The state of phosphorylation of these transcription factors can affect several functions, including their binding to DNA and transactivation. We have confirmed the activation of the p42/44 MAPK pathway in SMCs by AA (35) by Western blotting experiments with the phosphospecific ERK1/2 antibody. We established that EPA and DHA failed to enhance IL1β-induced ERK phosphorylation. The n-3 PUFAs have also been shown to inhibit the MAPK pathway in macrophages (45), endothelial cells (49), and T-cells (50). Interestingly, cooperation and physical interactions between NF-κB, C/EBPβ, and Ets-1 have been demonstrated for the synergistic regulation of various specific genes, including many inflammatory genes (28, 32). These interac-

tions may be a suitable target for the development of novel antiinflammatory therapies. Therefore, EPA and DHA could decrease proinflammatory gene transcription such as sPLA2 and COX-2 by reducing the activation of these multiple transcription factors by inhibiting p42/p44 MAPK pathway.

Our findings thus provide new information on the biological and molecular mechanisms whereby dietary fish oils exert their beneficial effects in atherosclerosis. Our current findings obtained in rat SMCs demonstrate that AA specifically stimulates the IL1β-induced inflammatory response, whereas n-3 PUFAs tend to be neutral or inhibit the process. The coordinated regulation of gene expression through modulation of NF-κB, C/EBPβ, and Ets-1 factors by EPA and DHA can help decrease SMC activation, probably by reducing MAPK ERK1/2 activity. Thus, changing the fatty acid balance in membrane phospholipids could modulate the responsiveness of SMCs to cytokines.

It was recently reported that n-3 PUFAs may act by altering membrane lipid microdomains, lipid rafts, of which caveolae are a subtype enriched with caveolin proteins (51–53). Caveolins inhibit signal transduction from the p42/p44 MAPK cascade, some of whose components are located in caveolae (54). Moreover, sPLA2 is functionally associated with glypican, a glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan found in caveolae and also in the perinuclear area close to COX-2 (55). Future studies are now needed to examine the way in which the changes in membrane lipid caused by EPA and DHA alter signal transduction.

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