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PGE_2 release is independent of upregulation of Group V phospholipase A_2 during long-term stimulation of $P388D_1$ cells with LPS[1]

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Abstract **$P388D_1$ cells release arachidonic acid (AA) and produce prostaglandin E_2 (PGE_2) upon long-term stimulation with lipopolysaccharide (LPS). The cytosolic Group IVA (GIVA) phospholipase A_2 (PLA_2) has been implicated in this pathway. LPS stimulation also results in increased expression and secretion of a secretory PLA_2, specifically GV PLA_2. To test whether GV PLA_2 contributes to PGE_2 production and whether GIVA PLA_2 activation increases the expression of GV PLA_2, we utilized the specific GIVA PLA_2 inhibitor pyrrophenone and *second generation* antisense oligonucleotides (AS-ONs) designed to specifically inhibit expression and activity of GV PLA_2. Treatment of $P388D_1$ cells with antisense caused a marked decrease in basal GV PLA_2 mRNA and prevented the LPS-induced increase in GV PLA_2 mRNA. LPS-stimulated cells release active GV PLA_2 into the medium, which is inhibited to background levels by antisense treatment. However, LPS-induced PGE_2 release by antisense-treated cells and by control cells are not significantly different. Collectively, the results suggest that the upregulation of GV PLA_2 during long-term LPS stimulation is not required for PGE_2 production by $P388D_1$ cells. Experiments employing pyrrophenone suggested that GIVA PLA_2 is the dominant player involved in AA release, but it appears not to be involved in the regulation of LPS-induced expression of GV PLA_2 or cyclooxygenase-2.**—Kessen, U. A., R. H. Schaloske, D. L. Stephens, K. Killermann Lucas, and E. A. Dennis. **PGE_2 release is independent of upregulation of Group V phospholipase A_2 during long-term stimulation of $P388D_1$ cells with LPS. *J. Lipid Res.* 2005.** 46: **2488–2496.**

Supplementary key words antisense inhibitor • macrophage • lipopolysaccharide • prostaglandin E_2 • arachidonic acid • eicosanoid

The phospholipase A_2 (PLA_2) superfamily encompasses a series of enzymes that catalyze the hydrolysis of the fatty acid esterified at the *sn-2* position of glycerophospholipids, producing free fatty acid and lysophospholipids (1). Any free arachidonic acid (AA) liberated by these enzymes can subsequently be converted into prostaglandins (PGs) through the action of cyclooxygenases (COXs) and PG synthases.

Prominent members of the PLA_2 superfamily are the secreted phospholipase A_2s ($sPLA_2$s), the cytosolic PLA_2s ($cPLA_2$s), and the calcium-independent PLA_2s ($iPLA_2$s). Common features of the $sPLA_2$s are their relatively low molecular mass (about 14 kDa), a high abundance of disulfide bonds, and their requirement for micromolar Ca^{2+} for activity. $cPLA_2$s are characterized by a higher molecular mass (about 85 kDa) and their preference for AA-containing phospholipids (2–4). The $iPLA_2$s are distinguished from the other PLA_2s in that they do not require Ca^{2+} for activity.

Previous work has demonstrated that the murine macrophage-like $P388D_1$ cells release prostaglandin E_2 (PGE_2) upon long-term stimulation with bacterial lipopolysaccharide (LPS). Shinohara et al. (5) and Balsinde et al. (6) have suggested possible roles for the c- and $sPLA_2$s in the release of AA, resulting in PGE_2 production. In a hypothetical model, activation of the Group IV (GIV) $cPLA_2$ results in an increased expression of Group V (GV) $sPLA_2$, which, in turn, is largely responsible for the release of the AA that is subsequently converted into PGE_2, and GV PLA_2 gives rise to the upregulation of COX-2 expression. The model is based on experiments utilizing the chemical inhibitors methyl arachidonyl fluorophosphonate (MAFP), affecting Group IVA (GIVA) PLA_2; 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy) propane sulfonic acid (LY311727), affecting $sPLA_2$; and first-generation antisense oligonucle-

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Abbreviations: AA, arachidonic acid; AS-ON, antisense oligonucleotide; GIVA, Group IVA; LPS, lipopolysaccharide; MAFP, methyl arachidonyl fluorophosphonate; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; S-ON, sense oligonucleotide; $sPLA_2$, secreted phospholipase A_2.

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otides (AS-ONs). All three of these inhibitors have drawbacks. MAFP is not specific, inasmuch as it inhibits a number of enzymes in addition to GIVA PLA_2, namely GroupVI PLA_2 (7, 8), anandamide amidase (9, 10), and platelet-activating factor-acetylhydrolase (11). It also binds to the CB1 cannabinoid receptor (12, 13). The $sPLA_2$ inhibitor LY311727 is structurally related to Me-Indoxam. Mounier et al. (14) described recently that Me-Indoxam does not pass through the plasma membrane of mammalian cells and that it does not inhibit AA release from human embryonic kidney cells that have been transfected with GIIA or GX PLA_2s. The authors concluded that the $sPLA_2$s act inside the cell prior to their secretion. It is probable that LY311727 is not able to act inside the cells. Recent experiments from our laboratory employing LY311727 in $P388D_1$ cells support this hypothesis. The first-generation AS-ONs suffer from their low affinity toward target RNA molecules and their toxic side effects (15–18).

To test the hypothetical model described above, we employed *second-generation* AS-ONs consisting of a 5-10-5 2′-*O*-methoxy-ethyl RNA gapmer with a phosphorothioate backbone. These modifications increase the binding of the oligonucleotide to its target, stabilize the ON by preventing nuclease degradation, and support RNase H-mediated cleavage of the targeted mRNA (15–18). We used second-generation AS-ONs specifically designed to inhibit expression of GV PLA_2, and thus its activity, in order to examine specifically the role of GV PLA_2 in PGE_2 production. We also utilized the novel GIVA PLA_2 inhibitor pyrrophenone, which is more specific and potent than MAFP (19, 20).

We now show that treatment of $P388D_1$ cells with AS-ONs resulted in a marked decrease of basal GV PLA_2 mRNA, prevented the LPS-induced increase in GV PLA_2 mRNA as measured with real-time quantitative PCR (Q-PCR), and also caused a significant decrease in basal and stimulated GV PLA_2 protein and activity levels. Basal and stimulated PGE_2 levels were not affected by AS-ON treatment. Treatment of the cells with the novel GIVA PLA_2 inhibitor pyrrophenone resulted in a 60–70% inhibition of PGE_2 production but did not affect COX-2 protein levels. We show that the upregulation of GV PLA_2 is *i*) not mandatory for PGE_2 production by $P388D_1$ cells during long-term stimulation with LPS and *ii*) not dependent on GIVA PLA_2 activity.

EXPERIMENTAL PROCEDURES

Materials

Iscove's modified Dulbecco's medium (IMDM), OPTI-MEM, and penicillin/streptomycin were obtained from Gibco (Grand Island, NY). Fetal bovine serum was from VWR International (Bristol, CT). LPS (*Escherichia coli* 0111:B4) was obtained from Sigma Chemical Co. (St. Louis, MO). Nonradioactive 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS) were purchased from Avanti Polar Lipids (Alabaster, AL). 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (radioactive DPPC) was purchased from Amersham Biosciences (Piscataway, NJ). [5,6,8,9,11,12,14,15-^{3}H]AA (specific activity 100 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA). The Complete Mini Protease Inhibitor Cocktail was from Roche Applied Science (Indianapolis, IN). Triton X-100 was purchased from Calbiochem (La Jolla, CA). The GV PLA_2 AS-ONs and control oligonucleotides were kindly provided by Dr. Frank Bennett of ISIS Pharmaceuticals (Carlsbad, CA). LY 311727 was the generous gift of Dr. Jerome Fleisch (Lilly Research Laboratories). Pyrrophenone was the generous gift of Dr. Takaski Ono (Shionogi Research Laboratories). MAFP and the polyclonal antibody directed against murine GV PLA_2 and the polyclonal antibody directed against COX-2 were purchased from Cayman Chemicals (Ann Arbor, MI). The monoclonal antibody against GAPDH was obtained from HyTest (Turku, Finland). Cytofectin was from Gene Therapy Systems (San Diego, CA). SYBR-Green PCR Master Mix was from Applied Biosystems (Foster City, CA). Primers used for the real-time Q-PCR were ordered from Proligo (Boulder, CO). M-MLV reverse transcriptase, RNase H, SeeBlue2 PreStained protein standard and the 12% Bis-Tris gels were from Invitrogen (Carlsbad, CA).

Cell culture

$P388D_1$ cells (MAB clone) (5), were maintained at 37°C in a humidified atmosphere at 90% air and 10% CO_2 in IMDM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete IMDM). For transfection experiments, the cells were seeded at a density of 10^6 cells/2 ml/well in 6-well plates and allowed to adhere overnight. For experiments using chemical inhibitors, cells were seeded either at a density of 10^6 cells/ml/well in 12-well plates or at a density of 3×10^6 cells/2 ml/well in 6-well plates and allowed to adhere overnight. For experiments assaying AA-derived radioactivity release, cells were seeded in 12-well plates in the presence of 0.5 μCi ^{3}H-labeled AA.

Stimulation of cells

Cells transfected with oligonucleotides were stimulated 5 h after transfection by adding 150 ng/ml LPS to the cells for 24 h. In experiments using chemical inhibitors assaying AA-derived radioactivity release or PGE_2 release, cells were washed three times in IMDM, incubated in the same medium for 1 h, and then stimulated by adding 100 ng/ml LPS for 18 h. Inhibitors were added during the starvation period 30 min prior to the addition of LPS.

Transient transfection of AS-ONs

The following ONs were used for transfection: GV sense oligonucleotide (S-ON) ISIS# 357 886 (5′-**TTC CG**G AGG AAG GGT **CTA GG**-3′) and GV AS-ON ISIS# 314 510 (5′-**CCT AG**A CCC TTC CTC **CGG AA**-3′). The nucleotides shown in bold and plain text represent 2′-*O*-methylribonucleotides and 2′-deoxyribonucleotides, respectively, and are linked through thioester bonds. All transfection solutions were prepared in polystyrol 6- or 12-well plates. The oligonucleotides were transfected into the cells by using the transfection reagent Cytofectin. Solution A contained 4 μg of Cytofectin diluted in 200 μl of OPTI-MEM, and solution B contained 3 μl of oligonucleotide (100 μM stock) diluted in 200 μl OPTI-MEM. Both solutions were incubated for 10 min at room temperature. Subsequently, solution B was added dropwise to solution A, and this transfection mix was incubated for another 15 min at room temperature. The cell medium was replaced by 600 μl fresh complete IMDM, and the transfection mix was then added to the cells. The cells were incubated for 5 h before stimulation with LPS.

Preparation of RNA and cDNA

Total cellular RNA was isolated from cells with the RNeasy Mini Kit from Qiagen (Valencia, CA) according to the manufacturer's instructions. The remaining DNA was digested by using

the RNase-Free DNase Set from Qiagen. cDNA was synthesized by using M-MLV reverse transcriptase and oligo dT primer following the manufacturer's protocol. The remaining RNA was digested by incubating the samples with 20 units of RNase H at 37°C for 20 min.

Design of primers used for real-time Q-PCR

All the primers used are listed in reference (21) and were selected using the Primer 3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). They encompassed at least one intron of the gene. To determine which mRNAs are expressed in $P388D_1$ cells, we performed PCR using cDNA from either nontreated cells or cells treated with LPS for 18 h. The reactions contained 100 ng of cDNA, 1 μM of gene-specific primers (described in Ref. 21) and the SYBR Green PCR Master Mix in a total volume of 25 μl. PCR was performed on a BioRad iCycler Thermal Cycler. PCR cycles were identical to those used for real-time Q-PCR as described below. Three microliters of the PCR product was applied to 4% agarose gels (E-gels, Invitrogen). In cases in which two different primer pairs for a given gene did not produce a detectable band, we assumed that the mRNA was not present. Occasionally, there was a faint band visible at the correct size of the GIIF amplicon. However, even when using 500 ng of cDNA template for the real-time Q-PCR, we could not quantitate the cDNA encoding GIIF PLA_2 (data not shown). Similarly, cDNA encoding GIII PLA_2 could only be detected occasionally. This suggests that the amounts of GIIF and GIII messages are close to the detection limit. We confirmed the functionality of the primers by using cDNAs that were synthesized from commercially available total murine tissue RNA (Ambion, Austin, TX).

Real-time Q-PCR

The real-time Q-PCR for all samples was performed on the ABI 7700 Sequence Detection System from Applied Biosystems using SYBR green detection. All primers were shown to yield a single product by performing a dissociation curve after each real-time Q-PCR run. The real-time Q-PCR consisted of an initial hold at 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 61°C for 1 min. The amount of template cDNA and the primer concentration that was used are described in reference (21). Gene expression was normalized to the house-keeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). PCR runs were performed at the Rebecca and John Moores UCSD Cancer Center, Molecular Pathology, Quantitative Real-Time PCR Shared Resource. The amplicons were cloned into the pCR2.1-TOPO vector (Invitrogen) and the sequences verified by sequencing. DNA sequencing was performed by the DNA Sequencing Shared Resource, Rebecca and John Moores UCSD Cancer Center.

Preparation of cell extracts and cell media for immunoblotting and PLA_2 assays

The cell media were cleared of detached cells by centrifuging for 4 min at 4,000 rpm in an Eppendorf centrifuge. The centrifuged supernatants were transferred into a fresh tube, stored at −80°C, and thawed right before the assay. The adherent cells on the plate were frozen at −80°C for 15–30 min and subsequently thawed. Lysis buffer (0.5% Triton X-100 and 1 × "Complete Mini" in PBS) was added, and lysates were transferred into Eppendorf reaction tubes and sonicated three times for 15 s at intervals of 15 s.

Immunoblotting

Cell media (22.5 μl from an original volume of 1 ml) were mixed with 7.5 μl 4× NuPAGE LDS sample buffer (Invitrogen) and 2% β-mercaptoethanol, boiled for 3 min, and subsequently run on 12% Bis-Tris SDS-polyacrylamide gels using MES buffer (Nupage, Invitrogen). Proteins were transferred onto nitrocellulose membranes. For detection of GV PLA_2, the membrane was blocked by incubating the membranes with 3% BSA and 1% goat serum in TBS buffer containing 0.05% Tween 20 (TBS-Tween) for 1 h before probing with a GV PLA_2-specific antibody (1:250 dilution in TBS-Tween-3% BSA) for 1 h. The membrane was washed three times in TBS-Tween and then incubated with secondary antibody (Biorad) for 1 h (goat anti-rabbit IgG-HRP conjugate in a 1:2,500 dilution in TBS-Tween-3% BSA). For detection of COX-2 and GAPDH protein, the membrane was blocked by incubating the membranes with 3% BSA in TBS-Tween for 1 h before probing with a COX-2-specific antibody (1:400 dilution in TBS-Tween-3% BSA) or a GAPDH-specific antibody (1:2,000 in TBS-Tween-3% BSA) for 1 h. The membrane was washed three times in TBS-Tween and then incubated with Protein A-HRP conjugate (Amersham) for 1 h in a 1:3,000 dilution in TBS-Tween-3% BSA.

For GV PLA_2, GAPDH, and COX-2 blots, the membrane was then washed three times with TBS-Tween and two times with 10 mM Tris-HCl, pH 7.4, and then developed using the Western Lightning ECL kit (Amersham Pharmacia Biotech).

PGE_2 assay

Cells were stimulated as described above. The media were removed and cleared by centrifugation, and the PGE_2 concentration was quantitated using a monoclonal PGE_2 EIA kit (Cayman Chemical, Ann Arbor, MI). The assays were conducted according to the manufacturer's protocol.

Lipid preparation

Lipid stock solutions were made by dispensing the volume of phospholipid solution in chloroform necessary for a total of 50 nmol per assay tube. The lipid was dried under nitrogen and lyophilized for at least 1 h to remove all traces of chloroform. Lipid was then resuspended in 3 ml 100 mM HEPES, pH 7.5, and small unilamellar vesicles (SUVs) were created by repeated sonication until the solution cleared. A small sample of the SUV preparation was then centrifuged at 15,000 *g* for 5 min to test for the precipitation of large lipid structures. The supernatant from the centrifuged sample was counted by liquid scintillation and compared with the counts derived from a noncentrifuged sample of equal volume. If the counts compared varied more than 10%, the sample was subjected to further sonication and the centrifugation test was repeated until the counts were in agreement. The SUV suspension (3 ml) was further diluted to 200 μl per assay tube by the addition of 100 mM HEPES, pH 7.5.

PLA_2 assay

In the GV PLA_2-specific assay (22) the lipids used contained 100 μM DPPC-POPS (3:1) doped with 100,000 cpm ^{14}C-labeled DPPC. The assay buffer contained 100 mM HEPES, pH 7.5, 4 mM $CaCl_2$, 1 mg/ml BSA, and 4 μM MAFP. The total volume for each assay was 500 μl (200 μl lipid, 250 μl assay buffer, and 50 μl cell lysate or cell media). The amount of calcium added was adjusted to account for the addition of EDTA in the lysis buffer to give the final concentrations listed above. For inhibitor studies, 1 μM LY311727 was added to the substrate preparation and mixed 5 min prior to the addition of samples. Samples were incubated with substrate for 1 h at 40°C in a shaking water bath. The assay was then terminated by the addition of 2.5 ml Dole Reagent (isopropyl alcohol-heptane-0.5 M sulfuric acid; 400:100:20; v/v/v). Silica gel (0.1–0.2 g) was added to each tube, followed by 1.5 ml heptane and 1.5 ml deionized water. Each tube was vortexed for 15 s. One milliliter of the organic phase was removed and passed through a glass wool-plugged Pasteur pipet containing silica gel (0.1–0.2 g). Radioactivity was then eluted with 1 ml diethyl ether.

Five milliliters of scintillation cocktail (Biosafe II, RPI, Mount Prospect, IL) was then added to the eluate, and the radioactivity was determined by scintillation counting.

Data presentation

All assays and real-time Q-PCR reactions were carried out in triplicate. Each set of experiments was performed at least three times with similar results. Unless otherwise indicated, the data presented are from representative experiments.

RESULTS

Abundance of mRNAs from sPLA$_2$s identified in P388D$_1$ cells stimulated with LPS

Using PCR, we amplified portions of genes encoding five different sPLA$_2$s in the P388D$_1$ cells: GIB, GIIE, GIID, GV, and GXII. Using real-time Q-PCR, we measured the relative abundance of these mRNA species after long-term LPS stimulation. Among these sPLA$_2$s, only the GV PLA$_2$ mRNA was significantly upregulated, resulting in a 5-fold increase in GV message after 13 h of LPS treatment (**Fig. 1**). We did not detect mRNA for GIIA, GIIC, and GX (data not shown). mRNAs encoding GIIF and GIII were close to the detection limit (see Experimental Procedures section).

Effect of second-generation AS-ONs on GV PLA$_2$ mRNA levels in P388D$_1$ cells

To investigate the role of GV PLA$_2$ in the release of PGE$_2$, we transfected P388D$_1$ macrophages with *second-generation* AS-ONs. Cells were either left untreated or were treated with transfection reagent alone (Mock), with cytofectin plus S-ON, or with cytofectin plus AS-ON. When the cells were treated with either cytofectin alone or S-ONs, there was no measurable effect on the untreated or LPS-stimulated GV PLA$_2$ mRNA levels as measured by Q-PCR (**Fig. 2A**). The transfection of the GV PLA$_2$ AS-ONs resulted in over 90% reduction of the GV PLA$_2$ mRNA in nonstimulated cells as well as in LPS-treated cells.

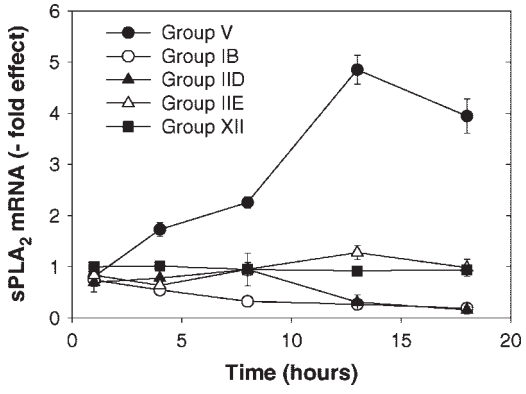

Fig. 1. Abundance of mRNAs from secreted phospholipase A$_2$s (PLA$_2$s) identified in P388D$_1$ cells. Cells (3×10^6) were plated in 6-well plates and allowed to adhere overnight. Cells were subsequently stimulated with 100 ng/ml lipopolysaccharide (LPS) for 18 h. Cells were lysed, RNA extracted, and reverse transcribed into cDNA. cDNA of the respective genes was quantified by real-time PCR. The abundance of PLA$_2$ mRNA was normalized to GAPDH mRNA. mRNA for GIIA, GIIC, and GX was not detected, and GIIF and GIII were close to the detection limit. Data represent one out of three experiments, each measured in triplicate. Error bars indicate SD.

GV PLA$_2$ protein levels in the presence of oligonucleotides

Previously, Shinohara et al. (5) found that sPLA$_2$ is secreted into the medium upon stimulation of the cells with LPS. Using a GV PLA$_2$-specific antibody, we investigated whether the reduction in GV PLA$_2$ mRNA would lead to a reduction in GV PLA$_2$ protein levels in the extracellular medium. Figure 2B shows a Western blot using media of cells that were either untreated or stimulated with LPS in the presence of either cytofectin alone (Mock), the S-ON, or the AS-ON. In media of cells that were not treated with LPS, the amount of GV protein appeared to be low. In

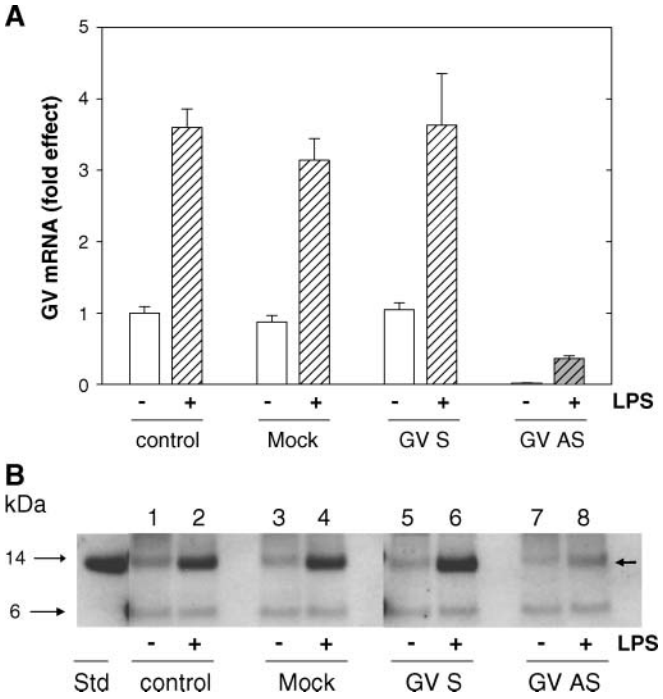

Fig. 2. Effect of antisense oligonucleotides (AS-ONs) on GV PLA$_2$ mRNA levels in P388D$_1$ cells. Cells (10^6) were plated in 6-well plates and allowed to adhere overnight. Cells were transfected with 300 nM oligonucleotide using Cytofectin as transfection reagent in a total volume of 1 ml, incubated for 5 h, and subsequently stimulated with 150 ng/ml LPS for 24 h. A: Cells were subsequently lysed, RNA extracted, and reverse transcribed into cDNA. cDNA was quantified by real-time PCR. The mRNA abundance of GV mRNA was normalized to GAPDH mRNA. Control: no addition; Mock: only transfection reagent; GV S: GV sense oligonucleotide (S-ON) + transfection reagent; GV AS: GV AS-ON + transfection reagent. Data represent one out of three experiments, each measured in triplicate. Error bars indicate SD. B: Media (22.5 μl) were loaded on a 14% polyacrylamide gel and transferred onto nitrocellulose, and the membrane was immunostained with anti-mouse GV antibody (Cayman). Std: 1.5 ng purified rat GV protein; control: media of untreated cells; Mock: media of cells treated with transfection reagent only; GV S: media of cells treated with GV S-ON + transfection reagent; GV AS: media of cells treated with GV AS-ON + transfection reagent. The GV PLA$_2$ band is indicated by the arrowhead. Data represent one out of three experiments.

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media of cells treated with cytofectin or transfected with S-ON in the presence of LPS, we measured a large increase in the amount of secreted GV PLA_2. In contrast, the LPS-induced increase of secreted protein was inhibited (Fig. 2B) in cells that were treated with AS-ON.

GV PLA_2 activity in $P388D_1$ cell lysates and cell media

To be sure that the reduction of GV PLA_2 mRNA results also in a reduction of GV PLA_2 enzyme as measured by activity, we measured GV activity in cell lysates as well as in the media of cells that were either untreated or stimulated with LPS in the presence of either cytofectin alone, the S-ON, or the AS-ON (**Fig. 3A**). In lysates of cells that were either untreated, treated with Cytofectin, or transfected with S-ON or AS-ON, we detected only minimal GV activity. In lysates of cells stimulated with LPS, we measured a 3-fold elevation in GV PLA_2 activity. Pretreatment of the cells with Cytofectin alone or Cytofectin plus S-ON did not alter this increase. However, lysates of cells stimulated with LPS in the presence of AS-ON showed a decrease in activity to a level comparable to that of nonstimulated cells.

In media of nonstimulated cells that were either untreated, treated with Cytofectin, or transfected with S-ON, we detected GV activity of only 37.5 ± 4.8, 44.1 ± 5.7, and 41.4 ± 2.6 pmol/min, respectively. Transfection of the cells with AS-ON significantly decreased this basal GV activity in the media by over 80%. Cells treated with LPS showed a 5-fold increase in the amount of secreted GV PLA_2. Pretreatment of the cells with Cytofectin alone or Cytofectin plus S-ON did not inhibit this increase. However, transfection of the cells with AS-ON inhibited the increase in the amount of secreted GV PLA_2 to the level of nonstimulated cells (Fig. 3A).

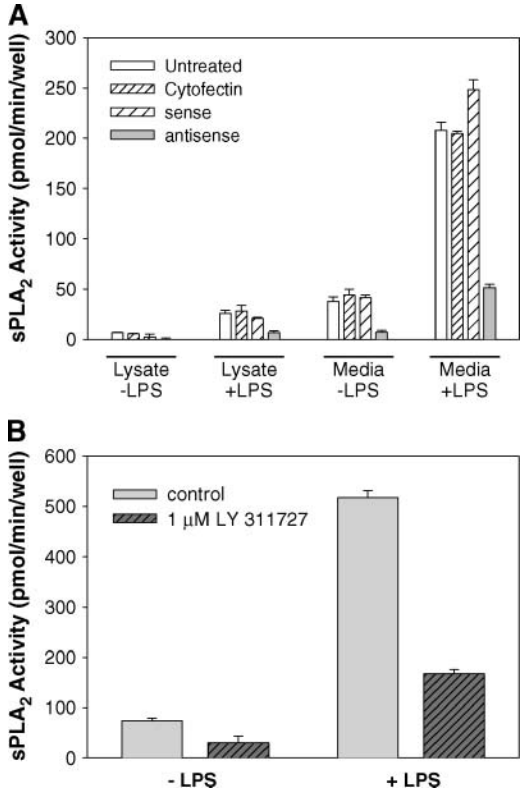

Fig. 3. GV PLA_2 activity measured in the media and lysate of GV antisense-treated $P388D_1$ cells. A: Cells (10^6) were plated in 6-well plates and allowed to adhere overnight. Cells were transfected with 300 nM oligonucleotide using Cytofectin as transfection reagent in a total volume of 1 ml, incubated for 5 h, and subsequently stimulated with 150 ng/ml LPS for 24 h. Cell lysates and the media were assayed for PLA_2 activity under conditions specific for the GV enzyme using the Dole assay. B: Effect of LY311727 on GV PLA_2 activity measured in the media of LPS-stimulated cells. Cells (2×10^6) cells were plated in 6-well plates and allowed to adhere overnight. Cells were then stimulated with 100 ng/ml LPS for 24 h. The media were assayed for GV PLA_2 activity in the presence (+) or absence (−) of the indicated amounts of LY311727. Data represent one out of three experiments, each measured in triplicate, and show the extrapolated total activity in the lysate and in the media. Error bars in A and B indicate SD.

Effect of second-generation AS-ONs on PGE_2 levels in $P388D_1$ cells

LPS induced a 30-fold increase in PGE_2 levels in the media of the cells. The transfection reagent alone and the S-ON had no effect on PGE_2 levels in stimulated or nonstimulated cells. Strikingly, the addition of the GV PLA_2 AS-ON did not reduce LPS-stimulated PGE_2 release in $P388D_1$ macrophages (**Fig. 4**).

Effect of LY311727 on AA and PGE_2 release in $P388D_1$ cells

The $sPLA_2$ inhibitor LY311727 at 1 μM concentration inhibited the GV activity that had been released into the medium by approximately 70% (Fig. 3B). Therefore, we stimulated $P388D_1$ cells with LPS in the absence or presence of either 25 or 50 μM LY311727. Up to 50 μM LY311727 did not inhibit basal or LPS-induced PGE_2 release (**Fig. 5B**). At 25 μM, LY311727 did not significantly decrease LPS-induced AA release. Only after increasing the con-

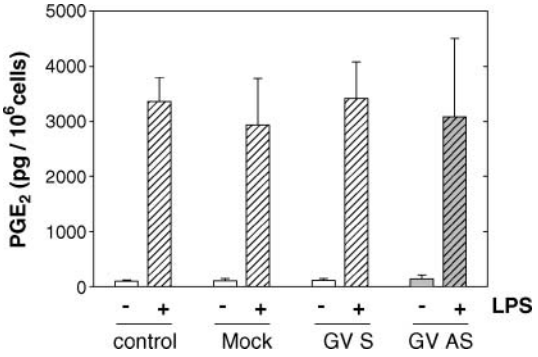

Fig. 4. Effect of AS-ONs against GV PLA_2 mRNA on prostaglandin E_2 (PGE_2) release from $P388D_1$ cells. Cells (10^6) were plated in 6-well plates and allowed to adhere overnight. Cells were transfected with 300 nM oligonucleotide using Cytofectin as transfection reagent in a total volume of 1 ml, incubated for 5 h, and subsequently stimulated with 150 ng/ml LPS for 24 h. The media were assayed for PGE_2 by enzyme immunoassay. Control: no addition; Mock: only transfection reagent; GV S: GV S-ON + transfection reagent; GV AS: GV AS-ON + transfection reagent. Data represent the mean of three independent experiments, each measured in triplicate. Error bars indicate SD.

centration to 50 μM LY311727 did we measure a moderate decrease in AA release in three independent experiments (39%, 47%, and 36%, respectively) (Fig. 5A). In contrast, Shinohara et al. (5) previously found a larger effect, in which the $sPLA_2$ inhibitor LY311727 at 25 μM suppressed AA release in LPS-treated $P388D_1$ cells by 70%.

Effect of the GIV PLA_2 inhibitor pyrrophenone on AA and PGE_2 release and COX-2 and GV PLA_2 expression in $P388D_1$ cells

Shinohara et al. (5) and Balsinde et al. (6) reported previously that COX-2 expression and the induction of GV PLA_2 was blunted by MAFP. This finding, combined with the observation that LY311727 and GV PLA_2 *first-generation* AS-ONs decreased COX-2 levels, led to the suggestion that GIV PLA_2 activation regulates the expression of GV PLA_2, which, in turn, could be responsible for delayed PG production by regulating COX-2 expression.

It has now been shown that MAFP inhibits a number of enzymes (7–13). To test whether the reported effect of MAFP on GV PLA_2 levels was indeed due to GIVA PLA_2 inhibition, we employed the newer, very potent and specific GIV PLA_2 inhibitor pyrrophenone (19, 20). The inhibitory effect of pyrrophenone on long-term AA release is shown in **Fig. 6A**. The maximal effect on [^{3}H]AA-derived radioactivity release was observed at a pyrrophenone concentration of 100 nM. Figure 6B shows the effects of 100 nM and 300 nM pyrrophenone on PGE_2 release. LPS stimulation of the cells led to an approximately 5-fold increase in AA-derived radioactivity release (Fig. 6A) and to a 12-fold increase in PGE_2 release (Fig. 6B). Both 100 nM and 300 nM pyrrophenone decreased the LPS-stimulated AA release by 80–90% and the LPS-stimulated PGE_2 release by 70%, indicating that GIVA PLA_2 plays a dominant role in the release of AA. Furthermore, long-term stimulation of cells with LPS induced the synthesis and secretion of catalytically active GV PLA_2 (Figs. 3, 6C) as well as the upregulation of COX-2 (Fig. 6D). However, neither 100 nM nor 300 nM pyrrophenone resulted in a pronounced decrease in the activity of GV PLA_2 (Fig. 6C), in the amount of secreted protein (data not shown), or in the upregulation of COX-2 (Fig. 6D).

We also tried to inhibit expression of GIVA PLA_2 using second-generation AS-ON to confirm the results found with pyrrophenone. Treatment of the cells with GIVA AS-ON greatly reduced the amount of GIVA mRNA but, unfortunately, over a 2 day period, had no effect on GIVA PLA_2 protein levels and GIVA PLA_2 activity in cell lysates as determined by Western blot and a group-specific activity assay, respectively (data not shown).

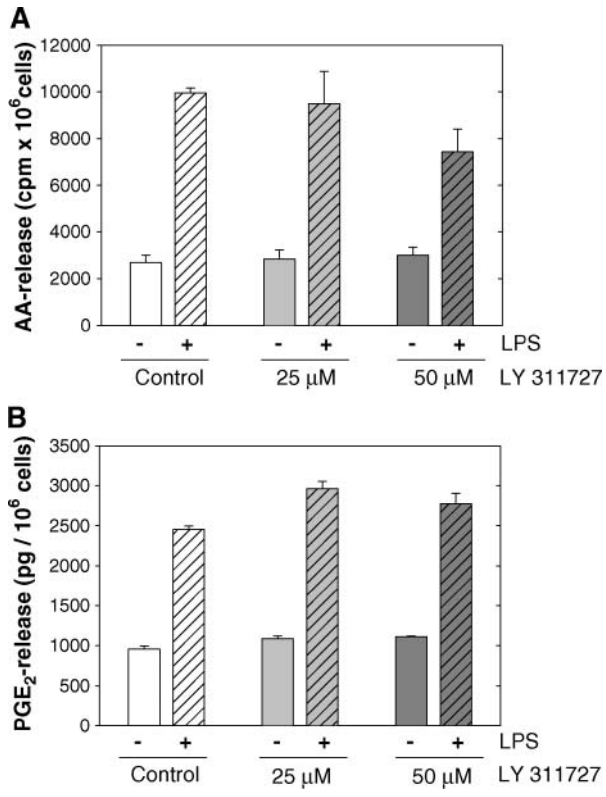

Fig. 5. Effect of LY311727 on AA and PGE_2 release. Cells (10^6) were plated in 12-well plates and allowed to adhere overnight in the presence of 0.5 μCi ^{3}H-labeled AA if necessary. Cells were then stimulated with 100 ng/ml LPS for 18 h in the presence (+) or absence (−) of LY311727. A: Determination of AA release by radioactivity detection of all arachidonic acid-derived products. Data represent one out of three experiments, each measured in triplicate. B: Media were assayed for PGE_2 by enzyme immunoassay. Data represent one out of three experiments, each measured in triplicate. Error bars in A and B indicate SD.

DISCUSSION

We have explored the role of the GV PLA_2 in the production of PGE_2 upon long-term stimulation of $P388D_1$ cells with LPS. Specific second-generation AS-ON directed against GV PLA_2 reduced the expression of the enzyme. We succeeded in decreasing the basal and LPS-induced levels of mRNA encoding GV PLA_2 as well as in reducing the amount of GV protein and activity measured in the cell lysate and cell media under both basal and LPS-stimulated conditions.

Although GV PLA_2 activity was reduced almost to background levels, there was no significant effect on the amount of PGE_2 produced. These results suggest that the upregulation of GV PLA_2 does not play a prominent role in the production of PGE_2. This appears to be in contrast to the findings by Shinohara et al. (5), where *first-generation* AS-ONs were used. However, this type of ON has been shown to have toxic effects (for review, see Ref. 23) and to bind nonspecifically to proteins (24), which might explain this discrepancy.

For comparison, we also tested the $sPLA_2$ inhibitor LY311727 and found that *i*) 1 μM LY311727 inhibits GV activity in the media and *ii*) PGE_2 release was not affected by treatment of the cells with up to 50 μM of the compound. At 25 μM, no significant reduction of LPS-induced AA-derived radioactivity release was detectable, but at 50 μM, a moderate inhibition of AA release was observed. At this high concentration, we cannot rule out nonspecific effects on the cells. Shinohara et al. (5) also utilized this inhibitor

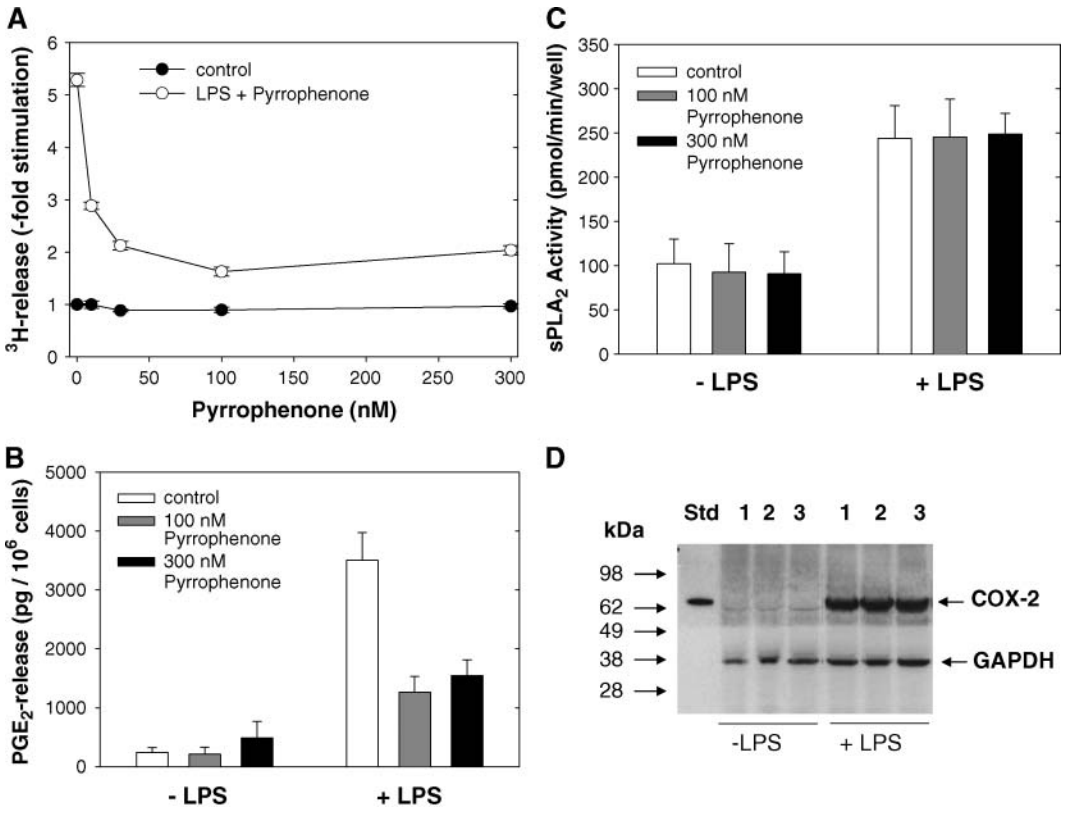

Fig. 6. Effect of pyrrophenone on AA and PGE_2 release, GV PLA_2 activity, and COX-2 expression. Cells (10^6) were plated in 12-well plates and allowed to adhere overnight in the presence of 0.5 μCi ^{3}H-labeled AA if necessary. Cells were then stimulated with 100 ng/ml LPS for 18 h. Pyrrophenone was added 30 min prior to LPS stimulation. A: Determination of AA release by scintillation counting of all radiolabeled AA-derived products. Data represent one out of two experiments, each measured in triplicate. Error bars indicate SD. B: Media were assayed for PGE_2 by enzyme immunoassay. Data represent one out of three experiments for 100 nM pyrrophenone (error bars indicate SD) and one out of two experiments for 300 nM pyrrophenone (error bars indicate SD), each measured in triplicate. C: Cells (3×10^6) were plated in 6-well plates and allowed to adhere overnight. Cells were then stimulated with 100 ng/ml LPS for 24 h in the presence (+) or absence (−) of the indicated amounts of pyrrophenone. Media were assayed for GV PLA_2 activity. Data represent the mean of three experiments for 100 nM pyrrophenone (error bars indicate SD) and the mean of two experiments for 300 nM pyrrophenone (error bars indicate SE), each measured in triplicate, and show the extrapolated total activity in the media. D: Protein (50 μg) from cell lysate was loaded on a 4–12% polyacrylamide gel, transferred onto nitrocellulose, and probed with anti-COX-2 and GAPDH antibody. Lane 1: control; lane 2: 100 nM pyrrophenone; lane 3: 300 nM pyrrophenone; Std: 20 ng purified ovine COX-2 protein (Cayman). The COX-2 and GAPDH bands are indicated by arrowheads. Data represent one out of three experiments.

and found a greater inhibition of AA release at a lower concentration. We do not have a good explanation for these differences. We did try several different batches of the inhibitor with essentially the same results. It is important to note that the LY311727 used in this study was efficient in in vitro experiments (Fig. 3B).

Mounier et al. (14) have recently shown that Me-Indoxam, a cell-impermeable $sPLA_2$ inhibitor structurally related to LY311727, does not have an effect on AA release from human embryonic kidney cells transfected with $sPLA_2$s GIIA or GX. The authors concluded that these $sPLA_2$s are active predominantly during the secretion process and not outside the cell. It is conceivable that LY311727 also is unable to cross the cell membrane and therefore cannot act inside the cell. From our results, we conclude that GV PLA_2 does not act on the cell surface of $P388D_1$ cells to release significant amounts of AA. We cannot exclude an intracellular role for GV PLA_2 in PGE_2 production that might occur prior to its secretion, similar to the mechanism suggested by Mounier et al. (14). However, the total amount of GV PLA_2 activity in lysates and media from cells stimulated with LPS was inhibited virtually to nonstimulated levels with GV AS-ON treatment. This result renders the aforementioned possibility unlikely, inasmuch as we would expect a decrease in PGE_2 production even if GV PLA_2 acted inside the cell. Low levels of GV PLA_2, however, might still contribute to some AA release in cells stimulated with LPS.

It is important to note that LPS-induced AA release is almost entirely blocked by the GIVA-specific inhibitor pyrrophenone. This suggests that GIVA PLA_2 is the dominant signaling component responsible for AA release and confirms previous results by our group utilizing the relatively nonspecific inhibitor MAFP (5).

We cannot rule out that by an unknown mechanism,

LPS-induced activation of GIVA PLA_2 results in the activation of low amounts of GV PLA_2 enzyme present in the cell, even after treatment with AS-ON, which might be responsible for some AA release intracellularly. Recently, Arm and coworkers (25) have shown that peritoneal macrophages from GV PLA_2 knock-out mice displayed a 50% reduction in the amount of eicosanoids released upon stimulation with zymosan, indicating a secondary role for the enzyme in this pathway. Previous studies by Leslie and coworkers (26) had shown that zymosan-induced AA release and eicosanoid production were abolished in peritoneal macrophages from GIVA PLA_2 knock-out mice. This suggests that GV PLA_2 is subordinate to GIVA PLA_2 and plays a rather augmentative role in this pathway in peritoneal macrophages. To test whether basal levels of GV PLA_2 are sufficient for AA release in a similar augmentative manner in the $P388D_1$ cell line would require a complete knock-out of the gene. Unfortunately, to date, utilizing several approaches, we have not succeeded in disrupting the GV PLA_2 gene in $P388D_1$ cells.

We have shown that considerable amounts of active GV PLA_2 are secreted into the extracellular fluid upon LPS stimulation. The secreted enzyme is inhibited in vitro by 1 μM LY311727. However, as mentioned earlier, even 25 μM LY311727 was not effective in inhibiting the LPS-induced release of AA-derived radioactivity, and 50 μM resulted in only a moderate inhibition.

Why does the secreted active enzyme not act on the plasma membrane of $P388D_1$ cells? Recently, it has been shown that extracellular addition of the human GV enzyme to $P388D_1$ cells leads to AA release (6). However, this AA release might be due to detached cells, considering the results from Bezzine et al. (27), which indicate that GV PLA_2 is virtually inactive on adherent HEK293 or CHO-K1 cells. However, when cells were dislodged from the surface, fatty acid release was readily detected (27). At high concentrations of murine GV PLA_2 (1 μg/ml), release of arachidonate from HEK293 cells but not from RBL-2H3 cells was detectable (28). We cannot offer an easy explanation as to why the endogenous secreted enzyme does not act on $P388D_1$ cells in our system. It is conceivable that the presentation of phospholipid substrate on the cell surface is not correct.

If the GV PLA_2 is only active inside the cell, as discussed earlier, is there a function for the protein once it is released into the extracellular space? There are several conceivable roles: *i*) $sPLA_2$ might act in a paracrine manner, as previously suggested (29, 30); *ii*) GV PLA_2 could bind with high affinity to the M-type $sPLA_2$ receptor (31) and might thus exert functions through a cytokine-like action rather than through its catalytic activity; *iii*) recently, a role in LDL hydrolysis and macrophage foam cell formation was attributed to GV PLA_2 (32); *iv*) GV PLA_2 could serve to kill Gram-positive bacteria (33); and *v*) secretion of the enzyme might simply be a means to clear the cells from its intracellular activity and direct it into the degradative pathway. A degradative pathway for GV PLA_2 in neutrophils has previously been suggested by Kim et al. (34).

The signal transduction model developed by Shinohara et al. (5) and Balsinde et al. (6) proposes that GIVA PLA_2 regulates expression of GV PLA_2, which, in turn, upregulates COX-2. These results were based mainly on the chemical inhibitors MAFP, LY311727, and first-generation AS-ON directed against GV PLA_2. Here we used pyrrophenone, which is more specific than MAFP in inhibiting the GIVA enzyme. Treatment of the cells with pyrrophenone did not result in an inhibition of the LPS-induced increase in GV PLA_2 activity or COX-2 protein level. We attempted to confirm the results found with pyrrophenone by utilizing second-generation AS-ON to inhibit expression of GIVA PLA_2. However, these experiments were not conclusive, inasmuch as GIVA PLA_2 protein levels and GIVA PLA_2 activity were not affected by treatment with AS-ON. The antisense technology in general has practical limitations, because reduction of protein levels depends on the half-life of the protein (15).

In cells treated with GV AS-ON we did not detect a decrease in COX-2 protein in three independent experiments. In one experiment, we detected only a slight decrease (data not shown). If GV PLA_2 were significantly involved in the regulation of COX-2 expression, we would expect a prominent decrease in the amount of COX-2 protein produced in the presence of GV AS-ON.

Altogether, our results suggest that the GIVA PLA_2 is indispensable for AA release. However, it does not appear to play a role in the regulation of the expression of GV PLA_2 or COX-2. The role of the GV PLA_2 in $P388D_1$ cells is elusive. However, from our results, it is clear that the upregulation of the enzyme upon stimulation with LPS is not required for PGE_2 production.

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