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Interleukin 1β Induces Type II-secreted Phospholipase A_2 Gene in Vascular Smooth Muscle Cells by a Nuclear Factor κB and Peroxisome Proliferator-activated Receptor-mediated Process*

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Type II-secreted phospholipase A_2 (type II-sPLA$_2$) is expressed in smooth muscle cells during atherosclerosis or in response to interleukin-1β. The present study shows that the induction of type II-sPLA$_2$ gene by interleukin-1β requires activation of the NFκB pathway and cytosolic PLA$_2$/PPARγ pathway, which are both necessary to achieve the transcriptional process. Interleukin-1β induced type II-sPLA$_2$ gene dose- and time-dependently and increased the binding of NFκB to a specific site of type II-sPLA$_2$ promoter. This effect was abolished by proteinase inhibitors that block the proteasome machinery and NFκB nuclear translocation. Type II-sPLA$_2$ induction was also obtained by free arachidonic acid and was blocked by either AACOCF$_3$, a specific cytosolic-PLA$_2$ inhibitor, PD98059, a mitogen-activated protein kinase kinase inhibitor which prevents cytosolic PLA$_2$ activation, or nordihydroguaiaretic acid, a lipoxygenase inhibitor, but not by the cyclooxygenase inhibitor indomethacin, suggesting a role for a lipoxygenase product. Type II-sPLA$_2$ induction was obtained after treatment of the cells by 15-deoxy-Δ^{12,14}-dehydroprostaglandin J$_2$, carbaprostacyclin, and 9-hydroxyoctadecadienoic acid, which are ligands of peroxisome proliferator-activated receptor (PPAR) γ, whereas PPARα ligands were ineffective. Interleukin-1β as well as PPARγ-ligands stimulated the activity of a reporter gene containing PPARγ-binding sites in its promoter. Binding of both NFκB and PPARγ to their promoter is required to stimulate the transcriptional process since inhibitors of each class block interleukin-1β-induced type II-sPLA$_2$ gene activation. We therefore suggest that NFκB and PPARγ cooperate at the enhanceosome-coactivator level to turn on transcription of the proinflammatory type II-sPLA$_2$ gene.

The structure of the arterial wall and its vasoactive properties are mainly supported by vascular smooth muscle cells, which represent a prominent cell type in this tissue. These cells are involved in major pathological conditions such as septic shock, neointima formation, and restenosis after acute vascular injury or chronic pathological processes such as atherosclerosis.

During severe infection, activation of inflammatory cells leads to the massive release of proinflammatory cytokines, including interleukin-1β (IL-1β)[1] and tumor necrosis factor-α (1). During the onset of atherosclerosis, vascular smooth muscle cells undergo phenotypic changes switching from a contractile phenotype to a proliferating and secretory phenotype. Early atherosclerotic lesions have many characteristics in common with the inflammatory reaction (2), and vascular smooth muscle cells participate in the local inflammatory process involved in the development of lesions and neointimal formation (3). IL-1β is thought to play an important role in this process since vascular smooth muscle cells express IL-1β receptors (4). Furthermore, in vascular smooth muscle cells, IL-1β induces expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (5) and IL-1β itself (6). IL-1β also stimulates extracellular matrix production by human smooth muscle cells (7).

Type II secreted phospholipase A_2 (type II-sPLA$_2$) has been implicated in the pathophysiological processes of sepsis syndrome as well as atherosclerosis. High levels of type II-sPLA$_2$ have been found in the plasma of patients with septic shock (8). In addition, high levels of this enzyme have been demonstrated in the aorta of endotoxin shock rats (9). Type II-sPLA$_2$ has been evidenced in human atherosclerotic plaques (10). A strong type II-sPLA$_2$ immunoreactivity colocalized with α-actin-positive vascular smooth muscle cells in both normal and atherosclerotic arteries (11). It has been suggested that type II-sPLA$_2$ could play an important role in early atherosclerosis because it is present in the pre-atherosclerotic arterial wall, where it may induce low density lipoprotein modification (12) and foam cell formation (13).

The precise physiological substrate of type II-sPLA$_2$ is still conjectural. Although only indirect evidence has been published to indicate that type II-sPLA$_2$ can hydrolyze mammalian cell phospholipids *in vivo* to generate lipid mediators (14–16), this enzyme seems involved in the specific synthesis of lyso-

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[1] The abbreviations used are: IL-1β, interleukin-1β; type II-sPLA$_2$, type II secreted phospholipase A_2; cPLA2, cytosolic phospholipase A_2; NFκB, nuclear transcription factor κB; AACOCF3, arachidonyltrifluoromethyl ketone; ALLN, *N*-acetyl-leucinyl-leucinyl-norleucinal; BEL, bromoenol lactone; CHX, cycloheximide; ETYA, 5,8,11,14-eicosatetraynoic acid; GAPDH, glyceraldehyde phosphate dehydrogenase; 9-HODE, 9-hydroxy octadecadienoic acid; INDO, indomethacin; NDGA, nordihydroguaiaretic acid; PD98059, 2′amino-3′-methoxyflavone; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; Z-AL, Z-Ile-Glu-(Ot-Bu)Ala-leucinal; 15-dPGJ$_2$, 15-deoxy-Δ^{12,14}-dehydroprostaglandin J$_2$; DMEM, Dulbecco's modified Eagle's medium; RT-PCR, reverse transcription-polymerase chain reaction; RXR, retinoid X receptor; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAPK/extracellular signal-regulated protein kinase; EMSA, electrophoretic mobility shift assay; VSMC, vascular smooth muscle cell; GC/MS, gas chromatography/mass spectrometry.

phosphatidic acid, a potent growth-promoting factor (17), and is inhibited by sphingomyelin, an important component of the outer leaflet of plasma cell membranes (18). Vascular smooth muscle cell proliferation is implicated in the pathogenesis of atherosclerosis and restenosis following interventional revascularization procedures (19). Several phospholipase A_2-derived mediators in addition to lysophosphatidic acid are thought to be involved in smooth muscle cell proliferation, *i.e.* lysophosphatidylcholine (20), cyclooxygenase, and lipoxygenase products (21) or arachidonic acid itself (22) and might mediate the proliferative effect of IL-1β (23).

IL-1β induces the synthesis and secretion of type II-sPLA$_2$ in various cell types (14, 15), as well as in vascular smooth muscle cells (24), but the precise signaling leading to type II-sPLA$_2$ gene expression remains to be elucidated. In most cells, IL-1β triggers its action by at least two main pathways involving specific receptors. IL-1β activates acid sphingomyelinase to initiate the ceramide cascade (25). IL-1β also induces signaling cascades which activate members of the nuclear transcription factor κB (NFκB) family in smooth muscle cells (26). NFκB activation has also been evidenced in vascular smooth muscle cells after rat arterial injury (27) or in human arteriosclerosis (28). In arterial smooth muscle cells, IL-1β-induced type II-sPLA$_2$ gene expression can therefore be expected to involve either the ceramide pathway or the NFκB pathway. In most cells, NFκB is retained in the cytoplasm by inhibitory proteins called IκBs. Signaling events that phosphorylate IκBs promote its degradation by the proteosome pathway and the subsequent nuclear translocation and activation of NFκB family members (29). A cytokine-responsive IκB kinase has been described (30), but the actual pathway by which IL-1β might induce phosphorylation of IκBs is still unknown.

Recently, cytosolic phospholipase A_2 (cPLA$_2$), an enzyme completely unrelated to type II-sPLA$_2$ (31), has been implicated in IL-1β-mediated type II-sPLA$_2$ gene induction in rat fibroblasts (32). This raises the possibility of a control of type II-sPLA$_2$ gene by free fatty acids or their derivatives via peroxisome proliferator-activated receptors (PPARs). PPARs are key players in lipid metabolism and are members of the nuclear receptor superfamily of transcription factors that regulate the pattern of gene expression in response to the binding of low molecular weight ligands (33). Three different subtypes have been described, PPARα, PPARβ/δ, and γ. Although the identities of the ligands that regulate *in vivo* activity remain to be established with certainty, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-dPGJ_2) (34), 13- or 9-hydroxyoctadecadienoic acid (HODE) (35), and certain polyunsaturated fatty acids (36) have been demonstrated to stimulate PPARγ-dependent transcription. Both PPARα and PPARγ are present in smooth muscle cells (37, 38), but there are conflicting views concerning their actual effect on the development of atherosclerosis (37, 39, 40).

In the present paper, we demonstrate that, in rat vascular smooth muscle cells, IL-1β induces type II-sPLA$_2$ gene via the NFκB pathway but not via the ceramide pathway. It can be mimicked by arachidonic acid as well as PPARγ agonists and blocked by cPLA$_2$ inhibitors and therefore involves the cPLA$_2$-PPARγ pathway. In addition, nuclear translocation of NFκB is required for the stimulation by PPARγ agonists, suggesting that both NFκB and PPARγ are required to mediate the IL-1β effect.

MATERIALS AND METHODS

Reagents—Type I collagen from calf skin, glutamine, penicillin, streptomycin, free fatty acid bovine serum albumin, and *Naja mossambica* type II-sPLA$_2$ were purchased from Sigma, Saint Quentin Fallavier, France. Fetal calf serum was from Roche Molecular Biochemicals. Antibodies against smooth muscle cell α-actin from hybridoma cells, clone 1A4, were from Dako S.A., Copenhagen, Denmark. Murine mammary lentivirus reverse transcriptase and Random Primers were from Life Technologies, Inc., and oligonucleotides were from Oligo Express; France. N+ nylon membranes, ECL direct nucleic acid labeling system, and ECL reagents kit for horseradish peroxidase were from Amersham Pharmacia Biotech. Fluorescent substrate 1-hexadecanoyl-2-(1-pyrenyldecanoyl)-*sn*-glycero-3-phosphoglycerol was from Interchim, Montluçon, France. IL-1β was purchased from Immugenex Corp. Carbaprostacyclin, 9-hydroxyoctadecadienoic acid (9-HODE), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-dPGJ_2) were from Cayman Chemical. Bromoenol lactone (BEL), arachidonyltrifluoromethyl ketone (AA-COCF$_3$) and Z-Ile-Glu-(Ot-Bu)-Ala-leucinal (Z-AL) were from Calbiochem. Arachidonic acid, *N*-acetyl-Leu-Leu-norleucinal (ALLN), 9-*cis*-retinoic acid, indomethacin, 5,8,11,14-eicosatetraynoic acid (ETYA), nordihydroguaiaretic acid (NDGA), and cycloheximide were from Sigma. The protein kinase C inhibitor GF109203X was a gift from Glaxo-Wellcome, Les Ulis, France. LipofectAMINE was from Life Technologies, Inc.; the pCMV-β-galactosidase plasmid was from CLONTECH Laboratories, and the luciferase reporter assay kit was from Promega, France.

Isolation and Culture of Rat Aortic Smooth Muscle Cells—Vascular smooth muscle cells were isolated by enzymatic digestion of thoracic aortic media from male Wistar rats (300 g, Elevage Janvier) according to the method of Michel and co-workers (41). The cells were seeded on dishes coated with type I collagen from calf skin and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 4 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin. The purity of the smooth muscle cell preparation was evaluated by staining the cells with monoclonal antibodies to smooth muscle cell α-actin. More than 96% of cells revealed immunoreactivity. Smooth muscle cells were subcultured every 7 days, and experiments were performed on cells at three to six passages after primary culture.

After confluence of the cells, quiescent mode was induced by incubation for 24 h in serum-free medium and then incubated with the same medium containing 0.2% free fatty acid bovine serum albumin and appropriate agents as described in the figure legends. At the end of incubation, the medium was removed for use in the phospholipase A_2 assay, and the cells were treated for RT-PCR analysis, protein determinations, or nuclear extract preparations.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—Total RNA was extracted according to Chomczynski and Sacchi (42), and 1.5 μg was used as template for the reverse transcription (RT) reaction. First strand cDNA was synthesized using reverse transcription reaction with murine mammary lentivirus reverse transcriptase and random primers. For semi-quantitative polymerase chain reaction (PCR) determinations, sPLA$_2$ cDNA was coamplified with GAPDH cDNA as an internal control, and the linear amplification was determined for each experiment (currently 20–24 cycles). The primers were synthesized according to published sequences for rat sPLA2 cDNA (43) corresponding to nucleotides 155–438 from start codon and rat GAPDH cDNA (44) corresponding to nucleotides 305–499 from start codon. The primers used for type II-sPLA$_2$ were GTG GCA GAG GAT CCC CCA AGG (CS 10, forward) and GCA ACT GGG CGT GTT CCC TCT GCA (CS 11, reverse), and those used for GAPDH were CCA TGG AGA AGG CTG GGG (GS, forward) and CAA AGT TGT CAT GGA TGA CC (GAS, reverse).

The following conditions were chosen as standard conditions for PCR reactions in a volume of 25 μl: 2.5 μl of cDNA template generated from RT reactions, 1.25 units of *Taq* DNA polymerase, 20–24 cycles of amplification in the presence of 160 nM CS 10 and CS 11 primers, 120 nM GS and GAS primers. PCR amplifications were performed using a thermocycler (Hybaid Omnigene) as follows: a denaturation step 3 min at 95 °C and then subsequent cycles of PCR using the following conditions: denaturation 1 min at 95 °C, annealing 1 min at 64 °C, extension 1 min at 72 °C. A final extension for 4 min at 72 °C was then performed.

The PCR products (5 μl each sample) were electrophoresed using 2% agarose gel, blotted, and fixed onto a Hybond N+ nylon membrane. The identity of amplified cDNA products was confirmed by hybridization with 5′-CAA CCG TCT GGA GAA ACG TGG ATG TGG CAC-3′ (nucleotides 216–245 from ATG) for sPLA$_2$ and 5′-GTG AAC CAC GAG AAA TAT GAC AAC TCC CTC-3′ (nucleotides 397–426 from ATG) for GAPDH. The oligonucleotide probes were labeled using ECL direct nucleic acid labeling system. After hybridization, membranes were washed, revealed using ECL reagents kit for horseradish peroxidase, and autoradiographed.

Phospholipase A_2 Assay—Phospholipase A_2 activity was measured using a selective fluorometric assay as described by Pernas *et al*. (45). The activity secreted into the medium was assayed on 400-μl samples using 4 nmol of fluorescent substrate 1-hexadecanoyl-2-(1-pyrenylde-

canoyl)-*sn*-glycero-3-phosphoglycerol. Total hydrolysis of the substrate obtained by 0.1 unit of phospholipase A_2 from *N. mossambica* was used as a reference to calculate phospholipase A_2 activity of the samples. Spontaneous hydrolysis of the substrate was assayed in the presence of fresh culture medium and subtracted.

Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared from smooth muscle cells by the method of Dignam *et al.* (46) with minor modifications. After washing in 5 ml of ice-cold phosphate-buffered saline, cells were harvested and centrifuged at 1000 × *g* for 5 min. The cell pellet was resuspended in 500 μl of buffer A (5 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% Nonidet P-40, 50 mM NaF, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin). Cell lysates were maintained for 10 min on ice and centrifuged at 3000 × *g* for 10 min. The nuclear pellet was then resuspended in 100 μl of buffer B (20 mM Hepes, pH 7.9, 25% glycerol, 0.5 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 50 mM NaF, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin). After 30 min at 4 °C, nuclear debris was removed by centrifugation at 45,000 × *g* for 30 min, and the supernatant (nuclear extract) was distributed into 15-μl aliquots that were stored at −80 °C until analysis by EMSA. Protein concentration was determined by the method of Lowry modified by Peterson (47).

The κB double-stranded oligonucleotides corresponded to either a, NFκB-binding site consensus sequence (48) 5′-GGG ACA GAG GGG ACT TTC CGA GAG G-3′ (NFκB consensus) or the sequence 5′-GTA TGA GGG CTT TTC CCT CGC CCT-3′ (NFκB sPLA2) corresponding to the region (−194, −174) of the rat type II-sPLA$_2$ promoter described by Walker *et al.* (49). The PPRE double-stranded oligonucleotides corresponded to a PPAR-binding site consensus sequence from acyl-CoA oxidase gene 5′-GGG AAC GTG ACC TTT GTC CTG GTC CC-3′ (PPRE consensus) (50) and to the sequence (−160, −133) of the rat type II-sPLA$_2$ promoter (51) 5′-C AGG CCT GTT GGG GGG AAA AGG GGA AAT T-3′ (PPRE sPLA$_2$) presenting extensive homology with the DR1 element of the PPAR-binding site. They were annealed and end-labeled using the T4 polynucleotide kinase in the presence of 50 μCi of [γ-^{32}P]dATP. Unincorporated nucleotides were removed using G-50 filtration. 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 of nuclear proteins and 50,000 cpm labeled (approximately 1 ng) with either the NFκB probe or the PPRE probe. Samples were incubated at room temperature for 15 min and fractionated by electrophoresis on 5% denaturing polyacrylamide gel in 0.25× TBE (45 mM Tris borate, 1 mM EDTA), after pre-electrophoresis for 30 min at 180 V. Gels were run at 180 V for 3 h and then transferred to 3MM paper (Whatman), dried in a gel dryer under vaccum at 80 °C, and then exposed to Amersham Pharmacia Biotech x-ray film. To determine the specificity of the DNA protein complexes, competition assays were performed using a 100-fold molar excess of an unlabeled double-stranded oligonucleotide corresponding to each of the probes (specific inhibitors) or a 100-fold molar excess of a double-stranded oligonucleotide corresponding to an AP1-binding site consensus sequence (52), 5′-GGG AGC CGC AAG TGA GTC AGC GCG GGG CTG GTG CA-3′ (nonspecific competitor).

Transfection and Luciferase Assays—Twenty four hours before transfection, cultured rat smooth muscle cells were seeded at a concentration achieving 70% confluence in 6-well dishes. Smooth muscle cells were cotransfected using 4 μl of LipofectAMINE, 800 ng of a plasmid construct containing the (PPRE consensus)×2 thymidine kinase promoter region fused to luciferase reporter gene (kindly provided by Walter Wahli) and 200 ng of pCMV-β-galactosidase per well (as a control of transfection efficiency). The reaction was carried out as recommended by the manufacturer (Life Technologies, Inc.). On the day after transfection, cells were washed twice with phosphate-buffered saline and subsequently cultured for 24 h in serum-free medium and incubated for 24 h in the same medium containing appropriate agents, as indicated in the figure legends.

The β-galactosidase activity in cell extracts was determined by an enzymatic assay with orthonitrophenyl phosphate as substrate. The luciferase activity was measured with a luciferase reporter assay kit, with detection of the signal for 12 s by a luminometer (Berthold, Inc.)

GC/MS Measurements—The non-esterified fatty acids were extracted and analyzed after methylation with diazomethane and separated by gas chromatography on a capillary column of Supelcowax-10 (Supelco)-bonded phase (diameter 0.32 mm, length 30 m) in a Hewlett-Packard 5890 Series II gas chromatograph as already described (18). Fatty acids were detected by mass spectrometry (Nermag 10–10C) in the chemical ionization mode with ammonia (10 kPa) as the reagent gas. The positive quasi-molecular ions were monitored and time-integrated. Quantification was performed with an internal standard of heptadecanoic methyl ester with response factors calculated for the various fatty acid methyl esters with calibrators.

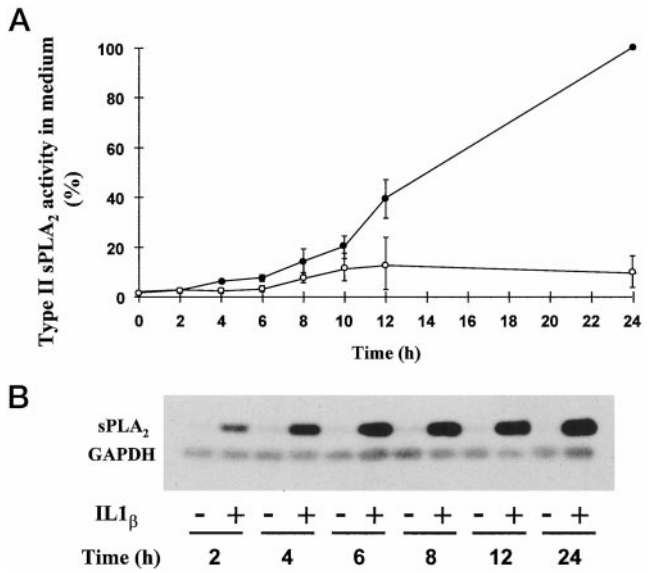

FIG. 1. **Time course of type II-sPLA$_2$ gene induction by IL-1β in VSMC.** Serum-starved cells were incubated in DMEM in the presence (●, +) or absence (○, −) of 10 ng/ml IL-1β for various times. *A,* phospholipase A_2 activity was determined in the extracellular medium by spectrofluorimetric assay (see "Materials and Methods"). Maximal activity obtained after 24 h was 2.7 ± 0.5 mmol/min/mg protein and is expressed as 100% in the figure. Results are means ± S.D. from three independent experiments performed in triplicate. *B,* the cells were washed at the end of each incubation time, and total RNA was extracted for mRNA analysis by RT-PCR as indicated under "Materials and Methods." A representative autoradiogram of three independent experiments is shown.

Statistical Analysis—The data illustrated in Table I were analyzed statistically using a Student's *t* test.

RESULTS

Time Course and Dose-dependent Effect of IL-1β on Type II-sPLA$_2$ Gene Expression in Vascular Smooth Muscle Cells—In rat aortic smooth muscle cells, as in other cells in primary culture, type II-sPLA$_2$ gene is not expressed under resting conditions. Interleukin 1β has been shown to induce type II-sPLA$_2$ gene expression in these cells (24). In order to identify the signal transduction events involved in IL-1β-mediated type II-sPLA$_2$ gene induction, we first characterized the kinetic parameters of its induction. Induction, which was nil at the onset of IL-1β addition, progressively increased with incubation time, as evidenced by RT-PCR analysis of type II-sPLA$_2$ mRNA and by measurement of sPLA$_2$ activity in the supernatant (Fig. 1). After addition of 10 ng/ml IL-1β, the presence of type II-sPLA$_2$ mRNA became obvious after 2 h and reached a maximum at 24 h (Fig. 1*B*). In contrast, the enzymatic activity was barely detected in the supernatant during the first 6 h and then increased linearly until 24 h (Fig. 1*A*). This result suggests that, in smooth muscle cells, as in chondrocytes (15), the secretion of type II-sPLA$_2$ is delayed after mRNA and protein synthesis. Type II-sPLA$_2$ mRNA can be evidenced 6 h after addition of as little as 0.1 ng/ml IL-1β, but the enzymatic activity is only detectable with 10 ng/ml IL-1β at this incubation time (Fig. 2). Stimulation by 10 ng/ml IL-1β was nearly maximal, and maximal stimulation was reached for 100 ng/ml. Most of the following experiments were therefore performed using 10 ng/ml IL-1β for 6 or 24 h of stimulation.

Effect of Actinomycin D and Cycloheximide on IL-1β-induced Type II-sPLA$_2$ Gene Expression in Vascular Smooth Muscle Cells—To investigate the mechanism by which IL-1β elicits

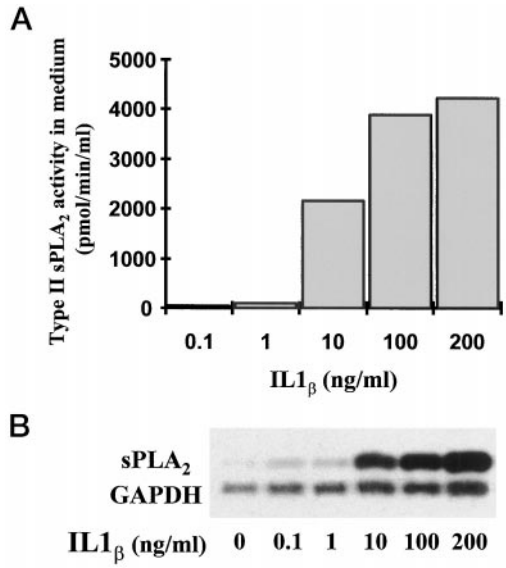

FIG. 2. **Dose-response of type II-sPLA$_2$ gene induction by IL-1β in VSMC.** Serum-starved cells were incubated for 6 h in DMEM containing increasing concentrations of IL-1β (0.1 to 200 ng/ml). *A,* phospholipase A_2 activity was measured as in Fig. 1, and results are expressed in pmol/min/ml and represent the mean of two independent experiments. *B,* RT-PCR analysis of mRNA as indicated in Fig. 1. A representative autoradiogram of three independent experiments is shown.

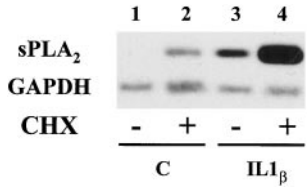

FIG. 3. **Cycloheximide effect on type II-sPLA$_2$ mRNA in VSMC.** Serum-starved cells were incubated for 6 h in DMEM in the presence (*lanes 3* and *4*) or absence (*lanes 1* and *2*) of 10 ng/ml IL-1β and in the presence (*lanes 2* and *4*) or absence (*lanes 1* and 3*)* of 10 μg/ml CHX. CHX was added 30 min before the addition of IL-1β. RT-PCR analysis of mRNA is as indicated in Fig. 1. A representative autoradiogram of three independent experiments is shown.

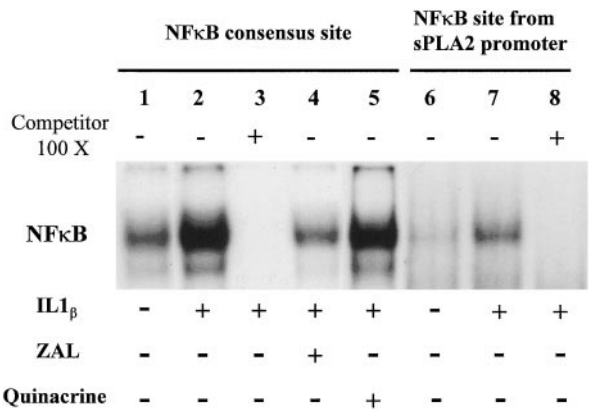

FIG. 4. **Effect of IL-1β and proteinase inhibitors on nuclear translocation of NFκB in VSMC.** Nuclear extracts were prepared from untreated cells and from cells treated for 24 h by 10 ng/ml IL-1β as indicated under "Materials and Methods." When required, proteinase inhibitors were added 1 h before IL-1β. Electromobility shift assays were performed to evidence NFκB binding using probes bearing either an NFκB consensus sequence (*lanes 1–5*) or an NFκB-binding site described on the type II-sPLA$_2$ promoter (*lanes 6–8*) as indicated under "Materials and Methods." Labeled probes were incubated with nuclear extracts from untreated cells (*lanes 1* and *6*) or from IL-1β-treated cells (*lanes 2–5* and *7–8*) in the presence of 1 μM ZAL (*lane 4*) or 5 μM quinacrine (lane 5). A large excess ($\times$100) of unlabeled κB probe was used to validate the specificity of binding (*lanes 3* and *8*).

type II-sPLA$_2$ mRNA in aortic smooth muscle cells, these cells were treated for 6 h with 10 ng/ml IL-1β in the presence or absence of the protein synthesis inhibitor cycloheximide or the transcriptional inhibitor actinomycin D. IL-1β-induced type II-sPLA$_2$ gene expression was inhibited by 5 μg/ml actinomycin D, as measured by either RT-PCR analysis of cellular RNA or enzymatic activity in the supernatant (result not shown). This confirms that synthesis of type II-sPLA$_2$ mRNA is a prerequisite to secretion of the enzyme. In contrast, cycloheximide alone induced a type II-sPLA$_2$ mRNA increase (Fig. 3), as observed for the expression of other genes (53). Furthermore, when IL-1β was coincubated for 6 h with cycloheximide, we observed a dramatic increase of type II-sPLA$_2$ mRNA as compared with IL-1β alone. This is in contrast to the situation observed in chondrocytes (15), in which cycloheximide inhibited the IL-1β effect on type II-sPLA$_2$ gene expression. This result indicated that the action of IL-1β does not need ongoing protein synthesis in smooth muscle cells. On the contrary, cycloheximide could inhibit a labile repressor molecule whose disappearance allows either a greater efficiency of IL-1β on gene transcription or an increased stability of type II-sPLA$_2$ mRNA.

Exploration of the Involvement of NFκB in IL-1β-induced Type II-sPLA$_2$ Gene Expression—In rat mesangial cells, Walker *et al.* (49) have previously found that NFκB is an essential component of the IL-1β-dependent up-regulation of type II-sPLA$_2$ gene transcription. We therefore used electrophoretic mobility shift assay (EMSA) to assess whether NFκB is involved in IL-1β responses in rat aortic smooth muscle cells. The nuclear extracts from untreated cells gave one major complex with labeled oligonucleotides bearing the κB site. This was obtained using oligonucleotides bearing either NFκB consensus or NFκB site of type II-sPLA$_2$ promoter (NFκB sPLA$_2$) (Fig. 4). Nuclear extracts from smooth muscle cells treated by 10 ng/ml IL-1β for 24 h gave much more intense complexes than those from control cells. These complexes were competed out by an excess of the corresponding unlabeled oligonucleotide but not by an excess of oligonucleotide bearing the AP1 site as nonspecific control (result not shown). Translocation of NFκB to the nucleus results from degradation of IκB inhibitory proteins by the proteasome machinery (54). We therefore tested the effect of proteinase inhibitors known to block proteasome activity. The specific proteasome inhibitor Z-IE(*O-t*-butyl)A-leucinal (ZAL) (Fig. 4) or the calpain inhibitor I *N*-acetyl-Leu-Leu-norleucinal (ALLN) (not shown) completely blocked the IL-1β-induced increase of NFκB binding. These two inhibitors also induced dose-dependent inhibition of the induction of type II-sPLA$_2$ gene expression by IL-1β (Fig. 5, *A* and *B*). Nuclear NFκB translocation and type II-sPLA$_2$ gene induction were completely blocked by 500 nM ZAL and 12 μM ALLN, concentrations in the range of those used to inhibit proteasome activities.

Arachidonic acid has been suggested to mediate nuclear NFκB translocation under certain experimental conditions (55). To examine whether this mechanism occurs in smooth muscle cells, we tested the ability of quinacrine, a broad phospholipase A_2 inhibitor, to suppress the IL-1β-induced nuclear translocation of NFκB. Treatment of the cells by 5 μM quinacrine for 24 h, inducing inhibition of IL-1β-stimulated type II-sPLA2 gene expression, did not prevent the increase in NFκB-DNA binding elicited by IL-1β in smooth muscle cells (Fig. 4).

Exploration of the Sphingomylinase/Ceramide Pathway and the Cytosolic PLA$_2$ Pathway in IL-1β-induced Type II-sPLA$_2$ Gene Induction—Several authors (25) have implicated the sphingomyelinase/ceramide pathway in the transcriptional ef-

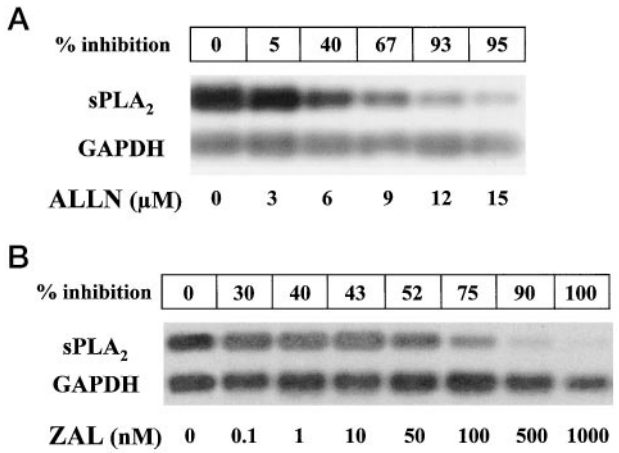

FIG. 5. **Effect of proteinase inhibitors on type II-sPLA$_2$ gene induction by IL-1β in VSMC.** Cells were preincubated for 1 h with increasing concentrations of ALLN (*A*) or ZAL (*B*) and then stimulated with 10 ng/ml IL-1β for 6 h. Cellular mRNA was assessed by RT-PCR as indicated in Fig. 1. Representative results of three (*A*) and two (*B*) independent experiments are shown. *Numbers* given in the frame are arbitrary units indicating inhibitory effect of each dose calculated as the percentage of each sPLA2 mRNA/GAPDH mRNA ratio obtained by densitometer scanning relative to the ratio obtained by IL-1β stimulation without inhibitors.

fect of IL-1β. To determine whether this pathway is relevant in IL-1β-induced type II-sPLA$_2$ gene induction, aortic smooth muscle cells were treated with bacterial sphingomyelinase and cell-permeable ceramide, *N*-acetylsphingosine (C_2-ceramide). RT-PCR analysis of cellular mRNA did not evidence any increase in type II-sPLA$_2$ mRNA in cells treated for 6 h by 10 units/ml sphingomyelinase or either 1 or 10 μM C_2-ceramide (results not shown).

Recently, the specific cytosolic-PLA$_2$ inhibitor, arachidonyltrifluoromethyl ketone (AACOCF$_3$), was shown to block IL-1β-induced type II sPLA$_2$ gene induction in rat fibroblasts (32). We therefore tested the ability of IL-1β to mobilize fatty acids from phospholipids in aortic smooth muscle cells by gas chromatography/mass spectrometry (GC/MS) measurement of cellular free fatty acids (Table I). After 6 h, IL-1β induced a marked increase in cellular unsaturated free fatty acids as follows: free oleic acid was increased 1.7-fold, free linoleic acid was increased 10-fold, and free arachidonic acid was increased 2.5-fold. This increase was completely blocked by AACOCF$_3$ (Table I). We then investigated the effect of several phospholipase A_2 inhibitors on IL-1β-induced type II-sPLA$_2$ gene expression. The nonspecific phospholipase A_2 inhibitor, quinacrine, as well as AACOCF$_3$ dose-dependently blocked the IL-1β-induced increase in type II-sPLA$_2$ mRNA (Fig. 6, *A* and *B*). Complete inhibition was achieved for concentrations (5 and 20 μM, respectively) known to inhibit phospholipase A_2 activities and smooth muscle cell proliferation (22). Conversely, bromoenol lactone (BEL), a specific inhibitor of the calcium-independent phospholipase A_2, only slightly inhibited IL-1β-induced type II-sPLA$_2$ mRNA increase at a very high concentration (Fig. 6, *lane 9*).

Activation of cPLA$_2$ was found to associate membrane translocation of the enzyme and phosphorylation at serine 505 by a MAP kinase (MAPK)-mediated process, and alternative pathways might also involve protein kinase C (56). We therefore tested the effects of inhibition of these two pathways on IL-1β-induced type II-sPLA$_2$ gene induction. Treatment of the cells by various concentrations (10, 100, and 500 nM) of the protein kinase C inhibitor GF109203X had no effect on IL-1β-induced type II-sPLA$_2$ mRNA increase (result not shown). This result ruled out any effect of the main protein kinase C species on

TABLE I

cPLA$_2$-mediated stimulation by IL-1β of intracellular free unsaturated fatty acids

Cells were incubated in serum-free DMEM for 24 h with or without 10 ng/ml IL-1β in the presence or in the absence of 20 μM AACOCF$_3$. AACOCF$_3$ was added 1 h before stimulation with IL-1β. Free fatty acids were extracted and quantified by GC/MS as described under "Materials and Methods." Values are means ± S.D. for three determinations. Results are expressed as nnmol/dish.

Fatty acid	Control	IL-1β	Control + AACOCF$_3$	IL-1β + AACOCF$_3$
C18:1 (n − 9)	7.08 ± 1.88	11.91 ± 0.38	4.77 ± 0.75	4.07 ± 0.84
C18:2 (n − 6)	0.11 ± 0.02	1.17 ± 0.21[a]	0.12 ± 0.02	0.12 ± 0.01
C20:4 (n − 6)	0.14 ± 0.05	0.35 ± 0.13[b]	0.18 ± 0.03	0.18 ± 0.27

[a] $p < 0.01$.
[b] $p < 0.05$.

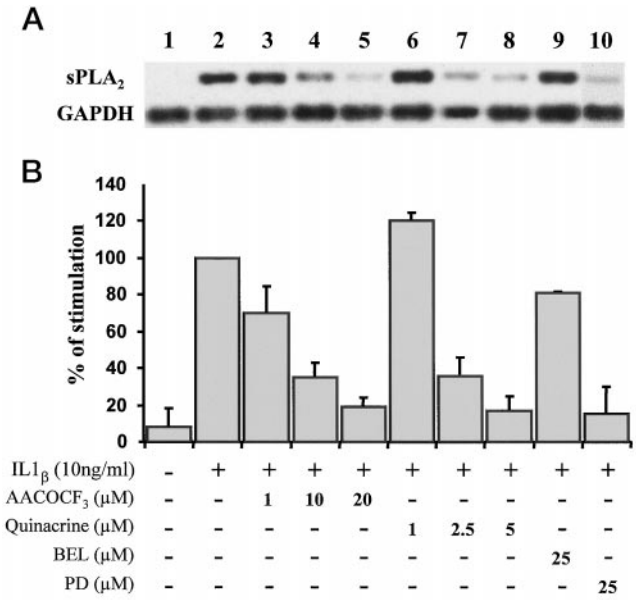

FIG. 6. **Inhibition of IL-1β induction of type II-sPLA$_2$ by cPLA$_2$ inhibitors and MEK inhibitor in VSMC.** Serum-starved cells were preincubated for 1 h with increasing concentrations of AACOCF$_3$ (*lanes 3–5*), quinacrine (*lanes 6–8*), BEL (*lane 9*), and PD98059 (*lane 10*) and then stimulated for 24 h with 10 ng/ml IL-1β (*lanes 2–10*). Control experiment in *lane 1*. Cellular mRNA was analyzed by RT-PCR as indicated in Fig. 1. *A,* autoradiogram displaying a representative experiment of the three experiments performed. *B,* quantification of the inhibitory effect by densitometer scanning of the sPLA2 mRNA/GAPDH mRNA ratio. Results are given as a percentage of the stimulatory effect obtained by IL-1β stimulation without inhibitors and are the mean ± S.E. from two experiments performed in triplicate.

IL-1β-induced type II-sPLA$_2$ gene induction. In most cells, MAPK is activated by a protein kinase cascade including MEK, a MAP kinase kinase and Raf-1, a MAP kinase kinase kinase, in which MAPK is directly phosphorylated and activated by MEK. Treatment of smooth muscle cells by the specific MEK inhibitor PD98059 leads to marked inhibition of IL-1β-induced increase in type II sPLA$_2$ mRNA (Fig. 6, *lane 10*), suggesting that the MAPK cascade may well be involved in IL-1β-induced type II-sPLA$_2$ gene transcription.

Effects of Ligands of Peroxisome Proliferator-activated Receptors (PPARs) and Retinoid X Receptor (RXR) on Type II-sPLA$_2$ mRNA Gene Expression—Among the products of cPLA$_2$-induced hydrolysis of cellular phospholipids, polyunsaturated free fatty acids have been shown to regulate the transcriptional activity of several genes. It has also been suggested that lysophosphatidylcholine (LPC) might regulate type II-sPLA$_2$ gene in rat fibroblasts (20). This LPC effect was never observed in rat aortic smooth muscle cells under our experimental conditions (result not shown). Since IL-1β increased free arachidonic

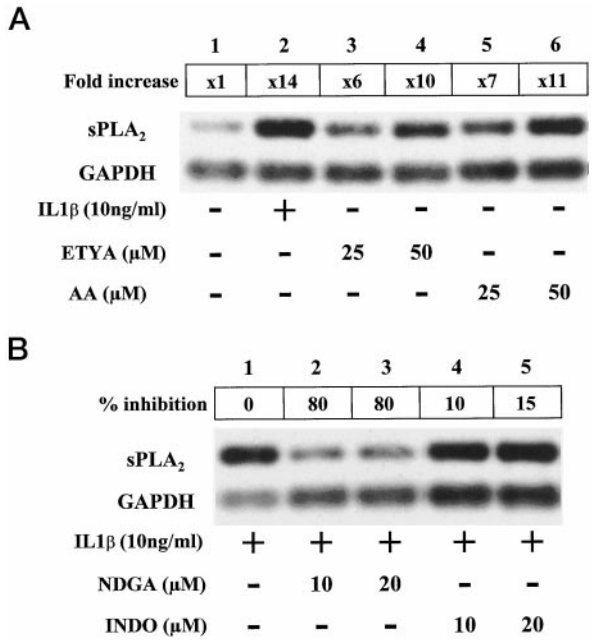

FIG. 7. **Effect of arachidonic acid, ETYA, and inhibitors of lipoxygenase and cyclooxygenase on type II-sPLA$_2$ gene expression in VSMC.** mRNA was analyzed by RT-PCR as indicated in Fig. 1. The autoradiogram displays a representative experiment of the two experiments performed in triplicate. *A,* cells were incubated in DMEM for 24 h in the absence (*lane 1*) or in the presence of IL-1β (*lane 2*), ETYA (*lanes 3* and *4*), or arachidonic acid (*lanes 5* and *6*). *Numbers* given in the frame are arbitrary units indicating stimulatory effect of the various agonists calculated as the increase of each sPLA2 mRNA/GAPDH mRNA ratio obtained by densitometer scanning relative to the ratio obtained without stimulation. *B,* cells were preincubated for 1 h with NDGA (*lanes 2* and *3*) or indomethacin (*lanes 4* and *5*) or vehicle alone (*lane 1*) and were stimulated by 10 ng/ml IL-1β (*lanes 1–5*). *Numbers* given in the frame are arbitrary units indicating inhibitory effect of each inhibitor calculated as the percentage of each sPLA2 mRNA/GAPDH mRNA ratio obtained by densitometer scanning relative to the ratio obtained by IL-1β stimulation without inhibitors.

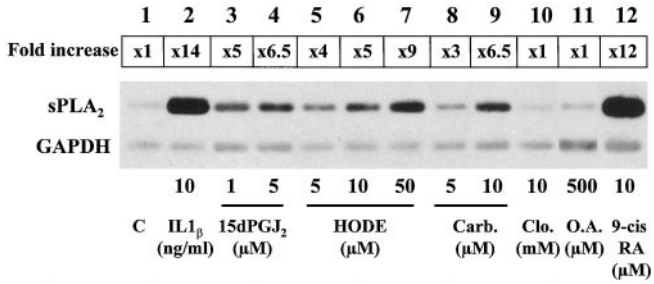

FIG. 8. **Effect of various PPAR ligands and 9-*cis*-retinoic acid on type II-sPLA$_2$ gene expression.** Cells were incubated in serum-free DMEM for 24 h in the absence (*lane 1*) or in the presence of IL-1β (*lane 2*), 15-dPGJ$_2$ (*lanes 3* and *4*), 9-HODE (*lanes 5–7*), carbaprostacyclin (*lanes 8* and *9*), clofibric acid (*lane 10*), oleic acid (*lane 11*), and 9-*cis*-retinoic acid (*lane 12*). Cellular mRNA was analyzed by RT-PCR as indicated in Fig. 1. The autoradiogram displays a representative example of the two experiments performed in triplicate. *Numbers* given in the frame are arbitrary units indicating the stimulatory effect of the various agonists calculated as the increase of each sPLA2 mRNA/GAPDH mRNA ratio obtained by densitometer scanning relative to the ratio obtained without stimulation.

acid in smooth muscle cells (Table I), we tested the ability of this fatty acid to induce the type II-sPLA$_2$ gene. We found a marked dose-dependent increase in type II-sPLA$_2$ mRNA when cells were treated with free arachidonic acid (Fig. 7*A*). This effect was reproduced by the non-metabolizable analogue, eicosatetraynoic acid (ETYA), at the same concentrations (Fig. 7*A*). However, the actual physiological inducer produced by IL-1β-stimulated cytosolic-PLA$_2$ activity might not be arachidonic acid itself, since nordihydroguaiaretic acid (NDGA), a well known lipoxygenase inhibitor, was also able to inhibit the IL-1β effect in smooth muscle cells (Fig. 7*B*). In contrast, the cyclooxygenase inhibitor, indomethacin, did not affect the IL-1β-induced response.

The transcriptional effect of fatty acids or their derivatives was thought to be mediated by peroxisome proliferator-activated receptors (PPARs) (34). We therefore investigated the ability of several putative PPAR ligands to reproduce the transcriptional effect of IL-1β. We tested the effect of 15-deoxy-$\Delta^{12,14}$-dehydro-prostaglandin J$_2$ (15-dPGJ$_2$) and 9-hydroxy-octadecadienoic acid (9-HODE), which are activators of PPARγ, clofibric acid, and oleic acid and which are activators of PPARα and carbaprostacyclin, a stable analogue of prostaglandin I$_2$, which is an activator of PPARα and PPAR β/δ (Fig. 8). Treatment of smooth muscle cells with both 15-dPGJ$_2$ and 9-HODE induced a dose-dependent increase in cellular type II-sPLA$_2$ mRNA (Fig. 8, *lanes 3–7*). The concentrations used are in the range of those known to stimulate PPARγ activation (34, 35). Neither clofibric acid nor oleic acid induced type II-sPLA$_2$ gene expression (Fig. 8, *lanes 10* and *11)*. In contrast, carbaprostacyclin induced the gene (Fig. 8, *lanes 8* and *9*) suggesting that PPARβ/δ but not PPARα may also transactivate.

To examine whether the effect of PPARγ ligands can be mediated by pathways independent of the IL-1β pathway, we costimulated smooth muscle cells using IL-1β and various PPARγ ligands. Neither additive nor synergistic effects were observed (results not shown).

PPARγ are a class of nuclear receptors that exert their action via heterodimerization with another nuclear receptor RXR. It has been shown that genes that respond to PPARγ/RXR heterodimers are also stimulated by the RXR ligand 9-*cis*-retinoic acid. We observed a marked increase in type II-sPLA$_2$ mRNA when rat aortic smooth muscle cells were stimulated for 24 h by 9-*cis*-retinoic acid (Fig. 8, *lane 12*).

IL-1β Stimulates PPARγ Activity in Smooth Muscle Cells, which Requires NFκB Nuclear Translocation—To demonstrate formally the involvement of PPARγ in the IL-1β effect, we first performed electrophoretic mobility shift assay (EMSA) to assess whether IL-1β modifies the binding of PPAR to type II-sPLA$_2$ promoter in rat aortic smooth muscle cells. The nuclear extracts from untreated cells gave one major complex with labeled oligonucleotides bearing either the PPAR-binding site consensus sequence from acyl-CoA oxidase gene (50) or the sequence of the rat type II-sPLA$_2$ promoter presenting extensive homology with the DR1 element of PPAR-binding sites (51) (Fig. 9*A*). Nuclear extracts from smooth muscle cells treated with 10 ng/ml IL-1β for 24 h gave more intense complexes than those from control cells (Fig. 9*A*). These complexes were competed out by an excess of the corresponding unlabeled oligonucleotide.

To confirm that the increased binding of nuclear factors to PPRE oligonucleotides induced by IL-1β reflects functional activation of PPARγ, we tested the effect of IL-1β on the transcriptional activity of a construct containing the (PPRE consensus) $\times$2 thymidine kinase promoter region fused to a luciferase reporter gene (57). LipofectAMINE-transfected vascular smooth muscle cells presented a 3-fold increase in luciferase activity when stimulated with 10 ng/ml IL-1β (Fig. 9*B*). This stimulatory effect is in the same range as the effect observed with 5 and 10 μM 15-dPGJ$_2$, a well known agonist of PPARγ.

Recent concepts concerning the activation of transcriptional machinery have emphasized the role of cooperation between nuclear receptors to achieve a complete transcriptional effect via coactivator molecules (58). We therefore wondered whether

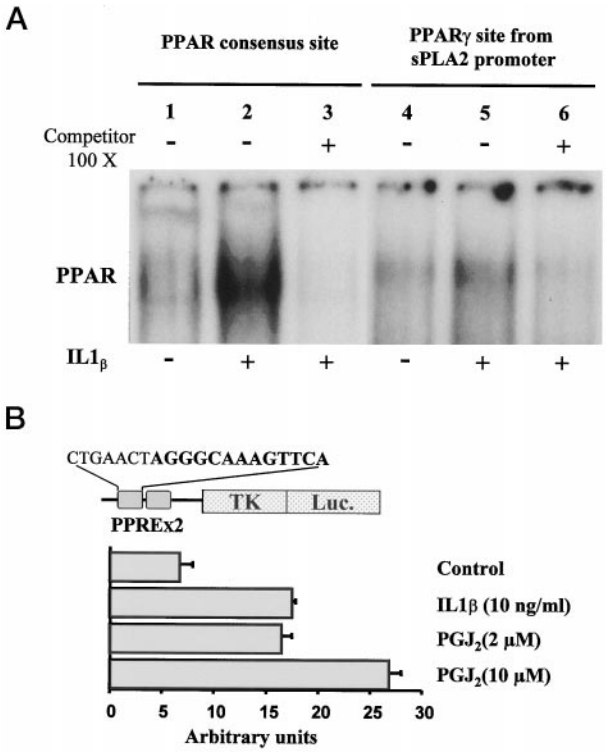

FIG. 9. **IL-1β-induced activation of PPARγ in smooth muscle cells.** *A,* vascular smooth muscle cell nuclear extracts were prepared as indicated under "Materials and Methods" from cells incubated for 24 h in the absence (*lanes 1* and *4*) or in the presence of 10 ng/ml IL-1β (*lanes 2, 3, 5,* and *6*). EMSA experiments were performed as in Fig. 4 and as described under "Materials and Methods" with oligonucleotides bearing either the PPAR-binding site consensus sequence from acyl-CoA oxidase gene (*lanes 1–3*) or a related PPAR-binding sequence of the type II-sPLA$_2$ promoter (*lanes 4–6*). *B,* vascular smooth muscle cells were transiently cotransfected by a plasmid construct containing the (PPRE consensus) $\times$2 thymidine kinase promoter region fused to luciferase reporter gene and pCMV-β-galactosidase as a control using the LipofectAMINE technique as described under "Materials and Methods." One day later, the cells were incubated for 24 h in the absence or in the presence of 10 ng/ml IL-1β or 5 or 10 μM 15-dPGJ$_2$, and luciferase activity was measured as indicated under "Materials and Methods." All luciferase activity values were reported to β-galactosidase activity and represent three independent experiments in which different conditions were tested in duplicate.

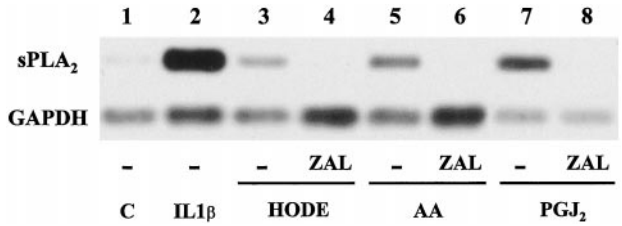

FIG. 10. **Effect of proteinase inhibitor on type II-sPLA$_2$ gene induction by PPARγ ligands.** Cells were incubated in serum-free DMEM for 24 h in the absence (*lanes 1* and *2*) or in the presence of 10 μM 9-HODE (*lanes 3* and *4*), 50 μM arachidonic acid (*lanes 5* and *6*), 5 μM 15-dPGJ$_2$ (*lanes 7* and *8*). 2 μM ZAL was added (*lanes 4, 6,* and *8*) or not (*lanes 1–3, 5,* and *7*) 1 h before stimulation by effectors. Positive control was obtained with 10 ng/ml IL-1β (*lane 2*). Cellular mRNA was analyzed by RT-PCR as indicated in Fig. 1. A representative autoradiogram of two independent experiments is shown.

NFκB, which is a ubiquitous nuclear factor activated by various inflammatory cytokines, is required to obtain the transcriptional response to PPARγ ligands. We found that the proteinase inhibitors ZAL and ALLN, at a concentration that completely blocks both NFκB nuclear translocation and IL-1β-induced type II-sPLA$_2$ gene activation, strongly inhibited 9-HODE-induced, arachidonic acid-induced and 15-dPGJ$_2$-induced type II-sPLA$_2$ gene transcription (Fig. 10). This effect is not mediated by inhibition of the binding of activated PPARγ to its nuclear target, since ZAL was unable to block the formation of PPAR oligonucleotide complexes (data not shown). This suggests that the binding of both NFκB and PPARγ to type II-sPLA$_2$ promoter is required to stimulate gene transcription.

DISCUSSION

Recent studies have shown that calcium-sensitive and calcium-insensitive cellular phospholipases A$_2$ are involved in regulating immediate eicosanoid gene generation (59). However, several pieces of evidence have shown that the secreted enzymes are also involved in the synthesis of lipid mediators such as eicosanoids or lyso-derivatives (14–17). Type II-sPLA$_2$ is a major secreted phospholipase A$_2$ found in many cell types when tissues are triggered by proinflammatory agents and are thought to participate in the development of the inflammatory process by generating delayed production of various lipid mediators (31). Our results confirm that type II-sPLA$_2$ gene, which is silent in rat aortic smooth muscle cells under resting conditions, is markedly induced by IL-1β in a time-dependent and dose-dependent manner (Figs. 1 and 2). This is an intense and sustained process that is maintained for 48 h (results not shown). In the present study, we explored the signaling pathways which lead from IL-1β receptor to the transcriptional machinery of the type II-sPLA$_2$ gene in rat aortic smooth muscle cells.

Since transcriptional up-regulation of proinflammatory genes is strongly dependent on NFκB activation, we first investigated NFκB involvement in type II-sPLA$_2$ gene induction in response to IL-1β. The rat type II-sPLA$_2$ promoters possess an NFκB-binding site (49). By using this site as well as consensus NFκB, we found a marked increase of active NFκB in nuclear extracts from IL-1β-treated cells in EMSA experiments (Fig. 4). Proteinase inhibitors (ZAL and ALLN), known to block proteasome and therefore the degradation of the NFκB inhibitors IκBα and β, decreased IL-1β-induced NFκB nuclear translocation and induction of type II-sPLA$_2$ gene (Figs. 4 and 5). We concluded that in rat aortic smooth muscle cells, as in rat mesangial cells (49), IL-1β induces type II-sPLA2 gene expression via an NFκB-mediated process. In unstimulated smooth muscle nuclear extracts, we found a basal NFκB binding activity (Fig. 4) that varied from one experiment to another (data not shown). The existence of such a basal activity has been challenged by Bourcier *et al.* (28) and might be reminiscent of the presence of growth factors from fetal calf serum added to the culture medium before starving the cells for 24 h. Stimulation of NFκB binding by IL-1β is observed after only 1 h and persists for at least 24 h (data not shown). This rather unusual sustained stimulation of NFκB binding might be related to a prolonged degradation of IκBs. In human smooth muscle cells, Bourcier *et al.* (28) demonstrated that IL-1β triggers a transient IκBα decrease and a sustained IκBβ decrease. The involvement of IκB turnover in the duration of IL-1β effects is supported by the results obtained with the protein synthesis inhibitor, cycloheximide, which superinduced both basal and IL-1β- induced type II-sPLA$_2$ gene expression (Fig. 3). This suggests that cycloheximide blocks the resynthesis of IκBs. Under unstimulated conditions, type II-sPLA$_2$ mRNA is poorly detectable in RT-PCR assays (Fig. 3). Although we cannot exclude that cycloheximide induces lasting stabilization of a weakly expressed mRNA, it more probably inhibits the synthesis of a labile repressor of type II-sPLA$_2$ gene transcription.

In several cell systems, it has been suggested that IL-1β exerts its effects on gene activation via activation of NFκB with or without involvement of the sphingomyelinase-ceramide

pathway (25, 60). In rat aortic smooth muscle cells, neither sphingomyelinase nor permeable C_2-ceramide was able to stimulate type II-sPLA$_2$ gene expression, excluding the participation of the sphingomyelinase-ceramide pathway in the IL-1β effect.

Kuwata *et al.* (32) recently demonstrated that IL-1β stimulates cPLA$_2$ in rat fibroblasts, inducing increased arachidonic acid release and synthesis of eicosanoids. IL-1β-induced cPLA$_2$ activation was responsible for stimulation of type II-sPLA$_2$ gene expression in this cell type. This is in agreement with our present results showing that, in smooth muscle cells, IL-1β-induced type II-sPLA$_2$ gene expression is blocked by both a broad phospholipase A$_2$ inhibitor and a specific cPLA$_2$ inhibitor (Fig. 6). In addition, inhibition of MEK by PD98059, which prevents MAPK activation necessary for the cPLA$_2$ stimulatory process (56), strongly inhibits the IL-1β-induced type II-sPLA$_2$ mRNA increase (Fig. 6). IL-1β is able to induce the release of free arachidonic acid, but the IL-1β effect is not restricted to this fatty acid, as a more marked release of free linoleic acid, also blocked by a specific cPLA$_2$ inhibitor, was observed (Table I). This might be controversial since cPLA$_2$ has been found to be very specific for phospholipids containing arachidonic acid (61). However, using the GC/MS methodology, we recently indicated that cPLA$_2$ is also able to hydrolyze phospholipids containing linoleic acid which are much more abundant than those containing arachidonic acid.[2]

In contrast with the results of Kuwata *et al.* (32), lysophosphatidylcholine failed to stimulate type II-sPLA$_2$ gene expression in smooth muscle cells, but we were able to induce this expression by exogenous arachidonic acid in a concentration range known to stimulate gene expression in other cell systems (Fig. 7*A*). As the lipoxygenase inhibitor, NGDA, but not the cyclooxygenase inhibitor, indomethacin, was found to block the IL-1β effect (Fig. 7*B*), one might therefore speculate whether arachidonic acid or its lipoxygenase metabolites are involved in the IL-1β effects. This was suggested by Kuwata *et al.* (32), who indicated preliminary results showing the stimulation of type II-sPLA$_2$ gene expression by 15-hydroxyeicosatetraenoic acid (15-HETE).

One mechanism by which polyunsaturated fatty acids or their derivatives activate gene expression is the binding of PPAR factors (36). Recently, controversy has arisen concerning the presence of various PPAR isoforms in smooth muscle cells (37, 38) and their actual effect on proinflammatory genes (35, 37, 38). We demonstrated a PPRE on the type II-sPLA$_2$ promoter that binds nuclear factors as does an oligonucleotide bearing a consensus PPRE sequence (Fig. 9*A*). Furthermore, the PPARγ ligand 15-dPGJ$_2$ activates a luciferase reporter gene whose promoter contains two consensus PPRE binding domains (Fig. 9*B*). To investigate a putative involvement of PPARs in type II-sPLA$_2$ gene expression in rat aortic smooth muscle cells, we tested the ability of various PPAR ligands to stimulate this gene. We found that clofibric acid and oleic acid, which are activators of PPARα (62), are unable to induce type II-sPLA$_2$ gene expression. Only PPARγ and PPARβ/δ ligands, *i.e.* 15-dPGJ$_2$, 9-HODE, and carbaprostacyclin, were able to induce type II-sPLA$_2$ gene expression (Fig. 8) at concentrations described to activate PPARγ effectively (34, 35). It has been demonstrated that PPARγ mediates its translocating action via heterodimers with RXR. RXR/PPARγ heterodimers can activate the transcriptional response to ligands specific for either subunit of the dimer (63). This is clearly the case for induction of type II-sPLA$_2$ in smooth muscle cells, since 15-dPGJ$_2$, 9-HODE, or 9-*cis*-retinoic acid alone induced sustained stimulation of the gene (Fig. 8). Mutation of PPARγ but not RXR in the hormone-dependent activation domain inhibits the ability of RXR/PPARγ heterodimers to respond to ligands specific for either subunit (63). This indicates that the presence of PPARγ ligands is required to obtain a functional heterodimer in the absence of 9-*cis*-retinoic acid.

The involvement of PPARγ in IL-1β stimulation of the type II-sPLA$_2$ gene is also consistent with the stimulatory effect of IL-1β on the binding of nuclear factors to a PPRE of the type II-sPLA$_2$ promoter (Fig. 9*A*) and with the stimulatory effect of IL-1β on a luciferase reporter gene whose promoter contains two consensus PPRE binding domains (Fig. 9*B*). This effect is of the same amplitude as those induced by the PPARγ ligand 15-dPGJ$_2$. This is also in line with the presence of PPARγ in rat aortic smooth muscle cells (Fig. 9*A*) (38) and with the result reported by Tontonoz *et al.* (64) indicating high levels of PPARγ in atherosclerotic lesions, using sections of aorta from spontaneously atherosclerotic mice.

Since the IL-1β effect is not inhibited by indomethacin (Fig. 7*B*), 15-dPGJ$_2$ cannot be the endogenous PPARγ ligand induced by cytosolic PLA$_2$ activation in rat aortic smooth muscle cells. On the contrary, 9-HODE, which strongly stimulates the type II-sPLA$_2$ gene (Fig. 8), may be produced from linoleic acid via the lipoxygenase pathway (12). This may be related to the marked increase in cellular free linoleic acid induced by IL-1β in smooth muscle cells (Table I). In preliminary experiments, we failed to demonstrate HODE synthesis in rat aortic smooth muscle cells in response to IL-1β stimulation (result not shown). However, type II-sPLA$_2$ has been recently shown to increase lipoxygenase-induced HODE production from low density lipoproteins (12). This raises the possibility that type II-sPLA$_2$-induced HODE synthesis constitutes a positive autocrine loop in the aorta during the inflammatory stage of atherosclerosis progression.

IL-1β stimulation induced only a slight increase in free arachidonic acid, but massive addition of exogenous arachidonic acid might drive type II-sPLA$_2$ gene expression either directly, since this action is mimicked by the non-metabolizable analogue ETYA (Fig. 7*A*), or indirectly via the production of lipoxygenase metabolites. Nagy *et al.* (35) have indeed indicated that 15-hydroxyeicosatetraenoic acid, a lipoxygenase derivative of arachidonic acid, is able to stimulate PPARγ-driven promoters albeit with a lower efficiency than HODEs.

The organization of an enhanceosome in the promoter region able to interact with the basic transcription machinery is an emerging concept in the regulation of gene expression (65). This complex recruits coactivator proteins, such as CREB-binding protein (CBP/p300), to stimulate the transcription rate (66). PPARγ was found to interact with CBP/p300 (67), and components of the USA coactivator are involved in transcriptional activation of the human immunodeficiency virus promoter by NFκB (68). NFκB was found to down-regulate PPARα-driven promoters in human smooth muscle cells (37). Nuclear translocation and binding of NFκB and binding of PPAR to their promoter targets can be independently achieved since proteasome inhibitors did not block PPAR binding and phospholipase A$_2$ inhibitor did not inhibit NFκB nuclear translocation.

In summary, in rat aortic smooth muscle cells, we demonstrated that IL-1β-induced type II-sPLA$_2$ gene transcription inevitably involves two complementary pathways as follows: the NFκB pathway and the cytosolic PLA$_2$ pathway, *e.g.* (i) independent inhibition of each pathway completely abolished the IL-1β effect, and (ii) IL-1β and PPARγ ligands are not additive or synergistic on type II-sPLA$_2$ gene transcription. We can therefore suggest that, in rat aortic smooth muscle cells in primary culture stimulated by IL-1β, NFκB and PPARγ coop-

[2] K. Koumanov, manuscript in preparation.

erate at the enhanceosome-coactivator level to turn on the expression of proinflammatory type II-sPLA$_2$ gene.

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