UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Function, Activation and Inhibition of Phosphatidic Acid Phosphohydrolase-1 and Group IVA Phospholipase A_2 in Toll-like Receptor-4 Activated Macrophages

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Chemistry

by

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The dissertation of Andrej Grkovich is approved, and it is acceptable in quality and form for publication on microfilm:

Chair

DEDICATION

Dedicated to My Family and Friends without whom this would not be possible.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AA	Arachidonic acid
AATFK	Arachidonyl trifluoromethyl ketone
BEL	Bromoenol lactone
C1P	Ceramide 1-phosphate
CERK	Ceramide kinase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
DAG	Diacylglycerol
DHA	Docosahexanoic acid
DMSO	Dimethyl sulfoxide
EDTA	(Ethylenedinitrilo)tetraacetic acid tetrasodium salt
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-tetraacetic acid
EPA	Eicosapentanoic acid
FFA	Free fatty acid
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GIVA PLA_2	Group IVA phospholipase A_2
HAD	Haloacid dehalogenase
HPLC-MS	HPLC-mass spectrometry
I-κB	Inhibitors of κB

IL	Interleukin
IP_3	1,4,5-trisphosphate
IRAK	Interleukin receptor associated kinase
KDO	Kdo_2-Lipid A
LBP	LPS binding protein
LPP	Lipid phosphate phosphohydrolase
LPS	Lipopolysaccharide
MAFP	Methyl arachidonylfluorophosphonate
Mal	MyD88-adaptor-like protein
MAPK	Mitogen activated protein kinase
MD-2	Myeloid differentiation protein-2
MeOH	Methanol
MyD88	Myeloid differentiation factor-88
NEM	*N*-ethyl maleimide
NF-κB	Nuclear factor-κB
NO	Nitrous oxide
PA	Phosphatidic acid
PAP-1	Phosphatidic acid phosphohydrolase-1
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol 4,5-phosphate

PKC	Protein kinase C
PL	Phospholipid
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol myristate acetate
PMN	Polymorphonuclear leukocytes
PPAR	Peroxisome proliferator activator receptor
Q-PCR	Real-time quantitative PCR
TICAM	TIR domain-containing adaptor molecule
TLC	Thin layer chromatography
TLR-4	Toll-like receptor-4
TNF-α	Tissue necrosis factor-α
TRAF 6	TNF receptor activated factor 6
TRIF	TIR domain-containing adaptor inducing IFN-β

LIST OF FIGURES

LIST OF TABLES

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Liu, Y., **Grkovich, A**., Baker, S., Johnson, C., and Dennis, E., Characterization of Group IVA phospholipase A_2 as a mediator in the LPS induced release of arachidonic acid in human U937 cells, ASBMB Conference (San Diego, CA, April 2003).

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ABSTRACT OF THE DISSERTATION

The Function, Activation and Inhibition of Phosphatidic Acid Phosphohydrolase-1 and Group IVA Phospholipase A_2 in Toll-like Receptor-4 Activated Macrophages

by

Andrej Grkovich

Doctor of Philosophy in Chemistry

University of California, San Diego, 2008

Professor Edward A. Dennis, Chair

Macrophages are large, single nucleated leukocytes that participate in the innate and adaptive immune systems through the release of bioactive agents, phagocytosis of unwanted particles and antigen presentation. The macrophage immune response to invading microorganisms begins with the binding of the bacterial endotoxin lipopolysacchride (LPS) to the toll-like receptor-4 (TLR-4), which initiates intracellular signal transduction cascades that subsequently activate a variety of inflammatory proteins, including phosphatidic acid phosphohydrolase-1 (PAP-1) and the group IVA phospholipase A_2 (GIVA PLA_2). PAP-1 is a Mg^{+2}-dependent membrane-associated enzyme that catalyzes the hydrolysis of phosphatidic acid (PA) into diacylglycerol (DAG), while GIVA PLA_2 catalyzes the release of arachidonate from the *sn*-2 position of membrane phospholipids yielding lysophospholipid and free arachidonic acid (AA). The

purpose of my research has been to better understand the molecular mechanism of the cellular regulation and function of PAP-1 and GIVA PLA_2 through the use of a macrophage model. We began by characterizing the expression and activation of key inflammatory proteins that participate in this pathway, including cyclooxygenase-2 (COX-2), PAP-1 and GIVA PLA_2. We observed that the chemical inhibition of cellular PAP-1 results in the loss of COX-2 upregulation in TLR-4 activated macrophages. We observed through HPLC-MS analysis of TLR-4 activated macrophage supernatants that PAP-1 was also necessary for the activation of cellular GIVA PLA_2. Supplementation of macrophages with exogenous DAG, the product of PAP-1 hydrolysis, restored COX-2 expression and GIVA PLA_2 activity. To ensure this was not the result of inhibitor promiscuity, we confirmed that PAP-1 inhibition does not result direct GIVA PLA_2 activation through cellular stimulation with Ca^{+2} agonists, such as ionomycin and ATP. Surprisingly, PAP-1 inhibition reduced the absolute amount of AA released from cells synergistically stimulated with ATP and Kdo_2-Lipid A, a TLR-4 specific agonist, while not affecting the magnitude of synergy enhancement. Furthermore, our data suggests that two bioactive lipids, phosphatidylinositol 4,5-bisphosphate and ceramide 1-phosphate, play regulatory roles in the activation of GIVA PLA_2 in TLR-4 activated macrophages. Since GIVA PLA_2 has become a target in the generation of future anti-inflammatory drugs, our laboratory has been developing a novel class of GIVA PLA_2 substrate-analogue chemical inhibitors. Using HPLC-MS to measure metabolite release for the purpose of evaluating inhibitor efficacy, we concluded that inhibitor structures that contain a 2-oxoamide moiety spaced at the δ or γ position relative to that of the carboxylic acid function group demonstrat e maximal cellular potency and specificity.

CHAPTER I

Introduction

A. Macrophages

Macrophages are large, single nucleated leukocytes that play an important role in the maintenance of the innate and adaptive immune systems. The term "macrophage" is derived from "large eater," as macrophages are members of the mononuclear phagocytic cell system that are derived from circulating blood monocytes which originate from hematopoietic progenitor cells within the bone marrow (1). Monocytes circulate within the blood stream for a number of days before extravating into the surrounding tissues where they differentiate into tissue specific macrophages. Once they have extravated from the blood vessel lumen into the surrounding tissues, macrophages are capable of participating in the innate and adaptive immune systems. Furthermore, macrophage morphology and physiological behavior tends to be distinctive within different host tissues, demonstrating that macrophages are able to adapt to the characteristic needs of the host environment (1).

Macrophages fulfill three principle immune functions, which include the removal of unwanted particles, presentation of antigens to T_H lymphocytes for the generation of antibody epitopes and the release of biologically active agents into the extracellular matrix (1-3). Through chemotaxis, macrophages are able to pursue invading organisms; once a target has been identified, the macrophage extends pseudopodia to surround and seize the target, which is subsequently engulfed and degraded. Portions of degraded targets can be displayed on the plasma membrane via Class II MHC receptors to be subsequently recognized by T_H lymphocytes for the purpose of antibody epitope design.

Additionally, macrophages release a variety of biologically active agents including interleukin-1 (IL-1), IL-6, tissue necrosis factor α (TNF-α), eicosanoids, nitric oxide (NO), epidermal growth factor (EGF), fibroblast growth factor (FGF) and matrix metalloproteases (MMP). These agents induce a variety of physiological effects including chemotaxis, activation of additional cells, fever, swelling, pain, destruction of bacteria, induction of apoptosis, induction of cellular growth and digestion of the extracellular matrix to assist mobilization of immune cells. Clearly, macrophages perform a diverse set of responsibilities in regards to immunity and maintenance of physiological homeostasis.

A macrophage becomes "activated" after encountering immunogenic particles such as bacterial endotoxin, host cytokines, complement proteins and cellular debris derived from damaged host cells (1,4). Activated macrophages display a number of distinctive morphological distinctions that basal cells do not; this includes enhanced phagocytosis and pseudopodia extension with an increased rate of cytokine and eicosanoid release. The focus of this dissertation is to better understand the molecular function of group IVA phospholipase A_2 (GIVA PLA_2) and phosphatidic acid phosphohydrolase 1 (PAP-1) in pro-inflammatory toll-like receptor (TLR)-4 activated macrophages. To conduct the necessary experiments, various macrophage-like mammalian cell lines were utilized, including the human U937, the murine $P388D_1$ and the murine RAW 264.7 cell lines.

B. LPS-TLR-4 signaling

Throughout the ages, humans have suffered from infection by microorganisms, often resulting in reduced life expectancies and living standards for the infected

individuals. The era of modern day microbiology began with the discovery of various microorganisms that included bacteria, nematodes and protists through the use of microscopy by the Dutch researcher Antonie van Leeuwenhoek in the 17th century (5). In 1876, Edwin Klebs theorized that different bacterial species were responsible for manifesting unique pathological disease states in humans. Nobel Prize winner Robert Koch verified this when he isolated and cultured tuberculosis, anthrax and cholera bacteria while recognizing that each species gave rise to a unique disease state. Additionally, he recognized that immunization of mice with serum derived from cattle that had been infected with anthrax prior to inoculation with anthrax spores enabled the mice to survive the infection (5). The next significant advancement was made by Richard Friedrich Johannes Pfeiffer, who identified endotoxin as the immunogenic component of bacteria, which he described as the bacterial poison inducing disease (5). The endotoxin lipopolysaccharide (LPS) is a major component of the outer bacterial membrane (6). LPS is not toxic to the mammalian immune system when it is located within an intact bacterium, however, it becomes immunogenic when bacteria lyse and the LPS molecule is released (7,8).

The mammalian immune system is composed of innate and adaptive defensive measures that the body utilizes to combat invading microorganisms. As previously mentioned, macrophages participate in the innate and adaptive immune responses to a bacterial infection. Furthermore, the manner in which macrophages respond to the presence of LPS within the body can depend upon the dosage of endotoxin encountered. Repeated exposure of macrophages to sublethal dosages can result in an unresponsive cellular state towards subsequent endotoxic challenge, during which macrophages do not

become fully activated and therefore an insufficient immune response is mobilized. On the opposite end of the spectrum, macrophages exposed to an excess of LPS can become hyperactivated to release greater than normal amounts of cytokines, which can result in septic shock to the host (9). Low doses of LPS are present in the serum of healthy individuals due to the entry of intestinal bacteria debris into the bloodstream through the portal vein of the liver. It is believed that low levels of LPS are necessary in a healthy individual to maintain macrophage sensitivity to the presence of elevated endotoxin levels during a potential pathological infection (5,10,11). Clearly, effective cellular mediated immunity consists of a delicate balance of destroying dangerous infectious agents while not causing unnecessary harm to host tissues.

After LPS has been released from the extracellular leaflet of the cell wall of a disintegrating bacterium, an intricate signaling cascade must be activated to enable the immune system to recognize the presence of the pathogen. The first event that takes is the binding of LPS monomers (Shown in Figure I.1) to the 60 kD LPS binding protein (LBP). LBP is present constitutively at low levels in the serum of healthy individuals, although serum levels are markedly increased during an infection (12). The LPS-induced activation of monocytes, macrophages and polymorphonuclear (PMN) leukocytes is enhanced by a factor of 100-1000 in response to the presence of LBP (13). Each LBP molecule is capable of binding to the Lipid A portion of an LPS molecule, stabilizing the monomeric form of LPS (14). After LBP has bound a molecule of LPS, it then catalyzes the transfer of LPS to CD14, a protein expressed by macrophages in both membrane bound and soluble forms (13). CD14 is a glycosylphosphatidylinositol-linked receptor that is expressed on the extracellular surface of macrophages, but lacks a

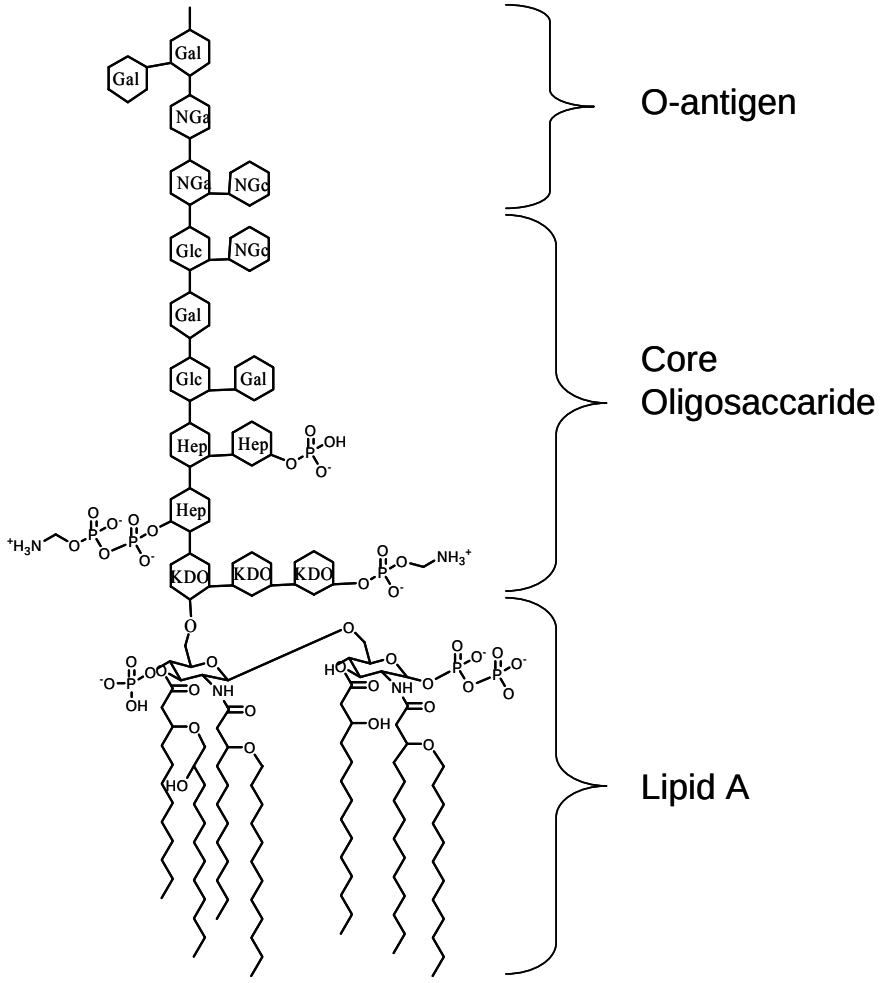


Figure I.1. Chemical structure of the bacterial endotoxin LPS molecule.

Schematic representation of E.coli O111:B4 LPS molecule. Hep, Gal, Glc,KDO, NGa and NGc denote the sugars: L-glycerol-D-manno-heptose, galactose, glucose, 2-keto-3- deoxyoctonic acid, N-acetyl-galactosamine and N-acetyl-glucosamine, respectively. Adapted from (15).

transmembrane domain; therefore, it requires the participation of toll-like receptor 4 (TLR-4) to initiate intracellular signaling cascades after binding to LPS (16).

The toll-like receptors were named after the homologous Toll protein discover in *Drosophilia melanogaster* and recognize a wide variety of pathogenic ligands derived from bacteria, yeast and fungi (17-19). TLR-4 is a 92 kD protein that is most predominantly expressed in monocytes, macrophages and PMNs and is regarded as the limiting factor in the LPS-induced activation of macrophages due to low expression levels and the fact that it is the only receptor in the transduction scheme that includes a transmembrane domain (20-23). Analogous to LBP, TLR-4 binds the fatty acid acyl chains within the Lipid A portion of the LPS molecule. Recent data suggests that TLR-4 can bind and be functionally activated by individual fatty acids, as NF-κB signaling was stimulated through a TLR-4 mechanism through the exposure of cells to fatty acids (24,25). In studies conducted with TLR-4 deficient mice, the animals did not exhibit a phenotypic response when challenged with a dose of LPS (26). RAW 264.7 macrophages transfected with a TLR-4 missense mutant were unresponsive to LPS stimulation (27). Clearly, TLR-4 is required for cellular recognition of LPS. However, TLR-4 functions within a complex with two other extracellular proteins, CD-14 and myeloid differentiation protein-2 (MD-2), to achieve the most optimal signaling response. CD14 is believed to be an accessory protein that complexes with TLR-4 during LPS signaling as it is known that CD-14 binds to LPS but does not directly participate in signaling (28). At low dosages of LPS, CD-14 knockout mice were highly resistant to

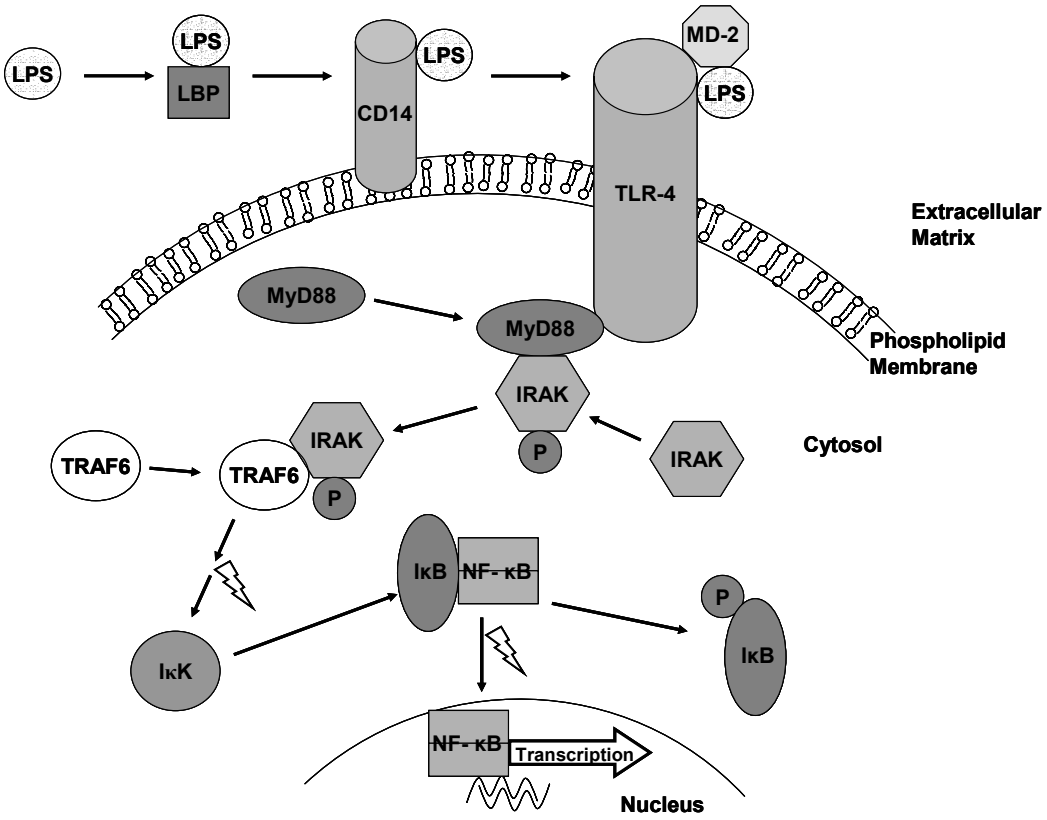


Figure I.2. Schematic of LPS/TLR-4 mediated signaling.

Schematic representation of the inflammatory signal transduction cascade that is activated through LPS agonization of TLR-4. Adapted from (5).

the LPS treatment, although they did respond to higher concentrations of LPS (29). The addition of soluble CD-14 to cultured cells enhanced NF-κB activation (30). Likewise, MD-2 has been found to be physically associated with TLR-4 during signaling and its presence is required for optimal response to LPS (31). A point mutation made on MD-2 expressed in Chinese hamster ovary cells resulted in loss of response to LPS stimulation (32). It is apparent that the presence of CD-14 and MD-2 are necessary to achieve maximal sensitivity to low doses of LPS.

Once the extracellular TLR-4 receptor complex has been activated by ligand binding of the LPS molecule, a signal transduction cascade is initiated in the cytosol through the intracellular domain of the TLR-4 receptor. The intracellular region of the TLR-4 receptor is homologous to that of the IL-1 receptor, and therefore both receptors activate the same kinases, such as the IL-1 receptor associated kinases (IRAK), and require participation of the same cytosolic accessory molecules, such as myeloid differentiation factor 88 (MyD88) and TNF receptor activated factor 6 (TRAF6) (33-35). MyD88 contains two protein interaction domains, an N-terminal death domain and a C-terminal TIR domain. The TIR domain is known to interact with TLR-4 and IL-1 receptors, while the N-terminal domain interacts and recruits IRAK to the intracellular receptor complex (34,35). After having been recruited to the receptor complex by MyD88, IRAK undergoes phosphorylation whereby it becomes activated, dissociates from the receptor complex and then associates with the adaptor protein TRAF6 (33,36,37). Once activated by association with IRAK, TRAF6 subsequently activates MAPK and inhibitors of κB (IκB) kinases. These kinases in turn then activate the transcription factors AP-1 and NF-κB, which ultimately activate inflammatory protein synthesis (38-43). Mice that are

deficient in any of the aforementioned signaling components demonstrate hyporesponsiveness to LPS challenges (44,45).

It should be addressed that TLR-4 receptor activation is capable of inducing inflammatory transcriptional activation through MyD88-independent mechanisms. One such mechanism proceeds through the activation of the TIR domain-containing adaptor protein (TIRAP) and MyD88-adaptor-like protein (Mal). Neither protein contains an N-terminal death domain. The two proteins are known to complex and then dock on the activated TLR-4 receptor at a different location from MyD88. Once activated through interaction on the TLR-4 receptor, the complex initiates MAPK and NF-κB signaling cascades (46,47). A second MyD88-independent mechanism involves a second protein complex comprising TIR domain-containing adaptor inducing IFN-β (TRIF) and TIR domain-containing adaptor molecule (TICAM)-1 (46,48). Like the TIRAP/Mal signaling complex, these proteins also do not contain an N-terminal death domain. TRIF gene knockouts inhibited the activation of NF-κB in experiments conducted through the activation of TLR-2, 3, 4 and 7 (46). Since the MyD88 dependent and independent pathways have the same general downstream regulatory targets, it is currently believed that they may have different temporal functions, with MyD88 dependent mechanisms acting early and independent acting later (49).

C. Group IVA Phospholipase A_2

Group IVA phospholipase A_2 (GIVA PLA_2), also known as the cytosolic phospholipase A_2 ($cPLA_2$), is a member of the phospholipase A_2 superfamily, which comprise a set of structurally distinctive enzymes that catalyze the hydrolysis of the ester

bond at the *sn*-2 position of membrane glycerophospholipids to produce free fatty acid and lysophospholipid (Figure I.3) (50,51). Since free fatty acids are the precursors of potent biochemical lipid messengers, such as eicosanoids, the regulation of their release is essential for the maintenance of normal physiology. GIVA PLA_2 is a ubiquitously expressed enzyme that has a substrate preference for arachidonic acid esterfied at the *sn*-2 position of membrane glycerophospholipids (50,51). The crystal structure of GIVA PLA_2 shows two distinctive domains within the protein, a Ca^{+2}-binding C2 domain and a catalytic domain containing the active site residues (52).

Experiments conducted in macrophages have shown that GIVA PLA_2 is required to elicit agonist-induced arachidonic acid (AA) release and the subsequent production of physiological lipid mediators, such as prostaglandins and leukotrienes (50,51). A substantial amount of evidence indicates that GIVA PLA_2 is the primary mammalian enzyme implicated in the release of membrane bound AA. GIVA PLA_2 knockout mice models have demonstrated that the enzyme participates in a range of physiological processes as knockout mice demonstrate renal dysfunction, lesions within the intestinal lumen, enlarged hearts and defective female reproduction (53-57). Additionally, knockout models have further implicated GIVA PLA_2 participation in a number of inflammatory disease models, including arthritis, encephalitis and allergic reactions. Removal of GIVA PLA_2 resulted in the loss of subsequent signaling eicosanoids, ultimately resulting in the alteration of the pathophysiological functions (58-61). Prediction of the physiological function of GIVA PLA_2 activity can be difficult due to the wide range of

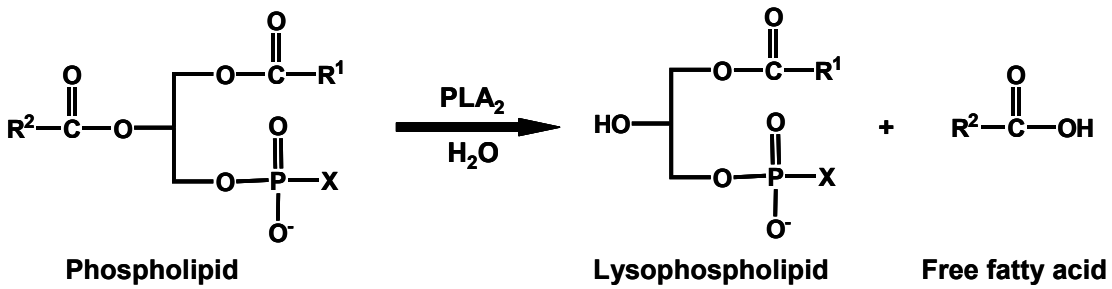


Figure I.3. Schematic diagram of PLA_2 mediated phospholipid hydrolysis.

R^1, R^2 represent fatty acyl chains; X represents a polar head group attached to the glycerol backbone through a phosphodiester bond. Potential head groups include: phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol and phosphatidylinositol.

eicosanoids that are produced from liberated AA and the tissue-specific effect that these mediators can elicit (51). Several distinctive cellular GIVA PLA_2 regulatory mechanisms have been identified, including Ca^{+2} levels, phosphorylation and bioactive lipid interactions that may regulate cellular activity. In the first regulatory mechanism, the translocation of GIVA PLA_2 from the cytosol to intracellular membranes is achieved through increased cytosolic Ca^{+2} (51,62). Studies using Chinese hamster ovary and rat leukemic cells demonstrated that Ca^{+2} ionophore induced the translocation of GIVA PLA_2 from the cytosol to the perinuclear membrane with increased AA release into the media (63,64). In the second regulatory mechanism, kinase-mediated phosphorylation on specific serine residues of GIVA PLA_2 results in enhanced GIVA PLA_2 catalysis (65-68). The manner in which phosphorylation regulates cellular GIVA PLA_2 function is not completely understood, as fully dephosphorylated GIVA PLA_2 protein exhibits maximal catalytic activity (69). One possible explanation is that phosphorylation may activate GIVA PLA_2 under conditions in which cytosolic Ca^{+2} concentrations are low (66). In the final regulatory mechanism, GIVA PLA_2 activity is enhanced through the interaction of GIVA PLA_2 with bioactive lipids, such as phosphatidylinositol 4,5-bisphosphate (PIP_2) and ceramide 1-phosphate (C-1-P). The introduction of PIP_2 into lipid vesicles has been reported to enhance *in vitro* GIVA PLA_2 activity by a factor of 20-fold (70). Cellular studies have demonstrated that elevated endogenous PIP_2 levels are located on the perinuclear region (71). Enhancement of cellular PIP_2 levels by the introduction of Rac1 resulted in increased AA release, while inhibition of PI-4 kinase led to a reduction of PIP_2 levels and AA release (72). Il-1β and Ca^{+2} ionophore induced increased C-1-P levels and AA release concomitantly, while RNA interference of CERK abolished this effect (73).

Clearly, C-1-P enhances GIVA PLA_2 mediated AA release, since the downregulation of GIVA PLA_2 resulted in the abrogation of C-1-P induced AA release from A549 cells (74). It is possible that GIVA PLA_2 regulation may be governed by a combination of these factors depending on physiological circumstances.

D. Cyclooxygenases

The cyclooxygenase (COX) enzyme is responsible for the conversion of free AA into prostaglandin H_2 (PGH_2), which can then be further metabolized into terminal prostaglandins by specialized prostaglandin synthases and subsequently released from the cell. The COX enzyme is a hemoprotein, which catalyzes two distinctive reactions. The first is the cyclooxygenase reaction in which COX incorporates two oxygen molecules into one molecule of arachidonic acid to yield one molecule of prostaglandin G_2 (PGG_2) and the second is a peroxidase reaction that further metabolizes the PGG_2 into PGH_2 (Figure I.4)(75).

Two isoforms of COX exist in mammals, termed COX-1 and COX-2, and possess a 60% structural homology despite performing disparate and distinctive physiological functions (76). Perhaps the most notable difference in the COX isoforms is the protein expression of the two isoforms. COX-1 is constitutively expressed in most mammalian cell types, while COX-2 levels are extremely low or undetectable at basal conditions, although heavily inducible in response to mitogen or cytokine stimulation in certain cell types (76-78). Perhaps the best studied model of COX-1 and COX-2 expression has been the macrophage, as macrophages constitutively expresses COX-1 while also dramatically upregulating COX-2 in response to a number of stimuli,

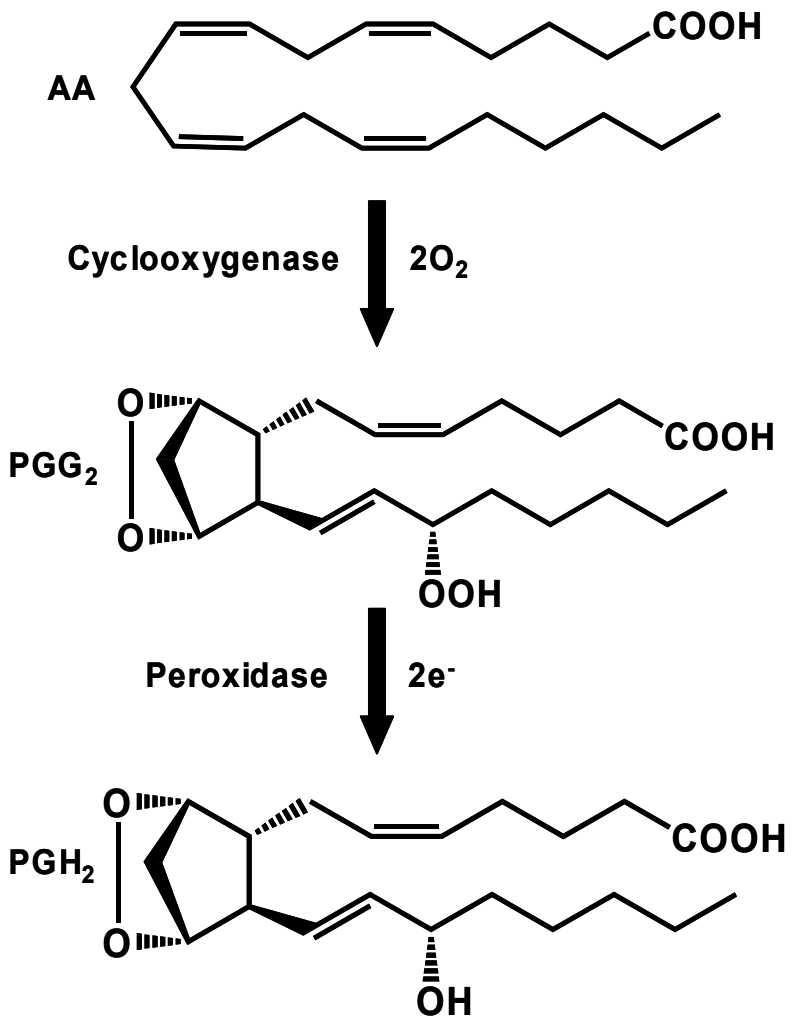


Figure I.4. Schematic diagram of COX mediated AA metabolism.

Schematic demonstrating the cyclooxygenase and peroxidase reactions catalyzed by COX enzymes.

including TLR agonists (79). Since COX-1 is constitutive and ubiquitous, it is generally regarded as a housekeeping enzyme performing roles in physiological functions such as renal homeostasis and gastric cytoprotection (77,80). On the contrary, COX-2 function is associated with mediating inflammatory and pathophysiological functions, such as vasodilation, swelling, vascular permeability, redness and pain (81). Another important difference between COX-1 and COX-2 is their differences in terms of substrate preferences. COX-1 has a preference for AA substrate, whereas COX-2 metabolizes a broader range of substrates, including AA, eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) (75,76). This difference is believed to be the molecular mechanism that enables COX-2 to participate in the resolution phase of inflammation. Both COX isoforms possess an elongated hydrophobic active site into which substrate enters during enzymatic catalysis. The amino acid composition of the channel is identical in the two isoforms with the exception of one residue, as isoleucine 523 in COX-1 is substituted for valine in COX-2 (75).

Due to its pathophysiological role, COX-2 has become a target in the production of anti-inflammatory drugs that alleviate pain, fever and swelling associated with inflammation while trying not to affect the homeostatic function of COX-1 (76,78). The active sites of the COX-1 and COX-2 enzymes are similar, with the exception that isoleucine 523 is substituted for a valine within the active site of COX-2; this difference has been utilized to develop COX-2 specific inhibitors that do not affect COX-1 significantly (75). A number of alternate methodologies have also been developed, including blocking the formation of the tyrosyl 385 radical that is required for COX-2 catalysis (75).

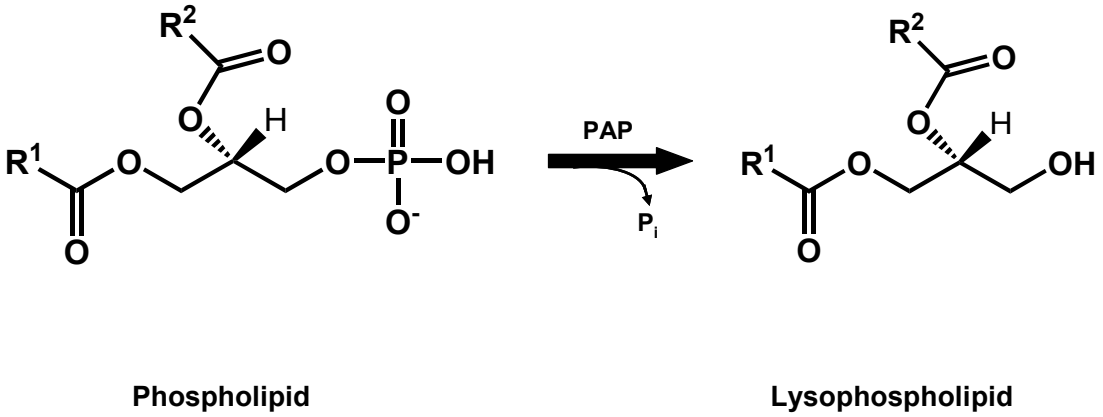


Figure I.5. Schematic diagram of PAP mediated hydrolysis.

Schematic demonstrating the dephosphorylation of PA by PAP; R1, R2 represent fatty acyl chains.

E. Phosphatidic Acid Phosphohydrolase-1

Phosphatidic acid phosphohydrolase (PAP) catalyzes the dephosphorylation of phosphatidic acid (PA), which results in the subsequent formation of the signaling molecule diacylglycerol (DAG) as well as inorganic phosphate (Figure I.5) (82). Cellular DAG can be produced directly from the hydrolysis of membrane phospholipids via the actions of phospholipase C (PLC), or through successive rounds of hydrolysis by phospholipase D (PLD) and PAP-1 enzymes. Current evidence suggests that at least two known classes of PAP enzymes exist within mammalian cells with experimental evidence indicating that additional PAP enzymes exist that have yet to be identified (83). PAP-1 is a soluble enzyme that undergoes translocation from the cytosol to the perinuclear membranes where it interacts with substrate; while PAP-2, which is also known as the lipid phosphate phosphohydrolase (LPP), is an integral plasma membrane protein (84). Besides subcellular location, PAP-1 and PAP-2 differ in a number of additional distinctive manners. The first is that PAP-1 has a distinctive substrate preference for the Mg^{+2} salt of PA, whereas PAP-2 is capable of dephosphorylating a number of substrates, including sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P), PA and lyso-PA (84). The second is that PAP-1 requires Mg^{+2} for catalytic activity and is sulphhydral sensitive, whereas PAP-2 does not require the presence of divalent cations nor is it affected by the presence of sulphhydrals.

Cytosolic PAP-1 activity was initially identified from preparations of liver, adipose and intestinal tissues as a factor that stimulated the formation of DAG and triacylglycerol (TAG) (84-88). Gaining a complete understanding of the molecular characteristics of the enzyme has been hindered until recently due to a number of difficulties that include the

contamination of samples preparations with Ca^{+2} which interferes with PAP-1 mediated hydrolysis as well as the presence of PAP-2 within samples, which has a much larger V_{MAX} (84,89). Recently, PAP-1 has been purified and sequenced from *S. cerevisiae*; it has been identified to be the mammalian homologue of Lipin-1 (83,90). Carman and colleagues have identified PAP-1 as the putative enzyme in the biosynthesis of phosphatidylethanol, phosphatidylcholine and TAG within the Kennedy pathway (84,90,91). In addition to playing a role in the biosynthesis of membrane glycerolipids, PAP-1 has been implicated in cellular growth, signal transduction, differentiation and survival (84,90,92-94).

The identification of Lipin-1 as a PAP-1 homologue has provided a wealth of information pertaining to the enzymatic catalysis, regulation and physiological function of PAP-1. Prior to having been identified as a PAP-1 homologue, Lipin-1 had been physiologically associated with triglyceride metabolism and adipocyte development within mammalian organisms (82). Lipin-1 is known to possess a catalytic sequence, DXDXT, which is conserved amongst lipid phosphatases within the haloacid dehalogenase (HAD) superfamily (95-97). Mutation of either aspartic acid residue results in the total loss of the proteins enzymatic activity (95). As previously mentioned, PAP-1 is known to require substrate to be presented as the Mg^{+2} salt of phosphatidate within a phospholipid membrane (84,91,98,99). Both PAP-1 and Lipin-1 are known to undergo kinase-mediated phosphorylation; although current evidence suggests that PAP-1 is functionally activated when it is phosphorylated by protein kinase A, whereas Lipin-1 is activated when it undergoes dephosphorylation (95,100). Further study is required to clarify this anomaly.

F. Eicosanoids

Eicosanoids are a dynamic class of AA-derived lipid mediators that have a wide range of physiological functions, including neurological development, reproductive development, cytoprotection of the digestive tract and participation within the inflammatory response (77). The first eicosanoids were identified after the discovery of prostaglandins from bovine prostate tissue extracts was described in a groundbreaking publication by von Euler (101). Subsequent crucial discoveries by Samuelsson and Bergström recognized that prostaglandins are metabolites of AA that have been released into the extracellular matrix (102). The etymology of the word eicosanoid is derived from the Greek "eicosa," meaning twenty; the relevance of which is that all eicosanoids are derivatives of the 20 carbon fatty acid AA. An understanding of the physiological function of eicosanoids was developed through advancements made by Vane when he had observed that aspirin treatment, which had been previously associated with analgesic and anti-inflammatory actions, also inhibited the biosynthesis of eicosanoids (103).

Eicosanoids are released from cells where they are synthesized through means of facilitated transport into the surrounding extracellular matrix. As described in figure I.6, eicosanoids are known to elicit a broad spectrum of physiological responses on affected host tissues. The anatomical distance that an eicosanoid induces an affect from the location where it is released depends upon a number of factors, including molecular lability, degradation and expression of the appropriate receptors. For example, PGI_2 and TxA_2 have half lives of only a few seconds, and therefore only induce localized physiological effects, whereas the more stable PGD_2 and PGE_2 are capable of eliciting

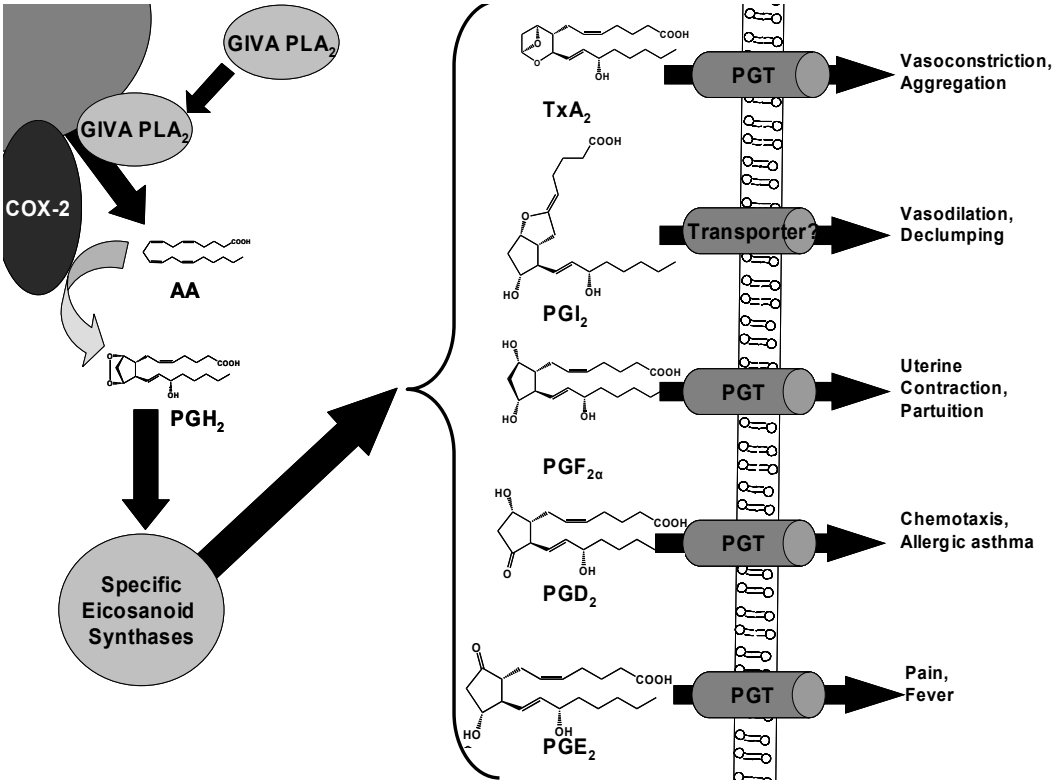


Figure I.6. Schematic diagram of the synthesis, release and physiological actions of eicosanoids.

Adapted from Funk, 2001 (77).

systemic responses. Eicosanoids modulate a range of physiological effects through interaction with ligand-specific G-protein coupled receptors, which subsequently results in the activation of signal transduction cascades (104). Interestingly, there are currently 4 known PGE_2 (EP_1-EP_4) and 2 PGD_2 (DP_1-DP_2) receptors. This indicates that it is possible that these two eicosanoids may initiate physiologically distinctive responses depending on the specific receptor that is expressed within a given tissue and therefore the potentially different signal transduction cascade that is subsequently activated (77,81). Clearly, eicosanoids are a dynamic class of biologically active lipid metabolites that are capable of eliciting a variety of physiological responses.

CHAPTER II

LPS Induced COX-2 Expression is PAP-1 dependent in Human U937 and Murine $P388D_1$ Macrophages

A. Background

Bacterial sepsis and septic shock result from the overproduction of inflammatory mediators as a consequence of the interaction of the immune system with bacterial cell components, such as lipopolysaccharide (LPS) (5). LPS activated macrophages produce and release a host of cytokines and eicosanoids in response to inflammatory stimulation, including prostaglandins (PG). PGs are involved in a number of physiological processes including inflammation, pain, and vascular permeability while also acting as major contributors of endotoxic shock (105).

Phospholipase A_2 (PLA_2) comprises a superfamily of enzymes that catalyze the hydrolysis of the *sn-2* ester bond in phospholipids, producing free fatty acid and lysophospholipid (106). PLA_2s regulate inflammation and intracellular signal transduction cascades by liberating arachidonic acid (AA) from membrane phospholipids. The AA that is released is subsequently metabolized by COX, producing prostaglandin H_2 (PGH_2). There are two known isoforms of cyclooxygenase, COX-1 and COX-2, COX-1 is usually a constitutively expressed enzyme that primarily plays a housekeeping role and has been implicated in a number of physiological functions, including platelet aggregation and parturition (107), while COX-2 is usually not expressed under basal conditions in macrophages, but is highly inducible by a number of pro-inflammatory agonists, such as LPS, interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) (108,109). In addition to inflammation, COX-2 expression is necessary for uterine

contractions during the birthing process and has been further implicated in a number of pathological conditions, including fever, pain and cancer (107,109). The PGH_2 produced by COX is further metabolized by specific downstream enzymes into prostaglandins, prostacyclins and thromboxanes, which are then subsequently secreted (110). When released, eicosanoids function as ligands in receptor-specific autocrine and paracrine signal transduction pathways leading to a diverse range of physiological effects (111).

Two forms of phosphatidic acid phosphohydrolase (PAP) have been identified, a Mg^{+2}-independent transmembrane isoform, lipid phosphate phosphohydrolase (LPP), and a cytosolic Mg^{+2}-dependent isoform that translocates to the ER when it hydrolyzes its substrate, known as phosphatidic acid phophohydrolase-1 (PAP-1). LPP is known to hydrolyze a host of substrates besides phosphatidic acid (PA), including lysophosphatidic acid, sphingosine-1-phosphate and ceramide-1-phosphate, while PAP-1 has been observed to have a preference for PA as substrate (112,113). Furthermore, PAP-1 enzymatic activity requires Mg^{+2} and is sensitive to the inhibition by *N*-ethyl maleimide (NEM), propranolol and bromoenol lactone (BEL), whereas LPP has no divalent cation requirements for activity and is not inhibited by these agents (114-119). LPS stimulation has been previously shown to induce the translocation of PAP-1 to the endoplasmic reticulum where it hydrolyzes PA to produce diacylglycerol (DAG) (84). Evidence has been presented that both the substrate and product of PAP-1, PA and DAG, respectively, may be essential signaling molecules in normal macrophage function (84,120). Previously, George Carman had succeeded in the purification and characterization of PAP-1 from *S. cerevisiae* (121,122). Recently, his laboratory has sequenced and cloned *S. cerevisiae* derived PAP-1, identifying it as the homologue of mammalian Lipin 1, which

has been associated with adipocyte development (83). In the present work, we will demonstrate that PAP-1 is necessary for the LPS induced expression of COX-2 in the human U937 cell line.

The human U937 macrophage-like cell line has been a widely characterized model of the mammalian cellular response to various inflammatory stimuli. It has been shown that U937 cells respond to the presence of LPS with a marked increase in the rate of cellular AA metabolism and cytokine release into the extracellular space (123).

B. Materials and Methods

1. *Reagents*

Human promonocytic leukemia U937 cells and murine $P388D_1$ were purchased from American Type Culture Collection (Manassas, VA). IMDM and RPMI 1640 cell culture medium were obtained from Gibco (Grand Island, NY). Fetal bovine serum was from VWR International (Bristol, CT). Phorbol myristate acetate (PMA) and lipopolysaccharide (LPS), from *E. coli* 0111:B4, were obtained from Sigma Chemical Company (St. Louis, MO). Dioctoyl-DAG was from Biomol (Plymouth Meeting, PA). Human heart PolyA+ RNA was purchased from CLONTECH (Palo Alto, CA). [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid (specific activity 100 Ci/mmol), [9,10-^{3}H] palmitic acid (specific acid 47.5 Ci/mmol) and L-α-dipalmitoyl,[glycerol-14C(U)] phosphatidic acid (specific activity 141 mCi/mmol) were obtained from NEN Life Science Products (Boston, MA). MAFP, NS-398, aspirin, and the COX-1 and COX-2 antibodies were purchased from Cayman Chemical (Ann Arbor, MI). The specific $cPLA_2$ inhibitor, pyrrophenone, was kindly provided by Dr. Kohji Hanasaki (Shionogi

Research Laboratories of Shionogi & Co., Ltd). The GIVA PLA_2 antibody was obtained from Cell Signaling (Beverly, MA) and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from BioTrend (Cologne, Germany). 20 cm x 20 cm x 250 μm K6 Silica gel thin layer chromatography plates were from Whatman (Clifton, New Jersey).

2. *Cell Culture and Stimulation Protocol*

The normal growth medium of the U937 cells contained RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37 °C in a humidified atmosphere of 95 % air and 5% CO_2. For experiments the cells were seeded at a density of 5 x 10^5 cells/well in 12-well plates (Corning Inc.) and differentiation was initiated by the addition of PMA into the cellular medium at a final concentration of 100 nM and allowed to proceed for 48 hrs (124). The cells were then washed once and incubated in normal growth medium for an additional 24 hrs prior to the addition of LPS (1 μg/ml). When inhibitors were used, they were added to the medium 30 min before LPS was added. When DAG was added to the cells, it was added immediately after the addition of the LPS. The DAG was initially dissolved in DMSO, and then further diluted into cellular medium contained prior to being dispensed into the medium of the cultured cells. Control cells were administered equivalent concentrations of DMSO. Cell viability was assessed visually by the Trypan Blue Dye exclusion assay (Gibco, Grand Island, NY) and through the usage of the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI).

$P388D_1$ cells were maintained at 37 °C in a humidified atmosphere at 90% air and 10% CO_2 IMDM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, 100 μg/ml streptomycin, and non-essential amino acids. Cells were plated at a confluency 10^6/well in 12-well tissue culture plates, allowed to adhere overnight, and then used for experiments the following day. All experiments were conducted in serum free IMDM medium.

3. *Preparation of RNA and Reverse-transcription*

Total cellular RNA was isolated from cells with the RNeasy Mini Kit from Qiagen (Valencia, CA), as described by the manufacturer's procedure. Any remaining DNA in the extract was removed with the DNA-*free* kit from Ambion (Austin, TX). The cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA), following the manufacturers printed protocol. Oligo dT primers (Invitrogen, Carlsbad, CA) were used during the reaction in order to produce cDNA. The remaining RNA was removed from the cDNA by incubating the samples with 20 U of RNAse H from Invitrogen (Carlsbad, CA) at 37 °C for 20 minutes.

4. *Real-time Quantitative PCR*

Primers used for the PCR are as follows: GAPDH forward primer 5' CCACCCAGAAGACTGTGGAT 3', reverse primer 5' TTCAGCTCAGGGATGACCTT 3'; GIVA PLA_2 forward primer 5' ACTGCACAATGCCCTTTACC 3', reverse primer 5' CGGGAGCCATAAAAGTACCA 3'; COX-1 forward primer 5' CAGTGGCTCGTATCCCAAAT 3', reverse primer 5' AGGCACAGATTCAGGGAATG 3'; COX-2 forward primer 5' CAGCACTTCACGCATCAGTT 3', reverse primer 5' CGCAGTTTACGCTGTCTAGC

3'. All of the primers were selected using the Primer 3 software (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and synthesized by Genset Corporation (La Jolla, CA). All primers were tested by conventional PCR and shown to give only one product visually on 4% agarose gels. Real-time quantitative PCR (Q-PCR) was performed on the ABI 7700 Sequence Detection System from Applied Biosystems (Foster City, CA) using SYBR Green PCR Master Mix detection as described in the manufacturer's procedure. The Q- PCR consisted of an initial hold at 95 °C for 10 minutes, and then 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. The amount of template cDNA used for each sample was 10 ng and 100 nM primer. Gene expression was normalized to the housekeeping gene, GAPDH.

5. *PGE_2 Assay*

The cellular media was cleared of detached cells by centrifugation and then the PGE_2 release was quantitated using a monoclonal PGE_2 EIA kit (Cayman Chemical, Ann Arbor, MI). The assays were conducted according to the manufacturer's protocol.

6. *Immunoblotting*

Cells were washed twice with cold PBS, and scraped free with a rubber policeman in 75 μL of Complete Mini protease cocktail solution (Roche, Mannheim, Germany). Protein concentrations were determined and normalized using the BioRad Protein Assay (BioRad, Hercules, CA). 35 μg total protein was loaded onto 4-12% Bis-Tris SDS-PAGE gels and then transferred onto nitrocellulose membrane. When blotting for the Group IVA PLA_2 the membrane was blocked with 5% milk protein in PBS buffer containing 0.1% Tween 20 for 1 hour before being probed with a GIVA PLA_2 specific antibody overnight. The membrane was then washed three times in PBS containing 0.1% Tween

20 before addition of a rabbit IgG-HRP conjugated secondary antibody (Cell Signaling, Beverly, MA) for one hour. The membrane was then washed three additional times and then developed using the Western Lightning ECL kit (Amersham Pharmacia Biotech). When blotting for COX-2 the membrane was blocked using 3% BSA (Sigma), 1% Casein (Vector, Burlingame, CA) in Tris buffered saline (TBS) buffer containing 0.05% Tween 20 for 1 hour before being probed with a COX-2 specific antibody overnight. The membrane was then washed three times in TBS containing 0.05% Tween 20 before the addition of a rabbit biotinylated IgG (Vector) secondary antibody for 30 min at room temperature. The membrane was again washed three times and exposed to a Streptavidin-HRP conjugated antibody (Vector) for 30 min at room temperature. The membrane was then washed three additional times and then developed using the Western Lightning ECL kit. When blotting for Lipin-1 protein the membrane was blocked with 5% milk protein in PBS buffer containing 0.1% Tween 20 for 1 hour before being probed with a specific Lipin-1 antibody (Alpha Diagnostic, San Antonio, TX) overnight. The membrane was then washed three times in PBS containing 0.1% Tween 20 before addition of a rabbit IgG-HRP conjugated secondary antibody (Alpha Diagnostic, San Antonio, TX) for one hour. The membrane was then washed three additional times and then developed using the Western Lightning ECL kit (Amersham Pharmacia Biotech).

7. *PA Phosphohydrolase Activity Assay*

PA phosphohydrolase activity was determined from U937 and P388D_1 cellular lysates according to the method of Day and Yeaman (125) as modified by Balboa et al (65). The substrate [^{14}C]glycerol-labeled PA was presented as mixed micelles with Triton X-100 at a detergent/phospholipids molar ratio of 10:1. Assays were conducted at 37 °C

for 1 hour. The assay mixture contained 100 μM PA substrate (.025 μCi/assay), 1 mM Triton X-100, 50 mM Tris-HCl, pH 7.1, 10 mM β-mercaptoethanol, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and the lysate to a final volume of 100 uL. The reaction was terminated by the addition of a chloroform/methanol system (60:40) to the reaction mixture. The organic phase was separated from the aqueous, placed into fresh microcentrifuge tubes and dried in a vacuum-centrifuge. The subsequent pellet was reconstituted in 20 uL of the (60:40) chloroform/methanol system, the resulting [^{14}C]PA and [^{14}C]DAG were then separated by thin layer chromatography using an *n*-hexane/ether/water (70:30:1). The TLC plate was then developed in an iodination chamber, and the DAG and PA spots were scraped and subsequently quantified by liquid scintillation counting. Additionally, to distinguish between the PAP-1 and LPP activities, the PAP-1 specific inhibitor, NEM, was added (8 mM) for a period of ten minutes prior to addition of the substrate (114). When PAP-1 assays were performed on the cytosolic or membrane fraction of lysates, lysate preparations were centrifuged at a speed of 100,000 x g for 30 minutes and the cytosolic and membrane fractions were then separated.

When quantifying the endogenous production of DAG, cells were incubated in 10% FCS RPMI medium supplemented with [9-10(n)-^{3}H]palmitic acid (1 μCi/mL) for 24 hours and the cells were washed, and serum free media was added for 1 hour prior beginning the experiment. Lipids were extracted according to the method of Bligh and Dyer (126). The extraction solution was placed into fresh microcentrifuge tubes and dried in a vacuum-centrifuge. The resulting pellet was reconstituted in 20 uL of the (60:40) chloroform/methanol system, the resulting [^{3}H]DAG was separated by thin layer chromatography using an *n*-hexane/ether/water (70:30:1). The TLC plate was then

developed in an iodination chamber, and the DAG and PA spots were scraped and subsequently quantified by liquid scintillation counting.

C. Results

1. LPS Upregulates Cyclooxygenase-2 Expression and Induces PGE_2 Production in a Time Dependent Manner.

Since LPS induced PGE_2 release is known to be dependent on the function of the PLA_2 and COX enzymes, we measured their expression over time with LPS stimulation to determine whether changes in their levels are a mechanism for controlling PGE_2 production. As has been previously described in the U937 cells (127), the levels of COX-2 mRNA transcript increased dramatically during the first four hours of LPS stimulation, reaching maximal levels at four hours, and then waning afterwards (Figure II.1.A). This result is corroborated by the LPS induced COX-2 protein expression profile, in which the protein was undetectable until 2 hours after LPS stimulation and then increased steadily until 20 hours (Figure II.1.B). Furthermore, 1 μg/mL LPS increased PGE_2 release from U937 macrophages in a time dependent manner. The PGE_2 release started between 2-4 hours after LPS stimulation and continued to increase with time for 20 hours, as shown in figure II.1.C. There was no change in PGE_2 production in control cells that were not exposed to LPS. Neither COX-1 nor GIVA PLA_2 expression was upregulated on the protein or messenger RNA levels in response to the addition of 1 μg/mL LPS agonist (Figure II.2).

Figure II.1. LPS stimulation induces COX-2 expression and PGE_2 release from U937 macrophages.

Time-course of LPS induced COX-2 mRNA transcript levels, COX-2 protein expression and PGE_2 release in U937 macrophages. A) U937 macrophages were incubated in the absence (●) and presence (○) of 1 μg/mL LPS and then subsequently COX-2 mRNA levels were determined at the indicated times over a 20 hour period by quantative PCR analysis. B) U937 macrophages were incubated in 1 μg/mL LPS for the indicated amounts of time before the cellular lysates were collected and blotted for COX-2 protein expression. C) U937 macrophages were incubated in the absence (●) and presence (○) of 1 μg/mL LPS and then subsequently PGE_2 release was determined at the indicated times over a 20 hour period. Representative experiments of at least three individual experiments are shown. Data is expressed as mean values ± S.D. of three individual replicates.

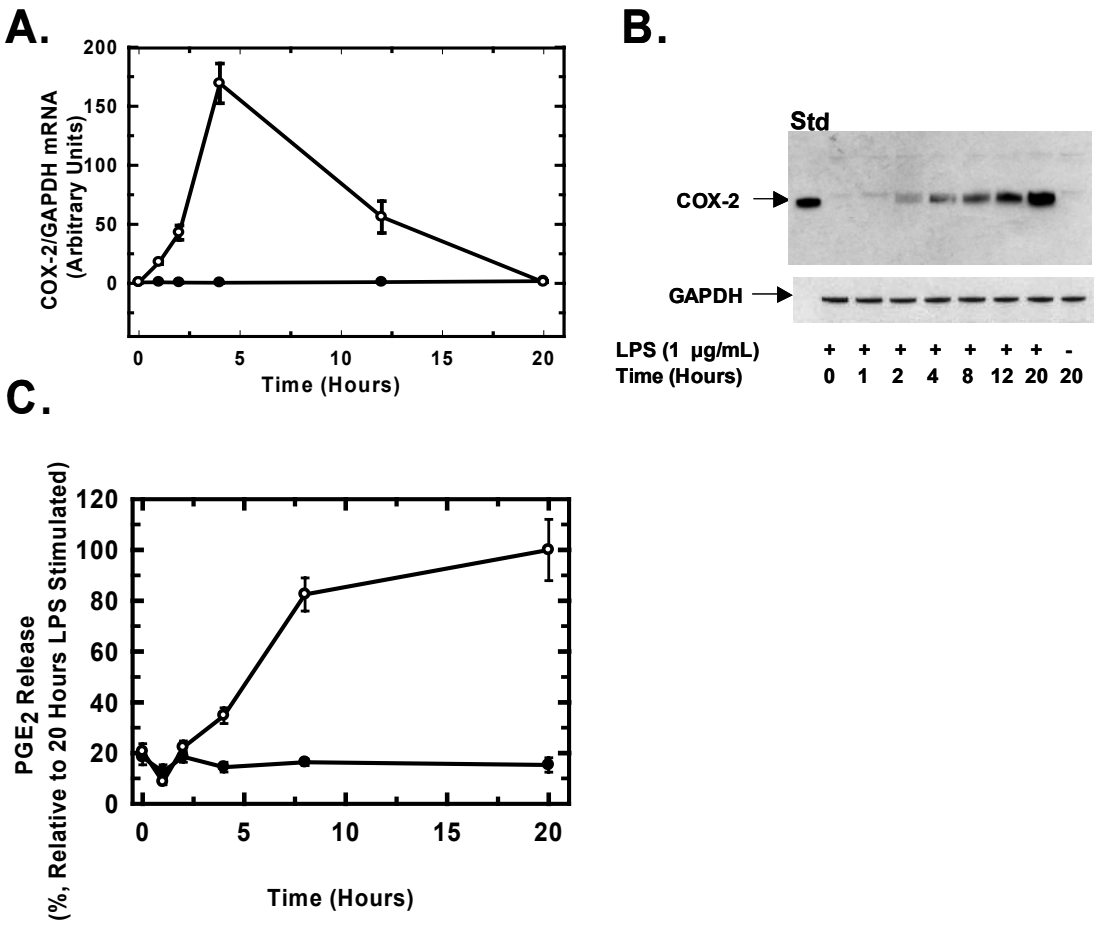
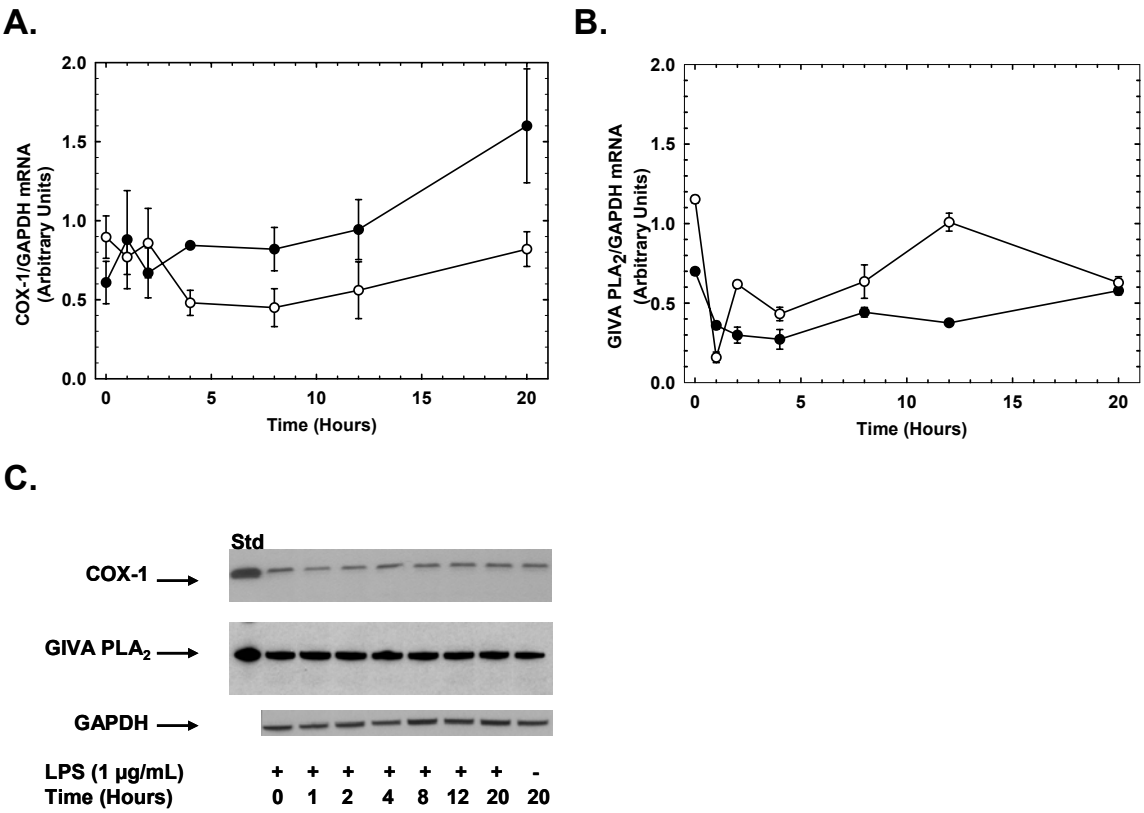

A.
COX-2/GAPDH mRNA
(Arbitrary Units)
200
150
100
50
0
0
5
10
15
20
Time (Hours)
B.
Std
COX-2
GAPDH
LPS (1 µg/mL) + + + + + + + -
Time (Hours) 0 1 2 4 8 12 20 20
C.
PGE2 Release
(%, Relative to 20 Hours LPS Stimulated)
120
100
80
60
40
20
0
0
5
10
15
20
Time (Hours)

Figure II.2. LPS stimulation does not upregulate COX-1 nor GIVA PLA_2 expression in U937 macrophages.

Time-course of LPS induced COX-1 and GIVA PLA_2 expression in U937 macrophages. A) U937 macrophages were incubated in the absence (●) and presence (○) of 1 μg/mL LPS and then subsequently COX-1 mRNA levels were determined at the indicated times over a 20 hour period by quantative PCR analysis. B). U937 macrophages were incubated in the absence (●) and presence (○) of 1 μg/mL LPS and then subsequently GIVA PLA_2 mRNA levels were determined at the indicated times over a 20 hour period by quantative PCR analysis. C). U937 macrophages were incubated in 1 μg/mL LPS for the indicated amounts of time before the cellular lysates were collected and blotted for COX-1 and GIVA PLA_2 protein expression. Representative experiments of at least three individual experiments are shown. Data is expressed as mean values ± S.D. of three individual replicates.


A.
COX-1/GAPDH mRNA (Arbitrary Units)
Time (Hours)
B.
GIVA PLA2/GAPDH mRNA (Arbitrary Units)
Time (Hours)
C.
Std
COX-1
GIVA PLA2
GAPDH
LPS (1 µg/mL) + + + + + + + -
Time (Hours) 0 1 2 4 8 12 20 20

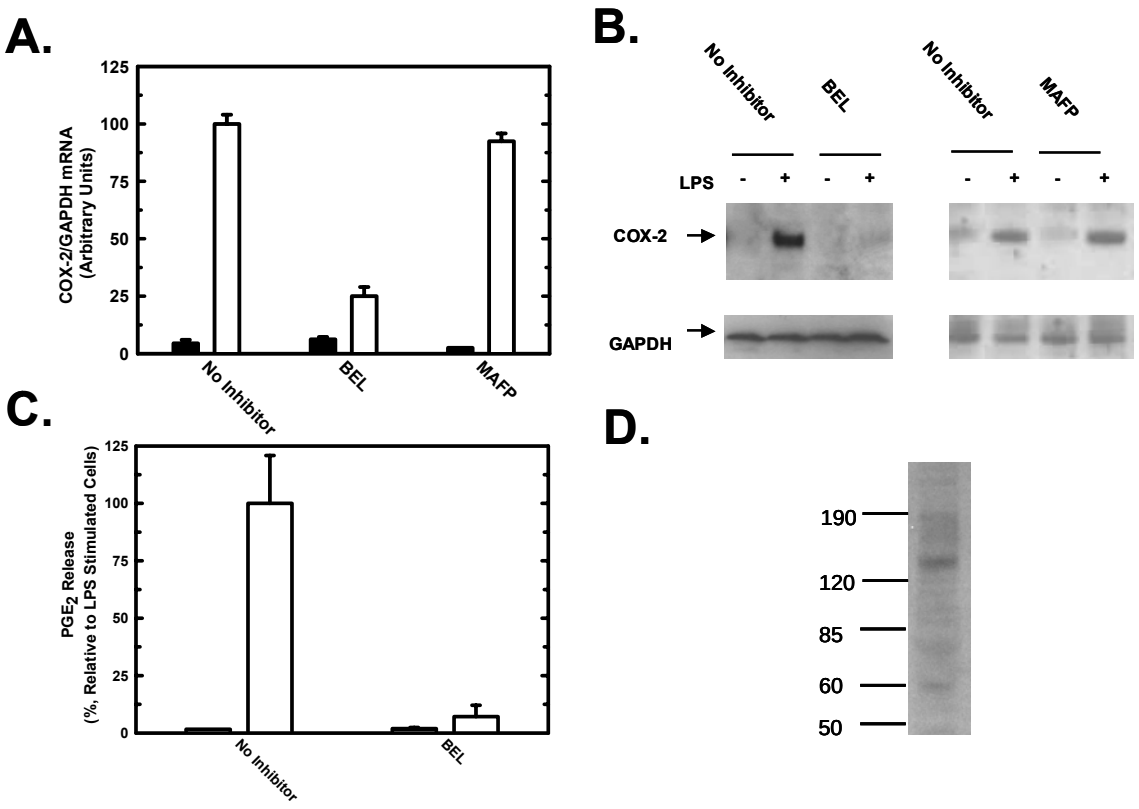
2. Inhibition of LPS Induced PGE_2 Production and COX-2 Expression by BEL

U937 cells that had been preincubated with 25 μM BEL, a dual GVI PLA_2/ PAP-1 inhibitor, prior to the addition of LPS had reduced COX-2 mRNA transcript levels relative to uninhibited cells (Figure II.3.A). The inhibition by BEL was observed to affect not only the levels of COX-2 mRNA transcript, but also the amount of COX-2 protein being expressed (Figure II.3.B) and the amount of PGE_2 being produced by the cells in response to LPS stimulation (Figure II.3.C). Since the BEL inhibitor is known to inhibit not only PAP-1, but also GVI PLA_2, it was imperative to discern which enzyme was responsible for the aforementioned phenomena. To clarify this issue, the chemical inhibitor MAFP, which inhibits the function of GVI PLA_2 but not PAP-1, was added to cellular experiments as had been done with the BEL inhibitor, As can be seen in figures 2A and 2B, MAFP had no effect on the induction of COX-2, at both the mRNA transcript and protein levels. It should be noted that PGE_2 release could not be determined for experiments conducted with MAFP, because addition of MAFP to the immunoassay gives a false positive measurement.

To ensure that PAP-1 is present in this cell line, U937 cell lysates were evaluated for the presence of Lipin-1 by Western blot. Lipin-1 is reported to run at a molecular weight of 140 kD on SDS-PAGE electrophoresis (128). We observed that the most prominent band from U937 cellular lysates corresponded to a molecular weight of 140 kD when run under similar conditions and then was western blotted using a Lipin-1 antibody (Figure II.3.D).

Figure II.3. BEL inhibits LPS induced COX-2 expression and PGE_2 release in U937 macrophages.

BEL inhibits the LPS induced production of COX-2 mRNA transcripts, COX-2 protein expression and release of PGE_2 while MAFP does not. A) The effect of the prior inhibition of BEL (25 μM) and MAFP (25 μM) on COX-2 mRNA transcript levels in U937 macrophages, in the absence (■) and presence (□) of 1 μg/mL LPS by quantative PCR analysis. B) Western blot analysis of COX-2 expression in U937 macrophages treated with BEL (25 μM) or MAFP (25 μM) prior to the addition of 1 μg/mL LPS for 20 hours. C) Effect of BEL (25 μM) on PGE_2 release in the absence (■) and presence (□) of 1 μg/mL LPS. D) Western blot analysis of U937 cellular lysate probed with Lipin 1 antibody. Representative experiments of at least three individual experiments are shown. Data is expressed as mean values ± S.D. of three individual replicates.


A.
COX-2/GAPDH mRNA (Arbitrary Units)
125
100
75
50
25
0
No Inhibitor
BEL
MAFP
B.
No Inhibitor
BEL
No Inhibitor
MAFP
LPS
- + - + - + - +
COX-2
GAPDH
C.
PGE2 Release (%, Relative to LPS Stimulated Cells)
125
100
75
50
25
0
No Inhibitor
BEL
D.
190
120
85
60
50

3. Inhibition of LPS Induced PGE_2 Production and COX-2 Expression by Propranolol, Ethanol and n-Butanol

To further confirm the role of PAP-1 in the LPS induced upregulation of COX-2 and subsequent release of PGE_2, U937 cells were cultured in the presence of another known inhibitor of PAP-1, propranolol. Addition of 150 μM propranolol to cells resulted in the reduction of LPS induced COX-2 transcript levels by 60% (Figure II.4.A), an equally pronounced reduction of COX-2 protein expression (Figure II.4.B) and near total abatement of PGE_2 release into the media (Figure II.4.C).

Addition of short chain, primary alcohols into the media results in their uptake by the cultured cells and subsequent participation in the PLD mediated transesterification of phospholipids, resulting in the production of the respective phosphatidyl-alcohols, which are not PAP-1 substrates (129). By competing with water, which is the normal PLD mediated reaction nucleophile, the primary alcohol inhibits PAP-1 cellular activity by reducing the availability of the substrate phosphatidic acid. Cells were preincubated for 30 minutes in the presence of 2% (volume/volume) ethanol, 0.6% *n*-butanol and 0.6% *t*-butanol, prior to the addition of LPS. The purpose of the tertiary alcohol, *t*-butanol, is to serve as a negative control, as it is not incorporated in PLD mediated phospholipid hydrolysis due to steric hindrance. Figure II.5.A demonstrates that the addition of ethanol or *n*-butanol to the supernatant resulted in a 50% reduction of COX-2 mRNA transcript levels in response to LPS stimulation. Importantly, *t*-butanol had no effect on the level of LPS induced COX-2 mRNA transcript levels relative to that of the uninhibited cells. The LPS induced COX-2 protein expression data corroborated the mRNA transcript results,

Figure II.4. Propranolol inhibits LPS induced COX-2 expression and PGE_2 release in U937 macrophages.

Propranolol inhibits the LPS induced production of COX-2 mRNA transcripts, COX-2 protein expression and release of PGE_2. A) Quantitative PCR analysis of the effect of propranolol (150 μM) on COX-2 mRNA transcript levels in U937 macrophages, in the absence (■) and presence (□) of 1 μg/mL LPS. B) Western blot analysis of COX-2 expression in U937 macrophages treated with propranolol (150 μM) prior to the addition of 1 μg/mL LPS for 20 hours. C) Effect of propranolol (150 μM) on PGE_2 release in the absence (■) and presence (□) of 1 μg/mL LPS. Representative experiments of at least three individual experiments are shown. Data is expressed as mean values ± S.D. of three individual replicates.

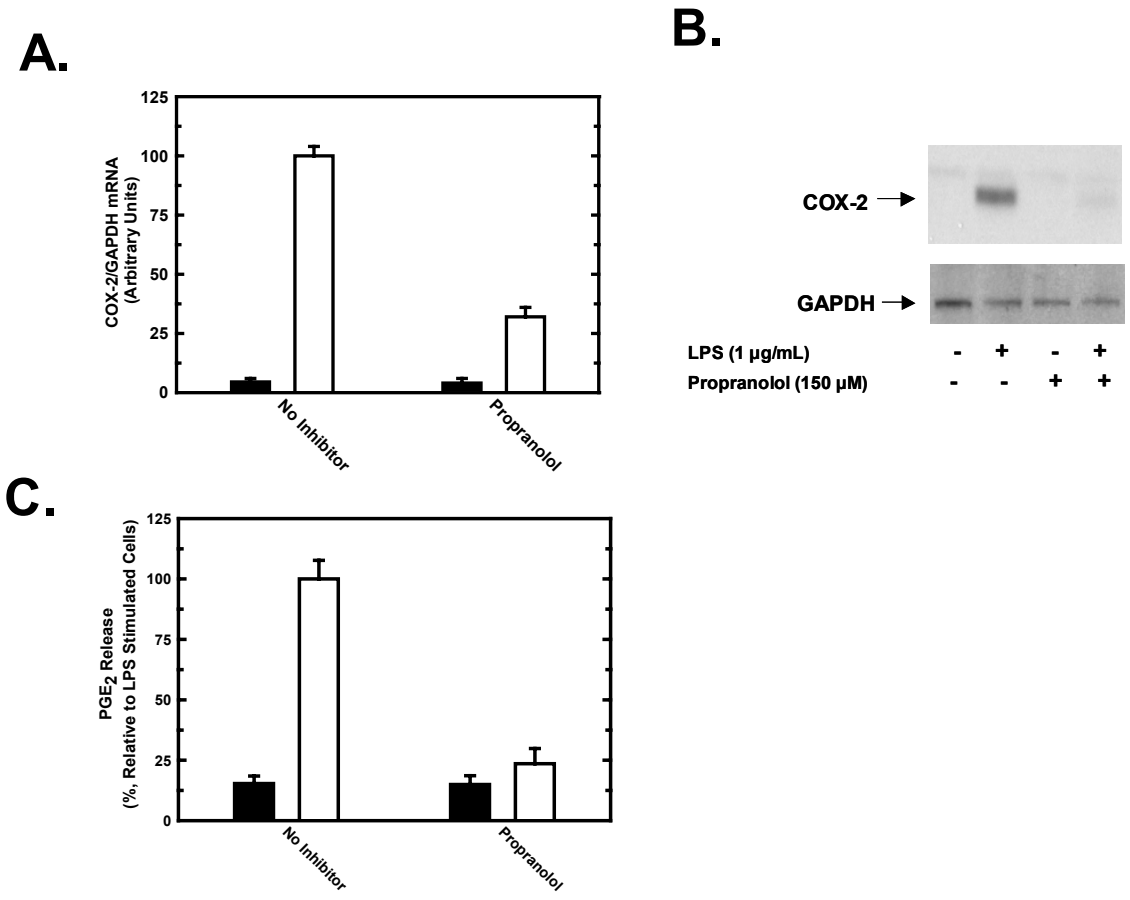
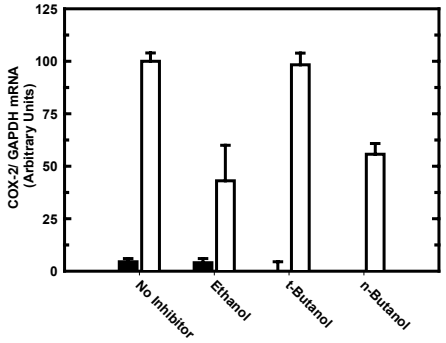

A.
COX-2/GAPDH mRNA (Arbitrary Units)
125
100
75
50
25
0
No Inhibitor
Propranolol
B.
COX-2
GAPDH
LPS (1 µg/mL) - + - +
Propranolol (150 µM) - - + +
C.
PGE2 Release (%, Relative to LPS Stimulated Cells)
125
100
75
50
25
0
No Inhibitor
Propranolol

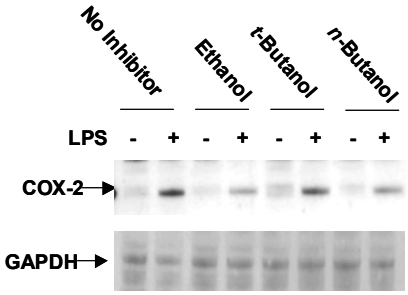
Figure II.5. Ethanol and *n*-butanol inhibit LPS induced COX-2 expression and PGE_2 release in U937 macrophages while *t*-butanol does not.

Ethanol and *n*-butanol inhibit the LPS induced production of COX-2 mRNA transcripts, COX-2 protein expression and release of PGE_2 while *t*-butanol does not. A) Quantitative PCR analysis of the effect of ethanol (2.0% vol/vol), *n*-butanol (0.6% vol/vol) and *t*-butanol (0.6% vol/vol) on COX-2 mRNA transcript levels in U937 macrophages, in the absence (■) and presence (□) of 1 μg/mL LPS. B) Western blot analysis of COX-2 expression in U937 macrophages treated with ethanol (2.0% vol/vol), *n*-butanol (0.6% vol/vol) and *t*-butanol (0.6% vol/vol) prior to the addition of 1 μg/mL LPS for 20 hours. C) Effect of increasing dosages of ethanol (0.5% to 2.0% vol/vol), *n*-butanol (0.6% vol/vol) and *t*-butanol (0.6% vol/vol) on PGE_2 release in the absence (■) and presence (□) of 1 μg/mL LPS. Representative experiments of at least three individual experiments are shown. Data is expressed as mean values ± S.D. of three individual replicates.

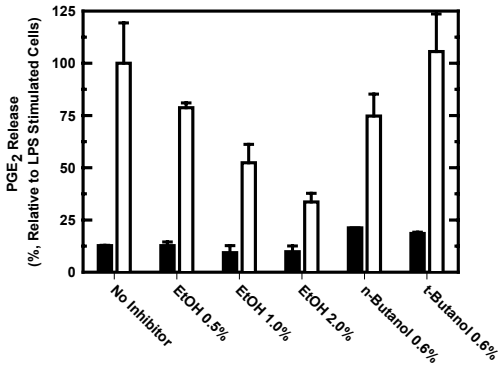
A.


COX-2/ GAPDH mRNA
(Arbitrary Units)
125
100
75
50
25
0
No Inhibitor
Ethanol
t-Butanol
n-Butanol

B.


No Inhibitor
Ethanol
t-Butanol
n-Butanol
LPS - + - + - + - +
COX-2
GAPDH

C.


PGE2 Release
(%, Relative to LPS Stimulated Cells)
125
100
75
50
25
0
No Inhibitor
EtOH 0.5%
EtOH 1.0%
EtOH 2.0%
n-Butanol 0.6%
t-Butanol 0.6%

as both ethanol and *n*-butanol were observed to reduce COX-2 protein expression by approximately 50% while *t*-butanol had no effect (Figure II.5.B). Additionally, the release of PGE_2 from LPS stimulated macrophages was blocked in cells in a dose-dependent fashion, while *n*-butanol reduced PGE release comparably to that of 0.5% ethanol and *t*-butanol had no effect (Figure II.5.C). Doses higher than 0.6% vol/vol of butanol were not utilized in cellular experiments due to lethal cytotoxicity to the macrophages at elevated concentrations.

4. DAG Production, PAP-1 Activity Reduced in PAP-1 Inhibited U937 Cells

To ensure that the chemical inhibitors had the desired effect of inhibiting PAP-1 activity in the cultured U937 cells, cells were tritium-radiolabeled with palmitic acid to study the production of endogenous DAG in response to LPS, in the presence and absence of BEL. DAG production increased by 20% to 50 % within the first two minutes of LPS stimulation, and then gradually returned to basal levels over 30 minutes following LPS stimulation (Figure II.6). However, the addition of BEL to cells resulted in a dramatic reduction of the DAG spike two minutes after LPS stimulation, while having an insignificant effect on the basal DAG levels. These results suggest that the LPS-induced DAG spike can be attributed to the BEL sensitive PAP-1, while the basal DAG level is not due to the activity of PAP-1. Presumably, this pool of DAG arises as a result of LPP activity associated with the basal metabolism of the cell or *de novo* DAG synthesis.

To confirm that the LPS-induced increase in DAG was due to PAP-1 activity, *in vitro* PAP assays of the cytosolic component of cellular lysates were performed to determine whether the levels of PAP-1 activity change after LPS stimulation. The PAP *in*

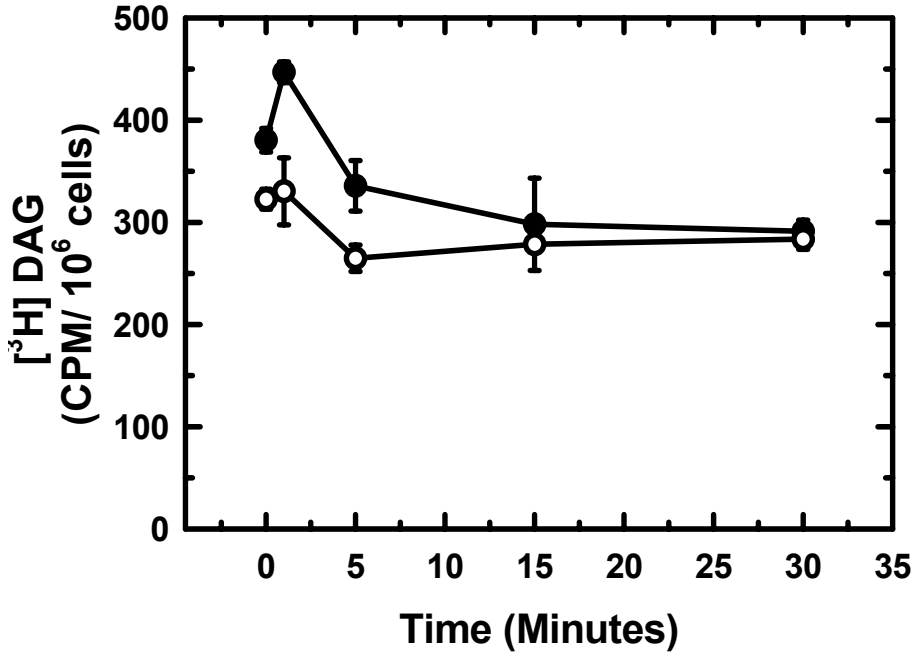


Figure II.6. LPS treatment of U937 macrophages increases cellular DAG.

Time course of DAG levels in U937 cells prelabelled with [9-10(n)-^{3}H]palmitic acid (1 μCi/mL) cultured in the absence (●) and presence (○) of 25 μM BEL for 30 minutes prior to the addition of 1 μg/mL LPS. Representative experiments of at least three individual experiments are shown. Data is expressed as mean values ± S.D. of three individual replicates.

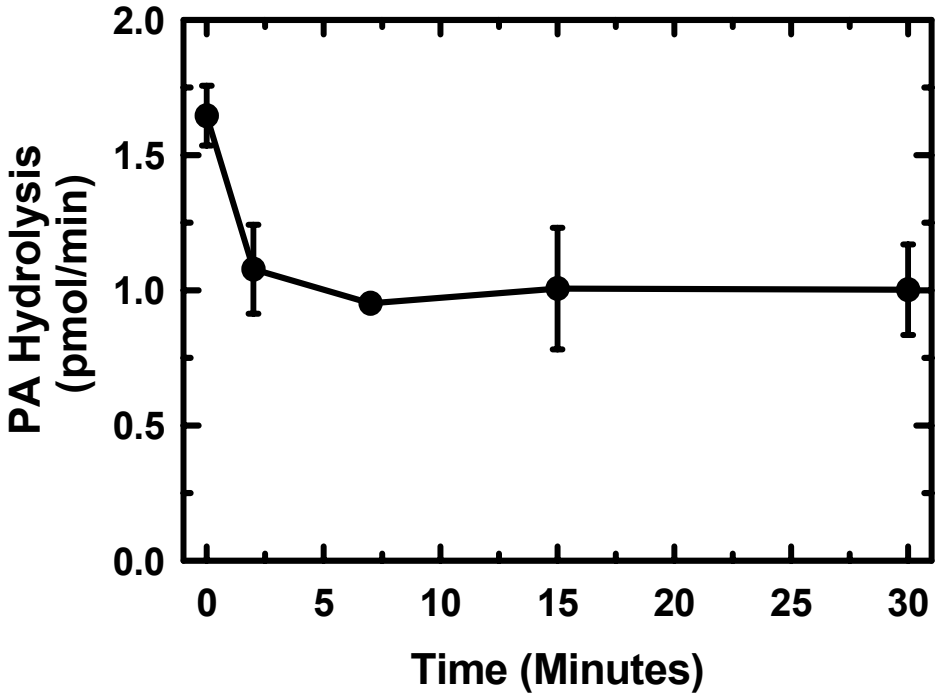


Figure II.7. LPS treatment of U937 macrophages decreases cytosolic PAP activity.

Time course of total PAP activity in the cytosolic fraction of U937 cell lysates cultured in the presence of 1 μg/mL LPS. Representative experiments of at least three individual experiments are shown. Data is expressed as mean values ± S.D. of three individual replicates.

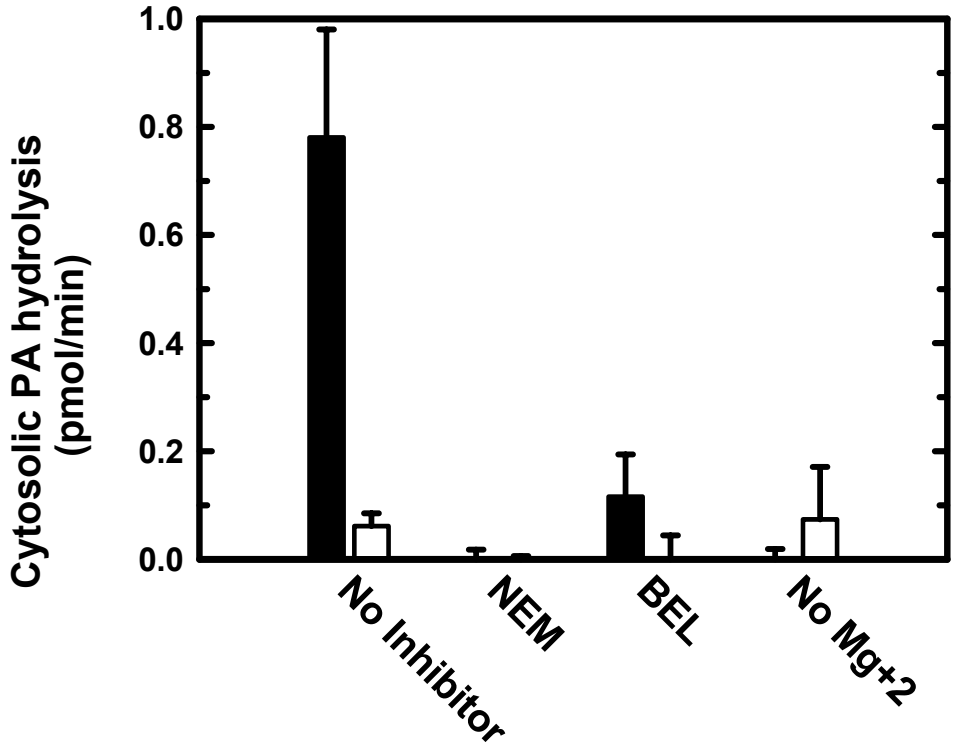


Figure II.8. Cytosolic PAP activity is sensitive to the presence of NEM and BEL while requiring Mg^{+2}.

Total PAP activity in the cytosolic fraction of U937 macrophages lysates was assayed with no inhibitor added, in the presence of *NEM* and BEL inhibitors, and in the absence of Mg^{+2}, utilizing the cytosolic fraction of cellular lysates from U937 cells that had been cultured in the absence (■) and presence (□) of 1 μg/mL LPS for two minutes of stimulation. Representative experiments of at least three individual experiments are shown. Data is expressed as mean values ± S.D. of three individual replicates.

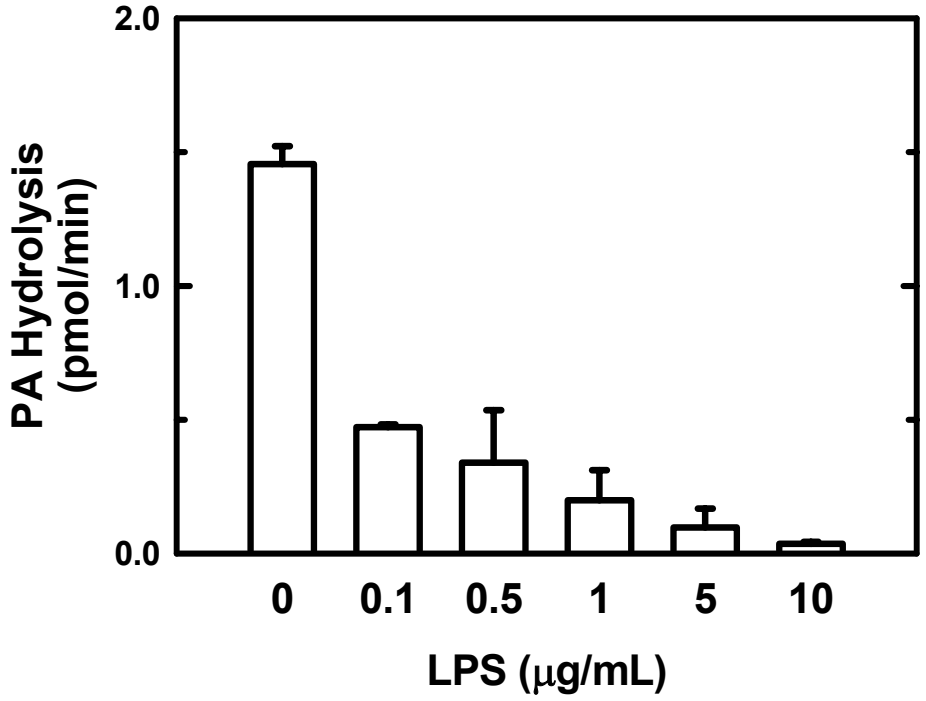


Figure II.9. Treatment of U937 macrophages with increasing dosages of LPS results in progressive reduction of cytosolic PAP activity.

LPS dose dependency of total PAP activity in the cytosolic fraction of U937 cell lysates cultured in the presence of 0.0, 0.1, 0.5, 1.0, 5.0 and 10.0 μg/mL LPS. Representative experiments of at least three individual experiments are shown. Data is expressed as mean values ± S.D. of three individual replicates.

vitro assay measures total PAP activity, which is the sum of all DAG produced from PA hydrolysis by PA dephosphorylating enzymes, including PAP-1 and LPP. Furthermore, in an effort to distinguish PAP-1 activity from the total PAP activity, we have taken advantage of the fact that PAP-1 requires the presence of Mg^{+2} for full enzymatic activity and is inhibited by the addition of the chemical inhibitors BEL and NEM. It is known that LPP is a transmembrane protein localized to the membrane fraction of the cellular lysate; therefore the only remaining PA dephosphorylating enzyme present in the cytosolic component of the cellular lysate is PAP-1. Cells that had been stimulated with LPS were subsequently lysed, and the cytosolic and membrane components were separated and isolated by centrifugation (100,000 x g, 1 hr, 4 °C). Since PAP-1 translocates from the cytosol to phospholipid membrane surfaces when it becomes activated, it was essential to first identify which time point following the addition of LPS agonist would yield the most pronounced change in PAP-1 activity. In Figure II.7, the change in total PAP-1 activity that was observed in the cytosolic fraction of the cell lysate in response to the addition of LPS is shown relative to basal levels in the cytosolic fraction over the course of one half-hour. The amount of total cytosolic PAP activity decreases by around 50% with LPS stimulation relative to basal activity during the first two minutes of stimulation, and was not observed to return to basal activity levels over the 30 minute time course of the experiment. Since two minutes of LPS stimulation was observed to yield the most dramatic DAG production and was seen to yield PAP-1 translocation, it was chosen as the representative time point from which to study chemical inhibition of PAP-1 activity in figure II.8.

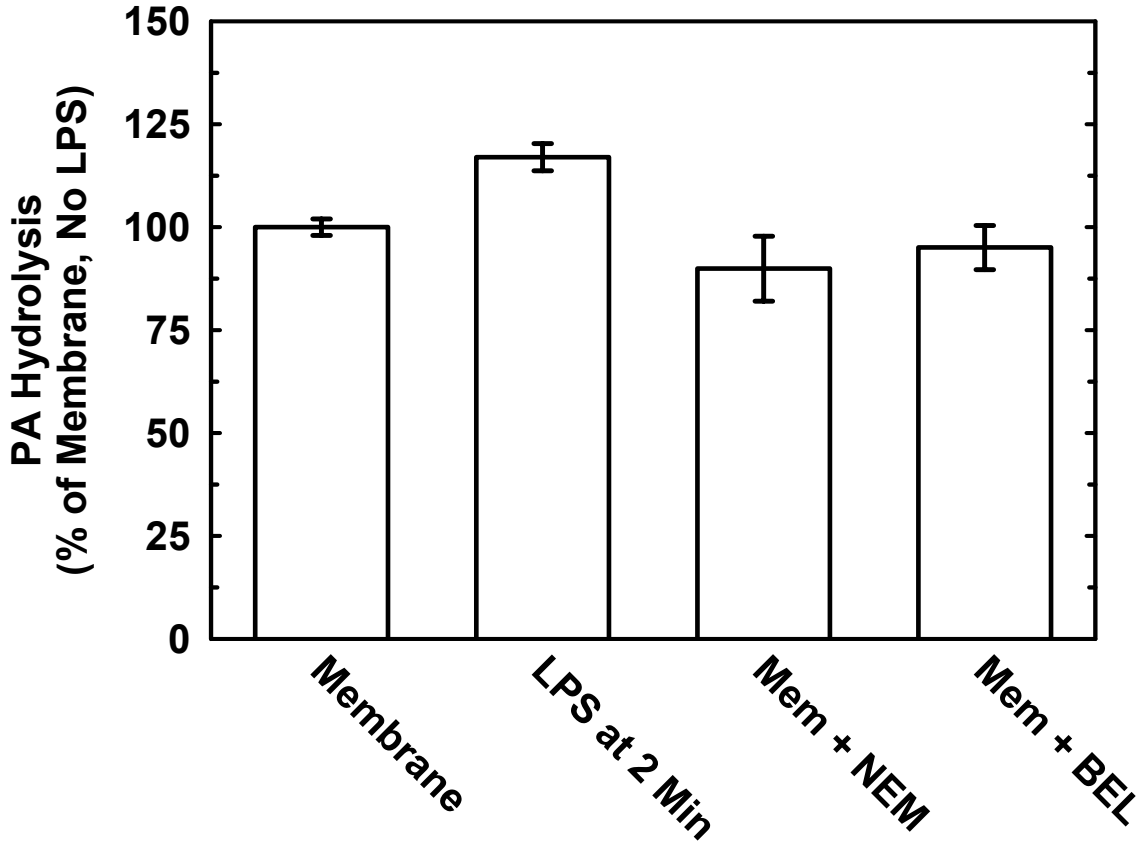


Figure II.10. Membrane PAP activity increases in response to LPS, is sensitive to the presence of NEM and BEL.

Total PAP activity in the membrane fraction of U937 macrophages lysates was assayed with no inhibitor added, in the presence of *NEM* and BEL inhibitors, and in the absence of Mg^{+2}, utilizing the cytosolic fraction of cellular lysates from U937 cells that had been cultured in the absence (■) and presence (□) of 1 μg/mL LPS for two minutes of stimulation. Representative experiments of at least three individual experiments are shown. Data is expressed as mean values ± S.D. of three individual replicates.

To confirm that the decrease in PAP activity observed at two minutes of LPS stimulation was due to PAP-1 translocation from the cytosol, chemical inhibitor studies were performed on the basal activity. The NEM and BEL inhibitors were added directly to the cellular lysates prior to being assayed, while Mg^{+2} was simply not added to a third condition which still included EDTA and EGTA to ensure that any Mg^{+2} or other divalent cation present in the lysate was chelated. The deprivation of Mg^{+2} and the addition of NEM and BEL all resulted in around 90% inhibition of the activity observed in the collected cytosolic fraction of the cellular lysates (Figure II.8). Additionally, we attempted to assay the membrane fraction of the cellular lysate to ensure that the PAP-1 activity could be accounted for as an increase in the total PAP activity of the membrane. While the total PA hydrolase activity increased after LPS stimulation and this increase was blunted by BEL and NEM (Figure II.10), the presence of LPP in the membrane overwhelmed the increase markedly. This factor made it impossible to accurately quantitate statistically significant changes in PAP-1 activity in the membrane fraction of cellular lysates.

Additionally, PAP activity was monitored in the cytosolic fractions of cellular lysates derived from cells that were challenged by increasing doses of LPS (Figure II.9). Macrophages were administered LPS for two minutes prior to the lysates being collected, and the cytosolic fraction was then assayed for PAP activity as described previously. PAP activity in the cell cytosol decreased in an LPS dose-dependent manner with increasing LPS.

5. Exogenous DAG Induces COX-2 Expression, PGE_2 Release

To simulate the physiological activity of PAP-1 in the cell, 50 μM exogenous DAG was added to cells to determine whether the addition of DAG would induce COX-2 expression and increased release of PGE_2. As shown in figure II.11.A, the addition of DAG to LPS primed cells increased COX-2 mRNA transcript levels two-fold relative to that of cells that were stimulated with LPS alone. The addition of DAG alone to cells without LPS appeared to have a negligible effect on COX-2 transcript levels. Concordantly, the addition of DAG to LPS stimulated cells had a similar effect on COX-2 protein production, as DAG primed LPS stimulated cells expressed approximately twice as much COX-2 protein as cells that were stimulated with LPS alone (Figure II.11.B). Furthermore, the addition of DAG enhanced the release of PGE_2 production with LPS stimulation by increasing PGE_2 three-fold relative to that of cells that were treated with LPS alone (Figure II.11.C). Addition of DAG to the cells alone had no significant effect on the release of the PGE_2 from the cells. This data provides evidence that DAG accentuates the expression of COX-2 and subsequent release of PGE_2 in response to the LPS priming of U937 cells. However, exogenous DAG addition appears not to be sufficient to elicit a pro-inflammatory response without LPS supplementation.

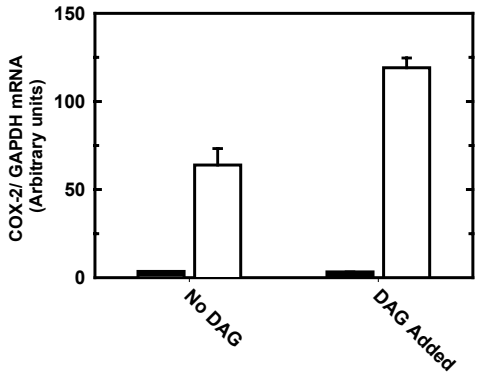
6. LPS Induced COX-2 Expression is PAP-1 Dependent in the $P388D_1$ Cell Line

To confirm that the results garnered in the U937 macrophages were not merely an anomaly of the cell line, or the result of direct PKC activation by the addition of PMA to the cells, similar experiments were conducted on the $P388D_1$ macrophage cell line, which

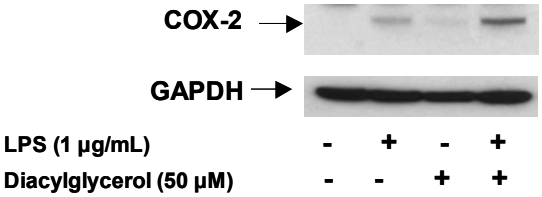
Figure II.11. Supplementation of exogenous DAG to LPS treated U937 macrophages enhances COX-2 expression and PGE_2 release.

DAG supplementation to LPS stimulated U937 macrophages augments COX-2 mRNA transcript production, COX-2 protein expression and PGE_2 release. A) Quantitative PCR analysis of the effect of 50 μM DAG supplementation on COX-2 mRNA transcript levels in the absence (■) and presence (□) of 1 μg/mL LPS. B) Western blot analysis of COX-2 expression in U937 macrophages treated with 50 μM DAG prior to the addition of 1 μg/mL LPS for an incubation of 20 hours. C) Effect of 50 μM DAG on PGE_2 production in the absence (■) and presence (□) of 1 μg/mL LPS. Representative experiments of at least three individual experiments are shown. Data is expressed as mean values ± S.D. of three individual replicates.

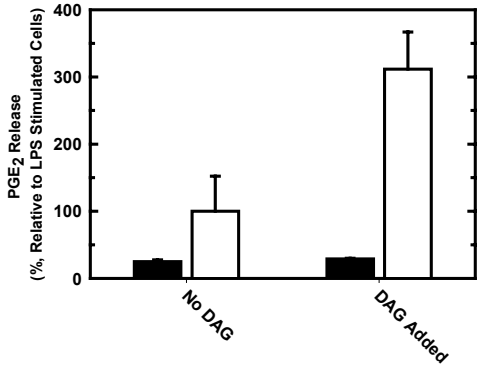
A.


150
100
50
0
COX-2/ GAPDH mRNA
(Arbitrary units)
No DAG
DAG Added

B.


COX-2
GAPDH
LPS (1 µg/mL) - + - +
Diacylglycerol (50 µM) - - + +

C.


400
300
200
100
0
PGE2 Release
(%, Relative to LPS Stimulated Cells)
No DAG
DAG Added

does not require the addition of PMA for cellular differentiation. All three inhibitors significantly reduced LPS induced COX-2 protein expression, COX-2 transcript levels and the release of PGE_2 into the supernatant (Figure II.12), confirming that the results are not simply an artifact of the U937 macrophages. Interestingly, one difference between the cell lines was that BEL and propranolol more potently inhibited LPS induced COX-2 expression in $P388D_1$ macrophages than in U937 macrophages. Consistent with the results that we had observed in the experiments conducted in the U937 macrophages, the addition of exogenous DAG to LPS stimulated $P388D_1$ macrophages resulted in augmented COX-2 protein expression and transcript levels relative to macrophages that had been stimulated with only LPS (Figure II.13.A and B). Addition of DAG to LPS stimulated cells also increased PGE_2 release in relative to cells that had not been treated with DAG (Figure II.13.C). The only difference between the U937 and $P388D_1$ cells that we had observed was that the addition of exogenous DAG seemed to increase the release of PGE_2 from cells that had not been treated with LPS. However, this cannot be attributed to COX-2 expression, as COX-2 mRNA transcripts levels and protein expression were not increased in cells that were treated with DAG alone.

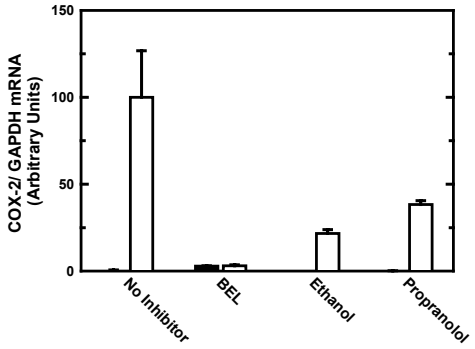
D. Discussion

The physiological function of the Mg^{+2}-dependent PAP-1 in mammalian cells has been previously associated with metabolism, apoptosis and inflammation. Until very recently, the PAP-1 enzyme had not been cloned nor sequenced, which had made it difficult to study its roll in regards to signal transduction and cellular physiology (84). In

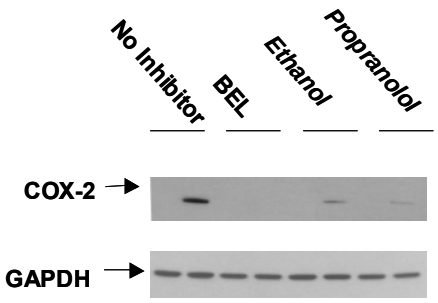
Figure II.12. Preincubation of P388D$_1$ macrophages with BEL, ethanol and propranolol results in decreased LPS induced COX-2 expression and PGE$_2$ release.

Addition of BEL, ethanol or propranolol to P388D$_1$ macrophages results in the inhibition of the LPS induced production of COX-2 mRNA transcripts, COX-2 protein expression and release of PGE$_2$. A) Quantitative PCR analysis of the addition of BEL (5 μM), ethanol (2.0%) or propranolol (100 μM) on COX-2 mRNA transcript levels in P388D$_1$ macrophages, both in the absence (■) and presence (□) of 1 μg/mL LPS. B) Western blot analysis of COX-2 expression in P388D1 macrophages treated with BEL (5 μM), ethanol (2.0%) or propranolol (100 μM) prior to the addition of 1 μg/mL LPS for 20 hours. C) Effect of BEL (5 μM), ethanol (2.0%) or propranolol (100 μM) on PGE$_2$ release in the absence (■) and presence (□) of 1 μg/mL LPS. Representative experiments of at least three individual experiments are shown. Data is expressed as mean values ± S.D. of three individual replicates.

A.


COX-2/ GAPDH mRNA
(Arbitrary Units)
150
100
50
0
No Inhibitor
BEL
Ethanol
Propranolol

B.


No Inhibitor
BEL
Ethanol
Propranolol
COX-2
GAPDH

C.

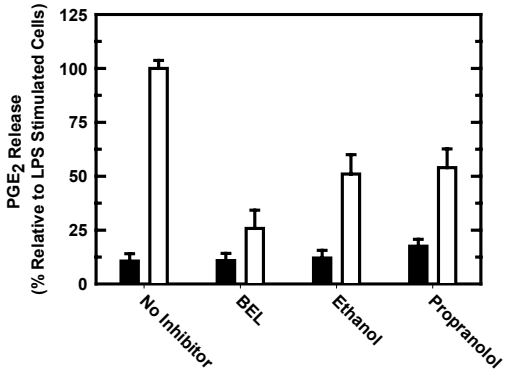
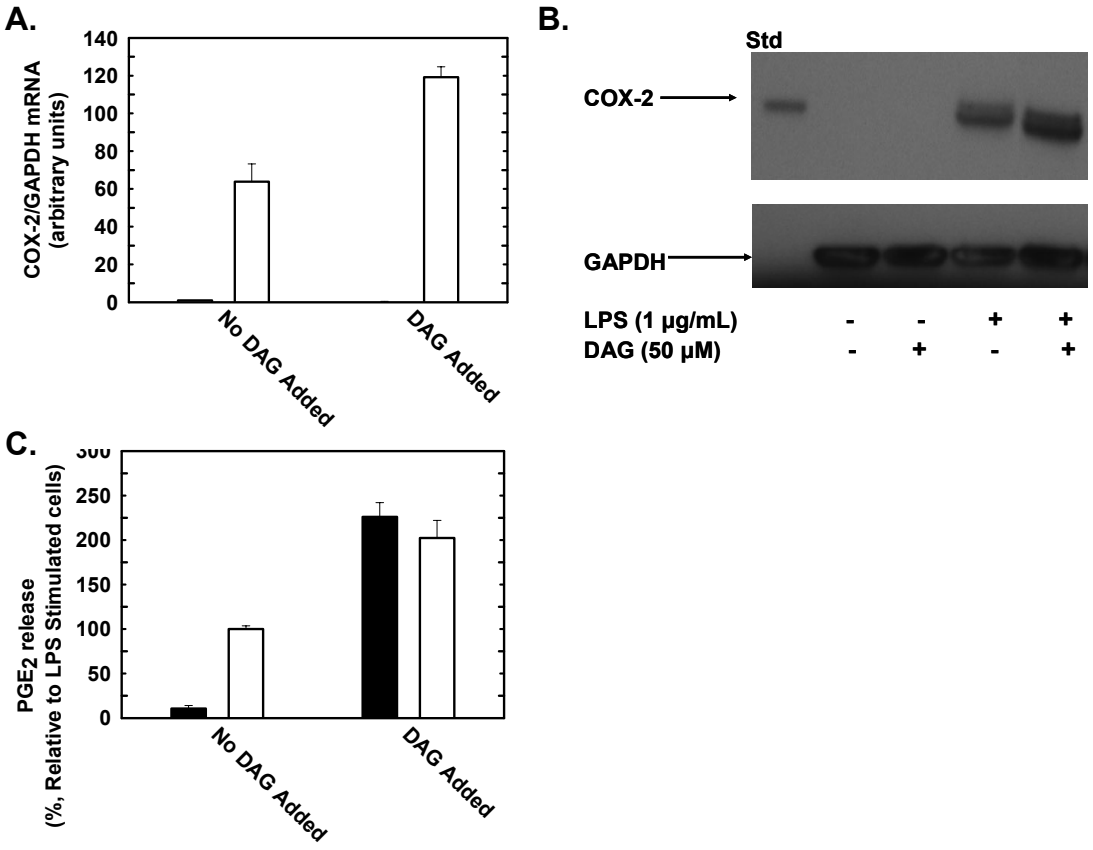

PGE2 Release
(% Relative to LPS Stimulated Cells)
125
100
75
50
25
0
No Inhibitor
BEL
Ethanol
Propranolol

Figure II.13. Supplementation of exogenous DAG to LPS treated P388D$_1$ macrophages enhances COX-2 expression and PGE$_2$ release.

DAG supplementation to LPS stimulated P388D$_1$ macrophages augments COX-2 mRNA transcript production, COX-2 protein expression and PGE$_2$ release. A) Quantitative PCR analysis of the effect of 50 μM DAG supplementation on COX-2 mRNA transcript levels in the absence (■) and presence (□) of 1 μg/mL LPS. B) Western blot analysis of COX-2 expression in P388D$_1$ macrophages treated with 50 μM DAG prior to the addition of 1 μg/mL LPS for an incubation of 20 hours. C) Effect of 50 μM DAG on PGE$_2$ production in the absence (■) and presence (□) of 1 μg/mL LPS. Representative experiments of at least three individual experiments are shown. Data is expressed as mean values ± S.D. of three individual replicates.


A.
COX-2/GAPDH mRNA (arbitrary units)
140
120
100
80
60
40
20
0
No DAG Added
DAG Added
B.
Std
COX-2
GAPDH
LPS (1 µg/mL) - - + +
DAG (50 µM) - + - +
C.
PGE2 release (%, Relative to LPS Stimulated cells)
300
250
200
150
100
50
0
No DAG Added
DAG Added

addition to having purified PAP-1 from *S. cerevisiae*, Carman's laboratory further showed that mutation of the gene resulted in an impaired cellular ability to produce triacylglycerol (121,122). PAP-1 involvement has been described in glycerolipid synthesis in lung tissue and to associate with PKC-α, although whether or not it undergoes phosphorylation remains to be determined (130,131). It has been reported that under starvation conditions, in which condition the body mobilizes fatty acids for β-oxidation, free fatty acids inhibit the action of PAP-1 in the liver (132). In phorbol ester stimulated human amnionic WISH cells, PAP-1 has been implicated in the upregulation of COX-2, the inactivation of which resulted in a loss of COX-2 expression (133). However, seemingly contradictory reports have been described in which the chemical inhibition of PAP-1 in interleukin 1-β (IL-1β) stimulated WISH cells or LPS-stimulated RAW 264.7 cells resulted in enhanced COX-2 expression (134,135). These contradictory results are difficult to interpret, although it may signify that the effect of the PLD/PAP-1 signaling pathway on the expression of COX-2 protein depends on the agonist stimulating the cells as well as the specific cell line.

Phospholipid derived DAG has been identified as a signaling molecule in the activation of various enzymes involved in macrophage differentiation and inflammatory activation, including protein kinase C (PKC), protein kinase D (PKD), RasGRP and chimerins (136,137). The chronology of DAG production in U937 macrophages from the hydrolysis of membrane phospholipids occurs in two distinctive waves. First, a transient wave attributed to phospholipid hydrolysis by phospholipase C (PLC) arises within seconds of LPS stimulation. Subsequently, a second, more prolonged DAG wave is then produced by the joint actions of PLD and PAP-1. PLD hydrolysis of phospholipids

results in PA release from membrane stores, which are then subsequently dephosphorylated by PAP-1 into DAG (138). The activation of PLD in differentiated U937 cells ultimately results in the enhanced release of AA from cells by a pathway involving a PLA_2 mediated mechanism (139). Our results support a PLD/PAP-1 regulatory mechanism that governs COX-2 expression suggesting a synergistic action of PLD/PAP-1 in the inflammatory response of U937 macrophages.

In this work, PAP-1 has been shown to be a required signaling component in the LPS stimulated upregulation of COX-2 protein in the human U937 macrophage-like cell line. LPS stimulation resulted in increased COX-2 mRNA transcript levels and protein expression levels, while having no effect on the levels of COX-1 mRNA and protein. Chemical inhibition of PAP-1 in U937 macrophages with BEL and propranolol resulted in reduced levels of LPS stimulated COX-2 mRNA transcript levels, COX-2 protein expression and prostaglandin release from U937 macrophages. Propranolol has long since been known to be an inhibitor of PAP-1, however recent advances by the Turk laboratory have shown that BEL has the ability to inhibit by binding thiol functional groups, which would explain the BEL sensitivity of PAP-1 (140). PAP-1 has been previously described as sensitive to thiol inhibitors, such as NEM and chlorpromazine. Since BEL is known to be an inhibitor of not only PAP-1, but also another possible pro-inflammatory enzyme, the GVI PLA_2, a second GVI PLA_2 inhibitor, MAFP was utilized in the study to verify that the GVI PLA_2 was not responsible for the COX-2 induction. Further evidence was garnered by the cellular deprivation of PAP-1 substrate PA by the addition of ethanol and *n*-butanol into the supernatant yielded inhibition of the LPS stimulated pathway, while the negative control *t*-butanol had no effect. Since U937 cells

are differentiated by PMA, a known activator of PKC, which is directly involved in the LPS signal transduction cascade by phosphorylating PLD, the results described in the U937 cells were confirmed by repetition in the murine $P388D_1$ macrophage cell line, which requires no chemical additives to differentiate the cells into an adherent morphology. The fact that the results were consistent in the $P388D_1$ cells suggest that PAP-1 may be a putative enzyme in a number of processes, as it has been shown to be involved in a number of different stimulation pathways leading to the expression of COX-2, including with LPS alone, PMA alone and with the two in combination.

In this study, we have observed an increase in the cellular endogenous concentrations of DAG within two minutes of LPS stimulation of the macrophages, and this increase is consistent with reports from other laboratories (141-143). The translocation of PAP-1 activity from the cytosolic fraction of the lysate to the membrane in the time course data is consistent with the temporal rise of endogenous DAG in the cells. Very importantly, the DAG spike was sensitive to the addition of BEL, while the PAP-1 activity could be blocked by the addition of the chemical inhibitors BEL or NEM, or removal of magnesium from the activity assay. Taken as a whole, these results demonstrate that PAP-1 is being mobilized from the cytosol in response to LPS signal transduction and is responsible for the generation of a DAG spike at two minutes following the addition of the stimulant. The fact that BEL abates the formation of the DAG spike while also decreasing the basal cytosolic total PAP activity by an amount that can be attributed to the LPS stimulated increase, further suggests that PAP-1 is responsible for these phenomena.

To simulate the activity of the endogenous PAP-1 enzyme in the cell during LPS transduction, exogenous DAG was added to the cells immediately after LPS priming, and was observed to bolster the induction of COX-2, while also increasing the release of eicosanoids into the supernatant. This suggests that the DAG produced by PAP-1 hydrolysis rather than the enzyme itself is the critical component in the LPS stimulated signaling cascade leading towards COX-2 expression. The fact that DAG alone did not have a significant effect upon PGE_2 release and COX-2 production in the absence of LPS supplementation suggests that DAG production is implicated in the pro-inflammatory response but is not sufficient without the activation of additional signaling components to elicit the entire LPS response.

It has been recently reported in U937 cells that the chemical inactivation of PAP-1 leads to development of hallmark characteristics of apoptosis, including loss of mitochondrial membrane integrity, caspase cleavage and the presence of phosphatidylserine on the extracellular cell membrane leaflet (144). Considering this result in tandem with the results presented in this paper, the PAP-1 enzyme may be a signaling component leading to the inflammatory response but also an essential regulatory element in the cellular function of macrophages. Previous studies have shown that protein kinase C (PKC) isoforms can be stimulated by the DAG produced by the action of PLC (134,145). One possible explanation that has been previously proposed is that a positive feedback loop may exist in which PKC would initially activate PLD/PAP-1, and subsequently be further activated by the DAG produced by way of PAP-1 (84,114). Such a model explains the manner in which the results described in this report can be

incorporated into the LPS signal transduction cascade to offer a more complete understanding of the roll of PAP-1 in cellular physiology.

While the physiological function of PAP-1 has yet to be fully elucidated, there is the additional ambiguity regarding the individual effects of the PA and DAG molecules on cell signaling. The production of PA in U937 macrophages has been shown to occur in two distinct waves, one derived from PLD activity occurring temporally before PAP-1 hydrolysis, and the second derived from DAG kinase activity, which utilizes the DAG that is produced by PAP-1 activity (142). Furthermore, PA and DAG are very easily interconverted by the actions of PAP and DAG kinases. The phosphatidic acid substrate of PAP-1 is derived from the action of PLD on phospholipids or DAGK phosphorylation of DAG. The existence of such a feedback mechanism would explain the durations of the two DAG pools in stimulated cells; the first being short and fleeting, the second longer. The production of DAG by the coupled activities of PLD and PAP-1 may lead to further PKC activation or to the activation of RasGRP. The induction of RasGRP, an activator of the MAPK pathway, would ultimately lead to activation of transcriptional factors, including AP-1 and NF-κB, which can result in the induction of COX-2 expression (146-148). Currently, our laboratory is conducting further research aimed at understanding both the regulation of PAP-1 leading to COX-2 expression as well as the relationship between PAP-1 function and the upregulation of COX-2 protein expression.

E.Chapter Acknowledgement

The material described in chapter II are published in the Journal of Biological Chemistry:

The Journal of Biological Chemistry, vol 281, pp 32978-32987. Grkovich, Andrej; Johnson, Christina A.; Buczynski, Matthew W.; Dennis, Edward A.

CHAPTER III

Kdo_2-Lipid A Stimulated GIVA PLA_2-mediated AA Release is PAP-1 Dependent in Macrophages

A. Background

Bacterial sepsis and septic shock result from the exacerbated production of inflammatory mediators by the immune system in response to bacterial endotoxins, such as lipopolysaccharide (LPS) (5). LPS-activated macrophages produce and release a host of cytokines and eicosanoids, including several prostaglandins (PGs). PGs are known to contribute to a number of normal physiological processes, including inflammation, pain, and vascular permeability while also being implicated as major contributors to endotoxic shock (105). A complete understanding of the regulation of cytokine and eicosanoid release should enhance the formulation of more efficient and safer therapeutic strategies to resolve pathological immune reactions.

Group IVA phospholipase A_2 (GIVA PLA_2) has been identified to be the primary phospholipase in LPS-mediated arachidonic acid metabolism resulting in the release of eicosanoids from macrophages (127,149). GIVA PLA_2 catalyzes the hydrolysis of arachidonic acid (AA) from the *sn*-2 position of membrane phospholipids, resulting in the release of free AA from the membrane and formation of lysophospholipid (150,151). GIVA PLA_2 has been implicated in a variety of inflammatory disease models, including collagen induced arthritis, autoimmune encephalomyelitis and acute lung injury (58,59,152). Gaining a complete understanding of the molecular mechanism of GIVA PLA_2 activation should assist in the development of more effective treatments of inflammatory diseases.

Phosphatidic acid phosphohydrolase-1 (PAP-1) is a Mg^{+2}-dependent, cytosolic enzyme that associates with lipid membrane surfaces where it is functionally active, metabolizing phosphatidic acid (PA) into inorganic phosphate and diacylglycerol (DAG) (153-158). PAP-1 has been purified and cloned from *Saccharomyces cerevisiae,* and was identified to be the homologue of the mammalian Lipin-1 protein (121,122,159). Lipin-1 has been associated with adipose tissue development and fat metabolism. The discovery that Lipin-1 is a PAP enzyme, known to contribute to triacylglycerol (TAG) formation clarifies the protein's molecular function (159-164). Our laboratory has previously characterized two signaling pathways in which PAP-1 inhibition leads to loss of cyclooxygenase-2 (COX-2) expression and PGE_2 release. The first study was conducted in the human amnionic WISH cell line utilizing the protein kinase C (PKC) activator phorbol myristate acetate (PMA) (133). The second study was conducted in the human U937 macrophage-like cell line stimulated with lipopolysaccharide (LPS) (165). In this manuscript, we provide evidence that the inhibition of PAP-1 results in loss of GIVA PLA_2 activation in response to the TLR-4 specific agonist, Kdo_2-Lipid A, while not reducing direct GIVA PLA_2 activation by ATP addition, via increased cytosolic Ca^{+2} levels. Furthermore, PAP-1 inhibition is shown to reduce the absolute amount of AA released in response to synergy, while not decreasing the synergistic activation value. Finally, we show that the addition of DAG to Kdo_2-Lipid A stimulated cells enhances AA release.

B. Materials and Methods

1. Reagents

Murine RAW 264.7 macrophages were purchased from American Type Culture Collection (Manassas, VA). DMEM cell culture medium was obtained from Gibco (Grand Island, NY). Fetal bovine serum was from VWR International (Bristol, CT). DTT, phorbol myristate acetate (PMA) and lipopolysaccharide (LPS), from *E. coli* 0111:B4, were obtained from Sigma Chemical Company (St. Louis, MO). Kdo_2-Lipid A, 1-palmitoyl-2-arachidonoyl phosphatidylcholine was from Avanti Polar Lipids (Alabaster, AL). Propranolol, Dioctoyl-DAG were from Biomol (Plymouth Meeting, PA). Bromoenol lactone (BEL) was from Cayman Chemical (Ann Arbor, MI). 1-palmitoyl-2-(1-^{14}C)-palmitoyl phosphatidylcholine, 1-palmitoyl-2-(1-^{14}C)-arachidonoyl phosphatidylcholine, [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid (specific activity = 100 Ci/mmol) and [9,10-^{3}H] oleic acid (specific activity = 23 Ci/mmol) were obtained from NEN Life Science Products (Boston, MA). Phosphatidylinositol 4,5-bisphosphate was from Roche (Basel, Switzerland). The specific $cPLA_2$ inhibitor, pyrrophenone, was kindly provided by Dr. Kohji Hanasaki (Shionogi Research Laboratories of Shionogi & Co., Ltd). 20 cm x 20 cm x 250 μm K6 Silica gel thin layer chromatography plates were from Whatman (Clifton, New Jersey).

2. Cell Culture and Stimulation Protocol

RAW 264.7 macrophages were maintained at 37 °C in a humidified atmosphere at 90% air and 5% CO_2 DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, 100 μg/ml streptomycin, and non-essential amino acids. Cells were plated at a confluency of $2x10^6$ cells/6 well tissue culture plate and

$1x10^6$/12 well tissue culture plate at the time of experimentation. Following plating, they were allowed to adhere overnight, and then used for experiments the following day, with the exception of cells that were radiolabelled, described below. When DAG was added to the cells, it was done immediately after the addition of Kdo_2-Lipid A. Whenever cellular experiments required the usage of chemical inhibitors, the inhibitors were initially diluted into media at a stock concentration that was 100 times more concentrated than that of the final cellular concentration. From this stock concentration, the inhibitors were then applied to the cells to achieve the desired final cellular concentration. The final concentration of DMSO in the supernatants never exceeded .05% (volume/volume). When synergy experiments were performed, ATP was added to cells treated with Kdo_2-Lipid A 50 minutes after Kdo_2-Lipid A stimulation, and supernatants were collected at 1 hour following stimulation. Cell viability was assessed visually by the Trypan Blue Dye exclusion assay (Gibco, Grand Island, NY) and through the usage of the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI).

3. Sample Preparation

Media was analyzed for extracellular eicosanoid release. After stimulation, 1.8 ml of media was removed and supplemented with 100 µl of internal standards (100 pg/µl, EtOH) and 100 µl EtOH to bring the total EtOH to 10% by volume. Samples were centrifuged for 5 minutes at 3000 rpm to remove cellular debris, and the supernatants were decanted into solid phase extraction columns. All eicosanoid extractions were conducted using Strata-X SPE columns (Phenomenex). The columns were washed with 2 ml MeOH and then 2 ml H_2O prior to the samples being applied. After applying the sample, the columns were washed with 10% MeOH to remove non-adherent debris, and

eicosanoids were then eluted off the column with 1 ml MeOH. The eluant was dried under vacuum and redissolved in 100 µl of solvent A [water-acetonitrile-formic acid (63:37:0.02; v/v/v)] for LC/MS analysis.

4. Cell Quantitation

Eicosanoid levels were normalized to cell number using DNA quantitation. After the extracellular media was removed, the cells were scraped in 500 µl PBS and stored at 4ºC for DNA quantitation using the Broad Range DNA Quant-Kit (Invitrogen).

5. HPLC and Mass Spectrometry

The analysis of eicosanoids was performed by LC/MS/MS. Eicosanoids were separated by reverse-phase HPLC on a C18 column (2.1 mm x 150 mm, Grace-Vydac) at a flow rate of 300 µl/min at 25ºC. The column was equilibrated in Solvent A [water-acetonitrile-formic acid (63:37:0.02; v/v/v)], and samples were injected using a 50 µl injection loop and eluted with a linear gradient from 0%-20% solvent B [acetonitrile-isopropyl alcohol (50:50; v/v)] between 0 to 6 min; solvent B was increased to 55% from 6 to 6.5 min and held until 10 min. Solvent B was increased to 100% from 10 to 12 min and held until 13 min; solvent B was dropped to 0% by 13.5 min and held until 16 min.

Eicosanoids were analyzed using a tandem quadrupole mass spectrometer (ABI 4000 Q Trap®, Applied Biosystems) via multiple-reaction monitoring (MRM) in negative-ion mode. The electrospray voltage was -4.5 kV, the turbo ion spray source temperature was 525ºC. Collisional activation of eicosanoid precursor ions used nitrogen as a collision gas.

Quantitative eicosanoid determination was performed by stable isotope dilution, as previously described (166). Results are reported as ng of eicosanoid per million cells (mean ± standard deviation).

6. GIVA PLA_2 Activity Assay

The GIVA PLA_2 assay was previously described (167,168). Briefly, following collection of cellular lysates, the samples were assayed for PLA_2 activity using the modified Dole assay. For each assay the final buffer conditions were: 100 mM Hepes, pH 7.5, 80 μM $CaCl_2$, .1 mg/ml BSA and 2 mM DTT. The PLA_2 assay also contained 97 μM PAPC doping with 1% ^{14}C labeled PAPC in 400 μM Triton X-100 mixed micelles. Lipid preparation: lipid was dried under N_2 and lyophilized for at least 1 h to remove all traces of chloroform. Lipid was then resuspended and mixed micelles were created by repeated vortexing and heating in hot water until the solution clarified. Samples were incubated with substrate for 30 min at 40 °C. The assay was then terminated by 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 mg) was added to each tube followed by 1.5 ml heptane and 1.5 ml deionized water. Each tube was vortexed 15 s. 1 ml of the organic phase was removed and passed through a Pasteur pipet filled with silica gel (0.1–0.2 mg). This column was then washed with 1 ml diethyl ether. Scintillation cocktail (5 ml, Biosafe II) was added to the eluent and the radioactivity was determined by scintillation counting.

7. Quantification of Lipids from RAW 264.7 Macrophages

When quantifying the endogenous metabolism of radiolabeled fatty acid or TAG, cells were incubated in 10% FCS RPMI medium supplemented with [5,6,8,9,11,12,14,15-^3H] arachidonic acid (specific activity = 100 Ci/mmol) and [9,10-^3H]

oleic acid for 48 hours (1 Ci/mL media for either fatty acid). After 48 hours, the experimentation was executed. Lipids were extracted according to the method of Bligh and Dyer (126). The extraction solution was placed into fresh microcentrifuge tubes and dried in a vacuum-centrifuge. The resulting pellet was reconstituted in 40 uL of the (70:30) chloroform/methanol system. The lipids were separated by thin layer chromatography using an n-hexane/ether/acetic acid (70:30:1). The TLC plate was then developed in an iodination chamber, and the FFA, TAG, DAG and PL spots were scraped and subsequently quantified by liquid scintillation counting.

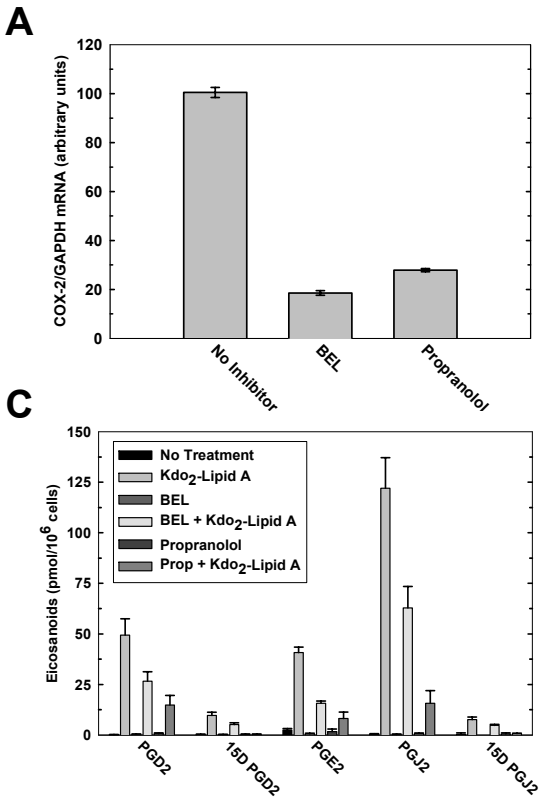
C. RESULTS

1. Kdo_2-Lipid A Stimulated COX-2 Expression and Eicosanoid Release is PAP-1 Dependent in RAW 264.7 Macrophages

Previous work in our laboratory has shown that PAP-1 chemical inhibition in PMA and LPS activated macrophages results in the loss of normal COX-2 upregulation and reduced prostaglandin E_2 release (133,165). Since PAP-1 inhibition blocked COX-2 upregulation, we concluded that the reduced PGE_2 release was a result of reduced COX-2 expression. We began this study by ensuring that RAW 264.7 macrophages preincubated with BEL or propranolol prior to treatment with Kdo_2-Lipid A behaved in a manner that is consistent with what was previously observed in WISH and U937 cells. Cells preincubated with propranolol or BEL prior to stimulation exhibited decreased COX-2 expression and mRNA transcript levels, consistent with our previous results (Figure III.1.A and B). In the present manuscript, we studied the effect of PAP-1 inhibition on the Kdo_2-Lipid A stimulated release of PGD_2, 15-deoxy-$\Delta^{12,14}$-PGD_2, PGE_2, PGJ_2 and 15-

Figure III.1. PAP-1 chemical inhibition blocks Kdo_2-Lipid A stimulated COX-2 expression in RAW 264.7 macrophages.

A) Quantitative PCR analysis of COX-2 expression in cells preincubated with 25 μM BEL or 50 μM propranolol prior to stimulation with 100 ng/mL Kdo_2-Lipid A. B) Western blot analysis of COX-2 expression in cells preincubated with 25 μM BEL or 50 μM propranolol prior to stimulation with 100 ng/mL Kdo_2-Lipid A. C) HPLC-MS detection of eicosanoids released from cells preincubated with 25 μM BEL or 50 μM propranolol for 30 minutes prior to stimulation with and without 100 ng/mL Kdo_2-Lipid A for 24 hours (as indicated in the figure legend). Representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.


A
COX-2/GAPDH mRNA (arbitrary units)
120
100
80
60
40
20
0
No Inhibitor
BEL
Propranolol
B
No Inhibitor
BEL
Propranolol
C
Eicosanoids (pmol/10^6 cells)
150
125
100
75
50
25
0
No Treatment
Kdo2-Lipid A
BEL
BEL + Kdo2-Lipid A
Propranolol
Prop + Kdo2-Lipid A
PGD2
15D PGD2
PGE2
PGJ2
15D PGJ2

deoxy-$\Delta^{12,14}$-PGJ_2 using HPLC-MS. We observed that both BEL and propranolol inhibited the release of all eicosanoid species by similar magnitudes (Figure III.1.C).

2. PAP-1 inhibition reduces Kdo_2-Lipid A stimulated AA release

We began this study by characterizing GIVA PLA_2 mediated AA release from macrophages stimulated with Kdo_2-Lipid A. We conducted AA release time-course experiments over a 24 hour time-course; as shown in figure III.2, the maximal release of AA is 300-400 pmol AA/10^6 cells at 1 hour after stimulation, after which levels gradually returned to baseline levels. We chose to study Kdo_2-Lipid A stimulated AA release at one hour following stimulation.

To ensure that the GIVA PLA_2 is responsible for mediating AA release under these conditions, cells were preincubated with the GIVA PLA_2 specific inhibitor pyrrophenone. Pyrrophenone reduced Kdo_2-Lipid A stimulated AA release to background levels, as shown in figure III.3.A; indicating that the Kdo_2-Lipid A stimulated AA release is mediated by GIVA PLA_2. To evaluate whether PAP-1 may participate in the activation of GIVA PLA_2, we preincubated cells with propranolol, which is known to inhibit PAP-1. This resulted in a ~70% decrease of Kdo_2-Lipid A stimulated AA release into the supernatant (Figure III.3.B). To corroborate this result, we repeated the experiment with another inhibitor of PAP-1, bromoenol lactone (BEL). Preincubation of cells with BEL reduced AA release similar to that of propranolol (Figure III.3.C).

It is known that the *R* and *S* enantiomers of BEL have ten-fold higher inhibitory potencies for the γ and β isoforms of GVI PLA_2, respectively (169). Therefore, cells

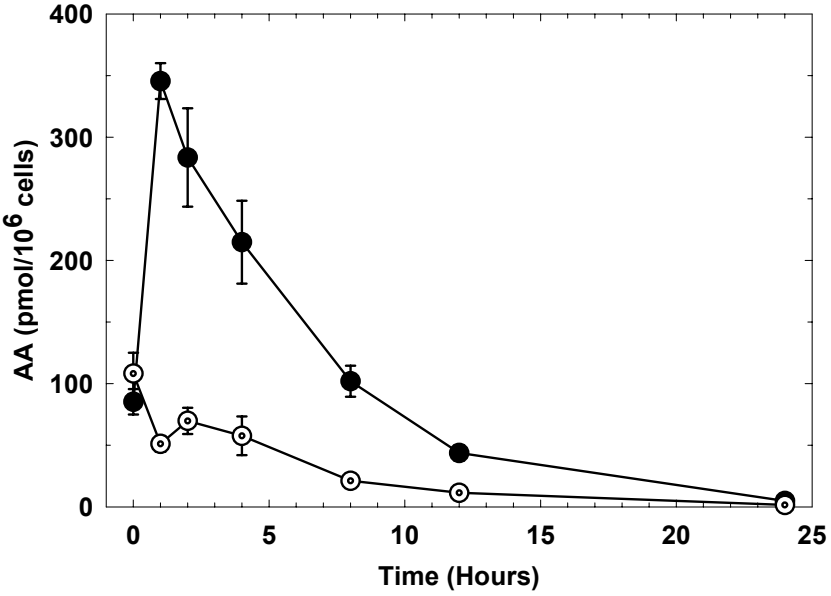


Figure III.2. Kdo_2-Lipid A stimulated AA release time-course.

RAW 264.7 macrophages were cultured in presence (●) or absence (○) of 100 ng/mL Kdo_2-Lipid A, the media was collected at the indicated time points and then subsequently analyzed for AA release by HPLC-MS. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

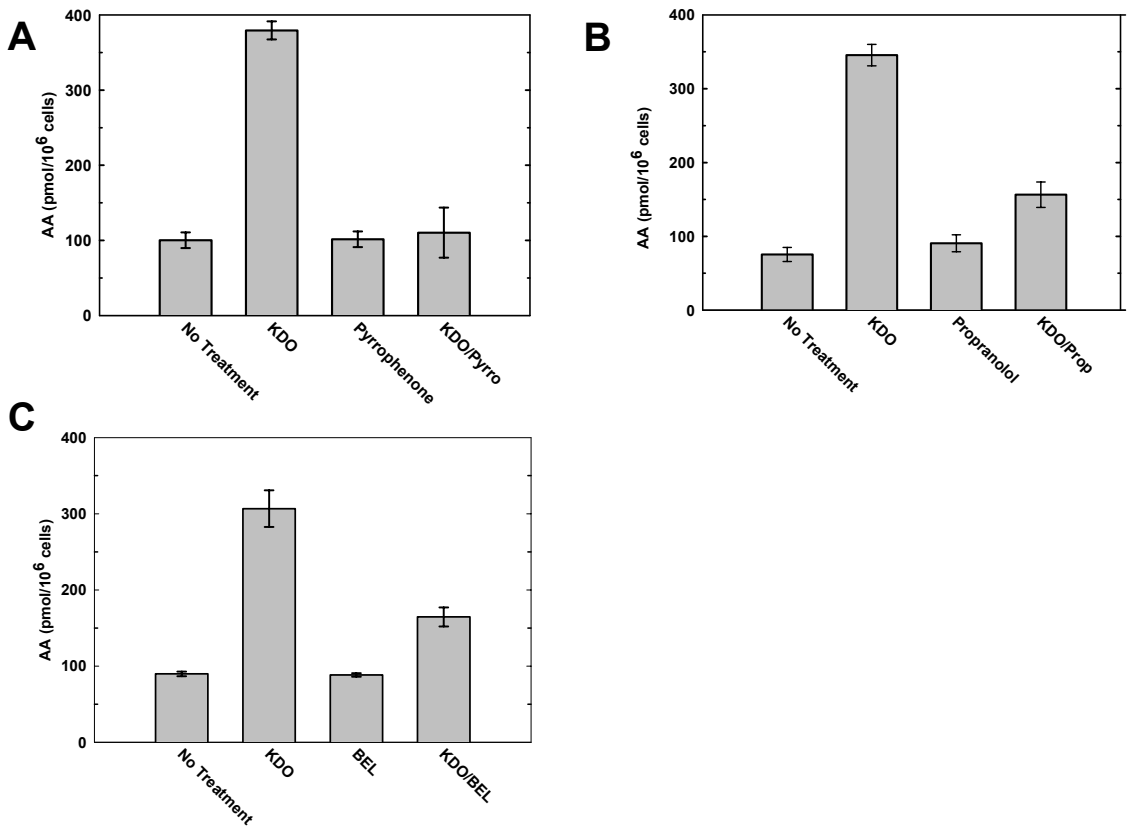


Figure III.3. Chemical inhibition of PAP-1 blocks Kdo_2-Lipid A stimulated AA release.

AA release was measured from RAW 264.7 macrophages preincubated with and without A) 1 μM pyrrophenone, B) 50 μM propranolol, or C) 25 μM BEL prior to stimulation with 100 ng/mL Kdo_2-Lipid A. The media was collected at 1 hour following Kdo_2-Lipid A stimulation and analyzed for AA release by HPLC-MS. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

were incubated with the *R* and *S* enantiomers of the BEL inhibitor to observe whether either enantiomer displayed a more pronounced inhibition of AA release; such a result would suggest that the observation is due to inhibition of a GVI PLA_2 isoform, whereas inhibition by both enantiomers would support involvement through a PAP-1 mediated mechanism. Both enantiomers inhibited AA release by similar magnitudes and approximately as potently as the racemic inhibitor (Figure III.4). Taken as a whole, these results demonstrate that Kdo_2-Lipid A stimulated AA release is mediated through GIVA PLA_2 in RAW 264.7 macrophages, and that the inhibition of PAP-1 results in reduction of Kdo_2-Lipid A stimulated AA release.

3. PAP-1 inhibition does not reduce ionomycin or ATP stimulated AA release

Our inhibitory data suggest that PAP-1 participates in Kdo_2-Lipid A stimulated GIVA PLA_2 activation. To verify that the reduced Kdo_2-Lipid A stimulated AA release is not due to direct inhibition of GIVA PLA_2 by BEL and propranolol, we stimulated cells with ionomycin and ATP in the presence and absence of these inhibitors. Both ionomycin and ATP induce GIVA PLA_2 activation by increasing cytosolic Ca^{+2} levels. Activation of GIVA PLA_2 through elevation of cytosolic Ca^{+2} levels does not require participation of the TLR-mediated signal transduction cascade; therefore, neither BEL nor propranolol should reduce AA release induced by these stimuli. Stimulation of macrophages with either ionomycin or ATP increased AA release into the supernatant; ionomycin treatment resulted in a 50% increase relative to control values, whereas ATP increased AA release by 100% (Figure III.4.A and B). Neither propranolol nor BEL reduced AA release induced by stimulation of the cells with ATP or ionomycin

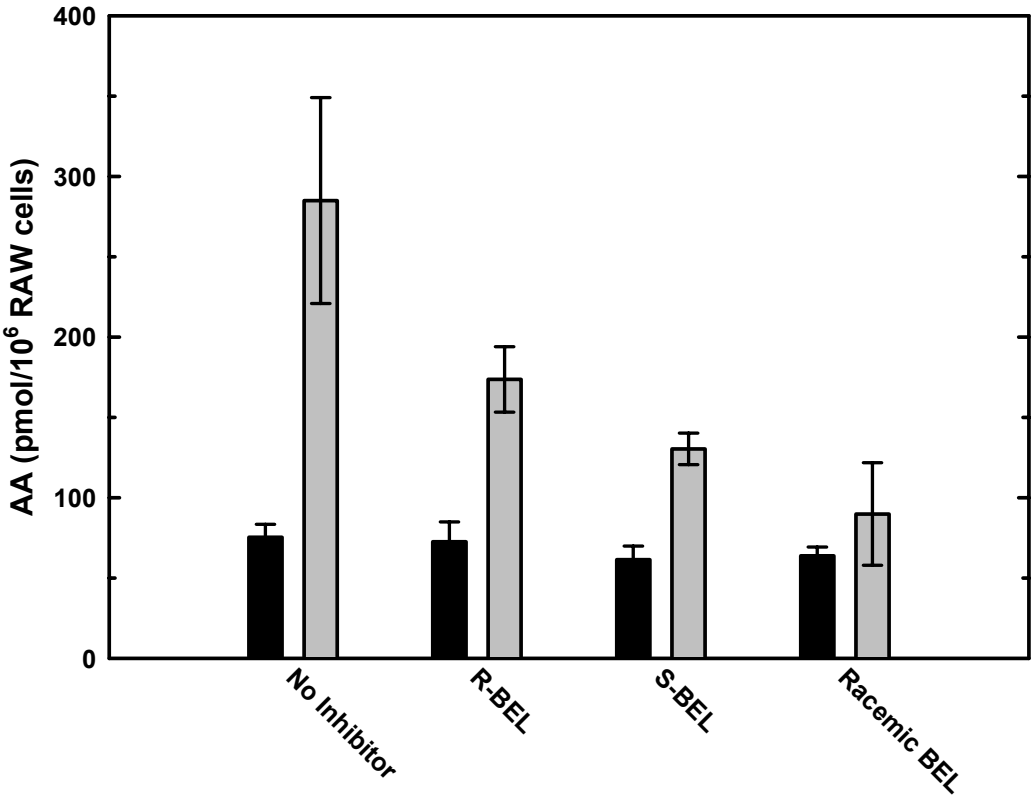


Figure III.4. *R*, *S* and racemic BEL inhibit Kdo_2-Lipid A stimulated AA release.

AA release was measured from RAW 264.7 macrophages preincubated with 25 μM BEL (*R*, *S* or racemic, as indicated) prior to stimulation with 100 ng/mL Kdo_2-Lipid A. The media was collected at 1 hour following Kdo_2-Lipid A stimulation and analyzed for AA release by HPLC-MS. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

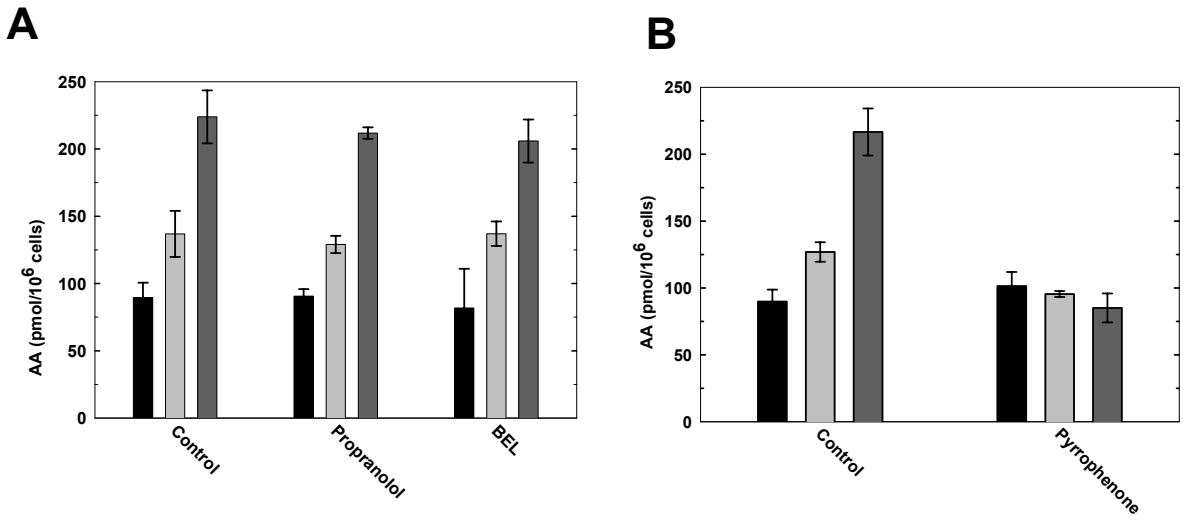


Figure III.5. Chemical inhibition of PAP-1 does not block ionomycin or ATP stimulated AA release.

AA release was measured from RAW 264.7 macrophages preincubated with and without A) 50 μM propranolol or 25 μM BEL, or B) 1 μM pyrrophenone prior to the addition of vehicle (black bars), 1 μM ionomycin (light grey bars) or 2 mM ATP (dark grey bars). The media was collected at 15 minutes following stimulation and analyzed for AA release. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

(Figure III.5.A). To ensure that the Ca^{+2} agonists had induced AA release via GIVA PLA_2, pyrrophenone was added to macrophages prior to stimulation with ionomycin and ATP. Pyrrophenone inhibited AA release induced by either ionomycin or ATP to background levels, indicating that both agonists induced AA release through GIVA PLA_2 involvement (Figure III.5.B).

4. PAP-1 Inhibition Reduces in vitro GIVA PLA_2 Activity in RAW 264.7 Cell Lysates

Our laboratory has previously developed a specific GIVA PLA_2 *in vitro* assay that enables the quantitation of cellular GIVA PLA_2 activity from lysates (167,170). Our laboratory has also shown that BEL does not inhibit *in vitro* GIVA PLA_2 activity (170). In the current study, we also verified that propranolol does not inhibit GIVA PLA_2 activity (Figure III.6). To evaluate whether Kdo_2-Lipid A stimulation increases GIVA PLA_2 activity in RAW 264.7 macrophages, we performed the *in vitro* GIVA PLA_2 activity assay on lysates derived from cells that were cultured in the absence and presence of Kdo_2-Lipid A; we observed a small, but significant increase with stimulation (Figure III.7.A). To ensure that the observed increase was significant, the experiment was repeated using U937 macrophages; in this situation, the increase was more pronounced (Figure III.7.B). Propranolol was added to cells to assess whether exposure of the cells to the PAP-1 inhibitor would subsequently reduce GIVA PLA_2 activity. Propranolol significantly reduced activity measured from RAW 264.7 and U937 lysates (Figures III.7.A and B).

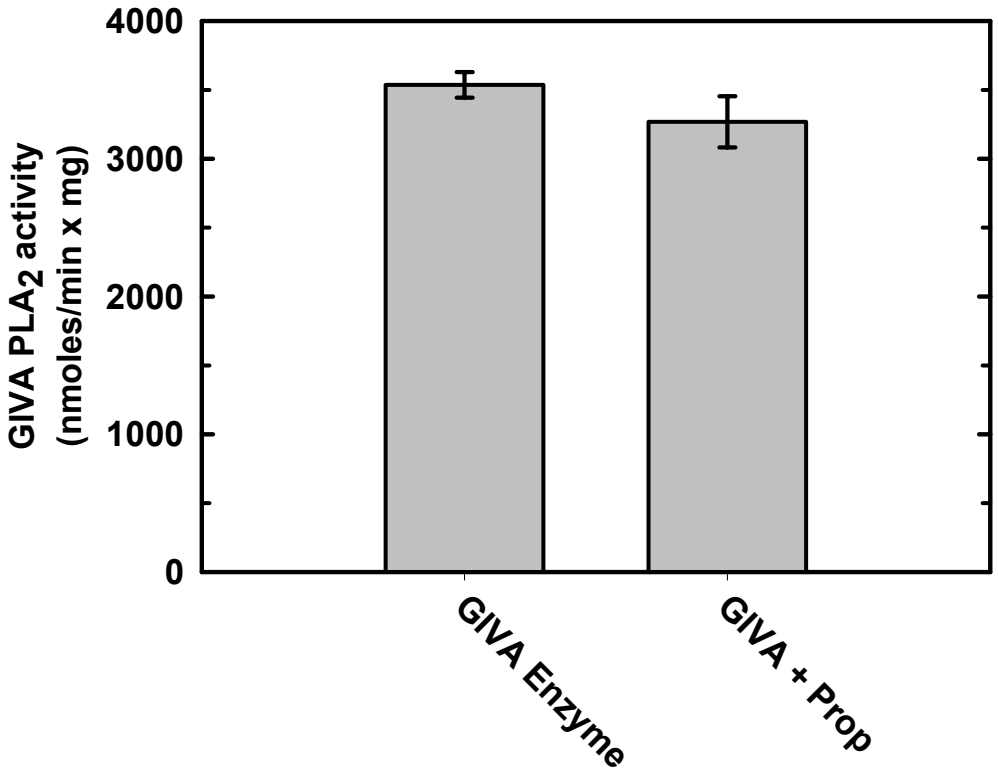


Figure III.6. Propranolol does not reduce recombinant GIVA PLA_2 *in vitro* activity.

Recombinant GIVA PLA_2 protein was incubated in the presence and absence of 100 μM propranolol prior to measurement of the *in vitro* GIVA PLA_2 activity. Data are expressed as mean values ± S.D. of three individual replicates.

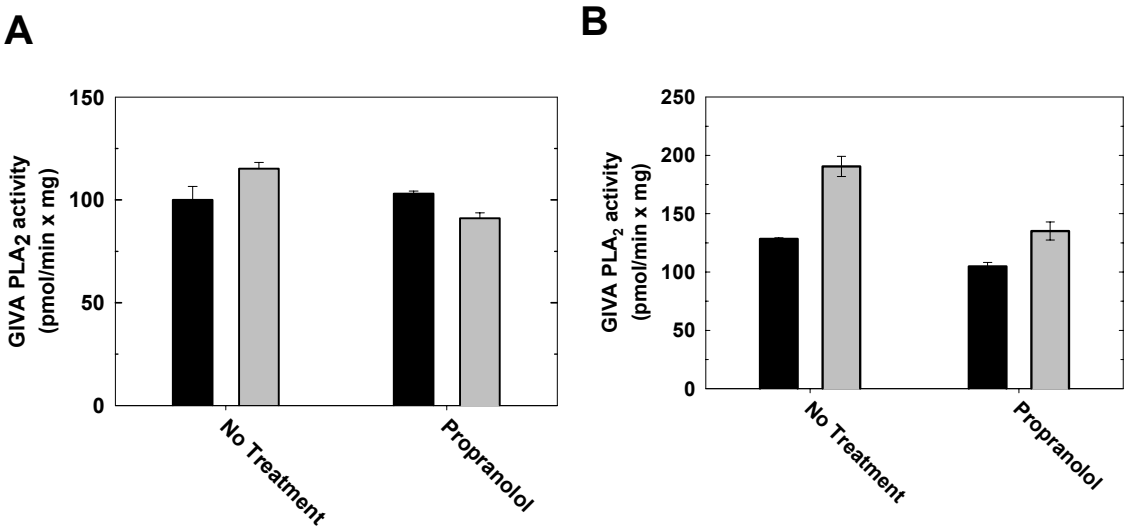


Figure III.7. Propranolol reduces Kdo_2-Lipid A stimulated GIVA PLA_2 *in vitro* activity in RAW 264.7 macrophages.

A) RAW 264.7 and B) U937 macrophages were cultured in presence and absence of 100 μM propranolol prior to the addition of 100 ng/mL Kdo_2-Lipid A. The media was removed and the cell lysates were collected and assayed for *in vitro* GIVA PLA_2 activity. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

5. PAP-1 inhibition reduces synergy-enhanced AA release, but does not affect synergistic activation

In this manuscript, we describe GIVA PLA_2 mediated AA release from RAW 264.7 macrophages stimulated by Kdo_2-Lipid A and by Ca^{+2} agonists. Recently, we reported that cells that were stimulated with Kdo_2-Lipid A and ATP sequentially released more AA than the sum of AA released from cells stimulated separately (171). The extent to which AA release is enhanced by the synergy of Kdo_2-Lipid A and ATP stimuli can be calculated from the following equation:

$$\textbf{Synergistic Activation} = \frac{\textbf{[Kdo}_2\textbf{-Lipid A + ATP]}}{\textbf{[Kdo}_2\textbf{-Lipid A] + [ATP]}}$$

Equation 1

When synergy is present, the synergistic activation value is greater than 1.0, as joint Kdo_2-Lipid A and ATP stimulation result in AA release that exceeds the sum of the individual stimulations. If no synergy is present, and therefore joint stimulation equals the sum total of separate stimulations, the value will equal 1.0. In the current study, sequential treatment of cells with Kdo_2-Lipid A and ATP resulted in synergistic activation of 2.5±0.3, as summarized in Figure III.8. Synergistically activated cells

released 1,600 pmol AA/10^6 cells, which is approximately four times greater than the amount of AA released by either Kdo_2-Lipid A or ATP alone induced.

As previously discussed, preincubation of macrophages with either BEL or propranolol prior to Kdo_2-Lipid A treatment reduced AA release by 70%, whereas the inhibitors did not affect ATP stimulated AA release. Preincubation of cells with BEL or propranolol reduced the synergy-enhanced AA released (Figure III.8.A). Surprisingly, despite the reduction in AA release, the synergistic activation value was not affected by the reduced AA release (Figure III.8.B). This suggests that PAP-1 inhibition results in reduced AA release by blocking the Kdo_2-Lipid A stimulation, while not affecting the magnitude by which synergy augments AA release.

6. Exogenous DAG enhances Kdo_2-Lipid A stimulated AA release through a GIVA PLA_2 dependent mechanism

Since PAP-1 converts cellular PA into DAG, macrophages were treated with exogenous DAG to assess whether this would elicit AA release. The addition of DAG to unstimulated cells did not increase AA release into the supernatant. However, the supplementation of DAG to Kdo_2-Lipid A treated cells increased AA release by 100% relative to that of cells treated with only Kdo_2-Lipid A (Figure III.9). To ensure that the enhanced AA release was due to additional activation of GIVA PLA_2, we preincubated cells with pyrrophenone prior to stimulation with Kdo_2-Lipid A and DAG. Pyrrophenone inhibited AA release to background levels, indicating that the enhanced AA release is through to the action of GIVA PLA_2.

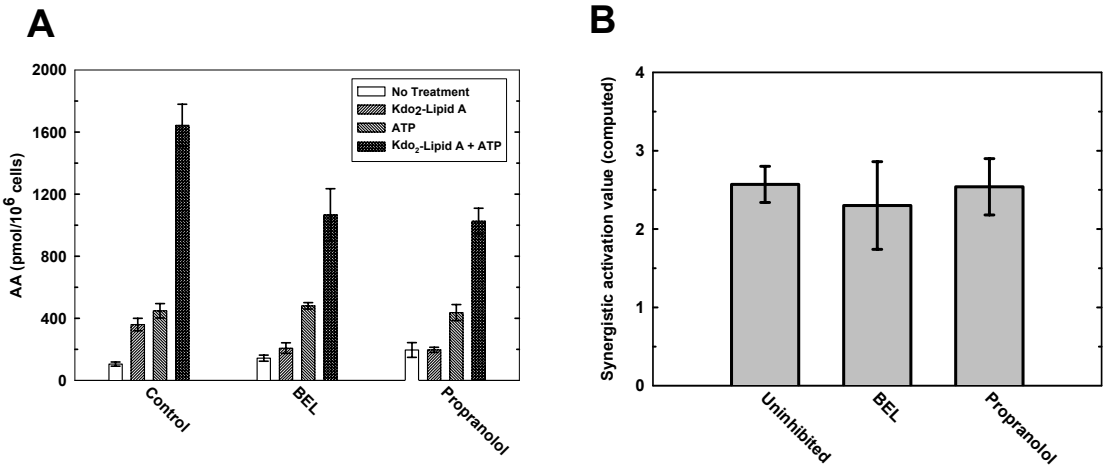


Figure III.8. Chemical inhibition of PAP-1 blocks synergy-enhanced AA release, but does not reduce the synergistic activation value.

A) AA release was measured from RAW 264.7 macrophages preincubated with and without 25 μM BEL or 50 μM propranolol prior to stimulation with and without 100 ng/mL Kdo_2-Lipid A for 50 minutes and with and without 2 mM ATP for 10 minutes (as indicated in the legend). The media was collected at 1 hour following the addition of the 100 ng/mL Kdo_2-Lipid A agonist and analyzed for AA release. B) Synergistic activation values were calculated for uninhibited cells, preincubated with 25 μM BEL or 50 μM propranolol. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

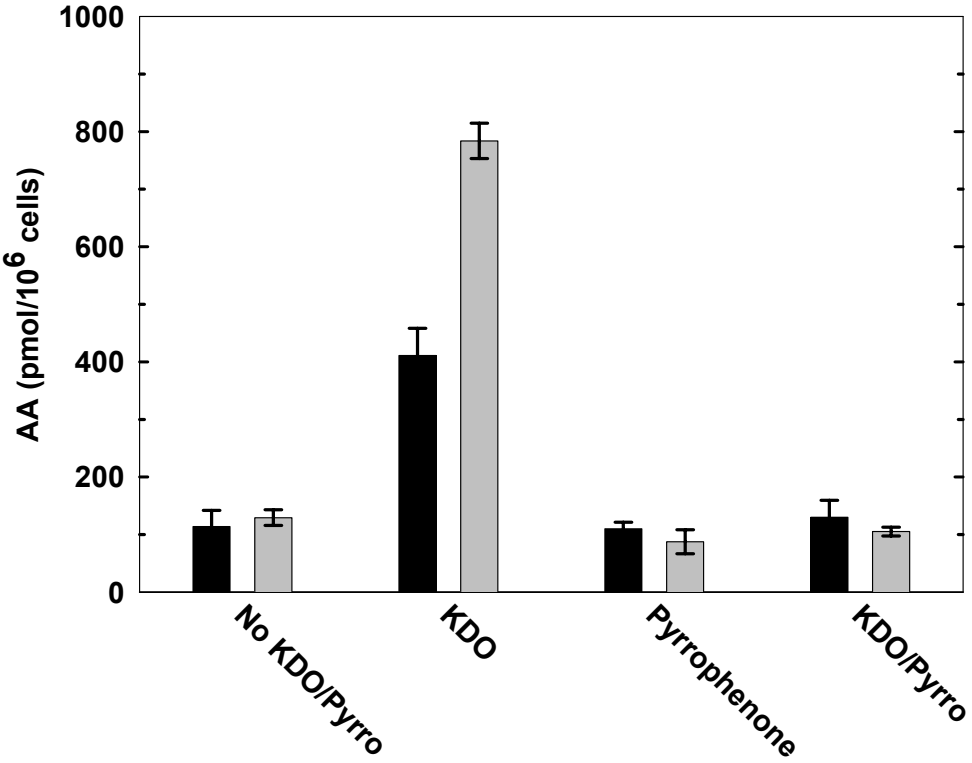


Figure III.9. Exogenous DAG bolsters the Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages.

AA release into the media was measured from RAW 264.7 macrophages that were stimulated with and without 100 ng/mL Kdo_2-Lipid A (as indicated) and in the absence (black bars) and presence (grey bars) of 50 μM DAG for 1 hour. Additionally, cells were preincubated with and without 1 μM pyrrophenone (as indicated) for 30 minutes prior to the addition of DAG and Kdo_2-Lipid A. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

7. Exogenous DAG restores AA release from cells inhibited by propranolol or BEL

Having observed that DAG enhanced Kdo_2-Lipid A stimulated AA release, we investigated whether the addition of exogenous DAG in an "add-back" fashion would restore AA release from cells pretreated with propranolol or BEL. The addition of DAG to cells pretreated with propranolol prior to stimulation with Kdo_2-Lipid A restored AA release to levels of that from uninhibited cellss that were treated with Kdo_2-Lipid A (Figure III.10.A). This experiment was then repeated utilizing BEL to inhibit PAP-1. The addition of DAG to cells that had been preincubated with BEL also restored AA release to uninhibited-Kdo_2-Lipid A treated AA release levels (Figure III.10.B). This experiment was then repeated utilizing BEL to inhibit PAP-1. The addition of DAG to cells that had been preincubated with BEL also restored uninhibited AA release levels (Figure III.10.B). Further supplementation of the cells with DAG resulted in a linear increase of AA release, both in the presence and the absence of the PAP-1 inhibitor BEL (Figure III.11).

8. Synergy is induced through treatment of macrophages with ATP and exogenous DAG

In this manuscript, we observed that the addition of DAG to Kdo_2-Lipid A stimulated macrophages resulted in enhanced GIVA PLA_2–mediated AA release; additionally, we have previously shown that DAG is formed in macrophages during TLR-4 signaling (25). Because DAG formation is a component of the TLR-4 pathway, we hypothesized that the increased GIVA PLA_2-mediated AA release following DAG exposure of the cells is attributed to TLR-4 activation of GIVA PLA_2. We then investigated whether synergy could be elicited after replacing the Kdo_2-Lipid A component of synergy with DAG. Therefore, Kdo_2-Lipid A was substituted with DAG in equation 1 to yield:

$$\text{Synergistic Activation} = \frac{[\text{DAG} + \text{ATP}]}{[\text{DAG}] + [\text{ATP}]}$$

Equation 2

Cells were treated with DAG alone, ATP alone or both ATP and DAG, after which AA release into the supernatant was quantitated; the results are summarized in figure III.12. Computing the synergistic activation value from equation 2, the synergistic activation value is calculated to be 2.6±0.3.

9. Propranolol Inhibits Kdo_2-Lipid A stimulated AA Release but not OA Release from RAW 264.7 Macrophages

Next, we wanted to explore whether PAP-1 participate in general phospholipase A_2 activation, or whether it specifically activates GIVA PLA_2. GIVA PLA_2 prefers substrate containing AA at the *sn*-2 position of membrane phospholipids; therefores, we compared the effect of PAP-1 inhibition on the intracellular metabolism of AA with that of oleic acid (OA), which is also released by $sPLA_2$ (172,173,174). RAW 264.7 macrophages were supplemented with radioactive AA or OA prior to exposure to propranolol and Kdo_2-Lipid A stimulation; membrane lipids were subsequently extracted for subsequent analysis, as described in the materials and methods section. We observed

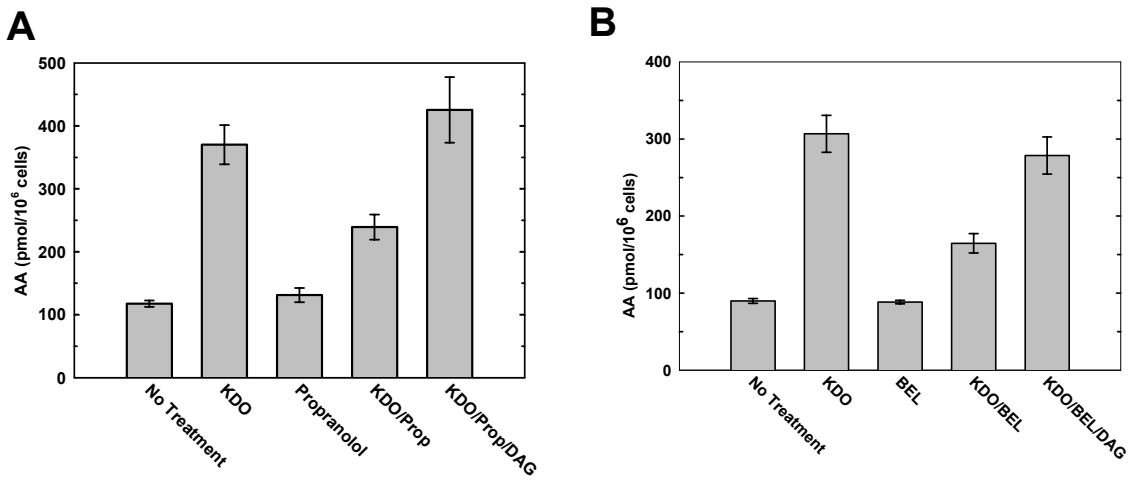


Figure III.10. DAG supplementation restores AA release from Kdo_2-Lipid A stimulated RAW 264.7 macrophages pretreated with PAP-1 inhibitors.

AA release was measured from cells preincubated with and without A) 50 μM propranolol or B) 25 μM BEL prior to stimulation with 100 ng/mL Kdo_2-Lipid A and 50 μM DAG. The media was collected at 1 hour following stimulation and analyzed for AA release. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

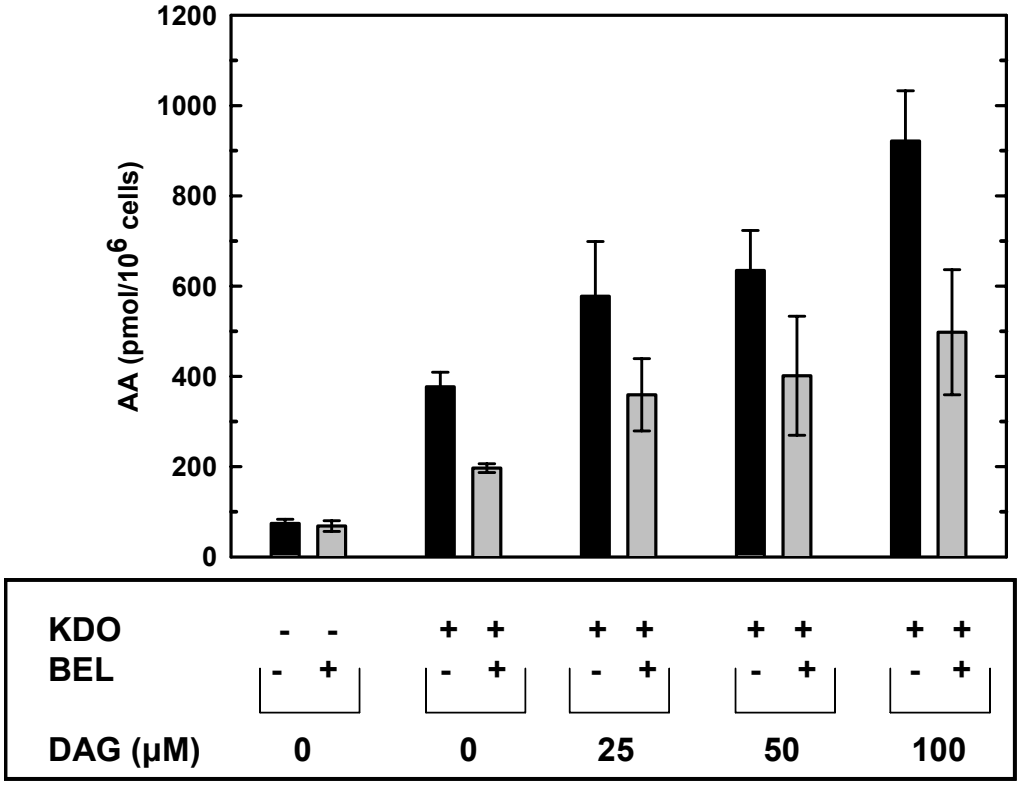


Figure III.11. Increasing doses of exogenous DAG bolster Kdo_2-Lipid A stimulated AA release in a linear fashion from RAW 264.7 macrophages.

AA release into the media was measured from RAW 264.7 macrophages that were incubated in the presence and absence of 25 μM BEL (as indicated) prior to stimulation with 100 ng/mL Kdo_2-Lipid A and 50 μM DAG (as indicated) for 1 hour. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

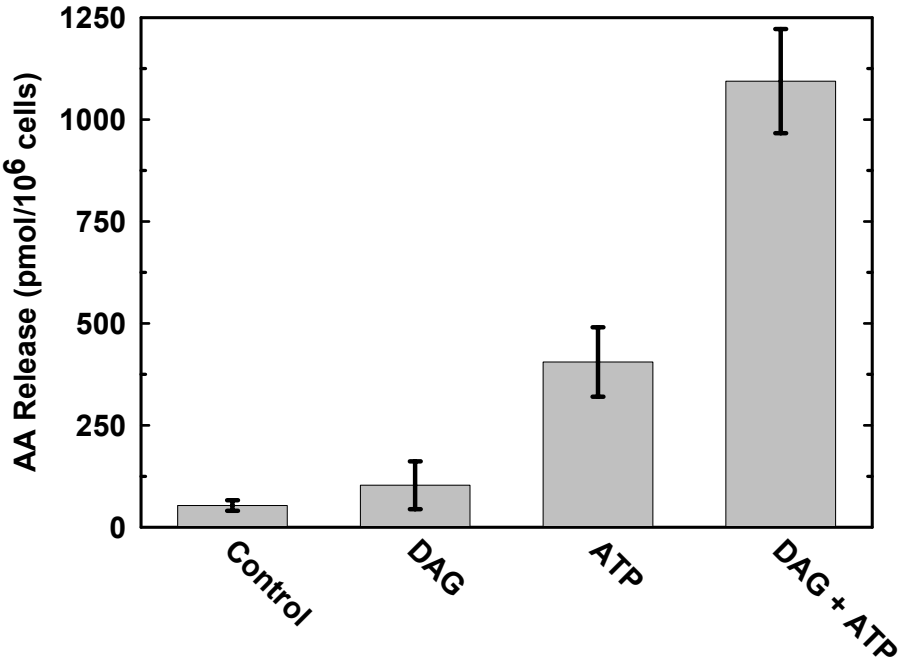


Figure III.12. Exogenous DAG bolsters the ATP stimulated release of AA.

AA release into the media was measured from RAW 264.7 macrophages that were stimulated with and without 2 mM ATP and 50 μM DAG (as indicated) as described in the materials and methods section. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

that in response to Kdo_2-Lipid A stimulation, cells showed a 75% increase in the membrane release of either free fatty acid, relative to that of the control cells (Figure III.13.A and B). When cells were preincubated with propranolol prior to stimulation with Kdo_2-Lipid A, we observed that the inhibitor abrogated the metabolism of AA, while not significantly blocking OA release.

Since propranolol did not inhibit the release of OA, it was necessary to ensure that PAP-1 was inhibited in cells that were supplemented with radioactive OA. To achieve this, TAG formation was measured. In response to Kdo_2-Lipid A stimulation, TAG levels including either fatty acid species increased by around five times relative to that of control levels. The Kdo_2-Lipid A increased TAG levels were abrogated to control levels in cells that had been preincubated with propranolol (Figure III.13.C and D).

Additionally, we quantitated the endogenous levels of DAG and phospholipid formed in response to stimulation. DAG levels decreased slightly in Kdo_2-Lipid A stimulated cells compared with that of controls, whereas phospholipid levels were not observed to change with stimulation (Figures III.13.E-H). There was no significant difference in AA-containing and OA-lipid metabolism amongst DAG and phospholipid species nor did propranolol significantly affect the levels of either lipid class.

Figure III.13.A-D. PAP-1 chemical inhibition in Kdo_2-Lipid A stimulated cells results in reduced free AA release, but not free OA while blocking TAG formation.

A) [^{3}H]AA, B) [^{3}H]OA, C) [^{3}H]AA-containing TAG and D) [^{3}H]OA-containing TAG were quantitated from RAW 264.7 macrophages stimulated with 100 ng/mL Kdo_2-Lipid A for 24 hours. Where indicated cells were preincubated with 50 μM propranolol prior to stimulation. The cells were radiolabelled with [5,6,8,9,11,12,14,15-^{3}H] AA or [9,10-^{3}H] OA as described in the Materials and Methods section prior to treatment with Kdo_2-Lipid A. The lipids were extracted from the cells, separated by TLC and quantitated by liquid scintillation. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

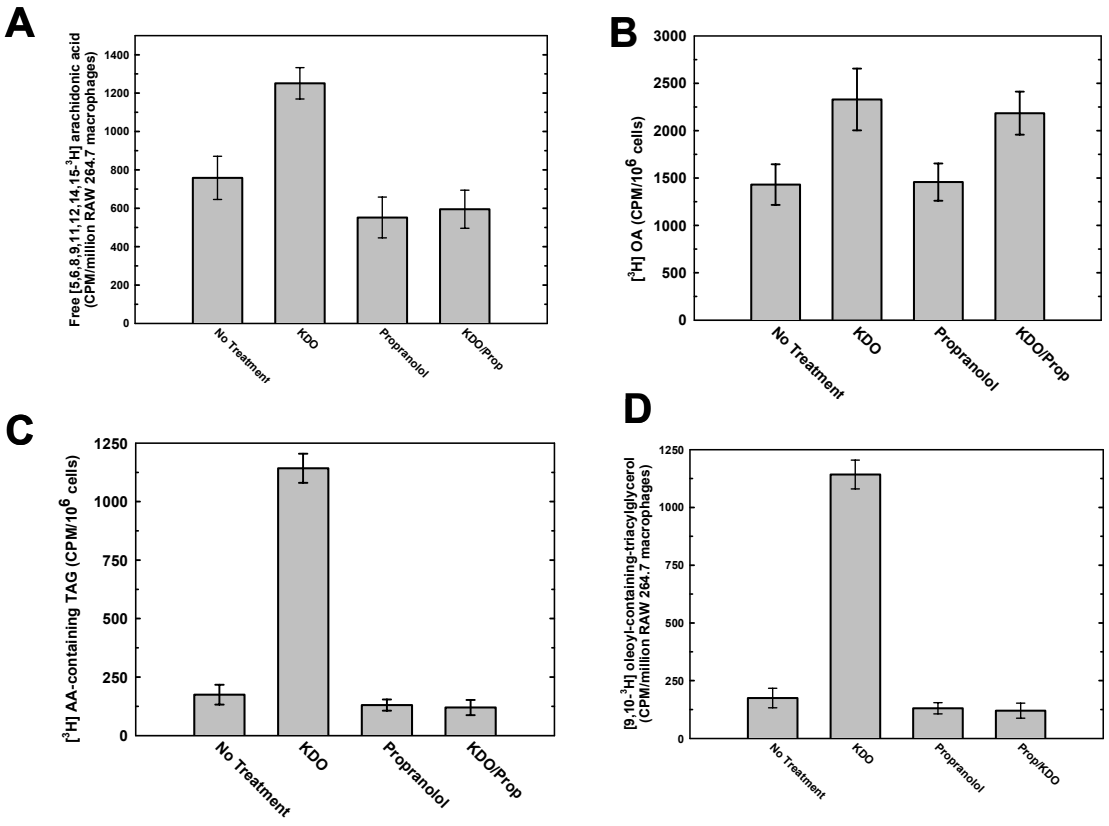
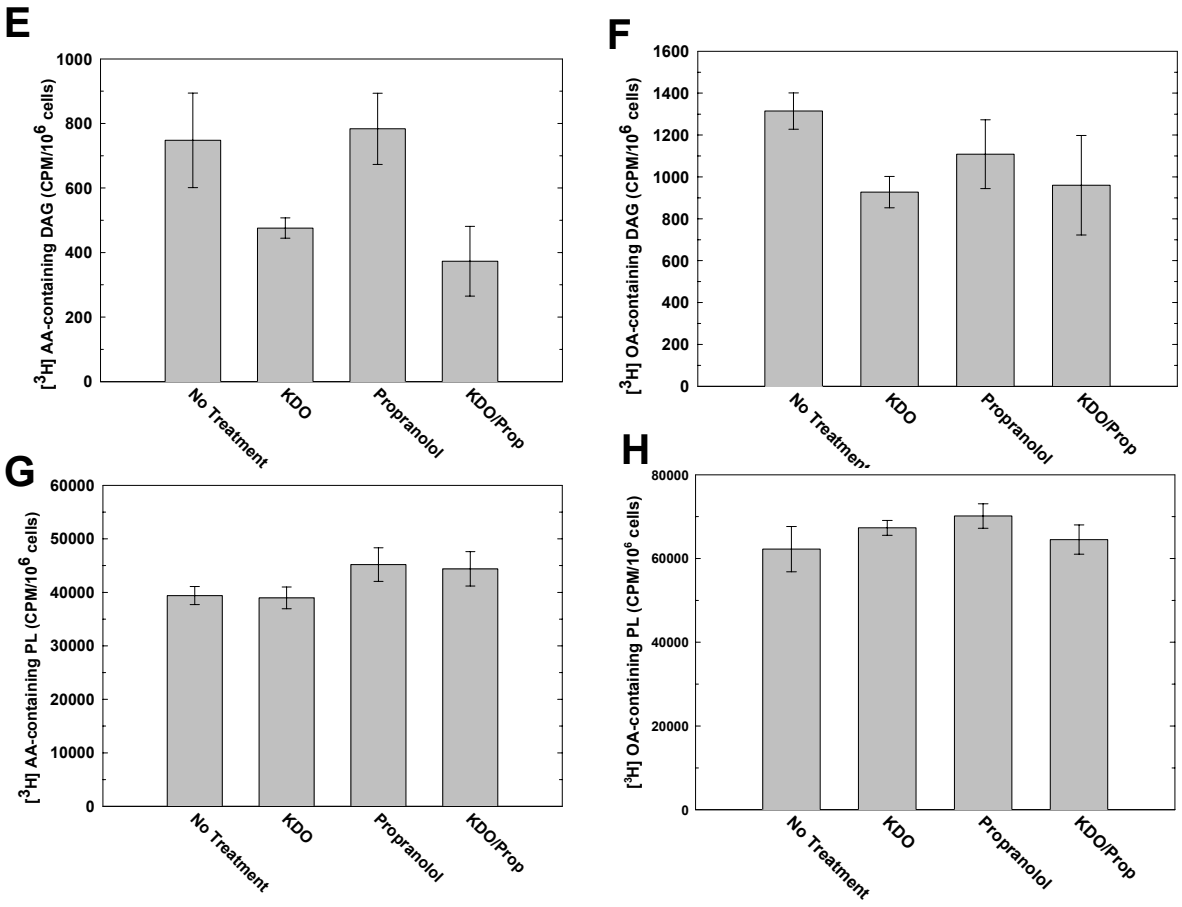

A
Free [5,6,8,9,11,12,14,15-3H] arachidonic acid (CPM/million RAW 264.7 macrophages)
0
200
400
600
800
1000
1200
1400
No Treatment
KDO
Propranolol
KDO/Prop
B
[3H] OA (CPM/10^6 cells)
0
500
1000
1500
2000
2500
3000
No Treatment
KDO
Propranolol
KDO/Prop
C
[3H] AA-containing TAG (CPM/10^6 cells)
0
250
500
750
1000
1250
No Treatment
KDO
Propranolol
KDO/Prop
D
[9,10-3H] oleoyl-containing-triacylglycerol (CPM/million RAW 264.7 macrophages)
0
250
500
750
1000
1250
No Treatment
KDO
Propranolol
Prop/KDO

Figure III.13.E-H. PAP-1 inhibition in Kdo_2-Lipid A stimulated cells does not affect DAG or phospholipid levels.

E) [^{3}H]AA-containing DAG, F) [^{3}H]OA-containing DAG, G) [^{3}H]AA-containing PL and H) [^{3}H]OA-containing PL were quantitated from RAW 264.7 macrophages stimulated with 100 ng/mL Kdo_2-Lipid A for 24 hours. Where indicated cells were preincubated with 50 μM propranolol prior to stimulation. The cells were radiolabelled with [5,6,8,9,11,12,14,15-^{3}H] AA or [9,10-^{3}H] OA as described in the Materials and Methods section prior to treatment with Kdo_2-Lipid A. The lipids were extracted from the cells, separated by TLC and quantitated by liquid scintillation. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.


E
[3H] AA-containing DAG (CPM/10^6 cells)
1000
800
600
400
200
0
No Treatment
KDO
Propranolol
KDO/Prop
F
[3H] OA-containing DAG (CPM/10^6 cells)
1600
1400
1200
1000
800
600
400
200
0
No Treatment
KDO
Propranolol
KDO/Prop
G
[3H] AA-containing PL (CPM/10^6 cells)
60000
50000
40000
30000
20000
10000
0
No Treatment
KDO
Propranolol
KDO/Prop
H
[3H] OA-containing PL (CPM/10^6 cells)
80000
60000
40000
20000
0
No Treatment
KDO
Propranolol
KDO/Prop

D. DISCUSSION

The focus of this research was to understand the relationship between PAP-1 and GIVA PLA_2 activation. To accomplish this, we studied the effect of PAP-1 inhibition on the Kdo_2-Lipid A stimulated release of AA from RAW 264.7 macrophages. In previous studies in which AA release was quantitated from macrophages, our laboratory and others made use of scintillation counting of radioactive AA as well as EIA quantitation of PGE_2 secreted to the supernatant as an indicator of cellular GIVA PLA_2 activity (123,165,175-177). Both techniques have important limitations. Regarding scintillation detection, in addition to measuring AA, it also accounts for AA-derived metabolites, such as eicosanoids. With regard to EIA measurement of PGE_2, the assumption is made that PGE_2 release is a direct indicator of AA release. In the current study, we make use of HPLC-MS methodology to directly quantitate AA release specifically.

We began this study by conducting Kdo_2-Lipid A stimulated AA release time-course experiments to identify the most optimal time point for further experimentation. Maximal AA release into the supernatant was observed at one hour following stimulation; therefore, this was the time point at which subsequent experiments were conducted. Since we utilized AA release as an indicator of cellular GIVA PLA_2 activity, it was imperative to ensure that Kdo_2-Lipid A stimulated AA release was mediated by GIVA PLA_2. To achieve this, macrophages were pretreated with the GIVA PLA_2 specific inhibitor, pyrrophenone, prior to stimulation with Kdo_2-Lipid A. Pyrrophenone reduced AA release to background levels, indicating that GIVA PLA_2 is responsible for Kdo_2-Lipid A stimulated AA release (figure 2A).

To investigate whether PAP-1 participates in the Kdo_2-Lipid A stimulated GIVA PLA_2 activation, PAP-1 inhibitors were employed in cellular experiments. Preincubation of cells with BEL or propranolol prior to Kdo_2-Lipid A stimulation resulted in a marked reduction of AA release into the supernatant. Since this could have resulted from the direct inhibition of GIVA PLA_2 by the inhibitors, we investigated the effect of propranolol and BEL on AA release from cells stimulated with ATP and ionomycin. These agonists activate GIVA PLA_2 directly by increasing the cellular concentration of Ca^{+2} rather than through a TLR-4 mediated mechanism. Neither BEL nor propranolol reduced AA release from cells stimulated with ATP or ionomycin. This result is significant, as it indicates that the reduced Kdo_2-Lipid A stimulated AA release is not due to direct inhibition of cellular GIVA PLA_2 activity. It is worth noting that we have shown that activation of TLR-4 in macrophages does not increase Ca^{+2} levels (178). Additionally, we confirmed that AA release from cells stimulated with ionomycin or ATP is mediated through GIVA PLA_2 by pyrrophenone inhibition.

Our laboratory has been investigating the molecular basis of GIVA PLA activation in the TLR-4 activated macrophage for a number of years. In the current study, we observed that Kdo_2-Lipid A stimulated AA release was reduced from PAP-1 inhibited cells while ATP stimulated AA release was unaffected by PAP-1 inhibition. We have previously described that PAP-1 participates in TLR-4 mediated COX-2 expression, while our current results suggest that PAP-1 also participates in the TLR-4 signaling mechanism resulting in activation of the GIVA PLA_2 (133,165). Collectively, these results suggest that PAP-1 plays a significant role in inflammatory signaling within the TLR-4 activated macrophage.

In a recent publication, our laboratory described AA release from macrophages treated with Kdo_2-Lipid A and ATP had greatly exceeded the sum total of cells stimulated separately; this phenomena has been referred to as synergy-enhanced AA release (171). We have concluded that the increased AA release results from enhanced cellular GIVA PLA_2 activation through complimentary molecular mechanisms – Kdo_2-Lipid A through the TLR-4 signaling and ATP through increased cellular Ca^{+2}. In this manuscript, we observed that PAP-1 inhibition blocked Kdo_2-Lipid A stimulated AA release although it did not affect ATP stimulated AA release. Furthermore, AA release was blunted from cells treated with propranolol and BEL prior to stimulation with both Kdo_2-Lipid A and ATP; however, synergistic activation was not affected by the inhibitors. It is likely that the reduced AA release from cells stimulated with Kdo_2-Lipid A and ATP is due to inhibition of PAP-1 in the TLR-4 portion of the signaling mechanism.

Several lines of evidence suggest that PAP-1 does not participate in synergy-enhanced GIVA PLA_2 activation and that there is likely a second source of DAG that plays a role in synergy. The first is that PAP-1 inhibition did not reduce the synergistic activation value. Secondly, our data suggests that PAP-1 functions in the TLR-4-mediated activation of GIVA PLA_2 and the addition of exogenous DAG to Kdo_2-Lipid A treated macrophages enhances AA release. This suggests that the exogenous DAG is stimulating a molecular target leading to GIVA PLA_2 activation distinctive from that targeted by PAP-1. The third is that the exogenous DAG enhances ATP stimulated AA release, which suggests that its effect is facilitated by elevated Ca^{+2}; whereas PAP-1 participates in TLR-4-mediated activation in which Ca^{+2} levels do not increase. Finally, exogenous DAG alone does not elicit AA release from unstimulated cells, nor does it

enhance AA release from cells stimulated with both Kdo_2-Lipid A and ATP; this suggests that exogenous DAG activates a molecular target that specifically functions in synergy-enhanced GIVA PLA_2 activation, whereas PAP-1 functions in the Kdo_2-Lipid A stimulated GIVA PLA_2 activation.

To evaluate whether DAG evolved from PAP-1 would directly activate cellular GIVA PLA_2, cells were supplemented with exogenous DAG prior to the measurement of AA release into the supernatant. The addition of DAG alone did not increase AA release, relative to that of control cells, whereas supplementation of DAG to macrophages that were also treated with Kdo_2-Lipid A resulted in enhanced AA release by an order of magnitude (Figure III.9). We confirmed that the increased AA release was mediated through GIVA PLA_2, as preincubation of cells with GIVA PLA_2 inhibitor pyrrophenone reduced AA release to background levels. The fact that DAG alone did not evoke AA release suggests that PAP-1 activation is insufficient to stimulate GIVA PLA_2 catalysis and therefore is likely to require the participation of additional factors to initiate GIVA PLA_2 function, possibly the bioactive lipids phosphatidylinositol bis-4,5-phosphate and ceramide 1-phosphate. It is likely that DAG evolved from PAP-1 function through in a molecular mechanism that activates PKC (130,185,227). However, it is possible that the DAG may induce GIVA PLA_2 signaling through other molecular targets, as the direct PKC activator PMA has been shown to elicit AA release in a number of studies whereas DAG did not in our the current manuscript.

Additionally, since PAP-1 inhibition reduced AA release, exogenous DAG was added to PAP-1 inhibited cells to discern whether this would restore AA release. Addition of exogenous DAG restored AA release from cells pretreated with PAP-1

inhibitors prior stimulation to levels released from uninhibited cells, suggesting that the exogenous DAG compensated for the inhibition of PAP-1.

After having recognized that PAP-1 participates in the activation of GIVA PLA_2, we wanted to explore whether PAP-1 regulates GIVA PLA_2 specifically or whether it may apply to a multitude of phospholipase A_2 proteins. To address this question, we made use of the fact that GIVA PLA_2 has a substrate preference for AA at the *sn*-2 position of membrane phospholipids (179). To address this question, we compared the effect of propranolol on AA and OA metabolism, since OA is believed to be released via a $sPLA_2$ enzyme. Propranolol dramatically reduced AA levels in response to Kdo_2-Lipid A, but did not significantly decrease OA release. To ensure that propranolol was targeting PAP-1, we quantitated TAG formation following Kdo_2-Lipid A stimulation in the presence and absence of propranolol to establish whether TAG formation had been blocked. PAP-1 has been described to be the next to the penultimate enzyme in TAG synthesis, and therefore the quantitation of this metabolite is an excellent indicator of the efficacy of PAP-1 chemical inhibition (82,159). We observed that TAG containing [^{3}H] labeled AA or OA increased in response to Kdo_2-Lipid A, and that this increase was blocked when the cells were preincubated with propranolol (Figure III.13). Taken as a whole, these results suggest that PAP-1 inhibition during Kdo_2-Lipid A stimulation results in decreased TAG formation and loss of GIVA PLA_2 activation.

In this manuscript, we present data that the activation of GIVA PLA_2 by Kdo_2-Lipid A requires the action of PAP-1. One possible mechanism of PAP-1 activation of GIVA PLA_2, is that DAG generated via PA hydrolysis would induce the activation and

translocation of PKC. The membrane-bound active PKC would then activate GIVA PLA_2 by phosphorylation. Three lines of evidence support such a theory.

First, it is known that GIVA PLA_2 undergoes phosphorylation and activation by PKC through a number of agonist-induced signaling pathways, including TLR-4 via LPS (180-183). Gene deletion or chemical inhibition of PKC in these studies resulted in loss of agonist-induced GIVA PLA_2 phosphorylation with reduced arachidonic acid release. Direct activation of PKC by the agonist PMA has been shown to result in release of arachidonic acid and GIVA PLA_2 activation in a number of macrophage cell lines, including RAW 264.7 and U937 (130,184,185).

Second, all three proteins have been observed to co-localize on the nuclear membrane surface. Lipin-1 protein is known to possess a nuclear localization sequence and the protein expression is concentrated to the nuclear membrane region of HEK293 and 3T3-L1 cells (186,187). PKC has been shown to translocate to the nuclear membrane with rising membrane levels of DAG (188,189). Likewise, GIVA PLA_2 associates with the nuclear membrane after phosphorylation (190).

Third, PAP-1 was found to be associated with PKCε and epidermal growth factor (EGF) receptor in EGF receptor immunoprecipitates. Following treatment with EGF and a second immunoprecipitation with an anti-PKCε antibody, PAP-1 was found to be associated with PKCε (191). GIVA PLA_2 is known to undergo phosphorylation and activation with subsequent AA release by way of an oxidative stress EGF receptor mediated pathway (192).

Recently, data has been published that describes the Lipin-1-dependent activation of peroxisome proliferator-activator receptors (PPAR) α and γ (163,193). PPARs are

ligand- dependent transcription factors, which play roles in regulation of lipid metabolism and inflammation (194). PPARs are known to bind and undergo ligand-activation by fatty acids, preferentially, by polyunsaturated fatty acids, such as AA as well as eicosanoids, such as 15-deoxy-Δ(12,14)-prostaglandin J_2 (195,196). Furthermore, GIVA PLA_2 has been implicated in the activation of both PPAR α and γ. Inhibition and gene deletion of GIVA PLA_2 led to the abrogation of PPAR activation, whereas GIVA PLA_2 overexpression resulted in enhanced activation (197-199). The data presented in this paper helps to clarify the aforementioned results. A model suggesting the PAP-1/Lipin-1 dependent regulation of agonist-induced arachidonic acid release by GIVA PLA_2 explains the molecular mechanism of Lipin-1 regulation of PPAR function.

CHAPTER IV

The Regulation of Cellular GIVA PLA_2 Activity by PIP_2 and C-1-P

A. Background

Macrophages release a host of arachidonic acid-derived metabolites, referred to as eicosanoids, in response to inflammatory stimuli. Two putative enzymes in the synthesis of eicosanoids are Group IV Phospholipase A_2 (GIVA PLA_2) and cyclooxygenase 2 (COX-2). GIVA PLA_2 is the enzyme responsible for arachidonic acid (AA) release from the *sn*-2 position of membrane phospholipids in Toll-like receptor (TLR) 4-activated macrophages, while COX-2 further metabolizes AA into prostaglandin H_2 (127,149-151). COX-2 is not expressed at basal conditions in macrophages, although it is significantly upregulated in inflammatory-activated macrophages. Unlike COX-2, GIVA PLA_2 is constitutively expressed in most cell types, although has been shown to be upregulated in response to agonist stimulation in a few cellular models (200-203). Several biochemical processes are believed to play a role in the cellular regulation of GIVA PLA_2, including intracellular Ca^{+2}, phosphorylation and lipid-protein interactions.

The first type of cellular GIVA PLA_2 regulatory mechanism is achieved through changes in cytosolic Ca^{+2} levels. Activation occurs through GIVA PLA_2 translocation from the cytosol to the membrane surface in response to increased cytosolic Ca^{+2} (51,62). GIVA PLA_2 does not require Ca^{+2} for catalysis, but rather for membrane association. The C2 domain interacts with two Ca^{+2} ions, resulting in translocation of the enzyme to the surface of membrane phospholipids, where it is catalytically active (52,203,204). In a number of studies conducted with Ca^{+2} mobilizing agents, GIVA PLA_2 was observed to translocate to the perinuclear region of the cell, including the nuclear envelope,

endoplasmic reticulum and Golgi apparatus (62,63,205). Induction of physiologically relevant Ca^{+2} levels resulted in the preferential translocation of GIVA PLA_2 to the surface of the Golgi apparatus; as levels decreased, GIVA PLA_2 dissociated (205). Additionally, the C2 domain alone was translocated in a manner resembling the wild-type protein, indicating that the C2 domain is sufficient to promote the cellular translocation of GIVA PLA_2 in response to increased Ca^{+2} (205).

In the second potential cellular regulatory mechanism, GIVA PLA_2 function would be activated through kinase-mediated phosphorylation. The catalytic domain of GIVA PLA_2 contains several specific serine residues, including serine 505, 515 and 727, which are known to be MAPK targeted phosphorylation sites, resulting in enhanced catalysis (65-68). The manner in which phosphorylation regulates cellular GIVA PLA_2 function is not completely understood, as fully dephosphorylated GIVA PLA_2 protein still exhibits maximal catalytic activity (69). One possible explanation is that GIVA PLA_2 phosphorylation may play a role at low Ca^{+2} levels by inducing a conformational change, resulting in increased interaction between the catalytic domain and membrane phospholipids; surface plasmon resonance data has shown that phosphorylation enhances the phospholipid binding affinity at low physiological Ca^{+2} concentrations (206). Very few cellular studies have been conducted to identify the cellular implications of GIVA PLA_2 phosphorylation. In cellular studies conducted with GIVA PLA_2 serine 505 mutants, the mutants translocated when the cells were treated with Ca^{+2} ionophore in the same manner as the wild-type protein (63,207). In a third study conducted with β–adrenergic stimulated cardiomyocytes, which do not exhibit the release of cytosolic Ca^{+2}, to achieve cellular GIVA PLA_2 activation, the participation of MAPK was required (208).

Collectively, these results are consistent with the notion that phosphorylation may activate GIVA PLA_2 at low concentrations of cellular Ca^{+2}.

In the third potential cellular mechanism, enhanced cellular GIVA PLA_2 activity would be achieved through the interaction of GIVA PLA_2 protein with phosphatidylinositol 4,5-bisphosphate (PIP_2). During the past ten years, substantial cellular and *in vitro* data has been published that indicates that PIP_2 may play roles such as an enhancer of GIVA PLA_2 activity. The introduction of PIP_2 into lipid vesicles has been reported to enhance *in vitro* GIVA PLA_2 activity by a factor of 20-fold (70). Cellular studies using astrocytoma and squamous carcinoma cell lines have demonstrated that elevated endogenous PIP_2 concentrations are localized to the perinuclear region, where it is believed that GIVA PLA_2 is most active (71). Colocalization experiments performed in Madin-Darby canine kidney cells have shown that the GIVA PLA_2 C2 domain localizes to PIP_2 rich membrane surfaces within the Golgi and ER (209). Later studies supported the notion that PIP_2 promotes the translocation of GIVA PLA_2 to the surface of perinuclear membranes in a Ca^{+2} independent manner (210). It is possible that endogenous PIP_2 functions as a cellular counterpart to that of Ca^{+2} in regards to GIVA PLA_2 activation, by enhancing GIVA PLA_2 catalysis at low levels of Ca^{+2} (168). Further study has shown that increased cellular PIP_2 levels result in enhanced AA release, while reduced PIP_2 levels result in decreased AA release (72).

In the final potential cellular mechanism, enhanced cellular GIVA PLA_2 activity would be achieved through the interaction of GIVA PLA_2 protein with ceramide 1-phosphate (C-1-P). Experiments in which exogenous C-1-P was added to cells demonstrated enhanced AA release into the media (73). Il-1β and Ca^{+2} ionophore induced

an increase of C-1-P levels and AA release concomitantly, while RNA interference of CERK abolished this effect (73). The addition of sphingomyelinase D to cells, which produces C-1-P, increased AA release, whereas the addition of sphingolmyelinase C, which produces ceramide, did not induce AA release (73). Clearly, C-1-P enhances GIVA PLA_2 mediated AA release, since the downregulation of GIVA PLA_2 resulted in the abrogation of C-1-P induced AA release from A549 cells (74). Earlier experiments had shown that the addition of exogenous ceramide to cells resulted in enhanced AA release (176,211). A likely explanation for this observation is that the introduced ceramide was converted into C-1-P by endogenous CERK, and that the enhanced AA release is to be attributed to the increased C-1-P levels.

It should be noted that PIP_2 and C-1-P are believed to interact with GIVA PLA_2 through very different molecular mechanisms. The GIVA PLA_2 catalytic domain is known to contain a cluster of basic residues that serve as the PIP_2 binding site (168,212). It has been suggested that PIP_2 may induce a conformational change in the catalytic domain of GIVA PLA_2 that leads to optimal interaction with the substrate (212). In contrast to PIP_2, evidence indicates that C-1-P interacts with the C2 domain of GIVA PLA_2, which induces protein translocation to membrane surfaces where subsequent hydrolysis ensues (213). Since C-1-P seems to function as an allosteric activator of GIVA PLA_2, it is thought that C-1-P probably recruits GIVA PLA_2 to the appropriate cellular lipid membrane for catalysis (214). An additional distinction is that the interaction of PIP_2 and GIVA PLA_2 does not require Ca^{+2}, whereas data shows that C-1-P requires submicromolar levels of Ca^{+2} to be present (214).

In the current study, the regulation of GIVA PLA_2 activity by the lipid mediators PIP_2 and C-1-P was explored in TLR-4 activated macrophages. Specifically, endogenous levels of PIP_2 and C-1-P were measured from RAW 264.7 cells treated with the TLR-4 specific agonist, Kdo_2-Lipid A, while simultaneously quantitating AA release as an indicator of GIVA PLA_2 activity. PIP_2 levels rose within moments after stimulation of the cells, suggesting that PIP_2 may participate in the initial activation of GIVA PLA_2. C-1-P levels were not initially elevated. However, levels were increased from two hours after stimulation, suggesting that C-1-P may help sustain prolonged GIVA PLA_2 activity following the initial activation. To generate as complete a profile as possible, ceramide, phosphatidylinositol (PI) and free inositol were also measured in conjunction to PIP_2 and C-1-P. Free inositol includes a number of inositol-based metabolites, including inositol 1,4,5-trisphosphate (Ins(1,4,5)P3), Ins(1,3,4)P3 and Ins(1,3,4,5)P4 (215). Surprisingly, free inositol levels increased steadily over the duration of the time course. A likely explanation is that the elevated free inositol levels are indicative of increased IP_3 levels, as IP_3 has been described to be the chief free inositol specie released in TLR activated macrophages (178). Since elevated IP_3 is indicative of increased cytosolic Ca^{+2}, and Ca^{+2} is a requirement of ceramide kinase (CERK) mediated phosphorylation, C-1-P mediated GIVA PLA_2 membrane binding and Ca^{+2}-dependent GIVA PLA_2 membrane binding, cells were treated with Ca^{+2} ionophore and C-1-P and AA release levels were subsequently measured. Since both C-1-P and AA release levels increased concomitantly when cells were treated with the Ca^{+2} agonist, it was necessary to ensure that this was relevant to the Kdo_2-Lipid A stimulated response also. To accomplish this, cells were preincubated with PI-PLC inhibitors prior to treatment with Kdo_2-Lipid A. The PI-PLC

chemical inhibitors markedly reduced C-1-P and AA release levels, supporting the notion that IP_3 plays a role in cellular GIVA PLA_2 activity as well. Collectively, the data suggests that PIP_2 and C-1-P may regulate GIVA PLA_2 in TLR-4 activated macrophages.

B. Materials and methods

1. Materials

Murine RAW 264.7 macrophages were purchased from American Type Culture Collection (Manassas, VA). DMEM cell culture medium was obtained from Gibco (Grand Island, NY). Fetal bovine serum was from VWR International (Bristol, CT). Kdo_2-Lipid A, 1-palmitoyl-2-arachidonoyl phosphatidylcholine was from Avanti Polar Lipids (Alabaster, AL). U-71322 and neomycin sulfate were from Biomol (Plymouth Meeting, PA). myo-[1,2-^{3}H(N)] inositol and L-[^{14}C(U)] serine were obtained from NEN Life Science Products (Boston, MA). The specific $cPLA_2$ inhibitor, pyrrophenone, was kindly provided by Dr. Kohji Hanasaki (Shionogi Research Laboratories of Shionogi & Co., Ltd). 20 cm x 20 cm x 250 μm K6 Silica gel thin layer chromatography plates were from Whatman (Clifton, New Jersey).

2. Cell Culture and Stimulation Protocol

RAW 264.7 cells were maintained at 37 °C in a humidified atmosphere at 90% air and 10% CO_2 DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, 100 μg/ml streptomycin, and non-essential amino acids. Cells were plated such that they were at a confluency of $2x10^6$ cells/6 well tissue culture plate and $1x10^6$/12 well tissue culture plate at the time of experimentation. Following plating, they were allowed to adhere overnight, and then used for experiments the following day, with

the exception being of cells that were radiolabelled, see below for details. The cells were incubated at 37 °C in a humidified atmosphere of 95 % air and 5% CO_2. When inhibitors were used, they were added to the medium 30 min before Kdo_2 Lipid A was added. Cell viability was assessed visually by the Trypan Blue Dye exclusion assay (Gibco, Grand Island, NY) and through the usage of the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI).

3. Sample Preparation

The media was analyzed for extracellular eicosanoid release. After stimulation, the entire 1.8 ml of media was removed and each sample was supplemented with 100 µl of internal standards (100 ng/µl, EtOH) and 100 µl of EtOH to bring the total concentration of EtOH to 10% by volume. Samples were centrifuged for 5 minutes at 3000 rpm to remove cellular debris, after which the supernatants were decanted into the solid phase extraction columns. When quantitating intracellular eicosanoids, cells were scraped into 500 µl MeOH, after which 1000 µl PBS and 100 µl of internal standards was added. Finally this mixture was added onto the solid phase extraction column. All eicosanoid extractions were conducted using Strata-X SPE columns (Phenomenex). The columns were washed with 2 ml MeOH and then 2 ml H_2O prior to the samples being applied. After applying the sample, the columns were washed with 10% MeOH to remove non-adherent debris, and eicosanoids were then eluted off the column with 1 ml MeOH. The eluant was dried under vacuum and redissolved in 100 µl of solvent A [water-acetonitrile-formic acid (63:37:0.02; v/v/v)] for LC/MS analysis.

4. Cell Quantitation

Eicosanoid levels were normalized to cell number using DNA quantitation. After the extracellular media was removed, the cells were scraped in 500 µl PBS and stored at 4ºC for DNA quantitation using the Broad Range DNA Quant-Kit (Invitrogen).

5. HPLC and Mass Spectrometry

The analysis of eicosanoids was performed by LC/MS/MS. Eicosanoids were separated by reverse-phase HPLC on a C18 column (2.1 mm x 150 mm, Grace-Vydac) at a flow rate of 300 µl/min at 25ºC. The column was equilibrated in Solvent A [water-acetonitrile-formic acid (63:37:0.02; v/v/v)], and samples were injected using a 50 µl injection loop and eluted with a linear gradient from 0%-20% solvent B [acetonitrile-isopropyl alcohol (50:50; v/v)] between 0 to 6 min; solvent B was increased to 55% from 6 to 6.5 min and held until 10 min. Solvent B was increased to 100% from 10 to 12 min and held until 13 min; solvent B was dropped to 0% by 13.5 min and held until 16 min. Eicosanoids were analyzed using a tandem quadrupole mass spectrometer (ABI 4000 Q Trap®, Applied Biosystems) via multiple-reaction monitoring (MRM) in negative-ion mode. The electrospray voltage was -4.5 kV, the turbo ion spray source temperature was 525ºC. Collisional activation of eicosanoid precursor ions used nitrogen as a collision gas. Quantitative eicosanoid determination was performed by stable isotope dilution curve method, as previously described (216). Results are reported as ng of eicosanoid per million cells (mean ± standard deviation).

6. Quantification of Lipids from RAW 264.7 Macrophages

When quantifying the endogenous metabolism of radiolabelled fatty acid or triacylglycerol, cells were incubated in 10% FCS RPMI medium supplemented with myo-[1,2-^{3}H(N)] inositol or L-[^{14}C(U)] serine for 48 hours and then concluded. Lipids

were extracted according to the method of Bligh and Dyer (126). The extraction solution was placed into fresh microcentrifuge tubes and dried in a vacuum-centrifuge. The resulting pellet was reconstituted in 40 uL of the (70:30) chloroform/methanol system. The lipids were then separated by thin layer chromatography. When separating lipids derived from cells that were labeled with myo-[1,2-^{3}H(N)] inositol, the lipids were separated using a mobile phase composed of chloroform/acetone/methanol/acetic acid/water (60:30:26:24:14). The TLC plates used for inositol separation were precoated with a 1% potassium oxalate solution (72). When separating lipids form cells that had been labeled with L-[^{14}C(U)] serine, a mobile phase composed of chloroform/benzene/ethanol (80:40:75) was used in an initial separation. The TLC plate was then dried, and developed with a second mobile phase composed of chloroform/methanol/28%ammonium hydroxide (65:25:5) (176). The TLC plates were then developed in an iodination chamber, and the lipids were scraped and subsequently quantified by liquid scintillation counting.

C. RESULTS

1. Cellular AA and PGD_2 release increases in response to Kdo_2-Lipid A stimulation

Initially, our goal was to identify metabolic markers of cellular GIVA PLA_2 activity in RAW 264.7 macrophages for the purpose of studying the mechanism of GIVA PLA_2 activation *in vivo*. Two metabolic markers of GIVA PLA_2 activity were identified and utilized to profile GIVA PLA_2 cellular activation, the release of AA and PGD_2 into the media. To this end, cells were stimulated with Kdo_2-Lipid A, and the media was collected at the indicated time points for subsequent analysis by LC/MS/MS (Figure

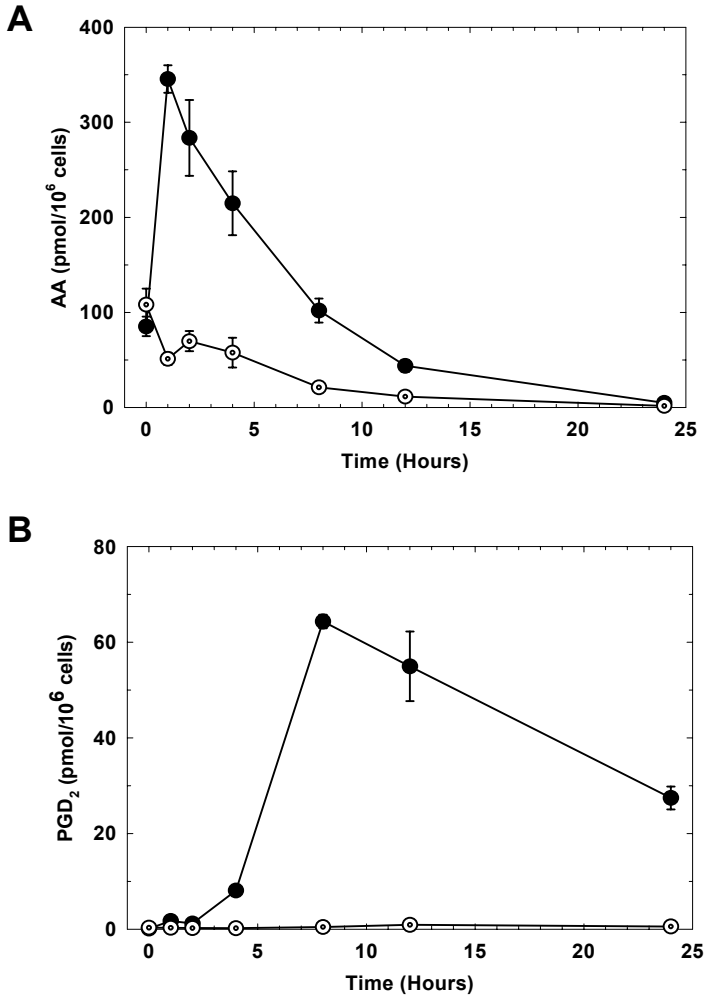


Figure IV.1. Kdo_2-Lipid A induces the release of arachidonic acid and PGD_2 from RAW 264.7 macrophages.

Time-course of A) arachidonic acid and B) PGD_2 release from cells treated with (filled circles) and without (empty circles) 100 ng/mL Kdo_2-Lipid A prior to collection of supernatants at the indicated time points. Eicosanoids were extracted and quantitated from the supernatants as described in the Materials and Methods section. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

IV.1.A and B). AA release increased markedly within the first hour of stimulation, reaching maximal levels of 350 pmol/million cells at 1 hour before waning at latter time points. PGD_2 release did not increase significantly until 4 hours after stimulation, and reached maximal levels at 8 hours. To more accurately define the initial AA release, and therefore identify the onset of GIVA PLA_2 activity, AA release was quantitated over the first hour of stimulation (Figure IV.2). From this time-course it was concluded that AA release begins at approximately 7 minutes after the cells were stimulated and increased continuously over the first hour.

2. GIVA PLA_2 mediates AA metabolism in RAW 264.7 macrophages

To correctly use AA release as an indicator of cellular GIVA PLA_2 activity, it is imperative to ensure that the AA release can be only attributed to the action of this enzyme. To accomplish this, AA release was quantitated from cells preincubated with a specific GIVA PLA_2 inhibitor, pyrrophenone, prior to stimulation. Pyrrophenone reduced AA release into the supernatant at 1 hour after stimulation to basal levels (Figure IV.3). This result indicates that Kdo_2-Lipid A stimulated AA release is mediated through GIVA PLA_2 in this model, and therefore that AA release can be used as a direct indicator of cellular GIVA PLA_2 activity.

3. GIVA PLA_2 mediates continuous AA release in RAW 264.7 macrophages

Significant PGD_2 release was not detected in the supernatant until 4 hours after stimulation, as seen in figure IV.1.B. Since AA release began within minutes of stimulation, the delay in PGD_2 release could be due to the initial absence of COX-2, which is upregulated in TLR-4 stimulated macrophages. Since there are 4 hours between the activation of GIVA PLA_2 and the initial release of eicosanoids, we postulated that

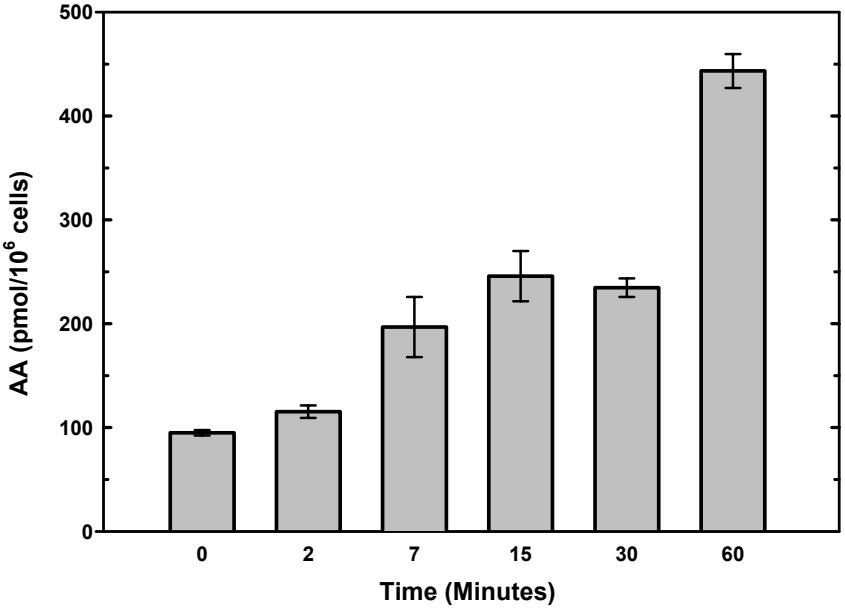


Figure IV.2. Kdo_2-Lipid A stimulated arachidonic acid release from RAW 264.7 macrophages.

Time-course of arachidonic acid release from RAW 264.7 macrophages treated with 100 ng/mL Kdo_2-Lipid A prior to collection of supernatants at the indicated time points. Arachidonic acid was extracted and quantitated from the supernatants as described in the Materials and Methods section. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

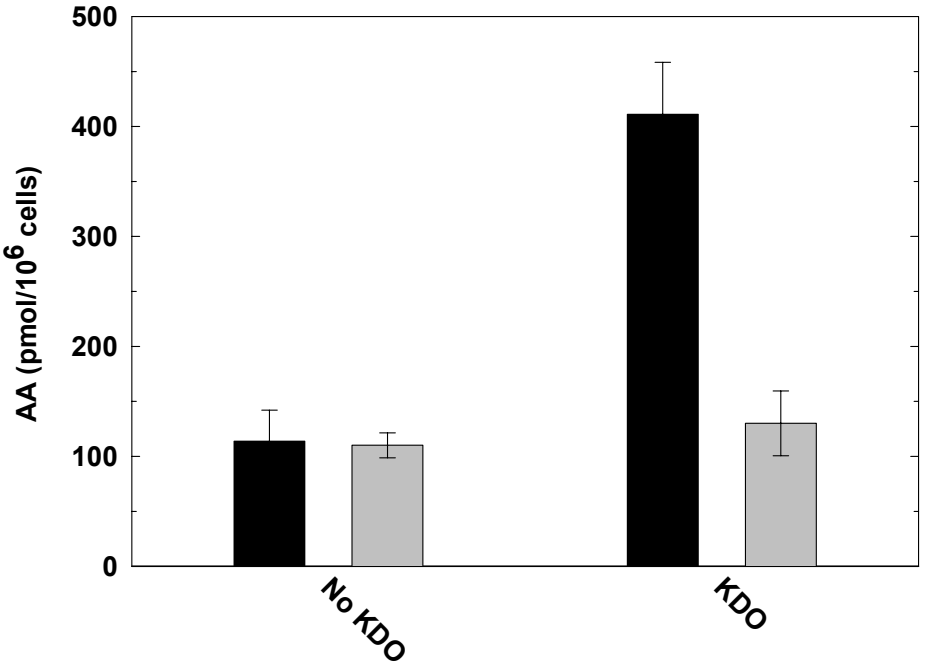


Figure IV.3. Pyrrophenone inhibits Kdo_2-Lipid A stimulated arachidonic acid release from RAW 264.7 macrophages.

Arachidonic acid release was quantitated from cells preincubated in the absence (black bars) and presence (grey bars) of 1 μM pyrrophenone and then treated with and without 100 ng/mL Kdo_2-Lipid A (as indicated) for 1 hour prior. Arachidonic acid was extracted and quantitated from the supernatants as described in the Materials and Methods section. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

GIVA PLA_2 must remain active from the onset of its activation until COX-2 is available to participate in eicosanoid synthesis. To discern whether GIVA PLA_2 remains active during eicosanoid synthesis, pyrrophenone was added at the indicated times after Kdo_2-Lipid A stimulation, and PGD_2 release was quantitated at 8 hours. Pyrrophenone reduced PGD_2 release to baseline values when it was administered to the cells at 0, 0.5, 1 and 2 hours after stimulation (Figure IV.4). AA released into the supernatant within the first two hours does not contribute to eicosanoid synthesis since pyrrophenone added two hours after stimulation still inhibited PGD_2 release to baseline values. At 4 hours after stimulation, the first significant increase of PGD_2 release was observed, relative to that of basal levels. The data indicate that AA release is a continuous process, and that AA released at one time period is available for further metabolism by COX-2 only within that respective period. Since cellular AA release is entirely attributed to GIVA PLA_2, the enzyme must remain active to provide AA to COX-2 during eicosanoid synthesis.

4. PIP_2 levels increase during the first hour of Kdo_2-Lipid A stimulation

We hypothesized that changes in cellular PIP_2 and C-1-P levels may be responsible for the increased GIVA PLA_2 activity in activated macrophages. Increased Ca^{+2} is not likely to be the initial activator, as published data has shown that Ca^{+2} levels do not increase within the first 6 minutes of TLR-4 activation in macrophages (178). It has been suggested that phosphorylation is not likely to be the initial activator in the RAW 264.7 cell model, as GIVA PLA_2 is already phosphorylated at serine 505 under basal conditions in this cell line (171). However, evidence suggests that phosphorylation may occur at other serine residues on GIVA PLA_2.

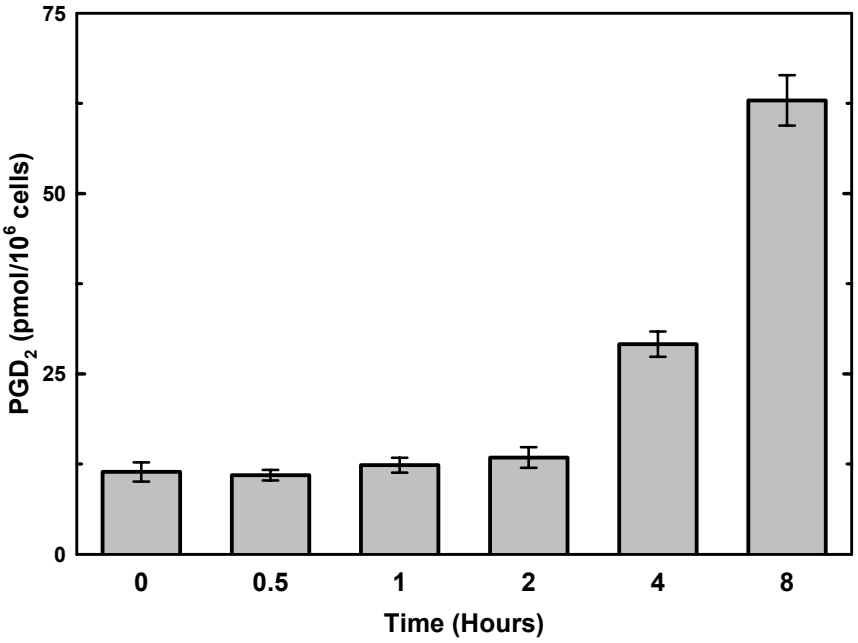


Figure IV.4. Pyrrophenone inhibits Kdo_2-Lipid A stimulated PGD_2 release from RAW 264.7 macrophages.

PGD_2 release was quantitated from RAW 264.7 macrophages incubated in the presence of 100 ng/mL Kdo_2-Lipid A (added at time = 0 minutes) and then treated with 1 μM pyrrophenone at the indicated time points. Eicosanoids were extracted and quantitated from the supernatants as described in the Materials and Methods section. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

To assess whether PIP_2 or C-1-P play a role in the initial activation of GIVA PLA_2, cellular PIP_2 and C-1-P levels were profiled during the first hour of Kdo_2-Lipid A stimulation. Furthermore, related lipid metabolites of PIP_2 and C-1-P, including PI, free inositol and ceramide were also quantitated. The profiles of PI and PIP_2 were similar, as levels of both metabolites were elevated from 7 to 30 minutes, before decreasing to basal levels at 60 minutes (Figure IV.5.A and B). Perhaps the most surprising result was that free inositol levels rose steadily during the 60 minute time-course (Figure IV.5.C). The free inositol measured in this figure included all liberated inositol species, including inositol 1,4,5-trisphosphate (IP_3). C-1-P levels did not increase during the first hour of stimulation. On the contrary, C-1-P levels decreased within the first 7 minutes of stimulation and then returned to basal levels at 60 minutes (Figure IV.6.A). Ceramide levels did not change during the first hour of stimulation (Figure IV.6.B). In comparison to AA release and PIP_2 profiles, it is apparent that the increased PIP_2 levels at 7 minutes correlate with the onset of AA release into the supernatant. Furthermore, both AA and free inositol increased by similar magnitudes, as free inositol levels were increased by 2-5 times that of basal levels while AA release increased by approximately four times basal levels.

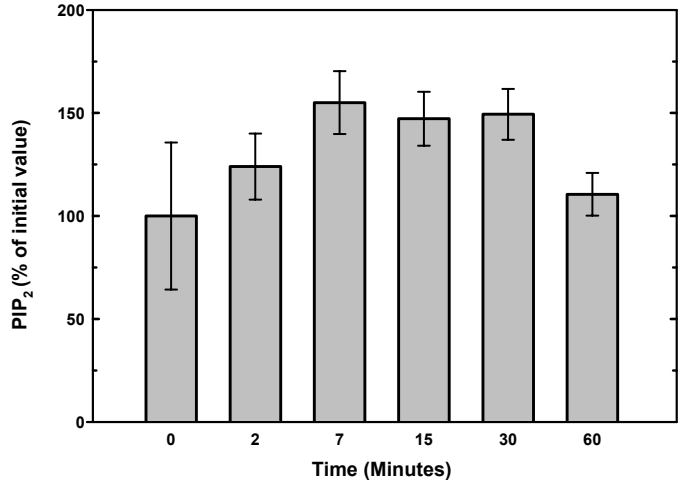
5. C-1-P levels are elevated from 2-8 hours after Kdo_2-Lipid A treatment

Eicosanoid release began at approximately 4 hours after Kdo_2-Lipid A stimulation. Therefore, we investigated the 2-8 hour time period after stimulation for elevated PIP_2 and C-1-P levels to evaluate whether either lipid may participate in the maintenance of GIVA PLA_2 activity during eicosanoid synthesis. PI and PIP_2 levels reach minimum values at 2-4 hours. However, after reaching minimum levels, PI and PIP_2

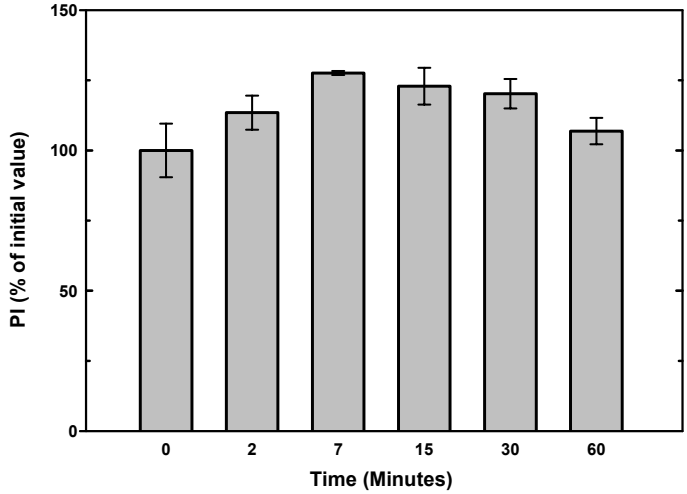
Figure IV.5. Kdo_2-Lipid A stimulated production of phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol and free inositol in RAW 264.7 macrophages.

A) PIP_2, B) PI and C) free inositol formation were measured at the indicated time points from myo-[1,2-^{3}H(N)] inositol labeled RAW 264.7 macrophages following stimulation with 100 ng/mL Kdo_2-Lipid A. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

A


200
150
100
50
0
PIP2 (% of initial value)
0
2
7
15
30
60
Time (Minutes)

B


150
100
50
0
PI (% of initial value)
0
2
7
15
30
60
Time (Minutes)

C

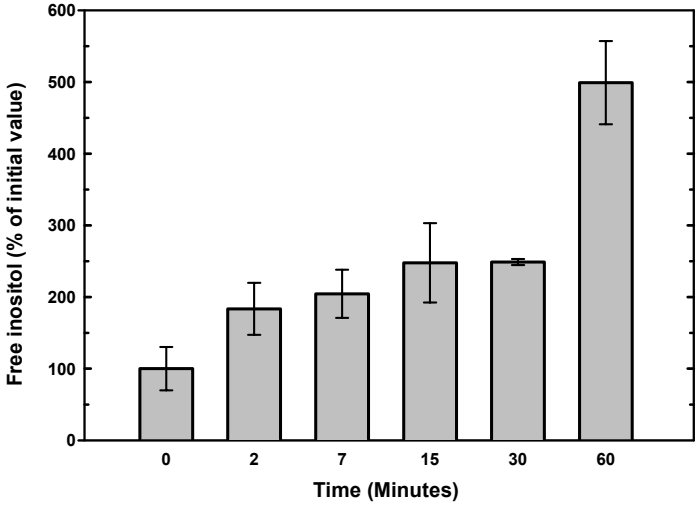
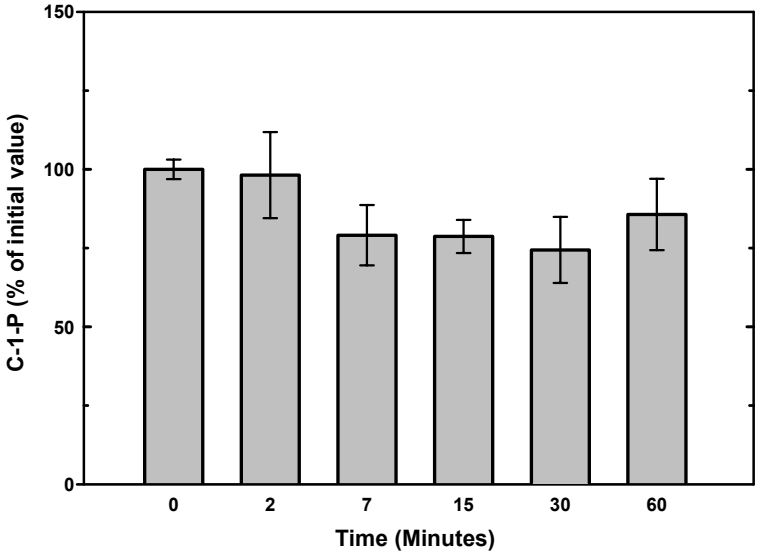

600
500
400
300
200
100
0
Free inositol (% of initial value)
0
2
7
15
30
60
Time (Minutes)

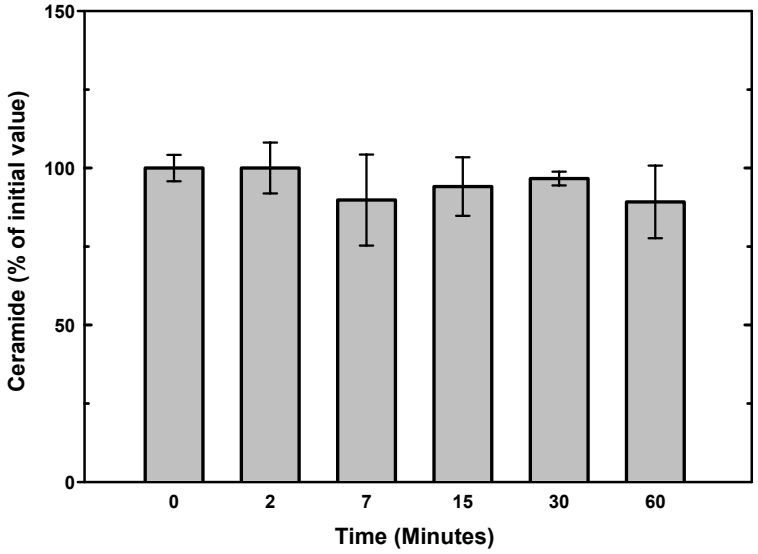
Figure IV.6. Kdo_2-Lipid A stimulated ceramide 1-phosphate and ceramide production in RAW 264.7 macrophages.

A) C-1-P and B) ceramide formation were measured at the indicated time points from L-[^{14}C(U)] serine labeled RAW 264.7 macrophages following stimulation with 100 ng/mL Kdo_2-Lipid A. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

A


150
100
50
0
C-1-P (% of initial value)
0
2
7
15
30
60
Time (Minutes)

B


150
100
50
0
Ceramide (% of initial value)
0
2
7
15
30
60
Time (Minutes)

levels rose by 50% from 6-8 hours (Figure IV.7.A and B). Free inositol levels, which rose throughout the first hour of stimulation, continued to rise through 8 hours, reaching maximal levels that were 5-10 times greater than that of basal levels by 4-8 (Figure IV.7.C).

C-1-P levels had decreased during the first hour of stimulation. However, at 2 hours they increased by approximately 50% and remained elevated throughout the remainder of the time-course (Figure IV.8.A). The ceramide profile resembled that of C-1-P, as levels were elevated from 2-8 hours after stimulation (Figure IV.8.B). The data indicates that C-1-P and ceramide levels are elevated during the time that cells are actively synthesizing eicosanoids. Furthermore, it is noteworthy that C-1-P levels rose when free inositol levels are maximal.

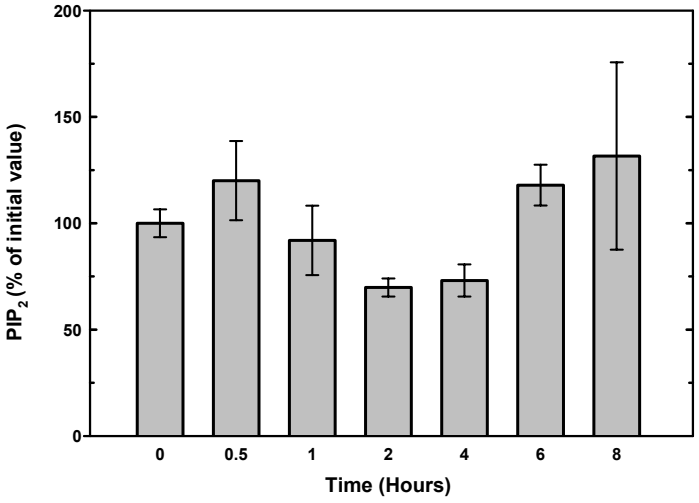
6. PI-PLC inhibition blocks Kdo_2-Lipid A C-1-P and AA release

Since ionomycin treatment had increased C-1-P and AA levels, we investigated whether blocking Kdo_2-Lipid A stimulated free inositol release would affect C-1-P and AA release levels. Two PI-PLC chemical inhibitors, U-71322 and neomycin sulfate, were utilized to conduct this line of investigation. All metabolite quantitation was done at 4 hours after Kdo_2-Lipid A stimulation, when free inositol and C-1-P levels peaked and eicosanoid production began. Both inhibitors reduced Kdo_2-Lipid A stimulated free inositol levels by 50%, confirming that PI-PLC function was impaired (Figure IV.9.A). Additionally, PI-PLC inhibition resulted in the reduction of cellular C-1-P levels beyond basal levels. Cellular C-1-P levels were inhibited to 50% of the basal levels (Figure IV.9.B). Finally, preincubation of the cells with the PI-PLC inhibitors reduced AA release by 50-75% (Figure IV.9.C).

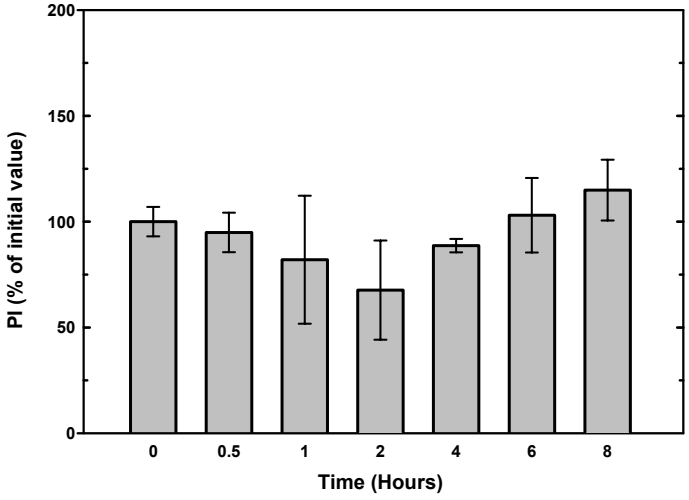
Figure IV.7. Kdo_2-Lipid A stimulated production of phosphatidylinositol 4,5-bisphosphate, phosphatidylinositol and free inositol in RAW 264.7 macrophages.

A) PIP_2, B) PI and C) free inositol formation were measured at the indicated time points from myo-[1,2-^{3}H(N)] inositol labeled RAW 264.7 macrophages following stimulation with 100 ng/mL Kdo_2-Lipid A. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

A


200
150
100
50
0
PIP2 (% of initial value)
0
0.5
1
2
4
6
8
Time (Hours)

B


200
150
100
50
0
PI (% of initial value)
0
0.5
1
2
4
6
8
Time (Hours)

C

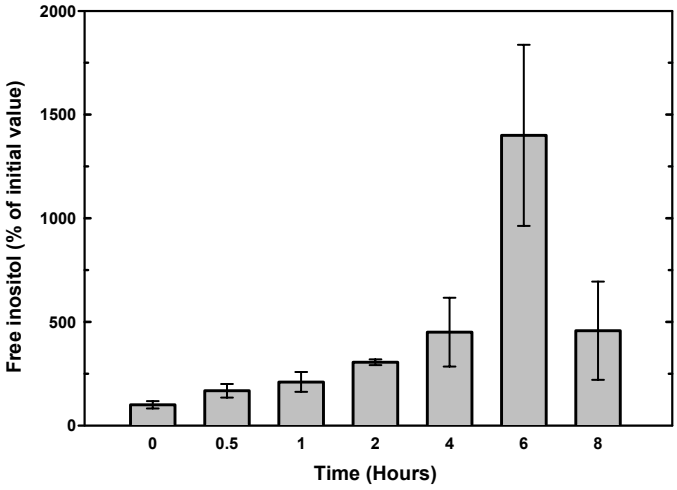
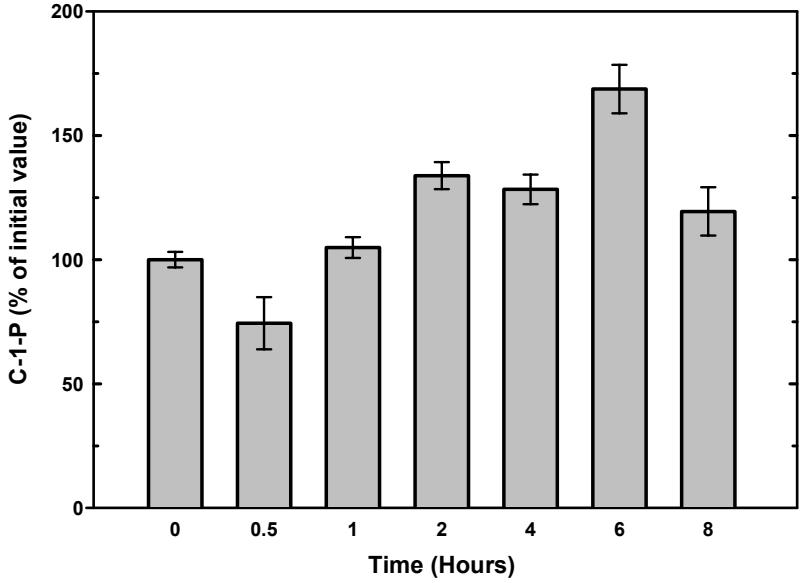

2000
1500
1000
500
0
Free inositol (% of initial value)
0
0.5
1
2
4
6
8
Time (Hours)

Figure IV.8. Kdo_2-Lipid A stimulated ceramide 1-phosphate and ceramide production in RAW 264.7 macrophages.

A) C-1-P and B) ceramide formation were measured at the indicated time points from L-[^{14}C(U)] serine labeled RAW 264.7 macrophages following stimulation with 100 ng/mL Kdo_2-Lipid A. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

A


C-1-P (% of initial value)
200
150
100
50
0
0
0.5
1
2
4
6
8
Time (Hours)

B

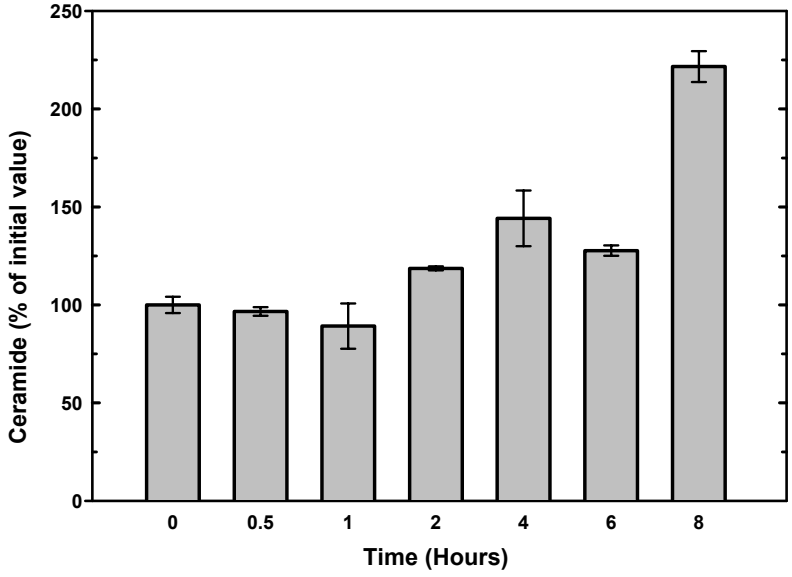
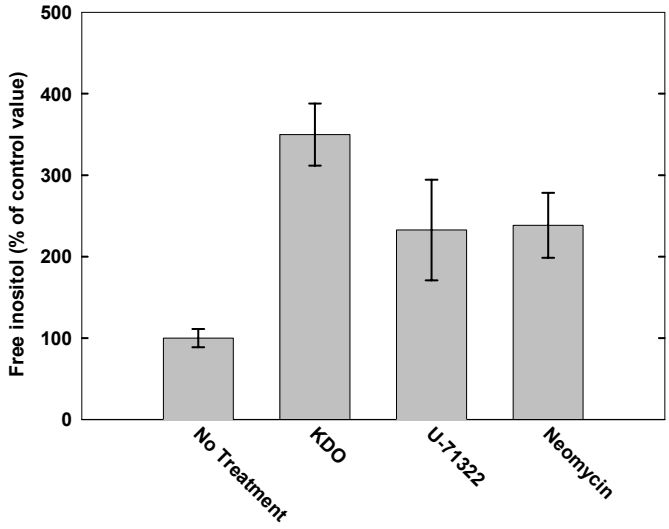

Ceramide (% of initial value)
250
200
150
100
50
0
0
0.5
1
2
4
6
8
Time (Hours)

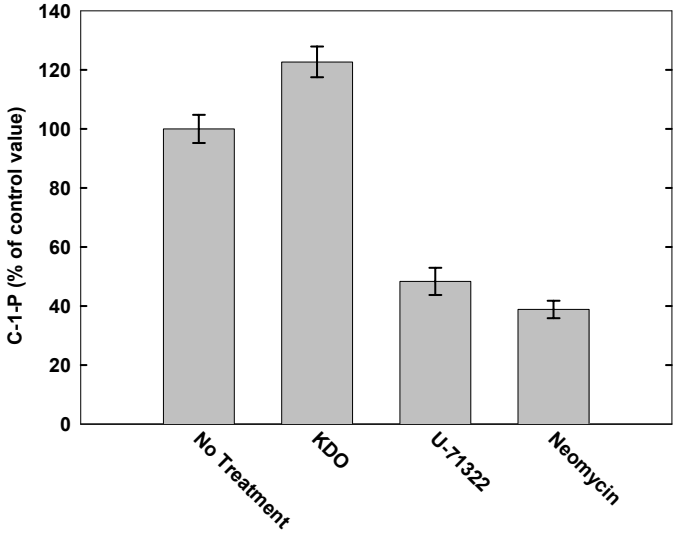
Figure IV.9. Kdo_2-Lipid A stimulated free inositol production in RAW 264.7 macrophages in the presence of PI-PLC inhibitors.

A) Free inositol formation and B) C-1-P were measured from myo-[1,2-^{3}H(N)] inositol and L-[^{14}C(U)] serine labeled RAW 264.7 macrophages after 6 hours of stimulation with 100 ng/mL Kdo_2-Lipid A, respectively. (*C*) Arachidonic acid was extracted and quantitated from the supernatants as described in the materials and methods section. Where indicated, cells were preincubated in the presence of 10 μM U-71322 or 3 mM neomycin sulfate prior to stimulation. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.

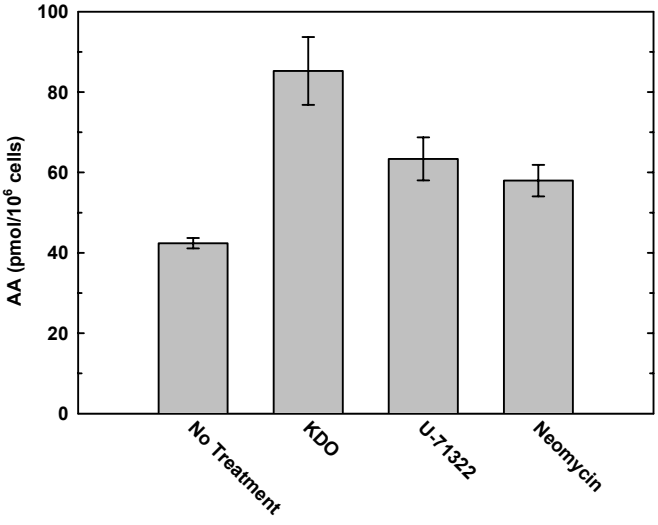
A


Free inositol (% of control value)
500
400
300
200
100
0
No Treatment
KDO
U-71322
Neomycin

B


C-1-P (% of control value)
140
120
100
80
60
40
20
0
No Treatment
KDO
U-71322
Neomycin

C


AA (pmol/10^6 cells)
100
80
60
40
20
0
No Treatment
KDO
U-71322
Neomycin

7. Ionomycin treatment increase C-1-P levels and GIVA PLA_2 dependent AA release

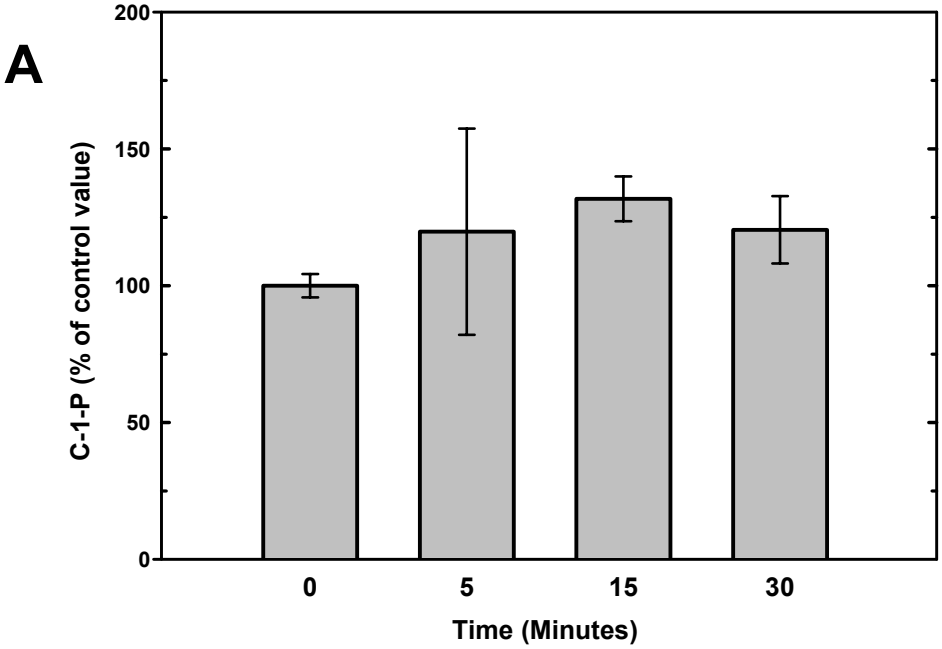
The C-1-P profile levels remained at basal levels during the initial two hours after stimulation of the macrophages. Levels increased at 2-8 hours after stimulation while free inositol levels also continued to rise. TLR-4 stimulation of macrophages is known not to increase cytosolic Ca^{+2} immediately after stimulation, but it is unknown whether an increase may occur at later times (178). Since both CERK phosphorylation and C-1-P mediated GIVA PLA_2 activation require elevated cytosolic Ca^{+2}, we hypothesized that the increased free inositol observed at the later time points of the Kdo_2-Lipid A stimulation may indicate an IP_3-mediated Ca^{+2} increase which activate the aforementioned cellular processes. To test this hypothesis, C-1-P levels and AA release were measured from cells treated with a direct cytosolic Ca^{+2} modulator, ionomycin. Fifteen minutes after treatment of the cells with ionomycin, C-1-P levels rose to a maximal value that was 50% greater than that of control levels (Figure IV.10.A). AA release levels were also increased by 50% relative to basal conditions at the same time (Figure IV.10.B). To ensure that AA release is GIVA PLA_2 mediated, pyrrophenone was added to ionomycin treated cells. The inhibitor reduced AA release to background levels, confirming that ionomycin-induced AA release is GIVA PLA_2 mediated.

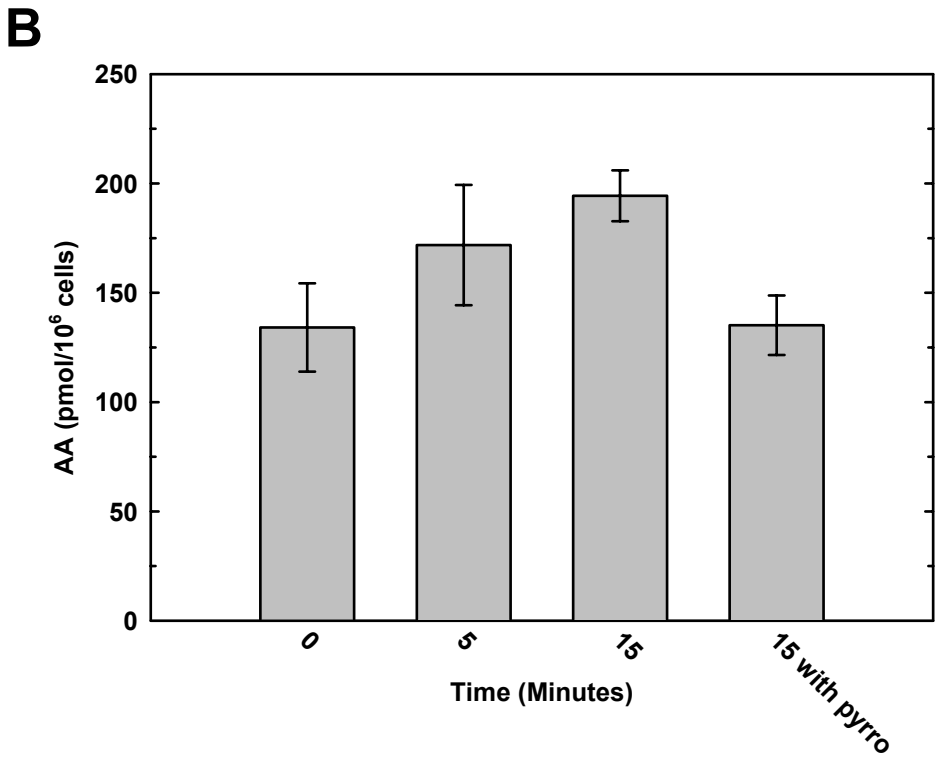
D. DISCUSSION

Macrophages release eicosanoids into the extracellular matrix in response to inflammatory stimuli, such as the activation of the TLR-4 signaling cascade through binding of the ligand Kdo_2-Lipid A. GIVA PLA_2 plays a pivotal role in mediating inflammatory eicosanoid synthesis, by releasing membrane-bound AA for the further

Figure IV.10. Ionomycin stimulated ceramide 1-phosphate and arachidonic acid production in RAW 264.7 macrophages.

A) C-1-P levels were measured at the indicated time points from L-[^{14}C(U)] serine labeled RAW 264.7 macrophages following stimulation with 1 μM ionomycin. B) Arachidonic acid was extracted and quantitated from the supernatants as described in the materials and methods section. A representative experiment of three individual experiments is shown. Data are expressed as mean values ± S.D. of three individual replicates.


A
200
150
100
50
0
C-1-P (% of control value)
0
5
15
30
Time (Minutes)


B
250
200
150
100
50
0
AA (pmol/10^6 cells)
0
5
15
15 with pyrro
Time (Minutes)

metabolism by downstream eicosanoid synthases. GIVA PLA_2 functions by catalyzing the hydrolysis of AA from the *sn*-2 position of membrane phospholipids, releasing free AA from the membrane while also producing membrane lysophospholipid (150,151). Although it is known that GIVA PLA_2 is the phospholipase responsible for mediating AA release, the molecular mechanism resulting in the activation of GIVA PLA_2 remains to be fully understood.

At basal conditions, mammalian GIVA PLA_2 is localized within the cellular cytosol, but undergoes translocation to the perinuclear envelope in response to activation of the TLR-mediated pathway. Past research has shown that cellular GIVA PLA_2 activation is mediated through several potential regulatory mechanisms. In the first regulatory mechanism, the translocation of GIVA PLA_2 from the cytosol to intracellular membranes is achieved through increased cytosolic Ca^{+2} (51,62). Studies using Chinese hamster ovary and rat leukemic cells demonstrated that Ca^{+2} ionophore induced the translocation of GIVA PLA_2 from the cytosol to the perinuclear membrane with increased AA release into the media (63,64). In the second regulatory mechanism, kinase-mediated phosphorylation on specific serine residues of GIVA PLA_2 results in enhanced GIVA PLA_2 catalysis (65-68). The manner in which phosphorylation regulates cellular GIVA PLA_2 function is not completely understood, as fully dephosphorylated GIVA PLA_2 protein exhibits maximal *in vitro* catalytic activity (69). Our recent results show that PAP-1 is necessary for normal TLR-4 initiated cellular GIVA PLA_2 activation (Manuscript in Submission). One possible explanation is that phosphorylation may activate GIVA PLA_2 under conditions in which cytosolic Ca^{+2} concentrations are low

(66). In the final regulatory mechanism, GIVA PLA_2 activity is enhanced through the interaction of GIVA PLA_2 with bioactive lipids, such as PIP_2 and C-1-P.

The purpose of this project was to investigate the participation of cellular PIP_2 or C-1-P in the Kdo_2-Lipid A stimulated activation of GIVA PLA_2 in macrophages. AA and PGD_2 were utilized as metabolic markers of cellular GIVA PLA_2 activity, as AA is the direct product of GIVA PLA_2 activity and PGD_2 is the primary eicosanoid released from RAW 264.7 macrophages under these stimulatory conditions. We began the project by studying the AA and PGD_2 time-course profiles with the purpose of understanding when cellular GIVA PLA_2 is activated within this pathway and for what duration it remains active. Considering the AA release time-course data summarized in figures IV.1.A and IV.2, cells began to release AA within 7 minutes of stimulation and maximal release levels were observed at 1 hour. Since AA release is attributed to GIVA PLA_2 activity, this sudden increase indicates that GIVA PLA_2 is undergoing activation at this time. It is likely that the reduction in AA release after the first hour of stimulation does not indicate reduced cellular GIVA PLA_2 activity after the first hour, but rather that the released AA is metabolized further into terminal eicosanoids or recycled into membrane stores. The synthesis of eicosanoids requires the expression of COX-2, which is not basally expressed in macrophages, but is heavily upregulated in Kdo_2-Lipid A stimulated cells. The upregulation of COX-2 is exemplified by the PGD_2 release profile, as PGD_2 levels are undetectable until four hours after stimulation, but levels rise steeply between 4-8 hours (Figure IV.1.B). The addition of pyrrophenone at 2 hours of stimulation, which is after the maximal AA release, fully inhibited PGD_2 release; this indicates that AA release within the first 2 hours does not contribute to eicosanoid synthesis (Figure IV.4). PGD_2

levels rose incrementally between the 2, 4 and 8 hour time points, indicating that AA is released between these time points and subsequently is metabolized into PGD_2. This data indicates that GIVA PLA_2 must remain active throughout the time course to make the AA available for further metabolism.

During the first hour of stimulation, cellular PI and PIP_2 levels were significantly increased at the 7 minute time point, and remained elevated until 30 minutes. The correlation between PI and PIP_2 profiles is probably due to phosphorylation of PI by PI kinases, yielding PIP_2. Interestingly, the levels of PIP_2 and AA had both increased at 7 minutes after stimulation. This data suggests that PIP_2 may play a role in the initial activation of GIVA PLA_2. This result complements preexisting PIP_2 data very well; PIP_2 is known to interact with the catalytic domain of GIVA PLA_2 under Ca^{+2} free conditions and is believed to induce a conformational change that enhances GIVA PLA_2 activity (168,209,210,212). Cytosolic Ca^{+2} levels have been reported not to increase within the first six minutes of TLR activation in macrophages despite the fact that GIVA PLA_2 is clearly activated shortly after TLR activation (178). Therefore, PIP_2 would be an appropriate "trigger activator" of GIVA PLA_2 under these conditions. These results suggest that PIP_2 is formed within the first hour of Kdo_2-Lipid A stimulation, but appears to be metabolized shortly thereafter, as PIP_2 levels wane and free inositol levels increase (Figures IV.5 and IV.7). It is important to note that C-1-P and ceramide levels did not increase during the first hour of stimulation. This suggests that C-1-P does not play a role in the initial activation of GIVA PLA_2.

PI and PIP_2 levels decreased after the first hour, before increasing again from 4-8 hours. The elevated PIP_2 levels observed at the latter time points may indicate that PIP_2 is

further activating GIVA PLA_2 during eicosanoid production through at least two mechanisms. First, it is possible that PIP_2 is directly activating GIVA PLA_2 by protein-lipid interaction. Secondly, it is possible that PIP_2 may undergo PI-PLC mediated hydrolysis, which would subsequently yield the cytosolic Ca^{+2}-mediator, IP_3. In this study we observed that PIP_2 levels increased prior to the continuously increasing free inositol levels, which supports this hypoethesis. Such a mechanism could enhance GIVA PLA_2 activity directly, via Ca^{+2} activation of the enzyme, or indirectly, through the subsequent actions of CERK and C-1-P. Published reports have shown that PI-PLC is activated in the TLR stimulation of macrophages, and that the principle free inositol species released is IP_3 (215,217-220). The ceramide and C-1-P profiles support the second mechanism, as levels of both lipids are elevated from 2-8 hours after stimulation of the cells at which times IP_3 levels are at maximal levels. Taken as a whole, it appears that cellular ceramide and C-1-P levels increased as IP_3 levels rose, suggesting that CERK phosphorylation has also been initiated. Taking into consideration the elevated levels of ceramide, C-1-P and free inositol with the CERK and C-1-P functional requirements for Ca^{+2}, it is likely that elevated cytosolic IP_3 levels evolved from PI-PLC hydrolysis modulate the release of Ca^{+2} at later time points (73,74,214,221-223). Considering the data as a whole, it appears that C-1-P may play a role in the continued activation of GIVA PLA_2 during eicosanoid synthesis.

Since we had garnered evidence that C-1-P and free inositol levels rose simultaneously, it was necessary to verify that Kdo_2-Lipid A stimulated IP_3 release contributes to C-1-P and GIVA PLA_2 activity. To accomplish this, C-1-P and AA levels were quantitated from macrophages that were cultured in the presence and the absence of

PI-PLC inhibitors prior to stimulation with Kdo_2-Lipid A. PI-PLC inhibition has been reported to block PGE_2 release from TLR-4 activated cells in a number of previous studies (224-226). However, this observation can be attributed to reduced COX-2 expression as well as decreased GIVA PLA_2 activity, as either factor would result in the abrogation of eicosanoid release into the media. Additional studies have shown that PI-PLC inhibition blocked agonist-induced AA release in models using UTP and fMLP/B stimulants (227,228). In the current study, preincubation of cells with PI-PLC inhibitors prior to Kdo_2-Lipid A treatment resulted in marked reduction of C-1-P and AA release in addition to the expected blockage of free inositol formation (Figure IV.9). This data suggests that PI-PLC mediated IP_3 release is required for Kdo_2-Lipid A stimulated GIVA PLA_2 activity and C-1-P formation. The ionomycin results described in figure IV.10 and the PI-PLC inhibitory results described in figure IV.9 collectively demonstrate a dependence of cellular C-1-P and GIVA PLA_2 activity on cellular Ca^{+2}. However, further study is required to deduce whether the increased GIVA PLA_2 activity with ionomycin treatment and decreased GIVA PLA_2 activity in the presence of PI-PLC inhibitors is to be attributed to cellular Ca^{+2} levels or C-1-P levels. It should be noted that PI-PLC catalysis will generate membrane DAG as well as IP_3. Since it is known that PKC phosphorylates GIVA PLA_2, it is possible that DAG evolved from PI-PLC hydrolysis may also play a role in the activation of GIVA PLA_2. It is very important here to note that all three proposed mechanisms of GIVA PLA_2 activation (Ca^{+2}, phosphorylation and bioactive lipids) may play a role through the PI-PLC catalyzed IP_3 mediated GIVA PLA_2 activation described herein.

Since we proposed that there is a correlation between IP_3-mediated Ca^{+2} release and C-1-P formation in the Kdo_2-Lipid A pathway, it was necessary to further explore the relationship between cellular Ca^{+2} and C-1-P modulated GIVA PLA_2 activity. Published reports have shown that the addition of Ca^{+2} ionophore to A549 lung adenocarcinoma cells resulted in enhanced cellular C-1-P levels and was accompanied by increased AA release into the media (73). In the current study, ionomycin was utilized to study the effect of directly increasing cellular Ca^{+2} in the macrophages on C-1-P and AA release levels (Figure IV.10). Ionomycin treatment of RAW 264.7 macrophages concomitantly increased cellular C-1-P levels and AA release within 15 minutes of stimulation, demonstrating that the increased cellular Ca^{+2} is sufficient to increase C-1-P levels and initiate GIVA PLA_2 activity. Because ionomycin does not activate the TLR pathway, this result also indicates that increased Ca^{+2} alone, without the activation of any other signaling cascades is sufficient to elicit this response. Interestingly, C-1-P and AA release levels rose simultaneously, suggesting additional correlation between GIVA PLA_2 activity and the presence of increased cellular C-1-P.

The data described in this study suggests that PIP_2 may play a role as an initial "trigger" activator of GIVA PLA_2, while C-1-P may play a role in the sustained binding of GIVA PLA_2 to the membrane surface during eicosanoid synthesis. This conclusion is consistent with published data on the interactions between the two bioactive lipids and *in vitro* GIVA PLA_2 activity. One of the distinctive characteristics of PIP_2-GIVA PLA_2 interactions is that PIP_2 is believed to induce a GIVA PLA_2 conformational change that results in enhanced catalytic activity in addition to facilitating membrane binding (168,212). Such an enhancement would increase the capacity of GIVA PLA_2 to mobilize

AA at the early stages of the stimulation when the cell is a transforming between basal and activated states. Furthermore, PIP_2 does not require Ca^{+2} for interaction with GIVA PLA_2. Since Ca^{+2} levels do not increase within the first several minutes of TLR stimulation, increased PIP_2 levels would still result in enhanced GIVA PLA_2 activity (178,210). We observed that C-1-P levels were elevated during later times, during which macrophages released eicosanoids and free inositol levels were maximized. This result compliments preexisting C-1-P data very well, since evidence indicates that Ca^{+2} is required for CERK catalysis and C-1-P interaction with GIVA PLA_2 (214). Furthermore, it should be noted that sphingolipids may play multiple signaling roles in the proinflammatory activation of macrophages. In addition to the C-1-P mediated activation of GIVA PLA_2, there is mounting evidence that sphingosine 1-phosphate (S-1-P) may play a role in the upregulation of COX-2 (229,230). If TLR activation were proven to lead to the simultaneous upregulation of COX-2 and the activation of GIVA PLA_2 through increased cellular S-1-P and C-1-P levels, this would indicate that macrophages mobilize both proteins through the metabolism of only one lipid class.

Further investigation is needed to address whether the reduced AA release is to be attributed to loss of IP_3, DAG or both metabolites, as any one of these regulators may be responsible for increased GIVA PLA_2 activity associated with TLR-4 activation. From the data presented in this study, it is very likely that Kdo_2-Lipid A stimulated GIVA PLA_2 activity is mediated by a combination of all four regulatory elements *in vivo*. Therefore, cellular GIVA PLA_2 activity is likely to be dependent upon the integration of regulatory effects derived from changes in cellular levels of Ca^{+2}, PIP_2, C-1-P and DAG in response to inflammatory stimulation. Further experimentation is necessary to study

each mechanism separately to discern what the effect of each component is on the activation of GIVA PLA_2 in the inflammatory system.

CHAPTER V

GIVA PLA_2 Chemical Inhibitor Studies

A. Background

The phospholipase A_2 (PLA_2) superfamily consists of a broad range of enzymes defined by their ability to catalyze the hydrolysis of the ester bond at the *sn*-2 position of membrane phospholipids (106). The group IVA PLA_2 (GIVA PLA_2) is known to play a key role in lipid mediator biosynthesis through the liberation of arachidonic acid (AA) from membrane phospholipids; once released from membrane stores, AA is subsequently converted into terminal signaling metabolites, known as eicosanoids. Loss of GIVA PLA_2 expression through gene deletion results in reduced eicosanoid biosynthesis in murine models, while also reducing the incidence of inflammatory responses to numerous disease states, stress and injury (53,55,56,58). Since GIVA PLA_2 is directly implicated in the synthesis of eicosanoids associated with inflammation and a variety of pathophysiological conditions, GIVA PLA_2 has become a molecular target for the development of potentially therapeutic chemical inhibitors.

The mechanism of GIVA PLA_2 hydrolysis is through nucleophilic attack S228 on the phospholipid substrate resulting in the formation of an acyl-enzyme intermediate in a reaction mechanism that is similar to that of other lipases utilizing a catalytic serine nucleophile (52,231,232). The acyl group is subsequently released from the enzyme by the incorporation of a water molecule, which replenishes the enzymatic activity (52,106,233). Chemical inhibitors have been developed for serine proteases and lipases through the utilization of activated carbonyl groups, such as fluorinated ketones and α–

ketoamides (233-238). The purpose of the current research was to develop a novel class of reversible GIVA PLA_2 chemical inhibitors that specifically inhibit the enzyme while showing minimal inhibitor promiscuity toward other PLA_2 enzymes. The chemical inhibitors were designed as analogues of the normal GIVA PLA_2 substrate phospholipid, which can be seen in figure V.1. The 2-oxoamide functionality was chosen as a replacement for the scissile ester bond of the substrate phospholipid, while ester and carboxylic acid functional groups were chosen as substitutions for the phosphate-ester moiety. The chemical synthesis of the inhibitors was carried out by Professor George Kokotos of the University of Athens and the *in vivo* rat model testing was conducted by associate professor Dimitra Hadjipavlou-Litina of the Aristotelian University of Thessaloniki. *In vitro* chemical inhibitor testing was accomplished by Daren Stephens, a staff research associate in the laboratory of Edward Dennis. My focus was to test the ex vivo activity of selected inhibitors for their ability to block AA release in RAW 264.7 macrophages; the results of these experiments are reported in this chapter.

B. Materials and Methods

1. Reagents

Murine RAW 264.7 macrophages were purchased from American Type Culture Collection (Manassas, VA). Kdo_2-Lipid A was from Avanti Polar Lipids (Alabaster, AL). 1-palmitoyl-2-(1-^{14}C)-palmitoyl phosphatidylcholine, 1-palmitoyl-2-(1-^{14}C)-arachidonoyl phosphatidylcholine, obtained from NEN Life Science Products (Boston, MA). Phosphatidylinositol 4,5-bisphosphate was from Roche (Basel, Switzerland). The specific GIVA PLA_2 inhibitor,

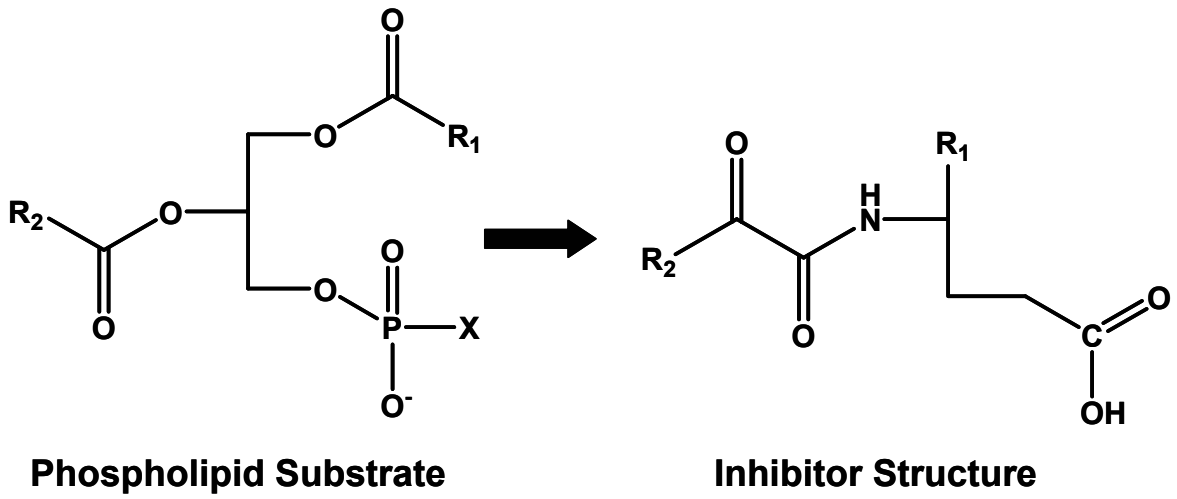


Figure V.1. Comparison of 2-oxoamide inhibitor structure to GIVA PLA_2 phospholipid substrate.

Schematic demonstrating the rationale design of the GIVA PLA_2 enzyme chemical inhibitors based on the natural phospholipid substrate (239).

pyrrophenone, was from Shionogi Research Laboratories of Shionogi & Co., Ltd (Japan).

2. Cell Culture and Stimulation Protocol

RAW 264.7 macrophages were maintained at 37 °C in a humidified atmosphere at 90% air and 5% CO_2 DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, 100 μg/ml streptomycin, and non-essential amino acids. Cells were plated at a confluency of $2x10^6$ cells/per well in a 6 well tissue culture plate and $1x10^6$/per well in a 12 well tissue culture plate at the time of experimentation. Following plating, they were allowed to adhere overnight, and then used for experiments the following day. Inhibitors were added to the medium 30 min before Kdo_2-Lipid A; DMSO Was added to control cells to equal that of the cells exposed to the chemical inhibitors. Supernatants were collected at 1 hour following stimulation. Cell viability was assessed visually by the Trypan Blue Dye exclusion assay (Gibco, Grand Island, NY) and through the usage of the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI).

3. Sample Preparation

Media was analyzed for extracellular eicosanoid release. After stimulation, 1.8 ml of media was removed and supplemented with 100 μl of internal standards (100 pg/μl, EtOH) and 100 μl EtOH to bring the total EtOH to 10% by volume. Samples were centrifuged for 5 minutes at 3000 rpm to remove cellular debris, and the supernatants were decanted into solid phase extraction columns. All eicosanoid extractions were conducted using Strata-X SPE columns (Phenomenex). The columns were washed with 2 ml MeOH and then 2 ml H_2O prior to the samples being applied. After applying the sample, the columns were washed with 10% MeOH to remove non-adherent debris, and eicosanoids were then eluted off the

column with 1 ml MeOH. The eluant was dried under vacuum and redissolved in 100 µl of solvent A [water-acetonitrile-formic acid (63:37:0.02; v/v/v)] for LC/MS analysis.

4. Cell Quantitation

Eicosanoid levels were normalized to cell number using DNA quantitation. After the extracellular media was removed, the cells were scraped in 500 µl PBS and stored at 4ºC for DNA quantitation using the Broad Range DNA Quant-Kit (Invitrogen).

5. HPLC and Mass Spectrometry

The analysis of eicosanoids was performed by LC/MS/MS. Eicosanoids were separated by reverse-phase HPLC on a C18 column (2.1 mm x 150 mm, Grace-Vydac) at a flow rate of 300 µl/min at 25ºC. The column was equilibrated in Solvent A [water-acetonitrile-formic acid (63:37:0.02; v/v/v)], and samples were injected using a 50 µl injection loop and eluted with a linear gradient from 0%-20% solvent B [acetonitrile-isopropyl alcohol (50:50; v/v)] between 0 to 6 min; solvent B was increased to 55% from 6 to 6.5 min and held until 10 min. Solvent B was increased to 100% from 10 to 12 min and held until 13 min; solvent B was dropped to 0% by 13.5 min and held until 16 min.

Eicosanoids were analyzed using a tandem quadrupole mass spectrometer (ABI 4000 Q Trap®, Applied Biosystems) via multiple-reaction monitoring (MRM) in negative-ion mode. The electrospray voltage was -4.5 kV, the turbo ion spray source temperature was 525ºC. Collisional activation of eicosanoid precursor ions used nitrogen as a collision gas.

6. GIVA, GV and GVI PLA_2 Activity Assay

The GIVA PLA_2 assay was previously described (168,168,170). Briefly, following collection of cellular lysates, the samples were assayed for PLA_2 activity using the modified Dole assay. For each assay the final buffer conditions were: 100 mM Hepes, pH 7.5, 80 µM

$CaCl_2$, 0.1 mg/ml BSA and 2 mM DTT. The PLA_2 assay also contained 97 μM PAPC doping with 1% ^{14}C labeled PAPC in 400 μM Triton X-100 mixed micelles. Lipid preparation: lipid was dried under N_2 and lyophilized for at least 1 h to remove all traces of chloroform. Lipid was then resuspended and mixed micelles were created by repeated vortexing and heating in hot water until the solution clarified. Samples were incubated with substrate for 30 min at 40 °C. The assay was then terminated by 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 mg) was added to each tube followed by 1.5 ml heptane and 1.5 ml deionized water. Each tube was vortexed 15 s. 1 ml of the organic phase was removed and passed through a Pasteur pipet filled with silica gel (0.1–0.2 mg). This column was then washed with 1 ml diethyl ether. Scintillation cocktail (5 ml, Biosafe II) was added to the eluent and the radioactivity was determined by scintillation counting.

The GV PLA_2 assay was previously described (168,168,170). Briefly, following collection of cellular lysates, the samples were assayed for PLA_2 activity using the modified Dole assay. For each assay the final buffer conditions were: 100 mM Hepes, pH 7.5, 5 mM $CaCl_2$, and 1 mg/ml BSA. The PLA_2 assay also contained 97 μM DPPC/POPS (3/1) doping with 1% ^{14}C labeled DPPC in 400 μM Triton X-100 mixed micelles. Lipid preparation: lipid was dried under N_2 and lyophilized for at least 1 h to remove all traces of chloroform. Lipid was then resuspended and mixed micelles were created by repeated vortexing and heating in hot water until the solution clarified. Samples were incubated with substrate for 30 min at 40 °C. The assay was then terminated by 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 mg) was added to each tube followed by 1.5 ml heptane and 1.5 ml deionized water. Each tube was vortexed

15 s. 1 ml of the organic phase was removed and passed through a Pasteur pipet filled with silica gel (0.1–0.2 mg). This column was then washed with 1 ml diethyl ether. Scintillation cocktail (5 ml, Biosafe II) was added to the eluent and the radioactivity was determined by scintillation counting.

The GVI PLA_2 assay was previously described (168,168,170). Briefly, following collection of cellular lysates, the samples were assayed for PLA_2 activity using the modified Dole assay. For each assay the final buffer conditions were: 100 mM Hepes, pH 7.5, 80 μM $CaCl_2$, 0.1 mg/ml BSA and 2 mM DTT. The PLA_2 assay also contained 97 μM DPPC doping with 1% ^{14}C labeled DPPC in 400 μM Triton X-100 mixed micelles. Lipid preparation: lipid was dried under N_2 and lyophilized for at least 1 h to remove all traces of chloroform. Lipid was then resuspended and mixed micelles were created by repeated vortexing and heating in hot water until the solution clarified. Samples were incubated with substrate for 30 min at 40 °C. The assay was then terminated by 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 mg) was added to each tube followed by 1.5 ml heptane and 1.5 ml deionized water. Each tube was vortexed 15 s. 1 ml of the organic phase was removed and passed through a Pasteur pipet filled with silica gel (0.1–0.2 mg). This column was then washed with 1 ml diethyl ether. Scintillation cocktail (5 ml, Biosafe II) was added to the eluent and the radioactivity was determined by scintillation counting.

C. Prior *in vitro* Experimental Results

1. Logistics and Design of Reversible, Specific GIVA PLA_2 Chemical Inhibitors

The development of GIVA PLA_2-specific inhibitors may have profound pharmacological value. The first significant step in the development of an effective, reversible GIVA PLA_2 inhibitor was the discovery of arachidonoyl trifluoromethyl ketone (AATFK) as a potent, tight and slow binding reversible inhibitor of the enzyme (240). Later, another GIVA PLA_2 inhibitor, methyl arachidonoyl fluorophosphonate (MAFP), was developed; although this inhibitor binds in an irreversible manner with the GIVA PLA_2 enzyme (241). Recently, a pyrrolidine structure, pyrrophenone, was developed as a potent and specific GIVA PLA_2 inhibitor; however, despite its potency, it has not been developed as a drug due to the irreversible binding of it to the enzyme and its cytotoxic properties *in vivo* (242). Despite the aforementioned advances, potent, specific and reversible inhibitors suitable to be utilized as pharmacological agents of GIVA PLA_2 are not currently available.

To design an effective GIVA PLA_2 inhibitor, one must first consider the GIVA PLA_2 active site and mechanism of enzymatic catalysis. GIVA PLA_2 activity proceeds through a catalytic serine, S228, which is located within a cleft at the center of a hydrophobic funnel (52). With consideration to the structure of the GIVA PLA_2 active site, potential GIVA PLA_2 inhibitors were designed to contain three basic characteristics. The first is to contain an activated electrophilic functional group that would potentially interact with S228. Second, an electronegative functional group is incorporated to interact with R200; R200 is believed to normally interact with the *sn*-3 head group of the substrate phospholipid. Finally, a lipophilic segment is included to enter the hydrophobic funnel portion of the active site and potentially orient the inhibitor into the correct spatial conformation. The lipophilic requirement of an effective GIVA PLA_2 inhibitor is the biggest challenge, since the lipophilic part of the structure markedly reduces the aqueous solubility of the inhibitor. Therefore, it is essential to

incorporate a hydrophobic side chain that will satisfy the enzyme while not cause precipitation of the inhibitor out of aqueous solutions. To satisfy these three requirements, a series of over 100 potential GIVA PLA_2 inhibitors were synthesized. All of these inhibitors possess a long, hydrophobic side chain on one side of the structure with a hydrophilic fluoroketone, carboxylic acid or ester located on the other end of the molecule. Additionally, some inhibitors contain a 2-oxoamide moiety within the structure, as it is thought that this feature may interact with S228 and therefore enhance interaction with the enzyme. A number of 2-oxoamide inhibitors have been developed for a variety of esterases that contain a putative catalytic serine (243). Finally, some of the inhibitors contained a short aliphatic side chain which is purported to potentially interact within a fourth potential substrate binding site within the GIVA PLA_2 active site that accommodates short hydrophobic side chains. The manner in which it was envisioned that these inhibitors might interact with the active site of GIVA PLA_2 is demonstrated in figure V.2 (241).

2. Evaluation of GIVA PLA_2 Chemical Inhibitor Potency through Utilization of the GIVA PLA_2 Activity Assay

To evaluate whether the potential inhibitors reduce *in vitro* GIVA PLA_2 enzyme activity, *in vitro* GIVA PLA_2 activity was assayed according to a procedure that the Dennis laboratory has developed (167,170). Our study focused on 29 inhibitors that reduced *in vitro* GIVA PLA_2 activity; the results are summarized in table V.1. From analysis of this data, there appears to be no one chemical structure that is clearly the most effective

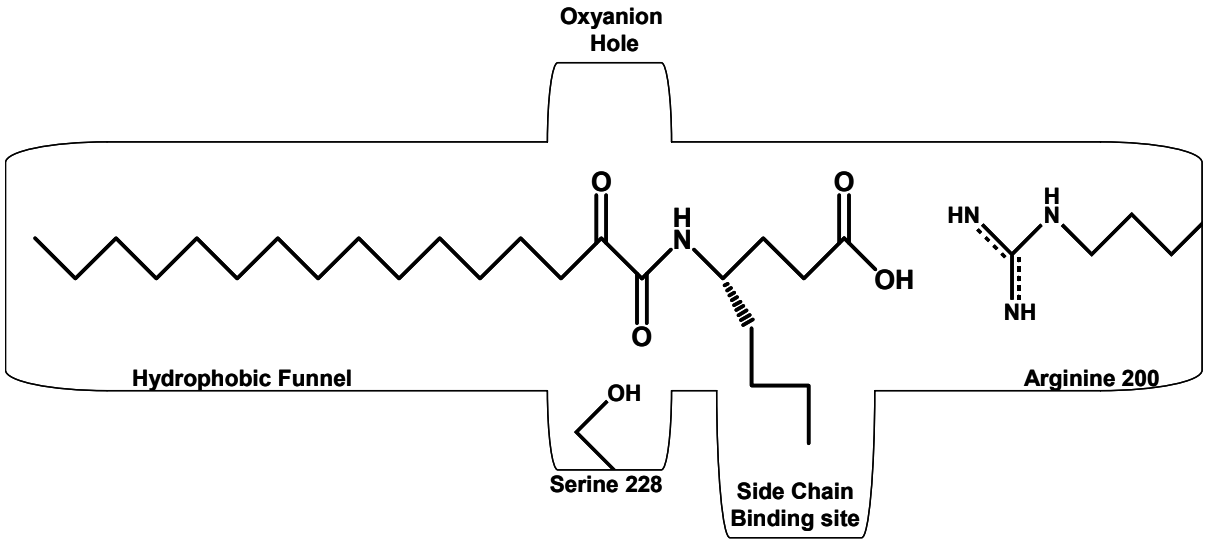


Figure V.2. Logistic design of 2-oxoamide moiety-based GIVA PLA_2 chemical inhibitors.

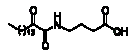
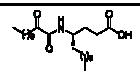
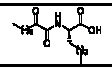
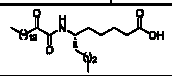
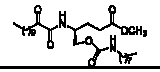
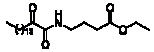
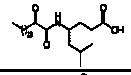
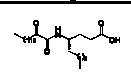
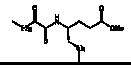
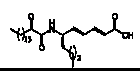
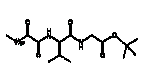
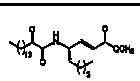
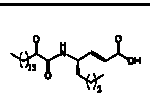
Schematic demonstrating the structural and logistic design of the GIVA PLA_2 enzyme chemical inhibitors (The inhibitor AX007 is depicted here) bound within the active site of GIVA PLA_2 (241).

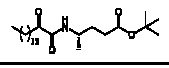
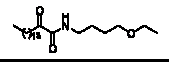
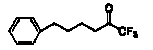
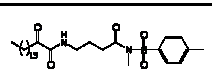
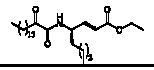
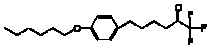
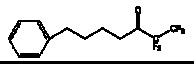
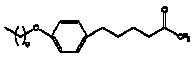
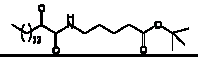
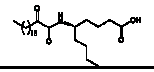
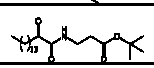
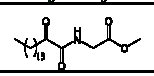
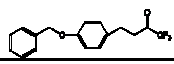
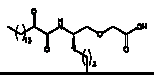
inhibitor of GIVA PLA_2 activity. A wide variety of hydrophilic functional groups were effective GIVA PLA_2 inhibitors, including fluoroketones, carboxylic acids and esters. Furthermore, a variety of ester-containing inhibitors were effective, including methyl esters, ethyl esters and *tert*-butyl esters; this adds further diversity to the range of effective inhibitor classes. Additionally, we see that there appears to be variance in the length, identity of both hydrophobic side chain and whether effective inhibitors contain the 2-oxoamide moiety.

It is essential that chemical inhibitors designed for GIVA PLA_2 be specific, as well as potent for the GIVA PLA_2 enzyme. Therefore, the effective GIVA PLA_2 inhibitors were further tested for inhibitory potency against *in vitro* GV and GVI PLA_2 enzyme activity to establish whether they are specific for GIVA PLA_2. Unlike GIVA PLA_2, GV PLA_2 operates through a catalytic histidine and requires Ca^{+2} for catalytic function (106). GVI PLA_2 and GIVA PLA_2 both possess an active site catalytic serine, but differ in regards to substrate specificity; whereas GIVA PLA_2 prefers AA containing phospholipids, GVI PLA_2 catalyzes the release of a diverse array of fatty acids that are esterified at the *sn*-2 position of phospholipids (106). It appears that inhibitors possessing the combination of a carboxylic acid functional group along with a 2-oxoamide moiety are the most specific for GIVA PLA_2. With the exception of AX025, none of these inhibitors demonstrated significant inhibition of *in vitro* GV or GVI PLA_2 activity, while potently inhibiting GIVA PLA_2 activity (Table V.1). The variance amongst these inhibitors was the length of the smaller lipophilic side chain, unsaturation within the molecule and the distance between the 2-oxoamide moiety and the carboxylic acid. The importance of this observation is that it demonstrates that there is a logical approach to distinguishing which inhibitor structures should demonstrate fidelity and potency for reduction of GIVA PLA_2 activity.

Table V.1. Summary of the effect of GIVA PLA_2 chemical inhibitors on *in vitro* GIVA PLA_2, GV PLA_2 and GVI PLA_2 activity.

The PLA_2 inhibition potency of the indicated chemical inhibitors is described. The data is presented as the XI_{50} when greater than 50%; otherwise, data is presented as the percent (%) inhibition at highest concentration of inhibitor tested (μM). Data adapted from previously unpublished data and the following references: a; (239,241,243,244), b; (243) and c; (244).

I.D.	STRUCTURE	Mol. Wt.	GIVA PLA_2 Inhibition[a]	GV PLA_2 Inhibition[b]	GVI PLA_2 Inhibition[c]
AX006		355.52	$X_i(50)$= 0.024 ± .015	N.D.	N.D.
AX007		355.51	$X_i(50)$= 0.012 ± .006	N.D.	N.D.
AX020		327.46	0.091; 61.3%	N.D.	N.D.
AX025		425.65	$X_i(50)$= 0.014 ± .006	0.091; 21.5%	N.D.
AX028		498.70	$X_i(50)$= 0.020 ± .011	$X_i(50)$= 0.035 ± .018	$X_i(50)$= 0.053 ± .005
AX048		383.57	$X_i(50)$= 0.022 ± .009	0.091; 61.5%	$X_i(50)$= 0.027 ± .009
AX059		411.33	$X_i(50)$= 0.008 ± .002	N.D.	N.D.
AX061		367.52	0.091; 67.9%	N.D.	0.091; 26.1%
AX062		411.62	$X_i(50)$= 0.008 ± .003	N.D.	N.D.
AX063		425.64	$X_i(50)$= 0.019 ± .010	$X_i(50)$= 0.034 ± .012	$X_i(50)$= 0.054 ± .004
AX066		435.64	$X_i(50)$= 0.029 ± .010	N.D.	N.D.
AX068		482.7	$X_i(50)$= 0.028 ± .015	0.091; 77.9%	$X_i(50)$= 0.044 ± .025
AX073		423.63	$X_i(50)$= 0.018 ± .010	0.091; 75.3%	$X_i(50)$= 0.032 ± .010
AX074		409.60	$X_i(50)$= 0.003 ± .001	N.D.	N.D.

I.D.	STRUCTURE	Mol. Wt.	GIVA PLA_2 Inhibition[a]	GV PLA_2 Inhibition[b]	GVI PLA_2 Inhibition[c]
AX076		425.64	$X_i(50)$= 0.018 ± .009	$X_i(50)$= 0.031 ± .016	$X_i(50)$= 0.067 ± 0.007
AX081		369.58	$X_i(50)$= 0.018 ± .016	$X_i(50)$= 0.056 ± .007	0.091; 50.9%
AX089		230.23	0.091; 37.5%	0.091; 17.7%	$X_i(50)$= 0.004 ± .002
AX092		522.74	$X_i(50)$= 0.033 ± .018	0.091; 67.5%	0.091; 52.8%
AX093		437.66	$X_i(50)$= 0.031 ± .011	0.091; 78.3%	0.091; 66.5%
AX095		330.39	$X_i(50)$= 0.009 ± .003	0.091; 85.5%	$X_i(50)$= 0.014 ± .008
AX099		280.23	0.091; 16.9%	0.091; 29.0%	$X_i(50)$= 0.007 ± .004
AX101		386.5	0.091; 93.5%	0.091; 95.8%	$X_i(50)$= 0.018 ± .003
AX105		425.64	0.091; 73.8%	0.091; 96.3%	0.091; 61.7%
AX109		425.64	$X_i(50)$= 0.005 ± .002	N.D.	N.D.
AX113		397.59	0.091; 79.2%	0.091; 100%	0.091; 54.0%
AX115		341.49	0.091; 62.2%	0.091; 95.5%	0.091; 44.8%
AX118		308.30	0.091; 86.1%	0.091; 49.2%	0.091; 70.6%
AX123		352.34	0.091 ; 72.6%	0.091 ; 93.5%	0.091; 96.1%
AX129		427.62	0.091; 91.1%	N.D.	N.D.

D. Results

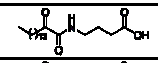
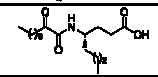
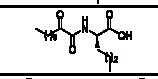
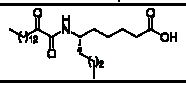
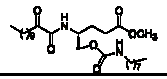
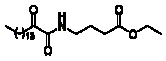
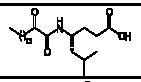
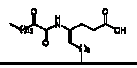
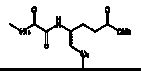
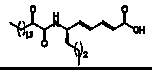
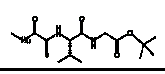
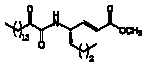
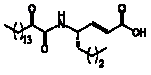
1. Evaluation of Cellular GIVA PLA_2 Chemical Inhibitor Potency

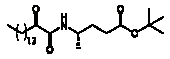
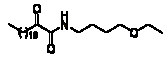
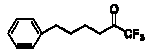
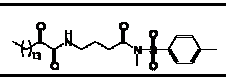
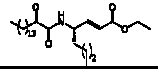
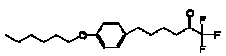
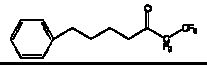
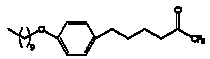
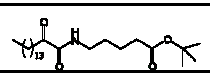
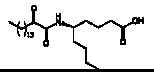
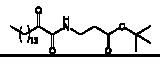
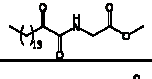
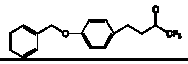
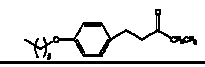
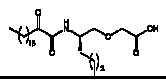
The 29 inhibitors described in Table V.1 that had shown significant *in vitro* GIVA PLA_2 activity inhibition were further tested in a cellular system to establish whether the inhibitors function *ex vivo*. To assess whether the inhibitors reduced cellular GIVA PLA_2 activity, RAW 264.7 macrophages were preincubated with 25 μM concentrations of the inhibitors prior to treatment of the cells with Kdo_2-Lipid A. Subsequently, AA release was quantitated from the supernatants to assess the effect of the inhibitors. AA is the ideal metabolic indicator of GIVA PLA_2 activity, since it is the product of GIVA PLA_2-mediated catalysis. Eight of the inhibitors reduced the cellular release of AA by greater than 50%; interestingly, the chemical structures of the inhibitors are very similar (Table V.2). Each inhibitor containing a carboxylic acid moiety at the δ or γ position relative to the 2-oxoamide moiety displayed inhibition of approximately 50% or greater (AX061 is presumed to function as a prodrug since it could become a carboxylic acid based inhibitor upon hydrolysis). Furthermore, all of the inhibitors that reduced cellular AA release contain the 2-oxoamide moiety, suggesting that this is important for cellular inhibition. Interestingly, the specific characteristics of the linkage between 2-oxoamide and the carboxylic acid appear to be unimportant. In this regard, AX074 has an unsaturated bond whereas the other seven inhibitors do not; AX129 contains an ether linkage, the rest do not; AX006 has no short aliphatic side chain, AX059 has a *tert*-butyl side chain and the rest have straight-chain butyl side chains.

Dose response curves were then generated for the eight carboxylic acid functional group containing GIVA PLA_2 chemical inhibitors that reduced AA release in table V.2 to

Table V.2. Summary of the effect of GIVA PLA_2 chemical inhibitors on Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages.

Data summary of the reduction of AA release from RAW 264.7 macrophages preincubated with a 25 μM concentration of the indicated inhibitors prior to stimulation with Kdo_2-Lipid A. Data is presented as percent reduction relative to uninhibited cells. Some chemical inhibitors increased AA release; the results are reported as "No Inhibition" in these cases and the percent increase of AA release is indicated in parenthesis. All data points were measured at 1 hour after stimulation of the cells.

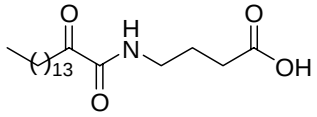
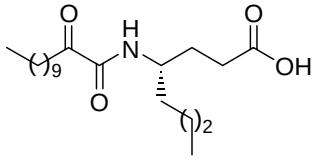
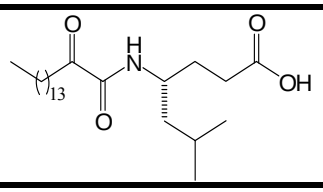
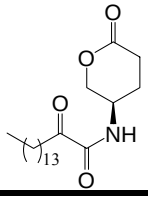
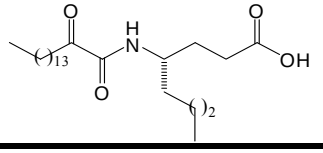
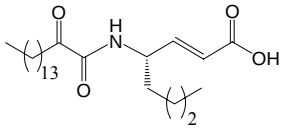
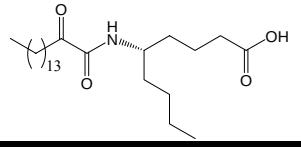
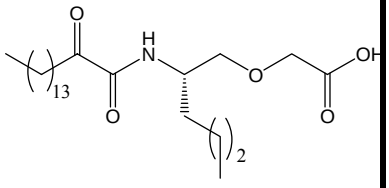
I.D.	STRUCTURE	Mol. Wt.	AA release Inhibition (%)
AX006		355.52	52 ± 5
AX007		355.51	56 ± 6
AX020		327.46	27 ± 6
AX025		425.65	No Inhibition (-25 ± 7)
AX028		498.70	No Inhibition (-142 ± 10)
AX048		383.57	No Inhibition (-30 ± 10)
AX059		411.33	62 ± 5
AX061		367.52	44 ± 3
AX062		411.62	79 ± 0.5
AX063		425.64	No Inhibition (-210 ± 20)
AX066		435.64	6 ± 10
AX068		482.7	30 ± 2
AX073		423.63	No Inhibition (-38 ± 10)
AX074		409.60	74 ± 8

I.D.	STRUCTURE	Mol. Wt.	AA release Inhibition (%)
AX076		425.64	No Inhibition (-172 ± 20)
AX081		369.58	No Inhibition (-243 ± 15)
AX089		230.23	No Inhibition (-36 ± 22)
AX092		522.74	No Inhibition (-23 ± 11)
AX093		437.66	16 ± 6
AX095		330.39	No Inhibition (-65 ± 12)
AX099		280.23	No Inhibition (-119 ± 10)
AX101		386.5	No Inhibition (-121 ± 12)
AX105		425.64	11 ± 6
AX109		425.64	59 ± 4
AX113		397.59	No Inhibition (-79 ± 12)
AX115		341.49	30 ± 2
AX118		308.30	No Inhibition (-88 ± 6)
AX123		352.34	No Inhibition (-99 ± 12)
AX129		427.62	68 ± 4

allow calculation of the IC_{50} values and enable structural comparison of the functional inhibitors. The IC_{50} values for the eight inhibitors are summarized in Table V.3. The individual dose response curves for the inhibition of AA release from RAW 264.7 cells stimulated with Kdo_2-Lipid A in the presence of AX006, AX007, AX059, AX061, AX062, AX074, AX109 and AX129 are depicted in figures V.3 through V.10. AX006, AX059, AX062 and AX074 all display IC_{50} values of approximately 25 μM whereas AX007, AX061, AX109 and AX129 display values of 10, 7, 7 and 2 μM, respectively. Interestingly, AX109 and AX129, which contains the carboxylic acid functional group spaced at the δ position relative to the 2-oxoamide moiety, demonstrated the lowest IC_{50} values at 7 and 2 μM, respectively. In contrast, the five γ linked inhibitors, AX006, AX007, AX059, AX062 and AX074, all displayed higher IC_{50} values at 25, 7, 25, 25 and 25 μM. This data suggests that inhibitors with δ structural spacing display more effective GIVA PLA_2 inhibition than γ structural spacing within this inflammatory cellular model. AX061 may undergo hydrolysis in aqueous environments to yield a similar structure to that of AX006, with an extra hydroxyl-methyl group side chain. This is significant, since the cyclic structure is not believed to be an effective inhibitory structure but could become a functional inhibitor after undergoing the ring-opening process. We observed that the IC_{50} value of AX061 in this experiment was estimated at 7 μM, whereas that of AX006 was 25 μM. The data clearly indicates that AX061 is a more efficient *in vivo* inhibitor of GIVA PLA_2 than AX006; assuming that AX061 undergoes ring opening prior to being functional, this data further suggests that the cyclic structure of AX061 may facilitate the delivery of the inhibitor to the correct intracellular location.

Table V.3. GIVA PLA_2 chemical inhibitor IC_{50} values.

Table summarizes the IC_{50} values for the indicated 2-oxoamide moiety containing chemical inhibitors.

I.D.	STRUCTURE	Mol. Wt.	IC_{50} (µM)
AX006		355.52	**25**
AX007		355.51	**10**
AX059		411.33	**25**
AX061		367.52	**7**
AX062		411.62	**25**
AX074		409.60	**25**
AX109		425.64	**7**
AX129		427.62	**2**

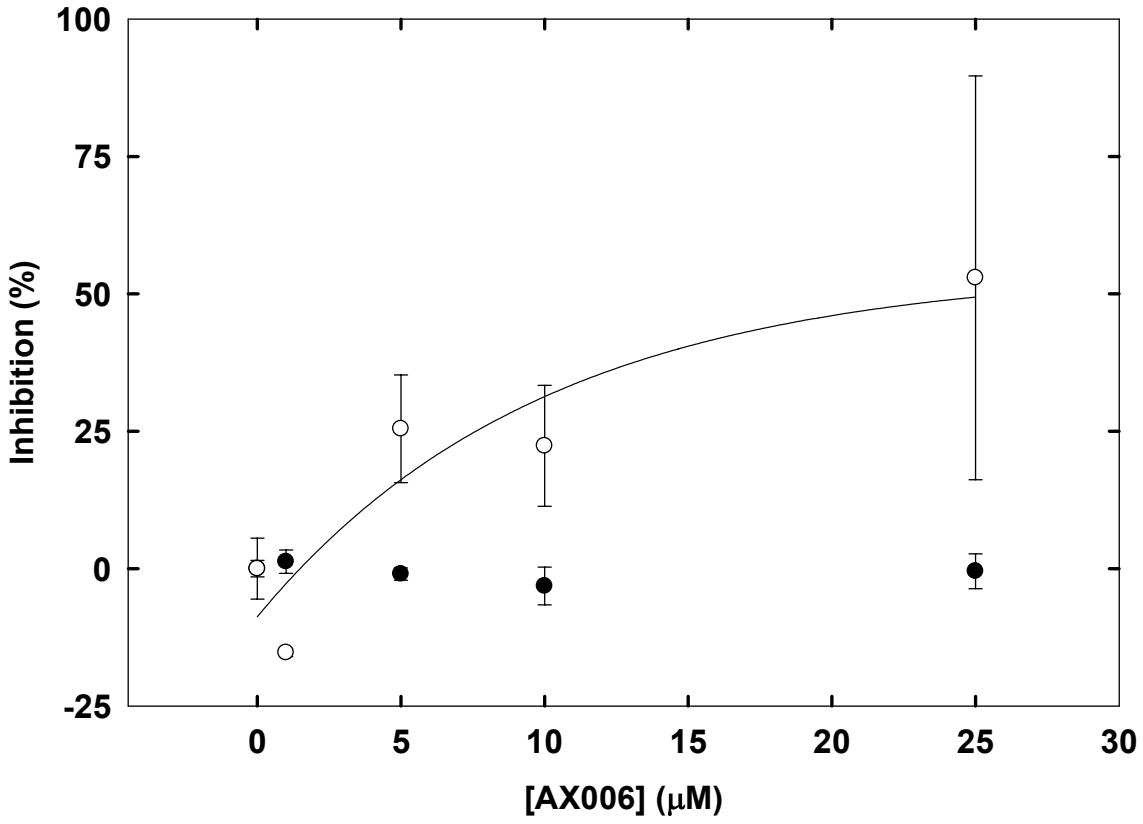


Figure V.3. AX006 inhibitory dose curve of Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages.

Figure depicts the effect of increasing doses of AX006 on Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages. Cells that were treated with Kdo_2-Lipid A are indicated with (○) and control cells that were not stimulated with Kdo_2-Lipid A are represented by (●). All data points were measured at 1 hour after stimulation of the cells.

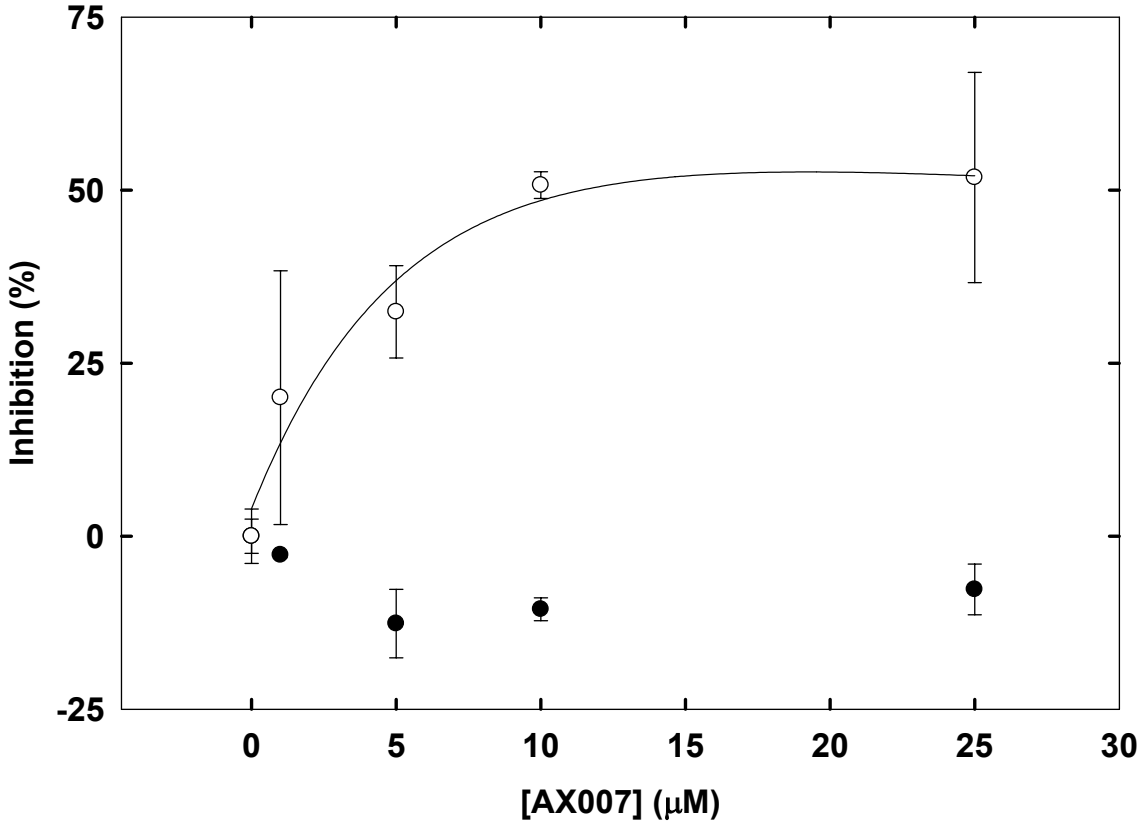


Figure V.4. AX007 inhibitory dose curve of Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages.

Figure depicts the effect of increasing doses of AX007 on Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages. Cells that were treated with Kdo_2-Lipid A are indicated with (○) and control cells that were not stimulated with Kdo_2-Lipid A are represented by (●). All data points were measured at 1 hour after stimulation of the cells.

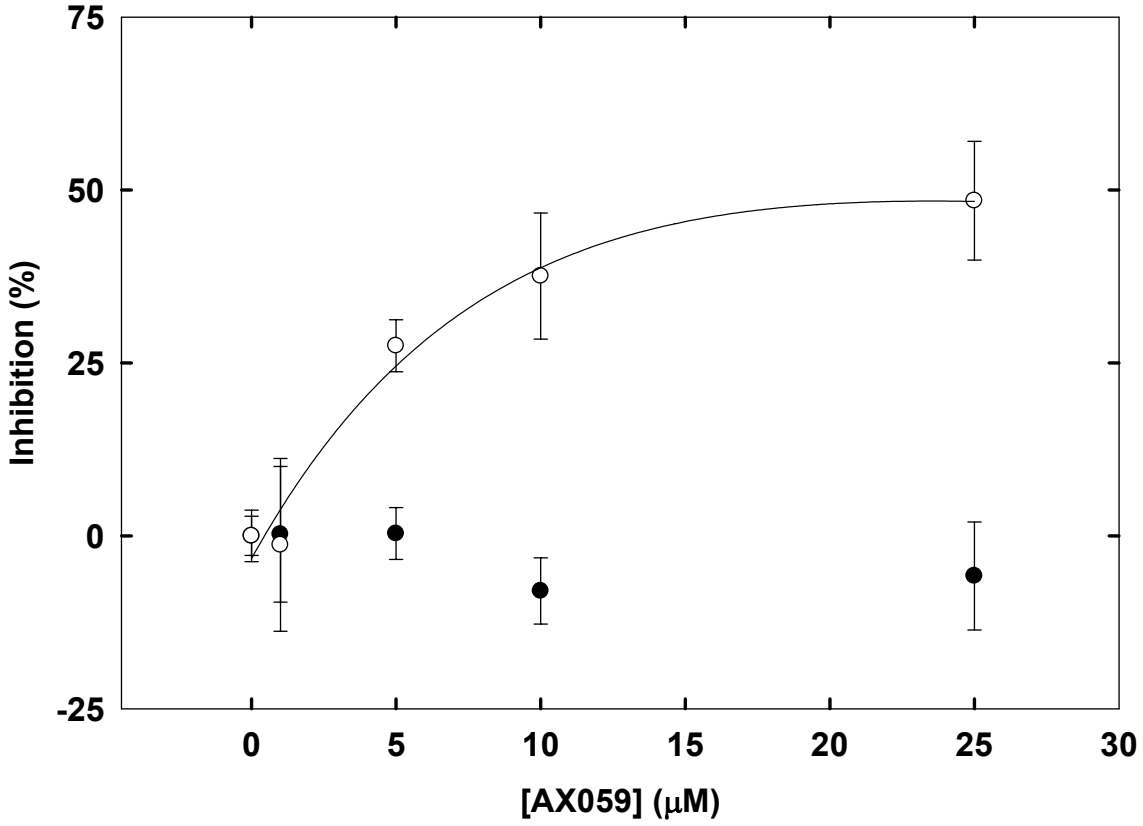


Figure V.5. AX059 inhibitory dose curve of Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages.

Figure depicts the effect of increasing doses of AX059 on Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages. Cells that were treated with Kdo_2-Lipid A are indicated with (○) and control cells that were not stimulated with Kdo_2-Lipid A are represented by (●). All data points were measured at 1 hour after stimulation of the cells.

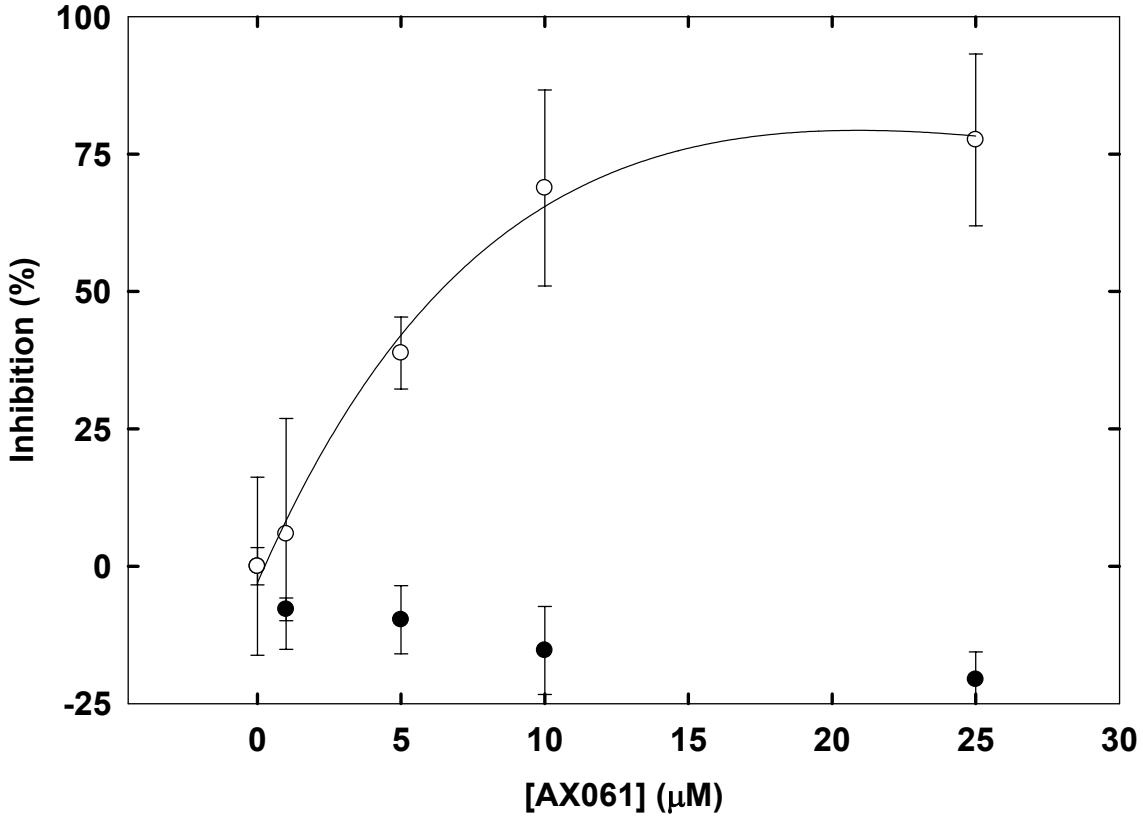


Figure V.6. AX061 inhibitory dose curve of Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages.

Figure depicts the effect of increasing doses of AX061 on Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages. Cells that were treated with Kdo_2-Lipid A are indicated with (○) and control cells that were not stimulated with Kdo_2-Lipid A are represented by (●). All data points were measured at 1 hour after stimulation of the cells.

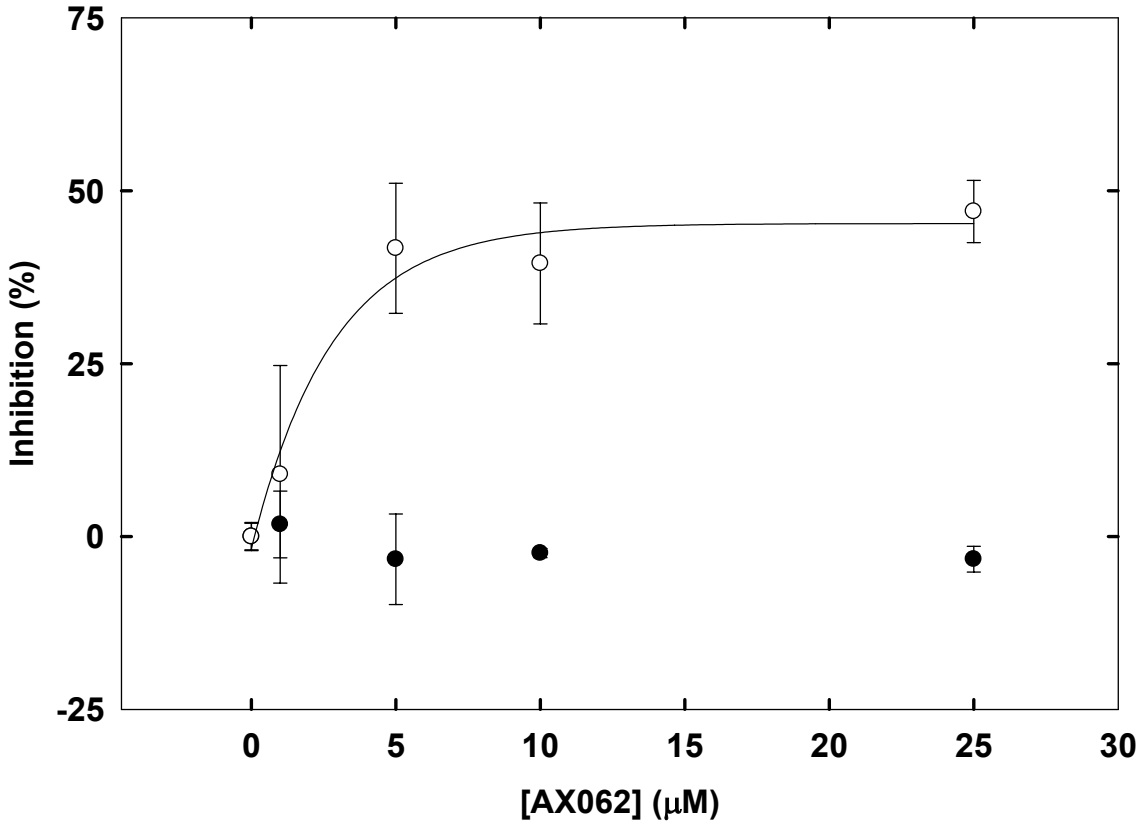


Figure V.7. AX062 inhibitory dose curve of Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages.

Figure depicts the effect of increasing doses of AX062 on Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages. Cells that were treated with Kdo_2-Lipid A are indicated with (○) and control cells that were not stimulated with Kdo_2-Lipid A are represented by (●). All data points were measured at 1 hour after stimulation of the cells.

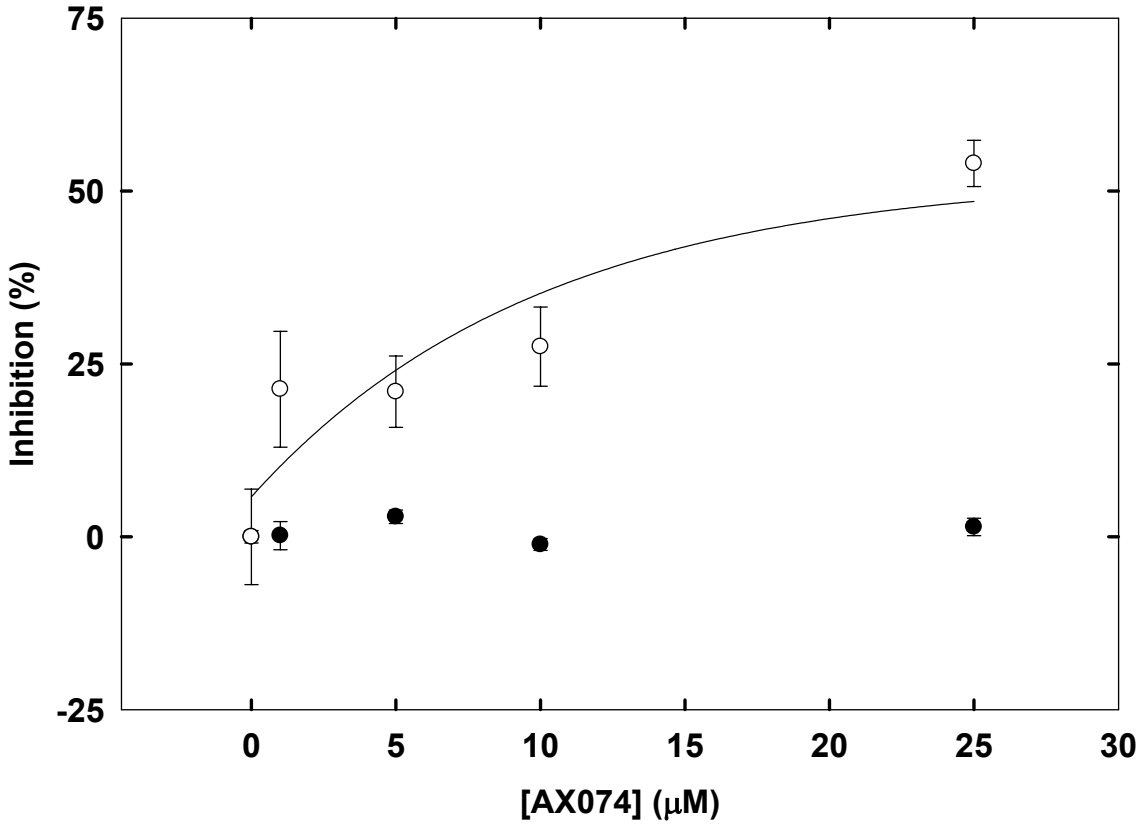


Figure V.8. AX074 inhibitory dose curve of Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages.

Figure depicts the effect of increasing doses of AX074 on Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages. Cells that were treated with Kdo_2-Lipid A are indicated with (○) and control cells that were not stimulated with Kdo_2-Lipid A are represented by (●). All data points were measured at 1 hour after stimulation of the cells.

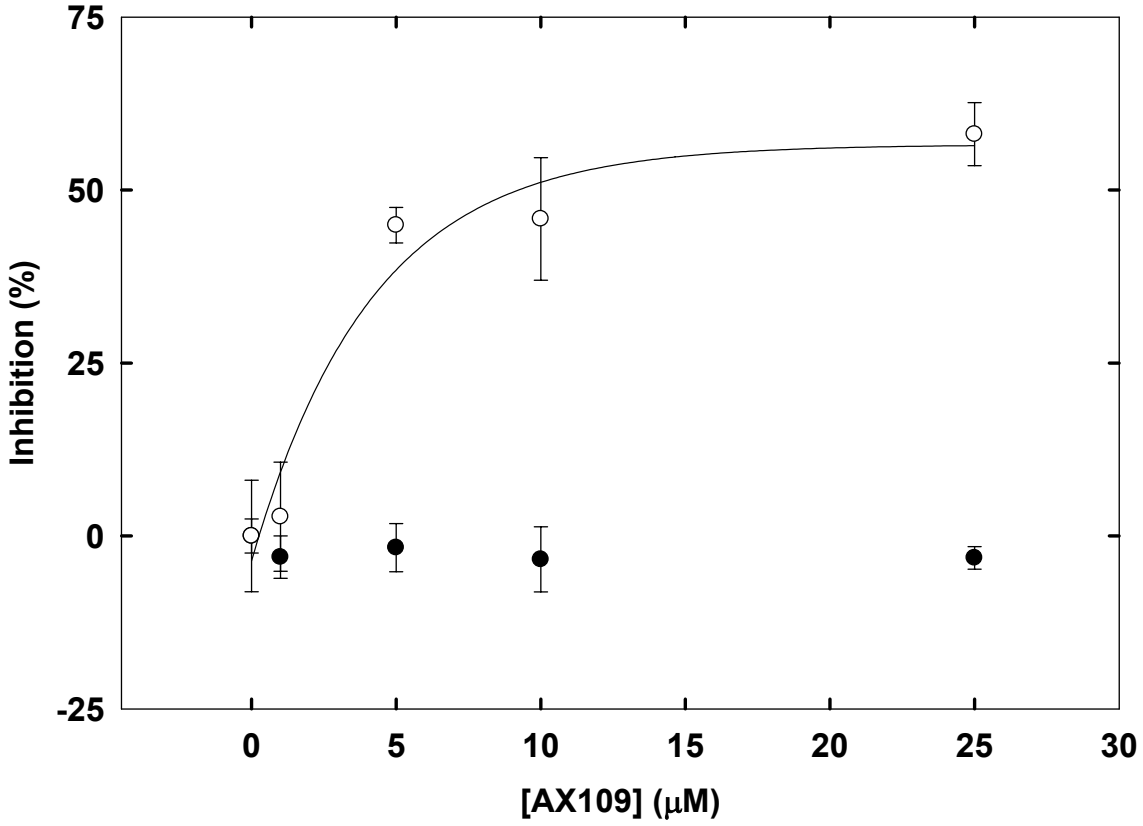


Figure V.9. AX109 inhibitory dose curve of Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages.

Figure depicts the effect of increasing doses of AX109 on Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages. Cells that were treated with Kdo_2-Lipid A are indicated with (○) and control cells that were not stimulated with Kdo_2-Lipid A are represented by (●). All data points were measured at 1 hour after stimulation of the cells.

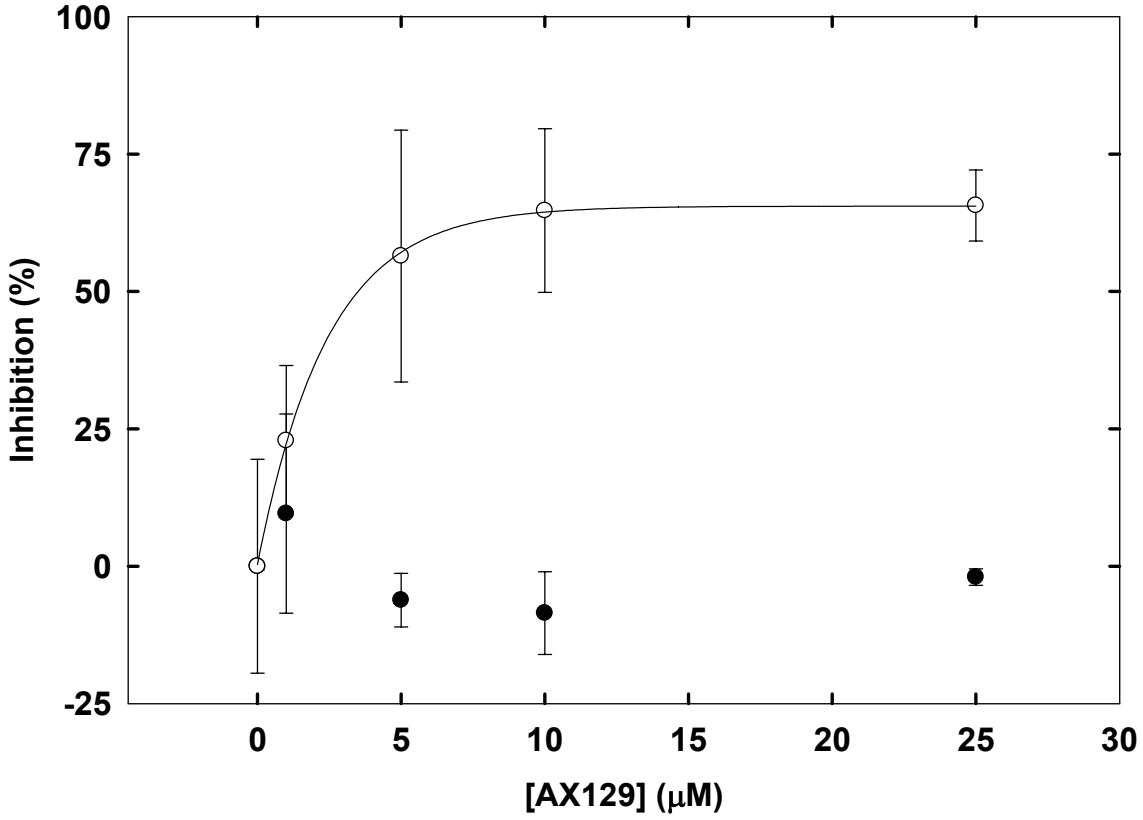


Figure V.10. AX129 inhibitory dose curve of Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages.

Figure depicts the effect of increasing doses of AX129 on Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages. Cells that were treated with Kdo_2-Lipid A are indicated with (○) and control cells that were not stimulated with Kdo_2-Lipid A are represented by (●). All data points were measured at 1 hour after stimulation of the cells.

2. Evaluation of the Analgesic and Anti-inflammatory Properties of the GIVA PLA_2 Chemical Inhibitors in an in vivo Rat Model

In an effort to establish whether the effective *ex vivo* inhibitors demonstrate potential therapeutic advantage, Dimitra Hadjipavlou-Litina employed the acetic acid writhing assay to evaluate the analgesic properties of the inhibitors, while the rat-paw carageenan assay was employed to determine the anti-inflammatory properties of the inhibitors (241,243,245). Four inhibitors were tested in these experiments, which included AX006, AX007, AX062 and AX109. All four inhibitors demonstrated analgesic properties of 60-90% when dosed at the ED_{50} within the animals. The anti-inflammatory properties of the inhibitors were significantly more variant than the analgesic characteristics; AX007 demonstrated the minimal anti-inflammtory potency amongst the inhibitors tested, with an ED_{50} of 0.008 mmol/kg, while AX109 showed a maximal value at 0.00005 mmol/kg.

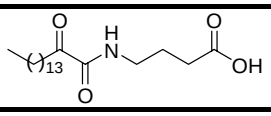
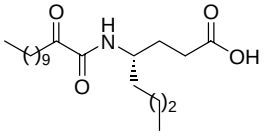
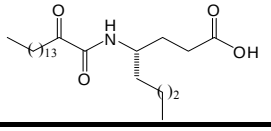
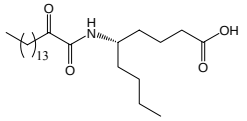
E. Discussion

1. Summary of 2-oxoamide structure GIVA PLA_2 Inhibitory Properties

In this study, we tested a range of GIVA PLA_2 inhibitors that are phospholipid substrate structural analogues in an *ex vivo* macrophage model to better understand cellular GIVA PLA_2 chemical inhibition. Our data suggest that structures containing a carboxylic acid functional group spaced at the δ or γ positions relative to the 2-oxoamide moiety are the most effective GIVA PLA_2 inhibitors. Structures containing the 2-oxoamide moiety and esters demonstrated *in vitro* and *in vivo* GIVA PLA_2 inhibitors, although these inhibitors did not display the same potency nor fidelity as the carboxylic acid functional

Table V.4. Summary of the anti-inflammatory and analgesic properties of the GIVA PLA_2 chemical inhibitors.

Data adapted from the following references: (241,243).

I.D.	STRUCTURE	Mol. Wt.	Anti-Inflammatory activity ED_{50} (mmol/kg)	Analgesic activity (%)
AX006		355.52	**0.01**	**93**
AX007		355.51	**0.1**	**63**
AX062		411.62	**0.008**	**65**
AX109		425.64	**0.00005**	**71**

group containing inhibitors did. Fluoroketone functional group-containing structures display *in vitro* inhibition but are not functional within the inflammatory RAW 264.7 cellular system.

The data suggest that the most effective inhibitors contain long, 2-oxoacyl chains; likely, this motif is essential for insertion into the hydrophobic funnel within the enzyme active site. This is best exemplified by the fact that all structures that reduced cellular AA release from the RAW 264.7 cells have lipophilic chain lengths of 14 carbons with the exception of AX007, which has only 10 (Table V.3). Furthermore, of the eight inhibitors described in table V.3 that had inhibited cellular AA release, four of the structures were tested in a rat model of inflammation (as described in table V.4). All four of these structures displayed anti-inflammatory and analgesic properties. Interestingly, AX007 displayed the most concentrated ED_{50} value amongst the four inhibitors that were tested. Since the AX007 structure contains the shortest 2-oxoacyl chain amongst these inhibitors, the data suggest that structures which contain longer hydrophobic side chains should display higher *in vivo* inhibitory potency in the rat model. Whether the shorter, secondary hydrophobic side chain is necessary to retain inhibitory properties is not clear from our current data. This is best exemplified by AX006, which has no secondary side chain, but inhibited cellular AA release and demonstrated *in vitro* inhibition of GIVA PLA_2 in a manner that was similar to the other seven effective inhibitors.

It is important to note that the structures that contain either a fluoroketone or ester functional group had shown significant inhibition of *in vitro* GIVA PLA_2 activity but did not reduce cellular AA release from Kdo_2-Lipid A stimulated RAW 264.7 macrophages. This observation may be the result of several potential issues associated with measuring

inhibition using a cellular model. The first possibility is that the inhibitors may not have translocated across the plasma membrane into the cellular cytosol and therefore were not been available to bind to GIVA PLA_2 protein. The second possibility is the inhibitors may undergo cellular metabolism within the cytosol, which would result in the inactivation of effective inhibitors. Third, it is possible that these inhibitors have a higher binding affinity for other cytosolic proteins that detracts from interaction with GIVA PLA_2.

2. Fidelity of Structural 2-oxoamide Inhibitors for blockage of GIVA PLA_2 Activity compared to GV and GVI PLA_2 Activity

Along with developing inhibitors that exhibit potent GIVA PLA_2 inhibition, it was equally important to identify which motifs would demonstrate fidelity and therefore not affect other PLA_2 enzymes. The current data suggest that 2-oxoamide inhibitors containing a charged functional group, such as a carboxylic acid, demonstrate the highest GIVA PLA_2 fidelity. Inhibitors containing the combination of the 2-oxoamide moiety with the carboxylic acid inhibited only GIVA PLA_2 *in vitro* activity, whereas 2-oxoamide structures that contained esters and fluorketones functional groups inhibited GIVA PLA_2 activity as well as GV and GVI PLA_2. Collectively, this data suggest that GIVA PLA_2 chemical inhibitors designed to resemble phospholipid substrates that carry an electrostatic charge display the highest preference for GIVA PLA_2 relative to that of other PLA_2 enzymes.

3. Challenge of the Balance of GIVA PLA_2 Inhibitory Design with Water Solubility

An additional consideration in the design of improved inhibitor structures is chemical solubility in aqueous solutions. Drug delivery is a serious challenge that pharmaceutical companies encounter while trying to devise physiologically relevant drugs, and the same has been true in the development of specific, effective GIVA PLA_2 inhibitors. For example, pyrrophenone and propane-2-one, which are two commercially available GIVA PLA_2 chemical inhibitors, are not readily soluble in aqueous solutions. This is an arduous challenge in the design of effective GIVA PLA_2 substrate-like inhibitors, as the hydrophobic side chains seem to be essential to inhibitory efficacy while also making the structure less water soluble. This statement is highlighted by the fact that the ED_{50} of AX007 is much larger than AX062 and AX109 despite containing the shortest 2-oxoacyl chain and being significantly more water soluble. In the design of effective new inhibitors, it will be crucial to design a side chain which will allow satisfactory water solubility in order to achieve delivery of the inhibitor to the cell membrane.

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