The Involvement of Phospholipase A_2 (PLA_2) in Acylation Stimulating Protein (ASP) Signaling

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ABSTRACT

The Involvement of PLA_2 in ASP Signaling

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Preliminary data suggests that the lipogenic factor Acylation Stimulating Protein (ASP), stimulates the activity of calcium-dependent phospholipase A_2 ($cPLA_2$) by increasing intracellular calcium levels $[Ca^{2+}]i$ and by activating extracellular-signal-regulated kinase 1/2 (ERK 1/2). The arachidonic acid (AA) generated by $cPLA_2$ action appears to function as a second messenger in ASP signaling.

ASP also blocks TG breakdown. The calcium-independent PLA_2 ($iPLA_2$) ζ has recently been identified as a novel TG-lipase in 3T3-L1 cells. Bromoenol lactone (BEL), a non-reversible $iPLA_2$ inhibitor, has been shown to specifically inhibit the TG-lipase activity of this enzyme. Preliminary data demonstrates that BEL stimulates basal TG synthesis, likely by inhibiting TG breakdown. The effects of BEL in combination with ASP are non-additive, suggesting they act through the same pathway. Furthermore, ASP appears to inhibit ^{3}H-AA release into the media in a concentration-dependent manner. We propose that ASP inhibits an $iPLA_2$ isoform with TG-lipase activity, an effect that can be mimicked by BEL.

RÉSUMÉ

Implication de PLA_2 dans la signalisation de l'ASP

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Des données préliminaires montrent que l'ASP (Acylation stimulating protein) stimule l'activité de la PLA_2 dependent du calcium ($cPLA_2$) en augmentant à la fois le niveau de calcium intracellulaire et l'activation de ERK1/2. L'acide arachidonique (AA) produit par la $cPLA_2$ fonctionne comme un second messager dans la signalisation par l'ASP.
Aussi, l'ASP inhibe la dégradation des triglycérides. La PLA_2 indépendant du calcium ($iPLA_2$) ζ a été récemment identifiée comme possédant une nouvelle activité lipase-TG dans des cellules 3T3-L1. Le Bromoenol lactone (BEL), un substrat inhibiteur de toutes les $iPLA_2$ connues, ihnibe l'activité lipase-TG de cet isoforme. Des données préliminaires montrent que le BEL stimule la synthèse basale de TG, probablement en limitant la dégradation des TG. Les effets du BEL en combinaison avec l'ASP sont non-additifs, suggérant qu'ils agissent via le même sentier de signalisation. L'ASP semble inhiber la libération de ^{3}H-AA dans le milieu d'une façon dépendante de la concentration. Nous proposons que l'ASP inhibe un isoforme de $iPLA_2$ avec une activité TG-lipase, un effet qui peut être imité par le BEL.

DEDICATION

I would like to dedicate this thesis to my family first and foremost, for their constant support and guidance during the writing of this thesis.

I also dedicate this thesis to Alex for truly being the inspiration in my life.

ACKNOWLEDGMENTS

I would like to thank Dr. Katherine Cianflone for all her intellectual discussions and positive energy.

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TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

CONTRIBUTION OF AUTHORS

All the experiments described herein were performed by myself, except in Chapter Three, where Dr.'s Katherine Cianflone, Magdalena Maslowska and myself, contributed equally to the writing of the manuscript. Both Dr. Magdalena Maslowska and I performed the westerns for $cPLA_2$ and MAPK. While, I perfomed the AACOCF3 and BAPTA-AM experiments, Dr. Magdalena Maslowska and Farzad Asadi are responsible for the remaining westerns and inhibitor studies. In addition, the BEL experiment presented in Chapter Five was perfomed by Dr. Magdalena Maslowska.

ABBREVIATIONS

AACOCF3- arachidonyltrifluoro-methyl ketone
Ang II- Angiotensin II
ASP- Acylation Stimulating Protein
ATGL- adipocyte TG lipase
BAPTA-AM- 1,2-bis-(o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic Acid Tetra-(acetoxymethyl) Ester
BEL- (E)-6-(Bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one or bromoenol lactone
Ca^{2+}- calcium
$[Ca^{2+}]i$- intracellular calcium
CaMK II- Ca^{2+}/Calmodulin-dependent Protein Kinase II
cAMP-cyclic 3'-5'-adenosine monophosphate
C/EBP- CCAAT/enhancer binding protein
cGMP- cyclic-guanosine monophosphate
COX- cyclo-oxygenase
$cPLA_2$- calcium-dependent phospholipase A_2
C3- Complement factor 3
DAG- Diacylglycerol
DGAT- Diacylglycerol- acyl- transferase
EGF- epidermal growth factor
ER- endoplasmic reticulum
ERK 1/2 – extracellular-signal-regulated kinase 1/2
GAS- one interferon -γ activated sequence
GluT- glucose transporter
GRE- glucocorticoid response element
HELSS- haloenol lactone suicide substrate
HSL- hormone sensitive lipase
IFNγ interferon -γ
γ-IRE- IFN-γ response elements
IP3- inositol-trisphosphate
$iPLA_2$- calcium-independent PLA_2
Lyso-PC- Lyso-phosphatidylcholine
Lyso-PA- Lyso-phosphatidic acid
MAFP- methyl arachidonyl fluorophosphonate
MAPK- Mitogen-activated protein kinase
MAPKAK- MAPK activated kinase
MNK1- MAP kinase-interacting kinase 1
MSK1- mitogen- and stress-activated protein kinase 1
NF-kB- Nuclear transcription factor-κB
OAG- 1-oleoyl-2-acetyl-rac-glycerol
PA-phosphatidic acid
PDE-phosphodiesterase
PGs- prostaglandins
PGI_2- Prostacyclin

PI- phosphatidylinositol
PI3K- phosphatidylinositol 3-kinase
PIP2- phosphatidylinositol-4,5-bisphosphate
PKA- cAMP-dependent protein kinase
PKC-protein kinase C
PLA_2- Phospholipase A_2
PLC- Phospholipase C
PLD- Phospholipase D
PMA- 4β-phorbol 12-myristate 13-acetate
PPARγ- peroxisome proliferator-activated receptor-gamma
PRAK1- p38-regulated and -activated kinase
PS- phosphatidylserine
TG- Triglyceride
TNFα- tumour necrosis factor-α
TTS-2.2- Transport secretion protein-2.2
TXAs- thromboxanes
UTR- untranslated region
VLDL-very low density lipoprotein

CHAPTER ONE
Regulation of Triglyceride (TG) Storage

Brown and white fat represent the two main types of adipose tissue in the body. While brown fat has a specialized function in energy metabolism in hibernating animals, it is almost absent in humans. White adipose tissue on the other hand is more abundant in humans and is the major storage site of excess energy in the form of triglycerides (1)

Triglyceride (TG) storage occurs when dietary energy supply goes beyond the energy demands of the body. During nutritional deprivation, TG can be hydrolyzed to free fatty acids, released for oxidation in other organs. Any disturbance to this steady state can lead to increased or decreased amounts of white adipose tissue, as can be seen in obesity and lipodystrophy respectively (2).

Obesity is an imbalance between energy expenditure and energy intake, which leads to excess adipose tissue. It is now well accepted that adipose tissue is a highly active metabolic and endocrine organ with lipogenesis and lipolysis regulating adipose tissue energy stores, along with the ability to constantly generate new adipose cells via adipogenesis (3). In addition to fatty acids, white adipose tissue has the capacity to generate a large number of hormones. These hormones can act at distant organs, such as the central nervous system, and can alter eating behavior, energy balance and hormone sensitivity (4).

Preadipocytes (non-fat containing cells) have the ability to differentiate to adipocytes (mature fat cells), which in turn have the ability to adjust (increase or decrease) their TG store size. As the adipocytes mature, they produce hormones, which can influence both TG storage and preadipocyte differentiation (5).

The fat storage process is very advanced in our species, if not we would not have survived the last Ice Age. Certainly, those who could not respond to alternating spells of feast and famine with long-term fat storage did not survive (6). The opposite is seen today, where an abundance of high-energy food is easily accesible, as well as a sedentary lifestyle. The development of obesity results from a combination of both environmental and genetic factors (7). Although various factors such as metabolic rate and adipokine release can predispose an individual to obesity, consumption of energy-rich foods and limited exercise are required for obesity to fully develop (7). Genetic abnormalities that

predispose to obesity, such as leptin receptor mutations have been identified (8). However, the lack of these mutations does not prevent obesity and suggests that many genes have yet to be discovered.

The most common index of weight is the body mass index. The body mass index is calculated by taking body weight (in kilograms), and dividing it by height squared (in meters) (kilograms / meters^2). A normal body mass index ranges from 18.5 to <25, overweight 25 to <30, and obesity $\geq$ 30 (9). Presently in Canada the percentage of people with a body mass index $\geq$ 30 has more than doubled in the past decade. In fact in 1997, the direct medical costs attributable to adult obesity in Canada are estimated to have been $1.8 billion, 2.4% of total direct medical costs (10).

Obesity is closely associated with a number of lipid dysmetabolism-related disorders such as type-2 diabetes (non-insulin-dependent diabetes), hypertension, and atherosclerosis, diseases currently considered epidemic in Western countries (11). As a result, the impact of adipocyte derived factors on both adipocyte differentiation and the development of obesity has become a focused topic in research.

De novo Triglyceride (TG) Synthesis

Triglycerides provide a concentrated form of metabolic energy, as they can be stored in the anhydrous form, as opposed to glycogen stores, which are heavily hydrated. Furthermore, glycogen is stored in tissue that cannot expand such as the liver or muscle, adipose tissue, however, is capable of vast expansion (1).

During the postprandial period (after a meal), dietary fat is absorbed in the intestine, packaged into lipoproteins, and secreted into the circulation. Circulating chylomicron-TG and very low density lipoprotein (VLDL) -TG are susceptible to cleavage by lipoprotein lipase, a protein secreted by adipocytes and expressed on the endothelial cell surface (12). The free fatty acids that are released can be taken up by adipose tissue in a passive fashion or via active free fatty acid transporters. In order for free fatty acids to be incorporated into TG they must first become activated to an acyl-CoA form, by the enzyme acyl-CoA synthase (13).

Like triglycerides, glucose levels also increase postprandially. Glucose can enter adipose tissue via specific glucose transporters, which are found across the cell

membrane. Once glucose is taken up in the cell it is phosphorylated and converted to glycerol 3-phosphate, the backbone of the TG molecule (Figure 1.1) (13).

The first committed step in TG synthesis is characterized by the acylation of glycerol-3-phosphate to lysophosphatidic acid. This reaction is catalyzed by the enzyme glycerol-3-phosphate acyl transferase. Phosphatidic acid (PA) is generated from the acylation of lysophosphatidic acid in a reaction catalyzed by lysophosphatidic acid-acyl transferase (14).

It is important to note that PA can also be synthesized from diacylglycerol (DAG) by DAG-kinase. However, this alternate route seems to generate PA for signaling purposes, rather than for storage into triglyceride (15).

The next step in TG synthesis involves the hydrolysis of the phosphate group on PA by PA-phosphatase, this reaction results in the generation of DAG. The final and committed step in TG synthesis involves the rate limiting enzyme diacylglycerol-acyl-kinase (DGAT), which acylates DAG to produce TG (16).

The de novo TG pathway provides a link between TG and phospholipid metabolism (17). In the early 1950's, Kennedy et al. demonstrated that both PA, and DAG, can be used as substrates in phospholipid synthesis. In the former, phosphatidylinositol (PI), phosphatidylglycerol, cardiolipin can be produced, while the more common lipid species phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine are generated from the latter (1, 14).

In addition to their function as storage molecules, the three intermediates produced during de novo TG synthesis can also function as signaling molecules. Both lysophosphatidic acid and PA can be produced externally from plasma membrane phospholipids where they can activate an intracellular signaling cascade upon binding to their corresponding G-protein coupled receptors (GPCRs) (18). Likewise, DAG can be produced from phospholipids intracellularly (see Chapter Two), resulting in the activation of protein kinase C (PKC) (19).

Lipolysis

The amount of TG stored in an adipocyte is determined by the balance between lipogenesis and lipolysis. Lipolysis is initiated by the activation of hormone-sensitive

lipase, the rate-determining enzyme in this pathway, resulting in the mobilization of fatty acids from TG stores (20). The regulation of TG hydrolysis begins with the ligand-dependent activation of β-adrenergic receptors by catecholamines. In white adipose tissue catecholamine activation of β-adrenergic receptors leads to the mobilization of stored fatty acids for energy when there is a demand for fatty acids elsewhere in the body, whereas in brown adipose tissue it generates heat for animals during their hibernating state, through a process called adaptive nonshivering thermogenesis (1, 21).

The three subtypes of β-adrenergic receptors (β1, β2 and β3), all belong to the GPCR family and couple to Gαs. The intracellular activation of the GTP-binding protein Gαs, signals membrane-bound adenylate cyclase to increase cyclic 3'-5'-adenosine monophosphate (cAMP) concentrations within the cell. The rise in cAMP levels in turn, leads to the activation of cAMP-dependent protein kinase (PKA), which can phosphorylate and increase the catalytic activity of hormone sensitive lipase, a known downstream target of PKA (Figure 1.2) (21).

The phosphorylation of hormone sensitive lipase results in its translocation from a cytosolic to a membrane fraction on the lipid droplet, whereupon it can gain access to the TG molecule, hydrolyzing it to fatty acids. Both in vivo and in vitro phosphorylation of hormone sensitive lipase by PKA has been shown to occur on serine (Ser) residues: 659, 660 and 563. Mutating either Ser 659 or Ser 660 completely abolishes the in vitro activation and translocation of hormone sensitive lipase. In contrast, no effect is observed when mutating Ser 563. This suggests that Ser 659 and Ser 660 are important targets for hormone sensitive lipase activity, whereas the role of the third PKA site is presently not clear (22).

Acylation Stimulating Protein (ASP) Introduction

Acylation Stimulating Protein (ASP) was named based on its ability to stimulate fatty acid esterification (or acylation) in cultured human skin fibroblasts and adipocytes (23, 24). ASP is a small (76 amino acid) and basic (pI 9.0) protein hormone identical to C3adesArg, a biological fragment of human complement factor C3 generated through the alternate complement pathway (23).

ASP secretion in the adipose tissue milieu increases postprandially and the degree to which they increase correlates directly with the rate at which triglycerides are cleared from the plasma (25, 26). Studies using cultured human skin fibroblasts and human adipose tissue microsomes have shown that ASP increases the Vmax of triglyceride synthesis through a co-ordinate effect on two separate aspects of the TG synthetic pathway. Firstly, like insulin ASP enhances the entry of glucose into cells. The translocation of glucose transporters 1, 3 and 4, from an intracellular pool to the plasma membrane results in enhanced TG substrate availability, as glucose is converted to glycerol-3-phosphate, the backbone of the TG molecule (27, 28). However, the effects of ASP and insulin on triglyceride synthesis are independent and additive to each other, suggesting that they work via separate mechanisms (27). Second, ASP enhances the overall rate at which fatty acids are esterified to the glycerol-3-phosphate backbone by increasing the activity of DGAT, the final and committed step in triglyceride synthesis (29). The mechanism by which ASP directly leads to DGAT activation is currently unknown, it has been shown however, in both human adipocytes and fibroblasts, that ASP mediates its lipogenic effects through the activation of protein kinase C and will be discussed in greater detail in Chapter Two (30).

In addition, ASP can inhibit TG hydrolysis. Similar to Insulin, ASP activation of phosphodiesterase (PDE) 3, and to a lesser extent PDE4 for ASP, prevents both the rise in intracellular cAMP levels and subsequent PKA activation (discussed in Chapter Five) (31).

ASP Production

ASP is identical to human complement C3adesArg in amino acid composition, molecular mass and NH2-terminal amino sequence (24). It is produced via a two-step process that involves three proteins of the alternate complement system: complement factor 3 (C3), factor B and adipsin (complement factor D). All these factors are synthesized and secreted by adipocytes, with ASP generated in the microenvironment. Initially, C3 is converted spontaneously to its active form C3*. This active analogue combines with factor B to form the C3*B complex. At this point adipsin comes into play, as it cleaves bound B from this complex generating C3*Bb and Ba. The enzymatic

activity of C3*Bb cleaves C3, generating C3a and C3b. The final product C3adesArg or ASP is produced by the action of Carboxypeptidase B (CpB) on C3a, which cleaves its C-terminal arginine to generate the 8932 Daltons protein ASP (24, 32).

While both ASP and C3a can stimulate TG synthesis, only C3a has the ability to function as an immune modulator (33). The carboxyl-terminal arginine, albeit essential for the immune function of C3a and binding to the C3a receptor, is not required for the signalling of TGS (34). In addition, the three disulfide-bridges forming part of the coiled region of the molecule, has been shown to be essential for stimulating TG synthesis. On the other hand, this coiled region does not appear to be necessary for mediating immune function (34). These structural differences provide further evidence of two separate receptors for C3a and ASP. The C3a receptor (C3aR) has been identified and cloned in human, mouse and rat (35, 36). Through northern blot analysis and immunofluorescence no C3a receptor mRNA or protein has been detected in adipose tissue (32).

ASP Expression

ASP levels appear to remain constant in the general circulation with alterations present in the adipose micro-environment (25, 37). The average plasma concentrations of ASP in normal healthy adults is 28.3 nM or 253 ng/mL. In contrast, its precursor C3 is present on average at 6.4 uM in plasma, a 225-fold greater molar concentration than ASP, suggesting that only a relatively small portion of C3 is used to generate ASP. The levels of the cleavage enzyme adipsin on average are 62.9 nM and are also much higher than ASP (32). The levels of C3, ASP and adipsin reported above do not vary between males and females, however, gender differences arise with obesity, as females have a reported two fold increase in circulating ASP levels compared to obese males (38). Plasma ASP levels in obese-subjects diminish with prolonged fasting and weight loss (39, 40, 41).

There is a differentiation-dependent increase in ASP binding and response in human adipocytes (42). Adipocytes are one of the few cells capable of producing all three factors (factor B, adipsin and C3) that are required for the production of ASP (32). The expression of, C3, adipsin and ASP accordingly, have been shown to increase with adipocyte differentiation (29, 42).

During the postprandial period, chylomicrons, in particular retinoic acid, stimulate ASP production by adipocytes. Interestingly, the increases in ASP production during this period correlates with maximal TG clearance and fatty acid uptake in adipocytes (43, 44).

ASP Knock-Out Studies

Lack of the C3 gene in mice, the precursor to ASP, results in ASP deficiency, reduced leptin levels, and delayed TG and free-fatty acid clearance after an oral fat load (45, 46). This delay in clearance results from the reduced uptake and esterification into TG. In addition, the lack of inhibition of lipolysis by ASP, results in the breakdown of stored TG. This effect is reflected by the increase in free-fatty acid in the circulation. Free-fatty acids, in turn, can inhibit the lipoprotein-lipase enzyme, via product inhibition. As a result, energy is re-directed into organs that are not equipped for energy storage, such as, the liver and muscle. Such a setting is observed in obesity and has been found to contribute to insulin resistance and ultimately adult-onset diabetes. In spite of this, glucose uptake and insulin sensitivity are enhanced in C3 knock-out mice (47). Likely, it is the decreased adipose tissue mass in these mice that leads to increased insulin sensitivity. Furthermore, decreases in body weight results in lower circulating leptin levels compared to wild-type mice. Despite a reported reduction in body fat, C3 knock-out mice eat more compared to their wild-type counterparts, an effect that was found to be independent of leptin. Their resistance to obesity, despite an increase in food intake, may be explained by their enhanced energy expenditure and is evidenced by their overall increase in oxygen consumption (45).

ASP Receptor, C5L2

Through the use of genomic sequence analysis, Lee et al. (2001) were able to identify a previously unrecognized intronless gene encoding the orphan receptor, GPCR 77. This receptor was localized on chromosome 19 and appeared to be a novel member of the C5a/C3a complement anaphylotoxin family, with 58% and 55% sequence identity in the transmembrane regions for C5a and C3a receptors respectively (48).

In 2003, Kalant et al. de-orphanized this receptor as the first ASP receptor, C5L2. C5L2 mRNA is found in the brain, liver, lung, spleen, testis, ovary, kidney, colon and

heart. In addition, the C5L2 receptor is expressed in human skin fibroblasts, 3T3-L1 and human adipose tissue, and is positive for binding complement fragments C5a, C4a and C3a, as well as their metabolites, C5adesArg, C4adesArg and C3adesArg respectively (49, 50). Despite binding to C5L2, there is no stimulation of TG synthesis in 3T3-L1 preadipocytes or in human skin fibroblasts with C5a, C5adesArg, C4a or C4adesArg. In contrast, both C3a and ASP are able to stimulate TG synthesis in both cell types (49). It has been shown that the C3a receptor and C5L2 bind C3a with similar affinities, whereas the C3a receptor has no detectable affinity for ASP (49, 51). While ASP and C3a can stimulate TG synthesis via C5L2, only C3a can induce an immune response by binding the C3a receptor (52).

Figure 1.1

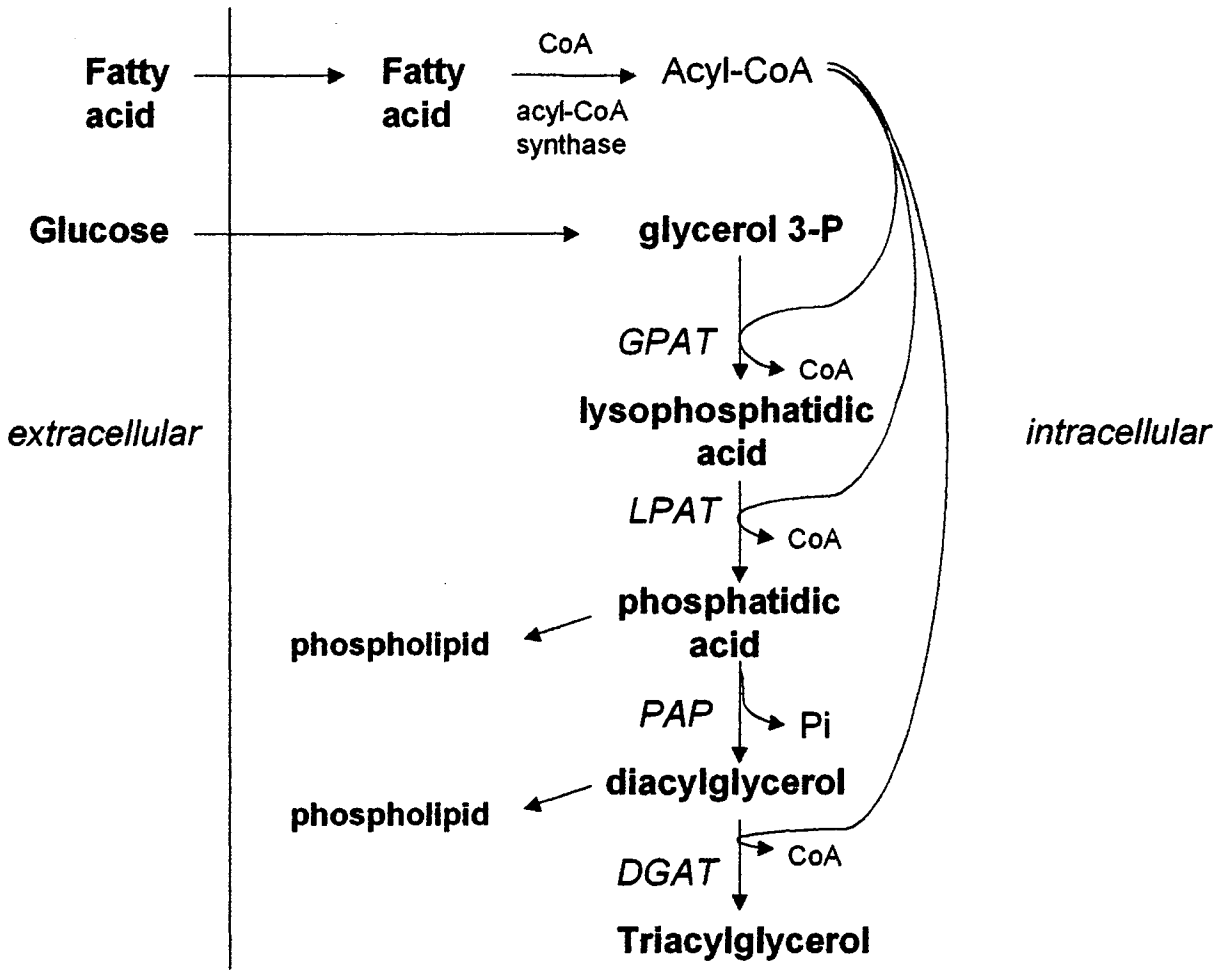

Fatty acid
Fatty acid
CoA
acyl-CoA synthase
Acyl-CoA
Glucose
glycerol 3-P
GPAT
CoA
extracellular
lysophosphatidic acid
intracellular
LPAT
CoA
phosphatidic acid
phospholipid
PAP
Pi
diacylglycerol
phospholipid
DGAT
CoA
Triacylglycerol

Figure 1.2

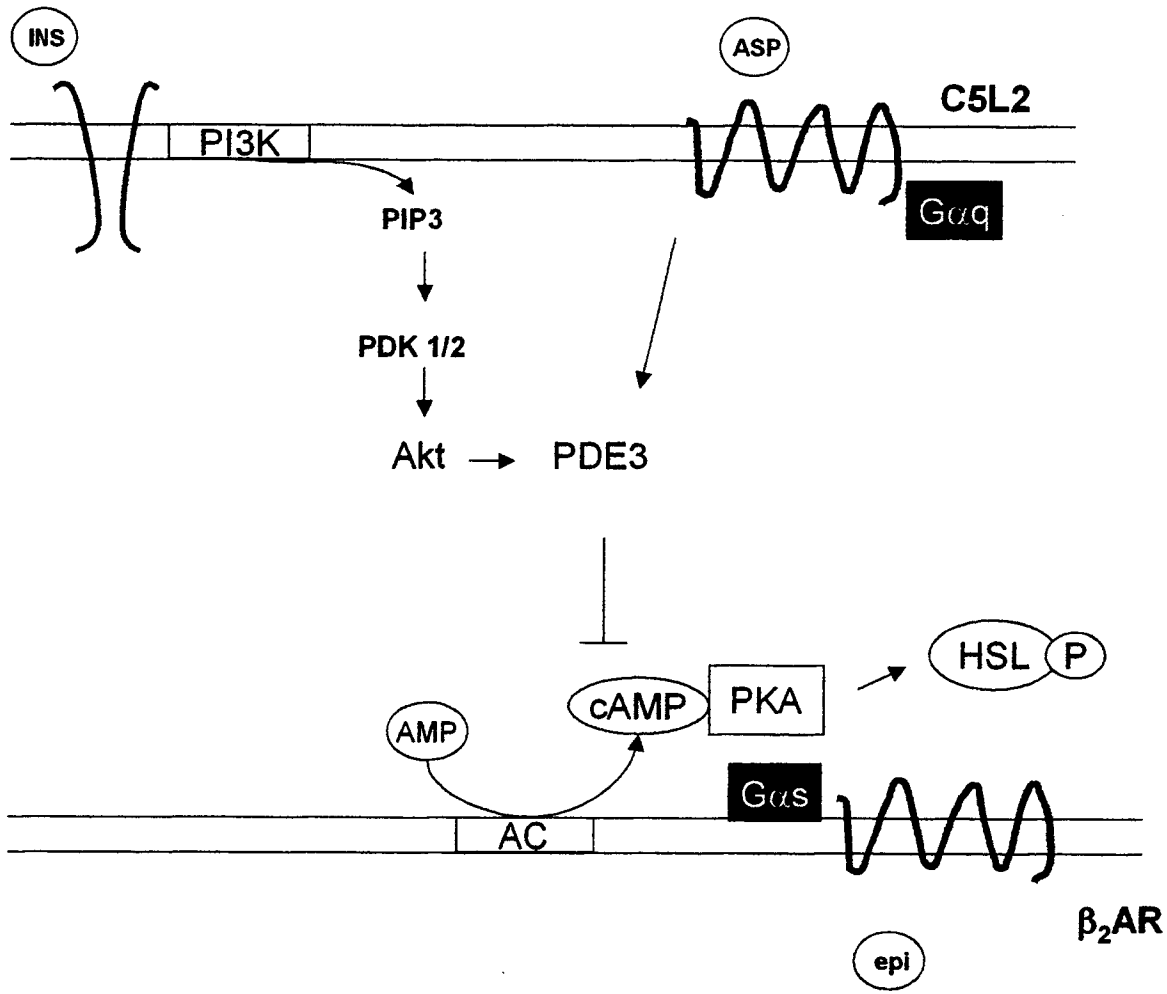

INS
ASP
C5L2
PI3K
PIP3
Gαq
PDK 1/2
Akt
PDE3
HSL
P
cAMP
PKA
AMP
Gαs
AC
β2AR
epi

Figure Legends:

Figure 1.1: De Novo Triglyceride (TG) Synthesis

De novo TG synthesis begins with the uptake of both fatty acid and glucose into the cell. Glucose is converted to glycerol-3-phosphate (glycerol 3-P), and fatty acid is activated to acyl-CoA by the enzyme acyl-CoA synthase. Glycerol 3-P is then acetylated by glycerol 3-P acyl-transferase (GPAT) to produce lysophosphatidic acid, and then to phosphatidic acid by the action of lysophosphatidic acid acyl-transferase (LPAT), the resultant phosphatidic acid is substrate to phospholipid synthesis. The phosphate head group of phosphatidic acid is hydrolysed by phosphatidic acid phosphatase (PAP) to produce diacylglycerol (DAG). This lipid is substrate to both phospholipid or TG synthesis, as it is substrate to the rate-limiting enzyme, DAG acyl-transferase (DGAT).

Figure 1.2: ASP and Insulin Inhibit Lipolysis through Phosphodiesterase (PDE).

Lipolysis is activated in a cell by catecholamine (e.g. epinephrine (epi)) binding to their respective G-protein coupled receptor (shown here β2-adrenergic receptor, β2-AR). The rises in cAMP that result from adenylate cyclase (AC) action, stimulate cAMP-dependent protein kinase (PKA), which phosphorylates and activates hormone sensitive lipase (HSL). ASP can inhibit HSL activity by PDE activation. Insulin has been shown to activate PDE through a phosphatidylinositol 3-kinase (PI3-K)-dependent mechanism, formation of phosphatidylinositol 3,4,5 triphosphate (PIP3). Binding of PIP3 to Phosphoinositide-dependant kinase-1/2 (PDK-1/2), results in the activation of the serine/ threonine kinase Akt, which in turn, can activate PDE3. PDEs catalyze the hydrolysis of the second messengers cAMP, and attenuates HSL activity.

CHAPTER TWO
ASP Signaling

Evidence suggests that ASP activates a downstream signaling pathway upon binding to its first identified receptor C5L2, a GPCR that couples to Gαq. Among the enzymes shown to be activated by ASP, phospholipase C (PLC), phosphatidylinositol 3-kinase (PI3-K) and PKC are discussed in this chapter.

The involvement of PKC in ASP action has been previously evaluated in human skin fibroblasts (30). PKC is a family of serine/threonine protein kinases, classified into three groups based on their structure and cofactor requirements (53-55). The first group, the conventional PKC family, is activated by DAG or its analogue 4β-phorbol 12-myristate 13-acetate (PMA), rises in intracellular calcium (Ca^{2+}), [Ca^{2+}]i, and the membrane phospholipid phosphatidylserine (PS). The novel PKC family is activated by PS, DAG and unsaturated fatty acids. Since these class of enzymes lack the calcium binding domain, found in the conventional family, they do not require this cofactor for activity. The atypical family of enzymes are activated by PS, phosphatidylinositol (PI), or unsaturated fatty acids (19).

1-oleoyl-2-acetyl-rac-glycerol (OAG) and PMA, both analogues of DAG and stimulators of PKC, potently stimulate TG synthesis in a concentration-dependent manner. The effects of PMA on TG synthesis are non-additive to those of ASP, suggesting that ASP action is mediated via a PKC- dependent mechanism (30). Furthermore, the two PKC inhibitors Calphostin C (a competive inhibitor of the DAG binding site on PKC), and Bisindoylmaleimide (a competitive inhibitor of the ATP binding site of PKC), effectively inhibit ASP effects on TG synthesis. A time-dependent translocation of PKC from the cytosol to membrane fraction is observed with ASP incubation between 30 to 50 minutes, with maximum effects attained at 40 minutes (30).

Biphasic DAG production with ASP

ASP stimulates a biphasic generation of DAG in human skin fibroblasts. The peak increases in DAG levels occur at both 5 and 35 minutes respectively (30). Although it is not clear which phospholipase is responsible for DAG generation, the biphasic nature

suggests that accumulation of DAG results from phospholipid- breakdown by the activation of different phospholipases.

The second prolonged peak of DAG generation with ASP clearly coincides with the translocation and activation of PKC (maximum at 40 minutes) (30). We propose that the first peak in DAG production may occur from the translocation of PI-specific PLCβ to the plasma membrane, an enzyme shown to be activated by ASP (discussed in Chapter 3), whereupon it can hydrolyze PI to produce inositol-trisphosphate (IP3) and DAG. The IP3 produces a release of Ca^{2+} from the endoplasmic reticulum (ER). The increase in DAG and possibly the rises in $[Ca^{2+}]i$ activate and translocate PKC, which further enhances phospholipase activity, phospholipid breakdown and subsequent DAG generation.

It has been suggested that DGAT is inactive when phosphorylated (56, 57). Stimulation of PKC by ASP may subsequently activate a phosphatase enzyme, resulting in the de-phosphorylation and activation of TG synthetic enzymes such as DGAT.

Sources of DAG production

The production of DAG in stimulated cells is often biphasic, with an initial, transient, rapid rise in DAG accumulation, then a slower accumulation that can be sustained for an hour or more. The initial peak is often attributed to PI-PLC activation, whereupon phosphoinositides on the membrane are hydrolysed to produce DAG. During the second phase, the DAG produced is derived mainly from the hydrolysis of phosphatidylcholine (PC), by PC-PLC, or through the sequential action of Phospholipase D (PLD) and PA- phosphatase (summarized in Table 2.1).

Phospholipases

The discovery that cellular phospholipids not only serve structural roles, but that they are also reservoirs of second messenger molecules, has led to great interest in the study of phospholipid metabolism. The generation of these second messenger molecules is a consequence of agonist-induced activation and secretion of intracellular and extracellular phospholipases respectively (14). A group of hydrolytic enzymes, termed phospholipases, are responsible for the breakdown of phospholipids. There are three

different types of phospholipases, these include phospholipase A_2 (PLA_2), PLC and PLD, each of which cleaves a specific bond of a phospholipid molecule (Figure 2.2). Phospholipases are thought to serve two general functions: by acting as enzymes that remodel complex lipids such as phospholipids and TG, and also as generators of cell signaling products. The latter concept is explored in ASP signaling, and is discussed in Chapter 5 and Chapters 3/ 4 respectively.

PLD is an enzyme that catalyzes the formation of PA and the release of the polar head group at the sn-3 position (64). The principal substrate is PC and its product PA, is believed to have a direct signaling role and can also be converted into DAG by the action of PA-phosphatase (65, 66).

The PI-specific isoform of PLC hydrolyzes the phosphor-ester bond at the sn-3 position. It is activated by GPCRs catalyzing the receptor-induced formation of DAG and IP3 (69). IP3 is able to mobilize intracellular stores of Ca^{2+} from the ER, while DAG can activate specific isoforms of PKC, which results in the activation of downstream signaling pathways such as the mitogen-activated protein kinase (MAPK) cascade (19, 69, 70).

PLA_2 is a specific sn-2 cleaving enzyme that releases mainly AA and lysophospholipid upon activation (71). There are approximately 15 genes that encode the structurally diverse PLA_2 enzymes in mammalian cells (66). There are three main types of PLA_2: the low-molecular-weight secreted PLA_2 ($sPLA_2$), and the intracellular Ca^{2+}-dependent ($cPLA_2$) and Ca^{2+}-independent ($iPLA_2$). There are 10 distinct mammalian $sPLA_2$s that have low MWs, require calcium for activity, and utilize a His-Asp dyad in the catalytic mechanism (67, 68). The intracellular PLA_2s, $iPLA_2$, and $cPLA_2$, have larger molecular masses and utilize an active site serine for catalysis (72, 73). In spite of the fact that each PLA_2 family member is suggested to have a role in receptor-mediated release of AA, the $cPLA_2\alpha$ isoform has been the most recognized in providing AA for eicosanoid production (74). Both Ca^{2+} binding and phosphorylation have been well documented as pathways that fully activate $cPLA_2$ (70). Thus, agonists that stimulate PI-PLC can activate $cPLA_2$ through this mechanism.

The $sPLA_2$ family have been associated with chronic inflammation (75). Although there is evidence *in vitro* that certain $sPLA_2$s can release AA for eicosanoid production, they do not exhibit sn-2 acyl chain specificity. Thus, for some $sPLA_2$s, their primary physiological function may not involve the specific release of AA. It is currently thought that once secreted, the $sPLA_2$s either act on the external leaflet of the plasma membrane or are taken up into cells and act at an intracellular site (66, 71).

There is evidence of a cross-talk mechanism between both $sPLA_2$s and $cPLA_2$. This has made it more difficult in defining the role of specific PLA_2 in regulating AA release. For example, $sPLA_2$ can act in an autocrine fashion and induce a rapid activation of PKC and extracellular-signal-regulated-kinase 1/2 (ERK1/2), resulting in $cPLA_2$ activation (76).

Two groups of $iPLA_2$s have been cloned i) GVIA $iPLA_2$ (also called $iPLA_2\beta$) and ii) GVIB $iPLA_2$ (also called $iPLA_2\gamma$). The $iPLA_2$ family contain a conserved nucleotide binding sequence (GXSXG) They have no apparent sn-2 fatty acid or phospholipid base group specificity (77). It was initially thought that GVIA $iPLA_2$ played a house-keeping role in the remodeling of sn-2 phospholipid fatty acids. However, current evidence suggests that it is also a regulated enzyme and plays a signaling role through production of lysophospholipid and AA (78, 79). GVIA $iPLA_2$ is widely expressed in tissues, most predominantly in liver, testis, muscle and kidney. It contains 8 ankyrin repeats and a calmodulin-binding domain, which in the Ca^{2+}-calmodulin state negatively regulates $iPLA_2$ activity (80). The most recently identified GVIB $iPLA_2$ is widely expressed in human tissues but is enriched in heart, placenta and skeletal muscle. It is tightly membrane bound and contains a C-terminal peroxisomal localization sequence (72).

The $cPLA_2$ family includes α, β and γ isoforms, classified into group IVA, IVB and IVC respectively (72, 74, 81). These three isoforms contain two catalytic domains and a lipase consensus sequence (GXSGS). The NH2-terminal of the α and β isoforms contains a C2 domain that associates with Ca^{2+} and phospholipid membranes. In contrast, $cPLA_2\gamma$ is constitutively membrane bound by its isoprenyl site found on its C-terminus (81). $cPLA_2\alpha$ appears to be more specific towards phospholipids bearing AA in the sn-2 position, while the β and γ isoforms are less strict, in fact they are reported to have strong sn-1 specificity (72, 81). The lipase motif of $cPLA_2\alpha$ contains a nucleophilic Ser- 228

that attacks the sn-2 ester bond of phospholipids, while Asp- 549 activates this catalytic center (66).

Transcriptional Regulation of $cPLA_2$

$cPLA_2$ is also subject to transcriptional regulation as the promoter region of the $cPLA_2\alpha$ gene contains potential binding sites for adaptor proteins (AP) 1 and 2, nuclear transcription factor-κB (NF-kB), CCAAT/enhancer binding protein (C/EBP) (82). Cytokines and mitogens (such as epidermal growth factor (EGF), tumour necrosis factor-α (TNFα) and interferon -γ (IFNγ)) have been documented in enhancing the expression of $cPLA_2$, while glucocorticoids have been shown to play an inhibitory role. The $cPLA_2\alpha$ promoter consists of CA repeats, five IFN-γ response elements (γ-IRE), one interferon -γ activated sequence (GAS) and two glucocorticoid response elements (GRE). Although the mechanism by which cytokines stimulate $cPLA_2$ expression is not clear, it is believed that the half-life of the cPLA2 mRNA is extended by stabilizing the 3'-prime untranslated region (UTR) of $cPLA_2$ (83).

Post-transcriptional Regulation of $cPLA_2$

i- Calcium (Ca^{2+}) binding

$cPLA_2\alpha$ can trap two Ca^{2+} ions in its C2 domain, and preferably binds to PC. Ca^{2+} allows $cPLA_2$ to translocate from the cytosol and become membrane bound (golgi, ER, or nuclear envelope). The binding of two Ca^{2+} ions to Asp-43 and Asp-93 in the NH2-terminal C2 domain of $cPLA_2$ is essential for the enzyme to gain access to phospholipid-containing membranes. In fact, inactive $cPLA_2$ results when mutating any one of these residues (66).

ii- Phosphatidylinositol-4,5-bisphosphate (PIP2)

In addition to DAG, PIP2 can facilitate the binding of $cPLA_2\alpha$ to membranes. Several cationic residues located on the surface of the C2 catalytic domains are critical for the binding of $cPLA_2\alpha$ to membranes containing PIP2 (84). It has been suggested that PIP2 levels can suprisingly regulate $cPLA_2$ activation in a Ca^{2+}-independent manner. Even at basal Ca^{2+} levels, $cPLA_2$ has been shown to bind vesicles containing PIP2,

leading to dramatic increases in enzyme activity. Thus higher levels of PIP2 in membranes can result in increased amounts of $cPLA_2$ bound to the membrane and enzyme activity (84, 85).

iii- Phosphorylation of $cPLA_2$

The $cPLA_2\alpha$ enzyme is phosphorylated on both Ser-505 and 727, located on its consensus phosphorylation motif, in response to agonist stimulation (86). The importance of phosphorylation of $cPLA_2$ on Ser-505 and other sites (Ser-727 and Ser-515) for agonist induced AA release has been confirmed by expression of phosphorylation-site mutants (87). Ser-505 resides within the sequence Pro-Leu-Ser-Pro and is typically recognized by proline directed kinases such as MAPKs. Ser-727 is flanked by arginines (Arg-Arg-(X)4-Arg-X-Ser-(X)8-Arg-Arg) and is a substrate of MAPK activated kinase (MAPKAK) enzymes (88, 89).

Agonist-induced phosphorylation of $cPLA_2$ appears to be mediated by either PKC-dependent and -independent activation of MAPKs (90, 91). Evidence suggests that PKC does not interact with $cPLA_2$ directly, but rather through the activation of MAPK (86).

ERK1/2, as well as the p38 MAPK family, are able to phosphorylate $cPLA_2$ on Ser-505 (66). It was not known until recently which enzymes are capable of phosphorylating $cPLA_2$ on Ser-727. Recent in vitro evidence suggests that the downstream target of MAPK, MAPKAK, can phosphorylate $cPLA_2$ on Ser-727. These enzymes include: the MAP kinase-interacting kinase 1 (MNK1), p38-regulated and -activated kinase (PRAK1) and the mitogen- and stress-activated protein kinase 1 (MSK1) (89). Thus, activation of MAPK can lead to the phosphorylation of $cPLA_2$ on Ser-505 and activate downstream enzymes that can phosphorylate Ser-727.

In contrast to the $iPLA_2$ family, both cAMP and cyclic-guanosine monophosphate (cGMP)-dependent protein kinases mediate an inhibitory effect on the 85-kDa $cPLA_2$. It is believed that PKA can phosphorylate $cPLA_2$ on Ser 727 in vitro, suggesting the presence of phosphorylation of $cPLA_2$ that is inhibitory (92).

iv- Ca^{2+}/Calmodulin-dependent Protein Kinase II (CaMK II)

CaMKII has been shown to phosphorylate cPLA2α on its Ser-515 residue, activating $cPLA_2$ independent of the MAPK pathway. At resting conditions, CaMKII is inactive due to its autoinhibitory domain, which blocks binding of both peptide and ATP substrate. Rises in Ca^{2+} or calmodulin binding are necessary for CaMKII to become active (93).

Arachidonic Acid (AA) as a signaling molecule

AA is an ω-6 polyunsaturated fatty acid with a number of physiological functions (discussed below). It is also the precursor for a number of biologically active metabolites formed by the oxygenation through cyclooxygenase and lipoxygenase pathways (94). The eicosanoids (prostanoids and leukotrienes) play important roles in regulating many physiological processes and acute inflammatory responses (Chapter 4) (95).

The levels of AA in serum are low compared to other fatty acids, except in obesity and diabetes, where levels are reportedly higher compared to normal weight and healthy subjects. (96, 97). Unsaturated fatty acids have been well documented in having stimulatory effects on PKC, whereas saturated fatty acids are ineffective. It is believed that in the presence of DAG, AA can enhance the affinity of conventional PKC for Ca^{2+}, thus stimulating its activity (19, 98, 99). AA has also been shown to stimulate the novel class of PKC even in soluble form. Thus, activation of PKC by AA appears to occur without relying on translocation to a membrane fraction, a step that is dependent on $[Ca^{2+}]i$ levels (19).

AA has been shown to increase both basal and insulin stimulated glucose uptake in both 3T3-L1 fibroblasts and adipocytes. AA increases the translocation of the major glucose transporters (GluT) 1 and 4 to the plasma membrane with no effect on total cellular levels of GluT after a 4 hour incubation period (100). Within this time frame, AA has been shown to double the Vmax of glucose uptake with no effects on Km. In addition, the acute effects of AA on glucose transport are enhanced with insulin. Furthermore, although translocation of both GluT 1 and 4 increases with AA, there is no reported effect on the total cellular levels of both transporters (100, 101).

Recently it was demonstrated that chronic exposure of AA in 3T3-L1 adipocytes was shown to inhibit GluT 4 gene expression. At incubation periods of 24- 48 hours, AA was shown to decrease total cellular and plasma membrane GluT 4 (102, 103). On the other hand, within this same time frame, AA increases total cellular and plasma membrane levels of GluT 1 (101). Thus, acutely, AA seem to stimulate the intrinsic activity of GluT 1 and longer exposure with AA seems to increase the overall content of GluT 1 in the cells. Although GluT4 translocation increases with AA after 4 hours, AA appears to negatively regulate GluT4 gene expression at longer incubation periods, possibly by de-sensitizing and down-regulating the transporter.

Although the direct mechanism by which AA enhances the overall uptake of glucose is unknown, it appears that AA either increases the translocation of both glucose transporters to the plasma membrane or attenuates their rate of internalization.

ASP, as mentioned in Chapter 1, increases GluT in 3T3-L1 cells, with effects additive to insulin (27, 28). It is believed that ASP stimulation of AA production may play a role in mediating this function and this is discussed in greater detail in Chapter 3 and 4.

Lyso-phosphatidylcholine (Lyso-PC) as a signaling molecule

Lyso-phospholipids are generated by the action of PLA_2 (71). The recent discovery of GPCRs for both Lyso-PA and Lyso-PC has established lysophospholipids as lipid mediators (104). Lyso-PC, like AA, can enhance DAG-dependent PKC activation. LysoPC has also been shown to stimulate glucose-induced insulin secretion from pancreatic β-cells by binding to GPCR 119, a receptor that is expressed in β-cells and couples to Gαs (105).

PLA_2 inhibitors

Presently two widely used mechanism-based $cPLA_2$ are used. Namely, arachidonyl trifluoromethyl ketone (AACOCF3) and methyl arachidonyl fluorophosphonate (MAFP) (106, 108). These two inhibitors react on activated serine groups via their arachidonyl tail and compete with phospholipid molecules for the catalytic site on the PLA_2 enzyme. MAFP has been shown to irreversibly inhibit both

$cPLA_2$ and $iPLA_2$ at similar concentrations, even though it was originally developed as a specific inhibitor for $cPLA_2$ (108). AACOCF3 is a slow, tight binding inhibitor of Group IV $cPLA_2$. Unfortunately AACOCF3 also potentially inhibits the Group VI $iPLA_2$ at higher concentrations (107). Lack of specificity relies on the fact that both $cPLA_2$ and $iPLA_2$ use a central Serine for catalysis and similar catalytic mechanisms (109).

In order to determine if the inhibitory effects are due to $cPLA_2$ or $iPLA_2$, then one must employ another inhibitor, such as bromoenol lactone (BEL), also referred to as haloenol lactone suicide substrate (HELSS). BEL is a selective, active site directed inhibitor that can irreversibly bind and inhibit both soluble, cytosolic and membrane associated $iPLA_2$ isoforms (110, 111). Therefore, if there is a given response to AACOCF3 and MAFP and not to BEL then one can conclude that $cPLA_2$ is involved.

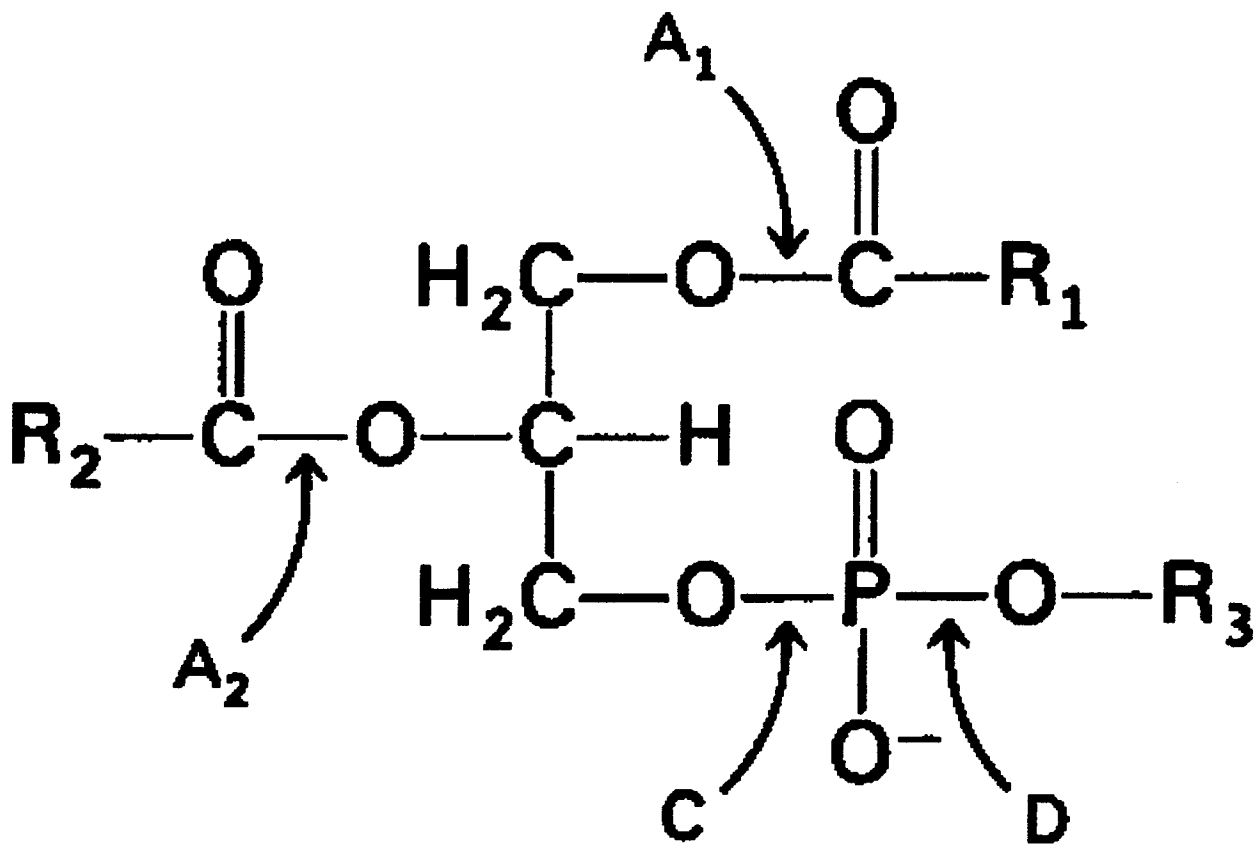
Isotetrandrine can indirectly inhibit PLA_2 activation without affecting PLC or PLD activity (112). Inhibition of PLA_2 activation by alkaloids, like isotetrandrine, results from the uncoupling of the GTP binding protein from the enzyme rather than affecting the enzyme directly. The effects of this inhibitor on ASP-stimulation of TG synthesis are discussed in Chapter 3.

Table 2.1 The Agonist-Stimulated Production of Diacylglycerol (DAG)

Stimulus	Lipid Species	DAG production	Target	Cell Type	Ref(s)
ASP	Early DAG (PI-PLCβ), late DAG (?)	biphasic	GPCR	HSF	30
PDGF	Early DAG (PI-PLCγ), late DAG (PC-PLD/ PAP)	biphasic	RTK	Swiss 3T3	58, 59, 61
Bradykinin	Early DAG (PI-PLC), late DAG (PC-PLD)	biphasic	GPCR	NIH-3T3, HSF	30, 60
Insulin	Early DAG (PI-PLCγ), late DAG (PC-PLC)	biphasic	RTK	Swiss 3T3, 3T3-L1	58, 63
PGF2α	Early DAG (PI-PLCβ), late DAG (PC-PLD/ PAP)	biphasic	GPCR	Swiss 3T3	58, 61
A23187	Early DAG (PI-PLCδ), late DAG (PC-PLD/ PAP)	biphasic	[Ca2+]i levels	Swiss 3T3	61
PMA	late DAG (PLD/ PAP)	monophasic (late peak)	PKC	Swiss 3T3, HSF	30, 58, 61

Abbreviations: ASP, acylation stimulating protein; PDGF, platelet derived growth factor; PGF2α, prostaglandin; PMA, phobol 12-myristate 13-acetate; HSF, human skin fibroblasts; RTK, receptor tyrosine kinase; GPCR, G-protein coupled receptor; PI-PLC, phosphoinositide-specific PLC, PC-PLC, phosphatidylcholine-specific PLC; PAP, phosphatidic-acid phosphatase; $[Ca^{2+}]i$, intracellular calcium levels.

Figure 2.2: Phospholipases Cleave Different Positions on the Phospholipid Molecule.



Phospholipase A1 and A2 enzymes can cleave the sn-1 and sn-2 position of phospholipids respectively. PLC cleaves the phospho-ester bond, while PLD cleaves the phospho-head group.

CHAPTER THREE

TARGETING THE SIGNALLING PATHWAY OF ACYLATION STIMULATING PROTEIN

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ABSTRACT

Acylation Stimulating Protein (ASP, C3adesArg) stimulates triglyceride synthesis (TGS) and glucose transport in preadipocytes and adipocytes. Recently, we have demonstrated that C5L2, a G protein coupled receptor, is functionally responsible for mediating ASP responses. However little is known of the intracellular signaling pathway involved. In the present study ASP signalling is compared to insulin-mediated stimulation of TGS in 3T3-L1 cells. ASP stimulation is not $G\alpha_s$ or $G\alpha_i$ mediated (neither pertussis toxin nor cholera toxin sensitive), suggesting that $G\alpha_q$ is a likely candidate. Phospholipase C (PLC) is required since Ca^{2+} chelator BAPTA-AM and PLC inhibitor U73122 decreased ASP stimulation of TGS by 93.1% ($p<0.001$) and 86.1% ($p<0.004$), respectively. Both wortmannin and LY294002 effectively blocked ASP effect by 69% ($p<0.001$) and 116.1% ($p<0.003$), respectively, supporting a role for phosphatidylinositol 3-kinase (PI3K). ASP treatment leads to rapid but transient 3.5 fold increase in Akt phosphorylation (maximal at 5 minutes, basal by 45 minutes), which is blocked by Akt inhibition, resembling that of insulin. Downstream of PI3K, the mTOR pathway is required for insulin but not ASP action. By contrast, both ASP and insulin activate the MAPK/$ERK_{1/2}$ pathway with rapid (10 minutes) and pronounced (30 fold) increases in $ERK_{1/2}$ phosphorylation, effects that are partially blocked by PD98059, a MAPK inhibitor (64.7% and 65.9% inhibition, respectively, $p<0.001$). Using anti-$cPLA_{2\text{-}Ser505}$ (phosphorylation site by MAPK/$ERK_{1/2}$), time-dependent (maximal at 30 minutes) and transient $cPLA_2$ phosphorylation was demonstrated by Western. Thus ASP signaling involves sequential activation of PI3K and PLC with downstream activation of PKC (as previously demonstrated), Akt, MAPK/$ERK_{1/2}$ and $cPLA_2$, all of which leads to an effective and prolonged stimulation of TG synthesis.

INTRODUCTION

Obesity is one of the most common health problems of our society (1) and today one of our biggest challenges is the understanding of how to limit its progress. Adipose tissue provides a long-term storage reservoir for the energy surplus in the form of triglycerides (lipogenesis), which in turn can be mobilized (lipolysis) when necessary to provide energy for much needed cellular processes (2). Normally, the balance between both lipogenesis and lipolysis is tightly controlled by numerous hormonal components. Predominance of the lipogenic state, whether driven by increases in food intake or lack of exercise is ultimately dependent on the activation of specific intracellular enzymatic pathways. Regrettably, the continuous augmentation of adipose tissue stores leads to obesity which, in turn, can lead to a number of metabolic perturbations such as diabetes, coronary artery disease (CAD) and hypertension.

In addition to being a storage organ, adipose tissue produces a variety of adipokines, some of which are closely involved in adipose tissue metabolism in an autocrine and paracrine manner (review (3)). Acylation Stimulating Protein (ASP) is generated by adipose tissue through the interaction of Factor B and adipsin with complement C3 and is identical to C3adesArg (4). ASP production, along with its precursor proteins Factor B, adipsin and C3, is increased during the differentiation of human and mouse adipocytes (5;6), a production which can be further augmented by insulin and dietary chylomicrons (7). *In vivo* production of ASP in the adipose environment has been elegantly demonstrated by studying arterial-venous differences across an adipose tissue bed (8;9). Local adipose ASP production increased postprandially and correlated with plasma triglyceride (TG) clearance. This correlation of ASP with postprandial TG clearance has been demonstrated across a wide range of fasting ASP in men and woman (10). Moreover, the circulating levels of ASP are increased in obesity, with greater increases observed in women than in men (11). Upon weight loss, ASP levels return to normal (12-14). Studies have shown that ASP levels are also significantly higher in diabetes and cardiovascular disease (15).

ASP plays a key role in the regulation of lipid storage in that it stimulates esterification of fatty acids onto a glycerol backbone resulting in augmentation of intracellular triglyceride depots in human preadipocytes, adipocytes and skin fibroblasts

(16;17). The effects of ASP on differentiated human adipocytes are much greater than those observed in preadipocytes (4). In stimulating TG synthesis, ASP increases activity of diacylglycerol acyltransferase (DGAT, the final enzyme in TG synthesis) in membrane preparations from adipocytes (18). ASP stimulates glucose transport in both adipocyte and muscle cells (19;20) through translocation of glucose transporters (GLUT1, GLUT4 and GLUT3) (21). Finally, as with insulin, ASP also inhibits lipolysis (22) and the effects of ASP and insulin are additive.

Recently, C5L2, an orphan receptor, has been identified as an ASP receptor (23;24). C5L2 is a seven transmembrane G protein belonging to the C5a, C3a and fMLP family of receptors. In HEK-293 cells transfected with the receptor, ASP binds C5L2 with high affinity, and cells become responsive to ASP (but not insulin) for TG synthesis and glucose transport (24). However, how the effects of ASP are signaled from the surface receptor is presently not well understood. Our initial study on ASP signaling demonstrated the involvement of protein kinase C (PKC) in ASP stimulated TG synthesis (25) Even though numerous studies have reported on the post-receptor signaling targets of the insulin pathway regulating glucose transport and lipolysis, the main components of the signaling cascade(s) resulting in the stimulation of TGS are unknown.

The aim of the present study was to identify the signaling pathway for ASP stimulation of TGS in comparison to insulin. 3T3-L1 preadipocytes were chosen as a cell model to evaluate the involvement of phospholipases (PLC, PLD and PLA_2), phosphatidylinositol 3-kinase (PI3K), Akt and mitogen-activated protein kinase pathway / extracellular-signal-regulated kinases ($MAPK/ERK_{1/2}$) in ASP signaling. This cell line was chosen based on previous reports that exposure to **ASP** stimulates both TGS and differentiation. Furthermore, in contrast to 3T3-L1 adipocytes which can generate their own ASP, one can control the concentration of ASP added to 3T3-L1 preadipocytes, as they lack the precursors necessary for ASP generation.

MATERIALS AND METHODS

Materials: 3T3-L1 preadipocytes were obtained from ATCC (Manassas, VA, USA). All tissue culture reagents such as Dulbecco's minimum essential medium (DMEM/F12), phosphate buffered saline (PBS), fetal bovine serum (FBS) and trypsin

were from Gibco (Burlington, On, Canada). Inhibitors used were pertussis toxin, isotetrandrine, PD098059, wortmannin, rapamycin, Bisindolylmaleimide V, Akt inhibitor, BAPTA-AM, $AACOCF_3$ (CalBiochem, LaJolla, CA, USA), LY294002 (Promega, Madison, WI, USA), U73122 (Sigma, Oakville, On, Canada). Stimulators of TG synthesis, insulin and phorbol 13-myristate 12-acetate (PMA), were from Sigma. Oleic acid [9,10-^{3}H(N)] was from DuPont-New England Nuclear (Mississauga, On, Canada) Thin-layer chromatography plates (silica gel 150A) came from Fisher (Nepean, On, Canada). Organic solvents, scintillation vials, general chemicals and tissue culture materials were from VWR (Montreal, QC, Canada). Scintillation fluid was from ICN (Costa Mesa, CA, USA). BioRad reagent for protein measurements was from BioRad (Mississauga, On, Canada).

ASP preparation: ASP was isolated and purified from human plasma as described previously (4). Each batch was verified for purity by ion-spray mass spectrometry at McGill University Mass Spectrometry Unit. The activity of each ASP preparation was checked by its ability to stimulate triglyceride synthesis in 3T3-L1 preadipocytes.

Cell culture and TG synthesis: 3T3-L1 preadipocytes, maintained at low passage number, were grown in DMEM/F12 media supplemented with 10% FBS. At 80% confluency, the cells were plated at 7,000 cells/ well on 24 well plates for experiments. On the 4th day after plating (at 80- 90% cell confluency), preadipocytes were switched to serum-free medium (DMEM/F12) for 2 hours followed by an incubation with various inhibitors for 30 minutes (U73122, n-butanol, wortmannin, LY294002, rapamycin, Bisindolylmaleimide V, PD98059, isotetrandrine, $AACOCF_3$, BAPTA-AM) or 4 hours (pertussis toxin). Subsequently, the media was changed to fresh serum-free DMEM/F12 supplemented with 100 μM [^{3}H] oleate:BSA (5:1 molar ratio, average specific activity 65 dpm/pmol), appropriate inhibitors, and stimulators (ASP, insulin or PMA). Inhibitors were reconstituted and stored (working solution) according to the manufacturer's instructions, and were added at appropriate concentrations from freshly prepared working solutions diluted in PBS. Triglyceride synthesis was measured over 4 hours at 37°C in the presence of inhibitors and hormones. Appropriate vehicle controls were performed in each experiment. After the incubation period, radioactive media was discarded and the

cells were washed two times in ice-cold PBS. The lipids were extracted for 1 hour in 1 ml of heptane:isopropanol (3:2 v/v) then rinsed with an additional 1 ml of the same solvent mix. Lipid extracts were evaporated to dryness in a centrifuge-evaporator (Canberra-Packard, Canada), redissolved in 100 ul of chloroform:methanol (2:1 v/v) and lipids were resolved by thin-layer chromatography(TLC) in hexane : ethyl ether : acetic acid (75:25:1 v/v/v) with reference lipids run concurrently. Separated lipids were visualized with iodine vapor and the spots corresponding to triglyceride were scraped into scintillation vials and counted by liquid scintillation counting (Beckman, CA). Cell proteins were solubilized in 0.1 N NaOH for 3 hours and measured by the method of Bradford (26). On average TGS stimulation with ASP was 168.65±24.17 % (range 132.7±8.5% to 209.4±17.0 %), insulin was 233.71±50.81 % (range 185.9±15.1% to 308.4±54.8 %) and that of PMA was 167.23±29.4 % (range 139.6±10.7% to 198.2±17.2%) where basal was always set as 100%.

Cell treatment, lysis and Western: Cells were grown to confluency on 60 mm dishes, preincubated in serum free media for 2 hrs and then stimulated with ASP or insulin in fresh serum free media for 0, 5, 15, 30, 45 and 60 minutes. The media was quickly removed and 500 ul of ice cold lysing buffer (50 mM Hepes, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 1 mM PMSF, 200 uM orthovanadate, 20 mM β-glycerophosphate, 100 uM AEBSF, 150 nM aprotinin, 1 uM E-64, 1 uM leupeptin, pH 7.5) was added to the plates for 10 minutes at 4^0C with gentle shaking. Total cell lysates were collected and centrifuged to remove particulate material (14,000 g, 10 minutes, 4^0C). Aliquots of the supernatant were stored at -80^0C for further analysis. Proteins were measured with BioRad assay. For Westerns, Laemmli sample buffer was added to the aliquots of cell lysates and the samples were boiled for 3 minutes. 25 ul of cell lysate was loaded per each lane and the proteins were resolved by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were then transferred to PVDF membrane and were immunoblotted with the appropriate antibody to phosphorylated forms of intracellular proteins and re-blotted with the antibodies directed to non-phosphorylated proteins in question. The immobilized proteins were detected by ECL Plus kit from Amersham Biosciences (Piscataway, NJ, USA) using Kodak film.

Statistical analysis: The results were normalized to basal TG synthesis in each experiment (set as 100%) and are presented as mean ± standard error of the mean. Differences between means were analyzed using Student's t-test or ANOVA, with $P<0.05$ considered to be significant. Western immunoblots were quantified by ChemiImager Ready System (San Leonardo, CA, USA).

RESULTS AND DISCUSSION

Immediate post-receptor events: involvement of G-proteins: The ASP receptor, C5L2, belongs to a large family of seven – transmembrane (7TM), G protein-coupled receptors (27). Ligand binding to 7TM receptors often couples to activation of G proteins. The Gα subunit (with 4 major groups $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$) dissociates from the receptor and from the β/γ complex, leading to activation of different intracellular enzymes and pathways (review (28)). Activation of the receptor coupled to the $G\alpha_s$, activates adenylyl cyclase and results in release of cAMP, which in turn, activates protein kinase A (PKA) (29). We have previously demonstrated that $G\alpha_s$ was not involved nor was PKA activated by ASP in TGS (25).

In the present study, pertussis toxin (PTX) was used to evaluate the involvement of the $G\alpha_i$ subunit in ASP action. Pertussis toxin modifies the C-terminal portion of $G\alpha_i$ and in so doing prevents its interaction with the receptor and its activation (30). Both the C3a stimulation of C3aR and C5a stimulation of CD88 (C5aR) are mediated through $G\alpha_i$ and are PTX sensitive (31;32). Confluent 3T3-L1 preadipocytes were pre-treated for 4 hours with 100 ng/ml of PTX prior to the stimulation with ASP or insulin. Shown in Figure 1A, ASP stimulation of TGS is unaltered by PTX treatment, differentiating the ASP activation of C5L2 from that of C3a on C3aR and C5a on C5aR activation (181.9±14.6% ASP alone *vs.* 216.3±10.7% ASP +PTX, where basal is set at 100%, P=NS). Insulin also stimulates TGS in 3T3-L1 preadipocytes (33) through the insulin receptor, which belongs to the family of tyrosine kinase receptors. As expected, PTX had no inhibitory effect on the TGS stimulatory action of insulin (302.1±2.1% insulin alone *vs.* 346.1±10.6% insulin + PTX; P=NS). PTX did not affect basal TG synthesis. This observation is in agreement with recent binding data showing that C5L2 couples poorly to $G\alpha_i$ (34), suggesting a possible link *via* $G\alpha_q$ or $G\alpha_{12/13}$.

Activation of phospholipase C but not phospholipase D: Although, to date, inhibitors for $G\alpha_q$ and $G\alpha_{12/13}$ families have not yet been identified, $G\alpha_q$ has been shown to activate specifically the PLCβ isoform (35). In addition, in the case of fMLP and C5a binding to their respective receptors it was demonstrated that the Gβ/γ subunit activates PLCβ2 (27;36). Activation of PLC results in generation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces calcium mobilization from the endoplasmic reticulum which further serves as a signaling molecule activating a number of intracellular enzymes including PKC, PI3K and PLA_2 (37-39), while DAG is a known activator of conventional and novel protein kinase C (PKC) isoforms. Based on our data demonstrating biphasic increases in intracellular DAG and PKC translocation by ASP (25) this suggests that PLC and/or PLD may be involved in ASP signaling (40).

To evaluate the involvement of PLC we tested the effect of U73122, a potent and widely used inhibitor of PLC (41). As shown in Figure 1B, in the presence of increasing concentrations of U73122 the ASP stimulatory effect was gradually blocked in a concentration dependent manner, reaching a maximum inhibition of 86.1% ($P<0.004$) and 151.8% ($P<0.0002$) at 10 and 20 μM U73122, respectively, leading to a complete loss of ASP stimulatory activity. Treatment with U73122 resulted in a slight, but significant, decrease in basal TG synthesis of 35.2% ($P<0.001$ at 20 uM U73122, where basal TGS = 100%). On the other hand, insulin stimulated TG synthesis (308.4±154.85%, $P< 0.0005$, where basal = 100%) was inhibited by only 7.1 % at 20 μM U73122. This data suggests that while both ASP and insulin can stimulate TGS, ASP, in contrast to insulin, mediates its effects *via* PLC. Similarly, insulin stimulated glucose transport is only attenuated by 25% with U73122 in studies in muscle (42) and adipocytes (43) indicating that the contribution of PLC in insulin signaling is relatively minor.

As PLC activation results in increased intracellular calcium $[Ca^{2+}]i$, an event required for activation of several signaling enzymes, ASP action was evaluated by employing a membrane permeable form of the Ca^{2+}-chelating agent 1,2-*bis*(*o*-Aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid Tetra (acetoxymethyl) Ester (BAPTA-AM). This cell-permeable molecule becomes hydrolyzed and trapped inside the cell as an active chelator BAPTA, thus only $[Ca^{2+}]i$ is chelated. As shown in Figure 1C, ASP stimulated TGS by 184.1±23 % ($P<0.001$). At 5 uM and 25 uM BAPTA-AM, 84.1% and

93.1%, respectively, of the ASP TG stimulatory effect was lost ($P<0.001$ compared to ASP alone). Basal TGS was not affected at low concentrations of chelator, however, a small (32.8%) decrease was observed at 25 uM BAPTA-AM ($P<0.01$ compared to basal alone). Insulin stimulated TGS by 203.8±9.6%, an effect that was attenuated by 56.6% and 62.8% with 5 uM and 25 uM BAPTA-AM, respectively ($P<0.001$ compared to insulin alone). Inhibition of insulin-stimulated glucose uptake using $[Ca^{2+}]i$ chelators has been reported previously in 3T3-L1 adipocytes (44-46). This is the first demonstration that $[Ca^{2+}]i$ - chelation not only inhibits insulin-stimulated TGS but also effectively blocks ASP- stimulated TGS, demonstrating a role for $[Ca^{2+}]i$ in ASP signaling and consistent with the observation that ASP activates PLC (as shown above).

Finally, we tested the involvement of phospholipase D (PLD) in the signaling of ASP. PLD hydrolyses phosphatidylcholine to choline and phosphatidic acid (which can subsequently be converted into DAG) which, in turn, are second messengers themselves (47). As well, PLD has been demonstrated to be activated by PKC (48) and has been shown to produce a prolonged rise in intracellular DAG. With ASP, the second prolonged rise in intracellular DAG suggests that phosphatidyl choline turnover may take place during ASP stimulation of TG synthesis (25). n-Butanol, a primary alcohol, serves as an artificial substrate for PLD, generating phosphatidylalcohol instead of phosphatidic acid (49). As the generated phosphatidylalcohol can no longer be converted to DAG by the enzyme phosphatidic acid phosphatase, n-butanol effectively prevents PLD generated 2^{nd} messengers. In the absence of n-butanol, ASP-stimulated TGS was 265.1±11.1% (where basal was set as 100%, $P<0.0001$), however, the ASP effect was not inhibited at any n-butanol concentrations tested (Figure 1D). In fact, the levels of TG synthesis rose significantly in the presence of increasing concentrations of n-butanol, but that increase was always additive to the ASP TGS stimulation. Based on this experimental data PLD does not seem to participate in ASP stimulated TGS.

Participation of PI3K/Akt pathway: Regulation of intracellular signaling involving inositol phospholipids extends beyond the action of PLC. Phosphatidylinositol 3-kinase (PI3K) is key to the production of 3-phospho derivatives of the inositol lipids. PI3K is not only activated by receptor tyrosine kinases (such as insulin receptor) but also by G protein-coupled receptors (50). The lipids produced by the action of PI3K can

interact directly with specific intracellular enzymes to regulate their activity (51). In addition, since BAPTA-AM treatment attenuates insulin Akt (PKB) stimulation (52;53), and BAPTA-AM blocks both insulin and ASP stimulation of TGS, this suggests that Akt may also be involved in the ASP pathway. The involvement of PI3K was analyzed using the two inhibitors, Wortmannin (cell permeable, irreversible) and LY294002 (reversible). Both compounds inhibit the catalytic site of PI3K and have been used extensively to demonstrate the role of PI3K in insulin action on glucose transport (54;55). The effects of wortmannin and LY294002 on stimulated and basal TGS were tested and the results are presented in Figure 2. As shown in Figure 2A, wortmannin inhibited the ASP stimulatory effect (258.3±24.7% ASP *vs.* basal, P<0.0001) by 70% (148.1±6.7% for ASP + 100 nM wortmannin, p<0.0001). Moreover, inhibition was evident at all concentrations tested. Wortmannin also inhibited 80% of the insulin stimulation of TG synthesis (360.8±20.1% insulin without inhibitor *vs.* 156.1±6.4% insulin with 100 nM of wortmannin, P<0.0001). Similarly, with LY294002 (Figure 2B), ASP-stimulated TGS (187.4±24.1%, P<0.003) was significantly reduced in a concentration dependent manner down to 85.89±19.1 % with 25 uM LY294002 (P<0.003, a reduction of 116.1% in TGS, below basal TGS). A similar effect was observed on insulin-stimulated TGS (219.83 ± 26.6%, where basal = 100%, P<0.001) although complete inhibition was already observed with concentrations as low as 10 uM LY294002 (136.5% inhibition, P<0.0001). The fact that inhibition of the ASP effect was observed at wortmannin concentrations as low as 25 nM suggests that class I (class IA and IB) PI3K is involved in ASP TG stimulatory action (56). Interestingly, the class IB PI3K, p110γ, is activated via the G protein-coupled receptors, more specifically, Gβ/γ subunit (50;57).

Once activated, PI3K can stimulate activation of various downstream effectors and signaling pathways including 3-phosphoinositide dependent protein kinase-1 (PDK-1) (58), Ras (59), PKC (60), and others. In turn, PDK-1 can signal to a number of different targets, one of which is Akt/PKB (61). Divergence downstream of this point is implicated in various signaling pathways ranging from mitogenesis to metabolism regulation (62;63). We have examined the phosphorylation level of PDK-1 in cells stimulated with ASP and insulin. Phosphorylation of PDK-1 on Ser241 remainsunchanged from basal, following ASP or insulin stimulation (5-60 minutes, data

not shown). It has been suggested that PDK-1 is constitutively phosphorylated and active (64). On the other hand Akt, the immediate target of PDK-1, is rapidly phosphorylated, an event necessary for activation. As shown in Figure 2D, phosphorylation of Akt on Ser473 (Akt-P) by ASP is rapid, reaching its maximum between 5-10 minutes and diminishing to basal levels by 45 minutes. Insulin activation of Akt phosphorylation was as rapid (5 minutes) but Akt remained phosphorylated over the entire time course with a slight decrease at 60 minutes (Figure 2E). Furthermore, treatment of cells with Akt inhibitor (1L-6-Hydroxymethyl-*chiro*-inositol 2-[(R)-2- O-methyl-3-O-octadecylcarbonate, Figure 2C) resulted in elimination of ASP stimulation in a concentration dependent manner (162.35±7.6 % ASP vs. 94.77±3.83 % ASP + 10uM inhibitor, $p< 0.00001$) reaching complete inhibition (108.4%) at 10 uM Akt inhibitor. A small decrease in basal TG at 10 uM Akt inhibitor was also observed (23.8 % decrease at 10uM, $p< 0.0005$). This finding further confirms that ASP stimulation of TGS is Akt dependent.

A key event in PKC regulation is the phosphorylation of the newly synthesized PKC by PDK-1 (60). This is true for all the known classes of PKC (conventional, novel and atypical) (60;65;66). We have shown previously that PKC is implicated in ASP signaling (25). In addition, PMA (phorbol 12-myristate 13-acetate), a known PKC activator, also stimulates TGS (25). 3T3-L1 cells were incubated with PMA (to stimulate TGS via PKC activation) and various inhibitors were tested. Although wortmannin and LY294002 inhibited both the ASP and insulin effects (Figure 2A and 2B), the TG stimulatory action of PMA was unaffected by LY294002 (Table 1, P=NS). Further, PMA treatment did not induce Akt phosphorylation (data not shown), suggesting that PKC acts downstream of PI3K as a stimulator of TGS. On the other hand, $[Ca^{2+}]$ chelation with 25 uM BAPTA-AM did decrease PMA stimulation of TGS from 145.5±19.5% down to 105.3%, an inhibition of 88.5% ($p<0.006$). To date, it is currently unknown as to which enzyme, PI3K or PLC, is responsible for PKC activation, or if both act in parallel. Since inhibition of either one results in a significant loss of ASP-stimulated TGS, this suggests that the latter is true, such that both PI3K and PLC are necessary. This will need to be confirmed by further evaluating the effects of a PI3K or PLC inhibitor on PKC activity specifically.

Downstream effectors of PI3K/Akt: One of the well characterized downstream targets of PI3K through Akt is the mTOR pathway which involves the activation of p70 S6 kinase and 4 E-binding protein (gene transcription/RNA translation pathway). We evaluated the mTOR pathway using two specific inhibitors, Rapamycin (inhibitor of mTOR) and Bisindolylmaleimide V (BisV, a p70 S6 kinase inhibitor). Rapamycin (Figure 3A) did not inhibit ASP TGS stimulatory activity at any concentration tested. Note, the PMA-induced TGS activity was also unaffected by mTOR inhibitor (see Table 1). However, the insulin-mediated stimulation of TGS was significantly reduced in the presence of Rapamycin (76.8%, 84.9% and 92.3% inhibition at 5, 50 and 500 nM Rapamycin, respectively, $P<0.0004$). This data indicates that mTOR does not participate in PKC driven TG synthesis stimulation (as demonstrated for ASP or PMA) but that insulin effects are in fact dependent on this pathway. To further exclude participation of p70 S6 kinase, which has been demonstrated to be dependent on PI3K(67;68), we have employed BisV, which blocks the activation of p70 S6 kinase but not that of PKC. As shown in Figure 3B, this inhibitor significantly inhibited basal TGS at all concentrations tested by 26.6%, 45.6% and 71.0% at 5, 10 and 25 uM concentrations of BisV, respectively ($P<0.05$). Although ASP stimulation of TGS was also reduced, the decrease paralleled those observed for basal TGS, suggesting that BisV does not influence the ASP pathway. By contrast, insulin stimulated TG synthesis was sharply decreased by 83.5%, 101.4% and 157.2% at 5, 10 and 25 uM BisV, decreases well beyond those observed for basal TG synthesis. These differences in ASP and insulin implicate a divergence of signaling pathways for ASP and insulin at this point. In addition, Western blot analysis of GSK-3β phosphorylation at Ser9 residue was without change for any of the stimulators (data not shown) indicating that GSK does not participate in the signaling pathway for TGS.

MAPK pathway: Both PI3K and PKC activation can lead to activation of mitogen-activated protein kinase / extracellular-signal-regulated kinase $MAPK/ERK_{1/2}$ (cellular proliferation/ differentiation pathways) (69;70). Using a $MAPK/ERK_{1/2}$ inhibitor, PD098059, significant inhibition of TGS was observed at all of the tested concentrations (224.45±16.5% ASP alone *vs.* 157.1±13.5% and 143.9±16.8% at 10 μM and at 25 μM of PD098059, Figure 4A). This inhibition was 54.1% and 64.7% at 10 uM

and 25 uM PD098059, respectively (P<0.001). Similarly, insulin TGS stimulation was inhibited by 59.4% and 65.9% (p<0.001) at those same concentrations of the inhibitor. Interestingly, PMA (Table 1) stimulation of TGS was also decreased from 289.3±57.5% (PMA alone) to 160.3±36.1% (PMA + 25 uM PD98059, P< 0.002) which translates into 68.1% of inhibition. Basal levels of TGS were not significantly reduced with inhibitor alone. Based on these results one can speculate that the PI3K pathway (ASP and insulin) as well as PKC pathway (ASP and PMA) converge with the MAPK/$ERK_{1/2}$ pathway. Several PKC family members have been shown previously to participate in the activation of MAPK/$ERK_{1/2}$ pathway via phosphorylation of Raf (70). However, it is clear that the involvement of the MAPK/$ERK_{1/2}$ pathway is only partly responsible for TGS stimulation, as the ASP TGS stimulation could not be further inhibited, even at higher concentrations of PD98059 (data not shown). Following ASP stimulation, $ERK_{1/2}$ is rapidly phosphorylated by 10 minutes (Figure 4B), an effect that is also seen with insulin (Figure 4C). These results further confirm the partial involvement of the MAPK/$ERK_{1/2}$ pathway that is required for the ASP activation of TGS in 3T3-L1 preadipocytes. Involvement of the MAPK pathway has also been previously implicated in induction of adipogenesis in 3T3-L1 preadipocytes (71).

Involvement of PLA_2 in ASP signaling: Arachidonic and lysophosphatidic acid are two second messengers ultimately produced by the action of PLA_2 (72) which, in turn, can be activated directly through G-protein activation (73), or, further downstream, through MAPK/$ERK_{1/2}$ activation (74). Isotetrandrine, an inhibitor of G-protein coupled phospholipase A_2 (PLA_2) (75), was not effective in inhibiting the stimulatory effect of ASP on TGS (Figure 5A). ASP stimulated TG synthesis was 190.3±14.7% (P<0.0005, *vs.* basal) and remained high after treatment with isotetrandrine (209.5±20.9% at 10 μM, P=NS). Also, basal TGS levels were unaffected by any concentration of isotetrandrine. The involvement of calcium-dependent phospholipase A2 ($cPLA_2$) was evaluated using the reversible-inhibitor arachidonyltrifluoromethyl ketone ($AACOCF_3$) (76-78). $cPLA_2$ is activated by $[Ca^{2+}]i$ increases, enhancing translocation from the cytosolic milieu to the membrane (nuclear, ER or Golgi). Following translocation, MAPK cascade enzymes phosphorylate $cPLA_2$, fully activating the enzyme (79-81). Increasing concentrations of $AACOCF_3$ significantly attenuate ASP - and insulin - stimulated TGS (Figure 5B).

Although basal TGS was slightly inhibited, the inhibition by $AACOCF_3$ was more pronounced on ASP - and insulin - stimulated TGS with complete inhibition of the ASP effect and 91.5% inhibition of the insulin effect. Using Western blot analysis with anti-$cPLA_2$ to Ser505 (a site phosphorylated by MAPK/$ERK_{1/2}$ enzymes), a time dependent phosphorylation of cPLA2 was demonstrated, reaching a maximal effect at 30 minutes. Thus ASP signaling involves rapid and transient phosphorylation of $ERK_{1/2}$ at 10 minutes which then leads to $cPLA_2$ phosphorylation (maximal effects at 30 minutes), a known downstream target of $ERK_{1/2}$. Interestingly, the results obtained here with $AACOCF_3$ and the pattern of $cPLA_2$ phosphorylation is very similar to that observed for Angiotensin II – a hormone which requires PLC activation (82).

Arachidonic acid (AA), as the product of $cPLA_2$ activity, can act as a signaling molecule directly, or can be utilized as a precursor for the production of prostaglandins (PG). PG have been implicated previously in differentiation (83-86) as well as in stimulation of TGS by angiotensin II (87;88). We used a general COX inhibitor (indomethacin) to examine this possibility. ASP and insulin stimulated TGS by 209.4±17% and 197.6±18% ($p<0.0001$), respectively, with no significant inhibition of TGS with any of the indomethacin concentrations tested (data not shown). Hence inhibiting the acute production of prostaglandin synthesis does not appear to have an effect on either ASP - or insulin - stimulated TGS. Rather, AA may be involved as a second messenger. It has been shown previously that AA stimulates both basal - and insulin - stimulated glucose uptake in 3T3-L1 adipocytes with maximal effects after 4 hours, effects mediated through increased translocation of GluT1 and GluT4 to the plasma membrane (89). Furthermore, in the presence of increased DAG, AA has been shown to increase the affinity of PKC for calcium which allows PKC to be fully active even at basal $[Ca^{2+}]i$ (90). Since ASP increases activation and translocation of PKC (25), this may partly be mediated through $cPLA_2$ activation.

Note that basal levels of TGS are significantly lowered in many scenarios in the presence of inhibitor (Table 1, Figure 2B, 2C, Figure 3A, 3B, Figure 5B). This suggests that the targeted enzyme is important for basal TGS, and implies that in the presence of an agonist (ASP, Insulin or PMA), the activity of the enzyme is increased from basal to stimulated levels of activity. In fact, the rate at which agonist-stimulated TGS decreases

in the presence of inhibitor, is greater than the decrease observed in basal TGS, further implicating an increase in enzyme activity in the presence of ASP, Insulin or PMA.

Based on the present study, and those published by Baldo *et al* (25), a scenario for the initial steps of the ASP intracellular signaling pathway is proposed in Figure 6. Following ASP binding to its receptor C5L2, a 7 transmembrane G protein-coupled receptor, activation of trimeric G protein ensues. In so doing, the Gα subunit dissociates from the Gβ/γ subunit. Gβ/γ could then activate PI3K, leading to PDK-1 activation, which phosphorylates PKC (PKC-P), releasing it into the cytoplasmic compartment. PLC activation (which in this case could be activated through $G\alpha_q$ or Gβ/γ subunit) results in the release of IP_3 and DAG. IP_3 stimulates the release of $[Ca^{2+}]i$ from the endoplasmic reticulum (ER). $[Ca^{2+}]i$ can then also be responsible for activation of PI3K and in addition both $[Ca^{2+}]i$ and DAG are now available for the activation of PKC. We have previously demonstrated that ASP treatment leads to transient increases in DAG, total PKC activity and PKC translocation to the membrane fraction (25). On the other hand, PI3K activation may further lead to MAPK/$ERK_{1/2}$ phosphorylation, and $cPLA_2$ activation. Intracellular AA can then enhance PKC activity and stimulate the translocation of appropriate glucose transporters, consequently increasing glucose transport into the cell. Note that while PI3K and PLC are proposed to be activated in parallel, activation of both appears to be necessary since blockage of one or the other results in the inhibition of ASP stimulated TGS. By contrast, inhibition of the $MAPKERK_{1/2}$ pathway, only appears to result in a partial blockage of the ASP effect. Downstream of this we can only speculate as to how PKC activation leads to increased TG synthesis. We have demonstrated that ASP increases glucose transport as well as fatty acid esterification onto the glycerol backbone through the activation of DGAT (an enzyme which is specific to TG synthesis) (18;91) but not phospholipid synthesis (24). Moreover, ASP stimulates glucose transport in the absence of TGS and glucose is not absolutely required for ASP stimulation of TGS (24). However, both effects are required for maximal ASP stimulation of TG synthesis. With respect to glucose transport, it has been demonstrated that PI3K and PKC activation lead to translocation of glucose transporters, and ASP may likely acts via this pathway (42;92). Two DGAT genes have recently been cloned (DGAT1 and DGAT2) (91;93) but there is little information yet

available on their regulation. Based on circumstantial evidence, and similarities to other endoplasmic reticulum associated anabolic enzymes, acyl-cholesterol-acyltransferase and glycerol-3-phosphate-acyltransferase, (93;94) it is reasonable to assume that this enzyme might be activated through dephosphorylation. In this case, downstream of PKC, we would invoke activation of a phosphatase enzyme, leading to activation of DGAT, although that intermediate step remains yet to be identified. Altogether, we have demonstrated that thesignaling pathway of ASP stimulated TGS involves PI3K, PLC, PKC, MAPK/$ERK_{1/2}$ and $cPLA_2$.

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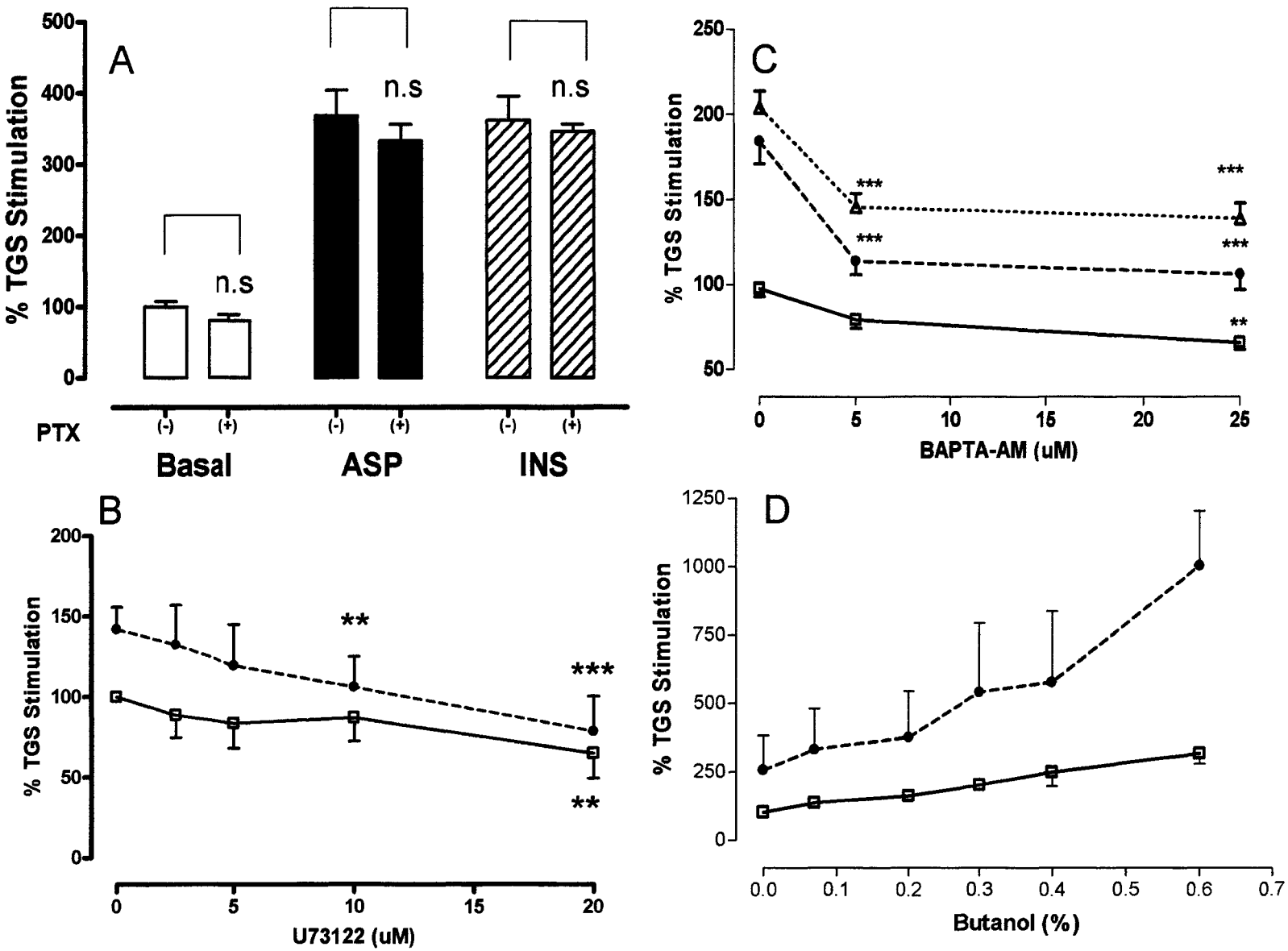
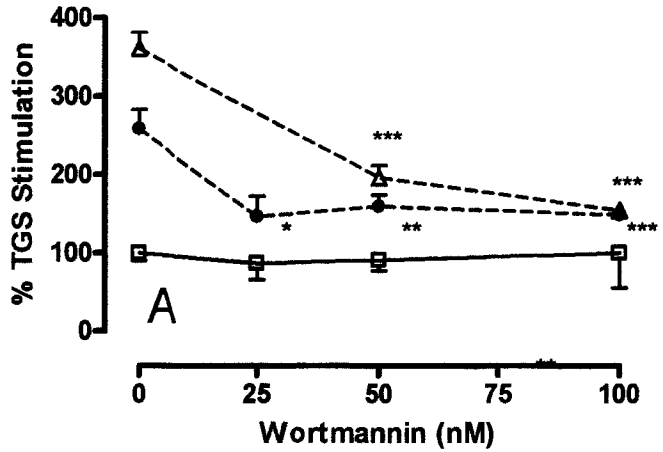
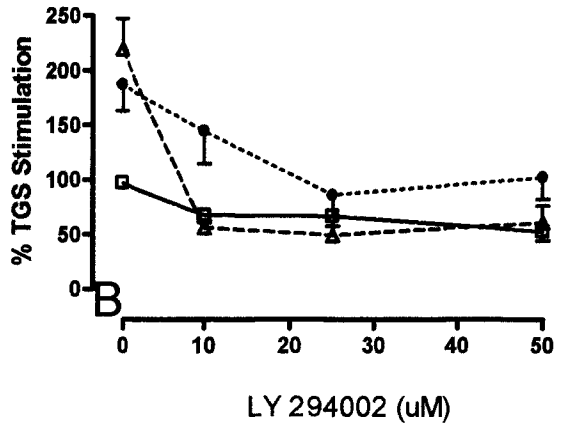
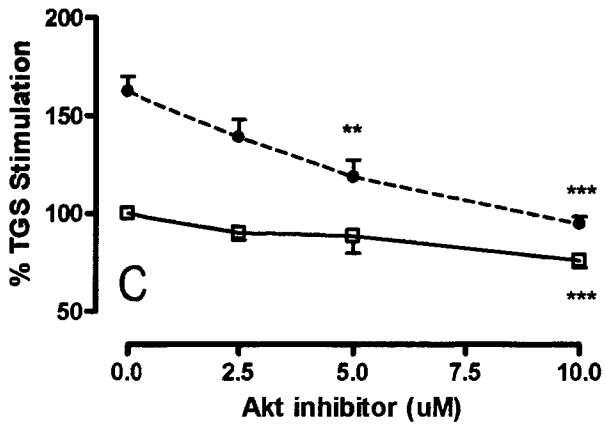

A
% TGS Stimulation
500
400
300
200
100
0
n.s
n.s
n.s
PTX
(-)
(+)
(-)
(+)
(-)
(+)
Basal
ASP
INS
B
% TGS Stimulation
200
150
100
50
0
**
***
**
0
5
10
15
20
U73122 (uM)
C
% TGS Stimulation
250
200
150
100
50
***
***
***
**
0
5
10
15
20
25
BAPTA-AM (uM)
D
% TGS Stimulation
1250
1000
750
500
250
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Butanol (%)

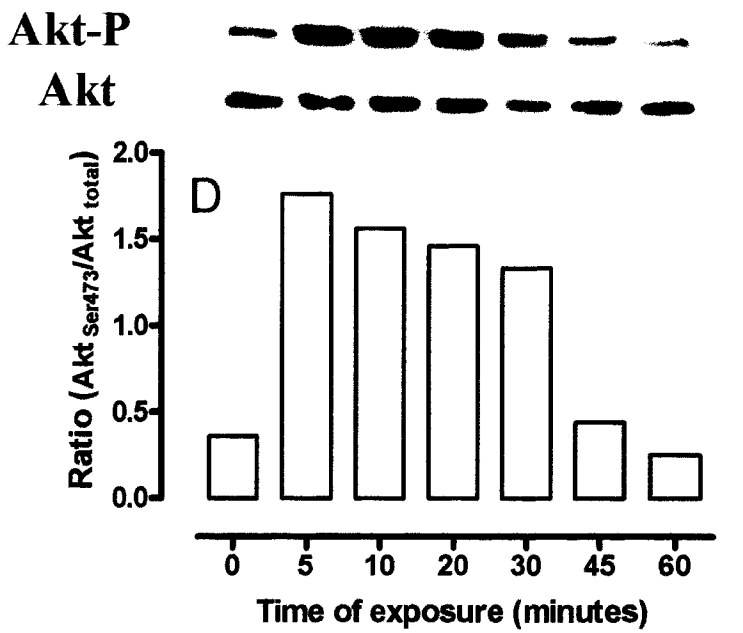
Figure 1

Figure 2


% TGS Stimulation
400
300
200
100
0
A
0
25
50
75
100
Wortmannin (nM)


% TGS Stimulation
250
200
150
100
50
0
B
0
10
20
30
40
50
LY 294002 (uM)


% TGS Stimulation
200
150
100
50
C
0.0
2.5
5.0
7.5
10.0
Akt inhibitor (uM)


Akt-P
Akt
Ratio (Akt Ser473/Akt total)
2.0
1.5
1.0
0.5
0.0
D
0
5
10
20
30
45
60
Time of exposure (minutes)

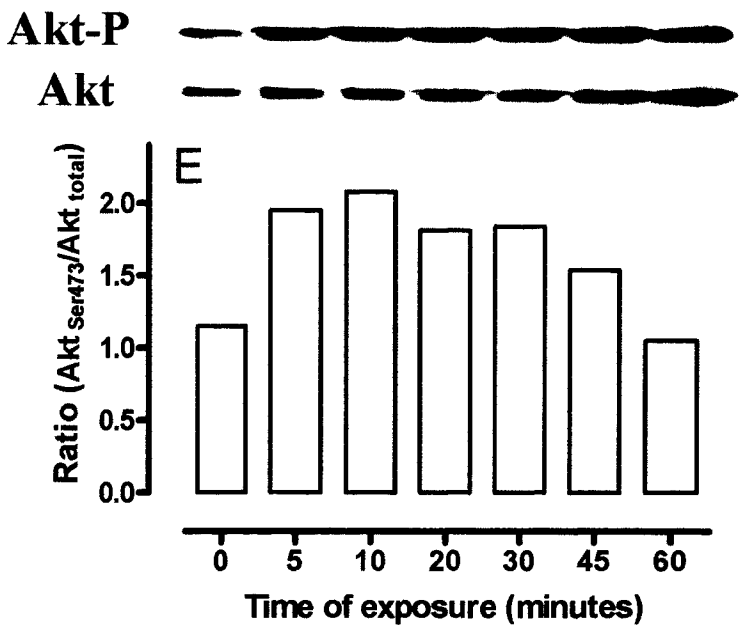

Akt-P
Akt
Ratio (Akt Ser473/Akt total)
2.0
1.5
1.0
0.5
0.0
E
0
5
10
20
30
45
60
Time of exposure (minutes)

Figure 3

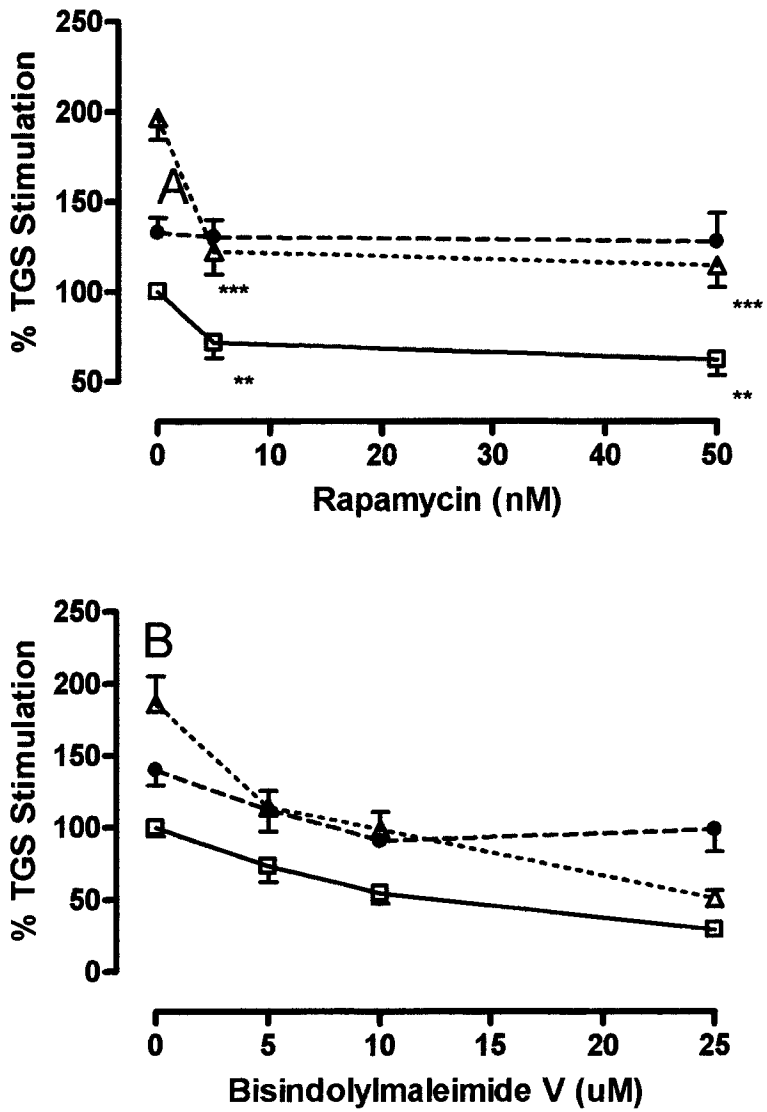
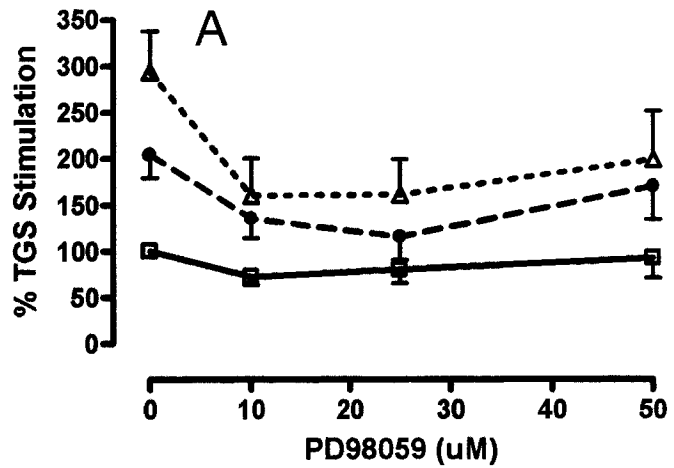
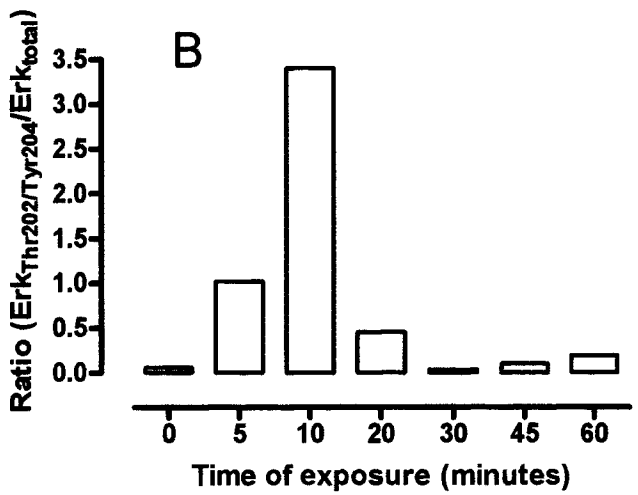

A
% TGS Stimulation
250
200
150
100
50
***
**
***
**
0
10
20
30
40
50
Rapamycin (nM)
B
% TGS Stimulation
250
200
150
100
50
0
0
5
10
15
20
25
Bisindolylmaleimide V (uM)

Figure 4


A
% TGS Stimulation
350
300
250
200
150
100
50
0
0
10
20
30
40
50
PD98059 (uM)


ERK-P
ERK
ERK-P
ERK


B
Ratio (Erk Thr202/Tyr204/Erk total)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
20
30
45
60
Time of exposure (minutes)

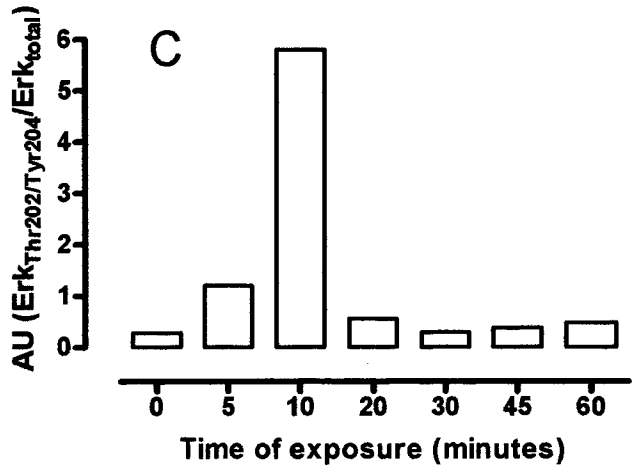
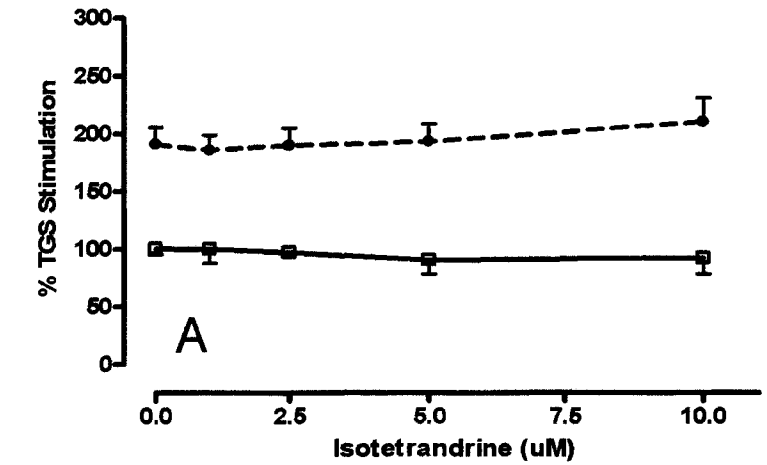
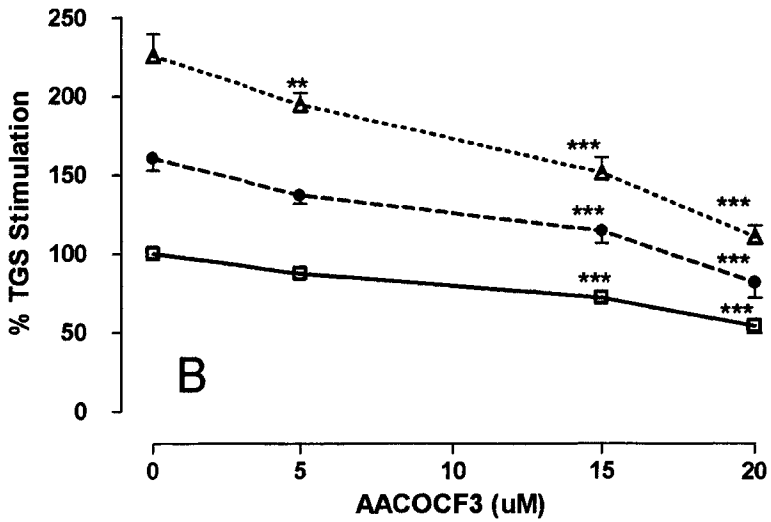

C
AU (Erk Thr202/Tyr204/Erk total)
6
5
4
3
2
1
0
0
5
10
20
30
45
60
Time of exposure (minutes)

Figure 5


% TGS Stimulation
300
250
200
150
100
50
0
A
0.0
2.5
5.0
7.5
10.0
Isotetrandrine (uM)


% TGS Stimulation
250
200
150
100
50
0
B
**
***
***
***
***
***
0
5
10
15
20
AACOCF3 (uM)

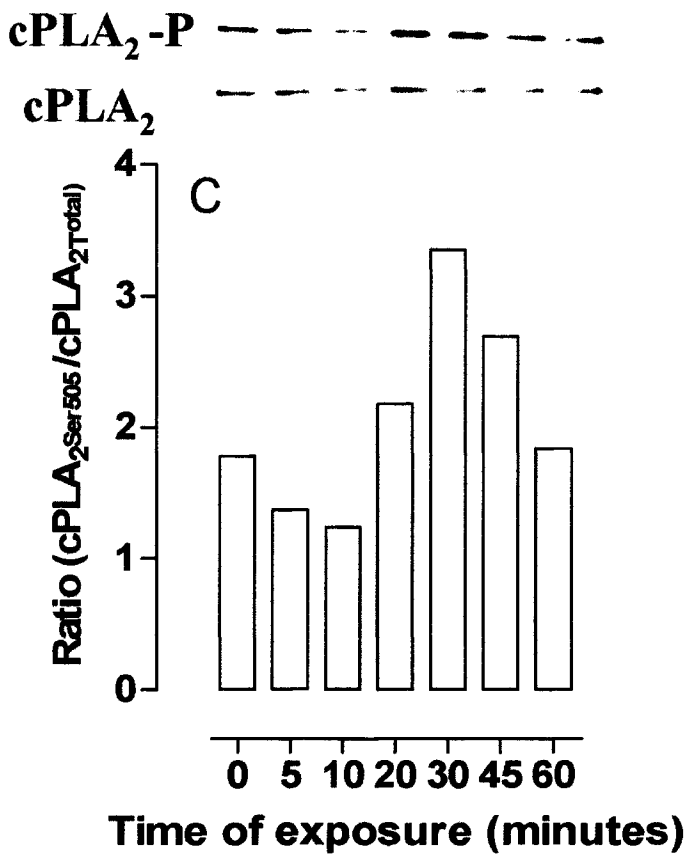

cPLA2-P
cPLA2
Ratio (cPLA2Ser505/cPLA2Total)
4
3
2
1
0
C
0 5 10 20 30 45 60
Time of exposure (minutes)

Figure 6

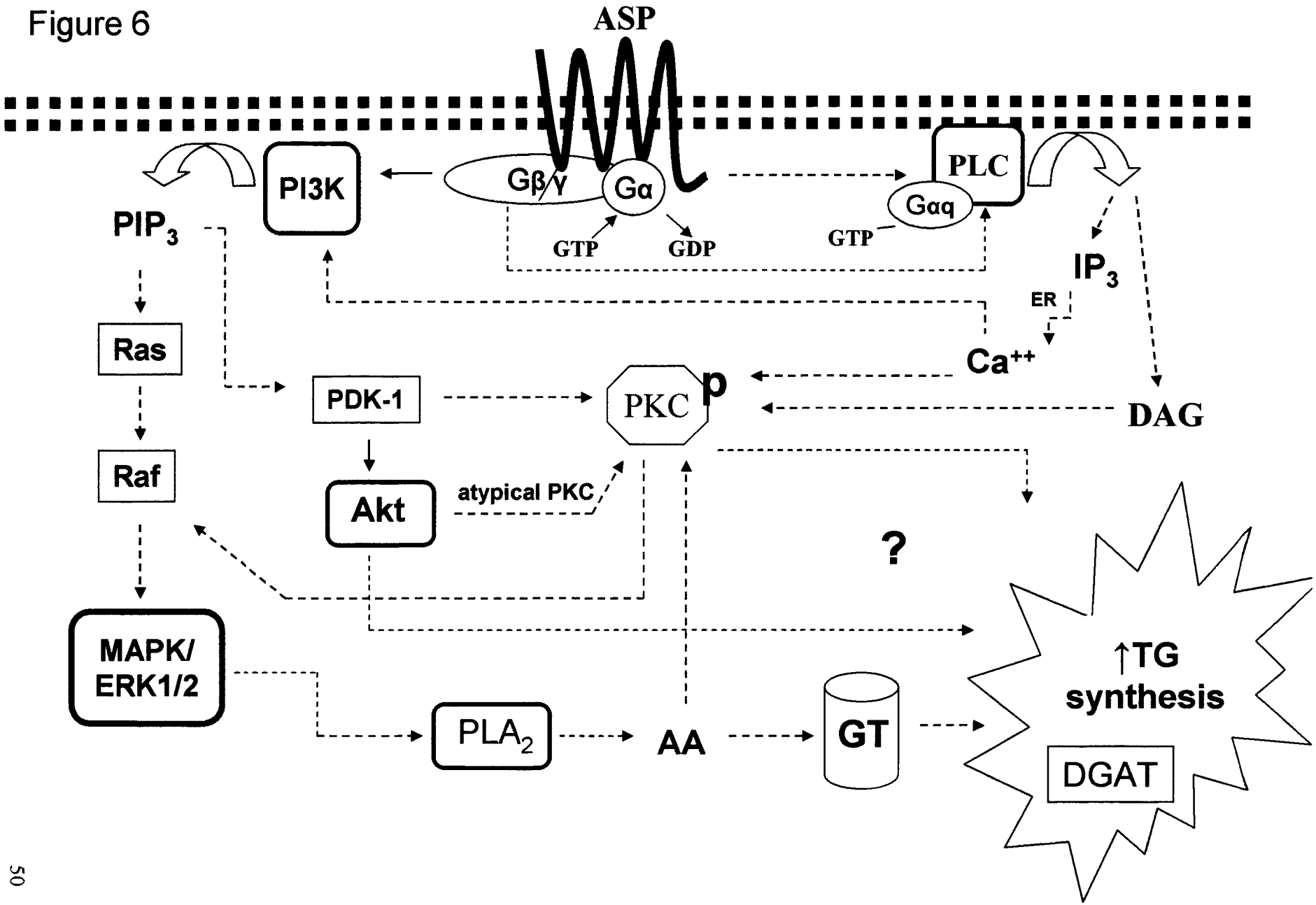

ASP
PI3K
Gβ/γ
Gα
PLC
Gαq
GTP
GDP
GTP
PIP3
IP3
ER
Ca++
Ras
PDK-1
PKC
p
DAG
Raf
Akt
atypical PKC
?
MAPK/
ERK1/2
PLA2
AA
GT
↑TG
synthesis
DGAT

Table 1: Effect of Inhibitors on PMA Stimulation of Triglyceride Synthesis

Inhibitor	Conc.	No Inhibitor			+ Inhibitor		
		-PMA	+PMA	p^1	-PMA	+PMA	p^2
LY294002	50 uM	100±3.6	163.9±16.3	0.001	51.9±7.9	134.4±20.1	NS
BAPTA-AM	25 uM	97.3±4.5	145.5±19.5	0.006	65.4±3.9	105.3±13.0	0.05
Rapamycin	50 nM	100±4.0	198.5±17.2	0.0004	69.9±4.4	203.1±12.3	NS
BisV	25 uM	100±5.5	139.6±10.7	0.02	28.9±4.4	111.4±11.4	NS
PD98059	25 uM	100±3.5	289.3±52.5	0.001	87.3±9.4	160.3±32.9	0.002

Confluent cells were pretreated with serum free media containing the indicated inhibitors at the indicated concentrations for 30 minutes and were then stimulated with 20 nM PMA for an additional 4 hours. All inhibitors were present throughout the experiment. Triglyceride synthesis was measured as pmols [^{3}H] oleate incorporated into triglyceride. The results were normalized to basal TG synthesis in each of the experiments performed and are presented as % TG stimulation, where basal (no addition and no treatment) was set as 100%. The appropriate amount of vehicle was included in the media for each of the inhibitors tested. The data was analyzed using ANOVA and the significance was set at $P<0.05$ for incubation [1] with PMA vs. without PMA (no inhibitor present) and for [2] PMA + inhibitor vs. PMA no inhibitor.

Figure Legends:

Figure 1. Effect of pertussis toxin and phospholipase inhibition on ASP stimulated TG synthesis in 3T3-L1 preadipocytes. Confluent cells were pretreated with serum free media containing A) 100 ng/ml of pertussis toxin, B) 2.5-20 uM U73122 (PLC inhibitor), C) 5 and 20 uM BAPT-AM (Ca^{++} chelator) and D) 0.1 – 0.6 % n-butanol (a PLD substrate substitute) for 30 minutes (with an exception of pertussis toxin which required 4 hours pretreatment) and were then stimulated with 5 μM ASP (circles) or 100 nM insulin (triangles) for an additional 4 hours. All inhibitors were present throughout the experiment. Triglyceride synthesis was measured as pmols [^{3}H] oleate incorporated into triglyceride. The results were normalized to basal TG synthesis (squares) in each of the experiments performed (minimum 3 experiments, each performed in triplicate) and are presented as % TG stimulation, where basal (no addition and no treatment) was set as 100%. The appropriate amount of vehicle was included in the media for each of the inhibitors tested. The data was analyzed using t-test and the significance was set at $P<0.05$.

Figure 2. Effect of PI3K inhibition and involvement of Akt in ASP stimulated TG synthesis in 3T3-L1 preadipocytes. Confluent cells were pretreated with serum free media containing various concentrations of A) Wortmannin, B) LY294002 and C) Akt inhibitor for 30 minutes and were then stimulated with 5 μM ASP (circles) or 100 nM insulin (triangles) for an additional 4 hours. All inhibitors were present throughout the experiment. Triglyceride synthesis was measured as pmols [^{3}H] oleate incorporated into triglyceride. The results were normalized to basal TG synthesis (squares) in each of the experiments performed (minimum 3 experiments were done, each performed in duplicate or triplicate) and are presented as mean ± SEM % TG stimulation, where basal (no addition and no treatment) was set as 100%. The appropriate amount of vehicle was included in the media for each of the inhibitors tested. The data was analyzed using t-test and the significance was set at $P<0.05$. In addition, 3T3-L1 preadipocytes were incubated with ASP (D) and insulin (E) for 5-60 minutes in serum free media, cells were lysed and

total cell protein was subjected to Western analysis. One Western blot was perfomed, and ratioofantibody binding to phosphorylated Ser473 of Akt versus total Akt was used.

Figure 3. Analysis of mTOR and p70S6 kinase involvement in ASP stimulated TG synthesis in 3T3-L1 preadipocytes

Confluent cells were pretreated with serum free media containing various concentrations of A) rapamycin (mTOR inhibitor) and B) Bisindolylmaleimide V (p70 S6 kinase inhibitor) for 30 minutes and were then stimulated with 5 μM ASP (circles) or 100 nM insulin (triangles) for an additional 4 hours. All the inhibitors were present throughout the experiment. Triglyceride synthesis was measured as pmols [^{3}H] oleate incorporated into triglyceride. The results were normalized to basal TG synthesis (squares) in each of the experiments performed (minimum 3 experiments, each in triplicate) and are presented as mean ± SEM % TG stimulation, where basal (no addition and no treatment) was set as 100%. The appropriate amount of vehicle was included in the media for each of the inhibitors tested. The data was analyzed using t-test and the significance was set at $P<0.05$.

Figure 4. Involvement of MAPK/ERK1/2 pathway in ASP stimulated TG synthesis in 3T3-L1 preadipocytes. Confluent cells were pretreated with serum free media containing A) PD098059 (MAPK/ERK1/2 inhibitor specific to MEK) for 30 minutes and were then stimulated with 5 μM ASP (circles) or 100 nM insulin (triangles) for an additional 4 hours. All inhibitors were present throughout the experiment. Triglyceride synthesis was measured as pmols [^{3}H] oleate incorporated into triglyceride. The results were normalized to basal TG synthesis (squares) in each of the experiments performed (n=3, performed in triplicate) and are presented as mean ± SEM % TG stimulation, where basal (no addition and no treatment) was set as 100%. The appropriate amount of vehicle was included in the media for each of the inhibitors tested. The data was analyzed using t-test and the significance was set at $P<0.05$. Furthermore, 3T3-L1 preadipocytes were incubated with ASP (B) and insulin (C) for 5-60 minutes in serum free media, were lysed and total cell protein were subjected to Western analysis. One Western blot was performed, and ratio of antibody binding to phosphorylated Thr202/Tyr204 of ERK versus total Erk was used.

Figure 5. Involvement of PLA_2 in ASP stimulation of TGS in 3T3-L1 preadipocytes. Confluent cells were pretreated with serum free media containing A) 1- 10 uM isotetrandrine, B) 5-20 uM AACOCF3 for 30 minutes and TG synthesis was then stimulated with 5 μM ASP (circles) or 100 nM insulin (triangles) for an additional 4 hours. Both inhibitors were present throughout the experiment. Triglyceride synthesis was measured as pmols [^{3}H] oleate incorporated into triglyceride. The results were normalized to basal TG synthesis (squares) in each of the experiments performed, where basal (no addition and no treatment) was set as 100% (n=3, performed in triplicate) and the results are presented as mean ± SEM. The data was analyzed using t-test and the significance was set at $P<0.05$. Furthermore, 3T3-L1 preadipocytes were incubated with ASP (C) for 5-60 minutes in serum free media, were lysed and total cell protein was subjected to Western analysis. One Western blot was performed, and ratio of antibody binding to phosphorylated Ser505 of $cPLA_2$ versus total $cPLA_2$ was used.

Figure 6. Proposed Intracellular Signaling Pathway for ASP Stimulated Triglyceride Synthesis. Acylation stimulating protein (ASP), receptor for ASP (C5L2), phosphatidylinositol 4,5-biphosphate (PIP_2), phosphatidylinositol 3,4,5-triphosphate (PIP_3), inositol 1,4,5-triphosphate (IP_3), diacylglycerol (DAG), diacylglycerol acyltransferase (DGAT), triglyceride (TG), endoplasmic reticulum (ER), protein kinase C (PKC), phosphatidyl inositol 3-kinase (PI3K), phospholipase C (PLC), 3-phosphoinositide dependent protein kinase-1 (PDK-1), mitogen-activated protein kinase (MAPK/ERK1/2).

CHAPTER FOUR

Arachidonic Acid (AA), a Signaling Molecule and Precursor to the Eicosanoids.

AA, a product of $cPLA_2$ activity, is metabolized to eicosanoids by the action of the lipoxygenase enzymes to yield leukotrienes, or by cyclo-oxygenase (COX) to produce prostaglandins (PGs) and thromboxanes (TXAs) (Figure 4.1) (94). Both AA and its PG products have been documented to play a role in adipocyte metabolism.

Angiotensin II (Ang II), a hormone better known for its ability to regulate blood pressure, has also been shown to stimulate adipocyte growth and development by PGs (113, 114). In addition, both Prostacyclin (PGI_2) and PGE_2 have been shown to exert their effects in a paracrine and autocrine fashion on adipocytes, stimulating adipogenesis (115, 116).

While $cPLA_2$ has been shown to be involved in ASP signaling of TG synthesis(Chapter 3), it is not clear whether it is AA or its metabolites that play a role in ASP-stimulated TG synthesis. The effects of blocking PG production were evaluated with ASP, by utilizing the general COX inhibitor indomethacin. Thus, while ASP can still activate $cPLA_2$, the subsequent generation of AA is no longer metabolized to PGs.

Prostaglandin (PG) production

Eicosanoid generation is dependent on AA-selective acyltransferase and transacylase reactions that transfer AA into specific phospholipid pools, and the release from these pools by the action of PLA_2s (95). Theoretically, AA could be produced by PLC, and the subsequent DAG could be cleaved to generate AA via a mono- or diacylglycerol lipase (Figure 4.1). In addition, PLD could generate PA that can be converted to DAG by PA- phosphatase and thus to AA (as seen with PLC) (117).

The production of PGs begins with the agonist-activation of PLA_2 and release of arachidonic acid (AA), which, in turn, is converted to PGs by COX enzymes (also called PGH_2 synthase) to form PGH_2, the rate-limiting step in PG synthesis (119).

PGH_2 is further metabolized by specific prostanoid synthases, producing the major prostanoids: PGD_2, PGE_2, PGF_2, PGI_2 (prostacyclin) and TXA_2 (Figure 4.1). Two isoforms of this enzyme have been well documented: COX-1 and COX-2 and both isoforms are expressed in 3T3-L1 preadipocytes (120).

The constitutively expressed COX-1 (PGHS-1) has been shown to play a role in the normal functioning of the kidneys, gastrointestinal tract and platelets. COX-2 (PGHS-2) is expressed in macrophages, monocytes and endothelial cells. In contrast to COX-1, COX-2 is virtually undetectable under normal physiological conditions, however, its expression is induced by growth factors, mitogens and proinflammatory stimuli, such as cytokines and tumour necrosis factor-alpha (TNF-α) (119). The receptors for the major prostanoids have been shown to be heptahelical (i.e seven trans-membrane receptors) and couple to heterotrimeric G-proteins (121).

Arachidonic Acid (AA) and Preadipocyte Differentiation.

Adipose tissue growth is accompanied by an increase in adipocyte size and formation of new adipocytes from precursor cells. Both CCAAT/enhancer binding protein (C/EBP) and peroxisome proliferator-activated receptor-gamma (PPARγ), can regulate adipocyte differentiation by binding to their respective response elements or by interacting with other intracellular signaling molecules, thus enhancing the overall expression of adipocyte genes, such as lipid-metabolizing enzymes (122).

Recent studies have shown that AA and its metabolites have implicated roles in adipocyte differentiation by interacting with the nuclear PPARs. For example, 15-deoxy-Δ12, 14 prostaglandin J2, is a potent ligand for PPARγ, subsequently stimulating preadipocyte differentiation (123, 124, 127). In addition, both PGI2 and PGE2 can act in a paracrine and autocrine manner to stimulate adipocyte growth by inhibiting lipolysis (115, 116). They mediate their effects by binding their respective receptors. The PGI2 receptor is not expressed in adipocytes, rather, it is expressed only in preadipocytes, whereupon it can stimulate adipocyte growth and development. In addition, it has recently been suggested that PGI2 may stimulate differentiation by interacting with PPARs, however, the exact isoform involved remains to be elucidated (125). The PGE2 receptor is expressed on mature adipocytes only, thus its antilipolytic action only works at the level of fully differentiated adipocytes (116).

Thus, the local production of PGs can act in concert, as PGE2 can stimulate adipose tissue mass (hypertrophy), and PGI2 can stimulate preadipocyte differentiation (hyperplasia) (116). In contrast, $PGF_{2\alpha}$ inhibits adipocyte differentiation by inhibiting

PPARγ, a mechanism that requires both the activation of MAPK and the phosphorylation of PPARγ (126).

These findings implicate an important role for AA metabolism in the regulation of intracellular signal events through activation of PPARs. Nuclear targeting of $cPLA_2$, and the activation of PPARs by AA and its metabolites in the nucleus, suggests that, $cPLA_2$ activation and translocation to the nuclear envelope, may lead to an increase in AA generation in the nucleus for PPARγ activation and preadipocyte differentiation.

The objective of this Chapter is to further extend the results obtained in Chapter Three, by providing evidence for the involvement of AA, produced by $cPLA_2$ activation, in ASP signaling of TG synthesis, and to rule out the involvement of its PG products. Since ASP stimulates TG synthesis within an acute time frame (4 hours), PGs seem to be unlikely candidates in ASP signaling as they require a longer time frame to elicit an effect (24-48 hours). In order to evaluate the role of PGs in ASP signaling, the general COX-inhibitor Indomethacin was employed. Indomethacin blocks the metabolism of AA to PGs, and evaluating its effects on ASP-stimulation of TG synthesis within this acute time frame was tested. .

MATERIALS AND METHODS

The materials, ASP preparation, cell culture and TG synthesis techniques are the same as those described in Chapter Three, with the addition of the general Cyclo-oxygenase (COX) inhibitor Indomethacin which was purchased from Sigma (Oakville, On, Canada).

Results and Discussion

AA has been documented to stimulate adipose tissue development. Similarly, PGs have been implicated in adipogenesis as various PGs, such as, PGI_2, PGJ_2 and PGE_2 have been documented as potentiating adipose growth, while $PGF_{2\alpha}$ inhibits adipose growth (115, 116, 123, 124, 126, 127).

ASP is able to significantly stimulate TG synthesis over a 4-hour period. Such an acute time frame is not likely to be sufficient for PGs to mediate their effects, as they must first be generated and secreted by adipocytes upon agonist stimulation. Then, PGs

must subsequently bind their corresponding receptor to elicit an effect. Such an effect by PGs requires chronic incubation (24-48 hours) and has been shown with Ang II, which mediates its effects through PGI2 during this time frame (128).

On the other hand, the anabolic effects seen with AA, such as stimulating glucose transport, are reported to occur with maximum effects at 4 hours (100). Thus, it appears more probable that AA is involved in ASP signaling and may not depend on its metabolism to PGs. This is further confirmed with the general COX-inhibitor indomethacin, as ASP-stimulation of TG synthesis was not affected with increasing concentrations of this inhibitor (Figure 4.2). Likewise, the potent stimulator of TG synthesis, insulin, was unaffected by indomethacin. ASP stimulated TG synthesis to 209.4 +/- 17% and insulin to 197.6 +/- 18% ($P< 0.001$ vs. control). Increasing concentrations of indomethacin were unable to significantly inhibit ASP- or Insulin-stimulation of TG synthesis. At 5 uM Indomethacin a small drop in ASP and Insulin-stimulated TG synthesis is observed, however, this effect was not significant. Furthermore, increasing concentrations of this inhibitor did not significantly inhibit the ASP or Insulin effect. Even at 20 uM indomethacin, ASP was still able to significantly stimulate TG synthesis to 179.6 +/- 17% ($P< 0.05$ vs. control) and Insulin to 194.8 +/- 25% ($P< 0.001$ vs. control). However, the reversible $cPLA_2$ inhibitor AACOCF3 was able to significantly inhibit both ASP- and Insulin-stimulated TG synthesis in a concentration dependent manner (Chapter Three). This suggests that the effects of either ASP or Insulin on TG synthesis are not PG-dependent, but rather may depend on the generation of AA.

Figure 4.1

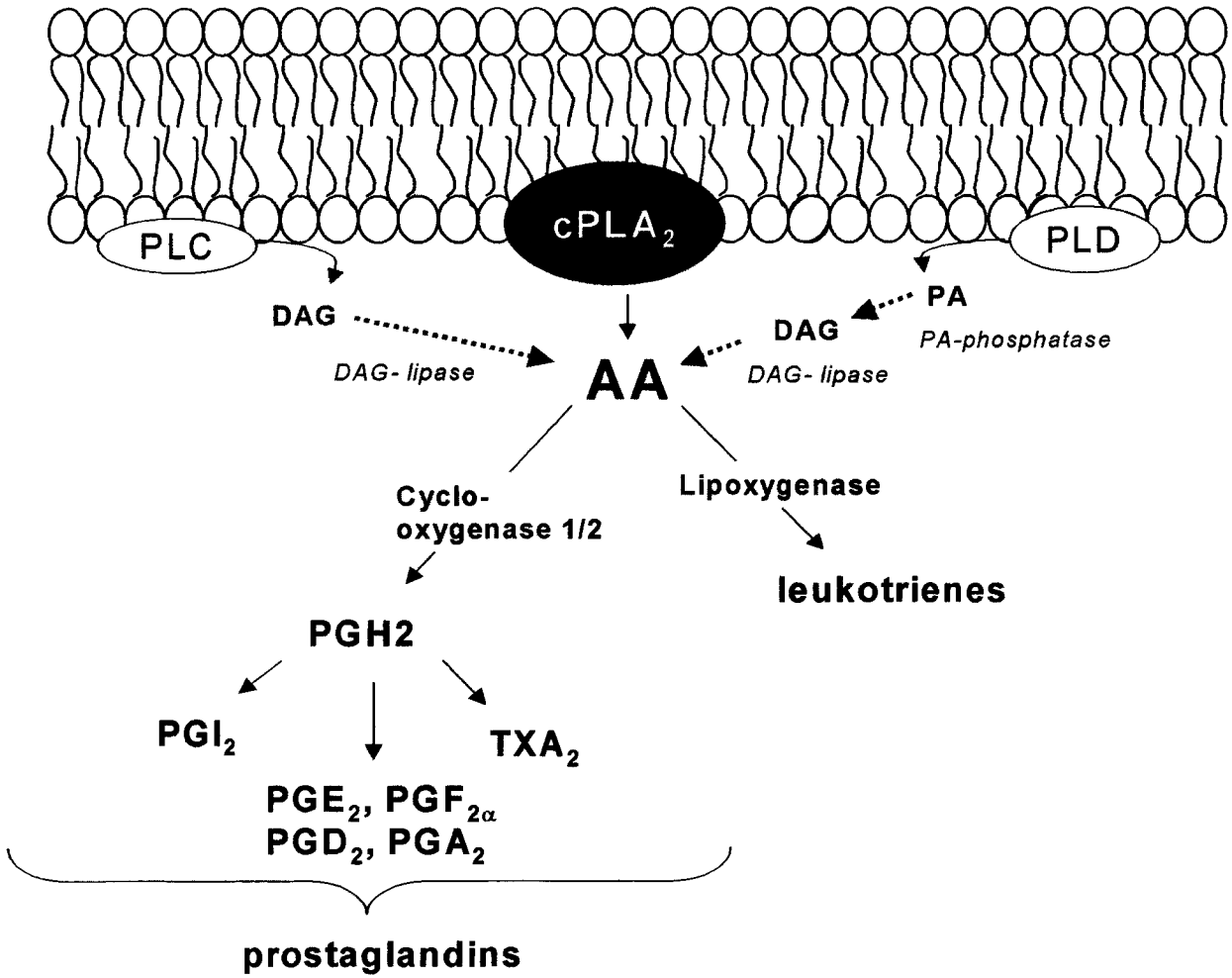

cPLA2
PLC
PLD
DAG
DAG- lipase
PA
PA-phosphatase
DAG
DAG- lipase
AA
Cyclo-
oxygenase 1/2
Lipoxygenase
leukotrienes
PGH2
PGI2
TXA2
PGE2, PGF2α
PGD2, PGA2
prostaglandins

Figure 4.2

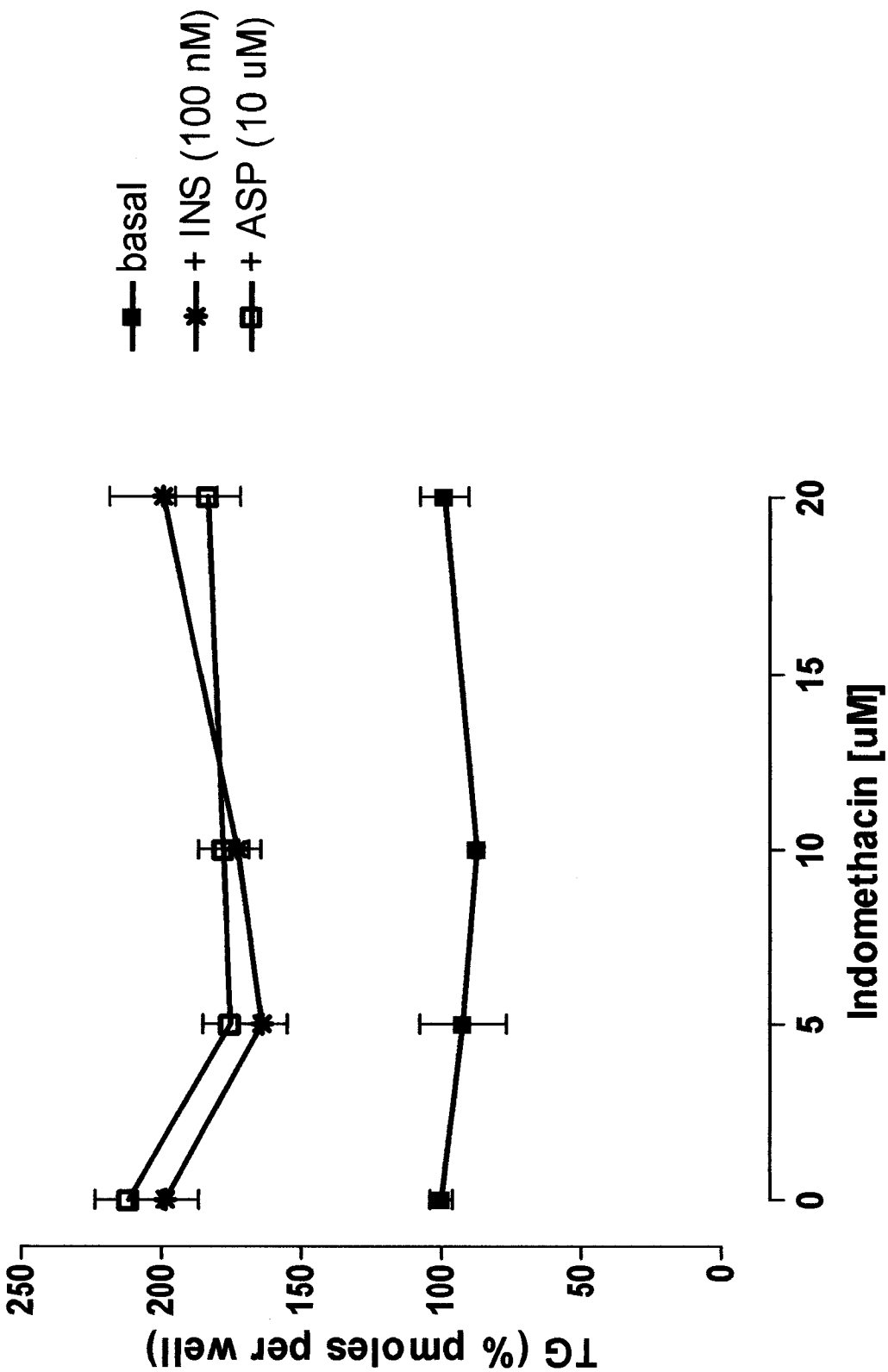

basal
+ INS (100 nM)
+ ASP (10 uM)
TG (% pmoles per well)
250
200
150
100
50
0
0
5
10
15
20
Indomethacin [uM]

Figure Legends:

Figure 4.1: The Generation of the Eicosanoids by Phospholipase A_2 (PLA_2).

The synthesis of eicosanoids first begins by the generation of arachidonic acid (AA) by PLA_2. Theoretically, both PLC and PLD can form AA by the action of diacylglycerol (DAG)-kinase and phosphatidicacid (PA)-phosphatase respectively. AA is then substrate to cyclo-oxygenase to produce the prostaglandins (PGs), or by lipo-oxygenase to form the leukotrienes.

Figure 4.2: The Effects of the General Cyclo-oxygenase (COX)-Inhibitor Indomethacin on ASP- and Insulin- Stimulated TG Synthesis.

Confluent 3T3-L1 preadipocytes were incubated in serum free media containing 5-20 uM indomethacin for 30 minutes. TG synthesis was stimulated with 10 uM ASP (open boxes) or 100 nM Insulin (astericks) for an addition 4 hours, in the presence of inhibitor. TG synthesis was measured as pmoles ^{3}H-oleate incorporated into TG. The results were normalized to basal TG synthesis (closed boxes) in each experiment performed, with basal (no addition, no indomethacin) taken as 100%. (n = 6-8 observations) and results presented as mean +/- SEM. The data were analyzed with two-way ANOVA and Bonferroni post-test, with significance set at $P<0.05$. ASP and Insulin stimulated TG synthesis to 209.4 +/-17 % and 197.6 +/- 18 % ($P< 0.0001$).

CHAPTER FIVE
The Discovery of a Novel Triglyceride (TG)-lipase

Perilipin and Lipolysis

Lipolysis is activated by catecholamines in human adipocytes, resulting in the release of free FAs and glycerol, which provide fuel and substrate to other organs, such as the muscle (FAs oxidation) and liver (glycerol for gluconeogenesis). Lipolysis has been thought to be mainly dependent on the phosphorylation of hormone sensitive lipase (Chapter One) by PKA, which results in its translocation from the cytosol to a lipid surface, whereupon it can hydrolyse TG (129).

Another target of PKA phosphorylation is the protein perilipin. Perilipin is found exclusively on the surface of a lipid droplet. In an unstimulated cell, the lipid droplet is coated with perilipin, which prevents the interaction of hormone sensitive lipase (HSL) with the lipid surface. Once perilipin is phosphorylated by PKA, the density of the perilipin coat decreases. Perilipin is then no longer able to block HSL, allowing it access to TG on the lipid surface (130, 131).

Hormone Sensitive Lipase (HSL) is not Alone

HSL knock-out mice have provided evidence of other possible TG lipases, since these mice are of normal body weight, and are still able to breakdown TG. Interestingly, there is an accumulation of DAG and not TG in these mice, suggesting that the major substrate of HSL is DAG (132). Furthermore, the discovery of new TG-specific lipases suggests that, although HSL is known to be the rate-limiting enzyme of lipolysis, non-HSL lipases specific to TG, can produce DAG that is further metabolized by HSL to produce free FAs. This is further supported by reports that suggest HSL has a ten fold higher specificity for DAG versus TG (133).

Ca^{2+}-independent PLA_2 ($iPLA_2$) and Lipolysis

In contrast to HSL, which is specific to acylglycerols and has no reported phospholipase activity, three individual papers have recently reported on $iPLA_2$ isoforms with potent TG-lipase activity, a novel function for this group of enzyme. The protein designated desnutrin, $iPLA_2\zeta$ or ATGL, was first identified based on its homology to the

patatin domain, specific to plant acyl-hydrolases. This protein is highly expressed in mouse and human adipose tissue and is under nutritional regulation, such that its expression goes up during fasting state and decreases with obesity.

While looking for protein markers of adipocyte differentiation, Vilena et al. (2004) identified a 486-aa protein, and denoted it desnutrin. The expression of desnutrin was induced in differentiating 3T3-L1 cells as early as day 2. In addition, transfected cells overexpressing this protein had enhanced TG breakdown (134).

Jenkins et al. (2004) identified desnutrin as the $iPLA_2\zeta$ isoform. In addition, they found that $iPLA_2\varepsilon$ (adiponutrin) and $iPLA_2\eta$, had potent TG-lipase function. Adiponutrin has been well documented in reflecting the energy status in rats (136-138). In addition, BEL, an irreversable inhibitor of all known $iPLA_2$s, inhibits the TG-lipase activity of each of these three isoforms. Furthermore, they confirmed that the expression of all three isoforms increased with adipocyte differentiation, however, only $iPLA_2\zeta$ mRNA could be detected in 3T3-L1 preadipocytes (135).

An inverse correlation was found between desnutrin and adiponectin gene expression. While the desnutrin gene was induced during fasting, the opposite was observed with adiponectin, as mice undergoing fasting had non-detectable levels, and was induced upon refeeding. Although desnutrin and adiponectin are mainly expressed in adipose tissue, low levels of desnutrin are also detected in other tissues, such as the heart and testis suggesting that desnutrin function is not restricted to adipose tissue alone (134).

Finally, Zimmerman et al. (2004) identified this lipase and called it adipocyte TG lipase (ATGL) also designated Transport secretion protein-2.2 (TTS-2.2). ATGL was found to be identical to the nutritionally regulated proteins desnutrin ($iPLA_2\zeta$). In addition, it was also found to share sequence similarity to adiponutrin ($iPLA_2\varepsilon$). Inhibition of ATGL was shown to block TG-breakdown and was found to work together with HSL, ensuring efficient TG-hydrolysis (149).

The aim of the current Chapter was to evaluate the effects of ASP on lipolysis in 3T3-L1 preadipocytes, by using BEL, an irreversible $iPLA_2$ inhibitor, that can block the activity of the novel TG-lipase $iPLA_2\zeta$. The results on lipolysis are extended in cells prelabeled with ^3H -AA, a fatty acid that readily incorporates into phospholipid, such

that, the effects of ASP on fatty acid release and phospholipid breakdown were investigated.

MATERIALS AND METHODS

ASP preparation, statistical analysis, cell culture and TG synthesis techniques are the same as described in Chapter Three, with the addition of the $iPLA_2$ inhibitor bromoenol-lactone (BEL), which was purchased from Sigma (Oakville, On, Canada).

Materials: 3T3-L1 preadipocytes were obtained from ATCC (Manassas, VA, USA). All tissue culture reagents such as Dulbecco's minimum essential medium (DMEM/F12), phosphate buffered saline (PBS), fetal bovine serum (FBS) and trypsin were from Gibco (Burlington, On, Canada). Stimulators of ^{3}H-arachidonic acid (^{3}H-AA) release, Bradykinin and Angiotensin II (Ang II), were from Sigma. [5, 6, 8, 9, 11, 12, 14, 15-^{3}H (N)]-Arachidonic acid was from Perkin Elmer (Woodbridge, On, Canada). Thin-layer chromatography plates (silica gel 150A) came from Fisher (Nepean, On, Canada). Organic solvents, scintillation vials, general chemicals and tissue culture materials were from VWR (Montreal, QC, Canada). Scintillation fluid was from ICN (Costa Mesa, CA, USA). BioRad reagent for protein measurements was from BioRad (Mississauga, On, Canada).

^{3}H-AA release procedure: 3T3-L1 preadipocytes, maintained at low passage number, were grown in DMEM/F12 media supplemented with 10% FBS. At 80% confluency, the cells were plated at 7,000 cells/ well on 24 well plates for experiments. When preadipocytes reached 60 % confluency they were switched to media (DMEM/F12 supplemented with 10% FBS) containing 0.2 uCi/mL ^{3}H-AA for 20 hours followed by a wash with buffer solution consisting of: 10 mM HEPES (pH = 7.4), 0.14 M NaCl, 3 mM $MgCl_2$ and 2 mM $CaCl_2$. The cells were then incubated in this buffer for 10 minutes at 30 °C. This is followed by an an incubation with additions (ASP, Bradykinin, Ang II) with buffer solution at different time intervals at 30 °C. Appropriate vehicle controls were performed in each experiment. After the incubation period, the cells are put on ice. An aliquot of media is taken and counted directly, representing released ^{3}H-AA. The cells were then washed three times in ice cold PBS, and lipids were extracted for 1 hour in 1 ml of heptane:isopropanol (3:2 v/v) then rinsed with an additional 1 ml of the same

solvent mix. Lipid extracts were evaporated to dryness in a centrifuge-evaporator (Canberra-Packard, Canada), redissolved in 100 ul of chloroform:methanol (2:1 v/v) and lipids were resolved by thin-layer chromatography (TLC) in hexane : ethyl ether : acetic acid (75:25:1 v/v/v) with reference lipids (TG, DAG and FA) run concurrently. Separated lipids were visualized with iodine vapor and the spots corresponding to lipid fractions were scraped into scintillation vials and counted by liquid scintillation counting (Beckman, CA). Cell proteins were solubilized in 0.1 N NaOH for 3 hours and quantified by the method of Bradford (139).

Results and Discussion

The Effects of the $iPLA_2$ inhibitor BEL on ASP-stimulated TG Synthesis

As mentioned above, the TG-lipase activity of $iPLA_2\zeta$ can be blocked with BEL, a suicide substrate inhibitor that is 1000-fold more selective for $iPLA_2$ versus $cPLA_2$ (110, 111). TG synthesis was measured in 3T3-L1 preadipocytes by their ability to incorporate ^{3}H-oleate into their TG stores. In this experiment ASP stimulated TG-synthesis ranged from 205.5 % to 148.0 %for an average of 177.0 +/- 15 % (versus control, set as 100 %, $P< 0.0001$) (Figure 5.1). Addition of BEL resulted in increased accumulation of TG in a concentration-dependent manner with initial effects seen at 2.5 uM BEL (533.0 +/- 3 % compared to control, $P< 0.0001$). In contrast to lower concentrations of BEL, both basal TG- and ASP-stimulation of TG-synthesis were stimulated to the same extent with 10 uM BEL (Figure 5.1), (906 +/- 20 % for basal versus 1014 +/- 21 % with ASP with 10 uM BEL vs. control, $P< 0.0001$). Thus, BEL, at 10 uM, appears to stimulate basal TG synthesis to the same extent as ASP and the effects of this inhibitor in combination with ASP are non-additive, likely mediating their effects through the same pathway, by inhibiting TG-breakdown.

ASP Inhibits the Activity of a Phospholipase Enzyme

In order to measure lipase activity specifically, ASP was further evaluated for its effects on AA release into the media of pre-labeled 3T3-L1 preadipocytes. Cells pre-treated with ^{3}H-AA can incorporate this fatty acid inside the cell. It is mainly incorporated into the sn-2 position of phospholipids, producing ^{3}H-arachidonyl

phospholipid, and to a lesser extent into TG and DAG fractions. After a 20-hour incubation with 0.2uCi/mL ^{3}H-AA, only 18 +/- 4 % of radioactivity was detected in the media (Figure 5.2). In other words, 82 +/- 4 % of total radioactivity was taken up inside the cells. AA was efficiently incorporated into phospholipid, 91 +/- 24 % (with 100 % taken as total radioactivity inside the cell) versus 6 +/- 8% in TG and less than 2 % in both DAG and fatty acid fractions (Figure 5.3).

Both bradykinin and Ang II were used as positive controls as they have been well documented in stimulating the release of this fatty acid into the media through PLA_2 activation, resulting in subsequent PG production, through which they are known to mediate their effects (140-144). It is important to note, depending on the PLA_2 isoform, not all activators of PLA_2 will produce a detectable release of AA into the extracellular milieu of pre-labeled cells. Rather, a local release of AA inside the cell also occurs. In this case, AA is released locally inside the cell, and is utilized for either second messenger function or is converted to PGs. For example, $cPLA_2\alpha$ has been well described in releasing AA from the membranes of the ER and nucleus. Since the ER and nuclear membrane are the sites of COX enzymes, then a local intracellular release of AA fascilitates coupling with COX and subsequent production of PGs (145).

A 50-minute time course with ASP had little effect on ^{3}H-AA release, in fact it appeared to play an inhibitory role as the release of AA with ASP was below basal levels (Figure 5.4). In contrast, cells incubated for 10 minutes with Ang II were able to significantly stimulate the release of ^{3}H-AA to 261.6 +/- 27 %, $P< 0.0351$ (Figure 5.4).

Furthermore, the inhibitory effects seen with ASP on AA release were also concentration-dependent, as little as 2.5 uM ASP was able to significantly inhibit ^{3}H-AA release to 54.2 +/- 37 % ($P< 0.0001$ vs control) (Figure 5.5). These effects were mirrored inside the cell, as both the total radioactivity inside the cell, and ^{3}H-arachidonyl phospholipid were enhanced to 161.0 and 148.2 +/- 26 % respectively ($P< 0.0094$ for total inside the cell and $P< 0.0355$ for phospholipid, vs control) (summarized in Table 5.6). This suggests that ASP inhibits an enzyme with phospholipase activity. Since AA is a fatty acid that is commonly found in the sn-2 position of phospholipids and is released by the action of PLA_2 enzymes, then one can speculate that ASP may mediate its anti-

lipogenic effects by blocking the action of a PLA_2 enzyme with TG-lipase function, such as $iPLA_2\zeta$.

The effects of ASP were further evaluated by looking at the changes in AA levels inside the cell. In contrast to the decrease in ^{3}H-AA release into the media, there was an increase in intracellular ^{3}H-AA. The statistical analysis on the potential inhibitory effects of ASP on AA release are summarized in Table 5.6

The changes seen inside the cell reflects the changes seen in the phospholipid fraction and was found to directly correlate when performing linear regression, slope = 0.9305, R = 0.926 (Figure 5.7). In contrast, ^{3}H-arachidonyl phospholipid, correlated negatively with extracellular ^{3}H-AA (slope = -0.704, R = 0.777, Fig 5.7). Thus, the increases in total intracellular ^{3}H-AA, reflects an inhibition of ^{3}H-arachidonyl phospholipid breakdown, and conversely, the total extracellular ^{3}H-AA decreases.

ASP has been well documented as an anti-lipolytic agent. The stimulation of phosphodiesterases PDE3 and PDE4 by ASP results in a reduction of cAMP levels, leading to a decrease in PKA activity and HSL phosphorylation (31). ASP has also been shown to stimulate $[Ca^{2+}]i$ through PLC activation (Chapter Three). Rises in $[Ca^{2+}]i$ have been shown to inhibit lipolysis by activating PDE3 (146). Although $iPLA_2$ has consensus phosphorylation sites for several serine PKAs, PKA has never been demonstrated to activate $iPLA_2$. On the other hand, the lysophospholipid products of $iPLA_2$, lysophosphatidylcholine and lysoplamsencholine, have been shown to effectively activate PKA in the heart during myocardial ischemia (147), although this has not yet been explored in adipocytes. One can speculate that the phospholipase activity of an $iPLA_2$ enzyme can produce lysophospholipid products to activate PKA and thus, lipolysis. Since rises in $[Ca^{2+}]i$ have been shown to inhibit $iPLA_2$ activity (Chapter Two), then the stimulation of $[Ca^{2+}]i$ by ASP, may be another mechanism by which ASP can effectively inhibit lipolysis (summarized in Figure 5.8).

BEL a specific $iPLA_2$ inhibitor, can enhance TG synthesis by preventing the action of an $iPLA_2$ with TG-lipase activity. Since preliminary data suggests that both ASP and BEL can enhance TG through the same mechanism, this suggests that ASP inhibits an $iPLA_2$ isoform with TG-lipase activity (possibly $iPLA_2\zeta$). However, further experiments will be needed to confirm the exact isoform involved.

Since no inhibitors to specific PLA_2 groups were used in the ^{3}H-AA experiments, then the rise of total ^{3}H-AA inside the cell may result from the inhibition of a lipase as an increase in ^{3}H-arachidonyl phospholipid occurred simultaneously In addition, the accumulation of ^{3}H-AA inside the cell, further implicates the involvement of $cPLA_2$ in ASP action, as only catalytic amounts of AA are released inside the cell that can be rapidly coupled to intracellular second messenger function. If the $iPLA_2$ inhibitor, BEL, was used in such an experiment, then the intracellular generation of fatty acid by $cPLA_2$ action specifically would be observed. Likewise, the inhibition of $cPLA_2$ with AACOCF3 would reveal the exclusive effects of ASP on $iPLA_2$. In addition, since ^{3}H-AA is not a good marker of TG (only 6 +/- 8% incorporation of total radioactivity inside the cell), then ^{3}H-oleate, a fatty acid commonly stored in TG, could be used to determine the effects of ASP on TG-lipase activity.

In summary, amphipatic lipids have been shown to function both as stimulators of signaling pathways and as storage molecules. It appears as though ASP has opposing effects on the PLA_2 family of lipases, as it can stimulate the release of AA through $cPLA_2$ activation, for the generation of AA as a signaling molecule. Conversely, ASP seems to block the release of fatty acid as a storage molecule by blocking the lipolytic action of an $iPLA_2$ isoform. Taken together, both these ASP-mediated mechanisms serve to enhance overall TG storage.

Figure 5.1

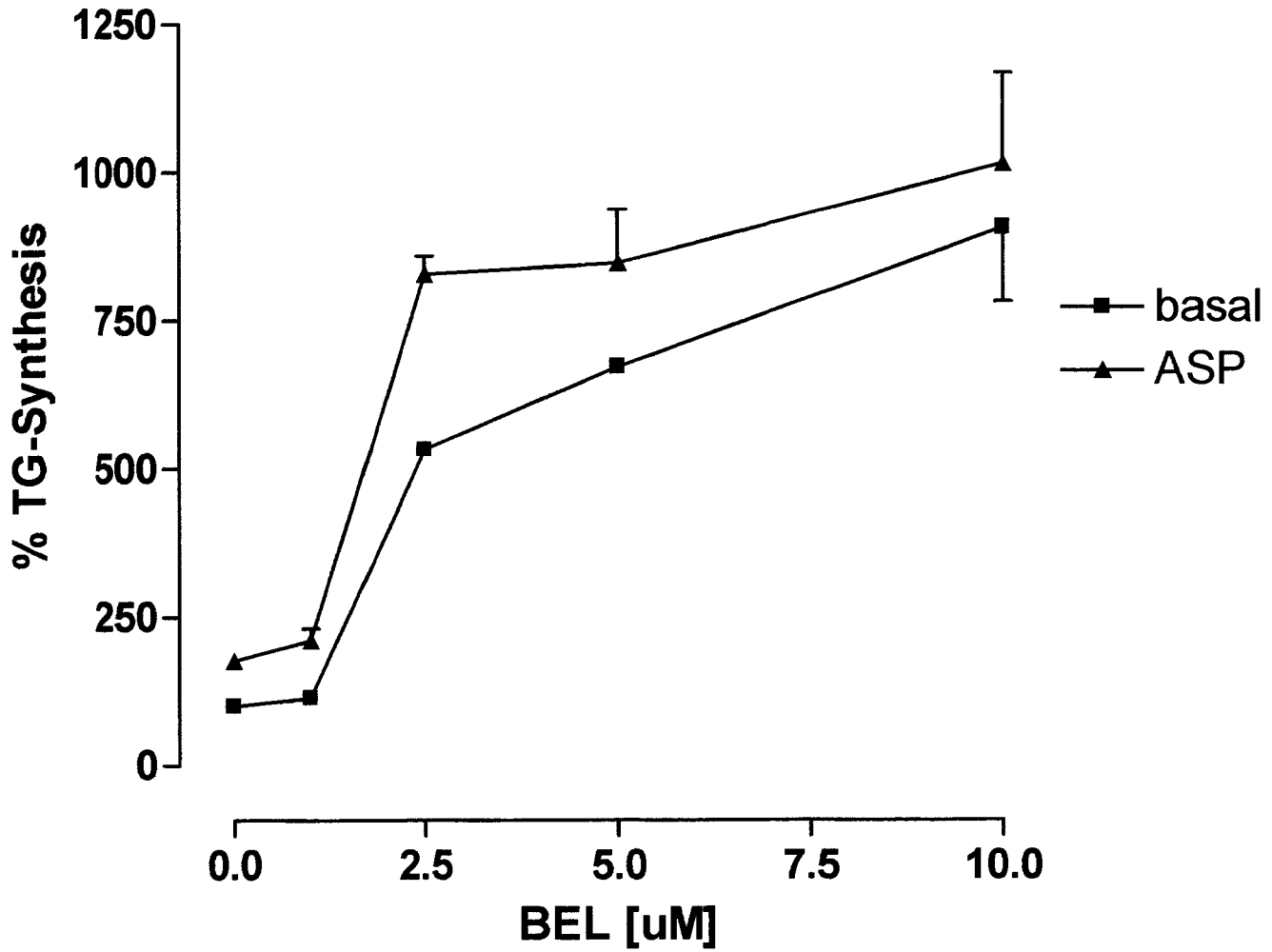

1250
1000
750
500
250
0
% TG-Synthesis
0.0
2.5
5.0
7.5
10.0
BEL [uM]
basal
ASP

Figure 5.2

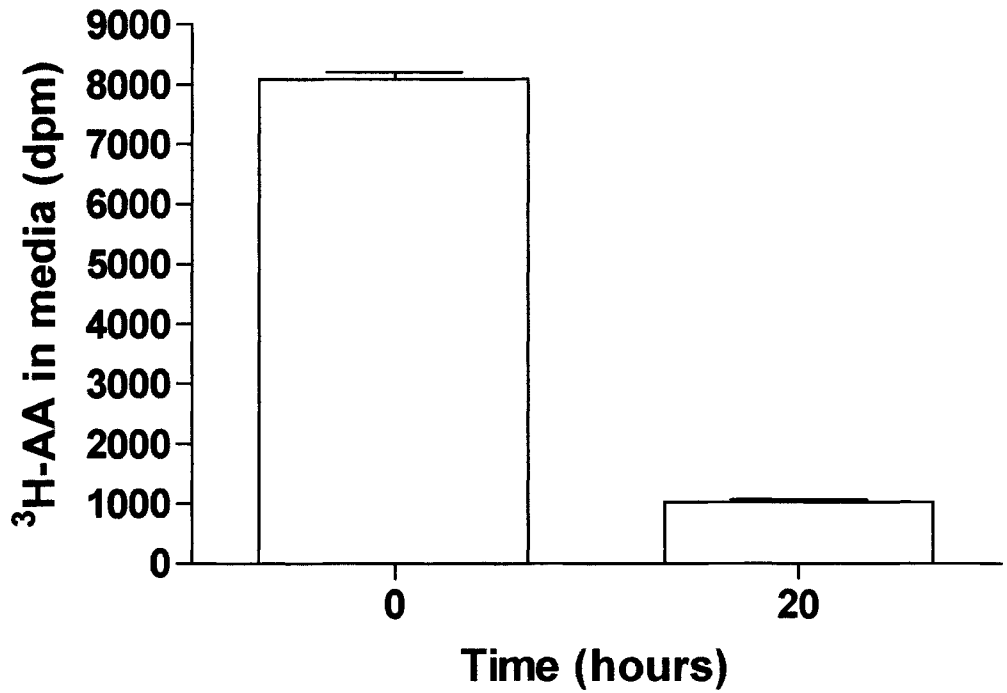

9000
8000
7000
6000
5000
4000
3000
2000
1000
0
^{3}H-AA in media (dpm)
0
20
Time (hours)

Figure 5.3

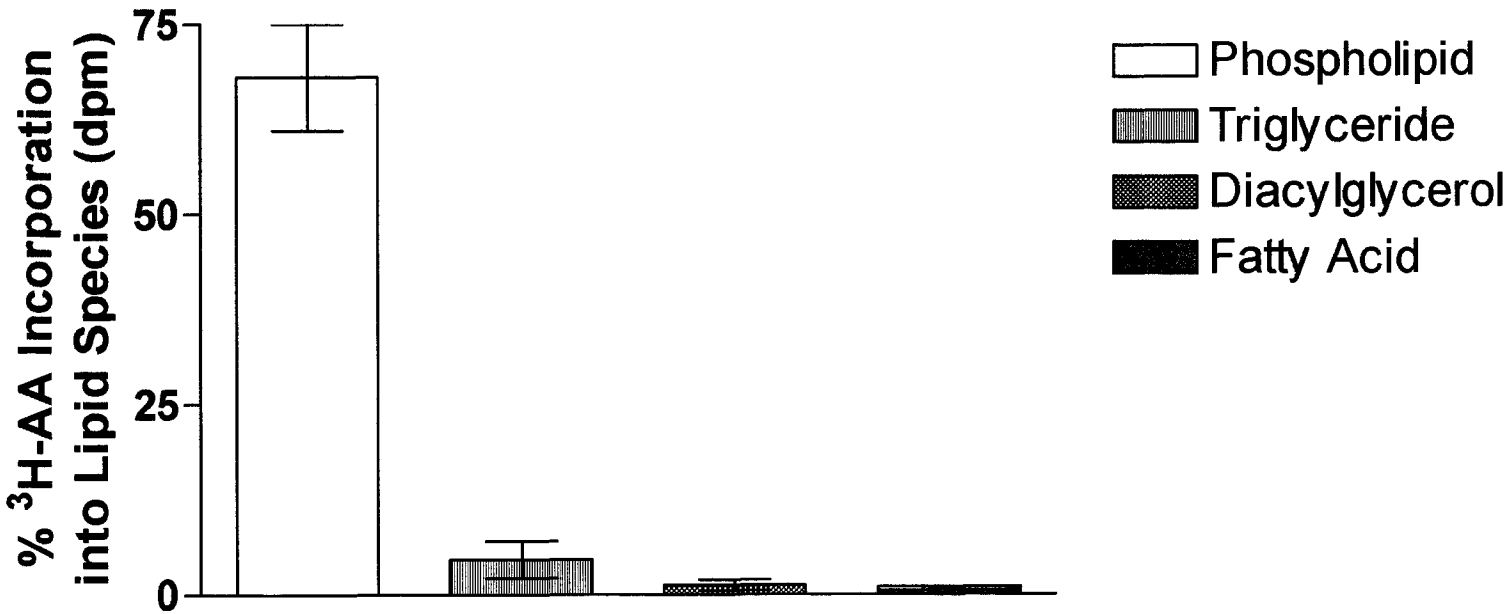

% ³H-AA Incorporation into Lipid Species (dpm)
75
50
25
0
Phospholipid
Triglyceride
Diacylglycerol
Fatty Acid

Figure 5.4

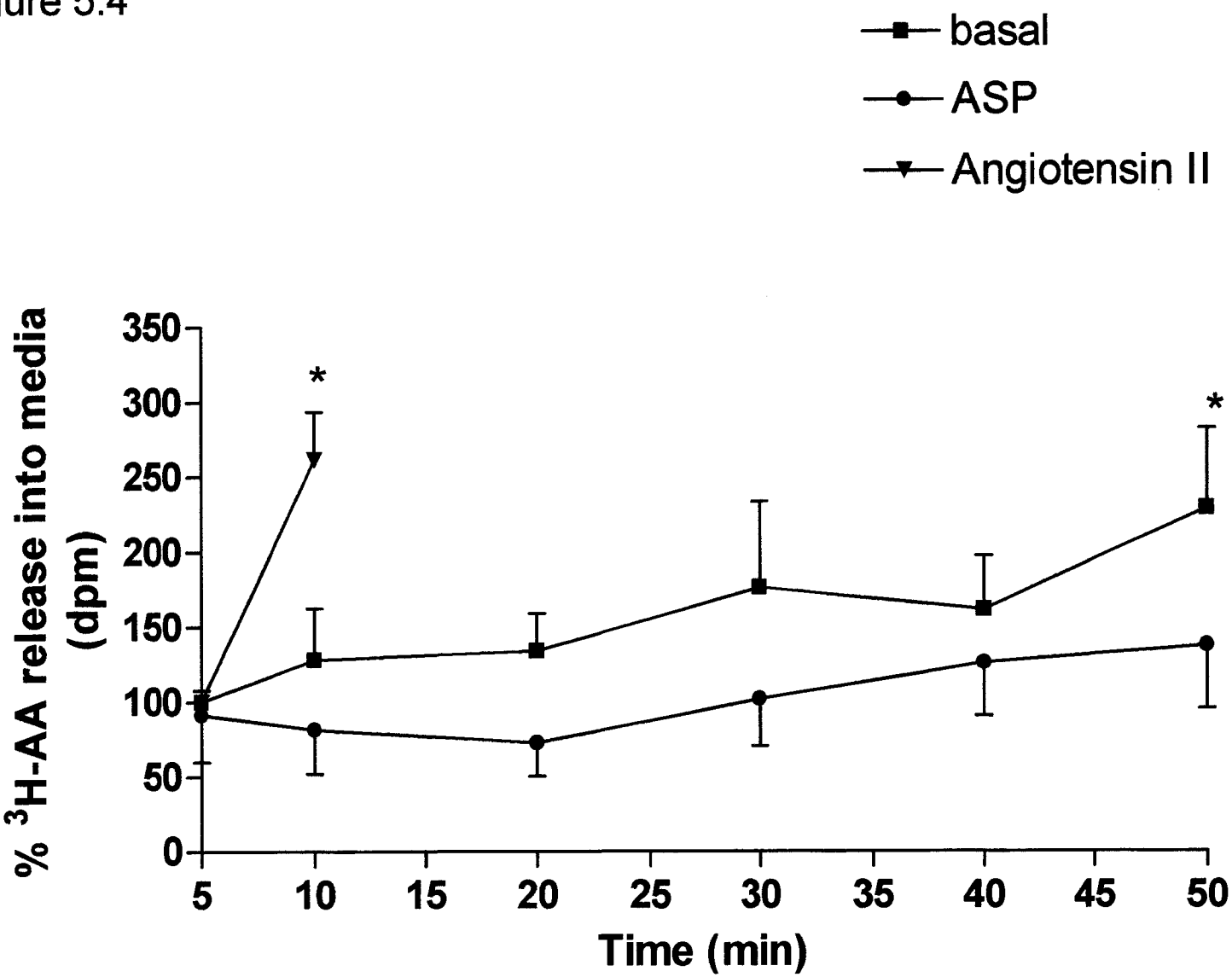

basal
ASP
Angiotensin II
% 3H-AA release into media (dpm)
350
300
250
200
150
100
50
0
*
*
5
10
15
20
25
30
35
40
45
50
Time (min)

Figure 5.5

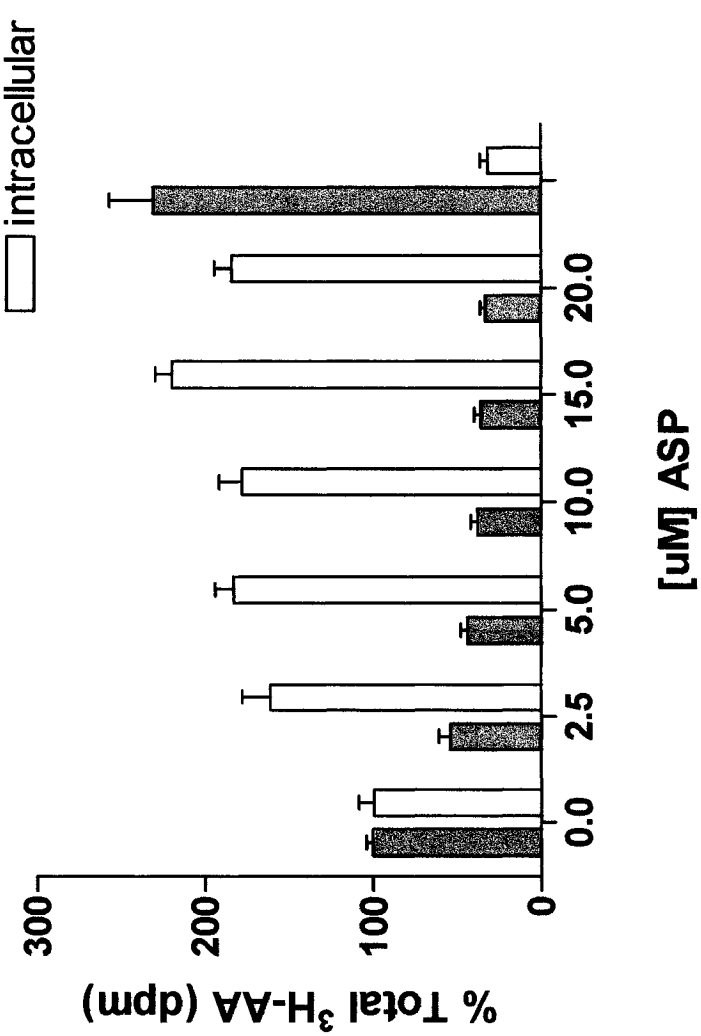

extracellular
intracellular
% Total 3H-AA (dpm)
300
200
100
0
0.0
2.5
5.0
10.0
15.0
20.0
[uM] ASP

Table 5.6: The Effects of ASP on 3T3-L1 Preadipocytes Pre-labeled with ^{3}H-AA

ASP (uM)	% 3H-AA					
	extracellular		Intracellular		Phospholipid	
		p		p		p
0	100 +/- 14 %		99.7 +/- 22%		100.2 +/- 30 %	
2.5	54.2 +/- 37 %	0.0001	161.0 +/- 26 %	0.0094	148.2 +/- 26 %	0.0355
5	43.8 +/-27 %	0.0001	183.0 +/- 15 %	0.0002	179.5 +/- 25 %	0.0047
10	37.8 +/- 38 %	0.0001	177.8 +/- 19 %	0.0008	178.5 +/- 31 %	0.013
15	35.8 +/- 38 %	0.0001	219.5 +/- 11 %	0.0001	209.0 +/- 18 %	0.0003
20	33.2 +/- 40 %	0.0001	184.0 +/- 20 %	0.0001	191.1 +/- 24 %	0.001
BRADY (30uM)	230.7 +/- 28 %	0.0001	31.7 +/- 37 %	0.0001	36.8 +/- 46 %	0.001

3T3-L1 preadipocytes were incubated in media containing 0.2 uCi/mL ^{3}H-AA when 60 % confluent, for 20 hours, and changed in serum free media for 2 hours. Then cells were incubated with increasing concentrations of ASP or with 30 uM bradykinin, for 40 minutes. An aliquot of media (extracellular) was counted directly, after 40-minute incubation period. Cells were then washed and lipids extracted with chloroform: methanol (2:1 v/v). An aliquot of this lipid extraction was counted directly (intracellular) while the phospholipid fraction was separated with thin layer chromatography. The results were normalized to basal ^{3}H-AA release in each experiment and are expressed as % ^{3}H-AA release, with basal (no ASP, no bradykinin) was set as 100 %. The data were analyzed using ANOVA, with significance set at $P<0.05$, compared to control (no ASP, no bradykinin).

Figure 5.7

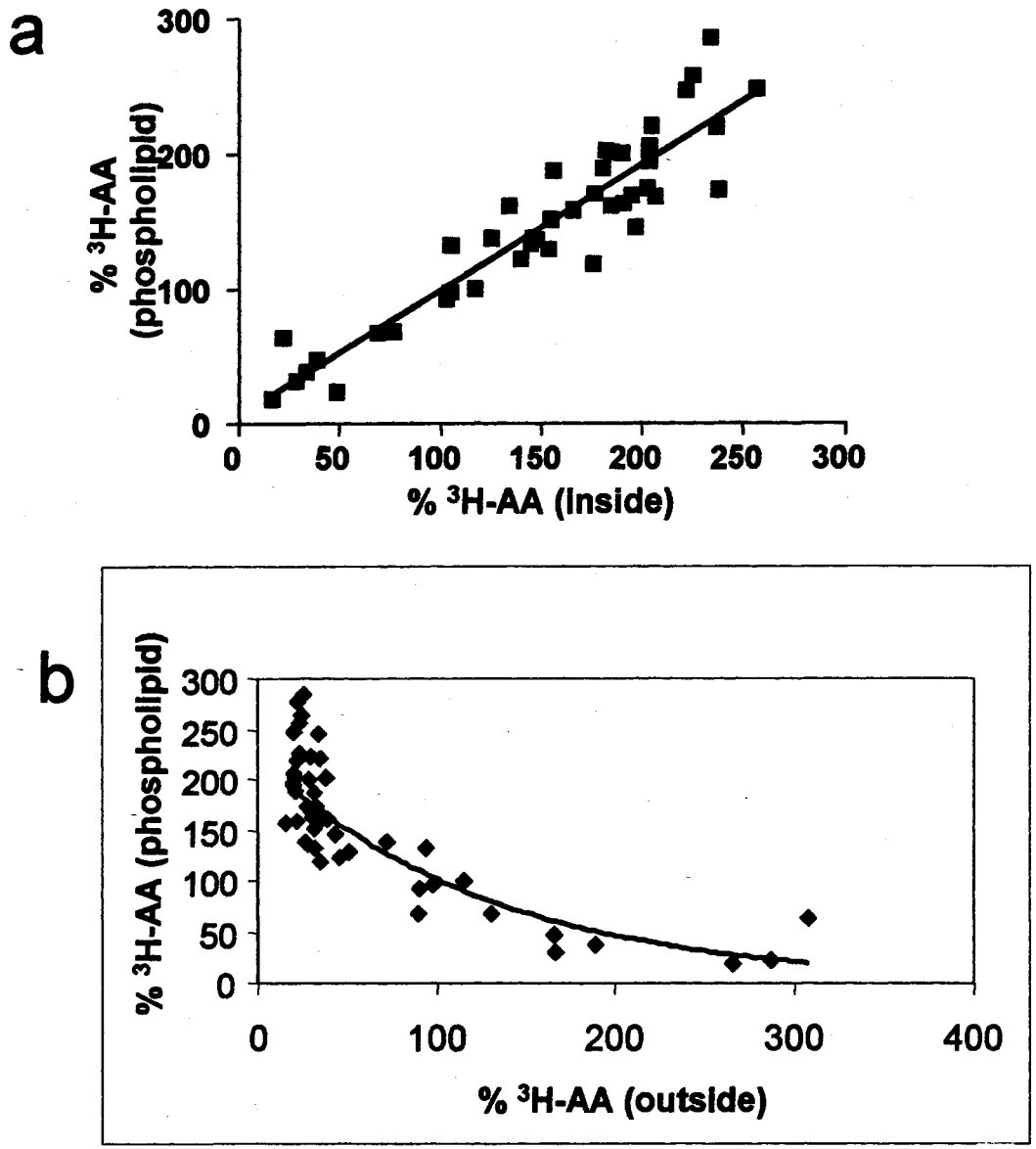

a
300
200
100
0
% ³H-AA (phospholipid)
0
50
100
150
200
250
300
% ³H-AA (inside)
b
300
250
200
150
100
50
0
% ³H-AA (phospholipid)
0
100
200
300
400
% ³H-AA (outside)

Figure 5.8

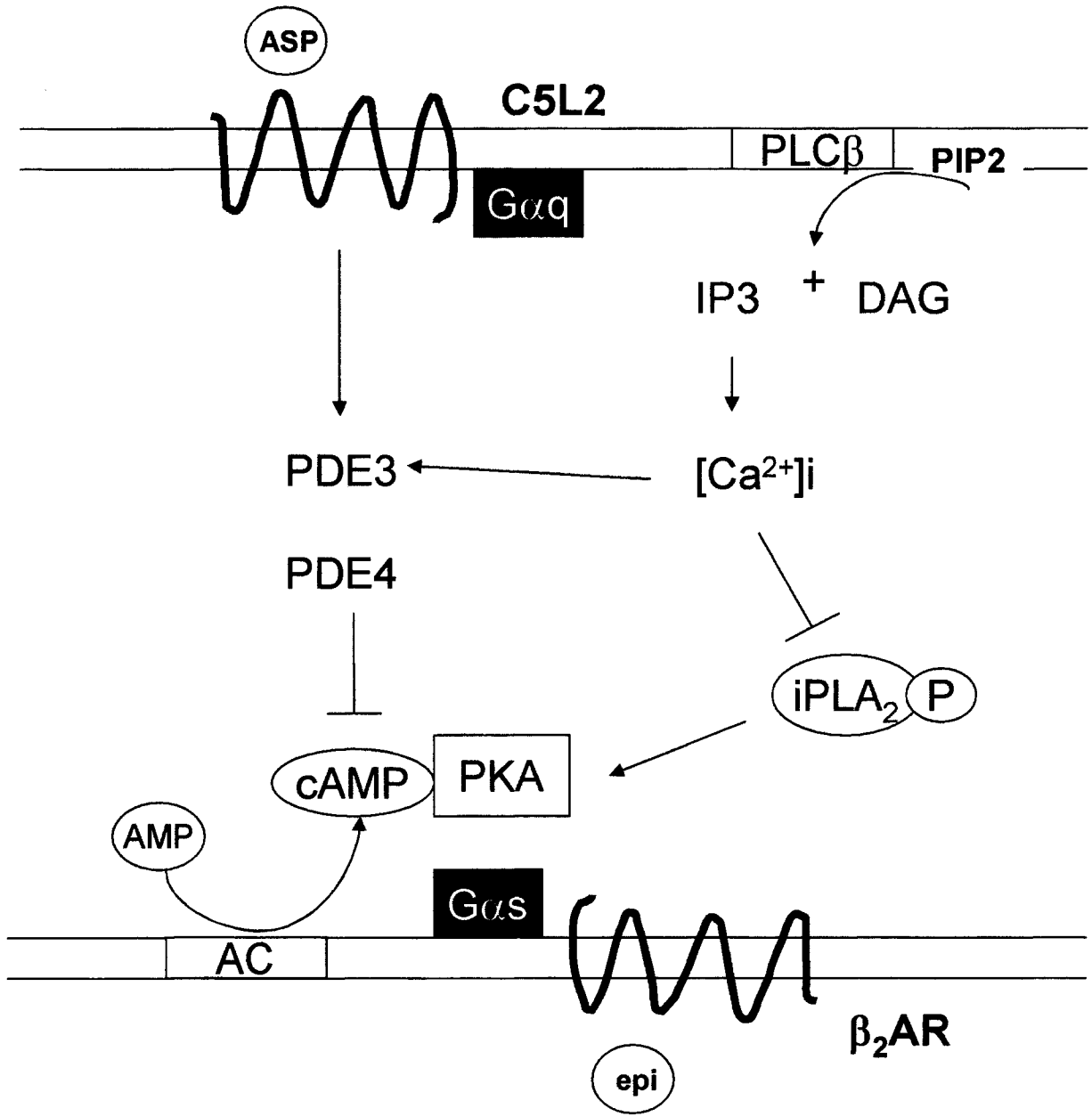

ASP
C5L2
PLCβ
PIP2
Gαq
IP3
+
DAG
PDE3
[Ca2+]i
PDE4
iPLA2
P
cAMP
PKA
AMP
Gαs
AC
β2AR
epi

Figure Legends:

Figure 5.1: The Effects of Bromoenol-lactone (BEL), the Irreversible $iPLA_2$ Inhibitor, on ASP-Stimulated TG Synthesis.

Confluent 3T3-L1 preadipocytes were incubated in serum free media containing 1-10 uM BEL for 30 minutes. TG synthesis was stimulated with 10 uM ASP (triangles) for an addition 4 hours, in the presence of inhibitor. TG synthesis was measured as pmoles ^{3}H-oleate incorporated into TG. The results were normalized to basal TG synthesis (boxes) in each experiment performed, with basal (no addition, no BEL) taken as 100%. (n = 6 observations) and results presented as mean +/- SEM. The data were analyzed with two-way ANOVA and Bonferroni post-test, with significance set at $P<0.05$. ASP stimulated TG synthesis to 177.0 +/- 15 % ($P< 0.0001$). BEL significantly stimulated both basal and ASP-stimulated TG synthesis to the same degree. For 2.5 uM BEL, basal TG synthesis was stimulated to 533.0 +/- 3 % and ASP to 828.0 +/- 5 % ($P< 0.0001$). For 5.0 uM, basal was raised to 671.5 +/- 0 % and ASP to 847.0 +/- 15 % ($P< 0.0001$). 10.0 uM BEL stimulated basal to 906.0 +/- 20 % and ASP to 1014.0 +/- 21 % ($P< 0.0001$).

Figure 5.2: The Average Uptake of ^{3}H-AA After a 20-hour Incubation

When 60 % confluent, 3T3-L1 preadipocytes were incubated in media containing 0.2 uCi/mL ^{3}H-AA for 20 hours. An aliquot of the original media (time = 0 hour) and an aliquot at time = 20 hours incubation was taken from the media and counted directly. The results presented as mean +/- SEM. The average uptake was 82.0 +/- 4 % for n = 9 observations.

Figure 5.3: The Incorporation of ^{3}H-AA into Different Lipid Fractions.

When 3T3-L1 preadipocytes reached 60 % confluency, they were incubated in media containing 0.2 uCi/mL ^{3}H-AA for 20 hours. After incubation, radioactive media was removed and cells were extracted for lipids with chloroform:methanol (2:1 v/v), and run on thin-layer chromatography to separate lipid fractions. The results were normalized to total radioactivity inside the cell (sum of all counts found in each lipid fraction) in each

experiment performed, taken as 100%. (n = 9 observations) and results presented as mean +/- SEM.

Figure 5.4: ASP Effectively Inhibits ^{3}H-AA Release in a Time-Dependent Fashion.
When 3T3-L1 preadipocytes reached 60 % confluency, they were incubated in media containing 0.2 uCi/mL ^{3}H-AA. Cells were changed into serum free media for 2 hours, and a time course with supliments (ASP or Angiotensin II) was preformed for 50 minutes. The results were normalized to basal ^{3}H-AA release synthesis (boxes) in each experiment performed, with basal (no ASP, no Angiotensin II) taken as 100%. (n = 9 observations) and results presented as mean +/- SEM. The data were analyzed with unpaired students t-test, with significance set at $P<0.05$. 20 nM Angiotensin II stimulated ^{3}H-AA release to 261.6 +/- 27 %, ($P< 0.0001$ versus 10 minutes without ASP or Angiotensin II). There was no significant change in ^{3}H-AA release with 10 uM ASP.

Figure 5.5: ASP Inhibits ^{3}H-AA Release in a Concentration-Dependent Manner
When 3T3-L1 preadipocytes reached 60 % confluency, they were incubated in media containing 0.2 uCi/mL ^{3}H-AA. Cells were changed into serum free media for 2 hours, and a time course with supliments (ASP or bradykinin) was preformed. The results were normalized to basal ^{3}H-AA release synthesis (boxes) in each experiment performed, with basal (no ASP, no bradykinin) taken as 100%. (n = 12-18 observations) and results presented as mean +/- SEM. The data were analyzed with ANOVA, with significance set at $P<0.05$. All results and statistical analyses are summarized in Table 5.6.

Figure 5.7: ^{3}H-AA: an Effective Marker of Phospholipid
Linear Regression based on data from Figure 5.5. Changes in total ^{3}H-AA inside (x-axis) against changes in ^{3}H-arachidonyl phospholipid (y-axis) gave the following equation: $y = 0.9305x + 6.002$, $R^2 = 0.857$, $R = 0.926$ ($P< 0.0001$). Changes in total ^{3}H-AA outside (x-axis) correlated negatively against changes in ^{3}H-arachidonyl phospholipid: $y = - 0.7402x + 203.9$, $R^2 = 0.604$, $R = 0.777$ ($P<0.0001$).

Figure 5.8: Proposed ASP-Mediated Inhibition of an $iPLA_2$ with TG-lipase Activity

Upon binding to its receptor, C5L2, ASP can stimulate PLC activity, and subsequently, the rise in intracellular calcium $[Ca^{2+}]i$ from the endoplasmic reticulum by inositol trispohosphate (IP3). Rises in $[Ca^{2+}]i$ can stimulate phosphodiesterase (PDE) activity, and inhibit the action of $iPLA_2$. PDEs can attenuate the levels of cAMP inside the cell, and thus inhibit PKA-mediated activation of lipolysis. The lysophospholipid products of $iPLA_2$ can activate PKA. ASP inhibition of $iPLA_2$ can result from rises in $[Ca^{2+}]i$ and activation of PDEs, and provides a mechanism by which ASP can inhibit lipolysis.

SUMMARY

It has become more apparent that amphipatic lipids, such as DAG, serve more than just structural roles. Not only are they reservoirs of energy in glycerolipids (phospholipid and TG), they are also important second messengers involved in cell signaling. DAG for example is a common substrate for both TG and phospholipid synthesis. Furthermore, this lipid can function as a signaling molecule upon agonist-stimulated release by PLC.

In fact, a recent paper by Bagnato et al. (2003) discovered that in cells overexpressing DGAT, not only was DAG substrate channelled into TG, there was a significant decrease in phospholipid synthesis (PC in particular). Furthermore, they also found that the growth rate of these DGAT cells was significantly decreased. This suggests that deprivation of DAG substrate into phospholipid not only attenuates membrane biogenesis, but also altered DAG-stimulation of signalling. This was further confirmed by the addition of PLA_2 and PLC, where the release of radiolabeled fatty acid and DAG by these enzymes respectively, was incorporated into TG. This indicates that DGAT can terminate intracellular lipids for storage into TG (148).

Like DAG, AA is a fatty acid that is substrate for energy storage in glycerolipids, as it is generally incorporated into the sn-2 position of phospholipids. Furthermore it can be released by the action of PLA_2 enzymes for signalling inside the cell (Chapter 3/4). ASP appears to have opposing effects on the PLA_2 family of lipases. First, ASP can stimulate the production of catalytic AA, which can signal as a second messenger for TG synthesis. This effect is mediated by $cPLA_2$, and evidence suggests that ASP activates $cPLA_2$ by stimulating MAPK and rises in $[Ca^{2+}]i$. Conversely, ASP also prevents the release of AA as a storage molecule, by blocking the lipolytic action of an enzyme with both sn-2 phospholipase- and TG-lipase activity. The recent identification of $iPLA_2\zeta$ and its expression in 3T3-L1 preadipocytes fits this criteria, however the exact $iPLA_2$ isoform involved is currently unknown. ASP may inhibit the action of this lipase by activating phosphodiesterase enzymes known to decrease cAMP in the cell, and thus attenuate HSL activity. Also the rises in $[Ca^{2+}]i$, mediated by ASP, has been shown to play an inhibitory role on the $iPLA_2$ family of enzymes. Both these ASP-mediated effects on PLA_2 enymes

globally lead to the accumulation of TG in the cell, and further provides evidence on the function of ASP as a potent lipogenic factor in adipose tissue.

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Centre universitaire de santé McGill
McGill University Health Centre

24-H Emergency number: RADIOPROTECTION ext. 53333

Internal Permit #: 5-0012-05

Permit Holder: Katherine Cianflone **Office:** H7.30

Department: RVH - Cardiology Research **Telephone:** 35426

A) Location

Room	Classification
7.25*	Intermediate
7.31*	Basic
7.33	Basic
7.36	Storage only
7.41*	Basic
7.88A*	High

Rooms identified by an asterix (*) must have a door warning sign

(B) Authorized Activity

Isotope	Max Manipulated (MBq)	Max Purchase (MBq)
C14	22.2	185
H3	22.2	185
I125	74	74
P32	74	74

C) Authorised Users

Last Name	First Name	P32	P33	S35	H3	C14	F659	I125	Ca45	Co57	Cr51	Na22	Rb86
anflone	Katherine	☐	☐	☐	☑	☑	☐	☑	☐	☐	☐	☐	☐
ollins	Melissa	☐	☐	☐	☐	☐	☐	☑	☐	☐	☐	☐	☐
ui	Wei	☑	☐	☐	☑	☑	☐	☑	☐	☐	☐	☐	☐
alant	David	☑	☐	☐	☑	☐	☐	☑	☐	☐	☐	☐	☐
gakis	Helen	☐	☐	☐	☑	☑	☐	☑	☐	☐	☐	☐	☐
aslowska	Magdalena	☑	☐	☐	☑	☑	☐	☑	☐	☐	☐	☐	☐
cLaren	Robin	☐	☐	☐	☑	☐	☐	☑	☐	☐	☐	☐	☐
aglialinga	Sabina	☐	☐	☐	☑	☑	☐	☐	☐	☐	☐	☐	☐
[illegible]	[illegible]	☐	☐	☐	☑	☐	☐	☐	☐	☐	☐	☐	☐
mith	Jessica	☐	☐	☐	☑	☐	☐	☑	☐	☐	☐	☐	☐

D) Conditions

Conditions
Only D. Kalant and Wei Cui are authorized to manipulate > 50 MBq of P-32 (ring TLD group # L19512)

The persons listed in section (C) are authorised to use the designated radioisotopes. The radioisotopes and their respective activities listed in section (B) can only be used in the boratories listed in section (A) in accordance with the conditions listed in section (D). Importation, storage, manipulation and disposition of radioactive material must be performed in nformity with our CNSC licence, with Federal regulations and with the MUHC Radiation Safety Policies and Procedures. A copy of the CNSC consolidated licence is available from the adiation Protection Service (ext. 43866).

Approved by: _____ **Date issued:** Wednesday, May 18, 2005

Expiration date: Feb 28, 2006

L'Institut de recherche du Centre Universitaire de Santé McGill
Research Institute of the McGill University Health Centre

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

)jects involving potentially biohazardous materials should not be commenced without approval from the Health and Safety fice. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the hazardous materials within existing projects.

PRINCIPAL INVESTIGATOR: Katherine Cianflone PHONE: 934-1934 #35426

EPARTMENT: Medicine / Cardiology FAX: 843-2843

DDRESS: RVH – MUHC, Room H7.30 E-MAIL: Katherine.Cianflone@mcgill.ca

ROJECT TITLE: Regulation of Triglyceride Storage and ASP Mechanism in ASP Deficient and Overexpressing Mice odels

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EMERGENCY: Person(s) designated to handle emergencies

ıme: Katherine Cianflone Phone No: work: 934-1934 #35426 home: 450-357-1904

ıme: David Kalant Phone No: work: 934-1934 #35425 home: 450-693-9423

FUNDING SOURCE OR AGENCY (specify): CIHR

ant No.: TBD Beginning date: 2005 End date: 2010

Indicate if this is

Renewal: procedures previously approved without alterations.

Approval End Date: _____

New funding source: project previously reviewed and approved under an application to another agency.

Agency: _____ Approval End Date: _____

New project: project not previously reviewed.

Approved project: change in biohazardous materials or procedures. (NEW APPLICATION- ONLY TITLE CHANGED)

Work/project involving biohazardous materials in teaching/diagnostics.

RTIFICATION STATEMENT: The Health and Safety Office approves the experimental procedures proposed and ifies with the applicant that the experiment will be in accordance with the principles outlined in Health Canada's boratory Biosafety Guidelines", the RI-MUHC's "Health and Safety policies" and the "McGill Laboratory Biosafety ıual".

ıtainment Level: ☐ 1 ☒ 2 / ☐ 2 with additional precautions ☐ 3

cipal Investigator: _____ date: 09/03/2005 (day / month / year)

roved by: Site Biosafety Officer: HEALTH AND SAFETY COORDINATOR _____ date: 15 / 03 / 05 (day / month / year)

Expiry: 31 / 03 / 10 (day / month / year)