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The omega-3 fatty acid docosahexaenoate attenuates endothelial cyclooxygenase-2 induction through both NADP(H) oxidase and PKCε inhibition

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A high intake of the omega-3 fatty acid docosahexaenoate [docosahexaenoic acid (DHA)] has been associated with systemic anti-inflammatory effects and cardiovascular protection. Cyclooxygenase (COX)-2 is responsible for the overproduction of prostaglandins (PG) at inflammatory sites, and its expression is increased in atheroma. We studied the effects of DHA on COX-2 expression and activity in human saphenous vein endothelial cells challenged with proinflammatory stimuli. A ≥24-h exposure to DHA reduced COX-2 expression and activity induced by IL-1, without affecting COX-1 expression. DHA effect depended on the NF-κB-binding site in the COX-2 promoter. EMSAs confirmed that DHA attenuated NF-κB activation. Because MAPK, PKC, and NAD(P)H oxidase all participate in IL-1-mediated COX-2 expression, we also tested whether these enzymes were involved in DHA effects. Western blots showed that DHA blocked nuclear p65 NF-κB subunit translocation by decreasing cytokine-stimulated reactive oxygen species and ERK1/2 activation by effects on both NAD(P)H oxidase and PKCε activities. Finally, to address the question whether DHA itself or DHA-derived products were responsible for these effects, we inhibited the most important enzymes involved in polyunsaturated fatty acid metabolism, showing that 15-lipoxygenase-1 products mediate part of DHA effects. These studies provide a mechanistic basis for antiinflammatory and possibly plaque-stabilizing effects of DHA

docosahexaenoic acid | endothelium | inflammation | NF-κB

Vascular endothelial cells (EC) play a key role in the inflammatory aspects of atherosclerosis, by sustaining monocyte recruitment via leukocyte adhesion molecules and chemoattractants and cooperating with macrophages in the release of inflammatory cytokines and matrix-degrading enzymes (1). Inflammatory cytokines stimulate the production from arachidonic acid (AA) of lipid mediators, such as prostanoids, that contribute both to inflammation and vascular homeostasis (2). Rate-limiting enzymes in prostanoid production include cyclooxygenases (COX)-1 and −2. Although COX-1 is constitutively expressed, COX-2 is rapidly induced after inflammatory challenges (3). Enhanced COX-2 expression is found in inflammation (3) and also in atherosclerotic lesions, where a pathogenetic role has been suggested (4). COX-2 induced in macrophages and EC by oxidized low-density lipoprotein or IL-1 indeed catalyzes massive formation of eicosanoids that enhance vascular permeability and promote monocyte chemotaxis, proliferation, and cholesterol ester retention (5). Diets rich in marine-derived omega-3 fatty acids, mainly docosahexaenoic acid (DHA, 22:6 n-3) and eicosapentaenoic acid (EPA, 20:5 n-3), can attenuate chronic inflammatory diseases (6) and protect against atherosclerosis (7). By competing with AA, DHA and EPA inhibit enzymatic activity of both COX isoforms (8, 9), but their effects on COX-2 expression in vascular endothelium are poorly understood. We showed that incorporation of DHA into EC decreases cytokine-stimulated expression of leukocyte adhesion molecules (10). We have now examined whether DHA can directly affect IL-1-induced expression of COX-2 in EC.

Results

DHA Inhibits Endothelial COX Activity. We previously showed that DHA (up to 25 μmol per liter) inhibited endothelial adhesion molecule expression without toxicity (10, 11). Because the effect of DHA was qualitatively similar to, but stronger than, the effect of EPA (11), all experiments were performed with DHA. We therefore first monitored the effects of prolonged (up to 48-h) exposure of human saphenous vein EC (HSVEC) to 25 μmol/liter DHA, by measuring production of 6-keto-$PGF_{1\alpha}$, the major metabolite of prostaglandin (PG) I_2 that is, in turn, the main product of COX in EC. 6-keto-$PGF_{1\alpha}$ increases in the presence of exogenous substrate, of stimuli for AA release and, more, of stimuli for EC activation, such as IL-1α or phorbol myristate acetate (PMA; Table 1, which is published as supporting information on the PNAS web site). Exposing HSVEC to DHA before stimulation with AA or thrombin significantly reduced 6-keto-$PGF_{1\alpha}$ (Table 1). EC exposed to IL-1α required >12 h before 6-keto-$PGF_{1\alpha}$ significantly rose (5-fold), and such production further increased after thrombin or AA addition (4- and 8-fold, respectively). DHA blocked thrombin or AA-stimulated PGI_2 40% more in the presence of IL-1α than without IL-1α, and PMA effects were even more strongly inhibited (Table 1). Similar results were obtained with human umbilical vein EC (not shown). Stearate, not a substrate for COX, was used as control, and up to 50 μmol/liter did not inhibit PGI_2 production.

Prolonged Exposure to DHA Is Necessary for Maximal Inhibition of COX Activity. We tested the time dependence of DHA inhibitory effect in HSVEC over 0–48 h before addition of IL-1α (Fig. 1*A*). With coexposure to DHA and IL-1α, inhibition of COX-activity was minimal, but inhibition increased >50% when cells were preexposed to DHA for 48 h before adding IL-1α. Similar results were obtained by using PMA (Fig. 1*B*). This suggested that DHA inhibits PGI_2 formation by two mechanisms: first as a competitive substrate and second by blocking COX-2 synthesis during EC activation.

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Abbreviations: DHA, docosahexaenoic acid; AA, arachidonic acid: EC, endothelial cell; HSVEC, human saphenous vein EC; COX, cyclooxygenase; PMA, phorbol myristate acetate; EPA, eicosapentaenoic acid; PG, prostaglandin; ROS, reactive oxygen species; DPI, diphenyl iodonium; LO, lipoxygenase.

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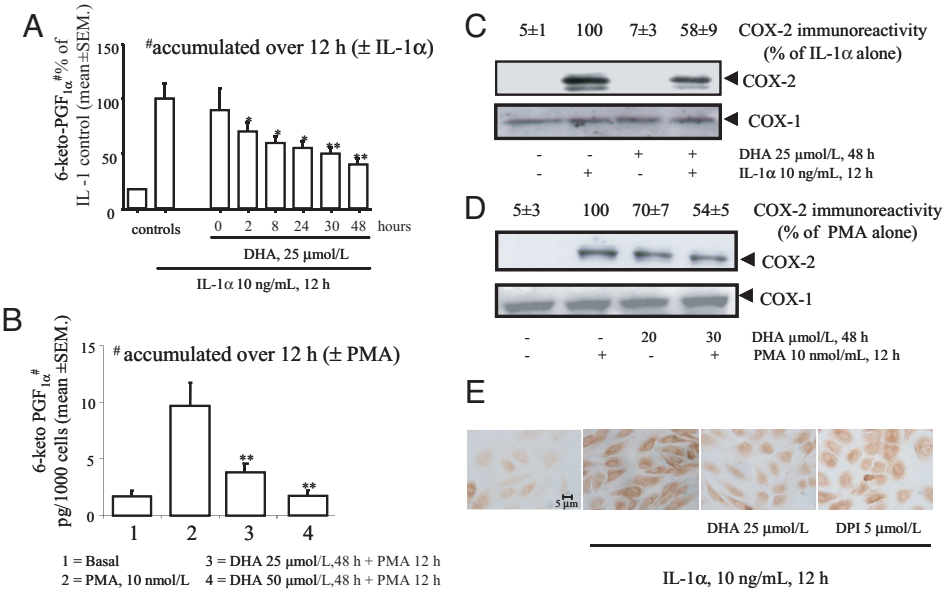

Fig. 1. Inhibition of IL-1α- and PMA-mediated induction of COX-2 activity and protein by DHA. (*A*) HSVEC were preincubated in the absence (vehicle) or presence of 25 μmol/liter DHA for 0–48 h before stimulation with 10 ng/ml IL-1α for 12 h, then medium was collected and 6-ketoPGF$_{1\alpha}$ measured by RIA. 6-ketoPGF$_{1\alpha}$ is shown as percent of maximum response to IL-1α as a function of DHA preincubation time. Each bar represents the mean of eight determinations repeated in three separate experiments. *, $P < 0.05$; **, $P < 0.01$ vs. stimulated control. (*B*) HSVEC were preincubated with DHA, stimulated with 10 PMA for 12 h, then medium was collected and 6-ketoPGF$_{1\alpha}$ measured and expressed as picogram per 1,000 cells for each of two DHA concentrations (25 and 50 μmol/liter). Each bar represents the mean of $n = 8$ determinations, repeated in three separate experiments. **, $P < 0.01$ vs. PMA alone. (*C*) HSVEC were preincubated with 25 μmol/liter DHA for 48 h, stimulated with 10 ng/ml IL-1α for 12 h, and then whole-cell lysates were analyzed by Western blot using antibodies specific for COX-1 and -2. Values of COX-2 are shown as percent of maximal control response (IL-1α alone). The blot depicted is representative of three similar ones. (*D*) HSVEC were preincubated with DHA for 48 h, stimulated with 10 nmol/liter PMA for 12 h, and cell lysates prepared and analyzed as in *C*. The blot depicted is representative of three similar ones. (*E*) HSVEC were preincubated with 25 μmol/liter for 48 h or 5 μmol/liter DPI, an inhibitor of NAD(P)H oxidase, for 30 min, then stimulated with 10 ng/ml IL-1α for 12 h, after which cells were fixed and immunostained as described in *Materials and Methods*. For *C* and *D*, densitometric values of COX-1 and -2 expression are reported as percent of maximal control response (IL-1α alone).

DHA Inhibits COX-2 Protein Expression. To test for a dual inhibitory effect of DHA on EC, we studied DHA effects on COX-2 protein expression by Western blot and immunocytochemistry in HSVEC stimulated with IL-1α and PMA. As shown in Fig. 1*C*, basal COX-2 was minimal in unstimulated EC and rose after exposure to IL-1α. Incubation of HSVEC with DHA alone did not affect COX-2 expression, but in DHA-preincubated HSVEC, IL-1α induced only 50% as much COX-2 compared with EC preincubated with stearate, whereas COX-1 expression was unchanged (Fig. 1*C Lower*). Similar results were obtained using PMA for both HSVEC (Fig. 1*D*) and human umbilical vein EC (not shown). Immunocytochemistry confirmed these effects (Fig. 1*E*). Production of PGI_2 was decreased in DHA-pretreated EC in a COX-2-dependent manner (Fig. 6, which is published as supporting information on the PNAS web site).

DHA Inhibits COX-2 Steady-State mRNA Levels Without Affecting mRNA Stability. Consistent with reduced COX-2 protein expression, DHA also reduced COX-2 steady-state mRNA levels at Northern blot. There were, however, no changes in COX-2 mRNA stability (half-life) in experiments using the transcription blocker actinomycin-D, indicating a transcriptional effect (Fig. 7, which is published as supporting information on the PNAS web site).

DHA Reduces COX-2 Promoter Activity. In transient transfection experiments using full-length or mutated human COX-2 promoter-luciferase constructs, we evaluated whether DHA regulated COX-2 promoter activity. We here used bovine aortic EC because of the difficulty in transfecting human EC, using PMA or LPS as stimuli. DHA pretreatment blocked COX-2 promoter activity after either stimulus (Fig. 8, which is published as supporting information on the PNAS web site). With a series of human COX-2 promoter/reporter constructs either deleted or site-mutated at specific transcriptionally active sites, we demonstrated that the proximal NF-κB-binding site is necessary for DHA inhibitory activity (Fig. 8).

DHA Reduces Activation of NF-κB and Nuclear Translocation of p65. Having determined that NF-κB-binding sites are a crucial target in down-regulation by DHA of COX-2 protein expression, we next examined DHA effects on NF-κB activation by EMSA. At all concentrations tested, DHA pretreatment decreased by ≈60% the amount of shifted complex induced by IL-1α (Fig. 9*A*, which is published as supporting information on the PNAS web site). Because nuclear translocation of the p65 NF-κB subunit is key for NF-κB activity, we tested whether DHA affected expression and nuclear translocation of p65. Western blots of nuclear proteins from DHA-pretreated cells before IL-1α stimulation showed significantly less p65 nuclear translocation into nuclei (but not total cellular p65) from cells pretreated with DHA than from nontreated cells (Fig. 9 *B* and *C*), confirming and expanding EMSA results.

DHA-Mediated Reduction in Cytokine-Induced COX-2 Expression and p65 NF-κB Subunit Translocation Involves ERK1/2 but Not p38 MAPK. IL-1 activates the MAPK pathway, including ERK1/2 and p38 (12). ERK1/2 acts upstream to NF-κB activation in the IL-1 signaling pathway leading to COX-2 expression (13). To demonstrate that ERK1/2 and NF-κB activations and COX-2 expression are linked, we first blocked ERK1/2 activation by PD98059, inhibiting a kinase (MAPK/ERK kinase 1) immediately upstream to ERK (14). This strategy (*i*) significantly inhibited IL-1α- and PMA-induced COX-2 expression (Fig. 2 *A* and *B*) and (*ii*) decreased translocation of p65 (Fig. 2*C*) and NF-κB activation (data not shown). We then evaluated whether DHA pretreatment affected IL-1α- and PMA-induced ERK1/2 phosphorylation (activation). In cells pretreated with DHA before IL-1α stimulation, ERK1/2 (but not p38 MAPK; data not shown) activation was significantly less than when no DHA was used (Fig. 2*D Upper*), suggesting that DHA blocks a molecular target upstream to ERK1/2. ERK total protein remained unchanged under all conditions tested (Fig. 2*D Lower*). Similar results (not shown) were obtained when ERK1/2 was activated by PMA.

DHA Reduces the Production of Intracellular Reactive Oxygen Species (ROS) Induced by IL-1α. Redox events are involved in IL-1 signaling (15), and exogenous oxidants can activate the ERK1/2 pathway (16, 17). We therefore tested in HSVEC whether IL-1α induces the production of ROS, whether ROS mediate the expression of COX-2, which enzymes are involved in ROS generation, and finally whether DHA modulates this process. The thiol donor and anti-

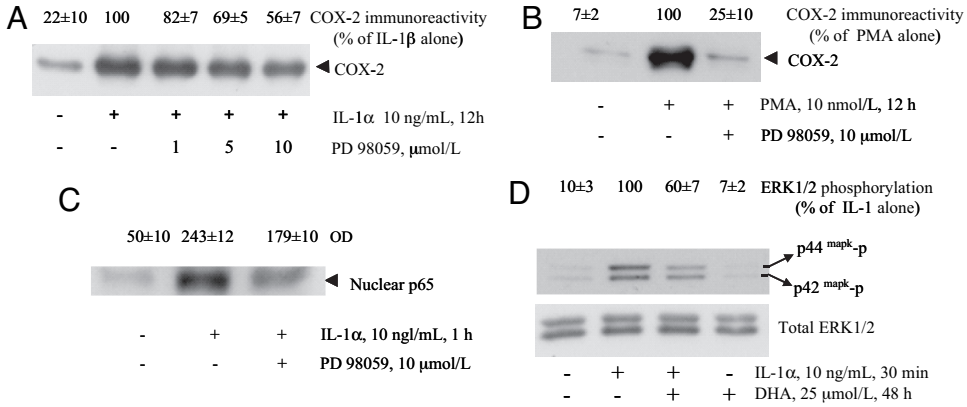

Fig. 2. Involvement of ERK1/2 in the stimulated expression of COX-2 and effect of DHA on ERK1/2 activation. (*A*) HSVEC were treated with MEK1 inhibitor PD 98059 at indicated concentrations for 30 min, then 10 ng/ml IL-1α was added for 12 h. Whole-cell lysates were analyzed by Western blot using an antibody specific for COX-2. Values of COX-2 are reported as percent of maximal control response (IL-1α alone). The blot shown is representative of three similar ones. (*B*) HSVEC were treated with 10 μmol/liter PD 98059 for 30 min, and then 10 nmol/liter of PMA was added for 12 h. Whole-cell lysates were used for Western blot as in *A*. The blot depicted is representative of three similar ones. (*C*) HSVEC were pretreated with 10 μmol/liter PD 98059 for 30 min and then stimulated with 10 ng/ml IL-1α. After 1 h, nuclear proteins were prepared and assayed by Western blot using a specific antibody against the p65 NF-κB subunit. Values of p65 expression are reported as units of OD. The blot shown is representative of three similar ones. (*D*) HSVEC were pretreated with 25 μmol/liter DHA for 48 h and then stimulated with 10 ng/ml IL-1α for 30 min. Whole-cell lysates were prepared and used for Western blot analysis using a specific antibody against phosphorylated ERK1/2. To ascertain that the total level of the ERK1/2 remained unchanged, the same blots were reprobed with an anti-ERK1/2 antibody that recognizes both phosphorylated and nonphosphorylated forms. Values of ERK1/2 phosphorylation are reported as percent of maximal control response (IL-1α alone). This blot is representative of three similar ones.

oxidant *N*-acetylcysteine dramatically attenuated IL-1α-induced expression of COX-2 (Fig. 3*A*). Similarly, diphenyl iodonium (DPI), an inhibitor of flavin-binding enzymes such as NAD(P)H oxidase, known to govern ROS generation in EC (18), concentration-dependently decreased IL-1α-stimulated COX-2 expression (Figs. 1*E* and 3*A*), without altering ERK1/2 activation (not shown). Correspondingly, we observed that IL-1α stimulates a strong production of ROS in HSVEC, as semiquantitatively assessed by dichlorofluorescein–fluorescence (Fig. 3 *C* and *D*). Here pretreatment with DHA before IL-1α decreased intracellular ROS (Fig. 3 *C* and *D*).

DHA Reduces Plasma Membrane Translocation of p47^{phox} and PKCε. Our results highlighted a crucial role for NADPH oxidase in the production of ROS in cells stimulated to express COX-2. Because the p47^{phox} component of NADPH oxidase is activated by its phosphorylation by PKC (19), which also activates ERK1/2 (20), we tested whether DHA, in altering NAD(P)H oxidase activity, alters membrane translocation of p47^{phox} or of conventional, novel, and atypical PKCs (α, ε, and ζ isoforms). We studied these links by two different approaches: (*i*) treating cells with the nonspecific PKC inhibitor Ro 318220 and (*ii*) using PMA pretreatment to down-regulate PKC before IL-1α or PMA stimulation (20). As shown in Fig. 4*A*, cell treatment with Ro 318220 abrogated IL-1α-induced COX-2 expression. Similarly, PKC down-regulation by PMA pretreatment similarly blocked COX-2 protein induction during subsequent stimulation by IL-1α or PMA (Fig. 4 *B* and *C*, respectively), indicating that PKC is involved in IL-1α-induced COX-2 expression. Western blots of cytosolic and plasma membrane-enriched fractions obtained from DHA-treated cells also showed that DHA reduces the stimulated membrane translocation of both p47^{phox} (Fig. 3*B*) and PKCε but not PKCα and -ζ (Fig. 4*C*).

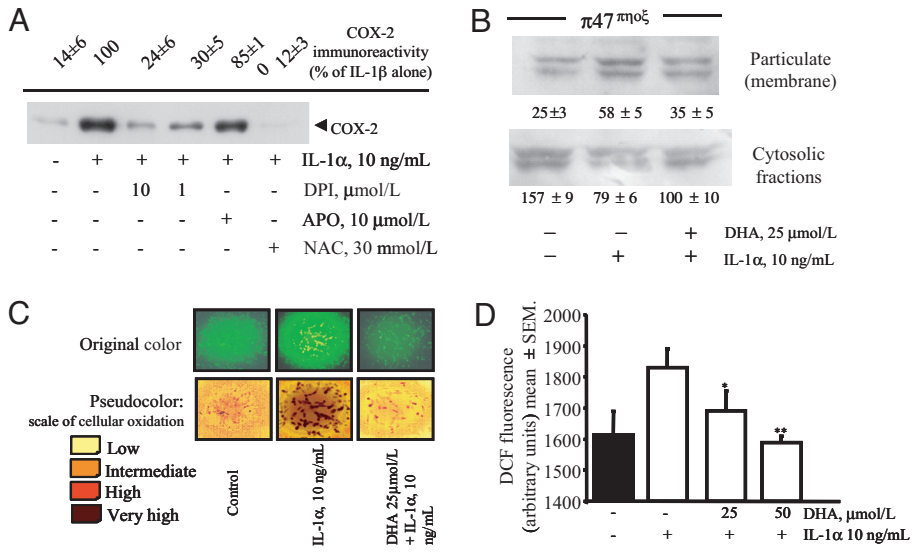

Fig. 3. Effect of DHA on ROS production and p47^{phox} translocation induced by IL-1α. (*A*) HSVEC were pretreated with DPI, apocynin, or *N*-acetylcysteine for 30 min before IL-1α stimulation for 12 h. Whole-cell lysates were subjected to Western blot for COX-2. Values of COX-2 are reported as percent of maximal control response (IL-1α alone). The blot shown is representative of three similar ones. (*B*) HSVEC were pretreated with 25 μmol/liter DHA for 48 h and then stimulated with 10 ng/ml IL-1α for 20 min. Subcellular fractions (soluble and particulate) were isolated and Western blots performed with an antibody specific for p47^{phox}. Values are in units of OD. The blot depicted is representative of two similar ones. (*C*) HSVEC were pretreated with 25 μmol/liter DHA for 48 h and then stimulated with 10 ng/ml IL-1α for 1 h. Monolayers were then washed and loaded with reduced dichlorofluorescein for 30 min and imaged as described in *Materials and Methods*. (*Upper*) Original microphotographs, where sides of each square are 300 μm long. (*Lower*) Corresponding pseudocolor transformation of digitalized images, where the yellow color indicates a low generation of ROS, and darker colors indicate increased ROS generation, proportional to color intensity. (*D*) Quantitative analysis of the effect of DHA on ROS production by IL-1α, as measured by dichlorofluorescein (DCF) fluorescence emission. Subconfluent HSVEC were treated with DHA at 25 and 50 μmol/liter for 48 h in 96-well plates, stimulated with 10 ng/ml IL-1α for 1 h, and finally loaded with reduced DCF. After 30 min, fluorescence was measured with a plate reader as described. More than eight replicates were used for each condition. Results are expressed as arbitrary fluorescence units ± SD. *, $P < 0.05$; **, $P < 0.01$ vs. IL-1α-stimulated control. This experiment is representative of a series of four, with similar results.

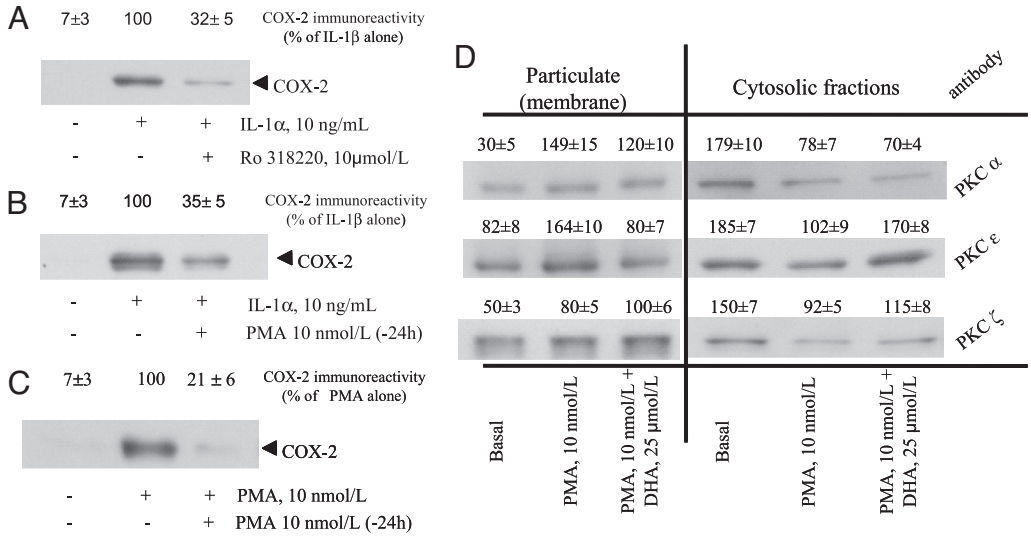

Fig. 4. Effects of DHA on PKC isoform translocations. (*A*) HSVEC were treated with Ro318220 for 30 min before IL-1α stimulation for 12 h. Whole-cell lysates were subjected to Western blot using an antibody specific for COX-2, and the value obtained at densitometric analysis is reported as percent of maximal control response (IL-1α alone). The blot is representative of three similar ones. (*B*) HSVEC were pretreated with PMA for 24 h before IL-1α stimulation for 12 h to down-regulate PKC activity. Whole-cell lysates were subjected to Western blot using an antibody specific for COX-2, as in *A*. The blot is representative of three similar ones. (*C*) HSVEC were pretreated as in *B* but then stimulated with PMA. The blot is representative of three similar ones. (*D*) HSVEC were pretreated with 25 μmol/liter DHA for 48 h and then stimulated with 10 nmol/liter PMA for 20 min. Subcellular fractions (soluble and particulate) were isolated, and Western blots were performed using anti-PKCα, -ε, or -ζ antibodies. Values of PKC translocations are reported as units of OD at densitometric analysis. The blot is representative of two similar ones.

Possible Role of Lipoxygenase (LO), P450 Epoxygenase, and COX Activities in DHA-Induced COX-2 Down-Regulation. To address the question of whether DHA itself or some DHA-derived metabolite (21) is causally involved in DHA-induced COX-2 expression down-regulation, we treated EC with pharmacological and molecular (siRNA) inhibitors of the most important routes involved in the metabolism of polyunsaturated fatty acids. The inhibition of LO activities, and in particular of 15-LO-1, partially reverted the protective effect of DHA, indicating that, at least in part, DHA acts by these metabolites (Table 2, which is published as supporting information on the PNAS web site).

Discussion

Here we show that exposure of EC to DHA, under conditions that efficiently incorporate DHA into membrane phospholipids (11), decreases stimulated COX-2 mRNA transcription, COX-2 protein expression and PG production. DHA blocks NF-κB-mediated transcriptional regulation of COX-2 by decreased activation of ERK1/2, diminished intracellular production of ROS, and inhibition of $p47^{phox}$ activation and PKCε translocation.

Antiinflammatory properties of n-3 fatty acids have been mostly attributed to competition with AA as substrates for COX or 5-LO (22). Here we demonstrate that exposure of EC to DHA alters gene expression of COX-2 independently of acting as a competitive substrate. Here prolonged incubation of human EC with DHA decreased expression of COX-2 protein and PG production upon cytokine/PMA stimulation, a setting in which COX-2 is induced. These results both complement and expand prior demonstrations by Spector *et al.* (8) of decreased PGI_2 production by human cultured EC briefly exposed to EPA or DHA (8). In Spector's experimental conditions, the decreased PGI_2 resulted from the inability of EC COX to metabolize EPA and DHA instead of AA (8). We confirm that DHA acutely reduces (by 27% on average) PGI_2 production when EC are stimulated by AA or thrombin, but we also demonstrate a stronger inhibition (40–71%) under conditions where COX-2 is induced. Our measurements likely underestimate DHA's inhibitory effect on PGI_2 production by stimulated EC, because our RIA is not totally specific for 6-keto-$PGF_{1\alpha}$ vs. Δ17–6-keto-$PGF_{1\alpha}$, the hydrolytic product of PGI_3 ($\approx$30% cross-reactivity in our assay; data on file), and some retroconversion of DHA to EPA likely occurs.

The time course of these inhibitory effects of DHA is fully compatible with that of DHA incorporation into EC membranes, previously shown to plateau after 48 h (11), and with the kinetics of DHA inhibition of endothelial activation products, such as vascular cell adhesion molecule-1, E-selectin, IL-6, and IL-8 (11). Measurements of COX-2 protein confirmed that DHA decreases induced COX-2 by $\approx$50%, whereas expression of COX-1 was unaffected. Both aspirin and NS-398, nonselective and selective inhibitors of COX-2 activity, respectively, augment DHA effects on PG production; these data support the hypothesis that DHA has a different action from aspirin or NS-398.

Regulation of COX-2 is both transcriptional and, mostly, post-transcriptional (23). Our results on mRNA stability and promoter transfection experiments indicate that DHA mainly affects transcriptional regulation. As in many other inflammatory early response genes, 5′ promoter regulatory sites are important in COX-2 transcriptional control. Binding sites for transcription factors NF-IL-6, AP-2, CRE, and the proximal NF-κB-binding site (23), located between -327 and -220 bp 5′ of the transcription start site, regulate COX-2 transcriptional activation by PMA and LPS. Of these promoter sites, only the NF-κB site is here shown to be essential for DHA modulation of COX-2 activity in EC, because only its deletion or mutation abolished DHA effects. The involvement of NF-κB in DHA regulation of COX-2 was further confirmed by EMSA and Western blot, showing reduced nuclear translocation of the p65 NF-κB subunit.

IL-1 receptor activation initiates a number of signaling pathways (15) involving ERK1/2, JNK, and p38 MAPK. ERK1/2, in particular, is clearly up-regulated *in vivo* in atherosclerosis (24) as well as in IL-1 (13)- and PMA (20)-induced COX-2 expression. Therefore, we examined whether DHA affected IL-1 and PMA-induced ERK1/2 activation. Having established a critical role for ERK1/2 in the expression of endothelial COX-2, because PD 98059 blocked p65 nuclear translocation and COX-2 protein induction, we then showed that DHA down-regulates IL-1α- and PMA-induced ERK1/2 activation. The effect of DHA on EC ERK1/2 confirms previous reports in non-EC (25, 26).

We next explored the possibility that DHA negatively affects one or more molecular target(s) upstream of ERK1/2. Because ROS production activates ERK-related pathways (27), as well as NF-κB (28), DHA might directly decrease IL-1-induced ROS by consuming superoxide anion by peroxidation of the double bonds in the

polyunsaturated fatty acid chain (29). In addition, DHA might interfere with some ROS-producing enzyme system in the endothelium, because n-3 fatty acids can alter membrane lipid microdomains, such as lipid rafts and caveolae (30), involved in the compartmentalization, modulation, and integration of cell signaling components upstream of NF-κB, which are sensitive to hydrogen peroxide (27). NADPH oxidase is the principal source of ROS in the endothelium (18). Upon stimulation, its $p47^{phox}$ component becomes phosphorylated, promoting its translocation, together with $p67^{phox}$, $p40^{phox}$, and the small GTP-binding protein Rac to the plasma membrane to form the active enzyme complex (18). Although endothelial NADPH oxidase is constitutively active, with a low-level constant intracellular production of ROS, upon stimulation by agonists (such as PMA and cytokines), NADPH oxidase activity is augmented. This occurs through an increased amount of assembled complexes and/or changes in the phosphorylation status of $p47^{phox}$. The phosphorylation of $p47^{phox}$ is mediated by PKC activation (19). We here demonstrate the involvement of NADPH oxidase activity in IL-1-mediated COX-2 expression by showing that DPI blocks IL-1-mediated COX-2 induction. Another NAD(P)H oxidase inhibitor, apocynin, had no effect on EC COX-2 expression, but it is known that apocynin inhibits NAD(P)H oxidase activity only in phagocytes, stimulating ROS production in nonphagocytic cells (31). We observed that DHA treatment of EC led to decreased $p47^{phox}$ membrane translocation, together with diminished NAD(P)H oxidase activity and intracellular ROS production. This effect is independent of the inhibition of ERK1/2 by DHA, because DPI alone does not alter ERK1/2 activation.

Because PKCε activity is also involved in the activation of NF-κB by ERK1/2 induction (32), and PKCs have been implicated in COX-2 expression (33), we explored the possibility that other molecular switches could be affected by DHA. We monitored membrane translocation of the main PKC isoforms in EC stimulated by PMA (34) in the presence of DHA. All such isoforms were activated by PMA, as demonstrated by their translocation to plasma membrane, but only the translocation of PKCε was reduced by DHA treatment. We conclude, therefore, that DHA inhibits at least two molecular switches, the activation of NAD(P)H oxidase and the activation of PKCε (which involves ERK1/2), both involved in COX-2 expression (Fig. 5).

We finally addressed the question whether DHA itself or some DHA-derived products are involved in this regulation of COX-2, because DHA can generate antiinflammatory lipid mediators through LO or COX pathways (21). We used pharmacological and molecular (siRNA) inhibitors of the main routes involved in polyunsaturated fatty acids metabilization. Here inhibition of LO activities, in particular 15-LO-1, partially reverted the DHA inhibitory effect on COX-2 expression. This suggests that DHA, after its incorporation in membrane lipids, acts at least in part through LO-derived structural rearrangements leading to antiinflammatory molecules, such as 10,17S-docosatriene, previously shown to inhibit oxidative stress in transformed human retinal pigment epithelium (35).

Overall, these findings provide insight into the mechanisms by which n-3 fatty acids may limit inflammation and atherogenesis. First, the effects shown here occur within a concentration range (≤25 μmol/liter) compatible with nutritional or pharmacological interventions *in vivo* (36). Second, inhibition of COX-2, which limits the amount of prostanoids released, may explain how n-3 fatty acids ameliorate rheumatoid arthritis, psoriasis, and inflammatory bowel disease (6). Third, in atherosclerotic vascular disease, COX-2 activation has been implicated in the growth of atherosclerotic plaques (37), as well as in plaque angiogenesis and the activation of matrix-metalloproteinases (38). Our results may therefore provide some explanation for the plaque-stabilizing effects recently ascribed to n-3 fatty acids on the basis of histological analyses (39). Although clinical results with selective COX-2 inhibitors in vascular disease have been recently found to be globally unfavorable (40), vascular effects of n-3 fatty acids on prostanoids are substantially different, because of milder effects on COX-2 and additional effects on thromboxane production. Thus, data presented here may explain part of the peculiar efficacy and safety profiles of these compounds in cardiovascular disease.

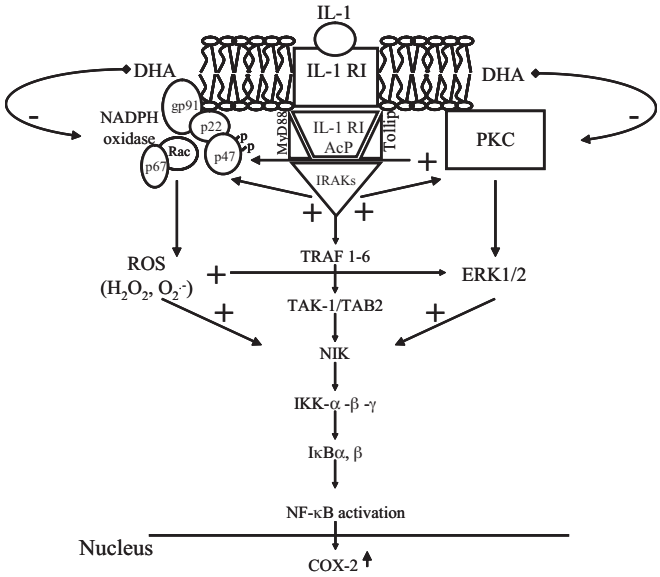

Fig. 5. Proposed molecular model of dietary omega-3 fatty acid interference with IL-1 signaling pathways leading to COX-2 induction in EC. IL-1 binds to the IL-1 receptor type I (IL-1RI), which heterodimerizes with the IL-1 receptor accessory protein (IL-1RAcP). The IL-1R-associated kinases (IRAK) are then recruited and associated by the adapter proteins myeloid differentiation factor(MyD)88 and Toll-interacting protein (Tollip). The signaling pathway also includes the production of ROS (H_2O_2) through the activation of NAD(P)H oxidase by IRAK activation, as well as the activation of PKC, both contributing to NF-κB activation. DHA, by interfering with the production of ROS (through the inhibition of $p47^{phox}$ translocation and/or the scavenging of ROS by its multiple double bonds), would prevent the formation of H_2O_2, thus limiting all of the downstream cascade leading to COX-2 gene expression. Furthermore, DHA reduces PKCε activation, thus inhibiting ERK1/2 activation, also leading to NF-κB activation and COX-2 expression. TRAF, TNF receptor-associated factor; TAK-1, TGFβ-activated kinase 1; TAB-2, TAK1-binding protein 2; NIK, NF-κB-inducing kinase; IKK, IκB kinase.

Materials and Methods

Materials. DHA (22:6 n-3 all cis), AA, (20:4 n-6, all cis), and stearate (18:0) were obtained as >99% pure sodium salts from Nu-Chek (Elysian, MN). DHA from two other commercial sources [Calbiochem (La Jolla, CA) and Sigma-Aldrich (St. Louis, MO)] was also used as control. IL-1α was obtained from Hoffmann-La Roche (Basel, Switzerland). The COX-2 inhibitor NS-398, the MEK1inhibitor PD 98059 (inhibiting a kinase upstream of ERK1/2), the 5-LO inhibitor MK886, and the cytochrome P450 epoxygenase inhibitor SKF-525A were from Calbiochem. The 5- and 12-LO inhibitor 5,8,11-eicosatriynoic acid was from Alexis (Lausen, Switzerland). All other reagents were purchased from Sigma.

Cell Cultures. We used two types of EC cultures: HSVEC and human umbilical vein EC (HUVEC), both harvested and maintained as described (41, 42). All experiments reported, when not otherwise specified, were performed in HSVEC, but control experiments were also done in HUVEC, with identical results. Cells were used at a time calibrated to reach confluence at the time of stimulation, and up to the fifth passage. For transfection assays, difficult in human EC, we used bovine aortic EC, as described (41).

Experimental Designs. All cultured EC were preincubated with DHA or other fatty acids, as control, for 0–48 h, followed by

stimulation with IL-1α, *Escherichia coli* LPS, or PMA for an additional 0–12 h, after which time cells and the supernatant medium were collected. In the experiments aimed at determining the production of 6-keto-$PGF_{1\alpha}$, some monolayers were also treated with aspirin or NS-398 for 30 min and then stimulated with AA or human thrombin for 5 min before medium collection.

Measurement of COX Activity. We determined the concentration of 6-keto-$PGF_{1\alpha}$, the stable nonenzymatic product of PGI_2, in the cell medium, by RIA (43).

Immunocytochemistry and Cell Lysis. Immunocytochemistry and cell lysis were done with routine methods (see *Supporting Text*, which is published as supporting information on the PNAS web site).

Assessment of $P47^{phox}$ and PKC Translocation. After exposure to IL-1α or PMA for 20 min, EC monolayers were washed three times in cold PBS and harvested by scraping. Cells were centrifuged, resuspended in extraction buffer, and disrupted by sonication on ice with three 15-s bursts. Sonicates were centrifuged at $500 \times g$ for 10 min, the nuclei-rich pellet discarded, and the supernatant fluid recentrifuged to obtain the plasma membrane and the cytosolic fractions, which were used to detect specific subunit translocation after protein separation by SDS/PAGE and subsequent immunoblotting. For details, see *Supporting Text.*

Immunoblotting. Equal amounts of proteins were separated by SDS/PAGE. The resolved proteins were transferred onto supported nitrocellulose sheets (Amersham Biosciences, Cardiff, U.K.) and, after saturation of nonspecific binding sites, incubated overnight with specific mono- and polyclonal antibodies against COX-1 and -2; various MAPK (also recognizing, in selected cases, specific activation epitopes); 15-LO-1; $p47^{phox}$; PKCα, -ε, and -ζ; and the p65 NF-κB subunit. For details, see *Supporting Text*.

Northern Blot. Northern blot was performed as published (41).

Transfections and Luciferase Assay. We used the 5′-flanking sequence from −1432 to +59 of the human COX-2 gene and a series of deleted promoters progressively eliminating the entire sequence upstream of the proximal NF-κB-binding site (−327/+59 bp), the proximal NF-κB-binding site (−220/+59 bp), as well as all regulatory sequences upstream of the TATA box (−52/+59 bp). We also used a promoter site mutated at −224/−214 for the proximal NF-κB site (κBM), all kindly provided by Hiroyasu Inoue (National Cardiovascular Center Research Institute, Osaka, Japan), and all inserted into the promoterless luciferase expression pGL2 basic plasmid (Promega, Madison, WI). For details, see *Supporting Text*.

Preparation of Nuclear Extracts and EMSA. After DHA treatment for 24–48 h and subsequent IL-1α stimulation for 1 h, nuclear proteins were purified as described (42). For the assay, we used the oligonucleotide 5′-AGTTGAGGGGACTTTCCCAGGC-3′ containing the consensus sequence for NF-κB (underlined; this is identical, with the only difference of a C→T substitution, to the proximal sequence of the NF-κB-binding site of the human COX-2 promoter (44), and a mutant oligonucleotide with a G→C substitution in the third nucleotide of the consensus sequence (5′-AGTTGAGGCGACTTTCCCAGGC-3′) (Santa Cruz Biotechnology, Santa Cruz, CA). For further details, see *Supporting Text.*

Measurement of Intracellular ROS. After DHA treatment and IL-1α stimulation for 1 h, we measured the intracellular production of ROS in ECs, as described by us (ref. 29; see *Supporting Text* for further details).

Statistical Analysis. Multiple comparisons were performed by one-way ANOVA, and individual differences then tested by the Fisher's protected least-significant difference test after demonstrating the existence of significant intergroup differences by ANOVA. Two-group comparisons were performed by unpaired Student's *t* test. Results are expressed as mean ± SEM, with a minimum of three separate experiments for each issue addressed.

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Supporting Figure 6

Fig. 6. Different effects of aspirin (ASA, *Left*) and NS-398 (*Right*), in the absence or presence of DHA, on unstimulated or stimulated prostacyclin production in HSVEC. HSVEC were preincubated in the absence (vehicle) or presence of 25 μmol per liter of DHA for 48 h in 24-well plates. After this time, 10 ng/ml IL-1α was added in selected wells for an additional 12 h to induce the expression of COX-2. Either the nonselective COX inhibitor ASA (100 μmol per liter, *Left*) or the selective COX-2 inhibitor NS-398 (at the IC_{50} of 1.77 μmol per liter, *Right*) was then added for an additional 25 min. After this time, medium was replaced with fresh medium containing, in addition to fresh aspirin or NS-398, 50 μmol AA per liter or human thrombin 10 units/ml, to provide exogenous substrate or to release endogenous substrate, respectively, for prostaglandin production through cyclooxygenases. After 5 min, the medium was collected from each well and snap-frozen for subsequent analysis of 6-keto-$PGF_{1\alpha}$, by RIA. 6-keto-$PGF_{1\alpha}$ is expressed as picogram per 1,000 cells. Bars represent mean ± SEM from eight or more replicates. All ASA-containing conditions are significantly ($P < 0.01$) different from corresponding conditions without ASA. (*Right*) Brackets link conditions for which statistically significant differences were found between the absence and the presence of NS-398 (* = $P < 0.05$; ** = $P < 0.01$). Here

aspirin strongly inhibited (>70%) prostacyclin production in all conditions tested, including those at which a partial inhibition already occurred as the result of DHA incubation. NS-398, here used at concentrations close to that producing 50% inhibition of the enzyme activity (IC_{50}) to avoid, as much as possible, nonselective inhibitory effects on COX-1, did not suppress prostacyclin production under basal conditions in which COX-2 activity is negligible, but strongly inhibited prostacyclin when COX-2 induction had occurred. These results suggest that DHA has a different site of action from aspirin or NS-398.

Supporting Figure 7

Fig. 7. Effect of DHA on IL-1α-mediated induction of COX-2 mRNA. (*A*) HSVEC were treated with 25 •mol per liter of DHA for 48 h and then stimulated with 10 ng/ml IL-1α for 3 h. Northern analysis of COX-2 mRNA is reported (*Upper*) and the ethidium bromide staining for the 28S rRNA, as control for equal loading of lanes (*Lower*). DHA treatment is associated with a 60% reduction of COX-2 mRNA at densitometric analysis. The blot depicted here is representative of a series of three similar ones. (*B*) HSVEC were treated with 25 •mol per liter DHA for 48 h and then stimulated with 10 ng/ml IL-1α for 4 h, in the absence (0 h) or the presence of the transcription inhibitor actinomycin-D (Act. D, 5 •g/ml) for 1, 2, and 6 h, after which total cellular RNAs were isolated. A representative Northern analysis of COX-2 mRNA is shown at various time points after actinomycin D addition (*Upper*). The corresponding ethidium bromide staining for the 28S rRNA, as a control for equal loading, is shown (*Lower*). Values of COX-2 mRNA are reported as units of OD at densitometric analysis. Calculation of mRNA half-lives in the presence of actinomycin D with or without DHA evaluates the effect of DHA on the rate of mRNA degradation. The similarity of mRNA half-lives in the absence or presence of DHA ($t_{1/2}$ = 5.6 ±1.4 h vs. 5.8 ± 1.2 h, respectively) indicates that DHA had no significant effect on the stability of COX-2 mRNA. The blot depicted here is representative of three similar ones.

Supporting Figure 8

Fig. 8. Effect of DHA on PMA- and LPS-mediated induction of COX-2 promoter activity. (*A*) BAEC were cotransfected with 2 μg of the full-length (-2,000 to +59 bp) human COX-2 promoter construct linked to the reporter gene luciferase and the pRSV.β-gal plasmid. Transfected cells were treated with DHA (0, 10, and 20 •mol per liter) for 48 h and subsequently stimulated with PMA (200 nmol per liter) or LPS (5 •g/ml) for a further 6 h, after which time reporter activities were measured in cellular extracts as described in *Materials and Methods*. The graph represents the means from three experiments, each run in duplicate. **P* < 0.05 and ***P* < 0.01 vs. the corresponding stimulated condition. (*Inset*) Structure of the transfected full-length COX-2 promoter, with the binding sites for various known transcription factors involved in COX-2 transcriptional regulation. Among these are the two NF-κB binding sites, termed "distal" and "proximal" (*Inset* from left to right) relative to the transcription start site (0). (*B*) BAEC were cotransfected with 2 •g of one of a series of human COX-2 promoter deletion- and/or site-mutated constructs linked to luciferase (-327/+59, -220/+59, κBM, -52/+59 bp), and with the pRSV.β-gal plasmid. Transfected cells were pretreated with 10 •mol per liter of DHA for 48 h and subsequently

stimulated with 200 nmol per liter of PMA for a further 6 h, after which time reporter activities were measured in cellular extracts as described in *Materials and Methods*. (*Inset*) Structure of the COX-2 promoters transfected. The graph represents the means from three experiments, each run in duplicate. Luciferase activity in each stimulated condition with -327/+59, -220/+59, and κBM constructs is significantly different from the corresponding control. Other statistical considerations are indicated.

Supporting Figure 9

Fig. 9. Effect of DHA on IL-1α-mediated induction of NF-κB and p65 nuclear translocation. (*A*) HSVEC were pretreated with DHA at concentrations of 10, 20, and 30 •mol per liter for 48 h and then stimulated with 1 ng/ml IL-1α. After 1 h, nuclear proteins were prepared and assayed for NF-κB by EMSA, as described. The intensity of the retarded band (lane 2), as an index of NF-κB activation, is significantly and concentration-dependently reduced by DHA pretreatment. This autoradiograph is representative of a series of three similar experiments. One additional gel-shift experiment was performed in HUVEC, with similar results. Values of retarded bands are reported as units of OD at densitometric analysis. (*B*) HSVEC were pretreated with DHA at concentrations of 20 μmol per liter for 48 h and then stimulated with IL-1α. After 1 h, nuclear proteins were prepared and assayed by Western analysis using a specific antibody against the p65 NF-κB subunit. Values of nuclear p65 are reported as units of OD. The blot depicted is representative of three similar blots. (*C*) HSVEC were treated as in *B* and, after stimulation with 1 ng/ml IL-1α for 1 h, total cellular proteins were extracted and assayed by Western analysis using a specific antibody against the p65 NF-κB subunit. Total cellular p65 is reported as units of OD. The blot depicted is representative of three similar ones. Overall, these results indicate that DHA inhibits the activation of the transcription factor NF-κB by IL-1α.

Supporting Text

DHA inhibits endothelial COX activity

We monitored the effects of prolonged (up to 48 h) exposure of human saphenous vein endothelial cells (EC) (HSVEC) to 25 •mol per liter of docosahexaenoic acid (DHA), by measuring production of 6-keto-$PGF_{1\alpha}$, the major metabolite of prostaglandin (PG) I_2 that is, in turn, the main product of cyclooxygenase (COX) in EC. 6-keto-$PGF_{1\alpha}$ increases in the presence of exogenous substrate, of stimuli for arachidonic acid (AA) release and, more, of stimuli for EC activation, such as IL-1α or phorbol myristate acetate (PMA; Table 1). Exposing HSVEC to DHA before stimulation with AA or thrombin significantly reduced 6-keto-$PGF_{1\alpha}$ (Table 1). EC exposed to IL-1α required >12 h before 6-keto-$PGF_{1\alpha}$ significantly rose (5-fold), and such production further increased after thrombin or AA addition (4- and 8-fold, respectively). DHA blocked thrombin or AA-stimulated PGI_2 40% more

in the presence of IL-1α than without IL-1α, and PMA effects were even more strongly inhibited (Table 1). Similar results were obtained with human umbilical vein EC (HUVEC; not shown). Stearate, not a substrate for COX, was used as control, and up to 50 •mol per liter did not inhibit PGI_2 production.

COX-2 dependence of DHA's effects on prostacyclin production: Experiments with aspirin and NS-398

To further verify the COX-2 dependence of the inhibitory effects of DHA on prostacyclin production, we assessed the effects of aspirin (a nonselective COX-1 and -2 inhibitor) and NS-398 (a selective COX-2 inhibitor) on the stimulated production of prostacyclin from HSVEC and HUVEC. As shown in Fig. 6*A*, the addition of aspirin 30 min before the acute stimulation of HSVEC with thrombin or AA always strongly decreased (by 70-98%) prostacyclin production, no matter what stimulus was used. The inhibition was quantitatively similar in the presence or absence of IL-1α, as expected, because aspirin inhibits both the constitutive and the inducible COX isoforms in the endothelium. The inhibitory effects of aspirin added 30 min before the acute stimulation were equally seen in cell monolayers pretreated with DHA, suggesting that DHA and aspirin act at different levels. Conversely, NS-398 reduced (by ≈50%) IL-1α-stimulated COX activity only when COX-2 was induced (Fig. 6*B*). Indeed, both aspirin and NS-398 were expected to inhibit COX activity, rather than the induction of COX-2 protein, whereas DHA mainly acted inhibiting COX-2 protein induction. Similar results were obtained in HUVEC (not shown).

DHA inhibits COX-2 steady-state mRNA levels without affecting mRNA stability

To better elucidate the mechanism of action of DHA on COX-2, we investigated its effect on steady-state COX-2 mRNA levels by Northern analysis. HSVEC monolayers were treated with 25 μmol/liter DHA for 48 h before the stimulation with IL-1α for 4 h. As shown in Fig. 7*A*, cell pretreatment with DHA was associated with a strong decrease of COX-2 mRNA. To assess whether DHA treatment modifies the stability of the IL-1α-induced COX-2 mRNA, we incubated monolayers treated with IL-1α for 3 h, in the presence or absence of DHA, with the transcription inhibitor actinomycin D, at 5 •g/ml, for 1, 2, and 6 h. Northern analysis showed similar mRNA half lives with or without DHA 25 •mol/liter (6.3 vs. 5.7 h at Northern densitometric analysis; P = NS), indicating no significant effect of DHA on COX-2 mRNA stability (Fig. 7*B*). Similar results were obtained in HUVEC (not shown).

DHA reduces COX-2 promoter activity

We conducted transient transfection experiments using full-length or mutated human COX-2 promoter-luciferase constructs to evaluate whether DHA regulated COX-2 promoter activity. Bovine aortic endothelial cells (BAEC) were used here because of the difficulty in transfecting human EC, using stimulatory concentrations of PMA or LPS, because human IL-1 is not active on BAEC. Stimulation with PMA or LPS increased COX-2 full-length promoter activity by 4- and 5-fold, respectively, and DHA pretreatment blocked COX-2 promoter activity after either stimulus (Fig. 8*A*). We further determined the responsible regions in the COX-2 promoter using a series of human COX-2 promoter/reporter constructs either deleted or site-mutated at specific transcriptionally active sites. As shown in Fig. 8*B*, PMA-mediated increases in COX-2 promoter activity were detectable with all the COX-2-deleted promoter constructs used, except for the -52/+59 construct containing only the TATA box. In contrast, constructs lacking the proximal NF-κB-binding site failed to show any inhibitory effect of DHA on reporter activity, suggesting that NF-κB binding to this site is critically impaired by DHA. Qualitatively similar results were obtained using LPS as a stimulus.

DHA reduces activation of transcription factor NF-κB and nuclear translocation of p65

Having determined that NF-κB-binding sites are a crucial target in the down-regulation by DHA of COX-2 protein expression, we next examined DHA effects on the activation of NF-κB using electrophoretic mobility-shift assays (EMSA). At all concentrations tested, DHA pretreatment decreased by ≈60% the amount of the shifted complex induced by IL-1α (Fig. 9*A*). Because nuclear translocation of the p65 NF-κB subunit is key for NF-κB activity, we tested whether DHA affected the expression and nuclear translocation of p65. Western blotting of nuclear proteins from DHA-pretreated cells before IL-1α stimulation showed significantly less p65 nuclear translocation into nuclei from cells pretreated with DHA than from nontreated cells (Fig. 9*B*), thus confirming and expanding the EMSA results. Western analyses on whole-cell lysates from both experimental conditions showed no difference in total cellular p65 in relation to DHA pretreatment (Fig. 9*C*).

Possible role of lipoxygenase, cytochrome p450 epoxygenase and COX activity in DHA-induced COX-2 down-regulation

DHA is now recognized to be the precursor of novel antiinflammatory lipid mediators such as 7S,8,17R-trihydroxydocosahexaenoic acid and 10,17S-docosatriene, obtained from acetylated COX-2 and/or lipoxygenase activities, respectively, in several cell types and tissues . To identify whether any of these metabolic pathways is involved in the observed DHA inhibitory effect on COX-2 expression, we tested whether the inhibition of several polyunsaturated fatty acid-metabolizing enzymes such as 5-, 12-, and 15-lipoxygenase and the cytocrome P450 epoxygenase [by pharmacological and molecular approaches (siRNA)] could reverse DHA inhibition of COX-2 expression and activity. We also tested whether the acetylation of COX-2 by ASA changed (potentiated) DHA inhibitory effects on COX-2 expression, as expected from previous findings.

We observed that inhibition of all lipoxygenase activities by NDGA partially reverted the DHA inhibitory effect on COX-2 expression and activity, with an average reversion of DHA inhibitory effect by 30% on COX-2 expression and 40% on COX activity (Table 2). Because the specific inhibition of 5- and 12-lipoxygenase by MK-886 and 5,8,11-eicosatriynoic acid (ETI) did not, however, affect the extent of the DHA effect, we postulated a predominant involvement of 15-lipoxygenase-1 (15-LO-1), in line with other recent findings . Having observed, by Western analysis, a clear basal expression of 15-LO-1 in HSVEC and HUVEC (data not shown), we specifically inhibited 15-LO-1 expression and activity by transfecting HSVEC with a mixture of siRNA specifically designed against 15-LO-1 . Such treatment, besides inhibiting 15-LO-1 protein expression (by ≈40 ± 5 % at Western analysis), also partially reverted DHA inhibitory effect on COX-2 expression and activity, although to a lesser extent than NDGA (Table 2). The inhibition of cytocrome P450 epoxygenase did not affect DHA inhibitory action on COX-2 expression. In addition, data obtained treating HSVEC with ASA failed to show any increase of DHA effect on COX-2 expression (total protein at Western analysis; data not shown), possibly expected on the basis of other recent findings on DHA-derived antiinflammatory mediators . This possibly occurred because we did not test any cell-cell interaction in our system.

METHODS

Immunocytochemistry

HSVEC were grown on gelatin- coverslips (Thermanox, ProSciTech, Thuringowa, Queensland, Australia). After

DHA treatment and IL-1α stimulation, monolayers were fixed with cold acetone and then incubated overnight at 4°C with a monoclonal anti-COX-2 antibody (Cayman Chemical, Ann Arbor, MI). After three washes, cell monolayers were incubated for 1 h with a biotinylated anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and for 1 h with extravidin peroxidase (Sigma, St. Louis, MO). Monolayers were then incubated with diaminobenzidine (Sigma) for 30 min, dehydrated, mounted, and stored at room temperature until analysis.

Cellular expression of COX-2

A modifed EIA procedure was used to measure the cellular expression of COX-2. Briefly, cells grown in 96-well plates and exposed to inhibitory drugs or 15-lipoxygenase siRNA ± DHA before IL-α stimulation were washed once with PBS before being fixed and permeabilized with cold acetone for 2 min on ice. EIA were then carried out by incubating monolayers first with saturating concentrations of a COX-2 monoclonal antibody (Cayman), then with biotinylated goat anti-mouse IgG (Immunotech, Marseille, France), and finally with streptavidin-alkaline phosphatase (Sigma-Aldrich, St. Louis, MO). Layers were washed three3 times between each incubation step, and integrity of the monolayer was monitored by phase-contrast microscopy. The COX-2 cellular content was quantified spectrophotometrically, reading the optical density of the wells (410 nm) 15-60 min after the addition of the chromogenic substrate para-nitrophenylphosphate (Sigma-Aldrich).

Transfections and luciferase assay

We used the 5'-flanking sequence from -1,432 to +59 of the human COX-2 gene, and a series of deleted promoters progressively eliminating the entire sequence upstream of the proximal NF-κB-binding site (-327/+59 bp) and the proximal NF-κB-binding site (-220/+59 bp), as well as all regulatory sequences upstream of the TATA box (-52/+59 bp). We also used a promoter site mutated at -224/-214 for the proximal NF-κB site (κBM), all kindly provided by Dr Hiroyasu Inoue, National Cardiovascular Center Research Institute, Osaka, Japan . These constructs were inserted into the promoterless luciferase expression pGL2 basic plasmid (Promega, Madison, WI). As control for transfection efficiency, the pRSV.β-gal plasmid was cotransfected in all experiments. BAEC were cotransfected with each reporter plasmid and the pRSV.β-gal plasmid (0.2 •g) by Fugene 6 transfection reagents (Roche, Indianapolis, IN), with DNA/Fugene ratio of 6 of 1:3. Briefly, 1.2 μg of the promoter constructs were mixed with 65 •l of complete DMEM culture medium containing serum and 3.6 •l of Fugene 6. The mixture was slowly added to single wells of cultured cells in a 12-well plate and incubated for 6 h. Fugene 6 and DNA plasmids were subsequently removed and replaced with complete medium overnight. DHA (10 or 20 •mol/liter) was added for 48 h prior to the addition of PMA 200 nmol/liter or LPS 5 •g/ml. The medium was removed and cells washed twice with PBS, lysed by adding reporter-lysis buffer (Promega, 150 μl per well), scraped off the well, and frozen at -70° C. Luciferase (Promega Italia, Milan, Italy) and β-gal (Roche) assays were performed according to the manufacturer's instructions using a luminometer (Thermolabsystems, Franklin, MA). The normalized reporter activity was calculated as the ratio of luciferase to β-gal activity. All experiments included transfections with both the promoterless -52/+59 bp construct and with the empty PGL-2 plasmid (Promega) as negative controls (data not shown).

Preparation of nuclear extracts and EMSA

After DHA treatment for 24-48 h and subsequent IL-1α stimulation for 1 h, nuclear proteins were purified as described . For the assay, we used the oligonucleotide 5'-AGTTGAGGGGACTTTCCCAGGC-3' containing the consensus sequence for NF-κB [underlined, with the only difference of a C→T substitution, to the proximal

sequence of the NF-κB-binding site of the human COX-2 promoter]), and a mutant oligonucleotide with a G→C substitution in the third nucleotide of the consensus sequence (5'-AGTTGAGG**C**GACTTTCCCAGGC-3') (Santa Cruz Biotechnology). Oligonucleotide probes were ^{32}P-end-labeled with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA), and unincorporated nucleotides were removed by column chromatography over a Sephadex G-50 column (Pharmacia, Piscataway, NJ). The DNA-binding reaction was performed at 30°C for 15 min in a volume of 20 •l containing 10 •g of nuclear extract, poly (dI-dC) (Roche) and glycerol. Samples were subjected to electrophoresis on native 5% 0.5× TRIS-borate-polyacrylamide gels. Specificity of the assay was determined by adding a 50- to 100-fold excess of unlabeled competing wild-type or mutant sequences in the binding mixture 10 min before the labeled oligonucleotide. Retarded bands disappear in the presence of a competing sequence but remain in the presence of the mutant sequence. After electrophoresis, gels were dried and autoradiographed by using Kodak X-AR films (Rochester, NY).

Immunoblotting

Equal amounts of proteins were separated by SDS/PAGE. The resolved proteins were transferred onto supported nitrocellulose sheets (Amersham Biosciences, Cardiff, U.K.) and, after saturation of nonspecific binding sites, incubated overnight with a monoclonal anti-COX-2 or a monoclonal anti-COX-1 antibody (Santa Cruz Biotechnology).

In the experiments investigating extracellular regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK) activation, we used monoclonal antibodies against a short amino acid sequence containing a phosphorylated Tyr-204 of ERK1 (and, correspondingly, in ERK2) and against a short amino acid sequence containing phosphorylated Tyr-182 of p38 MAPK, thus recognizing the activated forms of these kinases. We also used antibodies directed against the nonphosphorylated forms of ERK and p38, thus recognizing both the activated and the nonactivated forms. In the experiments investigating the membrane translocation of p47^{phox}, we used a monoclonal antibody raised against amino acids 196-390 of p47^{phox} of human origin, whereas, to study the membrane translocation of PKCα, ε, and ζ, we used rabbit polyclonal antibodies, all raised against peptides mapping at C terminus of the respective human antigens. All such antibodies were obtained from Santa Cruz Biotechnology and used with an overnight incubation. We also performed Western analysis using nuclear extracts (obtained as indicated below) to detect the p65 NF-κB subunit, again with a monoclonal antibody obtained from Santa Cruz Biotechnology. To investigate the endothelial expression of 15-LO-1, we carried out Western analyses using a rabbit polyclonal antibody (Cayman) against this protein. After incubation with the primary antibody, blots were incubated for 1 h with the pertinent secondary antibody consisting of an anti-mouse or -rabbit polyclonal IgG coupled with horseradish peroxidase (Sigma and Santa Cruz Biotechnology, respectively) diluted 1:1,000 for COX-1, -2; ERK1/2; PKCα, ε, ζ; p47^{phox}, 15-LO-1, and the p65 NF-κ B subunit; and a polyclonal goat anti-mouse biotinylated IgG (Dako, Glostrup, Denmark) diluted 1:1,500 for p38 MAPK. In this last case, such incubation was followed by a further incubation with extravidin peroxidase (Sigma) diluted 1:1,500 for 1 additional hour. Protein bands were visualized by an enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ). Blots were scanned using an Agfa (Mortsel, Belgium) Arcus II scanner.

Cell lysis

To obtain total cellular extracts, HSVEC were cultured and treated in six-well plates, then washed twice with ice-cold PBS and lysed in 150 ml of lysis buffer [150 mmol/liter HEPES, pH 7.9/150 mmol/liter NaCl/1 mmol/liter

EDTA/1% Triton-X 100/10% glycerol/1 mmol/liter diethyldithiocarbamate (DTT)/1 mmol/liter PMSF/1 mg/ml aprotinin/1 mg/ml leupeptin]. Lysates were vortexed ×3 for 10 s and incubated on ice for 30 min, after which they were centrifuged at 10,000 × *g* for 20 min at 4°C. After determination of protein concentration , supernatants were stored at -20°C until analysis.

Measurement of intracellular reactive oxygen species (ROS)

After DHA treatment and IL-1α stimulation for 1 h, we measured the intracellular production of ROS in endothelial cells, using a modification of the method originally described by Royal and Ischiropoulos , as described by us . This method is based on the oxidation of 2',7'-diclorofluorescin diacetate (DCFH-DA) by ROS, resulting in the formation of the fluorescent compound 2',7'-dichlorofluorescein (DCF). Treated endothelial cell monolayers, grown in 96-well plates, were stimulated in 2% serum-containing medium for 1 h. Monolayers were washed and then incubated with 50 •mol/liter DCFH-DA in Krebs-Ringer buffer for 20 min at 37°C. After this step, cells were washed, incubated in Krebs-Ringer buffer, and the fluorescence immediately read on a fluorescent plate reader (Fluoroskan II, Labsystem, Global Medical Instrumentation, Ramsey, MN) at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. Fluorescence microscopy, in six-well plates, was used in some experiments to qualitatively assess the formation of intracellular ROS in response to treatment with cytokines and fatty acids. In this case, samples were epi-illuminated with a 100-watt halogen lamp and photographed by using 490-nm excitation and 520-nm emission filters, respectively. Because illumination causes increased fluorescence emission due to the oxidation of the fluorescent dye, each field was exposed to light for exactly the same time (30 s).

Assessment of $P47^{phox}$ and PKC translocation

After exposure to IL-1α or PMA for 20 min, EC monolayers were washed ×3 in cold PBS and harvested by scraping. Cells were centrifuged, resuspended in extraction buffer (7.5 mmol/liter Tris-HCl, pH 7.5/2 mmol/liter EGTA/2 mmo/liter EDTA/0.25 mol/liter sucrose/1 mmol/liter DTT/1 mmol/liter PMSF/1 mg/ml aprotinin/1 mg/ml leupeptin) and disrupted by sonication on ice with three 15-s bursts. Sonicates were centrifuged at 500 × *g* for 10 min, the nuclei-rich pellet discarded, and the supernatant fluid recentrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant fluid (cytosolic fraction) was removed and stored at -20°C, while the pellet, containing the particulate fraction, was resuspended in extraction buffer containing 1% Triton X-100 and recentrifuged at 100,000 × *g* for 1 h. The resultant supernatant fluid (now membrane-enriched) was removed and stored at -20°C. The plasma membrane and cytosolic fractions were used to detect specific subunit translocation after protein separation by SDS/PAGE and subsequent immunoblotting.

Targeted silencing of 15-LO-1 by siRNA

The expression of 15-LO-1 in EC was silenced with three different siRNA sequences obtained from Ambion (Austin, TX) corresponding to the ID numbers 2306, 2309, and 2486. Briefly, EC were detached and seeded in the appropriate (6- or 96-well) multiwell plates, already containing 36 pmol/liter of a mixture of three different siRNA against 15-LO-1 or 36 pmol/liter negative control siRNA (Ambion), having no homology to known sequences from mice, rats, or humans, using the siPort NeoFX reagent (Ambion), and following the manufacturer's instructions. After their adhesion to the well, EC were treated with DHA30 •mol/liter for 48 h, with daily additions of the same siRNA species. Afterwards, EC were challenged with IL-1α 1 ng/ml for 12 h. Monolayers in six-well plates were then harvested to test COX-2 and 15-LO-1 expressions by Western analysis,

while monolayers in 96-well plates were fixed and subjected to EIA as described above.

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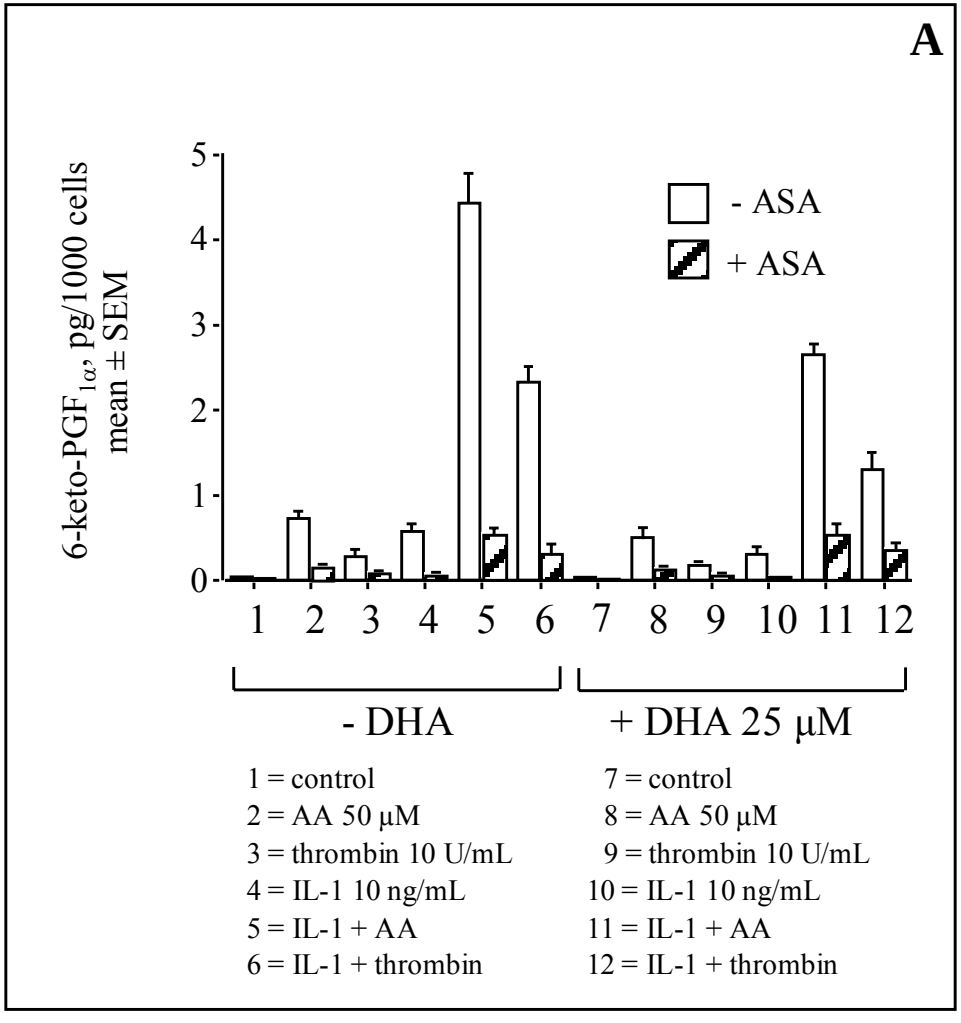
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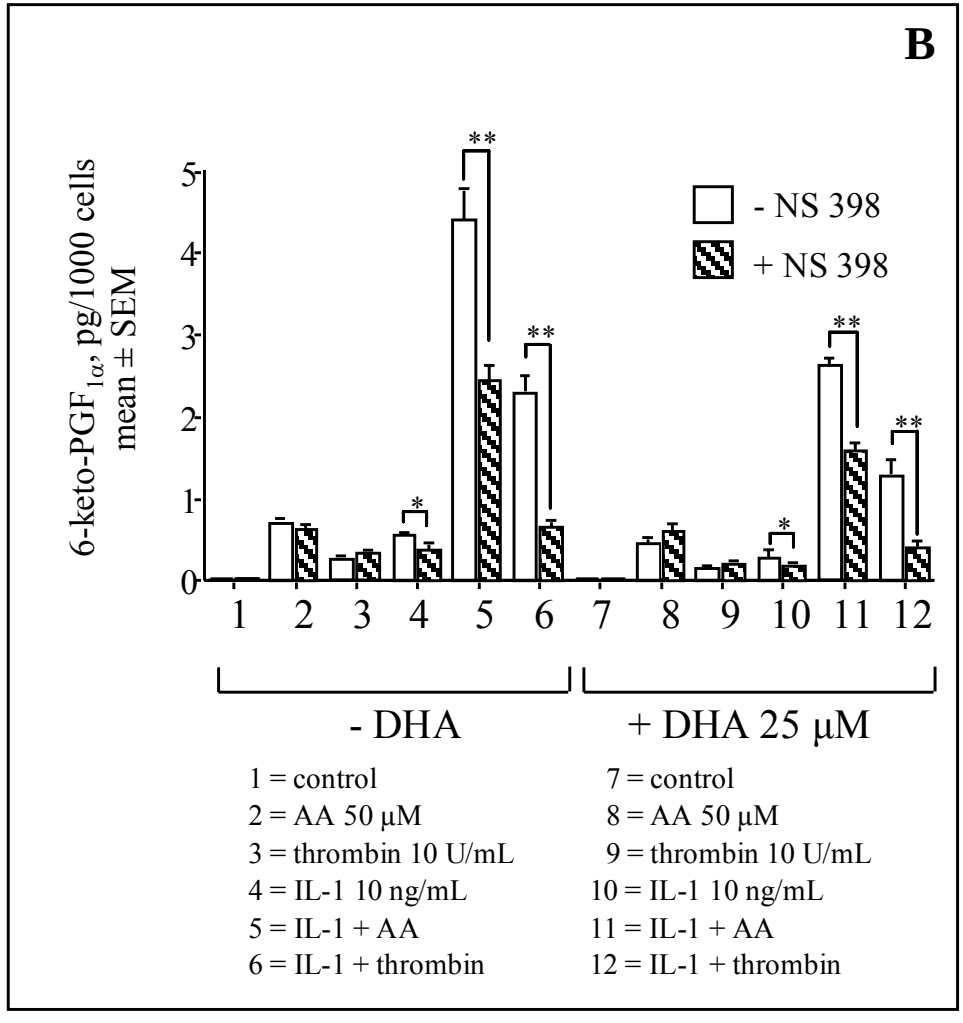
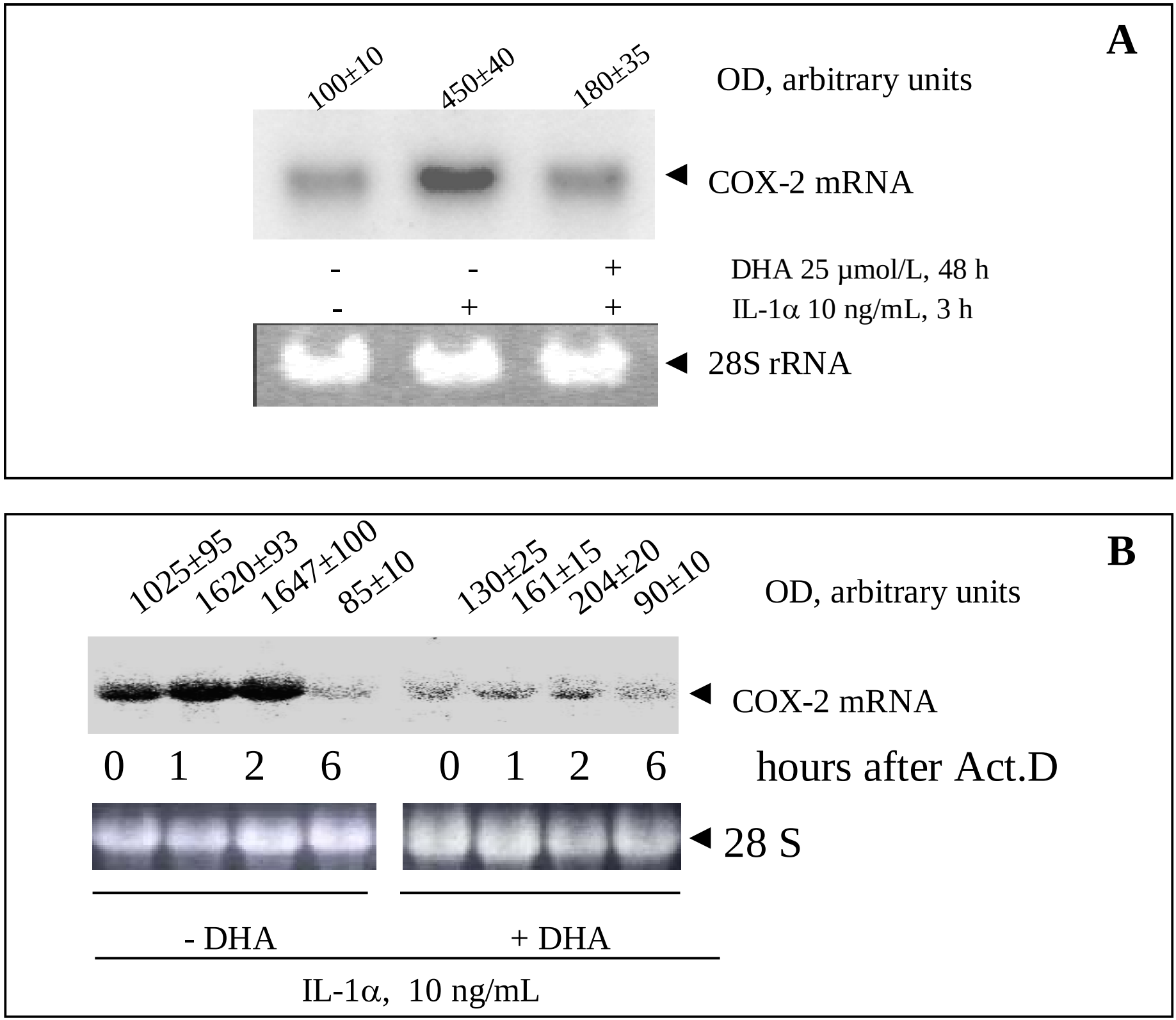
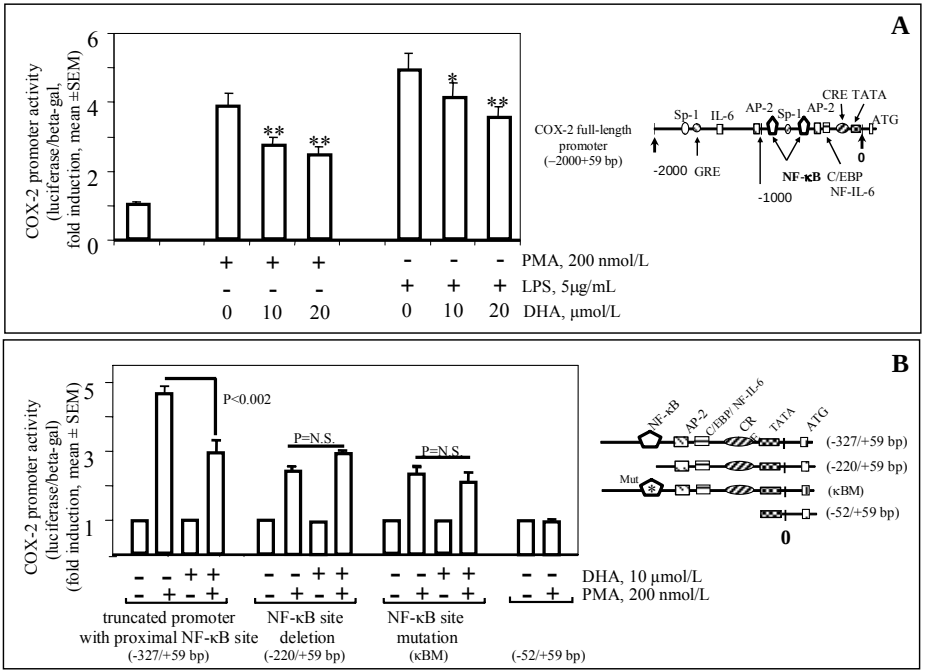
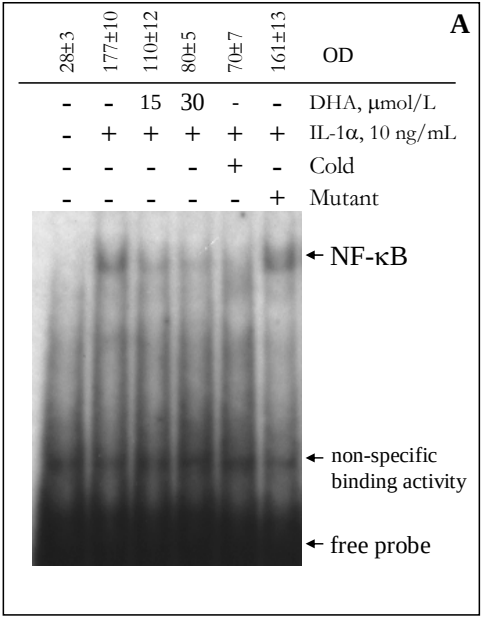
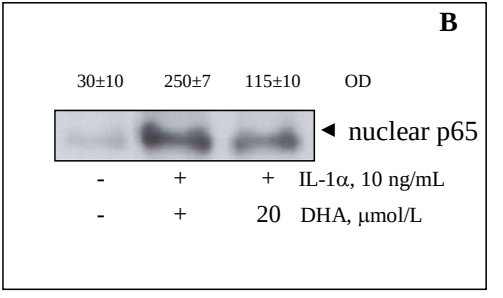
6-keto-PGF1α, pg/1000 cells
mean ± SEM
- ASA
+ ASA
1 2 3 4 5 6 7 8 9 10 11 12
- DHA
+ DHA 25 µM
1 = control
2 = AA 50 µM
3 = thrombin 10 U/mL
4 = IL-1 10 ng/mL
5 = IL-1 + AA
6 = IL-1 + thrombin
7 = control
8 = AA 50 µM
9 = thrombin 10 U/mL
10 = IL-1 10 ng/mL
11 = IL-1 + AA
12 = IL-1 + thrombin

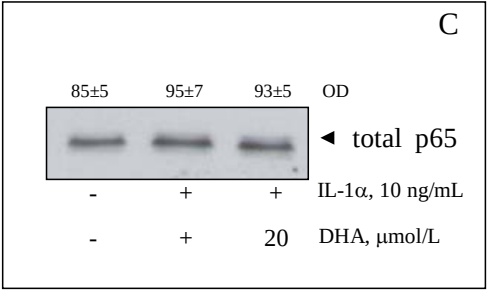

B
6-keto-PGF1α, pg/1000 cells
mean ± SEM
- NS 398
+ NS 398
1 2 3 4 5 6 7 8 9 10 11 12
- DHA
+ DHA 25 µM
1 = control
2 = AA 50 µM
3 = thrombin 10 U/mL
4 = IL-1 10 ng/mL
5 = IL-1 + AA
6 = IL-1 + thrombin
7 = control
8 = AA 50 µM
9 = thrombin 10 U/mL
10 = IL-1 10 ng/mL
11 = IL-1 + AA
12 = IL-1 + thrombin


A
100±10
450±40
180±35
OD, arbitrary units
COX-2 mRNA
-
-
+
DHA 25 μmol/L, 48 h
-
+
+
IL-1α 10 ng/mL, 3 h
28S rRNA
B
1025±95
1620±93
1647±100
85±10
130±25
161±15
204±20
90±10
OD, arbitrary units
COX-2 mRNA
0 1 2 6
0 1 2 6
hours after Act.D
28 S
- DHA
+ DHA
IL-1α, 10 ng/mL


A
COX-2 promoter activity
(luciferase/beta-gal,
fold induction, mean ±SEM)
6
4
2
0
**
**
*
**
+ + + - - - PMA, 200 nmol/L
- - - + + + LPS, 5μg/mL
0 10 20 0 10 20 DHA, μmol/L
COX-2 full-length
promoter
(–2000+59 bp)
Sp-1
IL-6
AP-2
Sp-1
AP-2
CRE TATA
ATG
-2000 GRE
-1000
NF-κB C/EBP
NF-IL-6
0
B
COX-2 promoter activity
(luciferase/beta-gal)
(fold induction, mean ± SEM)
5
3
1
P<0.002
P=N.S.
P=N.S.
- - + + - - + + - - + + - - DHA, 10 μmol/L
- + - + - + - + - + - + - + PMA, 200 nmol/L
truncated promoter
with proximal NF-κB site
(-327/+59 bp)
NF-κB site
deletion
(-220/+59 bp)
NF-κB site
mutation
(κBM)
(-52/+59 bp)
NF-κB
AP-2
C/EBP/ NF-IL-6
CR
TATA
ATG
(-327/+59 bp)
(-220/+59 bp)
Mut
(κBM)
(-52/+59 bp)
0


A
28±3
177±10
110±12
80±5
70±7
161±13
OD
- - 15 30 - - DHA, μmol/L
- + + + + + IL-1α, 10 ng/mL
- - - - + - Cold
- - - - - + Mutant
NF-κB
non-specific binding activity
free probe


B
30±10
250±7
115±10
OD
nuclear p65
- + + IL-1α, 10 ng/mL
- + 20 DHA, μmol/L


C
85±5
95±7
93±5
OD
total p65
- + + IL-1α, 10 ng/mL
- + 20 DHA, μmol/L