To the graduate Council.

I am submitting herewith a thesis written by Melissa Kay Standridge entitled "Angiotensin II regulation of adipocyte metabolism." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirement for the degree of Master of Science in Nutrition.

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Associate Vice Chancellor and
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ANGIOTENSIN II REGULATION OF ADIPOCYTE METABOLISM

A Thesis
Presented for the
Master of Science in Nutrition
Degree
The University of Tennessee, Knoxville

Melissa Kay Standridge
August 2000

DEDICATION

This thesis is dedicated to my parents Mr Jack Thomas Standridge, Mrs Brenda O'Kelley Standridge and my sister Mrs Jackie Standridge Hurst. Their loving support, patience and encouragement enabled me to complete this thesis I would like to thank my parents for teaching me to believe in myself even in the face of adversity. From an early age, my parents instilled in me that it is most important to act with dignity and honor when faced with adversity, for those are the situations that define one's character.

ACKNOWLEDGEMENTS

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Finally I want to express my appreciation for my friends. Without their endless support through bad times and good, I could not have reached this moment

ABSTRACT

Recent studies showed that adipocytes secrete angiotensin II (AII) and express AII receptors, suggesting a role of adipose tissue in obesity-associated hypertension. However, the physiological actions of AII in adipocytes remain unclear This thesis study was designed to investigate the role of angiotensin II, a hypertensive hormone, in adipose tissue metabolism. We hypothesized that AII may exert these effects by directly stimulating lipogenesis To address this issue, we used murine 3T3-L1 as a cell model and the fatty acid synthase (FAS) gene as a lipogenic marker. We demonstrated that AII up-regulates the FAS gene at the transcriptional level In order to identify AII regulatory sequences in the FAS promoter, we transfected fusion constructs linking various deletions of the FAS promoter to the luciferase reporter gene into 3T3-L1 adipocytes We mapped the AII responsive sequences to the previously identified insulin responsive sequences (-62 to –46) in the proximal region of the FAS promoter. Furthermore, we demonstrated that a mutation in the insulin response element abolishes not only insulin but also AII responsiveness of the FAS gene Since Adipocyte Determination and Differentiation factor 1, ADD1, has been postulated as a transcription factor in the regulation of adipocyte genes, we investigated the role of ADD1 in AII regulation of the FAS gene. We demonstrated that overexpression of a dominant negative form of ADD1 in 3T3-L1 adipocytes markedly attenuates FAS induction by AII In conclusion, our data indicate that AII exerts insulin like effects on the FAS gene by targeting similar regulatory sequences and transcription factors as insulin and suggest that paracrine effects of AII in adipocytes contribute to adipocyte hypertrophy.

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LIST OF ABBREVIATIONS

adipocyte differentiation determination factor 1 : ADD1

angiotensin converting enzyme: ACE

angiotensin II : AII

angiotensinogen : ANG

angiotensin II type-1 receptor AT1

angiotensin II type-2 receptor · AT2

CCAAT/enhancer binding protein alpha C/EBPα

Cyclic adenosine 3',5'-monophosphate . cAMP

fatty acid synthase . FAS

nicotinamide adenine dinucleotide phosphate NADPH

hormone sensitive lipase HSL

insulin responsive sequence . IRS

insulin response element IRE

lipoprotein lipase . LPL

messenger RNA · mRNA

preadipocyte factor-1 pref-1

polyunsaturated fatty acids . PUFA

renin-angiotensin system RAS

tumor necrosis factor alpha . TNFα

uncoupling protein · UCP

upstream stimulatory factors USF

PART 1

Introduction

AII is a vasoactive hormone that primarily regulates blood pressure Angiotensin II (AII) is generated in the classical renin angiotensin system (RAS) known to be expressed in the liver, kidney and lungs Recent studies demonstrated that components of the RAS are expressed in several peripheral tissues, including adipose tissue. Because of the importance of adipose tissue mass in obese patients, AII-derived from adipose tissue may contribute to obesity-related hypertension, indeed a significant relationship between angiotensinogen, leptin (a marker of adiposity), and blood pressure has been reported.

Adipose tissue expresses all components of RAS, including angiotensinogen, renin and angiotensin converting enzyme necessary for the generation of AII. Angiotensinogen, the only known precursor of AII is nutritionally and hormonally regulated in adipose tissue Adipocytes produce and secrete AII and they also express AII receptors Several studies, including from our lab, have shown that adipose tissue derived AII may act in a paracrine manner via these receptors to regulate adipocyte metabolism and differentiation

In vitro studies demonstrate that AII induces differentiation of preadipocytes into mature adipocytes, in part, via a prostaglandin-dependent mechanism. Our lab has determined that angiotensin type II receptors are present in differentiated 3T3-L1 and human adipocytes and mediate AII-induced lipogenesis and triglyceride accumulation in these cells Increased triglyceride accumulation in adipocytes was due, in part, to increased activity and expression of a key lipogenic marker, fatty acid synthase (FAS).

We determined that the FAS gene is primarily regulated at the transcriptional level The objectives of this thesis research are. 1) to investigate the mechanism of AII

regulation of FAS gene transcription by identifying the AII response elements within the FAS gene and 2) determine potential transcription factors involved in this regulation.

PART 2

Literature Review

Obesity, Adipocytes and Fatty Acid Biosynthesis

INTRODUCTION

Excess body fat characterizes obesity. An approximate ninety-seven million adults in the United States are over-weight or obese (1) Obesity substantially raises the relative risk for hypertension, dyslipidemia, type 2 diabetes as well as coronary heart disease (Table 1). Obesity is assessed by body mass index (BMI), which is weight of an individual in kg divided by height squared in meters (kg/m^2) (Table 1). Evidence exists that decreasing abdominal fat will reduce blood pressure levels (64) Based on forty-five randomized controlled studies, the World Health Organization concluded that weight loss is recommended to lower elevated blood pressure in overweight and obese persons with high blood pressure (1).

THE ROLE OF ADIPOSE TISSUE IN OBESITY AND CO-MORBIDTY

Human obesity is a multifactorial problem that is modulated by genetic influences as well as environmental influences Several genetic animal models have been used to study obesity (6, 20-23). However, these models are single gene mutations (Table 2) and are rare in the human syndromes of obesity (19).

Several genetic animal models have been used to study obesity. However, these models are single gene mutations, which have been demonstrated to be rare in the human syndrome of obesity (19) Two documented forms of mild obesity result from single gene mutations in *tubby* and *fat* (20-23), while other single gene mutation result in a more pronounced obesity syndrome as is found in leptin related mutations of *ob/ob*, *db/db* mice and *fa/fa* Zucker rats (23). Tubby, which is an autosomal recessive mutation, produced from a splicing defect of a gene product on mouse chromosome 7 (21) Even though the

Table 1: Classifications of obesity and degree of relative risk for development of disease. Classifications of overweight and obese individuals are based on body mass index (BMI). Obese individuals may be divided into three subcategories. As BMI and obesity increases, there in an increase in risk for hypertension, dyslipidemia, type 2 diabetes as well as coronary heart disease

Classification of Overweight	Obesity Class	BMI (kg/m^2)	Risk
Normal		18.5-24.9	----------
Overweight		25.0-29.9	Increased
Obesity	I	30.0-34.5	High
	II	35.0-39.9	Very High
	III	≥40	Extremely High

Table 2: Documented forms of obesity resulting from single gene mutations in rodents.

Gene Mutant in Rodents	Obesity	Types of Mutations	References
tubby	mild	Autosomal recessive mutation of a gene product in chromosome 7.	20-22
fat	mild	Carboxypeptidase mutation	20,23
ob/ob	severe	Lack of a functional leptin (OB) protein in mice	24, 26
db/db	severe	Lack of a functional leptin receptor (OB) protein in mice	27
fat/fat	severe	Lack of a functional leptin receptor (OB) protein in Zucker rats	27
A^{vy}	severe	Ubiquitous expression of the Agouti protein.	30

function of this gene product has not been defined, it has been implicated in obesity due to the fact that mice lacking normal transcripts which are abundantly expressed in the hypothalamus under normal conditions develop obesity (22). Also, carboxypeptidase mutation (*fat*), which normally functions to excise paired dibasic residues in carboxyl terminus of prohormones such as proinsulin, produces mild obesity (23). However; the molecular mechanisms by which *fat* mutations results in obesity has yet to be defined. Several of these obesity models have been linked to proteins that are secreted by adipocytes (58)

Adipose tissue and the hypothalamus are two tissues implicated in the control of obesity It was previously thought that adipose tissue was primarily a storage depot for lipids controlled by central mechanisms However, it is now well documented that adipose tissue produces and secretes many factors that may potentially regulate adiposity and whole body homeostasis (2)

White and brown adipose tissue are two distinct types of adipose tissue in mammals (2) Brown adipose tissue (BAT) is distinct from white in that it contains a preponderance of mitochondria and is involved primarily in the generation of heat (thermogensis) to maintain whole body homeostasis primarily in cold-dwelling, hibernating animals (3-5) White adipose tissue represents the majority of adipose tissue found in rodents and humans

ADIPOCYTE DIFFERENTIATION

White adipose tissue's primary function is storage of energy as triglycerides Recent knowledge of mechanisms of adipocyte development is derived primarily from

the development of several in vitro models of adipocytes (35-37). These include adipose tissue explants, isolated adipocytes maintained in primary culture, and established preadipocyte cell lines. Established cell lines (multipotent stem cells of mesenchymal origin or adipose precursor cells) are capable of differentiation in vitro into a mature adipocyte phenotype (36,37) The adipocyte precursor cells are committed to an adipocyte fate, and when stimulated to differentiate by a cocktail mix of 10% fetal bovine serum, 0 5mM isobutylmethylxanthine and 250nM dexamethasone they display the morphological and biochemical features of mature adipose cells

We chose 3T3-L1 as our adipocyte model based on their ability to accumulate lipid droplets in their cytoplasm and their morphological and biochemical features that resemble mature adipocytes (35-37) (Figure 1A and B) 3T3-L1 sublines were originally cloned from disaggregated Swiss mouse embryo cells (35) 3T3-L1 preadipocytes undergo several stages to reach a fully differentiated state (Figure 1A) Cell-to-cell contact at confluence is required for subsequent differentiation of preadipocytes (38) Preadipocytes arrest at the G_0/G_1 boundary of the cell cycle and begin to express early markers of differentiation such as lipoprotein lipase, the α chain of collagen VI, and fatty acid transporters (2) Following confluence, preadipocytes must undergo an additional round of mitosis (mitotic clonal expansion) in order to differentiate into mature adipocytes (2) (Figure 1A). However, mitosis alone is not sufficient to induce terminal differentiation (39). Following the mitotic clonal expansion, dramatic changes in gene expression begin to occur. Transcription rates of up to 200 different genes are altered during the process of differentiation (reviewed in 40). The genes regulated during differentiation encode proteins involved in 1) lipogenesis (i e acetyl-CoA carboxylase

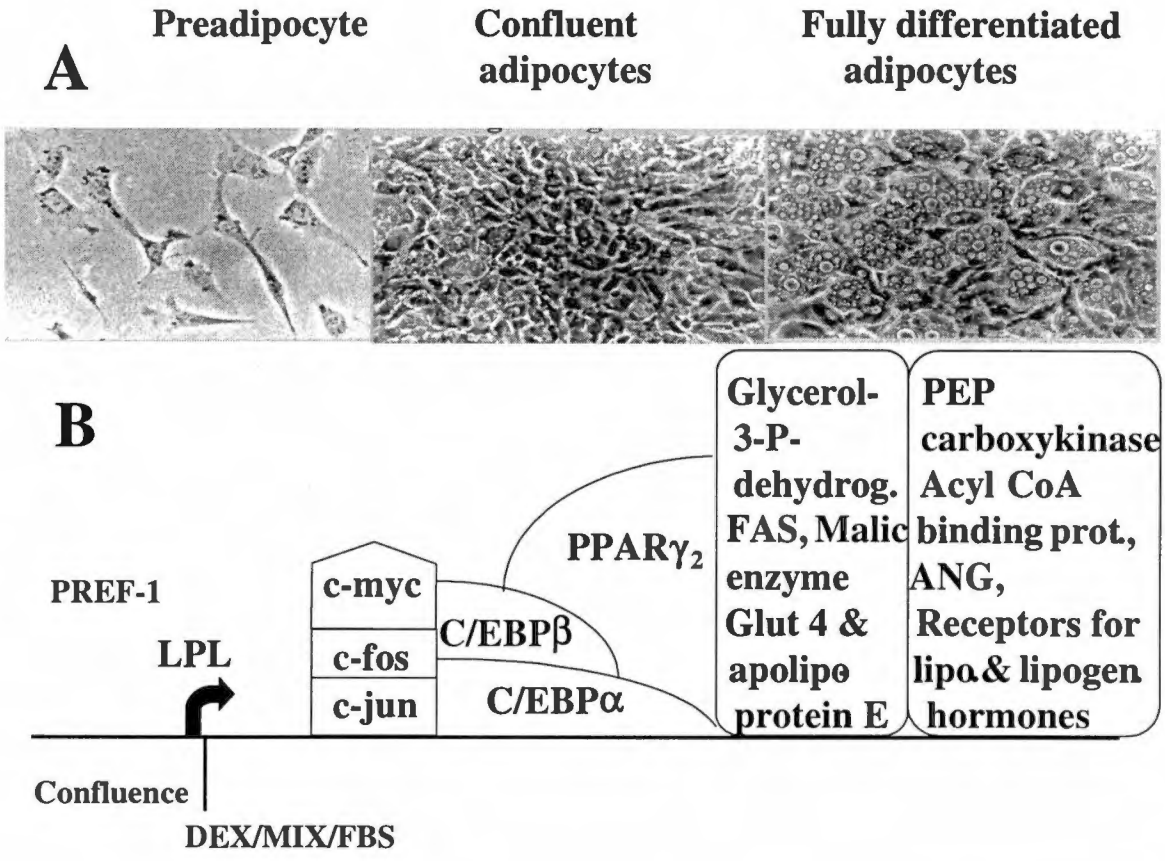


Figure 1: Sequential expression of markers during differentiation of 3T3-L1 preadipocytes to mature adipocytes. Figure 1A illustrates growing fibroblast (left), confluent cells (middle) and differentiated adipocytes (right). Several markers of preadipocytes such as lipoprotein lipase and preadipocyte determination differentiation factor-1 are present at confluence (Figure 1B). While other transcription factors such as CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferating activating receptors (PPARs) are sequentially expressed, following induction of adipocytes with 10% fetal bovine serum, 0.5mM isobutylmethylxanthine and 250nM dexamethasone (DEX/ MIX/ FBS) (Figure 1B). DEX/ MIX/ FBS promotes terminal differentiation via activation of several adipocyte markers.

and fatty acid synthase), 2) lipolysis (i.e. hormone sensitive lipase), 3) hormone action and signaling (i.e. G-proteins and insulin receptor), 4) secretory proteins (i e. angiotensinogen and adipsin) and 5) cytoskeletal and extracellular matrix proteins (i.e. actin, tubulin and collagens) (reviewed in 40) (Figure 1B). When adipocytes are terminally differentiated they are morphologically distinct from their preadipocyte precursors (2)

FATTY ACID BIOSYNTHESIS

Storage of triacylglycerols in mature adipocytes reflects a balance between the process known as lipogenesis (7) and lipolysis . There are several sources of fatty acids for triglyceride storage in adipose tissue Fatty acids can be derived from circulating lipoproteins, through the action of membrane-bound lipoprotein lipase (LPL) (7) or from de novo fatty acid biosynthesis The rate-limiting step in fatty acid biosynthesis is formation of malonyl CoA from acetyl CoA catalyzed by acetyl CoA carboxylase, a nutritionally regulated enzyme (8) Regulation of acetyl CoA carboxylase occurs primarily at the posttranscriptional level (47)

All subsequent steps for synthesis of fatty acids are catalyzed by FAS, a multifunctional cytosolic enzyme, which controls long term regulation of fatty acid biosynthesis (8). In higher eukaryotes, FAS catalyzes the synthesis of long chain saturated fatty acids, palmitate, in seven catalytic steps within the same polypeptide from acetyl–CoA and malonyl-CoA in the presence of NADPH (8). FAS is composed of seven enzymatic proteins· β-ketoacyl synthatase, acetyl-CoA transacylase, maloyl-CoA transacylase, dehydratase, enoyl reductase, ketoacyl reductase, acyl carrier protein, and

thioeterase activities which are organized into discrete domains in the order indicated (11) Multifunctional FAS requires reducing equivalents in the form of dihydroxynicotinamide adenine dinucleotide phosphate (NADPH) which are generated in the pentose phosphate pathway and by the malic enzyme reaction (7).

FAS concentrations in hepatic and adipose tissue are highly sensitive to nutritional, hormonal and developmental states (9-13,50). FAS is regulated primarily at the level of gene transcription, and there are no known allosteric regulators of FAS (41) Fasting in rats leads to diminished synthesis of FAS while refeeding a high carbohydrate diet or insulin treatment in vivo increases FAS synthesis (11) However, administration of glucagon or dibutyryl cAMP during refeeding of animals that had been fasting, completely blocked elevation of the FAS trancription rate, which suggests that glucagon, via cAMP, antagonizes the insulin effect and inhibits gene transcription (13, 50)

Diets high in simple carbohydrates and low in fats lead to induction of enzymes involved in lipogenesis Studies suggest that glucose-6-phosphate or xylulose-5-phosphate may be intracellular mediators of the signaling pathway for regulation of lipogenic genes by carbohydrates (reviewed in 52). FAS regulation by carbohydrates and insulin occurs at the transcriptional level (12,14) and glucose regulates FAS in an insulin-dependent as well as insulin-independent manner (48)

While carbohydrates and insulin have been shown to increase FAS expression, polyunsaturated fatty acids (PUFAs) have been shown to decrease FAS activity (a dominant effect over carbohydrates) (43) PUFA regulation of FAS occurs at the level of transcriptional repression (43), and peroxisome proliferator-activated receptor (PPAR)

has been implicated in this regulation (49). However, the exact mechanism (s) and identity of the putative repressor activity by PUFA remains to be determined.

Insulin was shown to exert its effects by increasing the transcription rate of the FAS gene (Figure 2) through an insulin responsive sequence (IRS) identified at –68 to –52 base pairs (GCC**CATGTG**GCGTGGCCGC) (bold letters represents essential E-box region for insulin regulation of the FAS gene) within the rat FAS gene promoter region (14) Insulin responsive sequences have been identified in numerous genes: collagenase AP-1, pyruvate kinase, glucagon, prolactin, phosphoenol-pyruvate carboxykinase and insulin-like growth factor binding protein genes (reviewed in 53) Insulin treatment of 3T3-L1 adipocytes expressing the FAS IRS reporter gene resulted in no change in the pattern of protein binding to the FAS IRS (14) However; this is opposite from results obtained for insulin responsive sequences (IRSs) of other genes, such as pyruvate kinase (reviewed in 53) In vivo, strepozotocin-diabetic mice where FAS mRNA levels were low, FAS mRNA levels increased by two fold within one hour after insulin injection and cyclohexamide abolished the insulin effect which indicates that ongoing protein synthesis is required for this action of insulin (50).

Several trans acting factors have been shown to bind IRS of the FAS gene in liver and adipose tissue Upstream stimulatory factors 1 and 2 (USF1 and 2) (15) as well as adipocyte determination and differentiation factor-1 (ADD-1) also known as sterol regulatory element binding protein-1 (SREBP-1) have been identified as trans acting factors that bind IRS or insulin responsive element of FAS (16) (Figure 2) Both USF and ADD-1 are members of the bHLH family of transcription factors (44). In the liver, USFs seem to be essential to sustain the dietary induction of the FAS gene (15) and USF1

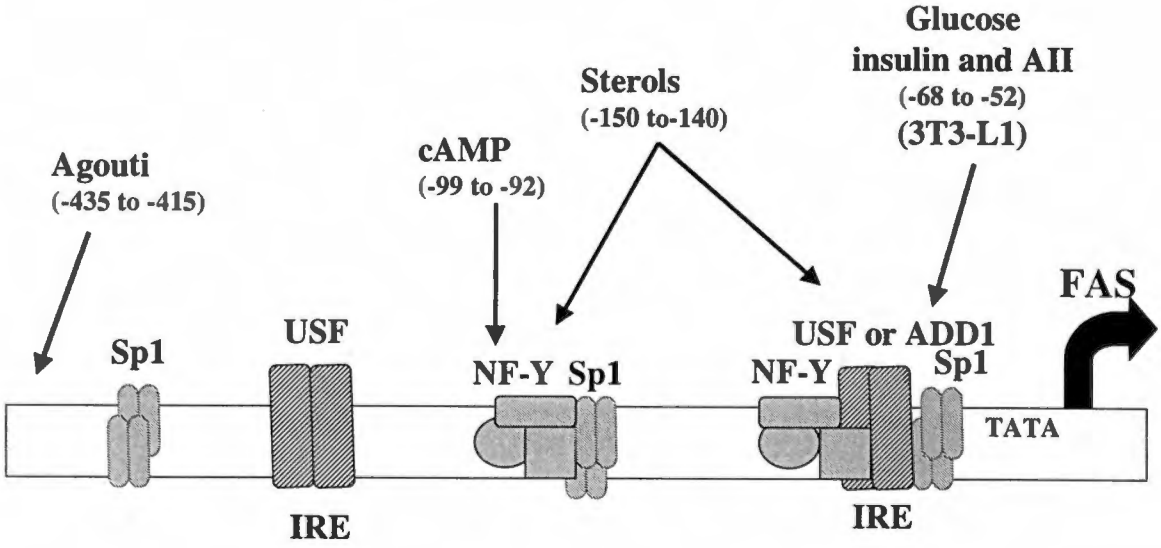


Figure 2: Regulatory sequences in the FAS promoter and transcription factors binding these sequences. Several regulatory sequences in the FAS promoter mediate regulation of the FAS gene transcription. These include an agouti response element at –435 to –415 region of the FAS gene, the insulin and glucose responsive element (IRE) or sequences (IRS) at –68 to -52, cyclic AMP response element at –99 to –140 and the sterol response elements at –150 to -140. Several trans acting factors have been shown to bind to these regulatory sequences as indicated in the figure. (reviewed in 53)

levels were dramatically increased in fasted rats upon refeeding indicating a molecular mechanism for FAS regulation by nutritional status (62).

ADD1 was originally cloned from rat adipose tissue (44) and has been shown to regulate the expression of several key genes of fatty acid and triglyceride metabolism in cultured fibroblasts, adipocytes, and livers of transgenic mice (16,54-55) When ADD1/SREBP1 is ectopically expressed in cultured cells and the livers of transgenic mice, it is sufficient to increase the expression of FAS and several other lipogenic genes (16, 54)

The basic domain of ADD1 bHLH protein binds to target DNA recognition sites termed E-boxes (CANNTG) (44) The HLH domain of these proteins controls homo- and/or heterodimerization (44). Unlike other bHLH transcription factors, ADD1 has dual DNA-binding specificity, interacting with E-box motif (44) and non-E-box sequences (ATCACCCCAC) (51) A single tyrosine residue in the basic domain controls this unique dual binding (51) Several genes (i e FAS, Leptin and S14) contain ADD1 consensus binding sequences in their promoter regions and these promoters can be transactivated by ADD1 (44,46)

ADD1 is expressed at high levels in white and brown adipose tissue as well as liver. ADD1 mRNA is elevated during differentiation (44) The human homolog of ADD1 (SREBP1) and a closely related factor (SREBP2) have been independently cloned and shown to be associated with the regulation of genes related to cholesterol metabolism (45) Data generated in vivo and in cultured cells suggest that SREBP2 is associated more specifically with cholesterol metabolism (56). Cholesterol depletion causes proteolytic release of the transcriptionally active amino-terminal portion of the molecules

from their embedded position in the endoplasmic reticulum membrane to allow movement to the nucleus (57).

ADD1/SREBP1 is required for the activation of hepatic lipogenic gene expression by glucose (18). ADD1 has been shown to promote adipocyte differentiation and expression of genes linked to fatty acid metabolism (16). Through the use of infection of a dominant negative form of ADD1 in adipocytes isolated from rats, FAS expression was determined to be ADD1 dependent (17) Insulin regulates ADD1/SREBP1 at a dose of insulin (1nM) consistent with an insulin action through the insulin receptor (46) ADD1/SREBP1, which has been shown to bind the previously identified E-box (14), is the strongest candidate as a transcription factor to participate in insulin regulation of the FAS gene in adipocytes (16-18).

FACTORS PRODUCED BY ADIPOCYTES THAT HAVE BEEN IMPLICATED IN REGULATION OF ADIPOCYTE MASS

In obese humans, several proteins are produced and secreted by adipocytes. In obese humans, leptin, tumor necrosis factor-alpha, angiotensinogen, acylation stimulating protein (ASP), cholesterol ester transfer protein (CETP) and phospholipid transfer protein (PLTP) are differentially produced and secreted according to depot and in some cases may be positively related to body mass index (BMI) (58) Plasminogen activator inhibitor type-1 (PAI-1) is a major inhibitor of fibrinolysis and is produced by endothelial cells as well as adipocytes (59). PAI-1 plasma levels correlate with the risk for myocardial infarction and venous thrombosis (59). PAI-1 is a possible common regulatory pathway between adipocytes and endothelial cells (59).

Leptin is the protein product of the *ob* gene that was originally cloned in mice (24). Leptin is produced and secreted by adipocytes in response to fat stores (25,26). Leptin is secreted into the circulation where it binds a leptin receptor in the hypothalamus and acts as a satiety factor by suppressing the production on neuropeptide Y (27) There are three animal models that have been associated with leptin production from adipocytes and interaction with leptin receptors in the hypothalamus. *ob/ob* mice lack the ability to produce a functional protein while *db/db* mice and *fa/fa* Zucker rats do not produce functional receptors

Agouti, which is a protein involved in coat color in many species (28), has a homologue in humans that has been cloned and is expressed in lean and obese humans (29) Mutation at the agouti gene leads to ubiquitous expression of Agouti in multiple tissue and causes obesity Agouti acts centrally to increase food intake via antagonizing the melanocortin receptor Peripherally agouti increases intracellular calcium, adipocytes lipogenesis and insulin secretion (30).

While several proteins that are produced and secreted by adipocytes are implicated in obesity, others such as tumor necrosis factor alpha (TNF-α) and interleukin-6, oppose increases in fat mass and have been implicated in inhibiting the differentiation of preadipocytes in vitro (31) Anti-inflammatory cytokines and transforming growth factor-beta increases steady state ob mRNA (60) Conversely, treatment of 3T3-L1 adipocytes with the pro-inflammatory cytokine, interleukin-6 and TNF-α decreases the leptin content and ob mRNA (60).

Another protein produced by adipocytes is adipsin that is a serine protease that has been proposed to participate in the regulation of systemic energy balance (32,33).

Several factors also have been shown to contribute to the adipocyte's supply of nutrients Monobutyrin is a fat-specific angiogenic factor produced by adipocytes (34) that acts to increase formation of blood vessels in developing clusters of adipocytes. Acylation stimulating protein (ASP) is a protein that is produced by adipocytes (61). In vitro studies have shown that ASP increases triglyceride synthesis and glucose transport (61) While, angiotensin II that is a classically known hypertensive hormone, has also been shown to also increase triglyceride accumulation in adipocytes and is a important factor in the metabolism of adipocytes (discussed below) (Table 3)

Table 3: Factors produced by adipocytes that have been implicated in regulation of adipocyte mass.

Key factors produced by adipocytes	Reference
Leptin	24-27
Agouti	28-30
Tumor necrosis factor alpha	31, 58
Interleukin-6	60
Adipsin	32-33
Monobutyrin	34
Acylation stimulating protein (ASP)	58, 61
Cholesterol ester transfer protein (CETP)	58
Phospholipid transfer protein (PLTP)	58
Plasminogen activator inhibitor type-1 (PAI-1)	59
Angiotensin II	10, 65-70

The Adipose Tissue Renin Angiotensin System: A link between obesity and hypertension?

INTRODUCTION

It is well recognized that obesity is a significant risk factor for hypertension and cardiovascular disease (63) and weight loss has been shown to be effective in lowering blood pressure in overweight and obese individuals (64). However, the pathophysiological mechanisms underlying the association between obesity and hypertension are still poorly understood. The discovery of the components of the renin angiotensin system (RAS) in adipose tissue (10,65-70) and its involvement in modulation of lipid metabolism (10,101) suggest that this local RAS may be involved in obesity-related disorders including hypertension and the metabolic syndrome.

CLASSICAL AND LOCAL RENIN ANGIOTENSIN SYSTEMS

The morbidity associated with the renin-angiotensin system (RAS) has been the topic of study for many years (71) It was previously known that angiotensinogen, a precursor hormone for the biologically active angiotensin II is produced by the liver in what is referred to as a classical RAS (72, Figure 3) In this system, angiotensinogen is produced by the liver and released into the circulation (72) Angiotensinogen is immediately cleaved into a decapeptide, angiotensin I, by renin, an enzyme produced by the kidneys. Angiotensin I is then cleaved by angiotensin converting enzyme, which is produced by the lungs, to form the octapeptide angiotensin II (73). Angiotensin II in the classical RAS causes a direct effect on blood vessels causing vasoconstriction, as well as, aldosterone release and sodium reabsorption in the kidney, with a net effect of increasing blood pressure (reviewed in 74). Sustained high blood pressure can lead to cardiac

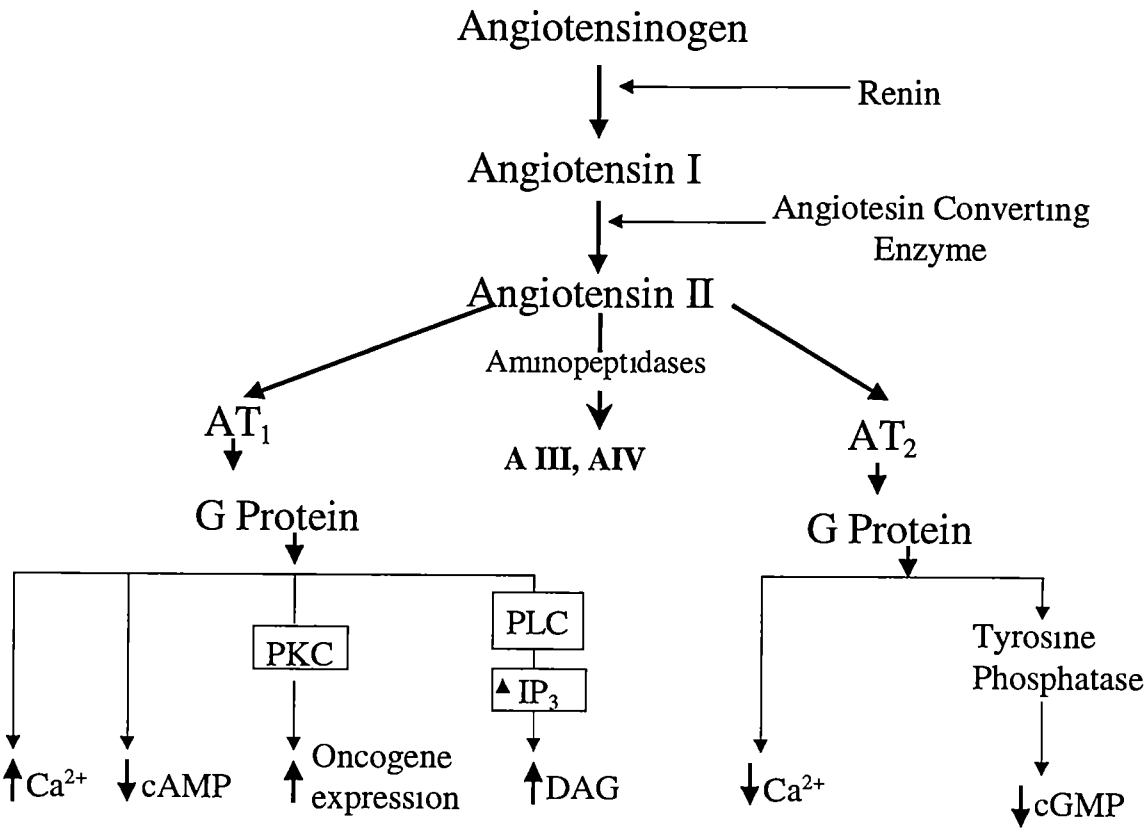


Figure 3: The renin angiotensin system: generation of angiotensin II and signaling.

Kidney renin cleaves angiotensinogen to form the decapeptide angiotensin I (AI). The lung Angiotensin converting enzyme (ACE) releases a dipeptide from AI to form the octapeptide AII Smaller peptides (AIII and AIV) are generated by aminopeptidases. The biologically active AII exerts multiple effects on several cell types via binding to its G-protein-linked receptors, primarily AT1 and AT2 receptors AT1 signaling mechanisms include increasing intracellular calcium, oncogenes and DAG as well as decreasing cAMP levels, while AT2 signaling involves decreasing calcium and cGMP.

hypertrophy, vascular endothelium damage and rupture of vessel walls which takes a toll on the cardiovascular system and other organs it perfuses.

Recent biochemical and molecular biological studies support the existence of local or tissue renin angiotensin systems in animals and humans (reviewed in 75-77) Local RAS has been identified in many tissues including liver, kidney, brain, spinal cord, aorta, mesentery, atria, lung, adrenal, large intestine, stomach, spleen (reviewed in 77-78), uterus (79), differentiated brown and white adipocytes and in fibroblast-like cells (10, 65-70) The first evidence for a local RAS was in the kidney where AII was proposed to control golmerular filtration rate by acting through what is now known as tubuloglomerular feedback (80) All components of local renin angiotensin system and AII effects have yet to be identified in each specific tissue that has been postulated to possess its own RAS However, there is considerable evidence based on research done to date on local renin angiotensin systems to support the link between hypertension, diabetes and cardiovascular disease (81-83).

AII RECEPTORS AND SIGNALING

The most predominant and well-studied receptors for angiotensin II are angiotensin type-1 (AT_1) and angiotensin type 2 (AT_2) receptors. AII signaling via these receptors is summarized in Figure 3 Other receptors for angiotensin 1-8 have been identified in non-mammalian species, but there is no compelling evidence from binding studies that indicates that another unique receptor for AII is present in mammals (84-85). There are two subtypes of angiotensin type 1 receptors that have been identified 1) AT_{1A} and 2) AT_{1B} AT_{1A} and AT_{1B} are 95-97% identical in amino acid sequence (86) There

are four documented cellular mechanisms associated with AT_1 signaling: 1) activation of phospholipase C (PLC) to form inositol triphosphate and diacylglycerol (DAG) as second messengers (87), 2) inhibition of adenylate cyclase activity (88), 3) changes in intracellular sodium which may result in opening of ion channels via L type calcium channel (89) and 4) activation of protein kinase C which stimulates the expression of several oncogenes (90) DAG can then serve as a second messenger for phospholipase A2 releasing arachidonic acid Arachidonic acid serves as substrate for prostaglandins, leukotrienes and other eicosanoids (reviewed in 91)

The second type of receptor for AII is angiotensin type 2 receptors (AT_2), primarily expressed in the brain and fetal tissue as well as some cell lines (92,101) The second messenger associated with the AT_2 receptor is of cyclic guanosine monophosphate (cGMP), which is inhibited by AII-AT_2 receptor binding through activation of a specific tyrosine phosphatase that dephosphorylates guanylate cyclase (93) AT_2 was also shown to reduce cytoplasmic calcium through inhibition of a T-type calcium channel (94)

The cellular responses brought about by AII appear to be different based on the receptor that binds AT_1 was shown to increase inositol triphosphate via activation of phospholipase C thereby leading to the conclusion that bound AT_1 receptors increase cytoplasmic calcium, whereas, bound AT_2 receptors are shown to reduce cytoplasmic calcium (reviewed in 91) Tissue dependent expression of receptors would therefore seem to regulate the response to AII in each individual tissue type. These responses have yet to be characterized for all tissues proposed to contain AII specific receptors. The

remainder of this review will focus on the adipose tissue RAS and the regulation of adipose tissue metabolism by AII.

Kidney renin cleaves angiotensinogen to form the decapeptide angiotensin I (AI). Angiotensin converting enzyme (ACE) produced by the lung releases a dipeptide from AI to form the octapeptide AII. Smaller peptides (AIII and AIV) are generated by aminopeptidases The biologically active AII exerts multiple effects on several cell types via binding to its G-protein-linked receptors, primarily AT_1 and AT_2 receptors. AT_1 signaling mechanisms include increasing intracellular calcium, oncogenes and DAG as well as decreasing cAMP levels, while AT_2 signaling involves decreasing calcium and cGMP

COMPONENTS OF THE RAS IN ADIPOSE TISSUE

Adipose tissue possesses its own non-classical RAS. The components of the renin angiotensin system have been identified in mammalian preadipocytes in primary culture as well as in rodent and human adipose tissues (65-70).

Adipose tissue can generate the angiotensin II precursor, angiotensinogen and can convert this precursor into the active hormone AII and related peptides. The majority of adipose tissue RAS components have first been identified in cell cultures of murine or rodent models However, angiotensinogen protein, renin and angiotensinogen converting enzyme were also documented in human adipose tissue by Karlson et al., confirming a complete human local adipose tissue renin angiotensin system (68). Expression of angiotensinogen, renin, renin binding protein, angiotensin converting enzyme (95) and AT_1 have been reported in primary cultures of human adipocytes (96) and human adipose

tissue (70). Many discrepancies have arisen in the literature surrounding differences in various components of RAS in adipose tissue, which have been attributed to cell type, species or age differences of the adipose tissue. A variety of renin isoforms as well as angiotensin converting enzyme have been identified in interscapular brown adipose tissue (67,98) but neither was found to be present in the preadipocyte cell line 3T3-F442A (98). Additionally, transcripts for cathepsin D and cathepsin G enzyme components of the non-renin angiotesin (NRAS) are detectable in human adipose tissue (68). Cathepsins are known to have an ACE-like activity and thus can lead to the generation of AII independent of ACE.

ANGIOTENSINOGEN EXPRESSION AND REGULATION IN ADIPOSE TISSUE

In adipose tissue, angiotensinogen is under hormonal and nutritional control (65, 99) Angiotensin II mRNA expression is differentiation-dependent (100) Hormonal differentiation of adipocytes requires a differentiation-specific element.which is also required for the sustained transcriptional expression of the angiotensingen gene (9) Isobutylmethylxanthine, a phosphodiesterase inhibitor that is commonly used to accelerate differentiation of preadipocytes into adipocytes, has been shown to increase levels of angiotensinogen mRNA (66, 100). Angiotensinogen mRNA is dramatically induced when adipocytes are differentiated in response to the combination treatment of dexamethasone, ethynylestradiol and T3 (99) (Table 4). AII has been shown to directly induce differentiation of preadipocytes to adipocytes (101), thereby increasing angiotensinogen levels in adipocytes, while adrenergic stimulation decreases

Table 4: Regulation of the angiotensinogen gene expression in adipose tissue.

	ANG mRNA Expression	Reference
Fasting	**Decrease**	**103**
Refeeding (High Carbohydrate diet or Fat diet)	**Increase**	**103**
Steroids	**Increase**	**65,66**
Glucocorticoids and Isobutylmethylxthanine (Dexamethasone)	**Increase**	**99**
Fatty Acids (long chain)	**Increase**	**105**
Angiotensin II	**Increase**	**101**
Insulin	**Increase/Decrease**	**10/102**
Isoproterenol	**Decrease**	**10**

angiotensinogen mRNA expression in differentiated cells (10) Insulin has been shown to increase angiotensinogen expression in 3T3-L1 adipocytes and cultured human adipocytes (10), while low concentrations of insulin were shown to decrease angiotensinogen expression in 3T3-F442A and Ob 1771 (102). Angiotensinogen expression is reduced by fasting and enhanced by high carbohydrate diet (10, 103-104) and high fat diets that stimulate angiotensinogen concomitant with enlarged fat mass (104-105).

Due to angiotensinogen's link with regulation of adiposity, angiotensinogen levels have been studied in many models of obesity. In obesity models, there are conflicting reports of angiotensinogen levels in lean and obese animals. *ob/ob* and *db/db* are mice with mutations in the leptin gene (ob) and leptin receptor gene, respectively. Leptin is secreted by adipocytes and overexpressed in most models of obesity and is proportional to the adipose tissue mass and thus used as a marker of adiposity (106). Angiotensinogen levels in adipose tissue of obese (*ob/ob* and *db/db*) mice were significantly higher than in

their respective controls (103). However; compared to *ob/ob* and *db/db*, other models of obesity such as the obese Zuckers (leptin receptor gene mutation) and A^{vy} (agouti mutation) mice exhibited opposite regulation (10). These obese animals displayed significantly lower levels of angiotensinogen mRNA when compared to their respective controls (10). In human adipose tissue, there was great variability among different patients (10) The differences seen in animals were reportedly based on species or age-related differences (10) or may be tissue specific differences (107).

While some studies preformed to date support the hypothesis that circulating angiotensinogen levels are positively correlated with adipose mass, further studies are warranted to determine the relationship between diabetes, hypertension and obesity and the role of AII receptors and signaling cascades in the pathology of these diseases.

Tissue specific differences in the production of bioactive molecules such as angiotensinogen mRNA have been demonstrated in abdominal subcutaneous and visceral adipose tissue of humans (107) Angiotensinogen mRNA levels are higher in the viseral fat, and this difference is more pronouned in subjects with body mass index (BMI) lower than $30kg/m^2$. An angiotensinogen polymorphism M235T was associated with body mass in women and a relationship between diastolic blood pressure and the AGT M235T polymorphism was dependent on fat mass in middle aged sedentary normotensive women (108) Polymorphisms at the renin, angiotensinogen and angiotensin-converting enzyme loci have also been shown to influence both blood pressure and hypertension (Reviewed in 109).

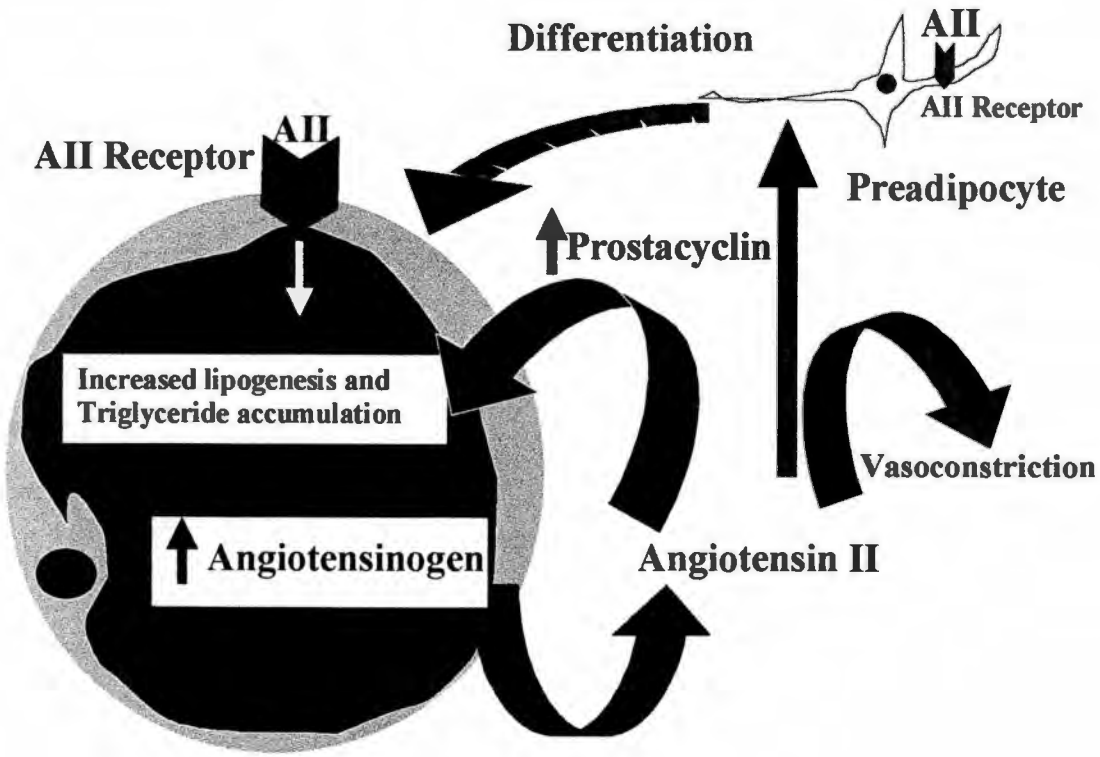
AII RECEPTORS AND SIGNALING IN ADIPOCYTES

AII is known to primarily mediate an intracellular signal via AT_1 or AT_2 receptors. In adipocytes of mammals, both AT_1 and AT_2 receptors have been reported, leading to confusion surrounding the function of AII in adipocyte regulation. In Ob 1771, using pharmacological inhibition of AII receptors the AT_2 was shown to mediate the effects of AII on these cells (101). In agreement with these findings, binding studies conducted in 3T3-L1 adipocytes revealed that these cells express predominantly the AT_2 form of angiotensin receptor (110), while adult rat and human adipocytes have been shown to express primarily AT_1 receptors (111-112). It has yet to be established whether this is an age-related or species specific difference with respect to AII receptors AII binding to the AT_2 receptor stimulates the production of prostacyclin (PGI_2) which is proposed to stimulate differentiation of preadipocytes into mature adipoctyes as well as stimulate growth of these adipocytes (101). By contrast, treatment of rats with the AT_1 antagonist, losartan, decreases fat pad weight and adipocyte size indicating that the AT_1 mediates AII effects on adiposity in rats (128).

EFFECTS OF AII ON ADIPOSITY AND ADIPOSE TISSUE METABOLISM

The function of AII in adipose tissue is unknown but the relationship between obesity and hypertension has been recognized for many years (71). However the mechanism that links these two diseases has yet to be determined. AII has been postulated to be involved in the control of adiposity (Figure 4). This hypothesis has evolved from in vitro studies in two murine cell lines. In Ob1771 cells, AII treatment enhances differentiation and lipid accumulation (101). In 3T3-L1 cells, AII significantly

Figure 4: Regulation of adipocyte differentiation and metabolism by angiotensin II. Differentiation of preadipocytes into adipocytes is accompanied by increased triglyceride accumulation concomitant with increased expression of angiotensinogen and AII production AII is known to cause vasoconstriction, but adipocyte-derived AII may also act in a paracrine fashion to regulate adipocyte metabolism by increasing lipogenic enzyme activities leading to increased fat storage Within adipose tissue, AII secreted by adipocytes can stimulate differentiation via AT_2 of preadipocytes into adipocytes mediated by prostacyclin (PGI_2) Both AT_1 and AT_2 have been documented in adipocytes and mediate AII effects on these cells


Differentiation
AII
AII Receptor
AII Receptor
AII
Preadipocyte
Prostacyclin
Increased lipogenesis and
Triglyceride accumulation
Vasoconstriction
Angiotensinogen
Angiotensin II

increases triglyceride accumulation and activities of two key lipogenic enzymes (fatty acid synthase and glycerol-3-phosphate dehydrogenase) (110). In agreement with these in vitro findings, treatment of mice with an AT_2 antagonist decreased adiposity and leptin (113). However; contradictory studies were performed in growing rats. Angiotensin II-infused rats (175ng/kg•min) did not gain weight over the fourteen-day study (114). Results of this study demonstrate that AII has the potential to reduce body weight, decrease white adipose tissue mass, and decrease plasma leptin levels (114). The lower levels of AII infused regulates body weight independent of blood pressure changes, showing that AII regulates body weight through mechanisms related to increased peripheral metabolism and independent of elevations in blood pressure (114). Furthermore, the components of the renin-angiotensin system in adipose tissue have been shown to change with maturation and adipose tissue enlargement in an age-dependent fashion (97).

ANGIOTENSIN II AND INSULIN/DIABETES:

Obesity is associated with activation of renal ACE in vivo and ACE inhibitors lead to weight loss but also improve insulin sensitivity in both rodents and humans (115-116) Combined exercise training and ACE inhibition was more efficient in improving glucose tolerance and insulin-stimulated glucose transport than either intervention alone in obese Zucker rats (117). Thus, there is an interaction between insulin and angiotensin II, and we have begun investigating this interaction. Our studies demonstrated that AII exerts an insulin-like effect on adipocyte by upregulating several markers of lipid metabolism including leptin (118) and the fatty acid synthase (119). AII and insulin

appear to regulate adipocyte gene transcription via similar mechanisms (119). AII signaling in muscle cells was shown to interfere with tyrosine phophorylation of the insulin receptor substrate one (IRS-1) to inhibit insulin-signaling (120). However, AII and insulin interactions in adipocytes have not been investigated.

RAS AND HUMAN OBESITY

Obesity is associated with several disorders including diabetes and hypertension and studies have shown that weight management is essential for improving or eliminating these diseases Several clinical and laboratory studies with either ACE inhibitors or AII receptor antagonists to control blood pressure have reported weight loss as a side effect (121,122) More recently, the hypertensive optimal treatment (HOT) study (123) reported a beneficial effect of weight loss on lowering the antihypertensive medication requirement, thus demonstrating the importance of weight loss in blood pressure management

The existence of a direct association between plasma renin, aldosterone and leptin (124-125) suggest that leptin may be an important regulator of blood pressure. Furthermore, body mass index (BMI) was found to be highly correlated with angiotensinogen levels across four black population samples (126) Leptin has also been shown to be significantly correlated with angiotensinogen levels and associated with hypertension in both lean and obese hypertensive subjects when compared to their respective normotensive controls (126). This study demonstrates that adipose mass is an important determinant of blood pressure, although the mechanism involved is not clearly understood.

In young normotensive men, plasma angiotensinogen was significantly correlated with both BMI and plasma leptin, while plasma angiotensinogen and blood pressure were positively correlated only in subjects with a positive family history of hypertension (127). In this same study, the insulin response to an oral glucose load was significantly related to both plasma angiotensinogen and plasma leptin (127).

SUMMARY AND CONCLUSIONS

Obesity has become an epidemic problem worldwide. Its management is necessary for the management of associated disorders such as diabetes and hypertension The expression of the renin angiotensin system in adipose tissue and the paracrine effect of angiotensin II on lipid metabolism suggest that this local system may be important in the pathogenesis of obesity-associated hypertension. While weight management is key to the management of diabetes and cardiovascular disease, studies of the function of adipose tissue RAS and its contribution to regulation of blood pressure may provide further insight into the role of this tissue in hypertension and the metabolic syndrome

Activation of the adipose tissue RAS and secretion of AII by fat cells may increase adiposity via paracrine effects of this hormone on triglyceride accumulation and differentiation of preadipocytes into mature adipocytes. Expansion of adipose tissue, especially with high fat diets may further potentiate accumulation of fat stores by AII via a positive feedback loop AII generated in this way could also contribute to the hypertension in obese patients. However, the contribution of adipose tissue RAS to the hypertension of the obese is not understood

We were therefore interested in investigating the mechanism of AII regulation of FAS gene transcription by identifying the AII response elements within the FAS gene and determining the potential transcription factors involved in this regulation.

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PART 3

The Angiotensin II Response Element is the Insulin Response Element in the Adipocyte Fatty Acid Synthase Gene: The Role of the ADD1/SREBP1c.

INTRODUCTION

Angiotensin II (AII) is a hypertensive hormone classically known to regulate blood pressure (1). However, studies from our laboratory and others have shown that this hormone also regulates adipocyte differentiation, metabolism and gene expression (2-4) Components of the renin angiotensin system (RAS) have been identified in several tissues including adipose tissue (2,5-7). Several lines of evidence indicate a role for the adipocyte derived AII in lipid metabolism (2,7). First, angiotensin II precursor, angiotensinogen, is expressed and, nutritionally and hormonally regulated in adipose tissue (8-11). Second, angiotensinogen gene expression is differentiation dependent and treatment of cultured adipocytes with AII increases the expression of differentiation markers (3) Furthermore, we have shown that treatment of cultured adipocytes with AII increases triglyceride storage and activities of lipogenic enzymes including the fatty acid synthase (FAS) (2)

FAS is a key lipogenic gene which catalyzes the synthesis of the long chain fatty acid, palmitate from acetyl-CoA and malonyl-CoA in the presence of NADPH (12) FAS concentrations in adipose tissue are highly sensitive to nutritional, hormonal and developmental states (12-13). Fasting and diabetes in rats lead to diminished synthesis of FAS, while obesity, a high carbohydrate diet or insulin treatment increase FAS synthesis (14) The FAS gene is primarily regulated at the transcriptional level (15-19), and we have previously identified an Insulin Response Element (IRE) in the FAS gene that mediates transcriptional upregulation by insulin in adipocytes (16). Because our previous studies indicate that AII is a lipogenic hormone that exerts an insulin like effect on

adipocyte gene expression, we hypothesized that AII and insulin regulate FAS gene expression via similar mechanisms

We report here that AII increases FAS gene transcription in adipocytes via an angiotensin responsive element (AIIRE) that is identical to the insulin response element (E box) in the proximal FAS promoter. Furthermore, we identify ADD1 as a transcription factor involved in the regulation of FAS gene by AII.

MATERIALS AND METHODS

3T3-LI Cell Culture

3T3-L1 cells were grown and differentiated as previously described (2,19) Briefly, cells were grown to confluence in standard medium (Dulbecco's Modified Eagle's medium, DMEM (25mM glocose)) supplemented with 10% fetal bovine serum, FBS) (Life Technologies/Gibco, Bethesda,MD) At confluency, cells were induced to differentiate by the addition of dexamethasone (250 nM) (Sigma, St Louise, M O.) and isobutylmethlyxanthine (0 5mM) (Sigma, St. Louis, M.O) to standard medium for 72 hours. Cells were then maintained for three additional days in standard medium, then changed to serum-free medium (containing 1% BSA) (Life Technologies/ Gibco, Bethesda,MD) for 12 hours followed by treatment with angiotensin II and/or insulin as indicated in the figure legends

FAS activity

3T3-L1 cells were scraped in 500mM sucrose buffer. Fatty acid synthase activity was assayed spectrophotometrically in cytosolic extracts of 3T3-L1 cells by measuring the oxidation rate of NADPH Data were expressed as nmol of NADPH

oxidized/min/mg of cytosolic protein, which was assayed by the method of Bradford (21).

RNA isolation and Northern analysis

RNA was isolated by the cesium chloride density gradient method (19). Individual culture dishes (100mm) of cells were used for each RNA sample within treatments, and three to four samples were included in each treatment group and each experiment was repeated three times Total RNA (~30μg) for each sample was eletrophorsed in a 0 7% agarose gel, transferred to nylon membrane and analyzed by Northern blotting, as previously described (19) Membranes were hybridized with ^{32}P-labeled cDNA probes for rat FAS (kindly provided by Dr. A G Goodridge) and 18S RNA (Promega, Madison, WI). Unbound probe was removed by washing membranes in 2X saline-sodium phosphate-EDTA (SSPE) for 45 min at 25°C and then in 0 1X SSPE-0 1% sodium dodecyl sulfate for 60 min at 65°C After washing, membranes were exposed to X-ray film (Dupont, Wilmington, Delaware). Autoradiograms were analyzed by densitometric scanning, and data were expressed as a ratio of FAS to 18S.

Nuclear run-on assay

Nuclei were isolated from control or AII treated 3T3-L1 cells, as previously described (19) Briefly, cells were homogenized in an NP-40 containing buffer, and nuclei were isolated by differential centrifugation Nuclear run-on assays and hybridizations were conducted as previously described (19). Labeled RNA was hybridized with plasmids containing cDNAs encoding FAS and β-actin, and with vector alone (pBS) immobilized on nylon membranes Membranes were then exposed to film

and the autoradiograms were quantitated by scanning laser densitometry. FAS transcription rates were normalized to those of β-actin

FAS-Luciferase fusion gene constructs

A fragment of the FAS 5'-flanking region spanning –2100 to +67 was generated by PCR using rat genomic DNA and subcloned into pGL-basic vector (Promega, Madison, WI). Various deletions of this fragment were subsequently generated using ExoIII/mung bean nuclease deletion (Stratagene, La Jolla, CA) or by PCR using previously published FAS promoter sequences (20) For fusion constructs containing two direct repeats of sequences spanning the insulin responsive region, –62 to –46 wild type (CAGCC***CATGTG***GCGTGG) and mutant (AGCC***TGTACG***GCGTGG), oligonucleotides of the specified sequences were first synthesized, annealed then subcloned into pGL2-SV promoter luciferase plasmid These FAS promoter-luciferase fusion constructs were used to transfect 3T3-L1 cells

Transfections

3T3-L1 fibroblast cells were transfected with FAS-luciferase fusion gene constructs and pSVneo plasmid that confers neomycin resistance using the calcium phosphate-DNA coprecipitation method (Life Technologies/Gibco, Bethesda,MD) as previously described (16). Stably transfected cells were selected by maintaining transfected cells in 500ug/ml neomycin for two weeks Surviving fibroblasts were cultured as described above for 3T3-L1 cells The differentiated stably transfected adipocytes were maintained overnight in serum-free medium before treating with AII and/or insulin as indicated in figure legends pGL2-control plasmid, which contains the SV-40 promoter linked to the luciferase gene was used as a control to determine the

specificity of the AII effect on the FAS promoter, and did not show responsiveness to insulin or AII (data not shown).

Luciferase assays

The cells were lysed in 100 mM potassium phosphate pH 7.8, 0.2% Triton X-100 and 1mM dithiothreitol. The cytosolic extracts of lysed cells were used to measure luciferase activities with a luminometer (Berthold, Nashua, NH) using a kit for luciferase assay (Tropix, Foster City, C A). Luciferase activity was normalized to protein measured using Bradford assay (21)

Adenovirus-mediated Overexpression of a Dominant Negative Form of ADD1 in 3T3-L1 Adipocytes

An adenoviral vector overexpressing a dominant negative version of rat ADD1 under the control of the cytomegalovirus immediate early promoter was previouly described (22) The adenovirus vector Adnull, whose expression cassette contains the major late promoter with no exogenous gene, was used as a control. The adenoviral vectors were propagated in 293 cells, purified by cesium chloride density centrifugation, and stored. Cells were grown as described above, and 35-mm dishes containing fully differentiated 3T3 L1 adipocytes were incubated overnight in serum free medium containing BSA Cells were transduced with 3×10^7 pfu in DMEM alone for 4 h at 37 °C and 1nM AII was added. Transduced cells were assayed for FAS enzyme activity 48 h after viral infection.

Statistical analysis

Data was tested for normality Levene's test of equality of variance was applied to all data. Where appropriate, the data was transformed (as noted in figure legends). The

general linear model univariate post hoc multiple comparisons for observed means was applied to data. The Dunnett's t pairwise multple comparison t test was used to compare a set of treatments against a single control. Scheffe test was used for all possible linear combinations of group means to be tested All tests were conducted using 95% confidence intervals.

RESULTS

Effects of AII on FAS activity, mRNA and gene transcription rate

We have previously reported that AII increases FAS gene expression in murine and human adipocytes (2) AII increases FAS enzyme activity in a dose responsive manner (Figure 5). Doses ranging from 0 1nM and 10nM AII significantly increased FAS enzyme activity An AII dose of 1nM was chosen as the minimal dose within physiological concentration that resulted in maximal dose response. Figure 6 shows the coordinated effects of AII on FAS activity, mRNA, and gene transcription rate AII increases FAS activity, mRNA and gene transcription by approximately 2 fold These data demonstrate that FAS gene is regulated by AII at the transcriptional level

Effects of All on FAS promoter activity. comparison with insulin.

To further investigate the mechanisms of transcriptional regulation of FAS by AII, we searched for AII responsive regions in the FAS gene AII significantly increases luciferase activity in 3T3-L1 cells that were transfected with the fusion constructs containing the regions -200 to +67 (Figure 7A) ($p<0.05$), and –70+67 (Figure 7B) ($p<0.05$) of the FAS promoter These fragments contain the insulin response element previously identified in the FAS gene (16).

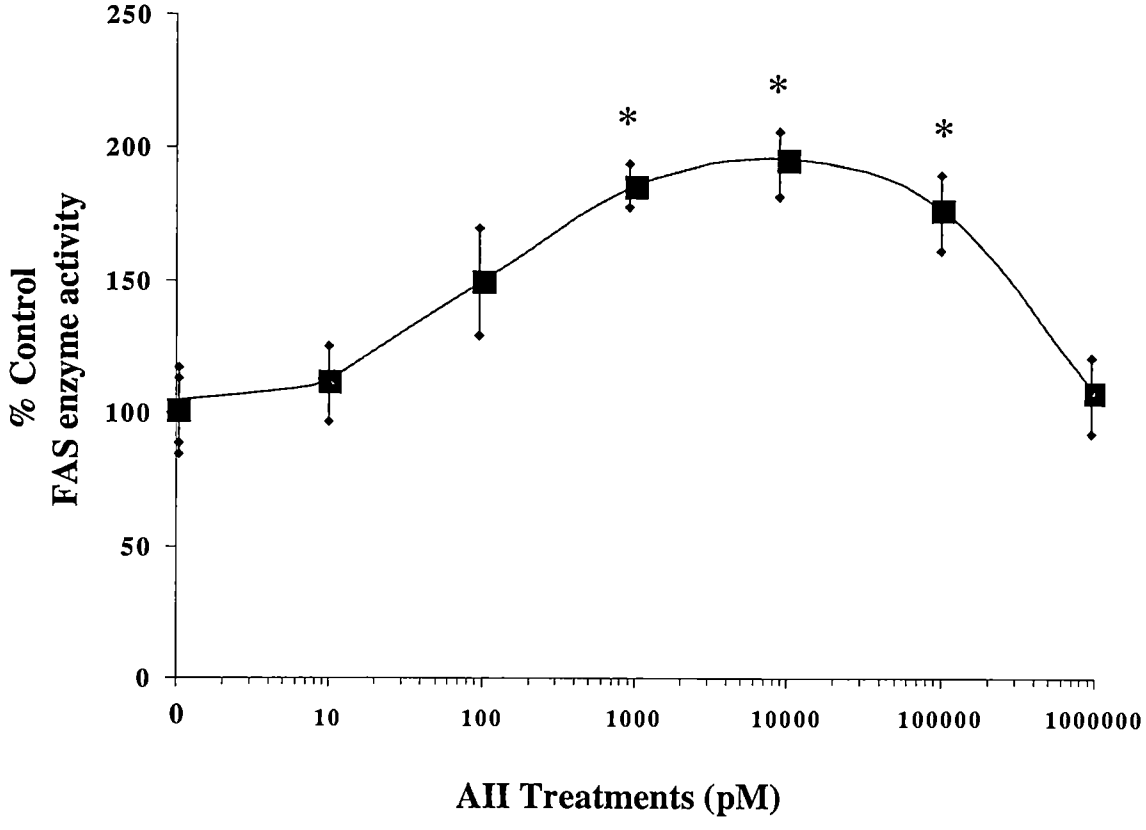

Figure 5. Effects of AII on FAS enzyme activity. 3T3-L1 adipocytes were treated overnight with serum free medium and then with 10pM-1μM AII. Cells were then harvested after 48 hrs and assayed for FAS enzyme activity, which is reported in nanomoles of NADPH oxidized/min/mg of protein. This figure represents the average for three experiments (n=5)

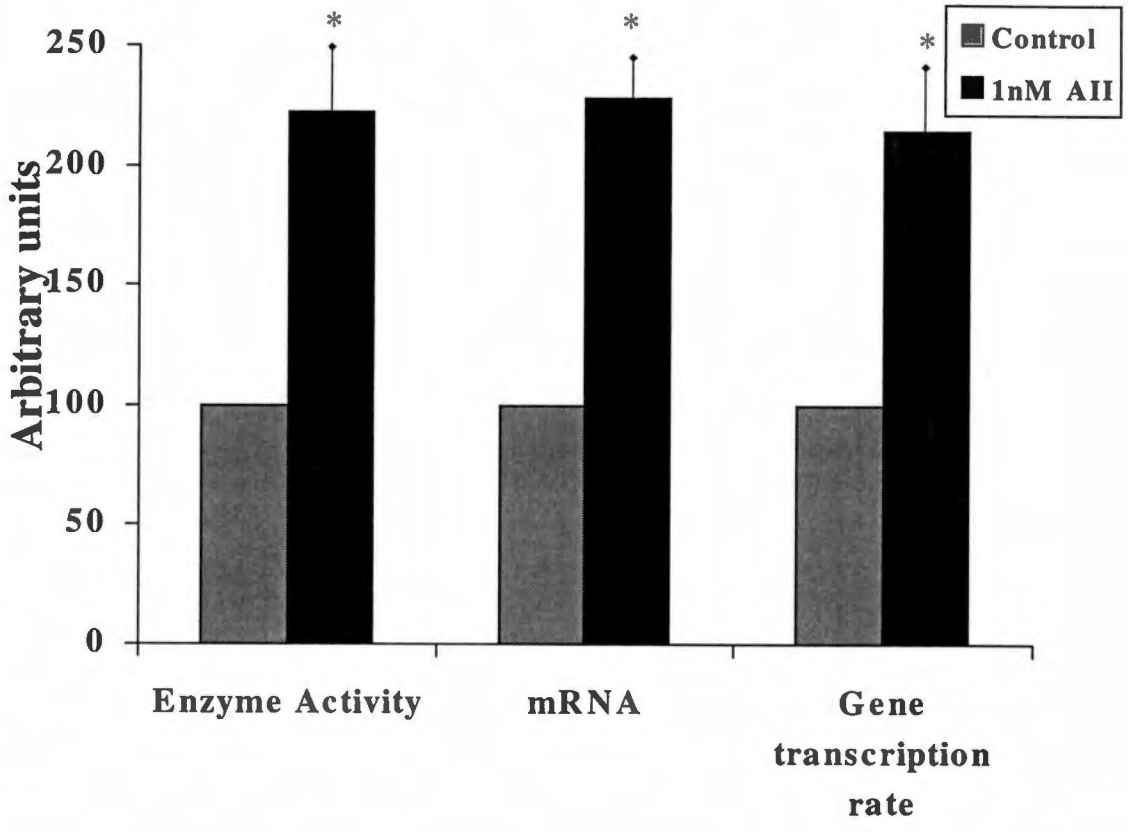

Figure 6. Effects of AII on FAS enzyme activity, mRNA and gene transcription rate. 3T3-L1 adipocytes were treated with 1nM AII. Cells were then harvested after 48 hrs for FAS enzyme activity reported in nanomoles of NADPH oxidized/min/mg of protein, or after 24 hours for mRNA analysis and gene transcription assays reported in arbitrary densitometric units normalized by actin. Triplicates were used in each experiment. Data are expressed as mean ± SD (% control). Figures represent 5, 4 and 3 experiments for FAS enzyme activity, mRNA and gene transcription respectively.

Figure 7A:

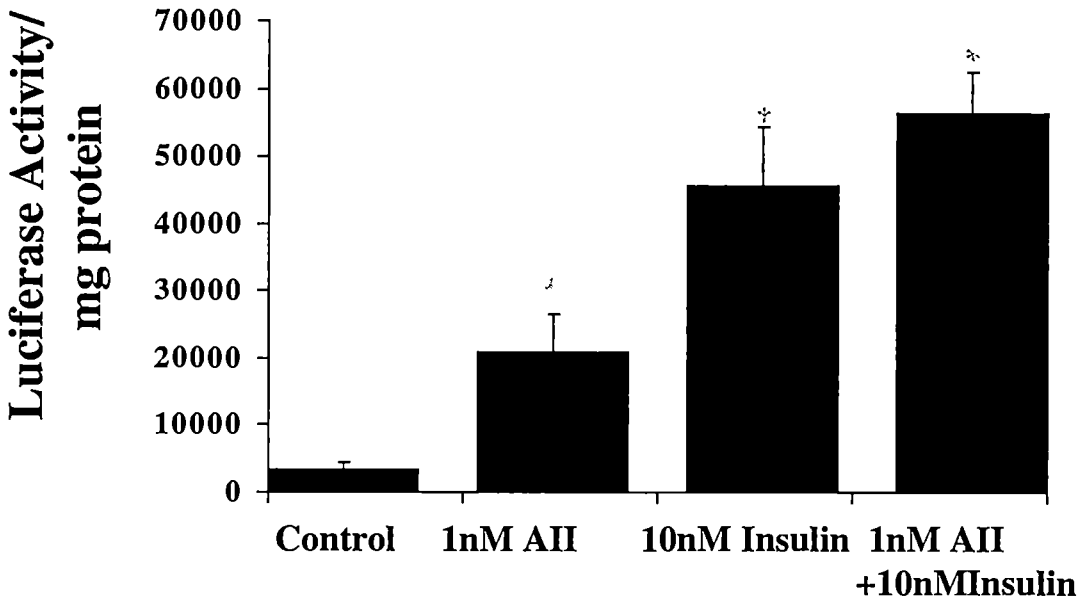

Figure 7. Effects of AII on luciferase activity in 3T3-L1 adipocytes transfected with various FAS promoter constructs 3T3-L1 preadipocytes were stably transfected with FAS-luciferase construct containing –200 to +67 region of the FAS promoter (A) or a construct containing –70 to +67 of the FAS promoter (B) We differentiated these stably transfected cells and treated for 48 hours with AII and/or insulin Luciferase activity was then measured, normalized to protein and reported in arbitrary units. Figure 7A shows . one experiment with n=4 dishes Figure 7B represents the combined averages of four different experiments Data was transformed for statistical analysis according to Box and Cox methods * indicates a significant difference between control and insulin or AII treated cells ($p<0.05$) Error bars represent standard deviation

Figure 7B:

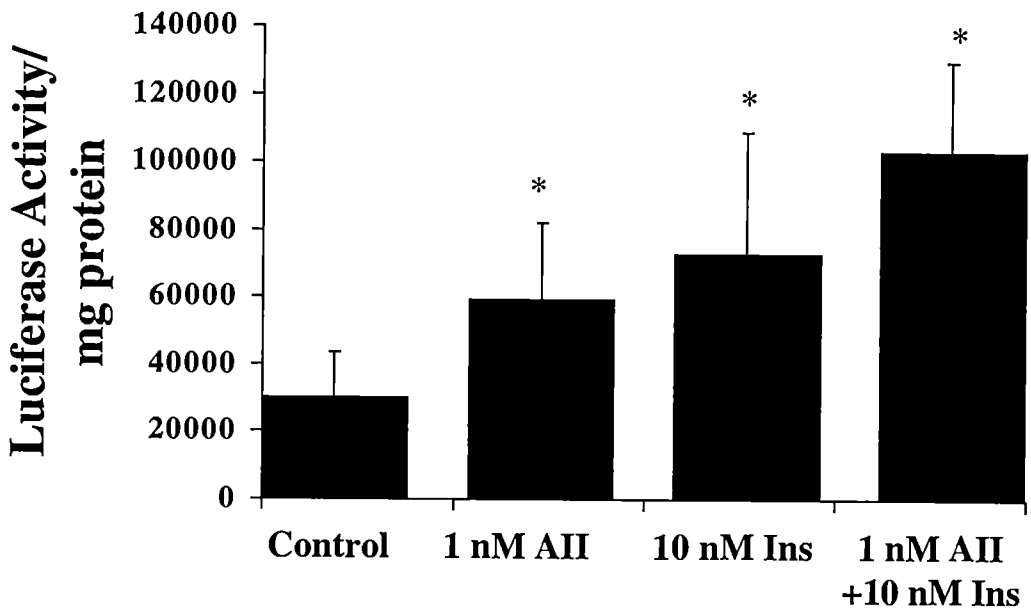

Figure 7 continued.

Adipocytes responded similarly to AII and insulin In addition, we show that AII and insulin in combination increase luciferase activity in 3T3-L1 cells transfected with the plasmids spanning -200 to +67 and –70+67 (Figure 7A and 7B, respectively) ($p<0.05$) regions of the FAS promoter. However, the increase in luciferase activity in the combined AII and insulin treatments was not found to be significantly different from insulin treatments alone suggesting that AII and insulin may target similar cis acting elements to regulate FAS gene transcription

AII responsive sequences are the insulin responsive sequences in the FAS gene

To determine whether the IRE may mediate AII responsiveness of the FAS gene, we tested the responsiveness of a construct containing only the –62 to –46 (IRE) motif linked to the SV40 heterologous promoter. Luciferase responsiveness to AII and insulin was tested in cells transfected with this construct We demonstrate (Figure 8) that AII and insulin significantly ($p<0.005$) increase luciferase activity This indicates that the –62 to –46 region alone is sufficient to confer AII responsiveness to the FAS promoter, with no additional sequence needed outside of this motif In addition, this indicates that AII and insulin both regulate FAS gene transcription through the same response sequences in the FAS promoter

To confirm this, we mutated the insulin responsive E box in the–62 to –46 region of the FAS promoter Figure 8 demonstrates that the mutation of this region causes a loss of stimulation of luciferase activity in response to AII treatment as it did for insulin when compared to control

Figure 8:

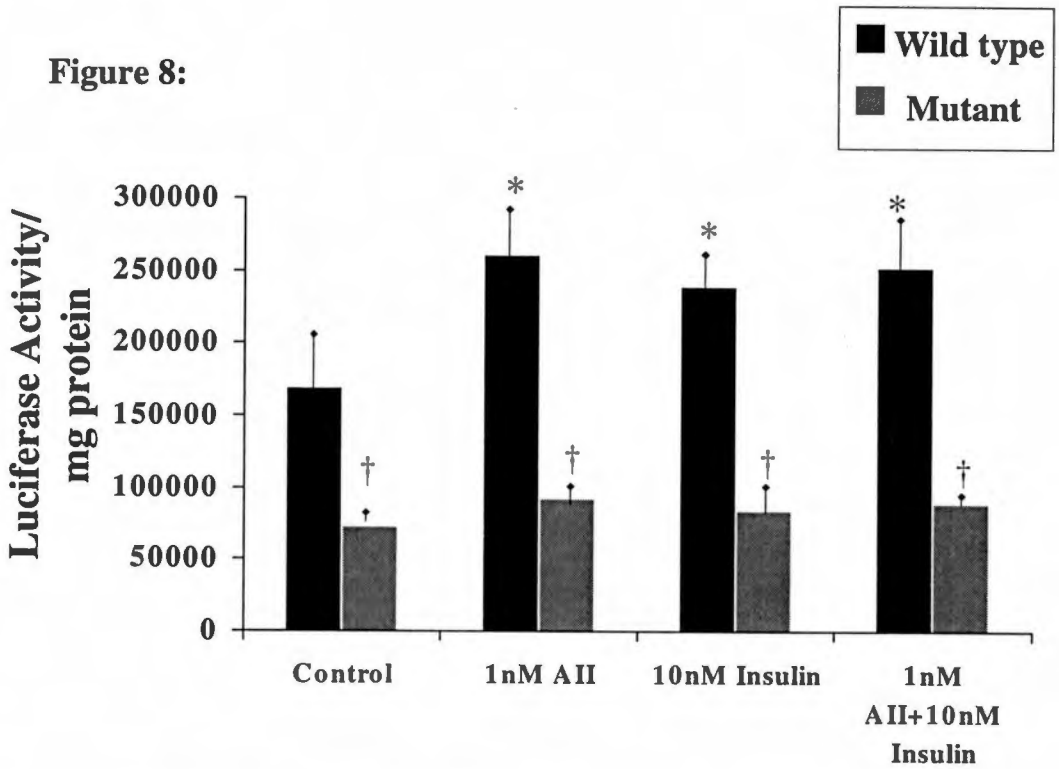

Figure 8. The insulin responsive element (E box) mediates AII effects on FAS activity. Fusion constructs containing two copies of the insulin responsive region (–62 to –46) with either the wild type or the mutated E box were subcloned into pGL2 SV-luciferase plasmid and transfected into 3T3-L1 adipocytes. Cells were then treated with or without 1nM AII and / or insulin for 48 hours then luciferase and proteins were measured. Data are reported in arbitrary units of luciferase/ mg of protein. This experiment was performed twice with n=6 dishes in each experiment. * indicates a significant difference between control and insulin (10nM) or AII (1nM) treated cells ($p<0.05$). † indicates significant difference when comparing wild type to mutant with the same treatments ($p<0.05$). Error bars represent standard deviation.

A proposed mechanism regulating the FAS gene transcription.

ADD1 has been proposed as a transcription factor that binds to the insulin responsive region (IRE), which is an E box in the FAS promoter (16,24). In order to determine if ADD1 is a possible transcription factor through which AII may affect FAS gene transcription, we infected 3T3-L1 cells with a dominant negative form of ADD1 (ADD1/DN) designed to induce the formation of transcriptionnally inactive ADD1 dimers unable to bind DNA target sequences. Figure 9 demonstrates that AII-induced FAS enzyme activity was eliminated by the infection of this dominant negative form of ADD1 ($p<0.05$). This data demonstrates that ADD1 is a key transcription factor involved in the regulation of the FAS gene by AII

DISCUSSION

Insulin is a well-known lipogenic hormone that regulates adipocyte metabolism and gene expression. Among these genes, FAS has been well studied for its transcriptional regulation by insulin (15-18). We have previously identified an insulin response element (IRE) in the FAS proximal promoter (16). This IRE is an E box that has been shown to bind both ADD1 (23,24) and upstream stimulating factor 1 (USF1) (25), both of which are members of the bHLH family. In the liver, USFs seem to be essential to sustain the dietary induction of the FAS gene (25), while ADD1 is required for the activation of hepatic lipogenic gene expression by glucose (25). ADD1 has been shown to promote adipocyte differentiation and expression of genes linked to fatty acid metabolism (26). In adipocytes isolated from rats, FAS expression as been shown to be ADD1 dependent through the use of infection of a dominant negative form of ADD1 (22)

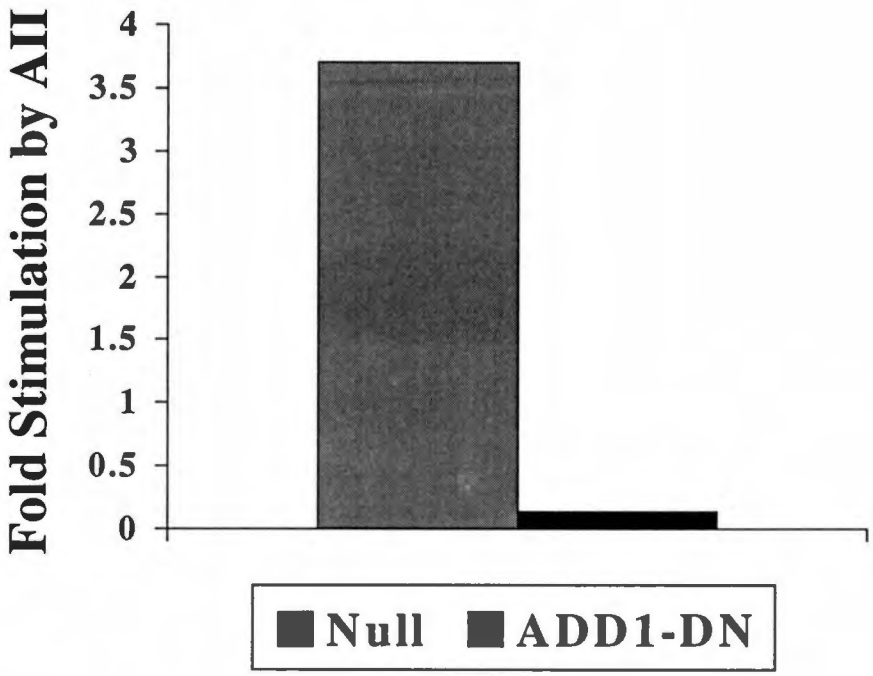

Figure 9. ADD1 is a potential transcription factor through which AII effects FAS gene transcription. As described in the methods, 3T3-L1 adipocytes were infected with adenovirus encoding or not (null) a dominant negative form of ADD1 (multiplicity of infection of 100) and treated with 1 nM AII. Duplicates were used. FAS activity was measured after 48 h and normalized per mg protein. This figure is representative of two experiments. In the other experiment 1nM treatment with AII resulted in a 3.75 fold increase and introduction of the dominant negative form of mutation completely eliminating this increase.

ADD1/SREBP1c is the strongest candidate as a transcription factor to participate in insulin regulation of the FAS gene in adipocytes (22,24,26)

We have previously shown that AII acts like insulin as an adipogenic hormone to regulate FAS and other lipogenic genes in adipocytes Here we demonstrate that AII regulates FAS at the transcriptional level in 3T3 adipocytes (2) and we searched for AII responsive sequences in the fatty acid synthase gene. Using transfection assays, we mapped the AII responsive region to the insulin responsive element (IRE). Furthermore, mutation in the E box abolished AII responsiveness as was seen in earlier work with the response to insulin when the E box was mutated (16,23) This is the first report on regulation of adipocyte FAS gene transcription by AII and identification of an AII response element in the FAS gene.

As discussed above, ADD1 and USF1 are transcription factors involved in regulation of FAS in adipocytes We tested the hypothesis that ADD1 may be a transcription factor involved in AII regulation of FAS Expression of ADD1/DN in 3T3-L1 adipocytes dramatically reduces FAS induction by AII, indicating that ADD1 is one of the transcription factors that predicts AII upregulation of FAS gene transcription This is the first evidence that insulin responsive element (E box) is also the AII responsive element and that ADD1 is a potential transcription factor involved in the regulation of FAS gene transcription by AII.

Very few AII responsive elements have been identified in other genes In smooth muscle, AII responsiveness is partially dependent on modulation of serum response factor binding to the smooth muscle α–actin CC(A/T-rich)$_6$GG The CC(A/T-rich)$_6$GG elements are found in the promoters of several immediate-early response genes (27).

These promoter response elements have been shown to confer serum- and growth factor-induced transcription activation of these various genes (28-32) However, in the current study, the AII response element is an E box (CATGTG) that is distinct from the serum response factor, and there is no evidence that such serum responsive elements are present in the 2kb promoter region located immediately upstream of the transcription start site of the FAS gene However, it is possible that such elements may be present at other sites in the FAS gene

The differences in the types of sequences targeted by AII may be due to the different types of receptors found in adipose versus muscle tissue There are several identified types of receptors through which AII can signal (33). Most studies have focused on the signaling of AII through an AT_1 that is primarily found in muscle cells (33-35) The AT_2 was identified by our lab and others as the primary receptor in murine adipocytes mediating AII regulation of lipogenic genes (2,3)

Although prostaglandin I_2 have been postulated as signaling molecules produced by adipocytes in response to AII binding to AT2 receptors (2-3), the signaling pathway of AII through this receptor in adipocytes is largely unknown Previous studies have linked AII and insulin signaling (36-37) However, these studies have focused on AII signaling through the angiotensin type I receptor of muscle In this cell type, AII negatively modulates insulin signaling by stimulating multiple serine phosphorylation events in the early components of the insulin signaling cascade (37) No studies have identified the signaling pathway of AII in murine adipocyte through the angiotensin type II receptor Since it appears that both insulin and AII stimulate lipogenesis through similar mechanisms regulating the FAS gene, it may be possible that the signaling cascade of AII

in murine adipocyte, is not inhibitory to insulin as has been found in muscle via the angiotensin type I receptor. Rather AII may signal through one or more factors in the insulin-signaling pathway via AT_2 receptors Taken together, these studies in addition to others show that AII mediates its cellular effects by insulin-like mechanisms, including signaling (36-37) and the insulin responsive sequences (present work).

Future investigations will determine the upstream mechanisms responsible for the activation of ADD1 by AII and the signaling mechanisms responsible for the AII regulation of the FAS gene.

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PART 4

SUMMARY AND CONCLUSIONS

The objectives of this research were to investigate the mechanism of AII regulation of the FAS gene transcription by identifying the AII response element within the FAS gene and determine potential transcription factors involved in this regulation

AII induced FAS activity in a dose-dependent manner at the transcriptional level (as previously reported). We determined that insulin and AII effects were not additive We demonstrated that AII uses IRS (E-box) to upregulate the FAS gene Like insulin, AII regulates the FAS gene via the transcription factor ADD1 that binds IRS Therefore, AII and insulin regulate lipogenesis via the same mechanism

In conclusion, this thesis has demonstrated that adipose tissue RAS is involved in control of murine adipocyte metabolism. Increases in AII secretion from adipocytes may lead to further fat deposition, as well as, adipocyte hypertrophy and possibly contributing to hypertension in obese individuals. This work also strengthens the link between obesity and diabetes by linking AII and insulin to the same regulatory sequences within the fatty acid synthase gene.

Further investigations are necessary to determine the upstream mechanisms responsible for the activation of ADD1 by AII and the signaling mechanisms responsible for the AII regulation of the FAS gene Further studies are also necessary to determine if insulin and AII signaling overlap.

VITA

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