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MMP production in human fibrosarcoma cells and their invasiveness are regulated by group IB secretory phospholipase A_2 receptor-mediated activation of cytosolic phospholipase A_2

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1. ABSTRACT

Matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, are expressed in most colonic, gastric, and ovarian carcinomas, and they play a key role in their invasiveness. Previous studies have shown the involvement of arachidonic acid (AA)-derived metabolites in the regulation of MMP expression and cancer dissemination, thus suggesting a role for phospholipase A_2, the AA producing enzymes, in these processes. The present study was undertaken to explore the role of phospholipases in MMP production and tumor cell invasiveness. Human fibrosarcoma cells were found to express and secrete type IB, IIA and V $sPLA_2$. The cells were found also to express the M-type $sPLA_2$ receptor. Treatment with an extracellular $sPLA_2$ inhibitor inhibited tumor cell's invasiveness concomitantly with MMP-2/9 production. Correspondingly, adding an exogenous $sPLA_2$-IB (but not IIA) resulted in significant elevation of MMP-2/9 secretion from the fibrosarcoma cells. Time-course determination of AA and oleic acid release by HT-1080 cells suggested that $cPLA_2$ is activated subsequently to $sPLA_2$ action. Accordingly, using Western blot analysis it was found that $sPLA_2$-IB induced $cPLA_2$ phosphorylation, a requirement for its activation, by a receptor-mediated activity, rather than its lipolytic activity. At the same time, $sPLA_2$-IIA did not induce either MMPs secretion or $cPLA_2$ phosphorylation. The results of this study show for the first time that MMP-2/9 production by human fibrosarcoma HT-1080 cells and their invasiveness is regulated by $sPLA_2$-IB acting as a receptor ligand to activate $cPLA_2$, which in turn provides the AA for production of eicosanoids required for MMP expression.

2. INTRODUCTION

A major cause of morbidity in patients with cancer is the metastatic spread of tumor cells, governed by a number of processes: invasiveness of tumor cells through the basement membrane, proliferation of the tumor cells in specific sites, and tumor vascularization which is essential for its growth (11, 2). The major components of the basement membrane, comprising the barrier to the invading tumor cells, are collagen IV, laminin and heparane sulfate proteoglycans (3). The degradation of extracellular matrix (ECM) in mammalian cells is regulated by a family of matrix metalloproteinases (MMP), including collagenases, gelatinases, stromelysins and membrane type MMPs. The passage of tumor cells through the basement membrane begins with the binding of the cell to laminin and subsequent activation of a protease cascade, leading to the production of active MMPs. These enzymes specifically degrade the major structural element in the ECM: collagen IV. The movement of cells across the basement membrane may occur in response to specific chemotactic and motility factors produced by the host tissue (4, 5) . Angiogenesis, beginning with endothelial cell proliferation, is an essential step in the development of primary tumors and of metastatic lesions. The initial step of angiogenesis is the migration of endothelial cells to the newly-formed tumor, and is dependent on MMP expression in the burrowing tip (6).

MMP production and cancer cell invasiveness have been shown to require the involvement of prostaglandins (PGs) and leukotrienes (LTs) produced from arachidonic acid (AA) via the cyclooxygenases (COX) and lipoxygenases

(LOX) pathways (7). Both PGs and LTs are involved in the development of several types of cancer in humans including: colon, breast, gastric and hepatocellular carcinomas. Different eicosanoids have been shown to facilitate the invasiveness of tumor cells, angiogenesis and tumor vascularization (8, 9). Previously, Reich and co-workers (10, 11) have shown that both tumor cell invasion through the basement membrane and metastasis formation *in vivo* require the involvement of eicosanoids; both COX and LOX – produced eicosanoids facilitate the production of MMP-2/9 and the respective invasive activity of several lines of malignant cells, and these can be attenuated by inhibitors of these enzymes.

The involvement of AA-derived lipid mediators of tumor cell invasion assigns an important role to phospholipase A_2 (PLA_2), which hydrolyzes membrane phospholipids to produce free fatty acids and lyso-phospholipids. The PLA_2 enzymes are divided into two major groups: the secretory ($sPLA_2$s) types, and the intracellular, which include the cytosolic ($cPLA_2$) and the calcium-independent ($iPLA_2$) types. Each group is further divided into several subgroups according to sequence and catalytic activities. At present, more than 10 distinct $sPLA_2$s have been identified in mammals and classified into different groups by their primary structures as characterized by the number and position of cysteine residues (12, 13).

In addition to generation of lipid mediators, some $sPLA_2$s may also act as a receptor ligand to initiate cell signaling, and both $sPLA_2$ lipolytic and receptor-mediated actions may lead to activation of $cPLA_2$ (14). In line with that, it has been reported that $sPLA_2$-IB stimulates extracellular matrix invasion by normal and cancer cells (15), and promotes MMP-2 production in fibroblasts.

Another group of substances that are well known to play an important role in cell invasiveness are the glycosaminoglycans (GAGs) which form part of the extracellular matrix (ECM). The passage through the ECM barrier, which requires GAG degradation, is a necessary step for cell invasion, and therefore can be attenuated by GAG enrichment (16). Accordingly, it has been demonstrated that phosphatidyl-ethanolamine (PE)–linked chondroitin sulfate (CS-PE) inhibits adhesion of cancer cells to ECM and metastases formation in mice (17). These effects were attributed to binding of CS-PE to cell surface and sub-endothelial matrix, thus inhibiting cancer cell adhesion. However, previous studies by Yedgar and coworkers have shown that phospholipids-conjugated GAGs are efficient inhibitors of $sPLA_2$, and protect the cell membrane from the action of exogenous and endogenous $sPLA_2$s (18, 19). Furthermore, the phospholipids-GAG conjugates, designed and synthesized to be extracellular cell-impermeable PLA_2 inhibitors (ExPLIs), effectively ameliorated inflammatory conditions that involves lipid mediators, including endotoxin-induced sepsis in rats (20), antigen-induced central nervous system inflammation (EAE) in rats and mice (21), and ovalbumin-induced asthma in rats (22, 23).

In the present study, we explored the mechanism by which $sPLA_2$ regulates MMP production and tumor cell invasion. We found that $sPLA_2$ type IB mediates MMP dependent activity via a receptor mediated pathway and activation of $cPLA_2$.

3. MATERIALS AND METHODS

3.1. Exogenous *PLA_2s*

Porcine pancreatic and Crotalus atrox PLA_2s were purchased from Sigma-Aldrich, St. Louis, MO, USA.

3.2. ExPLI

The ExPLI employed in the present study was Hyaluronic acid-N-conjugated phosphatidyl-ethanolamine (HyPE, M.W. *cr*. 50 kD), synthesized in the laboratory of S. Yedgar (18, 20, 23).

3.3. Cell culture

Human fibrosarcoma HT-1080 cells (CCL 121, ATCC, Rockville MD) were maintained in DMEM supplemented with calf serum, 10%. Glutamine, pyruvate, non-essential amino acids, vitamins and antibiotics (Biological Industries, Kibbutz Beth HaEmek, Israel) were added as additional supplements.

3.4. Determination of basement membrane invasiveness

Boyden chamber chemoinvasion assays were performed as previously described (24, 25). Matrigel (reconstituted basement membrane; 25 microgram) was dried on a polycarbonated filter (Nucleopore® Polyester PVP free; Whatman International Ltd., UK). Fibroblast-conditioned medium (obtained from confluent NIH-3T3 cells cultured in serum free DMEM) was used as the chemoattractant. Cells were harvested by brief exposure to 1mM EDTA, washed with DMEM containing 0.1% BSA, and added to the Boyden chambers (200.000 cells). The chambers were incubated at 37oC in humidified atmosphere of 5% CO2/95% air for 6h. The cells that have traversed the Matrigel layer and attached to the lower surface of the filter and stained with Diff Quick (Dade Diagnostics, USA) and counted in five random fields. The mean of the counts was calculated and values expressed in terms of non-treated HT-1080 cells normalized to 100%.

3.5. Determination of MMP activity (Zymography)

Sub-confluent cell cultures were incubated for 6/24h in serum-free DMEM and the resulted supernatant was analyzed for collagenolytic activity. The collagenolytic activity was determined on a gelatin impregnated (1 mg/ml, Difco, USA), SDS-PAGE 8% gel, as previously described (26).

3.6. Determination of cell chemotaxis

To rule out the possibility that the used inhibitors affect cell motility, chemotaxis evaluation was performed in a similar way to basement membrane invasion, with the exception that the filters were coated with 5 microgram collagen IV instead of Matrigel. This amount of collagen does not form a barrier to the migrating cells, but rather an attachment substratum, and thus serves to measure cell motility.

3.7. Determination of cell PLA$_2$ activity

Confluent HT-1080 cells were metabolically labeled with either [^{3}H-AA] or [^{3}H-OA] (0.5 microCi/24 well plate) (Amersham Biosciences, UK), by overnight incubation with the radioactive fatty acid, then washed and the temporal release of the labeled fatty acid to the culture medium was monitored under the different treatments (27).

3.8. Determination of exogenous PLA$_2$ activity

Lipolytic activity of exogenous PLA$_2$ was determined by using 4N3OBA (4-nitro-3-hydroxy-benzoic acid) (Sigma-Aldrich, St. Louis, MO, USA) as a substrate. 10microliter of PLA$_2$ [1u/ml and 0.5u/ml] in Tris-HCl (pH=8 100nM) was incubated with 190 microliter substrate solution (4N3OBA resuspended in 150mM KCl, 10mM $CaCl_2$, 50mM Tris-HCl, pH=7.5) at room temperature for 1h. The PLA$_2$ activity was calculated as:

$(A_{425nm}-A_{600nm})[OD_{425}/h]$ x 0.07862 [micromol/OD_{425nm}] x (1/sample volume [1/ml]).

3.9. Identification of cell sPLA$_2$ and sPLA$_2$ receptor expression

Cultured HT-1080 cells were assessed for the expression of mRNA for sPLA$_2$s. Total RNA was isolated from the cells using Tri-reagent (Sigma-Aldrich, St. Louis, MO, USA). First strand cDNA was transcribed with M-MLV reverse transcriptase (RT) (Promega, Madison, USA). Each cDNA (5 microgram) was amplified in standard PCR reaction (30-35 cycles) containing ReddyMix™ Master Mix (1.5mM $MgCl_2$) (ABgene®, UK) and 1.5mM oligonucleotide primers. The PCR was carried out in an Eppendorf Mastercycler with an initial 5 min denaturing at 94^{0}C, followed by the sequence of denaturation (95^{0}C, 30 s), annealing (50^{0}C, 30 s), and extension (72^{0}C, 2min). A final extension of 20 min at 72^{0}C ended the reaction.

PCR analysis was performed on reversed transcribed mRNA using 5'CTT-GAC-TGC-AAG-ATG-AAA-CTC as sense and 5'CTG-ACA-ATA-CTT-CTT-GGT-GTC as antisense primers for sPLA$_2$-IB to give a 455 bp; 5'ACC-ATG-AAG-ACC-CTC-CTA-CT as sense and 5'gaa-gag-ggg-act-cag-caa-cg as antisense primers for sPLA$_2$-IIA to give a 449 bp; 5'CAG-GGG-GCT-TGC-TAG-AAC-TGA-A as sense and 5'AAG-ACG-GTT-GTA-ACT-CCA-GAG-G as antisense primers for sPLA$_2$-V to give a 329 bp; 5'CGC-GCC-CGG-CCA-AAT-AAA-ATA-A as sense and 5'CAG-CGA-CGG-CAG-TAG-CAG-GAG-CAG as antisense primers for sPLA$_2$-X to give a 410bp; 5'CAG-AAG-AAA-GGC-AGT-TCT-GGA-TTG as sense and 5'AAA-GCC-ACA-TCC-TGG-CTC-TGA-TT as antisense for sPLA$_2$ receptor to give a 565 bp. The products were separated on 1.5% agarose gels.

3.10. Determination of cPLA$_2$ and its phosphorylation

Cells (150.000) were plated on a 6-well plate. Twenty-four h later, the culture medium was changed to a serum-free medium containing various treatments (intact or denatured porcine pancreatic PLA$_2$ (10u/ml) with/without HyPE (10microM)). After incubation for 15min, the cells were washed with cold PBS and lysed in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM sodium orthovanadate, 1microgram/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. Forty microgram of protein of each sample, under reducing conditions, were loaded on 8% SDS-PAGE. After electrophoresis, the proteins were transferred to PVDF membrane, Immobilone™-P (Millipore, USA). The blots were probed with the rabbit polyclonal phospho-cPLA$_2$ (Ser505) antibody (Cell Signaling Technology, Inc., USA). Apparent molecular weight of the enzyme was 110 kDa. The membranes were probed with the respective antibodies overnight, followed by incubation with peroxidase-conjugated AffiniPure goat anti-rabbit IgG (1:5,000 dilution) (Jackson ImmunoResearch, West Grove, PA) for 1 h, and visualized using the ECL Western blot system (Pierce, Rockford, IL). Membranes were stripped, blocked, and then probed again with anti-cPLA$_2$ antibody (Cell Signaling Technology, Inc., USA). The bands were scanned (Epson Perfection 3200 Photo), and the intensity was determined with the NIH Image 1.63 software. All values are expressed in terms of untreated HT-1080 cells normalized to 100%.

3.11. Statistics

Statistical analysis was performed by student's *t* test and by Dunn test using ANOVA program.

4. RESULTS

To elaborate on the involvement of PLA2 activity in cancer cell invasion, we first examined whether the ExPLI being a sPLA2 inhibitor, affects the invasion of HT-1080 fibrosarcoma cells through a reconstituted basement membrane. To this end, the cells were incubated for 24 h with HyPE, then washed, and challenged to cross through a Matrigel layer coated filter in a Boyden chamber.

As shown in Figure 1**,** pre-treatment of the cancer cells with HyPE, effectively inhibited cell invasiveness, without affecting the cell viability or its motility (not shown). It should be emphasized that cells were treated with HyPE prior to interaction with Matrigel and no ExPLI was added during the invasion assay. In addition, as shown in Figure 1, the hyaluronic acid (HA) alone (without the lipidic part of the ExPLI) did not affect the cell invasiveness. This implies that the reduced invasiveness is not due to possible ExPLI-exerted steric hindrance between cells and Matrigel, but rather due to ExPLI effect on the invasion capacity of the cancer cells.

Since invasion of basement membrane is dependent on the presence of collagen type IV degrading enzymes, as discussed in the Introduction, we subsequently examined the HyPE effect on MMP-2 and MMP-9 secretion by the tumor cells. Culture medium of HyPE-treated HT-1080 cells was collected and subjected to determination of its collagenolytic activity, as described in Methods. Figure 2 shows that the collagenolytic activity of both enzymes in the medium of

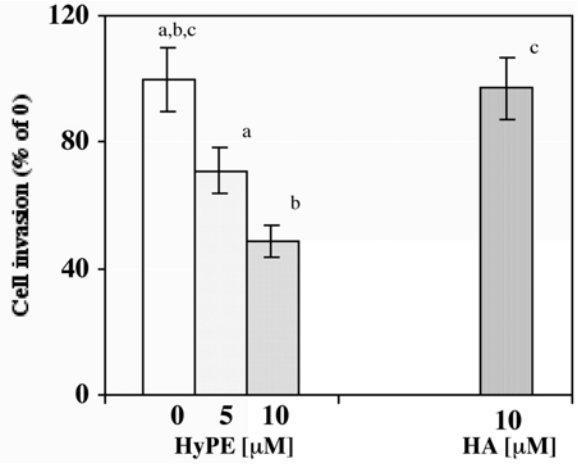

Figure 1. Inhibition of HT-1080 cell invasion by sPLA$_2$ inhibitor. HT-1080 cells were treated with the extracellular sPLA$_2$ inhibitor (ExPLI) HyPE, composed of Hyaluronic acid (HA) conjugated PE and with HA alone, at the indicated concentrations, for 24 h, than washed and placed on a Matrigel membrane. Cell invasion through the Matrigel was determined as described in Materials and methods. Each datum is Mean and SD for 3 replications (*a, b,* P less then 0.05).

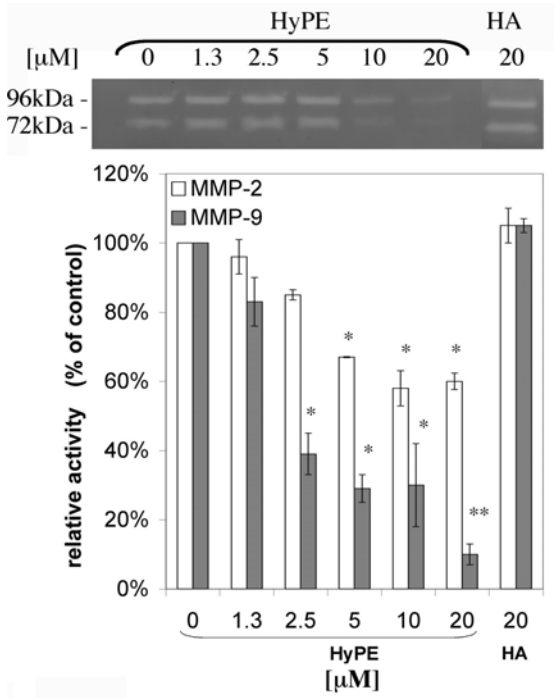

Figure 2. Inhibition of MMP-2 and MMP-9 secretion from HT-1080 cells by sPLA$_2$ inhibitor. HT-1080 were incubated for 24 h with either HyPE or HA. The cultured medium was then collected and subjected to determination of MMP-2 (72kDa) and MMP-9 (96kDa) content by their collagenolytic activity, using zymography as described in Materials and methods. Each datum is Mean and SD for 4 replications (**,* P less then 0.05, **, P less then 0.01).

HyPE-treated cells was reduced as a function of the PLA$_2$ inhibitor concentration. Here too, treatment of the cells with the GAG moiety alone did not inhibit MMP production.

To further examine the relationship of cancer cell invasion to PLA$_2$, we determined the direct effect of HyPE on PLA$_2$ activity in HT-1080 cells, in parallel to cell invasion and MMP production. Since cPLA$_2$ is specific to AA-carrying phospholipids, while sPLA$_2$ has no fatty acid preference, the cell membrane phospholipids were metabolically pre-labeled with radioactively-labeled either AA or oleic acid (OA), and the temporal fatty acid secretion to the culture medium was determined. Figure 3 shows that treatment of HT-1080 cells with ExPLI inhibited the release of both AA and OA, concomitantly with inhibiting MMP production and cell invasion (Figure 1 and 2). These findings may suggest that both sPLA$_2$ and cPLA$_2$ are involved in these processes, but since both activities are inhibited by the cell-impermeable inhibitor, it appears that they are controlled by sPLA$_2$.

As noted above, sPLA$_2$ may act as a lipolytic enzyme and/or as a receptor ligand. To explore these possibilities in the MMP production and corresponding cell invasion, we determined sPLA$_2$ types that are expressed in the HT-1080 cells (using RT-PCR). This search was confined to two receptor-ligand sPLA$_2$s, specifically IB and X, reported to act via M-type receptor, and two that are known to act mainly by lipolytic, specifically IIA and V (28). It was found that human HT-1080 fibrosarcoma cells express sPLA$_2$-IB, sPLA$_2$-IIA and sPLA$_2$-V as shown in Figure 4**.** The cytosolic cPLA$_2$-IV alpha was identified as well. Figure 4 also shows that HT-1080 cells express the receptor to sPLA$_2$-IB, thus implying the presence of all the components required for a PLA$_2$–mediated cell signaling.

Examination of the time course of the fatty acid release depicted in Figure 3 shows that at 1 h, OA production, catalyzed by sPLA$_2$, is higher than that of AA, while the reverse is observed at 2 h. In addition, AA production is significantly enhanced at 2 h, while that of OA is relatively higher at 1 h. Moreover, at both time points, treatment with sPLA$_2$ inhibitor suppressed AA production to the level of the control, untreated cells. This may suggest that in HT-1080 cells the activity of sPLA$_2$ (producing both OA and AA) precedes that of cPLA$_2$ (producing only AA), and raises the possibility that a cPLA$_2$ is activated subsequent to sPLA$_2$ action.

Exogenous sPLA$_2$ may act as a lipolytic enzyme, hydrolyzing cell membrane phospholipids, and also as receptor ligand, independent of its lipolytic activity (29). Both these activities may lead to cPLA$_2$ activation, as sPLA$_2$-produced lyso-phospholipids and receptor-mediated cell signaling lead to cPLA$_2$ phosphorylation, which is required for its activation (30). To differentiate between the two potential mechanisms for the activation of MMP production, exogenous sPLA$_2$s were subjected to boiling, which is expected to inactivate their lipolytic activity, and MMP production by HT-1080 was determined following treatment with the native and boiled sPLA$_2$s. Two commercially-available sPLA$_2$s were employed in this test; porcine

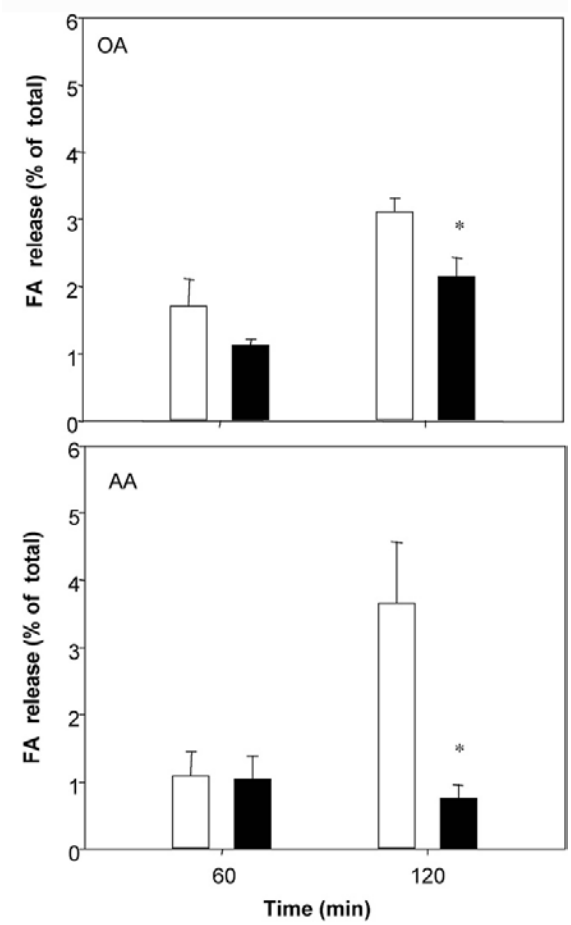

Figure 3. Inhibition of PLA$_2$ activity by sPLA$_2$ inhibitor in HT-1080 cells. HT-1080 cells were metabolically labeled by overnight incubation with either ^{3}H-arachidonic acid (AA) or ^{3}H-oleic acid (OA), then washed and the release of the labeled AA or OA into the culture medium during the indicated time, in the absence (□) or presence (■) of HyPE was measured. Each datum is Mean and SD for 3 replications. (*, P less then 0.05).

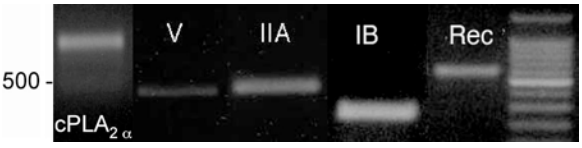

Figure 4. Expression of PLA$_2$s and PLA$_2$ receptor by HT-1080 cells. mRNA expression of the indicated PLA$_2$ was determined by RT-PCR, using the primers described in Materials and methods. The figure depicts RT-PCR of cPLA$_{2\alpha}$ (988bp); sPLA$_2$ Types V, IIA and IB (329bp, 449bp and 243bp respectively); and M-Type sPLA$_2$ receptor (Rec, 565bp).

pancreatic (Type-IB), for which HT-1080 cells express a receptor, and Crotalos atrox (Type-IIA) which has no receptor in these cells (Type V was not commercially available). As shown in Figure 5, boiling considerably suppressed the lipolytic activity of Type-IIA PLA$_2$, but had a small inhibitory effect on that of Type-IB (about 20%). On the other hand, production of both MMP-2 and MMP-9 was elevated by Type-IB PLA$_2$, in a concentration-dependent manner, as shown in Figure 6. However, it seems that heating impaired the enzyme-receptor recognition, as heat-inactivation of sPLA$_2$-IB considerably suppressed its capacity to induce MMP production (to a much larger extent than the boiling effect on its lipolytic activity). At the same time MMP production was not affected by sPLA$_2$- IIA (not shown), nor was it attenuated by its heat inactivation, which inhibited its lipolytic activity (Figure 5). These findings suggest that the induction of MMP production by sPLA$_2$ is mainly by a receptor-mediated process, rather than phospholipids-hydrolysis dependent.

Yet, as noted in the Introduction, AA derived eicosanoids are required for MMP production (7), and this is supported in the present study by the concomitant production of MMP and AA and inhibition of MMP secretion by the ExPLI (Figure 2 and 3 above). Since sPLA$_2$-dependent lipolysis does not contribute significantly to MMP production, one would assume that the required AA is provided by cPLA$_2$. This enzyme can be activated by phosphorylation that is induced by sPLA$_2$ receptor-mediated signaling, as has been previously reported for IB-sPLA$_2$ (14). To examine this possibility in the present system, we determined the phosphorylation status of cPLA$_2$ by native and heat-inactivated types IB and IIA sPLA$_2$, and its inhibition by ExPLI. As shown in Figure 7, sPLA$_2$-IB strongly enhanced cPLA$_2$ phosphorylation, and this was reduced to the basal level by heat inactivation of the enzyme or treatment with ExPLI. At the same time, sPLA$_2$-IIA did not lead to any cPLA$_2$ phosphorylation (not shown).

To further elaborate on the specific involvement of IB-PLA$_2$ in induction of MMP production, we determined the ExPLI effect on PLA$_2$ mRNA expression, using RT-PCR. As shown in Figure 8, treatment of HT-1080 cells with ExPLI had no effect on IIA-PLA$_2$ expression, but considerably reduced (by 50%) the expression of PLA$_2$-IB, concomitantly with the above shown inhibition of cell invasiveness (Figure 1), MMP production (Figure 2), and cPLA$_2$ phosphorylation (Figure 6).

5. DISCUSSION

Growing evidence directly implicates members of the metalloproteinase superfamily in tumor cell invasion and metastasis formation. Most invasive colonic, gastric, and ovarian carcinomas have shown to express MMPs, especially MMP-2 and MMP-9. It is therefore of major importance to understand the mechanism governing the production and action of these enzymes. As discussed in the Introduction, previous studies by Reich et al**.,** in cell cultures and animal models, have demonstrated the involvement of arachidonic acid metabolites in the regulation of MMP activity and metastasis formation (10, 11, 31). Accordingly, recent studies have demonstrated that extended treatment with COX inhibitors (suppressing PG production) reduces the incidence of certain cancers in humans (32). However, as noted above, AA derivatives that are produced by both COX and LOX pathways (PGs and LTs, respectively) take part in MMP production/action, and

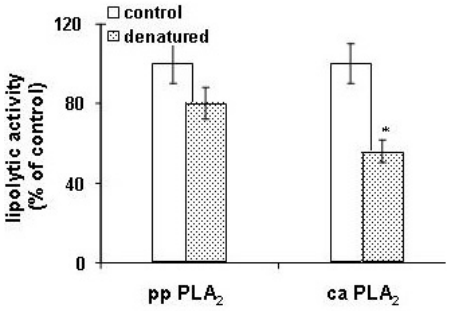

Figure 5. Effect of heat inactivation on lipolytic activity of porcine pancreatic and crotalus atrox sPLA$_2$s (ppPLA$_2$ and caPLA$_2$, respectively). The enzymes were denatured by heating at 95^{0}C for 15 min, and their lipolytic capacity was determined by their ability to hydrolyze 4N3OBA, as described in Materials and methods. Each datum is Mean and SD for 3 replications. (*, P less then 0.05).

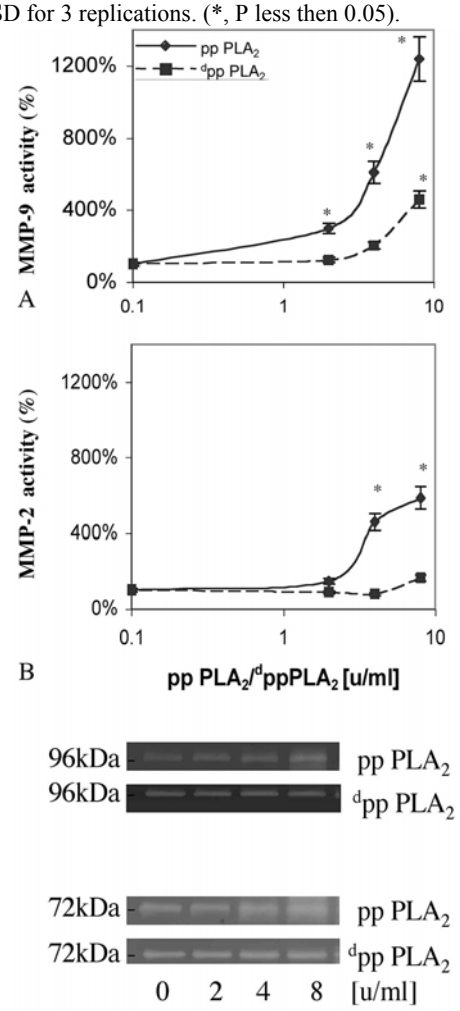

Figure 6. Effect of heat inactivation of ppPLA$_2$ on its ability to induce MMP production. HT-1080 cells were treated with either intact or denatured (^{d}) ppPLA$_2$ for 6 h, and the secretion of MMP-9 (A) and MMP-2 (B) secreted to the culture medium was determined by zymography. Each datum is Mean and SD for 3 replications (*, P less then 0.01).

blocking one pathway would divert the AA pool to the other pathway and would be insufficient or even counteracting (33). It has thus long been proposed that upstream control of AA production is a preferable approach to treatment of lipid mediator-related pathologies, which of course assigns a key role to PLA$_2$, the AA producing enzyme (34) In accord with this, previous studies have demonstrated a role for sPLA$_2$ as a mediator of fibroblast migration (35) and prostate cell's invasion (32).

This hypothesis was tested in the present study and is strongly supported by its findings: Three types of sPLA$_2$s are expressed in HT-1080 cells: IB, IIA and V. These cells also express the M-type sPLA$_2$ receptor. These enzymes differ in their mode of action. IB exhibits low catalytic activity along with independent high affinity for M-type sPLA$_2$ receptor. The receptor-mediated signaling reportedly leads to activation of cPLA$_2$, which is a major source of cellular AA release. The IIA and V are structurally close heparin-binding isoforms participating in stimulus-induced AA release. In the present study we employed exogenous enzymes that represent the two sPLA$_2$ types, namely porcine pancreatic-derived (Type IB) and crotalos atrox venom-derived (Type IIA) forms, to differentiate between the lipolytic and receptor-mediated contributions to MMP production and cell invasiveness.

The results of this study show for the first time that MMP-2/9 production by human fibrosarcoma HT-1080 cells and their invasiveness (Figure 1 and 2) correspond to AA production (Figure 3), and these activities are concomitantly inhibited by the cell-impermeable sPLA$_2$ inhibitor (ExPLI). It further shows that sPLA$_2$-IB activates MMP production (Figure 8) via a receptor-mediated process, rather than its lipolytic activity (Figure 5 and 6). Concomitantly, sPLA$_2$-IB activates cPLA$_2$ by its phosphorylation (Figure 7), which is in accordance with previous reports that intracellular cPLA$_2$ phosphorylation is induced by M-type sPLA$_2$ receptor interaction (14). All the above processes are inhibited by the ExPLI, thus assigning a pivotal role for sPLA$_2$- IB in MMP activation and subsequent cancer cell invasiveness. Taken together, the findings of the present and previous studies suggest that sPLA$_2$-IB-mediated MMP activation is compatible with the sequence of events illustrated in Figure 9: sPLA$_2$-IB secreted to the extracellular medium interacts with its membrane receptor (on its own and neighboring cells), signals the phosphorylation and subsequent activation of the cytosolic cPLA$_2$, which provides the AA for production of the eicosanoids required for MMP production/action.

Of specific interest is the finding that although sPLA$_2$-IB induces MMP production by acting as a receptor-ligand, rather by its lipolytic activity, its effect is suppressed by the ExPLI, which is designed to inhibit membrane phospholipids hydrolysis. This raises questions regarding the ExPLI's inhibitory mechanism(s). As noted above, the ExPLIs consist of a lipid component, which incorporates into the cell membrane, linked to a polymeric GAG, which prevent its internalization. It is not unlikely that the membrane-anchored GAG interferes with the sPLA$_2$-receptor interaction. On the other hand, we also find

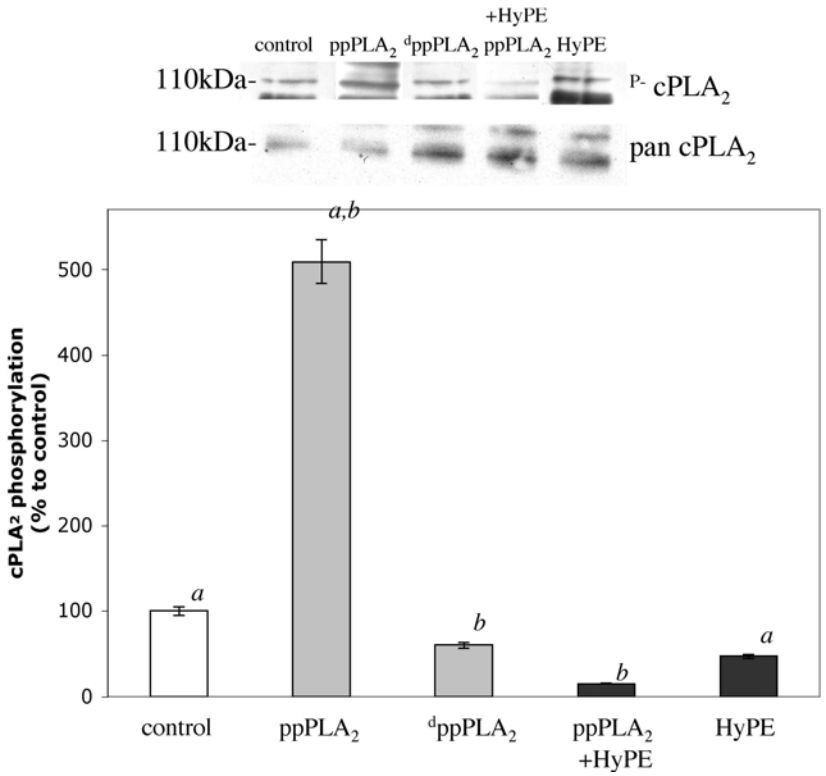

Figure 7. cPLA$_2$ Phosphorylation by ppPLA$_2$ and its suppression by heat inactivation or sPLA$_2$ inhibitor. HT-1080 cells were treated with ppPLA$_2$ in the absence (□) or presence (■) of HyPE, or with denatured ppPLA$_2$, for 15 min prior to protein isolation. The extent of cPLA$_2$ phosphorylation was determined by Western blot analysis with specific antibody directed against cPLA$_2$ phosphorylated on Ser505 and with specific antibody directed against the total (phosphorylated and non-phosphorylated) cPLA$_2$. Each datum is Mean and SD for 2 replications (*a, b,* P less then 0.05).

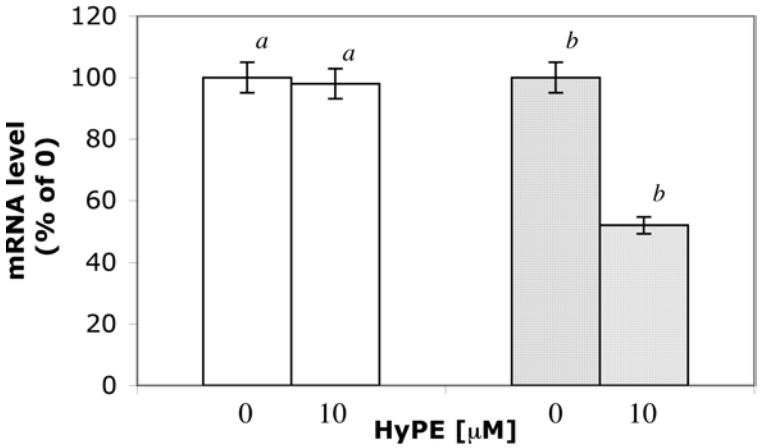

Figure 8. Effect of HyPE on expression of sPLA$_2$ Types IIA and IB by HT-1080 cells. HT-1080 cells were treated for 24 h in the absence or presence of HyPE (10 microM) prior to RNA extraction. The expression of sPLA$_2$-IB and IIA in these cells was analyzed by RT-PCR using the primers described in Methods. Sample loading was verified by 28s expression. Each datum is Mean and SD for 3 replications (*, P less then 0.05).

that in parallel to inhibition of MMP production, the ExPLI reduced the production of AA, which can be attributed to cPLA$_2$, and also OA, which is a product of sPLA$_2$ and other PLA (but not cPLA$_2$). It is thus possible that lipolytic activity of sPLA$_2$ and/or PLA$_2$ also take part in sPLA$_2$-IB-induced MMP production. These and possibly other mechanisms have yet to be explored in further studies. The present study was confined to investigating the way by

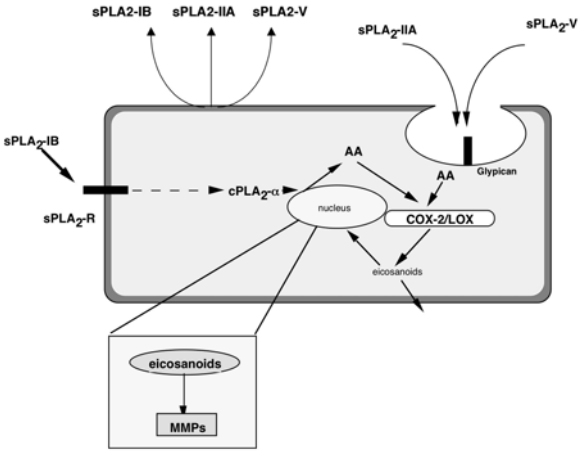

Figure 9. sPLA$_2$-IB induces MMP activity. Working hypothesis. sPLA$_2$ binds to a membranal receptor and activates intracellular cPLA$_2$. cPLA$_2$ in its turn, releases AA that is converted into eicosanoids. sPLA$_2$-induced eicosanoids eventually induce MMP expression.

which PLA$_2$ enzymes contributes to metastatic processes, specifically MMP production in cancer cells and their subsequent invasiveness, using HT-1080 human fibrosarcoma cell cultures. As illustrated in Figure 9, this study shows that these processes are activated predominantly by sPLA$_2$-IB acting as a receptor ligand, while the subsequently activated cPLA$_2$ provides the AA for production of eicosanoids required for MMP activation/action.

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Abbreviations: MMP: matrix metalloproteinase, sPLA$_2$: secretory phospholipase A$_2$, cPLA$_2$: cytosolic phospholipase A$_2$, iPLA2: calcium-independent phospholipase A$_2$, ExPLI: extracellular cell-impermeable PLA$_2$ inhibitors, ppPLA$_2$: porcine pancreatic phospholipase A$_2$, caPLA$_2$: crotalus atrox phospholipase A$_2$, AA: arachidonic acid, ECM: extracellular matrix, PGs: prostaglandins, LTs: leukotrienes, COX: cyclooxygenases, LOX: lipoxygenases, GAGs: glycosaminoglycans, CS-PE: phosphatidyl-ethanolamine–linked chondroitin sulfate, HA: hyaluronic acid, HyPE: Hyaluronic acid-N-conjugated phosphatidyl-ethanolamine

Key Words: sPLA$_2$, cPLA$_2$, MMP, ExPLI, Invasion

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