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Potentiation of Tumor Necrosis Factor α-induced Secreted Phospholipase A_2 ($sPLA_2$)-IIA Expression in Mesangial Cells by an Autocrine Loop Involving $sPLA_2$ and Peroxisome Proliferator-activated Receptor α Activation*

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In rat mesangial cells, exogenously added secreted phospholipases A_2 ($sPLA_2$s) potentiate the expression of pro-inflammatory $sPLA_2$-IIA first induced by cytokines like tumor necrosis factor-α (TNFα) and interleukin-1β. The transcriptional pathway mediating this effect is, however, unknown. Because products of PLA_2 activity are endogenous activators of peroxisome proliferator-activated receptor α (PPARα), we postulated that $sPLA_2$s mediate their effects on $sPLA_2$-IIA expression via $sPLA_2$ activity and subsequent PPARα activation. This study shows that various $sPLA_2$s, including venom enzymes, human $sPLA_2$-IIA, and wild-type and catalytically inactive H48Q mutant of porcine pancreatic $sPLA_2$-IB, enhance the TNFα-induced $sPLA_2$-IIA expression at the mRNA and protein levels. In cells transfected with luciferase $sPLA_2$-IIA promoter constructs, $sPLA_2$s are active only when the promoter contains a functional PPRE-1 site. The effect of exogenous $sPLA_2$s is also blocked by the PPARα inhibitor MK886. Interestingly, the expression of $sPLA_2$-IIA induced by TNFα alone is also attenuated by MK886, by the $sPLA_2$-IIA inhibitor LY311727, by heparinase, which prevents the binding of $sPLA_2$-IIA to heparan sulfate proteoglycans, and by the specific $cPLA_2$-α inhibitor pyrrolidine-1. Together, these data indicate that $sPLA_2$-IIA released from mesangial cells by TNFα stimulates its own expression via an autocrine loop involving $cPLA_2$ and PPARα. This signaling pathway is also used by exogenously added $sPLA_2$s including pancreatic $sPLA_2$-IB and is distinct from that used by TNFα .

During the last decade, increasing evidence has been obtained that secreted phospholipases A_2 ($sPLA_2$)[1] are important players in inflammatory diseases. Among the various $sPLA_2$s that have now been identified (1–3), group IIA $sPLA_2$ ($sPLA_2$-IIA) has been found to be expressed at very high levels in various acute and chronic inflammatory diseases like sepsis (4), asthma (5), and rheumatoid arthritis (6). $sPLA_2$-IIA is also highly expressed in the kidney during experimental and human acute pancreatitis (7), which can lead to post-injury multiple organ failure (8). In rat renal mesangial cells, which are used as a cell model system to study inflammatory processes, it was shown earlier that cAMP-elevating agents and proinflammatory cytokines such as interleukin-1β and TNFα stimulate the gene expression and secretion of $sPLA_2$-IIA by different transcriptional activation pathways (9–11). The functional role of the $sPLA_2$-IIA released from mesangial cells is, however, less clear. In rat mesangial cells, it has been shown that exogenously added $sPLA_2$-IIA acts as a growth factor mediating the action of IL-1β on cell proliferation, and that this effect is mimicked by lysophospholipids (12). Exogenous $sPLA_2$-IIA and lysophospholipids were also found to rapidly stimulate the mitogen activated protein kinase cascade in mesangial cells, leading to early activation of $cPLA_2$ (13, 14). It is, however, unclear whether $sPLA_2$-IIA released by mesangial cells after cytokine induction acts like exogenously added $sPLA_2$-IIA and, in particular, whether $sPLA_2$-IIA can exert a positive feedback amplification loop on its own gene expression by activating one of the above signaling pathways and/or other pathways (see below).

High levels of another $sPLA_2$ subtype, the so-called pancreatic-type $sPLA_2$-IB, are also found in kidney during acute pancreatitis (7, 15), suggesting that $sPLA_2$-IB may also contribute to the pathophysiological effects in such conditions. In rat mesangial cells, exogenously added pancreatic $sPLA_2$-IB can stimulate the mRNA and protein expression of $sPLA_2$-IIA, as well as prostaglandin biosynthesis (16, 17). This effect is thought to involve binding of $sPLA_2$-IB to the M-type $sPLA_2$ receptor expressed in mesangial cells (17). This view is strengthened by the fact that a catalytically inactive mutant of

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[1] The abbreviations used are: $sPLA_2$, secreted phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; TNFα, tumor necrosis factor-α; IL, interleukin; PPARα, peroxisome proliferator-activated receptor α; PPRE, peroxisome proliferator-responsive element; kbp, kilobase pair(s); HSPG, heparan sulfate proteoglycan; 5-LOX, 5-lipoxygenase; FLAP, 5-lipoxygenase-activating protein; BSA, bovine serum albumin; PBS, phosphate-buffered saline; RXRα, 9-*cis*-retinoic acid receptor-α; EMSA, electrophoretic mobility shift assay; RT, reverse transcription.

sPLA$_2$-IB, which still binds to the M-type receptor, has effects similar to those of the wild-type enzyme (16). Interestingly, sPLA$_2$-IB from different species including rat, but not rat sPLA$_2$-IIA, binds to the M-type receptor expressed in rat mesangial cells (17–19), suggesting that sPLA$_2$-IIA acts through binding to a different cell membrane target. When exogenously added to other cell types, sPLA$_2$-IB was also found to activate the expression of a number of pro-inflammatory genes including cyclooxygenase-2 (20, 21), sphingomyelinase, and ceramidase (22). On the other hand, exogenously added sPLA$_2$-IIA can induce the activation of cPLA$_2$ and cyclooxygenase-2 (3, 23, 24), the release of elastase (25) and β-glucuronidase (26), and the expression of Mac-1 (27), IL-6 (26), CD-69 (28), inducible nitric-oxide synthase (29), and Fas ligand (23) on different cell types. The nature of the sPLA$_2$-IIA cellular target involved in these biological effects remains, however, to be clearly identified. Although human sPLA$_2$-IIA does not bind to the human M-type receptor (18), it has been proposed that this receptor or a related receptor may be involved, whereas the sPLA$_2$ activity may not play a major role. Heparan sulfate proteoglycans (HSPG) including glypican-1 may also contribute to the effects of sPLA$_2$-IIA (30, 31).

Besides activation of p38, p42/44, and c-Jun N-terminal kinase kinases by sPLA$_2$-IB or -IIA (13, 23, 32), little is known about the transcriptional pathways activated by exogenously added sPLA$_2$s. It has been shown that sPLA$_2$-IB enhances the expression of COX-2 through activation of the transcription factor CCAT/enhancer-binding protein β in NIH3T3 and MC3T3E1 cells (21). Because sPLA$_2$s can produce lipid mediators such as free fatty acids and prostaglandins that are peroxisome proliferator-activated receptors (PPARs) ligands (33), another attractive hypothesis is that sPLA$_2$s induce the expression of genes containing PPAR-responsive promoters by activating PPAR nuclear receptors. Interestingly, a recent work has shown that sPLA$_2$-IB may exert its proliferative effects via hydrolysis of nuclear phospholipids and activation of PPARα (34). More recently, we and others found that the rat sPLA$_2$-IIA promoter contains peroxisome proliferator-responsive elements (PPRE) (35, 36), suggesting that PPAR activation in rat mesangial cells might be involved in the induction of sPLA$_2$-IIA by exogenously added sPLA$_2$s including sPLA$_2$-IB, and also by rat sPLA$_2$-IIA endogenously produced after cytokine treatment.

The purpose of this study was to analyze the role of PPARα in the induction of sPLA$_2$-IIA transcription by cytokines and by exogenous sPLA$_2$s in rat mesangial cells. To determine the role of PPARα and the sPLA$_2$ signaling pathways, we used rat sPLA$_2$-IIA promoter constructs containing functional or mutated PPARα binding sites, as well as PPARα and various PLA$_2$ inhibitors. Altogether, our data indicate that sPLA$_2$-IIA released by mesangial cells after treatment with cytokines potentiates its own expression in a positive feedback loop via activation of cPLA$_2$ and PPARα. Exogenously added sPLA$_2$s including sPLA$_2$-IB also use this transcriptional mechanism to enhance sPLA$_2$-IIA gene expression, although the pathways used at the plasma membrane may differ among sPLA$_2$s.

EXPERIMENTAL PROCEDURES

Materials—TNFα was a generous gift from Knoll AG (Ludwigshafen, Germany). IL-1β was obtained from Cell Concept (Umkirch, Germany). [1-^{14}C]Oleic acid, [γ-^{32}P]ATP (185 TBq/mmol), and [α-^{32}P]dCTP (110 TBq/mmol) were from Amersham Biosciences (Freiburg, Germany). Kidneys of rats with Thy-1 glomerulonephritis were a generous gift from Dr. T. Ostendorf (Rheinisch-Westfälische Technische Hochschule, Aachen, Germany). Immobilon-PVDF membranes were purchased from Millipore (Eschborn, Germany), and nylon membranes (GeneScreen) were purchased from PerkinElmer Life Sciences (Köln, Germany). 18 S RNA probe from mouse as well as specific antibodies against PPARα and 9-*cis*-retinoic acid receptor-α (RXRα) were purchased from Ambion (Wiesbaden, Germany). Pyrrolidine-1 was prepared as described by Ghomashchi *et al.* (37). LY311727 was a generous gift from Eli Lilly (Indianapolis, IN). MK886 was from Biomol (Hamburg, Germany), heparinase-1 was from Sigma (Deisenhofen, Germany), and all other chemicals used were from Sigma, Biomol, or Calbiochem (Bad Soden, Germany). All cell culture media and nutrients were from Invitrogen (Eggenstein, Germany). sPLA$_2$s used for the treatment of mesangial cells are listed in Table I. All enzymes were endotoxin-free as tested by the *Limulus* amebocyte assay from BioWhittaker (Walkersville, MD). Human sPLA$_2$-IIA was a generous gift of Prof. Tibes, Roche Diagnostics (Penzberg, Germany). sPLA$_2$s from Taipan snake venom, bee venom, *Naja mossambica mossambica* venom, and porcine pancreas were obtained as described (18, 38). The construction of the H48Q mutant of porcine sPLA$_2$-IB, and the H48Q and H48N mutants of human sPLA$_2$-IIA have been described elsewhere (39, 40).

Cell Culture—Rat mesangial cells were cultured and characterized as described (41). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and bovine insulin (0.66 units/ml). Twenty-four hours prior to stimulation and during the experiments, cells were incubated in Dulbecco's modified Eagle's medium containing 0.1 mg/ml fatty acid-free BSA.

Northern Blot Analysis—Confluent mesangial cells were cultured in 100-mm diameter culture dishes. After stimulation for 24 h, cells were washed with PBS and harvested using a rubber policeman. Total cellular RNA was extracted from the cell pellet using the guanidinium isothiocyanate/phenol/chloroform method. Ten μg of total RNA was separated on a 1.4% agarose/formaldehyde gel, transferred to GeneScreen membranes, and hybridized with the radiolabeled cDNA probes for sPLA$_2$-IIA or 18 S RNA. For quantification the signals of the filters were scanned and evaluated densitometrically using a BAS 1500 phosphorimager from Fuji (Raytest, Straubenhardt, Germany). The signal obtained with the sPLA$_2$-IIA probe was normalized to that obtained with the 18 S RNA probe.

Western Blot Analysis—sPLA$_2$-IIA protein secreted by mesangial cells was measured by precipitating 500 μl of the culture supernatant with 200 μl of 20% trichloroacetic acid. SDS-PAGE using a 15% polyacrylamide gel was performed under nonreducing conditions. The proteins were transferred to Immobilon-PVDF membranes for 30 min at 0.7 mA/cm^2. Nonspecific binding was blocked with 2% BSA in PBS plus 0.05% Tween 20 for 1 h at room temperature, followed by incubation with a mouse monoclonal antibody against rat sPLA$_2$-IIA (generous gift from Prof. Henk van den Bosch, Utrecht, The Netherlands) at a 1:100 dilution in 0.01% milk powder in PBS. This rat sPLA$_2$-IIA antibody cross-reacts with neither the human recombinant sPLA$_2$-IIA nor the other sPLA$_2$s used in this study. Indeed, Fig. 1*B* shows that the antibody detected the exogenously added recombinant rat enzyme (100 nM) as a thick band, but does not recognize the human sPLA$_2$-IIA and the other sPLA$_2$s. This clearly demonstrates that this antibody can be specifically used to detect the sPLA$_2$-stimulated release of rat sPLA$_2$-IIA from mesangial cells. Blots were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Amersham Biosciences, Freiburg, Germany) at a 1:15,000 dilution in blocking buffer for 1 h at room temperature. The washing steps were performed in 0.05% Tween 20 in PBS. After washing, peroxidase activity was detected using ECL (Amersham Biosciences).

sPLA$_2$ Assay—sPLA$_2$ activity in supernatants of mesangial cells was determined with [1-^{14}C]oleate-labeled *Escherichia coli* membranes as substrate (42). Briefly, assay mixtures (1 ml) contained 100 mM Tris-HCl (pH 7.0), 10 mM $CaCl_2$, [1-^{14}C]oleate-labeled *E. coli* (≈10,000 cpm), and 5 μl of cell supernatants, which produces less than 5% of substrate hydrolysis. Reaction mixtures were incubated for 30 min at 37 °C in a thermomixer. The extraction of the lipids was performed by Dole's method exactly as described (42). Free [1-^{14}C]oleate was measured in a β-counter.

Construction of Reporter Gene Fusions—A *Bam*HI/*Kpn*I fragment (2.67 kbp) of the rat sPLA$_2$-IIA promoter (accession no. AF375595) was fused to the luciferase gene by cloning this fragment to the respective sites in the pGL3 basic vector (Promega, Mannheim, Germany). Unidirectional nested deletions of this construct were performed with the Erase-a-Base system (Promega) as described previously (35).

Site-directed Mutagenesis—Mutations within the putative PPAR binding site −909 to −888 (5′-AGGTTGTCCTCTGAACTCCACA-3′ in the rat sPLA$_2$-IIA promoter fragment were introduced by PCR-based site-directed mutagenesis according to the instructions from the manufacturer (Stratagene) as described previously (35); the changes in

TABLE I
sPLA$_2$s used for treatment of rat mesangial cells

Abbreviation	Type	Source	Binding to the rat M-type sPLA$_2$ receptor ($K_{0.5}$ values)[a]
			nM
WT-IB	Wild-type porcine sPLA$_2$-IB	Porcine pancreas	0.5
IB-H48Q	Catalytically inactive porcine sPLA$_2$-IB mutant	Recombinant	0.5
hIIA	Wild-type human sPLA$_2$-IIA	Recombinant	>500
hIIA-H48N	Mutant human sPLA$_2$-IIA with 0.2 % residual catalytic activity	Recombinant	>500
RatIIA	Rat sPLA$_2$-IIA	Recombinant	>500
Bee	Bee venom sPLA$_2$-III	Bee venom	>500
Naja	Snake venom sPLA$_2$	*N. mossambica mossambica*	>500
OS1	Snake venom sPLA$_2$	*O. scutellatus sc.*	0.3
OS2	Snake venom sPLA$_2$	*O. scutellatus sc.*	0.1

[a] $K_{0.5}$ values were determined by competition binding assays with labeled OS1 on rat mesangial cell membranes as described (38).

the obtained sequence 5′-AGGTTGTGTTCTGCGCTCCACA-3′ are underlined.

Transfection and Luciferase Reporter Gene Assay—For transfection, cells were seeded in 35-mm culture dishes and incubated for 24 h at 37 °C in RPMI containing 10% fetal calf serum. The cells were then incubated in Dulbecco's modified Eagle's medium containing 0.1 mg/ml BSA and transfected with 400 ng of plasmid DNA and 40 ng of *Renilla* luciferase DNA (pRL-TK vector) per well using the Effectene transfection reagent from Qiagen (Hilden, Germany). After 16 h, cells were stimulated with the different effectors for another 24 h. The cells were then washed with ice-cold PBS, lysed in 250 μl of lysis buffer from the dual luciferase reporter assay system (Promega), scraped with a rubber policeman, and transferred into 1.5-ml vials. The cell lysates were subjected to two freeze/thaw cycles for complete lysis of cells. After short centrifugation, the assays for firefly luciferase activity and *Renilla* luciferase activity were performed sequentially by using a luminometer (Autolumat from Berthold, Wildbad, Germany). Values for the sPLA$_2$-IIA promoter activity were divided by those obtained from *Renilla* luciferase activity. The mean values ± S.D. obtained for the control cells were set as 1. Values obtained with treated cells are expressed as -fold increase in luciferase activity (relative units) compared with control.

Electrophoretic Mobility Shift Assay (EMSA)—The sequences of the double-strand oligonucleotides used to detect the DNA binding activities of PPAR were chosen as described previously (35). The complementary DNA strands were labeled with T4 polynucleotide kinase using [γ-^{32}P]ATP. Nuclear extracts from stimulated cells were isolated as described previously (35). Binding reactions with radioactive oligonucleotides were performed for 30 min at room temperature with 5 μg of total protein in 25 μl of 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 10% glycerol, 1 μg of acetylated bovine serum albumin, 2 μg of poly(dI-dC), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 50,000 dpm of ^{32}P-labeled oligonucleotides. For competition experiments, nuclear extracts were pre-incubated with a 100-fold excess of cold PPRE-1 oligonucleotide or with antibodies against PPARα (4 μg/onset) and RXRα (3 μg/onset) for 30 min at room temperature before addition of the labeled oligonucleotides.

DNA-protein complexes were separated from unbound DNA probe on native 8% polyacrylamide gels at 20 mA in 34 mM Tris-HCl (pH 7.5), 17 mM sodium acetate, and 0.5 mM EDTA (pH 8.0). Gels were vacuum-dried and analyzed with a phosphorimager.

RT-PCR Analysis of 5-Lipoxygenase (5-LOX) and 5-LOX-activating Protein (FLAP)—Expression of mRNA for 5-LOX and FLAP was analyzed by RT-PCR using a total of 5 μg of RNA. As positive controls, RNA extracts from kidneys of rats sacrificed at 6 and 24 h after induction of Thy-1 nephritis were used (43). First strand cDNA was transcribed with Superscript II RNase H-RT obtained from Invitrogen and oligo(dT)$_{15}$ primer (Promega). PCR was performed on a PerkinElmer Thermal Cycler with specific primers as follows: arachidonate 5-lipoxygenase (Alox5), sense (5′-CTGGTAGCCCATGTGAGGTT-3′) and antisense (5′-GCACAGGGAGGAATAGGTCA-3′) (product, 162 bp); rat FLAP sense (5′-CGTAGATGCGTACCCCACTT-3′) and antisense (5′-CGCTTCCGAAGAAGAAGATG-3′) (product, 245 bp); 18 S RNA, sense (5′-GCGGTAATTCCAGCTCCAATAG-3′) and antisense (5′-CCCTCT-TAATCATGGCCTCAGT-3′) (product, 289 bp).

The different cDNA probes were amplified in a prepared Mastermix containing dNTPs, specific primers, and Red *Taq* polymerase (Sigma) in the corresponding PCR buffer. For the PCR reactions, the following sequences were performed.

For 5-LOX, sequence was 95 °C for 4 min (1 cycle) followed immediately by 95 °C for 50 s, 55 °C for 30 s, and 72 °C for 20 s (36 cycles) and a final extension phase at 72 °C for 10 min.

For FLAP, sequence was 95 °C for 4 min (1 cycle) followed immediately by 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 20 s (36 cycles) and a final extension phase at 72 °C for 10 min.

For 18 S RNA, sequence was 95 °C for 4 min (1 cycle) followed immediately by 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min (24 cycles) and a final extension phase at 72 °C for 10 min.

Amplified PCR products were separated on 1% agarose gels containing 0.5 μg/ml ethidium bromide.

The PCR products from the rat kidneys were purified with the QIAquick PCR purification kit (Qiagen) for sequencing using a kit based on the dye terminator technology (PerkinElmer Applied Biosystems, Weiterstadt, Germany) in combination with the automated sequence analyzer A310 (PerkinElmer Applied Biosystems).

Statistical Analysis—Data are represented as means ± S.D. (n = 3; in transfection experiments, n = 6) showing one representative experiment of three with similar results. Statistical analysis was performed by Student's t test. A probability < 0.05 was considered as significant.

RESULTS

Effect of Exogenous sPLA$_2$s on sPLA$_2$-IIA Expression in Rat Mesangial Cells—The scope of this study was to identify the transcriptional regulatory mechanisms of sPLA$_2$-IIA expression in rat mesangial cells triggered by exogenous sPLA$_2$s. For this purpose, we treated rat mesangial cells with various sPLA$_2$s from mammalian and venom origins in the absence or presence of TNFα (Table I). The effects of a catalytically inactive mutant of the porcine pancreatic sPLA$_2$-IB containing a single amino acid mutation at position 48 (H48Q) was also studied. This mutant has less than 0.02% of wild-type PLA$_2$ activity (39). Rat mesangial cells were treated for 24 h with sPLA$_2$s, at a concentration of 100 nM, which was shown previously (17) to stimulate sPLA$_2$-IIA mRNA expression and prostaglandin synthesis in these cells. The expression of rat sPLA$_2$-IIA was investigated by Northern and Western blot analyses.

In the presence of TNFα, all the enzymes tested were able to enhance the cytokine-stimulated mRNA expression of sPLA$_2$-IIA in mesangial cells (Fig. 1*A*). This enhancement was accompanied by an increased secretion of the sPLA$_2$-IIA protein into the cell culture medium (Fig. 1*B*). Interestingly, the H48Q catalytic mutant of sPLA$_2$-IB also potentiated the expression of rat sPLA$_2$-IIA. The relative increase in sPLA$_2$-IIA mRNA and protein after costimulation with TNFα and exogenous sPLA$_2$s varied between 1.5- and 3.5-fold over TNFα alone in at least five independent experiments, and the values obtained by densitometric analysis showed a significant increase ($p < 0.05$) compared with TNFα alone. Finally, similar results were obtained in the presence of IL-1β (data not shown).

In contrast to the study by Kishino *et al.* (16, 17), we were unable to detect an increase in sPLA$_2$-IIA expression by RT-PCR (data not shown), Northern blot, and Western blot analysis when cells were treated with sPLA$_2$s alone, *i.e.* in the absence of TNFα or IL-1β.

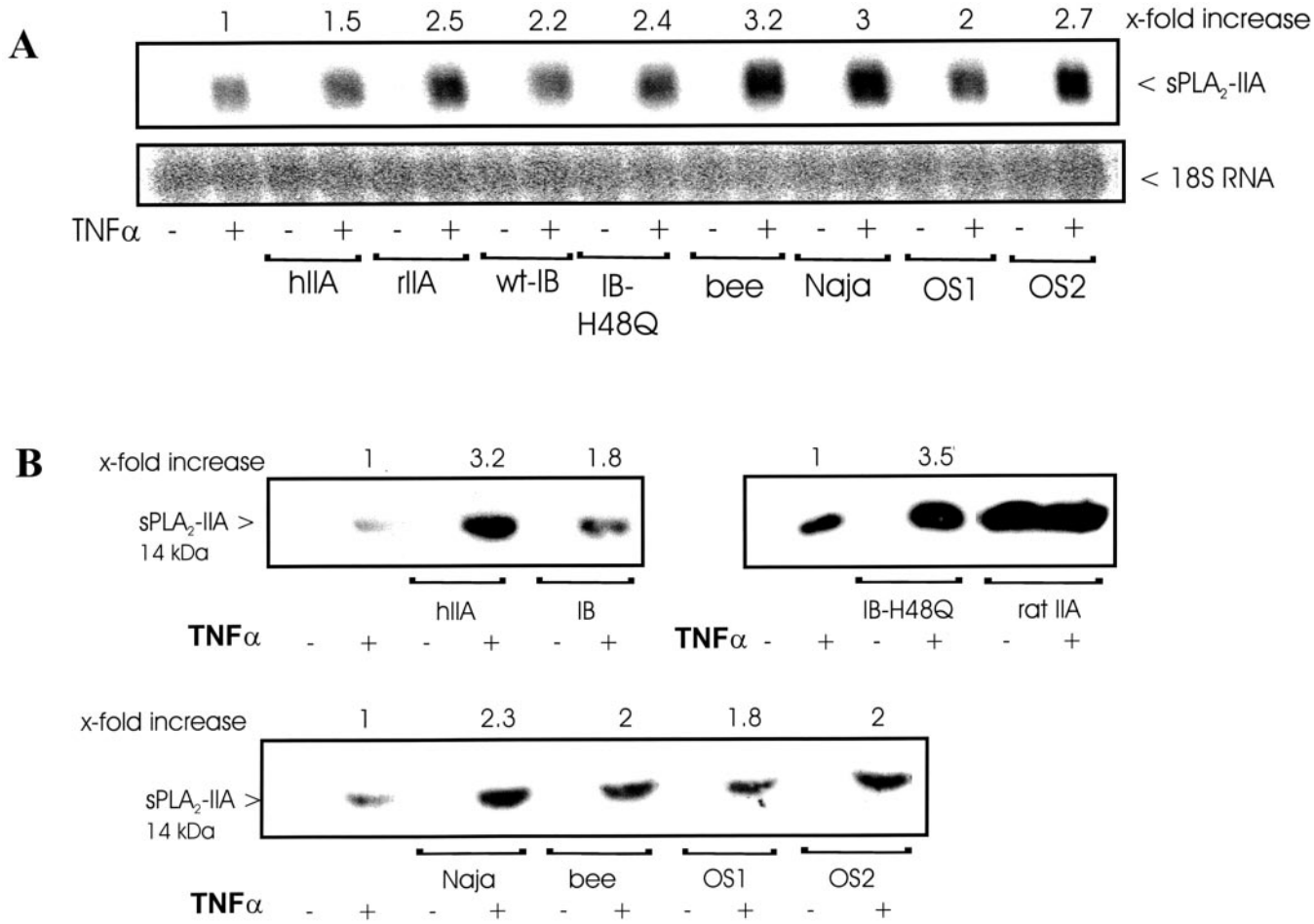

FIG. 1. **Influence of exogenous sPLA$_2$s on sPLA$_2$-IIA mRNA and protein expression in rat mesangial cells.** Mesangial cells were treated for 24 h with TNFα (0.5 nM). In parallel, cells were incubated with 100 nM human sPLA$_2$-IIA (*hIIA*), porcine sPLA$_2$-IB (*IB*), the catalytically inactive mutant sPLA$_2$-IB-H48Q (*H48Q* in *panel B*), rat sPLA$_2$-IIA (*rIIA*), sPLA$_2$ from *N. mossambica mossambica* (*Naja*), sPLA$_2$-III from bee venom (*bee*), and snake venom sPLA$_2$s from *Oxyuranus scutellatus sc.* (*OS1*, *OS2*). *wt*, wild-type. mRNA was extracted from cell lysates, and Northern blot analysis (*A*) was performed to detect sPLA$_2$-IIA mRNA expression as described under "Experimental Procedures." The signal density for each RNA sample was normalized to that obtained with the 18 S RNA probe. The amount of mRNA calculated for sPLA$_2$-IIA in IL-1β-stimulated cells is expressed as 100%. The experiments were repeated three times with similar results, and representative data are shown. From cell culture supernatants, Western blot analysis with a specific antibody was performed as described under "Experimental Procedures" to detect sPLA$_2$-IIA protein secretion (*B*). The *numbers* at the *top* represent the relative increase in sPLA$_2$-IIA protein compared with TNF, which is indicated as 1. The values are calculated as means of three independent experiments with comparable results and vary between 1.5- and 3-fold between the single experiments with a significant increase compared with TNFα ($p < 0.05$).

Rat mesangial cells were shown previously (16) to express relatively large amounts of the M-type 180-kDa receptor. The expression of this receptor in our rat mesangial cells was confirmed by RT-PCR, Western blot analysis with a specific antibody (44), as well as binding studies using iodinated OS1 as ligand (data not shown; Ref. 18). The maximal number of M-type receptor expressed in rat mesangial cells was found to be 0.3 pmol/mg of total cell membrane protein. The K_d value for labeled OS1 was 0.3 nM. The affinities of the various sPLA$_2$s (Table I) for the M-type receptor expressed in mesangial cells were measured by competition binding assays with iodinated OS1 as described previously (18). We found no obvious correlation between their affinities for the M-type receptor and their ability to enhance the TNFα-stimulated sPLA$_2$-IIA induction, because sPLA$_2$s that bind or do not bind to the receptor similarly activate sPLA$_2$-IIA expression. The catalytically inactive H48Q mutant of sPLA$_2$-IB, which also binds to the M-type receptor (Table I), could enhance the expression of sPLA$_2$-IIA induced by TNFα (Fig. 1), suggesting that the intrinsic sPLA$_2$ activity of sPLA$_2$-IB does not play a major role in this effect.

Effect of Exogenous sPLA$_2$s on the Rat sPLA$_2$-IIA Promoter Activity—The observation that the TNFα-stimulated sPLA$_2$-IIA mRNA induction is markedly enhanced by exogenous sPLA$_2$s suggests that TNFα and sPLA$_2$s act in a synergistic fashion using different signaling pathways and transcription factors. In a recent study we have shown that activators of PPARα (WY14643, LY171883, clofibrate) enhanced the transcription of sPLA$_2$-IIA in rat mesangial cells via the PPRE-1 site (−909 to −888) in the rat sPLA$_2$-IIA promoter (35). Because fatty acids and derivatives are potent activators of PPARs, an attractive hypothesis is that sPLA$_2$s activate PPARs by producing fatty acids and other derivatives, which will in turn lead to enhanced transcription of sPLA$_2$-IIA in the presence of TNFα.

To address this hypothesis, we first investigated the effects of the different exogenous sPLA$_2$s in the absence or presence of TNFα on the activity of a 2.67-kbp construct of the rat sPLA$_2$-IIA promoter fused to a luciferase reporter gene (35). Mesangial cells transfected with the wild-type 2.67-kbp promoter construct responded to treatment with different sPLA$_2$s with a marked increase in luciferase activity (Fig. 2). The promoter activity was also activated by TNFα, and this effect was further potentiated by treatment with the different sPLA$_2$s. Similar results were obtained with IL-1β (data not shown).

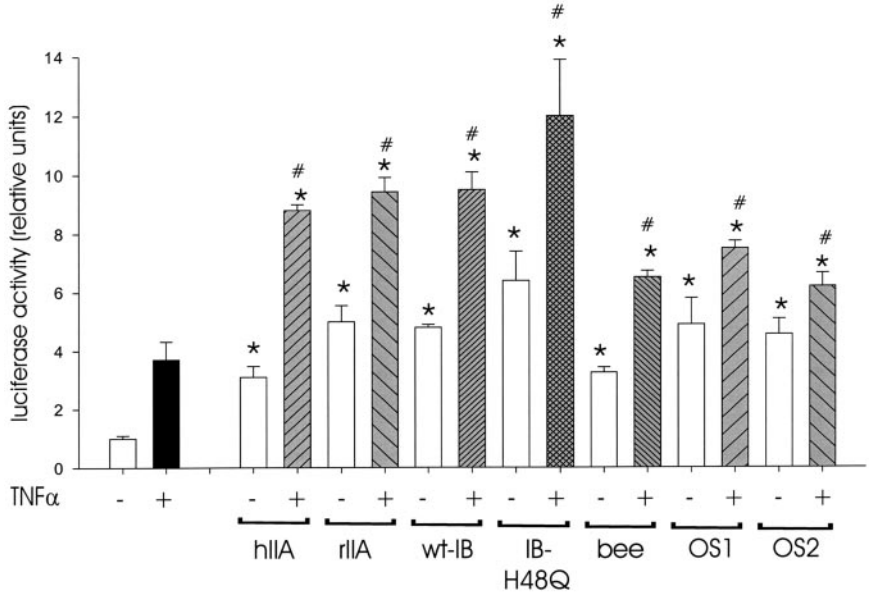

FIG. 2. **Modulation of the TNFα-stimulated activity of the rat sPLA$_2$-IIA promoter by exogenous sPLA$_2$s.** Mesangial cells were transfected with 0.4 μg of the 2.67-kbp rat sPLA$_2$-IIA promoter DNA and additionally with plasmid DNA plus 40 ng of *Renilla* luciferase DNA that contained the gene for *Renilla* luciferase fused to a constitutive promoter of the cytomegalovirus. After 16 h, cells were washed and treated with or without TNFα (0.5 nM) in the absence or presence of the indicated sPLA$_2$s (each 100 nM). After 24 h, dual luciferase assays were performed as described under "Experimental Procedures." Values for beetle luciferase were normalized to those for *Renilla* luciferase. The mean of the sPLA$_2$-IIA promoter activity in control cells is set as 1. Abbreviations are defined in Fig. 1 legend. Data are means ± S.D. ($n = 6$). Significant differences from the control group: *, $p < 0.05$; significant differences from the corresponding TNFα group: #, $p < 0.05$.

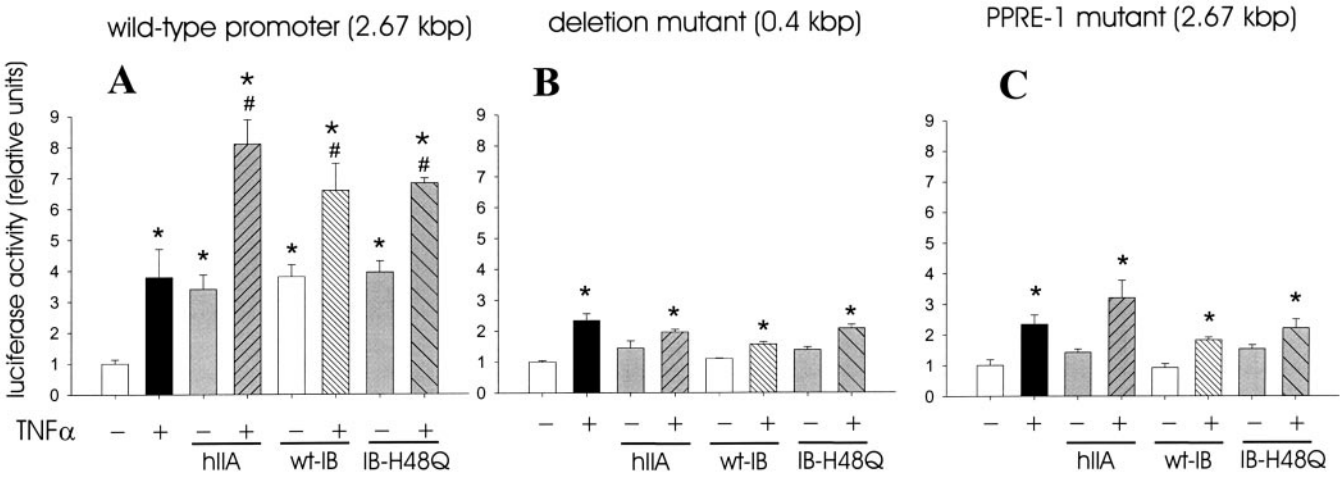

FIG. 3. **Effect of exogenous sPLA$_2$s on mesangial cells transfected with different sPLA$_2$-IIA promoter constructs.** Mesangial cells were transfected for 16 h with the wild-type 2.67-kbp promoter construct (*A*), with a 0.4-kbp deletion mutant without the PPRE-1 site (*B*), or with a PPRE-1 mutant promoter construct (2.67 kbp; *C*). Base exchanges in the PPRE-1 site by site-directed mutagenesis as well as deletion of the 2.67-kbp promoter construct are described under "Experimental Procedures." The cells were then treated with TNFα (0.5 nM) in the absence or presence of human sPLA$_2$-IIA (*hIIA*), wild-type sPLA$_2$-IB (*wt-IB*), or the catalytically inactive mutant sPLA$_2$-IB-H48Q (*H48Q*; each 100 nM). Dual luciferase assays were performed as described under "Experimental Procedures." Data are means ± S.D. ($n = 6$). Significant differences from the control group: *, $p < 0.05$; significant differences from the corresponding TNFα-treated cells: #, $p < 0.05$.

We next investigated whether the PPAR-responsive element 1 (PPRE-1) site found in the sPLA$_2$-IIA promoter is essential to the sPLA$_2$ effects. For this purpose, mesangial cells were transfected with promoter constructs deleted from a 0.4-kbp fragment containing the PPRE-1 site or with a promoter containing point mutations in the PPRE-1 site (35). The transfected cells were then treated with human sPLA$_2$-IIA and the wild-type or H48Q mutant of porcine sPLA$_2$-IB. In cells transfected with the wild-type sPLA$_2$-IIA promoter construct, TNFα and sPLA$_2$s alone stimulated luciferase activity, whereas the addition of both effectors led to a potentiation of luciferase activity (Fig. 3*A*). In contrast, when cells were transfected with the promoter lacking the PPRE-1 site (Fig. 3*B*) or containing a mutated PPRE-1 site (Fig. 3*C*), exogenous sPLA$_2$s were unable to activate the promoter or to enhance the TNFα-stimulated effect. In addition, we observed that the TNFα stimulation was reduced by ~3-fold in cells transfected with the PPRE-1 mutant or the 0.4-kb deletion mutant. From this experiment, we inferred that PPAR activation might not only be involved in the promoter activity enhanced by exogenously added sPLA$_2$s, but also in the TNFα-stimulated activation of sPLA$_2$-IIA transcription. This is partially the result of the autocrine loop involving the endogenous sPLA$_2$-IIA initially secreted by TNFα (see Fig. 10).

To confirm that fatty acids could act as PPAR ligands in our mesangial cells, we treated cells with docosahexaenoic acid or linoleic acid, which are known to preferentially activate PPARα (33). In the presence of a functional PPRE-1 site, the TNFα-dependent sPLA$_2$-IIA promoter activity was found to be markedly enhanced by the different fatty acids (Fig. 4*A*), which was reflected by a more pronounced sPLA$_2$-IIA protein secretion (Fig. 4*C*). In the absence of a functional PPRE-1 site, the potentiating effect of the fatty acids was completely abolished (Fig. 4*B*). These results suggest that sPLA$_2$s may potentiate the cytokine-stimulated sPLA$_2$-IIA expression by producing fatty acids capable of activating PPARα in mesangial cells.

Electrophoretic Mobility Shift Analysis of PPAR Binding by Exogenous sPLA$_2$s—To further support a role of PPAR activation in the transcriptional regulation of sPLA$_2$-IIA induction by TNFα, as well as by exogenous sPLA$_2$s, we performed electrophoretic mobility shift analyses using a radioactively labeled

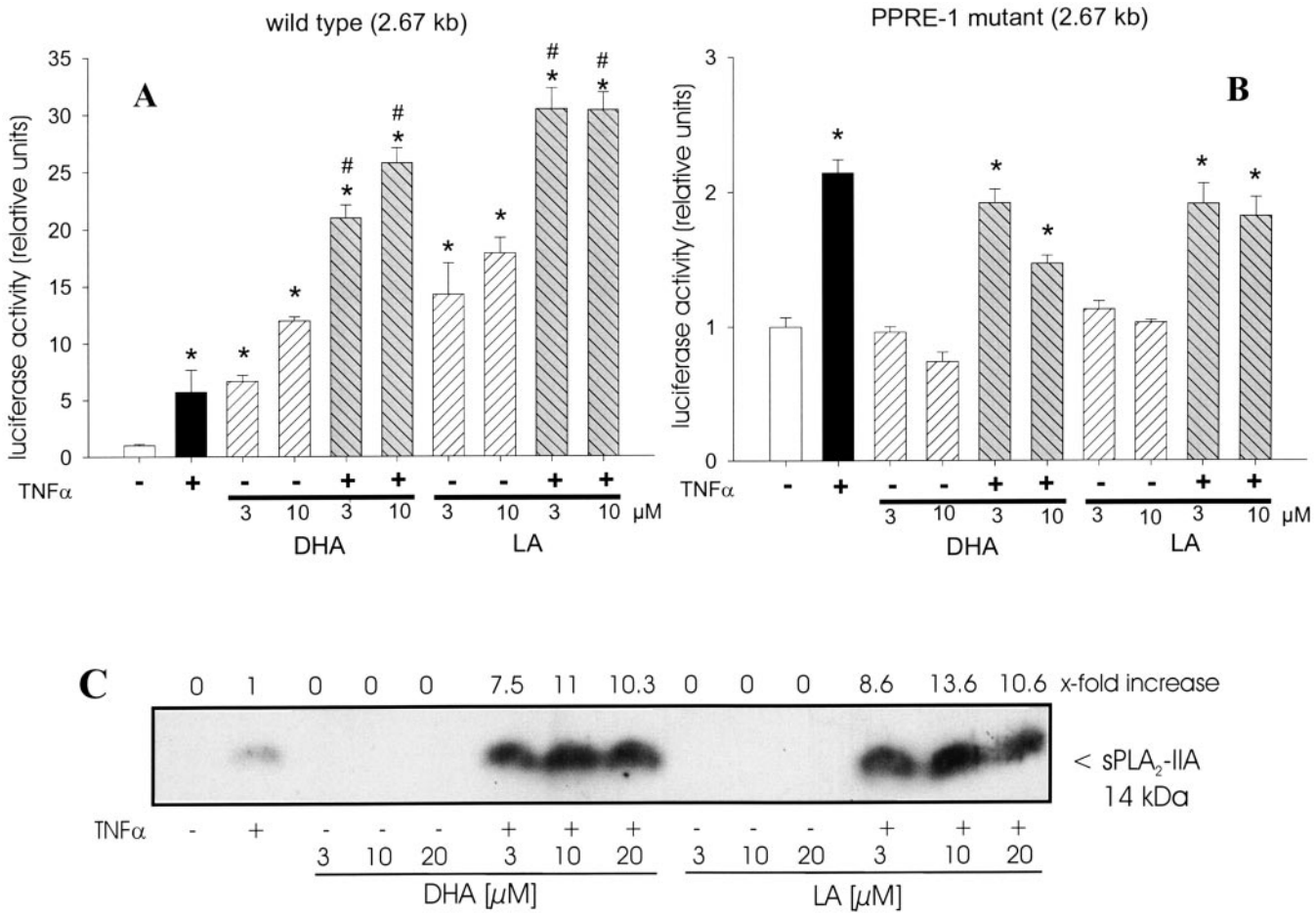

FIG. 4. **Effect of docosahexaenoic acid and linoleic acid on TNFα-stimulated sPLA$_2$-IIA promoter activity and protein expression.** Mesangial cells were transfected with the wild-type 2.67-kbp promoter construct (*A*) or with a PPRE-1 mutant-promoter construct (*B*) as described under "Experimental Procedures." The cells were then treated for 24 h with TNFα in the absence or presence of the indicated concentrations of docosahexaenoic acid (*DHA*) or linoleic acid (*LA*). Dual luciferase assays were performed as described under "Experimental Procedures." Data are means ± S.D. ($n = 6$). Significant differences from the control group: *, $p < 0.05$; significant differences from the corresponding TNFα-treated cells: #, $p < 0.05$. *C*, in parallel experiments cell culture supernatants were analyzed for sPLA$_2$-IIA protein by Western blot with a specific antibody as described under "Experimental Procedures." *Numbers* at the *top* of a representative blot of three with comparable results represent the increase in protein amount compared with TNFα, which is set as 1.

oligonucleotide comprising the PPRE-1 site from −909 to −888 of the rat sPLA$_2$-IIA promoter (35). Nuclear extracts were prepared from mesangial cells treated for 8 h with human sPLA$_2$-IIA and porcine sPLA$_2$-IB, as well as the mutant sPLA$_2$-IB-H48Q in the absence or presence of TNFα. As described previously (18), TNFα alone stimulated the formation of specific complexes (Fig. 5*A*). This effect was also observed after treatment with exogenous sPLA$_2$s, and an enhanced complex formation was obtained after co-incubation with TNFα and sPLA$_2$s. To demonstrate that this binding is specific for PPAR, extracts were co-incubated in the presence of radioactively labeled PPRE-1 oligonucleotide together with a 100-fold excess of cold PPRE-1 oligonucleotide. This competition completely abolished the binding of the radioactively labeled oligonucleotide to nuclear extracts obtained from TNFα- and sPLA$_2$-treated cells, implicating PPAR in sPLA$_2$-IIA expression mediated by TNFα alone or in combination with sPLA$_2$s (Fig. 5*A*). Competition with a nonrelevant oligonucleotide specific for the NF-κB consensus sequence did not prevent PPAR binding.

To identify PPARα-specific complexes, we also performed competition experiments using a specific PPARα antibody, which blocks the binding of PPARα to DNA and which does not cross-react with the other PPAR subtypes. As shown in Fig. 5*B*, both TNFα and exogenous sPLA$_2$s induced the formation of specific complexes. In the presence of antibody, the formation of the complex indicated by an *arrow* was nearly completely abolished (Fig. 5*B*). This effect was also observed with the combination of TNFα and sPLA$_2$s. The same complex also disappeared when nuclear extracts were incubated with a specific antibody against RXRα (Fig. 5*C*), which binds specifically to PPARα and acts as a coactivator (45, 46). Together, these data show that in rat mesangial cells TNFα and exogenous sPLA$_2$s induce the formation of a nuclear complex that specifically comprises PPARα and RXRa.

Effect of the PPARα Antagonist MK886 on sPLA$_2$-IIA Gene Expression—To further demonstrate the role of PPARα in sPLA$_2$-IIA gene expression, we used MK886, which was shown to act as a non-competitive inhibitor of PPARα binding (47). This compound was originally described as an inhibitor of the 5-lipoxygenase-activating protein FLAP (48). However, as shown in Fig. 6*A*, rat mesangial cells do not express FLAP or 5-lipoxygenase (5-LOX). As positive controls, we performed RT-PCR experiments with total RNA extracts from kidneys of rats sacrificed at 6 and 24 h after induction of a Thy-1 glomerulonephritis. It was shown previously (43) that there is an invasion of leukocytes into the glomerulus and production of lipoxygenase products such as leukotriene B$_4$ during this animal model disease. As expected, PCR products for 5-LOX and FLAP were amplified from the kidney samples, and sequencing of the fragments confirmed the identity of the PCR products. In

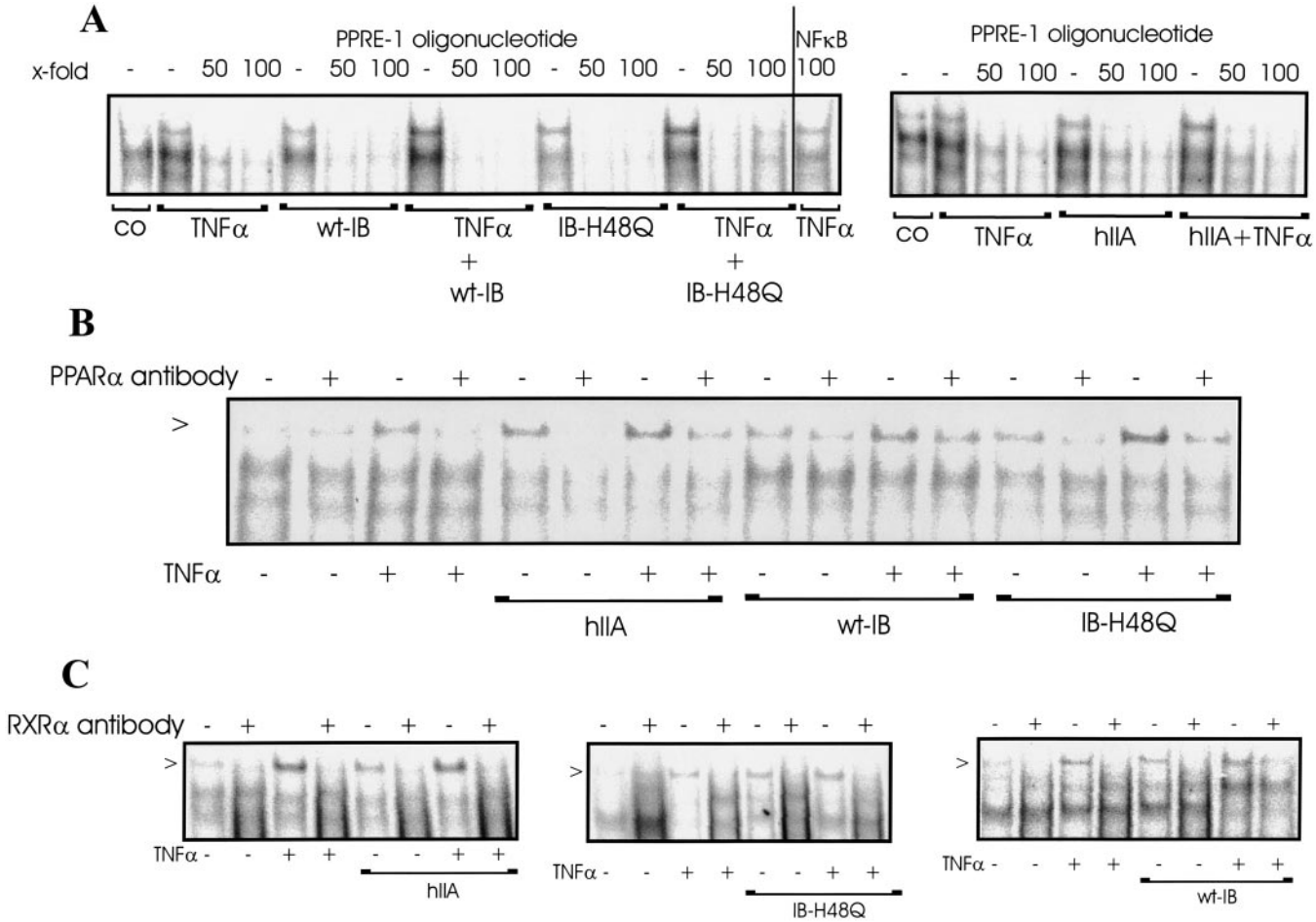

FIG. 5. **EMSA of PPAR binding.** *A*, mesangial cells were stimulated for 8 h with vehicle (*co*) or TNFα (0.5 nM) in the absence or presence of different sPLA$_2$s (100 nM) as indicated. ^{32}P-Labeled double-stranded PPRE-1 oligonucleotide from the sPLA$_2$-IIA promoter was incubated with nuclear extracts prepared from stimulated mesangial cells, and EMSA was performed as described under "Experimental Procedures." For competition, nuclear extracts were co-incubated with a 50- or 100-fold excess of unlabeled PPRE-1 oligonucleotide from the sPLA$_2$-IIA promoter. As a negative control, nuclear extracts from TNFα-treated cells were co-incubated with a 100-fold excess of unlabeled NF-κB consensus oligonucleotide. This experiment was performed three times, and a representative experiment is shown. *B*, mesangial cells were stimulated for 8 h with or without TNFα in the absence or presence of the indicated sPLA$_2$s (100 nM). EMSA with ^{32}P-labeled double-stranded PPRE-1 oligonucleotide from the sPLA$_2$-IIA promoter was performed in the absence or presence of a specific PPARα antibody described under "Experimental Procedures." *C*, EMSA was performed with a specific antibody against RXRα under the same conditions as described in *B*. Abbreviations are defined in Fig. 1 legend.

addition, we measured the release of leukotriene B$_4$ in cell culture supernatants by a specific enzyme immunoassay, and did not detect product formation under the above conditions.[2] Thus, we can exclude that the observed effects of MK886 on sPLA$_2$-IIA expression are produced by blocking the leukotriene pathway.

Cells were preincubated for 30 min with 3 μM MK886, and then treated with TNFα for 24 h. We have shown previously (35) earlier that, at this concentration, MK886 exerts in rat mesangial cells a maximal reducing effect on PPARα-dependent expression of sPLA$_2$-IIA without cytotoxic effects. MK886 partially attenuated the TNFα-stimulated sPLA$_2$-IIA secretion and activity and completely abolished the potentiating effects of sPLA$_2$s (Fig. 6, *B* and *C*). In transfection experiments with the wild-type sPLA$_2$-IIA promoter construct, MK886 also partially reduced the sPLA$_2$-IIA promoter activity stimulated by TNFα and dramatically decreased the effect of exogenous sPLA$_2$s (Fig. 6*D*). Together with the EMSA analysis, these results indicate that PPARα is involved in the transcriptional control of sPLA$_2$-IIA expression mediated by TNFα and sPLA$_2$s.

Effect of the sPLA$_2$-IIA Inhibitor LY311727 and of the cPLA$_2$ Inhibitor Pyrrolidine-1—Because MK886 strongly reduced the TNFα-mediated sPLA$_2$-IIA induction, we postulated that sPLA$_2$-IIA released by mesangial cells after TNFα treatment enhanced its own expression in a positive feedback loop, possibly through fatty acid release and PPAR activation. Consistent with this hypothesis, we considered the possibility that sPLA$_2$ produces fatty acids via its intrinsic sPLA$_2$ activity or after cPLA$_2$ activation. To address this possibility, we first incubated mesangial cells with TNFα in the absence or presence of LY311727. This compound was shown to inhibit the activity of group IIA and several other sPLA$_2$s, but not that of cPLA$_2$ (49, 50). In rat mesangial cells, the rat sPLA$_2$-IIA activity stimulated by cytokine was completely abolished by 10 μM LY311727 (data not shown).

LY311727 was found to markedly reduce the protein secretion of sPLA$_2$-IIA elicited by TNFα and human sPLA$_2$-IIA (Fig. 7*A*). Interestingly, the enhanced protein secretion induced by sPLA$_2$-IB or the catalytically inactive sPLA$_2$-IB-H48Q mutant was also markedly reduced by LY311727.

LY311727 was also able to reduce the sPLA$_2$-IIA promoter activity of cells transfected with the wild-type 2.67-kbp construct and stimulated with TNFα and exogenous sPLA$_2$s (Fig. 7*B*).

[2] S. Beck, J. Pfeilschifter, and M. Kaszkin, unpublished observation.

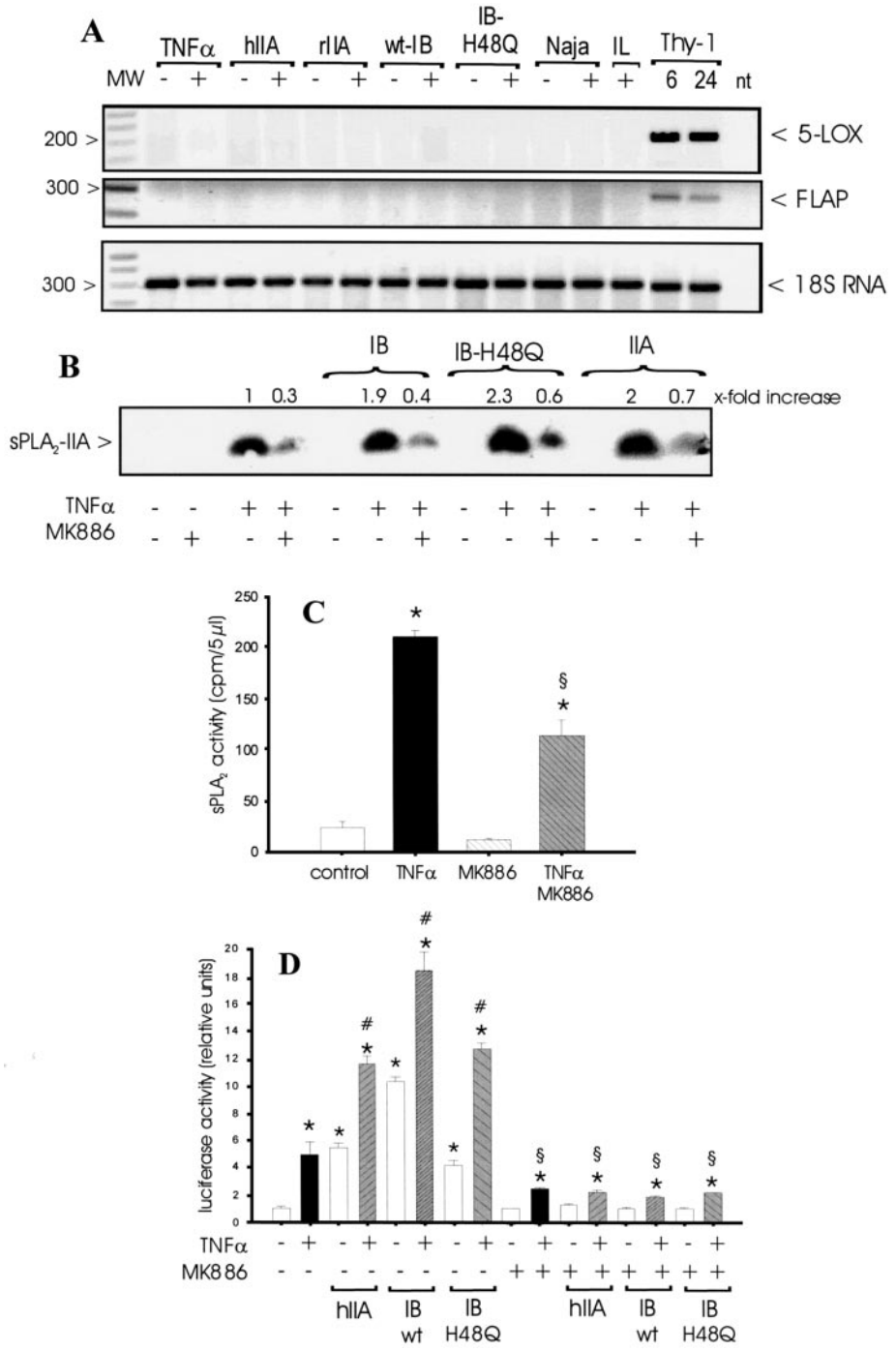

FIG. 6. **Expression of FLAP and 5-LOX in rat mesangial cells and effects of the PPARα antagonist MK886.** *A*, RNA extracts from TNFα-, IL-1β- (*IL*), and sPLA$_2$-treated mesangial cells were analyzed for 5-LOX and FLAP expression by performing RT-PCR with specific primers as described under "Experimental Procedures." As positive controls, RNA extracts from kidneys of rats, sacrificed at 6 and 24 h after induction of a Thy-1 glomerulonephritis were used. *nt*, no template. For abbreviations for sPLA$_2$s, see Fig. 1 legend. Mesangial cells were pretreated for 30 min with MK886 (3 μM), and cells were subsequently incubated for 24 h with TNFα in the absence or presence of different sPLA$_2$s. From the cell culture supernatants, Western blot analysis of secreted sPLA$_2$-IIA (*B*) as well as sPLA$_2$ activity assays (*C*) were performed as described under "Experimental Procedures." For analysis of the sPLA$_2$-IIA promoter activity (*D*), cells were transfected for 16 h with the wild-type 2.67-kbp construct of the rat sPLA$_2$-IIA promoter. Then cells were treated for 24 h with TNFα and human sPLA$_2$-IIA, sPLA$_2$-IB, or the mutant sPLA$_2$-IB-H48Q in the absence or presence of MK886 (3 μM). Dual luciferase assays were performed as described under "Experimental Procedures." Data are means ± S.D. ($n = 6$). Significant differences from the control group: *, $p < 0.05$; significant inhibition compared with TNFα-treated cells: §, $p < 0.05$.

These data suggest that sPLA$_2$-IIA, which is released by TNFα- and sPLA$_2$-treated rat mesangial cells, promotes its own expression.

In contrast, the activity of sPLA$_2$-IIA seems to be crucial. To support this, we compared the effects of the active wild-type sPLA$_2$-IIA with a H48N mutant, which shows only 0.22% of the activity of the wild-type enzyme (40). Western blot analysis in Fig. 7*C* shows that, compared with the wild-type enzyme, the H48N mutant had no potentiating effect.

Together, these data show that, in contrast to sPLA$_2$-IB, only the catalytically active forms of sPLA$_2$-IIA, either released by

mesangial cells, or added exogenously, mediate sPLA$_2$-IIA expression.

Because it was shown previously (14) that treatment of rat mesangial cells with extracellular sPLA$_2$s leads to an early activation of cPLA$_2$, we analyzed the role of cPLA$_2$ in sPLA$_2$-IIA expression. In this regard, cytokines differ significantly and cause a delayed increase in cPLA$_2$ by up-regulating its mRNA expression (51, 52). To elucidate whether cPLA$_2$ activation is involved in sPLA$_2$-IIA expression, we analyzed the effect of pyrrolidine-1, a recently characterized specific inhibitor of cPLA$_2$α (53, 54). At concentrations up to 1 μM, this inhibitor does not inhibit the calcium-independent PLA$_2$ or various sPLA$_2$s. Cells were pre-incubated with pyrrolidine-1 for 30 min prior to treatment with TNFα and different sPLA$_2$s. Pyrrolidine-1 only weakly reduced the TNFα-stimulated sPLA$_2$-IIA protein secretion (Fig. 8*A*). However, when cells were co-incubated with TNFα and sPLA$_2$s, pyrrolidine-1 markedly reduced sPLA$_2$-IIA protein secretion, suggesting that an early as well as a delayed activation of cPLA$_2$ was inhibited. These data indicate that activation of cPLA$_2$ plays a central role in the induction of rat sPLA$_2$-IIA mediated by exogenous sPLA$_2$s.

Pyrrolidine-1 also markedly reduced the sPLA$_2$-IIA protein level measured after co-incubation with TNFα and sPLA$_2$-IB or the H48Q catalytic mutant of sPLA$_2$-IB (Fig. 8*B*), suggesting that the IB mutant can also activate cPLA$_2$, which finally results in activation of PPARα. These data also explain how this mutant, although devoid of significant sPLA$_2$ activity, could enhance the expression of sPLA$_2$-IIA. Finally, these data indicate that sPLA$_2$-IB can be a potent activator of cPLA$_2$, independently of its catalytic activity.

We also found that pyrrolidine-1 inhibited the cytokine as well as the sPLA$_2$-stimulated sPLA$_2$-IIA promoter activity, indicating that, in rat mesangial cells, cPLA$_2$ is an important player in the transcriptional regulation of sPLA$_2$-IIA induced by PPARα (Fig. 8*C*).

Involvement of Heparan Sulfate Proteoglycans in TNFα- and sPLA$_2$-mediated sPLA$_2$-IIA Induction—To further confirm the positive feedback regulation of released sPLA$_2$-IIA on its own expression, we incubated mesangial cells with heparinase-1 to prevent binding of released sPLA$_2$-IIA to HSPG (30). Heparinase I treatment attenuated the stimulatory effects of TNFα as well as those of exogenous human sPLA$_2$-IIA on sPLA$_2$-IIA protein secretion (Fig. 9*A*) and promoter activity. This suggests that the action of sPLA$_2$-IIA released by TNFα on its own expression is at least in part mediated through HSPG binding. Interestingly, the effects of the wild-type sPLA$_2$-IB and of the inactive mutant sPLA$_2$-IB-H48Q on sPLA$_2$-IIA protein secretion (Fig. 9*A*) and promoter activity (Fig. 9*B*) were not abolished by heparinase-1 treatment, indicating that sPLA$_2$-IB regulates sPLA$_2$-IIA gene expression via mechanisms independent of HSPG binding.

DISCUSSION

The results presented in this paper lead to four main conclusions. First, this paper shows that various sPLA$_2$s, when added exogenously to rat mesangial cells, can dramatically up-regulate the expression of sPLA$_2$-IIA induced by the potent pro-inflammatory cytokines such as TNFα. Second, this paper shows that the endogenous rat sPLA$_2$-IIA initially induced by TNFα can enhance its own expression through an autocrine loop. Third, this paper shows that both exogenous and TNFα-induced, *i.e.* endogenous rat sPLA$_2$-IIA, increase the expression of this later sPLA$_2$ by activating a signaling pathway that is distinct from that of TNFα and that involves cPLA$_2$ and intracellular lipid mediator formation to activate the nuclear receptor PPARα. Fourth, our results suggest that the various sPLA$_2$s used in this study associate to different plasma membrane targets and/or use different mechanisms that eventually lead to cPLA$_2$ and PPARα activation.

The possible mechanisms by which cytokines like TNFα and exogenously added sPLA$_2$s or endogenously produced rat group IIA sPLA$_2$ trigger the expression of rat sPLA$_2$-IIA are depicted in Fig. 10. In this model, TNFα can be considered as a "primary" potent cytokine able to initiate by itself the expression of rat sPLA$_2$-IIA through NFKB signaling. The released rat sPLA$_2$-IIA eventually activates PPARα and its own secretion through an autocrine loop. On the other hand, exogenous sPLA$_2$s like IB (or sPLA$_2$-IIA secreted by neighboring rat mesangial cells) may be considered as "secondary cytokines," which require the presence of TNFα to further activate the autocrine loop, and their effect occurs via cPLA$_2$ activation. In the future, it will be interesting to know whether other sPLA$_2$s such as group IIF, III, V, and XII sPLA$_2$s, which are known to be expressed in the kidney or mesangial cells (2, 3, 54, 56), can also enhance TNFα-induced rat sPLA$_2$-IIA expression.

According to Fig. 10, both exogenous and endogenous sPLA$_2$s are acting extracellularly, and, therefore, they should first bind to the cell surface before activating the PPARα transcriptional pathway. Because rat mesangial cells are known to express the M-type receptor (17) and various HSPGs including glypican-1 (57), which are binding proteins for different sPLA$_2$s (2, 19, 57), sPLA$_2$s may increase sPLA$_2$-IIA expression through binding to one of these proteins and/or through their catalytic activity on cellular phospholipids.

Role of PPARα in sPLA$_2$-IIA Expression—By investigating the mechanisms involved in the potentiation of sPLA$_2$-IIA gene expression by exogenous sPLA$_2$s, we found a significant increase in the sPLA$_2$-IIA promoter activity. Because this promoter contains a PPRE-1 element and because PPARs may be activated by PLA$_2$ lipid products, we postulated that PPARs might be involved in the up-regulation of sPLA$_2$-IIA expression by sPLA$_2$s. In contrast to a previous study (17), we were unable to detect an increased expression of sPLA$_2$-IIA at the mRNA or protein level when rat mesangial cells were treated with exogenous sPLA$_2$s alone. The reason for this discrepancy is unknown but might be a result of differences in the "inflammatory status" or "priming" between the different rat mesangial cell cultures. From our results we conclude that PPARα alone might not be sufficient for an efficient sPLA$_2$-IIA mRNA expression. In our hand, the effect of exogenous sPLA$_2$s could only be observed in the presence of a potent cytokine like TNFα or IL-1β. We previously found that PPARα activators such as WY14643, LY171883, or clofibrate have strong stimulatory effects on the rat sPLA$_2$-IIA promoter activity in mesangial cells, but do not elicit on their own an equivalent increase in sPLA$_2$-IIA mRNA and protein (35). A similar effect was observed in the present study for docosahexaenoic acid or linoleic acid as potential PPARα agonists. This suggests that PPARα acts synergistically with other cytokine-activated transcription factors such as NF-κB, which was shown earlier to be a major player in the cytokine-dependent induction of sPLA$_2$-IIA gene expression in rat mesangial cells (10, 11). A further possibility is that the cytokine treatment might be important for the stabilization of the sPLA$_2$-IIA mRNA, which would otherwise be rapidly degraded. Finally, we cannot exclude that the endogenous sPLA$_2$-IIA promoter contains some inhibitory elements that suppress the promoter activation unless the cells are treated with cytokines, and that the promoter constructs used in our studies lack such inhibitory elements.

Testing specific PPARα and PPARγ activators for sPLA$_2$-IIA induction, we found that only PPARα activators like unsaturated long chain fatty acids had a potentiating effect (Ref. 35 and Fig. 4), whereas the putative PPARγ activator 15-deoxy-

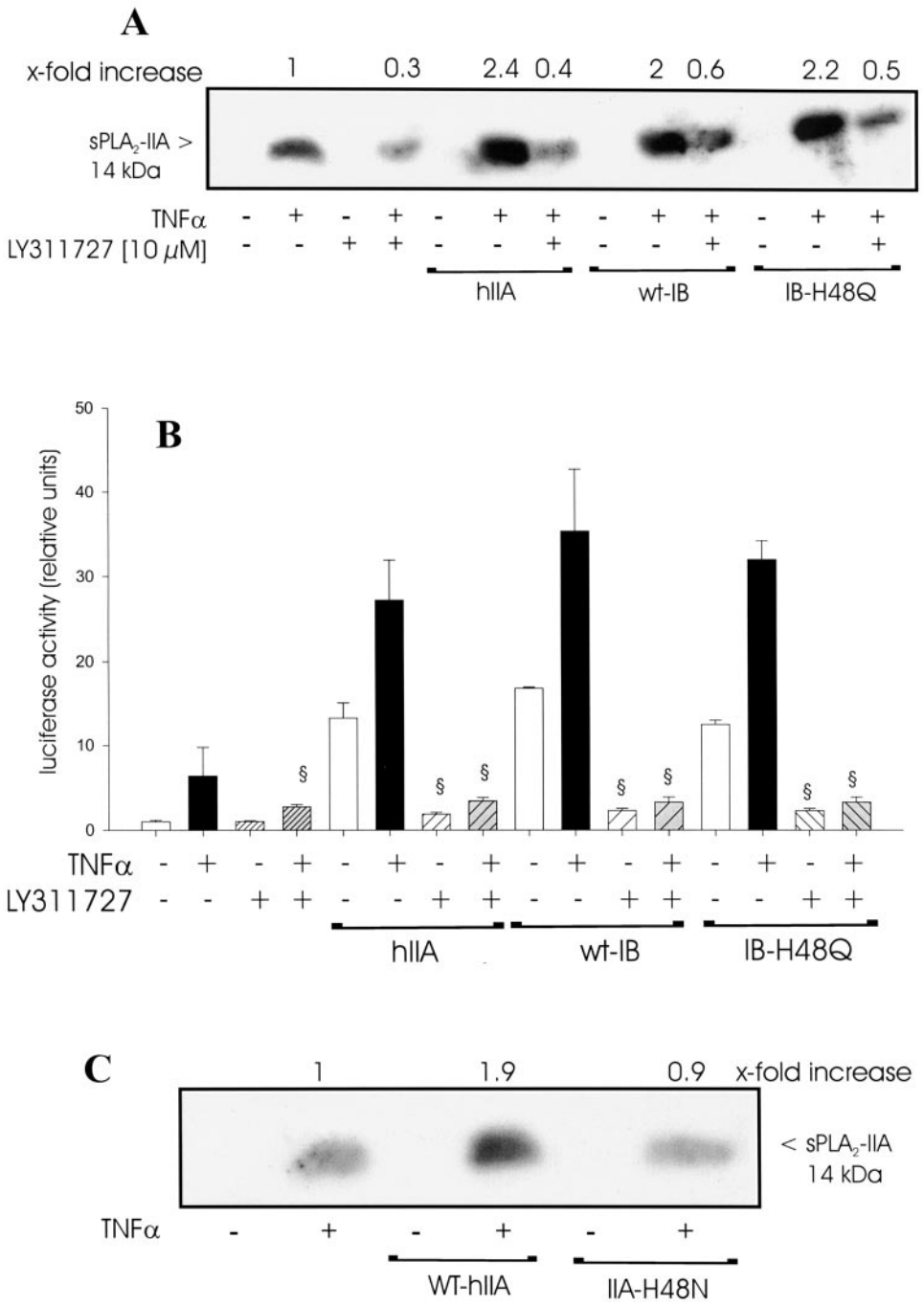

FIG. 7. **Effects of the sPLA$_2$ inhibitor LY311727 and the inactive mutant sPLA$_2$-IIA-H48N.** *A* and *B*, mesangial cells were treated for 24 h with or without TNFα as well as with human sPLA$_2$-IIA (*hIIA*), wild-type sPLA$_2$-IB (*wt-IB*), or the inactive mutant sPLA$_2$-IB-H48Q in the absence or presence of different concentrations of LY311727 (30-min preincubation) as indicated. *C*, cells were treated with or without TNFα in the absence or presence of the wild-type human sPLA$_2$-IIA (*WT-hIIA*) or the inactive mutant sPLA$_2$-IIA-H48N. Western blot analysis for detection of released sPLA$_2$-IIA in the cell culture supernatants (*A* and *C*) and luciferase assay (*B*) were performed as described under "Experimental Procedures." Data are means ± S.D. (n = 3).

$\Delta^{12,14}$- prostaglandin J$_2$ inhibited sPLA$_2$-IIA promoter activity and protein expression (data not shown). Using the PPARα antagonist MK886, various sPLA$_2$-IIA promoter constructs with and without the PPRE-1 element, and specific PPARα and RXRα antibodies, we found that the effect of exogenous and endogenous sPLA$_2$s clearly involves PPARα activation. Interestingly, more DNA-protein complexes disappeared when the competition was done with the PPRE-1 oligonucleotide compared with competition with the PPARα antibody. It is possible that the oligonucleotide, but not the antibody, prevents the formation of complexes between PPARα and coactivators. In this respect, 9-*cis*-retinoic acid receptor-α (RXRα) is known to act as a coactivator by forming a specific complex with PPARα (for reviews, see Refs. 45, 46, and 60). The fact that the same complex disappeared with the antibodies against RXRα and PPARα indicates that this complex is a PPARα/RXRα heterodimer.

Role of sPLA$_2$ Activity in sPLA$_2$-IIA Expression—Based on the results obtained with the inactive H48Q mutant of sPLA$_2$-IB, we concluded that the intrinsic enzymatic activity of this sPLA$_2$ is not crucial for the effect on rat sPLA$_2$-IIA expression. Interestingly, the sPLA$_2$ inhibitor LY311727 also reduced the

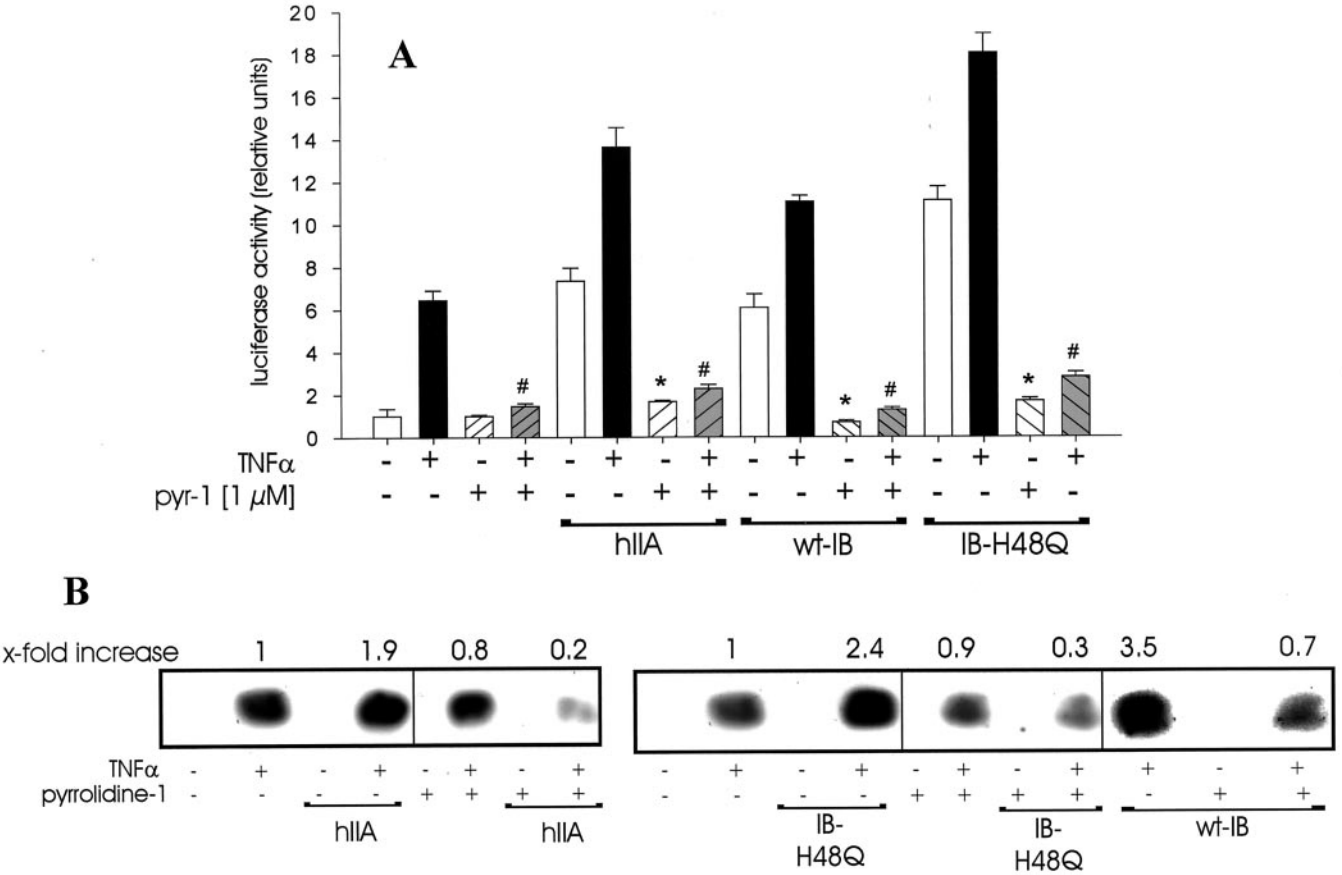

FIG. 8. **Effects of the cPLA$_2$ inhibitor pyrrolidine-1.** Cells were treated for 24 h with TNFα and human sPLA$_2$-IIA, sPLA$_2$-IB, or the inactive mutant sPLA$_2$-IB-H48Q in the absence or presence of pyrrolidine-1 (1 μM, 30-min preincubation). Luciferase assay (*A*) and Western blot analysis (*B*) were performed as described under "Experimental Procedures." Abbreviations are defined in Fig. 1 legend.

enhancing effect of sPLA$_2$-IB and of its inactive mutant on sPLA$_2$-IIA secretion. LY311727 also blunted the effect of exogenous human sPLA$_2$-IIA and endogenous rat sPLA$_2$-IIA, suggesting that the sPLA$_2$-IIA activity may be important for their effects. Furthermore, we found that Me-indoxam, which also blocks sPLA$_2$-IIA and sPLA$_2$-IB (50), could also block the enhancing effect of porcine sPLA$_2$-IB, human sPLA$_2$-IIA, and endogenous rat sPLA$_2$-IIA (data not shown). Together, these results suggest that the enzymatic activity of sPLA$_2$-IB is not important, whereas that of sPLA$_2$-IIA (either released by the cells or added exogenously) may be required for the enhanced expression of rat sPLA$_2$-IIA. This was confirmed by the observation that a H48N mutant of sPLA$_2$-IIA with only 0.2% the activity of the wild-type did not enhance the TNFα-induced expression of endogenous sPLA$_2$-IIA. A way to reconcile these results would be in fact to consider that LY311727 and Me-indoxam not only inhibit the sPLA$_2$ activity of sPLA$_2$-IB and IIA, but also affect their sPLA$_2$ binding properties to putative protein membrane targets distinct from phospholipids. This hypothesis is supported by the fact that these inhibitors bind tightly to sPLA$_2$s and may protrude out of the active site of the sPLA$_2$ molecule, as shown by the co-crystal structure of sPLA$_2$-IIA with a related inhibitor (61). If sPLA$_2$ residues located at the active site and/or at the interfacial binding surface are implicated in the binding properties to membrane proteins, then the binding of the inhibitor to the sPLA$_2$ would in turn prevent the binding of the sPLA$_2$ to the membrane protein targets. This view is supported by the fact that residues close to the active site and the Ca^{2+} loop are involved in the binding of sPLA$_2$-IB to the M-type receptor (38) and by the fact that Me-indoxam potently inhibits the binding of sPLA$_2$-IB to the 180-kDa M-type receptor (62).

Role of the M-type Receptor in sPLA$_2$-IIA Expression—Based on the use of various mutants of sPLA$_2$-IB, Kishino *et al.* (17) proposed that the M-type receptor, but not the sPLA$_2$ activity, is involved in the effect of sPLA$_2$-IB on prostaglandin E$_2$ release in rat mesangial cells. Here, we found that all the sPLA$_2$s assayed were able to enhance the expression of rat sPLA$_2$-IIA, whether or not they bound to the M-type receptor expressed in our mesangial cells (Table I). First, this suggests that a cellular target distinct from the M-type receptor is used by sPLA$_2$s that do not bind to the M-receptor. Indeed, sPLA$_2$s like rat sPLA$_2$-IIA may use the heparan sulfate proteoglycan pathway (see below). On the other hand, the fact that sPLA$_2$-IB including the rat enzyme (18) binds to the M-type receptor does not imply that this receptor is involved in the induction of rat sPLA$_2$-IIA by sPLA$_2$-IB. However, we currently cannot rule out this possibility. The use of siRNA or antisense oligonucleotides decreasing the level of the M-type receptor in mesangial cells would help to determine the role of this receptor in the sPLA$_2$-IB mediated effects. Another way to evaluate the contribution of the M-type receptor would be to use a specific antagonist that binds to the receptor, and not to the sPLA$_2$, as LY311727 or Me-indoxam do. Unfortunately, such inhibitors are not yet available. Finally, assuming that the M-type receptor is involved in the effect of sPLA$_2$-IB, it will be important to consider the possibility that sPLA$_2$-IB, after its binding and internalization through the receptor, is targeted to nuclear membranes and/or to the nucleus (33, 63), where it may activate cPLA$_2$ (Fig. 10).

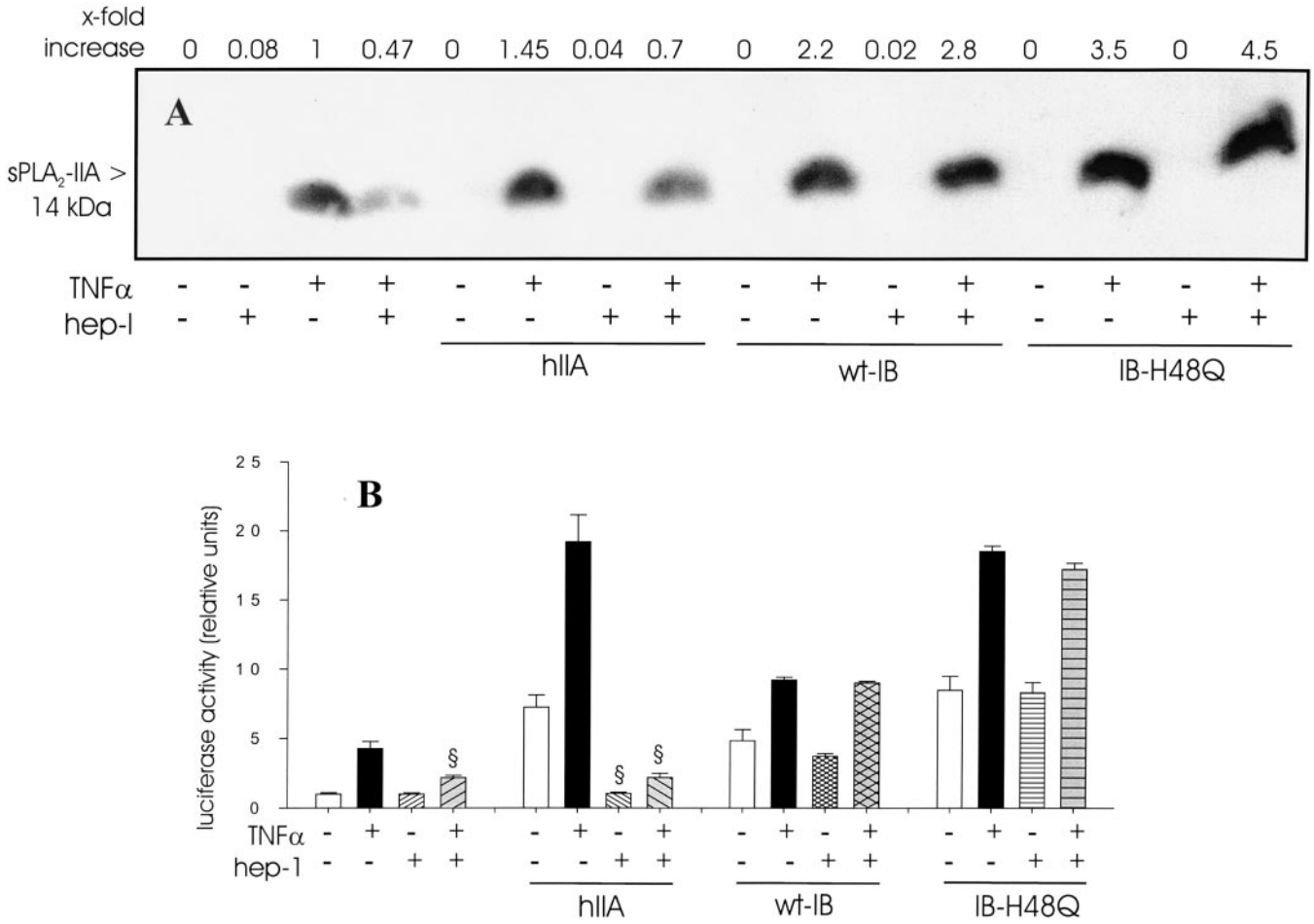

FIG. 9. **Effect of heparinase-1 (*hep-1*) on sPLA$_2$-IIA secretion and promoter activity in rat mesangial cells.** *A*, mesangial cells were preincubated for 30 min with 0.4 unit/ml heparinase I and then treated for 24 h with TNFα (0.5 nM) in the absence or presence of human sPLA$_2$-IIA (*hIIA*), wild-type sPLA$_2$-IB (*wt-IB*), or the inactive mutant sPLA$_2$-IB-H48Q (*H48Q*; each 100 nM). Western blot analysis of secreted sPLA$_2$-IIA protein in the cell culture supernatants was performed with a specific antibody as described under "Experimental Procedures." This experiment was performed three times, and a representative experiment is shown. The *numbers* at the *top* of the blot represent the increase in protein amount compared with TNFα, which is set as 1. *B*, mesangial cells were transfected for 16 h with the wild-type 2.67-kbp construct of the rat sPLA$_2$-IIA promoter. After washing cells were preincubated for 30 min with heparinase-1 (0.4 unit/ml) and then treated for 24 h with TNFα in the absence or presence of the exogenous sPLA$_2$s as described in *A*. Dual luciferase assays were performed as described under "Experimental Procedures." Data are means ± S.D. ($n = 6$). Significant inhibitory effect compared with the control group: *, $p < 0.05$; significant inhibitory effect compared with the corresponding TNFα-treated cells: #, $p < 0.05$.

Role of Heparan Sulfate Proteoglycans in sPLA$_2$-IIA Expression—The results obtained with heparinase-1 treatment suggest that some heparan sulfate proteoglycans including glypican-1 (59) are involved in the effect of exogenously added and secreted sPLA$_2$-IIA. On the other hand, proteoglycans might not be important for the action of sPLA$_2$-IB, because heparinase-1 had no effect on the action of this later enzyme. It is also important to consider that sPLA$_2$s like sPLA$_2$-IIA may bind to other yet unidentified receptors or to cell surface molecules such as decorin, as was shown for the human sPLA$_2$-IIA in atherosclerotic lesions (64). Indeed, rat mesangial cells express small proteoglycans such as decorin and biglycan (57), which are potent modulators of signaling cascades during glomerulonephritis (57), and which might represent alternative cellular targets for the rat sPLA$_2$-IIA. Heparan sulfate-dependent shuttling of sPLA$_2$-IIA into transfected HEK293 cells has been proposed as a mechanism for augmenting its ability to release arachidonic acid in these cells (30). Heparinase-1 treatment leads only to a partial reduction in the ability of sPLA$_2$-IIA to induce its own expression, and we have not established the mechanisms of this partial inhibition. It is possible that heparan sulfate proteoglycan is responsible for shuttling sPLA$_2$-IIA into mesangial cells, but this has not been investigated further with these cells. We cannot rule out the possibility that treatment with heparinase-1 results in the release of truncated heparan sulfate chains from the cell surface into the culture medium and that such chains capture sPLA$_2$-IIA in the medium, thus preventing it from acting on the plasma membrane. sPLA$_2$-IB and sPLA$_2$-IB-H48Q do not bind to heparan sulfate, which probably explains why treatment of mesangial cells with heparinase-1 has no effect on the induction of sPLA$_2$-IIA by these other sPLA$_2$s.

Role of cPLA$_2$ in sPLA$_2$-IIA Expression—It was shown previously (13, 14) that exogenously added sPLA$_2$-IIA activates the mitogen-activated protein kinase cascade and also leads to phosphorylation and activation of cPLA$_2$ in rat mesangial cells. Activation of cPLA$_2$ by various sPLA$_2$s has also been reported in many other cells (65–67). cPLA$_2$ is a major producer of arachidonic acid, which can then be converted to eicosanoids, which are potent PPAR ligands (33). We found here that pyrrolidine-1, a specific inhibitor of cPLA$_2$α (37, 53, 54), markedly reduced the expression of sPLA$_2$-IIA triggered by human sPLA$_2$-IIA or sPLA$_2$-IB and its catalytically inactive H48Q mutant. The same results were obtained with another recently described cPLA$_2$ inhibitor (68) from Astra Zeneca called AZ-1 (data not shown). These results indicate that both sPLA$_2$-IB

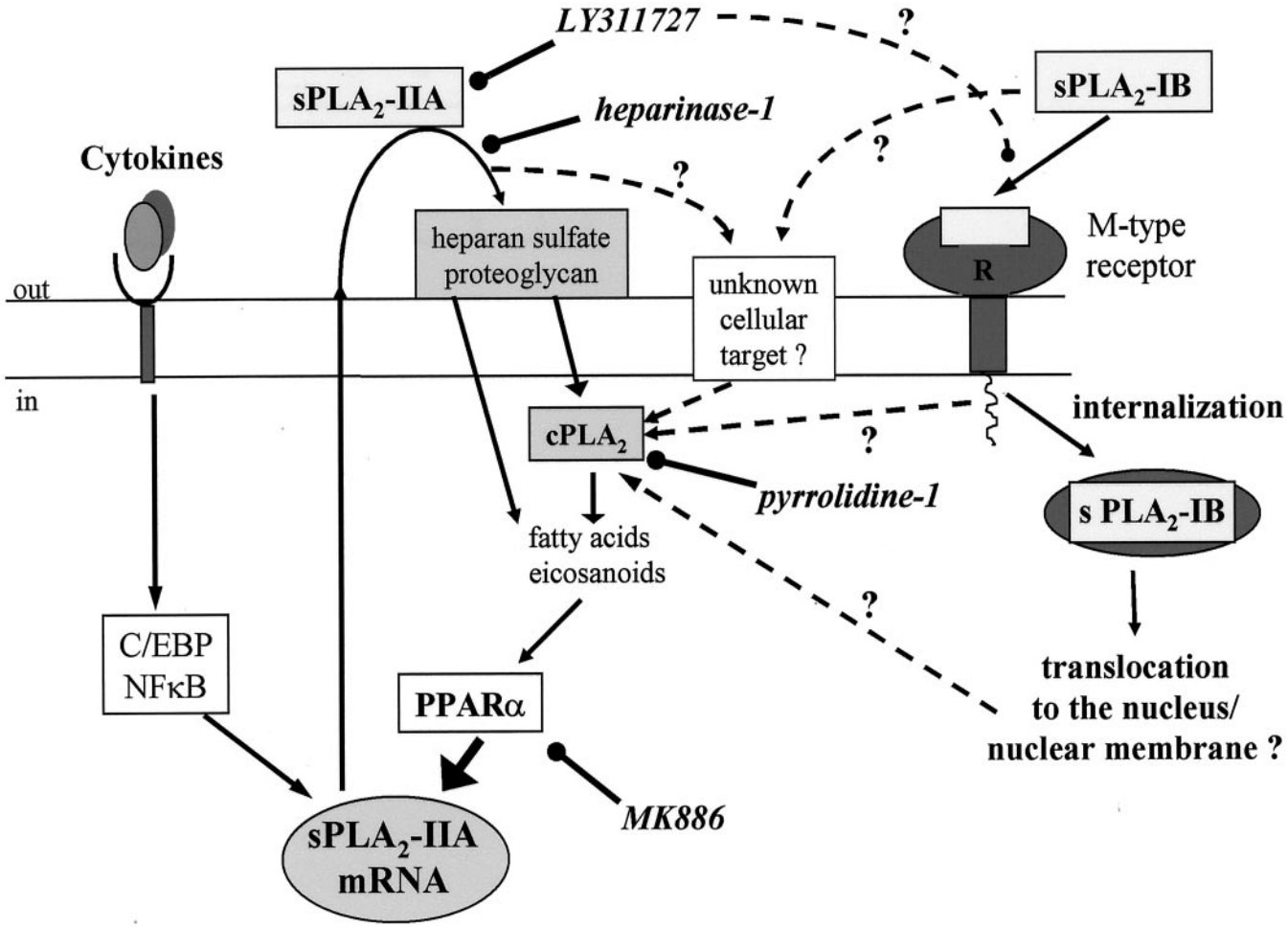

FIG. 10. **Mechanisms by which exogenous sPLA$_2$s and endogenous sPLA$_2$-IIA enhance the cytokine-stimulated sPLA$_2$-IIA expression by PPARα activation.** The *solid lines* indicate well known pathways or mechanisms; the *dotted lines* indicate putative stimulatory mechanisms (*arrows*) or interfering actions (*bullets*), which may represent further pathways in the regulation of sPLA$_2$-IIA expression via PPARα.

(independently of its enzyme activity) and the active form of sPLA$_2$-IIA activate cPLA$_2$α, which in turn produces fatty acids or eicosanoids as ligands for the PPARα receptor (Fig. 10).

Interestingly, the expression of sPLA$_2$-IIA induced by TNFα was only weakly reduced by pyrrolidine-1, although proinflammatory cytokines are known to produce a delayed activation of cPLA$_2$ in rat mesangial cells (51, 52). This suggests that the delayed activation of cPLA$_2$ is not a major route in the TNFα signaling pathway leading to sPLA$_2$-IIA expression. This view also fits with the fact that the sPLA$_2$-IIA promoter activity stimulated by TNFα is only partially reduced in cells transfected with the PPRE-1 mutant constructs. Together, these results support the view that TNFα activates transcription factors such as NF-κB, which act synergistically with PPARα to fully induce the sPLA$_2$-IIA gene (Fig. 10).

Role of Endogenous sPLA$_2$-IIA in sPLA$_2$-IIA Expression—An important finding of this study is that sPLA$_2$-IIA released by rat mesangial cells after cytokine treatment induces its own expression via PPARα activation, *i.e.* via a transcription pathway distinct from that used by cytokines. The activity of the sPLA$_2$-IIA promoter stimulated by TNFα was only partially reduced when transfection experiments were performed with the PPRE-1 mutant constructs or when the PPARα antagonist MK886 was used with the wild-type promoter, suggesting that TNFα led to the subsequent activation of two transcriptional pathways (Fig. 10). It is therefore tempting to propose that TNFα is required to initiate the transcription of sPLA$_2$-IIA via NF-κB activation, but as soon as sPLA$_2$-IIA is released from the cells, this later activates TNFα-independent signaling pathways, which lead to cPLA$_2$ activation, to PPARα activation, and finally to a dramatic increase of its own mRNA expression.

In summary, we have shown here that various exogenous sPLA$_2$s as well as endogenous rat sPLA$_2$-IIA can dramatically enhance the secretion of sPLA$_2$-IIA first activated by TNFα in rat mesangial cells. When used at 100 nM, we estimated that the contribution of exogenous sPLA$_2$s in the up-regulation of sPLA$_2$-IIA accounts for ~50%, that of TNFα corresponding to the remaining half. The role of exogenous sPLA$_2$s in the expression of sPLA$_2$-IIA therefore appears quite important, at least in this model of rat mesangial cells. Of particular interest was the effect of exogenous sPLA$_2$-IB, which is present at increased levels in inflammatory kidney diseases such as acute pancreatitis (7) and which might be a major cause of systemic complications in this disease (58). An important remaining question that remains to be answered concerns the identification of the early cellular events that are used by the different sPLA$_2$s and that link their initial binding to the plasma membrane to the activation of cPLA$_2$ and PPARα. It will also be interesting to see whether the promoters of the other sPLA$_2$s also contain PPRE-1 sites.

It has been so far proposed that PPARα activation leads to anti-inflammatory effects (60). However, our studies now provide evidence for a pro-inflammatory role of this transcription factor. Besides this study, we recently found that PPARα activators can also up-regulate the mRNA level of the inducible

nitric-oxide synthase, which is another pro-inflammatory enzyme expressed in rat mesangial cells.[2] This effect was mediated by a PPAR-binding site present in the inducible nitric-oxide synthase promoter. PPARα may thus represent a specific transcriptional pathway by which various extracellular sPLA$_2$s, acting as acute phase proteins during the early phases of inflammation, may modulate the expression of several pro-inflammatory genes containing a functional PPAR binding site in their promoters. Development and use of specific PPARα antagonists may thus provide novel approaches in the treatment of inflammatory diseases.

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