

A Bifunctional Role for Group IIA Secreted Phospholipase A₂ in Human Rheumatoid Fibroblast-like Synoviocyte Arachidonic Acid Metabolism*

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Human group IIA-secreted phospholipase A₂ (sPLA₂-IIA) is an important regulator of cytokine-mediated inflammatory responses in both *in vitro* and *in vivo* models of rheumatoid arthritis (RA). However, treatment of RA patients with sPLA₂-IIA inhibitors shows only transient benefit. Using an activity-impaired sPLA₂-IIA mutant protein (H48Q), we show that up-regulation of TNF-dependent PGE₂ production and cyclooxygenase-2 (COX-2) induction by exogenous sPLA₂-IIA in RA fibroblast-like synoviocytes (FLSs) is independent of its enzyme function. Selective cytosolic phospholipase A₂-α (cPLA₂-α) inhibitors abrogate TNF/sPLA₂-IIA-mediated PGE₂ production without affecting COX-2 levels, indicating arachidonic acid (AA) flux to COX-2 occurs exclusively through TNF-mediated activation of cPLA₂-α. Nonetheless, exogenous sPLA₂-IIA, but not H48Q, stimulates both AA mobilization from FLSs and microparticle-derived AA release that is not used for COX-2-dependent PGE₂ production. sPLA₂-IIA-mediated AA production is inhibited by pharmacological blockade of sPLA₂-IIA but not cPLA₂-α. Exogenous H48Q alone, like sPLA₂-IIA, increases COX-2 protein levels without inducing PGE₂ production. Unlike TNF, sPLA₂-IIA alone does not rapidly mobilize NF-κB or activate phosphorylation of p38 MAPK, two key regulators of COX-2 protein expression, but does activate the ERK1/2 pathway. Thus, sPLA₂-IIA regulates AA flux through the cPLA₂-α/COX-2 pathway in RA FLSs by up-regulating steady state levels of these biosynthetic enzymes through an indirect mechanism, rather than direct provision of substrate to the pathway. Inhibitors that have been optimized for their potency in enzyme activity inhibition alone may not adequately block the activity-independent function of sPLA₂-IIA.

Phospholipase A₂ (PLA₂)² enzymes regulate the provision of arachidonic acid (AA) to the cyclooxygenase (COX) and lipoxygenase biosynthetic pathways, the products of which, in turn, are critical autocrine and paracrine regulators of diverse physiological processes in mammals. Of the 23 currently known mammalian PLA₂ enzymes, *in vivo* gene deletion studies in mice have established the widely expressed intracellular enzyme cytosolic PLA₂-α (cPLA₂-α, Group IVA PLA₂) as an important enzyme in providing AA substrate to COX and lipoxygenase because deletion of this gene product abrogates eicosanoid production in cells stimulated *ex vivo* (1, 2). Significantly, cPLA₂-α gene deletion markedly reduces disease severity in the collagen-induced arthritis model of rheumatoid arthritis (RA), suggesting cPLA₂-α has a key role in the pathogenesis of RA (2).

The contribution of the remaining 18 PLA₂ enzymes to AA metabolism and to immune-mediated, inflammatory pathology is less clear. Macrophages from Group V secreted PLA₂ (sPLA₂)-deficient mice show impaired production of both COX- and lipoxygenase-derived eicosanoid products in response to the inflammatory stimulus zymosan (3), whereas deletion of Group X sPLA₂ results in impaired eicosanoid release into the lungs following ovalbumin challenge (4). sPLA₂-IIA, the best studied of the 10 mammalian sPLA₂ enzymes, is not expressed in certain mouse strains with restricted expression in others compared with either rats or humans (5, 6), making classical genetic deletion experiments impractical for this enzyme. Despite this, a proinflammatory role for sPLA₂-IIA in arthritis has been confirmed by recent genetic studies showing that arthritis is attenuated in sPLA₂-IIA knock-out mice, relative to congenic wild-type mice, in a K/BxN serum transfer model of arthritis (7). Surprisingly, these studies also showed that Group V sPLA₂ has an anti-inflammatory role in this model of arthritis (7). Transgenic

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² The abbreviations used are: PLA₂, phospholipase A₂; sPLA₂, secreted phospholipase A₂; cPLA₂, cytosolic phospholipase A₂; sPLA₂-IIA, group IIA-secreted phospholipase A₂; RA, rheumatoid arthritis; FLS, fibroblast-like synoviocyte; cPLA₂-α, cytosolic phospholipase A₂-α; AA, arachidonic acid; iPLA₂β, group VIB calcium-independent phospholipase A₂; Ab, antibody; BK, bradykinin; S.E., standard error of the mean; EMSA, electromobility shift assay.

expression of human sPLA₂-IIA in mice results in spontaneous atherosclerosis (8) that is transferable to non-transgenic mice by transplantation of transgenic bone marrow (9). Thus, aberrant expression of the human enzyme, *in vivo*, induces inflammatory pathology. These animals do not develop spontaneous arthritis (10), however, transgenic expression of human sPLA₂-IIA leads to earlier onset and more severe arthritis in a TNF transgenic, spontaneous arthritis model (11) implicating aberrant expression of sPLA₂-IIA as a positive regulator of cytokine-mediated joint inflammation. Furthermore, transgenic expression of human sPLA₂-IIA in mice results in increased severity in the K/BxN serum transfer arthritis model (7).

sPLA₂-IIA is markedly induced in the serum of patients with immune-mediated conditions, including RA and in tissues of patients with certain cancers (12–14). Serum enzyme activity and concentration correlate with disease severity in RA (15), synovial tissue expression of sPLA₂-IIA correlates with histological markers of inflammation (16), and several other sPLA₂ enzymes are also expressed in RA synovial tissue (17) and synovial fluid (7). Exogenous addition of sPLA₂-IIA to cultured RA synovial cells, at concentrations found in the synovial fluids of RA patients, enhances both TNF-stimulated PGE₂ production and up-regulation of the inducible cyclooxygenase, COX-2 by an unknown mechanism (18). However, blockade of enzyme activity with a potent inhibitor of sPLA₂-IIA, Group V and Group X sPLA₂ (in a randomized, double-blinded, placebo-controlled study) shows only transient benefit in patients with active RA (19)). Thus, despite compelling preclinical and early phase clinical data (19), the utility of sPLA₂-IIA blockade in the treatment of arthritis is not well supported by the most recent clinical evidence.

Here we show for the first time in cells that mediate inflammatory synovitis in RA that although exogenous sPLA₂-IIA contributes to AA flux in these cells in culture, exogenous sPLA₂-IIA-amplified cytokine-mediated PGE₂ production is sPLA₂-IIA enzyme activity-independent and is thus mediated by a signaling function of the enzyme that indirectly up-regulates levels of the cPLA₂- α /COX-2 pathway enzymes.

EXPERIMENTAL PROCEDURES

Materials—sPLA₂-IIA protein was expressed, purified, and quantified as described (18). cPLA₂- α inhibitors pyrrolidine-1 (20, 21) and pyrrophenone (22, 23) were synthesized as described. Pyrrolidine-1 inhibited purified, recombinant cPLA₂- α in a vesicle assay with an IC₅₀ of 70 nM, and AA release in ionomycin-stimulated Madin-Darby canine kidney cells with an IC₅₀ of 800 nM. It showed no detectable inhibition of purified, recombinant human sPLA₂-IIA, Group V or Group X sPLA₂ at 10 μ M concentration, and no physiologically relevant inhibition of recombinant cytosolic PLA₂- γ or the calcium-independent PLA₂, iPLA₂ β (20). Pyrrophenone inhibited recombinant cPLA₂- α in a mixed-micelle assay with an IC₅₀ of 80 nM (24) without significant inhibition of all five remaining human cPLA₂ isoforms in this assay.³ It inhibited AA release and PGE₂ production in ionophore-stimulated

THP-1 cells with an IC₅₀ of 25 nM (25) and had no appreciable inhibition of murine cytosolic PLA₂- β (24) or purified, recombinant human Group IB sPLA₂ or sPLA₂-IIA at 200 μ M (25). The sPLA₂ inhibitor c(2NapA)LS(2NapA)R was synthesized as previously described (Auspep, Melbourne, Australia) (26). LY311727 was a kind gift from Eli Lilly and Co. (Indianapolis, IN). The iPLA₂- β inhibitor, bromoenol lactone, was obtained from Sigma.

Construction of sPLA₂-IIA Catalytic Site Mutant H48Q—The sPLA₂-IIA cDNA (a kind gift from J. Seilhamer) (27) was subcloned into pBlueScribe(+) and histidine 48 was substituted for glutamine by oligonucleotide-directed mutagenesis of the His codon (non-coding strand oligonucleotide sequence 5'-AGCAACAGTCCTGAGTGACAC-3'). Mutagenesis was carried out with an *in vitro* mutagenesis kit (Amersham Biosciences) based on the method of Eckstein and co-workers (28). The nucleotide sequence of the mutagenized construct was confirmed and the cDNA was cloned into the zinc-inducible mammalian expression vector pMTSV40polyABam (pLEN) (29). The resultant plasmid (pMIK-1) was co-transfected with pRSV2-neo, carrying a G418 resistance gene, into Chinese hamster ovary (CHO) cells by calcium phosphate precipitation. Following several rounds of G418 selection, the resultant cell culture pool was used to express H48Q, and the protein was purified from conditioned medium by affinity chromatography (AKTA Explorer purification system, GE Healthcare) as described for sPLA₂-IIA (18), and quantified by ELISA (12).

sPLA₂ Enzyme Activity Assay—sPLA₂ enzyme activity was measured with a colorimetric microtiter plate, mixed micelle assay (Cayman Chemical, Ann Arbor, MI) using diheptanoylthiophosphatidylcholine as substrate (30) with the following modifications. Briefly, enzyme (10 μ l, 2.5 μ g/ml, sPLA₂-IIA or H₄₈Q) diluted in assay buffer (10 mM CaCl₂, 100 mM KCl, 0.3 mM Triton X-100, 1 mg/ml of BSA, 25 mM Tris-HCl, pH 7.5) was added to each well containing the free-thiol detection reagent 5,5'-dithio-bis-(2-nitrobenzoic acid) (10 μ l, 10 mM 5,5'-dithio-bis-(2-nitrobenzoic acid) in 0.4 M Tris-HCl, pH 8.0) and 5 μ l of assay buffer. Phospholipid substrate was reconstituted in assay buffer to a final concentration of 1.66 mM with vortexing until the solution was clear, then preheated to 40 °C. Assays were performed at 40 °C, started by addition of substrate (200 μ l/well) and A₄₀₅ was measured every 3 min over a 60-min time course (Spectramax 250 microtiter plate reader, Molecular Devices, Sunnyvale CA). Assays were performed in triplicate relative to blank wells containing assay buffer and data were analyzed using SoftMax Pro version 1.1 software in kinetic mode.

Fibroblast-like Synoviocytes—Synovial tissue was obtained from patients undergoing joint surgery and who were diagnosed with RA according to American Rheumatism Association criteria (31) using procedures approved by the St. Vincent's Hospital Ethics Committee. Fibroblast-like synoviocyte (FLS) cultures were established as described (18) and used between passages 3 and 10. Cells, CD14-negative and 4-prolylhydroxylase-positive by immunohistochemistry and CD21-negative by RT-PCR, were grown in Ham's/DMEM containing 10% FBS and used at 80–90% confluence.

³ M. H. Gelb, unpublished data.

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PGE₂ Assay—Cells, grown in 96-well plates were stimulated, medium was harvested and stored at -80°C prior to PGE₂ assay. Cells were lysed in wells by resuspension in ice-cold lysis buffer (40 μl) containing 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM EGTA, 50 $\mu\text{g}/\text{ml}$ of aprotinin, 200 μM leupeptin, 1 mM PMSF in PBS. Lysates from triplicate experiments were combined and stored at -80°C prior to protein determination (Bio-Rad DC Protein Assay, Bio-Rad). PGE₂ in medium was determined by enzyme immunoassay (Cayman Chemical) as previously described (18) and expressed as picograms of PGE₂/mg of total cellular protein.

Nuclear Protein Extracts—FLSs, grown in 150-cm² flasks, were stimulated and nuclear extracts were prepared as described (32) with minor modification as follows. Cells were harvested with trypsin/EDTA, centrifuged (4000 \times g, 1 min), supernatants were discarded, cell pellets were washed with PBS (1 ml), recentrifuged, and placed on ice. Cells were resuspended in ice-cold Buffer A (175 μl , 10 mM KCl, 1.5 mM MgCl₂, 100 mM EDTA, 1 mM DTT, 1 μM PMSF, 100 $\mu\text{g}/\text{ml}$ of aprotinin, 100 mg/ml of leupeptin, 10 mM HEPES, pH 7.9) and incubated on ice for 5 min. Nonidet P-40 (9 μl , 10% v/v) was added, samples were vortexed for 10 s, centrifuged (20 s, 13,790 \times g), and supernatants were discarded. Pellets were washed gently with ice-cold buffer A (150 μl), centrifuged, and supernatants were discarded. Buffer C (40 μl , 420 mM KCl, 1.5 mM MgCl₂, 100 mM EDTA, 1 mM DTT, 1 mM PMSF, 100 $\mu\text{g}/\text{ml}$ of aprotinin, 100 $\mu\text{g}/\text{ml}$ of leupeptin, 10 mM HEPES, pH 7.9) was added, samples were vortexed (10 s) and incubated with orbital shaking for 30 min on ice. Samples were centrifuged (13,790 \times g, 15 min, 4 $^{\circ}\text{C}$), supernatants were divided into aliquots and stored at -80°C prior to use. Protein concentration was determined by the Bradford protein assay (Bio-Rad).

Western Blot Analysis—Cells were grown in 24-well plates, stimulated, and medium was harvested and stored at -80°C . Cells were lysed, triplicate wells were combined and protein determinations made as described above. For phosphoprotein determination experiments, cells were grown in 75-cm² flasks, treated, and washed once with ice-cold PBS (10 ml/flask) containing 10 mM orthovanadate. Cells were scraped into ice-cold PBS (1 ml) containing 5.3 mM EDTA, 10 mM sodium orthovanadate, 50 mM disodium β -glycerophosphate, and 10 mM NaF. Following centrifugation the cells were lysed in 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM EGTA, 50 $\mu\text{g}/\text{ml}$ of aprotinin, 200 μM leupeptin, 1 mM PMSF, 10 mM sodium orthovanadate, 50 mM disodium β -glycerophosphate, and 10 mM NaF in PBS. Protein (15–20 $\mu\text{g}/\text{well}$) was electrophoresed on polyacrylamide gels (4–20% BisTris (2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol) gels, Novex) according to the manufacturer's instructions and transferred to nitrocellulose as described (18). Primary Abs for cPLA₂- α , COX-1, COX-2, and β -actin and secondary Abs were used as described previously (18). Murine monoclonal anti-human c-Jun Ab (Santa Cruz Biotechnology, Santa Cruz, CA; number sc-822) was used at 0.2 $\mu\text{g}/\text{ml}$ and detected with sheep anti-mouse IgG-horseradish peroxidase (HRP) (GE Healthcare, 1/3000). Rab-

bit polyclonal Abs were used at the following concentrations: anti-human I κ B- α (Santa Cruz, number sc-371), 10 ng/ml; anti-mouse I κ B- β (Santa Cruz, number sc-945), 400 ng/ml; anti-human NF- κ B p50 (Santa Cruz, number sc-114), 4 $\mu\text{g}/\text{ml}$; anti-human NF- κ B p65 (Santa Cruz, number sc-109), 1 $\mu\text{g}/\text{ml}$; anti-human phospho-p38 MAP kinase Ab (phospho-Thr¹⁸⁰/phospho-Tyr¹⁸², New England Biolabs, number 9211), 1/1000 dilution; anti-human p38 α MAP kinase (Santa Cruz, number sc-535), 33 ng/ml, anti-human phospho-ERK (phospho-Thr²⁰²/phospho-Tyr²⁰⁴) (Cell Signaling Technologies, number 9101), 1/10,000 dilution; and anti-rat ERK1 (Santa Cruz, number sc-94, ERK-2 cross-reactive), 10 ng/ml. These Abs were detected with donkey anti-rabbit IgG horseradish peroxidase conjugate (GE Healthcare, 1/3000 dilution). Bands were visualized using chemiluminescence (Renaissance chemiluminescent reagent Plus, New England Nuclear) with detection on x-ray film (Hyperfilm ECL, GE Healthcare). Bands were scanned (PDI densitometer, Molecular Dynamics) and density was quantified with IPLabelH software (Macintosh version 1.5g).

NF- κ B Gel Shift Assays—NF- κ B binding to a double-stranded consensus NF- κ B binding site oligonucleotide with top strand sequence (5'-AGTTGAGGGGACTTTC-CCAGGC-3'), 5'-end-labeled with [γ -³²P]ATP (GE Healthcare), and T4 polynucleotide kinase (Promega) was measured by electromobility shift assay (EMSA) as described (33). Briefly, nuclear extracts (\sim 5 μg of total protein), prepared as described above, were added to a binding reaction (20 μl) containing (polydeoxyinosine (dI)-deoxycytidine (dC)) \cdot (polydI-dC) (GE Healthcare) at 0.25 $\mu\text{g}/\mu\text{g}$ of total protein, 1–2 ng of ³²P-labeled double-stranded oligonucleotide probe and DNA binding buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, pH 8.0, 60 mM KCl, 1 mM DTT, glycerol (12% v/v)). The binding reaction was incubated at room temperature for 30 min prior to electrophoresis on non-denaturing polyacrylamide gels (5% polyacrylamide) in 0.25 \times Tris borate EDTA (TBE) buffer, pH 8.3, at 150 V for 2–3 h. Gels were dried and bands were imaged with x-ray film (X-Omat AR, Kodak, Sydney, Australia). NF- κ B EMSA bands were confirmed by NF- κ B cold-competitor studies and supershift of NF- κ B EMSA bands, using anti-p65 and anti-p50 Abs, were performed on nuclear extracts from TNF- α -stimulated FLSs.

AA Mobilization Assays—Cells were grown in 96-well plates and labeled with [5,6,8,9,11,12,14,15-³H]AA (PerkinElmer Life Sciences; NET-298Z) in Ham's/DMEM containing 10% FBS for 16 h at 37 $^{\circ}\text{C}$. Cells were washed 3 times in PBS containing 0.1% (w/v) BSA (fatty acid free, Sigma) and stimulated for the indicated times in Ham's/DMEM containing 0.1% (w/v) BSA. Treatments were added simultaneously with stimulants. Medium was harvested, cells were detached with trypsin/EDTA, and tritium was determined in medium and cells by scintillation counting (LS6000 TA, Beckman, Sydney, Australia). Data are expressed as % total radioactivity (cells plus medium) released into medium.

Thin Layer Chromatography—Samples (50 μl), to which unlabeled AA (1 μg) had been added, were spotted onto silica gel plates (Merck, Darmstadt, Germany), air dried, and eluted in chloroform:methanol:acetic acid:water (90:8:1:0.8) (34).

Plates were air-dried and developed in iodine vapor to identify the AA spot. Eluted samples were cut into seven equal segments in order of increasing R_f value and tritium was determined by scintillation counting (LS6000 TA, Beckman, Sydney, Australia). Data for each segment were expressed as % total radioactivity recovered from each sample for each segment.

Statistical Analysis—Data were analyzed and plotted using Prism Graphpad version 4.0. Statistical significance was determined using the Student's paired *t* test unless otherwise stated.

RESULTS

Exogenous sPLA₂-IIA Up-regulates TNF-mediated PGE₂ Production and COX-2 Protein by an Activity-independent Mechanism—To determine whether sPLA₂-IIA enzyme activity was necessary for up-regulation of TNF-dependent PGE₂ production and COX-2, we constructed an “activity-impaired” mutant of sPLA₂-IIA (H48Q) by site-directed mutagenesis as described under “Experimental Procedures.” Quantification of H48Q was determined relative to a sPLA₂-IIA standard by ELISA (35). The ELISA was validated for H48Q by quantitative amino acid analysis of 2 independent samples of sPLA₂-IIA and H48Q, followed by quantitation of each sample in the same ELISA. Equivalent concentrations were obtained for each sample by both methods (data not shown). Importantly, the mol % amino acid composition for each amino acid obtained for both sPLA₂-IIA and H48Q in the amino acid analysis was not significantly different from the theoretical composition calculated from their known protein sequences ($p = 1.0000$, $p = 0.9926$ for sPLA₂-IIA and H48Q respectively, Student's paired *t* test), confirming that both proteins were >99% pure. This mutation has been reported by others to have 2–4% residual enzyme activity (36). In our hands purified H48Q had 1% residual sPLA₂-IIA activity, with a specific activity of 0.28 ± 0.12 μmol of diheptanoylthiophosphatidylcholine/min/mg of protein (data are mean \pm S.D. of three experiments performed in triplicate) relative to 27.8 ± 2.3 μmol of diheptanoylthiophosphatidylcholine/min/mg of protein for sPLA₂-IIA. Our purified H48Q protein lacks the proliferative capacity of purified sPLA₂-IIA in the LNCaP prostate cancer cell line (37), confirming that the 1% residual enzyme activity we measure in our H48Q preparations is insufficient to recapitulate the effects of fully active sPLA₂-IIA in these cells. In FLSs, H48Q alone like sPLA₂-IIA, had no effect on PGE₂ production (Fig. 1A) at enzyme concentrations that are found in synovial fluid (18). TNF alone resulted in a 15-fold stimulation of PGE₂. H48Q increased this stimulation to ~30-fold, as did sPLA₂-IIA. In both cases PGE₂ production was completely abrogated by the COX-2-selective inhibitor NS-398. In a side by side experiment with sPLA₂-IIA, H48Q-mediated PGE₂ production was dose-dependent with both mutant and native enzyme having no effect at concentrations below 100 ng/ml (7 nM) (data not shown). In agreement with our earlier work (18), TNF alone up-regulated steady state COX-2 protein levels (Fig. 1B). H48Q alone (Fig. 1B), as with sPLA₂-IIA (18), also increased COX-2 protein, despite having no effect on PGE₂ production

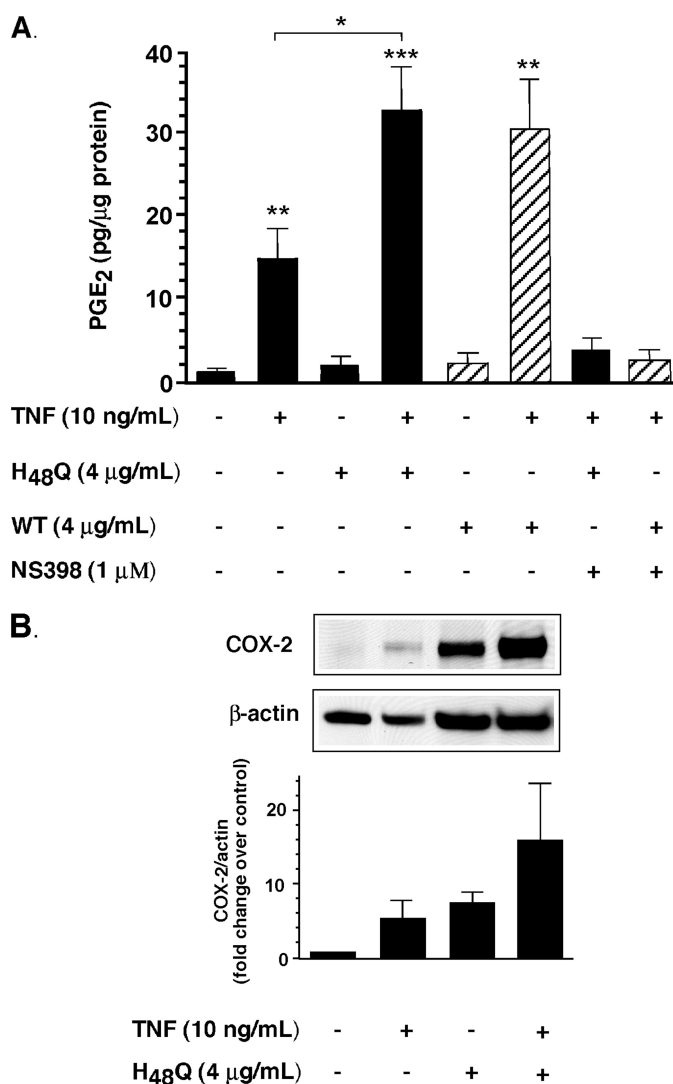


FIGURE 1. Up-regulation of cytokine-dependent PG production and COX-2 does not require sPLA₂-IIA enzyme activity. *A*, FLS cells, grown to 80–90% confluence, were stimulated with TNF (10 ng/ml), alone or in combination with the activity-impaired mutant of sPLA₂-IIA (H48Q) (4 $\mu\text{g/ml}$), or with sPLA₂-IIA (WT) (4 $\mu\text{g/ml}$) in the presence or absence of the COX-2-selective inhibitor NS-398 (1 μM) for 16 h in DMEM/Ham's F-12 containing 0.1% BSA. PGE₂ concentration was measured in cell culture supernatants and total cellular protein was determined as described under “Experimental Procedures.” Data are combined mean \pm S.E. of triplicate determinations from cell cultures derived from each of 4 patients. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student's unpaired *t* test) relative to unstimulated cells unless indicated. *B*, cells were treated for 16 h as indicated, lysed, and protein extracts were subjected to electrophoresis and Western blot analysis as described under “Experimental Procedures.” A representative Western blot from one cell culture (RA79) is shown. Bands were quantified by densitometry as described. COX-2 density for each sample was normalized relative to β -actin density and the COX-2/ β -actin ratio for each treatment was then normalized relative to control for each cell culture. Data are mean \pm S.E. of three independent cell cultures.

(Fig. 1A). As we have also shown for sPLA₂-IIA, H48Q, in combination with TNF, synergistically up-regulated COX-2 protein (Fig. 1B) without any effect on COX-1 (data not shown).

cPLA₂- α Mediates sPLA₂-IIA-dependent PGE₂ Production but Not COX-2 Up-regulation—To determine whether PGE₂ production in response to TNF and sPLA₂-IIA was dependent on cPLA₂- α , we used a pharmacological approach with well

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characterized pyrrolidine inhibitors that selectively block human cPLA₂- α activity over other human cPLA₂ isoforms, iPLA₂- β or sPLA₂ activities (20–25). Pyrrophenone (5 μ M) completely blocked the PGE₂ response to TNF alone, substantially suppressed the response to TNF/sPLA₂-IIA and showed a small but significant inhibition of the sPLA₂-IIA/TNF response at 1 μ M (Fig. 2A). Pyrrolidine-1 abrogated PGE₂ production in response to sPLA₂-IIA/TNF stimulation at all concentrations tested (Fig. 2B), without any effect on basal PGE₂ production. However, pyrrophenone, at concentrations that abrogate PGE₂ production, did not significantly block sPLA₂-IIA-mediated COX-2 up-regulation (Fig. 2C). Thus provision of AA to COX-2 for both TNF-dependent and sPLA₂-IIA up-regulated PGE₂ production appears to be mediated by cPLA₂- α .

Effect of sPLA₂-IIA on AA Mobilization—In light of these data and reports that exogenous sPLA₂-IIA does not efficiently mobilize AA in attachment dependent cells in culture (38), the effect of TNF and exogenous sPLA₂-IIA on AA mobilization was examined by [³H]AA release assays. First, the assay was validated by examining the response of FLSs to the known AA-mobilizing agonist bradykinin (BK) (Fig. 3A). BK stimulation (10 nM, 15 min) resulted in increased [³H]AA release from 8.0 \pm 0.8% (mean \pm S.E. of duplicate experiments from 4 independent cell cultures) in untreated cells to 11.3 \pm 0.6% total counts incorporated (p < 0.05), consistent with a previous report (39). Release peaked by 2 h post-stimulation (21.0 \pm 1.2% total counts, p < 0.05 relative to unstimulated cells). The basal level of AA release also increased rapidly with time peaking at 2 h (12.9 \pm 1.8% total counts) with similar kinetics to stimulated cells. Subsequent studies were terminated 2 h post-stimulation. Under these conditions, pyrrophenone (5 μ M) blocked BK-dependent AA release (Fig. 3B).

We next examined the effect of sPLA₂-IIA, alone or in combination with TNF in the presence or absence of pyrrophenone on AA release. Pyrrophenone alone showed a small but significant reduction in basal AA release (Fig. 3C). Basal AA release was unaffected by the calcium-independent Group VIA PLA₂ (iPLA₂- β) inhibitor, bromoenol lactone (40) (10 μ M) (data not shown). TNF stimulation resulted in a 1.2-fold increase in AA release that was abrogated by pyrrophenone. Surprisingly, at concentrations that fail to stimulate PGE₂ production, sPLA₂-IIA alone increased AA release by 1.5-fold and the increase was not inhibited by pyrrophenone. sPLA₂-IIA in combination with TNF resulted in a 1.7-fold increase over untreated cells that was not significantly affected by pyrrophenone. As with previous experiments, BK stimulated AA release by 1.3-fold and this was inhibited by pyrrophenone.

The dose responsiveness of sPLA₂-IIA-mediated AA release and the effect of selective sPLA₂ inhibition on the response were then determined (Fig. 4A). sPLA₂-IIA dose dependently induced AA release at concentrations above 1 μ g/ml (71 nM) but was ineffective at 100 ng/ml (7 nM) concentration. Co-incubation with the selective sPLA₂ inhibitor c(2NapA)LS(2NapA)R (26) (1 μ M) resulted in significant inhibition of the response at a molar ratio of inhibitor to enzyme approaching 1:1 and complete inhibition of the response at a

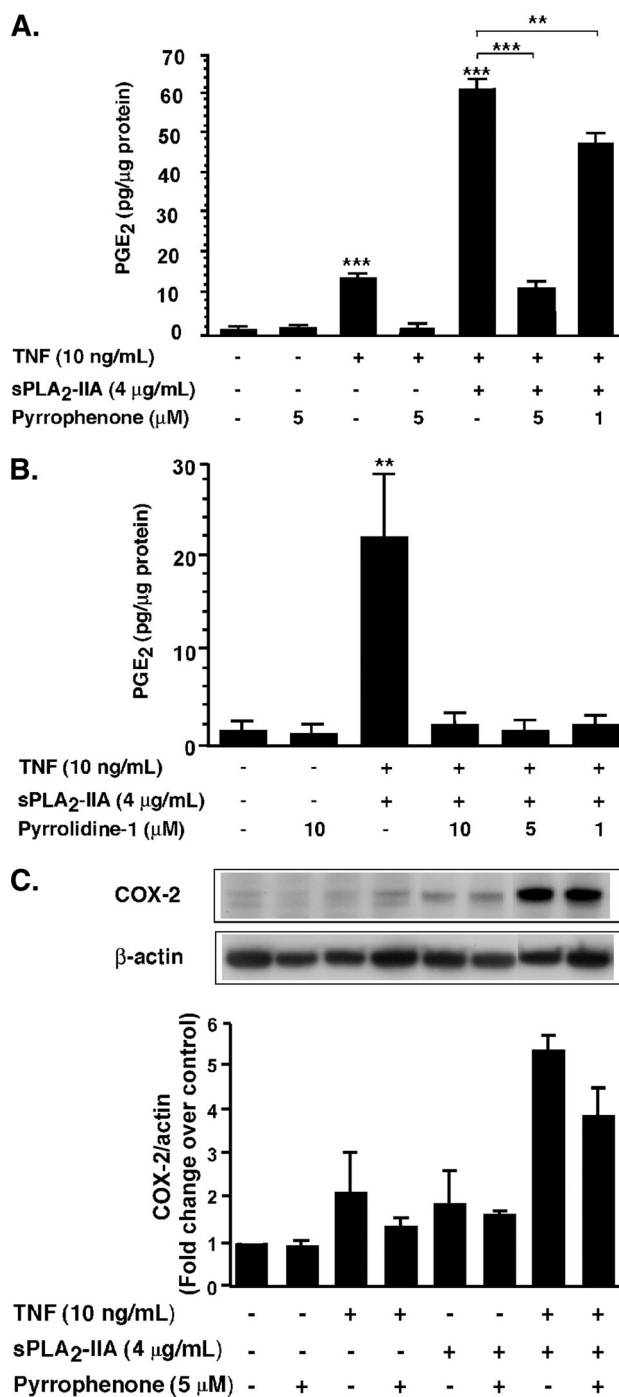


FIGURE 2. PGE₂ production, but not COX-2 up-regulation is dependent on cPLA₂- α enzyme activity. 80–90% confluent FLSs were stimulated with TNF (10 ng/ml), sPLA₂-IIA (4 μ g/ml) either alone or in combination in the presence or absence of the cPLA₂- α -selective inhibitor (A) pyrrophenone or (B) pyrrolidine-1 at the concentrations shown for 16 h in Ham's/DMEM medium containing 0.1% BSA. Medium and cells were harvested, PGE₂ in medium was determined and the protein concentration in cell lysates determined as described under "Experimental Procedures." Data are mean \pm S.E. of triplicate determinations from cell cultures derived from each of four patients. **, p < 0.01; ***, p < 0.001 (Student's unpaired t test) relative to unstimulated cells unless indicated. C, cells were grown in 24-well plates and stimulated as described above. Cells were harvested, lysates were electrophoresed, transferred to nitrocellulose, probed with Abs, and labeled proteins were visualized on x-ray film by enhanced chemiluminescence, blots were scanned and densitometry performed and analyzed as described under "Experimental Procedures." Representative blots from one cell culture are shown. Densitometry data are mean \pm S.E. normalized relative to unstimulated cells from experiments performed on cell cultures derived from three separate patients.

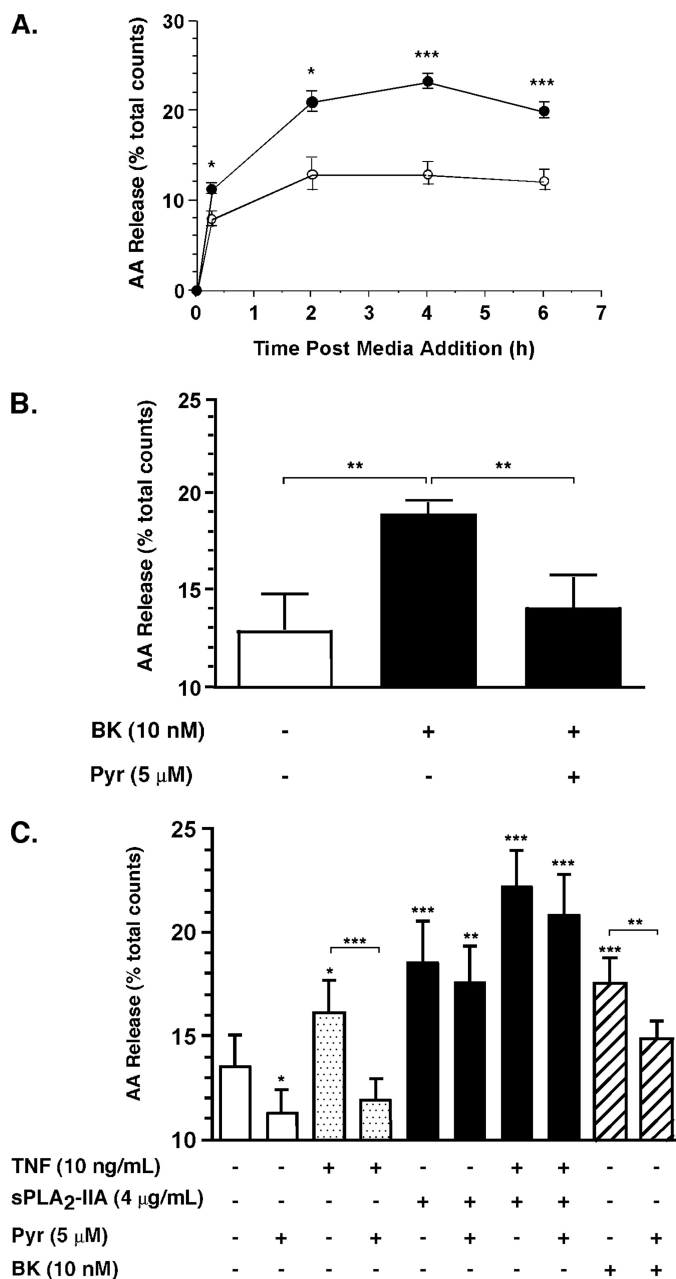


FIGURE 3. Effect of sPLA₂-IIA on AA mobilization in RA FLSs. Human synovial FLSs were labeled with [³H]AA and stimulated (A) with (closed circles) or without (open circles) BK (10 nM) or (B, C) as indicated in the presence or absence of pyrrophenone (Pyr) for 2 h prior to harvesting medium and cells for AA release determination as described under "Experimental Procedures." Data are mean ± S.E. of two independent experiments combined, each comprising duplicate determinations from four independent cultures. *, *p* < 0.05; **, *p* < 0.05; ***, *p* < 0.001 (Student's paired *t* test) relative to control unless indicated. Total radioactivity incorporated into cells ranged from 8,185–38,367 dpm.

molar ratio of 3.5:1. Because this inhibitor also suppresses the activity-independent functions of exogenous sPLA₂-IIA, viz. sPLA₂-IIA-mediated up-regulation of cytokine-dependent PGE₂ production (26), the effect of H48Q on AA release was determined in a side by side experiment with sPLA₂-IIA. H48Q was ineffective at concentrations where sPLA₂-IIA stimulates AA mobilization (Fig. 4B), confirming that sPLA₂-IIA enzyme activity mediates the response.

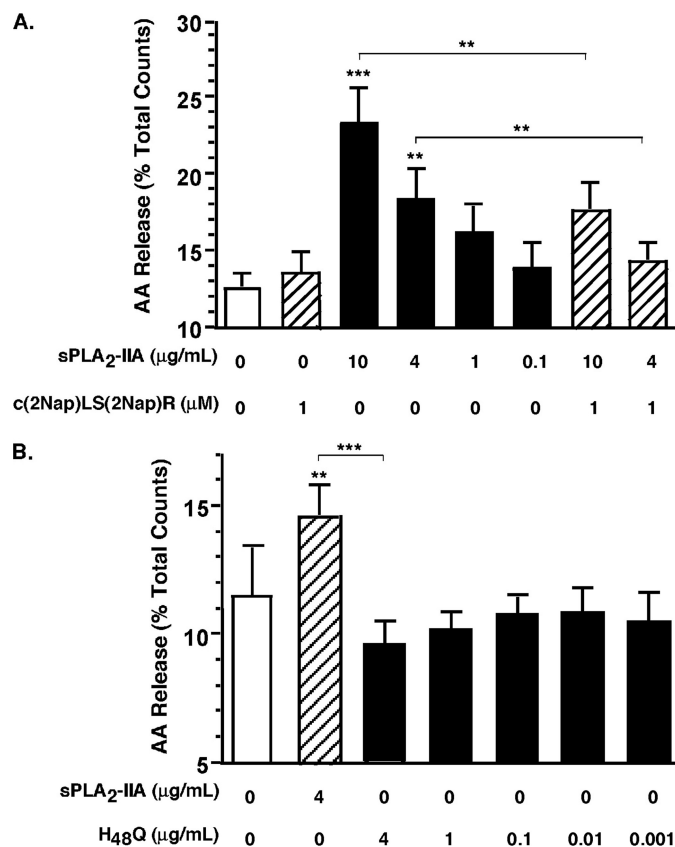


FIGURE 4. sPLA₂-IIA-dependent AA mobilization requires sPLA₂-IIA enzyme activity. FLSs were labeled with [³H]AA, incubated for 2 h: A, in the presence or absence of the sPLA₂-IIA inhibitor c(2Nap)LS(2Nap)R (C2), at 1 μM concentration (26) and in the presence or absence of sPLA₂-IIA as indicated; or B, in the presence or absence of sPLA₂-IIA or the activity-impaired mutant H48Q. AA release was measured as described under "Experimental Procedures." Data are mean ± S.E. of duplicate determinations from three to four cell cultures derived from separate patients and are representative of two independent experiments. **, *p* < 0.01; ***, *p* < 0.001 (Student's unpaired *t* test) relative to unstimulated cells unless indicated. Total radioactivity incorporated into cells ranged from: A, 18,733–40,749 dpm; B, 12,523–35,086 dpm.

FLSs are known to spontaneously release microparticles into culture medium (41), and purified microparticles derived from other cell types, particularly platelets, are known to amplify inflammation in arthritis models (42). It is possible that the AA mobilization measured here could reflect microparticle release from FLSs in addition to free AA. To evaluate this possibility, we examined the distribution of tritium in phospholipid (PL), arachidonic acid, and other lipid mediator fractions of conditioned medium derived from labeled cells following 2 h stimulation, by thin layer chromatography (TLC). More than half (63%) of the tritium released into supernatants of resting labeled cells remained associated with phospholipids on TLC (Table 1), whereas 20% coeluted with AA. The remaining 17% was evenly distributed between these fractions. TNF stimulation showed no change in the distribution of tritium in phospholipids and a trend to increased tritium in AA. In the presence of sPLA₂-IIA, the distribution of AA in phospholipids was significantly reduced relative to unstimulated cells with a trend to increased tritium distributed evenly between AA and other lipid mediators. This distribution pat-

TABLE 1

Distribution of ³H in supernatants of FLSs as measured by thin layer chromatography

Sample	³ H in TLC fractions (% total ³ H)		
	PL ($R_f < 0.07$)	AA ($R_f > 0.81$)	Other ($0.07 \leq R_f \leq 0.81$)
[³ H]AA control ^a	4.6 ± 0.2 ^b	86 ± 1	9.6 ± 0.8
No stimulus control	63 ± 5	20 ± 9	17 ± 8
TNF	52 ± 5	39 ± 6	8 ± 2
sPLA ₂ -IIA	16 ± 1 ^c	43 ± 17	41 ± 16
TNF + sPLA ₂ -IIA	15 ± 2 ^c	60 ± 18	25 ± 16

^a Purified [³H]AA standard. Total ³H in sample ~6800 dpm for each experiment.^b Data are mean ± S.E. of data from three separate experiments. In the case of FLSs, data represent 3 independent FLS cultures. Total ³H in FLS samples ranged from 1169 to 2781 dpm.^c $p < 0.001$ relative to "no stimulus" control (two-way analysis of variance using data from cell culture experiments, Bonferroni's multiple comparison test).

tern was largely recapitulated in cells stimulated with TNF + sPLA₂-IIA (Table 1).

Effect of sPLA₂-IIA on NF-κB Mobilization, p38 MAPK, and ERK MAPK Activation—Enhanced TNF-dependent PGE₂ production in the presence of sPLA₂-IIA appears to result from induction of COX-2 rather than sPLA₂-IIA-mediated increased AA flux through the COX-2 pathway. COX-2 protein induction in FLSs is regulated in response to certain agonists at the level of transcription via NF-κB activation (43), ERK MAPK activation (44), and/or post-transcriptionally through regulation of mRNA stability that requires phosphorylation of the MAPK p38 (45). To determine whether sPLA₂-IIA was activating these pathways in FLSs, the effect of exogenous enzyme on rapid activation of the NF-κB pathway, p38, and ERK phosphorylation was determined. An EMSA was established to measure direct binding of nuclear proteins to a consensus NF-κB DNA binding sequence. Supershift assays with anti-p65 and anti-p50 Abs (Fig. 5A) and competition experiments with cold binding sequence (data not shown) demonstrated the specificity of this assay for NF-κB subunits. Unlike TNF, sPLA₂-IIA alone had no effect on NF-κB binding to DNA (Fig. 5A), the mobilization of NF-κB subunits in the cytoplasm as measured by IκB-α degradation (Fig. 5B), or NF-κB subunit accumulation in the nucleus (Fig. 5C). Furthermore, sPLA₂-IIA in combination with TNF had no additional effect over stimulation with TNF alone in these assays. The potent and selective sPLA₂ enzyme activity inhibitor LY311727, at a concentration (10 μM) that blocks enzyme activity and also blocks COX-2 up-regulation in FLSs (17), did not modulate NF-κB mobilization by TNF/sPLA₂-IIA (Fig. 5). In addition, sPLA₂-IIA alone, again unlike TNF, did not induce p38 phosphorylation (Fig. 6A), nor did it modulate TNF-dependent phosphorylation. As with NF-κB mobilization, blockade of sPLA₂-IIA with LY311727 had no effect on p38 phosphorylation in the presence of TNF/sPLA₂-IIA (Fig. 6A). Unstimulated FLSs show significant basal ERK activation that was further stimulated by treatment with sPLA₂-IIA or TNF alone (Fig. 6B). However, sPLA₂-IIA did not augment TNF-dependent ERK phosphorylation, despite TNF being at a low (submaximal) concentration (50 pg/ml). In contrast to its effect of abrogating COX-2 up-regulation (18), LY311727 had no effect on TNF/sPLA₂-IIA-mediated ERK activation (Fig. 6B).

DISCUSSION

These data establish for the first time in cultured cells relevant to the pathogenesis of RA that the regulation of TNF-dependent PG production by exogenous sPLA₂-IIA does not depend on its enzyme function. sPLA₂-IIA mutant, H48Q, which retains only 1% of sPLA₂-IIA enzyme activity, is as effective as the fully functional enzyme in up-regulating PGE₂ production and in superinducing TNF-mediated COX-2 production (Fig. 1). H48Q alone up-regulates the production of COX-2 without increasing PGE₂ production (Fig. 1B), as does sPLA₂-IIA (18). It is very unlikely that the sPLA₂-IIA and H48Q effects are mediated by low-level contaminants because the effects we measure on PGE₂ production and COX-2 up-regulation by sPLA₂-IIA are completely abrogated by LY311727 (10 μM) (18), indicating that they are intrinsic to sPLA₂-IIA. Importantly, our earlier study also showed that the augmentation of TNF-induced PGE₂ production by wild-type sPLA₂-IIA depends on the amount of sPLA₂-IIA added. For example, addition of 1 μg/ml of sPLA₂-IIA together with TNF led to only 50% as much PGE₂ production as did addition of 10 μg/ml of sPLA₂-IIA together with TNF (18). Our observations that 4 μg/ml of H48Q gives the same level of PGE₂ production as 4 μg/ml of wild-type sPLA₂-IIA (Fig. 1A) and that the H48Q response is dose-dependent, shows that the augmentation of PGE₂ production is not due to the residual 1% enzymatic activity of H48Q. We have recently shown⁴ that complete abrogation of sPLA₂-IIA enzyme activity with the covalent active site modifier bromophenacylbromide also did not affect PGE₂ production. Although these findings may appear to conflict with our observation that LY311727 (a potent inhibitor of sPLA₂-IIA catalytic activity) also blocks PGE₂ production and COX-2 up-regulation (18), they argue that LY311727 acts as a dual-function sPLA₂-IIA inhibitor. Our finding is consistent with other observations that LY311727 can inhibit other catalytic activity-independent functions of sPLA₂-IIA such as M-type receptor binding (46). Interestingly, the cyclic peptide inhibitor c2, demonstrated to block AA mobilization here, also blocks PGE₂ production in FLS (26), indicating that it is a dual-function sPLA₂-IIA inhibitor also.

Our studies provide important and unexpected insights into the regulation of the AA metabolism in RA FLSs. First (Fig. 7), AA mobilization in resting FLSs appears high (10–15% over 2 h) in comparison to other resting cell lines (1–2%) (47). Basal AA mobilization is partially suppressible by inhibitors of cPLA₂-α, (Fig. 3C), but not by inhibitors of sPLA₂-IIA (Fig. 4A) or iPLA₂-β (data not shown). Analysis of the distribution of tritium in conditioned medium from these cells (Table 1) suggests that the majority of mobilized AA remains esterified in phospholipids. The apparently high basal AA mobilization is thus likely due to microparticle release. The 20% of tritium coeluting with AA correlates well with the proportion of AA mobilization that is suppressible by cPLA₂-α inhibition (Fig. 3C) suggesting that basal AA release is likely

⁴ L. Lee, P.-W. Lei, K. J. Bryant, E. P. Huang, S. Harrop, P. M. Curmi, A. P. Duff, W. B. Church, and K. F. Scott, manuscript in preparation.

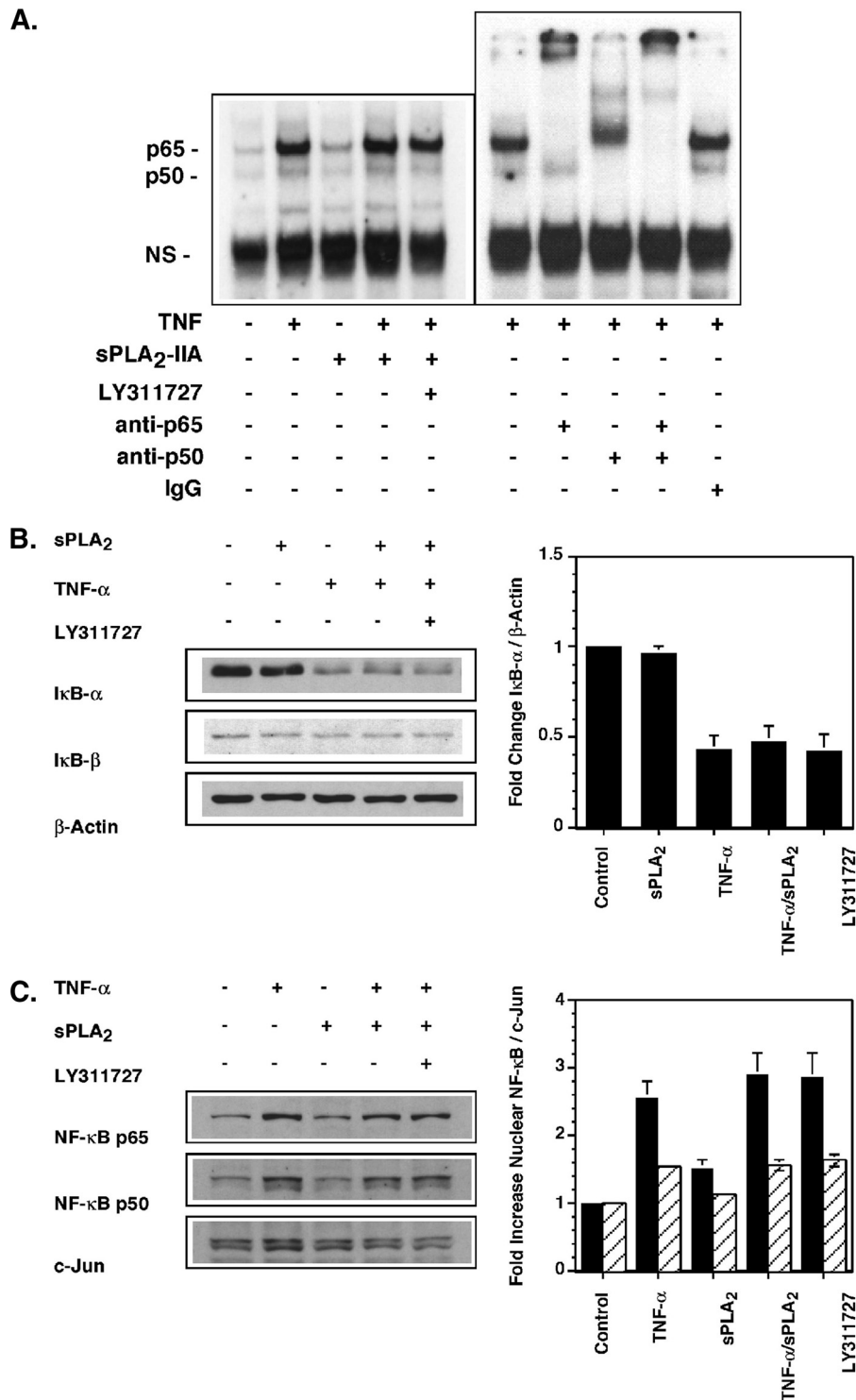


FIGURE 5. sPLA₂-IIA does not activate or enhance TNF activation of the NF- κ B pathway in RSF. *A*, EMSA. Single flasks of 90% confluent FLSs were stimulated for 1 h in DMEM/Ham's F-12 containing 0.1% BSA with TNF (10 pg/ml), sPLA₂-IIA (5 μ g/ml), TNF/sPLA₂-IIA or TNF/sPLA₂-IIA with LY311727 (10 μ M). Nuclear protein extracts were prepared and binding to a radiolabeled NF- κ B consensus binding sequence was determined by EMSA as described under "Experimental Procedures." Binding specificity to detected bands was confirmed by supershift assays with anti-p65 or anti-p50 Abs relative to an isotype-matched control Ab in extracts of TNF-stimulated cells as indicated. *B*, I κ B α degradation. Single flasks of 90% confluent FLSs ($n = 3$) in DMEM/Ham's F-12 containing 0.1% BSA were stimulated for 15 min with sPLA₂-IIA (5 μ g/ml), TNF (50 pg/ml), TNF/sPLA₂-IIA or TNF/sPLA₂-IIA with LY311727 (10 μ M). Total cell lysates were prepared. I κ B α , I κ B β , and β -actin protein were detected by Western blot analysis. The ratio of I κ B α to β -actin protein was quantified by densitometry and is normalized to the ratio measured in unstimulated cells. Data are mean \pm S.E. ($n = 3$). *C*, nuclear NF- κ B p65 and p50 protein in FLSs. Single flasks of 90% confluent RSF ($n = 3$) in DMEM/Ham's F-12 containing 0.1% BSA were stimulated for 1 h with TNF (10 pg/ml), sPLA₂-IIA (5 μ g/ml), TNF/sPLA₂-IIA or TNF/sPLA₂-IIA with LY311727 (10 μ M). Nuclear protein extracts were prepared. Nuclear c-Jun, NF- κ B p65 and p50 protein were detected by Western blot analysis. The ratio of nuclear NF- κ B p65 and p50 to c-Jun protein was quantified by densitometry and normalized to the ratio measured in unstimulated cells. Data are mean \pm S.E. ($n = 3$ independent FLS cultures).

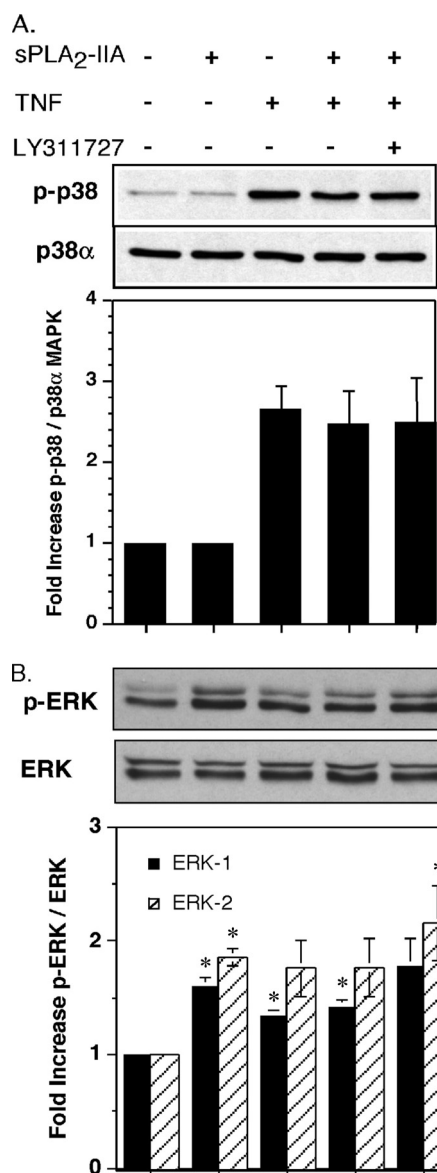


FIGURE 6. sPLA₂-IIA does not activate p38 MAP kinase in FLSs but activates ERK. Single flasks of 90% confluent FLSs ($n = 3$) in DMEM/Ham's F-12 containing 0.1% BSA were stimulated for 15 min with sPLA₂-IIA (5 μ g/ml), TNF (50 pg/ml), TNF/sPLA₂-IIA or TNF/sPLA₂-IIA with LY311727 (10 μ M). Total cell lysates were prepared, and *A*, phosphorylated p38 and total p38 α MAP kinase protein, or *B*, p-ERK and total ERK were detected by Western blot analysis as described under "Experimental Procedures." Representative Western blots and the ratio of phospho- to total MAP kinase protein normalized relative to unstimulated cells is shown. Data are mean \pm S.E. ($n = 3$ independent FLS cultures). Control phospho-MAPK/total MAPK ratios varied between cultures from 0.281 to 0.455 for p38, 0.432 to 0.671 for ERK-1, and 0.454 to 0.831 for ERK-2 (*, $p < 0.05$ relative to control, Student's paired t test).

cPLA₂- α -dependent. Under resting conditions, a small amount of PGE₂ production is detectable, which is not suppressible by COX-2 selective inhibitors (18), suggesting PGE₂ production likely couples to COX-1 in these circumstances.

Second (Fig. 7), AA mobilization following stimulation with TNF or BK is dependent on enhanced cPLA₂- α activity because pyrrophenone blocks agonist-induced AA mobilization (Fig. 3C). Thus, TNF alone, although inefficient at rapidly mobilizing calcium in most cells, activates cPLA₂- α , probably via enhancing cPLA₂- α phosphorylation, as established in other

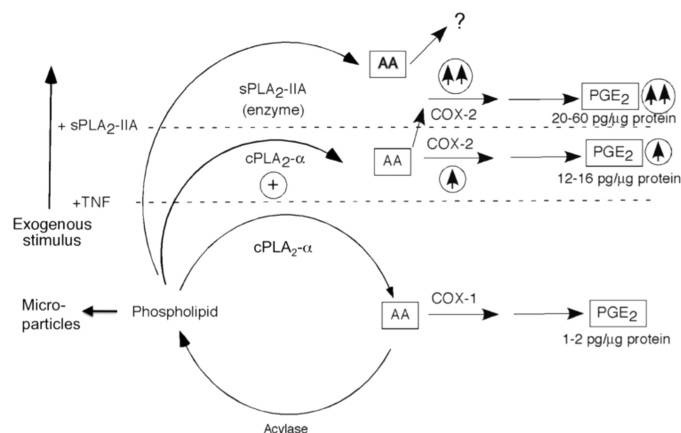


FIGURE 7. Model of sPLA₂-IIA function in FLS AA metabolism. Unstimulated cells, AA is mobilized in microparticles esterified to phospholipids. Low level AA release is mediated by cPLA₂- α and is reincorporated into phospholipid pools by acylase(s). AA flux to PGE₂ is very low and is via COX-1. TNF-stimulated cells, TNF. cPLA₂- α is activated (\oplus in circle), stimulating AA flux through the COX-2 pathway to stimulate PGE₂ production. TNF + sPLA₂-IIA, sPLA₂-IIA enzyme activity increases AA mobilization that is not coupled to PGE₂ production. sPLA₂-IIA-mediated signaling superinduces COX-2 (\uparrow in circle) resulting in increased cPLA₂- α -dependent AA flux through COX-2 to PGE₂. sPLA₂-IIA alone increases AA release and induces COX-2 without increasing PGE₂ production. The figure is modeled after Fitzpatrick and Soberman (51).

model cell lines (2). cPLA₂- α activation results in an \sim 50% increase over basal in AA mobilization (Fig. 3C) with this increase likely distributed to AA, because no change is seen in the distribution of tritium in phospholipid relative to unstimulated cells (Table 1). Increased steady-state levels of COX-2 protein (Figs. 1B and 2C) also contribute to the 8–10-fold increase in COX-2-dependent PGE₂ production (Figs. 1A and 2B) seen on TNF stimulation.

Third (Fig. 7), increased AA mobilization by sPLA₂-IIA (Figs. 3C and 4) is mediated directly by its enzyme function not by cPLA₂- α . H48Q fails to mobilize AA (Fig. 4B) and pyrrophenone does not block the effect (Fig. 3C). Importantly the distribution of mobilized tritium esterified in phospholipids is significantly lower in sPLA₂-IIA-stimulated cells than that seen in unstimulated cells, indicating that exogenous sPLA₂-IIA mobilizes AA from microparticle phospholipid pools. The trend to increased tritium distribution into other lipid mediators suggests that sPLA₂-IIA-derived AA may be metabolized into eicosanoids. Because sPLA₂-IIA alone does not increase PGE₂ production (Fig. 1A) and the majority of the tritium eluted at R_f values between 0.67 and 0.81 (data not shown), these data are consistent with the metabolites being hydroxyeicosatetraenoic acids, although further work is necessary to confirm this. Importantly, increased AA mobilization in FLSs requires concentrations of exogenous sPLA₂-IIA above 70 nM (1 μ g/ml), 7–70-fold higher than the concentrations required for activity-dependent enhanced proliferation in prostate cancer cells (37). Exogenous sPLA₂-IIA is not as potent in AA mobilization from resting cells as some other sPLA₂ forms present in RA synovial tissue, notably Group X sPLA₂ (38). However, effective sPLA₂-IIA concentrations are within the range of concentrations measured in RA synovial fluids suggesting that sPLA₂-IIA may contribute to AA mobi-

lization in pathological conditions such as RA, where enzyme concentrations are high.

FLSs appear more sensitive to AA mobilization by exogenous sPLA₂-IIA than other attachment-dependent cells. No detectable AA release was observed in CHO cells (38) or HEK 293 cells (47) at sPLA₂-IIA concentrations up to 1 or 10 μg/ml, respectively, even following 6 h stimulation. Thus, FLSs have a stable metabolic “phenotype” not seen in other attachment-dependent cells that allows exogenous sPLA₂-IIA-mediated AA mobilization. Although it is known that microparticle membranes express phosphatidylserine on their surface, the structural characteristics of FLS membranes have not yet been studied in detail, so whether membrane lipid asymmetry has been stably altered in FLSs remains to be determined.

AA mobilized by sPLA₂-IIA, either alone or in combination with TNF, is not utilized for PGE₂ production, despite the induction of COX-2. In the case of sPLA₂-IIA stimulation alone, no increased PGE₂ production is observed (Fig. 1A) and in the case of costimulation with TNF, abrogation of sPLA₂-IIA enzyme activity by mutagenesis does not affect PGE₂ production (Fig. 1A) and all of the observed increase in PGE₂ production is suppressible by cPLA₂-α inhibitors (Fig. 2, A and B). Thus under all conditions examined, only cPLA₂-α activation can account for AA flux to PGE₂.

The functional coupling of cPLA₂-α and COX-2, also seen in other cell lines (48), is particularly striking in FLSs: despite effectively blocking TNF or bradykinin-mediated AA mobilization, inhibition of cPLA₂-α in the presence of both TNF and sPLA₂-IIA has no significant effect on AA mobilization (Fig. 3C), although effectively blocking all PGE₂ production (Fig. 2). Under these conditions, over 20% of incorporated AA is mobilized from cells, yet only a very small proportion of released AA contributes to PGE₂ production, all of it generated by cPLA₂-α activity. Exogenous sPLA₂-IIA is thus not functionally coupled to the COX pathway as has been commonly proposed (49–51), but rather indirectly regulates PG production pathways in these cells (Fig. 7). Our data also argue against regulation of cPLA₂-α activity by sPLA₂-IIA as has been found with other cell types (52), because sPLA₂-IIA alone is unable to induce PGE₂ production, despite up-regulating COX-2. In the presence of sPLA₂-IIA alone, provision of AA to COX-2 by cPLA₂-α is the rate-limiting step in PGE₂ production. In contrast, in the presence of TNF, PGE₂ production is limited by the amount of COX-2. Thus the rate-limiting step in the pathway may be either cPLA₂-α or COX-2 depending on the cellular context.

Fourth (Fig. 7), the contribution of sPLA₂-IIA to the 10–60-fold increased PGE₂ production over basal levels seen on costimulation with TNF can be fully explained by enzyme activity-independent superinduction of the steady state levels of COX-2. The H48Q mutation does not affect the ability of sPLA₂-IIA to induce COX-2 (Fig. 1B). As with NS-398 (18), concentrations of pyrrophenone that completely suppress PGE₂ production do not affect the induction of COX-2 by sPLA₂-IIA (Fig. 2C). It follows then, that cPLA₂-α or sPLA₂-IIA-derived AA or its metabolites do not regulate COX-2 protein levels in sPLA₂-IIA-stimulated RA FLSs. This is in contrast to COX-2 up-regulation by IL-15 (53) or by certain

agonists in other cell types (54, 55) whereby stimulus-induced PGE₂ further up-regulates COX-2.

Although exogenous sPLA₂-IIA up-regulates COX-2 protein in some model cell lines, the effect is cell-type specific (50), and apart from one case, nerve growth factor-stimulated rat serosal mast cells, where up-regulation also appears independent of enzyme activity (56), the mechanism is unknown. We have ruled out rapid activation of NF-κB or p38 MAPK by sPLA₂-IIA, two pathways known to mediate agonist-dependent COX-2 up-regulation in FLSs (43, 57); sPLA₂-IIA alone activates the ERK MAPK pathway. Although modest, ERK activation demonstrates that sPLA₂-IIA regulates intracellular signaling and suggests broader effects on RA FLS function than the regulation of AA metabolism alone. In addition, this finding together with our observation that sPLA₂-IIA, in the absence of cytokine stimulation, does not induce PGE₂ production (Fig. 1A) (18), indicates that sPLA₂-IIA-mediated ERK activation alone is insufficient to stimulate prostaglandin production in these cells, despite induction of COX-2. In our hands, MEK inhibitors PD98059 and UO126, whereas completely suppressing TNF/sPLA₂-IIA-mediated PGE₂ production did not suppress COX-2 induction (data not shown), suggesting that blockade of the ERK pathway alone is insufficient to affect COX-2 induction under these conditions. In contrast, we have previously shown that blockade of sPLA₂-IIA function with LY311727 is sufficient to suppress both PGE₂ production and COX-2 induction in the presence of TNF (18), yet LY311727 is unable to suppress ERK phosphorylation under these conditions (Fig. 6B). It is thus likely that TNF is sufficient to stimulate ERK and that sPLA₂-IIA effects on PGE₂ production and COX-2 expression occur “downstream” of ERK activation. However, the importance of sPLA₂-IIA-mediated ERK activation in COX-2 up-regulation remains to be determined.

Our data predict that sPLA₂-IIA induces COX-2 expression via an indirect signaling mechanism mediated through direct interaction with a cellular component(s). The identity of this component(s) in RA FLSs is unknown at present, however, in our hands, immunofluorescence studies demonstrate that exogenous sPLA₂-IIA binds to the RA FLS cell surface and is very rapidly (within seconds) internalized demonstrating that the enzyme does bind to FLS cellular components.⁴ Receptor-mediated sPLA₂ function has been best established for Group IB sPLA₂ in mice using both biochemical and genetic approaches (58, 59). Murine Group IB sPLA₂ and sPLA₂-IIA both bind the murine 180-kDa M-type sPLA₂ receptor with high affinity (1–10 nM) (59). However, human sPLA₂-IIA is reported to have a binding affinity for the human M-type receptor that is too weak for sPLA₂-IIA to be a physiological ligand in human cells (60). An alternative model is that sPLA₂-IIA is internalized via binding to heparan sulfate proteoglycans, particularly glypican-1 in caveolae, followed by subsequent AA release and/or up-regulation of COX-2 (50, 61). It has been reported that sPLA₂-IIA localizes to caveolin-containing vesicles as well as the Golgi apparatus in one “normal” synovial cell line following adenoviral transfection with the sPLA₂-IIA cDNA, suggesting that this internalization pathway may be operative in these cells (17). However, there

is no evidence that perturbation of this pathway has any effect on PGE₂ production. Infection of FLs with adenoviral vectors alone induces both COX-2 and PGE₂ production (44) further complicating the interpretation of viral overexpression approaches.

In summary, our data show that human sPLA₂-IIA, when added with TNF to RA FLs, results in enhanced PGE₂ production that does not require the enzyme activity of sPLA₂-IIA. This finding, coupled with recent findings that sPLA₂-IIA can participate in intracellular AA release when stably expressed at lower concentrations than those required exogenously (23, 47) and that some indole inhibitors are cell impermeable (47) and therefore incapable of blocking intracellular effects, suggest that clinical studies with inhibitors that are known to be both cell permeable and to potentially block sPLA₂-IIA-dependent signaling, may show greater benefit in the treatment of RA.

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