2010

ADENOVIRUS-5 INFECTION AFFECTS LIPID METABOLISM IN HEPATIC AND ADIPOSE TISSUES

Marianna Sukholutsky
Virginia Commonwealth University

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ADENOVIRUS-5 EFFECT ON LIPID METABOLISM IN HEPATIC AND ADIPOSE TISSUES

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Physiology and Biophysics at Virginia Commonwealth University.

By
Marianna Sukholutsky, M.S.
B.A., Virginia Commonwealth University, 2004

Director: Suzanne E. Barbour, Ph.D.
Professor, Department of Biochemistry and Molecular Biology

Virginia Commonwealth University
Richmond, Virginia
December, 2010
ACKNOWLEDGEMENT

I would like to thank Dr. Suzanne Barbour for her unyielding support, guidance, and encouragement throughout my graduate career. Her passion for teaching and research was instilled into all of her students, including myself. I would also like to give a big thanks to Dr. Palmer Wilkins; without his help and knowledge this research would not have been made possible. I am also very grateful to meeting and having the pleasure of working with Dr. Rachael Griffiths. Her constant support and unwavering passion guided me in my studies and research. I would also like to thank my parents, Leonid and Ella Sukholutsky, for always pushing me to pursue my goals. To all of my close friends and family, thank you for all the good times and for helping me get through the tough times. To my committee members, Dr. Gil and Dr. Hylemon, thank you for your encouragements and advice. Finally, I would like to give a big thanks to my fellow labmate and friend Bhargavi Emani – thank you for being my partner in crime. A big thanks to Maimuna Bruce, Elizabeth Nelson, Jennifer Bradley, Vasudha Surampudi, and Caitlin Flora for always being there for me. Last but not least, thank you so much to Shane Garcia for always supporting and loving me during this important time in my life.
TABLE OF CONTENTS

Acknowledgements........................................................................................................................................ ii

List of Figures.................................................................................................................................................. iv

Chapter

1 INTRODUCTION .............................................................................................................................................1

   1.1 Sterol Regulatory Element Binding Proteins ................................................................. 2

   1.2 Characterization of Adenovirus-5 ...................................................................................... 7

   1.3 Adenovirus-5 and Gluconeogenesis .............................................................................. 12

2 METHODS AND MATERIALS .................................................................................................................. 19

3 RESULTS ...................................................................................................................................................... 23

   3.1 Ad-5 induces SREBP-1 .................................................................................................... 23

   3.2 ACUTE VS. CHRONIC Ad-5 INDUCTION OF SREBP-1 .............................................. 25

   3.3 Mechanism of Ad-5 Induction .......................................................................................... 31

   3.4 Induction of SREBP-1 through PKC λ/ζ/τ ........................................................................ 35

   3.5 PKC λ/ζ/τ AND AKT KNOCK DOWN IN HEPG2 CELLS ........................................ 40

   3.6 Ad-5 AND ADIPOGENESIS ............................................................................................ 42

   3.7 MECHANISM OF AD-5 AND GLUCONEOGENESIS .............................................. 43

4 DISCUSSION ................................................................................................................................................ 51

References ...................................................................................................................................................... 59

VITA .............................................................................................................................................................. 64
LIST OF FIGURES

Page

Figure 1: Summary of SREBP-1 and SREBP2 downstream targets ...................................5
Figure 2: SREBP-1a and SREBP2 domain structures ............................................................6
Figure 3: Ad-5 infection augments liver and plasma triglycerides .......................................27
Figure 4: Ad-5 infection induces SREBP-1 protein in infected liver tissue ..........................28
Figure 5: SREBP-1 and ACC are induced in Ad-5 infected livers .........................................29
Figure 6: Increased SREBP-1 is sustained in Ad-5 infected livers .......................................30
Figure 7: Ad-5 infection induces SREBP-1 in HepG2 cells .................................................33
Figure 8: The PI3K pathway if required for the optimal induction of SREBP-1 .................34
Figure 9: PKC λ/ζ/t and Akt activation in Ad-5 infected HepG2 cells .................................38
Figure 10: PKC λ/ζ/t is required for the optimal induction of SREBP-1 upon Ad-5 infection ..................................................................................................................39
Figure 11: Knock down of PKC λ/ζ/t and Akt .................................................................41
Figure 12: Ad-5 reduces blood glucose levels in infected mice ..........................................47
Figure 13: PEPCK expression is reduced in Ad-5 infected livers ........................................48
Figure 14: Akt activation in Ad-5 infected livers .................................................................49
Figure 15: Ad-5 infection augments expression of adiponectin in adipose tissue ............50
Figure 16: Model of Ad-5 infection .....................................................................................58
ABSTRACT

ADENOVIRUS-5 INFECTION AFFECTS LIPID METABOLISM IN HEPATIC AND ADIPOSE TISSUES

By Marianna Sukholutsky, M.S.

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Major Director: Suzanne E. Barbour, Ph.D.
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Our recent studies have shown a link between Adenovirus-5 (Ad-5) and elevated lipids, which prompted the hypothesis that Ad-5 infection might augment hepatic and/or adipose tissue lipid metabolism. To test our hypothesis, mice were infected with Ad-5 and screened for changes in lipogenesis and plasma markers associated with the metabolic syndrome. We observed increased expression of sterol regulatory element binding protein 1 (SREBP-1) in infected liver tissues, but not in adipose tissues and this correlated with elevated plasma and hepatic triglyceride levels. Elevated expression of adiponectin was seen in Ad-5 infected adipose tissues and this correlated with phosphorylated AMPK in infected liver tissues. These data suggested that the AMPK pathway was activated in livers of Ad-5 infected mice. Indeed, we observed reduced expression of PEPCK, a downstream target of AMPK in livers of Ad-5 infected mice. As PEPCK is an enzyme essential for gluconeogenesis, we hypothesized that Ad-5 infection would reduce blood sugar. Indeed, infected mice exhibited a transient decline in plasma glucose.

The increase in SREBP1 levels in Ad-5 infected hepatic tissues was evaluated by looking at Ad-5 infected HepG2 cells. Ad-5 is thought to mimic insulin’s actions in which the PI3K pathway is activated. We hypothesized that Ad5-induced SREBP-1 expression levels are mediated through the induction of PKC λ/ξ/τ, and not through Akt because it has been shown that PKC λ/ξ/τ mediates insulin-dependent lipogenesis. To test our hypothesis,
HepG2 cells were infected with Ad-5 and screened for downstream targets of the PI3K pathway. Through western blot analyses, we observed increased levels of phosphorylated PKC $\lambda/\zeta/\eta$. These results prompted the use of PKC pan inhibitor to see whether Ad-5 induced SREBP-1 levels would be down regulated. Indeed, with the presence of the PKC pan inhibitor, SREBP-1 expression levels were reduced.

Together, these studies suggest that Ad-5 induces changes in gene expression, glucose, and lipid metabolism; which prompts the hypothesis that this common respiratory pathogen may be associated with the Metabolic Syndrome, and this may preclude its use as a vector for gene therapy.
CHAPTER 1: INTRODUCTION

A group of metabolic risk factors referred to as Metabolic Syndrome (MS) were officially defined by the World Health Organization in 1998. MS is comprised of five risk factors which include obesity, high serum triglyceride levels, low serum high-density lipoprotein (HDL) cholesterol levels, hypertension, and increased fasting blood glucose levels. It has been noted that MS is a huge risk factor for diabetes, stroke, cardiovascular disease, infertility, cancer, liver disease, and cerebrovascular disease (Cornier et al., 2008). The underlying factor that contributes to MS is aberrant lipid metabolism that further plays a role in contributing to these metabolic diseases. The major focus of this study is to investigate alterations in lipid metabolism by looking at cellular signaling components in the liver, as well as in adipose tissue.

Studies have shown that genetic, behavioral, and environmental factors may all account as causative factors of dyslipidemia (So et al., 2005). Recent studies have associated viral, parasitic, and bacterial infections to changes in lipid metabolism, obesity, and fatty liver disease. Most importantly, viral infections have been overlooked as potential contributory factors affecting metabolic syndrome (So et al, 2005). It has been studied that enteroviruses, such as the coxsackie B4 virus, can infect human islet cells and trigger the destruction of β cells (Berg et al., 2007). This can lead to Type I diabetes, as well as to decreased levels of insulin secretion (Dotta F. et al., 2007). One of the most studied viruses associated with chronic liver disease, which exemplifies symptoms such as insulin resistance and visceral fat obesity is the Hepatitis C virus.
(HCV) (Saito et al., 2007). It is known that HCV infection leads to lipid accumulation in hepatocytes due to increased expression of lipogenic genes, which includes Sterol Regulatory Element Binding Protein 1 (SREBP-1) (Siddiqui et al., 2007).

1.1 STEROL REGULATORY ELEMENT BINDING PROTEINS

Sterol Regulatory Element Binding Proteins (SREBPs) are members of the basic-helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors that encode genes involved in triglyceride, cholesterol, as well as fatty acid synthesis (Figure 1) (Horton et al., 2002). Fatty acid synthesis associated genes such as fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC), as well as cholesterol associated genes such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) and squalene synthase are some of the genes regulated by mature SREBP (Eberle et al., 2004). There are three SREBP isoforms: SREBP-1a, SREBP-1c, and SREBP2. SREBP-1 has two alternative transcriptional start sites, which result in production of SREBP-1a and SREBP-1c. SREBP-1a can activate all SREBP-responsive genes, including genes for cholesterol, fatty acid, and triglyceride synthesis. SREBP-1c, on the other hand, is more specific and can only enhance transcription of genes responsible for fatty acid and triglyceride synthesis. SREBP2 is restricted to cholesterol synthesis and is the predominant form in the liver, along with SREBP-1c (Horton et al., 2002).

SREBPs are activated through a series of proteolytic processing steps. Once they are synthesized as inactive precursors of about 1150 amino acids, they are bound to the
endoplasmic reticulum (ER). The inactive precursor form of SREBP has three domains: an NH2-terminal domain that binds DNA, two hydrophobic transmembrane-spanning segments, and a COOH-terminal domain, which performs the essential regulatory function (Figure 2) (Goldstein and Brown, 1997). Processing of SREBP occurs with the help of three proteins, namely an escort protein called SREBP cleavage-activating protein (SCAP), and two proteases Site-1 protease (S1P) and Site-2 protease (S2P). Once the newly synthesized precursor SREBP is bound to the ER, its COOH-terminal regulatory domain binds to COOH-terminal domain of SCAP. SCAP is known to interact with insulin induced gene proteins (Insig), which help retain SCAP/SREBP complex in the ER (Eberle et al., 2004). SCAP not only acts as an escort of SREBP from the ER, but it is also a sensor of sterols in the cells. Therefore, once the cells are depleted of sterols, SCAP’s association with Insig decreases, and this allows SCAP to bring about translocation of SREBP from the ER to the golgi apparatus, in which S1P and S2P reside. S1P cleaves SREBP at the luminal loop between its two membrane-spanning segments, and consequently a second cleavage by S2P occurs in which the NH2-terminal domain (nuclear SREBP – nSREBP) is released. The “activated” nSREBP is then translocated to the nucleus, where it is able to bind to genes containing sterol response elements (SREs), thus promoting gene expression. If cells are in excess of sterols, then SCAP senses this in the cells and changes its conformation so that SCAP/SREBP complex is not translocated from the ER to the golgi apparatus because SCAP is unable to dissociate from Insig (Eberle et al., 2004). SREBP, therefore, cannot be cleaved by proteases residing in golgi apparatus, and thus transcription of target genes does not occur.
(Goldstein and Brown, 1999). Thus, the levels of sterols in the cells control the mature nuclear pool of SREBP in terms of expression at the precursor level, which then gets processed to its mature form. Overall, SREBP is essential in lipid metabolism and fatty acid synthesis in the liver.
SUMMARY OF SREBP-1C AND SREBP-2

Figure 1. Summary of SREBP-1 and SREBP-2 downstream targets (Horton et al., 2002).
Figure 2. SREBP-1a and SREBP2 domain structures (Goldstein and Brown, 1997).
1.2 CHARACTERIZATION OF ADENOVIRUS -5

As mentioned previously, several viruses elicit different responses such as targeting pancreatic islet cells leading to type I diabetes, or targeting the liver and leading to lipid accumulation and visceral fat obesity. Recently, immense amount of studies have focused on viral infections as a potential cause for changes in lipid metabolism that can lead to metabolic syndrome. It is of great interest to study adenovirus infection as a link to changes in lipid metabolism, as viral vectors are often used in gene therapy.

Adenovirus was first isolated and cultured from tonsils and adenoid tissue in 1953 (Shayakhmetov and Di Paolo, 2009). Currently, there are 52 identified human adenovirus serotypes, divided into six species (A-F). Adenovirus is commonly known to cause infections of the upper respiratory tract, as well as the gastrointestinal tract (Goncalves and Vries, 2006). There are many widely used applications for adenovirus vectors, but mainly they have been used for gene transfer studies. One of the main adenoviruses studied to date is Adenovirus-5 (Ad-5), which has been mostly used as a vector for gene delivery. It belongs to subgroup C of the adenoviruses, and commonly causes upper respiratory tract infections. It is a non-enveloped virus with double-stranded DNA of about 36 kilobases in size (Greber and Meier, 2004). Ad-5 has been proposed for use in human gene therapy, mainly because it can be easily produced in large quantities and large gene constructs can be inserted into the deleted regions of the virus (Young et al., 2006).

Ad-5 genes are divided into early (E) and late (L) regions. There are five early
genes, each of which is comprised of a transcriptional unit with sites for transcriptional initiation, termination, and splicing. The early genes are responsible for initiation of viral replication, as well as for expression of non-structural, regulatory proteins (Curiel et al., 2006; Templeton, 2008). The late genes are transcribed post beginning of DNA replication stage and are responsible for encoding the structural proteins for the virus. The E1A coding region of the adenovirus is required for transcription of early genes such as E1B, E2, and E4. This helps ensure that proteins required for viral replication are properly expressed. E2 and E4 regions promote expression of proteins necessary for late gene transcription, while the E3 region of the adenovirus codes for proteins that help the virus evade host defenses. The E1B region products are also able to transiently bind to apoptotic genes and inhibit the apoptotic pathway of the host cell ensuring that viral replication occurs (Templeton, 2008).

As mentioned earlier, Ad-5 of the group C adenoviruses, is the mostly commonly used adenovirus for transgene expression because large constructs can be inserted into its genome (Young et al., 2006). Ad-5 is rendered a replication deficient virus due to a deletion in the E1 coding region, as well as a partial or a complete deletion of the E3 region (McCaffrey et al., 2008). It is in the E1 and E3 deleted regions where the gene of choice is inserted. Numerous clinical trials have been conducted with the use of Ad-5 for transgene expression, but several issues with implementing this system have also occurred. In one of the earliest studies using Ad-5 mediated vector system complications occurred in which there was only a transient expression of the transgene, and almost all of the patients elicited an immune response, but had no benefit from the trial overall.
(Young et al., 2006). An explanation for this occurrence might be the fact that early in life, humans develop antibodies to group C adenoviruses, which include Ad-5, and thus the virus may potentially be neutralized (Young et al., 2006). Also, majority of the adenoviruses end up in the liver, and may not reach their target tissues; that is one of the biggest challenges presented with using Ad-5 as a vector is tissue specificity and delivery of the transgene (Bergelson and Zhang, 2005).

The structure of Ad-5 is quite complex and its attachment and internalization into non-target host cells presents a problem with using Ad-5 as a gene delivery system. Ad-5 has an icosahedral capsid, which contains viral DNA and core proteins, and is mainly composed of hexon protein. Each vertex of the icosahedron is capped by a penton base, from which an elongated trimeric penton fiber projects. The fiber has two ends, a proximal and a distal end. The proximal end is the end attached to the penton base, but it is the distal end that forms a globular “knob” which serves as an attachment site of adenoviruses to the host cell. The globular “knob” attaches to the Coxsackie and adenovirus receptor (CAR) with high affinity, which is followed by penton proteins, containing Arginine-Glycine-Aspartate (RGD) motif that interact with integrin co-receptors αv β3 and αv β5, and thus trigger intergrin-mediated endocytosis (Bergelson and Zhang, 2005). Once this process occurs, the virus enters the cell in a clathrin-coated vesicle, from which it escapes and goes to the cytoplasm and eventually enters the nucleus. This internalization of the adenovirus into the host cells has been shown to lead to activation of the PI3K pathway and subsequent activation of its downstream factors, such as Akt and PKC (Nemerow et al., 1998). Studies have also shown that virus
internalization and nuclear localization is rapid, occurring as fast as one-hour post infection (Wickham et al., 1993; Templeton, 2008).

It is suggested that adenovirus vectors elicit an early inflammatory response that is transcriptionally independent, meaning it is not due to viral gene transcription (Muruve, 2004). In clinical studies, it has been proven difficult to target the adenovirus vector to specific tissue types, mainly due to the fact that most adenovirus serotypes bind to the widely distributed CAR receptor. CAR serves as an attachment site for the adenovirus, while αv integrins are used for viral internalization into the cell. Also, since CAR is so widely distributed, it presents a challenging task for adenovirus-mediated gene therapy as the virus can target non-target tissues, and cause broad tissue tropism (Greber and Meier, 2004). However, in vivo mouse studies have revealed that adenovirus protein levels are two fold higher in the liver, compared to other organs such as the spleen, lungs, kidneys, stomach, and the heart. It was also concluded that the liver is the major organ to sequester adenovirus particles from the blood (Jiang et al., 2010). Since the liver is the major target for adenovirus infection, it is of great interest to study the effects adenovirus might potentially have on the liver and its surrounding organs.

Since Ad-5 has been widely used for gene therapy and it is known to target the liver, there have been several studies characterizing the effects of viral infections on liver and adipose tissues. Hepatitis C Virus (HCV) has been associated with liver cirrhosis, as well as hepatosteatosis (Saito et al., 2007). More importantly, it has been characterized to cause increased lipogenesis through the increase in expression of SREBP-1 and SREBP2 transcription factors (Siddiqui et al., 2009; Koike et al., 2010). Not only does HCV
infection cause altered lipid metabolism, but there is evidence which links adenovirus infection to aberrant lipid metabolism as well. One of the most prominent studies by Dr. Nikhil Dhurandhar focuses on the characterization of Adenovirus-36 (Ad-36) and its effects on adipose tissue functions. His studies lead to a belief that there is a correlation between Ad-36 infection and aberrant lipid metabolism, as he has shown that wild-type Ad-36 induces adiposity, leading to an accumulation of triglycerides in adipose tissue (Dhurandhar et al., 2004; Dhurandhar et al., 1999). As mentioned previously, FAS and ACC are downstream targets of SREBP-1, which are involved in the fatty acid synthesis pathway. It was shown that ACC and FAS levels are significantly increased in Ad-36 infected rat adipose tissues (Vangipuram et al., 2007). Atkinson’s group focused on studying adenovirus effects on adipose tissues as well, in which they demonstrate that Ad-37, but not Ad-2 increase adiposity in mice (Atkinson et al., 2005). Ad-36 and Ad-37 are of group D adenoviruses, while Ad-2 is of the group C human adenoviruses, which may potentially explain the difference in their findings. Another interesting finding by So et al, has revealed that Ad-5 increases adiposity (So et al., 2005). So far evidence points out that of group C adenoviruses, Ad-5, but not Ad-2, increase adiposity. First generation replication deficient Ad-5 is commonly employed as an expression vector for gene transfer, and is often used in gene therapy clinical trials (Muruve, 2004, Young et al., 2006). This may potentially become a problem in clinical trials, if replication deficient Ad-5 is linked to changes in lipid metabolism. Therefore, further studies need to be conducted to test whether Ad-5 may be a potential explanation for aberrant lipid metabolism in obese and diabetic individuals.
1.3 ADENOVIRUS-5 AND GLUCONEOGENESIS

As just mentioned, Ad-5 has been shown to be associated with increased adiposity, which prompts the hypothesis that it promotes aberrant or excessive adipogenesis. These events may lead to increased insulin secretion, or impaired lipid and glucose metabolism. It has been shown that Ad-36 acts as an adipogenic agent, increasing adiposity in infected chickens, mice, and rat animal models (Dhurandhar et al., 2000c). Proadipogenic genes such as fatty acid synthase and adiponectin are upregulated with Ad-36 infection in mice (Dhurandhar et al., 2008). As mentioned previously, fatty acid synthase is a target gene for SREBP-1, which is important in the fatty acid synthesis pathway. Adiponectin, on the other hand, is an “adipokine” produced by the adipose tissue that is essential in anti-diabetic and anti-inflammatory effects (Korbut et al., 2006).

Adipose tissue is thought of as not only a major organ for energy storage, but also as an important endocrine organ, which produces a number of hormones and cytokines that regulate insulin sensitivity (Haluzik et al., 2004). Some of these include leptin, adiponectin, resistin, which fall under the “adipokines” category, as well as some classical cytokines such as TNF-alpha, IL-6, and IL-1 that are most likely released by inflammatory cells which have infiltrated the adipose tissue (Korbut et al., 2006). Of all the bioactive mediators released from adipose tissue, leptin and adiponectin are most abundantly expressed within the adipose tissue, and have been the most studied.

Adiponectin, also known as adipocyte complement-related protein (Acrp30) is a 30kD in size protein, which can oligomerize into one of three major forms: a low
molecular weight (LMW) trimer, a medium molecular weight (MMW) 180kD hexamer, or a high molecular weight (HMW) 400-600kD multimer (Korbut et al., 2006; You et al., 2008). Interestingly, trimers have been shown to have the highest biological activity in adipose tissues (Korbut et al., 2006). Adiponectin consists of four domains including a signal peptide at the N terminus, a short variable region, a collagenous domain, and a C-terminal globular domain (Haluzik et al., 2008; You et al., 2008). In the blood, adiponectin levels are relatively high ranging from 5-10 ug/ml, which comprises about 0.01% of total plasma proteins (Korbut et al, 2006; You et al., 2008). There are two forms of adiponectin, a full-length adiponectin and a C-terminal globular adiponectin domain (gAd) that was generated from the full-length protein through a series of proteolytic processing steps. As there are two forms of adiponectin, there are also two major adiponectin receptors (You et al., 2008). They are structurally related integral plasma membrane proteins with seven membrane-spanning domains. The two receptors are termed adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2) (You et al., 2008; Korbut et al., 2006). AdipoR1 has a high affinity for the globular form of adiponectin and a low affinity for full-length adiponectin, whereas AdipoR2 is intermediate in its preference. Of the two receptors, AdipoR2 is primarily expressed in the liver, but AdipoR1 is mostly expressed in skeletal muscle (Korbut et al., 2006).

Until recently, not much was known about adiponectin and its signaling effects in adipose tissue as well as other target tissues. It is known that adiponectin is released by adipocytes and has major anti-diabetic properties, in which it regulates energy homeostasis and lipid metabolism (Musi et al., 2006). Obesity and type 2 diabetes have
been associated with decreased levels of adiponectin and it is known that adiponectin decreases insulin resistance, and suppresses hepatic glucose production (Mazzone and Fantuzzi, 2007). Adiponectin also acts as a major ‘adipokine’ and is related to several metabolic processes in the liver.

Adiponectin signaling has been associated with the activation of AMP-activated protein kinase (AMPK). Once adiponectin is released from adipose tissue, it interacts with adiponectin receptors (AdipoRs) in the liver and implements its effects on lipid metabolism by stimulating several signaling cascades including hepatic AMPK, PPAR-gamma coactivator alpha (PGC-1alpha), and SREBP-1 (Musi et al., 2006; Viollet et al., 2006). It has been suggested that in the liver AMPK is activated with full-length adiponectin. When adiponectin stimulates AMPK signaling, several downstream signaling events occur which bring out a reduction in lipid synthesis, and an indirect enhanced fatty acid oxidation (Kadowaki et al., 2002). So once AMPK is activated, it phosphorylates one of its downstream targets acetyl CoA carboxylase (ACC). Upon this phosphorylation, ACC becomes inactive and this results in a decrease in production of malonyl-CoA, which acts as an inhibitor of the carnitine palmitoyltransferase I (CPT-1). Once the inhibition of CPT-1 is relieved, it is able to transport fatty acids into the mitochondria for β-oxidation (Kadowaki et al., 2002; You et al., 2008; Violet et al., 2007). These events lead to lipid breakdown, and a decrease in lipid content in the liver. Not only does this signaling cascade increase lipid breakdown, but it also improves insulin sensitivity (Kadowaki et al., 2007).

AMPK activation also has affects on SREBP-1 expression in the liver. When
adiponectin activates AMPK in the liver, it leads to decreased expression of SREBP-1c mRNA and protein levels, which results in downregulation of genes regulated by SREBP-1c (Viollet et al., 2006). These genes primarily include ACC and FAS, which are lipogenic enzymes in the liver. Overall, this contributes to a reduction in hepatic lipid synthesis and has implications in insulin resistance, and dyslipidemia.

Adiponectin roles are not limited to reduction in hepatic lipid content, but also extend their role in gluconeogenesis through the activation of AMPK signaling cascade. It has been shown that upon activation of AMPK, there is a reduction in expression levels of molecules such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) in hepatocytes (Chung et al., 2010). These molecules are the rate-limiting enzymes involved in hepatic gluconeogenesis, and their decrease marks an overall decrease in hepatic glucose production (Viollet et al., 2006). Studies have shown that activation of AMPK mimics insulin’s effects on hepatic gluconeogenesis, in which a reduction in gluconeogenic genes is observed (Lochhead et al., 2000).

Reduction of gluconeogenesis via activation of AMPK is currently under study, but some research has shown that an important downstream factor of AMPK has emerged as a critical regulator of gluconeogenesis. Transducer of regulated CREB activity 2 (TORC2) has been shown to be the critical regulator of gluconeogenesis in mice (Viollet et al., 2006). It acts as an activator during fasting conditions, where it leads to recruitment of peroxisome proliferator-activated receptor-coactivator (PGC-1alpha), thus driving transcription of PEPCK and G6Pase in association with hepatocyte nuclear factor 4 (HNF4alpha) and the forkhead family activator FoxO1. AMPK was found to
phosphorylate TORC2, which promotes its binding to the 14-3-3 proteins. These events block TORC2 translocation to the nucleus, so it is sequestered in the cytoplasm and is unavailable for the activation of its downstream targets such as PEPCK and G6Pase (Viollet et al., 2006; Weickert and Pfeiffer, 2006). Interestingly, it is worth mentioning that there is evidence for repression of G6Pase during glucose starvation or treatment with aminoimidazole carboxamide ribonucleotide (AICAR) due to repression of cellular levels of FoxO1 protein (Barthel et al., 2002). FoxO1 is part of a family of forkhead transcription factors that behave similarly to TORC2, in which when phosphorylated they are sequestered from the nucleus through interaction with 14-3-3 proteins (Unterman et al., 2006). Further evidence suggests that once FoxO1 is sequestered in the cytosol, they are subjected to polyubiquitination and degradation (Carlsson and Mahlapuu, 2002). There is not a lot of evidence to confirm that AMPK directly phosphorylates FoxO1, thus driving the reduction of genes associated with gluconeogenesis, but of the research currently published, these events are suggestive (Fisslthaler et al., 2007).

Although FoxO1 is suggested to be directly phosphorylated through AMPK activation, it is also activated through the phosphoinositide 3-kinase/Akt (PI3K/Akt) pathway (Carlsson and Mahlapuu, 2002). Hepatic PI3K/Akt phosphorylates FoxO1, which contains 3 Akt phosphorylation sites. This results in FoxO1 exclusion from the nucleus with the help of 14-3-3 binding to the phosphorylated Akt site on FoxO1 and leading to masking of the nuclear localization sequence (Weickert and Pfeiffer, 2006). Overall, these events lead to a decrease in PEPCK and G6Pase gene expression.

The PI3K/Akt pathway is not only important in gluconeogenesis in which it can
dampen expression of gluconeogenic genes, but it is also a crucial pathway in lipid metabolism. Immense amount of research has been conducted to link insulin regulation of hepatic lipid metabolism through the induction of SREBP-1c. Insulin induces hepatic SREBP-1c expression through the PI3K/Akt pathway, leading to increased synthesis of fatty acids and triglycerides (Taniguchi et al., 2006). Effectors of the PI3K pathway include Akt and the atypical protein kinase C (PKC λ/ζ/ι), which are both essential for insulin-stimulated lipid synthesis (Taniguchi et al., 2007). The mechanism of how PI3K pathway generates the upregulation of SREBP-1c is not well understood, but it has been shown that Akt is responsible for mediation of insulin-dependent hepatic glucose metabolism, while PKC λ/ζ/ι has been linked to the insulin-dependent induction of expression of SREBP-1c (Taniguchi et al., 2006).

The liver is an organ that plays a crucial role in regulation of glucose and lipid metabolism. On the molecular level, these mechanisms all work together to create a balance between lipid breakdown and accumulation. Along with the liver, adipose tissue has emerged as an important endocrine organ that plays a role in not only energy storage, but also in production of hormones that can act as ‘adipokines’ to regulate cellular processes in the liver. Ad-5 has been linked to aberrant lipid metabolism, as well as to tropism to the liver. It is based on the aforementioned studies and research that we attempted to characterize the effects of Ad-5 infection on metabolism in hepatic and/or adipose tissues.
HYPOTHESIS

Based on the aforementioned studies and previous studies conducted in our lab, we hypothesize that Ad-5 infection induces changes in gene expression, glucose, and lipid metabolism. Here we investigate the role of Ad-5 in liver and adipose tissues, and try to characterize the mechanism of Ad-5 in aberrant lipid metabolism. It is suggestive that this common respiratory pathogen may be associated with the Metabolic Syndrome, and this may preclude its use as a vector for gene therapy.
CHAPTER 2: METHODS AND MATERIALS

Cell Culture - Human hepatoma cells (HepG2 cells) obtained from ATCC were maintained in Minimum Essential Medium (MEM) supplemented with 10% heat inactivated fetal calf serum, 2mM glutamine, 1% penicillin/streptomycin, 1mM nonessential amino acids, and 0.1mM sodium pyruvate. All cells were maintained at 37°C at 7.5% CO₂ humidity.

Infection of HepG2 cells - HepG2 cells were plated at 5 x 10⁶ cells/well in 6 well plates. Following adherence to the plates, HepG2 cells were infected with Ad-5 virus at multiplicity of infection (MOI) of 15 and harvested up to 72 hours post infection. For cells not harvested at 16 hours post infection, the virus was removed and cells were replenished with fresh media 16 hours post infection for harvesting at later time points.

PKC zeta inhibition - HepG2 cells were plated at 5 x 10⁶ cells/well in 6 well plates. Following adherence to plates, cells were pre-treated with myristoylated PKC zeta peptide inhibitor at 25uM per well for 1 to 2 hours. Following pre-treatment, cells were then infected with Ad-5 at MOI of 15. Virus and PKC zeta inhibitor containing media was removed 16 hours post infection and cells were replenished with fresh media or with fresh PKC zeta inhibitor. Cells were then harvested at 72 hours post infection.
**PKC inhibition using siRNA** – siRNA oligos against PKCi were obtained from Ambion. The sense sequence was 5’-GAGACCUAAUGUUUCAUAUtt-3’, while the antisense was 5’-AUAUGAAACAUUAGGUCUCct-3’. Silencer Validated siRNA, Silencer negative siRNA control were purchased from Ambion. HepG2 cells were plated at $5 \times 10^6$ cells/well in 6 well plates and transfected with validated siRNA and negative siRNA control at final concentration of 100nM using siPORTNeoFX Transfection Agent according to manufacturer’s instructions (Ambion). Plates were incubated at 37°C until harvest. Cells were harvested at 24 and 48 hours post transfection, using RIPA buffer. Cells harvested at 72 hours post transfection were re-transfected at 48 hours following the same protocol, and harvested at 72 hours post transfection.

**Adenovirus Infection** - C57BL/6 mice were infected by tail-veil injection with $1 \times 10^{11}$ particles (~3.2 x $10^8$ PFU) of first generation replication-deficient adenovirus-5 which lacks E1A, E1B and E3 regions. Mice were then sacrificed at indicated times post-infection. Adipose and liver tissues were extracted and flash frozen.

**Adipose and Liver Tissue Homogenate** - Mouse adipose and liver tissue homogenates were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Piez Biotechnology). Livers and adipose tissue were flash frozen post extraction. Liver and adipose tissue homogenates were prepared using ~15mg of tissue. Mouse livers were disrupted using a dounce homogenizer in a buffer containing 10mM HEPES (pH 7.4),
10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 50mM NaF, 1mM activated Na$_3$VO$_4$, 30mM Na$_4$P$_2$O$_7$, 20mM 2-glycerophosphate, and 1% SDS with phosphatase inhibitor cocktail I (Sigma) and a complete protease inhibitor cocktail (Roche).

**HepG2 Cell Homogenates** - HepG2 cells were incubated with calpain inhibitor I (25ug/ml) for two hours prior to harvest. Cell homogenates were prepared in a buffer containing 20mM Tris-HCl (pH 7.5), 50mM NaCl, 50mM NaF, 30mM Na$_4$P$_2$O$_7$, 20mM 2-glycerophosphate, 1mM activated Na$_3$VO$_4$, 5mM EDTA, 2mM EGTA, pefabloc 100mg/ml, PMSF 0.1M, and a complete protease inhibitor cocktail (Roche).

**Protein Analysis by Immunoblot** - Proteins were separated by 10% SDS-PAGE and immunoblotted with antibodies such as pan p- PKC $\lambda/\zeta$, total PCK $\lambda/\zeta$, SREBP1, p-AMPK, total AMPK, ERK1/2, GAPDH, HDAC1, or actin. Signals were detected with HRP-coupled secondary antibodies (Pierce) and developed with chemiluminescent detection system from Amersham. Signals were quantified using densitometric analysis (Alpha Inotech FC8800).

**qPCR Analysis** – Mouse liver RNA was extracted using SV Total RNA Isolation System (Promega). mRNA encoding PEPCK, G6Pase, SREBP1, SREBP2 and ACC were quantified by SYBR Green qPCR using the Brilliant QRT-PCR Core Reagent Kit, 1-Step (Stratagene). Target gene expression was normalized to 18S RNA (control), quantified with TaqMan, One Step RT-PCR Master Mix Reagents were also used (Applied Biosystems).
Primers used were as follows:

PEPCK Reverse: 5’-CTTCACCTGAGGTCCAGGAG-3’
PEPCK Forward: 5’-CTAACTTGCCATGATGAAACC-3’
SREBP1 sense: 5’-CAGGAAGCAAGCTGAATAATCTG-3’
SREBP1 antisense: 5’-AGTGATTTTGCTTTTGTGTCGACTT-3’
SREBP2 sense: 5’-CAAGAGAAAGTTCCATCAAGCAAGTG-3’
SREBP2 antisense: 5’-GTCCTTCAACTCTATGATTTTGTGCTGT-3’
ACC sense: 5’-AGGATTTGCTGTTTTCAGAGCTT-3’
ACC antisense: 5’-CAGGATCTACCCAGGCCACAT-3’
G6Pase Reverse: 5’-ACAGGTGACAGGGAACGTGCT-3’
G6Pase Forward: 5’-TCGGAGACTTGTTCAACCTC-3’
18S sense: 5’-AAAATTAGAGTGTCCAACAGCGGC-3’
18S antisense: 5’-CCTCAGTTCCGAAAACCAACAA-3’
18S probe: 5’-/56-FAM/CGAGCCGCCTGGATACCGCAGC/36-TAMSp/-3’

**Plasma analyses** - Plasma lipids and glucose were measured by the Diagnostic Laboratories of the McGuire VA Hospital, Richmond, VA. Thank you so much to Dr. W. Michael Pandak, who generously arranged for these analyses.

**Statistical Analysis** - Data were analyzed with two-sided Student’s t-test with p < 0.05 taken as the limit for statistical significance. Some *in vitro* experiments were replicated at least three times.
CHAPTER 3: RESULTS

There have been numerous studies conducted implicating a correlation between viral infection and aberrant lipid metabolism. Specifically, studies have shown that upon infection with wild-type Adenovirus-36 (Ad-36), mice exhibit an increase in body mass index due to accumulation of triglycerides (Dhurandhar et al., 2004). More importantly, Ad-36 infected rat adipose tissues have elevated FAS and ACC levels, which are important downstream targets of SREBP-1, and are essential in the fatty acid synthesis pathway (Vangipuram et al., 2007). Also, Adenovirus-5 (Ad-5) has been shown to increase adiposity, which has led us to hypothesize that replication deficient Ad-5 plays a role in lipid metabolism (So et al., 2005). Therefore we wanted to investigate the role of Ad-5 in liver and adipose tissues, and characterize the mechanism by which it brings about aberrant lipid metabolism. We hypothesized that Ad-5 infection would increase hepatic lipid synthesis and genes associated with lipid metabolism.

3.1 AD-5 INDUCES SREBP-1

One of the first indications we received about the role of Ad-5 and its link to lipid metabolism was upon infection of C57BL/6 mice with replication deficient Ad-5. Mice were sacrificed 4- and 5-days post infection, and livers and plasma were screened for genes associated with lipid metabolism. Triglyceride levels in the plasma, as well as in the liver were significantly elevated in day-4 infected mice compared to uninfected mice (Figure 3A
and 3B). On day-5 post infection, there was a significant increase in liver triglyceride levels, but not in the plasma (Figure 3A and 3B). Cholesterol levels were also analyzed, and showed no change on days 4 and 5 when compared to uninfected mice in both plasma and liver tissue (Figure 3A and 3B). An increase in triglycerides, but not cholesterol levels is an indication that there may be changes in genes associated with lipid metabolism. One of these genes is Sterol Regulatory Element Binding Protein-1 (SREBP-1), which is involved in synthesis of fatty acids and triglycerides. We wanted to see whether SREBP-1 levels are increased in Ad-5 infected livers, which would be an indication that the increase in triglyceride levels upon Ad-5 infection is mediated through SREBP-1c. We screened for SREBP-1 protein in liver homogenates, and observed an increase in SREBP-1 precursor (125kd) levels in infected livers compared to uninfected livers, suggesting that Ad-5 induces SREBP-1 expression levels, thus driving an increase in triglyceride synthesis in infected mice (Figure 4). To confirm our results that SREBP-1 protein is significantly increased in Ad-5 infected mice, we performed real time PCR (qPCR) to measure SREBP-1 transcripts in livers of infected and uninfected mice. SREBP-1 transcripts are significantly elevated in day-4 livers of infected mice, but this effect is transient as the levels begin to decline on day-5 post infection (Figure 5A). To reiterate the importance of SREBP-1, we also measured acetyl CoA-carboxylase (ACC) transcript levels, as ACC is a downstream target of SREBP-1 and is involved in synthesis of fatty acids and triglycerides. We observed a significant increase in ACC transcript levels on day-4 and -5 post infection, suggesting that Ad-5 infection leads to an increase in transcriptionally active
mature form of SREBP1 protein (Figure 5A). Overall, the transient elevation in triglyceride levels observed is most likely due to the fact that SREBP-1 is transiently induced in Ad-5 infected mice.

3.2 ACUTE VS. CHRONIC AD-5 INDUCTION OF SREBP-1

Since SREBP-1 levels are transiently increased in Ad-5 infected mice, we wanted to determine if the response to Ad-5 is acute rather than chronic. We looked at SREBP-1 expression levels at later time points post infection. Livers of Ad-5 infected mice sacrificed on day-4, -7, -10, and -14 post infection were screened for SREBP-1 transcripts using qPCR. Compared to uninfected livers, there is an increase in SREBP-1 expression in day-4 infected livers, but this increase is transient, as we observe a decline in levels of SREBP-1 on day-7, -10, and -14 post infection compared to the uninfected livers (Figure 6A). This suggests that the effect of Ad-5 on lipid metabolism and SREBP-1 is more prominent on day-4 post infection, and not the later time points. This is most likely due to the fact that the mice were only subjected to one infection of Ad-5, and since Ad-5 is a replication incompetent virus, it can only undergo one round of infection. However, we also observed a sustained increase in active mature SREBP-1 in nuclear extracts of these infected livers as early as day-7 post infection and up until day-14 post infection (Figure 6B). These results suggest that the SREBP-1 mRNA levels are transiently expressed with Ad-5 infection, but the mature SREBP-1 protein is stably expressed at least until day-14 post infection. Also, these results show that Ad-5 induces chronic increase in nuclear pool
of SREBP-1, suggesting a chronic effect on systemic lipid metabolism. This is somewhat surprising, as Ad-5 is a replication incompetent virus and is only able to have one round of infection, but this one round appears to have lasting effects on systemic lipid metabolism.

Our results showed no difference in plasma and liver cholesterol levels in infected mice compared to uninfected mice, so we screened these mice for SREBP-2 transcript levels using qPCR, as SREBP-2 is a transcription factor that is responsible for activation of enzymes involved in synthesis of cholesterol. SREBP-2 transcript levels were not significantly changed in Ad-5 infected mice either on day-4 or day-5 post infection (Figure 5B). These results correlate with the lack of changes seen in cholesterol at the plasma and hepatic levels.
AD-5 INFECTION AUGMENTS PLASMA AND LIVER TRIGLYCERIDE

Figure 3. Quantification of triglycerides and cholesterol in control (uninfected) or Ad-5 infected mice. (A) Plasma lipids, expressed in mg/dl. (B) Liver lipids, expressed as mg per g of liver. * p < 0.05 compared to control. n=4 mice in each group. This data is provided by courtesy of Dr. Palmer Wilkins (PostDoc in Department of Biochemistry).
**AD-5 INFECTION INDUCES SREBP-1 PROTEIN IN INFECTED LIVER TISSUE**

**Figure 4.** Liver homogenates from control and Ad-5 mice sacrificed day-4 post infection were subjected to western blot analysis and probed for SREBP-1 (~125 kD precursor form). SREBP-1 signal was normalized to actin. The antibody used detects both forms of SREBP-1 – SREBP-1a and SREBP-1c; but mostly SREBP-1c in the liver. * p < 0.05 compared to control. This figure is provided by courtesy of Dr. Palmer Wilkins (Post Doc in Department of Biochemistry).
SREBP-1 and ACC ARE INDUCED IN AD-5 INFECTED LIVERS

**Figure 5.** Real time qPCR quantifying mRNA expression level of (A) SREBP-1 and ACC. SREBP-1 and ACC are significantly increased on day-4; ACC is significantly increased on day-4 and -5. (B) Quantification of SREBP-2 mRNA. SREBP-2 is not significantly different from control on either day-4 or -5. * p < 0.05 compared to control. n=4 mice in each group. This data is provided by courtesy of Dr. Palmer Wilkins (PostDoc in Department of Biochemistry).
INCREASED SREBP-1 IS SUSTAINED IN AD-5 INFECTED LIVERS

Figure 6. (A). Quantification of hepatic SREBP-1 mRNA expression levels. SREBP-1 is transiently expressed on day-4 post infection. (B). Nuclear extracts were subjected to western blot analysis and probed for nuclear SREBP-1 (~65kD in size). SREBP-1 protein is sustained up until day-14 post infection. n=1.
3.3 MECHANISM OF AD-5 INDUCTION

Our next goal was to characterize the mechanism by which Ad-5 induces SREBP-1, leading to an overall increase in triglyceride synthesis. To do this, we wanted to employ a human cultured cell line that could be readily manipulated, both genetically and with small molecule inhibitors. This cell line is the hepatocellular carcinoma cell line (HepG2 cells), which comes from the liver. Our first step was to see if we can replicate the results we observed in Ad-5 infected mice to determine whether SREBP-1 is directly induced as a result of the Ad-5 infection. HepG2 cells were infected with Ad-5 at multiplicity of infection (MOI) of 15, and then harvested at 72 hours post infection. Cell homogenates were subjected to western blot analysis, and then screened for SREBP-1 protein. We observed that SREBP-1 was significantly increased in Ad-5 infected HepG2 cells (Figure 7). This was an indication that we could use HepG2 cells to elucidate the mechanism of Ad-5.

It is known that insulin induces hepatic SREBP-1, but not SREBP-2 expression levels through the induction of the PI3K pathway, leading to an overall increase in fatty acid synthesis (Taniguchi et al., 2006). HCV infection has also been shown to mediate its effects through the PI3K pathway (Banerjee et al., 2010). More importantly, it has been pointed out that Ad-5 brings about activation of the PI3K pathway upon internalization into its target cells (Nemerow et al., 1998). Based on these observations, we hypothesized that Ad-5 mediates its effects through PI3K, and induces SREBP-1 expression.
In order to look at the mechanism by which Ad-5 induces SREBP-1, we wanted to employ inhibitors of PI3K. PX-866 is a wortmannin analog that inhibits the p110 alpha unit of PI3K, thus preventing activation of its downstream targets such as Akt and PKC. LY924002 is another PI3K inhibitor that acts as a competitive inhibitor by competing for the ATP binding site on PI3K (Howes et al., 2007). Although LY294002 has been shown to cause high hepatotoxicity, we still wanted to employ both of the inhibitors in our study, but LY294002 was used in lower concentrations.

Our first step was to see if inhibition of PI3K, using either of the two inhibitors would suppress Ad-5 induction of SREBP-1. HepG2 cells were pretreated for 2 hours with either the PX-866 or LY294002 inhibitors at concentrations of 15uM and 30uM, and 10uM and 20uM, respectively. Following pretreatment, cells were infected with Ad-5 at MOI of 15 and harvested at 72 hours post infection. Again, cell homogenates were subjected to western blot analysis, and probed for SREBP-1. With treatment of PX-866 at 15uM, we observed about 42% suppression of Ad-5 induced SREBP-1, while at 30uM of PX-866, there was about 52% suppression of Ad-5 induced SREBP-1 levels (Figure 8). Similar results were obtained with the use of LY294002 at 10uM, in which we see 40% suppression of SREBP-1, and at 20uM there is about 45% suppression of Ad-5 induced SREBP-1 (Figure 8). Overall, we concluded that PI3K is required for the optimal induction of SREBP-1. These results implicate that Ad-5 mediates its effects through the activation of PI3K, thus bringing about an increase in SREBP-1.
AD-5 INFECTION INDUCES SREBP-1 IN HEPG2 CELLS

**Figure 7.** HepG2 cells were infected with Ad-5 at MOI of 5 or 15 and harvested 4-days post infection. Homogenates were subjected to western blot analysis and probed for SREBP-1 (precursor form ~125kD in size). SREBP-1 is significantly increased in MOI 5 or MOI 15 infected HepG2 cells compared to uninfected cells. SREBP-1 protein was normalized to actin. Fold change in SREBP-1 from three different experiments. * p < 0.05 compared to uninfected. This figure is provided by courtesy of Dr. Palmer Wilkins (Post Doc in Department of Biochemistry).
THE PI3K PATHWAY IS REQUIRED FOR THE OPTIMAL INDUCTION OF SREBP-1

**Figure 8.** HepG2 cells were treated either with PX866, LY294002, or DMSO vehicle at indicated concentrations. Two hours post treatment, cells were infected with Ad-5 at MOI of 15 and harvested 72 hours post infection. Homogenates were subjected to analysis and probed for SREBP-1 (precursor form ~125kD in size). SREBP-1 was normalized to actin. Percent inhibition of induced SREBP-1 are as indicated. n=2. This figure is provided by courtesy of Dr. Palmer Wilkins (Post Doc in Department of Biochemistry).
3.4 INDUCTION OF SREBP-1 THROUGH PKC λ/ζ/λ

So far we have observed an increase in SREBP-1 in Ad-5 infected HepG2 cells, as well as a suppression of induced levels of SREBP-1 with the use of a PI3K inhibitor, either PX-866 or LY294002. Our next goal was to see whether Ad-5 promotes activation of downstream effectors of PI3K pathway. These downstream effectors include Akt and the atypical PKC λ/ζ/λ. One way to look at the activation of these effectors is to look for their phosphorylated levels. We infected HepG2 cells with Ad-5 at MOI of 15, and then harvested them at 72 hours post infection. We screened cell homogenates for phosphorylated levels of Akt, as well as the atypical PKC λ/ζ/λ. Both Akt and PKC λ/ζ/λ are activated with Ad-5 infection (Figure 9A and 9B). PKCλ/ζ/λ is phosphorylated as early as 20 minutes post infection, but begins to decline at around 24 hours post infection (Figure 9A). Akt is also phosphorylated as early as 20 minutes post infection, but this phosphorylation is sustained up until 72 hours post infection (Figure 9B). These results strengthen our hypothesis that Ad-5 brings about activation of PI3K and its downstream targets. This was another indication that Ad-5 mediates its effects through the PI3K pathway, thus inducing Akt and PKCλ/ζ/λ.

Up until now, our data points out that Ad-5 selectively induces SREBP-1 and brings about an increase in hepatic and lipid triglyceride levels, and this is potentially conducted through the induction of PI3K and its effectors Akt and PKCλ/ζ/λ. PKCλ/ζ/λ is essential for insulin-stimulated lipid synthesis. The mechanism of how PI3K pathway generates the
upregulation of SREBP-1 is yet not well understood, but it has been shown that Akt is responsible for mediation of insulin-dependent hepatic glucose metabolism, while PKC \(\lambda/\zeta/t\) has been linked to the insulin-dependent induction of expression of SREBP-1 (Taniguchi et al., 2006). Since we observed activation of both Akt and PKC\(\lambda/\zeta/t\), perhaps we could delineate the mechanism of Ad-5 and potentially explain the role Akt and PKC\(\lambda/\zeta/t\) play in inducing SREBP-1 and its targets.

With the use of PI3K inhibitors, we were able to demonstrate that PI3K is needed for the optimal induction of SREBP-1 upon Ad-5 infection. Work conducted by Dr. Kahn delineates the roles the effectors of the PI3K pathway play in hepatic metabolism (Taniguchi et al., 2007). Since PKC \(\lambda/\zeta/t\) is important in regulation of insulin-dependent induction of SREBP-1, we wanted to explore this further in our study by using a specific inhibitor of PKC \(\lambda/\zeta/t\). One such specific inhibitor is myristoylated PKC\(\lambda/\zeta/t\) peptide inhibitor. It acts as a pseudosubstrate peptide to inhibit PKC\(\lambda/\zeta/t\) activity. We took HepG2 cells and pretreated them with 25uM of the inhibitor for 2 hours, followed by Ad-5 infection at MOI of 15. Next day, cells were replenished with new media or with the inhibitor. At 72 hours post infection, cells were harvested and subjected to analysis. Inhibition of PKC \(\lambda/\zeta/t\) suppressed SREBP-1 expression levels by about 60% in Ad-5 infected HepG2 cells (Figure 10). These results are comparable to the effects we see with the use of a specific PI3K inhibitor, in which we observe about 50% suppression of Ad-5 induced SREBP-1 levels (Figure 8). Our results not only strengthen our hypothesis that
Ad-5 mediates its effects through PI3K and PKCζ/ι, thus inducing SREBP-1, but it also supports work by Dr. Kahn in which they have shown active PKCζ/ι induces SREBP-1 levels in WT mice (Taniguchi et al., 2006).
PKC\(\lambda/\zeta\) and AKT ACTIVATION IN AD-5 INFECTED HEPG2 CELLS

A.

![Graph showing phosphorylated levels of PKC over time](image)

**Figure 9A** and 9B are representative of three different experiments. Figure 9B is provided by Dr. Palmer Wilkins (PostDoc in Biochemistry Department, VCU).

---

B.

![Graph showing phosphorylated levels of ACT over time](image)

**Figure 9**. Ad-5 infected hepatic homogenates were screened for phosphorylated levels of (A) PKC and (B) AKT. (A) Phosphorylated levels of PKC are increased as early as 20 minutes post infection, but begin to decline at around 12 hours post infection. (B) Phosphorylated levels of Akt are elevated as early as 20 minutes and this increase is sustained at 72 hours post infection. Figure 9A and 9B are representative of three different experiments.
**PKCα/λ/ζ is REQUIRED FOR THE OPTIMAL INDUCTION OF SREBP-1 UPON AD-5 INFECTION**

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**Figure 10.** HepG2 cells were pretreated with myristoylated PKC pan inhibitor, then infected with Ad-5 at MOI of 15. HepG2 cells were harvested at 72 hours post infection, subjected to western blot analysis and probed for SREBP-1 (precursor form ~125kD in size). Induced SREBP-1 levels are suppressed by about 100% with the presence of the PKC pan inhibitor. Percent inhibition relative to control. n=2.
3.5 PKC \(\lambda/\zeta/\iota\) AND AKT KNOCK DOWN IN HEPG2 CELLS

Although inhibition of PKC\(\lambda/\zeta/\iota\) resulted in suppression of Ad-5 induced SREBP-1 levels, we wanted to employ a different method since we had several problems with using the myristoylated PKC\(\lambda/\zeta/\iota\) inhibitor. In order to do that, we turned to using siRNA targeting PKC\(\lambda/\zeta/\iota\). We were able to successfully knock down expression of PKC\(\lambda/\zeta/\iota\) by about 60% using siRNA targeting PKC\(\lambda/\zeta/\iota\) (Figure 11A). We also employed siRNA targeting Akt I and II and were able to knock down the expression of Akt by about 40% in HepG2 cells (Figure 11B).

Once we had established the protocol for knocking down PKC\(\lambda/\zeta/\iota\) and Akt in HepG2 cells, we were ready to infect HepG2 cells with Ad-5 following the use of siRNA. Unfortunately, we ran into a problem with our stock of Ad-5, and are currently working on resolving these issues. So far, that is our last step to delineate the mechanism by which Ad-5 brings about activation of SREBP-1, thus altering lipid metabolism. We believe that once we knock down PKC\(\lambda/\zeta/\iota\) using siRNA, and then infect cells with Ad-5, we will be able to suppress Ad-5 induced SREBP-1, and demonstrate that this is mediated through PKC\(\lambda/\zeta/\iota\) and not Akt, because we believe that with the knock down of Akt, Ad-5 induced levels of SREBP-1 would not change. Therefore, we will be able to show the mechanism by which Ad-5 induces SREBP-1.
KNOCK DOWN OF PKCγ/λ/ζ and AKT

A.

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<th>48h</th>
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<td>PKC siRNA</td>
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PKC

ERK 1/2

1 | 0.54 | 1 | 0.47 | 1 | 0.46

B.

<table>
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Akt

Actin

1 | 0.57 | 1 | 0.69

Figure 11. HepG2 cells were subjected to knockdown of (A) PKCγ/λ/ζ or (B) Akt.
3.6 AD-5 AND GLUCONEOGENESIS

A lot of research has been conducted showing that Ad-36 acts as an adipogenic agent, increasing adiposity in chickens, rats, and mice (Dhurandhar et al., 2000). Dhurandhar et al. group has also shown that Ad-36 upregulates genes such as FAS and adiponectin (Dhurandhar et al., 2008). Our group wanted to study whether the replication incompetent Ad-5 would have similar effects on adipogenesis.

Our first indication that Ad-5 may play a role in adipogenesis was by measuring blood glucose levels of Ad-5 infected mice. Compared to uninfected mice, mice infected with Ad-5 and sacrificed 3-days post infection exhibited significantly lower amounts of plasma glucose (Figure 12). This cannot be said for glucose levels measured in day-2 infected mice, as those levels were only slightly lower than that of uninfected mice (Figure 12). This prompted us to look at genes associated with gluconeogenesis, as glucose levels are significantly decreased in day-3 Ad-5 infected mice. One of the critical enzymes in the gluconeogenesis pathway is PEPCK (Viollet et al., 2006). Livers of Ad-5 infected mice sacrificed 2- and 3-days post infection were screened for PEPCK expression using qPCR. Compared to uninfected livers, there is a slight decrease in PEPCK expression in day-2 mice, but there is a significant decrease in PEPCK expression in day-3 infected mice (Figure 13). This correlates with a significant decrease in glucose levels in day-3 infected mice (Figure 12). These results prompted us to look at the mechanism by which Ad-5 potentially decreases gluconeogenesis.
3.7 MECHANISM OF AD-5 AND GLUCONEOGENESIS

Since we have observed decreased blood glucose levels, as well as decreased PEPCK expression levels in Ad-5 infected mice, we were prompted to look at the mechanism by which Ad-5 mediates these effects. We wanted to look at the PI3K pathway again, because the effects of PI3K have different roles upon activation. Akt and PKC\(\lambda/\zeta/\iota\) are both effectors of the PI3K pathway that are activated upon Ad-5 infection. Their roles diverge upon PI3K activation in which both Akt and PKC\(\lambda/\zeta/\iota\) serve as distinct mediators in lipid and glucose homeostasis. PKC\(\lambda/\zeta/\iota\) is involved in insulin-dependent induction of SREBP-1, while Akt is involved in insulin-dependent hepatic glucose metabolism (Taniguchi et al., 2006). Since we have already established that Ad-5 drives the induction of SREBP-1 through PKC\(\lambda/\zeta/\iota\), we wanted to look further at the effects of Ad-5 activation of Akt and its role in glucose metabolism. It has been shown that knock down of Akt activity leads to glucose intolerance, and increased expression of gluconeogenic genes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) (Yang et al., 2004). More importantly Akt suppresses gluconeogenesis via phosphorylation of FoxO1 (Carlsson and Mahlapuu, 2002). Taken together, these evidences prompted our hypothesis that Ad-5 activation of Akt brings about suppression of gluconeogenesis.

Our first step in looking at the mechanism by which Ad-5 mediates reduced blood glucose levels was by looking at Akt activation. We have observed Akt activation with
Ad-5 infection in HepG2 cells (Figure 9B), but we wanted to see if the same would hold true when we looked at Akt phosphorylation in Ad-5 infected livers. Activation of Akt in Ad-5 infected livers is about 2-fold higher than in uninfected control livers (Figure 14). Despite an elevation in total mass of Akt, phosphorylated levels of Akt are significantly increased in Ad-5 infected livers, suggesting that Akt activation could be the path forward to delineating the mechanism by which reduced blood glucose is observed in Ad-5 infected mice. This would also support Dr. Kahn’s research, which points out that Akt is responsible for mediating insulin-induced glucose metabolism (Taniguchi et al., 2006).

Overall, we were prompted to look at Akt downstream targets that are involved in glucose metabolism. One such target is FoxO1. It is known that once FoxO1 is phosphorylated, it is sequestered from the nucleus in the cytoplasm, and therefore is unavailable for activation of gluconeogenic genes (Weickert and Pfeiffer, 2006). So our next goal was to look at FoxO1 expression levels in Ad-5 infected and uninfected livers. Unfortunately, much of our attempts were unsuccessful, and we were unable to target FoxO1 in Ad-5 infected and uninfected livers. We attempted to probe for FoxO1 in nuclear extracts, cytosolic extracts, and whole cell extracts, but our attempts were unsuccessful. This posed a problem, because we wanted to determine if indeed activation of Akt brings about sequestration of FoxO1 in Ad-5 infected livers.

Since we were unable to probe for FoxO1 in Ad-5 infected livers, we wanted to look at an alternative novel mechanism of AMPK. Research has suggested that AMPK can
directly phosphorylate FoxO1 and bring about a decrease in gluconeogenic genes such as PEPCK and G6Pase (Barthel et al., 2002). So our next step was to look at phosphorylated levels of AMPK in the liver. This turned out to be more doable as we observed an increase in p-AMPK levels in Ad-5 infected mice (data not shown). Although the increase is modest, it implicates that even the slightest changes in p-AMPK can bring about changes to overall glucose levels.

Next, we wanted to explore a mechanism by which AMPK gets activated. Research conducted by Dhurandhar et al., suggests that Ad-36 infected adipose tissue has increased transcript levels of adiponectin (Dhurandhar, et al., 2008). Adiponectin is an adipokine produced in adipose tissue that is usually associated with decreased adiposity. In findings of Dhurandhar et al., Ad-36 infection promotes the increase in adiponectin, and is associated with an activation of adipogenesis and increased glucose disposal (Rogers et al., 2008). Since our data shows decreased levels of glucose on day-3 post infection (Figure 12), we wanted to look at adiponectin levels in Ad-5 infected livers to determine whether there is a link between adiponectin activating AMPK, thus bringing about changes in blood glucose. Thankfully, we were able to send our samples to Dr. Dhurandhar to screen for mRNA transcripts of adiponectin. Based on our preliminary results, there is a trend towards increase in adiponectin in Ad-5 infected adipose tissue compared to uninfected tissue (Figure 15). This was an indication that adiponectin gets released from adipose tissue, binds to its receptor on the liver, and brings about activation of AMPK, which has
further implications in glucose metabolism.

Overall, we believe that there are two working mechanisms that drive the reduction of glucose. One of these mechanisms is the activation of Akt, which then brings about phosphorylation of FoxO1, and a decrease in PEPCK expression, while the other mechanism is more indirect in which adiponectin gets released from adipose tissue and acts on the liver by activating AMPK, which phosphorylates FoxO1, thus decreasing the expression of PEPCK. These two mechanisms converge at FoxO1, and may potentially work together to reduce gluconeogenic gene expression and decrease overall blood glucose levels. Although these results are not yet conclusive, they are suggestive that there are two mechanisms that potentially drive the reduction of blood glucose in Ad-5 infected mice. Further experiments need to be conducted to solidify these results.
**AD-5 REDUCES BLOOD GLUCOSE IN INFECTED MICE**

![Graph showing blood glucose levels](image)

**Figure 12.** Mice were infected at $1 \times 10^{11}$ particles of replication deficient Ad-5 and sacrificed at day-2 and -3 post infection. There is a significant decrease of blood glucose on day-3 post infection compared to control mice. n=3 or 4 mice in each group. Error bars are mean ± standard deviation. * p < 0.05 compared to control.
PEPCK mRNA expression was quantified using qPCR. There is a significant decrease of PEPCK in day-3 infected livers compared to control. n=4 mice in each group. Error bars are mean ± standard deviation. * p < 0.05 compared to control.
AKT ACTIVATION IN AD-5 INFECTED LIVERS

Figure 14. Mice infected with Ad-5 were harvested at 72 hours post infection. Liver homogenates were screened for phosphorylated levels of Akt. There is a significant increase in p-Akt in Ad-5 infected livers compared to control. This figure is provided by courtesy of Dr. Palmer Wilkins (PostDoc in Department of Biochemistry, VCU).
AD-5 INFECTION AUGMENTS EXPRESSION OF ADIPONECTIN IN ADIPOSE TISSUE

Figure 15. Adiponectin mRNA expression was quantified using qPCR. Data was normalized to actin. There is a trend for increased adiponectin in Ad-5 infected adipose tissue. n=2 or 3 mice in each group.
CHAPTER 4: DISCUSSION

This ongoing study encompasses the effects of replication deficient Ad-5 on hepatic lipid synthesis, as well as on adipose tissue lipid metabolism. Based on previous studies and several key observations conducted in our laboratory, we hypothesized that replication deficient Ad-5 has an effect on lipid metabolism in hepatic and adipose tissues.

We have observed increased triglyceride levels in Ad-5 infected livers and plasma, while cholesterol levels remained unchanged (Figure 3A and 3B). This observation prompted us to look further at SREBP-1 expression levels because SREBP-1 is responsible for synthesis of fatty acids and triglycerides (Horton et al., 2002). Interestingly, SREBP-1 mRNA levels were significantly increased in Ad-5 infected livers, compared to uninfected livers (Figure 5A). We also screened Ad-5 infected livers for SREBP-2 mRNA expression, which remained unchanged; this correlated with the lack of changes seen in cholesterol levels in both the liver and plasma (Figure 5B; Figure 3A and 3B). Ad-5 infection resembles HCV infection, in which there is an increase in SREBP-1. Where the two viruses differ, is in the fact that HCV infection has been shown to alter expression of not only SREBP-1, but SREBP-2 as well (Siddiqui et al., 2009; Koike et al., 2010). HCV is also associated with reduced serum cholesterol levels, but no significant change in triglyceride levels. Overall, these changes lead to lipid accumulation, visceral fat obesity, and insulin resistance (Saito et al., 2007).
Taken together, our data points to the fact that Ad-5 has implications in aberrant lipid metabolism in hepatic tissues. More importantly, based on Ad-5 infection in cultured hepatocytes, we believe that the increase in SREBP-1 is a direct measure of Ad-5 interaction with the liver, and is most likely not a due to a systemic effect that the virus might potentially have on the organism. This is in contrast to the Ad-5 effect on gluconeogenesis, which is potentially mediated indirectly through adipose tissue. Another interesting observation in this study was that even though SREBP-1 mRNA levels are transiently expressed in Ad-5 infected livers, mature SREBP-1 protein was still stably expressed as far as 14-days post infection. Despite this stable expression, there were no stable increases in plasma triglyceride levels (data not shown). This is a puzzling finding, but a plausible explanation could be based on the fact that other factors are driving triglyceride levels back to baseline. For instance, we have observed an increase in phosphorylated levels of AMPK (p-AMPK) due to adiponectin signaling from adipose tissue (Figure 14). p-AMPK could then phosphorylate one of its downstream factors, ACC, therefore inhibiting its activity, and thereby fatty acid synthesis; this inhibition favors the β-oxidation pathway (Viollet et al., 2006). This would drive break down of fatty acids, therefore suppress Ad-5 induced triglyceride levels. Further long-term infections need to be conducted to see whether this is a reproducible effect. It would also be interesting to determine whether p-ACC is upregulated in Ad-5 infected livers.

Based on our findings, we wanted to characterize the mechanism by which Ad-5 induces these changes and brings about an overall increase in SREBP-1. It is known that
insulin mediates its effects through the PI3K pathway, in which it induces hepatic SREBP-1 expression, leading to an increase in fatty acids and triglyceride synthesis (Taniguchi et al., 2006). It has also been shown that upon internalization of Ad-5 into the target cells, PI3K becomes activated (Nemerow et al., 1998). We wanted to explore the idea that Ad-5 mediates its effects through the activation of PI3K, thus leading to an increase in SREBP-1. With the use of the novel PI3K inhibitor, such as LY294002 or PX866, we were able to demonstrate a decrease in the Ad-5 induced levels of SREBP-1 by about 50% (Figure 8). This led us to believe that PI3K is required for the optimal induction of SREBP-1.

Research has shown that not only Ad-5 activates the PI3K pathway, but other adenoviruses such as Ad-2 of group C adenoviruses, as well as Ad-36 of group D adenoviruses (Rogers et al., 2008; Li et al, 1998). Perhaps by activating PI3K, Ad-2 and Ad-36 are also able to activate SREBP-1 and have similar effects to Ad-5 on lipid metabolism, but as research already pointed out Ad-2 does not induce adipogenesis (Rogers et al., 2008). Therefore, the effects that we have characterized may not only be limited to Ad-5, but could extend to other adenoviruses, such as Ad-2 and Ad-36.

Since we had evidence that PI3K is needed for the optimal induction of SREBP-1, we wanted to delineate the pathway even further to figure out whether SREBP-1 induction is conducted through Akt or PKCζ, both of which are downstream effectors of PI3K. Dr. Kahn’s research points out that upon activation of Akt and PKCζ, these two mechanisms diverge and the effectors are responsible for different systemic effects. Akt is
thought to mediate insulin-dependent hepatic glucose metabolism, while PKC\(\lambda/\zeta/\iota\) is linked to the insulin-dependent induction of expression of SREBP1 (Taniguchi et al., 2006). It has been shown that upon activation of PKC\(\lambda/\zeta/\iota\), SREBP-1 is induced in wild type livers. This knowledge led us to believe that we were on the right path and we explored the idea that SREBP-1 is induced through activation of PKC\(\lambda/\zeta/\iota\), due to Ad-5 infection and activation of PI3K.

With the use of a human hepatoma cell model (HepG2 cells), we have been able to demonstrated elevated levels of phosphorylated PKC \(\lambda/\zeta/\iota\) in Ad-5 infected HepG2 cells. Along with this knowledge, we employed a PKC pan inhibitor. Ad-5 infected HepG2 cells in the presence of PKC zeta inhibitor had decreased levels of SREBP-1, which made us believe that we were on the right path and that SREBP-1 is induced through the activation of PI3K, and its downstream target PKC \(\lambda/\zeta/\iota\). Since we had several issues with using the PKC pan inhibitor, we wanted to use a different method to test our hypothesis. We have developed protocols for knocking down both PKC \(\lambda/\zeta/\iota\) and Akt in HepG2 cells, but unfortunately we could not test our hypothesis because we are currently having issues with our stock of Ad-5, although we are trying to resolve them. Also, further experiments are necessary to evaluate the effects of Akt knock down on Ad-5 infected Hepg2 cells to confirm that atypical PKC \(\lambda/\zeta/\iota\) is involved in Ad-5 mediated induction of SREBP-1. We believe that once we are able to knock down PKC \(\lambda/\zeta/\iota\) and infect cells with Ad-5, we will be able to prove our postulated mechanism that Ad-5 mediates its effects through PI3K,
and more importantly through PKC \( \lambda/\zeta/\iota \), thus bringing about induction of SREBP-1 and triglycerides. We believe that Ad-5 effect on SREBP-1 induced expression is selective and mediated through PKC \( \lambda/\zeta/\iota \). With that, if we knock down Akt using siRNA, we also believe that it will have no effect on Ad-5 SREBP-1 induced levels.

Not only does Ad-5 infection play a role in hepatic lipid synthesis, but as we have demonstrated, it also plays a vital role in adipose lipid metabolism. Low blood glucose levels in Ad-5 infected mice promoted us to look at the effects of Ad-5 infection on adipose tissue (Figure 12). We have observed that Akt is activated upon Ad-5 infection, which is involved in hepatic glucose metabolism (Figure 13). Studies have also shown that adiponectin, an adipocyte-derived hormone, is able to suppress hepatic glucose production (Yamauchi et al., 2002). The Akt pathway and the adiponectin pathway, which activates AMPK in the liver, converge at phosphorylation of FoxO1, therefore blocking activation of gluconeogenic factors such as PEPCK. Based on our observed results, we believe that Ad-5 blocks gluconeogenesis due to activation of Akt through the PI3K pathway, and AMPK through adiponectin signaling from adipose tissue. Our data correlates with published data suggesting that in the presence of Ad-36 infection, there is an increase in adiponectin levels, as well as reduced blood glucose levels (Rogers et al., 2008; Dhurandhar et al., 2000).

Together, these findings characterize the effects of Ad-5 infection on lipid metabolism. Replication deficient Ad-5 is an important tool because it is often used as a
vector in gene therapy trials. It is important to further study the effects Ad-5 might have on lipid metabolism and adiposity, because they could either interfere with the gene therapy trial, or cause secondary effects that would be unwarranted. Based on our findings, we propose that Ad-5 could potentially be one of the factors contributing to fatty liver disease, as it has similar effects as HCV, which has been known to cause liver cirrhosis and lead to fatty acid accumulation. Since Ad-5 causes common respiratory infections, it is easy to say that many people could be infected with the virus. Research has shown that about 50% of people in the U.S. have antibodies against Ad-5, which is a sign that they have been previously infected with the virus (Wang et al., 2008). Their exposure to the virus could lead to increased levels of SREBP-1, and cause an increase in triglycerides and fatty acids. Since our virus is replication incompetent, it merits further studies as a replication competent Ad-5 could potentially be more potent because it can undergo multiple rounds of infection. Further studies need to be conducted to test whether Ad-5 could potentially be a contributing factor to fatty liver disease, but our research points out that Ad-5 is definitely a contributing factor that not only affects lipid metabolism in the liver, but also has implications in obesity and Metabolic Syndrome.

Overall, we believe that Ad-5 infection mediates its effects through the PI3K pathway, and activates Akt and PKC \( \lambda/\zeta/t \). PKC \( \lambda/\zeta/t \) further upregulates SREBP-1, which drives the increase in triglycerides. Akt, on the other hand potentially phosphorylates FoxO1, which inhibits PEPCK expression and reduces blood glucose levels. Another piece of the puzzle involves the adipose tissue, which upon Ad-5 infection, releases
increased levels of adiponectin. Upon adiponectin expression, it binds to its receptor on the liver and activates AMPK. Further, p-AMPK is able to phosphorylate Fox-O1 and again reduce blood glucose levels (Figure 16).

Together, this research has shown that replication deficient Ad-5, which is often used in clinical gene therapy trials, has effects on hepatic lipid synthesis and adipose tissue lipid metabolism, which leads to conclude that this common respiratory pathogen may be associated with the Metabolic Syndrome, and like HCV, it could be causative for fatty liver disease.
MODEL OF AD-5 INFECTION

Figure 16. Model of Ad-5 infection and its effects on hepatic and adipose tissues.
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VITA

Marianna Sukholutsky was born in Dnepropetrovsk, Ukraine on October 20th, 1985. She emigrated from Ukraine to United States in 1997, where she resided in Newport News, Virginia. In 2004, she attended Virginia Commonwealth University, in Richmond, VA, and graduated with a Bachelor’s degree in Biology. In 2008, she began to pursue her Master’s degree in the department of Biochemistry and Molecular Biology at Virginia Commonwealth University School of Medicine. In January 2010, she will begin to pursue a Master’s degree at Eastern Virginia Medical School to become a Physician Assistant.