Autocrine and Paracrine Transcriptional Regulation of Type IIA Secretory Phospholipase A2 Gene in Vascular Smooth Muscle Cells

Amandine Jaulmes, Brigitte Janvier, Marise Andreani, Michel Raymondjean

Objective—The inflammation that occurs during the development of atherosclerosis is characterized by a massive release of sPLA2-IIA (group IIA secretory phospholipase A2) from vascular smooth muscle cells (VSMCs). We have investigated the autocrine function of sPLA2-IIA in rat aortic and human VSMCs.

Methods and Results—We found that the transcription of the endogenous sPLA2-IIA gene increased by adding a cell supernatant containing human sPLA2-IIA proteins. We show that this effect was independent of the sPLA2 activity using sPLA2-IIA proteins lacking enzyme activity. Transient transfections with various sPLA2-IIA rat promoter-luciferase constructs demonstrated that the C/EBP, NF-kB, and Ets transcription factors are involved in the increase in sPLA2-IIA gene transcription. We also found the M-type sPLA2 receptor mRNA in VSMCs, and we showed that the sPLA2-luciferase reporter gene was induced by the specific agonist of the sPLA2 receptor, aminophenylmannopyranoside (APMP), and that this induction was mediated by the same transcription factor-binding sites. Finally, we used a sPLA2-IIA mutant unable to bind heparan-sulfate proteoglycans to show that the binding of wild-type sPLA2-IIA to proteoglycans is essential for the induction of an autocrine loop.

Conclusions—We have thus identified new autocrine and paracrine pathways activating sPLA2-IIA gene expression in rat and human VSMCs. (Arterioscler Thromb Vasc Biol. 2004;25:1161-1167.)

Key Words: atherosclerosis ■ autocrine/paracrine effects ■ type IIA sPLA2 ■ vascular smooth muscle cells

Phospholipase A2 (PLA2) enzymes (EC 3.1.1.4) hydrolyze ester bonds at the sn-2 position of glycerophospholipids to produce lysophospholipids and nonesterified fatty acids such as arachidonic acid (C20:4 n-6). The latter is further metabolized, contributing to the proinflammatory effects. Several studies have shown that sPLA2-IB and sPLA2-IIA act via specific receptors characterized in several species (M-type receptors) to play roles that are independent of their catalytic activity.8–13 Studies on mice lacking the M-type PLA2 receptor have demonstrated the involvement of the sPLA2-IB/sPLA2 receptor system for the production of proinflammatory cytokines during the endotoxic shock.14 Alternatively, the messenger sPLA2-IIA function may be caused by its binding to proteoglycans. VSMCs produce proteoglycans in vitro and in vivo and this production is increased in proliferating cells.15 sPLA2-IIA is anchored to cell surfaces via its C-terminal heparan sulfate proteoglycans (HSPG)-binding domain.16,17 Some of the functions of sPLA2-IIA9,18–19 depend on its binding to proteoglycans, and the sPLA2 enzymatic activity and/or the M-type sPLA2 receptor binding can also be involved.

This study was performed to identify the molecular events responsible for the amplification of sPLA2-IIA gene expression during atherosclerosis. We analyzed the effect of adding soluble human sPLA2-IIA proteins on sPLA2-IIA gene transcription.
expression in 2 complementary cell culture models of VSMCs. One was VSMCs isolated from rat aortas, previously used to study the interplay of transcription factors involved in the activation of the rat sPLA2-IIA promoter. The other one was a line of human VSMCs (AU-1 cells), used as a species homologous model. We demonstrate here that sPLA2-IIA has paracrine/autocrine functions, independent of its catalytic activity and involving the PLA2 receptor and/or proteoglycans.

Methods
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Aortic Smooth Muscle Cells
Rat VSMCs were isolated from male Wistar rats by enzymatic digestion of thoracic aortic media (method described previously). AU-1 cells are a cell line derived from a human aorta. Their morphology and the staining with monoclonal antibodies to α-actin and desmin confirmed the smooth muscle cell phenotype.

Phospholipase A2 Assay
Phospholipase A2 activity was measured using a selective fluorometric assay as described.

Plasmid Constructs
The constructs containing the V5-His tagged human sPLA2-IIA (hsPLA2-IIA) wild-type or mutated were obtained by polymerase chain reaction (PCR) as described online. [−488; +46] sPLA2-luc and mutant constructs were obtained by site-directed mutagenesis as previously described.

VSMCs Transfections for Tagged Protein Expression and Protein Purification From the Supernatants
70% confluent P100 dishes (Corning) were transfected by lipofectAMINE plus as recommended by the manufacturer (Life Technologies). The tagged proteins present in the supernatants of transfected cells were purified with Ni-NTA agarose nickel-charged beads as recommended by the manufacturer (Quiagen). The eluted product was concentrated with acetone and prepared for SDS-PAGE.

RNA Extraction and Reverse-Transcription PCR
Total RNAs were extracted from P100 dishes with the RNeasyMini Kit (Quiagen) and 1 μg was used as template for retrotranscription. RT-PCR was performed as described previously using GAPDH cDNA as internal control.

Ni and V5 Depletions of the Supernatants
8 μg of the V5 antibody (In Vitrogen) were incubated 1 night with 50 μL of proteins Ag/G agarose beads (Santa Cruz). Supernatants were depleted by a 2-hour incubation with these beads or Ni-NTA agarose nickel-charged beads (Quiagen), filtered, and used to induce VSMCs. We checked the good depletion by measuring sPLA2 activity in the supernatants.

Heparin-Binding Assay
hsPLA2-IIA and b3E supernatants were incubated 1 hour at 4°C with 50 μL of heparin-Sepharose (Pharmacia). NaCl was added to 0.4 mol/L final when necessary. After centrifugation, the sPLA2 activities remaining in the supernatants were assayed.

Results
Influence of a Supernatant Containing hsPLA2-IIA Proteins on the sPLA2-IIA Gene Transcription
Secondary cultures of rat VSMCs were transiently transfected with a human sPLA2-IIA V5-His tagged (hsPLA2-IIA) construct (Figure 1A). The supernatant was collected 24 hours later and added onto untransfected cells (Figure 1B). We checked that the hsPLA2-IIA construct was expressed in supernatants. 24 hours after induction, cells were lysed and total RNA extracted. C, Western blot of the transfected cell supernatants. 24 hours after transfection by a mock vector (mock) or the hsPLA2-IIA construct (hsPLA2-IIA), supernatants were purified with a Ni-NTA resin and total elution products were separated by 15% SDS-PAGE, transferred, and the membrane was blotted with an anti-V5 antibody.

Statistical Analysis
The data represent the mean ± standard deviation (SD) of at least 3 independent experiments. The Student t test was used to compare 2 means of different experimental conditions. Multiple comparisons were performed by 1-way ANOVA followed by the Student-Newman-Keuls comparison procedure. P<0.05 was considered to be significant.
No Need for sPLA2 Enzymatic Activity for the Induction of the sPLA2-IIA Gene Transcription

Because many of the effects of sPLA2s are thought to be unrelated to their enzymatic activity, we determined whether the induction of sPLA2-IIA transcription depended on sPLA2 catalytic activity in VSMCs. We constructed 2 human sPLA2-IIA mutants (Figure 1A). The calcium-binding loop of the sPLA2-IIA protein contains a Glycine (Gly29) essential for Ca2+ binding. In the mutant designated G29S, this Gly was replaced by Ser. The second catalytically inactive human sPLA2-IIA mutant, H47E, contained a Glu instead of the His47, which is the catalytic center of the protein. We verified that the supernatants of cells transfected with mutant constructs contained no sPLA2 enzymatic activity (Figure 3A), and confirmed the lack of enzymatic activity by a more functional method (Figure III, available online at http://atvb.ahajournals.org). We also checked that these 2 constructs were produced and secreted by rat aortic smooth muscle cells (Figure IV, available online at http://atvb.ahajournals.org).

The supernatants from cells overproducing the G29S or the H47E proteins stimulated sPLA2-IIA gene transcription as the supernatant of cells overproducing the wild-type protein (Figure 3B). The mRNA/GAPDH ratio was equally increased in rat smooth muscle cells and human smooth muscle cells (Figure V, available online at http://atvb.ahajournals.org). The removal of G29S or H47E by passing the supernatants of transfected cells through a nickel column almost completely prevented endogenous sPLA2-IIA gene transcription (results not shown). We conclude that the induction of endogenous sPLA2-IIA gene expression by sPLA2-IIA proteins does not depend on the sPLA2 enzymatic activity.

Involvement of CAAT/Enhancer-binding Protein, Nuclear Factor-kB, and Winged Helix-Turn-Helix Transcription Factors in the Induction of sPLA2-IIA Transcription by hsPLA2-IIA Proteins

We have previously demonstrated that a 5' flanking region of the rat sPLA2-IIA promoter, up to 500 bp (−488;+46), is
Mediation of sPLA2-IIA Gene Transcription by sPLA2 Receptors

The effects of the sPLA2-IIA proteins on VSMCs could be mediated by the M-type sPLA2 receptor located in cellular plasma membranes. M-type sPLA2 receptor mRNAs were detected in our secondary cultures of rat VSMCs and in AU1 cells by reverse-transcription PCR. A single 488-bp cDNA identified by automatic sequencing as the M-type sPLA2 receptor was found (result not shown). This indirectly suggested that M-type sPLA2 receptors could mediate some of the effects of sPLA2-IIA. We investigated the consequences of inducing M-type receptors on sPLA2 gene expression by transfecting VSMCs with the [-488; +46] luc construct and inducing them 24 hours later with a specific agonist of the receptor, p-amino-phenyl-α-D-mannopyranoside (APMP),10,25,26 APMP significantly increased the reporter gene activity in a dose-dependent manner. APMP already enhanced transcription at a concentration of 20 μmol/L (1.7-fold induction) and had a maximal effect at 200 μmol/L (3.1-fold induction). An almost maximal effect at ≈50 μmol/L was observed (Figure 5). The induction by APMP was abolished when the mutated [-488; +46] luc constructs were used (Figure VI, available online at http://atvb.ahajournals.org). Thus, the transcription of sPLA2-IIA is induced after stimulation of the M-type sPLA2 receptor, via the same transcription factors binding sites.

Need for sPLA2 Protein Binding to Proteoglycans for Increased sPLA2-IIA Gene Expression by Cell Supernatant

We constructed a sPLA2-IIA mutant that has normal sPLA2 enzyme activity but impaired proteoglycan binding. This b3E construction contains 3 mutations: K56E, K102E, and R118E (Figure 1A). The mutated amino acids of the resulting protein correspond to the amino acids necessary for the binding of sPLA2 to proteoglycans.17 The b3E protein was correctly synthesized and secreted by VSMCs (Figure IV). The sPLA2 activity in b3E supernatant correlated with the amount of protein visualized by immunoblotting. Incubating transfected cells for 20 minutes with a medium containing 1 mol/L NaCl abolished the interaction of sPLA2 with proteoglycans and released cell surface-associated sPLA2 proteins.15 As can be seen on Figure 6A, the 3 mutations in b3E resulted in the accumulation of more sPLA2 activity in the supernatants,
used supernatants from cells transfected with b3E to induce nontransfected cells and investigated the endogenous transcription of the sPLA2-IIA gene. The b3E supernatants stimulated the transcription of the endogenous sPLA2-IIA gene less than did wild-type supernatant in rat VSMCs (Figure 6C) as in human VSMCs (Figure VII, available online at http://atvb.ahajournals.org). Thus, sPLA2-IIA must be bound to proteoglycans to allow stimulation of sPLA2-IIA gene transcription.

Discussion

The phenotypic switch of VSMCs occurring early in atherogenesis is accompanied by an increased production of several extracellular matrix molecules such as vascular cell adhesion molecule-1 and proteoglycans having chemoattractant properties and facilitating the sequestration of secreted proteins at the cell surface.\textsuperscript{27} The synthesis of sPLA2-IIA is stimulated by pro-inflammatory cytokines and the enzyme is responsible for the production of pro-inflammatory mediators promoting atherogenesis. sPLA2-IIA is thus at the edge of an inflammation amplification mechanism.\textsuperscript{3} Our previous studies on the pathophysiological functions of sPLA2-IIA in inflammation of the arterial wall have included work on the stimulation of sPLA2-IIA gene expression via the IL-1β and cAMP pathways. We have now examined the autocrine role of sPLA2-IIA together with the mechanisms by which it regulates its own synthesis. We used 2 cell systems: rat vascular VSMCs and a human cell line (AU-1) developed by the group of Bonnet.\textsuperscript{24} These secondary cultures are good physiological models of the inflammatory process because they express almost all the main markers of inflammation. The use of these 2 types of cells shows that sPLA2-IIA action is not species-specific, because it has been shown that some sPLA2 isoforms may have different functions depending on the species.\textsuperscript{10}

The present study shows that a cell supernatant containing human sPLA2-IIA proteins stimulates the transcription of the sPLA2-IIA gene in rat and human VSMCs, and that this effect is independent of its enzymatic activity. For the first time to our knowledge in VSMCs, we thus demonstrate an autocrine/paracrine role of sPLA2-IIA potentially implicated in the delayed and amplified inflammation observed in atherosclerotic and restenotic vessels. sPLA2-IIA could stimulate activation of VSMCs via 2 mechanisms: interaction with membrane receptors analogous to those binding other sPLA2s, and binding to HSPG accumulated on the cell surface.\textsuperscript{27} Upregulation of the endogenous sPLA2-IIA gene was reproduced with the sPLA2-IIA gene promoter region [−488; +46] fused to an exogene luciferase reporter. The 500-bp promoter seems to contain sufficient sequences to enable the enhancement of sPLA2-IIA transcription by the supernatant containing sPLA2-IIA proteins. Because mutation of the C/EBP, NF-\textit{k}B, and Ets transcription factor binding sites contributed equally to a dramatic reduction of the promoter activity, we propose that these 3 factors cooperate to stimulate gene transcription. Cooperation and physical interactions between these 3 transcription factors occur in the regulation of several inflammatory genes.\textsuperscript{29–32} Our results emphasize the
interplay of concerted signaling pathways. The present study also demonstrates that the M-type sPLA2 receptor is expressed in VSMCs, and that a specific agonist of the sPLA2 receptor (APMP) can also increase the transcription of the luciferase reporter gene via the same transcription factor binding sites.

Besides, it is known that VSMCs express different subtypes of proteoglycans, and that HSPG secretion by proliferating cells is implicated in atherosogenesis. Binding of sPLA2-IIA to HSPG may account for the autocrine/paracrine action of sPLA2-IIA proteins. Murakami et al demonstrated that sPLA2-IIA is bound to glypicans, and Sartipy et al described the binding of sPLA2-IIA to a cell surface molecule, decorin, in atherosclerotic lesions. Our results using cell supernatant containing sPLA2-IIA proteins unable to bind proteoglycans (mutant b3E) demonstrated the involvement of proteoglycan interactions in the induction of sPLA2-IIA gene transcription. This finding corroborates recent results obtained in mesangial cells using heparinase-1. Unlike this pharmacological compound, the use of sPLA2-IIA mutants eliminates the possibility of a release of truncated heparin sulfate chains from the cell surface. Proteoglycans function as a type of receptor system for fibroblast growth factor and for members of the opiate receptor family. Proteoglycans are thus able to potentiate transmembrane signaling pathways.

Whereas sPLA2-IIA is the most abundant sPLA2 isoform in atherosclerotic arteries, other sPLA2 isoforms can also bind to proteoglycans (eg, group V) and could have functions similar to those of sPLA2-IIA. However, we did not observe a significant upregulation of group V sPLA2 transcription by IL-1β plus forskolin in rat VSMCs and AU-1 cells (result not shown).

sPLA2s could then be implicated in the pathogenesis of atherosclerosis via 2 mechanisms. First, sPLA2s may interact with the cell surface of VSMCs and hydrolyze phospholipids of apolipoprotein B100 lipoproteins bound to the extracellular proteoglycans. This could facilitate the binding of aggregated low-density lipoproteins to VSMCs and would lead to the accumulation of pro-atherogenic oxidized lipids in the arterial intima. Second, sPLA2s absorbed onto the cell surface could increase the susceptibility to atherosclerosis by activating the transcription not only of the sPLA2-IIA gene, but also that of genes involved in proliferation. These autocrine/paracrine mechanisms should thus worsen atherosclerotic disease and prevent any resolution in inflammation.

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References


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METHODS ON LINE

Constructs of human sPLA2-IIA wild-type and mutants

A construct containing the full length cDNA of the sPLA2-IIA from human placenta was described previously (Seilhamer et al., J. Biol. Chem. 1989;264:5335-8).

A 453 pb-fragment containing the human sPLA2-IIA coding sequence was obtained by a polymerase chain reaction with the following oligonucleotide primers: 5'-CGGGAAGCTTACCATGAAGAC-3' (sense) and 5'-GGGGAATTCGCAACGAGGGGT-3' (antisense). The fragment obtained was then inserted in the pcDNA3.1/V5-His TOPO (Invitrogen). Confirmation of the correct clone was obtained by automatic sequencing.

Site-directed mutagenesis was performed by two series of PCR from the sPLA2-IIA human cDNA construct in pcDNA3.1/V5-His TOPO. For the 5' segment of the G29S mutant, we used the primers: 5'-CGGGAAGCTTACCATGAAGAC-3' (hPLA2-S) and 5'-GCCACCCACGGAACAGTGGCGACC-3' (G29S-AS), and for the 3' segment the primers 5'-GGCTGCCACTGTTCCGTGGGTGGC-3' (G29S-S) and 5'-GGGGAATTCGCAACGAGGGGT-3' (hPLA2-AS). In the second step, the mutant fragment was amplified by mixing an aliquot of each primary fragment with the primers hPLA2-S and hPLA2-AS. The H47E mutant was generated by using the primers hPLA2-S and 5'-CAACAGTCCTCATGACACAGC-3' (H47E-AS), and the primers 5'-GCTGTGTCAGGAGACGGTGTTG-3' (H47E-S) and hPLA2-AS. In a second step, the mutant fragment was amplified as described above. For the first mutation of the b3E mutant (K56E), we used for the 5' segment the primers hPLA2-S and 5'-GCCACATCCACGTTCCTCAGAC-3' (K56E-AS), and for the 3' segment the primers 5'-GTCTGGAGGAACGTGGATGTGGC-3' (K56E-S) and hPLA2-AS. The mutant fragment was amplified as described above and this mutant was used as frame to make the second mutation of the b3E mutant (K102E) with the primers 5'-GTAGGTGTCTCCTCTTGTTCTAGC-3' (K102E-AS) and 5'-GCTAGAAACGAGACGCATCGTAC-3' (K102E-S). The double mutant fragment was obtained with the primers hPLA2-S and hPLA2-AS and was also used as frame to make the third mutation of the b3E mutant (R118E) with the primers hPLA2-S and 5'-GGGGTGTCTCCCTCCAGTGGTT-3' (R118E-AS), and 5'-AACACTGCGAAGGGAGCACC-3' (R118E-S) and 5'-GGGTTAGGGATAGGCTACCTTC-3' (reverse). The triple mutant fragment was amplified with the primers hPLA2-S and hPLA2-AS.

Western blot

The eluates obtained after tagged protein purification were separated by SDS/PAGE on 15% (w/v) polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher & Schull). The membranes were then placed with the primary anti-V5 antibody (Invitrogen) diluted to 1/1000 for 2h, and a following 1-hour incubation with secondary anti-mouse antibody coupled to the rabbit peroxidase (P.A.R.I.S. Compiègne, France) diluted to 1/5000 was realized. The complexes were located using the ECL Western Blotting kit (Amersham Pharmacia Biotech).
Normalized sPLA2-IIA transcriptional induction by the hsPLA2-IIA supernatant in an inflammatory context. Cells were pre-treated 24 hours with IL-1beta 5 ng/ml and induced as described in Fig.1B. sPLA2-IIA and GAPDH RNA were analyzed by RT-PCR 24 hours after induction. ** P<0.05 significantly different compared with the control supernatant.
**Figure II**

**Induction of the endogenous sPLA2-IIA gene by the hsPLA2-IIA supernatant in AU-1 cells.** AU-1 cells were induced as described in Fig. 1B. sPLA2-IIA and GAPDH RNA were amplified by RT-PCR. The results are expressed as the ratio of sPLA2-IIA RNA by GAPDH RNA. The ratio obtained with the hsPLA2-IIA supernatant was normalized to 100%. **P<0.05 significantly different compared with the hsPLA2-IIA supernatant. IL-1=interleukin-1beta 10 ng/ml; FK=Forskolin 10 mM**
**Release of $^3$H oleic acid.** The release of $^3$H oleic acid was measured after 30 minute-incubation of *E.coli* membranes enriched into $^3$H-oleic acid (Franson et al J Lipid Res. 15: 380-388) with the supernatants of VSMCs over-expressing wild-type hsPLA2-IIA, G29S or H47E. **P<0.05** significantly different compared with the control supernatant.
**FIGURE ON LINE**

**Figure IV**

**A-**

anti-V5 antibody

hsPLA2-IIA G29S H47E

**B-**

anti-V5 antibody

hsPLA2-IIA b3E

**Figure IV-Western blot of the transfected cell supernatants.**
24 hours after transfection by a mock vector (mock), the wild-type hsPLA2-IIA, the mutated constructs G29S and H47E (A) or b3E (B), supernatants were purified with a Ni-NTA resin and total elution products were separated by 15% SDS-PAGE, transferred, and the membrane was blotted with an anti-V5 antibody.
sPLA2-IIA gene expression in AU-1 cells. Cells were induced as described in Fig.1B and sPLA2-IIA and GAPDH RNA were analyzed by RT-PCR. ** P<0.05 significantly different compared with the hsPLA2-IIA supernatant
Involvement of the C/EBP1, Ets and NF-κB transcription factor binding sites in the induction by APMP. Rat VSMCs were co-transfected with the wild-type or mutated sPLA2 promoter-luciferase constructs and the β-galactosidase expression vector. Results are expressed as the luciferase activity/β-galactosidase activity ratio in comparison with the control (non induced). ** P<0.05 significantly different compared with the wild-type non induced.
Inability of the b3E supernatant (supern.) to induce sPLA2-IIA transcription. AU-1 cells were induced as described in Fig. 1B and sPLA2-IIA and GAPDH RNA were analyzed by RT-PCR. The ratio sPLA2-IIA/GAPDH RNAs obtained for the hsPLA2-IIA supernatant was normalized to 100%. ** P<0.05 significantly different compared with the hsPLA2-IIA supernatant.