

## A BIFUNCTIONAL ROLE FOR GROUP IIA SECRETED PHOSPHOLIPASE A<sub>2</sub> IN HUMAN RHEUMATOID FIBROBLAST-LIKE SYNOVIOCYTE ARACHIDONIC ACID METABOLISM.

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Human group IIA secreted phospholipase A<sub>2</sub> (sPLA<sub>2</sub>-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 sPLA<sub>2</sub>-IIA inhibitors shows only transient benefit. Using an activity-impaired sPLA<sub>2</sub>-IIA mutant protein (H<sub>48</sub>Q), we show that upregulation of TNF-dependent PGE<sub>2</sub> production and cyclooxygenase-2 (COX-2) induction by exogenous sPLA<sub>2</sub>-IIA in RA fibroblast-like synoviocytes (FLSs) is independent of its enzyme function. Selective cytosolic phospholipase A<sub>2</sub>- $\alpha$  (cPLA<sub>2</sub>- $\alpha$ ) inhibitors abrogate TNF/sPLA<sub>2</sub>-IIA-mediated PGE<sub>2</sub> production without affecting COX-2 levels, indicating arachidonic acid (AA) flux to COX-2 occurs exclusively through TNF-mediated activation of cPLA<sub>2</sub>- $\alpha$ . Nonetheless, exogenous sPLA<sub>2</sub>-IIA, but not H<sub>48</sub>Q, stimulates both AA mobilization from FLSs and microparticle-derived AA release that is not used for COX-2-dependent PGE<sub>2</sub> production. sPLA<sub>2</sub>-IIA-mediated AA production is inhibited by pharmacological blockade of sPLA<sub>2</sub>-IIA but not cPLA<sub>2</sub>- $\alpha$ . Exogenous H<sub>48</sub>Q alone, like sPLA<sub>2</sub>-IIA, increases COX-2 protein levels without inducing PGE<sub>2</sub> production. Unlike TNF, sPLA<sub>2</sub>-IIA alone, does not rapidly mobilize NF- $\kappa$ B or activate phosphorylation of p38 MAPK, two key regulators of COX-2 protein expression, but does activate the

ERK1/2 pathway. Thus, sPLA<sub>2</sub>-IIA regulates AA flux through the cPLA<sub>2</sub>- $\alpha$  /COX-2 pathway in RA FLSs by upregulating 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 sPLA<sub>2</sub>-IIA.

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) enzymes regulate the provision of arachidonic acid (AA) to the cyclooxygenase (COX) and lipoxygenase (LOX) 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 PLA<sub>2</sub> enzymes, *in vivo* gene deletion studies in mice have established the widely-expressed intracellular enzyme cytosolic PLA<sub>2</sub>- $\alpha$  (cPLA<sub>2</sub>- $\alpha$ ) as an important enzyme in providing AA substrate to COX and LOX because deletion of this gene product abrogates eicosanoid production in cells stimulated *ex vivo* (1, 2). Significantly, cPLA<sub>2</sub>- $\alpha$  gene deletion markedly reduces disease severity in the collagen-induced arthritis model of rheumatoid arthritis (RA), suggesting cPLA<sub>2</sub>- $\alpha$  has a key role in the pathogenesis of RA (2).

The contribution of the remaining 18 PLA<sub>2</sub> enzymes to AA metabolism and to immune-mediated, inflammatory pathology is less clear.

Macrophages from Group V sPLA<sub>2</sub> (sPLA<sub>2</sub>-V)-deficient mice show impaired production of both COX- and LOX-derived eicosanoid products in response to the inflammatory stimulus zymosan (3), while deletion of Group X sPLA<sub>2</sub> (sPLA<sub>2</sub>-X) results in impaired eicosanoid release into the lungs following ovalbumin challenge (4). sPLA<sub>2</sub>-IIA, the best-studied of the 10 mammalian secreted PLA<sub>2</sub> enzymes, is not expressed in certain mouse strains with restricted expression in others compared to either rats or humans (5, 6), making classical genetic deletion experiments impractical for this enzyme. Despite this, a proinflammatory role for sPLA<sub>2</sub>-IIA in arthritis has been confirmed by recent genetic studies showing that arthritis is attenuated in sPLA<sub>2</sub>-IIA knockout mice, relative to congenic wild-type mice, in a K/BxN serum transfer model of arthritis (7). Surprisingly, these studies also showed that sPLA<sub>2</sub>-V has an anti-inflammatory role in this model of arthritis (7). Transgenic expression of human sPLA<sub>2</sub>-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 sPLA<sub>2</sub>-IIA leads to earlier onset and more severe arthritis in a TNF transgenic, spontaneous arthritis model (11) implicating aberrant expression of sPLA<sub>2</sub>-IIA as a positive regulator of cytokine-mediated joint inflammation. Further, transgenic expression of human sPLA<sub>2</sub>-IIA in mice results in increased severity in the K/BxN serum transfer arthritis model (7).

sPLA<sub>2</sub>-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 sPLA<sub>2</sub>-IIA correlates with histological markers of inflammation (16) and several other sPLA<sub>2</sub> enzymes are also expressed in RA synovial tissue (17) and synovial fluid (7). Exogenous addition of sPLA<sub>2</sub>-IIA to cultured RA synovial cells, at concentrations found in the synovial fluids of RA patients, enhances both TNF-stimulated PGE<sub>2</sub> production and upregulation of the inducible cyclooxygenase, COX-2 by an unknown

mechanism (18). However, blockade of sPLA<sub>2</sub> enzyme activity with potent inhibitors that blocked, sPLA<sub>2</sub>-IIA, sPLA<sub>2</sub>-V and sPLA<sub>2</sub>-X (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 sPLA<sub>2</sub>-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 sPLA<sub>2</sub>-IIA contributes to AA flux in these cells in culture, exogenous sPLA<sub>2</sub>-IIA-amplified, cytokine-mediated PGE<sub>2</sub> production is sPLA<sub>2</sub>-IIA enzyme activity-independent and is thus mediated by a signaling function of the enzyme that indirectly upregulates levels of the cPLA<sub>2</sub>- $\alpha$ /COX-2 pathway enzymes.

## EXPERIMENTAL PROCEDURES.

*Materials.* sPLA<sub>2</sub>-IIA protein was expressed, purified and quantified as described (18). cPLA<sub>2</sub>- $\alpha$  inhibitors pyrrolidine-1 (20, 21) and pyrrophenone (22, 23) were synthesized as described. Pyrrolidine-1 inhibited purified, recombinant cPLA<sub>2</sub>- $\alpha$  in a vesicle assay with an IC<sub>50</sub> of 70 nM, and AA release in ionomycin-stimulated MDCK cells with an IC<sub>50</sub> of 800 nM. It showed no detectable inhibition of purified, recombinant human sPLA<sub>2</sub>-IIA, -V or -X at 10  $\mu$ M concentration and no physiologically relevant inhibition of recombinant cPLA<sub>2</sub>- $\gamma$  or the calcium independent PLA<sub>2</sub>, iPLA<sub>2</sub> $\beta$  (20). Pyrrophenone inhibited recombinant cPLA<sub>2</sub>- $\alpha$  in a mixed-micelle assay with an IC<sub>50</sub> of 80 nM (24) without significant inhibition of all five remaining human cPLA<sub>2</sub> isoforms in this assay (Gelb, M. H., unpublished). It inhibited AA release and PGE<sub>2</sub> production in ionophore-stimulated THP-1 cells with an IC<sub>50</sub> of 25 nM (25) and had no appreciable inhibition of murine cPLA<sub>2</sub>- $\beta$  (24) or purified, recombinant human sPLA<sub>2</sub>-IB or -IIA at 200  $\mu$ M (25). The sPLA<sub>2</sub> 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 iPLA<sub>2</sub>- $\beta$  inhibitor, bromoenol lactone, was obtained from Sigma (Sydney, Australia).

*Construction of sPLA<sub>2</sub>-IIA catalytic site mutant H<sub>48</sub>Q.* The sPLA<sub>2</sub>-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, Sydney, Australia) based on the method of Eckstein and coworkers (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 H<sub>48</sub>Q and protein was purified from conditioned media by affinity chromatography (AKTA Explorer purification system, GE Healthcare, Sydney, Australia) as described for sPLA<sub>2</sub>-IIA (18), and quantified by ELISA (12).

*sPLA<sub>2</sub> enzyme activity assay.* sPLA<sub>2</sub> enzyme activity was measured with a colorimetric microtitre-plate, mixed-micelle assay (Cayman Chemical, Ann Arbor, MI) using diheptanoylthiophosphatidylcholine as substrate (30) with the following modifications. Briefly, enzyme (10 µL, 2.5 µg/mL, sPLA<sub>2</sub>-IIA or H<sub>48</sub>Q) diluted in assay buffer (10 mM CaCl<sub>2</sub>, 100 mM KCl, 0.3 mM Triton X-100, 1 mg/mL 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) (DTNB) (10 µL, 10 mM DTNB in 0.4 M Tris-HCl, pH 8.0) and 5 µL 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 µL/well) and OD<sub>405</sub> measured every 3 mins over a 60 min. time course (Spectramax 250 microtitre plate reader, Molecular Devices, Sunnyvale CA). Assays were performed in triplicate relative to blank wells containing assay buffer and data was analyzed using SoftMax Pro v1.1 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 passage three and ten. Cells, CD14-negative and 4-prolylhydroxylase-positive by immunohistochemistry and CD21-negative by RT-PCR, were grown in Hams/DMEM media containing 10 % FBS and used at 80-90% confluence.

*PGE<sub>2</sub> assay.* Cells, grown in 96 well plates were stimulated, media harvested and stored at -80°C prior to PGE<sub>2</sub> 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 µg/mL 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, Hercules, CA). PGE<sub>2</sub> in media was determined by enzyme-immunoassay (Cayman Chemical) as previously described (18) and expressed as pg PGE<sub>2</sub>/mg total cellular protein.

*Nuclear Protein Extracts.* FLSs, grown in 150 cm<sup>2</sup> flasks, were stimulated and nuclear extracts prepared as described (32) with minor modification as follows. Cells were harvested with trypsin/EDTA, centrifuged (4000 x g, 1 min.), supernatants discarded, cell pellets 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 MgCl<sub>2</sub>, 100 mM EDTA, 1 mM DTT, 1 µM PMSF, 100 µg/mL aprotinin, 100 mg/mL 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 sec, centrifuged (20 sec, 13,790 x g) and supernatants discarded. Pellets were washed gently with ice-cold buffer A (150 µL), centrifuged and supernatants discarded. Buffer C (40 µL, 420 mM KCl, 1.5 mM MgCl<sub>2</sub>, 100 mM EDTA, 1 mM DTT, 1 mM PMSF, 100 µg/mL aprotinin, 100 µg/mL leupeptin, 10 mM HEPES, pH 7.9) was added, samples were vortexed (10 secs) and incubated

with orbital shaking for 30 min on ice. Samples were centrifuged (13,790 x g, 15 min, 4°C), supernatants 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, media harvested and stored at -80°C. Cells were lysed, triplicate wells combined and protein determinations made as described above. For phospho-protein determination experiments, cells were grown in 75 cm<sup>2</sup> 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 μg/mL 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 μg/well) was electrophoresed on polyacrylamide gels (4-20% Bis-Tris gels, Novex) according to the manufacturer's instructions and transferred to nitrocellulose as described (18). Primary Abs for cPLA<sub>2</sub>-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, #sc-822) was used at 0.2 μg/mL and detected with sheep anti-mouse IgG-horseradish peroxidase (HRP) (GE Healthcare, 1/3000). Rabbit polyclonal Abs were used at the following concentrations: anti-human IκB-α (Santa Cruz, #sc-371), 10 ng/mL; anti-mouse IκB-β (Santa Cruz, #sc-945), 400 ng/mL; anti-human NF-κB p50 (Santa Cruz, #sc-114), 4 μg/mL; anti-human NF-κB p65 (Santa Cruz, #sc-109), 1 μg/mL; anti-human phospho-p38 MAP kinase Ab (phospho-Thr<sup>180</sup>/phospho-Tyr<sup>182</sup>, New England Biolabs, #9211), 1/1000 dilution; anti-human p38α MAP kinase (Santa Cruz, #sc-535), 33 ng/mL, anti-human phospho-ERK (phospho-Thr<sup>202</sup>/phosphoTyr<sup>204</sup>) (Cell Signalling Technologies #9101), 1/10,000 dilution; anti-rat ERK1 (Santa Cruz #sc-94, ERK-2 cross-reactive), 10 ng/mL. These Abs were detected with donkey anti-rabbit-IgG-horse radish

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 quantified with IPLabelH software (Macintosh v1.5g).

*NF-κB gel shift assays.* NF-κB binding to a double-stranded consensus NF-κB binding site oligonucleotide with top-strand sequence (5'-AGTTGAGGGGACTTTCCAGGC-3'), 5'-end-labeled with [ $\gamma$ -<sup>32</sup>P]-ATP (GE Healthcare) and T4 polynucleotide kinase (Promega), was measured by EMSA as described (33). Briefly, nuclear extracts (~5 μg total protein) prepared as described above, were added to a binding reaction (20 μL) containing (polydeoxyinosine (dI)-deoxycytidine (dC))(polydI-dC) (GE Healthcare) at 0.25 μg/μg total protein, 1-2 ng <sup>32</sup>P-labelled 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 x Tris-borate EDTA (TBE) buffer, pH 8.3 at 150 V for 2-3 hrs. Gels were dried and bands imaged with X-ray film (X-Omat-AR, Kodak, Sydney, Australia). NF-κB EMSA bands were confirmed by NF-κB cold-competitor studies and supershift of NF-κB EMSA bands, using anti-p65 and anti-p50 Abs, performed on nuclear extracts from TNF-α stimulated FLSs.

*AA mobilization assays.* Cells were grown in 96 well plates and labeled with [<sup>5,6,8,9,11,12,14,15-<sup>3</sup>H(N)]-AA (Perkin Elmer NET-298Z, Wellesley, MA) in Hams/DMEM media containing 10 % FBS for 16 hrs at 37°C. Cells were washed 3 times in PBS containing 0.1% (w/v) BSA (Fatty acid free, Sigma-Aldrich, Sydney, Australia) and stimulated for indicated times in Hams/DMEM media containing 0.1% (w/v) BSA. Treatments were added simultaneously with stimulants. Media was harvested, cells were detached with trypsin/EDTA and tritium was determined in media and cells by scintillation counting (LS6000 TA, Beckman, Sydney, Australia). Data are</sup>

expressed as % total radioactivity (cells plus media) released into medium.

*Thin layer chromatography.* Samples (50  $\mu$ L), to which unlabelled AA (1  $\mu$ g) had been added, were spotted on to 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 vapour to identify the AA spot. Eluted samples were cut into seven equal segments in order of increasing  $R_f$  value and tritium 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 V4.0. Statistical significance was determined using the Student's paired t-test unless otherwise stated.

## RESULTS.

*Exogenous sPLA<sub>2</sub>-IIA upregulates TNF-mediated PGE<sub>2</sub> production and COX-2 protein by an activity-independent mechanism.* To determine whether sPLA<sub>2</sub>-IIA enzyme activity was necessary for upregulation of TNF-dependent PGE<sub>2</sub> production and COX-2, we constructed an "activity-impaired" mutant of sPLA<sub>2</sub>-IIA (H<sub>48</sub>Q) by site-directed mutagenesis as described in Materials and Methods. Quantification of H<sub>48</sub>Q was determined relative to a sPLA<sub>2</sub>-IIA standard by ELISA (35). The ELISA assay was validated for H<sub>48</sub>Q by quantitative amino acid analysis of 2 independent samples of sPLA<sub>2</sub>-IIA and H<sub>48</sub>Q, followed by quantitation of each sample in the same ELISA assay. 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 sPLA<sub>2</sub>-IIA and H<sub>48</sub>Q 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 sPLA<sub>2</sub>-IIA and H<sub>48</sub>Q 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 H<sub>48</sub>Q had 1% residual sPLA<sub>2</sub>-IIA activity, with a specific activity of 0.28

$\pm 0.12$   $\mu$ mol diheptanoylthiophosphatidylcholine / min / mg protein (data are mean  $\pm$  SD of three experiments performed in triplicate) relative to  $27.8 \pm 2.3$   $\mu$ mol diheptanoylthiophosphatidylcholine / min / mg protein for sPLA<sub>2</sub>-IIA. Our purified H<sub>48</sub>Q protein lacks the proliferative capacity of purified sPLA<sub>2</sub>-IIA- in the LNCaP prostate cancer cell line (37), confirming that the 1% residual enzyme activity we measure in our H<sub>48</sub>Q preparations is insufficient to recapitulate the effects of fully-active sPLA<sub>2</sub>-IIA in these cells. In FLSs, H<sub>48</sub>Q alone like sPLA<sub>2</sub>-IIA, had no effect on PGE<sub>2</sub> production (Fig. 1A) at enzyme concentrations that are found in synovial fluid (18). TNF alone resulted in a 15-fold stimulation of PGE<sub>2</sub>. H<sub>48</sub>Q increased this stimulation to  $\sim 30$ -fold, as did sPLA<sub>2</sub>-IIA. In both cases PGE<sub>2</sub> production was completely abrogated by the COX-2-selective inhibitor NS-398. In a side-by side experiment with sPLA<sub>2</sub>-IIA, H<sub>48</sub>Q-mediated PGE<sub>2</sub> 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 upregulated steady state COX-2 protein levels (Fig. 1B). H<sub>48</sub>Q alone (Fig. 1B), as with sPLA<sub>2</sub>-IIA (18) also increased COX-2 protein, despite having no effect on PGE<sub>2</sub> production (Fig. 1A). As we have also shown for sPLA<sub>2</sub>-IIA, H<sub>48</sub>Q, in combination with TNF, synergistically upregulated COX-2 protein (Fig. 1B) without any effect on COX-1 (data not shown).

*cPLA<sub>2</sub>- $\alpha$  mediates sPLA<sub>2</sub>-IIA-dependent PGE<sub>2</sub> production but not COX-2 upregulation.*

To determine whether PGE<sub>2</sub> production in response to TNF and sPLA<sub>2</sub>-IIA was dependent on cPLA<sub>2</sub>- $\alpha$ , we used a pharmacological approach with well-characterized pyrrolidine inhibitors that selectively block human cPLA<sub>2</sub>- $\alpha$  activity over other human cPLA<sub>2</sub> isoforms, iPLA<sub>2</sub>- $\beta$  or sPLA<sub>2</sub> activities. Pyrrophenone (5  $\mu$ M) completely blocked the PGE<sub>2</sub> response to TNF alone, substantially suppressed the response to TNF/sPLA<sub>2</sub>-IIA and showed a small but significant inhibition of the sPLA<sub>2</sub>-IIA/TNF response at 1  $\mu$ M (Fig. 2A). Pyrrolidine-1 abrogated PGE<sub>2</sub> production in response to sPLA<sub>2</sub>-IIA/ TNF stimulation at all concentrations tested (Fig. 2B), without any effect on basal PGE<sub>2</sub>

production. However, pyrrophenone, at concentrations that abrogate PGE<sub>2</sub> production, did not significantly block sPLA<sub>2</sub>-IIA-mediated COX-2 upregulation (Fig. 2C). Thus provision of AA to COX-2 for both TNF-dependent and sPLA<sub>2</sub>-IIA-upregulated PGE<sub>2</sub> production appears to be mediated by cPLA<sub>2</sub>- $\alpha$ .

*Effect of sPLA<sub>2</sub>-IIA on AA mobilization.* In light of these data and reports that exogenous sPLA<sub>2</sub>-IIA does not efficiently mobilize AA in attachment-dependent cells in culture (38), the effect of TNF and exogenous sPLA<sub>2</sub>-IIA on AA mobilization was examined by <sup>3</sup>H-AA release assays. First, the assay was validated by examining the response of FLSs to the known AA-mobilizing agonist bradykinin (BK). BK stimulation (10 nM, 15 minutes) resulted in increased <sup>3</sup>H-AA release from 8.0  $\pm$  0.8 % (mean  $\pm$  SE 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 hrs 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 hrs (12.9  $\pm$  1.8 % total counts) with similar kinetics to stimulated cells. Subsequent studies were terminated 2 hrs post-stimulation. Under these conditions, pyrrophenone (5  $\mu$ M) blocked BK-dependent AA release (data not shown).

We next examined the effect of sPLA<sub>2</sub>-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. 3). Basal AA release was unaffected by the calcium-independent Group VIA PLA<sub>2</sub> (iPLA<sub>2</sub>- $\beta$ ) inhibitor, bromoenol lactone (40) (10  $\mu$ 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 PGE<sub>2</sub> production, sPLA<sub>2</sub>-IIA alone increased AA release by 1.5-fold and the increase was not inhibited by pyrrophenone. sPLA<sub>2</sub>-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 sPLA<sub>2</sub>-IIA-mediated AA release and the effect of selective sPLA<sub>2</sub> inhibition on the response were then determined (Fig. 4A). sPLA<sub>2</sub>-IIA dose-dependently induced AA-release at concentrations above 1  $\mu$ g/mL (71 nM) but was ineffective at 100 ng/mL (7 nM) concentration. Coincubation with the selective sPLA<sub>2</sub> inhibitor c(2NapA)LS(2NapA)R (26) (1  $\mu$ 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 molar ratio of 3.5:1. Because this inhibitor also suppresses the activity-independent functions of exogenous sPLA<sub>2</sub>-IIA, *viz*, sPLA<sub>2</sub>-IIA-mediated upregulation of cytokine-dependent PGE<sub>2</sub> production (26), the effect of H<sub>48</sub>Q on AA release was determined in a side-by-side experiment with sPLA<sub>2</sub>-IIA. H<sub>48</sub>Q was ineffective at concentrations where sPLA<sub>2</sub>-IIA stimulates AA mobilization (Fig. 4B), confirming that sPLA<sub>2</sub>-IIA enzyme activity mediates the response.

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, arachidonic acid and other lipid mediator fractions of conditioned media derived from labeled cells following 2 hours 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 I), while 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 sPLA<sub>2</sub>-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 pattern was largely recapitulated in cells stimulated with TNF + sPLA<sub>2</sub>-IIA (Table I).

*Effect of sPLA<sub>2</sub>-IIA on NF- $\kappa$ B mobilization, p38 MAPK and ERK MAPK activation.* Enhanced

TNF-dependent PGE<sub>2</sub> production in the presence of sPLA<sub>2</sub>-IIA appears to result from induction of COX-2 rather than sPLA<sub>2</sub>-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 if sPLA<sub>2</sub>-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 assay 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, sPLA<sub>2</sub>-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). Further, sPLA<sub>2</sub>-IIA in combination with TNF had no additional effect over stimulation with TNF alone in these assays. The potent and selective sPLA<sub>2</sub> enzyme activity inhibitor LY311727, at a concentration (10 μM) that blocks enzyme activity and also blocks COX-2 upregulation in FLSs (17), did not modulate NF-κB mobilization by TNF/sPLA<sub>2</sub>-IIA (Fig. 5). In addition, sPLA<sub>2</sub>-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 sPLA<sub>2</sub>-IIA with LY311727 had no effect on p38 phosphorylation in the presence of TNF/sPLA<sub>2</sub>-IIA (Fig. 6A). Unstimulated FLSs show significant basal ERK activation that was further stimulated by treatment with sPLA<sub>2</sub>-IIA or TNF alone (Fig 6B). However, sPLA<sub>2</sub>-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 upregulation (18), LY311727 had no effect on TNF/sPLA<sub>2</sub>-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 sPLA<sub>2</sub>-IIA does not depend on its enzyme function. sPLA<sub>2</sub>-IIA mutant, H<sub>48</sub>Q, which retains only 1% of sPLA<sub>2</sub>-IIA enzyme activity, is as effective as the fully functional enzyme in upregulating PGE<sub>2</sub> production and in superinducing TNF-mediated COX-2 production (Fig. 1). H<sub>48</sub>Q alone upregulates the production of COX-2 without increasing PGE<sub>2</sub> production (Fig. 1B), as does sPLA<sub>2</sub>-IIA (18). It is very unlikely that the sPLA<sub>2</sub>-IIA and H<sub>48</sub>Q effects are mediated by low-level contaminants because the effects we measure on PGE<sub>2</sub> production and COX-2 upregulation by sPLA<sub>2</sub>-IIA are completely abrogated by LY311727 (10 μM) (18), indicating that they are intrinsic to sPLA<sub>2</sub>-IIA. Importantly, our earlier study also showed that the augmentation of TNF-induced PGE<sub>2</sub> production by wild-type sPLA<sub>2</sub>-IIA depends on the amount of sPLA<sub>2</sub>-IIA added. For example, addition of 1 μg/mL sPLA<sub>2</sub>-IIA together with TNF led to only 50% as much PGE<sub>2</sub> production as did addition of 10 μg/mL sPLA<sub>2</sub>-IIA together with TNF (18). Our observations that 4 μg/mL H<sub>48</sub>Q gives the same level of PGE<sub>2</sub> production as 4 μg/mL wild-type sPLA<sub>2</sub>-IIA (Fig. 1A) and that the H<sub>48</sub>Q response is dose-dependent, shows that the augmentation of PGE<sub>2</sub> production is not due to the residual 1% enzymatic activity of H<sub>48</sub>Q. We have recently shown (Lee *et al*, in preparation) that complete abrogation of sPLA<sub>2</sub>-IIA enzyme activity with the covalent active-site modifier bromophenacylbromide also did not affect PGE<sub>2</sub> production. While these findings may appear to conflict with our observation that LY311727 (a potent inhibitor of sPLA<sub>2</sub>-IIA catalytic activity) also blocks PGE<sub>2</sub> production and COX-2 upregulation (18), they argue that LY311727 acts as a dual-function sPLA<sub>2</sub>-IIA inhibitor. Our finding is consistent with other observations that LY311727 can inhibit other catalytic activity-independent functions of sPLA<sub>2</sub>-IIA such as M-type receptor binding (46). Interestingly, the cyclic peptide inhibitor c2, demonstrated to block AA mobilization here, also

blocks PGE<sub>2</sub> production in FLS (26), indicating that it is a dual-function sPLA<sub>2</sub>-IIA inhibitor also.

Our studies provide important and unexpected insights into the regulation of AA metabolism in RA FLSs. Firstly (Fig 7), AA mobilization in resting FLSs appears high (10-15% over 2 hours) in comparison to other resting cell lines (1-2%) (47). Basal AA mobilization is partially suppressible by inhibitors of cPLA<sub>2</sub>- $\alpha$ , (Fig. 3), but not by inhibitors of sPLA<sub>2</sub>-IIA (Fig. 4A) or iPLA<sub>2</sub>- $\beta$  (data not shown). Analysis of the distribution of tritium in conditioned media from these cells (Table I) 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 cPLA<sub>2</sub>- $\alpha$  inhibition (Fig 3) suggesting that basal AA release is likely cPLA<sub>2</sub>- $\alpha$ -dependent. Under resting conditions, a small amount of PGE<sub>2</sub> production is detectable which is not suppressible by COX-2 selective inhibitors (18), suggesting PGE<sub>2</sub> production likely couples to COX-1 in these circumstances.

Secondly (Fig 7), AA mobilization following stimulation with TNF or BK is dependent on enhanced cPLA<sub>2</sub>- $\alpha$  activity because pyrrophenone blocks agonist-induced AA mobilization (Fig. 3). Thus, TNF alone, though inefficient at rapidly mobilizing calcium in most cells, activates cPLA<sub>2</sub>- $\alpha$ , probably *via* enhancing cPLA<sub>2</sub>- $\alpha$  phosphorylation, as established in other model cell lines (2). cPLA<sub>2</sub>- $\alpha$  activation results in an ~50% increase over basal in AA mobilization (Fig 3) with this increase likely distributed to AA, since no change is seen in the distribution of tritium in phospholipid relative to unstimulated cells (Table I). Increased steady-state levels of COX-2 protein (Fig. 1B, Fig 2C.) also contribute to the 8-10-fold increase in COX-2-dependent PGE<sub>2</sub> production (Fig 1A, Fig 2B) seen on TNF stimulation.

Thirdly (Fig 7), increased AA mobilization by sPLA<sub>2</sub>-IIA (Fig 3C, Fig 4) is mediated directly by its enzyme function not by cPLA<sub>2</sub>- $\alpha$ . H<sub>48</sub>Q 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

sPLA<sub>2</sub>-IIA-stimulated cells than that seen in unstimulated cells, indicating that exogenous sPLA<sub>2</sub>-IIA mobilizes AA from microparticle phospholipid pools. The trend to increased tritium distribution into other lipid mediators suggests that sPLA<sub>2</sub>-IIA derived AA may be metabolized into eicosanoids. Since sPLA<sub>2</sub>-IIA alone does not increase PGE<sub>2</sub> production (Fig 1A) and the majority of the tritium eluted at R<sub>f</sub> values between 0.67 and 0.81 (data not shown) these data are consistent with the metabolites being HETES, although further work is necessary to confirm this. Importantly, increased AA mobilization in FLSs requires concentrations of exogenous sPLA<sub>2</sub>-IIA above 70 nM (1  $\mu$ g/mL), 7-70-fold higher than the concentrations required for activity-dependent enhanced proliferation in prostate cancer cells (37). Exogenous sPLA<sub>2</sub>-IIA is not as potent in AA mobilization from resting cells as some other sPLA<sub>2</sub> forms present in RA synovial tissue, notably sPLA<sub>2</sub>-X (38). However, effective sPLA<sub>2</sub>-IIA concentrations are within the range of concentrations measured in RA synovial fluids suggesting that sPLA<sub>2</sub>-IIA may contribute to AA mobilization in pathological conditions such as RA, where enzyme concentrations are high.

FLSs appear more sensitive to AA mobilization by exogenous sPLA<sub>2</sub>-IIA than other attachment-dependent cells. No detectable AA release was observed in CHO cells (38) or HEK 293 cells (47) at sPLA<sub>2</sub>-IIA concentrations up to 1  $\mu$ g/mL or 10  $\mu$ g/mL respectively even following 6 hrs stimulation. Thus, FLSs have a stable metabolic “phenotype” not seen in other attachment-dependent cells that allows exogenous sPLA<sub>2</sub>-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 sPLA<sub>2</sub>-IIA, either alone or in combination with TNF, is not utilized for PGE<sub>2</sub> production, despite the induction of COX-2. In the case of sPLA<sub>2</sub>-IIA stimulation alone, no increased PGE<sub>2</sub> production is observed (Fig 1A, Fig 2B) and in the case of costimulation with TNF, abrogation of sPLA<sub>2</sub>-IIA enzyme activity by mutagenesis does not affect PGE<sub>2</sub> production (Fig.

1A) and all of the observed increase in PGE<sub>2</sub> production is suppressible by cPLA<sub>2</sub>- $\alpha$  inhibitors (Fig 2A, B). Thus under all conditions examined, only cPLA<sub>2</sub>- $\alpha$  activation can account for AA flux to PGE<sub>2</sub>.

The functional coupling of cPLA<sub>2</sub>- $\alpha$  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 cPLA<sub>2</sub>- $\alpha$  in the presence of both TNF and sPLA<sub>2</sub>-IIA has no significant effect on AA mobilization (Fig. 3C) while effectively blocking all PGE<sub>2</sub> 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 PGE<sub>2</sub> production, all of it generated by cPLA<sub>2</sub>- $\alpha$  activity. Exogenous sPLA<sub>2</sub>-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 cPLA<sub>2</sub>- $\alpha$  activity by sPLA<sub>2</sub>-IIA as has been found with other cell types (52), since sPLA<sub>2</sub>-IIA alone is unable to induce PGE<sub>2</sub> production, despite upregulating COX-2. In the presence of sPLA<sub>2</sub>-IIA alone, provision of AA to COX-2 by cPLA<sub>2</sub>- $\alpha$  is the rate-limiting step in PGE<sub>2</sub> production. In contrast, in the presence of TNF, PGE<sub>2</sub> production is limited by the amount of COX-2. Thus the rate-limiting step in the pathway may be either cPLA<sub>2</sub>- $\alpha$  or COX-2 depending on the cellular context.

Fourthly (Fig 7), the contribution of sPLA<sub>2</sub>-IIA to the 10-60-fold increased PGE<sub>2</sub> 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 H<sub>48</sub>Q mutation does not affect the ability of sPLA<sub>2</sub>-IIA to induce COX-2 (Fig 1B). As with NS398 (18), concentrations of pyrrophenone that completely suppress PGE<sub>2</sub> production, do not affect the induction of COX-2 by sPLA<sub>2</sub>-IIA (Fig. 2C). It follows then, that cPLA<sub>2</sub>- $\alpha$  or sPLA<sub>2</sub>-IIA-derived AA or its metabolites, do not regulate COX-2 protein levels in sPLA<sub>2</sub>-IIA-stimulated RA FLSs. This is in contrast to COX-2 upregulation by IL-15 (53) or by certain agonists in other cell types (54, 55)

whereby stimulus-induced PGE<sub>2</sub> further upregulates COX-2.

Although exogenous sPLA<sub>2</sub>-IIA upregulates 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 upregulation also appears independent of enzyme activity (56), the mechanism is unknown. We have ruled out rapid activation of NF- $\kappa$ B or p38 MAPK by sPLA<sub>2</sub>-IIA, two pathways known to mediate agonist-dependent COX-2 upregulation in FLSs (43, 57); sPLA<sub>2</sub>-IIA alone, activates the ERK MAPK pathway. Though modest, ERK activation demonstrates that sPLA<sub>2</sub>-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 sPLA<sub>2</sub>-IIA, in the absence of cytokine stimulation, does not induce PGE<sub>2</sub> production (Fig 1A, 18), indicates that sPLA<sub>2</sub>-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, while completely suppressing TNF/sPLA<sub>2</sub>-IIA-mediated PGE<sub>2</sub> 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 sPLA<sub>2</sub>-IIA function with LY311727 is sufficient to suppress both PGE<sub>2</sub> 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 sPLA<sub>2</sub>-IIA effects on PGE<sub>2</sub> production and COX-2 expression occur “downstream” of ERK activation. However the importance of sPLA<sub>2</sub>-IIA-mediated ERK activation in COX-2 upregulation remains to be determined.

Our data predict that sPLA<sub>2</sub>-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 sPLA<sub>2</sub>-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 (Lee *et al.*, manuscript in preparation). Receptor-mediated sPLA<sub>2</sub> function has been best established for sPLA<sub>2</sub>-IB in mice using both biochemical and genetic approaches (58, 59). Murine sPLA<sub>2</sub>-IB and sPLA<sub>2</sub>-IIA both bind the murine 180 kD M-type sPLA<sub>2</sub> receptor with high affinity (1-10 nM) (59). However, human sPLA<sub>2</sub>-IIA is reported to have a binding affinity for the human M-type receptor that is too weak for sPLA<sub>2</sub>-IIA to be a physiological ligand in human cells (60). An alternative model is that sPLA<sub>2</sub>-IIA is internalized *via* binding to heparan sulfate proteoglycans, particularly glypican-1 in caveolae, followed by subsequent AA release and/or upregulation of COX-2 (50, 61). It has been reported that sPLA<sub>2</sub>-IIA localizes to caveolin-containing vesicles as well as the Golgi apparatus in one "normal" synovial cell line following adenoviral transfection with the sPLA<sub>2</sub>-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 PGE<sub>2</sub> production. Infection of FLSs with adenoviral vectors alone induces both COX-2 and PGE<sub>2</sub> production (44) further complicating the interpretation of viral overexpression approaches.

In summary our data show that human sPLA<sub>2</sub>-IIA, when added with TNF to RA FLSs, results in enhanced PGE<sub>2</sub> production that does not require the enzyme activity of sPLA<sub>2</sub>-IIA. This finding, coupled with recent findings that sPLA<sub>2</sub>-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 sPLA<sub>2</sub>-IIA-dependent signaling, may show greater benefit in the treatment of RA.

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## FOOTNOTES

We are grateful to Ms Chitra De Silva for excellent assistance with cell culture and to Dr Siiri E. Iismaa for helpful discussions. Quantitative amino acid analysis was performed by the Australian Proteome Analysis Facility (Macquarie University node), Sydney. This work was supported by Grants 980263 [KFS, P. M. Brooks] and 222870 [KFS, Garry G. Graham, HPMcN] from the National Health and Medical Research Council, Grant RG07-17 from the Cancer Council NSW [KFS, Garry G. Graham, Q. Dong, P. J. Russell], a grant from the Rebecca Cooper Foundation [KFS], Australia and National Institutes of Health Grants HL50040 and HL3625 [MHG].

Abbreviations. sPLA<sub>2</sub>-IIA, group IIA secreted phospholipase A<sub>2</sub>; RA, rheumatoid arthritis; COX-2, cyclooxygenase-2; FLS fibroblast-like synoviocyte; cPLA<sub>2</sub>, cytosolic phospholipase A<sub>2</sub>; cPLA<sub>2</sub>-α,

cytosolic phospholipase A<sub>2</sub>- $\alpha$ ; AA, arachidonic acid; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; COX, cyclooxygenase; LOX, lipoxygenase; cPLA<sub>2</sub>- $\gamma$ , cytosolic phospholipase A<sub>2</sub>- $\gamma$ ; iPLA<sub>2</sub> $\beta$ , group VIB calcium-independent phospholipase A<sub>2</sub>; sPLA<sub>2</sub>-IB, group IB secreted phospholipase A<sub>2</sub>; CHO, Chinese hamster ovary.

TABLES.

Table 1. Distribution of  $^3\text{H}$  in supernatants of FLSs as measured by thin layer chromatography.

Sample	$^3\text{H}$ in TLC Fractions (% Total $^3\text{H}$ )		
	PL ( $R_f < 0.07$ )	AA ( $R_f > 0.81$ )	Other ( $0.07 \leq R_f \leq 0.81$ )
$^3\text{H}$ AA control <sup>1</sup>	4.6 ± 0.2 <sup>2</sup>	86 ± 1	9.6 ± 0.8
No stimulus control	63 ± 5	20 ± 9	17 ± 8
TNF	52 ± 5	39 ± 6	8 ± 2
sPLA <sub>2</sub> -IIA	16 ± 1 <sup>***3</sup>	43 ± 17	41 ± 16
TNF + sPLA <sub>2</sub> -IIA	15 ± 2 <sup>***</sup>	60 ± 18	25 ± 16

1. Purified  $^3\text{H}$  AA standard. Total  $^3\text{H}$  in sample ~6800 DPM for each experiment
2. Data are mean ± SE of data from three separate experiments. In the case of FLSs, data represent 3 independent FLS cultures. Total  $^3\text{H}$  in FLS samples ranged from 1169 dpm – 2781 dpm.
3. \*\*\*,  $p < 0.001$  relative to “No stimulus” control (two-way ANOVA using data from cell culture experiments, Bonferonni’s multiple comparison test).

FIGURE LEGENDS.

**Fig. 1.** Upregulation of cytokine-dependent PG production and COX-2 does not require sPLA<sub>2</sub>-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 sPLA<sub>2</sub>-IIA (H<sub>48</sub>Q) (4 μg/mL), or with sPLA<sub>2</sub>-IIA (WT) (4 μg/mL) in the presence or absence of the COX-2-selective inhibitor NS398 (1 μM) for 16 hrs in DMEM/Ham’s F-12 containing 0.1% BSA. PGE<sub>2</sub> concentration was measured in cell culture supernatants and total cellular protein determined as described in Materials and Methods. Data are combined mean ± SE of triplicate determinations from cell cultures derived from each of 4 patients. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  (Students unpaired t-test) relative to unstimulated cells unless indicated. B. Cells were treated for 16 hrs as indicated, lysed and protein extracts were subjected to electrophoresis and Western blot analysis as described in Materials and Methods. 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 ± SE of 3 independent cell cultures.

**Fig. 2.** PGE<sub>2</sub> production, but not COX-2 upregulation is dependent on cPLA<sub>2</sub>-α enzyme activity. 80-90% confluent FLSs were stimulated with TNF (10 ng/mL), sPLA<sub>2</sub>-IIA (4 μg/mL) either alone or in combination in the presence or absence of the cPLA<sub>2</sub>-α-selective inhibitor A. pyrrophenone or B. pyrrolidine-1 at the concentrations shown for 16 hrs in Hams/DMEM medium containing 0.1% BSA. Media and cells were harvested, PGE<sub>2</sub> in media determined and protein concentration in cell lysates determined as described in Materials and Methods. Data are mean ± SE of triplicate determinations from cell cultures derived from each of four patients. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  (Students 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 electrophoresed, transferred to nitrocellulose, probed with Abs, labeled proteins visualized on X-ray film by enhanced chemiluminescence, blots scanned and densitometry performed and analyzed as described in Materials and Methods. Representative blots from one cell culture are shown. Densitometry data are mean ± SE normalized relative to unstimulated cells from experiments performed on cell cultures derived from 3 separate patients.

**Fig. 3.** Effect of sPLA<sub>2</sub>-IIA on AA mobilization in RA FLSs. Human synovial FLSs were labelled with <sup>3</sup>H-AA and stimulated as indicated in the presence or absence of pyrrophenone (Pyr) for 2 hrs prior to harvesting media and cells for AA release determination as described in Materials and Methods. Data are mean ± SE of two independent experiments combined, each comprising duplicate determinations from four independent cultures. \*; p < 0.05, \*\*; p < 0.05, (Students paired t-test) relative to control unless indicated. Total radioactivity incorporated into cells ranged from 8,185 dpm – 38,367 dpm.

**Fig. 4.** sPLA<sub>2</sub>-IIA-dependent AA mobilization requires sPLA<sub>2</sub>-IIA enzyme activity. FLSs were labeled with <sup>3</sup>H-AA, incubated for 2 hrs A. in the presence or absence of the sPLA<sub>2</sub>-IIA inhibitor c(2Nap)LS(2Nap)R, (C2) at 1 μM concentration, (26) and in the presence or absence of sPLA<sub>2</sub>-IIA as indicated or B. in the presence or absence of sPLA<sub>2</sub>-IIA or the activity-impaired mutant H<sub>48</sub>Q. AA release was measured as described in Materials and Methods. Data are mean ± SE of duplicate determinations from 3-4 cell cultures derived from separate patients and are representative of two independent experiments. \*\* p < 0.01, \*\*\* p < 0.001 (Students unpaired t-test) relative to unstimulated cells unless indicated. Total radioactivity incorporated into cells ranged from A. 18,733 dpm- 40,749 dpm, B. 12,523 dpm – 35,086 dpm.

**Fig. 5.** sPLA<sub>2</sub>-IIA does not activate or enhance TNF activation of the NF-κB pathway in RSF. A. EMSA. Single flasks of 90% confluent FLSs were stimulated for 15 min in DMEM/Ham's F-12 containing 0.1% BSA with TNF (10 pg/mL), sPLA<sub>2</sub>-IIA (5 μg/mL), TNF/sPLA<sub>2</sub>-IIA or TNF/sPLA<sub>2</sub>-IIA with LY311727 (10 μM). Nuclear protein extracts were prepared and binding to a radiolabeled NF-κB consensus binding sequence determined by EMSA as described in Materials and Methods. 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. IκB-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 sPLA<sub>2</sub>-IIA (5 μg/mL), TNF (50 pg/mL), TNF/sPLA<sub>2</sub>-IIA or TNF/sPLA<sub>2</sub>-IIA with LY311727 (10 μM). Total cell lysates were prepared. IκB-α, IκB-β and β-actin protein were detected by Western blot analysis. The ratio of IκB-α to β-actin protein was quantified by densitometry and is normalized to the ratio measured in unstimulated cells. Data are mean ± SE (n=3). C. Nuclear NF-κB 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), sPLA<sub>2</sub>-IIA (5 μg/mL), TNF/sPLA<sub>2</sub>-IIA or TNF/sPLA<sub>2</sub>-IIA with LY311727 (10 μM). Nuclear protein extracts were prepared. Nuclear c-Jun, NF-κB p65 and p50 protein were detected by Western blot analysis. The ratio of nuclear NF-κB p65 and p50 to c-Jun protein was quantified by densitometry and normalized to the ratio measured in unstimulated cells. Data are mean ± SE (n=3 independent FLS cultures).

**Fig. 6.** sPLA<sub>2</sub>-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 sPLA<sub>2</sub>-IIA (5 μg/mL), TNF (50 pg/mL), TNF/sPLA<sub>2</sub>-IIA or TNF/sPLA<sub>2</sub>-IIA with LY311727 (10 μM). Total cell lysates were prepared and A. phosphorylated p38 and total p38α MAP kinase protein or B. p-ERK and total ERK were detected by Western blot analysis as described in Materials and Methods. Representative Western blots and the ratio of phospho- to total MAP kinase protein normalized relative to unstimulated cells is shown. Data are mean ± SE (n=3 independent FLS cultures). Control Phospho-MAPK/Total MAPK ratios varied between cultures from 0.281 – 0.455 for p38, 0.432 - 0.671 for ERK-1 and 0.454 - 0.831 for ERK-2. (\*; p<0.05 relative to control, Student's paired t-test).

**Fig. 7.** Model of sPLA<sub>2</sub>-IIA function in FLS AA metabolism. Unstimulated cells; AA is mobilized in microparticles esterified to phospholipids. Low level AA release is mediated by cPLA<sub>2</sub>-α and is reincorporated into phospholipid pools by acylase(s). AA flux to PGE<sub>2</sub> is very low and is *via* COX-1.

TNF stimulated cells. TNF; cPLA<sub>2</sub>- $\alpha$  is activated ( $\oplus$ ) and COX-2 expression is induced ( $\uparrow$ ), stimulating AA flux through the COX-2 pathway to stimulate PGE<sub>2</sub> production. TNF + sPLA<sub>2</sub>-IIA; sPLA<sub>2</sub>-IIA enzyme activity increases AA mobilization that is not coupled to PGE<sub>2</sub> production. sPLA<sub>2</sub>-IIA-mediated signaling superinduces COX-2 ( $\uparrow$ ) resulting in increased cPLA<sub>2</sub>- $\alpha$ - dependent AA flux through COX-2 to PGE<sub>2</sub>. sPLA<sub>2</sub>-IIA alone increases AA release and induces COX-2 without increasing PGE<sub>2</sub> production. Figure is modeled after Fitzpatrick and Soberman (51).

Bryant *et al.* Figure 1.











