Potentiation of TNFα-induced sPLA2-IIA expression in mesangial cells by an autocrine loop involving secreted phospholipase A2 and PPARα activation

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ABSTRACT

In rat mesangial cells, exogenously added secreted phospholipases A₂ (sPLA₂s) potentiate the expression of pro-inflammatory sPLA₂-IIA first induced by cytokines like tumor necrosis factor-α (TNFα) and interleukin-1β. The transcriptional pathway mediating this effect is however unknown. Since products of PLA₂ activity are endogenous activators of peroxisome proliferator-activated receptor α (PPARα), we postulated that sPLA₂s mediate their effects on sPLA₂-IIA expression via sPLA₂ activity and subsequent PPARα activation. This study shows that various sPLA₂s, including venom enzymes, human sPLA₂-IIA, wild-type and catalytically inactive H48Q mutant of porcine pancreatic sPLA₂-IB, enhance the TNFα-induced sPLA₂-IIA expression at the mRNA and protein levels. In cells transfected with luciferase sPLA₂-IIA promoter constructs, sPLA₂s are active only when the promoter contains a functional PPRE-1 site. The effect of exogenous sPLA₂s is also blocked by the PPARα inhibitor MK886. Interestingly, the expression of sPLA₂-IIA induced by TNFα alone is also attenuated by MK886, by the sPLA₂-IIA inhibitor LY311727, by heparinase, which prevents the binding of sPLA₂-IIA to heparan sulfate proteoglycans, and by the specific cPLA₂-α inhibitor pyrrolidine-1. Together, these data indicate that sPLA₂-IIA released from mesangial cells by TNFα stimulates its own expression via an autocrine loop involving cPLA₂ and PPARα. This signaling pathway is also used by exogenously added sPLA₂s including pancreatic sPLA₂-IB and is distinct from that used by TNFα.
INTRODUCTION

During the last decade, increasing evidence has been obtained that secreted phospholipases A\textsubscript{2} (sPLA\textsubscript{2}) are important players in inflammatory diseases. Among the various sPLA\textsubscript{2}s which have now been identified (1-3), group IIA sPLA\textsubscript{2} (sPLA\textsubscript{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\textsubscript{2-}IIA is also highly expressed in the kidney during experimental and human acute pancreatitis (7), which can lead to postinjury multiple organ failure (8). In rat renal mesangial cells which is used as a cell model system to study inflammatory processes, it was shown earlier that cAMP-elevating agents and proinflammatory cytokines such as interleukin-1\textbeta and TNF\textalpha stimulate the gene expression and secretion of sPLA\textsubscript{2}-IIA by different transcriptional activation pathways (9-11). The functional role of the sPLA\textsubscript{2}-IIA released from mesangial cells is however less clear. In rat mesangial cells, it has been shown that exogenously added sPLA\textsubscript{2}-IIA acts as a growth factor mediating the action of IL-1\textbeta on cell proliferation, and that this effect is mimicked by lysophospholipids (12). Exogenous sPLA\textsubscript{2}-IIA and lysophospholipids were also found to rapidly stimulate the mitogen activated protein kinase (MAPK) cascade in mesangial cells, leading to early activation of cPLA\textsubscript{2} (13,14). It is however unclear if sPLA\textsubscript{2}-IIA released by mesangial cells after cytokine induction acts like exogenously added sPLA\textsubscript{2}-IIA, and in particular, if sPLA\textsubscript{2}-IIA can exert a positive feed-back 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 sPLA₂ subtype, the so-called pancreatic-type sPLA₂-IB are also found in kidney during acute pancreatitis (7,15), suggesting that sPLA₂-IB may also contribute to the pathophysiological effects in such conditions. In rat mesangial cells, exogenously added pancreatic sPLA₂-IB can stimulate the mRNA and protein expression of sPLA₂-IIA, as well as prostaglandin biosynthesis (16, 17). This effect is thought to involve binding of sPLA₂-IB to the M-type sPLA₂ receptor expressed in mesangial cells (17). This view is strengthened by the fact that a catalytically inactive mutant of sPLA₂-IB, which still binds to the M-type receptor, has effects similar to those of the wild-type enzyme (16). Interestingly, sPLA₂-IB from different species including rat, but not rat sPLA₂-IIA, binds to the M-type receptor expressed in rat mesangial cells (17-19), suggesting that sPLA₂-IIA acts through binding to a different cell membrane target. When exogenously added to other cell types, sPLA₂-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 sPLA₂-IIA can induce the activation of cPLA₂ and cyclooxygenase-2 (3, 23, 24), the release of elastase (25) and β-glucuronidase (26), the expression of Mac-1 (27), IL-6 (26), CD-69 (28), iNOS (29) and Fas ligand (23) on different cell types. The nature of the sPLA₂-IIA cellular target involved in these biological effects remains however to be clearly identified. Although human sPLA₂-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, while the sPLA₂ activity may not play a major role. Heparan sulfate proteoglycans (HSPG) including glypican-1 may also contribute to the effects of sPLA₂-IIA (30, 31).
Besides activation of p38, p42/44 and JNK kinases by sPLA₂-IB or IIA (13, 23, 32), little is known about the transcriptional pathways activated by exogenously added sPLA₂s. It has been shown that sPLA₂-IB enhances the expression of COX-2 through activation of the transcription factor CCAT/enhancer-binding protein β (C/EBP-β) in NIH3T3 and MC3T3E1 cells (21). Because sPLA₂s 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 sPLA₂s induce the expression of genes containing PPAR responsive promoters by activating PPAR nuclear receptors. Interestingly, a recent work has shown that sPLA₂-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 sPLA₂-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 sPLA₂-IIA by exogenously added sPLA₂s including sPLA₂-IB, and also by rat sPLA₂-IIA endogenously produced after cytokine treatment.

The purpose of this study was to analyze the role of PPARα in the induction of sPLA₂-IIA transcription by cytokines and by exogenous sPLA₂s in rat mesangial cells. To determine the role of PPARα and the sPLA₂ signaling pathways, we used rat sPLA₂-IIA promoter constructs containing functional or mutated PPARα binding sites, as well as PPARα and various PLA₂ inhibitors. All together, our data indicate that sPLA₂-IIA released by mesangial cells after treatment with cytokines potentiates its own expression in a positive feedback loop via activation
of cPLA$_2$ and PPAR$\alpha$. 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). 1-14C]oleic acid, γ-[^32P]ATP (185 TBq/mmol) and α-[^32P]dCTP (110 TBq/mmol) were from Amersham-Pharmacia (Freiburg, Germany). Kidneys of rats with Thy-1 glomerulonephritis were a generous gift from Dr. T. Ostendorf (RWTH Aachen, Germany). Immobilon-PVDF-membranes were purchased from Millipore (Eschborn, Germany) and Nylon membranes (Gene Screen) were purchased from NEN Life Science (Köln, Germany). 18S RNA probe from mouse as well as specific antibodies against PPARα and 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, USA). MK886 was from Biomol (Hamburg, Germany), heparinase-1 was from Sigma (Deisenhofen, Germany), and all other chemicals used were from either Sigma, Biomol or Calbiochem (Bad Soden, Germany). All cell culture media and nutrients were from Invitrogen (Eggenstein, Germany). sPLA2s 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, USA). Human sPLA2-IIA was a generous gift of Prof. Tibes, Roche Diagnostics (Penzberg, Germany). sPLA2s 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 sPLA2-IB, and the H48Q and H48N mutants of human sPLA2-IIA were 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 essential medium (DMEM) 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 sPLA2-IIA or 18S RNA. For quantification the signals of the filters were scanned and evaluated densitometrically using a phosphoimager BAS 1500 from Fuji (Raytest, Straubenhardt, Germany). The signal obtained with the sPLA2-IIA probe was normalized to that obtained with the 18S RNA probe.

**Western Blot Analysis** - sPLA2-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 non reducing conditions. The proteins were transferred to Immobilon-PVDF membranes for 30 min at 0.7 mA/cm². Non specific binding was blocked with 2% BSA in PBS/0.05% Tween-20 for 1 hr at room temperature followed by incubation with a mouse monoclonal antibody against rat sPLA2-IIA (generous gift from Prof. Henk van den Bosch, Utrecht) at a 1:100 dilution in 0.01% milk powder in PBS. This rat sPLA2-IIA antibody cross-reacts neither the human recombinant
sPLA2-IIA nor the other sPLA2s used in this study. Indeed, Fig. 1B shows that the antibody detected the exogenously added recombinant rat enzyme (100 nM) as a thick band, but does not recognize the human sPLA2-IIA and the other sPLA2s. This clearly demonstrates that this antibody can be specifically used to detect the sPLA2-stimulated release of rat sPLA2-IIA from mesangial cells.

Blots were incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Amersham Pharmacia Biotech, Freiburg, Germany) at a 1:15000 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 Pharmacia Biotech).

*sPLA2 assay* - sPLA2 activity in supernatants of mesangial cells was determined with [1-14C]-oleate-labeled *E. coli* membranes as substrate (42). Briefly, assay mixtures (1 ml) contained 100 mM Tris/HCl (pH 7.0), 10 mM CaCl2, [1-14C]-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 the Dole’s method exactly as described (42). Free [1-14C]-oleate was measured in a β-counter.

*Construction of reporter gene fusions* - A BamHI/KpnI fragment (2.67 kbp) of the rat sPLA2-IIA promoter (accession number 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, Mannheim, Germany) as described earlier (35).
Site-directed mutagenesis - Mutations within the putative PPAR binding site –909 to –888 (5’-AGGTTGGTCTCTGAACCCACA-3’) in the rat sPLA₂-IIA promoter fragment were introduced by PCR-based site-directed mutagenesis according to the manufacturer’s instructions (Stratagene) as described earlier (35); the changes in 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 hr at 37°C in RPMI containing 10% FCS. The cells were then incubated in DMEM containing 0.1 mg/ml BSA and transfected with 400 ng of plasmid DNA and 40 ng 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 hr. The cells were then washed with ice-cold PBS, lysed in 250 μl of lysis buffer from the dual-luciferase reporter assay system (Promega, Mannheim, Germany), scraped with a rubber policeman, and transferred into 1.5 ml vials. The cell lysates were subjected to 2 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 sPLA₂-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 to control.

Electrophoretic Mobility Shift Assay - The sequences of the double strand oligonucleotides used to detect the DNA binding activities of PPAR were chosen as described earlier (35). The
complementary DNA strands were labeled with T4 Polynucleotide Kinase using $\gamma^{32}$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 acetylated bovine serum albumin, 2 µg poly[d(I-C)], 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 50,000 d.p.m. 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 PPAR$\alpha$ (4 µg/onset) and RXR$\alpha$ (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 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 Gibco BRL and Oligo (dT) 15-Primer (Promega). PCR was performed on a Perkin Elmer Thermal Cycler with specific primers as follows:

- arachidonate five-lipoxygenase (Alox5)–sense: 5’- CTGGTAGCCCATGTGAGGTT-3’;
  antisense: 5’- GCACAGGGAGGAATAGGTCA-3’; product 162 bp;
- rat FLAP –sense: 5’- CGTAGATGCGTACCCCACTT-3’;
  antisense: 5’- CGCTTCCGAAGAAGAAGATG-3’; product 245 bp;
18S RNA-sense: 5’- GCGGTAATTCCAGCTCCAATAG – 3’;
antisense: 5’- CCCTCTTAATCATGGCCTCAGT – 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:

5-LOX: 95°C for 4 min (1 cycle) followed immediately by 95°C for 50 sec, 55°C for 30 sec and
72°C for 20 sec (36 cycles) and a final extension phase at 72°C for 10 min.
FLAP: 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 sec (36 cycles) and a final extension phase at 72°C for 10 min.
18S RNA: 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, Hilden, Germany) for sequencing using a kit based on the dye terminator technology
(Perkin Elmer Applied Biosystems, Weiterstadt, Germany) in combination with the automated
sequence analyzer A310 (Perkin Elmer Applied Biosystems).

Statistical analysis - Data are represented as means ± SD (n = 3; in transfection experiments:
n = 6) showing one representative experiment out of 3 with similar results. Statistical analysis
was performed by Student’s t-test. A probability < 0.05 was considered as significant.
RESULTS

Effect of exogenous sPLA2s on sPLA2-IIA expression in rat mesangial cells

The scope of this study was to identify the transcriptional regulatory mechanisms of sPLA2-IIA expression in rat mesangial cells triggered by exogenous sPLA2s. For this purpose, we treated rat mesangial cells with various sPLA2s 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 sPLA2-IB containing a single amino acid mutation at position 48 (H48Q) was also studied. This mutant has less than 0.02% of wild-type PLA2 activity (39). Rat mesangial cells were treated for 24 h with sPLA2s, at a concentration of 100 nM which was shown earlier to stimulate sPLA2-IIA mRNA expression and prostaglandin synthesis in these cells (17). The expression of rat sPLA2-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 sPLA2-IIA in mesangial cells (Fig. 1A). This enhancement was accompanied by an increased secretion of the sPLA2-IIA protein into the cell culture medium (Fig. 1B). Interestingly, the H48Q catalytic mutant of sPLA2-IB also potentiated the expression of rat sPLA2-IIA. The relative increase in sPLA2-IIA mRNA and protein after costimulation with TNFα and exogenous sPLA2s varied between 1.5- and 3.5-fold over TNFα alone in at least 5 independent experiments, and the values obtained by densitometrical analysis showed a significant increase (p < 0.05) compared to 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
sPLA2-IIA expression by RT-PCR (not shown), northern blot and western blot analysis when cells were treated with sPLA2s alone, i.e. in the absence of TNFα or IL-1β.

Rat mesangial cells were previously shown to express relatively large amounts of the M-type 180 kDa receptor (16). 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 sPLA2s (Table I) for the M-type receptor expressed in mesangial cells were measured by competition binding assays with iodinated OS1 as previously described (18). We found no obvious correlation between their affinities for the M-type receptor and their ability to enhance the TNFα-stimulated sPLA2-IIA induction, since sPLA2s that bind or do not bind to the receptor similarly activate sPLA2-IIA expression. The catalytically inactive H48Q mutant of sPLA2-IB, which also binds to the M-type receptor (Table I), could enhance the expression of sPLA2-IIA induced by TNFα (Fig. 1), suggesting that the intrinsic sPLA2 activity of sPLA2-IB does not play a major role in this effect.

Effect of exogenous sPLA2s on the rat sPLA2-IIA promoter activity

The observation that the TNFα-stimulated sPLA2-IIA mRNA induction is markedly enhanced by exogenous sPLA2s suggests that TNFα and sPLA2s 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 sPLA2-IIA in rat mesangial cells via the PPRE-1 site (−909 to −888) in the rat sPLA2-IIA promoter (35). Since fatty acids and derivatives are potent activators of PPARs, an attractive hypothesis is that sPLA2s activate PPARs by producing fatty acids and other derivatives, which will in turn lead to enhanced transcription of sPLA2-IIA in the presence of TNFα.

To address this hypothesis, we first investigated the effects of the different exogenous sPLA2s in the absence or presence of TNFα on the activity of a 2.67 kbp construct of the rat sPLA2-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 sPLA2s 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 sPLA2s. Similar results were obtained with IL-1β (not shown).

We next investigated if the PPAR responsive element 1 (PPRE-1) site found in the sPLA2-IIA promoter is essential to the sPLA2 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 sPLA2-IIA and the wild-type or H48Q mutant of porcine sPLA2-IB. In cells transfected with the wild-type sPLA2-IIA promoter construct, TNFα and sPLA2s alone stimulated luciferase activity, while the addition of both effectors led to a potentiation of luciferase activity (Fig. 3A). In contrast, when cells were transfected with the promoter lacking the PPRE-1 site (Fig. 3B) or containing a mutated PPRE-1 site (Fig. 3C), exogenous sPLA2s
were unable to activate the promoter or to enhance the TNFα-stimulated effect. In addition, we observed that the TNFα stimulation was reduced by about 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 sPLA2s, but also in the TNFα-stimulated activation of sPLA2-IIA transcription. This is partially due to the autocrine loop involving the endogenous sPLA2-IIA initially secreted by TNFα (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 sPLA2-IIA promoter activity was found to be markedly enhanced by the different fatty acids (Fig. 4A), which was reflected by a more pronounced sPLA2-IIA protein secretion (Fig. 4C). In the absence of a functional PPRE-1 site, the potentiating effect of the fatty acids was completely abolished (Fig. 4B). These results suggest that sPLA2s may potentiate the cytokine-stimulated sPLA2-IIA expression by producing fatty acids capable of activating PPARα in mesangial cells.

Electrophoretic mobility shift analysis of PPAR binding by exogenous sPLA2s

To further support a role of PPAR activation in the transcriptional regulation of sPLA2-IIA induction by TNFα as well as by exogenous sPLA2s, we performed electrophoretic mobility shift analyses using a radioactively labeled oligonucleotide comprising the PPRE-1 site from -909 to –888 of the rat sPLA2-IIA promoter (35). Nuclear extracts were prepared from mesangial cells treated for 8 h with human sPLA2-IIA, porcine sPLA2-IB as well as the mutant sPLA2-
IB-H48Q in the absence or presence of TNFα. As described earlier (18), TNFα alone stimulated the formation of specific complexes (Fig. 5A). This effect was also observed after treatment with exogenous sPLA2s, and an enhanced complex formation was obtained after co-incubation with TNFα and sPLA2s. 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 sPLA2-treated cells, implicating PPAR in sPLA2-IIA expression mediated by TNFα alone or in combination with sPLA2s (Fig. 5A). Competition with a non relevant 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. 5B, both TNFα and exogenous sPLA2s 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. 5B). This effect was also observed with the combination of TNFα and sPLA2s. The same complex also disappeared when nuclear extracts were incubated with a specific antibody against the 9-cis retinoic acid receptor-α (RXRα; Fig. 5C), which binds specifically to PPARα and acts as a coactivator (45, 46). Together, these data show that in rat mesangial cells TNFα and exogenous sPLA2s induce the formation of a nuclear complex that specifically comprises PPARα and RXRα.

*Effect of the PPARα antagonist MK886 on sPLA2-IIA gene expression*
To further demonstrate the role of PPARα in sPLA2-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. 6A, 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 hours after induction of a Thy-1 glomerulonephritis. It was shown earlier that there is an invasion of leukocytes into the glomerulus and production of lipoxygenase products such as LTB₄ during this animal model disease (43). 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 addition, we measured the release of LTB₄ in cell culture supernatants by a specific enzyme-immuno-assay, and did not detect product formation under the above conditions (unpublished observation). Thus, we can exclude that the observed effects of MK886 on sPLA2-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 hours. We have shown earlier that at this concentration, MK886 exerts in rat mesangial cells a maximal reducing effect on PPARα-dependent expression of sPLA2-IIA without cytotoxic effects (35). MK886 partially attenuated the TNFα-stimulated sPLA2-IIA secretion and activity and completely abolished the potentiating effects of sPLA2s (Fig. 6B, C). In transfection experiments with the wild-type sPLA2-IIA promoter construct, MK886 also partially reduced the sPLA2-IIA promoter activity stimulated by TNFα and dramatically decreased the effect of exogenous sPLA2s (Fig. 6D). Together with the EMSA analysis, these results indicate that PPARα is
involved in the transcriptional control of sPLA₂-IIA expression mediated by TNFα and sPLA₂s.

Effect of the sPLA₂-IIA inhibitor LY311727 and of the cPLA₂ inhibitor pyrrolidine-1

Since MK886 strongly reduced the TNFα-mediated sPLA₂-IIA induction, we postulated that sPLA₂-IIA released by mesangial cells after TNFα treatment enhanced its own expression in a positive feed-back loop, possibly through fatty acid release and PPAR activation. Consistent with this hypothesis, we considered the possibility that sPLA₂ produces fatty acids via its intrinsic sPLA₂ activity or after cPLA₂ 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 sPLA₂s, but not that of cPLA₂ (49, 50). In rat mesangial cells, the rat sPLA₂-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 sPLA₂-IIA elicited by TNFα and human sPLA₂-IIA (Fig. 7A). Interestingly, the enhanced protein secretion induced by sPLA₂-IB or the catalytically inactive sPLA₂-IB-H48Q mutant was also markedly reduced by LY311727.

LY311727 was also able to reduce the sPLA₂-IIA promoter activity of cells transfected with the wild-type 2.67 kbp construct and stimulated with TNFα and exogenous sPLA₂s (Fig. 7B).

These data suggest that sPLA₂-IIA, which is released by TNFα- and sPLA₂-treated rat mesangial cells, promotes its own expression.

In contrast, the activity of sPLA₂-IIA seems to be crucial. To support this, we compared the
effects of the active wild type sPLA2-IIA with a H48N mutant, which shows only 0.22 % of the activity of the wild type enzyme (40). Western blot analysis in Fig. 7C shows that compared to the wild-type enzyme, the H48N mutant had no potentiating effect.

Together, these data show that in contrast to sPLA2-IB, only the catalytically active forms of sPLA2-IIA, either released by mesangial cells, or added exogenously, mediate sPLA2-IIA expression.

Because it was shown earlier that treatment of rat mesangial cells with extracellular sPLA2s leads to an early activation of cPLA2 (14), we analyzed the role of cPLA2 in sPLA2-IIA expression. In this regard, cytokines differ significantly and cause a delayed increase in cPLA2 by upregulating its mRNA expression (51, 52). To elucidate whether cPLA2 activation is involved in sPLA2-IIA expression, we analyzed the effect of pyrrolidine-1, a recently characterized specific inhibitor of cPLA2α (53, 54). At concentrations up to 1 µM, this inhibitor does not inhibit the calcium-independent PLA2 or various sPLA2s. Cells were pre-incubated with pyrrolidine-1 for 30 min prior to treatment with TNFα and different sPLA2s. Pyrrolidine-1 only weakly reduced the TNFα-stimulated sPLA2-IIA protein secretion (Fig. 8A). However, when cells were co-incubated with TNFα and sPLA2s, pyrrolidine-1 markedly reduced sPLA2-IIA protein secretion, suggesting that an early as well as a delayed activation of cPLA2 was inhibited. These data indicate that activation of cPLA2 plays a central role in the induction of rat sPLA2-IIA mediated by exogenous sPLA2s.
Pyrrolidine-1 also markedly reduced the sPLA2-IIA protein level measured after co-incubation with TNFα and sPLA2-IB or the H48Q catalytic mutant of sPLA2-IB (Fig. 8B), suggesting that the IB mutant can also activate cPLA2, which finally results in activation of PPARα. These data also explain how this mutant, although devoid of significant sPLA2 activity, could enhance the expression of sPLA2-IIA. Finally, these data indicate that sPLA2-IB can be a potent activator of cPLA2, independently of its catalytic activity.

We also found that pyrrolidine-1 inhibited the cytokine as well as the sPLA2-stimulated sPLA2-IIA promoter activity, indicating that in rat mesangial cells, cPLA2 is an important player in the transcriptional regulation of sPLA2-IIA induced by PPARα (Fig. 8C).

Involvement of heparan sulfate proteoglycans in TNFα- and sPLA2-mediated sPLA2-IIA induction

To further confirm the positive feed back regulation of released sPLA2-IIA on its own expression, we incubated mesangial cells with heparinase-1 to prevent binding of released sPLA2-IIA to heparan sulfate proteoglycans (HSPG; 30). Heparinase I treatment attenuated the stimulatory effects of TNFα as well as those of exogenous human sPLA2-IIA on sPLA2-IIA protein secretion (Fig. 9A) and promoter activity. This suggests that the action of sPLA2-IIA released by TNFα on its own expression is at least in part mediated through HSPG binding. Interestingly, the effects of the wild-type sPLA2-IB and of the inactive mutant sPLA2-IB-H48Q on sPLA2-IIA protein secretion (Fig. 9A) and promoter activity (Fig. 9B) 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\textsubscript{2}s, when added exogenously to rat mesangial cells, can dramatically upregulate the expression of sPLA\textsubscript{2}-IIA induced by the potent pro-inflammatory cytokines such as TNF\textgreek{a}.

Second, this paper shows that the endogenous rat sPLA\textsubscript{2}-IIA initially induced by TNF\textgreek{a} can enhance its own expression through an autocrine loop. Third, this paper shows that both exogenous and TNF\textgreek{a}-induced, \textit{i.e.} endogenous rat sPLA\textsubscript{2}-IIA, increase the expression of this later sPLA\textsubscript{2} by activating a signaling pathway which is distinct from that of TNF\textgreek{a} and which involves cPLA\textsubscript{2} and intracellular lipid mediator formation to activate the nuclear receptor PPAR\textgreek{a}. Fourth, our results suggest that the various sPLA\textsubscript{2}s used in this study associate to different plasma membrane targets and/or use different mechanisms that eventually lead to cPLA\textsubscript{2} and PPAR\textgreek{a} activation.

The possible mechanisms by which cytokines like TNF\textgreek{a} and exogenously added sPLA\textsubscript{2}s or endogenously produced rat group IIA sPLA\textsubscript{2} trigger the expression of rat sPLA\textsubscript{2}-IIA are depicted in Fig. 10. In this model, TNF\textgreek{a} can be considered as a primary potent cytokine able to initiate by itself the expression of rat sPLA2-IIA through NFkB signaling. The released rat sPLA\textsubscript{2}-IIA eventually activates PPAR\textgreek{a} and its own secretion through an autocrine loop. On the other hand, exogenous sPLA\textsubscript{2}s like IB (or sPLA\textsubscript{2}-IIA secreted by neighboring rat mesangial cells) may be considered as secondary cytokines which require the presence of TNF\textgreek{a} to further activate the autocrine loop, and their effect occurs via cPLA\textsubscript{2} activation. In the future, it will be
interesting to know if other sPLA\textsubscript{2}s such as group IIF, III, V and XII sPLA\textsubscript{2}s, which are known to be expressed in the kidney or mesangial cells (2, 3, 54, 56), can also enhance TNF\textalpha-induced rat sPLA\textsubscript{2}-IIA expression.

According to Fig. 10 both exogenous and endogenous sPLA\textsubscript{2}s are acting extracellularly, and therefore, they should first bind to the cell surface before activating the PPAR\textalpha transcriptional pathway. Since rat mesangial cells are known to express the M-type receptor (17) and various HSPG including glypican-1 (57), which are binding proteins for different sPLA\textsubscript{2}s (2, 57, 58), sPLA\textsubscript{2}s may increase sPLA\textsubscript{2}-IIA expression through binding to one of these proteins and/or through their catalytic activity on cellular phospholipids.

*Role of PPAR\textalpha in sPLA\textsubscript{2}-IIA expression* By investigating the mechanisms involved in the potentiation of sPLA\textsubscript{2}-IIA gene expression by exogenous sPLA\textsubscript{2}s, we found a significant increase in the sPLA\textsubscript{2}-IIA promoter activity. Because this promoter contains a PPRE-1 element and because PPARs may be activated by PLA\textsubscript{2} lipid products, we postulated that PPARs might be involved in the upregulation of sPLA\textsubscript{2}-IIA expression by sPLA\textsubscript{2}s. In contrast to a previous study (17), we were unable to detect an increased expression of sPLA\textsubscript{2}-IIA at the mRNA or protein level when rat mesangial cells were treated with exogenous sPLA\textsubscript{2}s alone. The reason for this discrepancy is unknown but might be due to differences in the inflammatory status or priming between the different rat mesangial cell cultures. From our results we conclude that PPAR\textalpha alone might not be sufficient for an efficient sPLA\textsubscript{2}-IIA mRNA expression. In our hand, the effect of exogenous sPLA\textsubscript{2}s could only be observed in the presence of a potent
cytokine like TNFα or IL1-β. We previously found that PPARα activators such as WY14643, LY171883 or clofibrate have strong stimulatory effects on the rat sPLA2-IIA promoter activity in mesangial cells, but do not elicit on their own an equivalent increase in sPLA2-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 sPLA2-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 sPLA2-IIA mRNA, which would otherwise be rapidly degraded. Finally, we cannot exclude that the endogenous sPLA2-IIA promoter contains some inhibitory elements which 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 sPLA2-IIA induction, we found that only PPARα activators like unsaturated long-chain fatty acids had a potentiating effect (35 and Fig. 4), whereas the putative PPARγ activator 15-deoxy-Δ12,14 prostaglandin J2 inhibited sPLA2-IIA promoter activity and protein expression (not shown). Using the PPARα antagonist MK886, various sPLA2-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 sPLA2s clearly involves PPARα activation. Interestingly, more DNA-protein complexes disappeared when the competition is done with the PPRE-1 oligonucleotide compared to 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 45, 46, 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 sPLA2 activity in sPLA2-IIA expression Based on the results obtained with the inactive H48Q mutant of sPLA2-IB, we concluded that the intrinsic enzymatic activity of this sPLA2 is not crucial for the effect on rat sPLA2-IIA expression. Interestingly, the sPLA2 inhibitor LY311727 also reduced the enhancing effect of sPLA2-IB and of its inactive mutant on sPLA2-IIA secretion. LY311727 also blunted the effect of exogenous human sPLA2-IIA and endogenous rat sPLA2-IIA, suggesting that the sPLA2-IIA activity may be important for their effects. Furthermore, we found that Me-indoxam, which also blocks sPLA2-IIA and sPLA2-IB (50), could also block the enhancing effect of porcine sPLA2-IB, of human sPLA2-IIA and endogenous rat sPLA2-IIA (not shown). Together, these results suggest that the enzymatic activity of sPLA2-IB is not important, whereas that of sPLA2-IIA – either released by the cells or added exogenously - may be required for the enhanced expression of rat sPLA2-IIA. This was confirmed by the observation that a H48N mutant of sPLA2-IIA with only 0.2 % activity of the wild-type did not enhance the TNFα-induced expression of endogenous sPLA2-IIA. A way to reconcile these results would be in fact to consider that LY311727 or Me-indoxam not only inhibit the sPLA2 activity of sPLA2-IB and IIA, but also affect their sPLA2 binding properties to putative protein membrane targets distinct from phospholipids. This hypothesis is supported by the fact that these inhibitors bind tightly to sPLA2s and may protrude out of the active site of
the sPLA₂ molecule, as shown by the co-crystal structure of sPLA₂-IIA with a related inhibitor (61). If sPLA₂ 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 sPLA₂ would in turn prevent the binding of the sPLA₂ to the membrane protein targets. This view is supported by the fact that residues close to the active site and the Ca²⁺-loop are involved in the binding of sPLA₂-IB to the M-type receptor (38) and by the fact that Me-indoxam potently inhibits the binding of sPLA₂-IB to the 180 kDa M-type receptor (62).

Role of the M-type receptor in sPLA₂-IIA expression — Based on the use of various mutants of sPLA₂-IB, Kishino et al. proposed that the M-type receptor, but not the sPLA₂ activity, is involved in the effect of sPLA₂-IB on prostaglandin E₂ release in rat mesangial cells (17). Here, we found that all the sPLA₂s assayed were able to enhance the expression of rat sPLA₂-IIA, whether or not they bind 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 sPLA₂s that do not bind to the M-receptor. Indeed, sPLA₂s like rat sPLA₂-IIA may use the heparan sulfate proteoglycan pathway (see below). On the other hand, the fact that sPLA₂-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 sPLA₂-IIA by sPLA₂-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 sPLA₂-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).

**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, since 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 sPLA₂-IIA in the medium thus preventing it from acting on the plasma membrane. sPLA₂-IB and sPLA₂-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 sPLA₂-IIA by these other sPLA₂s.

Role of cPLA₂ in sPLA₂-IIA expression — It was shown earlier that exogenously added sPLA₂-IIA activates the mitogen-activated protein kinase (MAPK) cascade and also leads to phosphorylation and activation of cPLA₂ in rat mesangial cells (13, 14). Activation of cPLA₂ by various sPLA₂s has also been reported in many other cells (65-67). cPLA₂ 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 cPLA₂α (37, 53, 54) markedly reduced the expression of sPLA₂-IIA triggered by human sPLA₂-IIA or sPLA₂-IB and its catalytically inactive H48Q mutant. The same results were obtained with another recently described cPLA₂ inhibitor (68) from Astra Zeneca called AZ-1 (not shown). These results indicate that both sPLA₂-IB (independently of its enzyme activity) and the active form of sPLA₂-IIA activate cPLA₂α, which in turn produces fatty acids or eicosanoids as ligands for the PPARα receptor (Fig. 10).

Interestingly, the expression of sPLA₂-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 TNF\(\alpha\) signaling pathway leading to sPLA\(_2\)-IIA expression. This view also fits with the fact that the sPLA\(_2\)-IIA promoter activity stimulated by TNF\(\alpha\) is only partially reduced in cells transfected with the PPRE-1 mutant constructs. Together, these results support the view that TNF\(\alpha\) activates transcription factors such as NF-\(\kappa\)B, which act synergistically with PPAR\(\alpha\) 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 PPAR\(\alpha\) activation, i.e. via a transcription pathway distinct from that used by cytokines. The activity of the sPLA\(_2\)-IIA promoter stimulated by TNF\(\alpha\) was only partially reduced when transfection experiments were performed with the PPRE-1 mutant constructs or when the PPAR\(\alpha\) antagonist MK886 was used with the wild-type promoter, suggesting that TNF\(\alpha\) lead to the subsequent activation of two transcriptional pathways (Fig. 10). It is therefore tempting to propose that TNF\(\alpha\) is required to initiate the transcription of sPLA\(_2\)-IIA via NF-\(\kappa\)B activation, but as soon as sPLA\(_2\)-IIA is released from the cells, this later activates TNF\(\alpha\) independent signaling pathways which lead to cPLA\(_2\) activation, PPAR\(\alpha\) 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
sPLA2-IIA can dramatically enhance the secretion of sPLA2-IIA first activated by TNFα in rat mesangial cells. When used at 100 nM, we estimated that the contribution of exogenous sPLA2s in the upregulation of sPLA2-IIA accounts for about 50%, that of TNFα corresponding to the remaining half. The role of exogenous sPLA2s in the expression of sPLA2-IIA therefore appears quite important, at least in this model of rat mesangial cells. Of particular interest was the effect of exogenous sPLA2-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 (69). An important remaining question that remains to be answered concerns the identification of the early cellular events which are used by the different sPLA2s and that link their initial binding to the plasma membrane to the activation of cPLA2 and PPARα. It will also be interesting to see if the promoters of the other sPLA2s 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 upregulate the mRNA level of the inducible nitric oxide synthase (iNOS), which is another pro-inflammatory enzyme expressed in rat mesangial cells (unpublished observations). This effect was mediated by a PPAR-binding site present in the iNOS promoter. PPARα may thus represent a specific transcriptional pathway by which various extracellular sPLA2s, 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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Table 1: sPLA2s used for treatment of rat mesangial cells

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Type</th>
<th>Source</th>
<th>Binding to the rat M-type sPLA2 Receptor (K&lt;sub&gt;0.5&lt;/sub&gt; values)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-IB</td>
<td>wild-type porcine sPLA2-IB</td>
<td>porcine pancreas</td>
<td>0.5 nM</td>
</tr>
<tr>
<td>IB-H48Q</td>
<td>catalytically inactive porcine sPLA2-IB mutant</td>
<td>recombinant</td>
<td>0.5 nM</td>
</tr>
<tr>
<td>hIIA</td>
<td>wild-type human sPLA2-IIA</td>
<td>recombinant</td>
<td>&gt; 500 nM</td>
</tr>
<tr>
<td>hIIA-H48N</td>
<td>mutant human sPLA2-IIA with 0.2 % residual catalytic activity</td>
<td>recombinant</td>
<td>&gt; 500 nM</td>
</tr>
<tr>
<td>ratIIA</td>
<td>rat sPLA2-IIA</td>
<td>recombinant</td>
<td>&gt; 500 nM</td>
</tr>
<tr>
<td>bee</td>
<td>bee venom sPLA2-III</td>
<td>bee venom</td>
<td>&gt; 500 nM</td>
</tr>
<tr>
<td>Naja</td>
<td>snake venom sPLA2</td>
<td>Naja mossambica mossambica</td>
<td>&gt; 500 nM</td>
</tr>
<tr>
<td>OS1</td>
<td>snake venom sPLA2</td>
<td>Oxyuranus scutellatus sc.</td>
<td>0.3 nM</td>
</tr>
<tr>
<td>OS2</td>
<td>snake venom sPLA2</td>
<td>Oxyuranus scutellatus sc.</td>
<td>0.1 nM</td>
</tr>
</tbody>
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* K<sub>0.5</sub> values were determined by competition binding assays with labeled OS1 on rat mesangial cell membranes as described (38).
LEGENDS TO FIGURES

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 TNF$\alpha$ (0.5 nM). In parallel, cells were incubated with 100 nM of human sPLA$_2$-IIA (hIIA), porcine sPLA$_2$-IB (IB), the catalytically inactive mutant sPLA$_2$-IB-H48Q (H48Q in Fig. 1B), rat sPLA$_2$-IIA (rIIA), sPLA$_2$ from *Naja mossambica mossambica* (Naja), sPLA$_2$-III from bee venom (bee), and snake venom sPLA$_2$s from *Oxyuranus scutellatus sc.*. mRNA was extracted from cell lysates, and Northern blot analysis (A) was performed to detect sPLA$_2$-IIA mRNA expression as described in Experimental procedures. The signal density for each RNA sample was normalized to that obtained with the 18S RNA probe. The amount of mRNA calculated for sPLA$_2$-IIA in IL-1$\beta$-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 in Experimental procedures to detect sPLA$_2$-IIA protein secretion (B). The numbers at the top represent the realive increase in sPLA$_2$-IIA protein compared to TNF, which is indicated as 1. The values are calculated as means of 3 independent experiments with comparable results and vary between 1.5- and 3-fold between the single experiments with a significant increase compared to TNF$\alpha$ ($p < 0.05$).

Fig. 2 Modulation of the TNF$\alpha$-stimulated activity of the rat sPLA$_2$-IIA promoter by exogenous sPLA$_2$s — Mesangial cells were transfected with 0.4 $\mu$g of the 2.67 kbp rat sPLA$_2$-IIA promoter DNA and additionally with plasmid DNA plus 40 ng 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 sPLA2s (each 100 nM). After 24 h, dual luciferase assays were performed as described in Experimental procedures. Values for beetle luciferase were normalized to those for Renilla luciferase. The mean of the sPLA2-IIA promoter activity in control cells is set as 1. Data are means +/- SD (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 sPLA2s on mesangial cells transfected with different sPLA2-IIA promoter constructs**—Mesangial cells were transfected for 16 h with the wild-type 2.67 kbp promoter construct (A), 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 were described in Experimental procedures. The cells were then treated with TNFα (0.5 nM) in the absence or presence of human sPLA2-IIA (hIIA), wild-type sPLA2-IB (WT-IB) or the catalytically inactive mutant sPLA2-IB-H48Q (H48Q; each 100 nM). Dual luciferase assays were performed as described in Experimental procedures. Data are means +/- SD (n = 6). Significant differences from the control group: *p < 0.05; significant differences from the corresponding TNFα-treated cells: # p < 0.05.

**Fig. 4** **Effect of docosahexaenoic acid and linoleic acid on TNFα-stimulated sPLA2-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 in 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 in Experimental procedures. Data are means +/- SD (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 sPLA2-IIA protein by western blot with a specific antibody as described in Experimental procedures. Numbers at the top of a representative blot out of 3 with comparable results represent the increase in protein amount compared to TNFα, which is set as 1.

Fig. 5 Electrophoretic mobility shift analysis (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 sPLA2s (100 nM) as indicated. 32P-labeled double stranded PPRE-1 oligonucleotide from the sPLA2-IIA promoter was incubated with nuclear extracts prepared from stimulated mesangial cells and EMSA was performed as described in Experimental procedures. For competition, nuclear extracts were co-incubated with a 50- or 100-fold excess of unlabeled PPRE-1 oligonucleotide from the sPLA2-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 3 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 sPLA2s (100 nM). EMSA with 32P-labeled double stranded PPRE-1 oligonucleotide from the sPLA2-IIA promoter was performed in the absence or presence of a specific PPARα antibody described in Experimental procedures. C)
EMSA was performed with a specific antibody against RXRα under the same conditions as described in (B).

**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 sPLA2-treated mesangial cells were analyzed for 5-LOX and FLAP expression by performing RT-PCR with specific primers as described in 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. Abbreviations for sPLA2s – see Legend of Fig. 1.

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 sPLA2s. From the cell culture supernatants, western blot analysis of secreted sPLA2-IIA (B) as well as sPLA2 activity assays (C) were performed as described in Experimental procedures. For analysis of the sPLA2-IIA promoter activity (D), cells were transfected for 16 h with the wild type 2.67 kbp construct of the rat sPLA2-IIA promoter. Then cells were treated for 24 h with TNFα and human sPLA2-IIA, sPLA2-IB or the mutant sPLA2-IB-H48Q in the absence or presence of MK886 (3 µM). Dual luciferase assays were performed as described in Methods. Data are means +/- SD (n = 6). Significant differences from the control group: *p < 0.05; significant inhibition compared to TNFα-treated cells: § p < 0.05.

**Fig. 7** Effects of the sPLA2 inhibitor LY311727 and the inactive mutant sPLA2-IIA-H48N A and B) Mesangial cells were treated for 24 h with or without TNFα as well as with human
sPLA2-IIA (hIIA), wild-type sPLA2-IB (wt-IB) or the inactive mutant sPLA2-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 sPLA2-IIA or the inactive mutant sPLA2-IIA-H48N. Western blot analysis for detection of released sPLA2-IIA in the cell culture supernatants (A, C) and luciferase assay (B) were performed as described in Experimental procedures. Data are means ± SD (n = 3).

Fig. 8 – Effects of the cPLA2 inhibitor pyrrolidine-1 — Cells were treated for 24 h with TNFα and human sPLA2-IIA, sPLA2-IB or the inactive mutant sPLA2-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 in Experimental procedures.

Fig. 9  Effect of heparinase-1 on sPLA2-IIA secretion and promoter activity in rat mesangial cells  A) Mesangial cells were preincubated for 30 min with 0.4 U/ml of heparinase I and then treated for 24 h with TNFα (0.5 nM) in the absence or presence of human sPLA2-IIA (hIIA), wild-type sPLA2-IB (wt-IB) or the inactive mutant sPLA2-IB-H48Q (H48Q; each 100 nM). Western blot analysis of secreted sPLA2-IIA protein in the cell culture supernatants was performed with a specific antibody as described in Experimental procedures. This experiment was performed 3 times, and a representative experiment is shown. The numbers at the top of the blot represent the increase in protein amount compared to 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 sPLA2-IIA
promoter. After washing cells were preincubated for 30 min with heparinase-1 (0.4 U/ml) and then treated for 24 h with TNFα in the absence or presence of the exogenous sPLA₂s as described in (A). Dual luciferase assays were performed as described in Experimental procedures. Data are means +/- SD (n = 6). Significant inhibitory effect compared to the control group: *p < 0.05; significant inhibitory effect compared to the corresponding TNFα-treated cells: # p < 0.05.

Fig. 10 Mechanisms by which exogenous sPLA₂s and endogenous sPLA₂-IIA enhance the cytokine-stimulated sPLA₂-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 sPLA₂-IIA expression via PPARα.
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Beck et al., Fig. 1
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C

![Graph showing sPLA2 activity (cpm/5µl) for various conditions.](image)

D

![Graph showing luciferase activity (relative units) for various conditions.](image)

Beck et al., Fig. 6
**Figure 9**

Panel A: Western blot analysis showing the expression of sPLA2-IIA (14 kDa) under different conditions of TNFα and hep-1.

Panel B: Graph illustrating the luciferase activity (relative units) under various combinations of TNFα and hep-1, comparing different constructs: hIIA, wt-IB, and IB-H48Q.