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Angiotensin IV and the Molecular Mechanisms Involved in the Development of Insulin Resistance in 3T3-L1 Adipocytes

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I am submitting herewith a thesis written by Julie Anne Jungwirth entitled "Angiotensin IV and the Molecular Mechanisms Involved in the Development of Insulin Resistance in 3T3-L1 Adipocytes." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

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Angiotensin IV and the Molecular Mechanisms Involved in the Development of Insulin Resistance in 3T3-L1 Adipocytes

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Dedication

This thesis is dedicated to my mother

Ms. Emilia M. Jungwirth

who has taught me the value of education, has shown how to persevere in even the toughest times, and has given me love and support to help me reach my goals

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Abstract

This study explored angiotensin IV's (Ang IV) affects on the signaling pathways involved in the development of insulin resistance in 3T3-L1 adipocytes. Ang IV, working through the AT_4 receptor, interferes with insulin signaling through the blockade of the phosphatidylinositol 3-kinases (PI3K)/Akt pathway and through activating mitogen activated protein kinases (MAPK): extracellular signal regulated kinase (ERK) and c-Jun-N-terminal kinase (JNK) which are known to impair insulin receptor substrate-1 (IRS-1) signaling.

The expression of AT_4 receptors was confirmed by reverse transcription polymerase chain reaction. Ang IV's effects were found by treating adipocytes with combinations of Ang IV, AT_4 receptor inhibitor Norleual, and insulin. Cell lysates were resolved by SDS-PAGE electrophoresis and immunoblotted.

Ang IV down-regulated the PI3K/Akt pathway. Insulin exerts its effects on adipocytes by activating this pathway, phosphorylating Akt (S473 and T308) residues. Pre-treatment with Ang IV blocked insulin's effects, reversing Akt activation. Addition of Norleual blocked Ang IV's inhibitory actions, leading to the phosphorylation of Akt residues.

Studies show elevated MAPK levels produced by angiotensin peptides catalyze the phosphorylation of serine residues on IRS-1, impairing insulin signal transduction. Ang IV increased the activation of ERK 1/2 and JNK. This was reversed by pretreatment with Norleual. Ang IV's effects on IRS-1 residues were found by immunoprecipitation of IRS-1 followed by SDS-PAGE immunoblotting. Ang IV increased the phosphorylation of IRS-1(S307 and S612). Pre-incubation with Norleual attenuated Ang IV's effects. Ang IV's stimulation of adipocytes quickly caused the phosphorylation of MAPK corresponding to serine residue phosphorylation

on IRS-1, which may implicate Ang IV activation of MAPK in the development of insulin resistance.

Ang IV is involved in the down-regulation of the insulin-signaling cascade by inhibiting insulin's phosphorylation on Akt (S473 and T308). Ang IV increased phosphorylation of ERK 1/2 and JNK, corresponding with increases in serine phosphorylation of IRS-1. Pre-treatment with Norleual inhibited Ang IV's negative effects on insulin signaling. This study elucidates a new mechanism that may lead to the development of insulin resistance in adipose tissue.

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Chapter I
Introduction

Insulin resistance, a key component of the metabolic syndrome, is characterized by an abnormal cellular response to blood insulin levels and an inability to maintain normal blood glucose levels [1]. Insulin resistance causes a variety of chronic disorders including: hypertension, heart disease, inflammation, obesity, and type II diabetes. In addition, insulin resistance is also linked to an increase in mortality and morbidity in critically ill patients due to hyperinsulinemia and hyperglycemia [2, 3]. While the physiological effects of insulin resistance are relatively well known, less is understood about the molecular mechanisms involved in the development of insulin resistance at the cellular level.

Over-activity in the classic renin angiotensin system (RAS) and a concomitant increase in angiotensin II (Ang II) levels are linked to an increase in insulin resistance in adipose and skeletal muscle tissues [2, 4-6]. Blocking the RAS improves insulin sensitivity in patients with thermal injury and type II diabetes [2, 7]. Preventing or controlling the increase of Ang II may consequently reduce insulin resistance and be beneficial to critically ill patients and patients with diseases related to the metabolic syndrome.

Recently, adipose tissue has been explored as an endocrine organ that regulates fat mass and nutrient homeostasis. It produces adipokines and other molecules, like the local RAS, that signal organs and systems- modulating many pathways including glucose metabolism and insulin signaling [8]. Many components of the local RAS were found to be produced in adipose tissue [9]. The local adipose RAS has been implicated in autocrine, paracrine, and endocrine actions [10-12].

Local RAS secretions play a critical role in the etiology of insulin resistance [2]. Ang II, the dominant effector of the RAS, is elevated in patients with obesity, diabetes, sepsis, trauma, and the metabolic syndrome [2, 13]. Elevated Ang II levels negatively regulate glucose metabolism and insulin signaling by interfering with the insulin signaling pathway in skeletal muscle and adipose tissue [5, 14]. The mechanisms behind this effect are partially attributed to the negative modulation of Ang II on several steps of the insulin signaling cascade including the down-regulation of the phosphatidylinositol 3-kinases **(**PI3K)/Akt pathway and insulin receptor substrate-1 (IRS-1) signaling, as well as the activation of various mitogen activated protein kinases (MAPK) [15].

Ang II's affects work through two cellular membrane-associated G-protein coupled receptors, AT_1 and AT_2. In some cells, certain functions attributed to Ang II and its receptors are also found to be regulated by angiotensin IV (Ang IV) and its receptor AT_4 [16]. This implies that Ang IV may also share pathways or cross-talk with the insulin signaling pathway.

It is important to differentiate between Ang IV and Ang II-mediated signaling to determine the possible role of Ang IV in insulin resistance. To do this, Ang IV's affect on the signaling pathways involved in the regulation of glucose metabolism and in the progression of insulin resistance in adipose tissue was explored by testing the peptide's effects on the PI3K/Akt pathway, the phosphorylation of specific IRS-1 residues, and the regulation of MAPK: ERK and JNK.

Ang IV peptides cross-talk with the insulin signaling pathway through signaling the up-regulation of several MAPK. These Ang IV activated MAPK may have a role in IRS-1 receptor phosphorylation. In fact, Ang IV stimulation of adipocytes was found to

increase the phosphorylation of serine residues on IRS-1. This peptide is also implicated in the inhibition of the PI3K/Akt pathway. Ang IV was found to block insulin induced phosphorylation on the activation sites of Akt. It is the goal of this study to improve our understanding of the molecular mechanisms involved in insulin resistance in adipose tissue.

Chapter II
Literature Review

Adipose as an Endocrine Organ

Introduction to Adipose Endocrinology:

Adipose tissue was once thought of only as a storage form for fat. Emerging studies are showing that adipose tissue has a wide variety of roles in the human body. Adipose tissue is now considered a major endocrine and secretory organ releasing different proteins, hormones, adipokines, and fatty acids. A hormonal crosstalk between adipose tissue and other tissues has now been established. In addition, adipose tissue is regarded as a key mediator of obesity and also inflammation

There are two major types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). BAT is primarily found in newborns. BAT is specialized for heat production, using its lipid droplets as a fuel for thermogenesis, via uncoupling protein-1 in its mitochondrial membrane [17]. WAT is the main adipose tissue in adult humans. WAT functions primarily as a long-term fuel reserve for the organism. WAT was thought to only regulate lipogenesis and lipolysis, now WAT is recognized as an endocrine tissue that interacts with other organs and has functions in metabolic and physiological control [18]. With obesity becoming an epidemic in the United States, it is now imperative to understand how increased quantities of adipose tissue, due to its endocrine function, can affect whole body homeostasis.

WAT Endocrinology:

The most important secretory product of adipose tissue are fatty acids. Fatty acids are released at periods of negative energy balance, especially when fasting or during acute cold exposure. In addition to fatty acids, a range of protein factors and signals, called adipokines, are also released from adipose cells [10]. Adipokines are a diverse group of secretions affecting different metabolic and physiologic functions. Some adipokines, like leptin, adiponectin, inflammation-related adipokines, and local renin angiotensin secretions, play major roles in insulin resistance and are associated with obesity and the metabolic syndrome [4, 11, 14, 18].

In 1994, leptin was the first adipokine to be discovered. WAT is the main source of this hormone, with a close correlation observed between increased body fat and increased circulating leptin levels [19]. It is released by adipose cells which signal the hypothalamus to control food intake and energy balance. Leptin was identified as the product of the *ob* gene described in mice. Total leptin deficiency, as a result of *ob* mutation, has been shown to lead to hyperphagia, decreased energy expenditure, and obesity [20].

Adiponectin, which was discovered in 1995, is a hormone found to be produced exclusively by adipocytes [21]. Unlike most adipokines, the circulating levels of adiponectin fall with increasing obesity [22]. High levels of adiponectin show anti-inflammatory functions and increased insulin sensitivity [23, 24].

Inflammation-related adipokines include tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), and plasminogen activator inhibitor-1 (PAI-1). These inflammatory peptides are normally released after a

trauma incident and are linked to immune system regulation. Information has recently emerged suggesting that obese patients show chronic mild inflammation due to elevated levels of these peptides, especially TNF-α and IL-6 [8]. There is a growing view that this inflammatory state, due to obesity, is one of the causes in the development of type II diabetes and the associated metabolic syndrome. Locally, IL-6 disrupts insulin signaling in fat cells and is systemically linked to the pathogenesis of diabetes mellitus and the development of obesity [18]. TNF-α levels, which increase in obesity, may also contribute to insulin resistance by inhibiting insulin stimulation of tyrosine activity on the insulin receptor [25]. This causes a decrease in the synthesis and translocation of glucose transporter type 4 (GLUT4) to the cellular membrane.

Adipose tissue is an endocrine organ that regulates fat mass and nutrient homeostasis. It also produces adipokines that signal other organs; modulating many pathways including glucose metabolism and insulin signaling. More recently, the local renin-angiotensin system was found to be produced in adipose tissue. This system has also been implicated in the development of insulin resistance and the metabolic syndrome.

Renin-Angiotensin System

Introduction to RAS:

In the classic view, the renin-angiotensin system (RAS) is a hormone system primarily regulating blood pressure and fluid balance [26]. The liver is the primary producer of the inactive peptide angiotensinogen (AGT), which is secreted into the blood

stream. When blood volume is low, the kidneys produce renin, which cleaves AGT to form angiotensin I (Ang I). Ang I is then circulated to the lungs where angiotensin converting enzyme (ACE), found in the capillaries of lungs, cleaves off two terminal residues from Ang I, converting Ang I to angiotensin II (Ang II). Ang II is the major effector of the RAS by acting on the pituitary gland, kidney, adrenal gland, and several other tissues. Ang II is degraded to angiotensin III (Ang III) by angiotensinases located on red blood cells and vascular beds of most tissues [27, 28]. Ang III has a diminished ability to cause vasoconstriction, but it can readily stimulate the production of aldosterone [29]. Angiotensin IV (Ang IV) is a degradation of Ang III and also has a range of central and peripheral activities throughout the body.

Classic RAS Effects:

The classic RAS is primarily known for its vascular affects. Ang II is a potent vasoconstrictor, causing a rise in systemic arterial blood pressure. It also stimulates the secretion of aldosterone, a hormone from the adrenal cortex [30, 31]. Aldosterone causes the proximal tubules of the kidneys to increase sodium re-absorption and therefore water re-absorption. This increases blood pressure by causing an increase in the volume of body fluid. RAS also has neural effects: Ang II causes an increase in the thirst sensation through the subfornical organ in the brain, while increasing the desire for salt. Ang II increases anti-diuretic hormone (ADH) secretion in the posterior pituitary and adrenocorticotropic hormone (ACTH) secretion in the anterior pituitary. Ang II also has been found to have a pro-thrombotic potential, through the adhesion of platelets and production of PAI-1.

Local RAS:

The idea of a local renin angiotensin system came about when the beneficial effects of ACE inhibitors and Ang II receptor blockers were found to be partially independent of the blood pressure effects of these drugs [32, 33]. The most plausible explanation was that Ang II was not only generated in the circulation by the classic RAS, but also locally in the brain, kidney, and heart cells.

A local RAS is characterized by the presence of RAS components at the tissue level. Such local systems are regulated independently of circulatory RAS but can also interact with the latter. Due to this, local RAS effects could occur in a paracrine fashion, i.e. the cell that produced the peptides, an adjacent cell, or through the blood stream affecting a specific organ. The local renin-angiotensin system can be activated in hypertension, metabolic disorders, and trauma.

Different tissues have varying abilities to synthesize the components of the RAS. For instance, cardiac tissue has low renin mRNA levels and cultured cardiac cells do not release renin. On the other hand, most of the Ang I and Ang II peptides found in the heart were synthesized by the heart tissue [32-35]. This is in contrast to the brain where more components of the RAS like: Ang II, Ang IV, renin, ACE, AT_1, AT_2, and AT_4 receptors have been shown to be synthesized.

Local Adipose RAS:

Adipose cells are capable of secreting AGT, the precursor to angiotensin. Overfeeding rodents causes an increase in the local formation of Ang II due to the increased secretion of AGT from adipocytes [2]. Adipose tissue also produces renin, which

acts on AGT to produce Ang I. Current literature indicates that adipose cells not only secrete renin; its secretion is independent of plasma renin levels except in very high quantities [36]. Adipose cells have been shown to produce ACE, which converts Ang I to Ang II, and also AT_1 and AT_2 receptors, which facilitate the actions of Ang II on adipocytes. Recently, these cells have also been found to secrete Ang IV, a degradation of Ang II. This peptide activates the AT_4 receptor, which has also been confirmed on adipocytes [37].

The local RAS secretions modulate a variety of functions in adipocytes. Ang II takes part in the differentiation of adipocytes and in lipogenesis. This indicates a potentially manipulatable involvement of the local renin angiotensin system with the accumulation of body fat. Because adipose is a metabolically active tissue, the discovery of the local adipose tissue RAS caused an interest in the system's effects on the pathophysiology of obesity, insulin resistance, and metabolic syndrome.

Angiotensinogen Secretions in Adipose Tissue:

AGT, the precursor to Ang II is a peptide that is primarily produced in the liver. Interestingly after the liver, WAT is the most abundant source of AGT. AGT mRNA levels were first found in rats in the late 1980's [38, 39, 39]. Later, abundant amounts of AGT mRNA were found in adipocytes from WAT and BAT taken from cultured fat pads [40]. Researchers found that the locations of the adipocytes made a difference in the amount of AGT mRNA expressed; levels were higher in visceral as compared with subcutaneous adipose tissue in both rodents and humans [41]. Results vary, but point to an intensification in adipocyte AGT formation in obesity-associated insulin resistance [42-45]. This suggests

that the local adipose RAS is more active in obese populations and its related peptides, like Ang II, may also have a greater role in the molecular signaling of these cells.

ACE:

ACE functions mainly as a peptidyl dipeptidase, removing dipeptides from the C-terminus of the peptide substrates [46]. ACE predominantly cleaves Ang I, processing the decapeptide to the 8 amino acid peptide Ang II [46]. ACE also inactivates bradykinin and kallidin, which helps to continue the vasopressor effects of the RAS. Tissue ACE is present in all major organs, including the heart, brain, and kidney, as well as adipose tissue [47, 48]. Attenuation of ACE by ACE inhibitors, like lisinopril, has a powerful effect by decreasing blood pressure [49, 50]. These inhibitors are often used in anti-hypertensive therapies and have been found to have beneficial effects on patients with diabetes [51, 52]. This is attributed to the inhibition of Ang II formation, therefore decreasing Ang II levels, attenuating its effect on the insulin signaling pathway.

Angiotensin II:

Ang II, an octapeptide, is the main effector in the RAS and is formed when ACE cleaves Ang I [53]. Its main function is to mediate blood pressure, and it is also implicated in inflammation, hypertension, heart failure, and diabetes. When Ang II binds to its receptor on the cellular membrane, it activates a series of signaling cascades, which regulate various physiological functions that include mediating blood pressure, inflammation, hypertension, heart failure, and diabetes. These effects vary depending on which cell type Ang II stimulates [4, 33, 44, 54, 55]. The pathways induced by Ang II have

been divided into two classifications: G-protein and non G-protein related signaling [53]. Ang II can also carry out its signaling through MAPK and receptor tyrosine kinases like the insulin receptor (IR) [53].

Ang II works through many pathways in adipocytes. It has been implicated in stimulating the production of prostacyclin, which promotes adipocyte differentiation [56]. Ang II may also be involved in the control of adiposity through the regulation of lipid synthesis and storage [44, 57]. Ang II has also been found to exert its affects on the insulin signaling and metabolic pathways of adipose tissue [4, 53, 58].

Ang II down-regulates the PI3K/Akt pathway by inhibiting the enzymatic activity of IRS-1/PI3K as well as blocking Akt activation [6, 58, 59]. This attenuates the actions of insulin, including inhibiting GLUT4 translocation to the cellular membrane. This action can be reversed with pre-treatment of Ang II receptor antagonists [60].

Ang II also activate serine-threonine kinases like protein kinase C (PKC) and MAPK, which inhibit insulin-induced IRS-1 activation. This was reversed by Ang II receptor inhibitors, and by antagonists of MAPK p38 and ERK1/2. This suggests that MAPK signaling is involved in inhibitory actions of Ang II on insulin signaling. Ang II-induced activation of ERK and JNK has also been implicated in the inhibition of insulin-induced nitric oxide (NO) production by promoting IRS-1 inhibiting phosphorylation [58]. Ang II also enhanced NADPH oxidase activity which increases reactive oxygen species (ROS). This is accompanied by a decrease in insulin-induced IRS-1 tyrosine phosphorylation, Akt activation, and GLUT4 translocation to the cellular membrane [61].

Impairing insulin induced GLUT4 translocation and glucose uptake can also be shown when intravenous infusion of Ang II in the heart caused a significant expression of

suppressor of cytokine signaling-2 (SOCS-3) [62, 63]. SOCS-3 can interact with insulin signaling pathway through IR, IRS-1, and JAK-2, impairing insulin induced signaling [64]. Inhibiting SOCS-3 pathway partially restored this insulin induced IRS-1 tyrosine phosphorylation, as well as PI3K and Akt activity [64].

AT_1 and AT_2 receptors:

Ang II receptors were some of the first peptide hormone receptors to be identified and characterized. To activate certain cellular functions, Ang II interacts with two cellular membrane-associated G-protein coupled receptors, AT_1 and AT_2. These receptors are responsible for the signal transduction of their main effector hormone, Ang II. Ang II receptors are distinguished according to inhibition by specific antagonists. AT_1 receptors are antagonized by biphenylimidazoles, while AT_2 receptors are inhibited by tetrahydroimidazopyridines [65].

The AT_1 receptor is the best elucidated angiotensin receptor [66, 67]. Most of the physiological effects of Ang II are mediated by this receptor. Two AT_1 receptor subtypes have been discovered in mice, the AT_{1A} and AT_{1B}, these receptors are mapped on chromosome 13 and 3 respectively. These receptors have a greater than 95% amino acid sequence identity [68].

The AT_1 receptor is found mainly in the heart, adrenal glands, brain, liver, kidneys, and adipose tissue. The receptor activates phospholipase C and increases the cytosolic Ca^{2+} concentrations, which in turn triggers cellular responses, like increasing PKC and MAPK activation. The activated receptor also inhibits adenylate cyclase and activates various tyrosine kinases.

The AT_1 receptor can provide a mechanistic link between hypertension and various disorders like insulin resistance. Recent studies have shown that Ang II can interact with the AT_1 receptor on the cellular membrane causing an inhibition of GLUT4 translocation to the cellular membrane [69]. Also, insulin increases AT_1 receptor gene expression by post-transcriptional mRNA stabilization, providing evidence of an association between hypertension and hyperinsulinemia [70].

AT_2 receptors are found in higher concentrations in fetus and neonates, declining rapidly after birth [37, 71, 72]. In adults, the AT_2 receptor is present in low levels and is located predominantly in the heart, adrenal glands, uterus, ovaries, kidneys, and brain. Effects mediated by the AT_2 receptor include the inhibition of cellular growth, fetal tissue development, neuronal regeneration, and cellular differentiation, although exact signaling pathways are unclear.

Angiotensin II works on adipose cells through both AT_1 and AT_2 receptors to mediate cellular growth and metabolism. Inhibitors of RAS like AT_1 receptor blockers, have been used as therapy for hypertension and diabetes. Blockade of the RAS with these inhibitors decreases body weight, improves insulin sensitivity, and prevents the development of insulin resistance in obese, insulin-resistant, or hypertensive animals and humans [73-76]. AT_1 receptor blockers are now recognized therapy in this patient population. More research must be done to see if blockade of the RAS at other sites may also be beneficial to these patients.

Angiotensin IV:

Ang IV is a fragment of Ang II. First, Ang II is metabolized to Ang III by aminopeptidase A. Then, aminopeptidase N metabolizes Ang III to Ang IV (Figure 1). Ang IV has drawn a lot of attention recently due to its wide range of central and peripheral effects like enhancing memory, increasing blood pressure, increasing the inflammatory response, and affecting insulin signaling.

Ang IV can affect different types of tissue in a variety of different ways. Working through brain cells, this hormone has been found to enhance learning and memory in rodents [77, 78]. Ang IV can affect the intracellular calcium concentration by rapid release from intracellular stores [79, 80] or influx from extracellular medium [81]. Ang IV is similar to Ang II in that it increases blood pressure after central administration to rats [55, 82]. Chronic elevation of Ang IV in a transgenic mouse model is also associated with an increase in blood pressure [83].

Ang IV has been shown to up-regulate several pro-inflammatory factors [16] and it participates in inflammatory response steps causing vessel inflammation and oxidative stress. This oxidative stress response via activation of endothelial NADPH oxidase and NF-κB leads to an increase in reactive oxygen species, inflammation, and insulin resistance [16].

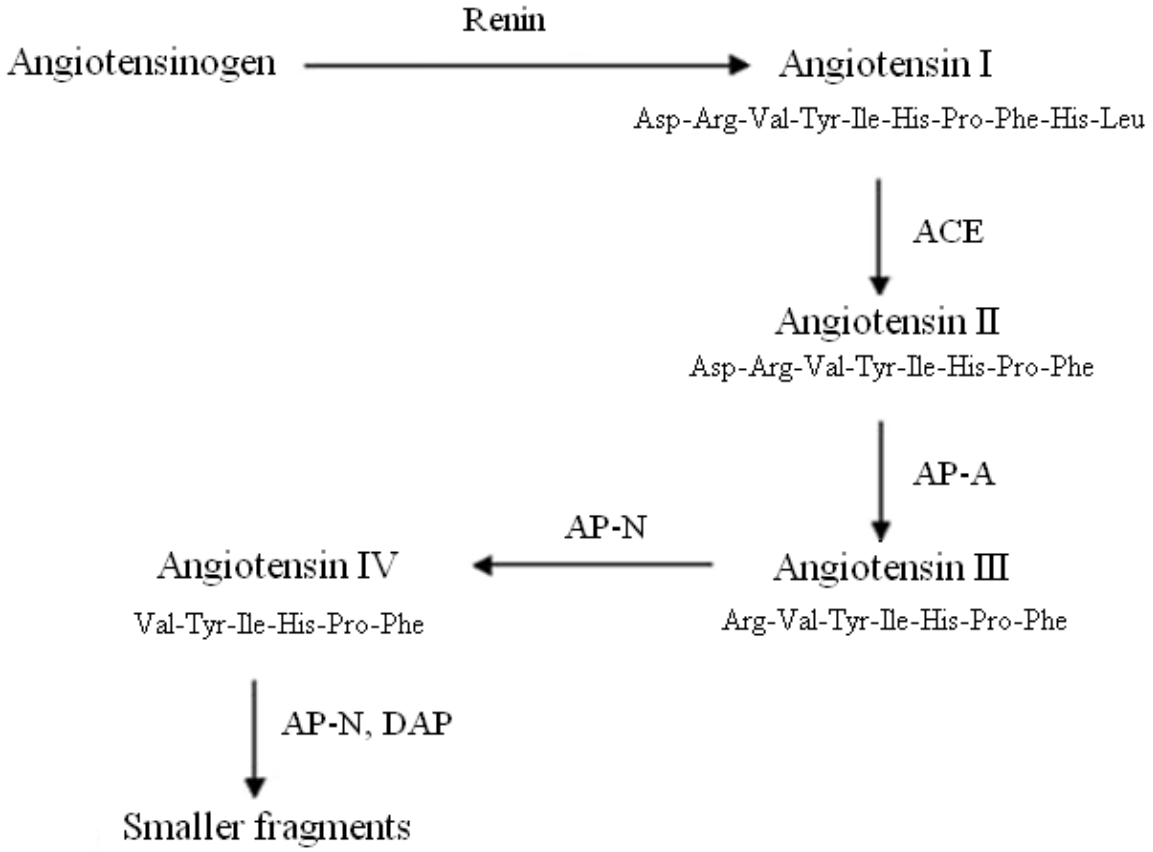


Figure 1: Overview of the formation and metabolism of Ang IV.

Ang IV activates certain mitogen-activated protein kinases (MAPK) like ERK1/2 and JNK [84]. This may have a role in cell proliferation [85, 86]. Ang IV interacts with the PI3K/Akt and ERK1/2 pathways to stimulate endothelial cell proliferation.

Numerous efforts to characterize the Ang IV-induced intracellular signaling in various cell types have been attempted. It has become clear that these effects are not only heterogeneous but very dependent on the cell type being studied.

Ang IV binding sites:

Some of the affects of Ang IV have been shown to be mediated by its interaction with AT_1 and AT_2 receptors in peripheral and central functions [87-90]; [83, 91-93]. But many of the effects induced by Ang IV are not blocked by classical AT_1 and AT_2 receptor antagonists like losartan and PD123.177 [16, 92-103]. Because there was an inability of AT_1 and AT_2 antagonists to block some effects induced by Ang IV and researchers have identified the binding sites with high affinity for Ang IV in different tissues that had low affinity for Ang II and AT_1 and AT_2 receptor antagonists, [71, 104-111] [86, 105, 112-114] the existence of a novel angiotensin receptor subtype was discovered, and called the AT_4 receptor [72, 115]. This receptor is not only displayed on a broad tissue distribution; in the brain, aorta, heart, kidney, liver, lung, uterus [109, 116], but has also been found in different mammalian species [105, 113].

AT_4 receptor:

Most Ang IV actions are mediated by a specific binding site called the AT_4 receptor. The AT_4 receptors have a pharmacological profile that deviate significantly

from that of AT_1 and AT_2 receptors. Structural studies provide evidence that, unlike AT_1 and AT_2 receptors, AT_4 receptors do not belong to the family of 7-trans-membrane domain receptors. The AT_4 receptor does not bind to AT_1/AT_2 receptor antagonists like losartan and PD123319.

The receptor is activated by Ang IV, and by synthetic peptides like Nle1-Ang IV and Norleucinal. Studies have shown that the first three amino acid residues of Ang IV are critical for the binding to the AT_4 receptor [117].

Photo-affinity labeling of Ang IV binding sites conveyed that this receptor exists as complexes formed of three different peptides, with molecular weights of 165 kDa, 50-60 kDa, and 70-80 kDa [108, 118, 119]. Albiston *et al* identified the 165 kDa peptide as insulin-regulated aminopeptidase (IRAP) [120]. Recombinant expression of this protein not only displayed the typical AT_4 receptor binding characteristics, it showed the distribution of the mRNA for IRAP and immunohistochemistry matching the AT_4 receptor binding site in brain slices [120]. Enzymatic activity of IRAP was inhibited by AT_4 receptor ligand Ang IV; this suggests that Ang IV may function by inhibiting IRAP activity.

Insulin-regulated aminopeptidase:

IRAP is a type II integral membrane spanning protein belonging to the M1 family of aminopeptidases and is predominantly found in GLUT4 vesicles in insulin-responsive cells. Northern blotting has shown a large tissue distribution of IRAP, including adipocytes, skeletal muscle, brain, heart, kidney, and lungs [80, 105, 111, 121-123]. IRAP consists of an acidic intracellular region, followed by a hydrophobic trans-membrane segment, and an extracellular domain possessing its aminopeptidase activity [122, 124-126].

Studies using IRAP-transfected cells show that it binds to radio-labeled Ang IV analogue [125 I] Nle1-Ang IV with high affinity, and it can be cross linked with [125I] Nle1-BzPhe6-Gly7-Ang IV. In adipocytes, IRAP is co-localized almost exclusively with GLUT4 in specialized vesicles [127]. IRAP has been implicated in the regulation of GLUT4 vesicle trafficking, which affects facilitative glucose uptake into the cells [127]. Ang IV may exert its affects by inhibiting the catalytic activity of IRAP. Researchers are now interested in the molecular mechanisms IRAP mediates, the range of intracellular signaling, and the *in vivo* affects triggered by the AT_4 ligands.

AT_4 Receptor Inhibitors:

As discussed previously, AT_1/AT_2 receptor inhibitors have been developed as a pharmacological treatment for hypertension and insulin resistance [49, 51, 52, 60, 75, 96, 128]. It is now important to investigate AT_4 receptor blockers as a new therapeutic target. The administration of AT_4 receptor antagonists drugs have appeared to be useful as a tool to treat the memory impairment that has been associated with Alzheimer's disease [91]. In this regard, the effect of Ang IV through the AT_4 receptor in patients with insulin resistance, hypertension, and the metabolic syndrome, may also be inhibited by AT_4 antagonists. It is now important to test the affect of the inhibition of Ang IV and its signaling in this population.

Angiotensin Receptors in 3T3-L1 Cells:

Local RAS components are present in preadipocytes and mature adipocytes. This system is involved in metabolic effects and tissue differentiation. AT_1 receptors are well

described in 3T3-L1 and human adipocytes by Western blotting, PCR, confocal laser microscopy, and binding studies [44, 129-133]. AT_2 receptors were identified in adipose tissue by expression cloning of cDNA [130, 134]. A study by Weiland and Verspohl showed that AT_1 and AT_2 receptors are present in preadipocytes, and coexist in preadipocytes in fairly equimolar ratios [37]. There was high affinity of Ang II to AT_2 binding sites when non-selective substances like radioactively-labeled Ang II were used [37].

AT_1 receptors are found in mature adipocytes, where as AT_2 receptors were not. This may suggest that AT_2 receptors are primarily necessary for developing tissues [37].

AT_4 receptors have been confirmed on adipocyte cells. Weiland and Verspohl also showed that AT_4 receptor density differs depending on the type of cells used and whether the cells were differentiated. AT_4 receptor density is lower in undifferentiated 3T3-L1 cells than in differentiated 3T3-L1 cells. In contrast, 3T3-F442A preadipocytes have enhanced AT_4 receptor densities comparable to mature adipocytes. During differentiation of 3T3-L1 there is increased intracellular sequestration of IRAP containing AT_4 receptors [135]. This may suggest that Ang IV may have a role in cellular growth.

Insulin Resistance

Introduction to Insulin Resistance:

Insulin resistance, a key component of the metabolic syndrome, is characterized by an abnormal cellular response to blood insulin levels and an inability to maintain normal blood glucose levels. Insulin, in addition to its effects on glucose metabolism, also has pleiotrophic actions that can modulate lipid and protein metabolism, amino acid

transport, cell cycle, apoptosis, and nitric oxide synthesis. Consequently, this modulation must also be considered in situations of insulin resistance. Insulin resistance causes a variety of disorders including: hypertension, heart disease, inflammation, obesity, and type II diabetes [4, 136]. In addition, insulin resistance is linked to an increase in mortality and morbidity in patients due to hyperinsulinemia and hyperglycemia [2, 3, 137]. While the affects of insulin resistance are relatively well known, less is understood about the exact molecular mechanisms involved in the development of insulin resistance at the cellular level.

RAS and Insulin Resistance:

The association of the RAS with the endocrine system is illustrated by the prominent role of Ang II in insulin resistance, diabetes, and the metabolic syndrome [4]. The recurrent association of diabetes mellitus with hypertension, nephropathy, and cardiovascular disease has implicated RAS in the initiation and progression of these disorders. Several groups have dissected the components of the cross-talk between insulin and RAS signaling pathways. This research has shown that the cross-talk occurs at multiple levels and encompasses several signaling proteins. It is made of multifaceted and multilayered events affecting cellular functions. Research has shown that Ang II infusion induced insulin resistance, and the blockade of the RAS improved insulin sensitivity. Renin knockout mice (Ren1c(-/-)) have been shown to be lean, insulin sensitive, and resistant to diet-induced obesity. These metabolic changes were reversed by Ang II administration, further implicating Ang II in the pathogenesis of diet-induced obesity and insulin resistance [138]. There have been many clinical trials where RAS

inhibitors significantly reduce the prevalence of vascular complications in DM patients [139-143]. These effects include protective actions upon skeletal muscle [144, 145], enhanced insulin sensitivity and reduced cell size in adipocytes [75], and increased trans-capillary glucose transport [144, 145]. Ang II has been implicated in causing insulin resistance by interfering with the insulin-stimulated increase in insulin receptor substrate 1 (IRS-1)/phosphatidylinositol 3-kinase (PI3K) activity [15]. Estaban *et al* shows research implying that Ang IV can also regulate some functional attributes to Ang II, like having a role in the inflammatory process via NF-κB, which may affect insulin resistance [16].

Local Adipose RAS and Insulin Resistance:

As previously stated, components of the RAS are present in adipose tissue [146]. Research has shown that Ang II, through its receptors, is able to modulate insulin actions in certain cells. This may be due to the cross-talk between Ang II signaling and insulin signaling in metabolic and vascular tissues [147]. Deletion of the AT_2 receptor in mice has been shown to lead to reduced adipose size and had protective effects on diet induced obesity and insulin resistance [44, 76]. Local Ang II secretions have also been shown to stimulate insulin resistance in adipose cells by increasing MAPK, which inhibit IRS-1 tyrosine phosphorylation, leading to the reduction of insulin signaling. In addition, adipose tissue secretes a variety of hormones, called adipokines, which can alter the insulin signaling cascade. For example TNF-alpha, a pro-inflammatory molecule which can be stimulated by Ang II, is secreted by adipose cells and may have a role in causing insulin resistance [25]. It is important to understand if other local RAS secretions, like Ang IV, also affect insulin signaling in similar ways.

Insulin Signaling Pathway

Introduction to Insulin Signaling Pathway:

Glucose transport and metabolism, protein synthesis, and cell cycle are all regulated by the activation of the insulin signaling pathway. Insulin binds to the insulin receptor (IR) on the cellular surface. The IR is from a family of cell surface receptors that have intrinsic tyrosine kinase activities. The IR is made of two extracellular α-subunits and two transmembrane β- subunits linked by disulfide bonds [4]. Insulin binds to the α-subunits which induces a tyrosine residue phosphorylation of the β-subunit, thus activating the IR. The IR subsequently phosphorylates its substrates, which include IRS [148]. IRS are regulatory docking proteins that associate with the IR. They play a central role in the selection and differentiation of the insulin signal toward metabolic or gene effects. The phosphorylated IRS subunits can dock downstream effectors which can activate different signaling pathways. For example IRS-1 can activate the ERK pathway involved in cellular growth and division [149]. IRS-1 can also activate PI3K, which is involved in the metabolic actions of insulin including propagating insulin's signal to downstream molecules like Akt [150].

RAS and IRS-1/PI3K:

Once IRS is phosphorylated by the insulin signaling cascade, there is a concomitant docking of this substrate to the p85 subunit of PI3K, causing a stimulation of PI3K and its downstream pathway. IRS associated PI3K activity can be decreased by Ang II [151]. *In vivo* studies have shown that Ang II can stimulate IRS docking with PI3K,

but the peptide reduces PI3K enzymatic activity in the heart. This effect was found in both the basal and insulin-stimulated states, and did not affect the tyrosine phosphorylation of IRS and the insulin receptor β-subunit [151]. Ang II stimulation of rat aortic smooth muscle cells showed increases in the phosphorylation of the IR and IRS-1 serine residues, attenuating insulin's activation of the insulin pathway [5]. This suggests that Ang II inhibits insulin signaling via the PI3K pathway.

Ang II stimulation of cells also can activate MAPK: ERK and JNK which can catalyze the serine phosphorylation of IR and associated IRS-1 and PI3K. This then impairs insulin's signal toward Akt and other downstream molecules of the insulin signaling cascade [5].

Studies have shown that Ang II impairs the insulin signaling pathway towards the production of NO by inducing serine phosphorylation of IRS-1 on Ser^{312} and Ser^{616} in rats via JNK and ERK respectively [58]. This then blocks the insulin signaling cascade which then impairs the vasodilator effects of insulin mediated by the IRS-1/PI3K/Akt/eNOS pathway [58]. This implies that Ang II negatively modulates the insulin signaling pathway by stimulating multiple serine phosphorylation events in the insulin-signaling cascade.

Akt:

Akt (also known as protein kinase B) is a serine/threonine kinase. It is activated by phospholipid binding and activation loop phosphorylation at threonine 308 residues by PDK1, and by the phosphorylation of its carboxy terminus at serine 473 residues by mTOR.

Akt serves as a multifaceted intermediary protein by propagating PI3K signaling from IR to diverse downstream biological effectors. Akt stimulates glucose uptake through the translocation of GLUT4, a major glucose transporter. In vascular smooth muscle cells insulin plays a role in the regulation of glucose metabolism and vasodilatation via signaling through the PI3K/Akt pathway. The affect of Ang II is targeted at the inhibition of PI3K/Akt pathway. Ang II opposes the action of insulin to enhance glucose uptake in cells and may lead to insulin resistance in cells [59]. In the heart, intravenous infusion of Ang II inhibits insulin-mediated activation of PI3K/Akt as well, and it promotes the expression of SOCS-3 [152]. SOCS-3 then interacts with key components of the insulin signaling system including IR, IRS, and impairs GLUT4 translocation and glucose uptake [64].

Mitogen Activated Protein Kinases

Introduction to Mitogen activated protein kinases:

MAPK are serine/threonine-specific protein kinases that are activated by dual phosphorylation. MAPK respond to extracellular stimuli like pro-inflammatory cytokines and osmotic stress to regulate various cellular activities including gene expression and cell survival. There are at least three different MAPK signaling pathways in mammalian cells. These are extracellular signal regulated kinases (ERK), c-Jun-N-terminal kinases (JNK) and p38 kinases. ERK1/2 was the first of the ERK/MAPK family to be cloned. It is stimulated by growth and differentiating factors like epidermal growth factor, platelet-derived growth factor and nerve growth factor, through receptor tyrosine

kinases, heterotrimeric G protein-coupled receptors, or cytokine receptors. JNKs and p38 kinases are implicated in responses to cellular stress, inflammation and apoptosis. They are activated by lipopolysaccharides, IL-1, TNF-α, ultraviolet radiation, heat shock or hyper-osmotic stress.

MAPK and RAS:

Ang II via AT_1 receptors carry out a variety of functions through MAPK. Ang II can stimulate MAPK quickly. For example, Ang II signaling through the AT_1 receptors activates ERK1/2 within 5 minutes. There are at least two mechanisms through which Ang II mediates ERK signaling in cells. Ang II through its AT_1 receptor activates Gq protein, which leads to an increase in cytosolic Ca^{2+} and activation of EGF receptor. This receptor recruits shc/Grb2 and induces the activation of the Ras-Raf-MEK-ERK pathway [153]. The second pathway depends on AT_1 activation of Gq and the subsequent increase of cytosolic Ca^{2+}. This then activates PKC with subsequent activation of Raf-1, MEK, and then ERK [154]. Ang II through its AT_1 receptor may activate JNK in cardiac myocytes through an increase in intracellular Ca^{2+} and the activation of protein kinase C [155].

Ang II causes insulin resistance through the up-regulation of certain MAPK. Chronic activation of ERK, JNK, or p38, due to prolonged Ang II activation, induces insulin resistance by affecting glucose transport and insulin signaling [149]. Ang II is known to impair insulin signaling through the activation of ERK and JNK, which phosphorylate IRS-1 on Ser residues (Figure 2). Additionally, Ang II is known to

activate JNK and ERK pathways via protein kinase C (PKC), which inhibits insulin signaling by Ser-612 phosphorylation of IRS-1.

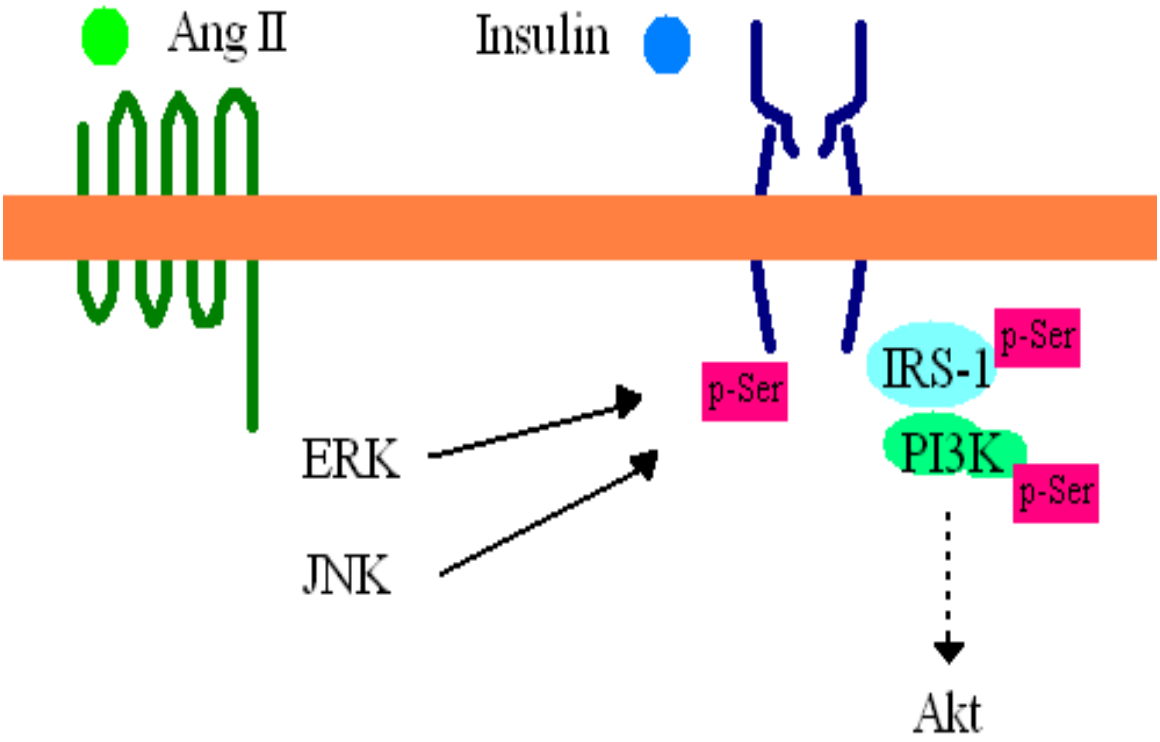


Figure 2: Early events in the insulin Ang II cross-talk.

MAPK and Insulin Resistance:

Researchers are interested in three MAPK and their affects on insulin resistance: ERK1/2, JNK, and p38. ERK1/2 activation was shown to suppress the expression of IRS-1 and IRS-2 and phosphorylate serine 612 residues on IRS-1, which would lead to difficulty phosphorylating tyrosine residues on IRS [58, 149]. This then decreases PI3K activation, a molecule involved in the metabolic actions of insulin, and its downstream signaling molecules [149].

JNK activation suppressed tyrosine phosphorylation of IRS-1 and IRS-2, while causing the serine phosphorylation of 307 residues on IRS-1. This in turn suppresses the activation of PI3K [149].

Finally, p38 pathway activation slightly reduces IRS-1 and IRS-2 expression, although not as much as ERK1/2 [149]. All three of these MAPK have also been found to inhibit glucose transport by up regulation of GLUT1 expression and down regulation of GLUT4 expression, in 3T3-L1 cells [149, 156, 157]. In certain cells, some functions attributed to Ang II and its receptors are also found to be regulated by Ang IV and its receptor AT_4. It is thus important to differentiate Ang IV mediated signaling from Ang II and determine its possible role in insulin resistance through MAPK.

Specific Aims

The purpose of this study is to investigate Ang IV effects on the signaling pathways that are involved in the regulation of glucose metabolism and insulin resistance in adipose tissue. Several studies indicate that Ang II impairs insulin signaling at various

stages of the insulin signaling pathway by inhibitory phosphorylation of several key proteins [4, 58, 59, 61]. Inactivation of these proteins is known to be mediated by several pathways like MAPK and PI3K/Akt pathway [4]. Insulin signaling, a critical player in insulin resistance, is regulated by a balance between positive IRS-1 tyrosine phosphorylation and negative IRS-1 serine phosphorylation. Many factors such as cellular stress, hyperinsulinemia, and MAPK induce insulin resistance by impairing insulin signaling (inhibiting IRS-1 function by serine phosphorylation) via PI3K/Akt pathway [4, 150]. Recent evidence suggests that Ang IV is also known to elicit diverse biological responses independently of Ang II and its receptors [16, 158]. Because of this, it is important to elucidate the role of Ang IV in the development of insulin resistance. Based on these findings we speculate that Ang IV induced up-regulation of MAPK may inhibit the insulin signaling pathway through IRS-1 serine phosphorylation and Ang IV may impair insulin signaling through the PI3K/Akt pathway in adipocytes.

Ang IV induced activation of ERK and JNK will be assessed by culturing differentiated 3T3-L1 cells in serum free media for 18 hours and treating with or without Ang IV, insulin, and AT_4 receptor blockers. Ang IV induced activation of MAPK will be determined by Western blots using MAPK specific phospho-antibodies. Phosphorylation of IRS-1 serine residues will be determined by immunoprecipitation with anti-IRS-1 and Western blot using specific phospho-IRS-1 serine antibodies.

To determine if Ang IV induced insulin resistance is mediated by the PI3K/Akt pathway, differentiated 3T3-L1 cells will be serum starved for 18 hours and treated with or without Ang IV, insulin, and AT_4 receptor blockers. Akt activation will be determined by Western blot using phospho-Akt antibody.

Chapter III
Materials and Methods

Reagents and Antibodies

Human Ang IV was purchased from Sigma (St. Louis, MO). AT_4 receptor inhibitor, Norleual, was kindly provided by Dr. Joseph Harding, Washington State University. Phospho-ERK1/2 also known as p42/44 MAPK, anti-ERK 1/2, phospho-JNK, anti-JNK, phospho- IRS-1(Ser612), phospho- IRS-1 (Ser 307), phospho- Akt (Thr308), and anti-Akt were purchased from Cell Signaling Technology (Beverly, MA). Phospho-Akt1/PKBα (Ser473) and anti-IRS-1 were obtained from Upstate Biotechnology (Lake Placid, NY). Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell System

Cell growth:

3T3-L1 pre-adipocyte cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were kept in freezing serum (Dulbecco's Modified Eagle Medium High Glucose 1x (DMEM+ GlutaMAX-1), Invitrogen, Carlsbad, CA, 10% Fetal Bovine Serum (FBS), 5% DMSO) in liquid nitrogen in a Dewar vessel. Once thawed the cells were kept in growth media (DMEM+ GlutaMAX-1, 10% FBS, 1% penicillin/streptomycin) in a NAPCO CO_2 incubator (Precision Scientific, Chicago, IL) at 37° C, 5% CO_2, and humidified balanced room air.

3T3-L1 cells were incubated in growth media until they were 90-95% confluent. Cells were trypsinized and plated (~38 x 10^5 cells/ 75cm^2 flask; 0.5 x 10^5 cells/100mm dish) onto flasks (75 cm^2) and/or petri dishes (100 mm) for culture. Flasks were trypsinized by aspirating growth media and rinsing the flask Hank's Balanced Salt Solution (HBSS). HBSS was aspirated and trypsin was added to the flask. Cell separation from the flask was monitored using an inverted microscope. Growth media was added to the mix to inhibit trypsin's actions. The cell mixture was plated onto flasks and/or petri dishes.

Dishes were left to grow to 100% confluence, changing the growth media every 48 hours. Cells were placed in differentiation media (DMEM+ GlutaMAX-1, 10% FBS, 1% penicillin/streptomycin, 5 mM dexamethasone, 0.25μM 3-isobutyl-methylxanthine) for 7 days. Differentiation was confirmed by visual reference. Once differentiated, the cells were placed in growth media for 2 days to further mature. Growth media was replaced by serum free media (0.1% BSA, 1% penicillin/streptomycin) for 15-24 hours before the experimental procedure.

Cell Lysis:

Treatments were incubated for specific amounts of time, and cell media was aspirated. After experimental treatments, the 3T3-L1 cells were lysed to obtain whole cell extracts. The plates were rinsed in ice cold phosphate buffered saline (PBS) and treated with lysis mixture (1 μM phenylmethylsulfonyl fluoride (PMSF), 10 μM sodium fluoride (NaF), 1 μM sodium orthovanadate ($NaVO_4$), 0.5 μM leupeptin, 25 μL Igpal, 10

mL lysis buffer (0.05% Triton X-100 in PBS). Lysates were collected and centrifuged 15 minutes. The cytosol samples were stored at -80º C.

Protein Quantification (BCA)

Protein concentration was determined in cytosolic extracts by the method of BCA. Each sample was measured in duplicate using 5 μL of cell lysate and 250 μL of dye in a 96-well plate. Tubes were incubated for 30 minutes at 37°C then cooled to room temperature. Protein concentrations were measured using Spectramax Plus (Molecular Devices Corporation, Sunnyvale, CA) at 562 nm. A standard curve was generated using serial dilutions of a BSA standard of known concentrations (ThermoScientific, Rockford, IL). Sample concentrations were extrapolated based on this standard curve.

Reverse transcription- polymerase chain reaction

Total RNA from 3T3-L1 cells was isolated with TRIzol according to manufacturer's directions (Invitrogen, Carlsbad, CA). The cell lysates were incubated for 5 minutes at 15-30° C. Chloroform was added and samples were centrifuged for 15 minutes at 2-8°C. RNA was precipitated by the addition of isopropyl alcohol. Samples were incubated for 10 minutes at 4°C. Supernatant was removed and the RNA pellet was washed with 75% ethanol. The sample was mixed by vortexing and centrifugation for 5 minutes at 2-8°C. The RNA pellet was air-dried for 5 minutes, dissolved in RNase-free water, and then incubated at 60°C. RNA concentrations were estimated based on

absorbance at 260 nm. PCR was performed using a RETROscript Kit according to manufacturer's directions (Ambion, Naugatuck, CT). The samples were kept at 70°C for 3 minutes, and then incubated at 42°C for an hour. Samples were incubated at 92°C to inactivate reverse transcriptase and then placed on ice. PCR was performed using specific primers designed by Dr. Jaya Desai. PCR ran in Gene Amp PCR System 2700 (Applied Biosystems). PCR reaction mixture was denatured at 95°C for 5 minutes and allowed to proceed for amplification. Each cycle consisted of denaturation at 94°C for 1 minutes, primer annealing at 55°C for 45 seconds, and primer extension at 72°C for 2 ½ minutes. A final extension step at 72°C for 7 minutes was performed. DNA generated was loaded into a 1% agrose gel and electrophoresed. Visualization of the bands was performed using ethidium bromide on an Alpha Innotech FluorChem 8000 imager.

Experimental Design

3T3-L1 cells were grown and differentiated as described above. Cells were treated with or without Norleual 10^{-10} M for one hour, Ang IV 10^{-10} M for 10 minutes, and insulin (Sigma-Aldrich, St. Louis, MO) 10^{-7} M for 5 minutes. For MAPK and IRS-1 studies, in plates treated with multiple stimulants Norleual was added first for the required time, followed by insulin, then Ang IV (Table 1). For cell treatments in the Akt experiment Norleual was added first for the required time, followed by Ang IV, then insulin (Table 2). Cells were lysed and cytosol was collected as described above. Protein concentrations were determined using the BCA procedure and Western blotting was performed probing for phospho-antibody. The membranes were stripped and re-probed

for the total antibody. Band density was determined on an Alpha Innotech FluorChem 8000 imager. For IRS-1 study, cell lysates were immnoprecipitated first then western blotting was performed. These experiments were repeated 3 times.

Table 1: Ang IV regulation of MAPK and IRS-1 cellular treatments.

Plate Number	Ang IV (10^{-10} M) Time	Norleual (10^{-10} M) Time	Insulin (10^{-7} M) Time
1 (Control)	--	--	--
2	5 minutes	--	--
3	10 minutes	--	--
4	60 minutes	--	--
5	--	60 minutes	--
6	--	--	5 minutes
7	--	60 minutes	5 minutes
8	10 minutes	--	5 minutes
9	10 minutes	60 minutes	--
10	10 minutes	60 minutes	5 minutes

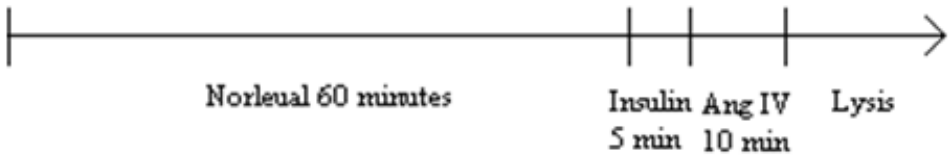
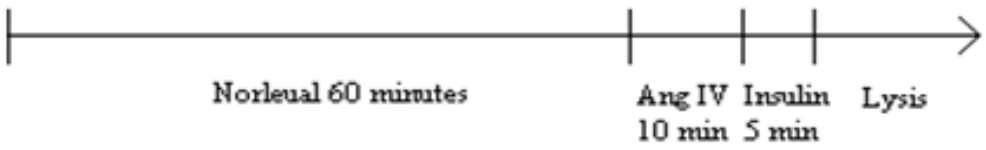


Table 2: Ang IV effects on the phosphorylation of Akt cellular treatments.

Plate Number	Ang IV (10^{-10} M) Time	Norleual (10^{-10} M) Time	Insulin (10^{-7} M) Time
1 (Control)	--	--	--
2	10 min	--	--
3	10 min	60 min	--
4	--	--	5 min
5	10 min	--	5 min
6	10 min	60 min	5 min
7	10 min	--	5 min
8	10 min	60 min	5 min



Western Blot:

Equal amounts (30 µg) of protein were mixed with 4x loading buffer and loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 1.5 mm thick gel (Invitrogen, Carlsbad, CA). The gels ran at 100 volts for 2 hours in running buffer (25mM Tris base, 190 mM glycine, 0.1% SDS) using an Invitrogen Novex Mini Cell and Fisher Scientific FB300 power supply. Proteins were transferred onto PVDF membranes at 200 mAmps for 2 hrs in transfer buffer (48 mM Tris base, 39mM glycine, 0.04% SDS, 20% methanol). The PVDF membranes were blocked in a 5% bovine serum albumin/Tris-Buffered Saline Tween-20 (BSA/TBST) blocking buffer for one hour at room temperature. The membranes were probed with specific phospho-antibodies using a 1:1,000 dilution factor (primary antibody: 5% BSA/TBST) overnight at 4°C. After primary incubation, the membranes were washed in TBST (10 mM Trizma Base, 150 mM NaCl, 0.05% Tween 20, pH to 8). Secondary antibody in a 1:50,000 dilution was added to the membranes for one hour at room temperature. The membranes were washed in TBST. ECL was used to visualize the bands on Kodak film developed using SRX-101A Medical Film Processor and band density was determined on an Alpha Innotech FluorChem 8000 imager.

Stripping Membranes:

Membranes that were probed for a specific anti-body were re-probed with a loading control to show equal loading of protein. Membranes were washed in TBST. The membranes were incubated in Restore Western Blot Stripping Buffer (ThermoScientific, Rockford, IL) for 15 minutes at room temperature (pH 3.0).

Membranes were washed in TBST then blocked with 5 % BSA/TBST for one hour at room temperature and blotted using the Western Blot procedure above.

Immunoprecipitation of IRS-1:

IP was obtained using manufacturer's directions (Pierce Classic IP Kit, Thermo Scientific, Rockford, IL). Once protein concentrations were determined using BCA, lysate (750 µg/µL) was incubated overnight at 4° C with 5 µg/µL of IRS-1 antibody. Pierce Protein A/G Agarose was centrifuged for one minute at 4° C then washed with IP Lysis/Wash Buffer. The antibody/lysate sample was added to the Protein A/G Agarose and mixed at 4° C for one hour. This was centrifuged at for one minute at 4° C. The resin was then washed with IP Lysis/Wash buffer and centrifuged for one minute at 4° C, then washed with Conditioning buffer. 2x reducing sample buffer was added to the resin and incubated at 100° C for 5 minutes. The tubes were centrifuged and cooled to room temperature before applying to the SDS-PAGE gel made in lab (Appendix).

Analysis of Western Blotting (Alpha-Innotech)

Western blot images were generated using FluorChem 8000 imager and band analysis was performed using the AlphaView- FluorChem Q software.

Statistical Methods

Data are reported as means ± SEM (standard error of the mean). Equality of variance in different samples was generated by Levene's test, $p \leq 0.05$ was considered

significant. For analysis that were significant, difference in mean percent were determined by Kruskal-Wallis non-parametric rank test with a Mann-Whitney pairwise group comparison to determine where differences lay. For data sets with equality of variance comparisons between groups were generated using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison procedure. Figures and statistical analyses were produced using GraphPad Prism (GraphPad Prism 5 for Windows/ GraphPad Software Inc.) and a $p \leq 0.05$ was considered significant.

Chapter IV
Results

AT_1, AT_2, and AT_4 Receptor Confirmation

RT-PCR confirmed the the expression of Angiotensin II type1a (AT_1a) and type1b (AT_1b), Angiotensin II type 2 (AT_2), and Angiotensin IV/Lnpep (AT_4/ aminopeptidase) receptors in adipocytes (Figure 3). AT_1a and AT_1b receptors are expressed at lower levels than AT_2 and AT_4 receptors in 3T3-L1 adipocytes.

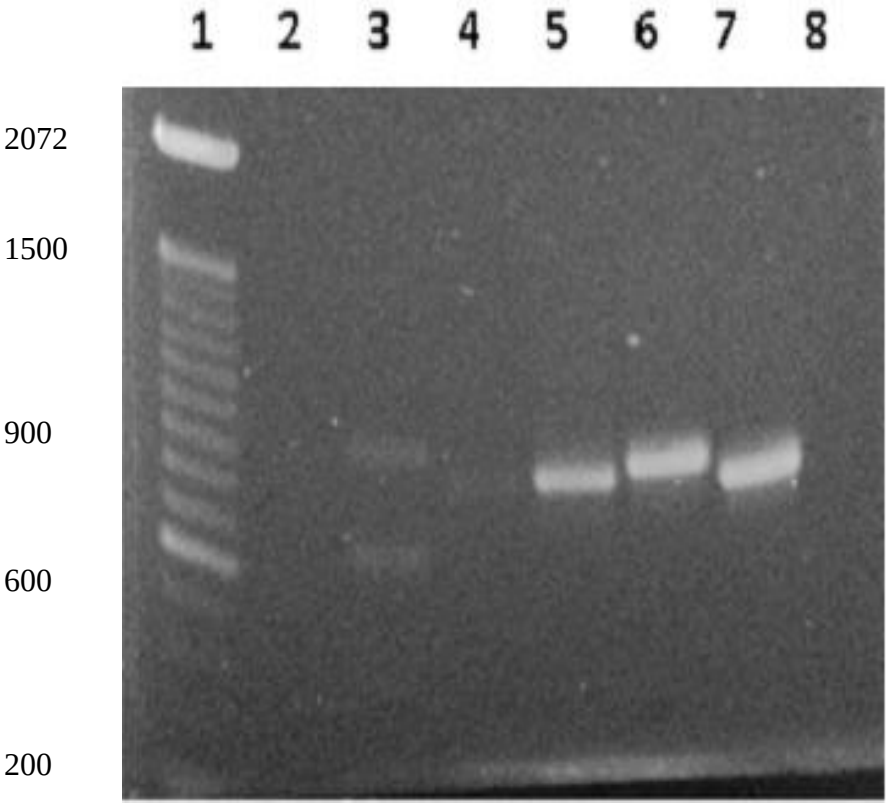


Figure 3: Expression of Angiotensin Receptors in 3T3-L1 Cells Adipocyte Cells. Lane 1: 100bp ladder, Lane 2: blank, Lane 3: AT_1a, Lane 4: AT_1b, Lane 5: AT_2, Lane 6 and 7: AT_4, Lane 8: blank. Each PCR amplicon is ~ 800-900 bp long.

Effects of Ang IV on the Phosphorylation of ERK

The amount of phosphorylated ERK from the stimulation of various combinations of Ang IV, insulin, and Norleual was detected using Western blotting (Figure 4). Bars 2, 3, and 4 show stimulation of Ang IV 10^{-10} M for 5, 10, and 60 minutes. The phosphorylation of ERK increases significantly over 5 and 10 minutes of Ang IV stimulation. By 60 minutes of Ang IV stimulation, the phosphorylation of ERK is no different than control levels. Cellular stimulation using Norleual, 10^{-10} M for 60 minutes, did not change the phosphorylation of ERK as compared with control. Insulin stimulation, 10^{-7} M for 5 minutes, caused three times the phosphorylation of ERK compared with control. Norleual did not attenuate insulin's actions on the phosphorylation of ERK; the stimulation of Norleual and insulin together caused an increase in the pERK levels compared with control. Ang IV and insulin in combination increased the phosphorylation of ERK 1/2 as compared to control, but had no additive effect when comparing Ang IV and insulin stimulation of pERK independently. Ang IV-induced phosphorylation of ERK 1/2 was reversed by pretreatment with the novel AT_4 receptor antagonist, Norleual. Norleual attenuated Ang IV phosphorylation of ERK1/2, whereas it did not affect ERK 1/2 phosphorylation with the combination of insulin and Ang IV.

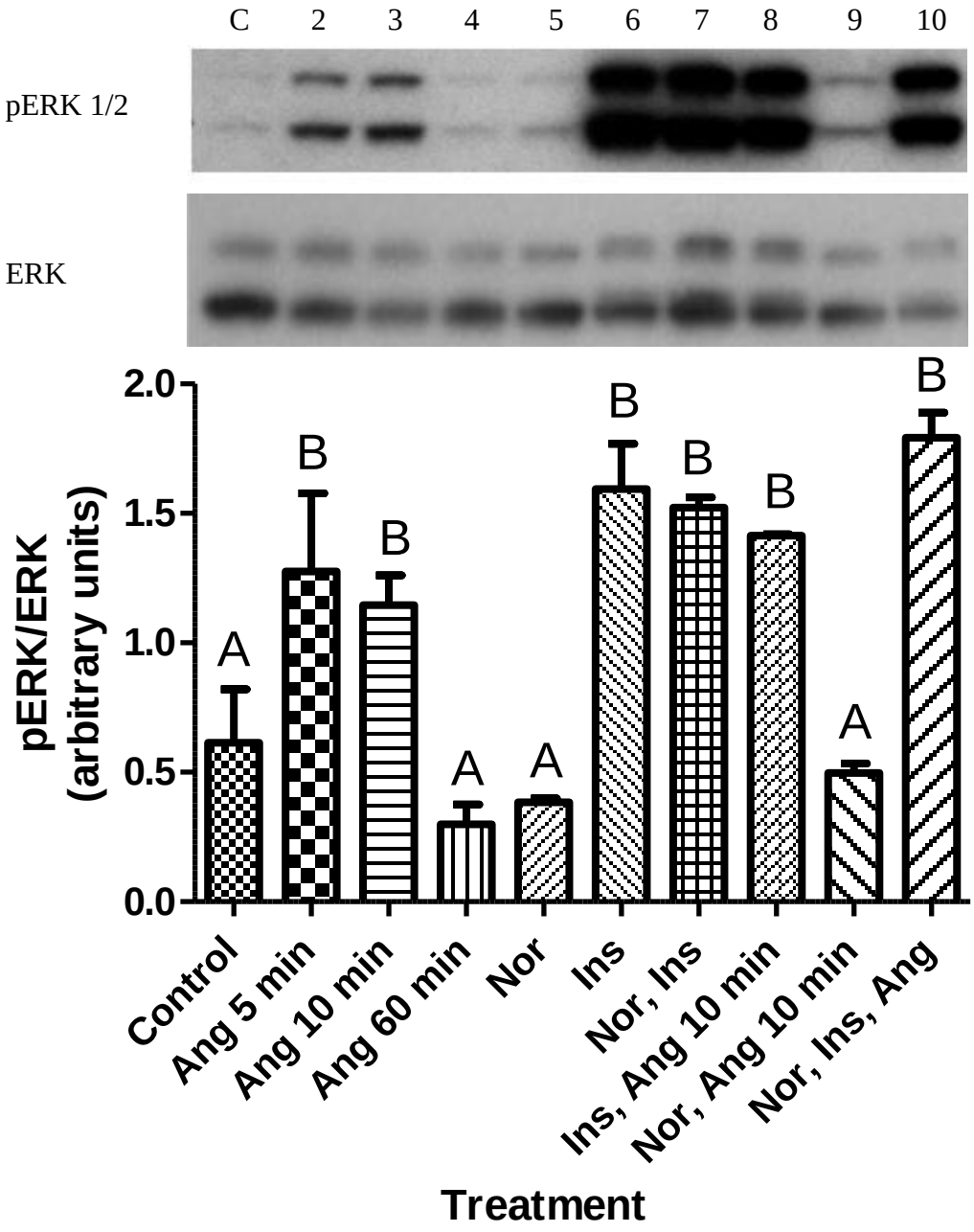


Figure 4: Expression of phosphorylated ERK. C: Control, 2: Ang IV 10^{-10} M 5 minutes, 3: Ang IV 10^{-10} M 10 minutes, 4: Ang IV 10^{-10} M 60 minutes, 5: Norleual 10^{-10} M 60 minutes, 6: Insulin 10^{-7} M 5 minutes, 7: Norleual 10^{-10} M 60 minutes, insulin 10^{-7} M 5 minutes, 8: Insulin 10^{-7} M 5 minutes, Ang IV 10^{-10} M 10 minutes, 9: Norleual 10^{-10} M 60 minutes, Ang IV 10^{-10} M 10 minutes, 10: Norleual 10^{-10} M 60 minutes, insulin 10^{-7} M 5 minutes , Ang IV 10^{-10} M 10 minutes. Values are means ± SE; Bars indicated with different letters are significantly different from each other ($p \leq 0.5$) n=3 experiments.

Effects of Ang IV on the Phosphorylation of JNK

The phosphorylation of JNK from various treatments of Ang IV, insulin, and Norleual stimulation was detected using Western blotting (Figure 5). Bars 2, 3, and 4 show the phosphorylation of JNK due to the stimulation of Ang IV 10^{-10} M for 5, 10, and 60 minutes. The phosphorylation of JNK increased significantly over 5 and 10 minutes of Ang IV stimulation. By 60 minutes of Ang IV stimulation, the phosphorylation of JNK is not significantly different from control levels. The stimulation of adipose cells with Norleual, 10^{-10} M for 60 minutes did not significantly change the levels of pJNK as compared with control. Insulin stimulation at 10^{-7} M for 5 minutes did not increase pJNK levels as compared with control. Stimulation with Norleual and insulin did not significantly increase the levels of phosphorylated JNK as compared with control. The combination of Ang IV for 10 minutes and insulin also did not significantly increase pJNK levels as compared with control. Ang IV-induced phosphorylation of JNK was reversed by pretreatment with the novel AT_4 receptor antagonist, Norleual. Stimulation with Norleual, Ang IV, and insulin did not significantly raise pJNK levels as compared with control.

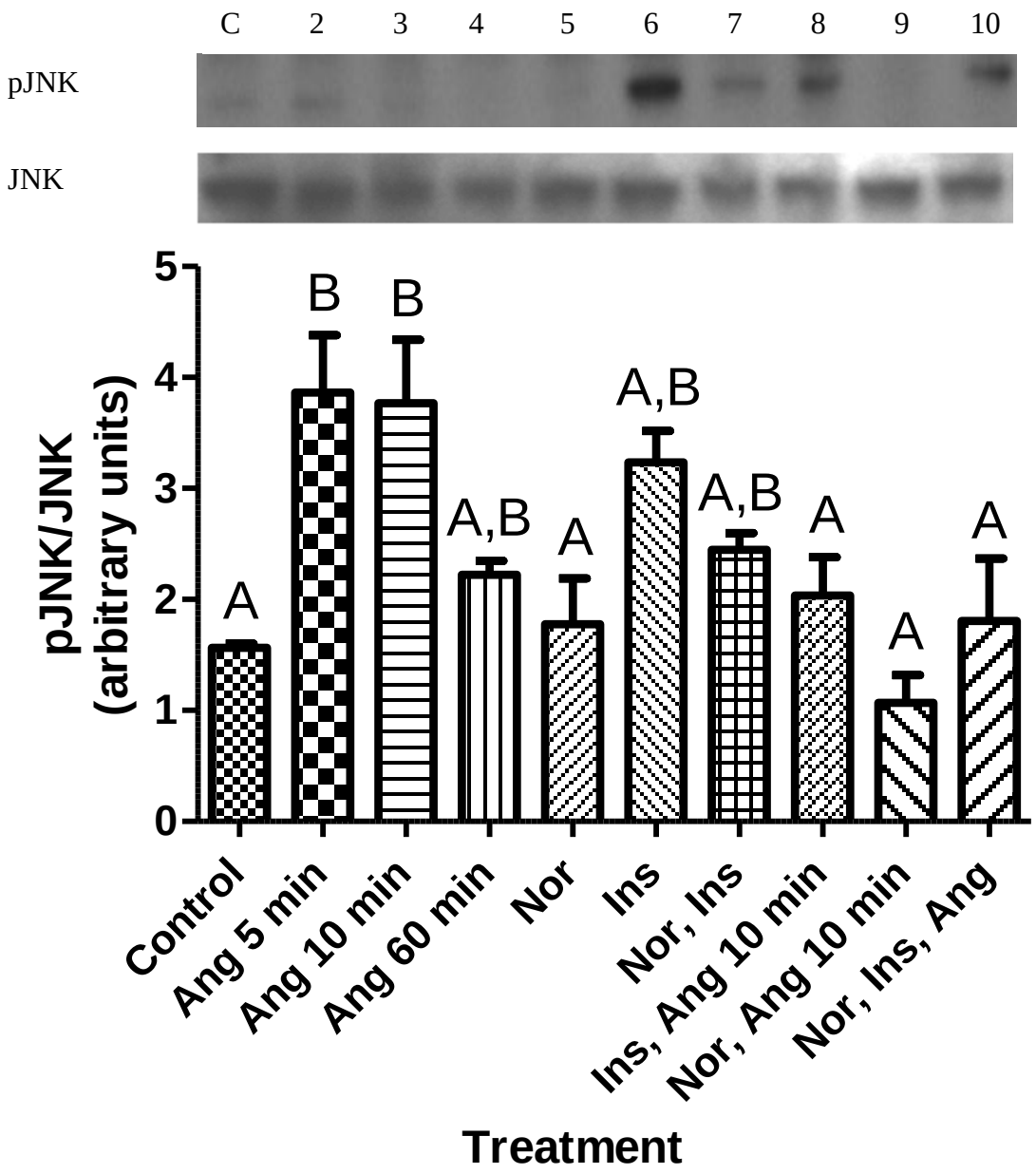


Figure 5: Expression of phosphorylated JNK. C: Control, 2: Ang IV 10^{-10} M 5 minutes, 3: Ang IV 10^{-10} M 10 minutes, 4: Ang IV 10^{-10} M 60 minutes, 5: Norleual 10^{-10} M 60 minutes, 6: Insulin 10^{-7} M 5 minutes, 7: Norleual 10^{-10} M 60 minutes, insulin 10^{-7} M 5 minutes, 8: Insulin 10^{-7} M 5 minutes, Ang IV 10^{-10} M 10 minutes, 9: Norleual 10^{-10} M 60 minutes, Ang IV 10^{-10} M 10 minutes, 10: Norleual 10^{-10} M 60 minutes, insulin 10^{-7} M 5 minutes , Ang IV 10^{-10} M 10 minutes. Values are means ± SE; Bars indicated with different letters are significantly different from each other ($p \leq 0.5$) n=3 experiments.

Effects of Ang IV on the Phosphorylation of Akt (S473 and T308)

The phosphorylation of Akt (T308 and S473) from Ang IV, insulin, and/or Norleual stimulation was found using Western blotting (Figures 6 and 7, respectivly). Ang IV at 10^{-10} M for 10 minutes did not increase the phosphorylation of Akt (T308 and S473). The combination of Norleual, 10^{-10} M for 60 minutes, and Ang IV did not increase the pAkt (T308 and S473) levels compared to control. Insulin at 10^{-7} M for 5 minutes stimulated the phosphorylation of both Akt residues as compared with control. Pre-treatment of Ang IV blocked insulin's upregulation of Akt (T308 and S473), making the phosphorylation of these residues not significantly different from control. This affect can be reversed by stimulating the cells first with Norleual, which blocks Ang IV inhibition of insulin. The stimulation with Norleual, Ang IV, and insulin caused an upregulation of Akt (T308 and S473) as compared with controls and comprable to insulin's stimulation of Akt.

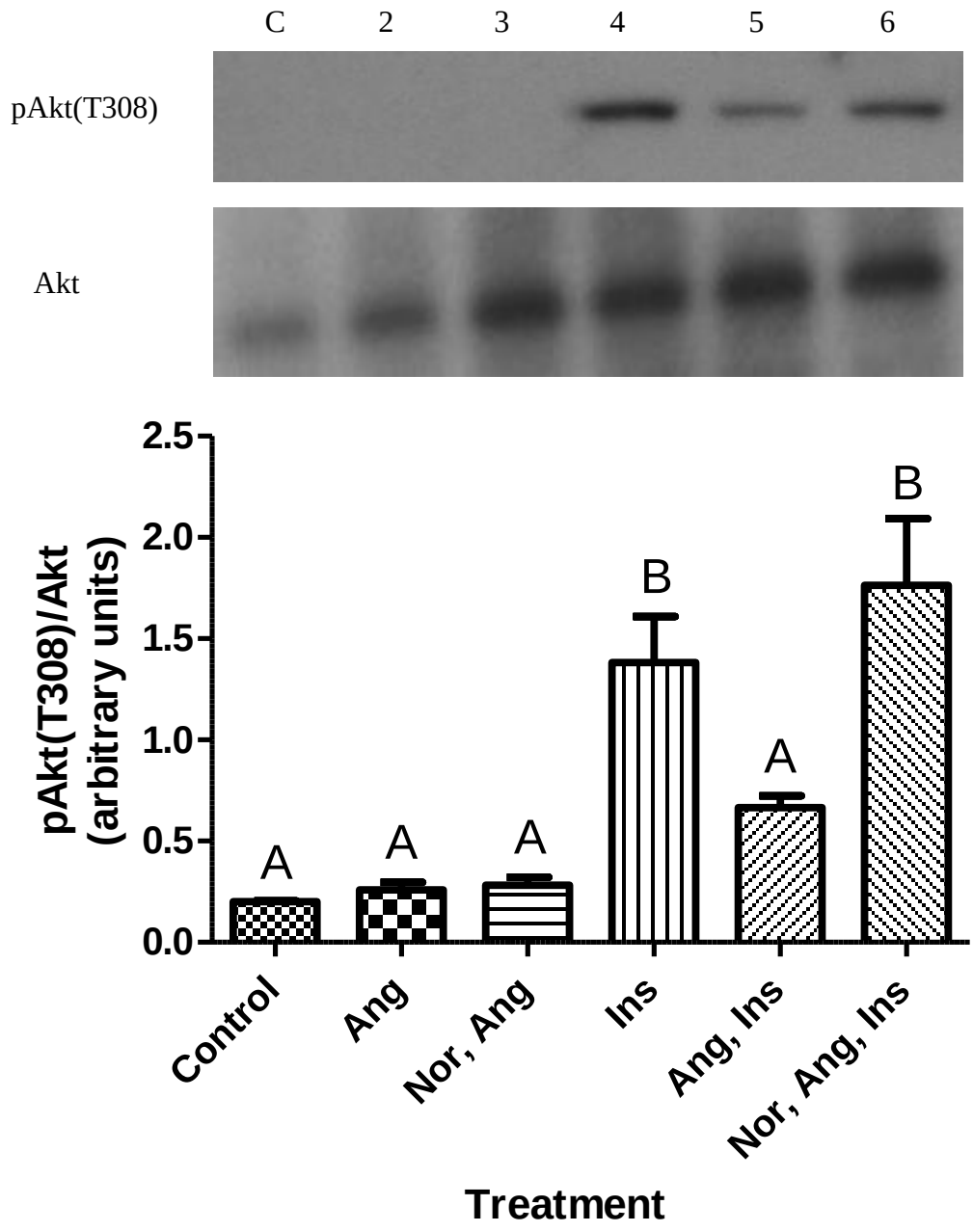


Figure 6: Expression of phosphorylated Akt (T308). C: Control, 2: Ang IV 10^{-10} M 10 minutes, 3: Norleual 10^{-10} M 60 minutes, Ang IV 10^{-10} M 10 minutes, 4: Insulin 10^{-7} M 5 minutes, 5: Ang IV10^{-10} M 10 minutes, insulin 10^{-7} M 5 minutes, 6: Norleual 10^{-10} M 60 minutes, Ang IV 10^{-10} M 10 minutes, insulin 10^{-7} M 5 min. Values are means ± SE; Bars indicated with different letters are significantly different from each other ($p \leq 0.5$) n=3 experiments.

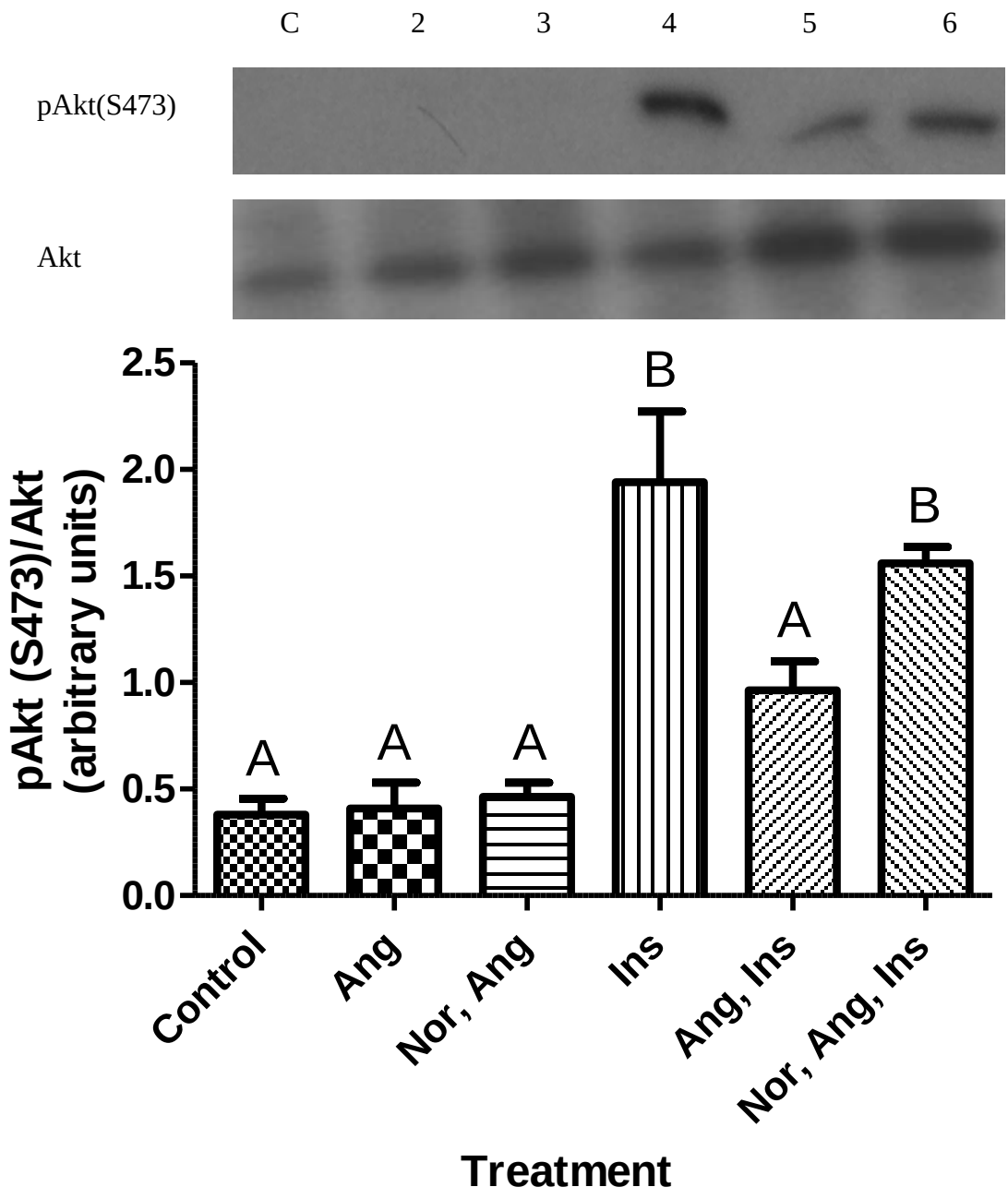


Figure 7: Expression of phosphorylated Akt (S473). C: Control, 2: Ang IV10^{-10} M 10 minutes, 3: Norleual 10^{-10} M 60 minutes, Ang IV 10^{-10} M 10 minutes, 4: Insulin 10^{-7} M 5 minutes, 5: Ang IV10^{-10} M 10 minutes, insulin 10^{-7} M 5 minutes, 6: Norleual 10^{-10} M 60 minutes, Ang IV 10^{-10} M 10 minutes, insulin 10^{-7} M 5 minutes. Values are means ± SE; Bars indicated with different letters are significantly different from each other ($p \leq 0.5$) n=3 experiments.

Effects of Ang IV on the Phosphorylation of IRS-1(S307 and S612)

The phosphorylation of IRS-1 (S307 and S612) from various treatments of Ang IV, insulin, and Norleual stimulation was found by immunoprecipitation followed by Western blotting (Figures 8 and 9, respectively). Treatment of 3T3-L1 adipocytes with Ang IV at 10^{-10} M for 10 minutes resulted in a statistically significant phosphorylation of serine 307 and serine 612 residues on the IRS-1 subunit as compared with the control. The pre-incubation of Norleual attenuated the effect of Ang IV; this stimulation did not cause the phosphorylation of IRS-1 serine residues. Insulin did not phosphorylate IRS-1 on serine 307 or serine 612 residues. Pre-incubation of the cells with insulin blocked the affects of Ang IV on the phosphorylation of serine 307 and 612 residues.

Cells were treated with a time course of Ang IV at 10^{-10} M for 5, 10, and 60 minutes. MAPK phosphorylation was found by western blot analysis. IRS-1 serine phosphorylation was found by immunoprecipitation followed by western blotting to compare the affects of different Ang IV time stimulations on the phosphorylation of ERK and IRS-1(S612) (Figure 10) and the phosphorylation of JNK and IRS-1(S307) (Figure 11). Ang IV activates the phosphorylation of MAPK quickly in 3T3-L1 adipocytes. Activation occurs within 5 minutes of Ang IV stimulation. This phosphorylation continues to be significantly increased over 10 minutes of Ang IV treatment. By 60 minutes of stimulation, the levels of phosphorylated MAPK are back to control levels. Ang IV stimulation of cells for 5 minutes did not significantly increase the phosphorylation of IRS-1 (S612). While Ang IV stimulation of cells for 5 minutes did significantly increase the phosphorylation of pIRS-1(S307) compared to control. By 10

minutes of Ang IV stimulation there was an increase in the phosphorylation of both serine residues on IRS-1. This activation was decreased by 60 minutes of Ang IV stimulation.

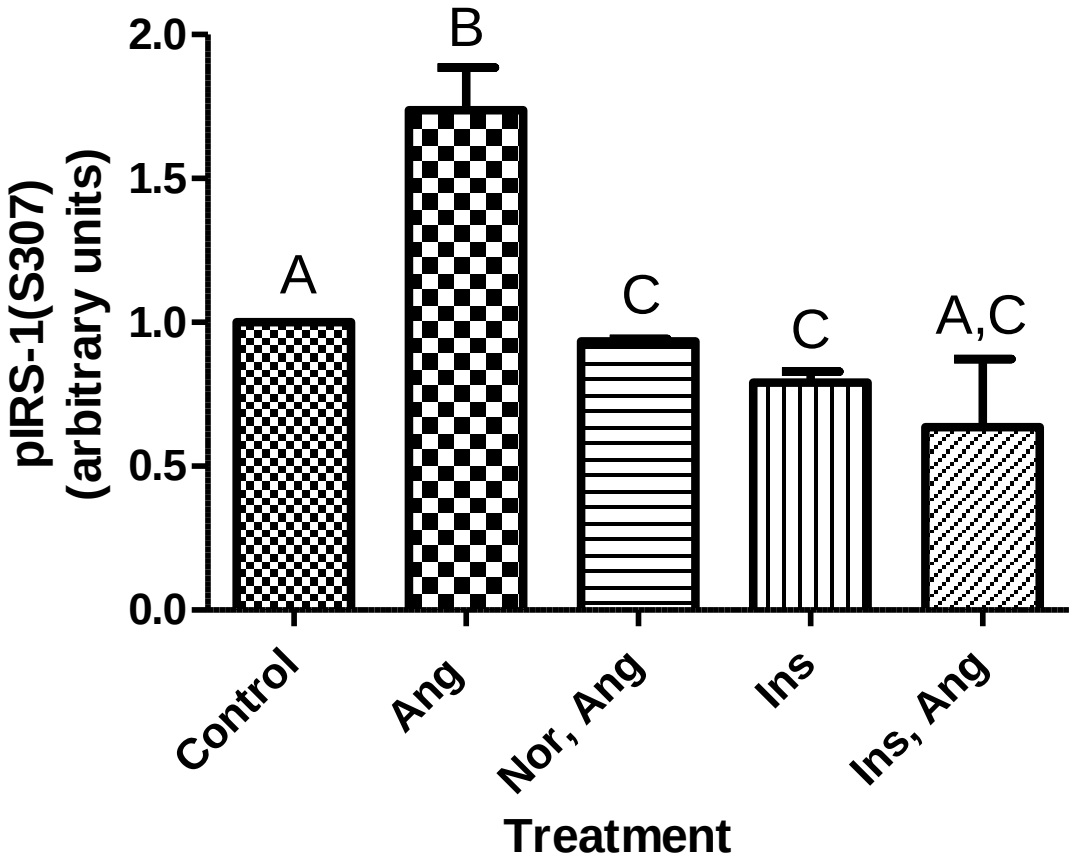


Figure 8: Expression of phosphorylated IRS-1(S307). C: Control, 2: Ang IV 10^{-10} M 10 minutes, 3: Norleual 10^{-10} M 60 minutes, Ang IV 10^{-10} M 10 minutes, 4: Insulin 10^{-7} M 5 minutes, 5: Insulin 10^{-7} M 5 minutes, Ang IV 10^{-10} M 10 minutes. Values are means ± SE; Bars indicated with different letters are significantly different from each other ($p \leq 0.5$) n=3 experiments.

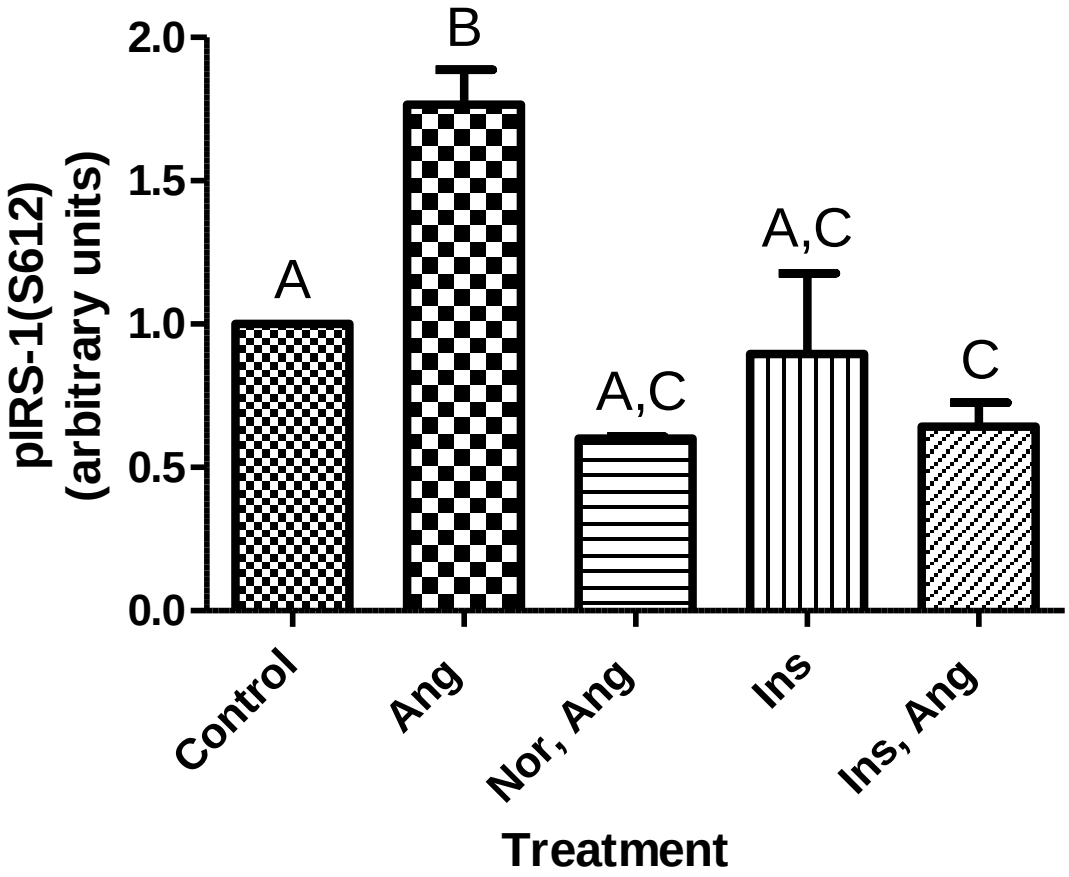


Figure 9: Expression of phosphorylated IRS-1(S612). C: Control, 2: Ang IV 10^{-10} M 10 minutes, 3: Norleual 10^{-10} M 60 minutes, Ang IV 10^{-10} M 10 minutes, 4: Insulin 10^{-7} M 5 minutes, 5: Insulin 10^{-7} M 5 minutes, Ang IV 10^{-10} M 10 minutes. Values are means ± SE; Bars indicated with different letters are significantly different from each other ($p \leq 0.5$) n=3 experiments.

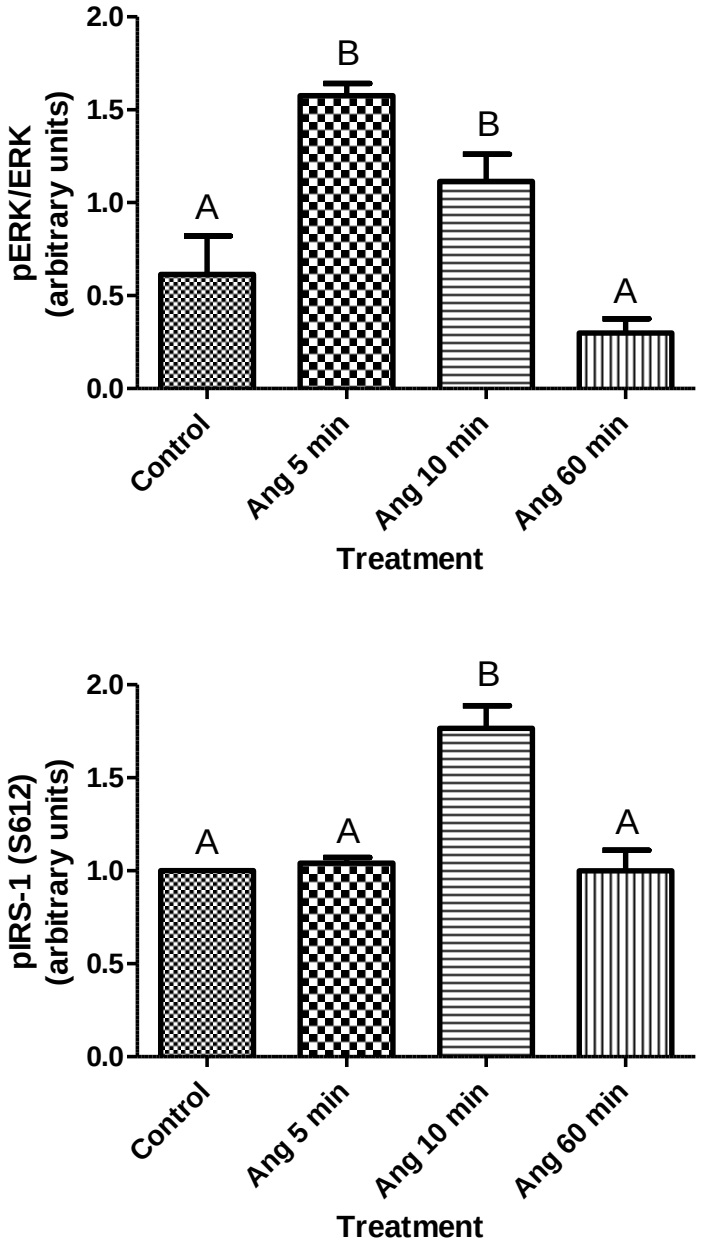
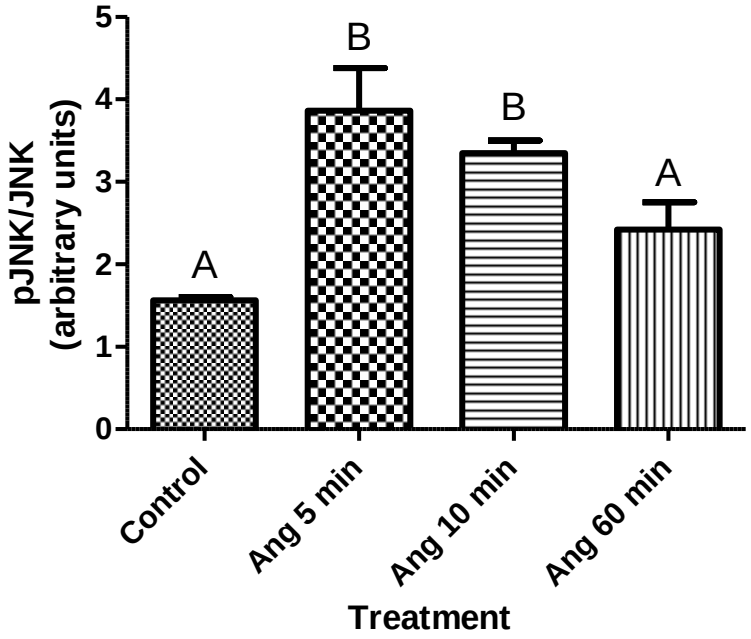


Figure 10: Expression of phosphorylated ERK and phosphorylated IRS-1(S612). C: Control, 2: Ang IV 10^{-10} M 5 minutes, 3: Ang IV 10^{-10} M 10 minutes, 4: Ang IV 10^{-10} M 60 minutes. Values are means ± SE; Bars indicated with different letters are significantly different from each other ($p \leq 0.5$) n=3 experiments.



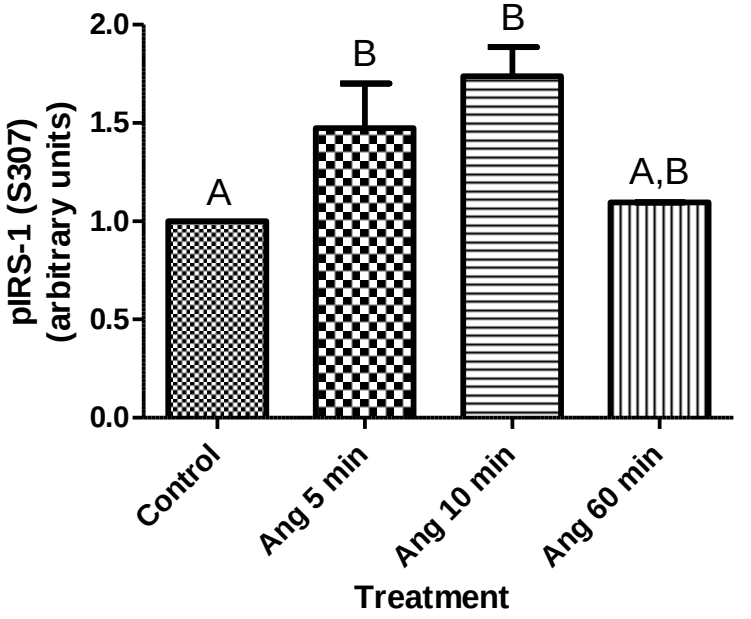


Figure 11: Expression of phosphorylated JNK and phosphorylated IRS-1(S307). C: Control, 2: Ang IV 10^{-10} M 5 minutes, 3: Ang IV 10^{-10} M 10 minutes, 4: Ang IV 10^{-10} M 60 minutes. Values are means ± SE; Bars indicated with different letters are significantly different from each other ($p \leq 0.5$) n=3 experiments.

Chapter V
Discussion

This study shows that Ang IV stimulates MAPK: ERK and JNK phosphorylation and also down-regulates Akt phosphorylation in 3T3-L1 adipocytes, which are possible mechanisms in the development of insulin resistance. Adipocytes incubated in the presence of Ang IV significantly increased the phosphorylation of ERK and JNK. These MAPK are known to phosphorylate IRS-1 residues on serine 612 and serine 307, respectively, which has been shown to block activation of regulatory molecules in the insulin signaling cascade [58]. In fact, Ang IV stimulation of adipocytes resulted in an increased phosphorylation of serine residues on IRS-1. Cellular stimulation of Ang IV also blocked insulin's activation of Akt residues. Numerous studies have found that blocking insulin's activation of Akt leads to insulin resistance by attenuating GLUT4 translocation to the cellular membrane and glucose uptake [15, 58, 59].

Ang IV and the Phosphorylation of MAPK

Ang II causes insulin resistance through the up-regulation of certain MAPK [149]. Ang II is known to impair insulin signaling through the activation of ERK and JNK, which phosphorylate IRS-1 on serine residues [58]. This then decreases the ability of IRS-1 to be phosphorylated on tyrosine residues- attenuating IRS-1 activation and impairing insulin's signaling to Akt and other downstream molecules [59]. In fact, chronic activation of ERK and JNK due to prolonged Ang II activation has been shown to induce insulin resistance by affecting glucose transport and insulin signaling [149].

Ang IV also causes an increase in the phosphorylation of ERK and JNK. Ang IV activates MAPK quickly in 3T3-L1 adipocytes. Within 5 minutes of Ang IV stimulation, the phosphorylation of ERK is 2 fold higher than control and JNK activation is 3 times higher compared with control levels. This stimulation continues to be significant over 10 minutes. By 60 minutes of Ang IV stimulation, the phosphorylated levels of MAPK are back to control. This confirms that Ang IV signals 3T3-L1 adipocytes to phosphorylate ERK and JNK.

This study found Norleual acted as a AT_4 receptor antagonist in 3T3-L1 adipocytes. Ang IV-induced phosphorylation of both MAPK was inhibited by pretreatment with the novel AT_4 receptor antagonist, Norleual. This effect suggests that there may be a role for AT_4 receptor inhibitors as a theraputic target to decrease the activation of insulin resistance inducing MAPK.

Cellular stimulation of insulin did not increase pJNK levels as compared with control. Insulin stimulation at 10^{-7} M for 5 minutes caused three times the phosphorylation of ERK compared with control. Insulin stimulation of certain cell types activate the Ras-Raf-ERK pathway, this activation of ERK has been shown to control mitogenesis and cellular growth [159-161] .

ERK has been found to be a distal point of interaction between insulin and Ang II signaling systems. Insulin and Ang II have both been found to up-regulate the phosphorylation of ERK. This is done through two separate receptors (AT_1 and IR) which activate cell pathways that converge onto the Ras/Raf/MEK/ERK system [5].

Ang IV and insulin in combination increased the phosphorylation of ERK 1/2 as compared with control. The combination of Ang IV and insulin did not have an additive

effect on pERK levels. As discussed earlier, Norleual attenuated Ang IV phosphorylation of ERK1/2, but Norleual did not attenuate insulin's actions on the phosphorylation of ERK; the stimulation of Norleual and insulin together caused an increase in the pERK levels as compared with control. Because AT_4 receptor antagonism did not interfere with insulin's actions, this infers that Ang IV and insulin work through separate receptors to phosphorylate ERK. This study suggests that Ang IV, signaling through the AT_4 receptor, and insulin, which stimulates cells through the IR, converge on the ERK pathway to phosphorylate ERK in adipocytes.

Effects of Ang IV on the Phosphorylation of IRS-1 (S307 and S612)

The regulation of insulin signaling, which is important in the formation of insulin resistance, is achieved through a balance of positive IRS-1 tyrosine phosphorylation and negative serine phosphorylation on IRS-1 [58, 151]. It is well established that insulin induces the phosphorylation of IRS-1 tyrosine residues which leads to the activation of the insulin signaling pathway in which insulin can exert its affects on adipocytes. Factors like Ang II, hyperinsulinemia, and cellular stress have been shown to activate serine kinases that phosphorylate IRS-1. This impairs insulin signaling.

Studies have shown that Ang II can activate MAPK, and this increase of ERK and JNK has been implicated in insulin resistance because they phosphorylate IRS-1 serine 612 and serine 307 residues, respectively [5, 58, 59]. This serine phosphorylation of IRS-1 decreases the ability of tyrosine phosphorylation on the substrate decreasing

insulin's signaling in cells. With the confirmation of Ang IV induced ERK and JNK phosphorylation, the affect of Ang IV on IRS-1 residues was explored.

Ang IV stimulation quickly activates many pathways in 3T3-L1 adipocytes. Ang IV stimulation for 10 minutes significantly increased the phosphorylation of IRS-1 serine residues as compared with controls. This time affect of Ang IV was also found to be similar in MAPK activation. The quick phosphorylation of ERK and JNK by 5 to 10 minutes of Ang IV stimulation mirrored the activation of serine residues on IRS-1 by 10 minutes of stimulation. This suggests that Ang IV plays a role in insulin resistance through the up regulation of MAPK, which may be interfering with IRS-1 signaling. Further studies are needed to confirm this effect.

Ang IV's effects on the phosphorylation of serine residues on IRS-1 were found to be inhibited by Norleual. These findings demonstrate a cross-talk between Ang IV and the insulin cascade that negatively modulates insulin receptor signaling. The blockade of Ang IV's effects by Norleual suggests a potential role of AT_4 receptor blockade as a therapeutic strategy to enhance insulin sensitivity.

Stimulation with insulin did not phosphorylate serine residues on IRS-1 in adipocytes, as expected. Interestingly, incubation of insulin before Ang IV did not cause the phosphorylation of serine residues on IRS-1. This suggests that insulin overshadows Ang IV's effects on IRS-1 and that Ang IV does not overcome insulin's ability (in high doses) to phosphorylate tyrosine residues.

Effects of Ang IV on the Phosphorylation of Akt (S473 and T308)

This study looked at Ang IV's direct effects on the insulin signaling pathway. Studies have shown that inhibition of various parts of this pathway lead to insulin resistance [15, 162]. One effect of Ang II is targeted at the inhibition of PI3K/Akt pathway. Ang II down regulation of this pathway opposes the action of insulin to enhance glucose uptake in cells and may lead to insulin resistance in cells [59].

Akt is an important downstream molecule in the insulin signaling pathway, and serves as a multifaceted intermediary protein by propagating PI3K signaling from IRS-1 to a wide range of biological effectors. Inhibition of Akt has been shown to induce insulin resistance [59, 152]. Ang IV's affect on the phosphorylation of Akt activation sites in adipocytes was explored.

Ang IV stimulation of 3T3-L1 adipocytes did not induce the phosphorylation of Akt. This suggests that Ang IV does not activate the insulin signaling pathway through Akt. Insulin, as expected, increased the phosphorylation of Akt at its activation sites, which would lead to Akt's activation of its downstream effectors to exert insulin's actions in adipocytes. Adipocytes that were pre-treated with Ang IV before insulin did not significantly increase the phosphorylation of Akt. This suggests that Ang IV can block insulin's activation of Akt in these cells. Blocking Ang IV signaling through its AT_4 receptor by Norleual attenuated this inhibition of insulin's signal. This again suggests a possible therapeutic role of AT_4 receptor antagonists in enhancing insulin sensitivity in adipocytes.

Ang IV attenuates insulin's signaling by blocking insulin-induced activation of Akt. This may cause a decrease in the activation of Akt's downstream effects including GLUT4 translocation and glucose uptake in cells.

AT_4 Receptor Inhibition

Most of Ang IV actions are mediated by a specific binding site called the AT_4 receptor. AT_4 receptors have a pharmacological profile that deviate significantly from that of AT_1 and AT_2 receptors [78, 163]. Structural studies provide evidence that, unlike AT_1 and AT_2 receptors, AT_4 receptors do not belong to the family of 7-trans-membrane domain receptors. The AT_4 receptor does not bind to AT_1/AT_2 receptor antagonists like losartan and PD123319.

As discussed previously, AT_1/AT_2 receptor inhibitors have been developed as a pharmacological treatment for hypertension and insulin resistance [49, 51, 52, 60, 75, 96, 128]. In this study we have found that pre-treatment with Norleual, an AT_4 receptor inhibitor, was able to block Ang IV's effects on the up regulation of MAPK, IRS-1 serine residue phosphorylation, as well as Ang IV's inhibition of Akt activation sites. Now, with increasing evidence of Ang IV cross-talk with the insulin signaling pathway, it is important to investigate AT_4 receptor blockers as a new therapeutic target.

The administration of AT_4 receptor antagonist drugs have appeared to be useful as a tool to treat the memory impairment that has been associated with Alzheimer's disease [91]. In this regard, blockade of Ang IV through the AT_4 receptor antagonists may have beneficial effects on other pathways that Ang IV controls. AT_4 receptor blockers could

be a novel therapeutic target in patients with insulin resistance, hypertension, and the metabolic syndrome.

Chapter VI
Conclusion

Obesity is a becoming a rampant problem throughout the United States and the world. It is estimated that almost 75% of adults in the United States are either overweight or obese [164]. Now, with the understanding that adipose tissue has endocrine functions, it is important to elucidate how the increased adipose mass found in obesity is affecting the body. Many studies have shown that obesity is linked to a variety of maladies, including the metabolic syndrome, type II diabetes, and hypertension [2, 7]. Data from studies demonstrates increased secretions of proteins and hormones from adipose tissue to the development of these diseases [10]. It is now imperative to understand the molecular mechanisms involved in adipose signaling and the development of insulin resistance in adipocytes.

This thesis explored a secretion of the local adipose RAS, Ang IV, and its effects on insulin resistance. Ang II, the main effector hormone of this system, has been linked to the attenuation of insulin signaling and glucose transport leading to type II diabetes [4]. We have shown that Ang IV, a fragment of Ang II, is also involved in the molecular mechanisms leading to insulin resistance.

We first confirmed the expression of Ang IV's receptor AT_4, in 3T3-L1 adipocytes by RT-PCR. Adipocytes were then stimulated with various combinations of Ang IV, Norleual, and insulin to better understand the role of these peptides in insulin resistance.

Ang IV, like its precursor Ang II, was found to up-regulate the activation of MAPK: ERK and JNK. These MAPK are implicated in the insulin signaling pathway by

their phosphorylation of serine residues on IRS-1. This phosphorylation interferes with tyrosine phosphorylation, and therefore activation of IRS-1, leading to the down-regulation of the insulin signaling pathway and insulin resistance. In fact, phosphorylation of IRS-1 (S307 and S612) was found to be up-regulated with the addition of Ang IV. This corresponded to the increased activation of MAPK caused by Ang IV in the adipocytes.

Ang IV also directly affected the PI3K/Akt pathway. This pathway has been found to regulate insulin-induced signaling in 3T3-L1 adipocytes. Ang IV stimulation inhibited the insulin-induced activation of Akt. Studies have confirmed that inhibition of Akt leads to the decrease of glucose uptake in cells, due to the attenuation of insulin's signal and the decreased ability for GLUT4 to translocate to the cellular membrane [152].

Obesity is linked to insulin resistance and the development of diabetes. This may be in part due to endocrine functions of adipose tissue, releasing Ang IV. Ang IV has been shown to affect various components of the insulin signaling cascade. Further studies on the regulation of Ang IV on downstream targets of the insulin signaling pathway, such as GLUT4 translocation, would be beneficial to elucidate the role of Ang IV crosstalk with the insulin signaling pathway. Also the physiological effects of Ang IV, like glucose uptake in adipocytes, should be explored to better understand the role of Ang IV in insulin resistance. Although this study has found that Ang IV negatively affects the insulin signaling pathway *in vivo*, there is no evidence on Ang IV effects *in vitro*. Therefore it is crucial to develop animal and human studies to explore Ang IV effects on the development of insulin resistance.

Another area of future research would be the affects of Ang IV inhibition through AT_4 receptor blockade on insulin resistance. Ang II receptor inhibitors and ACE inhibitors have already been found to be beneficial in patients with type II diabetes and hypertension [49]. It is possible that AT_4 receptor antagonist, Norleual, may also be a therapeutic target for patients with these diseases. Ang IV is a novel peptide of the RAS, further studies are needed to understand if it also affects other pathways that Ang II is known to work through on adipocytes, including its role in adipocyte differentiation and lipid metabolism [44].

This study addressed the role of Ang IV in insulin resistance, an underlying defect of the metabolic syndrome and the cause of increased morbidity and mortality [2, 7]. We have found that Ang IV can inhibit the insulin signaling cascade through multiple mechanisms *in vitro.* The data gathered from this study contributes to a better understanding of the molecular mechanisms involved in the development of insulin resistance. This in turn may lead to the identification of novel molecular targets and the development of new therapeutic approaches to improve insulin sensitivity and correct glucose impaired metabolism.

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Appendix

Whole Cell Culture Buffers

Lysis buffer
100 μL phenylmethylsulfonyl fluoride (PMSF)
100 μL sodium fluoride (NaF)
50 μL sodium orthovanadate ($NaVO_4$)
50 μL leupeptin
25 μL Igpal
10 mL lysis buffer (0.05% Triton X-100 in PBS)

Phosphate buffered saline 1x (PBS)
8 g of NaCl
0.2 g of KCl
1.44 g of Na_2HPO_4
0.24 g of KH_2PO_4
Adjust pH to 7.4
Distilled H_2O up to 1L

Western Blotting Buffers

4x Loading buffer (1L)
2.4 mL Tris HCl
0.8 g SDS
4 mg Bromophenol blue
5 mL glycerol
Distilled H_2O up to 10 mL

Running buffer 1x (5L)
15 g Tris base
72 g glycine
5 g SDS
Distilled H_2O up to 5 L

Transfer buffer 1x (5L)
15 g Tris
72 g glycine
1.25 g SDS
Distilled H_2O up to 4 L
20% methanol (add when using buffer)

Blocking buffer (per membrane)
0.5 g BSA
10 mL TBST

TBS 10x
24.23 g Trizma HCl
80.06 g NaCl
Mix with 800 mL ultra pure water
pH to 7.6 with HCl
Distilled H_2O up to 1 L

TBST
For 1 L: 100 mL of TBS + 900 mL ultra pure water + 1 mL Tween20

Primary and secondary antibody
Concentration depend on antibody
Dilute antibody in blocking buffer
Dilution ranges are 1:1,000- 1:50,000

ECL (per membrane)
2 mL solution A
50 µL solution B

Gel Preparation for IRS-1 Western Blots

Solution A
Acrylamide/Bis-acryl amide 40% solution

Solution B
7.28 g Trizma Base
92 µL TEMED
pH to 8.9 with HCl and add ultra pure water to 40 mL final volume

Solution C
1.21 g Trizma Base
92 µL TEMED
pH to 6.9 with HCl and add ultra pure to 20 mL final volume

Solution D
Dissolve 1 g of sodium dodecyl sulfate (SDS) in 10 mL of ultra pure water

Solution E
Dissolve 60 mg of ammonium persulfate (APS) in 5 mL of ultra pure water

Gel Pouring Procedure

Set up plates in BioRad plate holder.
For separating gel, add water, Solution A, B, D, and E to a 50 mL Falcon tube (Table 3).
Pour gel approx 2/3 of the way full; slowly add water on top of gel to even.
Let gel set for 30 minutes
After 30 minutes, pour off water by tilting the whole casting stand over sink; use Kimwipe to siphon the rest of water from between plates.
For stacking gel add water, Solutions A, C, D, and E to a 50 mL Falcon tube (Table 4).
Pour gel to the top, and then place the 15 well comb between the plates
Allow to set for 30 minutes

Table 3: Separating Gel

Separating Gel (8%)	(mL)
Ultra Pure Water	9.80
Solution A	4.00
Solution B	5.00
Solution D	0.20
Solution E	1.0

Table 4: Stacking Gel

Stacking Gel (3%)	(mL)
Ultra Pure Water	5.72
Solution A	0.60
Solution C	1.00
Solution D	0.08
Solution E	0.60

Vita

Julie Ann Jungwirth was born in La Jolla, California on April 14, 1986, and is the daughter of Raymond M. and Emilia M. Jungwirth. Julie has one sibling, Michael. She received her high school diploma from Keene High School, in Keene, New Hampshire in June 2004. She then entered the University of Connecticut majoring in Nutritional Science. She received a Bachelor of Science degree in Nutritional Science in May 2008. Julie then began her Masters work at the University of Tennessee, Knoxville in Nutritional Science. She became a Registered Dietitian after completing the University of Tennessee's Dietetic Internship in 2010. Julie received her Master of Science degree in Nutritional Science in August 2011.