Oxysterol and 9-*cis*-retinoic acid stimulate the group IIA secretory phospholipase A₂ gene in rat smooth-muscle cells

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The inflammation that occurs during rheumatoid arthritis or atherosclerosis is characterized by the release of large amounts of sPLA₂ (group IIA secretory phospholipase A₂). We have shown previously that the sPLA₂ promoter in SMC (smooth-muscle cells) is activated by interleukin-1 β and cAMP-signalling pathways, through the interplay of multiple transcription factors [Antonio, Brouillet, Janvier, Monne, Bereziat, Andreani, and Raymondjean (2002) *Biochem. J.* **368**, 415–424]. In the present study, we have investigated the regulation of sPLA₂ gene expression in rat aortic SMCs by oxysterols. We found that oxysterol ligands that bind to the LXR (liver X receptor), including 25-HC (25-hydroxycholesterol) and 22(*R*)-HC, cause the accumulation of sPLA₂ mRNA and an increased enzyme activity. Transient transfection experiments demonstrated that the sPLA₂ promoter is synergistically activated by 22(*R*)-HC in combination with 9-*cis*-retinoic acid, a ligand for the LXR heterodimeric partner RXR (retinoid X receptor). Promoter activity was also increased in a sterol-responsive fashion when cells were co-transfected with

LXR α /RXR α or LXR β /RXR α . Mutagenesis studies and gel mobility-shift assays revealed that LXR/RXR heterodimers regulate sPLA₂ transcription directly, by interacting with a degenerated LXRE (LXR response element) at position [–421/–406] of the sPLA₂ promoter. Chromatin immunoprecipitation revealed the *in vivo* occupancy of LXR on the sPLA₂ promoter. In addition, the orphan nuclear receptor LRH-1 (liver receptor homologue-1) potentiated the sterol-dependent regulation of the sPLA₂ promoter by binding to an identified promoter element (TCAAGGCTG). Finally, we have demonstrated that oxysterols act independent of interleukin-1 β and cAMP pathways to activate the sPLA₂ promoter. In the present study, we have identified a new pathway activating sPLA₂ gene expression in SMCs.

Key words: chromatin immunoprecipitation, 9-*cis*-retinoic acid, secretory phospholipase A₂, liver X receptor, oxysterol, smooth-muscle cell.

INTRODUCTION

sPLA₂ (group IIA secretory phospholipase A₂) is a low-molecular-mass secreted phospholipase that hydrolyses the acyl group at the *sn*-2 position of phospholipids to give a non-esterified fatty acid and a lysophospholipid [1]. The fatty acid is probably arachidonic acid (20:4 *n*–6), the precursor of many pro-inflammatory lipid mediators, the eicosanoids [2]. The sPLA₂ enzyme can be produced and secreted in response to pro-inflammatory cytokines and contributes to the pathogenesis of various inflammatory diseases, including cancer, septic shock, rheumatoid arthritis and atherosclerosis [3]. In atherosclerotic plaques, enhanced sPLA₂ expression has been detected in SMCs (smooth-muscle cells) [4]. The enzyme seems to play a crucial role in atherosclerosis as it catalyses the production of precursors of lipid mediators which, in turn, stimulate the proliferation of SMCs in response to inflammatory cytokines [4].

sPLA₂ has been reported recently to have other atherogenic effects, linked to its ability to hydrolyse the phospholipid monolayers of HDL and LDL (high- and low-density lipoproteins respectively) [5]. sPLA₂-modified LDLs have an increased affinity for glycosaminoglycans and proteoglycans, a great tendency to be entrapped in the arterial extracellular matrix and an enhanced ability to deliver cholesterol to cells [6]. Cultured macrophages incubated with sPLA₂-treated LDL and HDL accumulate intra-

cellular lipids and form foam cells [5]. The non-esterified fatty acid, liberated from HDL and LDL by sPLA₂, also increases the formation of ox-LDLs (oxidized LDLs), which are believed to be the main cause of LDL atherogenicity [7]. Thus sPLA₂ appears to be a major link between the lipid and the inflammation that is part of atherosclerosis.

Ox-LDLs are rich in oxysterols, oxidized derivatives of cholesterol, and are avidly taken up by macrophages and SMCs, resulting in significant sterol loading [8,9]. Oxysterols are found in arterial foam cells [10] and may be generated by the oxidation of lipoproteins by SMCs, endothelial cells and macrophages [11]. Exposure of SMCs and other vascular cells to ox-LDL and oxysterols exerts pleiotropic effects on gene expression and leads to cellular changes that are collectively thought to influence the development of the atherosclerotic lesion [12]. Oxysterols, including 25-HC (25-hydroxycholesterol) and 22(*R*)-HC, appear to modify fatty acid distribution and lipid synthesis in cultured bovine aortic SMCs [13]. 25-HC-treated SMCs undergo morphological changes similar to those described for activated SMCs after injury. These changes of phenotype are concomitant with increased eicosanoid release [14]. The enrichment of arterial SMCs with cholesterol stimulates a pathway that enhances phospholipase A₂ activity independent of mitogen-activated protein kinase [15]. Panini et al. [16] have shown that fibroblasts and macrophages treated with oxysterol or ox-LDL release arachidonate.

Abbreviations used: C/EBP, CCAAT-enhancer-binding protein; ChIP, chromatin immunoprecipitation; 9cRA, 9-*cis*-retinoic acid; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; Ets, erythroblastosis virus twenty-six; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HC, hydroxycholesterol; HDL and LDL, high- and low-density lipoproteins; ox-LDL, oxidized LDL; IL-1 β , interleukin-1 β ; LRH-1, liver receptor homologue-1; LRHE, LRH-1-responsive element; LXR, liver X receptor; LXRE, LXR response element; NF-1, nuclear factor 1; RXR, retinoid X receptor; RT, reverse transcriptase; SMC, smooth-muscle cell; sPLA₂, group IIA secretory phospholipase A₂.

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Recently, ox-LDL has been shown to induce strongly sPLA₂ mRNA production by macrophages [17]. Consequently, the oxysterols derived from ox-LDL may be involved in the initial stages of atherosclerosis, but how they act in vascular cells, particularly SMCs, is poorly understood.

A number of oxysterols from ox-LDL, such as 22(R)-HC, 25-HC and 27-HC, are natural ligands for the nuclear transcription factors, LXRs (liver X receptors) [9]. LXRs are members of the nuclear receptor superfamily that includes the LXR α and LXR β isoforms [18]. These receptors regulate the expression of target genes by binding to a specific promoter response element, i.e. LXRE (LXR response element), in association with the obligate heterodimerization partner RXR (retinoid X receptor), which is the receptor for 9cRA (9-*cis*-retinoic acid) [9]. Activation of LXR transcription factors results in increased expression of the genes involved in the regulation of cholesterol and lipid metabolisms in the liver and also cholesterol efflux from lipid-loaded macrophages. This includes the genes encoding cholesterol 7 α -hydroxylase (CYP7A), ATP-binding cassette protein A1 (ABCA1), cholesteryl ester transfer protein (CETP) and lipoprotein lipase (LPL) [19–22]. The CYP7A and CETP genes are also transactivated by LRH-1 (liver receptor homologue-1), a mouse homologue of the orphan nuclear receptor *fushi tarazu F1* (Ftz-1) from *Drosophila*, which potentiates the sterol induction mediated by the binding of LXRs to an extended nuclear-receptor half-site as monomers [23].

Thus we have investigated the regulation of the sPLA₂ gene by oxysterols in SMCs. Induction of SMCs by oxysterols and retinoic acid ligands results in the specific, selective production of sPLA₂. Also, sPLA₂ is produced when oxysterols stimulate the production of sPLA₂ mRNA after the binding of LXR/RXR to an LXRE in the sPLA₂ promoter. Finally, the cells release sPLA₂ protein. This sterol induction is also potentiated by LRH-1. Hence, sPLA₂ appears to be a new direct target gene for the LXR-signalling pathway.

Therefore the sPLA₂ promoter activity may be supported, *in fine*, by the influence of several signal-transduction pathways. One is the classical proliferative pathway triggered by inflammation [e.g. NF- κ B (nuclear factor κ B), C/EBP (CCAAT-enhancer-binding protein) and Ets (erythroblastosis virus twenty-six)] [24,25], and a second pathway is generated by the accumulation of oxysterols.

EXPERIMENTAL

Reagents

22(R)-HC, 22(S)-HC, 25-HC, cholesterol, 9cRA and bee venom phospholipase A₂ were obtained from Sigma. The cell culture was prepared from DMEM (Dulbecco's modified Eagle's medium), Dulbecco's PBS, trypsin, type I collagen, glutamine, penicillin, streptomycin and fatty acid-free BSA. Materials for cell culture were purchased from Sigma. Foetal calf serum was obtained from Gibco BRL (Cergy-Pontoise, France), and flasks and Petri dishes were from Costar (Costar VWR, France). Synthetic oligonucleotides were provided by Genome Express (France). Mouse mammary lentivirus RT (reverse transcriptase), random primers, LIPOFECTAMINE™ Plus and OptiMEM were obtained from Life Technologies (Life Technologies SARL, France). The fluorescent substrate 1-hexadecanoyl-2-(1-pyrenyldecanoyl)-sn-glycero-3-phosphoglycerol was from Interchim (Interbioch, France). The pCMV- β -galactosidase plasmid was obtained from ClonTech Laboratories (Erembodegem, Belgium) and the pGL3-Basic plasmid, the 100 bp DNA Ladder and the luciferase reporter assay kit was from Promega (Charbonnières, France). Antibodies

were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Isolation and culture of SMCs from rat aortas

Vascular SMCs were isolated from the thoracic aortas of adult male Wistar rats as described previously [25]. Cells were seeded on dishes coated with type I collagen from calf skin and were cultured in DMEM supplemented with 10% (v/v) foetal calf serum, 4 mM glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. Cells were subcultured every 5 days, and experiments were performed on cells between passages 3 and 12 after primary culture. Confluent cells were made quiescent by incubating them for 24 h in serum-free medium containing 0.2% fatty-acid-free BSA and appropriate agents (see the Figure legends). The medium was then removed and assayed for phospholipase A₂ activity. The cells were treated for RT-PCR analysis or for the preparation of nuclear extracts.

Phospholipase A₂ activity

Phospholipase A₂ activity was measured using a selective fluorimetric assay [24]. The activity secreted into 0.4 ml samples of the medium was assayed with 4 nmol of the fluorescent substrate 1-hexadecanoyl-2-(1-pyrenyldecanoyl)-sn-glycero-3-phosphoglycerol. Total hydrolysis of the substrate obtained by 0.1 unit of phospholipase A₂ from bee venom was used as a reference to calculate the phospholipase A₂ activity in the samples. Spontaneous substrate hydrolysis was assayed in fresh culture medium and subtracted from the test values. Results are the means $\pm$ S.E.M. from four independent experiments.

RNA extraction and reverse transcription

Total RNA was extracted from rat SMCs using RNeasy kit columns (Qiagen, Courtaboeuf, France) according to the supplier's instructions. RNA (1.5 μ g) was reverse-transcribed for 1 h at 37 °C with 200 units of mouse mammary lentivirus RT, random primers and the buffer supplied by the manufacturer, in a total volume of 20 μ l. The reaction was terminated by heating to 95 °C for 5 min.

Non-quantitative RT-PCR analysis

After reverse transcription, 2.5 μ l of the first-strand cDNA was added to each PCR. The primers used to amplify sPLA₂ and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) have been described previously [24]. The primers for rat LXR α were rLXR α forward 5'-CAACCGGAAGACTTTGCC-3' and rLXR α reverse 5'-TGCAGGCGAAGTGCAAACA-3' (GenBank® Nucleotide Sequence Database accession no. U11685; 329 bp expected). For rat LXR β , the primers were rLXR β forward 5'-AGTGCCAGCTCTGCAGACT-3' and rLXR β reverse 5'-CCTGGACTGATGATGGC-3' (GenBank® accession no. U20389; 404 bp expected). The cDNA of these genes were amplified together with GAPDH cDNA, which was used as an internal control for semi-quantitative PCR, and the linear amplification was experimentally determined for each experiment (currently 20–24 cycles). The conditions used for PCR were as described in [24]. PCR amplifications were performed in a thermocycler (Hybaid Omnigene, Syngene Ozyme): 3 min at 95 °C followed by cycles of PCR (1 min at 95 °C, 1 min at 64 °C for sPLA₂ or 60 °C for LXR α

and LXR β and 1 min at 72 °C) and a final extension for 4 min at 72 °C.

Products were size-separated by electrophoresis on 2% (w/v) agarose gel and visualized by ethidium bromide staining. PCR bands were quantified using the Genegenius system (Syngene, Ozyme). The relative amount of PCR product was normalized to the corresponding amount of GAPDH PCR product, and values are expressed relative to the control samples. To ensure that the correct sequences had been amplified, all fragments were then sequenced.

Real-time quantitative RT-PCR

Quantitative RT-PCR was performed using the qPCR core kit for SYBR Green I-No ROX (Eurogentec, Angers, France). Reactions were performed in 25 μ l of total volume containing SYBR Green PCR core reagents with 8 ng of the first-strand cDNA and 300 nM primers. Amplification was performed on an iCycler (Bio-Rad Laboratories) according to the manufacturer's instructions, and cycle parameters were: 50 °C (2 min) and 95 °C (10 min), followed by 40 cycles of 95 °C (15 s), 64 °C (30 s) and 72 °C (30 s). To normalize variations in the levels of total cDNA templates across different samples, expression levels of GAPDH were determined. Oligonucleotide primers, used to quantify sPLA₂ and GAPDH mRNAs, were identical with those used in non-quantitative RT-PCR (see above).

Plasmid constructions and transfection

The cloning of the rat sPLA₂ promoter (−488 to +46 bp) into pGL3-basic luciferase plasmid to create [−488; +46]sPLA₂-Luc, and preparation of the deleted constructs ([−156; +46]sPLA₂-Luc, Δ [−156; −42]sPLA₂-Luc and [−42; +46]sPLA₂-Luc) used in Figure 4, have been described previously [25]. The [−398; +46]sPLA₂-Luc construct was obtained by PCR amplification of −398 to +46 bp of the sPLA₂ promoter. The mLXRE-sPLA₂ mutant construct lacks the LXR-binding element of the sPLA₂ promoter, whereas mLRE-sPLA₂ lacks the LRH-1-binding element. They were replaced by *Bgl*III restriction site PCR-based site-directed mutagenesis.

SMCs were seeded, 24 h before transfection, in 6-well plates at a concentration giving 70% confluence. Cells were transfected using 2 μ l of LIPOFECTAMINE™ Plus, 0.8 μ g of reporter DNA and 0.2 μ g of pCMV- β -galactosidase per well. For transactivation studies, 50 ng of either CMX-hLXR α or CMX-hLXR β expression vectors and 10 ng of CMX-hRXR α were added. The cells were re-fed with DMEM containing 0.2% BSA, 3 h after adding the DNA, and incubated for 24 h. Then, 1 μ M 22(R)-HC, 0.1 μ M 25-HC and 10 μ M 9cRA, or ethanol alone were added and incubation continued for a further 24 h. Luciferase activity was measured using a luciferase reporter assay kit, with signal detection for 12 s by a luminometer (Berthold, Pforzheim, Germany), and normalized by dividing the relative light units by β -galactosidase activity. The degree of induction was calculated relative to the control. Results are the means $\pm$ S.E.M. from three independent duplicate experiments with two different plasmid preparations.

EMSAs (electrophoretic mobility-shift assays)

EMSAs were performed as described previously [25]. Sequences of the oligonucleotides used are shown in Figure 5. Competition assays were performed using a 100-fold molar excess of an unlabelled oligonucleotide. Polyclonal antibodies against pep-

tides from LXR α / β (C19, sc-2101X), RXR α (D20, sc-553X) or ROR α (retinoid-related orphan receptor α) (K-20, sc-6063X) were used for supershift experiments. Samples were electrophoresed on 0.5 $\times$ TBE (Tris/borate/EDTA) and 6% polyacrylamide gel. After electrophoresis, the gel was dried at 80 °C and autoradiographed overnight at room temperature.

ChIP (chromatin immunoprecipitation) assays

ChIP assays were performed as described by Forsberg et al. [26] with minor modifications. Protein–DNA cross-linking was performed by incubating rat SMCs (2×10^7 /condition) with formaldehyde at a final concentration of 1% for 10 min at 37 °C. Glycine (125 mM) was added to stop the reaction. Cells were then washed with cold PBS, scraped into PBS and then centrifuged at 2000 *g* for 3 min at 4 °C. Nuclear extracts were prepared as described previously [26]. The lysate was sonicated to shear the chromatin to an average length of 500–1000 bp and the nuclear debris was removed by centrifugation at 10 000 *g* for 10 min at 4 °C. The chromatin solution was precleared by adding 50 μ l of Protein A/G PLUS-agarose (Santa Cruz Biotechnology) for 1 h at 4 °C. An aliquot of precleared chromatin was removed (input) and used in the subsequent PCR analysis. The remainder of the chromatin was first incubated overnight with 5 μ g of goat polyclonal anti-LXR α / β antibody at 4 °C with gentle agitation and consecutively incubated with anti-goat IgG for 6 h to immunoprecipitate LXR protein–DNA complexes. Negative controls were realized where chromatin was incubated either without antibody or with the anti-goat IgG alone to control non-specific binding. Immune complexes were collected by incubation with 50 μ l of Protein A/G PLUS-agarose for 2 h at 4 °C. After a series of washes and elution of DNA–protein complexes [26], cross-links were reversed by heating the samples at 65 °C for 4 h, followed by proteinase K digestion for 1 h at 45 °C. DNA was purified by two phenol/chloroform extractions and ethanol precipitations. Purified DNA was resuspended in 40 μ l of water and 5 μ l aliquots were used as the template for PCRs. Immunoprecipitated DNA was amplified by PCR using RedTaq and primers designed to amplify the [−488; +46] sPLA₂ promoter (forward: 5'-CGGCGCTCGAGATGGAATGAATGAC-3'; reverse: 5'-GGCGGAAGCTTCTCTTGGATGGGAT-3'). The thermocycling conditions were 90 °C for 5 min, 30 cycles of 1 min at 90 °C, 1 min at 60 °C and 1 min at 72 °C, followed by a 10 min extension at 72 °C. PCR products were resolved on 1.5% agarose gel containing ethidium bromide.

RESULTS

A previous study using rat SMCs in primary culture revealed the interplay of transcription factors implicated in the activation of the rat sPLA₂ promoter by IL-1 β (interleukin-1 β), a pro-inflammatory cytokine and by cAMP [25]. We have now investigated the influence of oxysterol compounds on sPLA₂ gene expression in SMCs during atherosclerosis.

Effect of oxysterols on sPLA₂ activity and mRNA accumulation

We stimulated cells with increasing doses of the oxysterols, 25-HC, 22(R)-HC and 22(S)-HC, or with free cholesterol, and measured the enzyme activity of sPLA₂ in the supernatant. The oxysterols, 25-HC and 22(R)-HC, caused significant secretion of sPLA₂ by SMCs into the medium (Figure 1). Increasing the amount of 25-HC above 0.1 μ M decreased the sPLA₂ activity,

Figure 1 sPLA₂ activity in response to oxysterols

SMCs were incubated for 24 h with the indicated concentrations of 25-HC, 22(R)-HC, 22(S)-HC or cholesterol. Supernatants were collected and the phospholipase A₂ activity was measured spectrofluorimetrically. Results are the means $\pm$ S.E.M. from four separate experiments and are expressed as an induction over the control value (=0).

probably by the toxicity of 25-HC on SMCs, as frequently quoted in the literature [25]. 25-HC has been shown to inhibit SMC proliferation in a dose-dependent manner and to be a potent inducer of SMC apoptosis [27,28]. In contrast, 22(S)-HC had relatively little effect on sPLA₂ secretion, and cholesterol had no effect. Thus, certain oxysterols can stimulate the activity and secretion of the sPLA₂ enzyme.

We then performed semi-quantitative RT-PCR analysis of total RNA from rat SMCs to determine whether the sPLA₂ activity stimulated by oxysterols correlated with increased sPLA₂ mRNA. Treating SMCs with 25-HC or 22(R)-HC led to significant increases in sPLA₂ mRNA (2- and 3-fold respectively; Figure 2A). The RXR ligand, 9cRA, also increased the concentration of sPLA₂ mRNA (up to 5-fold), whereas 25-HC or 22(R)-HC acted in synergy with 9cRA. 22(S)-HC or cholesterol did not alter sPLA₂ expression significantly, either alone or in combination with 9cRA. Thus incubation for 24 h with exogenous oxysterols [25-HC or 22(R)-HC] activates the sPLA₂ gene in SMCs, whereas 22(S)-HC and cholesterol are inactive. We also realized a quantitative real-time RT-PCR analysis of RNA sPLA₂ from rat SMCs and observed that sPLA₂ expression is increased by 2-fold in SMCs treated with 22(R)-HC, by 4.2-fold with 9cRA and by 10-fold in the presence of the two ligands (Figure 2B). This experiment was also performed in human aortic immortalized SMCs (AU1; a gift from Jacques Bonnet, INSERM U441, Bordeaux, France). We observed the same induction by LXR and RXR ligands at the same concentrations (results not shown). These results suggest the physiological relevance of the induction of sPLA₂ expression by sterol and 9cRA in SMCs.

Many oxysterols bind to and activate the nuclear receptors LXR α and LXR β [29,30], whereas 22(S)-HC binds to LXRs without activating them [31]. The transcription of the sPLA₂ gene strongly correlated with the relative potency of these oxysterols that are known to activate LXRs. Therefore these results suggest that not only the transcription factors LXR α and/or LXR β , but also RXR, play a role in the oxysterol-dependent activation of sPLA₂.

Transactivation of the sPLA₂ promoter activity in SMCs by LXR/RXR

LXRs consist of two closely related protein isoforms LXR α and LXR β encoded by separate genes [18]. The rat homologues of these two receptors are RLD-1 and orphan receptor-1 (OR1) [32]. LXR α is found primarily in liver, intestine, kidney, adipose tissue and macrophages, whereas LXR β is ubiquitous [33]. We looked

Figure 2 Effects of oxysterols and 9cRA on the amount of sPLA₂ mRNA in SMCs

(A) SMCs were treated for 24 h with ethanol ('Control'), 25-HC (0.1 μ M), 22(R)-HC (1 μ M), 22(S)-HC (1 μ M), cholesterol ('Chol.'; 10 μ M) and/or 9cRA (10 μ M). Semi-quantitative RT-PCR was performed on 1.5 μ g of total RNA from each sample. PCR was performed with sPLA₂ primers and with GAPDH used as an internal standard. mRNA was quantified by the Genegenius system and normalized to GAPDH. Results are representative of two independent experiments. (B) SMCs were treated for 24 h with ethanol ('Control'), 22(R)-HC (1 μ M) and/or 9cRA (10 μ M). Expression of sPLA₂ mRNA, normalized to the expression of GAPDH mRNA, was determined by real-time quantitative RT-PCR. Each quantitative RT-PCR experiment was performed twice using triplicate samples from two independently generated cDNA templates.

for the mRNAs of these nuclear receptors in SMCs to check the potential role of LXR α and LXR β in sterol-mediated activation of sPLA₂ (Figure 3A). The mRNAs encoding both receptors were detected in SMCs, but oxysterols did not increase the LXR α / β mRNA in SMCs (results not shown). We performed the same experiment with liver mRNA as a control, as this organ contains a high concentration of LXRs (Figure 3A).

The ability of oxysterol LXR ligands to modulate sPLA₂ gene expression suggested that the sPLA₂ gene was a target of the LXR-signalling pathway in SMCs. LXRs form heterodimers with RXR to activate their target genes. These heterodimers are permissive, since they are activated by agonists of both LXR and RXR [32]. The RXR family contains three members (RXR α , RXR β and RXR γ) that preferentially bind to 9cRA. Two of the three RXRs, RXR α and the widespread RXR β , are found in the aorta and in cultured rat aortic SMCs [34]. We examined the ability of LXRs to regulate the sPLA₂ promoter activity as a way of assessing the regulation of sPLA₂ by LXR pathways. A pGL3-basic luciferase reporter construct containing -488 to +46 bp of the sPLA₂ promoter ([-488; +46]sPLA₂-Luc) was transiently transfected into SMCs, either alone or with pCMX expression vectors encoding LXR α , LXR β and/or RXR α . The transfected cells were incubated with 22(R)-HC and/or 9cRA, or ethanol for 24 h. The activity of the sPLA₂ promoter in SMCs not containing a co-transfected receptor was induced by 22(R)-HC (3.5-fold) and 9cRA (5-fold) ligands alone (Figure 3B; no receptor). The combined actions of 9cRA and 22(R)-HC agonists were

Figure 3 Involvement of LXRα/β and RXRα in the stimulation of the sPLA₂ promoter

(A) RT-PCR using rat LXRα primers was performed with 1.5 μg of total mRNA SMCs (lane 1) or liver (lane 2). The amplified 329 bp product corresponded to nucleotides 981–1310 of the rat LXRα cDNA. The same experiment was performed with rat LXRβ primers to amplify a 404 bp fragment corresponding to nucleotides 582–986 of the rat LXRβ cDNA (lanes 3 and 4). An amplified 194 bp GAPDH fragment was used as internal standard. A 100 bp DNA ladder (L) was used to verify the size of DNA fragments. (B) SMCs were transfected with a rat sPLA₂ promoter/luciferase reporter gene (–488; +46)sPLA₂-Luc) with or without expression vectors for human LXRα or LXRβ and human RXRα along with 22(R)-HC (1 μM) and/or 9cRA (10 μM) for 24 h. Luciferase activities were standardized with respect to β-galactosidase activity and represent the means ± S.E.M. from three independent duplicate experiments. Results are expressed as the increase in activity over the control value. (C) SMCs were transfected with a construct containing three copies of the LXR/RXR-binding site of the ΔMTV promoter plus the LXRα and RXRα expression vectors. The transfected cells were then incubated for 24 h with 22(R)-HC (1 μM) and/or 9cRA (10 μM).

synergistic (22-fold). This was presumably due to the presence of the endogenous transcription factors LXR and RXR in SMCs. Co-transfection of LXRα and RXRα caused a 6-fold increase in basal enzyme activity, an 11-fold increase in the presence of 22(R)-HC and a 20-fold increase with 9cRA. The two ligands acted in synergy to give a 42-fold increase. Similarly, the co-transfection of LXRβ and RXRα caused a 4-fold increase in basal activity and 9cRA and 22(R)-HC acted in synergy (28-fold increase). The same results were obtained using 25-HC instead of 22(R)-HC (results not shown). We used a construct containing three copies of the LXRα/RXRα-binding site of the ΔMTV promoter derived from the mouse mammary tumour virus LTR (LXRE3-TK-Luc) [35] as a positive control for these experiments (Figure 3C). These

results reveal that the LXR and RXR in SMCs and their ligands activate the sPLA₂ promoter activity synergistically. Along with the results presented below for the chimaeric construct LXRE3-TK-Luc, the experiment performed with the sPLA₂ promoter strongly indicates that SMCs contain all the co-activators required for full transactivation by the nuclear receptors LXR and RXR. Thus the effects of LXR and RXR on the activation of the sPLA₂ promoter in transient transfection assays are consistent with the ability of their ligands to stimulate sPLA₂ gene expression.

Characterization of the oxysterol response elements of the sPLA₂ promoter

We identified the regions of the sPLA₂ promoter involved in sterol induction by a series of progressive deletions (Figure 4). We determined the ability of 22(R)-HC and 9cRA to activate luciferase reporters lacking various parts of the promoter (Figure 4B). Deletion of –488 to –398 bp resulted in a complete loss of response to oxysterol or retinoic acid. Similar results were obtained with other deleted constructs of the promoter lacking this distal region. The construct lacking only the proximal region (–156 to –42 bp) was still activated by 22(R)-HC and 9cRA. However, the basal activity of this construct was clearly lower without the ligands, suggesting that this promoter is fully active in co-operation with other factors of the basal promoter, e.g. Sp1 (specificity protein 1) and NF-1 (nuclear factor-1) (see [25]). The same results were obtained using the 25-HC agonist instead of 22(R)-HC (results not shown). These observations suggest that the region between –488 and –398 bp is required for induction of the sPLA₂ promoter by LXR and RXR ligands.

Nuclear receptors that form heterodimers with RXR interact with a DNA response element built from two repeats of a core sequence, AGGTCA, separated by 1–5 bp [36]. The LXR/RXR heterodimers bind to a direct repeat sequence that is separated by 4 bp (DR4) [22]. The first proposed target gene for LXR is the CYP7A gene, which encodes the enzyme cholesterol 7α-hydroxylase [29]. Analysis of the –488 to –398 bp sequence of the sPLA₂ promoter led to the identification of a putative degenerated LXR-binding site (LXRE) located between –421 and –406 bp (see Figure 4A). We also identified a putative binding site from the nuclear receptor LRH-1 in the region from –175 to –167 bp, which is identical with the identified LRHE (LRH-1 response element) from the hamster CYP7A gene [37]. The LRH-1 monomer binds to an extended nuclear receptor half-site (TCAAGGCCA) in DNA and acts as a competence factor, enhancing the ability of LXRα/RXRα to mediate the responses of the CYP7A and CETP genes to sterol [23]. We tested the role played by the putative LXR- and LRH-1-binding sites in the activation of the sPLA₂ promoter by sterol by introducing a mutated version of these elements into the [–488/+46] sPLA₂ promoter (mLXRE-sPLA₂ and mLRHE-sPLA₂). SMCs were transfected with these mutants and then treated with LXR and RXR ligands (Figure 4C). The LXRE mutation decreased the basal activity of the promoter (by 30%) and abolished the response to 22(R)-HC, either alone or in combination with 9cRA. The LRHE mutation decreased the basal and sterol-induced activities by 20%.

Binding of LXR/RXR heterodimers and LRH-1 to the sPLA₂ promoter

We checked that the putative LXRE of the sPLA₂ promoter was a binding site for LXR/RXR heterodimers using oligonucleotides matching the putative wild-type LXRE of the sPLA₂ promoter (sPLA₂-LXRE) in EMSA (Figure 5A). EMSA using SMC

Figure 4 Analysis of deleted and mutated constructs of the sPLA₂ promoter

(A) Diagram of the rat sPLA₂ promoter, showing the putative transcription binding sites for LXR and LRH-1. Wild-type and mutated sequences of these binding sites are indicated. Binding sites for transcription factors involved in the stimulation of promoter activity by IL-1 β (NF- κ B and Ets) and cAMP (C/EBP) are also indicated [25]. The arrow indicates the transcription start site. (B) Various deletions were performed by enzymic digestion or PCR amplification of the sPLA₂ promoter. SMCs transfected with these constructs were then treated with 22(R)-HC (1 μ M) and/or 9cRA (10 μ M) for 24 h. The results are representative of two independent duplicate experiments. The induction was calculated relative to untreated cells ('Control'). (C) SMCs were transfected with the wild-type sPLA₂ promoter ([-488; +46]sPLA₂-Luc) or its LXRE- or LRHE-mutated versions. SMCs were then treated with 22(R)-HC (1 μ M) and/or 9cRA (10 μ M) for 24 h. The induction was calculated relative to untreated cells.

proteins and radiolabelled sPLA₂-LXRE oligonucleotide showed a strong single shift (Figure 5A, lane 1), which disappeared in the presence of excess unlabelled sPLA₂-LXRE, indicating the specificity of this interaction (Figure 5A, lane 2). This specific complex was not displaced by the mutated version of sPLA₂-LXRE (mLXRE), but was displaced by the identified LXRE of the rat CYP7A gene (CYP-LXRE) [19] (Figure 5A, lanes 3 and 4). Polyclonal antibodies directed against LXR α / β ('LXR') and

RXR α ('RXR') markedly decreased binding activity (Figure 5A, lanes 5 and 6). As expected, antibody specific for the orphan receptor ROR α ('ROR') had no significant effect (Figure 5A, lane 7). These results demonstrate that the putative LXRE from the rat sPLA₂ gene binds LXR/RXR. The affinity of this site for LXR/RXR seems to be similar to that of the CYP-LXRE identified previously (Figure 5A, lanes 8–10). LXRE is well conserved between rat and human sPLA₂ genes (human LXRE, -435

Figure 5 Binding of LXR/RXR and LRH-1 to the sPLA₂ promoter

(A) A radiolabelled DNA probe corresponding to nucleotides -421 to -406 of the rat sPLA₂ promoter (sPLA₂-LXRE) was incubated with SMC nuclear extracts, with or without excess unlabelled probe corresponding to sPLA₂-LXRE, the mutated LXRE motif (mLXRE) or the identified LXRE from the rat CYP7A gene (CYP-LXRE) (lanes 1–4). Supershift experiments were performed with 2 µg of anti-LXRα/β polyclonal antibodies (LXR), RXRα (RXR) or RORα (ROR) (lanes 5–7). A radiolabelled probe corresponding to the CYP-LXRE was used as a positive control for LXR/RXR binding (lanes 8–10). (B) A radiolabelled DNA probe encompassing nucleotides -435 to -420 of the human sPLA₂ promoter (H-sPLA₂-LXRE) was incubated with rat SMC nuclear extracts, with or without excess unlabelled probe corresponding to H-sPLA₂-LXRE, the mutated human LXRE motif (H-mLXRE) or the identified LXRE from the rat CYP7A gene (CYP-LXRE) (lanes 1–4). Supershift experiments were performed with anti-LXRα/β polyclonal antibodies (LXR) or RXRα (RXR) (lanes 5 and 6). (C) An EMSA was performed using a radiolabelled DNA probe corresponding to nucleotides -160 to -184 of the sPLA₂ promoter (sPLA₂-LRHE) incubated with SMC nuclear extracts. Competition experiments were performed using an excess of unlabelled probe corresponding to sPLA₂-LRHE, mutated LRHE motif (mLRHE) or the LRHE from the rat CYP7A gene (CYP-LRHE) (lanes 2–4). Radiolabelled CYP-LRHE was used as a positive control for LRH-1 binding (lanes 5–8).

TGAGCAGaagGGAGCA) and we observed *in vitro* the binding of LXR/RXR heterodimers on the human sPLA₂ promoter (Figure 5B), suggesting that it is probably an important regulatory element.

We next determined whether a putative LRHE (from -175 to -167 bp) of the sPLA₂ promoter binds the transcription factor LRH-1. We performed an EMSA using oligonucleotides containing the wild-type LRHE of the sPLA₂ promoter (sPLA₂-LRHE) or the mutated version (mLRHE) and SMC nuclear proteins. The radiolabelled sPLA₂-LRHE probe used alone gave a single major shift (Figure 5C, lane 1), which disappears when excess unlabelled competitor was added, revealing the specificity of the binding (Figure 5C, lane 2). This complex was also displaced by the oligonucleotide corresponding to the LRHE from the rat CYP7A gene (CYP-LRHE), whereas the oligonucleotide corresponding to the mutated LRHE of sPLA₂ did not compete for binding (Figure 5C, lanes 3 and 4). Thus the LRH-1 binding to the LRHE of the sPLA₂ gene has an affinity similar to that observed with CYP-LRHE (Figure 5C, lanes 5–8).

LXRs are recruited by the sPLA₂ promoter *in vivo*

The *in vitro* results and site-directed mutagenesis studies strongly suggest that LXRE may control the sterol induction of sPLA₂ in rat SMCs. Accordingly, we performed ChIP assays in rat SMCs for direct confirmation that binding to this site occurs *in vivo* (Figure 6). Fragmented chromatin from formaldehyde-cross-linked SMCs was incubated without antibody ('No Ab'), with goat antibody raised against LXR and anti-goat IgG

Figure 6 Interaction of LXR *in vivo* with the rat sPLA₂ promoter in SMCs in a ChIP experiment

SMCs were grown under standard conditions and cross-linking was induced with formaldehyde. After fragmentation, the chromatin was precipitated without antibody ('No Ab') or with goat antibody against LXR followed by anti-goat IgG ('αLXR') or anti-goat IgG alone ('αIgG') to control non-specific precipitation of chromatin. Immunoprecipitated chromatin was amplified by PCR using primers designed to amplify the rat sPLA₂ promoter (-488/+46). As a positive control, lysate chromatin ('Input') was also amplified.

('αLXR') or with anti-goat IgG alone ('αIgG'), and the presence of the sPLA₂ promoter in the immunoprecipitates was then analysed by PCR using primers designed to amplify the [-488; +46] region of the sPLA₂ gene promoter. We found that addition of goat anti-LXR (αLXR) to cross-linked protein-DNA complexes resulted in the immunoprecipitation of the sPLA₂ gene sequence encompassing the LXRE site. Likewise, chromatin that had not been subjected to immunoprecipitation ('Input') was readily amplified. In contrast, chromatin incubated without antibody ('No Ab') or with anti-goat IgG alone ('αIgG'), used as a control against non-specific precipitation, did not amplify the PCR product. The same ChIP experiment realized with SMCs treated with 22(R)-HC and 9cRA showed identical results (not shown). These results indicate that LXR nuclear receptors are bound to the rat sPLA₂ gene promoter *in vivo*.

Figure 7 Effect of IL-1 β and cAMP on the oxysterol-stimulated rat sPLA₂ promoter activity

SMCs were transfected with the [–488; +46]sPLA₂-Luc construct and then treated with 22(R)-HC (1 μ M) and/or 9cRA (10 μ M) for 24 h with or without IL-1 β (10 ng/ml) or forskolin (FK; 10 μ M). The induction was calculated relative to the activity of control cells not stimulated by IL-1 β or FK.

Induction of the sPLA₂ promoter activity by sterol independent of IL-1 β and cAMP pathways

We have shown previously that the sPLA₂ promoter activity is stimulated by the IL-1 β and cAMP-signalling pathways, involving the transcription factors NF- κ B, Ets and C/EBP [25]. We transfected the [–488; +46]sPLA₂-Luc construct into SMCs treated with 22(R)-HC and/or 9cRA in the presence of IL-1 β or cAMP effectors to determine whether the sterol regulation of the sPLA₂ promoter is modified in an inflammatory context (Figure 7). The activity of the sPLA₂ promoter was increased by IL-1 β (3.3-fold) and by forskolin (5.2-fold), an agent increasing intracellular cAMP. Addition of IL-1 β or forskolin to SMCs treated with 22(R)-HC or 9cRA activated the sPLA₂ promoter in an additive manner. The synergistic induction of the promoter activity by the combination of 9cRA and 22(R)-HC was not increased by IL-1 β or forskolin.

DISCUSSION

The abundant secretion of sPLA₂ is a major feature of the inflammatory process. sPLA₂ is crucial for the development of inflammation, as it catalyses the production of lipid mediators and then induces the proliferation of SMCs [38]. We have demonstrated previously the interplay of various transcription factors (NF- κ B, Ets and C/EBP- β) involved in the induction of the sPLA₂ promoter activity in the inflammatory context [25].

In the present study, we have investigated the regulation of sPLA₂ gene expression by oxysterols in SMCs. We find that the amounts of sPLA₂ mRNA and protein in SMCs are increased by oxysterol LXR ligands such as 25-HC and 22(R)-HC. In contrast, the 22(S)-HC enantiomer, which binds to LXRs but does not activate it [31], and free cholesterol had no effect. We also show for the first time that both LXR α and LXR β are present in SMCs, indicating that these cells are a potential target for LXR drugs. Our transient transfection experiments demonstrate that the sPLA₂ promoter is a direct target for transactivation by LXR/RXR heterodimers. LXR α or LXR β , in association with the obligate heterodimerization partner RXR α , binds to an LXRE at position –421/–406 of the rat sPLA₂ promoter. Mutations in this region abolished the action of sterol on the promoter. By performing ChIP assays, we demonstrated the occupancy of this site by LXR in rat SMCs *in vivo*. This sequence is well conserved between rat and human sPLA₂ genes, and we show the binding of LXR/RXR heterodimers on the human sPLA₂ promoter *in vitro*,

suggesting that it is probably an important regulatory element. We also confirm the sPLA₂ up-regulation by oxysterols and 9cRA in immortalized AU1 cells (results not shown).

Another region, in addition to the sPLA₂-LXRE, seems to be required for full LXR responsiveness. Thus we have identified a sequence (–175/–167) that binds LRH-1, a monomeric nuclear receptor (sPLA₂-LRHE). This sequence is highly conserved and is identical with the LRHE in the hamster CYP7A gene promoter [37]. Alignment of rat and human promoter sequences revealed that this sequence is also found in the human sPLA₂ promoter at position –135 TCAACTCTG (cf. with TCAAGGCTG from rat). LRH-1 factor seems to be limited to liver, pancreas, intestine and ovary [35]. Our results suggest that LRH-1 factor is also produced in rat SMCs. Although no ligand has yet been found for LRH-1 receptor, several target genes have been identified, including CYP7A and CETP [23,37]. LRH-1 acts as a competence factor, enhancing the ability of LXR α /RXR α to mediate the responses of the CYP7A and CETP genes to sterol. Mutagenesis analyses indicate that LRH-1 is also involved in the potentiation of LXR/RXR induction of the sPLA₂ promoter. LRH-1 action may be inhibited in SMCs by a potent repressor, the orphan receptor SHP (small heterodimer partner), thus limiting cholesterol absorption [39].

Deletion of the –156 to –42 region decreased the sPLA₂ promoter activity by approx. 60%, suggesting that ubiquitous factors in the vicinity of the proximal promoter are also required to facilitate the recruitment of the RNA polymerase II. We have shown previously that the two signalling pathways, IL-1 β and cAMP, act together on the promoter activity through the interplay of multiple transcription factors [25]. Therefore we sought to determine whether the activation of the two pathways orchestrated by the transcription factors NF- κ B, Ets and C/EBP favoured the association of a co-activator like CBP/p300 [where CBP stands for cAMP-response-element-binding protein (CREB)-binding protein] in a co-operative manner. It was thought that many sterol receptors are complexed with a co-repressor and that their binding to the ligand caused a change in the conformation of the receptor, resulting in recruitment of the co-activator. We have now demonstrated that LXR and RXR ligands act independent of these pathways to stimulate the sPLA₂ promoter activity in an inflammatory context. Clearly, the co-activators required for enhancing the transcription by IL-1 β and cAMP do not stimulate the activation of the promoter by oxysterols and retinoic acid in the sPLA₂ promoter. This suggests that several kinds of co-activators are associated in an additive fashion. We propose here a new molecular mechanism leading to the up-regulation of the sPLA₂ gene in SMCs by oxysterols.

Whereas much progress has been made in the understanding of the molecular mechanisms that lead to up-regulation of the major inflammatory pathway, i.e. sPLA₂ $\rightarrow$ cyclo-oxygenase-2 $\rightarrow$ microsomal prostaglandin E synthase (mPGE2S), the activation of sPLA₂ gene expression by oxysterols and retinoic acid was unexpected.

It is known that LXRs are nuclear receptors that function as cholesterol sensors. This key role of LXR is based principally on the inability of LXR α knockout mice to maintain normal cholesterol homeostasis when given a high-cholesterol diet [18] and on the findings that genes regulating cholesterol homeostasis are targets of LXR. This includes (i) the CYP7A gene which is suggested to play a role in cholesterol clearance via its conversion into bile acids [19,39], (ii) the CYP27 gene, whose mutation, cerebrotendinous xanthomatosis, causes accelerated atherosclerosis [40], (iii) the ABCA1 gene and (iv) the ApoE and CETP genes involved in reverse cholesterol transport [21,22,41,42]. LXRs are also involved in fatty acid synthesis.

They stimulate fatty acid synthase and sterol-regulated-element-binding protein-1c (SREBP-1c) genes [33]. Retinoids are also involved in the regulation of cell growth, morphogenesis, differentiation and especially lipid homeostasis in association with other heterodimerization partners [43]. Both ligands may completely regulate cholesterol and lipid homeostasis in fully differentiated cells under these conditions [44].

The most potent LXR ligands are 22(R)-HC, 24(S)-HC and 24(S), 25-epoxycholesterol. However, the most abundant oxysterols in the circulation, 27-HC and 25-HC, also bind to LXR and may be the most relevant natural LXR ligands. Concentrations of 25-HC and other oxysterols, which are very low in normal vascular wall, are increased in atherosclerotic plaques [45]. Indeed, genes involved in the progression of atherosclerotic plaque have been shown recently to be LXR targets. One of them is the gene encoding lipoprotein lipase (LPL). Lipoprotein lipase, similar to sPLA₂, promotes the retention of LDL and the accumulation of extracellular lipid in the arterial intima, contributing to foam-cell formation [46]. The LPL gene is also regulated by mediators that are present in the lesion. This is also the case with the tumour necrosis factor- α gene, an inflammatory cytokine present in atherosclerotic lesions, which has been identified as an LXR target gene [9].

In the present study, we clearly show the up-regulation of sPLA₂ gene expression by oxysterols via the LXR/RXR pathway in SMCs. These results corroborate previous work showing the pleiotropic effects of oxysterols on gene expression in SMCs. These effects appear to be related to phenotypic changes of the cells [12]. We propose that activated LXRs by oxysterols in response to cellular sterol loading could stimulate strongly IL-1 β -induced sPLA₂ gene expression before contributing to the extended production of pro-inflammatory mediators [47]. Among them, PGE₂ (prostaglandin E₂) and PGF₂ modify cellular functions by increasing vascular permeability and decreasing vascular tone [48]. Recently, we have observed a PGE₂-dependent secretion of sPLA₂ in SMCs, which results from the activation of the cAMP pathway. Therefore oxysterol may contribute to auto-/paracrine activation induced by IL-1 β . These results emphasize the interplay of concerted signalling pathways and strengthen the interest in identifying the role of lipid mediators in this auto-crine loop. Therefore, one physiopathological function of the stimulation of the sPLA₂ gene by oxysterols may be the release of large amounts of inflammatory lipid mediators.

Our findings identify SMCs as a potential target for LXR antagonists. Further investigations will help to elucidate the biological consequence of increasing sPLA₂ gene activity in SMCs by LXR in terms of cholesterol homeostasis.

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