The Influence of Adenoviral Infection and the Group VIA Calcium-Independent Phospholipase A2 on Hepatic Lipid Metabolism

William Palmer Wilkins III
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THE INFLUENCE OF ADENOVIRAL INFECTION AND THE GROUP VIA
CALCIUM-INDEPENDENT
PHOSPHOLIPASE A₂ ON HEPATIC LIPID METABOLISM

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy at Virginia Commonwealth University.

by

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<td>arachidonic acid</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl-CoA carboxylase</td>
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<tr>
<td>Ad</td>
<td>adenovirus</td>
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<tr>
<td>Ad-5</td>
<td>adenovirus-5</td>
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<tr>
<td>alkphos</td>
<td>alkaline phosphatase</td>
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<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>BEL</td>
<td>bromoenol lactone</td>
</tr>
<tr>
<td>bHLHLZ</td>
<td>basic helix-loop-helix leucine zipper</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackie and adenovirus receptor</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
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<tr>
<td>CT alpha</td>
<td>CTP: phosphocholine cytidylyltransferase-alpha</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DPPC</td>
<td>1-palmitoyl-2-palmitoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>E1, E2, E3</td>
<td>early gene 1, early gene 2, early gene 3, etc.</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acid</td>
</tr>
<tr>
<td>FAS</td>
<td>fatty acid synthase</td>
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<tr>
<td>FOXO1</td>
<td>the forkhead box O transcription factor FOXO1</td>
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Beta-gal  beta-glactosidase
GFP    green fluorescent protein
G6Pases glucose-6-phosphatase
HCV    hepatitis C virus
HMG CoA reductase 3-hydroxy-3-methylglutaryl CoA reductase
iPLA$_2$ Group VIA calcium-independent phospholipase A$_2$
LDL    low density lipoprotein
LPA    lysophosphatidic acid
LPC    lysophosphatidylcholine
LXR    liver X receptor
lysoPC lysophosphatidylcholine
lysoPL lysophospholipid
MOI    multiplicity of infection
MTD    maximum tolerated dose
MTP    microsomal transport protein
NAFLD non-alcoholic fatty liver disease
ORO    oil red o
PC     phosphatidylcholine
PEPCK  phosphoenolpyruvate carboxykinase
PPAR   peroxisome proliferator-activated receptor
PI     phosphatidylinositol
PI3K   phosphatidylinositol 3-kinase
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<td>PL</td>
<td>phospholipid</td>
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<td>PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
</tr>
<tr>
<td>S1P</td>
<td>site-1 protease</td>
</tr>
<tr>
<td>S2P</td>
<td>site-2 protease</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage-activating protein</td>
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<tr>
<td>SRE</td>
<td>sterol response element</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>UFA</td>
<td>unsaturated fatty acid</td>
</tr>
<tr>
<td>UPR</td>
<td>unfolded protein response</td>
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<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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Abstract

THE INFLUENCE OF ADENOVIRAL INFECTION AND THE GROUP VIA CALCIUM-INDEPENDENT PHOSPHOLIPASE A2 ON HEPATIC LIPID METABOLISM

By William Palmer Wilkins, III, Bachelor of Science

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2008

Major Director: Suzanne E. Barbour, PhD
Professor, Department of Biochemistry and Molecular Biology

Sterol regulatory element-binding proteins (SREBP) are transcription factors that regulate genes involved in lipid metabolism especially in the liver. Therefore, hepatic SREBP is a significant regulator of systemic lipid metabolism. Evidence demonstrates that insulin and dietary unsaturated fatty acid (UFA) regulate SREBP1 expression and subsequent SREBP1-mediated gene transcription, events that in many instances result in modulation of systemic fatty acid and triglyceride (TG) homeostasis. A series of investigations was designed to uncover novel regulators of SREBP1. Dietary and exogenous addition of UFA has been shown to regulate SREBP function; yet, an
endogenous source of UFA capable of modulating SREBP remains elusive. Group VIA calcium-independent phospholipase A\textsubscript{2} (iPLA\textsubscript{2}) releases UFA from the sn-2 position of glycerophospholipids. We hypothesized that iPLA\textsubscript{2} provides UFA to suppress SREBP. iPLA\textsubscript{2} overexpression and inhibition studies were implemented. iPLA\textsubscript{2} inhibition increased SREBP1 expression, SREBP-mediated transcription and the expression of SREBP1 gene targets \textit{in vitro}. \textit{In vivo} overexpression of iPLA\textsubscript{2} resulted in decreased expression of SREBP1 protein and plasma triglyceride. In contrast, iPLA\textsubscript{2} overexpression attenuated SREBP1 expression, SREBP-mediated transcription and expression of SREBP1 targets genes. These data support the hypothesis that iPLA\textsubscript{2} generates endogenous UFA that limit SREBP function. Use of a replication-deficient adenovirus 5 (Ad-5) expression vector in the iPLA\textsubscript{2} study led to the unexpected observation of hepatic SREBP1 activation following Ad-5 infection. Because of this observation, we tested the hypothesis that replication-deficient Ad-5 might augment lipid synthesis in liver. We demonstrate that first generation Ad-5, a ubiquitous transgene expression vector, induces expression of SREBP1 and its target genes and leads to increases in fatty acid synthesis \textit{in vivo} and \textit{in vitro}. The phosphatidylinositol 3-kinase (PI3K) inhibitor, PX-866, suppressed Ad-5-induced SREBP1 expression and hypertriglyceridemia implicating the PI3K/Akt pathway in Ad-5 activation of SREBP1. Use of PX-866 led to the discovery of a third mechanism of SREBP1 regulation. \textit{In vivo} studies demonstrate that PX-866 modulates basal lipid metabolism in part through decreasing plasma TG, an increased trend toward decreased SREBP1 expression and a
significant increase in plasma cholesterol. These studies characterize three distinct novel regulatory mechanisms of SREBP1.
CHAPTER 1: General Introduction

Cellular lipids serve several roles beyond providing a source of energy and the building blocks of the cell-membrane architecture (1). It is now well appreciated that lipids themselves greatly impact cellular function and homeostasis as studies provide ample evidence that lipids are often bioactive and via intracellular signaling and/or endocrine, paracrine or autocrine action are capable of influencing diverse cellular processes often facilitated by modulation of gene expression (2-9). Consequently, aberrant lipid metabolism itself plays an etiological role in many disease states including diabetes, liver disease, cancer, nervous system dysfunction and atherosclerosis (10-15). The investigation of several distinct elements and their mechanisms that impact lipid metabolism and/or regulate the expression of proteins involved in lipid metabolism is warranted and is the major focus of these studies. The following studies characterize novel regulation of lipid metabolism by first-generation adenovirus-5 (Ad-5), commonly used as an expression vector in research (16-19) and gene therapy (20); the Group VIA calcium-independent phospholipase A$_2$ (iPLA$_2$), an enzyme already appreciated for its roles in lipid metabolism and a novel phosphatidylinositol 3-kinase (PI3K) inhibitor PX-866 (21).
Examples of the influence of lipids on cellular processes and signaling

Proper cell signaling and function rely on all classes of lipids. For instance, cholesterol-derived steroid hormones diffuse across the plasma membrane to subsequently bind intracellular steroid hormone receptors which translocate to the cell nucleus and act as nuclear transcription factors (22). Other steroid hormones modulate cellular processes that can result in inflammation, suppression of the immune response, allergic reactions, proper or improper organ function, blood-pressure maintenance and energy mobilization (23). Cholesterol itself can modulate cellular function and homeostasis. Enrichment of cholesterol in the ER can initiate the unfolded protein response (UPR) in macrophages, an event capable of resulting in cell apoptosis (24). Cholesterol can also affect transcription via its inhibition of the transcription factor sterol regulatory element binding proteins (SREBP), a fundamental regulator of genes involved in cholesterol, fatty acid and triglyceride synthesis in the liver and other tissues (25).

The sphingolipids, sphingosine-1-phosphate and ceramide, synthesized via the sphingomyelinase (Smase) hydrolysis of sphingomyelin or de novo synthesis (1), are highly bioactive and enormously impact cell motility, proliferation and apoptosis, survival and cancer cell biology (26;27). Beyond its prosurvival and mitogenic influence, sphingosine-1-phosphate has been implicated as a molecular player in immune system function (28), cardiac development and angiogenesis (29). Ceramide on the other hand has been implicated in cell growth arrest (29) the stress response and
subsequent results including apoptosis and growth suppression (30). The cellular implications of this lipid are clinically relevant in cancer biology. Studies demonstrate that chemotherapeutic agents often increase levels of ceramide, an event that has prompted research into its potential in the treatment of cancer (31). In contrast, sphingosine kinase, responsible for the conversion of sphingosine to sphingosine-1-phosphate (1), is arguably an oncogene as its generation of sphingosine-1-phosphate promotes cell proliferation (32).

Like the sphingolipids, glycerophospholipid derivatives are often exceptionally bioactive lipids capable of guiding cellular function. Phospholipase C catabolism of phosphatidylinositol-bisphosphate generates diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP$_3$) (33;34). The release of IP$_3$ can induce the release of calcium stores from the endoplasmic reticulum (ER), an event central to many cellular processes. Released ER calcium facilitates the translocation of protein kinase C (PKC) to the plasma membrane where it is activated by DAG and, in turn, can regulate many second-messenger proteins (34). Furthermore, there is evidence to suggest a role for IP$_3$-mediated calcium release in the secretion of insulin from the pancreas (35;36), thus implicating a bioactive role for IP$_3$ and lipids in general in the control of glucose levels and in the pathology of diabetes. Phospholipase A$_2$ (PLA$_2$) enzymes catabolize membrane glycerophospholipids to release unsaturated fatty acid from the sn-2 position and a lysophospholipid (37). Further enzymatic action by lysophospholipase D on the lysophospholipid released by phospholipase A$_2$ yields mitogenic lysophosphatidic acid (LPA), an extremely potent lipid signaling molecule as virtually all mammalian cells
respond to this lipid (38). Polyunsaturated fatty acid (PUFA) released from the sn-2 position of membrane glycerophospholipids is bioactive in many respects. Exogenous unsaturated fatty acids, like cholesterol, can modulate SREBP regulation of lipogenesis (39), thus providing a role for fatty acid bioactivity in atherosclerosis and lipid disorders. As will be mentioned, PLA₂-generated free fatty acid can potentially influence lipid metabolism via SREBP. In inflammatory cells, arachidonic acid commonly occupies the sn-2 glycerophospholipid position (40;41). Following PLA₂-mediated hydrolysis, released arachidonic acid can be further metabolized to produce eicosanoids, leukotrienes and other inflammatory mediators (42). PUFA has also been shown to increase sphingomyelinase (Smase) activity resulting in the generation of ceramide (43) as well as serving as a ligand for peroxisome-proliferator-activated receptors (PPAR) which can impact cancer cell biology and lipid metabolism (44). Another phosphoglyceride species, platelet-activating factor (PAF), is potently bioactive as it, among other activities, is an inflammatory mediator to which macrophages, platelets, neutrophils and other cells respond (45) and plays a role in liver damage (46). While incomplete, these cursory details above underscore the significance of lipids to proper—and improper—cell function and can provide an initial framework to understanding the etiological role that aberrant lipid metabolism plays in many diseases. The studies presented here will examine the effect of Ad-5, iPLA₂ and PX-866 on lipid metabolism with an emphasis on SREBP and the liver.
Sterol regulatory element-binding proteins

Many of the genes encoding proteins involved in fatty acid (FA), triglyceride (TG), and cholesterol synthesis contain sterol response elements (SRE) that allow for feedback regulation of expression (25;47;48). SREs promote gene expression when bound by sterol regulatory element binding proteins (SREBPs), members of the basic-helix-loop-helix family of transcription factors. Genes regulated by mature active SREBP include many of the genes encoding proteins involved in the synthesis and uptake of cholesterol, triglycerides and fatty acids (25) (Figure 1). These genes include the group VIA calcium-independent phospholipase A₂ (49), cholesterol-associated proteins such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), low-density-lipoprotein (LDL) receptor, and squalene synthase, fatty acid-associated synthesis genes including fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC) (50). SREBPs are characterized by their dual-DNA-binding specificity—the ability to recognize both a unique sequence and a common structural motif of DNA (51)--and in their initial expression as an immature precursor protein bound to the ER. These transcription factors bind the canonical inverted repeat E-box sequence 5’-CAXXTG-3’, typically recognized by bHLHLZ family members and the SRE, the direct repeat sequence 5’-TCACXCCAC-3’ (52).

The SREBPs are encoded by two genes, generating SREBP1 and SREBP2 proteins. The SREBP1 gene has two transcription start sites, which generate two forms of SREBP1: SREBP1a and SREBP1c. In general, SREBP2 regulates genes involved in cholesterol synthesis and metabolism, SREBP1c is more closely associated with the
synthesis of fatty acids and triglycerides and SREBP1a induces both families of genes when its mature form is over expressed in mouse liver (53;54). SREBP1c appears to be the most relevant isoform in vivo especially in the liver and in primary cells while cultured cells express primarily SREBP1a. However, transcriptional activity of SREBP1c is less robust than that of SREBP1a, likely due to the truncated acidic domain at the N-terminus of SREBP1c.

The nuclear pool of SREBP is controlled at two levels: expression of precursor SREBP protein and processing to its mature, transcriptionally active form (25;48). Processive regulation of SREBP has been characterized as follows (25): In sterol-loaded cells, SREBP is bound to the membrane of the ER in its inactive precursor form. The regulatory domain of approximately 590 amino acids at the SREBP C-terminus binds to the SREBP cleavage-activating protein (SCAP) which in the absence of sterols associates with COPII proteins, form COPII-coated vesicles and bud from the ER and subsequently fuse to the golgi (55;56). SCAP acts as a sterol sensor. When sterol levels decline, SCAP favors a conformation that dissociates it from the ER resident protein Insig and promotes translocation of SREBP1 from the ER to the golgi apparatus (57;58). SCAP and Insig have been shown to bind cholesterol and oxysterols, respectively, and the binding of either sterol induces association between the two proteins resulting in the retention of SREBP in the ER (59-61). Recently, Du et al demonstrated that Akt activation is required for SCAP to facilitate the translocation of SREBP2 and presumably SREBP1 from the ER to the golgi (62). Two proteases in the Golgi membrane designated site-1 protease (S1P) and site-2 protease (S2P) release the
N-terminal “business end” of SREBP yielding the processed mature transcription factor. This “activated” form of SREBP translocates to the nucleus to effect transcription of its target genes. SREBP processing is an essential event in the regulation of lipid synthesis and metabolism in the liver. This event is subject to feedback regulation by both sterols and polyunsaturated fatty acids which suppress translocation and processing (39), though currently no evidence indicates the ability of PUFA to directly bind SCAP or Insig to inhibit SREBP processing.
**Figure 1:** Gene targets regulated by SREBP in cholesterol and fatty acid metabolism
**Early studies characterizing SREBP**

The use of knockout and transgenic mice led to the characterization of SREBP, its targets and highlighted its critical role in lipid metabolism especially in the liver and adipose tissue. Dietary manipulation in mouse and hamster models also contributed to the early characterization of SREBP1 and 2 in the liver. Hamster hepatic SREBP1 and 2 was shown to decrease in response to a high-cholesterol diet (63). Hamsters fed a low-fat, high-carbohydrate diet showed increases in the transcriptionally active nuclear form of SREBP1 (64) whereas in hamsters treated with the HMG CoA synthesis inhibitor lovastatin, hepatic, nuclear SREBP2 increased (65). Although germline deletions of SREBP1, which deleted both SREBP1a and 1c, and deletions of SREBP1c only resulted in partial lethality, surviving SREBP1 and SREBP1c specific knockout mice showed decreased hepatic FAS expression, hepatic FA synthesis and decreased circulating TG (66;67). Because germline deletion of SREBP2, 1c or 1a resulted in complete lethality or partial lethality, respectively (25;66-68), the laboratory of Brown and Goldstein resorted to a Cre recombinase system in order to disrupt the S1P and SCAP genes in adult mice which resulted in adequate inhibition of the transcriptionally active nuclear forms of SREBP1 and 2 (68;69). Analysis of the livers of these knockout mouse demonstrated decreased expression of the SREBP targets HMG CoA reductase and FAS and a 70 to 80% decrease in fatty acid and cholesterol content. Identification of several mutant Chinese hamster ovary (CHO) cell lines with deficiency in the S1P and S2P further support the *in vivo* results as these mutations created cholesterol
auxotrophs incapable of processing SREBP2 and subsequent transcription of its targets HMG CoA reductase, HMG CoA synthase and the LDL receptor (50;70-72).

Transgenic mice overexpressing nuclear SREBP1a and c and SREBP2 lacking the transmembrane domains that tether precursor SREBP to the ER and mice overexpressing a dominant-negative SCAP protein support conclusions drawn from the aforementioned gene deletion studies (25;50). Dominant-negative SCAP mice exhibit elevated hepatic nuclear SREBP1a and 1c and SREBP2, increased expression of SREBP1 and 2 target genes, increased FA and cholesterol synthesis and increased liver FA and cholesterol mass (73). Overexpression of SREBP1a resulted in increases in cholesterol and FA synthesis, liver cholesterol and FA mass and increases in cholesterol and FA biosynthetic gene expression (54). Furthermore, due to the extreme increases in hepatic lipid mass, these mice developed significantly larger livers. As expected, overexpression of SREBP1c increased FAS gene expression, increased in FA synthesis and hepatic FA mass by four-fold whereas overexpression of SREBP2 increased cholesterol synthetic gene expression, hepatic cholesterol synthesis and mass with moderate elevations in FA and FA synthetic genes indicating a slight ability of SREBP2 to regulate genes involved in FA metabolism (53;74).

**Regulation of SREBP by PUFA**

As mentioned, sterols have been shown to inhibit SREBP processing and thus the transcription of its target genes and other studies indicate that exogenous unsaturated fatty acids demonstrate this inhibitory role as well (39;75;76). Expression
of SREBP is thought to be controlled in several ways. The promoter of the SREBP gene itself contains SREs to provide what has been termed a “feed-forward” mechanism of regulation (25,77,78). In addition to sterols, polyunsaturated fatty acids (PUFAs) also suppress SREBP processing and translocation (39,43,79). The effect of PUFAs appears to be largely specific for SREBP1 (76,80) and therefore effects of PUFAs are most apparent on fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), and other lipogenic genes associated with triglyceride synthesis. SREBP1c expression is another target for PUFA, which antagonizes LXR-mediated induction of SREBP1c expression (81-84). Proposed mechanisms include reduced LXR binding to the LXR response element (LXRE) in the SREBP1c promoter and reduced association with coactivators (81,82) in the presence of PUFA, although a recent study suggests that dietary PUFA may regulate SREBP1c in a LXR-independent manner (84). To this end, there is also evidence that PUFA promote increased turnover of SREBP1c mRNA and proteasome-mediated catabolism of mature SREBP1c (85,86). Together, these data indicate that PUFA likely suppress hepatic lipogenesis by regulating SREBP1c at a variety of levels.

**SREBP and insulin signaling**

SREBP1c is also regulated at the expression level by insulin which promotes lipogenesis largely through induction of this protein (25,83). Notably, activation of SREBP1c by insulin is crucial to lipid metabolism in the liver (87). In primary rat hepatocytes, inhibition of the PI3K/Akt pathway with LY294002 and wortmannin attenuates SREBP1c expression in response to insulin (88). Further characterizing the
activation of SREBP1c expression by insulin is a recent study demonstrating that atypical protein kinases C ζ and λ play roles as well (89). This study found that disrupted insulin-activated signaling via atypical protein kinases C ζ and λ results in decreased SREBP1c expression. Other studies have further demonstrated that liver X receptors (LXR) are essential for basal and as well as insulin-stimulated SREBP1c expression (83;90;91). LXR induces SREBP1c transcription by binding to a ACCTCA direct repeat (DR-4) together with retinoid X receptor (RXR) (92). Ligand binding (LXR: oxysterol; RXR: retinoic acid) induces dissociation of the corepressors SMRT and NCoR followed by association of coactivators (SRC-1) that drive transcription of the SREBP1c gene (93;94). In addition, the SREBP1c promoter has a SRE that provides positive feedback regulation of its expression (95).

Influence of microorganisms on lipid metabolism and the biology of Adenovirus-5

A growing body of literature relates viral (96-100), bacterial (101;102), and parasite (103) infections to changes in metabolism, dyslipidemia, fatty liver disease, and obesity. For example, enteroviruses, particularly coxsackie B4 virus, target pancreatic β cells, leading to type I diabetes secondary to reduced insulin secretion (96;97). Perhaps the best characterized example is that of chronic hepatitis C virus (HCV) infection which has been strongly associated with insulin resistance, non-alcoholic fatty liver disease (NAFLD) and increased expression of lipogenic genes in hepatocytes (98;99;104). Because viral expression vectors are often used for transgene expression and gene therapy, the potential for these viral expression vectors,
specifically Ad-5 based vectors, to induce aberrant lipid metabolism is an attractive and necessary investigation.

Since the discovery of the human adenovirus in 1953 (105), an etiological factor in upper respiratory tract infection (106), replication-deficient adenoviruses have been widely employed for gene transfer in vitro and in vivo studies (20). While their efficacy in gene therapy presents many challenges, adenoviruses are currently used in clinical trials to treat cancer, cardiovascular disease and several inherited disorders (20). Notably, the potential for a severe immune response as illustrated by the death of Jesse Gelsinger is one of several obstacles facing researchers in the field of gene therapy (20;107). Another challenge to adenovirus-mediated gene therapy is the ability to target specific tissues as most adenovirus serotypes initially associate with the Coxsackie and adenovirus receptor (CAR), a receptor expressed broadly among many cell types (20). Though modifications to adenoviruses have improved tissue specificity, there is the propensity for significant natural tropism of the adenovirus to the liver in vivo (108). Adenoviruses have also been employed to study liver function in vivo and may hold promise as a vector to treat liver disorders (16;18).

Unlike retroviruses, adenoviruses do not integrate into the host cell genome. Ad-5 and Ad-2 of subgroup C of the adenoviruses are two of the most extensively studied adenoviruses and in humans are typically associated with upper respiratory tract infections (109). Crucial to adenovirus structure is the fiber protein containing a distal fiber knob that interacts with host cell CAR and the penton base adjacent to where the
fiber shaft meets the icosahedral protein capsid. The penton base contains an arginine-glycine-aspartate (RGD) motif that interacts with host cell alpha and beta integrins and with CAR binding to the fiber knob, the adenovirus enters the host cell (109;110). Intracellularly, infection by Ad-5 and other adenoviruses has been shown to activate the PI3K/Akt pathway (111;112)

Of the adenoviruses, Ad-5 is most often employed for transgene expression. Ad-5 has a genome consisting of linear double-stranded DNA molecule of 36 kb in which transgenes typically are inserted in place of either early gene 1 (E1) and early gene 3 (E3) (113). There are two “sets” or regions of genes in the genome of Ad-5 and other adenoviruses. The early genes are involved in what could be considered “priming” the host cell for viral replication whereas the late genes are responsible for encoding viral structural proteins (109). The E1A coding region of E1 interacts with host cell transcription factors to facilitate the transcription of other early genes and promotes expression of proteins critical to adenoviral replication. E1B is responsible for sequestering p53 to promote cell survival during viral replication. The E3 region acts to suppress the host immune response as well as inhibit host-cell apoptosis (109).

First-generation Ad-5 used for transgene expression are replication-deficient due to deletions in of the E1 region and/or a complete or partial deletion of region E3 (20). Studies using these vectors have uncovered several shortcomings of their use. Replication deficient Ad-5 was used in clinical trials for the first time to treat cystic fibrosis and due to poor transgene expression prompted the use of second generation
Ad-5 with E4 deleted or E3 returned to the Ad-5 genome (20;114). Transgene expression using first-generation Ad-5 was shown to be limited with undetectable expression occurring at 2 to 3 weeks post infection \textit{in vivo} (115). As mentioned, \textit{in vivo} use of Ad-5 results in significant tropism to the liver and therefore represents an obstacle to targeting extrahepatic tissues (109).

Despite the significant liver tropism of Ad-5, few studies have characterized the effect of adenovirus infection on hepatocyte function aside from adenovirus-induced hepatotoxicity. A recent report demonstrated that disruption of lipid metabolism plays a role in liver pathology following liver infection by hepatitis C virus (HCV) (98). Other studies allude to a correlation between adenoviral infection and aberrant lipid metabolism in other tissues. Several serotypes of adenovirus have been shown to increase adipocyte differentiation and triglyceride accumulation in adipocytes (116). Rats infected with adenovirus-36 (Ad-36) showed an increase the expression of adipocyte acetyl CoA-carboxylase (ACC) and fatty acid synthase (FAS) (117) in adipose tissue while Ad-5 infection has been linked to increased adiposity in mice (118). There are 51 identified serotypes of adenoviruses. Of those, Ad-5 has been exploited in its replication-deficient form as an \textit{in vivo} and \textit{in vitro} expression vector. Additionally, Ad-5 is currently a candidate gene-therapy vector for several clinical trials (119). Based on the aforementioned studies, the potential for adenovirus tropism to the liver, and the use of adenoviruses to study hepatocyte function and potentially treat liver
disorders, studies were designed to test investigate the potential for Ad-5 to influence lipid metabolism via SREBP.
I. Glycerophospholipids and Phospholipases

Glycerophospholipids (GPL) are major components of cell membranes. Until the mid 1980’s their function in cell biology was thought to be limited to a “bricks and mortar” role as constituents of cell membranes. However, the pioneering work of Irvine, Nishizuka, Berridge, and others (120-122) demonstrated that GPL have information content that is unleashed when these molecules are metabolized by phospholipases, a family of enzymes that hydrolyze ester bonds in GPL. The phospholipases $A_2$ (PLA$_2$) are defined as acylhydrolases that add water across the $sn$-$2$ ester bond, generating lysophospholipid and unesterified fatty acid products, both of which have the potential to be metabolized into biologically active signaling molecules. Although some members of this family also hydrolyze lysophospholipid (generating glycerophosphocholine and unesterified fatty acid products), the enzymes are classified together based on the phospholipase reaction.

PLA$_2$ enzymes have been categorized into 15 groups based on sequence homology and catalytic mechanism (123). Although the group classification scheme has been extremely useful for investigators in the PLA$_2$ field, a streamlined and more simplified categorization has gained popularity among researchers in general. In this scheme, PLA$_2$ enzymes are classified into four categories: secreted PLA$_2$ (sPLA$_2$), cytosolic...
calcium dependent PLA₂ (cPLA₂), platelet-activating factor acetylhydrolase (PAFAH), and cytosolic calcium independent PLA₂ (iPLA₂). The sPLA₂s are relatively small (14-18 kDa) enzymes with rigid tertiary structures largely dictated by 5-7 disulfide bonds. These enzymes are strictly dependent on calcium which is involved in their catalytic mechanisms. Given the calcium requirement of sPLA₂s (millimolar range) and the requirement for disulfide bonds in their tertiary structures, it has long been proposed that these are secreted enzymes that hydrolyze substrate in the extracellular space (124). However, a recent study shows that sPLA₂ activity in gastric mucosal cells is not suppressed by a membrane impermeant inhibitor suggesting that the enzyme is active in an intracellular compartment (125). The cPLA₂s are a broad class of enzymes which display both phospholipase and lysophospholipase activity (126). The best studied of these enzymes, cPLA₂α (also known as the group IVA PLA₂), exhibits the unique property of selective hydrolysis of arachidonyl GPL making it a key player in arachidonic acid (AA) mobilization and eicosanoid production (123;126). In addition, some members of the cPLA₂ family translocate to membranes in response to increases in cytosolic calcium. This effect is mediated through the C2 domain, a motif that directs calcium-dependent binding to membrane GPL. PAFAH enzymes are distinguished from the other PLA₂s by their substrate specificity which is focused on GPL containing relatively short native sn-2 acyl chains or oxidatively fragmented fatty acids such as those in oxidized low density lipoprotein (LDL) and damaged cell membranes (127). Both intracellular and secreted PAFAH isoforms have been identified. Importantly, the secreted group VIIA enzyme associates with lipoproteins and is the lipoprotein
associated PLA$_2$ (LpPLA$_2$) that has been connected with atherosclerosis and other vascular diseases (128;129).

The iPLA$_2$s are among the most recently described and least well-characterized of the PLA$_2$s. These enzymes have the unique property that they are active in the absence of calcium, as this divalent cation is neither required for catalytic activity nor for translocation to membrane. Indeed, some studies suggest that iPLA$_2$ activity is induced upon calcium depletion (130), suggesting that intracellular calcium may negatively regulate iPLA$_2$ enzymes (see below). Interestingly, several recent studies suggest essential roles for iPLA$_2$ in the regulation of store operated calcium channels (131). Like cPLA$_2$, these enzymes exhibit significant homology to patatin, a lipase from potato (126). Homology searches have identified iPLA$_2$ enzymes in a variety of species ranging from \textit{Pseudomonas aeruginosa} bacteria which express ExoU, a phospholipase virulence factor that is cytotoxic to mammalian host cells (132) to Arabidopsis in which AtPLA1 regulates basal jasmonic acid synthesis and is involved in resistance to fungal infection (133), to the potato enzyme patatin, to human where iPLA$_2$ has been implicated in ischemia-reperfusion injury following myocardial infarction (134;135), insulin sensitivity (136;137), and other biomedically relevant conditions (see below). Until recently, it was thought that the family of iPLA$_2$ enzymes was derived from only two genes which encoded the group VIA (also known as iPLA$_2$$\beta$) and group VIB (iPLA$_2$$\gamma$) enzymes. However, recent sequence homology searches have identified additional members of the family (group VIC, group VID, group VIE, and group VIF
enzymes) that have the signature nucleotide binding motif (GxGxxG) and lipase consensus sequence (GxSxG) (138;139). iPLA₂β exhibits broad tissue distribution, is constitutively expressed, and has important roles in basal GPL metabolism (123;140). In contrast, although iPLA₂γ is expressed in a variety of tissues, especially high expression has been observed in heart and kidney (141-143). As mentioned above, iPLA₂ is involved in pathogenesis of several medically relevant disorders. Hence, the iPLA₂s may be important targets for drug development. The group VIC, VID, VIE, and VIF enzymes have only recently been described and relatively little is known about them.

II. iPLA₂ Structure

The iPLA₂ enzymes are classified in group VI based on conserved structural characteristics including a C-terminal lipase consensus motif (GXSXG). The active site serine is the nucleophile that attacks the sn-2 carbonyl, generating an acyl-enzyme intermediate. iPLA₂ enzymes also exhibit lysophospholipase and transacylase activity (144) and a recent report indicates that iPLA₂β also hydrolyzes long chain acyl-CoA, indicating it has thioesterase activity as well (145). The group VI D, E, and F enzymes are triacylglycerol lipases and acylglycerol transacylases in addition to PLA₂ activity (138). All of these activities are suppressed by bromoenol lactone (BEL), a suicide inhibitor of iPLA₂ (146). This observation suggests that all of the activities are mediated by the same active site. In addition, several members of the family contain additional structural features that impinge on their subcellular localization and function. Prominent
among these is a series of 7-8 ankyrin repeats at the N-terminus of the group VIA (iPLA$_2$$\beta$) enzymes (Figure 2). Ankyrin repeats are involved in protein-protein interactions (147) and these motifs are believed to mediate oligomerization of iPLA$_2$$\beta$ into active tetramers (148;149) (Figure 3). Although iPLA$_2$$\beta$ also contains a putative bipartite nuclear localization sequence (123;140), only a few studies have localized it to the nucleus (150). Most evidence suggests that iPLA$_2$$\beta$ is likely in the endoplasmic reticulum and therefore associated with perinuclear compartments (123;151;152). Both group VIA (iPLA$_2$$\beta$) and group VIB (iPLA$_2$$\gamma$) PLA$_2$s also contain a consensus nucleotide binding motif (GxGxxG) which exhibits homology to those of protein kinases (141;153). This feature is important, given the well-established regulation of the enzymes by ATP (154-156) (and see below). The C-terminus of iPLA$_2$$\beta$ contains a calmodulin binding motif (AWSEMVGIQYFR in hamster iPLA$_2$) that is implicated in the regulation of iPLA$_2$ activity through its association with calmodulin (157) as will be explained further below. The group VIB enzyme (iPLA$_2$$\gamma$) is distinguished by its C-terminal SKL peroxisome localization sequence (141). iPLA$_2$$\gamma$ also contains a N-terminal mitochondrial targeting sequence and exhibits dual subcellular localization to the two compartments (158;159). Recent reports suggest that the mitochondrial localization of iPLA$_2$$\gamma$ has important implications for mitochondrial function and bioenergetic efficiency (143;159). Although iPLA$_2$$\gamma$ has also been observed in rat liver peroxisomes (160), the functional consequence of this localization remains somewhat obscure. As rat liver peroxisomes contain large amounts of arachidonyl GPL, it has
been suggested that this pool of enzyme is involved in AA release and eicosanoid production (160).
Figure 2: Depiction of structure of (A) Group VIA PLA$_2$ (iPLA$_2\beta$) and (B) Group VIB PLA$_2$ (iPLA$_2\gamma$). The active site serine is highlighted.

A: iPLA$_2\beta$

- Caspase-3 cleavage site: DVTDY
- Lipase consensus motif: GTSTG

B: iPLA$_2\gamma$

- Lipase consensus motif: GTSTG
- N-terminal mitochondrial localization sequence
- Ankyrin-repeat domain
- ATP binding domain
- Bipartite nuclear localization signal
Figure 3: Regulation of iPLA\textsubscript{2}\textbeta\ Activity by Oligomerization. Active iPLA\textsubscript{2}\textbeta is proposed to be a homotetramer, formed through protein-protein interactions between the ankyrin repeat domains of iPLA\textsubscript{2}\textbeta monomers. Splice variants of the iPLA\textsubscript{2}\textbeta pre-mRNA encode truncated proteins that retain the ankyrin repeat domain but lack the active site and C-terminus. These proteins interact with full length iPLA\textsubscript{2}\textbeta monomers and prevent oligomerization into active tetramers.
III. Alternative Splicing and iPLA$_2$ isoforms

The group VIA PLA$_2$ (iPLA$_2\beta$) gene undergoes a variety of alternative splicing events, generating variants that differ in their subcellular localization, catalytic activity, and likely cellular function (148;149;161). Two of the splice variants, group VIA-1 and group VIA-2 are known to encode functional PLA$_2$ enzymes that differ in the number of ankryrin repeats (8 in group VIA-1, 7 in group VIA-2). The group VIA-2 isoform has only 7 ankryrin repeats due to a 54 amino acid insertion in the eight ankryrin repeat. This isoform results from an alternatively spliced exon (exon 8). The group VIA-2 isoform is slightly larger than group VIA-1 iPLA$_2$ (752 amino acids versus 806 amino acids; 83 kDa versus 88 kDa) and is proposed to be membrane-associated based on hydropathy plots (148;162). Another splice variant that encodes the group VIA-3 protein arises due to a 158 nucleotide insertion derived from an alternatively spliced exon that generates a premature stop codon. This protein contains the ankryrin repeats, nucleotide binding motif and consensus lipase consensus sequence but does not include the C-terminal amino acids. The function of this ~70 kDa protein remains elusive. Two additional splice variants also encode premature stop codons due to alternatively spliced exon 10a. These insertions result in premature stop codons that terminate the proteins after the ankryrin repeat domain but before the active site. As such, group VIA ankryrin-1 and group VIA ankryrin-2 proteins are catalytically inactive. However, because these
proteins contain the ankryin repeat domain, they retain the ability to oligomerize with full length monomers (149;161). This property has important implications for the regulation of iPLA\textsubscript{2}\textgreek{b} activity as will be examined below.

There are two major isoforms of group VIB PLA\textsubscript{2} (iPLA\textsubscript{2}\textgreek{g}) which arise from different start codons and result in proteins of \~77 and \~63 kDa (141). When over expressed in the myocardium, the enzyme is subject to a multitude of posttranslational modifications, as indicated by the large variety of isoforms observed in 2 dimensional gel analyses (159). The iPLA\textsubscript{2}\textgreek{g} gene is also subject to alternative splicing (141;158). At present, it is not certain if the various isoforms of iPLA\textsubscript{2}\textgreek{g} differ in their relative catalytic efficiencies or substrate specificities, or other characteristics but a recent report indicates that they may have different subcellular localization (158).

IV. Regulation of iPLA\textsubscript{2} Activity

Although early studies suggested that iPLA\textsubscript{2} activity was not regulated by “classic” mediators of signal transduction (cytosolic calcium, phosphorylation), more recent studies have identified these and other factors as important modulators of iPLA\textsubscript{2} activity. One published purification protocol for iPLA\textsubscript{2} includes a calmodulin affinity column (163). The Gross laboratory mapped the site of calmodulin binding to a peptide (AWSEMVGIQYFR) within 15 kDa of the C-terminus of iPLA\textsubscript{2}\textgreek{b} and showed that this interaction suppresses catalytic activity (157;164). As calmodulin interacts with client proteins in a calcium-dependent manner, these data are consistent with the observation
that iPLA$_2$ activity increases in calcium-depleted cells (130;131;165). As discussed below, the negative regulation of iPLA$_2$$\beta$ by Ca$^{2+}$/calmodulin complexes has important implications for activation of store operated calcium channels (SOC) (131). In contrast, iPLA$_2$ activity is induced when the enzyme binds Ca$^{2+}$/calmodulin dependent protein kinase $\beta$II in pancreatic $\beta$ cells (166). This interaction is calmodulin-independent and results in activation of both enzymes.

The original purification protocols for iPLA$_2$ included ATP affinity columns (154;167), indicating that the enzyme binds ATP. For many years, it has been accepted that ATP is required for optimal iPLA$_2$ activity (154-156). However recent studies indicate that ATP activates group VIA-2 iPLA$_2$ but not the group VIA-1 protein (162). This observation is of particular interest because the 54 amino acid insertion that distinguishes these two isoforms does not disrupt the putative nucleotide binding site (G/AxGxxG). However, the insertion is highly enriched in proline residues (PX$_3$PX$_8$HHPX$_{12}$NX$_4$Q) making it likely that conformational changes in this part of the protein modulate ATP binding to the adjacent nucleotide binding site. Although the nucleotide binding site is highly homologous to those of protein kinases (153), ATP binding to this site has not been confirmed experimentally. Our preliminary experiments indicate that a mutant iPLA$_2$$\beta$ protein lacking a glycine in the putative nucleotide binding site exhibits reduced binding to ATP agarose and minimal catalytic activity (Figure 4). Non-hydrolyzable analogs of ATP and other nucleotide triphosphates can substitute for ATP, suggesting that this effect is not mediated through phosphorylation
of the enzyme (154-156). ATP protects iPLA₂ from thermal denaturation, an indication that it stabilizes iPLA₂ proteins and/or oligomers (144;155;156). However, the detailed molecular mechanism of iPLA₂ activation by ATP remains elusive at present.

As noted above, iPLA₂ activity is induced by non-hydrolyzable analogs of ATP (154;155;155) suggesting that this effect is not mediated through phosphorylation of the enzyme. However, several investigators have proposed that iPLA₂ is activated downstream of serine-threonine protein kinases, PKC and p38 in particular (151;168-171). Direct evidence that active iPLA₂ is a phosphoprotein remains elusive. To date, only one study has demonstrated stimulus-dependent phosphorylation of iPLA₂β in response to activation through FcγRI (172).
Figure 4: iPLA₂β ATP Binding Mutant has minimal catalytic activity. (A) HEK 293 cells were transiently transfected with vector encoding wild type iPLA₂β (wt) or mutant iPLA₂β (mt) in which G433 was replaced with aspartate. Whole cell homogenates were prepared and incubated with ATP agarose. The unbound fraction and the ATP agarose eluent (bound fraction) of each preparation were subjected to SDS-PAGE followed by immunoblot analysis for iPLA₂β. (B) iPLA₂ activity was quantified in whole cell homogenates of HEK cells transiently transfected with wild type iPLA₂β or mutant iPLA₂β. The data shown are compiled from four independent experiments. iPLA₂ activity (expressed as pmol substrate hydrolyzed/ min/ mg cell protein) was lower in homogenates expressing mutant iPLA₂β (p < 0.0001).
Radiation inactivation studies indicate that active iPLA₂β is a homotetramer (154). This observation indicates oligomerization as another mechanism for the regulation of iPLA₂ activity. The N-terminal half of iPLA₂β protein is largely composed of a series of ankryin repeats (163;173), a motif known to be involved in protein-protein interactions and that is likely to mediate oligomerization of iPLA₂ monomers. Several recent studies suggest that the group VIA ankryin-1 and group VIA ankryin-2 proteins encoded by alternatively spliced iPLA₂β mRNAs (148;149;161) are endogenous dominant negative proteins that suppress iPLA₂β activity (Figure 3). These proteins contain the N-terminal ankryin repeats but lack the C-terminal active site. As such, ankryin iPLA₂ proteins retain the ability to associate with full length iPLA₂β. Importantly, group VIA ankryin-1 coimmunoprecipitates with full length iPLA₂β and suppresses catalytic activity when the two proteins are co-expressed (148;149;161). Together these observations support the hypothesis that group VIA ankryin-1 is an endogenous dominant negative inhibitor of iPLA₂β, a characteristic that may be important for cell cycle-dependent regulation of the enzyme as will be explained below.

V. Regulation of iPLA₂ Expression

Although iPLA₂ was once thought to be a constitutively expressed enzyme, strictly involved in basal GPL metabolism, a growing body of evidence suggests that its expression is regulated in response to a variety of stimuli. Several recent studies indicate that iPLA₂β expression is induced in retina, both during phagocytosis of
photoreceptor outer segments and in endothelial cells stimulated to proliferate upon interaction with retinal pericytes (174;175). iPLA$_2$$\beta$ is expressed in the cerebral cortex and hippocampus and its expression declines during aging (176). Expression of the enzyme is also down regulated in myocardial tissues of rats undergoing congestive heart failure (177). Several recent studies indicate that iPLA$_2$ expression is regulated by lipids and in tissues involved in systemic lipid metabolism. Enzyme expression and transcriptional activity of the iPLA$_2$$\beta$ promoter are induced lipid depleted Chinese hamster ovary (CHO) cells cultured in lipoprotein-depleted serum (LpDS) (49). Both iPLA$_2$$\beta$ and iPLA$_2$$\gamma$ expression increases during adipocyte differentiation, a response that likely has important functional consequences for adipocyte development (178).

Relatively little is known about the molecular mechanisms that regulate iPLA$_2$ expression. The 5’ flanking region of the human iPLA$_2$$\beta$ gene contains a sterol response element (SRE). Sterol response element binding protein-2 (SREBP2) has been shown to bind this element and is likely the mechanism for iPLA$_2$ induction in lipid-depleted cells (49). The iPLA$_2$$\gamma$ gene contains a proximal E-box that interacts with MyoD, thereby augmenting transcription of the gene (158). Given the mounting evidence for iPLA$_2$ involvement in a variety of biomedically relevant conditions, a more detailed understanding of the mechanisms regulating iPLA$_2$ expression is warranted and is almost certain to be an active area of research in the future.

VI. Biological Functions of iPLA$_2$
Most studies of the biological functions of iPLA₂ have focused on iPLA₂β. However in recent years there has been increased interest in the functions of iPLA₂γ. (141;179). The observation that the R enantiomer of BEL (R-BEL) inhibits predominantly iPLA₂γ, whereas S-BEL selectively inhibits iPLA₂β (180), has provided researchers with a pharmacological means to distinguish between the activities of the two major forms of iPLA₂. The remainder of the attention of this section will distinguish between iPLA₂β and iPLA₂γ when possible. However, it must be noted that many studies have used racemic BEL to study iPLA₂ function and/ or were conducted before gene silencing tools were available. Such studies therefore cannot distinguish between the two forms of the enzyme, though it is generally assumed that the majority of them are focused on iPLA₂β.

A. Glycerophospholipid (GPL) Metabolism

1. Acyl chain remodeling

For many years, iPLA₂ has been proposed as a major regulator of basal GPL metabolism. Among the roles proposed for iPLA₂ in this regard is regulation of the acyl chain composition of GPL. In mammalian cells, long chain polyunsaturated fatty acids (PUFA) are typically esterified to the $sn$-2 carbon and are incorporated into GPL through a remodeling process (41;181-183). The proposed mechanism for this process is GPL hydrolysis by a PLA₂, generating a lysophospholipid acceptor for PUFA. Once incorporated into GPL (typically phosphatidylcholine (PC) initially), PUFA are remodeled into other GPL classes (primarily phosphatidylethanolamine, PE). This
process is especially important in inflammatory cells (macrophages, neutrophils, for example) that are highly enriched in arachidonyl GPL and are sources of AA for eicosanoid production (40;41). Balsinde et al (184;185) were among the first to demonstrate a role for iPLA$_2^\beta$ in remodeling, as this process was inhibited in P388D$_1$ mouse macrophages by bromoenol lactone (selective iPLA$_2$ inhibitor) and an antisense oligonucleotide targeting iPLA$_2^\beta$. Similar results have been reported in uterine stromal cells (186). In both uterine stromal cells and P388D$_1$ macrophages, iPLA$_2$ inhibition is associated with an acute reduction in the lysophospholipid acceptors required for AA remodeling. Conversely, over expression of iPLA$_2^\beta$ or iPLA$_2^\gamma$ results in accumulation of select species of lysophosphatidylcholine (LPC) (159;187). Together, these data suggest that iPLA$_2$ may control the incorporation of AA into GPL, thereby making the enzyme an attractive candidate for drugs to control eicosanoid production. In addition, a recent study indicates that iPLA$_2^\gamma$ is involved in the acyl chain remodeling that generates the tetra C18:2 species of cardiolipin that is essential for Complex IV function and mitochondrial respiration (143). However, it must be noted that studies in variety of cells and tissues, including pancreatic islet cells, an insulinoma cell line, T cells, and most recently in macrophages of iPLA$_2^\beta$-null mice suggest that the acyl chain composition of GPL does not change when iPLA$_2^\beta$ expression or iPLA$_2$ activity are modulated (136;188-191). Together, these studies suggest that iPLA$_2$ likely regulates acyl chain remodeling in a select subset of cells and tissues.

2. Regulation of GPL Mass
The other major role of iPLA₂ in basal GPL metabolism is related to the regulation of phosphatidylcholine (PC) mass (PC homoestasis). This function is essential for cell biology not only because PC is a reservoir for important bioactive lipids and signaling molecules, but also because PC is the most abundant GPL in mammalian cells and therefore has a vital role in membrane integrity. An early study by Walkey et al (192) demonstrated that over expression of the rate limiting enzyme in PC synthesis (CTP: phosphocholine cytidylyltransferase, CCT) resulted in only modest increases in PC mass due to increased turnover of this GPL in HeLa cells. Subsequently, several groups showed that this turnover was associated with an accumulation of glycerophosphocholine (GPC, the product of PLA₂ followed by lysophospholipase activity) and that this was blocked when cells were treated with bromoenol lactone (193;194). Importantly, iPLA₂ activity and expression were elevated in response to CCT over expression (193). Together, these observations suggest that PC mass is maintained through a dynamic balance between CCT and iPLA₂ activities. More recent studies suggest that iPLA₂β also regulates accumulation of PC mass for daughter cell membranes (161), an observation that is especially interesting given recent studies linking iPLA₂β to cell cycle progression and cell proliferation (195-198). This topic will be treated below and suggests iPLA₂ as a potential target for chemotherapeutic drugs.

3. Arachidonic acid metabolism and eicosanoid production

Like other long chain polyunsaturated fatty acids, arachidonic acid (AA) is primarily esterified in the sn-2 position of mammalian GPL. Upon its release from GPL by
phospholipase A₂, AA can be shuttled to the cyclooxygenase (COX) and lipoxygenase pathways for eicosanoid synthesis ((37). It is well-established that cPLA₂ and sPLA₂ are responsible for AA release and subsequent eicosanoid production in response to a variety of stimuli and in many cell types (199-201). This is likely related to the substrate specificity of cPLA₂, which preferentially targets arachidonate-containing GPL (202;203). However, evidence has accumulated that iPLA₂ can participate in AA release and eicosanoid production under certain conditions. For example, HEK293 cells cotransfected with iPLA₂ and COX-1 exhibit AA release and prostaglandin E₂ (PGE₂) production when stimulated with calcium ionophore A23187 but not in response to IL1β (204). In phagocytes, AA release in response to oxidative stress has been shown to rely on iPLA₂ activity (205;206) and leukotriene B₄ (LTB₄) production by zymosan-stimulated granulocytes is attenuated by the iPLA₂ inhibitor BEL (207). iPLA₂ is also involved in eicosanoid synthesis in mesanglial cells, as BEL partially blocks AA release and subsequent prostaglandin E₂ (PGE₂) production by rat mesangial cells stimulated with interleukin-1β (IL-1β) and dibutyryl cAMP (208). Although iPLA₂ is involved in zymosan-stimulated AA release in P388D₁ cells, (171;209), PAF-induced AA release is iPLA₂-independent in the same cell line (210). Similarly, iPLA₂ is not involved in PAF-stimulated AA release in U937 cells, but these cells exhibit iPLA₂-dependent AA release in response to activation through FcγRI (172). Together, these observations indicate that cell type and stimulus dictate whether iPLA₂ is involved in AA release and eicosanoid production. AA is also metabolized by the CYP epoxygenase and CYP hydroxygenase pathways to generate the eicosanoids epoxideicosatrienoic acid (EET)
and hydroxyeicosatetraenoic acid (20-HETE), respectively (211). A recent study indicates that iPLA$_2$-generated AA may be metabolized by these pathways, resulting in EET production that in turn induces calcium influx (212). Taken together, these studies indicate that sPLA$_2$ and cPLA$_2$ are not the only mediators of AA release. Hence, iPLA$_2$ should be considered as a potential target for anti-inflammatory drugs designed to block eicosanoid production.

B. Pathogenesis and Innate Immune Response

1. Innate Immune response

Recent studies suggest several important roles for iPLA$_2$ in the host immune response. In plants, an iPLA$_2$ homolog (AtPLA1 in Arabidopsis) is associated with basal production of jasmonic acid and resistance to infection by the fungus Botrytis cinerea (133). iPLA$_2$ homologs in tobacco (NtPat) have also been associated with jasmonic acid production in response to fungal infection (213). iPLA$_2$ is also important for the innate immune response in humans and other mammals. In addition to its established role in AA release and eicosanoid production (171;205;206), iPLA$_2$ is involved in a variety of additional functions associated with macrophage biology. iPLA$_2$-derived AA is required for adherence and spreading of primary mouse macrophages (214). Lysosyme secretion by U937 human monocytes is dependent on iPLA$_2$-derived lysophosphatidylcholine (LPC) and suppressed by BEL and antisense oligonucleotides targeted to iPLA$_2$ (215). LPC is also essential for clearance of apoptotic cells, as iPLA$_2$ inhibitors suppress both macrophage chemotaxis to and phagocytosis of apoptotic cells.
In contrast although BEL also inhibits phagocytosis of red blood cells opsonized with IgG, AA restores this activity, suggesting that it is mediated by the fatty acid product of \( \text{iPLA}_2 \) not the lysophospholipid (218). \( \text{iPLA}_2 \) is also involved in recognition of apoptotic cells, as \( \text{iPLA}_2 \)-derived LPC in the outer leaflet of the plasma membrane is an epitope recognized by IgM antibodies (219). Bromoenol lactone and \( \text{iPLA}_2 \) antisense oligonucleotides attenuate macrophage chemotaxis in response to monocyte chemoattractant protein-1 (MCP-1) (220). Infection with encephalomyocarditis virus (EMCV) stimulates expression of inducible nitric oxide synthase (iNOS) in mouse macrophages. Experiments with the \( R \)- and \( S \)-enantiomers of BEL indicate that this induction occurs downstream of \( \text{iPLA}_2 \beta \)-mediated activation of cyclic AMP response element binding protein (CREB) (221). This observation is consistent with other reports that double-stranded RNAs induce phosphorylation of CREB in an \( \text{iPLA}_2 \)-dependent manner (222,223). Other lines of study suggest that \( \text{iPLA}_2 \) plays an essential role in protecting host cells against the innate immune response. For instance, overexpression of \( \text{iPLA}_2 \gamma \) and \( \beta \) in glomerular epithelial cells reduces cellular damage and proteinuria induced upon complement activation (224). Taken together, these studies indicate that \( \text{iPLA}_2 \) plays essential roles in macrophage biology and the innate immune response. This is an important consideration in the design and implementation of drugs targeting \( \text{iPLA}_2 \) activity as such compounds are likely to be immunosuppressive if administered systemically or for extended periods of time.
2. Pathogenesis of *Pseudomonas aeruginosa*

As noted above mammalian iPLA\(_2\) is highly homologous to ExoU, a phospholipase A\(_2\) and virulence factor expressed by *Pseudomonas aeruginosa* that is directly exported into the cytosol of mammalian host cells through a type III secretion system (132;225). ExoU shares a variety of characteristics with iPLA\(_2\) including hydrolysis of zwitterionic GPL *in vitro*, induction of AA release when injected into the cytosol of CHO cells, calcium-independent activity, lysophospholipase activity, and an active site serine (226;227) (227;228) (229). However, although ExoU activity is suppressed by methyl arachidonyl fluorophosphonate (MAFP), an inhibitor of iPLA\(_2\) and cPLA\(_2\), BEL (a selective inhibitor of iPLA\(_2\)) only partially suppresses ExoU activity (227;228). These data indicate that the ExoU PLA\(_2\) is similar but not identical to mammalian iPLA\(_2\). Importantly, a variety of studies indicate that ExoU is an essential virulence factor for *P. aeruginosa* (225) (230) (227) (231) (232). These observations suggest that selective pharmacological inhibitors of the phospholipase activity of ExoU may be effective treatments for *P. aeruginosa* infection.

C. Calcium Signaling and Homeostasis

Another emerging role for iPLA\(_2\) relates to calcium homeostasis, specifically the replenishment of depleted intracellular calcium stores. The mechanism of this replenishment has been hotly debated for many years, with two major models emerging: the “conformational coupling” model in which IP\(_3\) receptors/ calcium channels in the ER membrane become physically associated with calcium channels in the plasma
membrane and the “calcium influx factor” (CIF) model which postulates that a
diffusible molecule generated upon depletion of calcium stores interacts with store
operated channels (SOC) in the plasma membrane and induces calcium influx
(233;234). Strong evidence of a role for iPLA₂ in the CIF model has emerged from
recent studies by the Bolotina group and others. These studies were prompted by the
observation that iPLA₂ activity is induced upon calcium depletion and that the enzyme
associates with calmodulin (157;164). Based on these observations, Smani et al treated
smooth muscle cells (SMC) with thapsigargin (TSG) to deplete ER calcium stores and
demonstrated an induction of iPLA₂ activity (235). iPLA₂ activity is also activated by
partially purified CIF and this is at least in part mediated through dissociation of
calmodulin, a negative regulator of the enzyme (131). Importantly, calcium influx
through calcium release-activated channels (CRAC) is blunted when SMC or rat
basophilic leukemia (RBL) cells are pretreated with the S- enantiomer of BEL (selective
iPLA₂β inhibitor) or antisense oligonucleotides targeting iPLA₂β (131;236). Similar
results have been observed in platelets, prostate cancer cells, cerebellar astrocytes, and
Jurkat T cells, suggesting that iPLA₂-dependent replenishment of calcium stores is a
commonly used mechanism for restoring calcium homeostasis in a variety of cells and
tissues (131;236-238). The lysophospholipid product of the iPLA₂ reaction is essential
for this process, as LPC restores activation of SOC in BEL-treated cells (131;237).
Based on these observations, Bolotina has proposed a model wherein CIF (still a poorly
defined entity) promotes dissociation of iPLA₂ from calmodulin thereby activating the
enzyme and prompting generation of LPC that then activates SOC and allows calcium
influx to replenish intracellular stores (233;234). Recent studies suggest that iPLA₂ may regulate calcium influx through transient receptor potential (TRP) channels as well (239), indicating the potential for a wider role of the enzyme in calcium homeostasis. There are a variety of potential applications for pharmacological interventions to regulate iPLA₂-dependent activation of SOC. Most notably, such strategies could be useful in treatment of Duchenne muscular dystrophy which is characterized by progressive muscle destruction secondary to uncontrolled calcium entry through SOC. Boittin et al have recently shown that iPLA₂ expression is 2-3 fold higher in dystrophic than normal skeletal muscle fibers and that BEL restores calcium entry to normal levels in these tissues (240). Increased cytosolic calcium is required for exocytosis of mast cell granules, making iPLA₂ an attractive target for controlling this process and thereby the contribution of mast cells to type I hypersensitivity (allergy). As replenishment of intracellular calcium stores is essential for homeostasis and given the diverse array of cells and tissues that mediate this process in an iPLA₂-dependent manner, any drugs designed to suppress iPLA₂ activity will require exquisite tissue selectivity to avoid global disruption of calcium homeostasis.

D. Cell Proliferation, Survival, and Apoptosis

1. iPLA₂ and apoptosis

Several lines of evidence have implicated free AA in apoptosis (241-243) and in doing so have prompted investigation into the relationship between iPLA₂ and apoptosis. Indeed, there are now numerous studies linking iPLA₂ to apoptosis secondary to
chemotherapeutic drugs, ER stress, and other agents. For example, iPLA$_2$ inhibition protects renal cells from apoptosis induced by the chemotherapeutics cisplatin and vincristine presumably through the retention of AA in GPL (244). A similar study in rabbit renal proximal tubule cells demonstrated that iPLA$_{2\gamma}$ works together with caspase 3 and p53 to facilitate cisplatin-induced apoptosis (151). Kinsey et al have recently extended this study and observed that iPLA$_{2\gamma}$-generated AA plays a central role in mediating the mitochondrial permeability transition that leads to cell death (245). Studies in U937 monoblastic leukemia cells indicate that iPLA$_2$ is involved in apoptosis induced by TNF$\alpha$ and anti-Fas antibodies as iPLA$_2$ inhibitors suppress both AA release and apoptosis induced by these agents (246;247). Caspase 3 cleaves iPLA$_{2\beta}$ at Asp$^{183}$ to produce a truncated enzyme with enhanced enzymatic activity that is likely responsible for AA release during apoptosis as well as the migration of non-apoptotic ovarian cancer cells (247;248). Polycyclic aromatic hydrocarbons in cigarette smoke induce AA release from and apoptosis of endothelial cells in an iPLA$_2$-dependent manner (249). While some studies have connected iPLA$_{2\beta}$ to apoptosis secondary to oxidative stress (250), others suggest that this mechanism of cell death, while iPLA$_2$-dependent, is not mediated through apoptosis (150). Whether apoptotic or non-apoptotic, cell death in response to oxidative stress may be controlled by feedback mechanisms that result in covalent modifications of iPLA$_{2\beta}$ that suppress its catalytic activity (251). Several recent studies connect iPLA$_2$ to apoptosis secondary to ER stress induced by free cholesterol loading, calcium depletion and other mechanisms. However, it is not yet clear whether these responses are mediated by iPLA$_{2\beta}$, iPLA$_{2\gamma}$, or both (151;191;252).
Together, these studies suggest iPLA$_2$ is a key player in programmed cell death of a variety of cells and tissues and in response to a variety of stimuli.

The mechanism by which iPLA$_2$ augments apoptosis is still under investigation, but is likely cell type- and/ or stimulus-specific. iPLA$_2$-dependent apoptosis may be secondary to changes in lipid metabolism and mass in apoptotic cells. As noted above, some studies suggest that apoptosis is induced due to AA accumulation, a likely outcome of iPLA$_2$ activity (241-243;253). There is evidence that iPLA$_2$ is involved in acyl chain remodeling and that apoptosis ensues when this process is inhibited (188;253). However, as the role for iPLA$_2$ in acyl chain remodeling appears to be limited to macrophage cell lines (136;188;190;191), this is likely not a major mechanism for iPLA$_2$-mediated apoptosis. Other studies suggest that apoptosis downstream of AA accumulation is mediated through the generation of ceramide (15), a proapoptotic sphingolipid derived from sphingomyelin hydrolysis (254). Much of this work stems from an early report that AA and other fatty acids mediate tumor necrosis factor-$\alpha$- (TNF$\alpha$-) induced sphingomyelin hydrolysis in a PLA$_2$-dependent manner (255). In recent years, iPLA$_2$$\beta$-dependent generation of ceramide has emerged as an important mechanism for apoptosis (252;256). One recent report demonstrates that iPLA$_2$$\beta$ increases ceramide through induction of neutral sphingomyelinase during ER-stress induced apoptosis in INS-1 insulinoma cells (252). As expected, BEL (a selective iPLA$_2$ inhibitor) blocks neutral sphingomyelinase activity, ceramide generation and apoptosis during ER stress in INS-1 cells. Conversely, iPLA$_2$$\beta$ over expression
exacerbates ER-stress induced apoptosis. BEL also attenuates apoptosis secondary to depletion of ER calcium stores whereas over expression of iPLA$_2$β exacerbates this process (256). In these studies, increased ceramide levels correlate with the accumulation of a 62-kDa iPLA$_2$β protein resulting from caspase-3 cleavage. This truncated iPLA$_2$β has increased catalytic activity, reminiscent of the highly active, caspase-processed iPLA$_2$β that is generated during Fas-mediated apoptosis of U937 cells (246;247).

It has also been suggested that iPLA$_2$ is involved in the intrinsic apoptosis pathway that involves cytochrome c release from the mitochondria and subsequent caspase activation and apoptosis. Both iPLA$_2$β and iPLA$_2$γ can associate with the mitochondria (158) (245;257-259). iPLA$_2$ accentuates mitochondrial permeability transition, thereby promoting cytochrome C release and the intrinsic pathway of apoptosis (260;261). The selective iPLA$_2$γ inhibitor R-BEL suppresses calcium-induced swelling of rabbit renal cortex mitochondria, suggesting a role for iPLA$_2$γ in this process. In contrast, iPLA$_2$β is down-regulated during staurosporine-induced apoptosis and over expression of the enzyme protects CHO and INS-1 cells from loss of mitochondrial potential and cytochrome C release (257). Although the reason for these disparate responses is unclear at present, they provide further evidence that the role of iPLA$_2$ in cell growth and death is likely to be cell type- and stimulus-specific.
Together, these studies suggest that iPLA$_2$ is involved in the induction of apoptosis in many cells and tissues. Other studies suggest that iPLA$_2$ also plays a vital role in clearance of apoptotic cells. iPLA$_2$-derived lysophosphatidylcholine is recognized by IgM antibodies, resulting in complement activation, opsonization, and clearance of apoptotic cells (219). There is also evidence that iPLA$_2$ activity may facilitate the externalization of phosphatidylserine, another marker of apoptotic cells that promotes their clearance (219;247). As noted above, caspase processed iPLA$_2$ is highly active, generating both free fatty acid and lysophospholipid products (217;247;248). Lysophosphatidylcholine (LPC), in particular, is a chemoattractant for monocytes which recruits these cells and thereby promotes phagocytosis and clearance of apoptotic cells (217).

In summary, there is considerable evidence that iPLA$_2$ promotes not only apoptosis but also clearance of apoptotic cells by the innate immune system. These studies suggest that products of the iPLA$_2$ reaction are potential drug targets, as these molecules or their mimetics might be useful as adjuvants together with standard approaches to chemotherapy.
2. *iPLA*$_{2}$ and cell survival, growth and proliferation

Although *iPLA*$_{2}$ has important roles in a variety of apoptotic responses (150;151;191;244;246-250;252), there is substantial evidence that the enzyme is required for proliferation as well. Cell type, the nature of the cell environment, and the absence or presence cellular stressors may determine whether *iPLA*$_{2}$ exerts antiapoptotic or proapoptotic pressure. For instance, although *iPLA*$_{2}$β contributes to apoptosis in ER-stressed INS-1 insulinoma cells (252), in the same cell line *iPLA*$_{2}$β is also required for optimal proliferation and prevents mitochondrial cytochrome c release, loss of mitochondrial membrane potential and apoptosis in response to staurosporine (189;257). BEL suppresses proliferation of mitogen-stimulated Jurkat T cells, endothelin-stimulated Schwann cells, and serum-activated fibroblasts (262-264). Importantly, ovarian cancer cell lines with attenuated *iPLA*$_{2}$β expression exhibit reduced tumorigenicity in nude mice (198). Both p53-dependent and independent mechanisms for *iPLA*$_{2}$ suppression of proliferation have been demonstrated (195;198). Although most studies suggest that inhibition of *iPLA*$_{2}$β by BEL or genetic knock down results in S phase arrest (195;197;262;264), there is also evidence that *iPLA*$_{3}$β is required for progression through the G2/M transition in Jurkat T cells and ovarian cancer cells (198;263).

The molecular connection between *iPLA*$_{2}$ and cell cycle progression is unclear at present and likely to vary with different cell types and stimuli. As noted above, *iPLA*$_{2}$ activity is cell cycle-dependent and regulated in a manner consistent with the
accumulation of lipid for daughter cell membranes (161). Arachidonic acid (AA) reverses the inhibition of HUVEC and Caco-2 cell proliferation by BEL (196;197). The effects of AA on proliferation are likely related to effects of AA-derived eicosanoids on cancer progression (14;265). Along these lines, over expression of iPLA2γ in human colorectal adenocarcinoma HCA-7 cells leads to increased AA and eicosanoid production resulting in increased proliferation (266). Evidence also suggests that the lysophospholipid product of iPLA2 is involved in cell growth and cancer cell progression. Lysophosphatidic acid (LPA) is a potent mitogen for many cell types and is readily exploited by transformed cells for proliferation and metastasis (267). For example, LPA generated by human peritoneal mesothelial cells induces chemotaxis of SKOV-3 ovarian cancer cells (268). In this system, BEL attenuates both the production of LPA and the chemotaxis of SKOV3 cells. There is also evidence that ovarian cancer cells synthesize LPA in an iPLA2-dependent manner and that this lipid mediator acts in an autocrine fashion to induce proliferation and migration (198;269). LPA releases BEL-treated ovarian cancer cells from S phase arrest as well (198).

Together, these studies demonstrate that iPLA2 activity is associated with both programmed cell death and proliferation of cancer and normal cells. As discussed previously, the pro-proliferative or pro-apoptotic effects of iPLA2 are likely dependent on cell type and the nature of external stimuli. Thus, although iPLA2 is an attractive candidate for development of anti-cancer drugs, such strategies must be carefully designed to balance these two opposing effects of the enzyme.
E. iPLA₂ in metabolism

1. iPLA₂ and glucose-stimulated insulin secretion

Dysfunction of and insufficient glucose-stimulated insulin secretion from pancreatic islet β cells are pathological manifestations of diabetes mellitus. Fatty acids are required for proper β cell function, as fatty acid deprivation of islet cells results in decreased insulin secretion in response to glucose (270). Plasma, lipoproteins, intracellular triglyceride and GPL have all been proposed as the source of fatty acid for proper islet function (271). Glucose stimulates both insulin secretion and AA release (272-274) and inhibitors of GPL hydrolysis suppress insulin secretion (275-281). These observations suggest PLA₂ as a potential regulator of β cell function and there is accumulating evidence to indicate that this regulation is mediated by iPLA₂. Early studies from the laboratories of Gross and Turk demonstrated that pancreatic islets contain a PLA₂ that has the same properties as the enzyme that has come to be called iPLA₂, including calcium-independence, inhibition by BEL, and activation/stabilization ATP and its non-hydrolyzable analogs (282;283). Although the islet cell enzyme has been definitively identified as iPLA₂β, a recent report indicates that this enzyme differs from other isoforms of iPLA₂β in that it is 70 kDa (versus “native” iPLA₂β which is 84-88 kDa) (137;284). The origin of the 70 kDa isoform of iPLA₂β in islet cells is still in question, but it does not appear to be encoded by a splice variant of the iPLA₂β pre-mRNA (137). The signaling pathways stimulated by glucose provide additional
evidence of a role for iPLA₂ in glucose-stimulated insulin secretion. Cyclic AMP (cAMP) signaling is required for glucose-stimulated insulin secretion from β cells (285;286). cAMP-elevating agents induce translocation of iPLA₂β to a perinuclear compartment (286) (287) and it has been proposed that this allows the enzyme to access and release AA from GPL in the ER (288;289). Like cAMP signaling, changes in intracellular calcium facilitate glucose-stimulated insulin secretion (290). PLA₂ products induce calcium release from ER stores in β cells (273) and BEL inhibits glucose-stimulated increases in intracellular calcium (276). The strongest evidence for involvement of iPLA₂ in insulin secretion comes from studies in which islet cells are genetically manipulated or treated with BEL before stimulation with glucose. Over expression of iPLA₂β in INS-1 insulinoma cells not only induces proliferation but also increases glucose-stimulated AA release (287;291). Conversely, both BEL and siRNA/shRNA targeted against iPLA₂β suppress glucose-stimulated insulin secretion (189;291). Exogenous AA reverses effects of BEL, consistent with previous evidence for AA involvement in insulin secretion (7;291). Studies in animal models provide additional support for a role for iPLA₂β in pancreatic β cell function and highlight the ability of the enzyme to influence systemic glucose homeostasis. Mice expressing an islet-specific iPLA₂β transgene have lower fasting glucose and higher insulin levels than do wild type mice. These mice also perform better in glucose tolerance tests (187). In contrast, mice treated with BEL or shRNA targeted to iPLA₂β demonstrate decreased in insulin secretion and impaired glucose tolerance (291). Furthermore, iPLA₂β knockout mice exhibit insufficient insulin secretion in response to glucose and forskolin
when maintained on chow diet and develop severe glucose intolerance on a high-fat diet (136;187). When treated with streptozotocin, iPLA$_2$$\beta$ knockout mice experience more severe hyperglycemia than do wild-type mice (136). Collectively, these findings provide strong evidence of a role for iPLA$_2$-derived bioactive lipids in glucose homeostasis and spotlight iPLA$_2$ as a possible therapeutic target for the treatment of diabetes.

2. iPLA$_2$ and adipocyte/hepatocyte biology

Adipogenesis, the differentiation of preadipocytes into mature adipocytes, relies on the broad and concerted regulation of genes by PPAR$_\gamma$, CCAAT/enhancer binding protein (C/EBP)$_\alpha$ and SREBP1, also referred to as adipocyte determination and differentiation-dependent factor 1 (ADD-1) (292;293). iPLA$_2$$\beta$ and iPLA$_2$$\gamma$ expression increases during adipocyte differentiation (178) and has been shown to play an essential role in adipogenesis. Inhibition of iPLA$_2$$\beta$ and iPLA$_2$$\gamma$ with BEL enantiomers selective for either of the two proteins blocks differentiation of 3T3-L1 preadipocytes (178). In addition, siRNA directed against either iPLA$_2$ attenuates PPAR$_\gamma$ and C/EBP$_\alpha$ induction by a cocktail of adipogenic hormones. Troglitazone, a PPAR$_\gamma$ agonist, reverses effects of iPLA$_2$ inhibition and restores adipogenesis. Citing evidence that eicosanoids and lysophospholipids promote adipocyte differentiation (294-297) and that these molecules and unesterified fatty acids can activate PPAR$_\gamma$ (294;298-301), Su et al speculate that iPLA$_2$ provides PPAR$_\gamma$ agonists that drive differentiation (178).
Many of the genes encoding enzymes involved in fatty acid, triglyceride, and cholesterol synthesis contain sterol response elements (SRE) that allow for feedback regulation of expression (25;48;302). SREs promote gene expression when they are bound by sterol regulatory element binding proteins (SREBPs), members of the basic-helix-loop-helix family of transcription factors. The nuclear pool of SREBP1 is controlled at two levels: expression of precursor SREBP1 protein and processing to its mature, transcriptionally active form (25;48). In addition to the well-established role for sterols in regulation of SREBP, polyunsaturated fatty acids (PUFAs) also suppress SREBP processing and translocation (39;43;79). The effect of PUFAs appears to be largely specific for SREBP1 (76;80) and therefore effects of PUFAs are most apparent on fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), and other lipogenic genes associated with triglyceride synthesis. To date, all of the published studies have been performed in cells treated with exogenous PUFA or animals maintained on PUFA-rich diets. We hypothesize that iPLA₂ is an endogenous source of PUFA capable of inhibiting SREBP. To test this hypothesis, we modulated iPLA₂β expression and activity in Chinese hamster ovary (CHO) cells and HepG2 human hepatoma cell lines and then measured SREBP1 expression, functional activity, and expression of SREBP1 target genes. As shown in (Figure 5), iPLA₂ inhibition and over expression result in respective increases and decreases in transcriptional activity of expression of SREBP. These findings not only indicate a role for iPLA₂ in hepatic lipid metabolism, but suggest a potential role for the enzyme in adipogenesis as PPARγ expression is transcriptionally controlled by SREBP in hepatocytes and adipocytes (303). Thus,
iPLA$_2$ has important roles in both hepatic and adipocyte biology. Given the relevance of these tissues to systemic lipid metabolism, iPLA$_2$ is likely to be an important target for drugs that control obesity, fatty liver disease, and other manifestations of the metabolic syndrome.
Figure 5: iPLA\(_{2}\)\(\beta\) Regulates Sterol Response Element Binding Protein (SREBP).

Chinese hamster ovary (CHO) cells were transiently transfected with expression vector to over express iPLA\(_{2}\)\(\beta\) (A) or siRNA to suppress endogenous iPLA\(_{2}\)\(\beta\) (B). Subsequently, cells were transfected with luciferase reporter construct (pSynSRE) containing sterol response elements (SRE). Luciferase activity was quantified as a measure of functional SREBP protein in the cells. SREBP function was reduced in cell over expressing iPLA\(_{2}\)\(\beta\) and augmented in cells with reduced iPLA\(_{2}\)\(\beta\) expression (p < 0.01).
Figure 5

A. 

![Bar graph showing promoter activity for empty and iPLA2 conditions. The graph indicates a significant difference in promoter activity between the two conditions, with the empty condition having a higher activity.

B. 

![Bar graph showing promoter activity for control siRNA and iPLA2 siRNA conditions. The graph shows a marked increase in promoter activity in the iPLA2 siRNA condition compared to the control siRNA condition.]
3. iPLA$_2$ in mitochondrial function and energy homeostasis

Several recent reports from Mancuso et al have suggested an important role for iPLA$_2^\gamma$ in mitochondrial functioning and energy metabolism in cardiac myocytes. Fasted transgenic mice over expressing iPLA$_2^\gamma$ in myocytes exhibit exercise-associated ventricular dysfunction (159). The onset of this dysfunction correlates with increased expression of diacylglycerol acyltransferase I (DGATI) and massive accumulation of triglyceride accumulation, a signature of lipotoxicity. Indeed, the mitochondria of iPLA$_2^\gamma$ transgenic animals are structurally compromised and exhibit reduced respiratory control quotient compared to wild type mitochondria. Mitochondrial function is also compromised in iPLA$_2^\gamma$ knock out mice which exhibit reduced Complex IV-mediated oxygen consumption, likely due to decreased levels of tetra C18:2 cardiolipin in mitochondria (143). These animals also exhibit reduced cold tolerance, decreased exercise capacity, and fail to gain weight after adolescence, all indications of compromised energy balance secondary to the loss of iPLA$_2^\gamma$. Together, these observations suggest that iPLA$_2^\gamma$ plays an important role in maintenance of proper mitochondrial function and energy homeostasis. As such, a more detailed understanding of this enzyme may be necessary for development of drugs designed to restore energy balance in diabetes and other disorders related to obesity and the metabolic syndrome.

F. iPLA$_2$ in Neurological Diseases
iPLA₂β is highly expressed in the brain and is the most highly expressed PLA₂ in the hippocampus (304). Several recent studies have implicated iPLA₂β in a variety of neurological processes, ranging from neural development to neuron survival, to neural transmission. For example, the selective iPLA₂ inhibitors BEL and palmitoyl trifluoromethyl ketone, (PACOCF3) increase the amplitude of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor-mediated excitatory postsynaptic currents in hippocampal CA1 pyramidal cells (305). iPLA₂ inhibitors suppress endothelin-induced Schwann cell proliferation and also reduce viability in primary cultures of rat cortical neurons, suggesting a role for the enzyme in survival of neuronal cells (305;306). It is well-documented that PLA₂ activity is elevated in patients with schizophrenia (307;308). A recent report has associated first-episode schizophrenia with increased calcium-independent PLA₂ activity in serum (309). At first glance, this observation appears to exclude iPLA₂, as the enzyme is cytosolic and not secreted by viable cells. However, the observation of significant allelic and genotypic associations between iPLA₂β polymorphisms and schizophrenia (307) underscore the possibility that iPLA₂ may be a susceptibility gene for the disease. Perhaps the strongest association between iPLA₂ and neurological disease is in diseases associated with iron accumulation in the brain. Both Infantile Neuroaxonal Dystrophy (INAD), a neurodegenerative disease associated with psychomotor regression and hypotonia and Neurodegeneration with Brain Iron Accumulation (NBIA) have been associated with inactivating mutations in the iPLA₂β gene (310;311). Importantly, iPLA₂β knockout mice develop neurological impairment by ~13 months of age (312). This impairment is
associated with the formation of spheroids containing tubulovesicular membranes, a characteristic of INAD in humans. These observations provide strong support for the hypothesis that iPLA\textsubscript{2} activity is essential for proper neurological development and brain function. They also underscore the need to identify the products of the iPLA\textsubscript{2} reaction that are involved in neurological development, as these molecules may be candidates for drug development in the future.

VII. Summary and Perspectives

There is accumulating evidence that the biological functions of iPLA\textsubscript{2} are not limited to GPL homeostasis (Table 1). As described, iPLA\textsubscript{2} has a variety of biomedically relevant activities, ranging from glucose and energy homeostasis to adipocyte and hepatic biology, to proliferation and chemotherapy-induced apoptosis of tumor cells, to the pathogenesis of \textit{Pseudomonas aeruginosa}, to sperm motility (313) among others (Figure 6). Most of these effects are likely due to bioactive lipids derived from the products of the iPLA\textsubscript{2} reaction, unesterified fatty acid and lysophospholipid. However, it is possible that the iPLA\textsubscript{2} protein has catalysis-independent functions due to protein-protein interactions mediated by its N-terminal ankyrin repeats. For applications in which enhanced iPLA\textsubscript{2} activity is likely to have therapeutic benefit (fatty liver, insulin sensitivity, apoptosis in response to chemotherapeutic drugs, INAD), detailed lipidomics analyses are warranted to identify the relevant bioactive lipids, their receptors, and the signaling mechanisms by which they control cell responses. In the design and implementation of such studies, it is imperative to recognize the relevant
bioactive lipids are likely to vary with cell/tissue type and also with the nature of the stimulus. For applications in which iPLA₂ inhibition is indicated (proliferation of cancer cells, *Pseudomonas aeruginosa* pathogenesis, chronic inflammation due to macrophage activation), it is important to develop more selective inhibitors, as BEL inhibits phosphatidate phosphohydrolase as well (314). Receptor antagonists are another potential strategy in cases where the relevant bioactive lipids have been identified. Known receptors for bioactive lipids range from G protein coupled plasma membrane receptors to nuclear hormone receptors that directly regulate transcription (315-317). As the iPLA₂ enzymes are ubiquitous and have essential roles in lipid homeostasis, any iPLA₂-directed drug strategy must be carefully designed and have exquisite selectivity for target cells and tissues. One must also consider the potential for iPLA₂ inhibitors to suppress innate immune responses by compromising macrophage function. Hence, although iPLA₂ is an attractive drug target for a variety of diseases, the design and implementation of such strategies is likely to be a technical and intellectual challenge.
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<td>Glycerophospholipid Metabolism</td>
<td>Acyl chain remodeling</td>
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<td>Regulation of Glycerophospholipid Mass</td>
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<td>Arachidonic acid metabolism and eicosanoid production</td>
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<td>Innate Immunity</td>
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<td>Calcium Signaling and Homeostasis</td>
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<td>Cell Proliferation, Survival, and Apoptosis</td>
<td>Apoptosis</td>
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<td>Adipocyte/ hepatocyte Biology</td>
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<td>Mitochondrial function and energy homeostasis</td>
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Figure 6: Biological Activities of iPLA$_2$ and Relevance for Drug Design and Targeting. Accumulating evidence indicates that the biological function of iPLA$_2$ is not limited to basal GPL metabolism. The enzyme impacts on several biomedically relevant processes and as such is an attractive drug target for a variety of disorders. However as iPLA$_2$ expression is ubiquitous and the enzyme has important roles in basal metabolism, such strategies must be carefully designed to ensure targeting to appropriate cells and tissues.
The novel PI3K inhibitor PX-866

PI3K phosphorylates membrane phosphatidylinositides (PI) to generate PI(3,4,5)P₃ that interacts with Akt via its PH domain and subsequently leads to activation of Akt by phosphorylation of ser473 (318;319). PI3K consists of a heterodimer formed by a catalytic p110 subunit and a regulatory p85 subunit that is activated by receptor tyrosine kinase or G-protein coupled receptors (318;320;321). Isoforms alpha and beta of the p110 subunit are distributed among many mammalian tissues (21). Three genes encode eight splice variants of the p85 subunit (322); however there is no preference of any of the p110 catalytic subunit for any of p85 subunit (21). Activation of the PI3K/Akt pathway is associated with cell survival and cell proliferation (318).

A variety of transformed cells frequently exploit the PI3K/Akt signaling pathway (318;323-327) often via constitutive activation of PI3K (328;329) as a means of survival. Downstream activation of Akt by PI3K confers resistance to apoptosis (330;331). Therefore considerable interest has accumulated to determine the efficacy of targeting this pathway with chemotherapeutics. Wortmannin, a fungal metabolite, and the flavonoid derivative LY294002 are PI3K inhibitors frequently used to inhibit the PI3K/Akt pathway and in turn, transformed cell growth and proliferation (318;332). LY294002 is a competitive inhibitor of the ATP-binding site on PI3K (318). Wortmannin inhibits the alpha and beta p110 subunit and has an IC₅₀ that is approximately 500-fold lower than LY294002 (318;333). While wortmannin is a potent inhibitor of PI3K, it and LY294002 demonstrate poor pharmacokinetics and high
hepatotoxicity thereby mitigating their efficacy as *in vivo* inhibitors of PI3K and diminishing their clinical value (334).

In the last several years, the Powis laboratory has synthesized the semisynthetic viridin PX-866, a wortmannin derivative (Figure 7) which exhibits potent antitumor activity and significantly lower toxicity in comparison to wortmannin (21;334-336). *In vivo*, PX-866, an inhibitor of the p110alpha subunit of PI3K, exhibited negligible hepatotoxicity and demonstrated 80% inhibition of ser473-Akt phosphorylation in HT-29 colon tumor xenografts at low nanomolar concentrations (21). The IC$_{50}$ of PI3K by PX-866 is approximately 10-fold lower than that of wortmannin and exhibits an intraperitoneal-injected maximum tolerated dose (MTD) of 19.5 mg/kg in C57BL/6 mice in comparison to an MTD of 3.0 by wortmannin (21). Hyperglycemia, reduced glucose tolerance and transient hyperinsulinemia lasting six hours following administration, however, were observed *in vivo* (335). Further studies demonstrated that PX-866 treatment induces decreases in cancer cell motility and growth (334) and potentiates the antitumor activity of the epidermal growth factor receptor inhibitor, gefitinib (335). In examining a potential role for activation of SREBP by the PI3K/Akt, we were prompted to employ this compound in several experiments designed to study its effects on lipid metabolism while appreciating the potential for its effect on insulin and glucose metabolism.
Figure 7: Chemical structures of Wortmannin and its derivative PX-866 (21)
Rationale for studies:

The ability of lipids to influence cell signaling and function highlights the importance of studies that characterize the mechanisms, whether biological or chemical, that influence lipid metabolism. At the systemic level, the liver is crucial to maintenance of lipid homeostasis and is the site at which these many of these biological processes occur. Clearly, the study of lipid metabolism and the effectors involved is broad in scope. Nonetheless, the studies presented here are an attempt to characterize the effect on hepatic lipid metabolism by several distinct biological entities: replication-deficient Adenovirus-5, the group VIA calcium-independent phospholipase A_2 and the novel phosphatidylinositol 3-kinase inhibitor PX-866.

Author’s note: Unless, otherwise noted, the following studies emphasize and refer to SREBP1c when the term “SREBP1” is used.
CHAPTER 2: The influence of replication-deficient adenovirus-5 on hepatic lipid metabolism

Abstract: Adenovirus 5- (Ad-5) based expression systems are commonly used to investigate the functions of gene products in cell lines and animal models. Recent reports have associated viral and other microbial infections with changes in metabolism and dyslipidemia. These observations prompted the hypothesis that replication-deficient Ad-5 might augment lipid synthesis in liver. To test this hypothesis, C57BL/6 mice were infected with first generation replication deficient Ad-5 and screened for hepatic and plasma triglyceride levels and expression of lipogenic genes and gene products. Ad-5 infection induced transient hypertriglyceridemia together with increased expression of sterol regulatory element binding protein-1 (SREBP1) and its target genes in the triglyceride synthesis pathway. Unlike Hepatitis C Virus (HCV), another liver-directed virus associated with hepatic dyslipidaemia, Ad-5 infection selectively targeted SREBP1, as no changes were observed in SREBP2 or cholesterol levels. Ad-5-infected HepG2 human hepatoma cells and primary rat hepatocytes also exhibited increased expression of SREBP1 and its targets, suggesting that the virus induced dyslipidemia through direct effects on the liver. Ad-5 infection induced phosphorylation of Akt in HepG2 cells and livers of infected mice. Selective inhibitors of phosphatidylinositol 3-kinase (PI3K) suppressed induction of SREBP1 and hypertriglyceridemia in cultured cells and Ad-5 infected mice. Together, these studies support the emerging concept of
virus-induced dyslipidemia and suggest a possible link between Ad-5 infection and hepatic steatosis. These data also raise concerns about use of replication-deficient Ad-5 expression systems to study lipid metabolism in the liver.
Introduction

There is accumulating evidence that supports a relationship between changes in metabolism, dyslipidemia, fatty liver disease, and increased obesity with viral (97;98;100), bacterial (101;102), and parasite (103) infections. Hepatitis C virus (HCV) infection, in particular is strongly associated with insulin resistance, non-alcoholic fatty liver disease (NAFLD) and increased expression of lipogenic genes in hepatocytes (98;104). Adenovirus-36 (Ad-36) infections are also correlated with aberrant lipid metabolism (116;117) and adenovirus-5 (Ad-5) induces adiposity in mice (118). Replication-deficient adenoviruses, particularly adenovirus-5 (Ad-5), are widely used as expression vectors for gene transfer in vitro and in vivo (20). Viral replication is not necessary for Ad-36 induction of adipogenesis (337), suggesting that replication-deficient adenovirus may have the same lipogenic effects as does wild type.

Replication-deficient adenoviruses have become indispensable tools for molecular biologists and are widely used as expression vectors in vitro and in vivo (20). Of the 51 currently identified serotypes of human adenoviruses, adenovirus-5 (Ad-5) has been extensively characterized and in its replication-deficient form is widely employed for transgene expression for in vitro and in vivo studies. Replication-deficient Ad-5 is the most commonly used adenovirus-based transgene expression vector (113). Like HCV, Ad-5 primarily targets the liver when administered intravenously (20). Given the major role of liver in systemic lipid metabolism, we
hypothesized that replication-deficient Ad-5 engineered for expression of exogenous genes would induce dyslipidemia in infected mice.

**Materials and Methods**

**Materials**

Cell culture reagents were obtained from Invitrogen or Hyclone. PX-866, Oil Red O and thyroxine were obtained from Sigma. Calpain Inhibitor I was obtained from Biomol. Antibodies were obtained from Cell Signaling Technology (total Akt and phospho-Akt, ser473), BD Biosciences (SREBP1 and FAS), Calbiochem (actin), and Pierce (HRP-coupled secondary antibodies). All qPCR primers and probes were purchased from Integrated DNA Technologies. All first-generation replication-deficient adenovirus-5 constructs lacking early genes E1 and E3 were obtained from the Massey Cancer Center Virus Vector Shared Resource facility of the Virginia Commonwealth University Medical Center.

**Mice**

C57BL/6 mice obtained from the Jackson Laboratory were housed on a reverse light/dark cycle and provided standard chow and water *ad libitum* except where indicated otherwise. Eight to ten-week-old mice weighing approximately 20-25 grams were infected by tail-vein injection with 1 x 10E11 particles of adenovirus-5 expressing beta-galactosidase. Mice were sacrificed at 2 to 5 days after infection. The inhibitor PX-866 at a dose of 12.5 mg/kg or vehicle was administered by tail-vein injection 3 to 4
hours prior to infection. Another injection of PX-866 or vehicle was administered 2 days after infection. PX-866 dissolved in DMSO was administered using a total vehicle volume of 100 uL PBS containing 20% DMSO. Mice were sacrificed by exsanguination. Livers were excised and flash frozen. Plasma was collected. Mice were maintained in accordance with Virginia Commonwealth University Institutional Animal Care and Use Committee standards.

**Plasma and lipid analysis**

~100 mg of liver tissue was homogenized in 1 mL of PBS. Lipids were extracted according to the method of Folch (338), dried under nitrogen, and dissolved in PBS containing 1% Triton X-100 (339). Triglyceride and total cholesterol were quantified with colorimetric assay kits from Wako Diagnostics: L-Type TG H and Cholesterol E, respectively and normalized to starting liver mass. Plasma triglyceride, cholesterol, ALT, AST, Alkaline Phosphatase and glucose were quantified by the diagnostic laboratories of the McGuire Veterans Affairs Hospital, Richmond, VA. Plasma insulin was analyzed with the Mercodia Mouse Insulin ELISA.

**Cell culture**

HepG2 human hepatoma cells (ATCC) were maintained in Dulbecco’s Modified Eagle Medium or Dulbecco’s Modified Eagle Medium without glucose for kinetic analysis of Akt phosphorylation (Gibco) supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin/streptomycin, 2 mM glutamine, 1 mM sodium pyruvate and 0.1 mM non-
essential amino acids. Primary rat hepatocytes were isolated from Harlan Male Sprague-Dawley rats (200-300 g) as described previously (340;341) and plated on collagen-coated plates. Rat hepatocytes were maintained in William’s E Media (Hyclone) supplemented with 1% penicillin, 0.1 µM dexamethasone, and 1 µM thyroxine. All cell cultures were maintained at 7.5% CO₂ humidity and 37°C. Rats used were maintained in accordance with Virginia Commonwealth University Institutional Animal Care and Use Committee standards.

**Infection and treatments of HepG2 and primary rat hepatocytes**

HepG2 cells were plated at 5 x 10⁵ to 10⁶ cells/well in 6-well plates. Primary rat hepatocytes were plated at 50% confluence on six-well collagen-coated plates. Cells were infected with cesium chloride purified empty replication-deficient adenovirus-5 at the indicated MOI. After 16-20 hours, virus-containing media was removed and cells were cultured in fresh media until approximately 72 hours post infection. For kinetic analysis of Akt phosphorylation, HepG2 cells were infected at an MOI of 15 and harvested at the indicated times for time points up 72 hours post infection. For cells not harvested in the first 15 hours, virus was removed and media was replenished at 15 hours post infection for the remaining time points. HepG2 cells or primary rat hepatocytes were pre-treated with the indicated concentrations of PX-866, Ly294002, or DMSO vehicle for one hour before infection with replication-deficient Ad-5 at MOI 15 and 2.5 for HepG2 cells and primary rat hepatocytes, respectively. After overnight infection in the presence of inhibitors or vehicle, Ad-5 was removed. Fresh media,
inhibitors and vehicle were replaced daily until harvest at approximately 72 hours post infection. DMSO concentration never exceeded 0.04 percent.

**Protein Analysis by immunoblot**

Mouse liver homogenates were prepared from ~15 mg liver. Using a dounce homogenizer, tissue was homogenized in a buffer containing 10 mM HEPES, pH 7.4, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM NaF, 30 mM Na$_4$P$_2$O$_7$, 20 mM 2-glycerophosphate, 1 mM activated Na$_3$VO$_4$, 1 % SDS supplemented with a phosphatase inhibitor cocktail I (Sigma) and a complete protease inhibitor cocktail (Roche). HepG2 cells and primary rat hepatocytes were incubated with 25 µg/ml calpain inhibitor I for two hours prior to harvest. Cell homogenates were prepared in 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM NaF, 30 mM Na$_4$P$_2$O$_7$, 20 mM 2-glycerophosphate, 1 mM activated Na$_3$VO$_4$, 5 mM EDTA, 2 mM EGTA, 1% SDS supplemented with a complete protease inhibitor cocktail (Roche). Proteins were separated by SDS-PAGE followed by immunoblotting with the antibodies described above. Immunoreactive proteins were detected with chemiluminescence (ECL kit from Amersham or Femto kit from Pierce) which was captured on X-ray film. Signals were quantified by densitometry (Alpha Inotech FC8800) and mean ± of the densitometry signals is shown below the blot.

**RNA Analysis by Quantitative reverse transcriptase PCR**
Mouse liver RNA was prepared using the SV Total RNA Isolation System (Promega). mRNA encoding SREBP1, SREBP2 and ACC were screened by SYBR Green qPCR using the Brilliant QRT-PCR Core Reagent Kit, 1-Step (Stratagene). rRNA encoding 18S served as an internal control and was screened using the TaqMan, One-Step RT-PCR Master Mix Reagents (Applied Biosystems). Primers described previously (342) were as follows: SREBP1, sense 5’-CACTGAAGCAAGCTGAATAAATCTG-3’, antisense 5’-AGTGATTTGCCTTTTGCTGCACTT-3’; SREBP2, sense 5’-CAAGAGAAAGTTCCTATCAAGCAAGTG-3’, antisense 5’-GTCCTTCAACTCTATGATTTCGTTGCTGTT-3’; ACC, sense 5’-AGGATTTTGCTGTTTCTCAGAGCTT-3’, antisense 5’-CAGGATCTACCCAGGCACAT-3’. Primers and probe for 18S described previously (343) were: sense 5’-AAAATTAGAGTGTCAAAAGCAGGC-3’, antisense 5’-CCTCAGTTCCGAAAAACCAACAA-3’, probe CY5-CGAGCCGCCTGGATACCGCAGC-BHQ-2. Differences in the fold levels of mRNA were calculated based on the standard curve method. The standard curve was generated using a mouse liver RNA sample from one mouse used in the experiment.

**Oil Red O staining**

HepG2 cells were fixed and stained with Oil Red O according to Laughton (344). In brief, cells were washed with PBS and fixed using 2% (v/v) perchloric acid. Fixed cells were stained with Oil Red O dissolved in 1,2-propanediol at 2 mg/mL and prepared as previously described (344). Images of stained cells at 20X magnification were collected.
with an Olympus DP12 digital microscope camera. The experiment was repeated three times.

**Statistical Analysis**

All *in vitro* experiments were repeated at least three times. The mean ± standard deviation of three determinations is shown. The student’s t-test with $p < 0.05$ as a cutoff for statistical significance was used. Three to eight mice were studied for each *in vivo* condition. The mean ± standard error is shown. Mann-Whitney two-sided U test with $p < 0.05$ as a cutoff for statistical significance was used to analyze data generated from *in vivo* experiments unless otherwise indicated.
Results

To begin to test the hypothesis the Ad-5 infection increases hepatic lipid synthesis, C57BL/6 mice were infected by tail-vein injection with replication-deficient Ad-5 expressing β-galactosidase (β-gal), a commonly used in vivo and in vitro control vector for exogenous gene expression (20). Mice were sacrificed 4 and 5 days post infection. Livers and plasma were screened for lipid and proteins associated with lipid metabolism. Compared to uninfected mice, mice infected with Ad-5 exhibited elevated plasma triglyceride on day 4 (Figure 8A) and elevated hepatic triglyceride (Figure 8C) on days 4 and 5 after infection with Ad-5. In contrast, infected and uninfected mice exhibited similar levels of plasma and hepatic cholesterol. Synthesis of fatty acids and triglycerides is regulated by sterol regulatory element binding protein-1 (SREBP1), a helix-loop-helix lipid-regulated transcriptional activator that is synthesized as a ~125 kDa precursor and processed to its ~68 kDa transcriptionally active form in lipid-depleted cells (25). Hence, Ad-5 could increase triglyceride synthesis through increased SREBP1 expression, processing, or both. To test the hypothesis that Ad-5 elevated triglyceride through induction of SREBP1, we screened liver homogenates for expression of SREBP1. Livers of Ad-5 infected mice expressed precursor SREBP1 protein at higher levels than did uninfected livers (inset, Figure 8B), suggesting that Ad-5 induced SREBP1 at the expression level.

To confirm this observation, we used real time PCR (qPCR) to quantify SREBP1 transcripts in livers of control and Ad-5 infected mice.
SREBP1 transcripts were significantly elevated in livers of infected mice on day 4 post infection (Figure 8B). The effect was transient, as SREBP1 expression returned to baseline by day 5. Transcripts encoding acetyl-CoA carboxylase (ACC), a SREBP1 target gene that encodes an essential enzyme in the fatty acid/ triglyceride biosynthetic pathway (25), were elevated in infected mice at day 4 and 5 (Figure 8B) suggesting that Ad-5 infection increased levels of transcriptionally active mature SREBP1 protein as well. Together, these data indicate that infection with replication-deficient Ad-5 induces a transient hypertriglyceridemia secondary to increased SREBP1 expression. These changes in lipid metabolism are in contrast to those in transgenic mice expressing mature SREBP1 which exhibit elevated hepatic triglyceride but reduced plasma levels of this lipid (54). Over expression of SREBP1 in the transgenic model is a chronic change that may prompt adaptive responses that temper plasma triglyceride levels. The increase in plasma triglyceride that we observe is likely an acute response to infection with replication-deficient Ad-5 that is transient because the replication-deficient virus induces only a single round of infection.

Control and infected livers exhibited similar levels of transcripts encoding SREBP2, a lipid-regulated transcriptional activator that induces enzymes associated with cholesterol synthesis (25) (Figure 8D). This observation was consistent with the unchanged cholesterol mass in Ad-5 infected livers (Figure 8B). Importantly, the Ad5-induced changes in hepatic lipid metabolism largely preceded frank hepatotoxicity, as plasma levels of alanine aminotransferase (ALT) and alkaline phosphatase (alkphos) were similar in control and Ad-5-infected mice on day 4 post infection (Figure 8E).
Aspartate aminotransferase (AST) was slightly elevated on day 4; however, this increase was modest compared to that observed on day 5. Effects of adenovirus infection on SREBP1 and lipid metabolism were more modest on day 5 than day 4. This could be related to the more substantial liver damage that occurs on day 5 post infection. Together, these data indicate a selective induction of triglyceride synthesis in Ad-5 infected livers. Furthermore, these observations clearly distinguish effects of Ad-5 from those of HCV, as HCV infection is associated with reduced serum cholesterol and no change in serum triglyceride levels (98;345). In contrast to our observations, another group has reported no changes in plasma or liver triglyceride in response to control Ad-5 vectors (346). The reason for this apparent contradiction is unclear at present, but may be related to differences in viral dose, laboratory chow, or other issues.
Figure 8: Replication-deficient Ad-5 induces SREBP1, its target genes, and hypertriglyceridemia in mice. C57BL/6 mice were infected with $10^{11}$ particles of first generation Ad-5 expressing β-gal or mock infected with saline and sacrificed at day 4 or 5 post infection. (A) Quantification of lipids in plasma. (B) Quantification of liver lipid, expressed as mg lipid per g of liver. (C) Quantification of hepatic SREBP1 and ACC RNA. Inset, liver homogenates from uninfected and Ad-5-infected mice sacrificed at day 4 were screened for SREBP1 by immunoblot. Mean ± standard deviation (SD) of the densitometry signals normalized to actin is shown above blot. (D) Quantification of hepatic SREBP2 RNA. SREBP1, SREBP2, and ACC RNA was normalized to 18S RNA and is expressed as relative mRNA expression, with uninfected mRNA set at 1.0. (E) Quantification of ALT, AST and Alk Phos in plasma. * p < 0.05 compared to uninfected. Three or four mice were studied in each group.
We next addressed the mechanism of replication-deficient Ad-5 induction of SREBP1 and hepatic triglyceride. Because reports suggest that HCV, another virus that targets liver and augments lipid synthesis, induces changes in adiponectin, insulin, and glucose levels (347-349) that could increase hepatic SREBP1 expression, we could not discount the possibility that Ad-5 induced hepatic SREBP1 by increasing glucose or insulin levels in mice. However, control and Ad-5 infected mice showed no difference in levels of insulin and glucose four days post infection. In fact, plasma glucose was significantly decreased at day 3 (Figure 9). At day 4, non-fasting mean plasma glucose was 280 ± 42 mg/ dl for uninfected mice and 285 ± 10 mg/ dl for Ad-5 infected mice (seven mice/ group), whereas non-fasting mean plasma insulin was 1.4 ± 0.9 mg/dL for uninfected mice and 1.3 ± 0.4 mg/dL for Ad-5 infected mice (three mice/ group). These observations prompted the hypothesis that Ad-5 directly interacts with liver cells and triggers signaling events that culminate in induction of SREBP1.
Figure 9: Replication-deficient Ad-5 decreases plasma glucose in mice. C57BL/6 mice were infected with $10^{11}$ particles of first generation Ad-5 expressing β-gal or mock infected with saline and sacrificed at day 2 or 3 post infection. Error bars represent mean ± standard deviation (n =4). * p < 0.05.
We performed experiments to determine whether Ad-5 directly interacts with liver cells and triggers signaling events that culminate in induction of SREBP1. HepG2 human hepatoma cells and rat primary hepatocytes were infected with replication-deficient Ad-5. Cells were cultured in the presence of Ad-5 for approximately 16 hours, subsequently cultured in the absence of virus and analyzed 72 hours from the start of infection. Fixed cells were stained with Oil Red O to detect triglycerides and other neutral lipids. As shown in Figure 10A, infected HepG2 cells exhibited increased Oil Red O staining at relatively modest multiplicity of infection (MOI). Whole cell homogenates were prepared and screened for SREBP1, SREBP2, and fatty acid synthase (FAS), another SREBP1 target and enzyme in the fatty acid/triglyceride synthetic pathway. HepG2 cells and primary hepatocytes infected with Ad-5 demonstrated robust increases in expression of SREBP1 (Figure 10B-C). Expression of FAS, a target of SREBP1 was also increased in infected cells (Figure 10D, data not shown) again indicating increased levels of the transcriptionally active mature form of SREBP1 in infected cells. Similar results were obtained with “empty” Ad-5 vector (shown) and Ad-5 expressing green fluorescent protein (data not shown). In contrast, Ad-5 infection had no effect on SREBP2 expression (data not shown), consistent with the observation that neither SREBP2 (Figure 8D) nor plasma cholesterol was elevated in Ad-5-infected mice (Figure 8A). Together these data indicate that like HCV, Ad-5 induces lipid synthesis through direct interaction with hepatocytes (350;351). However, they also further underscore the differences between Ad-5 and HCV, as HCV
infection induces both SREBP1 and SREBP2 in Huh7 human hepatoma cells (350). HCV core protein induces expression, processing, and phosphorylation of both SREBP1 and SREBP2 and thereby expression of their target genes (350-352). In addition, HCV core protein suppresses the activity and/or expression of microsomal transport protein (MTP), thereby reducing the assembly and secretion of VLDL (353). Although we cannot rule out an effect of replication-deficient Ad-5 on MTP at present, elevated plasma triglyceride levels suggest that the SREBP1 is a major target of the virus and that induction of this protein results in the hypertriglyceridemia observed in Ad-5 infected mice.
Figure 10: Replication-deficient Ad-5 induces SREBP1, its target genes, and neutral lipid accumulation in cells. HepG2 cells and primary rat hepatocytes were infected with replication-deficient Ad-5 at various multiplicity of infection (MOI, shown). (A) HepG2 cells were stained with Oil Red O to detect neutral lipids. Shown are fields from a representative experiment. The experiment was performed three times. (B and C) Immunoblot of SREBP1 in homogenates of (B) HepG2 cells and (C) primary rat hepatocytes. (D) Immunoblot analysis of FAS in homogenates of HepG2 cells. All immunoblot signals were quantified by densitometry and normalized to actin. Fold change in SREBP1 or FAS (mean ± SD relative to uninfected cells) from three separate experiments is shown below each blot. A representative blot is shown. * p < 0.05, ** p < 0.01, *** p < 0.001 compared to uninfected.
Figure 10B

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Figure 10C

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fold change

SREBP1

**

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Figure 10D

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FAS fold change
We next addressed the molecular mechanism of replication-deficient Ad-5 induction of SREBP1. Ad-5 infection has a selective effect on SREBP1 and its targets and does not impact SREBP2 expression or function. These observations are reminiscent of the effects of insulin, another selective inducer of SREBP1. Phosphatidylinositol 3-kinase (PI3K) and its downstream effectors Akt and the atypical protein kinases Cζ and λ are components of the insulin signaling pathway that are essential for insulin-stimulated lipid and glycogen synthesis as well as the inhibition of gluconeogenesis (88;89). HCV infection activates the PI3K/ Akt pathway in Huh 7 hepatoma cells (350). Notably, other investigators have observed PI3K/Akt activation in response to replication-deficient Ad-5 infection (111;112). These observations prompted the hypothesis that Ad-5 induces SREBP1 by activating the PI3K/ Akt pathway. To test this hypothesis, HepG2 cells were infected with replication-deficient Ad-5 at MOI 15 and screened for phosphorylated Akt as a measure of PI3K/ Akt activation. A sustained increase in Akt phosphorylation was observed starting at 12 hours and extending through at least 72 hours post infection (Figure 11A). Levels of phosphorylated Akt were also elevated in livers of Ad-5 infected mice (Figure 11B) due to both increased Akt mass (ratio total Akt/ actin = 0.16 ± 0.01, uninfected; 0.26 ± 0.02, infected, p = 0.029) and increased phosphorylation of Akt protein (ratio phosphoAkt/ total Akt= 17± 0.6, uninfected and 27 ± 1, infected, p= 0.057). To our knowledge, the induction of Akt expression is unique to Ad-5 and is not observed in other viral infections. At present, we are uncertain why Akt protein increases in the livers of Ad-5 infected mice but not in infected HepG2 cells. However, the data suggest
that Ad-5 infection increases the pool of activated Akt both infected mice and cultured cells. To directly test the hypothesis that Ad-5 induces SREBP through PI3K/Akt, we treated hepatocytes and C57BL/6 mice with Ly294002 or PX-866, a wortmannin analog (21;336) before infection with replication-deficient Ad-5. Both Ly294002 and PX-866 suppressed Ad-5 induction of SREBP1 in cultured hepatocytes (Figure 11C and D).
Figure 11: Replication-deficient Ad-5 activates SREBP1 via the PI3K/Akt pathway which is involved in induction of lipogenesis. (A) HepG2 cells were infected with replication-deficient Ad-5 (MOI 15) and harvested at the indicated times. Homogenates were screened for total and phosphorylated Akt. A representative experiment is shown. A representative experiment is shown. The bar graph below shows mean and standard deviation (error bars) of the ratio of phosphorylated Akt/total Akt (white) or total Akt/actin (black) in three experiments. * p < 0.05 compared to uninfected; ** p < 0.01 compared to uninfected. (B) Liver homogenates from vehicle-treated mice were screened for total and phosphorylated Akt and actin by immunoblot. The ratios of phosphorylated/total Akt, phosphorylated Akt/actin and total Akt/actin (relative to uninfected mice) are shown below the blots. (C) HepG2 pre-treated with the indicated concentrations of PX-866, Ly294002, or DMSO vehicle for one hour before infection with replication-deficient Ad-5 at MOI 15. Cells were cultured in the presence of inhibitors or vehicle for the duration of the experiment and were harvested at approximately 72 hpi. Cell homogenates were prepared and screened for SREBP1 by immunoblot. Signals were quantified by densitometry and normalized to actin (loading control). Percent inhibition of Ad-5-induced SREBP1 activation is shown below blot. A representative experiment is shown (n = 2). (D) Immunoblot analysis of SREBP1 in primary rat hepatocytes treated as in (C) normalized to actin. Infection MOI = 2.5. Lanes: 1: vehicle, uninfected; 2: vehicle, Ad-5; 3: 30 µM PX-866, Ad-5; 4: 15 µM PX-866, Ad-5; 5: 20 µM Ly294002, Ad-5; 6: 10 µM Ly294002, Ad-5. Percent inhibition of
Ad-5-induced SREBP1 activation is shown below blot. A representative experiment is shown (n = 2).
Figure 11A
Figure 11B

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<td>actin</td>
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<tr>
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<th>Ad-5</th>
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<tbody>
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<td>6.8 ± 1</td>
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<tr>
<td>total Akt/ actin</td>
<td>0.16 ± 0.01</td>
<td>0.26 ± 0.02*</td>
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Figure 11C

Figure 11D
PX-866 induces transient increases in plasma insulin (21). Despite these potential confounding effects that would be expected to increase SREBP1 expression, PX-866 suppressed SREBP1 (p=0.0058, Figure 12A) and hypertriglyceridemia (p=0.006, Figure 12B) induction by Ad-5 to levels that were not statistically different from uninfected controls. Furthermore, PX-866 treatment induced little or no liver damage, as assessed by plasma ALT, AST, and alk phos levels (Figure 12C).
Figure 12: Inhibition of the PI3K/Akt pathway blocks Ad-5 induced lipogenesis in vivo. (A-C) C57BL/6 mice were pretreated with 12.5 mg/kg PX-866 or DMSO before infection with replication-deficient Ad-5 and sacrificed on day 4 post infection (A) Quantification of hepatic SREBP1 RNA, normalized to 18S RNA. The data are expressed as relative mRNA expression, with uninfected, vehicle-treated mice set at 1.0. (B) Quantification of plasma triglyceride (mg/ dl). Each symbol represents one mouse. Horizontal bar is mean of the group. * p < 0.05 compared to Ad-5, vehicle-treated; # p < 0.05 compared to uninfected, vehicle-treated. (C) C57BL/6 mice were treated as shown. Plasma was harvested 4 days post infection and assessed for ALT, AST, and Alkphos. Data presented are the mean of “n” determinations and error bars are the standard error of the mean. * statistically different from vehicle, uninfected (p < 0.05).
Figure 12C

[Bar chart showing enzyme levels with error bars.]

- ALT
- AST
- Alk phos

- Vehicle (n=7)
- Uninfected (n=7)
- PX-866 (n=6)

* Indicates significant difference.
Discussion

This report characterizes previously unreported effects of replication-deficient Ad-5 on hepatic lipid metabolism: induction of the PI3K/ Akt pathway, increased expression of SREBP1 and its targets, increased hepatic triglyceride, and hypertriglyceridemia. These observations distinguish effects of Ad-5 from those of HCV, as HCV infection is associated with reduced serum cholesterol and no change in serum triglyceride (98;345). Ad-5-induced hyperlipidemia also differs from that of transgenic mice expressing mature SREBP1 which exhibit elevated hepatic triglyceride but reduced plasma levels of this lipid (54). Over expression of SREBP1 in the transgenic model is a chronic change that may prompt adaptive responses that temper plasma triglyceride levels. In contrast, the increase in plasma triglyceride that we observe is likely an acute response to Ad-5 infection.

The molecular mechanism of PI3K activation by replication-deficient Ad-5 is unclear at present. The observations that circulating insulin levels are reduced in infected mice and that Ad-5 induces SREBP1 in cultured hepatocytes argue against a systemic effect and suggest that Ad-5 induces SREBP1 through direct interaction with hepatocytes. Another group C adenovirus, adenovirus-2 (Ad-2), activates PI3K and Akt when its penton base binds αv integrin (354;355). Other studies suggest that E4-ORF1 protein is required for PI3K activation by Ad-5 (356). As our replication-deficient adenoviruses retain both the penton base and E4-ORF1, additional experiments are necessary to delineate the mechanism of PI3K activation in our system.
We are not certain if induction of SREBP1 is common feature of adenovirus infection or a specific response to infection with Ad-5. Like Ad-5, Ad-2 activates PI3K making it likely that this group C adenovirus also induces SREBP1 and hepatic lipid synthesis (354;355). Ad-9 and Ad-36 (group D adenoviruses) also activate the PI3K pathway through E4-ORF1 (357;358), suggesting that SREBP1 may be induced by group D adenoviruses as well. Group D adenoviruses have other well-established effects on systemic lipid metabolism including induction of adipogenesis and obesity in a variety of animal models (358-360). These observations suggest that Ad-5 could promote obesity as well, a hypothesis that is supported by recent studies with wild-type Ad-5 in outbred CD1 mice (118). Thus, Ad-5 has a variety of effects on systemic biology that could contribute to development of the metabolic syndrome. This hypothesis merits further investigation.

Activation of the PI3K targets PKCζ and PKCλ is necessary for insulin-mediated induction of SREBP1 (89;361). Constitutively active PKCλ but not Akt induces SREBP1 in livers of wild type mice and restores SREBP1 expression in livers of p85α/ p85β double knock out mice to wild type levels (89). These observations prompt the hypothesis that the induction of SREBP1 by Ad-5 may be mediated through PKCζ/ λ. Although classical PKC is activated in Ad-2-infected HeLa cells (362), there are no reports of PKCζ/ λ activation in response to Ad-5 infection. Our preliminary experiments indicate that neither PKCζ nor PKCλ is phosphorylated upon Ad-5 infection (data not shown), but additional experiments are necessary to verify these
observations and confirm that $\text{PKC}_\zeta/\lambda$ is not involved in Ad-5-mediated induction of SREBP1.

Replication-deficient Ad-5 has become an indispensable expression vector for gene transfer and has been used in gene-therapy clinical trials (20). Our observations prompt concerns regarding the use of Ad-5 expression vectors. The negative control for such studies is often “empty” Ad-5 or Ad-5 expressing irrelevant protein. Our data highlight the importance of uninfected controls in these experiments.

Other investigators have demonstrated increased adiposity in Ad-5 infected mice and that other serotypes of adenovirus, infect adipocytes and induce adipogenesis and obesity (118;360). Our work is an important extension of these studies, as we demonstrate that adenovirus infection also alters lipid metabolism in the liver. These observations prompt the hypothesis that wild-type Ad-5 contributes to the etiology of fatty liver disease. Nearly 50% American adults have antibodies against Ad-5, indicating previous infection with the virus (363). It is tempting to speculate that some fatty liver disease patients develop this disorder secondary to adenovirus infection. Transgenic mice expressing transcriptionally active SREBP1 develop fatty liver due to increased synthesis of fatty acids and triglycerides (53;54). Patients infected with replication-competent Ad-5 could have chronically high levels of SREBP1, resulting in increased hepatic triglyceride synthesis and fatty liver. Additional studies are required to test this hypothesis and determine whether adenovirus infection is a risk factor for fatty liver disease.
Chapter 3: The group VIA calcium-independent phospholipase A\textsubscript{2} as a novel regulator of sterol regulatory-element binding proteins

Abstract:
Aberrant lipid metabolism underlies the complications of lipid disorders. Sterol regulatory element-binding proteins (SREBP) are transcription factors that regulate the transcription of genes involved in lipid metabolism. SREBP undergoes posttranslational processing from an inactive precursor to an active mature form. Exogenous unsaturated fatty acids (UFA) suppress SREBP processing, expression and SREBP-mediated gene expression. To date, an endogenous source of UFA capable of modulating SREBP is elusive. Localized to the same compartment as precursor SREBP, Group VIA calcium-independent phospholipase A\textsubscript{2} (iPLA\textsubscript{2}) releases UFA from the \textit{sn}-2 position of glycerophospholipids. We hypothesized that iPLA\textsubscript{2} provides endogenous UFA to suppress SREBP-mediated transcription. siRNA and chemical inhibitors were used to suppress iPLA\textsubscript{2} and plasmid and adenovirus strategies were used to overexpress iPLA\textsubscript{2} in CHO and HepG2 cells. iPLA\textsubscript{2} inhibition increased both SREBP expression and
SREBP-mediated transcription. In contrast, iPLA₂ overexpression attenuated SREBP expression and SREBP-mediated transcription. This decline in SREBP correlated with reduced expression of SREBP target genes and attenuated overall fatty acid synthesis in HepG2 cells. These data support the hypothesis that iPLA₂ generates endogenous UFA that limit SREBP function and suggest that this enzyme may hold therapeutic promise to treat the various diseases that stem from lipid dysregulation.
Introduction

Many of the genes encoding enzymes involved in fatty acid, triglyceride, and cholesterol synthesis contain sterol response elements (SRE) that allow for feedback regulation of expression (25;47;48). SREs promote gene expression when they are bound by sterol regulatory element binding proteins (SREBPs), members of the basic-helix-loop-helix family of transcription factors. The SREBPs are encoded by two genes, generating SREBP1 and SREBP2 proteins. The SREBP1 gene has two transcription start sites, which generate two forms of SREBP1: SREBP1a and SREBP1c. In general, SREBP2 regulates genes involved in cholesterol synthesis and metabolism while SREBP1a and 1c are more closely associated with the synthesis of fatty acids and triglycerides. SREBP1c appears to be the most relevant isoform in primary cells while cultured cells express primarily SREBP1a. In sterol loaded cells, SREBP proteins are inactive and tethered to the ER membrane through two transmembrane segments and an association with SREBP cleavage activating protein, SCAP. When sterol levels decline, SCAP dissociates from the ER resident protein Insig and promotes translocation of SREBP from the ER to the golgi apparatus (57;58). Two golgi-associated proteases, the site-1 and site-2 proteases, release the basic-helix-loop-helix portion of SREBP that then translocates to the nucleus to induce gene expression. Hence, SREBP processing is an essential event in the regulation of lipid synthesis and metabolism in the liver and this event is subject to feedback regulation by sterols.

Other investigators have demonstrated that SREBP processing is suppressed by exogenous polyunsaturated fatty acids (PUFA), that PUFA augments sterol suppression
of SREBP processing, and that SREBP1c expression is suppressed when PPAR-α binds PUFA derivatives (39;43;79;84;364). Like sterols, PUFAs suppress SREBP translocation from ER to golgi and thereby prevent processing and generation of mature, transcriptionally active SREBP (39;43;79). The effect of PUFAs appears to be largely specific for SREBP1 (76;80), although fish oil, a rich source of PUFA, also suppresses SREBP2 (365). Thus, PUFAs selectively suppress expression of SREBP1 targets, including fatty acid synthase (FAS), acetyl CoA carboxylase (ACC), and other genes associated with fatty acid and triglyceride synthesis. SREBP1c expression is another potential target for PUFAs and their derivatives through PPAR-α-dependent suppression of liver X receptor- (LXR)-mediated transcription of the SREBP1 gene (84;364). Together, these data indicate PUFA as a potential mechanism for controlling SREBP1 activation and thereby disorders associated with dyslipidemia such as fatty liver disease, cardiovascular disease, diabetes, and metabolic syndrome. Indeed, diets rich in PUFAs have been associated with decreased risk for atherosclerosis and cardiovascular disease (366;367).

All of these observations are derived from studies in which exogenous PUFAs are added to cultured cells or to the diets of laboratory animals. Although mammalian cells do not synthesize PUFA, these molecules are taken up from the diet and stored in neutral lipids and glycerophospholipids. Thus, glycerophospholipid can be viewed as a reservoir of PUFAs that might be released to regulate lipid synthesis and lipoprotein assembly by hepatocytes. The group VIA phospholipase A₂ (iPLA₂), is a lipid hydrolase
that attacks the \textit{sn}-2 ester bond of glycerophospholipids releasing lysophospholipid and unesterified fatty acid, often PUFA (37;368).

Recent reports suggest that iPLA$_2$ has major roles in insulin mobilization and fat metabolism. Glucose-stimulated insulin production is suppressed when insulinoma cells are treated with an inhibitor of iPLA$_2$ (287). In addition, iPLA$_2$ knock out mice exhibit impaired insulin secretion in response to glucose and forskolin and exaggerated hyperglycemia when treated with streptozotocin (136). Furthermore, these mice exhibit increased glucose intolerance when fed a high-fat diet (136). Conversely, iPLA$_2$ overexpressing cells exhibit increased insulin secretion in response to glucose stimulation (287;291). iPLA$_2$ overexpression augments ER stress in insulinoma cells thereby promoting apoptosis of a major source of insulin (369). Furthermore, recent studies suggest a role for iPLA$_2$ in adipogenesis, as terminal differentiation of 3T3-L1 preadipocytes is blocked when iPLA$_2$ expression is suppressed with siRNA and iPLA$_2$ expression is elevated in the adipose tissues of obese Zucker rats (178).

Given its connection to lipid and insulin metabolism, it seems reasonable to propose a role for iPLA$_2$ in the regulation of SREBP. We hypothesize that the reaction catalyzed by iPLA$_2$ is an endogenous source of PUFAs that suppress SREBP and thereby regulate the expression of genes that are controlled by SREBP (153;368). To test this hypothesis, we generated HepG2 human hepatoma and Chinese hamster ovary (CHO) cells with enhanced or reduced iPLA$_2$ activity. Our studies indicate that SREBP1 expression, transcriptional activity, and expression of SREBP1 target genes is suppressed in cells with high levels of iPLA$_2$ and that reduced iPLA$_2$ activity is
associated with increased SREBP1 expression, activity and target gene expression. Together, these studies suggest iPLA$_2$ as a novel target for the control of SREBP1 and thereby lipid disorders associated with its target genes.
Materials and Methods

Materials

Chinese Hamster Ovary (CHO) 7 cells were received from Dr. Joseph L. Goldstein (University of Texas Southwestern Medical Center). HepG2 cells were obtained from the ATCC. All siRNA was constructed using an online siRNA design engine provided by Qiagen and obtained from Qiagen-Xeragon. Primers employed in the design of adenoviral vectors for overexpression of iPLA$_2$ were obtained from Integrated DNA Technologies. The pSyn SRE luciferase construct was provided by Dr. Gregorio Gil (VCU Medical Center) and Dr. Timothy Osborne (University of California at Irvine). Luciferase and β-galactosidase activity was measured using the Tropix Dual-Light Kit from Applied Biosystems. N-acetyl-leucyl-leucyl-norleucinal (Calpain Inhibitor 1) and bromoenol lactone (BEL) were purchased from Biomol International. 1-Palmitoyl-2-$[^{14}\text{C}]$ palmitoyl-sn-glycero-3-phosphocholine (DPPC) and $[^{14}\text{C}]$ acetate were purchased from Amersham Biosciences and New England Nuclear, respectively. Unlabeled DPPC was purchased from Avanti Polar Lipids. Cell culture reagents were obtained from Invitrogen. Fatty acid-free bovine serum albumin and all organic reagents were purchased from Sigma Chemical Company. iPLA$_2$ polyclonal antiserum, monoclonal anti-SREBP-1 and 2 and polyclonal anti-ERK 1 were obtained from Cayman Chemical, BD Pharmingen/BD Biosciences and Santa Cruz Biotechnology, Inc., respectively. Secondary antibodies conjugated to horseradish-peroxidase were obtained from Pierce. All first-generation replication-deficient adenovirus-5 constructs were obtained from the
Massey Cancer Center Virus Vector Shared Resource facility of the Virginia Commonwealth University Medical Center.

**Cell Culture**

CHO7 cells were maintained in DMEM/F12 supplemented with 10% heat inactivated fetal calf serum, 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mM glutamine. For lipid depletion of CHO7 cells, cells were cultured in 1% BSA in lieu of 10% heat inactivated fetal calf serum. HepG2 human hepatoma cells (ATCC) were maintained in Dulbecco’s Modified Eagle Medium or Dulbecco’s Modified Eagle Medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum, 1% penicillin/streptomycin, 2 mM glutamine, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. All cell cultures were maintained at 7.5% CO$_2$ humidity and 37 degrees C.

**Mice**

C57BL/6 mice obtained from the Jackson Laboratory were housed on a reverse light/dark cycle and provided standard chow and water *ad libitum* except where indicated otherwise. Eight to ten-week-old mice weighing approximately 20-25 grams were infected by tail-vein injection with 1 x 10E11 particles of adenovirus-5 expressing beta-galactosidase or iPLA$_2$. Mice were sacrificed at 4 days after infection by exsanguinations. Livers were excised and flash frozen. Mice were maintained in
accordance with Virginia Commonwealth University Institutional Animal Care and Use Committee standards.

**Inhibition and overexpression of iPLA₂**

iPLA₂ was inhibited with BEL, a suicide inhibitor highly specific for iPLA₂ (146;210) and siRNA. BEL was resuspended in DMSO at a stock concentration of 25 mg/mL. Cells were incubated with BEL for the indicated times and concentrations of BEL. The siRNA for iPLA₂ targets the CHO iPLA₂ sequence 5’-AACCCACATAGTGGCTTCCGA-3’. Control siRNA was used to control for the effects of exogenous oligonucleotides. siRNA was transfected per the manufacturer’s instructions using Lipofectamine 2000 (Qiagen). Cells treated with siRNA were plated, allowed to adhere and treated with siRNA at time zero and retransfected 48 hours later with harvest for western, iPLA₂ activity assay or luciferase assay occurring at approximately 72 hours. iPLA₂ overexpression in HepG2 cells was achieved using replication deficient Ad-5 lacking E1 and E3. In brief, CHO iPLA₂ was cloned into the pZero tg-CMV vector that was used for insertion into replication-deficient Ad-5 performed by the Massey Cancer Center Virus Vector Shared Resource facility of the Virginia Commonwealth University Medical Center. HepG2 cells were plated at 5 x 10⁵ to 10⁶ cells/well in 6-well plates. Cells were infected with cesium chloride purified empty Ad-5 or Ad-5 expressing GFP or expressing iPLA₂ at the indicated MOI in a volume of 700 uL. After 16-20 hours, virus-containing media was removed and cells were cultured in fresh media until time of harvest. iPLA₂ overexpression was achieved
in CHO7 cells using a “CFP-iPLA<sub>2</sub>” construct in which CHO iPLA<sub>2</sub> was inserted into the pECFP-c1 vector provided by Dr. Robert Tombes, Virginia Commonwealth University, Dept of Biology. CHO7 cells were plated in 6-well plates at 5 x 10<sup>5</sup> cells per well and transfected in accordance with manufacturer’s instructions with CFP-iPLA<sub>2</sub> using Fugene 6 (Roche).

**iPLA<sub>2</sub> activity assay**

CHO7 cells were harvested and cell homogenates were prepared by resuspending pelleted cells with a buffer consisting of 10 mM HEPES, pH 7.4, 0.34 M sucrose, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 1 mM PMSF followed by sonication on ice. iPLA<sub>2</sub> activity was analyzed by an activity assay as described (161). Briefly, equal amounts of protein were incubated for two hours at 40 degrees C with a substrate consisting of 100 µM DPPC, 400 µM Triton-X 100, with 0.02 µCi labeled DPPC added as a tracer. Assays were processed and released palmitic acid was quantified according to the method of Dole (370). Each experimental sample was run in duplicate.

**Measurement of Luciferase activity**

The pSyn SRE construct containing a generic TATA element and three SREs of the hamster HMG CoA synthase gene fused into a luciferase vector was cotransfected with a B-galactosidase reporter to control for transfection efficiency. The pSyn SRE and β-gal constructs were transfected per the manufacturer’s instructions using Lipofectamine 2000 (Qiagen). For experimentation using siRNA, the pSYN SRE construct was
cotransfected with siRNA at 48 hours (siRNA treatment alone at time zero and cotransfected at 48 hours with the pSYN SRE construct). Cells were then harvested for luciferase analysis at approximately 24 hours after transfection with the pSYN SRE construct. For overexpression studies, the pSYN SRE construct was cotransfected with the overexpression construct and cells were assayed approximately 16 hours after transfection. Following the indicated treatment and transfection and incubation times of CHO7 cells with the pSYN SRE reporter and β-galactosidase (β-gal) construct, luciferase and β-gal activity were assayed per the manufacturer’s instructions using the Tropix Dual-Light Kit (Applied Biosystems).

**Protein extraction and immunoblotting**

Mouse liver homogenates were prepared from ~15 mg liver. Using a dounce homogenizer, tissue was homogenized in a buffer containing 10 mM HEPES, pH 7.4, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM NaF, 30 mM NaH₂PO₄, 20 mM 2-glycerophosphate, 1 mM activated Na₃VO₄, 1 % SDS supplemented with a phosphatase inhibitor cocktail I (Sigma) and a complete protease inhibitor cocktail (Roche). Following the indicated treatment, CHO7 or HepG2 cells received 25 µg/mL calpain inhibitor 1 and were harvested as described (43). Equal amounts of protein in whole cell homogenates were resolved on a 10% SDS-polyacrylamide gel (tris-glycine gel) and transferred to a supported nitrocellulose membrane (Bio-Rad) followed by blocking 1 hr to overnight in 5% non-fat dry milk suspended in tris-buffered saline (TBS) with 0.02% Tween 20. Following blocking, blots were incubated overnight with primary
antibodies, washed 3 times with TBS, incubated 2 hr with a secondary horseradish peroxidase conjugated antibody and washed 3 times. An enhanced chemilluminescent detection reagent (Amersham Biosciences) and subsequent detection of chemiluminescence using x-ray film were used to analyze the immunoblot. Primary antibodies for SREBP and iPLA\textsubscript{2} were obtained from BD Pharmingen and Cayman Chemical, respectively. ERK 1 antibody used to control for protein loading was obtained from Santa Cruz Biotechnology.

**Measurement of Fatty Acid Synthesis**

To analyze fatty acid synthesis as described, HepG2 cells were plated at 5 x 10\textsuperscript{5} cells per well in 6-well plates, with three wells/ treatment group. Cells were pulsed with 0.5 μCi/mL [\textsuperscript{14}C] acetate for two hours and harvested as described previously (371). Radiolabeled cells were washed one time with PBS, solubilized with 500 μL of 0.5M NaOH, and transferred to glass tubes. Saponification was achieved using 1.5 mL of EtOH and 250 μL of 50% (w/v) KOH. 100 μL aliquot was removed for protein quantification and analysis of total cell [\textsuperscript{14}C] acetate uptake. The remaining sample was incubated 1 hr at 60 degrees C. 500 uL were then acidified (pH < 3.0)—approximately 65 μL 10N HCL--and followed by extraction with 1 mL of hexane. Following vortexing, the organic layer was extracted and subjected to scintillation counting. The raw data were normalized by protein mass and are expressed as the cpm/μg protein.

**Statistical Analyses**
Experiments were repeated three times in most instances. A representative example is shown. Where applicable, the mean ± standard deviation of three determinations is shown and the student’s t-test with $p < 0.05$ as a cutoff for statistical significance was used to analyze the data shown.
Results

iPLA₂ over expression suppresses SREBP1 expression and activity

Other investigators have demonstrated suppression of SREBP1 expression and processing by exogenous unsaturated fatty acids (39;75). The goal of this investigation was to determine if endogenous iPLA₂-derived fatty acids had the same effect. To test this hypothesis, we developed a recombinant adenovirus expressing CHO iPLA₂ and examined the effects of its overexpression in HepG2 human hepatocarcinoma cells. HepG2 cells have relatively modest levels of endogenous iPLA₂ (Figure 13A). However, the adenovirus increased iPLA₂ expression in a dose dependent manner and increased catalytic activity was evident at relatively modest multiplicity of infection (MOI) (Figure 13A and B). HepG2 cells were infected with wild type adenovirus (MOI 50) or iPLA₂ adenovirus, cell homogenates were prepared, and SREBP1 protein expression assessed by immunoblot analysis. As shown in Figure 14, SREBP1 expression inversely correlated with expression of iPLA₂. Despite the increases in SREBP1 following Ad-5 infection of hepatocytes as detailed in Chapter 2, the data in Figure 14 indicate that iPLA₂ overexpression using Ad-5 attenuates this Ad-5-induced expression of SREBP1. It can be assumed that Ad-5 lacking the iPLA₂ gene is itself increasing SREBP1 expression. C57BL/6 mice infected with Ad-5 expressing iPLA₂ also demonstrated decreased hepatic expression of SREBP1 in response to iPLA₂ overexpression (Figure 15). Again, while Ad-5 is shown to increase SREBP1 in vivo, insertion of the iPLA₂ gene in Ad-5 inhibits this outcome. Interestingly, of mature and
precursor SREBP1, levels of precursor SREBP1 were the most profoundly reduced in response to iPLA$_2$ overexpression. This observation prompted experiments to determine if iPLA$_2$ overexpression correlated with reduced SREBP transcriptional activity that is facilitated by mature SREBP. Experiments to determine whether iPLA$_2$ overexpression inhibits SREBP-mediated transcription are ongoing. Preliminary findings begin to support that hypothesis that iPLA$_2$ overexpression indeed inhibits SREBP transcriptional activity.
Figure 13: Overexpression of iPLA$_2$ in HepG2 cells with recombinant adenovirus

Adenovirus-mediated over expression of iPLA$_2$ in HepG2 cells. HepG2 human hepatoma cells were infected with empty adenovirus or recombinant adenovirus expressing iPLA$_2$ and harvested approximately 72 hours post infection. Cell homogenates were prepared and screened for iPLA$_2$ expression (A) or catalytic activity (B). Expression of ERK 1/2 was quantified as a loading control for the immunoblot. A representative experiment is shown (n=2).
Figure 14: iPLA₂ suppresses SREBP1 expression in HepG2 cells

HepG2 cells were infected with control adenovirus expressing GFP (MOI 50, lane 1) or adenovirus expressing iPLA₂ at MOI 5 (lane 2), MOI 20 (lane 3), or MOI 50 (lane 4). Cell homogenates were prepared and screened for SREBP1 by immunoblot. ERK1/2 is a loading control (n=1).
Figure 15: Overexpression of iPLA$_2$ in C57BL/6 mice results in decreased hepatic SREBP1 expression

C57BL/6 mice were infected with $1 \times 10^{11}$ particles of Ad-5 expressing iPLA$_2$ or beta-gal (control). Mice were sacrificed at 4 days post infection and liver homogenates were prepared and analyzed by immunoblot for SREBP1. Each lane represents one mouse of the indicated experimental group.
iPLA2 suppresses expression of SREBP1 target genes

The observations in Figures 14 and 15 prompted experiments to determine if iPLA2 overexpression correlated with reduced SREBP transcriptional activity. Therefore, as a surrogate marker for SREBP1 transcriptional activity, an immunoblot strategy was used to screen HepG2 cells infected with empty control Ad-5 or recombinant Ad-5 overexpressing iPLA2 for expression of FAS, an SREBP1 target associated with synthesis of fatty acids and triglycerides (53). As shown in Figure 16, iPLA2 overexpression suppressed FAS expression. Importantly, the decline in FAS expression correlated with reduced fatty acid synthesis in HepG2 cells, as assessed by incorporation of [14C]-acetate into fatty acid (Table 2). Shown are data from two independent experiments: an experiment in which cells were incubated in FCS-containing media and one in which cells were incubated in delipidated media.
Figure 16: iPLA$_2$ overexpression suppresses SREBP1 target genes in HepG2 cells

HepG2 cells were infected with empty (MT) adenovirus or adenovirus expressing recombinant iPLA$_2$. Cell homogenates were prepared and screened for expression of the SREBP1 targets FAS 72 hours post infection (n=1).
Table 2: iPLA₂ overexpression decreases fatty acid synthesis in HepG2 cells

HepG2 cells were infected by with control empty Ad-5 or Ad-5 overexpressing iPLA₂. Cells were assayed as described in “Materials and Methods” at 72 hours post infection. Cells subject to lipid-free conditions were incubated in delipidated media for 16 hours prior to incubation with [¹⁴C]-acetate. *fatty acid synthesis expressed as cpm of incorporated [¹⁴C]-acetate /ug protein (n = 1 for experiment performed in presence and absence of lipid). The experimental groups indicated as “lipid free” (second data set of three experimental groups) were cultured in lipoprotein-depleted serum containing media supplemented with 1% fatty-acid free BSA.

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</tr>
<tr>
<td>iPLA₂ virus</td>
<td>137</td>
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Analysis of SREBP1 in CHO cells overexpressing iPLA₂ and in CHO cells with low iPLA₂ activity

Together, these data in vivo and in HepG2 cells support the hypothesis that bioactive lipids generated downstream of the iPLA₂ reaction suppress SREBP1 expression and presumably processing based on the effect of iPLA₂ overexpression on the SREBP1 gene target FAS and fatty acid synthesis. These observations prompted additional experiments to determine if the converse was true: if SREBP1 was induced in cells with low iPLA₂ activity. As endogenous iPLA₂ activity is low in HepG2 cells, an additional model was used to test this hypothesis, CHO cells. Other investigators have used this model extensively to delineate the mechanisms controlling SREBP processing and a variety of mutant CHO cell lines are available for the study of SREBP (39;371;372). In addition, overexpression of iPLA₂ in CHO cells as shown in Figure 17A suppresses SREBP transcriptional activity in lipid-free conditions and FCS-supplemented conditions (Figure 17B). Thus, CHO cells are an appropriate model to test the hypothesis that iPLA₂ modulates SREBP1 expression and processing.

Two strategies were used to suppress iPLA₂ in CHO cells: iPLA₂-specific siRNA and an iPLA₂-selective inhibitor, bromoeno lactone (BEL). The siRNA strategy strongly reduced iPLA₂ expression and activity by approximately 80% vs. the enzymatic activity of control siRNA-treated cells (Figures 18A and 18B) and treatment with BEL, an suicide substrate inhibitor of iPLA₂(184), suppressed iPLA₂ activity in CHO cells to a similar degree (data not shown).
Figure 17: iPLA₂ overexpression suppresses SREBP transcriptional activity in CHO cells.

(A) CHO cells were transiently transfected with plasmid encoding CFP-iPLA₂ or control CFP. Cell homogenates were prepared approximately 20 hours later and screened for iPLA₂ activity. A representative experiment is shown (B) CHO cells were transiently cotransfected with plasmid encoding CFP-iPLA₂ or control CFP and the pSynSRE reporter construct and β-gal control plasmid. Luciferase activity was quantified and normalized to β-gal control plasmid activity (promoter activity) 18 hours after transfection. (Open bars represent culture conditions supplemented with fetal calf serum. Closed bars represent culture conditions using media supplemented with fatty-acid free BSA in lieu of fetal calf serum.) Error bars represent the standard deviation of the mean of a representative experiment run in triplicate (experiment n=2).
Figure 17B

[Bar chart showing pSYN promoter activity for empty and iPLA2 conditions with FCS and lipid-free conditions.]

- Empty condition with FCS and lipid-free conditions.
- iPLA2 condition with FCS and lipid-free conditions.
Figure 18: iPLA₂ inhibition in CHO cells by siRNA

Cells were transfected with control siRNA or siRNA targeted to iPLA₂. Cells were treated with control or iPLA₂-directed siRNA at time zero and 48 hours later. Cells were harvested at approximately 72 hours. (A) Immunoblot analysis of iPLA₂ protein in cells transfected with control or iPLA₂ siRNA. (B) iPLA₂ activity in cells transfected with control or iPLA₂ siRNA. Representative experiments are shown.
Control siRNA-treated, iPLA$_2$-treated cells, and vehicle-treated and BEL-treated cells were transfected with the pSynSRE reporter and luciferase assays were performed to quantify SREBP-dependent transcriptional activity. As shown in Figure 19, pSynSRE reporter activity was ~ three-fold higher in CHO cells treated with iPLA$_2$-specific siRNA compared to cells treated with control siRNA. Similar results were obtained when CHO cells were treated with BEL (Table 3), indicating that SREBP modulation by iPLA$_2$ is dependent on its catalytic activity. Emphasizing the potential relationship between iPLA$_2$ catalytic activity and SREBP, supplementing BEL-treated cells with oleate, an unsaturated fatty acid added to mimic the presence of an iPLA$_2$ catalytic product, reversed the effect of BEL and blocked the increase in transcriptionally active SREBP (Table 3). As expected, effects of BEL were reversed when cells were treated with exogenous oleic acid, an unsaturated fatty acid (data not shown). The induction of SREBP activity indicated that SREBP1 expression and/or processing might be elevated in CHO cells with low iPLA$_2$ activity. To test this hypothesis, CHO cells were treated with control siRNA or iPLA$_2$-specific siRNA, cell homogenates were prepared, and SREBP1 expression was assessed by immunoblot. As shown in Figure 20, SREBP1 expression was induced in CHO cells treated with iPLA$_2$-specific siRNA. iPLA$_2$ overexpression in HepG2 cells (Figure 14) primarily affected levels of precursor SREBP1 and a similar effect was observed in siRNA-treated CHO cells. To better elucidate the effect on mSREBP1, experiments are planned to examine mSREBP1 in nuclear extracts. However, the robust induction of SREBP transcriptional activity (Figure 19) suggests that modest changes in mature SREBP1 have significant impact on
transcriptional activity measured in the luciferase assay.
Figure 19: PSYN in CHO siRNAiPLA₂-targeted siRNA decreases SREBP transcriptional activity in CHO cells

CHO cells were transfected with control or iPLA₂ siRNA at time zero and cotransfected with the same siRNA treatments and the pSynSRE reporter and β-gal control constructs at 48 hours. Approximately 15 hours later, cells were harvested and luciferase activity was quantified and normalized to β-gal activity (promoter activity). A representative experiment is shown. Error bars represent the standard deviation of the mean of an experiment run in triplicate.
Table 3: Chemical inhibition of iPLA₂ increases SREBP1-mediated gene expression in CHO cells

CHO cells were cotransfected with the pSYN SRE luciferase and β-gal control constructs. Following transfection, cell were treated with 10 µM BEL and incubated in serum-supplemented media or delipidated media containing 1% fatty-acid free BSA for 15 hours. Cells were harvested and luciferase activity was analyzed and normalized to β-gal control promoter activity. *Fold induction expressed as the ratio of the response in lipid-free conditions to FCS-supplemented conditions. A representative experiment run in triplicate is shown.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fold Induction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEL</td>
<td>11.5</td>
</tr>
<tr>
<td>BEL + oleate</td>
<td>8.4</td>
</tr>
<tr>
<td>vehicle</td>
<td>7.0</td>
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</tbody>
</table>
Figure 20: iPLA$_2$ suppression by siRNA induces SREBP1 expression in CHO cells

CHO cells were transfected with control siRNA or siRNA targeted to iPLA$_2$ at time zero and 48 hours later. Cell homogenates were prepared at approximately 72 hours later and screened for SREBP1 protein by immunoblot. A representative experiment is shown.
Expression of the SREBP1 target gene FAS was suppressed when iPLA$_2$ was over expressed in HepG2 cells (Figure 16). These observations prompted the hypothesis that SREBP target genes would be induced in CHO cells with low iPLA$_2$ activity. To test this hypothesis, homogenates of cells treated with control siRNA or iPLA$_2$-specific siRNA were screened for FAS and ACC expression by immunoblot. As shown in Figure 21, expression of both gene products was induced in CHO cells with low iPLA$_2$. Together, these data support the hypothesis that bioactive lipids derived from the iPLA$_2$ reaction suppress SREBP1 expression and subsequently the SREBP1 transcriptional regulation of its gene targets.
Figure 21: iPLA$_2$ suppression induces SREBP1 target genes

CHO cells were transfected with control siRNA or siRNA targeted to iPLA$_2$ at time zero and 48 hours later. Cell homogenates were prepared at 72 hours and screened for the SREBP1 targets FAS and ACC (n=1).
Discussion

Other investigators have demonstrated that unsaturated fatty acids suppress expression of SREBP1 target genes (39,75). We hypothesized that the unsaturated fatty acid products of iPLA2 would suppress SREBP1. In CHO and HepG2 cells, overexpression of iPLA2 suppresses SREBP-mediated transcription (Figure 17B) and expression of the SREBP1-target FAS (Figure 16), respectively. Conversely, SREBP1-mediated transcription and expression of target genes was induced when iPLA2 expression or catalytic activity was suppressed in CHO cells. Together, these data implicate iPLA2 as an endogenous source of unsaturated fatty acids that regulate SREBP1.

Evidence from several groups and from studies in various mammalian cells and organisms have characterized the effect of unsaturated fatty acids on SREBP1. Using CHO, CV-1 and HepG2 cells, Worgall et al demonstrated that oleate and other UFA attenuated the transcriptional activation of SREBP (39). The authors concluded that the effect of UFA on SREBP occurred at the level of processing from the transcriptionally inactive 125 kDa isoform to the transcriptionally active isoform. Another study in HEK293 cells, reached a similar conclusion with data that also provide evidence for a prefential inhibitory effect by UFA on SREBP1 rather than SREBP2 (373). In vivo studies also demonstrated similar effects on SREBP1 upon feeding of Sprague Dawley Rats diets enriched in UFA (374). Based on these studies, (39) and preliminary fluorescence microscopy images alluding to localization of CFP-iPLA2 at the ER, the organelle to which precursor SREBP1 is bound, we originally hypothesized that iPLA2
overexpression would inhibit SREBP1 at the level of processing. However, our results indicate an effect of iPLA2 largely on the expression of SREBP1. Others have seen the effect of UFA on expression of SREBP1. In an vivo study, Xu et al. (374), observed decreases in hepatic SREBP1 mRNA in response to an UFA-enriched diet. Furthermore, a study using human colonic carcinoma CaCo-2 cells uncovered the effect of UFA on SREBP1 at the expression level (375). Of course, cell type may play a role in whether the effect by UFA is at the level of expression or processing; however, our data from two distinct cell types indicate an effect at the level of SREBP1 expression that subsequently results in an effect on SREBP1-mediated transcription of its gene targets.

Although we hypothesize that iPLA2-derived UFA regulate SREBP1, these data do not rule out the possibility that the lysophospholipid released from iPLA2 catabolic activity participates partially or fully on the effect on SREBP1. To test this possibility, we can determine whether the exogenous addition of lysophosphatidylcholine (LysoPC) or another lysophospholipid can attenuate the increase in SREBP1 expression in response to BEL treatment. Currently, there is no evidence to demonstrate an effect by lysoPL on any SREBP isoform. On the other hand, there is evidence that lysoPL could potentially have the opposite effect, as one study in HepG2 cells has demonstrated that treatment with lysoPC increased TG synthesis (376). In light of this finding, our data argue that if iPLA2-generated lysoPC activates SREBP1, this effect is negligible compared to the suppressive effect of iPLA2-derived UFA. Conversion of LPC to LPA by a lysophospholipase D such as autotaxin could potentially mediate the
effects on SREBP1 via LPA binding to LPA receptors (267). However, as LPA is a potent mitogen to which a majority of mammalian cells respond (38) it may be a poor inhibitor of SREBP1 because increases in cell growth require concomitant increases in lipid mass for daughter cells. On the other hand, LPA binding to PPARγ, could limit LXR activation of SREBP1 expression via competition between PPARγ and LXR for RXR (90;364). One phenotype of cancer cell progression gaining broader interest is overexpression of the SREBP1 target FAS (377). In summary, while there is evidence to support the notion that iPLA2-generated lysoPC is not involved in inhibiting SREBP1, further experimentation is required to examine the relationships between SREBP1, iPLA2, LPA, LXR and PPARγ.

In characterizing the relationship between SREBP regulation and iPLA2, we questioned whether the catalytic activity resulting in iPLA2 catabolic products or the mere presence of iPLA2 influences SREBP1. The data in CHO cells treated with BEL (Table 3) indicate that iPLA2 enzymatic activity is required to affect SREBP1 transcriptional activity. Immunoblot analysis of CHO cells treated with BEL will indicate if indeed the effect remains at the level of expression. To supplement the experiments using BEL, our laboratory has a catalytically inactive, dominant negative CHO iPLA2 mutant that can be overexpressed in CHO and HepG2 cells to test the hypothesis that iPLA2 catalytic activity is responsible for the effect on SREBP1. Implementation of this strategy to further characterize the relationship between iPLA2 and SREBP1 is planned. Alternatively, transient transfection of the endogenous
dominant alternatively spliced ankyrin protein can be used to study whether catalytic iPLA₂ activity is responsible for the effect of this enzyme on SREBP.

Confusion occasionally arises in distinguishing SREBP1a from 1c. Many papers refer only to SREBP1 presumably referring to SREBP1c. SREBP1a is the isoform with the longer acidic N-terminal domain that is found in cultured cell lines and without preference activates both fatty acid and cholesterol synthetic genes (25). SREBP1c is highly expressed in vivo and in liver in particular and, as mentioned, preferentially activates genes in the fatty acid synthetic pathway (25). Our data does not distinguish between SREBP1a and SREBP1c. Several studies can be employed if it becomes necessary to distinguish between the two isoforms. To truly determine a direct effect on SREBP1a expression, an RNAse protection assay can be employed as has been described (91). Despite this unanswered question and because SREBP1c is the relevant and predominate isoform in liver and in vivo, it is clear that SREBP1c expression is inhibited by iPLA₂ as indicated by our in vivo data (Figure 15). To further characterize the effect of iPLA₂ on SREBP, studies are planned to determine the effect, if any, of iPLA₂ on SREBP2 and its gene targets employing similar methods detailed in this study. This is an important follow up study as it could uncover a role for iPLA₂ in the regulation of cholesterol.

There are several lines of evidence to support the mechanisms by which iPLA₂ may regulate SREBP1 as shown in this study. Studies to determine the effect of UFA on SREBP found that exogenous UFA induce sphingomyelinase activity and therefore decrease sphingomyelin mass in the cell membrane (43). This finding prompted
speculation that loss of sphingomyelin mass in the cell membrane could lead to the release of free cholesterol from the cell membrane to the cytoplasm as sphingomyelin in the cell membrane stabilizes and acts to retain cholesterol in membranes (43). Therefore, released cholesterol due to increased sphingomyelinase activity could, in turn inhibit SREBP (43). However, this SREBP regulation of cholesterol occurs at the level of processing and not expression. Again, this mechanism may be one of the ways in which iPLA₂ affects SREBP1. Nonetheless, it cannot account for the effect we and others have observed at the level of SREBP1 expression.

Several studies allude to the mechanism by which iPLA₂-generated UFA might impact SREBP1 expression, specifically in the liver in which SREBP1c is the prominent isoform (25)(39;75;76). It was discovered \textit{in vivo} using LXR knockout mice, that LXR alpha and beta, which are activated by oxysterols, can selectively control the expression of SREBP1c (91). A study concluded that SREBP1c gene expression mediated by the Liver X Receptor (LXR)/retinoid X receptor heterodimer may be inhibited by exogenous PUFA through activation of the nuclear receptor peroxisome proliferator-activated receptor (PPAR) alpha (364), a nuclear hormone receptor associated with activation of fatty acid beta-oxidation. This study concluded that the presence PUFA and other PPAR-alpha agonists potentially result in competition of PPAR-alpha with LXR for RXR dimerization, which is necessary for LXR to mediate SREBP-1c transcription. Nonetheless, these findings have not been confirmed by other investigators. The laboratory of Brown and Goldstein has presented evidence that UFA antagonize the agonist-induced activation of LXR that normally leads to increased
SREBP1 expression in cultured rat hepatoma cells (82). These data prompt future studies to determine if iPLA\(_2\) suppress SREBP1 by targeting LXR alpha and beta and potentially via PPAR alpha.

Finally, it is necessary to reconcile the data in Figures 14 and 15 with data presented in Chapter 2. Figure 14 and 15 have no uninfected controls. Based on the findings in Chapter 2, it can be assumed that Ad-5 itself is increasing SREBP1 expression. Therefore the baseline expression of SREBP1 is elevated in these experiments in which iPLA\(_2\) is overexpressed using Ad-5. These findings may lead one to question whether iPLA\(_2\) can attenuate SREBP1 expression in the absence of Ad-5 infection. The use of conventional transfection methods to overexpress iPLA\(_2\) have been used as shown in Figure 17A and 17B and demonstrate that iPLA\(_2\) overexpression indeed attenuates, in this case, SREBP-mediated transcription. Likely, overexpression of iPLA\(_2\) using conventional transfection methods to avoid elevating baseline SREBP1 expression in HepG2 cells would result in decreased SREBP1 expression; however, these experiments have not been performed.

Studies in iPLA\(_2\) knockout mice and INS-1 insulinoma cells have demonstrated the importance iPLA\(_2\) to proper glucose-stimulated insulin secretion in pancreatic islet cells and allude to the potential of iPLA\(_2\) modulation as a treatment for diabetes. Our data begin to highlight the potential therapeutic role for iPLA\(_2\) in the liver, specifically pathological aberrant lipid as in steatohepatitis. iPLA\(_2\) generates unesterified arachidonic acid that can be metabolized into bioactive prostaglandins and leukotrienes (9). These oxygenated derivatives could bind to cell surface receptors and augment the
inflammatory response that constitutes the “second hit” required for the development of steatohepatitis (9;378;379). However, derivatives of arachidonic acid and other PUFA also bind the nuclear hormone receptor PPAR-γ and can suppress inflammation through this mechanism thereby limiting hit 2 (380;381). In addition, iPLA₂-derived PUFAs suppress SREBP1 and expression of FAS, ACC, and other proteins involved in fatty acid and triglyceride synthesis, essential components of the first “hit” (25;79;364). Thus iPLA₂-derived PUFA have the potential to block one of the processes that leads to triglyceride accumulation, steatosis and the dyslipidaemic pathologies arising from accumulation of triglycerides. In support of these speculations, other investigators have shown that dietary PUFA both suppress induction and reverse established steatosis (76;80;382;383). We hypothesize that iPLA₂ will be even more effective in this regard when it is activated in hepatocytes, as it will provide a continuous, local source of PUFA to suppress SREBP1c. This study details the first endogenous source of UFA capable of regulating SREBP1 and, in turn, may allude to a new therapeutic target to treat dyslipidemia and its complications.
Chapter 4: The influence of the novel phosphatidylinositol 3-kinase inhibitor PX-866 on systemic and hepatic basal lipid metabolism

Abstract:
Insulin stimulates SREBP1 expression via the PI3K/Akt pathway in hepatocytes. Researchers relied on the use of phosphatidylinositol 3-kinase (PI3K) inhibitors to characterize this insulin-induced signaling event in hepatocytes. The novel PI3K inhibitor PX-866, a wortmannin analog, is gaining recognition for its potency, potential as a chemotherapeutic and low in vivo toxicity relative to wortmannin and LY294002, two commonly employed PI3K inhibitors. We recently showed that PX-866 attenuates replication-deficient adenovirus-5 induced activation of hepatic SREBP1 activation. This finding prompted us to hypothesize that PX-866 would influence basal hepatic
lipid metabolism and result in changes in circulating lipid levels. In C57/BL6 mice administered PX-866 we observed decreased plasma triglyceride and a trend towards decreased hepatic SREBP1 expression without the presence of hepatotoxicity. These mice also exhibited increases in circulating cholesterol, an event that requires further examination. These findings suggest that PX-866 is a novel regulator of basal lipid metabolism.
Introduction

Insulin regulates hepatic lipid metabolism through the induction of SREBP1 (87;88). This event promotes fatty acid and triglyceride synthesis and dampens glucose synthesis in the liver (25;87). The role of PI3-kinase (PI3K) in insulin signaling has been well established (89;384). Inhibitors of this enzyme not only block SREBP1 induction by insulin, but also prevent the down regulation of genes associated with gluconeogenesis, resulting in impaired glucose tolerance and hyperinsulinemia (89). Coupled with other studies, the use of these inhibitors has been responsible for uncovering the participation of the PI3K/Akt pathway in SREBP biology (62) and in insulin-induced activation of SREBP1 expression (88;385). The two commonly used chemical inhibitors of PI3K are LY294003 and Wortmannin. Wortmannin is among the most potent of these inhibitors. It is a pan-specific PI3K inhibitor that irreversibly suppresses catalytic activity by modifying a lysine in the active site (21).

PX-866, an analog of wortmannin, represents a new PI3K inhibitor that exhibits increased biological stability, reduced hepatotoxicity, and 10-fold higher inhibitory activity against the enzyme (21). PX-866 selectively inhibits the p110 alpha subunit isoform of PI3K. While PI3K signaling is ubiquitous among cell types and has several roles in facilitating proper cell function, there is evidence to suggest that the catalytic p110 alpha isoform is involved in insulin activation of Akt (386). Of the few published studies using PX-866, the focus has been on its potential as a novel chemotherapeutic as it demonstrates significant inhibition of Akt phosphorylation and cancer cell motility.
and shows promise as an enhancer of epidermal growth factor receptor inhibitors used in the treatment of non-small cell lung cancer (21;334-336). Despite this focus, only a few studies have begun to characterize systemic effects of PX-866 in vivo. Ihle et al showed that PX-866 induces transient hyperinsulinemia and impaired glucose tolerance, conditions that were reversed upon administration of metformin or when drug treatment was stopped (335). This finding suggests that the compound may be an unsuitable candidate to study liver lipid metabolism as the inadvertent increases in circulating insulin due to PX-866 administration would, in effect, induce PI3K signaling in the hepatocyte. On the other hand, it was possible that, at a proper dose, the increase in plasma insulin would be insignificant enough to allow for adequate in vivo inhibition of PI3K in the liver despite increases in plasma insulin. Other studies in our laboratory showed that replication-deficient adenovirus-5 increases Akt activation (Chapter 2, Figure 11B) with increases in SREBP1 induction (Chapter 2, Figure 10C). We also demonstrated that PX-866 was able to antagonize SREBP1 induction and increases in triglyceride by replication-deficient adenovirus (Chapter 2, Figure 12A,B). As such, we hypothesized that PX-866 treatment would suppress basal lipid metabolism in C57BL/6 mice.
Materials and Methods:

Materials

PX-866 and DMSO was obtained from Sigma. All qPCR primers and probes were purchased from Integrated DNA Technologies.

Mice

C57BL/6 mice obtained from the Jackson Laboratory were housed on a reverse light/dark cycle and provided standard chow and water *ad libitum* except where indicated otherwise, 12.5 mg/kg PX-866 or DMSO vehicle was administered by tail-vein injection to eight to ten-week-old mice weighing approximately 20-25 grams. Inhibitor or DMSO control were administered in the a.m. at time zero and again at 48 hours in the a.m. Non-fasted mice were sacrificed at 96 hours from the beginning of treatment. PX-866 dissolved in DMSO was administered using a total vehicle volume of 100 µL PBS containing 20% DMSO. Mice were sacrificed by exsanguination. Livers were excised and flash frozen. Plasma was collected. Mice were maintained in accordance with Virginia Commonwealth University Institutional Animal Care and Use Committee standards.

Plasma and lipid analysis

Plasma triglyceride, cholesterol, ALT, AST, Alkaline Phosphatase and glucose were quantified by the diagnostic laboratories of the McGuire Veterans Affairs Hospital, Richmond, VA.
**RNA Analysis by Quantitative reverse transcriptase PCR**

Mouse liver RNA was prepared using the SV Total RNA Isolation System (Promega). mRNA encoding SREBP1, SREBP2 and ACC were screened by SYBR Green qPCR using the Brilliant QRT-PCR Core Reagent Kit, 1-Step (Stratagene). mRNA encoding 18S served as an internal control and was screened using the TaqMan, One-Step RT-PCR Master Mix Reagents (Applied Biosystems). Primers described previously (342) were as follows: SREBP1, sense 5’-CACTGAAGCAAAGCTGAATAAATCTG-3’, antisense 5’-AGTGATTTGCTTTTGTGTGCACTT-3’; Primers and probe for 18S described previously (343) were: sense 5’-AAAATTAGAGTGGTTAAAGCAGGC-3’, antisense 5’- CCTCAGTTCCGAAAAACCAACAA-3’, probe CY5-CGAGCCGCCTGGATACCGCAGC-BHQ-2. Differences in the fold levels of mRNA were calculated based on the standard curve method.

**Statistical Analysis**

Three to eight mice were studied for each *in vivo* condition. The mean ± standard error is shown. Mann-Whitney two-sided U test with p < 0.05 as a cutoff for statistical significance was used.
Results

To test the hypothesis that PX-866 would influence basal lipid metabolism, we treated mice with 12.5 mg/kg PX-866 or DMSO vehicle by tail-vein injection every 48 hours for four days (two doses over four days). Upon sacrifice, livers were harvested and plasma collected and screened for aminotransferases and lipids. As shown in Figure 22, triglyceride was reduced by ~29% (from 113 ± 9 mg/dl to 80 ± 4 mg/dl) in plasma of PX-866 treated mice (p=0.0122). We hypothesized that this reduction might be due to reduced expression of SREBP1. To test this hypothesis, SREBP1 mRNA in livers of control and PX-866-treated mice was quantified with the qPCR strategy used in Chapter 2. The PX-866-treated livers exhibited a trend towards reduced SREBP1 expression (Figure 23) that did not reach statistical significance due to single outlier (p = 0.32). Interestingly, PX-866-treated mice also exhibited increased plasma cholesterol (p=0.0006) (Figure 24). As high density lipoprotein is the major cholesterol transporter in mice, this suggests an increase in HDL cholesterol (HDL-C), a hypothesis that is currently being tested in the laboratory. PX-866 also increased plasma glucose (311 ± 12 versus 374 ± 8, 20%, p= 0.0012, (Figure 25) in these non-fasted mice.
Figure 22: Mice treated with PX-866 exhibit decreased plasma triglyceride

Mice treated as described in “materials and methods.” Each symbol represents one mouse. The horizontal bar depicts the mean for the mice of the experimental group. p=0.0122. n=7.
Figure 23: Mice treated with PX-866 exhibit trend towards decreased hepatic SREBP1

Mice treated as described in “materials and methods.” Each symbol represents one mouse. The horizontal bar depicts the mean for the mice of the experimental group. p=0.32. n=7.
Figure 24: PX-866 increases plasma cholesterol

Mice treated as described in “materials and methods.” Each symbol represents one mouse. The horizontal bar depicts the mean for the mice of the experimental group. p=0.0006. n=7.
Figure 25: PX-866 increases plasma glucose (non-fasted mice)

Mice treated as described in “materials and methods.” Each symbol represents one mouse. The horizontal bar depicts the mean for the mice of the experimental group. p=0.0012.
Importantly, PX-866 induced changes in lipid metabolism occurred in the absence of hepatotoxicity, as assessed by plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (alk phos) levels (Figure 26). The dose of 12.5 mg/kg is well below the reported maximum tolerated dose (MTD) of 19.5 mg/kg via i.p. injection (21). Together, these data indicate that PX-866 alters basal lipid metabolism resulting in changes in resting plasma lipid levels. The reduction in baseline triglyceride levels prompted us to re-examine effects of PX-866 on Ad-5 induced hypertriglyceridemia. As shown in Figure 27, PX-866-treated mice still exhibited increased plasma triglyceride upon infection with replication-deficient adenovirus. However, PX-866 significantly attenuated Ad-5 induced hypertriglyceridemia (1.76 ± .042 fold versus 1.49 ± .164 fold induction, p = 0.047).
Figure 26: PX-866 has modest effects on plasma aminotransferases

Mice treated as described in “materials and methods.” ALT $p=0.0099$, $n=7$; AST $p=0.0262$, $n=7$; alkphos $p=0.8$, $n=3$. * $p < 0.05$, ** $p < 0.01$. 
Figure 27: PX-866 suppresses Ad-5-induced hypertriglyceridemia

C57BL/6 mice were pretreated with 12.5 mg/kg PX-866 or DMSO before infection with replication-deficient Ad-5 and sacrificed on day 4 post infection. Quantification of plasma triglyceride (mg/dl). Each symbol represents one mouse. Horizontal bar is mean of the group. * p < 0.05 compared to Ad-5, vehicle-treated; # p < 0.05 compared to uninfected, vehicle-treated.
Discussion:

Currently, there is no published research demonstrating a relationship between the novel PI3K inhibitor PX-866 and hepatic lipid metabolism. Furthermore, a literature search reveals an absence of data and/or study concerning the ability of the popular PI3K inhibitors LY294002 and wortmannin to decrease triglyceride. The participation of the PI3K/Akt pathway in insulin-induced SREBP1 expression and the inhibition of Ad-5-induced SREBP1 activation by PX-866 observed in our other studies prompted the hypothesis that this compound could, like wortmannin and LY294002, be used to elucidate the role of the PI3K/Akt pathway in lipid metabolism. Despite the observations by others that PX-866 increases plasma insulin and leads to glucose intolerance in mice (335), we were curious to determine if PX-866 would decrease basal hepatic SREBP1 and influence systemic lipid metabolism based on our previous observation that PX-866 inhibits Ad-5-induced SREBP1 expression and activation. The unexpected increase in plasma glucose in response to PX-866 is perplexing in light of observations that PX-866 induced hyperinsulinaemia. Insulin affects the liver by not only inducing increases in SREBP1 leading to increased fatty acid synthesis, but also dampens glucose production by the liver (87). One explanation for our observation could be based on the fact that in an hyperinsulinaemic insulin resistant state, such as diabetes, insulin does not suppress liver gluconeogenesis (87). Perhaps this is partially responsible for our observation of increased plasma glucose following PX-866 administration. Another plausible scenario responsible for the observed increase in
glucose could involve the relationship between PI3K/Akt and the Forkhead transcription factor FOXO1 and the FOXO1 gene targets phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). Hepatic PI3K/Akt signaling results in the phosphorylation of FOXO1, a phosphorylation event leading to subsequent inhibition of the expression of the gluconeogenic genes PEPCK and G6Pase (387). With this in mind, the observed increase in glucose could feasibly be in response to hepatic inhibition of PI3K/Akt signaling by PX-866, thus allowing for unchecked signaling activation of gluconeogenesis via FOXO1. Despite the potential explanations for this observation, the increase in plasma glucose in response to PX-866 may well diminish its potential in the clinic as this effect could disrupt proper systemic glucose and lipid metabolism as glucose, like insulin, can activate SREBP1 (87).

On the other hand, plasma TG was decreased in PX-866-treated mice. This was accompanied by a trend toward a decrease in hepatic SREBP1 expression. Presumably this is due to the location of hepatic PI3K signaling downstream of glucose and insulin signaling in the hepatocyte, an event likely inhibited by PX-866. Therefore, despite the PX-866-mediated increases in plasma glucose that can 1) lead to increases in pancreatic insulin secretion and 2) itself activate hepatic SREBP1 expression and activation, the outcome of blatant increases in SREBP1 expression and increases in systemic TG were not observed in these PX-866-treated mice. This alludes to the possibility that this compound might have a clinical use in treating hyperlipidemia. Furthermore, the absence of frank hepatotoxicity supports this and suggests that the proper dose could sidestep even modest increases in hepatotoxicity. Nonetheless, further investigation is
required in this and other *in vivo* models before speculating as to what lipid-lowering benefits PX-866 might have in humans as it is argued that there is no one appropriate animal model from which conclusions can be drawn concerning the potential efficacy of a compound in humans (388).

A possible explanation for decreased SREBP1 and plasma TG upon administration of this compound is that a spike in plasma insulin resulting from PX-866 in mice exhibiting normal plasma insulin prior to treatment can paradoxically reduce hepatic fatty acid synthesis. Previous evidence showed that a transient spike in insulin can reduce liver fat mass (389). It can be assumed that this is a compensatory mechanism to safeguard the liver from becoming overstimulated in response to an acute spike in insulin.

Another interesting observation in this study was the significant increase in plasma cholesterol following PX-866 administration. As mentioned, this increase may be due to an increase in HDL, the primary transporter of cholesterol in mice. Unquestionably, further investigation of this event is required if PX-866 is ever destined for the clinic. For instance, in other mammals relying on LDL as a TG transporter, would PX-866 increase LDL and, in effect, promote atherogenesis? These questions and our observation also prompts the hypothesis that SREBP2 is affected by PX-866 as it is the primary SREBP isoform involved in cholesterol metabolism (25). Further experimentation is necessary to determine whether PX-866 can regulate also SREBP2. In several organisms, the antiatherogenic action of the statins class of compounds is primarily due to decreases in plasma TG (388). In light of these data, PX-866 could be
a beneficial lipid-lowering therapy. Further extension of our findings is imperative. In conclusion, our data indicate that PX-866 modulates basal lipid metabolism in mice. Whether PX-866 will be of clinical value excluding its chemotheurapeutic benefit is uncertain at this juncture.
Chapter 5: Concluding remarks

It is appreciated that abnormal lipid metabolism underlies several disease states including diabetes, atherosclerosis, the metabolic syndrome and cancer. In order to develop new and effective strategies to treat these diseases, a greater understanding of the mechanisms that contribute to aberrant lipid metabolism and subsequent cellular dysfunction is imperative. Systemic lipid metabolism depends significantly on proper lipid metabolism in the liver, specifically as directed by SREBP. The SREBP family of transcription factors is responsible for the regulation of cholesterol and fatty acid biosynthesis in the liver and other tissues. SREBP proteins respond to and, in effect, are regulated by the cellular environment to maintain lipid homeostasis.

The studies presented here characterize three novel regulators of SREBP1, the isoform of SREBP involved in the control of fatty acid and TG metabolism that subsequently impact SREBP1-directed regulation of lipid metabolism. The second study presented here provides evidence that iPLA2 can inversely influence SREBP1. Our experiments using the suicide substrate inhibitor of iPLA2, BEL, implicate iPLA2 catalytic activity in modulating SREBP1. Furthermore, it can be inferred that iPLA2-generated products, especially UFA, participate in regulating SREBP1. An unexpected finding in these studies was the effect of iPLA2 on SREBP1 at the level of expression as previous research showed a relationship between PUFA and SREBP at the level of SREBP processing (39). Nonetheless, the effect of iPLA2 at the level of expression still
resulted in downstream effects at the level of SREBP-mediated transcription and at the expression level of SREBP1 targets.

These data allude to the potential targeting of iPLA2 in the clinic. Overexpression of iPLA2 in hepatocytes could, based on these data, lead to decreased SREBP1 activity and subsequent decreases in hepatic TG. Liver hypertriglyceridemia is an etiological player in several diseases such as NAFLD and is one manifestation of the metabolic syndrome. Overexpression if iPLA2 could antagonize the characteristics of this syndrome. Because iPLA2 is involved in a variety of cell types and demonstrates diverse cellular roles (see general introduction), overexpression of this enzyme in several cell types could prove to be detrimental. For instance, it has been shown by the Tabas group that atherosclerotic plaque stability depends on macrophage foam cell survival (24;191;390). His group determined that macrophage foam cells will undergo apoptosis if a threshold ratio of cholesterol to the glycerophospholipid PC is exceeded (390). With this in mind, inhibition of SREBP1, which facilitates expression of genes involved in glycerophospholipid synthesis, by iPLA2 could exacerbate macrophage foam cell death by increasing the cholesterol to PC ratio. In this instance, iPLA2 inhibition would be the desired regulatory “direction.” However, inhibition of the iPLA2 could prove detrimental, again underscoring the significance of cell context, as Bao et al, in an extension of previous studies in INS-1 cells that demonstrated that iPLA2 overexpression enhanced glucose-induced insulin secretion (190;287), recently observed impaired glucose tolerance and decreased insulin secretion in iPLA2 knockout mice (187). Thus, while iPLA2 can regulate SREBP1, whether or not a cell or organism
benefits from iPLA₂ modulation is inherently cell-type specific. Furthermore it is necessary to determine if iPLA₂ can modulate SREBP2 as well. These experiments are planned.

An unexpected discovery during studies that employed iPLA₂ overexpression by an adenovirus strategy was the ability of replication-deficient Ad-5 to activate SREBP1 and result in increases in liver and circulating TG in vivo. This finding has not been reported to our knowledge though it is becoming well understood that HCV can influence hepatic lipid metabolism (99;349-351;391). However, our investigation of Ad-5 demonstrated its effect is selective for SREBP1, whereas HCV activates SREBP1 and SREBP2 (350). Our data indicate that Ad-5 signaling mimics insulin signaling in the hepatocyte by activating the PI3K/Akt pathway. The PI3K inhibitors LY294002 and PX-866 were able to block activation of SREBP1 by first-generation Ad-5. This study is significant for several reasons. It characterizes a potential role of viral infection in dyslipidemia, fatty liver disease and NAFLD and raises questions concerning the use of these vectors to express transgenes in gene therapy as replication-deficient Ad-5 is commonly exploited for gene therapy. It also provides an explanation for the visible increase in liver mass of infected C57BL/6 mice other researchers have witnessed (personal communication with Gregorio Gil, PhD). However, it should be noted that all effects in response to replication-deficient Ad-5 were transient. Nonetheless, there is no reason to assume that this transient spike in lipid metabolism is precluded from affecting other systemic processes, especially those connected to lipid metabolism, following this transient spike and once hepatic lipid metabolism has returned to basal
condition. This study was initiated through the rigorous use of controls for several initial experiments and therefore highlights the importance of controlling for even the “not-so-obvious” aspects of an experiment. This study also underscores the need for the search for “negligible” transgene vectors. HepG2 cells are infected with lentivirus indicating that lentiviral strategies may be a more suitable alternative to transgene expression; however, these studies have not been performed. The question remains as to whether lentivirus would induce similar effects on hepatic lipid metabolism. Studies using the protocols developed in Chapter 2 to assess hepatic and systemic lipid metabolism should be designed to screen for the affects of various viral vectors including lentiviruses, other adenovirus serotypes, Ad-5 vectors with different deletions and/or additions of the early genes on lipid metabolism.

It is possible, however, that, in the case of Ad-5 infection, the association of the penton base RGD domain with alpha and beta integrins of the hepatocyte is the culprit responsible for initiating PI3K signaling leading to modulation of hepatic lipid metabolism. If, indeed, this is the case, infection by internalization of Ad-5 into the host cell arguably is not required to impact SREBP1 and lipid metabolism. Instead the mere association of the penton base of Ad-5 with alpha and beta integrins of the host cell could be the minimum requirement to induce PI3K activation and hence the induction of SREBP1. Were studies to find that the mere association of the penton base with host cell integrins and/or the internalization of the Ad-5 into the host cell to be the minimum requirement(s) to induce hepatic lipid biosynthesis, experiments screening for the effects of the adenoviral early genes might be unnecessary and irrelevant. On the
other hand, the internalization of Ad-5 into the host cell could be required. Recent evidence from Rogers et al demonstrates that the early gene E4 open reading frame 1 (E4ORF1) of Ad-36 influences lipid metabolism by inducing adipogenic genes in 3T3-L1 preadipocytes (358) and therefore prompts speculation as to whether the genetic composition of Ad-5 induces the effects presented in Chapter 2.

The Ad-5 study of Chapter 2 led to another exciting discovery that characterizes “chemical” regulation of hepatic lipid metabolism. Currently, there are only four studies in the literature characterizing PX-866. All of these focus on its efficacy as an antitumor agent. The study detailed in Chapter 4 further characterizes the compound and demonstrates its value beyond cancer research. It is interesting that there have not been more studies focused on the potential for PI3K inhibitors to treat hyperlipidemia despite their being used to uncover the relationship among insulin, PI3K/Akt and SREBP1 biology (62;88). There is evidence, at least in the case of wortmannin, to suggest that the compounds are either unstable and/or induce hepatotoxicity thus precluding their clinical value (392). Despite this, a literature search of the contributions of LY294002 and wortmannin to hypotriglyceridemia indicates that area is not a focus of current research. Perhaps systemic administration of a PI3K inhibitor would be tantamount to a dose of chemotherapy. In other words, in vivo PX-866 treatment might not only attenuate SREBP1 activation, but might prove deleterious to other cells in the organism as the PI3K/Akt pathway is critical to cell growth and proliferation (318). Potentially the long-term effects may prove to outweigh the benefits of the compound. For instance, would long-term use of PX-866 lead to bona
fide insulin resistance as it has been shown to lead to decreased glucose tolerance and hyperinsulinemia (335)?

On the other hand, it remains tempting to consider the potential for PX-866 to treat insulin resistance based on the following: Insulin resistance is a complex pathology. Diet, plasma glucose, pancreatic insulin secretion, plasma insulin levels and relative function or dysfunction of the hepatocyte and its ability to respond to insulin are disparate manifestations contributing to the disease. Nonetheless, a simple truth is that there is often hyperactivity of SREBP-mediated transcription due to the hyperinsulinemic state of the disease. Based on this information, it is reasonable to speculate that PX-866 could, in a sense, provide a therapeutic “short” in the circuit between increased insulin signaling and subsequent SREBP1 activation. Yet, as observed in our study, plasma glucose increased in response to PX-866 treatment (Figure 25) potentially due to undampened signaling through FOXO1 to induce gluconeogenesis during PI3K/Akt inhibition by PX-866. Perhaps it is for this reason PI3K inhibitors are not used in the clinic to treat dyslipidemia, diabetes and/or other disorders of lipid metabolism. Pioneering the development of PX-866, the Powis group attempted to blunt PX-866 induced glucose intolerance and hyperglycemia by administering insulin coupled with pioglitazone, a PPARγ agonist and member of the thiazolidinedione class of drugs used to treat hyperglycemia in Type 2 Diabetes by enhancing peripheral glucose disposal (393), and found that this regimen attenuated PX-866-induced hyperglycemia (335). Therefore, before more progress can be made in determining the efficacy of PX-866 to treat lipid disorders, extensive review of systemic
glucose biology and signaling processes must be implemented while bearing in mind that, as the Powis group determined (335), successful use of PX-866 in the clinic may require coadministration of other compounds to mitigate the undesired “side effects of PX-866.

And what about the increases in cholesterol upon PX-866 administration observed in the study presented here? Perhaps this is a negligible increase? Perhaps in organisms using HDL and LDL as lipid transporters, there would be a shift from LDL to HDL species resulting in a phenotype resistant to atherosclerosis and its complications. However, might the shift favor LDL and thereby promote a dyslipidemic phenotype? Understandably, based on the scarcity of literature characterizing this compound and the questions raised by available literature and those presented here, further investigation of the effect of this compound on lipid metabolism, and in this case on lipoprotein profile, must be explored further to determine whether its impact on lipid metabolism will be a benefit or detriment to the host.

It is interesting to consider that SREBP1 can be influenced by such disparate elements: a foreign virus, an enzyme already involved in lipid metabolism and a compound destined for chemotherapy. Certainly the classical feedback mechanisms can begin to explain the reason \( iPLA_2 \) can inhibit SREBP1. However, why would an adenovirus induce SREBP1 and TG synthesis? Perhaps this Ad-5-induced activation of SREBP1 is a mechanism exploited by wild-type adenovirus. What purpose it serves is uncertain. For example, if the adenovirus needed energy to replicate, it might be assumed that the balance would tilt toward beta-oxidation of fatty acids rather than lipid
synthesis. On the other hand, adenoviral replication and the subsequent generation of daughter adenoviruses likely depend on lipid synthesis for daughter adenoviruses. In this case, SREBP1 activation to promote lipid synthesis for daughter adenoviruses is a logical reason for increased SREBP1 activation following adenovirus infection. Again, these studies, while discovering and characterizing novel mechanisms of lipid regulation, have raised additional questions that can only be answered by further experimentation. In conclusion, these findings further support the importance of SREBP, especially SREBP1, to the regulation of lipid metabolism and characterize novel regulatory mechanisms of SREBP1 that, in the case of iPLA2 and PX-866, may prove useful in the clinical setting.
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