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I am submitting herewith a dissertation written by Suyeon Kim entitled "Mechanisms of paracrine and endocrine actions of Angiotensin II." I have examined the final paper copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Human Ecology.

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MECHANISMS OF PARACRINE AND ENDOCRINE ACTIONS OF ANGIOTENSIN II

A Dissertation
Presented for the Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Suyeon Kim
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DEDICATION

This dissertation is dedicated to my parents, Mr. Kim, Kun-Tae and Mrs. Kang, Jo-Ja and sister, Ms. Kim, Ji-Yeon

For their ongoing support and encouragement throughout my life

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ABSTRACT

Adipose tissue expresses high levels of angiotensinogen (agt), the only known precursor of Angiotensin II (Ang II) and we have previously shown that this hormone plays a paracrine role in regulation of adipocyte metabolism by increasing activity and gene expression of two key lipogenic enzymes, fatty acid synthase (FAS) and glycerol-3-phosphate dehydrogenase (GPDH). However, molecular mechanisms by which Ang II regulates an adipocyte lipogenic gene, namely FAS and transcription factors mediating this regulation are largely unknown. Furthermore, signaling mechanisms whereby Ang II induces adiposity need to be further explored. Accordingly, our studies were designed to investigate transcriptional regulation of FAS gene by Ang II and determine signaling mechanisms of Ang II leading to activate FAS gene transcription. We demonstrated that Ang II responsive element is an E box within adipocyte FAS gene and the adipocyte determination and differentiation factor1/sterol-regulatory element binding protein 1c (ADD1/SERBP1c) functions as a transcription factor mediating these actions. Ang II induced FAS transcription as well as ADD1 gene expression in a glucose-dependent manner. Using immunoprecipitation and western blot analyses, we found that Ang II regulates adipocyte metabolism via activation of insulin signaling molecules including IRβ, IRS, PI-3K and Akt. This signaling mechanism was mainly mediated by angiotensin receptor type 1 receptors (AT1) and in part by angiotensin receptor type 2 receptors (AT2). Next, with agt knockout (agt -/-, KO) and transgenic (Tg-KO mice expressing agt exclusively in adipose tissue and Tg-WT mice overexpressing agt in adipose tissue) mouse models, we examined changes in adipose tissue metabolism *in vivo* and renal gene regulation in these mice and further dissected endocrine effects of adipocyte agt. We found that compared with those of WT mice, body weight gain and fat pad weight were lower in response to high fat diet in KO mice exhibiting hypotension and renal

abnormalities. Targeted expression of the agt gene only in adipose tissue (Tg-KO) partly rescued these phenotypes whereas agt overexpression in adipose tissue of transgenic mice (Tg-WT) was associated with increased epididymal fat pad mass and blood pressure. Subsequent western blot and renal gene expression analyses indicated that adipocyte agt participates in systemic blood pressure regulation at least partly by increasing renal agt and AT1 receptor production in Tg-WT mice. Additionally, microarray data revealed that adipose tissue-specific agt restoration was able to correct altered expression of genes associated with blood pressure homeostasis and renal function in KO mice. In conclusion, Ang II plays a hypertrophic role in adipocytes by a paracrine/autocrine mechanism. Furthermore, adipocyte Ang II exerts endocrine effects on renal function and gene expression, thereby contributing to systemic blood pressure regulation and kidney homeostasis. This study may provide valuable approaches in treatments of obesity and obesity-associated metabolic alternations such as insulin resistance and hypertension.

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AA: Arachidonic acid	CEBP: cAMP response element binding protein	IR: Insulin receptor
ACC: Acetyl CoA carboxylase	CaM Kinase: Calcium/calmodulin-dependent protein kinase	IRS: Insulin receptor substrate
ACE: Angiotensin converting enzyme	CPT: Carnitine palmitoyltransferase	IRE: Insulin response element
ACEH: Angiotensin converting enzyme homolog	DAG: Diacylglycerol	IRAP: Insulin regulated aminopeptidase
ADD1: Adipocyte determination and differentiation dependent factor 1	Dex: Dexamethasone	ISBAT: Intrascapular brown adipose tissue
AGCE1: hAG core promoter element 1	DSE: Differentiation specific element	JAK: Janus kinase
AGCF1: hAG core promoter binding factor 1	DSEBP: Differentiation specific element binding protein	JC: Juxtaglomerular cells
AGE: Angiotensinogen gene activating element	EGF: Epidermal growth factor	JNK: c-Jun N-terminal protein kinase
AGF: Angiotensinogen gene activating factor	EGFR: Epidermal growth factor receptor	MAP: Mean arterial pressure
Agt: Angiotensinogen	ERK: Extracellular regulated kinases	MAPK: Mitogen activated protein kinases
AMPK: AMP activated protein kinase	FAK: Focal adhesion kinase	MCP: Monocyte chemoattractant protein
Ang I: Angiotensin I	FAS: Fatty acid synthase	MC4R: Melanocortin 4 receptor
Ang II: Angiotensin II	FATP: Fatty acid transport protein	MKP: MAP kinase phosphatase 1
Ang III: Angiotensin III	FFA: Free fatty acids	MIX: Methylisobutylxanthine
Ang IV: Angiotensin IV	FGF: Fibroblast growth factor	NHANES III: National Health and Nutrition Examination Survey
APRE: Acute-phase response element	GFR: Glomerular filtration rate	NEP: Neural endopeptidase
ASP: Acylation stimulating protein	GPCR: G-protein coupling receptor	NE: Norepinephrine
ATF-2: Activating transcription factor 2	GPDH: Glycerol-3 phosphate dehydrogenase	NFκB: Nuclear factor-κB
BAT: Brown adipose tissue	GRE: Glucocorticoid receptor element	PA: Phosphatidic acid
BBB: Blood brain barrier	HSL: Hormone sensitive lipase	αPAK: p21-activated kinase
BK: Bradykinin	ICAM: Intercellular adhesion molecule	PAI-1: Plasminogen activator inhibitor-1
BMI: Body mass index	IGF-1: Insulin-like growth factor-1	PC1: Proconvertase 1
CAP: Cbl-associated protein	IGFR: Insulin growth factor receptor	PDGF-A: Platelet-derived growth factor A chain

PGC-1: Peroxisome proliferators-activated receptor gamma coactivator 1	RAR: Retinoid acid receptor
PGE2: Prostaglandin E2	RXR: Retinoid X receptor
PGI2: Prostacyclin	RASM: Rat aortic smooth muscle
PI3K: Phosphatidylinositol-3 kinase	STAT: Signal transducers and activators of transcription
PKA: Protein kinase A	SAPK: Stress activated protein kinase
PKC: Protein kinase C	SER: Sterol element response
PLA2 : Phospholipase A2	SERBP1: Sterol regulatory element-binding protein 1
PLC : Phospholipase C	SHP: Src homology (SH2)-domain containing protein tyrosine phosphatase
PLD : Phospholipase D	SIM1: Single minded drosophila homolog 1
POMC: Pro-opiomelanocortin	SNS: Sympathetic nervous system
PPARγ: Peroxisome proliferator-activated receptor γ	SR: Sarcoplasmic reticulum
PPA: Plasma renin activity	TGFβ: Transforming growth factorβ
PP2A: Serine/threonine phosphatase 2A	TNFα: Tumor necrosis factor α
PTP: Protein tyrosine phosphatase	TRE; Thyroid response element
PTP1B: Protein tyrosine phosphatase 1B	TZD: Thiazolidinedione
PUFA: Polyunsaturated fatty acids	UCP: Uncoupling protein
PYK2: Proline-rich tyrosine kinase 2	USF: Upstream stimulating factor
QTL: Quantitative trait loci	VCAM: Vascular cell adhesion molecules-1
ROS: Reactive oxygen species	VLDL: Very low density lipoproteins
RTK: Receptor tyrosine kinase	VSMC: Vascular smooth muscle cells
RAS: Renin angiotensin system	WAT: White adipose tissue

PART 1

INTRODUCTION

Angiotensin II (Ang II), a main effector of the renin-angiotensin system (RAS), is an important regulator of vascular tone and electrolyte and water homeostasis and is locally synthesized in various tissues including adipose tissue (1). In addition to its classical function, increasing evidence has suggested putative roles of adipose RAS in the development of obesity, which is widespread and increasingly prevalent disease as well as may cause or exacerbate many health problems including insulin resistance, type II diabetes, and hypertension (2). Several epidemiological studies have reported positive correlations between plasma angiotensinogen (agt) levels, plasma renin activity (PRA) and plasma angiotensin-converting enzyme (ACE) activity with body mass index (BMI) in different human populations (3-12). Of note, a positive relationship between plasma leptin and agt levels in humans was observed and a similar relationship with plasma leptin was also described for plasma renin activity, indicating a positive association of systemic RAS with obesity (10, 12). Genetic studies have also demonstrated an association between the agt locus and obesity-related phenotypes in humans (13, 14). Especially, the AGT235T variant has been associated with body fatness in women (14). Moreover, a significant association of adipose agt expression with obesity has been illustrated. In obese humans, agt mRNA expression in subcutaneous and omental adipose tissues is strongly associated with increased body mass index and waist-to-hip ratio index (a index of central obesity) (15-17). The expression of other RAS component genes, renin and ACE in adipose tissue was also upregulated in obese subjects (18). This is also true in some animal models of obesity (19, 20). Agt secretion in adipose tissue from genetically obese mice (*ob/ob* and *db/db* mice) was higher than in lean control animals (21). A very recent study has reported that diet-induced obesity induces agt expression in a depot-specific manner (22). Of importance, multiple molecular and clinical studies have emphasized possible roles of RAS in insulin resistance and type II diabetes. Specifically, Ang II has been shown to interfere with insulin signaling pathways

at multiple levels in vascular smooth muscle cells (VSMC) (23-27). In support of negative effects of RAS on insulin sensitivity, the results of two recent clinical trials, Captopril Prevention Project (CPP) and Heart Outcomes Prevention Evaluation (HOPE), demonstrated that blockade of the RAS, beyond lowering blood pressure, prevent the development of type 2 diabetes (28, 29). Very recently, a clinical study has shown that RAS blockade improves insulin sensitivity with increased adiponectin plasma levels, insulin sensitizer (30). Moreover, the positive link between systemic RAS and obesity-related hypertension has been described in several epidemiological studies (4, 5, 10, 12). The effects of adipose RAS on obesity-induced hypertension have not been addressed until recently. Earlier data from study of Frederich et al. have suggested that changes in adipose agt expression contribute to changes in systemic blood pressure associated with fasting and refeeding (21). This mechanism provides a possible explanation for the refeeding hypertension models. Couple of years ago, availability of agt knockout and transgenic models provided mechanisms underlying involvement of adipose RAS in systemic blood pressure regulation; it was clearly shown that adipose tissue itself contributes to systemic agt concentrations. Agt knockout mice are hypotensive, lose large amounts of sodium from the kidney and have no detectable agt plasma levels (31-34). Adipose tissue specific reexpression of agt results in detectable plasma agt levels, blood pressure normalization and significant enhanced ability of sodium reabsorption (31). Overexpression of agt in adipose tissue leads to hypertension concomitant with elevated adiposity (31). Taken together, these observations indicate the close association of systemic RAS with obesity and obesity-associated complications. Especially, adipose RAS appears to be implicated in the development of obesity and obesity-associated insulin resistance and hypertension, although the precise mechanisms responsible for functional roles of RAS in obesity and its related alternations are largely unknown.

To date, the pivotal actions of Ang II in adipose tissue including growth, metabolism and

thermoregulation of brown adipose tissue have been demonstrated by our studies as well as others (35-37). Previous data from our lab have shown that Ang II controls fat mass via upregulating activities and gene expression of two key lipogenic enzymes, fatty acid synthase (FAS) and glycerol-3 phosphate dehydrogenase (GPDH) in human and murine adipose cells (37). In addition, Ang II has been demonstrated to increase gene expression and secretion of leptin, a well-known adiposity marker, by adipocytes as well. However, molecular and signaling mechanisms underlying Ang II-induced adiposity remain unresolved.

The purpose of this study was to define potential mechanisms by which Ang II induces adiposity and hypertension associated with obesity in a paracrine/autocrine manner. Accordingly, our studies were specifically designed to address the following aims:

Aim1: Investigate the mechanisms by which Ang II regulates adipocyte lipogenic gene, FAS and transcription factors mediating this regulation.

Aim 2: Investigate potential interactions of Ang II signaling with insulin signaling pathways.

Aim 3: Investigate endocrine effects of adipose agt on changes in lipid metabolism *in vivo* and renal gene regulation as well as systemic blood pressure regulation using agt knockout and transgenic mouse models.

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PART II

LITERATURE REVIEW

A. OBESITY

Obesity is a complex and multifactoral disorder. Its onset and progression have been ascribed to genetics, lifestyle and endocrine disorders (1). Of note, obesity is a major health problem in developed countries and is rapidly becoming a significant health problem in developing countries. In the United States, obesity is reaching epidemic proportions: more than 31% of adults are clinically obese as defined by a body mass index (BMI) of 30 kg/m^2 or higher and an additional 33% are overweight. In addition, the number of children and adolescents with obesity and type II diabetes has dramatically increased: 11% of children and adolescents are overweight (1, 2). Obesity increases the risk for many disorders that are associated with high mortality and morbidity, including diabetes, hypertension, coronary heart disease, dyslipidemia, several forms of cancer and gallbladder disease (1).

1. OBESITY AND MORTALITY

It has been reported that obesity-associated diseases cause more than 400,000 deaths annually in the United States alone. Previous data from the Nurses' Health study have demonstrated a J-shaped relationship between BMI and overall mortality (3). The lowest mortality was found among women with BMI of 19.0 to 26.9 kg/m^2, but then mortality increased steadily as BMI levels rose above 27 kg/m^2. Similarly, another cohort study has illustrated that obesity and overweight in adulthood are associated with large decreases in life expectancy and increases in early mortality (4).

2. OBESITY AND MORBIDITY

Excessive adipose tissue, particularly caused by abdominal or upper-body obesity, has been associated with a number of chronic diseases: insulin resistance, hypertension, hyperlipidemia, diabetes, cancer, restrictive lung diseases, gout and degenerative arthritis. The

precise association between obesity and each above chronic disease remains poorly understood (1).

2.1 Obesity and Insulin Resistance/Diabetes

Obesity is associated with insulin resistance and type 2 diabetes. Insulin resistance, a defect in the response of muscle, adipose tissue and liver to insulin-mediated glucose transport, metabolism and storage, is a prediabetic status. Insulin resistance associated with obesity is manifested by decreased insulin-induced glucose transport and utilization in skeletal muscle and adipose tissue. These functional defects may be in part due to impaired insulin signaling pathways such as insulin binding to its receptors, receptor and insulin receptor substrate (IRS) phosphorylation and phosphatidylinositol 3-kinase (PI3K) activation, thus subsequently downregulating insulin-responsive glucose transporter, Glut 4 (5). Indeed, decreased expression of IRS-1 and subsequently reduced PI3K activity in adipocytes are evident in obese humans (6). In morbid obesity, the expression of insulin signaling molecules is reduced in skeletal muscle (7). Additionally, diminished Glut 4 expression or impaired translocation, docking and vesicle fusion of Glut 4-containing vesicles is shown in skeletal muscle and adipose tissue of obese subjects (8). Further, one of the potential mechanisms for the signaling defects in obesity has been proposed. Increases in expression and activity of protein tyrosine phosphatase 1B (PTP1B) and src homology 2 domain-containing protein tyrosine phosphatase (SHP), which have shown to dephosphorylate the insulin receptor (IR) and IRS-1, were observed in adipose tissue and muscle of obese humans and rodents. Accordingly, the obesity-associated insulin signaling defect may be in part mediated by activation of these protein phosphatases (9, 10). However, mechanisms whereby increased fat mass induces insulin resistance in insulin-sensitive tissues, including liver and muscle, remain unclear. To date, two paradigms have been suggested to explain link between obesity and insulin resistance: ectopic fat storage syndrome and adipocyte-secreted factors.

2.1.1 Ectopic Fat Storage Syndrome

Lipodystrophy syndrome is characterized by a decrease in adipose tissue mass and diabetes. Insufficient adipose tissue leads to excess energy storage as triglycerides in the liver and skeletal muscle, and consequently to insulin resistance and diabetes (11). Recently, transgenic animals that block adipose tissue development revealed lipid infiltration of skeletal muscle and fatty liver along with insulin resistance, glucose intolerance and diabetes (12, 13). The transplantation of adipose tissue back into lipoatrophic animals reverses the elevated glucose levels (14). In this context, obesity has been suggested to be another ectopic fat storage syndrome (15). Massive obesity also proceeds the progressive overaccumulation of lipid in nonadipose tissues including the liver, skeletal muscle and the pancreas, leading to impairment of functions, steatosis, and increased ceramide formation, which triggers nitric oxide-mediated lipotoxicity and ultimately lipoapoptosis, causing severe diabetes (16, 17). In contrast to lipodystrophic diabetes, adipose tissue stores are adequate or even large in these patients, reflecting that adipose cells fail to maintain their leptin-mediated liporegulatory effect on confining the storage of triglycerides to adipocytes. Current study has proposed that the lipid storage in the wrong places may result from increased fat cell size, which is highly associated with insulin resistance and the development of diabetes (18). The enlarged fat cells, a characteristic of obesity are indicative of a failure of proliferation and/or differentiation of adipocytes (18, 19). In other words, increased fat cell size represents the failure of the adipose tissue to expand and subsequently induces lipid accumulation in extra-adipose tissues like the liver, muscle and the pancreas, contributing to the development of diabetes. This was also supported by recent evidence demonstrating that single nucleotide substitutions in the peroxisome proliferator activated receptorγ (PPARγ) gene involved in adipose tissue development are associated with the development of diabetes (20). Additionally, cross-sectional studies have presented the adverse effect of increased intracellular lipid in peripheral

nonadipose tissues on insulin sensitivity. The elevated myocyte lipid represses the activity of carnitine palmitoyltransferase-1 (CPT-1), a limiting enzyme of fat oxidation, and in turn enlarges the intracellular free fatty acid (FFA) pool, thereby leading to a vicious cycle of continuous deterioration (21).

2.1.2 Endocrine Function of Adipose Tissue

Adipose tissue functions as an endocrine organ that secretes several biological molecules that exert multiple endocrine effects. Several factors including tumor necrosis factor-α (TNF-α), adiponectin and resistin are associated with insulin resistance in obesity (22).

TNF-α is overexpressed in enlarged adipocytes from obese animals (23). TNF-α inhibits lipogenesis and increases lipolysis, resulting in increasing mobilization of FFA, which are closely related to insulin resistance (24). TNF-α signaling has been shown to impair insulin signaling, in part through serine phosphorylation of IRS-1 (25). This cytokine reduces Glut 4 gene expression thorough inhibition of CCAAT/enhancer binding protein α (C/EBPα) homodimerization which is required for transcriptional activity of GLUT 4 gene, consequently decreasing insulin-induced glucose transport (26, 27). Further supporting evidence derives from TNF-α knockout studies demonstrating that TNF-α inactivation improves insulin sensitivity (28).

Adiponectin, also termed as Acrp30 and adipoQ, is a newly discovered plasma protein which is produced from adipocytes (29). Independent of body adiposity, circulating adiponectin levels are positively associated with insulin sensitivity as assessed with fasting insulin levels, hyperinsulinemic-euglycemic clamps or frequently sampled intravenous glucose tolerance tests (18, 30-32). In diabetic animals, reduced adiponectin expression and circulating levels correlated with insulin resistance (33). Decreased tyrosine phosphorylation of muscle insulin receptors is related to lower plasma adiponectin concentrations (34). Further, adiponectin administration reduces insulin resistance and improves glucose tolerance in mice with low adiponectin levels

resulting from lipoatrophy-or obesity-induced insulin resistance (29). Adiponectin decreases insulin resistance by reducing triglyceride content and increasing fatty acid oxidation in muscle and the liver. These insulin-sensitizing effects of adiponectin appear to be mediated by activation of AMP activated protein kinase (AMPK) (35, 36). In addition, thiazolidinediones (TZDs) of PPARγ agonist have shown to increase adiponectin expression in adipocytes, suggesting that adiponectin mediates anti-diabetic effects of TZDs (37).

Resistin is an adipocyte-derived hormone and has been suggested to potentially link obesity to diabetes (38). Resistin is expressed in white adipose tissue with higher levels in female gonadal adipose tissue and resistin mRNA levels have been reported to be markedly increased during 3T3-L1 and primary preadipocyte differentiation into adipocytes (39). However, resistin had inhibitory effects on adipose conversion, suggesting that resistin may be a feedback regulator in adipogenesis and thus signal to restrict adipose tissue formation (39). Genetic-and diet-induced obese mice showed markedly increased resistin levels in the circulation (38). Treatment of normal mice with recombinant resistin has been demonstrated to impair glucose tolerance and insulin action (38). In contrast to adiponectin, rosiglitazone treatment has been shown to decrease resistin mRNA and serum levels, thus improving insulin sensitivity (38).

2.2 Obesity and Hypertension

The increase in the prevalence of obesity is a global phenomenon associated with increased risk for the development of cardiovascular disease (40). Recent study has demonstrated that both systolic and diastolic blood pressure increase in a linear manner over the whole range of BMI or waist circumference, an index of visceral obesity (41). The National Health and Nutrition Examination Survey III (NHANES III) data also show that obesity increases the risk of hypertension. Respondents with a BMI of 30 kg/m^2 or greater were twice as likely to have hypertension compared with non-obese subjects. Similarly, subjects with abdominal obesity,

defined by a waist circumference of at least 102 cm for men and 88 cm for women, were twice as likely to have hypertension (1). Of significance, an increase in blood pressure following a gain in body weight is associated with profound changes in cardiovascular-renal physiology, including high cardiac output, increased plasma volume, increased tubular sodium retention, inappropriately normal to total peripheral resistance, increased heart rate, activations of the sympathetic nervous system (SNS) and of the systemic renin-angiotensin system (RAS) (42). Although the mechanisms leading to these obesity-associated changes are not fully understood, alternations in renal mechanisms, increased sympathetic activity and systemic RAS activation seem to be especially important contributors to the development of hypertension in relation to obesity.

2.2.1 Renal Mechanisms

Animal models of obesity induced by high fat feeding have displayed renal structural changes including increases in renal weight largely attributable to augmented mesangial and capillary endothelial cell proliferation, elevated deposition of hyaluronate in the inner medulla and higher intrarenal lipid levels (43-46). Increased Bowman's capsule space area, glomerular basement thickening and increased focal formation of transforming growth factorβ (TGFβ) have also been observed (44). Of note, the alternations in renal structure result in increased intrarenal pressure. In fact, intrarenal pressure is about 10 mmHg higher in the kidneys of obese dogs than in those of lean dogs (47). The rise in interstitial fluid pressure of the kidney reduces medullary blood flow and causes tubular compression, consequently slowing tubular flow rate and increasing fraction tubular sodium reabsorption (47). Collectively, these obesity-associated changes in renal structure induce elevated tubular sodium absorption and a rightward shift of the pressure-natriuresis curve, ultimately raising arterial blood pressure. In addition to renal structural changes, the increase in renal sympathetic nerve activity has been suggested as a contributing

factor in obesity-related sodium retention (48, 49). Renal denervation has been demonstrated to attenuate sodium retention and hypertension associated with obesity (48). Consistently, combined systemic α- and β-adrenergic blockade prevented the development of obesity-associated hypertension in rabbits fed a high-fat diet (49).

2.2.2 Sympathetic Nervous System (SNS)

SNS hyperactivity has been considered a hallmark of obesity-associated hypertension. Obese subjects have a higher peripheral tissue norepinephrine turnover and increased muscle sympathetic activity (50). Kassab et al. illustrated that the impact of sympathetic activation on kidney function is due to the activation of renal efferent nerves, when they studied dogs fed a high fat diet for five weeks: the kidneys with intact renal nerves retained almost twice as much sodium as the denervated kidneys (51). The mechanisms by which obesity activates the SNS are still unknown, but hyperinsulinemia, fatty acids, Angiotensin (Ang II), and hyperleptinemia are proposed as putative mediators (52). Commonly, circulating leptin increases proportionately with adiposity. Corica et al. reported that leptin plasma levels are higher in hypertensive than in normotensive obese women. This study showed positive association between plasma leptin and norepinephrine levels in obese but not lean subjects (53). This finding is in line with the observation in a cross-sectional study showing higher norepinephrine and leptin plasma levels in obese hypertensive volunteers than in age-and sex-matched obese normotensive participants (54). A study by Fogteloo et al. has further demonstrated that higher leptin levels associated with hypertension in humans appear likely to be due to increased secretion from adipose tissue rather than to impaired leptin clearance by the kidneys (55). Experimental studies by Paolisso et al. confirmed the possibility that increase in blood pressure associated with higher leptin levels is caused by an increase in SNS activity (56). Intracerebroventricular (ICV) administration of leptin has been shown to rapidly increase blood pressure, renal SNS activity and plasma catecholamine

levels (57). In addition, the stimulatory effects of chronic venous infusion of leptin on heart rate and blood pressure have been shown to be markedly enhanced by concomitant blockade of nitric oxide (NO) synthesis with L-NAME (58). Moreover, a recent study by Correia et al. suggested a new concept of selective leptin resistance. The leptin resistant state in obese agouti yellow mice could stimulate sympathetic outflow, thus elevating arterial pressure despite resistance to the weight-reducing effects of leptin. This finding indicates that there is a selective resistance to the metabolic actions of leptin but not to its sympathoexcitatory effects in obese mice (59).

2.2.3 Adipose Tissue Renin Angiotensin System (RAS)

Enhanced activity of systemic RAS has been reported in obese humans (60-63). Epidemiological studies have shown a positive correlation between angiotensinogen (agt) and plasma leptin levels, suggesting a direct contribution of adipose tissue to plasma agt levels (60). This notion is supported by recent agt knockout and transgenic studies showing that in Tg-KO mice reexpressing agt only in adipose tissue, circulating agt returned to detectable levels (~20% of levels in WT) and blood pressure was restored to the levels comparable to those seen in WT mice (64). Additionally, adipose tissue agt contributions to systemic agt levels seen in Tg-KO mice could explain the significantly elevated plasma renin activity (PPA) phenomenon in obese people despite their marked sodium retention and greater extracellular fluid volume; plasma concentration is close to the Michaelis constant of enzymatic reaction between agt and renin (52). In mature adipocytes, Ang II induces leptin gene expression and production, increases prostacyclin (PGI2) and prostaglandin E2 (PGE2) production and induces norepinephrine release in brown adipose tissue. It suggests that all of these mechanisms may account for the regulation of adipose tissue blood flow or systemic blood pressure. The earlier study from Soltis and Cassis provides evidence for functional role of perivascular adipose tissue RAS in vascular responsiveness. Contractile responses of adipose tissue-embedded aortic ring preparation to

electrical stimulation were increased compared with denuded aortic ring preparations. This effect was blunted by AT1 receptor antagonist saralasin. Accordingly, increased vascular constriction appears to be most likely due to Ang II-stimulated norepinephrine (NE) release from adipose tissue, suggesting that perivascular adipose tissue locally modulates vascular reactivity, compared to large adipose tissue depots (65). In addition, the relationship among adipose tissue agt expression, hypertension and body weight has been investigated in several animal models and humans. Although studies have yielded inconsistent findings, positive correlation between agt expression in adipose tissue and BMI or waist-to-hip ratio in humans has been reported (66-68). Gene expression of other RAS components like AT1 receptor, ACE and renin in adipose tissue, was significantly increased in hypertensive obese individuals, suggesting potential association of adipose tissue RAS with obesity-associated hypertension (69).

B. GENETICIS OF OBESITY

Obesity arises as results of numerous behavioral, environmental and genetic factors (70). Obesity is a complex trait, reflecting the effect of a network of genes. Evidence for genetic influences on obesity emerged in twin, adoption and family studies; 40-70% of the variation in obesity-related phenotypes such as BMI, sum of skinfold thickness, fat mass and leptin levels in humans is heritable (71). Although mutations of mouse obesity genes displaying a Mendelian inheritance pattern have been cloned, it is very rare in common human obesity (70). To date, two general approaches have been performed in the search for potential genes underlying common polygenic obesity in humans;1) candidate genes approach focusing the search for specific obesity susceptibility mutations in genes selected on the basis of their known or presumed biological roles in energy homeostasis and 2) genome scanning approach to detect chromosomal regions showing linkage with obesity using inbred strains of mice and human population. These

approaches provide information for obesity gene map in humans and rodents and identifying quantitative trait loci (QTL) associated with obesity and human population with a genetic susceptibility to obesity (72).

1. GENETICS OF HUMAN OBESITY

The role of genetic factors in common human obesity is complex, being determined by the interaction of several genes (polygenic), each of which may have relatively small effects. They are susceptibility genes and work in combination with each other as well as with environmental factors such as nutrients, physical activity and smoking (70, 72). Most of the syndromic forms of obesity like Prader-Willi, Achondroplasia, Angelman, Alstrom, Cohen, Bardet-Biedle, Fanconi Biekel, Borjeson Forssman Lehmann and Simpson-Golabi Behmel have been genetically mapped, but causative genes have not yet been isolated because of the extreme rarity of these mutations (73). Using the strategy focused on mutations in candidate genes that are homologous to murine genes known to be involved in energy balance and mutated, thus causing obesity in rodents, six different human obesity genes have been identified (Table 1): *LEP*, *LEPR*, proopiomelanocortin (*POMC*), proconvertase 1 (*PC1*), single minded drosophila homolog 1(*SIM1*) and melanocortin 4 receptor (*MC4R*) (74-78). Significantly, except for *SIM1*, all of the proteins encoded by these five genes are part of the same pathway regulating food intake in the brain. In human monogenic obesity, *LEP*, *LEPR* and *POMC* gene mutations are very rare and recessive form of obesity. On the other hand, the *MC4R* gene is the most prevalent obesity gene to date, being involved in 1-4% of very obese individuals (79). MC4R mutations generally segregate in families via an autosomal dominant mode of inheritance with variable penetrance (79). In some consanguineous pedigrees, MC4R mutations with relatively modest loss of function appear to be codominantly or even recessively associated with obesity. Further, the

Table 1. ***Single gene mutations for obesity from humans***

Mutation	*Inheritance*	*Gene Product*	*Function*	*Phenotype*
LEP	Recessive	Leptin	1.Satiety hormone (mainly produced by fat cells) 2.Increased energy expenditure	1.Early-onset obesity 2. Hyperphagia 3. Hypogonatropic hypogonadism
LEPR	Recessive	Leptin Receptor	1.Receptor of leptin	1. Massive obesity 2. Growth retardation
POMC	Recessive	Pro-opiomelano-cortin	1.Decreased food intake	1.Obesity 2.ACTH deficiency 3. Altered pigmentation and red hair
PC1	Recessive	Proprotein convertase 1	1.Conversion of POMC and proinsulin to α-MSH and insulin, respectively	1.Hyperproinsulinemia 2.Early-onset obesity 3.ACTH deficiency
SIM1	Recessive	Single minded Drosophila homolog 1	1. SIM1 is involved in paramentricular nucleus development and neurogenesis	1. Profound obesity 2. Increased linear growth 3. Normal energy expenditure
MC4R	Dominant or Recessive	Melacortin receptor 4	1. Decreased food intake	1.Mild severe and morbid obesity 2. Hyperphagia 3. No effect on thyroid and gonadal function

strategies of genetic linkage and association analyses have been performed to identify chromosome regions containing candidate genes underlying common polygenic obesity. Statistical probability of linkage is typically presented as a logarithm of the likelihood ratio for the linkage score (LOD). A number of DNA polymorphisms and susceptibility loci associated with obesity and related phenotypic traits have been identified over the whole genome (72). Thus far, genome-wide scans for obesity susceptibility genes have been performed in several populations of diverse ethnic background including Mexican American families, French pedigrees, Pima Indians and in White Americans. Loci at chromosome 2 and 8, as well as to a lesser extent, loci on chromosome 5 have been described to contribute to the genetic risk for obesity in humans (80).

2. ANIMAL MODELS OF OBESITY

Several spontaneous single-gene mutations causing obesity have been identified in inbred mice. Mouse models of obesity having single-gene mutations include obese (*ob*), diabetic (*db*), fat (*fat*), tubby (*tub*) and yellow agouti (A^y) mutant mouse strains (81-86) (Table 2). These models are very useful for elucidating genetic mechanisms underlying the development of obesity. For instance, *agouti and fat* mice provide models to investigate later onset and moderate obesity, in contrast with other monogenic obese mice characterized by early onset obesity. *Tubby* mice are a good model to study late onset obesity that is not associated with diabetes. In addition, numerous knockout and transgenic models resulting in the development of obesity have been generated. Studies on knockout and transgenic mice revealed additional genes causing body weight dysregulation by hyperphagia or changes in behavioral and metabolic responses (87, 88). Similar to human obesity which is polygenic and genetically heterogeneous, polygenic mouse models of obesity are unique resources to study the genetic effects on obesity caused by gene-gene interactions or gene-environment interactions. Using molecular markers (microsatellites,

Table 2. ***Single gene mutations for obesity from mouse models***

Mutation	*Gene product*	*Function*	*Phenotype*
Obese (ob)	Leptin	1. Satiety hormone (mainly produced by fat cells) 2. Increased energy expenditure	1.Early-onset obesity 2. Hyperphagia 3. Growth hormone deficiency 4. Decreased linear growth
Diabetes (db)	Leptin receptor	1.Receptor of leptin	1. Early-onset obesity 2. Diabetes
Fat (fat)	Carboxypeptidase E	1.Posttranslational processing of prohorrmone (cleaves basic amino acids from C-terminal)	1.Late-onset obesity 2. Infertility 3. Hyperglycemia
Tubby (tub)	Tub protein	1. Transcription factor	1.Late-onset obesity 2.Retinal degeneration 3.Deafness 4.Impaired infertility
Agouti yellow obese (A^y)	Agouti signaling Protein	1.Antagonist of melanocortin receptor 4	1.Yellow pigment 2. Late-onset obesity 3. Immune defect 4. Increased linear growth 5. Decreased infertility

restriction fragment length polymorphisms or single nucleotide polymorphisms) linked to segregation of quantitative obesity phenotypes (*e.g.* BMI, % of body fat, fat mass), 75 QTLs for multigenic obesity and 85 QTLs for body weight have been mapped in crosses between various mouse lines (80). One set of QTLs was mapped by their effects on high fat diet-induced obesity following crosses between the strains ARK/J and SWR/J. These QTLs appear to be active only under the environmental effects of a high-fat diet (30% fat) (89). Additional QTLs for obesity-related sub-phenotypes (*e.g.* increased serum levels of leptin and insulin) and influence of age and gender have also been identified (88).

C. ROLE OF ADIPOSE TISSUE IN OBSITY

The primary role of adipocytes is to store triglycerides during periods of caloric excess and to mobilize this reserve when expenditure exceeds intake. Mature adipocytes possess the full complement of enzymes and regulatory proteins needed to carry out both lipolysis and *de novo* lipogenesis. Of significance, white adipose tissue has been recently considered as an endocrine secretory organ. Adipose tissue lies at the center of a network of autocrine, paracrine and endocrine signals, thereby dynamically influencing systemic metabolism. Further, metabolic alternations in adipose tissue is associated with the development of obesity and obesity-associated metabolic syndromes (90).

1. ORIGIN OF ADIPOCYTES AND ADIPOSE TISSUE

Despite the fact that the developmental origin of the adipose cells is still unknown, studies on multipoint stem cell lines have shown that adipocyte lineage derives from an embryonic stem (ES) cell precursor which is able to differentiate into the mesodermal cell types of adipocytes, chondrocytes, osteoblasts and myocytes (91). Development of adipose tissue occurs at different states in various species (92). In most species, white adipose tissue (WAT)

formation begins before birth and rapidly expands postnatally. Although genes that commit progression from pluripotent mesodermal stem cells to the adipoblast stage of development have not yet been identified, it has been demonstrated that transcriptional factor GATA downregulation induces the transition of embryonic stem cells to adipocytes (93). In contrast to the initial steps of adipogenesis, transcriptional controls in late stages of adipogenesis (conversion of preadipose cells to adipocytes) have been well established using preadipocyte cell lines (*e.g.* 3T3-L1, 3T3-F442 A and Ob 1771) which are already committed solely to the adipocyte lineage(94).

Brown adipose tissue (BAT) develops earlier than WAT. With respect to morphology and physiology, BAT is distinct form WAT; they store lipid in multiple small droplets instead of a single large droplet, and BAT serves primarily to dissipate energy instead of storing it though the expression of uncoupling proteins (UCP) (94). BAT stores are not present in humans, although the expression UCP-1 in WAT reflects the presence of some BAT cells in WAT deposits (95). Additional evidence indicates a transition of BAT to WAT at birth (96). In spite of the fact that no transcription factors have been identified and specifically expressed in brown fat cells, PPARγ and C/EBP have been described to be involved in a induction of brown adipogenesis in a similar fashion to white cell differentiation (94). Peroxisome proliferator activated receptor γ coactivator-1 (PGC-1) was also shown to preferentially direct preadipocytes to a brown fat phenotype (97).

Fat mass expansion occurs as a consequence of increased fat cell size, increased number of fat cells arising from differentiation of preadipocytes into mature adipocytes or both throughout life span (90). Several experimental studies have supported this notion. Studies of rats fed a high-calorie diet have shown that ^{3}H-thymidine incorporates into new fat cells throughout adulthood (81). Adipocyte precursor cells from human adipose tissue can be fully differentiated

into mature adipose cells *in vitro*, (98). Further, Kras et al. have demonstrated that adipocyte acquisition is the result of preadipocyte proliferation, not stem cell recruitment, to become adipocytes (99). Especially, the development of hyperplastic adipose tissue has been implicated in the most severe forms of obesity (90). However, the relative contribution of adipose hypertrophy and hyperplasia to adipose tissue expansion and development of obesity remain still elusive.

On the other hand, adipose tissue does not respond uniformly to stimuli that trigger lipid storage or mobilization, implying local depot-specific growth regulation. Indeed, recent studies have reported differential adipose tissue growth and its potential regulators in various adipose tissue regions (90, 100). For example, there is an inverse relation between the degree of SNS innervation and the propensity for increases in fat cell proliferation. The order of density of SNS innervation of adipose tissue depots from richest to poorest is mesenteric, epididymal, retroperitoneal and inguinal, but proliferative capacity and hyperplasic growth, from greatest to least, is just the opposite (100). The precursor cell population, perfusion capacity, innervation density and some hormones including insulin (hypertrophy inducer), androgen (proliferation promoter) and testosterone (proliferation promoter) could contribute to the site-dependent patterns of adipose tissue growth (90).

2. PROCESS ADIPOCYTE DIFFERENTIATION

Adipocyte differentiation is the transition from undifferentiated fibroblast-like preadipocytes into mature round lipid-filled fat cells. The adipogenic program in preadipocyte cell lines consists of several sequential steps that have been well characterized (94). Briefly, the sequence of events that precedes terminal differentiation includes; 1) Growth arrest at confluence: when confluence is reached, cells arrest in the G_0/G_1 stage of the cell cycle. The step is required for the initiation of differentiation event 2) Clonal expansion: the process involves synchronous entry of all cells into S phase of cell cycle, leading to one or two rounds of mitosis. It occurs

when appropriate mitogenic or adipogenic stimuli (*e.g.* methylisobutylxanthine (MIX) and dexamethasone (Dex)) are presented to the cells and is characterized by the expression of C/EBPβ and C/EBPδ 3) Terminal differentiation: this process is accompanied by an exit from the cell cycle. PPARγ, C/EBPα and many of the adipocyte markers are expressed at this stage and the cells assume their characteristics rounded morphology and visibly accumulate lipid droplets (94).

2.1 Transcriptional Regulation of Adipocyte Differentiation

Adipose differentiation is the result of transcriptional remodeling that leads to activation of a considerable number of adipose-related genes. This process is controlled by various positive and negative signals changing the activity of a variety of transcription factors. In an early adipogenic process, the master transcription factor for adipocytic commitment of mesodermal stem cells remains unknown. Two transcription factors, twist and scleraxis, have been shown to be important for the formation of tissues of mesodermal lineage. However, the roles of these factors in fat development are unknown (101, 102). Further evidence has shown that GATA 2 and 3 (zinc-finger DNA binding proteins) likely act as inhibitors of preadipocyte-adipocyte transition, and their inhibitory effects are mediated by inhibition of PPARγ. GATA 3-defiecient ES cells display an enhanced capacity to differentiate into adipocytes, indicating the role GATA as a determination factor in the transition of pluripotent stem cells to a preadipocytic lineage (93). Similarly, activation of wnt signaling has been reported to prevent preadipose-adipose transition (103).

In a later stage of adipogenesis (preadipocyte-mature adipocyte), three transcription factor families, C/EBPs, PPARs and adipocyte determination and differentiation dependent factor 1(ADD1)/sterol regulatory element-binding protein 1 (SREBP1), have emerged as critical adipogenic regulators (94). In the transcriptional cascade of adipogenesis, hormonal signals

initiate transient increases in expression of C/EBPβ and C/EBPδ. These factors stimulate the expression of PPARγ though the C/EBP binding site in the PPARγ promoter region and also induce C/EBPα via its transcription activation. ADD1/SERBP1, in addition to regulating genes important in fatty acid metabolism, increases the activity of PPARγ, possibly through the production of the endogenous ligands. Activation of PPARγ by ligands allows the differentiation process to proceed. Subsequently, the expression of C/EBPα is induced and C/EBPα also allows for the continued expression of PPARγ once the levels of C/EBPβ and C/EBPδ have decreased after early transient expression. It is likely that both C/EBPα and PPARγ are important for maintaining the fully differentiated state (94). Recently reported FOXC2, a winged helix/forkhead transcription factor, has been shown to be implicated in adipocyte differentiation through the activations of PPARγ, C/EBPα and ADD1/SREBP1 genes (104).

Of note, three key adipogenic transcriptional factors, C/EBP, PPARγ and ADD1/SERBP1, are also responsible for the expression of many adipocyte marker genes. Accordingly, the adipogenesis is accompanied with complex changes in the expression of fat-specific genes. Lipoprotein lipase (LPL) is one of early genes expressed during differentiation process; its expression appears in the confluent stage of growth arrest (105). The expression of C/EBPβ, C/EBPδ, PPARγ and ADD1/SERBP1 is also induced early in the differentiation program. PPARγ expression is induced by transient expression of C/EBPβ and C/EBPδ genes. Following the expression of these early transcriptional factors, preadipocytes enter the terminal phase of differentiation (94). C/EBPα is expressed late in the adipogenesis, concomitant with decreases in C/EBPβ and C/EBPδ gene expression. Several adipocyte-specific genes associated with adipocyte function and phenotype are directly induced by PPARγ, C/EBPα and ADD1/SERBP1; PPARγ activates the transcription of fatty acid transport protein (FATP), phosphoenol-pyruvate

carboxykinase (PEPCK), adipocyte specific fatty acid binding protein (aP2) and acyl CoA synthase (ACS) genes; C/EBPα induces expression of Glut4, UCP1, IR, and IRS1 genes; ADD1/SERBP1 enhances fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC) gene expression (94, 105).

In addition, C/EBPα and PPARγ exerts antimitotic activity in the control of adipocyte differentiation (second and permanent period of growth arrest). This action is related to both a transcriptional effect and interaction with proteins involved in cell cycle control. PPARγ activation leads to a loss of DNA binding activity of E2F/DP, a central transcription factor in the regulation of many genes involved in cell growth and differentiation (106). C/EBPα inhibits the growth of fibroblasts via a direct repression of E2F/DP activity *in vivo* and *in vitro* (107, 108). A subsequent study has illustrated that C/EBPα interacts directly with the cyclin dependent kinases 2 and 4, preventing cyclin binding and thus inducing growth arrest (109). Further, PPARγ induces the expression of several cyclin-dependent kinase inhibitors such as p18 and p21, and C/EBPα stabilizes p21 and thus inhibits adipocyte proliferation (110, 111).

Recent gain-and loss-function studies on these adipogenic transcriptional factors have presented the profound impacts of these proteins on fat cell development. *In vivo*, invalidation of C/EBPβ and C/EBPδ genes impairs severely viability, but live double knockout mice (C/EBPβ$^{-/-}$ and C/EBPδ$^{-/-}$) exhibited defects in adipocyte maturation despite normal expression of C/EBPα and PPARγ, suggesting that C/EBPβ and C/EBPδ can synergistically promote terminal adipocyte differentiation (112). The C/EBPα-null mice did not exhibit any subcutaneous inguinal WAT along with decreased UCP expression (113). Similarly, transgenic mice expressing a dominant-negative bZIP protein under the control of adipocyte specific aP2 promoter, which induces inhibition of C/EBP transcriptional activity, exhibit a lipodystrophy phenotype, emphasizing the

importance of C/EBP proteins in adipogenesis *in vivo* (114). The ablation of the PPARγ gene in mice results in placental-lethal phenotypes. A live PPARγ $^{-/-}$ pup by aggregation of two-celled PPARγ embryos with wild-type tetraploid embryo shows a lack of adipose tissue, proving that PPARγ is required for fat cell development *in vivo* (115). Of interest, PPARγ $^{+/-}$ mice were less prone to develop insulin resistance when fed a high fat diet than were their wild-type littermates, possibly owing to the reduced adipocyte size and decreased production of TNFα and fatty acids in the heterozygotes (116). Surprisingly, ADD1/SERBP1-null mice were normal without alternations in their adipose mass and the levels of lipogenic genes. It was suggested that this may be due to the presence of a low level of SERBP2 in fat, which compensated for the lack of SREBP1(117). Overexpression of ADD1/SERBP1 in fat tissue *in vivo* has been demonstrated to result in mice with severely compromised levels of fat; these animals have fatty liver to compensate for reduced adipose mass (118).

2.2 Modulator of Adipocyte Differentiation

Adipocyte differentiation is modulated by several hormones, cytokines and growth factors (94, 105).

2.2.1 Inducer of Adipogenesis

Insulin and insulin-like growth factor (IGF) stimulate adipogenesis, and ras and Akt/protein kinase B(PKB) have been suggested to play as mediators in the stimulatory effects of these hormones on adipocyte differentiation (119, 120). Glucocorticoids and Dex (a synthetic glucocorticoid) induce preadipocyte differentiation (98). Dex was shown to increase adipogenic activity through increasing expression of C/EBP and transcriptionally repress preadipocyte factor 1 (pref-1), which is a negative regulator of adipogenesis and is found in preadipocytes (98, 121). Retinoic acids (RA) in physiological concentrations promote the transition of OB17 preadipocyte into mature adipocytes (122). However, pharmacological doses of RA inhibit adipogenesis (123).

An intracellular secondary messenger, cAMP promotes adipogenesis and its stimulatory effect was demonstrated in MIX application; MIX inhibits phosphodiesterase concurrent with stimulating adenylyl cyclase via blocking protein Gi and thus accumulates intracellular cAMP. Thus, MIX triggers fat cell differentiation via increasing cellular cAMP accumulation (98). Mechanistically, cAMP-activated transcription factor, cAMP response element binding protein (CEBP) has been shown to mediate cAMP-enhanced adipogenesis (124). One of the prostaglandins, PGI2 is an endogenous ligand for PPARγ thereby leading to adipogenesis in a PPARγ-mediated manner. Alternatively, PGI2 promotes preadipocyte differentiation by increasing cAMP (125). Recent studies on the differential effects of long chain fatty acids (*e.g.* ω-6 *vs* ω-3) on adipogenesis have illustrated that polyunsaturated fatty acids (PUFA) of the ω-6 series are more adipogenic both *in vitro* and *in vivo* compared with their ω-3 counterparts. ω-6 arachidonic acids induce adipocyte development via prostacyclin-activated protein kinase A (PKA) pathways, which upreguate C/EBPβ and C/EBPδ gene transcription (126).

2.2.2 Inhibitor of Adipogenesis

TNFα suppresses fat cell differentiation through downregulation of C/EBPα and PPARγ genes (98). Several growth factors including epidermal growth factor (EGF), TGFα and TGFβ have been shown to inhibit adipogenesis in various preadipocyte cell lines and primary rat preadipocytes (127). This inhibitory effect on adipogenesis is likely to be mediated through activation of mitogen-activated protein kinase (MAPK). This kinase and JNK MAPK directly serine phosphorylate PPARγ 2 and thus inhibit its adipogenic activity (128). In 3T3-L1 cells, prostaglandin F2α (PGF2α) exerts its inhibitory effects on adipogenesis in a FP receptor-dependent manner. FP receptor stimulation causes a transient increase in intracellular calcium, activation of calcium/calmodulin-dependent protein kinase (CaM kinase) and an increase in DNA synthesis associated with the inhibition of differentiation (129). CaM kinase has been shown to

repress differentiation. However, the precise role of CaM kinases in this process remains to be clarified (130).

3. METABOLISM OF ADIPOSE TISSUE

The adipose tissue is the major store of lipids. These stores are constitutively remodeled by control of lipogenesis and lipolysis. The adipose tissue is primarily responsible for the conversion of excess dietary carbohydrate to triglycerides, a process known as lipogenesis (131, 132). This process includes enzymes of glycolysis, fatty acid biosynthesis, and triglyceride synthesis.

FAS and ACC play central roles in *de novo* lipogenesis (131). ACC catalyzes acetyl CoA into malonyl CoA and is a rate limiting enzyme in *de novo* fatty acid biosynthesis (132). ACC is primarily regulated via a short-term allosteric effectors or covalent modifications. Hormonal and nutritional manipulations influence the activity of this enzyme (131). FAS catalyzes all the reactions in the conversion of acetyl-CoA and malonyl-CoA to palmitate by the action of its seven active sites (131, 132).Unlike ACC, FAS activity has been shown to be regulated at the transcriptional levels; *cis*-and *trans*- acting factors. FAS concentration is exquisitely sensitive to nutritional and hormonal status in lipogenic tissues such as liver and adipose tissue (131). FAS transcription was undetectable in the lipogenic tissue of fasted mice and was dramatically induced upon refeeding of a high carbohydrate, fat-free diet (133). Recent studies have reported that the binding of SREBP-1 to a sterol response element (SRE:-150) is mediated by refeeding-induced FAS promoter activation. Upstream stimulatory factor 1 (USF-1) binding to the -65 E box is necessary for SREBP binding and activation of the FAS promoter in *in vivo* context (134). Changes in nutrient intake bring changes in circulating glucose, which in turn signal the secretion of hormones, insulin and glucagon. Glucose and insulin induce FAS promoter activity dependently and independently. Glucose directly activates the FAS gene trough a glucose

response element (GRE, the first intron of the FAS gene) (135). Alternatively, rather than glucose itself, glucose-6-phosphate and its metabolites have been described as a mediator in glucose-induced FAS transcription regulation (136). By using adipocytes in culture and transgenic mice expressing the reporter gene driven by the FAS promoter, the cis-acting insulin response element (IRE, E box) that mediates insulin regulation of the FAS promoter was defined. In addition, USF and SREBP (the basic helix-loop-helix leucine family of transcription factors) have been presented to be key players in such regulation through both *in vivo* and *in vitro* studies. USF-1 and-2 that bind to the -65 E box are required for insulin-mediated transcriptional activation of FAS gene(137, 138). ADD1/SREBP-1 was also shown to be involved in induction of this gene by insulin though binding to the E box (-65/-60) (139). Of note, glucose was definitely required in insulin-induced FAS transcription mediated by both USF and ADD-1/SERBP-1 (140, 141). Further, using specific inhibitors and expressing various signaling molecules, the cellular signaling pathway was defined. Insulin regulation of the FAS promoter is mediated by PI3K and Akt/ PKB, a downstream effector of PI3K signaling pathway in adipocytes (142). Other hormones such as T3, glucagon and Ang II also influence activation of the FAS promoter. The inhibitory effect of glucagon on FAS expression is mediated by cAMP and a cAMP-response element (-149/+68) has been identified in the FAS gene (143). In addition, the -99/-92 region of the FAS promoter was recognized as a core region for cAMP responsiveness (143). Mutation of this box has been shown to prevent cAMP-mediated inhibitory effect on the FAS gene as well. T3 has been demonstrated to enhance FAS expression. FAS promoter region contains two thyroid response elements (TRE):TRE1 (-870/-650) and TRE2 (-272/-40) (144). Ang II has been reported to increase FAS transcriptional activity via an E box, and this stimulatory effect of this hormone was dependent on ADD1/SERBP1 and glucose in 3T3-L1 adipocyte cells (145). Agouti stimulates FAS transcription in a Ca^{+2}-dependent mechanism. An agouti/Ca^{+2}-response element (-

435/-415) within FAS gene has been identified (146). Additionally, Moon et al. have recently examined *in vivo* and *in vitro* the specific FAS promoter elements implicated in PUFA suppression. They found that the promoter region required for PUFA suppression *in vivo* is located in the -150 sterol response element (SRE) where SREBP functions and the -65 E Box which contributes to PUFA suppression of the FAS promoter, at least *in vitro* (147).

Hormone-sensitive lipase (HSL), the rate-limiting enzyme of intracellular triglyceride hydrolysis, is a major determinant of fatty acid mobilization in adipose tissue. Adipocyte lipolysis is also subject to hormonal and neuronal regulation (148). Catecholamines are considered as major stimulators while insulin is accepted as the most important physiological inhibitor of catecholamine-induced lipolysis. SNS activation induces the release of catecholamines, which triggers an elevation of cAMP concentration that activates PKA, resulting in the phosphorylation of HSL and the perilipins (148). Thus, phosphorylation of these proteins dramatically increases lipolysis. By contrast, insulin exerts its antilipolytic actions by inducing phosphorylation and activation of the phosphodiesterase type 3B (PDE3B), resulting in a decrease in cAMP levels and concomitant decrease of PKA activity (148). Molecular mechanisms underlying activation of lipolysis have been provided. On stimulation of the adipocytes, PKA-mediated phosphorylation triggers the translocation of HSL from the cytoplasmatic compartment to the surface of the lipid droplets (149). Perilipins, which is located on the surface of the lipid droplets in adipocytes and precludes HSL binding to lipid droplets in resting cells, are phosphorylated and probably relieve the restraint and allow the translocation of phosphorylated HSL to the lipid droplet (150). Compelling evidence for this barrier role of perilipins has recently been reported (151). In nutritional regulation of HSL, prolonged fasting in rats induces HSL transcription, protein and enzyme activity, collectively elevating basal lipolysis activity (152). Of importance, *in vivo and in vitro* studies have presented that catecholamine-induced lipolytic effects as well as antilipolytic

effects of insulin are blunted in obese subjects, thereby contributing to considerable increases in the net adipocyte lipolytic rate of obese subjects compared to lean individuals (153). The altered lipolysis of catecholamine could be attributed to a decreased number of β2 adrenergic receptors, changes in the functional balance between α2- and β-adrenergic receptors, or postreceptor alterations. Other studies have provided evidence that this impaired lipolytic action of catecholamines was due mainly to reduced HSL activity (154). A dysfunction in lipolysis due to impaired HSL expression and a decrease in enzyme activity could precede the actual development of obesity. Furthermore, a marked resistance to the lipolytic effect of noradrenaline in adipocytes was also observed in type II diabetic subjects who were characterized with elevated FFA levels, due to a decrease in the number of β2-adrenergic receptors (155, 156).

4. FUNCTION OF ADIPOSE TISSUE

Adipose tissue is actively involved in cell function regulation through a complex network of endocrine, paracrine and autocrine signals that influence the response of many tissues including the hypothalamus, liver and skeletal muscle. Adipocyte-secreted biological molecules have been shown to be implicated in obesity and obesity-related metabolic alternations including insulin resistance, diabetes and hypertension (Table 3) (22).

D. ROLE OF ANGIOTENSIN II IN OBESITY

1. SYSTEMIC RENIN ANGIOTENSIN SYSTEM (RAS)

1.1 Overview

Ang II, a vasoactive hormone, is the active component of the RAS. Ang II is a multifunctional peptide hormone that not only controls blood pressure homeostasis but also regulates cardiovascular and renal functions. Ang II is produced systemically via classical or circulating RAS and locally via tissue RAS (157). The classical pathway of RAS is composed of

Table 3. ***Adipocyte-associated biological factors***

	Molecule	***Function***	***References***
Adipocyte-secreted Proteins	Leptin	Signals to the brain about body fat stores Regulation of appetite and energy expenditure	(82, 158)
	TNFα	Interferes with insulin receptor signaling and is a possible causes of the development of insulin resistance in obesity	(159, 160)
	IL-6	Implicated in host defense and in glucose and lipid metabolism	(161)
	PAI-1	Potent inhibitor of the fibrinolytic system	(162)
	Tissue Factor	Major cellular initiator of the coagulation cascade	(22)
	Agt	Precursor of angiotensin II and regulator of pressure and electrolyte homeostasis	(163)
	Adipsin	Possible link between the activation of the alternative complement pathway and adipose tissue metabolism	(164)
	ASP	Influences the rate of triacylglycerol synthesis in adipose tissue	(164)
	Adipophilin	May be a specific marker for lipid accumulation in the cells	(165)
	Adiponectin	Associated with insulin sensitivity	(166)
	PGI2, PGF2α	Implicated in regulatory function such as inflammation and blood clotting	(161)
	TGFβ	Regulates a wide variety of biological responses including proliferation, differentiation, apoptosis, and development	(167)
	Resistin	Associated with insulin resistance	(38)
	Receptor	***Main effects of receptor activation on adipocyte metabolism***	***References***
White adipocyte receptors	Leptin (OB-R)	Stimulation of lipolysis Autocrine regulation of leptin expression	(22, 168)
	Insulin	Inhibition of lipolysis and stimulation of lipogenesis Induction of glucose uptake and oxidation	(22)
	Prostaglandin	Strong antilipolytic effects (PGE2) Modulation of preadipocyte differentiation (PGF2α and PGI2)	(22)
	TNFα	Stimulation of lipolysis, Regulation of leptin secretion, Potent inhibition of adipocyte differentiation, involvement in development of insulin resistance	(26, 169)
	IL-6	LPL activity inhibition, induction of lipolysis	(161)
	Ang II	Increase lipogenesis, Stimulation of prostacyclin and PGE2 by mature fat cells, interaction with insulin in regulation of adipocyte metabolism	(163, 170)
	β1,β2, β3	Stimulation of lipolysis, Induction of thermogenesis, Reduction of leptin mRNA levels	(171)
	α1	Induction of inositol phosphate production and PKC activation	(171)
	α2	Inhibition of lipolysis	(171)
	PPARγ	Induction of adipocyte differentiation and insulin sensitivity	(172)
	PAR/PXR	Regulation of adipocyte differentiation	(173)
	Vitamin D	Inhibition of adipocyte differentiation	(174)
	VLDL	Binding and internalization of VLDL particles	(175)

a sequential reaction of two critical enzymes that convert hepatic-derived precursor agt into Ang II. The first enzyme, renin, is secreted from juxtaglomerular (JG) cells in the kidney and splits a specific leucine-leucine peptide bond in circulating angiotensinogen, resulting in the formation of decapeptide angiotensin I. Biologically inactive angiotensin I (Ang I) is then carried via the blood to the lung, where the second enzyme, angiotensin converting enzyme (ACE), removes two amino acids from the carboxy terminus of angiotensin I to generate the active octapeptide Ang II (176). ACE is a monomeric, membrane-bound, zinc-and chloride-dependent peptidyl dipeptidase anchored on the endothelium of the lung that degrades bradykinin and is involved in the biosynthesis of different neuropeptides such as substance P. In addition to renin and ACE-dependent pathways of Ang II formation, the existence of non-renin and ACE pathways have been also demonstrated (177, 178). Ang II can be formed via several non-renin and non-ACE enzymes including chymase, kallikrein, cathepsin D, cathepsin G, tissue plasminogen activator and tonin. Alternatively, Ang I can be processed into heptapeptide Ang $_{1-7}$ by neural endopeptidase (NEP). Unlike renin and agt, which have relatively longer plasma half-lives, Ang II is degraded by angiotensinases to angiotensin III (Ang III), angiotensin IV (Ang $_{3-8}$, Ang IV) or Ang $_{1-7}$ within few seconds. Circulating Ang II and agt-derived bioactive peptide fragments are dispersed to the target tissues of body, where they can exert diverse physiological effects via their interactions with specific angiotensin receptors, such as AT1a, AT1b, AT2 and AT4, which induce different signal cascades (176). The well-known biological actions of Ang II such as vasoconstriction, antinatriuresis and cell proliferation are mediated via the classic AT1 receptor(176). Paradoxically, other Ang II-mediated effects, including cell death, vasodilation and natriuresis are mediated by AT2 receptor activation. Currently, the physiological functions of Ang II-derived peptides have been intensely investigated in different cell types. Ang III has been shown to signal via both AT1 and AT2 receptors with a similar affinity to that of Ang II. Ang III

appears to have a key role in the brain RAS. It participates in the central control of blood pressure and the release of vasopressin. Ang IV regulates renal blood flow and facilitates memory retention and retrieval via AT4 receptors (179). Ang $_{1-7}$ promotes vasodilatation, antiproliferation and apoptosis possibly through interacting with AT1 and AT2 receptors (Fig 1).

1.2 Angiotensin Receptors: Structure and Distribution (Table 4)

Ang II exerts its effects via at least two plasma membrane receptors: AT1 and AT2 (180, 181). Most of the physiological actions of Ang II are mediated via the AT1 receptors. Ang III is a full agonist at AT1 receptors and binds with high affinity at AT2 receptors. Ang IV and Ang $_{1-7}$ display lower affinity for AT1 and AT2 receptors. AT4 receptor, a novel Ang IV receptor, was recently purified and identified by mass spectrometry as insulin-regulated amino-peptidase (IRAP), a transmembrane-anchored metallopeptidase that co-translocates in vesicles with GLUT4 to the cell surface in response to insulin (182). Very recently, Santos et al demonstrated that Ang $_{1-7}$ mediates vasodilatory actions by direct interactions with the G-protein-coupled receptor Mas in mouse kidneys, suggesting Mas as a functional receptor for Ang $_{1-7}$ although a specific Ang $_{1-7}$ receptor has not been cloned (183). Additionally, an AT3 receptor has also been identified in cultured mouse neuroblastoma cells, but not yet characterized (176).

1.2.1 AT1 and AT2 receptors

Both receptors belong to the seven transmembrane spanning G-protein-coupled receptor family (176). The receptors comprise extracellular, transmembrane and carboxyl-terminal domains; the extracellular loop and the transmembrane domain play an important role in Ang II binding whereas carboxyl-terminal domain is the cytoplasmic and regulatory site, often containing sites for phosphorylation and cellular trafficking. Like most G-protein-coupled receptors (GPCR), the AT1 receptor, but not the AT2 receptor, undergoes Ang II-stimulated internalization. The internalized receptors are either trafficked to lyzosome (degradation) or

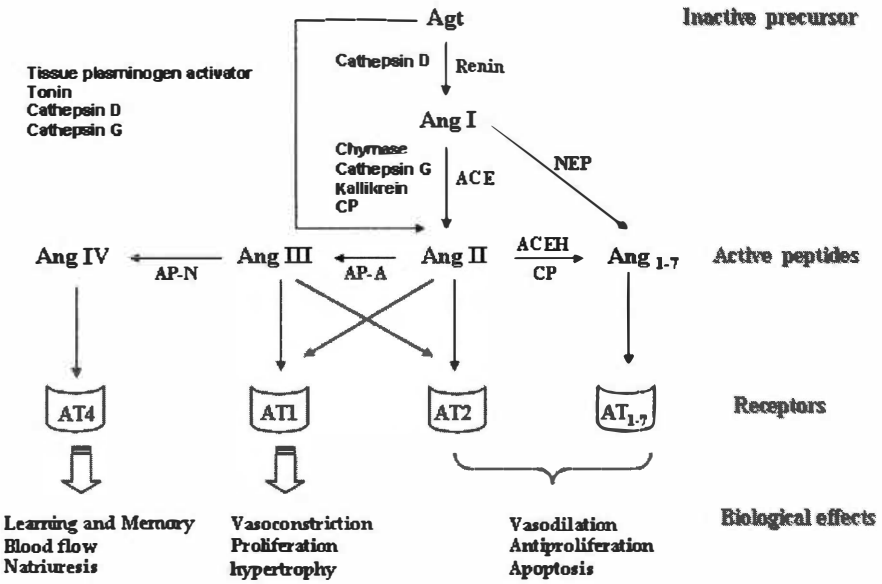

Fig. 1 ***Scheme of the systemic RAS.*** The diverse biological effects of active peptides (Ang II, Ang III and Ang $_{1-7}$) that are derived from Ang I and the principal enzymes involved in their production are illustrated, including the possible sites of action of the human homologue of ACE (ACEH or ACE2). The angiotensin AT1 and AT2 receptors, which are G-protein-coupled receptors (GPCRs), have been cloned and characterized in detail. The AT4 receptor has recently been identified as insulin-regulated aminopeptidase (IRAP), a membrane-bound enzyme and AT_{1-7} receptor has not been cloned. AP, aminopeptidase; CP, carboxypeptidase; NEP, neural endopeptidase (neprilysin)

Table 4. ***Properties of angiotensin receptor subtypes***

Characteristics	*AT1*	*AT2*	*AT4*
Affinity	Ang II>Ang III>Ang I	Ang III> Ang II>Ang I	Ang IV
Selective antagonists	Losartan (DuP753), CGP4627 DuP532, EXP3174, L158809 SK1F108566	PD121981, PD123319, PD124125, CGP42112A PD 123177	Divalinal-Ang IV
Coupling to G-protein	YES (Gi, Gq, Gα)	YES (Gi ?)	NO
Signal transduction	Increase Ca^{+2} and IP3 Decrease cAMP Increased prostaglandins Tyrosine phosphorylation	Decrease cGMP Increase prostaglandins Tyrosine dephosphorylation	Tyrosine phosphorylation Increase Ca^{+2} in kidney
Structure	359 amino acids Seven transmembrane domains	363 amino acids Seven transmembrane domains	Trimer (α,β,γ)
Internalizations	YES	NO	YES (?)

dephosphorylated and recycled to the cell surface. AT1 and AT2 receptors, with ~ 32 % amino acid sequence homology are single copy genes found on chromosome 3 and X, respectively. In rodents, the two distinct subtypes (AT1a and AT1b) of AT1 receptor share 94 % similarity and are pharmacologically indistinguishable but are differentially expressed and regulated. AT1 receptors are antagonized by biphenylimidazoles such as losartan whereas tetrahydroimidazopyridines such as PD 123319 specifically block AT2 receptors (176).

Northern blot and RT-PCR analyses have identified mRNA for AT1 receptor in most Ang II target tissues (176). AT1 receptors are expressed in blood vessels, the adrenal cortex, liver, kidney and brain. In rats and mice, AT1a is the predominant receptor in most organs whereas AT1b is most abundant in the adrenal and pituitary glands. AT2 receptors are ubiquitously expressed in human fetal mesenchymal tissues. However, the expression of the receptors rapidly declines at birth. In adults, AT2 receptor expression is detectable in pancreas, heart, kidney, adrenals, brain and vasculature (184). After vascular and cardiac injury and during wound healing and renal obstruction, AT2 receptors are reexpressed in adults, suggesting a possible role of AT2 receptors in tissue remodeling, repair, growth and development (176).

1.2.2 AT4 receptors

In contrast to the other angiotensin receptors associated with G proteins, AT4 receptor/IRAP is a type II membrane-spanning protein and a member of the M1 family of zinc-dependent metallopeptidases (185). Besides the endogenous ligand Ang IV, other peptides like LVV-hemorphin-7 (the endogenous globin fragment) are also capable of binding and activating AT4 receptors (185). AT4 receptor signals via its homodimers: 1) the intracellular amino-terminus contains multiple serine/threonine residues indicative of phosphorylation and two dileucine internalization motifs 2) the single transmembrane-spanning region is connected to an extracellular domain containing N-linked glycosylation sites and a putative cleavage site. Three

isoforms of AT4/IRAP are synthesized from a single gene on chromosome 5 (176). The binding of angiotensin IV to the AT4 receptor is insensitive to both losartan and PD123319, but is selectively inhibited by the peptide antagonist divalinal-Ang IV (186). High levels of AT4/IRAP are expressed in heart, placenta, skeletal muscle, kidney and small intestine whereas lower expression is found in brain, liver, testes, colon and spleen (185).

1.3 Physiological Function of Systemic RAS

Ang II, a physiologically active effector of circulatory RAS, controls blood pressure, body fluid homeostasis and cardiovascular homeostasis primarily due to its potent vasoconstrictor actions. The mechanisms of blood pressure regulation by Ang II are well-established. In addition to its documented vasoconstrictive effects, growing evidence has shown that Ang II may play a central role in cardiovascular and renal diseases. Overactivity of the RAS has been implicated in the development of various cardiovascular diseases, such as hypertension, congestive heart failure and renal insufficiency (176)

1.3.1 Regulation of Blood Pressure

Circulating Ang II is a main regulator of systemic and local blood pressure, as well as electrolyte and fluid homeostasis. It acts through its vasoconstrictive effects on the vascular system and by promoting renal sodium and water absorption in the kidneys in both acute and chronic manners (187).

1.3.1.1 Short-term Regulation

In the vascular system, Ang II constricts the capillary arterioles, leading to an increase in total peripheral resistance. Ang II enhances the peripheral effector cell response to noradrenalin and has a positive inotrophic effect on the heart due to an increase of calcium-ion influx during the plateau phase of action potential. In the central nervous system, Ang II stimulates sympathetic

nervous activity, thus inducing peripheral resistance which contributes to increasing body blood pressure (187).

1.3.1.2 Long-term Regulation

In its chronic actions, Ang II regulates blood pressure through direct effects in the kidneys and indirect effects mediated through its interactions with other hormones such as aldosterone and vasopressin. Ang II directly causes contraction of mesangial cells and constriction of the afferent and efferent arterioles in the glomerulus and thus reduces blood flow in the kidney. The sum of these changes reduces the glomerular filtration rate (GFR). This decreased GFR results in decreasing formation of urine and increasing sodium and water retention, thereby elevating extracellular volume and blood pressure. In addition to its GFR regulation, Ang II augments proximal tubular sodium and bicarbonate reabsorption primarily by enhancing tubular sodium transporter activities (e.g. increasing Na^+/ H^+ exchanger, Na^+/HCO^- co-transporter and Na^+/K^+ ATPase activities) and changes in starling forces (187). Ang II indirectly stimulates the synthesis and secretion of aldosterone in the adrenal gland, the release of vasopressin (antidiuretic hormone) in the pituitary and the sensation of thirst and salt appetite in the brain (187).

1.3.2 Regulation of Gene Expression

Independent of its vasopressor effects, Ang II directly causes cellular phenotypic changes, growth and migration and regulates the gene expression of growth factors, extracelluar matrix components and cytokines in renal mesangial, endothelial and vascular smooth muscle cells (VSMC) (176). These actions are proposed to participate in the pathophsiology of cardiac hypertrophy and remodeling, vascular thickening, thrombosis and glomerulosclerosis (188).

Ang II induces expression of the several proto-oncogenes in human and rat vascular smooth muscle cells, including c-*fos*, c-*jun*, and c-*myc*. The study of Chen et al has shown that

Ang II induces vascular c-*fos* gene expression in a protein kinase C (PKC)- and Ca^{2+}-dependent manner (189). Ang II increases the expression and production of several growth factors such as insulin-like growth factor-1 (IGF-1), platelet-derived growth factor A chain (PDGF-A), basic fibroblast growth factor (FGF) and adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1(VCAM-1) as well as chemotactic factors such as monocyte chemoattractant protein (MCP-1)(188). In vascular smooth muscle and mesangial cells, Ang II, time and dose dependently increases TGF-β mRNA, which is associated with increase in mRNA for matrix proteins fibronectin and collagen type 1(190). Ang II also stimulates expression and production of cytokine TNF-α in renal tissue(191). Additionally, Ang II enhances levels and activity of plasminogen activator inhibitor-1 (PAI-1) in vascular smooth muscle, mesangial, endothelial cells, thereby influencing fibrinolysis, extracellular matrix turnover, degradation and regulation of cell migration (192, 193). Consistently, Ang II and its metabolites such as Ang III and IV promote PAI-1 production and release by human fat cells in a AT1 receptor-mediated manner, suggesting a potential prothrombotic effect of Ang II in human adipose tissue (194).

Ang II is a powerful mitogen for many cells types. Ang II stimulates transition from the G_0-G_1 phase in the cell cycle, which leads to increased DNA synthesis and to mitogenesis in combination with other growth factors (195). In adrenal cortical cells and human preadipose cells, Ang II increases cell cycle regulatory protein cyclin D1 expression and cyclin D1-dependent kinase activity, thus promoting cell cycle progression (196, 197). Additionally, Ang II upregulates transcription of genes for later markers of adipocyte differentiation such as FAS and leptin as well as expression of adipogenic transcription factor, ADD1, indicating a significant role of Ang II in adipose growth and development (145, 198).

1.4 Regulation of Systemic RAS Components

The rate-limiting step in the cascade of enzymatic events that make up the circulating RAS is the first step, the cleavage of agt by renin. The plasma agt micromolar range (~1μM) whereas the affinity of renin for its substrate (Km) is about 1.25 μM in humans, indicating that the agt concentration is a determinant of Ang I production *in vivo* (199). Accordingly, like the classical controller of plasma renin activity (PRA), agt availability is important in circulating RAS activation. Evidence from clinical studies has noted that ACE activity is also a rate-limiting factor that influences the activity of RAS. Treatment with ACE inhibitors (such as captopril) has been clinically shown to significantly reduce blood pressure (200). In addition, a relationship between RAS component polymorphisms and essential hypertension has been reported previously. Genetic background has been shown to highly influence the activity and expression of RAS. Thus, it appears that genetic factors can be a critical determinant in circulating RAS activity (201).

1.4.1 Angiotensinogen (agt)

The human agt gene contains five exons and four introns, which span 13 kb and is located on chromosome 1q42-3(202). In term of exon number, size and splicing sites, the agt gene belongs to the serpin (serine protease inhibitor) superfamily. Human agt protein is a globular glycoprotein with a molecular mass between 55 and 65 kDa, depending on its state of glycosylation (203). Despite the unknown function of glycosylation in humans, it has been shown that the glycosylation status influences the agt clearance rate in rats; the more highly glycosylated form is secreted faster by the liver and eliminated more rapidly by the kidney than is the less glycosylated form (203). Mature agt contains 453 amino acid residues (~50kD): the first ten amino acids correspond to Ang I and the other larger portion corresponds to des(AngI)agt. Plasma agt concentration reflects mainly hepatic agt synthesis since agt is constitutively released from hepatocytes. The plasma and tissue agt concentrations both contribute directly to the

circulating amounts of Ang I and Ang II, ultimately influencing the degree of RAS activation (176). Agt synthesis and secretion are regulated by several hormone factors. In its transcriptional regulation, some of the *cis*- and *trans*- regulatory elements of promoter regions of agt gene have been identified.

Evidence from i*n vivo* and *in vitro* experiments has demonstrated that glucocorticoids (especially dexamethasone), estrogens, Ang II and thyroid hormones all increase the synthesis and release of agt from hepatic and nonhepatic cells (204). Agt production is mainly regulated by controlling gene transcription, although it has been shown that Ang II posttranscriptionally regulates agt gene expression by increasing the stability of agt mRNA (205). It has been found that the transcriptional *cis* elements, angiotensinogen-gene activating element 1 (ACE1, from -339 to-139), AGE2 (from -96 to -52) and AGE3 (-6 to +22) of the proximal promoter of agt gene have pivotal roles in the regulation of hepatic angiotensinogen production *in vivo* (206). Novel transcriptional factors such as angiotensinogen gene-activating factor (AGF) 1 to 3 have been identified and regulate agt expression levels in human hepatocytes (207). The agt expression is tightly controlled through the actions of a multihormone-inducible enhancer (from -615 to -470). Glucocorticoids stimulate agt transcription by binding directly to the glucocorticoid receptor (GR) which is translocated into the nucleus to interact with glucocorticoid receptor element 1 (GRE1: from -548 to -570) and glucocorticoid receptor element 2 (GRE2: from -472 to -477) within the agt promoter (208, 209). Cytokine TNF-α induces agt transcription by nuclear factor-κB (NF-κB) transcription factor which binds to acute-phase response element (APRE: 5' to the transcriptional state site), which is the binding site for C/EBP (210, 211). The critical core promoter region of agt gene has been analyzed. A ubiquitously expressed nuclear factor, hAG core promoter binding factor 1 (AGCF1), binds to hAG core promoter element 1 (AGCE1: from -25 to -1) which appears to play a major role in activating agt transcription. Several natural

variants of agt gene have been detected in this regulatory region. A variant in the proximal promoter of the agt, an adenine instead of a guanine six nucleotide upstream from transcription initiation site, A at (-6) has been suggested to be causally related to a genetic predisposition to essential hypertension (212).

1.4.2 Renin

Renin is encoded by a single gene and renin mRNA is translated into the preprorenin. In JG cells, preprorenin is converted into mature and active renin by a series of proteolytic cleavages and glycosylation (213). Stored active renin is released from JG cells by an exocytic process involving stimulus-secretion coupling. Plasma renin depends on 1) transcriptional control of the renin gene;2) posttranscriptional control at the mRNA level;3) posttranslational processing of the preprorenin protein, and;4) the uptake of renin into vesicles and its release (214, 215).

With regard to the regulation of renin transcription, two enhancer regions have been identified that markedly increase renin synthesis. In the renin enhancer, cAMP responsive elements and several important transcription factors (retinoid acid receptor, RAR/retinoid X receptor RXR), E box proteins USF1/USF2 and Hox/Pbx have been found (215, 216). Vitamin D_3 and its receptor have been suggested as a negative regulator of renin expression (217). This was also supported by clinical observations that there is an inverse relationship between plasma vitamin D_3 levels and plasma renin activity as well as with blood pressure. Vitamin D_3 supplementation has been shown to lower blood pressure in hypertensive subjects (218).

In addition to transcriptional regulation in renin expression, posttranscriptional control at the renin mRNA level has been demonstrated. In particular, cAMP has been known as an inducer of renin synthesis via prolonging its mRNA half-life (213). More recently, the studies from Skalweit et al have identified several RNA-binding regulatory proteins (hnRNP K and E1, Dynamin, Nucleolin, Y-Box1 and MINT homologous protein) involved in 3'-untranslated regions

(UTR)-mediated control of renin mRNA stability and also illustrated that cAMP-based renin mRNA stabilization is accompanied by upregulation of these renin mRNA binding proteins (219).

Renin release from the kidney JG cells is affected by several systemic and intrarenal factors (213). Systemic factors include sympathetic nerves (stimulator), circulating Ang II (inhibitor), blood pressure (inhibitor) and salt balance (inhibitor). Intrarenal factors are renal nitric oxide and prostaglandins which stimulate renin secretion (213). At the cellular level, renin secretion is controlled by classic signaling systems, including cAMP, Ca^{2+}/PKC and cGMP pathways (213). While cAMP and Ca^{2+}/PKC appear to be direct antagonists in the regulation of renin secretion, cGMP plays a more versatile role by directly inhibiting renin secretion via protein kinase GII (PKGII) and by indirectly stimulating renin secretion via inhibition of cAMP degradation (220) Renin metabolic clearance rate (MCR) in plasma has been shown to be decreased by elevated agt concentration. The studies pointed out that higher agt concentration increases agt-renin complex stability and thus elevates PRA, thereby increasing blood pressure independent of renin secretion and Ang II-mediated feedback (220). Interestingly, the studies of Nguyen et al have implicated renin receptor as an important cofactor in PRA since it increases the catalytic efficiency of agt cleavage by receptor-bound renin, thus facilitating Ang II generation and action on a cell surface (221).

1.4.3 ACE

The ACE gene produces two mRNA species from tissue-specific promoters and two isoforms of ACE (222). The ubiquitous somatic ACE and the sperm-specific germinal ACE have identical enzymatic activity. ACE converts Ang I into Ang II and inactivates two vasodilator peptides, bradykinin (BK) and kallidin (176). ACE exists as soluble and membrane-bound forms but most of ACE is membrane-bound (222). ACE2 (an ACE homolog, ACEH) has recently been identified in humans that differs from ACE in specificity and physiological roles (223). ACE2

cleaves a single residue from Ang I, generating Ang $_{1-9}$ and a single residue from Ang II to generate Ang $_{1-7}$. ACE2 has been shown to play as a critical regulator of cardiac function (224).

Pharmacological stimulators and pathological conditions modulate ACE expression and secretion (200, 225-228). Several agents, such as cAMP analogues and calcium ionophore A23187 have been shown to induce ACE secretion from cultured bovine endothelial cells (225, 226). ACE secretion was also increased by glucocorticoid hormone on cultured endothelial cells and VSMC (227). Fibroblast growth factor increases ACE gene expression in cultured rat aortic smooth muscle (RASM) (228). Pathophysiologic conditions such as hypertension and heart failure induce an increase in tissue ACE activity. Additionally, ACE insertion (I)/deletion (D) polymorphism has been shown to influence plasma ACE level; subjects with the D allele have higher plasma ACE levels and blood pressure than subjects with the I allele (200).

1.5 Signaling Mechanisms of Angiotensin Receptors

1.5.1 Overview

The interaction of Ang II with AT1 and AT2 receptors activates a complex series of intracellular signaling events (176). The physiological functions of AT1 receptors are 1) cell growth and proliferation 2) vasoconstriction and 3) salt and water retention. AT1 receptor-induced effects are mediated via complex and interacting signaling pathways involving 1) stimulation of phospholipase C (PLC)/PKC and Ca^{+2} mobilization 2) activation of phospholipase D (PLD)/phospholipase A_2 (PLA_2) 3) activation of MAPK/signal transducers and activators of transcription (STAT)/ Janus kinase (JAK) and 4) transactivation of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), insulin growth factor receptor (IGFR) and platelet-derived growth factor receptor (PDGFR) (Fig 2). By comparison, the cell signaling pathways involved in AT2 receptor activation are not fully clarified but appear to involve G protein-

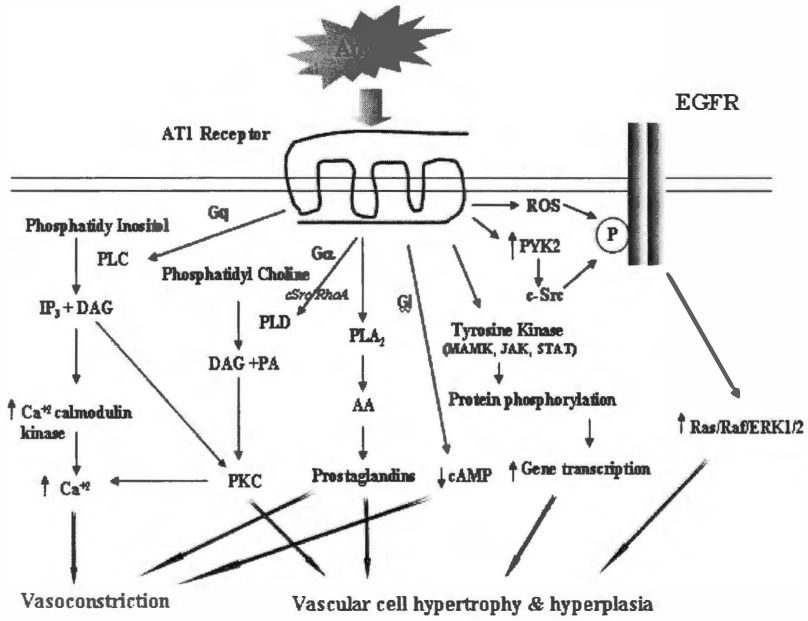

Fig. 2 ***Signal transduction mechanisms and physiological effects mediated by the AT1 receptor.***

dependent and independent pathways. Current evidence has elucidated four major signal transduction cascades induced by AT2 receptor activation: 1) activation of the phosphotyrosine phosphatase and protein dephosphorylation 2) regulation of BK-nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) system 3) stimulation of PLA_2 and release of arachidonic acid (AA) and 4) sphingolipid-derived ceramide (Fig 3). Functionally, the AT2 receptor exerts vasodilatory, antihypertrophic and proapoptotic effects, which antagonize the AT1 receptor-mediated physiological actions. Like AT2 receptors, Ang IV-activated AT 4 receptors may oppose the effect of AT1 receptor by modulating renal blood flow, inhibiting tubular Na^+ reabsorption and blocking cardiac hypertrophy. The initial events that characterize the intracellular signaling mechanisms of the AT4 receptor are presently unknown, although downstream target genes (c-Fos, c-Jun, PAI-1) have been found (176).

1.5.2 Signaling Mechanisms of AT1 Receptors

AT1 receptor expression is physiologically and hormonally regulated in a transcriptional or posttranslational-dependent mechanism. The expression of AT1 receptors is tissue-specific. Ang II downregulates the expression of vascular AT1 receptors by a negative feedback mechanism (176). Recent studies have provided evidence that AT2 receptor is a natural antagonist of AT1 receptor in a ligand-independent manner. AT1/AT2 receptor heterodimerization increases structural stability of AT1 receptors and thus diminishes its sensitivity to Ang II. Overexpression of AT2 receptors decreases AT1 receptor expression (229). Like GPCR signaling cascades, AT1 receptors interact with different G protein isoforms including Gq/11, Gi and Gα12 and Gα13 which couple to distinct signal cascades. One of the earliest events triggered by Ang II-AT1 receptor stimulation is a rapid, PLC-dependent hydrolysis of phophatidylinositol-4,5-bisphophate. PLC activation results in the production of 1,4,5-inositol

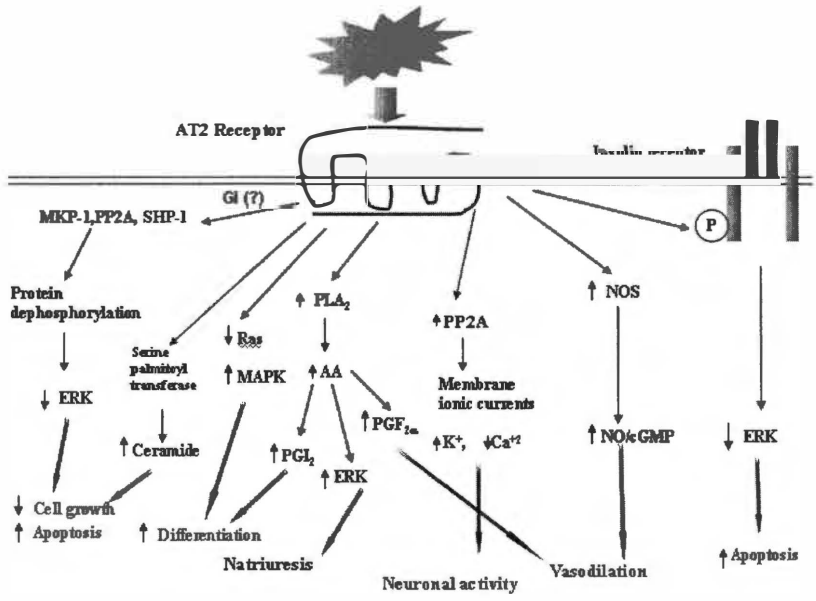

Fig. 3 ***Signal transduction mechanisms and physiological effects mediated by the AT2 receptor.***

triphosphate (IP3) and diacylglycerol (DAG), which are involved in Ca^{+2} mobilization from the sarcoplasmic reticulum (SR) and stimulation of PKC, respectively (230). Increased intracellular Ca^{+2} induces VSMC contraction, whereas PKC activation regulates intracellular pH through the Na^{+}/H^{+} exchanger and induces voltage-opening Ca^{+2} channel activation on plasma membrane via phosphorylation, thereby leading to cellular contraction (231, 232).

Ang II-induced PLD activation induces the formation of phosphatidic acid and subsequent generation of DAG which is the physiological activator of PKC and is also a source of arachidonic acid production. Earlier studies reported that molecular mechanisms coupling AT1 receptors to PLD were independent of Gq and Gi proteins (176). These observations are supported by studies from Ushio-Fukai et al, which has illustrated that AT1 receptor activation of PLD is mediated by $G\alpha12$ and $G\beta\gamma$ subunits, Src and RhoA (233). AT1 receptor-stimulated PLD signaling has been implicated in vascular contractility, cardiac hypertrophy and VSMC proliferation. These effects have been revealed to be mediated via phosphatidic acid (PA) and other PLD metabolites (234), which affect vascular generation of superoxide anion by activating NAD(P)H oxidase (233), stimulate tyrosine kinases and Raf/Ras, and regulate intracellular Ca^{+2} signaling (235).

Ang II activates PLA_2, which is responsible for the release of arachidonic acid from cell membrane phospholipids. Released arachidonic acid (AA) is metabolized by cyclooxygenases, lipoxygenases or cytochrome P450 oxygenases to many different eicosanoids in vascular and renal tissues. PLA_2-derived eicosanoids have been recognized as important regulators in vascular and renal mechanisms of blood pressure regulation (236). AT1 receptor-induced activation of vascular PLA_2 in response to Ang II is dependent on intracellular Ca^{+2} and Ca^{+2}-calmodulin-dependent protein kinase II and MAPK (237). On the contrary, in renal epithelial cells, Ang II-evoked PLA_2 stimulation has been shown be mediated via AT2 receptors and a Ca^{+2}-independent

mechanism (238). Ang II-generated eicosanoids through PLA_2 activation have been demonstrated to modulate vascular contraction and growth possibly via MAPK and redox-sensitive pathways(236).Vasorelaxant prostaglandins such as PGI2 and PGE2 attenuate Ang II-mediated vasoconstriction in vascular tissues whereby thromboxanes are involved in Ang II-activated contraction (236). Lipoxygenase-derived eicosanoids appear to facilitate Ang II-elicited vasoconstriction in VSMC. Lipoxygenase inhibitors decrease the vasoconstrictor effect of Ang II and attenuate blood pressure in spontaneously hypertensive rats (SHR) (236).

AT1 receptor-Gi coupled signaling inhibits adenylate cyclase in several Ang II target tissues including liver, kidney and adrenal glomerulosa, thus decreasing formation of the vasodilator cAMP and consequently inducing vasoconstriction (176). Further, the early event of AT1 receptor signaling is also associated with stimulation of non-receptor tyrosine kinase phosphorylation including JAK, Src family kinase, Ca^{+2}-dependent and redox sensitive tyrosine kinases (*e.g.* proline-rich tyrosine kinase, PYK2), focal adhesion kinase (FAK), p130Cas, PI3K and MAPK. These processes are linked to mitogenic-and inflammatory-like characteristics of Ang II (176).

Ang II rapidly activates c-Src in VSMC. Src plays a pivotal role in AT1 receptor-induced activation of PLC-γ and IP3 formation. Src has also been associated with AT1-induced stimulation of PYK2 and extracellar signal regulated kinase (ERK) as well as activation of other downstream proteins including JAK2, STAT1 and adaptor protein, Shc (239). Studies on VSMC from human resistance arteries suggested an important role of Src in the regulation of Ang II-induced Ca^{+2} mobilization (240). Further, the studies of Sayeski et al have illustrated that c-Src activation is required for Ang II-induced activation of p130Cas which is important in cytoskeletal reorganization, focal adhesion formation and cell migration (241).

Similar to classical cytokine receptors, AT1 receptors also activate JAK2 and TYK2, member of the JAK family in VSMC, cardiac myocytes, and renal mesangial cells, which may partly explain the cytokine-like actions of Ang II in mediating cardiovascular remodeling (242). Mechanically, AT1 receptor-derived messengers (Ca^{+2}, DAG, PKCδ) signal to JAK2 through PYK2 in VSMC. The JAK2 phosphorylation in response to Ang II is also associated with Gq/PLC pathways (243). In other studies in RASM cells, Schieffer et al proposed a distinct pathway for Ang II-induced JAK2 activation. Ang II-stimulation of the AT1 receptors increases JAK2 activity via Gβ-subunit association (244). The AT1 receptor activated by Ang II induces rapid tyrosine phosphorylation of JAK2 and subsequently the phosphorylation of its substrates STAT proteins, which are transcription factors and are involved in mRNA expression of early growth response genes such as c-fos, c-jun and c-myc (245). Using electroporation techniques with anti-STAT proteins, Marrero et al demonstrated an essential role of STAT proteins in Ang II-induced cell proliferation, but not to other growth factors (245). Further evidence has revealed that NADPH oxidase-generated O_2^- is required for AT1 receptor activation of the JAK/STAT cascade participating in Ang II-induced inflammation in RASM cells. This study implies additional functional roles of the Ang II-stimulated JAK/STAT signaling pathway (246).

Ang II promotes cell migration and changes in cell shape and volume in FAK-dependent signaling pathways (247). It has been shown that Ang II-activated FAK results in its translocation to sites of focal adhesion with extracelluar matrix and phosphorylation of paxillin and talin, which may be involved in the regulation of cell morphology and movement (248). Previous studies have reported that Ang II /AT1 receptor-mediated stimulation of PYK2 (also called FAK2) is implicated in the regulation of ion channel, cell adhesion, motility as well as mitogenic and hypertrophic reactions (248).This PYK2 activation was associated with Fyn (Src family member). Alternatively, other studies have demonstrated that PYK2 accounts for Ang II-induced

EGFR transactivation, leading to stimulation of ERK cascade (Ras/Raf/MEK/ERK). AT1 receptors activate tyrosine phosphorylation of PYK2 which increase c-Src phosphorylation. PYK2 associated c-Src activation induces the direct recruitment of Grb2/Sos complex for ERK activation (249). In endothelial cells, Ang II-induced tyrosine phosphorylation of PYK2 was regulated by a Yes tyrosine kinase (Src family member) and a tyrosine phosphatase SHP-1 (250). The study by Tang et al has emphasized that PYK2 functions as an important mediator in protein tyrosine kinase-mediated contractility when stimulated with Ang II in rat VSMC (250).

PI3K, characteristically associated with tyrosine kinase receptors, influences cell survival, metabolism, cytoskeletal reorganization and membrane trafficking (251). In VSMC, Ang II has been shown to stimulate activity, phosphorylation and migration of PI3K by AT1 receptors, leading to DNA synthesis and proliferation (252, 253). PI3K inhibition by wortmannin and LY 294002 abolished Ang II-stimulated hyperplasia in cultured rat VSMC, implicating a regulatory role in VSMC growth. Further, Akt/PKB has been identified as a PI3K downstream target in Ang II-activated VSMC (253). However, the cellular mechanisms whereby the AT1 receptor mediates activation of PI3K-dependent Akt/PKB remain unclear. Further elucidation of the mechanisms of AT1 receptor-mediated PI3K activation in hypertrophy of mesangial cells has revealed arachidonic acid/redox-dependent and PI3K-independent pathways, suggesting that reactive oxygen species (ROS), a critical factor in the development of cardiovascular diseases, may be an important component of Ang II-mediated growth (254). A very recent study has pointed out that Akt/PKB is necessary for Ang II-AT1-induced hyperplastic actions and this effect is mediated by a Gq protein-dependent Ca^{+2}-dependent pathway in RASM cells (255). In addition, Ang II-activation of PI3K/Akt has also been implicated in the prevention of cell apoptosis through inhibition of caspases (253). Despite the mitogenic effect of PI3K in Ang II target tissues, the exact role of PI3K in Ang II signaling has not yet been established.

Ang II differentially activates the major members of the MAPK family, ERK1/2, c-Jun N-terminal protein kinase (JNK) and p38 MAPK at various intracellular levels via AT1 receptor mediation (256, 257). Ang II-stimulated ERK1/2 is associated with increased expression of the early response genes like c-fos, c-myc and c-jun involved in DNA synthesis, cell growth and differentiation and cytoskeletal organization(258). Src, Ras-protein kinas C-ζ (PKC-ζ) and PI3K have been identified as upstream linker molecules of ERK1/2 stimulation (258). In addition to these upstream modulators, Ang II-induced oxygen radicals have been shown to induce phosphorylation of ERK1/2, which is responsible for enhanced $p27^{kip1}$ protein (an inhibitor of Gi phase cyclin-dependent kinase) expression and cellular hypertrophy, implicating an essential role of reactive oxygen species (ROS) in ERK phosphorylation in response to Ang II in renal proximal tubular cells (259). In addition to ERK activation, Ang II activates JNK/Stress activated protein kinase (SAPK), which control VSMC growth by promoting apoptosis or inhibiting mitogenesis via p21-activated kinase (αPAK) (260). Following phosphorylation, JNK1/2 translocate to the nucleus to activate transcription factors such as c-Jun and activating transcription factor 2 (ATF-2). It appears that Ang II exerts growth-facilitative effects via ERK1/2 activation as well as growth-inhibitory effects mediated via JNK/SAPK stimulation (261). Ang II also phosphorylates vascular p38 MAPK, which is associated with inflammatory responses, apoptosis, arterial remodeling in hypertension and cellular stresses including heat and osmotic shock (262). A recent study has observed that Ang II-induced p38 MAPK activation along with ERK1/2 activation is required for hypertrophic action mediated by AT1 receptors in VSMC (262). Ang II-induced ROS also play a role as second messenger in this signal cascade (262).

Significantly, it has become apparent that mitogenic responses to the AT1 receptor are mediated by activation of receptor tyrosine kinase (RTK), even though it does not directly bind to

RTK (261). Two major possible mechanisms of Ang II-induced EGFR and/or PDGFR transactivation have been proposed; 1) tyrosine kinase (c-Src/PYK2) or JAK mediation and 2) ROS mediation. Ang II-induced EGFR transactivation has been shown to require Ca^{+2}, Ca^{+2}-sensitive tyrosine kinases (PYK2 and Src) and linker proteins such as Grb2/Sos complex, which promotes tyrosine receptor dimerization and, subsequently ERK activation, ultimately leading to VSMC hyperplasia (249). EGFR transactivation also participates in the signaling pathway of Ang II-stimulated c-fos gene expression, DNA synthesis, and protein synthesis (263). Previous studies have provided strong evidence of a key role for ROS (e.g. H_2O_2) in ERK1/2 and p38 MAPK activation by Ang II and growth factors (264). Similarly, other studies have demonstrated that ROS mediate EGF-induced phosphorylation of its receptor as well as PDGF stimulation of STAT tyrosine phosphorylation (265, 266). Further study provided direct evidence that the transactivation of PDGFR by Ang II required activation of RAS as a second messenger (267). Alternatively, the studies from Prenzel et al have demonstrated another mechanism for cross-talk between GPCR and RTK. Requirement of a metalloproteinase mediated cleavage of pro-heparin binding (HB)-EGF (a ligand of EGFR) for GRCR-induced EGFR transactivation has been reported. Further, metalloproteinase was activated by Ca^{+2} or PKC leading to formation of active HB-EGF, suggesting a plausible mechanism of Ang II-induced transactivation mediated by metalloproteinase (268). A similar process was found in insulin-like growth factor 1(IGF-1) transactivation with Ang II in VSMC (269). However, the role of this mechanism in RTK transactivation by Ang II is still unclear as some studies failed to observe inhibition of Ang II-mediated EGFR transactivation by metalloproteinase inhibitor (261). Like EGFR transactivation, Ang II-transactivated PDGFR has been reported. PDGFR transactivation by Ang II has been shown to be a Ca^{+2} independent and redox-sensitive mechanism (261). However, the exact mechanism by which ROS mediated this mechanism is still unknown. Collectively, the AT1

receptor-induced signal transduction cascade is mainly mediated through phospholipids, non-receptor tyrosine kinase phosphorylation and receptor tyrosine kinase transactivation, leading to cell proliferation, growth and vasoconstriction in a variety of cells.

1.5.3 Signaling Mechanisms of AT2 Receptors

Although the regulation of AT2 receptor is poorly understood, AT2 receptor expression may be controlled at the transcription, post-transcription (mRNA stability) and translation levels (176). AT2 receptor expression is upregulated by sodium depletion and is suppressed by Ang II and growth factors such as PDGF and EGF. Interestingly, growth factors like insulin and IGF upregulate AT2 receptor expression. Currently, much less is known about the intracellular mechanism of AT2 receptors. However, recent studies have demonstrated that its signaling pathways are associated with serine and tyrosine phosphatases, NO, PLA_2 (prostaglandins) and cGMP (176).

Current evidence indicates that AT2 receptor stimulation leads to activation of various phosphatases, especially serine/threonine phosphatase 2A (PP2A), protein tyrosine phosphatase (PTP), MAP kinase phosphatase1 (MKP-1) and SH2-domain-containing phosphatase 1 (SHP-1), resulting in the inactivation of MAPK, especially ERK1/2 and consequently inhibition of growth in different types of cells (176). Earlier studies have demonstrated that AT2 receptor stimulation antagonizes the growth-stimulatory effect via AT1 receptors. AT2 receptor activation in cultured rat neonatal cardiomyocytes and fibroblasts inhibited AT1 receptor-dependent growth (270, 271). Further reports have elucidated that AT2 receptor activation inhibited growth factor-induced ERK1/2 activities through dephosphorylation of serine/threonine phosphate by PP2A or MKP-1 in cultured rat neurons. The activation of PP2A by AT2 receptors has been shown to be associated with $G_{\alpha i}$ proteins. These findings suggest that AT2 receptor activation involves antiproliferative/antigrowth effects (272). Consistently, growth-inhibitory effects by AT2 receptor

activation have also been reported in cultured coronary endothelial cells, VSMC and PC12W cells (176). In addition to AT2 receptor-mediated inactivation of ERK1/2 via PP2A, an alternative tyrosine kinase phosphatase-dependent pathway was observed in growth-suppressing effects of AT2 receptor activation. Growth factor-induced ERK1/2 activities were attenuated by AT2-induced tyrosine kinase phosphatase, SHP-1. The inhibitory effect was mediated by non-Gi/Gq pathways (273). Interestingly, the study by Dulin et al provided evidence that AT2 receptors increased ERK1/2 phosphorylation by PLA_2-dependent release of Arachidonic acid (AA) and cytochrome P450-dependent production of epoxy derivatives of AA in renal proximal tubule epithelial cells (274). Additional evidence of AT2-dependent MAPK cascade activation mediated by AA was demonstrated in kidney epithelial cells. Ang II/AT2's activation of $p21^{ras}$ has been shown to be mediated by the Shc/Grb2/Sos complex and AA(275). The MAPK cascade activation by Ang II in the kidney has been suggested to be responsible for the AT2-induced natriuretic effect. Further, AT2 receptor-stimulatory effects on differentiation were reported in neuronal NG108-5 cells, PC12W cells and VSMC. Neuronal differentiation by Ang II/AT2 receptors was mediated by a decrease in $p21^{ras}$ activity, together with an increase in MAPK activity (276). A subsequent study from the same group has also reported that AT2-mediated neuronal differentiation occurs through an increase in NO production(277). These findings were confirmed by another study on VEGF-induced endothelial cell differentiation. They found that the MAPK cascade was activated the by NO/cGMP pathway (278). Very recently, the same group of researchers has examined whether Rap1 (a small guanine nucleotide-binding protein of the Ras family)/B-Raf (a neuron-specific member of the Raf family of kinases) may involve Ang II-induced MAPK activities participating in the morphological differentiation of NG108-15 cells. They found that the AT2 receptors of Ang II induce differentiation of NG108-15 cells in cAMP-independent and Rap1/B-Raf-dependent manners (279). An AT2 receptor-mediated proapoptotic

effect has been observed in R3T3, VSMC and PC12W cells. The mechanism by which AT2 receptor promotes apoptosis has been demonstrated to be associated with ERK inactivation mediated by bcl-2 dephosphorylation via MKP-1 and SHP-1 activation (280). Recently, ceramides, which are possible caspase 3 and phosphatase activators have been proposed to be mediators in AT2 receptor-stimulated apoptosis (281). Like AT1 transactivation with RTK, a recent study has shown that AT2 activation cross-talks with the insulin signal cascade and thus induces apoptosis in Ang II-responsive cells (176).

In addition to AT2 receptor's apoptotic effect, AT2 receptor activation influences neuronal cell activity via modulating ion channel activity. Kang et al showed that AT2 receptor stimulation increased neuronal delayed rectifier K^+ current through Gi protein and PP2A activation (282). In non-differentiated NG 108-15 cells, AT2 receptors mediate inhibition of T-type Ca^{+2} currents via activation of a tyrosine phosphatase (283). Neuronal membrane ionic current changes by AT2 receptors may result in hyperpolarization of membranes that suppress cellular activities stimulated by depolarization.

It has been shown that AT2 receptors activate PLA_2 and stimulate AA and prostaglandin generation, which are linked to AT2 receptor-induced vasoactive effects. The study by Kohout et al has demonstrated that the AT2 receptor-mediated release of arachidonic acid upregulates ion transporter system (Na^+/H^+ exchanger and Na^+/HCO^- symporter system), which influences pH in cardiac cells leading to heart contractility (284). Recently, AA has been documented as a second messenger in AT2 receptor-induced activation of $p21^{Ras}$ in renal tubule epithelial cells (275). AT2 receptor-dependent production ofPGI2 was reported in differentiated adipocytes Ob 1771 in culture. PGI2 facilitates differentiation of undifferentiated Ob1771 cells in a coculture system by a paracrine-dependent mechanism. This action was blocked by PD1231771, but not the AT1 receptor antagonist losartan, suggesting that prostacyclin production is dependent on AT2

receptor activation (285). However, the mechanism involved in this signaling pathway remains to be defined. In the kidney, the AT2 receptor has been shown to stimulate the conversion of renal PGE2 to PGF2α and subsequently increase the production of vasodilator prostanoids, contributing to its vasodilatory actions (176).

Moreover, AT2 receptor was subsequently shown to mediate vasodilation via stimulation of the BK-NO-cGMP pathway in the vasculature and kidney. In aortic VSMC, the AT2 receptor causes intracellular acidosis by inhibition of an amiloride-sensitive epithelial sodium channel, thus activating kininogenase, which cleaves BK from intracellularly stored kininogen. The AT2 receptor-induced BK release triggers the formation NO/cGMP in a paracrine manner to promote vasodilation and natriuresis thus leading to antihypertensive effects (286, 287). Collectively, the AT2 receptors exert an anti-AT1 receptor effect in VSMC, PC12W cells and neuronal cells. In response to Ang II, AT2 receptor-induced antigrowth, vasorelaxation, sodium excretion, differentiation and apoptosis are mediated by inhibition of AT1 receptor or RTK-mediated MAPK via activation of various phosphatases (protein dephosphorylation), stimulation of BK-NO-cGMP pathway and release of prostaglandins in G protein-dependent and-independent manners.

1.5.4 Signaling Mechanisms of AT4 Receptors

AT4 receptor is new Ang-receptor type that has recently been discovered and characterized that preferentially binds to Ang IV. AT4 receptor stimulation by Ang IV is suggested to mediate both peripheral and central actions. To date, intracellular signaling mechanisms of AT4 receptors remain largely unknown. Peripherally, Ang IV has been shown to elicit a vasodilatory effect by increasing renal blood and flow and natriuresis in an AT4 receptor-dependent manner. In a related study, Patel et al have noted that the vasodilatory effects of AT4 receptors in response to Ang IV may be attributed to Ang II-induced NO effects (288). The study

by Handa et al has found that Ang IV acts on tubular epithelium to inhibit sodium transport via a decrease in Na^+-K^+ ATPase activity without changes of mean arterial pressure (MAP), GFR and urine volume, thereby contributing to natriuretic action of AT4 receptors in the kidney (289). Additionally, the activation of the renal AT4 receptor has been reported to result in increased PAI-1 expression in proximal tubule cells and an increase in intracellular calcium signaling activity in mesangial, proximal tubule and distal/collecting duct cells. Further, AT4 receptor activation has been shown to inhibit DNA and protein synthesis in chick cardiocytes, thus blocking hypertrophy of cardiac cells (176). Interestingly, in the brain, Ang IV/AT4 receptor stimulation has been demonstrated to activate *c-fos* expression and facilitate learning and memory processes (176).

1.5.5 Cross-talk between Ang II and Insulin Signaling Systems

1.5.5.1 Overview

As mentioned above, Ang II and Ang II-derived biologically active products of RAS have a vast range of actions throughout the body. Importantly, the AT1 or AT2 receptor activations in response to Ang II have been documented to negatively or positively cross-talk RTK signaling in a variety of cells, thus contributing to Ang II's divergent effects related to health and cardiovascular diseases (176). Several lines of evidence have demonstrated cross-talk between Ang II and insulin signaling systems, emphasizing an association between hypertension and insulin resistance which are risk factors for cardiovascular diseases (290, 291). Epidemiological studies have shown that hypertensive individuals are more likely to become diabetic than normotensive ones (292).

Additionally, it has been suggested that pharmacological inhibition of RAS, as with ACE inhibitors and AT1 receptor antagonists, has beneficial effects on both hypertension and insulin resistance (293). This suggests a role for RAS in the regulation of insulin action, even though

results of experimental and clinical studies on the effect of AT1 receptor antagonists on insulin sensitivity have been conflicting. Some studies show no influence on insulin sensitivity and others demonstrate an improvement of insulin sensitivity (293, 294). In support of positive effects of AT1 antagonists on insulin sensitivity, Umeda et al demonstrated that AT1 receptor antagonists improve insulin sensitivity in hypertensive animals with insulin resistance. However, the mechanism underlying this effect is unknown (295). Recently, Sharma et al proposed a mechanism for prevention of diabetes by RAS blockade. Inhibition of RAS promotes recruitment of preadipocytes which are sensitive to insulin because Ang II has been shown to act as an antiadipogenic substance in human adipose tissue. This induces redistribution of lipids from muscle and other tissue to adipose tissue, subsequently resulting in improved insulin sensitivity (296). Additionally, Henriksen et al have reported that AT1 receptor antagonism improves insulin resistance due to the effect on skeletal-muscle glucose uptake associated with an increase in GLUT4 protein expression (297).

On the other hand, it has been observed that hyperinsulinemia and insulin resistance participate in the pathogenesis of hypertension (298). In this context, it has been described that hyperinsulinemia is closely associated with reduced renal sodium excretion and stimulation of the SNS (299). Insulin has direct effects on blood vessels modulating vascular tone via alterations in NO and cytosolic Ca^{+2} levels (300). The growth promoting effects of insulin have been proposed to be the most critical factors in increased cardiovascular risk (301). The different forms of insulin like IGF and proinsulin have also been shown to be involved, along with insulin, in vascular growth stimulation and atherosclerosis (301). Further, insulin activates the vascular RAS via stimulation of agt or Ang II production and upregulation of AT1 receptor expression, thus resulting in enhanced cell growth of VSMC on stimulation with Ang II. These results provide evidence that the action of insulin on blood vessels, especially its effects on cell growth, is

mediated through activation of RAS components (302, 303).Very recently, Harte et al showed that insulin increases agt expression and production in human abdominal subcutaneous adipocytes in a dose-dependent manner, suggesting that hyperinsulinemia in obesity may be a contributing factor in obesity-associated hypertension (304).

Consequently, potential interplays between insulin and the RAS (e.g. combined effects of insulin and the RAS) may account for the pathogenesis of hypertension/atherosclerosis and insulin resistance/diabetes.

1.5.5.2 Insulin Signaling Systems

Insulin is a key hormone implicated in wide biological effects on metabolism, cellular growth and differentiation. Insulin stimulates the synthesis and storage of carbohydrates, lipids and proteins in insulin-sensitive tissues such as fat, liver and muscle by promoting glycogen and protein synthesis and lipogenesis while inhibiting glycogenolysis, protein breakdown and lipolysis (251). Insulin resistance or deficiency is a defect in the response of muscle, adipose tissue and liver to insulin-mediated glucose transport, production of metabolism, and storage, thereby resulting in elevations in fasting and postprandial glucose and lipid levels. Insulin resistance is also linked to other common health problems such as obesity, diabetes, hyperlipidemia, hypertension and atherosclerosis. The pathophysiology of insulin resistance may be ascribed to impaired insulin signaling including insulin binding to its receptor, receptor phosphorylation and kinase activity, phosphorylation of IRS, activation of PI3K and subsequent down-regulation of GLUT4 (major insulin-responsive glucose transporter) (251).

Binding of insulin to the extracellular α-subunit of the IR leads to IR autophosphorylation and subsequently tyrosine phosphorylation of IRSs. Phosphorylated IRS-1 and IRS-2 act in turn as docking proteins, recruiting and activating divergent signaling molecules, including those in the Ras-Raf-MAPK and PI3K pathways. The PI3-K pathway triggers several

cellular responses such as 1) glucose transport 2) glycogen synthesis 3) protein synthesis 4) antiapoptosis and 5) gene expression while the Ras-Raf-MAPK pathway is important for mitogenic effects of insulin (Fig 4) (251).

To date, two signaling pathways for insulin-stimulated glucose transport have been proposed in most insulin-responsive cells: the classic-PI3K-dependent pathway and the proposed PI3K-independent, alternative pathway (251). In classic PI3K-dependent pathway, the activated IR catalyzes IRS tyrosine phosphorylation, leading to recruitment of downstream effectors, the most important for glucose transport being PI3K. Akt/PKB
and the atypical PKC isoforms have been both implicated as PI3K-induced PtdIns $(3,4,5)P_3$ effectors involved in GLUT4 translocation (251). In a parallel pathway, the Cbl-associated protein (CAP)-Cas B-lineage lymphoma (Cbl) dimeric complex is recruited to the IR, and Cbl is also tyrosine phosphorylated by IR kinase. The CAP-Cbl complex is recruited to lipid raft domains in the plasma membrane, and phosphorylated Cbl assembles a signaling complex consisting of the scaffolding protein CrkII, the guanine nucleotide exchange factor C3G, and the small GTP-binding protein TC10. Activation of TC10 is proposed to mediate insulin-stimulated actin rearrangement which seems to be important in GLUT4 translocation to the plasma membrane (251) (Fig 5).

1.5.5.3 Interactions between Ang II and Insulin Signaling Systems

In view of the pivotal association between insulin resistance, hypertension and cardiovascular disease, several groups have explored the interactions between Ang II and insulin-signal transduction using both *in vivo* and *in vitro* systems (305-310). Several lines of evidence have revealed that positive or negative cross-talk between Ang II and insulin signaling occur at several different levels of these two hormone response pathways in muscle and heart tissues.

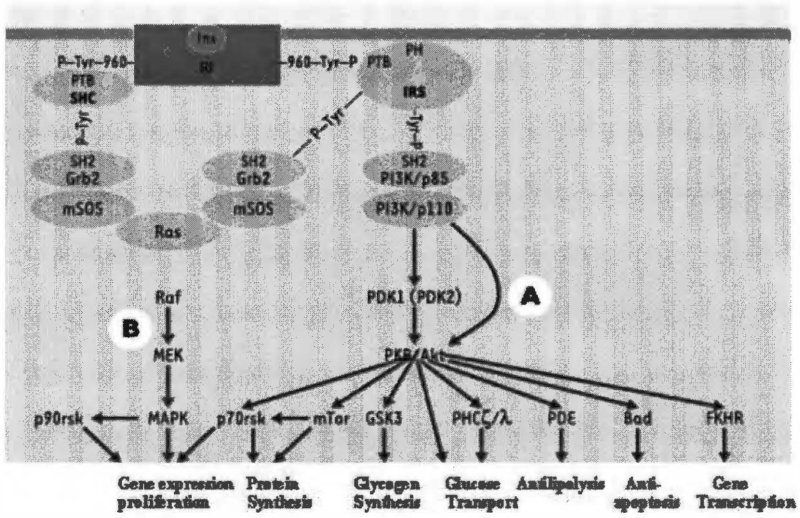

Fig. 4 ***Multifunctional actions of insulin signal transduction.*** Insulin is the most potent anabolic agent known and exerts its numerous effects on insulin-responsive cells through two main signaling pathways, A (PI3K pathway) and B (MAPK pathway). Two signaling pathways regulate diverse functions including metabolic activity, growth, and differentiation (251).

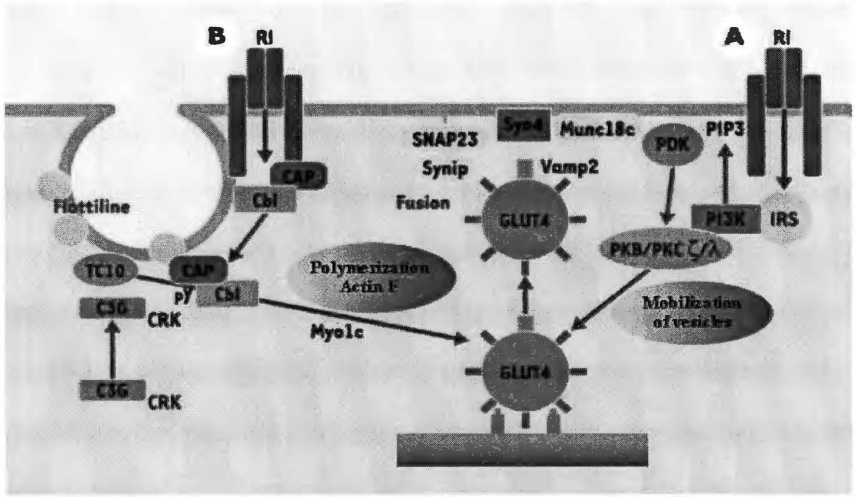

Fig. 5 ***The mechanisms of insulin-stimulated glucose transport in insulin-sensitive tissues such as muscle and adipose tissue.*** It is likely that at least two signaling pathways (e.g. classic PI3K-dependent pathway (A) and alternative-PI3K-independent pathway (B)) are required for the stimulation of Glut 4 translocation in response to insulin (251).

An earlier *in vivo* study from Saad et al. has shown that Ang II stimulates IRS and its associated PI3K activations in rat heart *in vivo*. This effect occurred at 1min after Ang II infusion and was mediated in an AT1 receptor-dependent manner (311). In a related study, Du et al. examined cross-talk between G-protein coupled and tyrosine kinase receptors in RASM cells. Consistent with previous findings, western blot analysis showed that Ang II (100nM, 5mins) caused a marked increase in IRS-1 and IGF IRβ chain tyrosine phosphorylation (269). Both studies have suggested additional Ang II signaling pathways which may interact with insulin signaling pathways. The study by Ali et al. has illustrated that protein tyrosine phosphatase 1D (PTP-1D) is a second mediator in the interaction of Ang II with insulin. The VSMC stimulation by Ang II was shown to induce tyrosine phosphorylation of IRS-1, PTP-1D and complex formation of PTP-1D with IRS-1 docking protein (312). Conversely, the study by Velloso et al has suggested potential mechanisms for negative relationship between Ang II and insulin signaling systems in rat heart. They found that Ang II stimulated IRS-1 and IRS-2 phosphorylation and IRS-1/p85 and pl 10α associations but inhibited PI3K enzyme activity, along with increased JAK2 phosphorylation in an AT1 receptor-mediated pathway. In this study, JAK2 may account for Ang-induced IRS-1/2 activations. In spite of the fact that Ang II treatment stimulates IRS activation and IRS-1/PI3K docking, it has been shown that Ang II attenuates insulin-induced PI3K activation via Ang II-induced serine phosphorylation of p85, thus inhibiting insulin signaling pathways (305). Additional mechanisms for inhibitory effects of Ang II on insulin signaling were proposed by Foli et al. They demonstrated that Ang II suppressed insulin signaling in VSMC at multiple steps (e.g. Ang II-induced IRβ, IRS-1 and p85 serine phosphorylation), suggesting that Ang II may contribute to insulin resistance in the vasculature (307). Subsequently, *in vivo* and *in vitro* studies from the same group have strongly supported their previous findings that Ang II negatively regulates insulin signaling via the PI3K pathway although Ang II exhibits opposing effects on IR

and IRS phosphorylation. These effects also were mediated via the AT1 receptors but not AT2 receptors in intact rat heart (306). In addition to acute effects of Ang II on insulin signaling, Ogihara et al have recently investigated the chronic *in vivo* effects (12-day infusion of Ang II) of Ang II on insulin signaling in insulin-sensitive tissues and the role of ROS in these effects (308). Chronic Ang II infusion into normal rats induced hypertension and insulin resistance. This Ang II action was mediated by activation of early insulin-signaling steps (IR/IRS/PI3K pathway) in fat, liver and muscle tissues which contrasts with earlier findings, suggesting that the sites where Ang II impairs the insulin signal pathway are possibly located in downstream from PI3K. Treatment with tempol (a stable spin trap of superoxide, ROS inhibitor) in these mice was shown to improve insulin resistance seen in Ang II-infused rats via increasing glucose uptake in soleus muscle tissue. This indicates that ROS may plausibly inhibit the downstream pathways of PI3K activation and thus mediate Ang II-induced insulin resistance (308). In addition to ROS-mediated negative interactions of Ang II with insulin, Fukuda et al demonstrated that Ang II impaired insulin signaling in VSMC from SHR in a MAPK/AT1 receptor-dependent manners (309). Likewise, in a very recent study, Carvalheira et al. examined whether Ang II directly interacts with insulin signaling through MAPK and PI3K pathways in cardiac tissues of control and obese Zucker rats. The results have revealed that Ang II elicits an inhibitory effect on insulin signaling at the level of PI3K/Akt pathways via AT1 receptor activation while Ang II has stimulatory effects on insulin signaling at the level of IRS/Grb2 association and ERK1/2 activations in an AT1 receptor-independent manner in hearts of obese Zucker rats. These findings suggest that imbalanced interactions of Ang II and insulin signaling may play roles in development of cardiovascular abnormalities observed in insulin resistant states, as in obese Zucker rats (310) (Fig. 6).

Furthermore, cross-talk of AT2 receptors with the insulin signaling pathways in regulating cell growth and proliferation has been reported recently. AT2 receptor activation

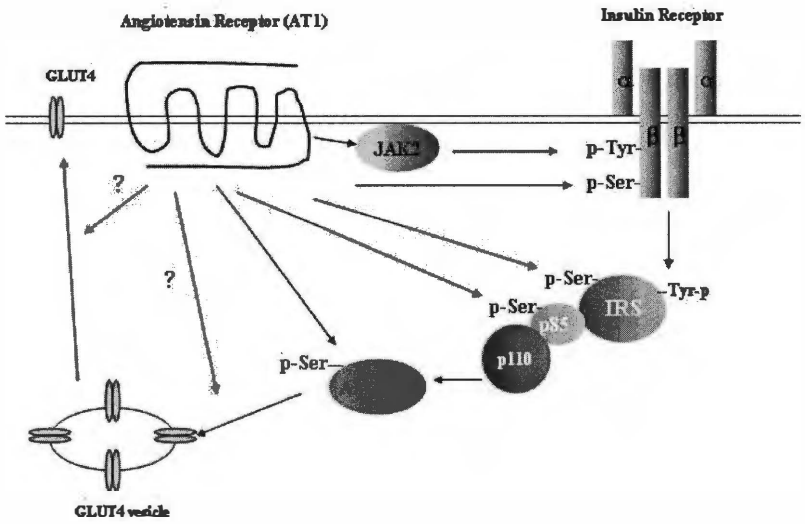

Fig. 6 ***Proposed interactions between Ang II and insulin signaling transduction.***

inhibited insulin-induced ERK2 activity and cell growth via impairment in the early event of insulin signaling pathways, implying a possible role of AT2 receptor in insulin-sensitive tissues and/or pathology of proliferative diseases(313). In a related study, AT2 receptor-induced apoptosis was mediated by negative cross-talk with insulin-induced PI3K/Akt pathways and activation of protein tyrosine phosphatase (SHP-1) in PC12W cells where AT2 receptors are predominantly expressed. Thus, Ang II displays apoptotic effects through previously reported MAPK inactivation as well as functional trans-inactivation of insulin signaling pathways in an AT2 receptor-dependent manner (314)

Altogether, Ang II plays a critical role in hypertension-associated insulin resistance, in part by its ability to differentially modulate insulin signaling pathways. However, the mechanisms that link Ang II-dependent hypertension with insulin resistance have not been fully elucidated.

1.6 Genetic Manipulation of Systemic RAS

Transgenic mice are created to overexpress a protein of interest either systemically or a cell specifically via random integration of the transgenic gene construct into the genome. In contrast, the inactivation of a target gene in knockout mice generally is accomplished by inserting a region of nonsense DNA, typically a selectable marker encoding antibiotic resistance, via homologous recombination into the target gene (315). Depending on the targeting constructs'design, the mutagenic process can result in transcription inhibition of the target gene or the generation of a transcript encoding a defective protein. Recently, the use of the cre-loxP recombinase system has provided a tool to induce the knockout of a gene in a specific tissue and under temporal control (315, 316).

Many factors contribute to the development of essential hypertension, including environment, diet, daily stress and genetics. Although several single gene disorders causing high blood pressure have been identified, the genetics of essential hypertension are much more

complicated. The transgenic and knockout strategies have been useful tools to evaluate the genetics of essential hypertension and to identify pathways regulating blood pressure(317).

1.6.1 Knockout Animal Models (Table 5)

As mentioned previously, apart from the classic action of the RAS in the control of arterial pressure, RAS is a key player in the regulation of kidney function and structure under basal conditions and in response to various hormonal and environment factors; RAS regulates GFR, in part through the tubuloglomerular feedback mechanism and its direct constrictor actions on renal arterioles, as well as is involved in mature formation of the papilla and glomerular structure, thereby increasing proximal tubular sodium reabsorption. The pivotal roles of RAS in the kidney function were also supported by the results from RAS knockout studies (187). Subsequent studies on tissue specific RAS knockout via the use of cre-loxP recombinases have confirmed essential roles of RAS in cardiac and renal development via its growth-promoting effects. Of interest, the significance of RAS in brain structure and blood brain barrier (BBB) function has been suggested. Accordingly, genetic manipulation of major components of RAS genes in a tissue-specific manner has provided new insights into additional physiological functions of the tissue RAS (316, 318).

1.6.1.1 Angiotensinogen (agt)

The agt encodes the major substrate for the RAS. A critical role for the RAS in renal development has been described (319, 320). Surprisingly, the results from the reports on agt inactivation were that mice appear normal at birth and have normal organs including the kidneys, indicating that the RAS is not essential for embryonic development (321, 322).

However, their survival in the perinatal period is reduced. Further, surviving adult $Agt^{-/-}$ mice have marked renal structural and vascular abnormalities. These vascular changes were not seen in other organs (322). Agt knockout mice elicited loss of GFR autoregulation in response to

Table 5. ***Genetic manipulation of RAS (Knockout mouse models)***

	Genotype	***Characteristics of Phenotype***
Monogenic knockout models	Agt $^{-/-}$	1. Postnatal mortality 2. Hypotension 3. Renal structural abnormalities 4 Renal dysfunction 5.decreased body weight 6.Reduced fat mass 7. Increased locomotor activity 8.Resistance to high-fat induced obesity
	Ren1d $^{-/-}$	1. Altered kidney morphology 2. Sexually dimorphic hypotension
	Ren 1c $^{-/-}$	1. Low blood pressure 2. Marked renal structural abnormality
	Ren 2$^{-/-}$	1. Normal blood pressure 2 Normal kidney morphology
	ACE$^{-/+}$ (a single copy deletion)	1. Normal blood pressure
	ACE 1 (both sACE and tACE null)	1. Hypotension 2. Renal structural abnormalities 3. Impaired renal function 4. Male infertility 5.Reduced hematocrit and hemoglobin
	ACE2$^{-/-}$	1. Normal blood pressure 2. Normal kidney function 3.impaired cardiac function
	Agtr 1a$^{-/-}$	1. Moderated hypotension 2. Normal cardiac and vascular development 3. Reduced ischemia-induced angiogenesis 4. Reduced Ang II-induced remodeling
	Agtr 1 b$^{-/-}$	1. Reduced blood pressure 2. Reduced dipsogenic effect of Ang II 3. Decreased response to epinephrine
	Agtr 2$^{-/-}$	1. Slight increased blood pressure 2. Hypersensitivity to Ang II-mediated vasoconstriction 3.Normal renal function 4. Behavioral changes (decreased spontaneous movement and impaired drinking response) 5. Decreased cardiac hypertrophy
Double knockout models	Agtr1a$^{-/-}$/Agtr1b$^{-/-}$	1.Smilar to phenotypes of Agt and Ace deficient mice
	B2$^{-/-}$/ACE$^{-/-}$	1. Hypertension 2. increased sensitive to Ang II
	ACE$^{-/-}$/ACE2$^{-/-}$	1.Similar to phenotypes of ACE4 mice
Tissue-specific knockout models	Agt$^{-/-}$(Liver-specific deletion of Agt)	1. No pressor response
	ACE2$^{-/-}$(Tissue null)	1. Hypotension 2. Abnormal renal structure 3. Reduced hemoglobin level and hematocrit

changes in sodium intake and hypoplastic renal papilla, inability in preservation of glomerular size in response to extracellular fluid changes and arterial wall thickening. Several lines of evidence have demonstrated abnormalities in the regulation of renal RAS genes and proteins of overt hypotensive $Agt^{-/-}$ mice (323, 324). Further, we and other illustrated additional characteristic phenotypes of agt knockout mice; reduced body weight, decreased fat mass, increased locomotor activity and resistance to high fat diet-induced obesity (325). In addition to renal dysfunction in these mice, the decreased density in granular layer cells of the hippocampus and an impaired blood brain barrier (BBB) function were reported in $Agt^{-/-}$ mice (326, 327). Numerous studies on agt null-mutant mice have confirmed that RAS is essential for normal blood pressure maintenance. In supporting of importance of systemic RAS in blood pressure regulation, studies on transgenic mice lacking only hepatic agt pointed out that extrahepatic-derived agt is not a significant source of the circulating pool of agt (328).

1.6.1.2 Renin

In contrast to most other species which only have a single renin gene (Ren-1^c), other strains such as 129 and DBA contain two renin genes (Ren-1^d and Ren-2). Ren 2-dificent mice showed normal blood pressure, plasma renin activity, and normal kidney morphology, perhaps due to compensation by the other remaining renin gene. Although Ren-1^d knockout mice exhibited altered kidney morphology, complete absence of juxtaglomerular cell granulation and sexually dimorphic hypotension, phenotypes observed in these mice were much less severe than those seen in agt and ace knockout mice (329, 330). Recently, mice completely lacking renin (Ren-1^c) have low blood pressures and marked renal structural abnormalities, similar to those seen in agt knockout mice, indicating a dependence of these phenotypes on the generation of Ang II. Interestingly, these knockout mice did not have the abnormal granular cell layer in the

hippocampus seen in agt knockout mice, suggesting that a non-renin enzyme (cathepsin D) is capable of producing angiotensin peptides in the brain of these animals (331).

1.6.1.3 ACE

There are two ACE isoforms which are encoded by the same genetic locus but are regulated by two different promoters: somatic ACE (sACE) and testis ACE (tACE). The majority of enzyme is bound to the tissue such as vascular endothelium. The tissue-bound form of the enzyme is cleaved to produce a soluble form of the enzyme that circulates in plasma. Deletion of a single copy the Ace gene have been demonstrated to have no effect on basal arterial pressure, suggesting that arterial pressure is not influenced by partial decreases in Ace gene expression (332). In contrast, the phenotype of $Ace^{-/-}$ mice lacking both sACE and tACE (termed ACE.1) is similar to that of $Agt^{-/-}$ mice, with prominent hypotension, vascular hyperplasia, atrophy of the renal papillae, and impaired renal function, male infertility (332-335). The sperm-specific expression of transgenic germinal Ace in $Ace^{-/-}$ male mice restored fertility without curing their other abnormalities (336). Using gene targeting, mice exhibiting one-third normal plasma ACE levels but no measurable ACE activity associated with tissues such as the lung or the kidney were generated (termed ACE.2). These mice have a truncated ACE protein that circulates in the plasma, hypotension and abnormally thickened renal arteriolar walls, similar to those of ACE.1 knockout mice. Interestingly, they did not develop characteristic atrophy of the renal papilla, suggesting the tissue and plasma forms of ACE differ in their contribution to the regulation of blood pressure and renal structure. Further, these studies strongly supported the concept that the tissue-bound ACE is essential for the control of blood pressure and the structure and function of the kidney. Both ACE.1 and ACE.2 knockout mice exhibited reduced hematocrit and hemoglobin levels, suggesting a direct role of the RAS in erythropoiesis. Recently, Cole group created new mice that selectively express ACE in the liver but lack all ACE within vasculature (termed ACE.3). These

mice were indistinguishable from their WT mice, suggesting that the circulating ACE in plasma presumably due to enzymatic release from hepatocytes can compensate for the absence of the endothelial ACE enzyme (337). Mice expressing ACE limited to the kidney were created by using a kidney androgen-regulated protein promoter (termed ACE.4). The mice displayed profound reduction of sACE but retained normal tACE expression. The phenotypes of these mice are nearly identical to those of the ACE.1 (null) mice: marked decreased blood pressure, impaired urine concentrating ability, renal morphological defects, and a decreased hematocrit (338). Additionally, double knockout mice lacking both bradykinin B_2 receptor (B_2R) and ACE also exhibited nearly identical phenotypes of ACE.4 mice, suggesting that the ACE inactivation develops the phenotypes independently of bradykinin (339).

Homologs of ACE have recently been described, one of these, ACE2 (also referred to as ACEH) shares more than 40 % homology at the protein level with the catalytic domains of ACE, and its expression appears to be limited to heart, kidney and testis (223, 340, 341). Studies on mice with targeted disruption of the ACE2 have demonstrated that ACE2 is a critical regulator of cardiac function. In comparison with Ace knockout mice, these mice displayed normal blood pressure and no altered kidney function (224). Of note, impaired cardiac function (*e.g.* left ventricular failure) concomitant with elevated plasma Ang II, was observed in these mice whereas neither Ace $^{-/-}$ nor Agt $^{-/-}$ null mice developed any overt heart disease. This congestive heart failure phenotype was rescued in double mutant mice that are deficient in both ACE and ACE2 (224). On the other hand, in the distinct lines of ACE2-deficient mice, different phenotypes have been reported (223). The differences in phenotypes observed have been explained by different genetic backgrounds or different assessments in each study.

1.6.1.4 Angiotensin receptors

The Ang II-induced vascular effect is primarily mediated by AT1 receptor. Homologous recombination has been used to generate mice lacking the type 1a receptor for Ang II (342-346). Agtr1a-deficient mice were moderately hypotensive but exhibited normal cardiac and vascular development. Their renal structure was intact except for hypertrophy of the juxtaglomerular apparatus and modest mesangial expansion. They lacked the atrophy of the renal inner medulla observed in Agt $^{-/-}$ and Ace$^{-/-}$ mice but had a renal concentration defect, impaired TGF response and a stepwise reduction in resting blood pressure associated with loss of Agtr-1a expression. This confirmed that the AT1a receptor plays an important role in blood pressure homeostasis. In addition, physiologic roles of AT1a receptor on cardiac growth have been investigated by loss-of-function studies (AT1a knockout mouse models). Of interest, pressure overload-induced cardiac hypertrophy was observed in mice lacking AT1a receptor, suggesting that AT1-mediated Ang II signaling is not essential for the development of pressure overload-induced cardiac growth *in vivo* (347, 348). Matsusaka et al. characterized the mode of action of Ang II in cardiac remodeling using Agtr-1a null mutant chimeras. They found that compared to Agtr-1a null mutant chimeras, control chimeras had more extensive cardiac fibrosis, most prominently in perivascular regions in repose to Ang II, indicating that Ang II is involved in cardiac remodeling through AT1 receptor signaling pathways (349). Further, Sasaki et al. provided evidence for regulatory roles of AT1a receptor in ischemia-induced angiogenesis. Agtr-1a knockout mice displayed decreased angiogenesis and fewer well-developed collateral vessels in response to hindlimb ischemia compared to WT mice. These homozygous mutant mice had decreased infiltration of inflammatory mononuclear cells in their ischemic tissue with concurrent reduced expression of MCP-1and VEGF genes (350).

Less was known about function of the AT1b receptor. Agtr-1b null mice have phenotypes indistinguishable from those of their WT mice (351, 352). Direct roles of AT1b in blood pressure control and vascular response to Ang II have been suggested by studies on Agtr-1a$^{-/-}$ animal models. Agtr-1a null mice displayed a modest pressor response to Ang II infusion. This response to Ang II was not affected by sympatholytic agents and chronic administration of losartan caused further reduction of systemic blood pressure, indicating that in the absence of the dominant angiotensin receptor (AT1a), AT1b could contribute to the regulation of blood pressure (344). Further, the function of the AT1b receptor in the brain was defined by examining the differential response to Ang II in Agtr1a$^{-/-}$ mice and Agtr1b$^{-/-}$ mice. The results from this study have shown that Ang II mediates its CNS effects on blood pressure control via mainly the AT1a receptor while the dipsogenic effect of Ang II is mediated by AT1b receptor (353). In contrast with the phenotypes seen in homozygous mutant mice of Agtr-1b, the homozygous, doubly mutant mice (Agtr1a$^{-/-}$; Agtr1b$^{-/-}$) showed virtually identical phenotypes to the Agt and Ace deficient mice, indicating that major biologic functions of endogenous Agt are mediated by the Agtr1a receptors. The mice have significantly reduced postnatal survival, reduced body weight gain, diminished growth, very low baseline blood pressure, renal vascular changes and marked hypoplasia of the renal papilla. Interesting features in the double knockout mice are that they responded normally to another vasoconstrictor, epinephrine, and following administration of an ACE inhibitor, their blood pressure increased paradoxically. This suggests that this may be a result of disruption of the AT2 receptor signaling pathway in theses mice (352, 354).

The AT2 receptor has served as a counter-regulator of the AT1 receptor. AT2 receptor-deficient mice displayed a slight elevation in resting blood pressure and hypersensitivity to Ang II-mediated vasoconstriction. Even though these animals exhibited no apparent abnormalities in either the kidney, the brain or the adrenal gland, they had behavioral changes with decreased

spontaneous movements and impaired drinking responses (355, 356). Recently, Gross et al. defined potential mechanism for elevated blood pressure seen in AT2 receptor knockout mice. They performed pressure-natriuresis-diuresis experiments. These mice exhibited rightward shift of pressure natriuresis, failure in medullary blood flow increase in response to the elevated renal perfusion pressure and increased renal vascular resistance along with an increment in renal AT1 receptor gene expression. These alterations in renal mechanisms lead to modification in renal sodium handling, thereby contributing to the development hypertension in these animals (357). With AT1 receptor, the role of the AT2 receptor in cardiac hypertrophy has also been studied. Of interest, recent reports using AT2 knockout mice have suggested that this receptor is necessary for hypertrophy induction by *in vivo* pressor overload or Ang II infusion. This effect was mediated by a reduced phosphorylation of $p70^{S6k}$, which plays a pivotal role in cardiac hypertrophy (358, 359).

1.6.2 Transgenic Animal Models (Table 6)

With other genetically manipulated animal models (gene targeting), the use of transgenic mouse models has become important tools to unravel the complex interplay of genetic and environmental influences causing essential hypertension in humans (316)

1.6.2.1 Angiotensinogen (agt)

Numerous studies in genetic animal models have provided several lines of evidence on a critical role of circulating agt in the pathogenesis of hypertension. In gene targeted mice, arterial pressure and plasma agt levels have been shown to vary proportionally with the number of functional copies of the mouse agt gene (321). Transgenic mice overexpressing rat Agt gene in liver and brain developed hypertension (360). The dependence of blood pressure on agt levels is analogous to what has been found in humans with increased circulating agt contents (361).

Table 6. ***Genetic manipulation of RAS (Transgenic mouse models)***

	Genotype	***Characteristics of Phenotype***
Overexpression Transgenic models	Agt (1-4 copies)	1. Increased 8mmHg per gene copy with plasma agt levels 35 % (1copy) of WT, 124% (3copy) of WT, 145% (4 copy) of WT
	Agt overexpression in liver and brain	1.Hypertension
	R^+/A^+ (human agt and renin overexpression in brain)	1. Chronic Hypertension 2. High Ang II plasma concentrations 3. Altered baroreflex activity 4. Cerebral arteriolar hypertrophy
	Agt overexpression in adipose tissue	1. Hypertension 2, Increased body weight 3. Increased fat mass 4 .Partially rescue abnormal renal function and structure.
	Cardiomyocyte overexpression Of AT1 receptor	1. Massive hyperplasia of arterial myocytes 2. Perinatal mortality
	Brain overexpression of AT1	1. Enhanced cardiovascular sensitivity 2. Normal blood pressure
	mRen-2 overexpression	1. Hypertension 2. Altered heart rate
	ACE (1-3 copies)	1.No changes in blood pressure
Tissue-specific transgenic models	Agt restoration in multi-tissue (MT-$Agt^{+/-}$/Agt-/-; MT-AGT/KO)	1.Partially rescue abnormal renal function and structure 2. Restored blood pressure
	Kidney -specific expression of Agt (using proximal tubule specific promoter)	1. Overproduction of renal Ang II production 2. Hypertension 3. Not able to rescue kidney abnormalities
	Adipose-specific expression of Agt	1. Increased systemic Ang II 2. Partially rescue abnormal renal function and structure 3. Restored blood pressure 4. Increased fat mass
	Brain-specific restoration of Ang II	1. Hypertension 2. Partially rescue abnormal renal function and development 3.Increased sympathetic nerve 4. Salt preference 5. Increased drinking intake
	$ACE3^{-/-}$(Liver- specific expression of ACE)	1. Normal
	$ACE4^{-/-}$(Kidney specific expression of ACE)	1 Similar to phenotype of ACE1

With another genetic animal model of hypertension, double transgenic mice harboring both human renin and agt (R^+/A^+), potential mechanisms of hypertension and hypertension-associated diseases (*e.g.* inflammation, renal necrosis and fibrosis, vascular hypertrophy) have been investigated. These animals exhibit high plasma Ang II concentrations, altered baroreflex activity, cerebral arteriolar hypertrophy, impaired endothelial function and chronic hypertension (362-364). In a separate series of studies, Davisson's group has presented possible mechanisms of hypertension in these transgenic mice. The results from the studies demonstrated that increased brain RAS activity in these animals augments local Ang II production and subsequently Ang II-mediated AT1 receptor activation stimulates vasopressin (antidiuretic hormone) secretion, collectively elevating blood pressure (365). In addition, Didion et al. have recently demonstrated the mechanisms responsible for endothelial dysfunction observed in R^+/A^+ mice. The mechanism of this impairment was mediated by production of an endothelium-derived contracting factor produced by the cyclooxygenase pathway (364).

Subsequent studies on several different double transgenic models, which were developed using tissue or cell-specific promoters (KAP for proximal tubule; albumin for hepatocytes; GFAP for glia in the central nervous system; synapsin I for neurons in the central nerve system; aP2 for adipocytes) have provided numerous insights into the functionality of the local tissue RAS (64, 366-369). Various transgenic studies have pointed out the importance of brain Ang II in the regulation of blood pressure and electrolyte balance. In particular, chronic overproduction of Ang II locally in the brain of transgenic mice have been shown to induce sympathetic nerve activity, drinking intake, salt preference and salt intake, thereby leading to hypertension (367, 368). These effects are AT1 receptor-dependent. Notably, a significant effect of brain RAS on blood pressure was independent of endocrine RAS, indicating that brain RAS *per se* plays a role as a determinant in systemic blood pressure regulation. In addition to brain RAS, the significance of renal RAS in

renal function and development as well as blood pressure homeostasis has also been appreciated with studies in kidney-specific models (specific targeting of RAS overexpression in the kidney and specific targeting of kidney RAS ablation). These transgenic mice exhibited overproduction of intrarenal Ang II and chronic hypertension. Mechanically, renal RAS increased blood pressure of these transgenic animals without increasing systemic Ang II levels, which was also observed in brain-specific models (370). Along with reports on other tissue-specific RAS models, studies from Davisson et al.'s group have clarified the function of the proximal tubule RAS (370). They demonstrated that human agt expression only in proximal tubule was not able to rescue renal abnormalities seen in agt null mice but elevated systemic blood pressure. In comparison with renal RAS effects, systemic expression of human agt and renin genes has been shown to partially rescue renal defects. Brain-specific restoration of Ang II and rat agt expression exclusively in adipose tissue prevented altered renal function such as hydronephorosis in agt knockout mice. Taken together, this suggests that renal RAS is an important regulator of arterial pressure and non-renal tissue RAS plays a significant role in renal function and development (64, 369). Of clinical relevance, the potential mechanisms of renal RAS-induced hypertension (*e.g.* alteration in sodium or fluid homeostasis) appear to be a common underlying mechanism causing high blood pressure in a number of human genetic syndromes (371, 372). Recently, adipose tissue-specific models of rat agt gene using aP2 promoter were created by Massiera et al's group (64). Using these transgenic models, ***in vivo*** paracrine and endocrine effects of adipose RAS have been elucidated. The lipogenic effects of adipose RAS reported previous studies ***in vitro*** were confirmed by these transgenic studies. In this context, another study demonstrating that expression of both transgene human and endogenous mouse agt genes in visceral fat depots (*e.g.* omental, reproductive and perirenal fats) was significantly increased by a high fat diet provided evidence for a key role of adipose RAS in diet-induced obesity (373). Of significance, agt

overexpression in adipose tissue contributes to the development of hypertension via elevated systemic agt levels (presumably increased plasma Ang II concentrations). In contrast with mechanisms accounting for brain RAS or renal RAS-induced hypertensive effect, adipose RAS effect on blood pressure appear to be in part mediated by endocrine RAS. In addition, restoration of agt only adipose tissue was able to prevent renal anomalies seen in agt-deficient mice, suggesting that adipose RAS play a significant role in renal function and blood pressure homeostasis in an endocrine manner (64, 325).

Further, with respect to functionality of human agt gene variants highly associated with the development of hypertension, Sigmund et al. have recently developed two different transgenic mice targeting a single copy of each human agt haplotypes to the mouse hypoxanthine phosphoribosyl transferase (HPRT) locus (-6A/235Thr (AT) and -6G/235Met (GM)). This study revealed that AT and GM haplotypes of the human agt have no effect on gene expression of agt, but regulate the cardiovascular system (*e.g.* heart weight) and the control of blood pressure differently; A small but significant increase in BP and relative heart weight was demonstrated by mice carrying the GM haplotypes (374).

1.6.2.2 Renin

To evaluate the role of an overexpression renin gene in the pathogenesis of hypertension, several investigators established transgenic models expressing the mouse Ren-2 gene (mRen-2) (375). Mullins and Ganten created transgenic rat models expressing the mRen-2 gene; three transgenic lines termed TGR (mRen-2) 25, 26 and 27 were derived (376). Of note, the fulminant hypertension developed in the TGR (mRen-2) 27 animals has been suggested to be partially dependent on the expression of the transgene in the brain. Crossbreeding with transgenic rats carrying a brain-specific deficiency of agt led to the blunt of the central Ang II generation in TGR (mRen-2) 27 and thus a significant reduction in blood pressure in the TGR (mRen-2) 27

animals (377). In addition, these transgenic mice displayed inversed circadian blood pressure rhythm and altered heart rate. Further studies by Morimoto et al showing that human renin transgene is expressed in brain area relevant for cardiovascular and fluid homeostasis supports direct involvement of brain RAS activation in the development of hypertension (378).

1.6.2.3 ACE

Krege et al.'s group studied the effect of quantitative changes in ACE gene function on blood pressure regulation using transgenic mice having from one to three copies of ACE gene. The one to three ACE copy mice did not show any significant changes in blood pressure although there was a linear relationship between ACE gene copy number and serum ACE activity (379). In contrast with previous observations, subsequent study has recently described an inverse relationship between the number of ACE gene copies and arterial pressure response to dietary NaCl, suggesting that ACE may become a more important regulator in arterial pressure (380).

1.6.2.4 Angiotensin receptors

Thu et al. generated transgenic mice harboring with 2, 3 or 4 copies of Agtr-la gene to determine the physiological effects of quantitative variation of AT1 receptor gene expression. In contrast with previous studies showing that deletion of AT1a receptor gene decreases arterial pressure in a copy-dependent manner, the results of this study have demonstrated that the impact of enhanced AT1 receptor expression on blood pressure was blunted by systemic compensations and altered other gene expression (kallikrein and aldosterone) involved in cardiovascular regulation (381). In addition to the effect of the AT1 on blood pressure, the role of AT1 receptor in cardiovascular homeostasis has been illustrated using transgenic mice with CNS-targeted AT1 receptors (neuron-specific enolase (NSE) promoter-AT1). Interestingly, brain-specific overexpression of AT1a receptor has been shown to enhance cardiovascular sensitivity (baroreflex sensitivity) to intracerebroventricular (ICV) Ang II concomitant with normal blood

pressure. These phenotypes seem to contradict earlier findings; activation of central AT1a receptor was responsible for increased blood pressure in various genetic and experimental models. Of interest, blockade of the central AT1 receptor with ICV losartan injection significantly diminished basal blood pressure and increased heart rate in these mice, suggesting that central AT1a receptors involve maintenance of basal blood pressure in part mediated by resetting baroreflex sensitivity. Also, this AT1a action in the brain may prevent hypertension in these animals (382). Further, transgenic mice overexpressing the AT1a receptor in the myocardium using the alpha-myosin heavy chain gene (α-MHC) promoter were generated to explore other noncardiovascular-related functions of Ang II in heart (355, 383, 384). Earlier studies have reported that transgenic mice overexpressing the mouse AT1 receptor in cardiomyocytes displayed massive hyperplasia of arterial myocytes at birth but died perinatally due to bradycardic heart failure despite normal systolic blood pressure and the heart rate (384). Consistently, different strain of transgenic mice overexpressing human AT1 receptor only in heart also developed similar but less severe phenotypes (385). In contrast with pronounced cardiac changes, cardiac hypertrophy and remodeling as well as enhanced mortality seen transgenic animals (α-MHC-AT1R), no cardiac morphological alterations were observed in transgenic mice overexpressing AT2 under the control of the same α-MHC promoter (355). Collectively, this implies that Ang II can directly induce classical features of cardiac hypertrophy, remodeling and failure via its myocardial AT1 receptor. On the other hand, mechanisms accounting for the anti-AT1 actions of the AT2 receptor such as vasodilation, cell differentiation and apoptosis have been proposed in several transgenic mice. Mechanically, expression of the AT2 receptor exclusively in the vasculature has been shown to induce activation of kinin system, thus causing vasodilation (386). Cardiac-specific overexpression of AT2 receptor resulted in attenuated

response to AT1 receptor-mediated chronotrophic and pressor effects (*e.g.* decreased AT1-induced MAPK) (387).

2. LOCAL RAS

2.1 Overview

Along with endocrine RAS, the whole or partial RAS system exists in many tissues including the brain, heart, kidney and pancreas. Recently, the focus has shifted from the classical role for RAS to an autocrine/paracrine role in specific tissue function; tissue RAS may either potentiate systemic function or have entirely separate activities meeting the specific needs of these individual tissues. These local systems are regulated at many levels ranging from the synthesis of renin to the dimerization of angiotensin receptors. Regulation of local RAS occurs in multiple tissues and, as a result, tissue concentrations of Ang II and the concentration of other RAS components and their active metabolites vary in each tissue independently of the circulating system. Further, therapeutic interventions involving ACE inhibitors and angiotensin receptor antagonists have shown to be likely to provide benefit at least in part through the interruption of local systems (388, 389). Accordingly, understanding the biological function of the multiple RASs may help the development in novel therapeutic strategies in the treatments of hypertension and hypertension-associated diseases

2.2 Physiological Function of Local RAS

2.2.1 Brain

Ang II actions in the central nerve system include arterial pressure regulation, promotion of thirst behavior and salt appetite, regulation of vasopressin secretion and sympathetic outflow, modulation of the sensitivity of the arterial baroreflex, thermoregulation, blood brain barrier function and memory mechanism (318).

2.2.2 Heart

Renin, agt ACE and Ang II receptors are all present in the myocardium. The accumulating evidence has demonstrated hypertrophic actions of cardiac Ang II on cardiomyocytes; RAS blockade (*e.g.* ACE inhibitors or AT1 receptor antagonists) prevent or decrease left ventricular hypertrophy independently of blood pressure. Of importance, cardiac RAS has been implicated in the remodeling of the myocardium after myocardial infarction as well as is directly involved in the development of cardiac fibrosis by induction of growth and fibrosis-related genes (388).

2.2.3 Kidney

All of the components of the RAS are present within the kidney and specifically, renin, agt and ACE mRNA are localized in a site-specific manner within the kidney. Most of the intrarenal agt mRNA and protein is localized in the proximal tubule and then directly secreted into the tubule lumen, where Ang I may be formed by renin or renin-like enzymes. ACE is located in on the proximal tubule cell brush border. In the interstitium, Ang II levels are 1000-fold higher than those in plasma, indicating that most of the intrarenal Ang II is formed within the kidney. Renal agt mRNA and protein biosynthesis are stimulated by Ang II; Ang II may autoamplify the activation of the intrarenal RAS. Intrarenal Ang II participates in renal function (e.g. sodium handling and water metabolism) and renal development. Collective evidence from the angiotensin receptor knockout mouse model has elucidated that the AT1 receptor but not the AT2 receptor plays a critical role in renal development (388). Additionally, *in vitro* Ang II induces hypertrophy or hyperplasia of various renal cell types, mainly proximal tubular cells and mesangial cells (176)

2.2.4 Pancreas

Emerging data have revealed the potential influence of pancreatic RAS on growth and free radical generation. Moreover, exogenous Ang II induced a marked vasoconstriction, which could delay the first phase of insulin release in response to glucose. These findings concluded that the pancreatic RAS may play a pivotal role in regulating islet blood perfusion, thereby, affecting insulin release by the islet cells. The significance of a local pancreatic RAS has been emphasized in view of therapeutic implications in diabetes, pancreatitis, cystic fibrosis as well as pancreatic cancer (388).

3. ADIPOSE TISSUE RAS

With other local tissue RAS, paracrine and endocrine effects of RAS in adipose tissue which hosts all RAS components have widely been investigated. Ang II has been elicited be a pivotal physiological regulator in adipocyte metabolism; adipocyte hypertrophy and adipogenesis. Accordingly, it has been suggested possible roles of Ang II in pathophysiology of obesity (170). However, functional studies on adipose RAS have yielded some conflicting results (*e.g.* Ang II roles in adipogenesis and angiotensin receptor-mediated functions). In particular, recent findings revealing that Ang II inhibits adipocyte differentiation in human adipose tissue supports a new concept that its antiadipogenic effect may lead to fat redistribution into liver and muscle cells, thereby contributing to the development of diabetes in obesity (170). Of note, *in vivo* data on endocrine effects of adipose tissue agt using agt knockout and transgenic mice have currently provided convincing evidence for the contribution of adipose Ang II to the development of hypertension in obesity (64).

3.1 Expression and Production of RAS Components

Agt gene expression has been identified in murine adipose cell lines, rat adipose tissue and human adipose tissue. The presence of adipose agt mRNA was first detected in periaortal

brown adipose tissue (BAT) and other adipocytes (390). Subsequently, the detection of agt mRNA in several rat adipose tissue depots and brown adipose tissue as well as agt secretion from cultured fat pads were demonstrated (391-393). In humans, agt expression has been shown in adipose tissue, in primary cultured adipocytes and in differentiating preadipocytes (163, 394-396). Of note, agt expression and secretion have been demonstrated to be differentiation-dependent and thus serve as a late marker of adipocyte differentiation (92, 397).

Renin mRNA expression in humane adipocytes and its increased expression during human preadipocyte differentiation were recently reported (396). Studies on hormonal regulation of RAS demonstrated the expression of renin and other RAS components in subcutaneous adipocytes isolated from both lean and obese patients (69). In contrast with this finding, studies by Faloia et al observed no detection in renin mRNA or protein expression in adipose tissue of either lean or obese normotensive subjects (398). In rodents, renin activity was detected in isolated rat BAT even after bilateral nephrectomy (399). Consistently, later studies in a comparative gene expression of RAS components in rat adipose tissue revealed the detection of agt, renin, and AT1 receptor expression in mature adipocytes (400). However, Harp et al. found no renin mRNA expression but was able to measure Ang I activity in adipose tissue (401).

Several reports have described ACE expression and activity in human preadipocyte and adipocytes. Further, stronger ACE expression was found in human visceral than in subcutaneous adipose tissue (394-396, 402). On the other hand, ACE gene was found in only in the stromal-vascular cells of the rat (400). In preadipocyte cell line (3T3-F442A), no ACE and its isoforms was found (403)

Ang I, Ang II and Ang II-derived biological active peptides such as Ang III, Ang IV and Ang $_{1\text{-}7}$ have been shown to be present in rat BAT than WAT (399, 404, 405). Ang II is also found in conditional media of 3T3-L1 cells and primary human preadipocytes (406). Further,

functional studies with differentiated human preadipocytes suggested that Ang II is endogenously formed by these cells (194, 407).

Collectively, the discrepancies in adipose RAS component presence of humans and rodents may be in part attributable to differences in cell types and age of animals. In addition, another possibility is that enzymes other than renin and ACE can form Ang I from agt or Ang II from agt in adipose tissue or enzyme like neprilysin can degrade Ang II in adipose tissue. In supporting of this notion, few studies have demonstrated the presence of cathepsin G, chymase and cathepsin D in human adipose tissue (395). Neprilysin was also found in rat adipose tissue and human stromavascular cells (408, 409). Of interest, in human adipocytes, renin binding protein gene expression was detected. This enzyme was known to function as a renin inhibitor (410, 411). Accordingly, modulation of renin activity in adipose tissue by this protein possibly contribute to some controversy on the presence of RAS components in fat tissue

Similarly, confusing findings have also been shown in Ang II receptors in adipose tissue. Along with northern and western analyses, functional and pharmacological studies have illustrated the presence of angiotensin receptors (AT1 and AT2). AT1 receptor was detected in adipocyte membrane from rat epididymal fat tissue, BAT and human subcutaneous and visceral adipose tissues whereas AT2 receptor was also present in human preadipocytes and adipocytes and murine clonal cell line including 3T3-L1 and Ob1771 cells (197, 285, 412-415). Higher density of AT1 receptor was particularly observed in epididymal adipocytes of adult *Sprague Dawley* and obese *Zucker* rats compared to those of retroperitoneal, mesenteric and interscapular adipose tissues (413, 414). In humans, the expression of AT1 mRNA was higher in visceral than subcutaneous adipose tissues, but no difference in total Ang II binding between depots was reported (67). On the other hand, the contradictory results on mouse angiotensin receptors have been described. The presence of AT1a receptor in adipose tissue of adult mice was found by the

study from Burson et al (416). Subsequent studies have also demonstrated the expression of AT1 receptor in 3T3-L1 adipose and preadipose cells (163, 198, 285, 417). Of note, available data including our results have suggested that AT2 receptor is a primary receptor for paracrine functions of Ang II in murine adipose cell lines (198, 285). By contrast, recent results have described AT1 as a being primary receptor type in fully differentiated adipose cells; AT1 protein expression was maintained but protein levels of AT2 was diminished during differentiation of 3T3-L1 preadipocytes (417). However, in humans, increased AGTR2 and decreased AGTR1 gene expression were observed during *in vitro* differentiation of human preadipocytes (170, 415). Possible explanations for the controversy have been suggested to be due to variance in receptor activity depending on species or strains, developmental stage and nutritional and hormonal states.

3.2 Regulation of Adipose RAS Components

Adipose RAS is controlled by a variety of factors including hormones, nutrients, sympathetic nerve activity, salt intake and pathophysiological conditions such as hypertension and obesity with its own distinct regulatory system (170). The confusing results have been obtained in adipose RAS regulation; species differences and various experimental conditions could in part explain these variations (170).

3.2.1 Hormone and Nutrition

Agt production is directly regulated at its transcriptional levels (205). In murine clonal cell lines, the expression level of agt mRNA was very low in preadipocytes but was increased during adipocyte differentiation(170). Several studies have identified differentiation specific elements (DSE) within agt promoter region and its corresponding binding protein (DSEBP) in hormonal differentiation of 3T3-L1 adipose cells (418, 419). Agt gene in Ob 1771 preadipocytes was upregulated by long-chain natural and non-metabolized fatty acids (known as PPAR natural ligands) and their stimulatory effects were abolished by actinomycin D (transcription inhibition)

(420). In addition, peroxisome proliferators such as rosiglitazone (a PPARγ activator) and bromopalmitate (a PPARδ activator) have also been elicited to activate agt gene (420). Together, this raises a possibility that PPAR mediates fatty acid-induced agt expression even though PPAR responsive elements have so far not been identified within mouse agt promoter region. Of interest, unlike hepatic agt gene, agt gene expression in rat adipose tissue was regulated by fasting or overfeeding; fasting reduces agt gene expression whereas overfeeding increases mRNA level of agt (421). Glucose also influences expression of agt gene. Plasma high glucose in the presence of an insulin clamp increased adipose agt expression in both lean and obese rats (422). However, agt expression in 3T3-L cells was not changed by glucose treatment (163). Intriguingly, studies from Hart et al have recently reported that insulin dose-dependently increases protein level of agt in isolated human abdominal fats (304). Consistently, in 3T3-L1 adipocytes, insulin-stimulatory effect on agt expression was obtained while in other murine cell lines, repressed agt gene expression by insulin was observed (163, 423). Hyperinsulinemia with euglycemia also decreased adipose and hepatic agt mRNA levels of lean animals (422). Glucocorticoid has been shown to increase agt expression in murine preadipocyte cell lines and rat adipose tissue (69, 424). Other hormones such as estrogen, triiodothyronine and Ang II, which was described as activators of hepatic agt mRNA, had no effect on Ob1771 cells (425). In human adipocytes, Ang II has been shown to negatively regulates agt at the transcriptional levels (69, 425, 426). Also, SNS contributes to modulate agt expression (163). The agt expression in adipose tissue of *Sprague Dawley* rats was increased in response to bilateral nephrectomy or treatment with ACE inhibitor (enalapril) but no change in agt mRNA level was observed by sodium restriction (392, 393). Adipose agt is also regulated by aging; agt gene expression was higher in adipocytes from younger rats but no differences in aging-associated agt expression was observed in obese *Zucker* rats (401). In addition to agt gene regulation, mechanisms responsible for agt secretion by

adipocytes have been recently elucidated. In epididymal adipocytes isolated from obese *Zucker* rats, agt was constitutively released from the intracellular stores whereas in Ob1771 adipocytes, agt production rather than storage affect agt secretion (420, 425, 427).

3.2.2 Obesity and Hypertension

Overall hormonal and metabolic alterations accompanying obesity, obesity-associated insulin resistance and hypertension could affect the expression and secretion of adipose RAS components. A decrease in adipose agt expression was observed in obese *Zucker* rats and viable yellow (A^{vy}) mice whereas other genetic mice (*ob/ob* mice, *db/db* mice, *fa/fa* rats) exhibited increased agt formation in adipose tissue (163, 421, 428). Further studies in agt secretion and expression in *fa/fa* rats have supported the concept that increased agt secretion is closely associated with the onset of adipose tissue hyperplasia and stimulation of hypertrophy (429). They found that agt protein levels and secretion per cell number were significantly increased in adipocytes from obese *fa/fa* rats, compared to those of lean rats even though these elevations were independent of cell size (429). By contrast, another study demonstrated that obesity-associated hypertrophic adipocytes contribute to an increase in adipose agt expression. This was mediated by altered cholesterol distribution, which is a feature characteristic of hypertrophic adipocytes (430). In obese humans, agt gene expression in subcutaneous and omental adipose tissues has been shown to be highly associated with increased body mass index (BMI) and waist-to-hip ratio (index of central obesity) (66-68). A positive relationship between agt secretions by isolated adipocytes and adipocyte volume has observed in obese subjects (170).

In addition to modulation of adipose agt in obesity, the impacts of systemic blood pressure on adipose RAS components have been delineated. Epidemiological studies have demonstrated that hypertensive or obese hypertensive subjects exhibited decreased or unchanged adipose agt gene expression compared to normotensive controls while AT1 receptor, renin and

ACE genes in abdominal subcutaneous adipocytes was exclusively expressed in hypertensive obese women (69, 398). Linkage among obesity, hypertension and RAS gene polymorphisms (agt, ACE, AT1 and AT2) has been found in several populations. In agt polymorphisms, agt promoter variants composing an A/G polymorphism at -217 are associated with hypertension (431). In addition, the DD allele of the ACE gene was closely related to abdominal adiposity and hypertension (432).Another study has recently illustrated female-specific association between the AT2 receptor C4599A polymorphism and hypertension in Japanese populations (433).

3.3 Physiological Roles of Adipose Tissue RAS

3.3.1 Autocrine/Paracrine Effects of Adipose Tissue RAS

3.3.1.1 Brown Adipose Tissue (BAT) Function

Brown adipose tissue plays a critical role in cold-thermogenesis, is capable of formation of Ang II (434). The thermogeic function of BAT is primarily mediated by norepinephrine (NE) release from sympathetic nerve densely innervating this tissue. Ang II has shown to increase evoked [^{3}H]-NE release from rat BAT following electric field stimulation. During cold acclimation, endogenous Ang II production and AT1 receptor density were elevated in rat intrascapular BAT (ISBAT) independent of systemic RAS (434). Further, Ang II-induced [^{3}H]-NE release was enhanced in cold-exposed rats compared to controls. These changes in NE turnover by cold exposure were completely prevented by treatment with losartan, suggesting potential role of Ang II in stimulating thermogenesis in BAT. In subsequent studies, decreased content of Ang II but increased NE activity by Ang II in ISBAT was observed in young obese *Zucker* rats. Conversely, in old *Zucker* animals, elevated Ang II content and decreased local SNS neurotransmission by Ang II were observed (435).

3.3.1.2 Adipose Tissue Growth and Differentiation

Agt expression is differentiation-dependent; spontaneous differentiation also increases agt mRNA levels while dexamethasone, which potentiates adipocyte differentiation, enhances expression of adipose agt (397, 420). Recent microarray data from 3T3-L1 cells have also addressed that adipose agt expression was higher levels in 3T3-L1 cells after several days of *in vitro* differentiation (436, 437). Earlier studies on co-culture of adipocytes and preadipocytes revealed adipogenetic effect of Ang II in Ob 1771 cells. This effect was PGI2-and AT2 receptor-dependent (285). Further studies using microdialysis technique have confirmed that Ang II-induced adipocyte differentiation is mediated by PGI2. *In vivo* and *ex vivo* rat epididymal fat pads with Ang II or prostacyclin exposure resulted in an increase in stromavascular cells undergoing adipogenesis and this effect was abolished by aspirin (inhibition of PG synthesis) (438). Study by Crandall et al. demonstrated mitogenic effects of Ang II, suggesting involvement of Ang II in early stages of adipocyte differentiation. Stimulation of human preadipocytes with Ang II resulted in an acceleration of the G1-phase of the cell cycle and increased expression of the cell cycle regulator cyclin D1(197). Taken together with the observation of differential expression of Ang II receptors during adipogenic conversion, these findings may suggest that in rodents and humans, Ang II exerts its adipogenic effects via interacting with different signaling pathways; Ang II promotes adipocyte hypertrophy by an adipogenic factor, prostacyclin in AT2-dependent mechanisms and induces hyperplasia in adipose cells by upregulation of cyclin D1 gene through AT1 receptor activation. In contrast with its stimulatory effects on adipogenesis, Janke et al. elicited inhibitory action of Ang II in differentiation of human preadipocytes (407). Ang II inhibited insulin-induced differentiation and this effect was blocked by treatment of AT1 receptor antagonist. Consistently, co-culture studies showed that mature adipocytes prevent preadipocyte differentiation in an AT1-dependent manner (407). On the basis of these results, they proposed a

possible mechanistic link between hypertension and insulin resistance; inhibition of preadipocyte recruitment by Ang II may result in lipotoxicity, thereby leading to the development of diabetes (407). Other study demonstrating that Ang II induces the formation of PAI-1, which inhibits cluster formation of human preadipocytes also support antiadipogenic effect of Ang II (194, 439).

3.3.1.3 Adipose Tissue Metabolism

In addition to Ang II's adipogenic actions in a paracrine manner, influences of Ang II on lipogenesis and lipolyisis in adipocytes have been studied (170). Previously, physiological concentration of Ang II significantly increased activity and gene expression of two key lipogenic enzymes, FAS and GPDH as well as *ob* gene activation concomitant with elevated triglyceride storage in 3T3-L1 cells and human adipocytes. Ang II-induced lipogenic effect was mediated by AT2 receptor (198). Specifically, ADD1 and glucose was involved in FAS promoter activity by Ang II. In addition, Ang II stimulated production of leptin (a adiposity marker) independent of prostacyclin (adipogenic effector) in human and murine adipose cells (440). Of note, several studies have illustrated lipolytic effects of Ang II through a pressor-independent mechanism. Infusion of Ang II at a pressor dose into the rats caused weight loss and reduction of adipose tissue mass over the course of one week, with concurrently marked changes in levels of circulating IGF-1 and its binding proteins. This weight-loss effect was predominately ascribed to decreased food intake and was prevented by blockade of AT1 receptor (441). The successive study has demonstrated that infusion of Ang II for 7 days into rats reduces weight loss compared to vehicle-infused controls in a dose-related manner. This response paralleled with site-specific reduction of white adipose tissue, alternation of plasma leptin levels and elevation in abdominal surface temperature (442). Collectively, long-term systemic infusions of Ang II led to its lipolytic effect and this impact was primarily mediated by decreased food intake and increased energy expenditure with metabolic alternations. In contrast, in humans, both Ang II infusion at

subpressor or pressor doses for short-terms and blockade of Ang II formation had no effect on whole lipolysis and lipid oxidation rate (443). Of interest, further studies have demonstrated local actions of Ang II on lipid and carbohydrate metabolism and blood flow of human adipose tissue in a site- and tissue-specific manner. Perfusion of Ang II dose-dependently decreased local blood flow, lipolysis and glucose uptake and this effect was more pronounced in the femoral fat depots than the abdominal fat depots (444). Boschmann et al. examined specific effects of interstitial Ang II on metabolism and local blood flow in muscle and adipose tissues. Interstitial Ang II stimulated lipolysis in a dose-dependent manner along with a minimal effect on local blood flow of human adipose tissue whereas in the muscle, this hormone dose-dependently inhibited lipolysis. This finding indicates that interstitial Ang II modulates metabolism in a tissue-specific fashion (445). Taken together, Ang II regulates adipose tissue metabolism with changes in local blood flow in a site-and species-specific manner.

3.3.2 Endocrine Effects of Adipose Tissue RAS

In addition to functional roles of Ang II in a paracrine mechanism, with agt knockout and transgenic mouse models, endocrine effects of adipose Ang II have been investigated (64, 325, 446). Agt knockout mice exhibited altered adipose tissue development including adipocyte hypotrophy and marked decreased lipogenesis with the resultant reduction of fat mass as well as increased locomotor activity. Body weight was considerably decreased in agt homozygous mutant mice compared to WT mice and this effect was mainly due to reduction in fat mass, especially epididymal fat pad weight with no changes in other Ang II-responsive organs. Fat mass reduction in KO mice was related to adipose tissue hypotrophy with a parallel decrease in FAS enzyme activity. Of note, KO mice were resistant to high fat diet-induced weight gain despite maintained FAS activity in adipose tissue (325, 446). They also generated two transgenic mouse models using an aP2 adipocyte-specific promoter; Tg-KO mice exclusively expressing agt in adipose

tissue and Tg-WT mice overexpressing agt in adipose tissue and investigated potential role of adipose agt in systemic blood pressure regulation. Tg-WT mice displayed body weight gain with dramatically increased fat mass, confirming *in vitro* lipogenic role of Ang II in adipocytes. Further, overexpression of agt in adipose tissue was able to raise blood pressure level by ~16% concurrent with elevated circulating agt level by ~20% compared to control mice, suggesting a potential mechanism responsible for obesity-induced hypertension; adipose tissue agt increases whole body blood pressure by activation of endocrine RAS. Tg-KO mice showed increased fat mass, restored renal morphology and function and blood pressure along with increased plasma agt concentrations (20 % of WT). Additionally, microarray-based data demonstrated that reexpression agt only in adipose tissue was able to correct altered expression of genes including ion channel and transporter, RAS components and Ang II signaling molecules seen in KO mice (325). Altogether, this indicates that adipose tissue agt plays pivotal roles in renal function and arterial pressure regulation.

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PART III

ANGIOTENSIN II RESPONSIVE ELEMENT IS THE INSULIN RESPONSIVE ELEMENT IN THE ADIPOCYTE FATTY ACID SNYTHASE GENE: ROLE OF ADIPOCYTE DETERMINATION AND DIFFERENTIATION FACTOR 1/STEROL REGULATORY ELEMENT BINDING PROTEIN 1C

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My primary contribution to this paper is to conduct experiments.

A. ABSTRACT

We have previously shown that angiotensin II (Ang II) increases the expression of the gene encoding adipocyte fatty acid synthase (FAS). Here we investigate the mechanism responsible for increased FAS gene transcription by Ang II. We demonstrate that Ang II increased luciferase activity by 3-fold in 3T3-L1 adipocytes transfected with fusion constructs linking the FAS promoter to the luciferase reporter gene. Interestingly, we mapped the Ang II regulatory sequences to the insulin-responsive region (E box) in the proximal FAS promoter. The E box alone was able to mediate Ang II responsiveness when linked to a heterologous promoter. However, this response was lost when mutations that abolished the binding of the E box to its transcription factors were introduced. Using adenoviral overexpression of a dominant-negative form of adipocyte determination and differentiation factor 1 (ADD1), a transcription factor that binds to the insulin-responsive E box, we demonstrated that ADD1 was required for Ang II regulation of the FAS gene in 3T3-L1 adipocytes. Furthermore, ADD1 expression was also up-regulated by Ang II. With the use of transfections as well as glucose transport assays, we further demonstrated that Ang II stimulation of the FAS gene was dependent on glucose. In conclusion, this is the first report that Ang II regulates adipocyte FAS gene transcription via insulin response sequences in a glucose-dependent manner and that this regulation is mediated at least in part via the ADD1 transcription factor.

B. INTRODUCTION

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

FAS is a key lipogenic gene that catalyses 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, whereas obesity, a high carbohydrate diet or insulin treatment increases FAS synthesis (14). The gene encoding FAS is primarily regulated at the transcriptional level (15–19) and we have previously identified an insulin-responsive element (IRE) in the FAS gene that mediates the transcriptional up-regulation of this gene by insulin in adipocytes (16). Because our previous studies indicate that Ang II is a lipogenic hormone that exerts an insulin-like effect on adipocyte gene expression, we hypothesized that Ang II and insulin regulate FAS gene expression via similar mechanisms.

We report here for the first time that Ang II increases FAS gene transcription in adipocytes via an angiotensin-responsive element that is identical with the insulin-responsive region (E box) in the proximal FAS promoter. Furthermore, we show that Ang II effect is dependent on glucose and we identify adipocyte determination and differentiation factor 1/sterol-regulatory-element-binding protein 1c (ADD1/SREBP1c) as a transcription factor involved in the regulation of the FAS gene by Ang II.

C. MATERIALS AND METHODS

1. 3T3-L1cell Culture

3T3-L1 cells were grown and differentiated as described previously (2, 19). In brief, cells were grown to confluence in standard DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum. At confluency, cells were induced to differentiate by the addition of dexamethasone (250nM) and isobutylmethylxanthine (0.5mM) to standard medium for 72h. Cells were maintained for a further 3 days in standard medium, then changed to serum-free medium containing 1% (w/v) BSA for 12h followed by treatment with Ang II and/or insulin as indicated in the figure legends. All treatments were performed in serum-free medium to test individual and direct hormone effects. However, the responsiveness of the FAS gene to both Ang II and insulin can be demonstrated in the presence of both 1% and 10% (v/v) serum (results not shown). Unless otherwise stated, cells were cultured in the presence of a high concentration of glucose (25mM).

2. RNA Isolation and Northern Analysis

RNA was isolated by the CsCl density gradient method (19). Individual culture dishes (100mm) of cells were used for each RNA sample within treatments; three or four samples were included in each treatment group and each experiment was repeated two or three times. Total RNA (approx. 30μg) for each sample was subjected to electrophoresis in a 0.7% agarose gel, transferred to nylon membrane and analyzed by Northern blotting, as described previously (19). Membranes were hybridized with ^{32}P-labelled cDNA probes for rat FAS (kindly provided by Dr A. G. Goodridge) or ADD1 (kindly provided by Dr B. M. Spiegelman) and 18S (purchased from Promega). Unbound probe was removed by washing membranes in 2×saline/sodium phosphate/EDTA (SSPE) for 45min at 25°C and then in 0.1×SSPE/0.1% SDS for 60min at 65°C. After being washed, membranes were exposed to X-ray film (Dupont). Autoradiograms were analyzed by densitometric scanning; data are expressed as a ratio of FAS to 18S.

3. Nuclear Run-On Assay

Nuclei were isolated from control or Ang II-treated 3T3-L1cells as described previously (19). In brief, cells were homogenized in a buffer containing Nonidet P40, and nuclei were isolated by differential centrifugation. Nuclear run-on assays and hybridizations were conducted as previously described (19). Labelled RNA was hybridized with plasmids containing cDNA species encoding FAS and β-actin, and with vector alone (pBS) immobilized on nylon membranes. Membranes were then exposed to film and the autoradiograms were quantified by scanning laser densitometry. FAS transcription rates were normalized to those of β-actin.

4. FAS-Reporter Gene Constructs

A fragment of the FAS 5′-flankng region spanning -2100 to +67 was generated by PCR with rat genomic DNA and subcloned into pGL-basic vector (Promega). Various deletions of this fragment were subsequently generated by ExoIII/mung bean nuclease deletion (Stratagene, La Jolla, CA, U.S.A.) or by PCR with previously published FAS promoter sequences (20). For fusion constructs containing two direct repeats of sequences spanning the E box, -70 to -54 wild-type (CAGCCCATGTGGCGTGG) and mutant -69 to -54 (AGCC***TGTACG***GCGTGG) oligonucleotides of the specified sequences were synthesized, annealed then subcloned into pGL2-SV promoter luciferase plasmid. These FAS-promoter–luciferase fusion constructs were used to transfect 3T3-L1cells. To determine the role of this IRE in the context of the native FAS promoter, we also used the native FAS promoter fragment -200 to +67 linked to the chloramphenicol acetyltransferase (CAT) reporter gene, containing either the wild-type or mutated E box (21).

5. Transfections

3T3-L1cells were transfected with FAS–luciferase gene fusion constructs and pSVneo plasmid that confers neomycin resistances using the calcium phosphate–DNA co-precipitation method (Gibco BRL, Bethesda, MD, U.S.A.) as described previously (16). Stably transfected cells were selected by maintaining transfected cells in 500μg/ml neomycin for 2 weeks. Surviving fibroblasts were cultured as described above for 3T3-L1cells. The differentiated stably transfected adipocytes were maintained overnight in serum-free medium before being treated with Ang II and/or insulin as indicated in figure legends. pGL2-control plasmid, which contains the simian virus 40 promoter linked to the luciferase gene, was used as a control to determine the specificity

of the effect of Ang II on FAS promoter and did not show responsiveness to insulin or Ang II (results not shown).

6. Reporter Gene Assays

For luciferase assays, the cells were lysed in 100mM potassium phosphate (pH7.8)/0.2% (w/v) Triton X-100/1mM dithiothreitol. The cytosolic extracts were used to measure luciferase activities with a luminometer (Berthold, Nashua, NH, U.S.A.) and a kit for luciferase assay (Tropix). Luciferase activity was normalized to protein measured with the Bradford assay (22). For CAT assays the cells were lysed in a buffer containing 250mM Tris/HCl, pH8, and 5mM dithiothreitol; a quantitative non-chromatographic assay was used to measure CAT activity in the cell extracts, as described previously (21).

7. 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 previously described (23). 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 HEK-293cells, purified by CsCl density centrifugation and stored. Cells were grown as described above, and 35mm dishes containing fully differentiated 3T3-L1 adipocytes were incubated overnight in serum-free medium containing BSA. Cells were transduced with 3×107 plaque-forming units in DMEM alone for 4h at 37°C; 1nM Ang II was then added. Transduced cells were assayed for FAS enzyme activity 48h after viral infection.

8. Uptake of 2-Deoxyglucose

3T3-L1cells were differentiated as described above and incubated overnight in serum-free medium containing 2% (w/v) BSA. Cells were then washed twice with Krebs–Ringer phosphate, pH7.4, and incubated for 30min at 37°C in the presence or absence of insulin (10nM) or Ang II (1nM). 2-Deoxyglucose uptake was initiated by the addition of 0.2mM 2-[^{3}H] deoxyglucose (500GBq/mmol). After 5min, cells were washed rapidly with ice-cold buffer and lysed with NaOH; radioactivity was then counted.

9. Statistical Analysis

Experiments were repeated two to eight times as indicated in the figure legends. Analysis of variance was used to compare overall group means. Student's t test was used for comparison between groups. All tests were conducted with 95% confidence intervals.

D. RESULTS

1. Effects of Ang II on FAS Activity, mRNA Level and Gene Transcription Rate

We have previously reported that Ang II increases FAS gene expression in murine and human adipocytes (2). Fig. 1 shows the coordinated effects of Ang II on FAS activity, mRNA level and the rate of gene transcription. Ang II increased FAS activity, mRNA level and gene transcription by approx. 2.5-fold, 4.5-fold and 2.5-fold respectively. These results demonstrate that FAS gene is regulated by Ang II at the transcriptional level.

2. Effects of Ang II on FAS Promoter Activity: Comparison with Insulin

To investigate further the mechanisms of transcriptional regulation of FAS by Ang II, we searched for Ang II-responsive regions in the FAS gene. Ang II significantly increases luciferase

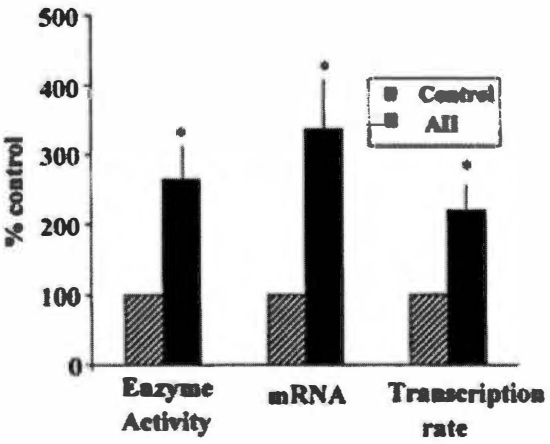

Fig. 1 ***Effects of Ang II on FAS enzyme activity, mRNA level and gene transcription rate.*** 3T3-L1 adipocytes were treated with 1nM Ang II (AII). Cells were then harvested after 48h for FAS enzyme activity (measured in nmol of NADPH oxidized/min per mg of protein) or after 24h for mRNA analysis and gene transcription assays (measured in arbitrary densitometric units). Results are presented as percentages of control. *$P < 0.01$ compared with controls from a total of eight experiments.

activity in 3T3-L1cells transfected with the fusion constructs containing the regions -200 to +67 (Fig. 2A) and -70 to +67 (Fig. 2B) of the FAS promoter. These fragments contain the IRE previously identified in the FAS gene (16). The magnitude of FAS promoter response to Ang II stimulation was half of that elicited by insulin but it is clear from Fig. 2 that adipocytes responded similarly to Ang II and insulin. In addition we show that Ang II and insulin in combination increased luciferase activity in 3T3-L1cells transfected with the plasmids spanning the regions -200 to +67 and -70 to +67 (Fig. 2A and 2B, respectively) of the FAS promoter. Importantly, the increase in luciferase activity in the combined Ang II and insulin treatments was not found to be significantly different from insulin treatments alone, suggesting that Ang II and insulin might target similar cis-acting elements to regulate FAS gene transcription.

3. Ang II-Responsive Sequences are the Insulin-Responsive Sequences in the FAS Gene

To determine whether the IRE might mediate the Ang II responsiveness of the FAS gene, we tested the responsiveness of this element to Ang II and insulin in the context of a heterologous promoter and when linked to the native FAS promoter. Using adipocytes transfected with a construct containing only the -70 to -54 (IRE) motif linked to the simian virus 40 heterologous promoter, we tested the responsiveness of luciferase to Ang II and to insulin. We demonstrate (Fig. 3, upper panel) that Ang II and insulin significantly increased luciferase activity. This indicates that the -70 to -54 region alone is sufficient to confer Ang II responsiveness on the FAS promoter and no additional sequence is needed outside this motif. In addition, this indicates that Ang II and insulin both regulate FAS gene transcription through the same responsive sequences in the FAS promoter. To confirm this we mutated the insulin-responsive E box in the -70 to -54 region of the FAS promoter. Fig. 3 (upper panel) demonstrates that the mutation of this region caused a loss of

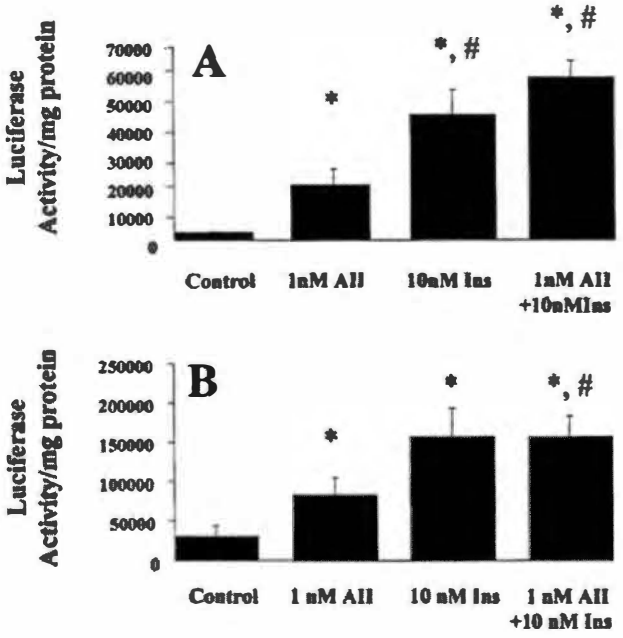

Fig. 2 ***Effects of Ang II on luciferase activity in 3T3-L1 adipocytes transfected with various FAS promoter constructs.*** Adipocytes were transfected with FAS–luciferase constructs containing the -200 to +67 region (A) or the -70 to +67 region (B) of the FAS promoter and treated for 48h with Ang II (AII) and/or insulin (Ins). Luciferase activity was then measured (in arbitrary units) and normalized to protein levels. Results are means for six different experiments. *P < 0.01 compared with control; #P < 0.01 compared with 1nM Ang II.

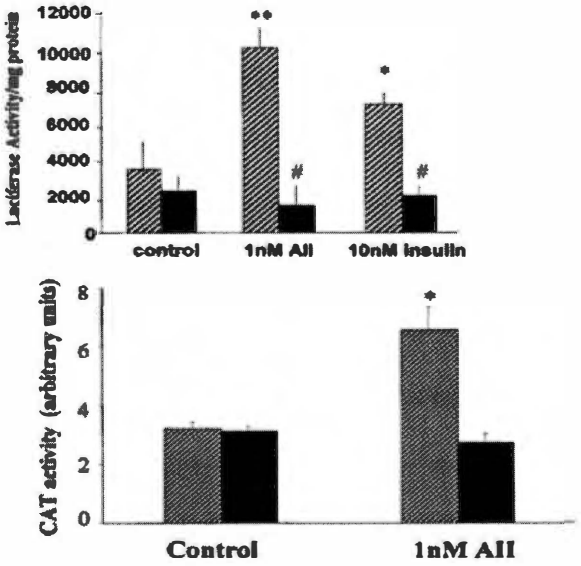

Fig. 3 ***Insulin-responsive E box mediates Ang II responsiveness of the FAS promoter.*** *Upper panel*: the IRE (E box) linked to a heterologous promoter mediates the effects of Ang II (AII) and insulin on luciferase activity. Two copies of the insulin-responsive region (-70 to -54) containing the wild-type (hatched columns) or mutated (black columns) E box were subcloned into pGL2 SV-luciferase plasmid and transfected into 3T3-L1 adipocytes. Cells were then treated with or without 1nM Ang II and/or insulin for 48h; luciferase activity and protein content were then measured. Results are reported in arbitrary units of luciferase/mg of protein. This experiment was performed in triplicate. **$P < 0.01$, Ang II effect compared with control; *$P < 0.05$, insulin effect compared with control; #$P < 0.05$, mutation effect compared with control. *Lower panel*: mutation of the IRE (E box) in the native FAS promoter abolishes the effects of Ang II (AII) on the CAT reporter gene. Fusion constructs containing the wild-type (hatched columns) or mutated (black columns) E box within the FAS promoter (-200 to +67) linked to the CAT reporter gene were reporter gene activity by Ang II was abolished (Fig. 3, lower panel).Cells were then treated with or without 1nM Ang II for 48h and harvested for assays. CAT activity was normalized to protein content. *$P < 0.01$ compared with control; this experiment was performed in triplicate.

stimulation of luciferase activity in response to Ang II treatment as it did for insulin when compared with control. Additionally, when IRE within the native -200 to +67 FAS promoter was mutated, stimulation of the CAT reporter gene activity by Ang II was abolished (Fig. 3, lower panel)

4. ADD1/SREBP1c is required for Ang II-Regulated FAS Gene Transcription

ADD1/SREBP1c has been proposed as a transcription factor mediating transcriptional effects of insulin on lipogenic genes (23, 25). Because Ang II exhibited insulin-like effects on FAS gene expression, we next investigated whether ADD1 might be the potential transcription factor through which Ang II might affect FAS gene transcription: we infected 3T3-L1cells with a dominant-negative form of ADD1 (ADD1/DN) designed to induce the formation of transcriptionally inactive ADD1 dimers that are unable to bind DNA target sequences. Fig. 4 demonstrates that Ang II-induced FAS enzyme activity was eliminated by the infection of this dominant-negative form of ADD1. This result demonstrates that ADD1, the insulin-induced transcription factor, is also required for the regulation of the FAS gene by Ang II.

5. Glucose-dependent Effect of Ang II on FAS Promoter: A Proposed Mechanism for Regulating FAS Gene Transcription

Because the above results demonstrated that Ang II regulates the FAS promoter activity through the same cis-active sequence as insulin, and targets the same transcription factor as insulin, we next addressed the question of whether Ang II might exert its effects by mimicking a common action of insulin on glucose entry within adipocytes. Because the regulation of lipogenic genes by insulin is also dependent on glucose, we next investigated whether the regulation of FAS

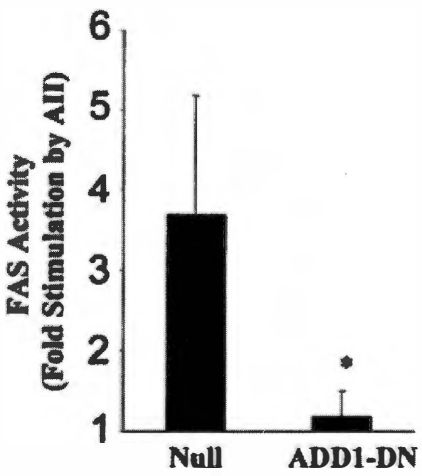

Fig. 4 ***ADD1 is a potential transcription factor through which Ang II effects FAS gene transcription.*** As described in the Materials and methods section, 3T3-L1 adipocytes were infected with adenovirus either encoding or not (null) a dominant-negative form of ADD1 (ADD1-DN; multiplicity of infection of 100) and treated with 1nM Ang II. FAS activity was measured after 48h and normalized to protein. *$P < 0.01$, ADD1/DN compared with null. This figure is representative of four experiments.

by Ang II still occurred when glucose was withdrawn from the culture medium and replaced by pyruvate as the energy source. 3T3-L1 adipocytes were transfected with the FAS-luciferase fusion construct used above (-70 to +67) and treated with or without glucose in the presence or absence of 1nM Ang II. Fig. 5A demonstrates that glucose alone increased FAS promoter activity by 50%. This small effect of glucose alone in adipocytes should be related to the low concentration of glucose transporters at the plasma membrane in the absence of insulin, leading to inefficient glucose entry. Interestingly, in the absence of glucose, Ang II was almost unable to increase luciferase activity (25% stimulation), whereas it elicited a 3-fold increase in the presence of glucose. This clearly indicates that the stimulation of FAS activity by Ang II is largely dependent on glucose. Because the stimulation of the FAS promoter by Ang II was dependent on the presence of glucose in the culture medium, we next investigated whether the effect of Ang II might be mediated through the stimulation of glucose uptake into adipocytes. Fig. 5B shows that Ang II does indeed stimulate 2-deoxyglucose uptake in 3T3-L1 adipocytes. Even though Ang II was less potent than insulin, this effect of Ang II can provide a potential mechanism through which Ang II might exert its insulin-like effects on FAS expression in fat cells.

6. Regulation of ADD1 Expression by Ang II and Glucose

Because ADD1 mediates the nutritional and hormonal regulation of several lipogenic genes (23, 25), we tested whether Ang II modulated ADD1 expression (Fig. 6). Adipocytes cultured in the absence of glucose expressed very low levels of ADD1mRNA. Consistent with the activatory effect of glucose on ADD1 expression in cultured hepatocytes (23) was the observation that glucose alone was able to induce ADD1 expression in adipocytes. This effect was further potentiated in the presence of Ang II (Fig. 6). This indicates that ADD1 expression is modulated

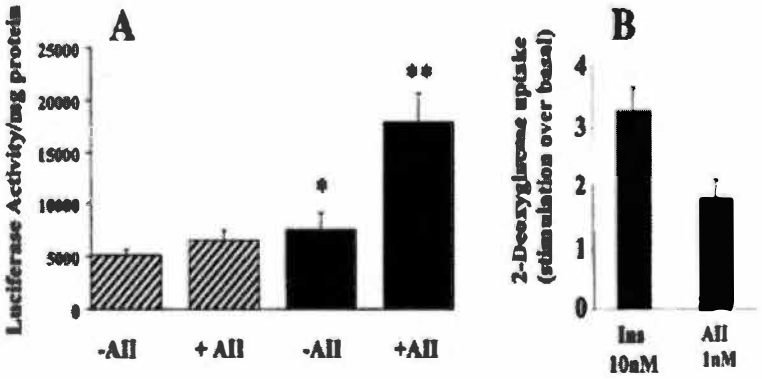

Fig. 5 ***Ang II- and glucose-dependent regulation of the FAS gene and glucose transport.***
(A) The effect of Ang II on promoter activity is dependent on glucose. Adipocytes were transfected with the SV-luciferase plasmid (described in the legend to Figure 3), which contains the insulin-responsive E box, then treated with glucose-free DMEM for 8h before treatment for an additional 48h with (black columns) or without (hatched columns) glucose in the presence or absence of Ang II. Luciferase activity was normalized to protein and is reported in arbitrary units. *P < 0.05 compared with no glucose; **P < 0.01 compared with all other treatments. This experiment was repeated twice. (B) Ang II stimulates 2-deoxyglucose uptake in 3T3-L1cells. 2-Deoxyglucose uptake was measured for 5min in differentiated 3T3-L1cells in the absence (basal conditions) or presence of insulin (Ins) or Ang II at the indicated concentrations. Values are presented as the effect of the hormones over basal unstimulated conditions. This experiment was repeated three times.

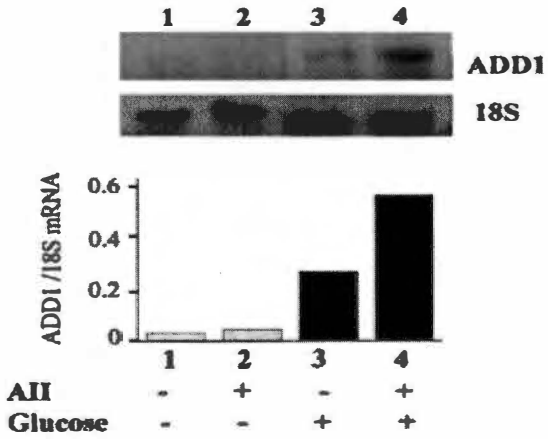

Fig. 6 ***ADD1 expression is increased by Ang II and glucose in 3T3-L1 adipose cells.***
Differentiated 3T3-L1 adipocytes were treated with or without Ang II (AII, 1nM) in the presence or absence of glucose (25mM) for 24h; ADD1mRNA expression was analyzed by Northern blotting as described in the Materials and methods section. ADD1mRNA levels were normalized to 18S RNA. This experiment was repeated twice.

by both glucose and Ang II.

E. 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 IRE in the FAS proximal promoter (16). This IRE is an E box that has been shown to bind ADD1/SREBP1c (23, 25, 26), a member of the bHLH family. In established adipose cell lines, ADD1 has been shown to promote adipocyte differentiation and the expression of genes linked to fatty acid metabolism (24). In isolated rat adipocytes, FAS expression has been shown to be dependent on ADD1 through the use of infection of a mutated dominant-negative form of ADD1 (23). Moreover, in the cultured hepatocyte system, ADD1/SREBP1c is required for the regulation of lipogenic genes by glucose (23) and its expression is nutritionally regulated (23,25) in hepatocytes and fat cells, establishing it as the strongest candidate for participation in the regulation of the FAS gene by insulin.

We have shown previously that Ang II acts like insulin as an adipogenic hormone to regulate FAS and other lipogenic genes in adipocytes. Here we demonstrate that Ang II regulates FAS at the transcriptional level in 3T3 adipocytes and we searched for Ang-II-responsive sequences in the FAS gene. By using transfection assays, we mapped the Ang-II-responsive region to the IRE. Furthermore, mutation in the E box motif in this sequence abolished Ang II responsiveness, as seen in earlier work with the response to insulin when the E box was mutated (16,25). This is the first report on the regulation of FAS gene transcription in adipocytes by Ang II and the identification of an Ang II-responsive element in the FAS gene. Very few Ang II-

responsive elements have been identified in other genes. In smooth muscle, responsiveness of the α-actin gene to Ang II is partly dependent on modulation of the serum response factor binding to CC(A/T-rich)6GG regions. The CC(A/T-rich)6GG elements are found in the promoters of several immediate-early response genes (27). These response elements have been shown to confer serum-induced and growth factor-induced transcription activation of these various genes (28–32). In the present study the Ang-II-responsive element is an E box (CATGTG) that is distinct from the serum response element; there is no evidence that such elements are present in the 2kb promoter region located immediately upstream of the transcription start site of the FAS gene.

As discussed above, the transcription factor ADD1/SREBP1c is critical in the regulation of FAS gene transcription in adipocytes. We tested the hypothesis that ADD1 might be a transcription factor mediating the regulation of FAS by Ang II. Expression of ADD1/DN in 3T3-L1 adipocytes markedly decreases FAS induction by Ang II, indicating that ADD1 is one of the transcription factors required for the up-regulation of FAS gene transcription by Ang II. This led to the conclusion that the Ang-II-responsive element, which is also the insulin-responsive sequence (E box), functions through the same transcription factor (ADD1) as insulin for the regulation of FAS gene transcription. This led us to investigate whether Ang II might produce its transcriptional effects through insulin-like effects on glucose utilization in adipocytes. Indeed, several studies have pointed out that glucose independently was able to regulate lipogenic gene expression, including FAS, and that the effect of insulin was in part dependent on glucose. Moreover, it has been demonstrated in the hepatic cell system that ADD1/SREBP1c was required for the transcriptional effects of glucose on the FAS gene (23). Here we have demonstrated that stimulation of the FAS promoter activity with Ang II was largely dependent on glucose. The

present results also indicate that Ang II stimulates 2-deoxyglucose uptake in adipocytes, which further supports the possibility that the effect of Ang II might be linked to glucose utilization. Together, these results provide evidence that, rather than a direct effect of Ang II on ADD1/SREBP1c, the induction of the FAS gene by Ang II is linked to increased glucose utilization, which in turn up-regulates FAS via ADD1. This led us to propose the following scheme for Ang II regulation of FAS transcription, in which the primary action of Ang II is exerted at the level of glucose entry within the adipocytes. Once transported within the fat cell, glucose can regulate the transcription of the FAS gene through ADD1/SREBP1c, which targets the IRE. According to this scheme, insulin is not required for Ang II effects because substantial levels of ADD1/SREBP1c are present in differentiated 3T3-L1 adipocytes.

There are at least two identified types of receptor through which Ang II can signal (33). Most studies have focused on the signaling of Ang II through the angiotensin type I receptor (AT1). The primary receptor mediating the regulation of lipogenic genes in adipocytes by Ang II identified in our laboratory and in others was the angiotensin type II (AT2) receptor (2, 3); the signaling pathway of Ang II through this receptor is largely unknown. Prostaglandin I2 has been postulated as a signaling molecule produced by adipocytes in response to Ang II binding to AT2 receptors (2, 3). One recent study has linked Ang II and glucose transport in vascular smooth cells (34). In that experiment, low concentrations of Ang II (0.1nM) stimulated 2-deoxyglucose uptake. Although the receptor subtype involved in this effect was not determined, this is reminiscent of our present results showing that the effects of Ang II on FAS gene transcription might be related primarily to the insulin-like effects of Ang II on glucose metabolism. Because changes in ADD1 expression have been involved in the regulation of lipogenic genes by both insulin and glucose

(23), we tested whether Ang II also modulates ADD1 expression in the presence or absence of glucose. The present study demonstrates that Ang II in the presence of glucose markedly induced ADD1 expression, suggesting that the regulation of the FAS gene in adipocytes by Ang II might be mediated by the direct modulation of ADD1 in the presence of glucose.

In conclusion, the present study demonstrates for the first time that Ang II can act on gene transcription through insulin-responsive sequences, extending results obtained by others on insulin-like mechanisms of intracellular signaling by Ang II.

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PART IV

EFFECTS OF HIGH FAT DIET, ANGIOTENSINOGEN GENE INACTIVATION AND TARGETED EXPRESSION TO ADIPOSE TISSUE ON LIPID METABOLISM AND RENAL GENE EXPRESSION

This manuscript has been published in similar form with co-authors Urs S, Massiera F, Wortmann P, Joshi R, Heo YR, Andersen B, Kobayashi H, Teboul M, Ailhaud G, Quignard-Boulange A, Fukamizu A, Jones BH, Kim JH, Moustaid-Moussa N. in Horm Metab Res 34: 721-725, 2002. My primary contributions to this paper include 1) conduction of experiments and 2) most of writing.

A. ABSTRACT

To address the role of angiotensinogen (agt) in lipid metabolism and its potential endocrine effects *in vivo,* we studied the effects of high fat diets (HFD) on adult, 28-week-old agt knockout (KO) mice compared to wild type (WT) mice. Since recent studies by Massiera et al. demonstrated that reexpression of agt in adipose tissue of KO mice normalized adiposity, blood pressure and kidney abnormalities, we used microarray analysis to investigate changes in gene expression profile in kidneys of KO *vs.* Tg-KO mice, where agt expression is restricted to adipose tissue. Body weight, adiposity and insulin levels were significantly decreased ($p<0.05$) in KO mice fed a chow diet (CD) compared to WT mice, while circulating leptin levels were similar. On a high fat diet, compared with WT mice, KO mice exhibited significantly lower body weight ($p< 0.05$), adiposity ($p<0.05$), leptin and insulin levels ($p<0.05$). In agreement with previously reported changes in kidney histology, agt KO mice displayed altered expressions of genes involved in blood pressure regulation and renal function, but these levels were corrected by reexpression of agt in adipose tissue. Collectively, these findings further document important endocrine roles of adipocyte agt, in part via regulation of lipid metabolism and kidney homeostasis.

B. INTRODUCTION

Obesity is a major health problem and a common risk factor for cardiovascular diseases. Adipose tissue is a central organ in glucose and lipid metabolism. In addition to its function as the major storage depot for triglycerides, adipose tissue is an active organ, sensing metabolic signals and secreting hormones that affect whole-body energy homeostasis (1-3). Adipose tissue possesses all components of the renin angiotensin system (RAS) that are necessary to generate the hypertensive hormone Angiotensin II (Ang II) (4, 5). Angiotensinogen (agt) expression is differentiation-dependent, nutritionally and hormonally regulated and differentially regulated in adipose tissue of lean *vs* obese mice and rats (6-8). Ang II regulates adipocyte metabolism by increasing lipogenic gene expression in a glucose dependent manner (9). The adipocyte determination and differentiation factor 1 (ADD1) / sterol regulatory element binding protein 1c (SREBP 1c) mediates these effects as a transcription factor binding the Ang II and insulin response E box (9). Furthermore, several epidemiological and genetic studies have suggested a potential role of adipose Ang II in obesity-related hypertension (10-12). These reports have shown an association between genetic polymorphism of human agt gene and variation in body fat distribution in men (13, 14). A positive correlation between plasma agt level, BMI and blood pressure in humans has also been reported (15). More recently, studies by Massiera et al demonstrated that the sole expression of agt in adipose tissue is sufficient to increase blood pressure, renal morphology and adiposity in young mice (12-20 weeks of age) (16,17). Thus, the purpose of the present work is to extend these studies to older mice (28 weeks of age) fed a standard chow diet or a high fat diet (45 % of calories as fat) and determine effects of agt inactivation or restricted expression to adipose tissue on lipid metabolism and renal gene

expression.

C. MATERIALS AND METHODS

1. Experimental Animals

The agt gene was inactivated in the mouse as previously described (16, 18). Transgenic mice (aP2-agt$^{+/-}$) were generated by using transgenic construct containing adipocyte P2 (aP2) gene promoter, intron, agt cDNA, polyadenylation site. Mice expressing agt only in adipose tissue (Tg-KO) were generated by crossing transgenic mice with agt-deficient mice (16). WT (ICR-CDl strain, Harlen, Gannat, France), KO and Tg-KO male mice provided by Dr. Gerard Ailhaud (University of Nice, France) were housed 4-6 per cage and fed either a chow diet or a high fat diet containing 3% corn oil, 21% lard (representing 45% of calories as fat), 35% carbohydrate, 20% protein and 1.2 % minerals (Research diets, NJ, USA), and water *ad libitum* in a 12:12 h light: dark cycle at constant temperature (22 °C). Body weight was measured and mice were sacrificed at 28 wk for body compositional analysis. Individual adipose depots were removed along with liver and kidney and the epididymal fat pad was weighed. Mice were generated at the CNRS 6543, Centre de Biochimie at Nice, France, and then shipped to the Department of Nutrition at the University of Tennessee, Knoxville. All experiments described were conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee.

2. Plasma Measurements

Following an overnight fast, blood was collected by cardiac puncture for glucose, insulin and leptin assay. The plasma was separated by centrifugation at 12,000 rpm for 20 min and stored at -80 ^{0}C. Plasma leptin and insulin levels were measured by RIA using a mouse leptin

and rat insulin RIA kits, respectively, obtained from Linco Research (St. Charles, MO). Blood glucose concentrations were measured using a glucose analyzer (LIFESCAN Co, MI, USA).

3. GeneChip Microarray Assay and Data Analysis

Total RNA from kidneys of KO and Tg-KO mice maintained on a Chow Diet (CD) was isolated by the cesium chloride density gradient method and further purified by phenol chloroform extraction. Microarray analysis was conducted using the Affymetrix Murine Genome U74v2 set that contains probe sets interrogating approximately 36,000 mouse genes and EST clusters and was performed at the Genome Explorations Inc. in Memphis, TN. Kidneys from four to six KO or Tg-KO mice were used and pooled into 2 sets each; 2 aliquots from the each pool for each genotype (see Appendix Fig 1). Total renal RNA (~20μg) from each of these genotypes was reverse-transcribed and the phycoerythrin dye was incorporated to the second strand. cRNA was synthesized from cDNA and biotinylated and hybridized to a set of Murine Genome GeneChips. Following hybridization and washing, the overall fluorescence intensity across each chip was scaled to a target intensity of 1500 using Affymetrix GeneChip Microarray Suite 5.0 software, and pairwise comparisons of mRNA levels were performed. Each experiment was performed twice in each genotype and a minimum expression ratio of 2-fold differences was used as the selection criteria for the differentially expressed genes between the KO (2 sets) and Tg-KO mice (2 sets). The fold changes in gene expression were presented as mean of the four datasets which are associated with renal function and blood pressure regulation.

4. Statistical Analysis

All data are expressed as the mean ± SEM. Student's test and ANOVA were used to compare overall group means. The values were examined by the Bonferroni test for multiple

comparison (SAS, Carry, NC) to compare differences among group means after a significant F-test and statistical significance was defined as $p<0.05$.

D. RESULTS

1. Effects of Diet and agt Inactivation on Metabolic Markers

The agt deficient mice fed a chow diet or high fat diet showed significantly decreased body weight, adiposity index and epididymal fat pad weight, compared to wild type mice (Fig 1). The adiposity of high fat diet fed WT and KO mice were increased 83 % and 106 %, respectively, compared with that of chow-fed WT and KO mice. Consistent with recent observations (16, 17), the epididymal fat pad mass of chow-fed KO mice was 4-fold lower than that of WT mice. Similarly, diet-induced fat pad mass was significantly decreased in KO mice ($p<0.05$). High fat-fed KO mice exhibited a decreased adiposity index, compared to WT mice fed a high fat diet (4.4 $\pm$ 0.9% vs 2.0 $\pm$ 0.1%, n=4-6), suggesting that the KO mice were more resistant to dietary obesity. Collectively, these results support the hypertrophic effect of Ang II on adipose tissue. Two key hormones involved in the regulation of energy homeostasis are insulin and leptin (20, 21). Plasma insulin levels of KO mice were lower than those of WT animals when fed a chow diet or a high fat diet (Fig 2). The high fat diet significantly increased circulating insulin levels as expected in the WT mice but not in the KO mice. Similar changes were seen for leptin (compared to insulin); however leptin levels were comparable between the WT and the KO mice but significantly increased in response to the HFD in the WT and to a lesser extent in the KO mice. Normally, plasma leptin levels closely correlate with body fat (20). However, changes in leptin levels did not strictly parallel the changes in adiposity. Circulating leptin levels were similar in chow-fed WT and KO mice in spite of the fact that epididymal fat mass of chow-fed KO was

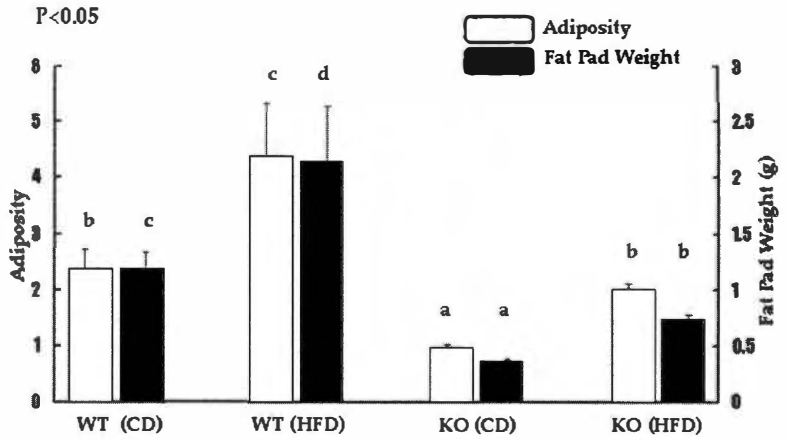

Fig. 1 ***Effects of agt inactivation and high fat diets on adiposity index and fat pad weight.*** 28-wk-old mice fed a high fat diet since weaning were used. Body weight and fat fad weight were measured. Adiposity index (x100) was calculated for each mouse as the ratio of epididymal fat weight divided by body weight minus epididymal fat fad weight. Data are expressed means ± SEM, n=4-6. The Bonferroni test was used to compare differences among group means after significant F-test. Means with different letters are significant different, $p<0.05$.

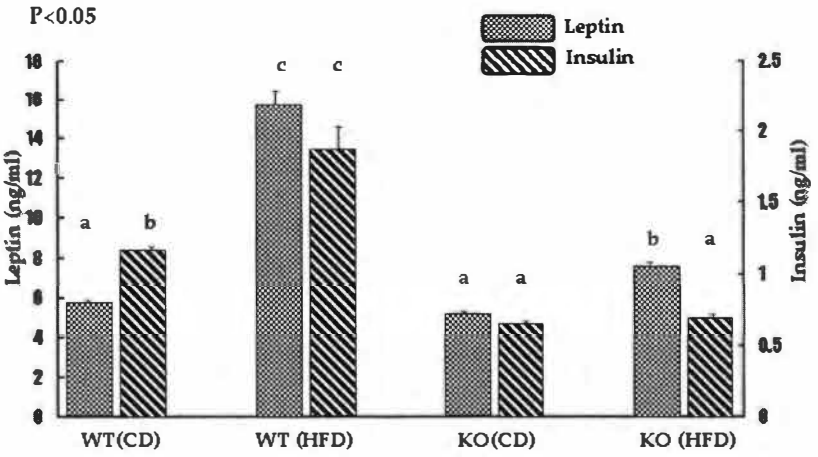

Fig. 2 ***Effects of agt inactivation and high fat diets on plasma leptin and insulin levels.*** 28-wk-old mice fed a high fat diet since weaning were used. Plasma parameters were measured as indicated in the method section. Data are expressed means ± SEM, n=4-6. The Bonferroni test was used to compare differences among group means after significant F-test. Means with different letters are significant different, $p<0.05$.

smaller. Further, leptin levels of high fat-fed KO mice were higher than that of chow-fed WT mice, although their epididymal fat mass was comparable. Consistent with previous observations in 6-week old KO mice (17), 28 week-old KO mice exhibited a lower plasma leptin levels in high fat fed animals, compared with high fat fed WT (Fig 2), suggesting that KO mice are less sensitive to high fat-induced hyperleptinemia and insensitive to HFD-induced hyperinuslinemia. Glucose levels in KO mice remain unchanged when fed a chow or high fat diet, compared with WT mice (data not shown).

2. Effect of Adipose agt on Renal Gene Expression

Previous studies have shown that the plasma agt levels of KO mice were normalized by reexpression of agt in adipose tissue (16). These studies have shown that in accordance with normalized agt levels in KO mice, the expression of adipose agt reversed hypotension and renal abnormalities resulting from agt inactivation (16). Further, agt reexpression in adipose tissue corrected increased water intake and urine output of altered renal functions at 12-wk-old mice (16). Tg-KO mice exhibited increased body weight and fat pad weight compared with KO mice, in agreement with the observation from recent study (16) (data not shown). Reduced leptin levels of KO mice were restored by repression of agt in adipose tissue of KO mice (Fig 3). However, while insulin levels were higher in Tg-KO mice versus KO mice, this difference was not statistically different (Fig 3). To investigate gene expression profiles of kidneys which are related to renal function and blood pressure regulation, we used Affymetrix microarrays to detect changes in mRNA expressions in kidneys of KO *vs.* Tg-KO mice fed a chow diet (Table 1). In agreement with increased renin mRNA and protein levels previously reported in KO mice compared to WT mice (16), our KO mice displayed increased renin (~11 fold) gene expression

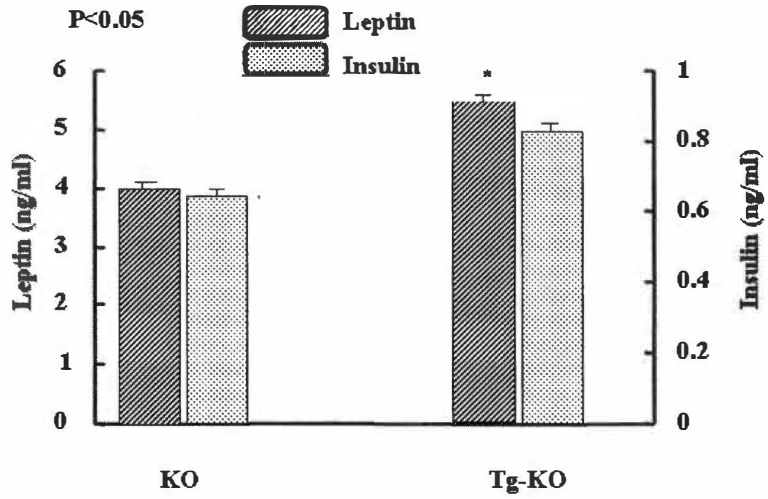

Fig. 3 ***Effect of agt inactivation or agt reexpression in adipose tissue on plasma leptin and insulin levels.*** 28-wk-old mice fed a chow diet were used and total RNA was prepared from kidneys. Differential gene expression was analyzed by gene microarray as indicated in the Appendix section. Data are expressed means ± SEM, n=4-6. Means with different letters are significantly different, $p<0.05$.

Table 1. ***GeneChip dataset of mRNAs related to renal function and blood pressure regulation, showing ≥2-fold differences in levels between 28 week old KO and Tg-KO mice***

Classification/Function	*GeneBank Accession Number*	*Genes*	*Fold changes in expressions (KO vs. Tg-KO)*
RAS	M32352	Renin (Ren-1-d)	**↑ (11)**
	J04946	Angiotensin converting enzyme (ACE)	**↑ (2)**
Ion channels	AF047838	Calcium-sensitive chloride conductance protein-1 (mCLCA1)	**↓ (1.9)**
	AF029347	Chloride channel protein 3 (CLCN3)	**↓ (2.5)**
	U61085	Thiazide-sensitive Na-Cl cotransporter	**↓ (2.4)**
	AF012834	Inwardly rectifying potassium channel ROMK-2	**↓ (2.8)**
Ang II signaling molecules	X58289	Protein tyrosine phosphatase receptor type B	**↓ (3.6)**
	D84376	mRNA for phosphatidic acid phosphatase	**↓ (2)**

↑: Increase, ↓: Decrease, ( ): Fold changes expressed as Mean

compared to Tg-KO mice. Along with upregulated renin expression in kidneys of KO mice, two-fold increased expression of angiotensin converting enzyme (ACE), which is one of renin angiotensin system (RAS) components, was found in KO mice. In addition to altered RAS component gene expressions, differences in ion transport/ channel gene expressions were observed between KO and Tg-KO mice. Calcium-sensitive chloride conductance protein-1, thiazide sensitive Na-Cl cotransporter, chloride channel protein 3 and potassium channel ROMK-2 expressions were down regulated by 2-3 folds in KO mice compared with those of Tg-KO mice. As anticipated, changes in Ang II signaling molecule gene expression were also observed in KO mice; protein tyrosine phosphatase receptor type B and mRNA for phosphatidic acid phosphatase gene expressions which are documented to mediate AT2 receptor signaling pathways (26), were decreased by ~4 folds and 2 folds in KO compared with Tg-KO mice, respectively. We were not able to detect expression of AT1 gene in these studies and no changes in AT2 receptor expression were observed. Taken together, these results confirm that adipose tissue agt contributes to kidney homeostasis via endocrine mechanisms that affect renal gene expressions.

E. DISCUSSION

In the present study, the endocrine roles of adipose agt were studied using agt null and transgenic mice. Several studies have suggested various roles of Ang II in adipose tissue: 1) control of local blood flow 2) stimulation of PGI_2 synthesis 3) lipogenesis 4) gene expression 5) cell cycle regulation 6) estrogen production (7, 27-30). Studies on agt knockout and transgenic mice have revealed that adipose agt acts as a trophic factor in body fat mass and a modulator in blood pressure, emphasizing the potential role of adipose agt as an endocrine effector of obesity-

associated hypertension (16). Subsequent comparison experiments of agt knockout and control wild-type mice have supported that adipose agt regulates adipose tissue development in young mice (6 week of age) by modulating endogenous lipogenesis as well as locomotor activity (17). Our studies also confirmed that agt is involved in the regulation of fat accretion in adipose tissue of older mice (28 weeks of age). In addition to decreased body fat mass in agt deficient mice, plasma concentrations for key metabolic hormones, leptin and insulin levels were reduced along with decreased body fat mass, indicating a trophic effect of adipose agt. However, in previous studies, similar levels of leptin and insulin in agt KO and control WT mice were observed in both chow-fed and high-fat fed animals (17). It is possible that this is an age-related resistance to diet-induced hyperleptinemia and hyperinsulinemia since younger mice (6 weeks of age) were used in these studies (16, 31). Further, the direct role of adipose agt in blood pressure regulation has been proposed (16). In the present study, as an approach to understand mechanisms, which account for changes in kidney homeostasis, we used microarray analysis to investigate differential gene expression in kidneys for KO and Tg-KO mice. Upregulated renin and ACE expressions in KO mice were in accordance with the observations of altered renin expression and protein levels in KO mice (16). Consistent with renal dysfunction observed in mice lacking agt, ion channel-related gene expressions in kidneys were suppressed in KO mice. These ion channel related genes have been identified as important blood pressure regulatory genes that are responsible for renal function and also associated with Bartter's syndrome which is a hyperkalemic alkalosis with dehydration, hypotension and severe polyuria and caused by null mutations in any of Na^+-K^+-$2Cl^-$ cotransporter, ROMK potassium channel and CLC-KB chloride channel (22-25). Additionally, Ang II signaling molecule expressions were also downregulated by agt inactivation. However, agt

deficiency-induced renal abnormalities and hypotension were restored by adipose agt reexpression, reflecting that adipose agt is released into the blood stream and mediates blood pressure regulation (16, 32). Ang II also modulates other physiological functions such as fibrinolysis, via upregulation of PAI-1 expression (33). Ang II receptors are expressed in several tissues including adipose tissue and kidney that express both AT1 and AT2 subtypes (28, 34). Future studies will determine Ang II receptor subtypes mediating the paracrine and endocrine effects of adipose Ang II in both adipose tissue and kidney. Collectively, our results confirm a critical role of agt in adipose tissue metabolism and further directly support a regulatory role of adipose agt in body blood pressure as well as kidney homeostasis.

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PART V

CROSSTALK BETWEEN ANGIOTENSIN II AND INSULIN SIGNALING IN 3T3-L1 ADIPOCYTES

The manuscript is being prepared for publication with Co-authors Jones Voy, B., Huang, T.Y., Koontz, J. and Moustaid-Moussa, N for the Biochem J

A. ABSTRACT

In adipocytes, Angiotensin II (Ang II) exerts actions on lipogenesis by increasing fatty acid synthase (FAS) gene transcription in a paracrine/autocrine manner. This effect was mediated by glucose and the transcription factor ADD1/SREBP1c. In the present study, we have elucidated the potential signaling mechanisms mediating FAS promoter and enzyme activity regulation by Ang II in adipocytes. Since Ang II was shown to mimic insulin actions in regulation of FAS gene and simultaneous stimulation of both hormones did not induce an additive effect on FAS transcription, we have proposed that the lipogenic hormone Ang II may act on the same signaling molecules as those used by insulin in adipocytes. Inhibition of phosphatidylinositol 3-kinase (PI3-K) abolished the Ang II-dependent increase in FAS enzyme and promoter activities whereas repression of the mitogen-activated protein kinase (MAPK) cascade was without effect or had a stimulatory effect. Ang II caused rapid activations of insulin receptor (IR)β subunit and insulin receptor substrate-1 (IRS-1) which are initial components in the insulin signaling cascade and induced the association of p85 with tyrosine-phosphorylated IRS-1, an indicator of PI3-K activation. Furthermore, Ang II stimulated the phosphorylation of the major PI3-K downstream effector, Akt at Ser ^{473}, the critical site for the kinase activation. This action of Ang II was completely abrogated by the AT1 receptor antagonist and only partially by the AT2 receptor antagonist. In summary, this study provides a new mechanistic insight by which Ang II may display its lipogenic effects in adipocytes through the activation of insulin signaling molecules including IRβ, IRS-1, PI3-K and Akt.

B. INTRODUCTION

Ang II, the dominant effector peptide of the systemic renin-angiotensin system (RAS) is a pleiotrophic hormone which plays a critical role in blood pressure regulation and fluid volume balance (1).Various tissues possess their own RAS, which seems to influence local tissue functions. Human and rodent adipose tissues have a functional RAS. The locally generated Ang II exerts its physiological effects via at least two G-protein coupled receptors (GPCR) subclassified as AT1 and AT2. Adipose Ang II has been shown to directly participate in adipose tissue growth and development by significantly increasing triglyceride stores as well as indirectly in adipogenic differentiation concomitant with stimulating release of the autocrine adipogenic effector prostacyclin (2, 3). Earlier studies from our lab have demonstrated that the lipogenic mechanisms of Ang II are linked to increasing activity and gene expression of the two key lipogenic enzymes, fatty acid synthase (FAS) and glycerol-3 phosphate dehydrogenase (GPDH) in an AT2 receptor-dependent manner, accumulating triglyceride content and ultimately increasing adiposity in human and 3T3-L1 adipose cells (2, 4). Of significance, Ang II exhibits insulin-like action in the regulation of FAS gene transcription in adipose cells. The molecular mechanisms underlying these effects by Ang II stimulation in 3T3-L1 adipocytes were dependent on glucose and a transcription factor ADD1 (adipocyte determination and differentiation factor 1) that binds to the insulin-responsive element (E-box) within the proximal FAS gene promoter (5). Further, the signaling mechanism for insulin regulation of FAS transcription has been shown to involve activation of phosphoinositide 3-kinase (PI3-K)-Akt pathways in 3T3-L1 adipose cells (6). Accordingly, we hypothesized that given the insulin-like effect of Ang II in adipocytes, this hormone is likely to mediate its effects on the activation of FAS transcription via insulin signaling

molecules such as PI3-K and mitogen-activiated protein kinase (MAPK), which play important roles as mediators in insulin-induced glucose transport, lipogenesis and mitogenesis. To test this hypothesis, we first investigated the effects of the inhibitors for well-documented insulin-stimulated kinases like PI3-K and MAPK on Ang II-induced FAS promoter and enzyme activities. Next, we examined the action of Ang II with signaling molecules (*e.g.* IR, IRS-1, PI-3K and Akt), which involve an early step of PI3-K cascade or MAPK cascade in insulin receptor signaling. Our results suggest that Ang II regulates adipocyte metabolism by positive crosstalk with the PI-3K mediated pathway of the insulin signaling cascade. This Ang II effect was mediated by both Ang II receptors (AT1 and AT2).

C. MATERIALS AND METHODS

1. Reagents and Antibodies

Human Ang II was purchased from Sigma (St. Louis, MO).Rabbit polyclonal anti-IR, rabbit polyclonal anti-IRS-1, phosphospecific anti-Akt/PKB, anti-Akt/PKB and secondary antibodies as well as protein A/G-Sepharose were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-phosphotyrosine antibody (clone 4G10) was from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibody against the p85α regulatory subunit of PI3-K was purchased from BioSource International (Camarillo, CA). Phosphospecific-ERK1/2, also known as p42/44 MAPK and anti-ERK1/2 were from Cell Signaling Technology (Beverly, MA). PI3-K inhibitor, LY294002 was purchased from Sigma (St. Louis, MO) and MEK1/2 inhibitor, PD 98059 was from New England Biolabs (Beverly, MA).

2. Cell Culture

3T3-L1 fibroblast cells (American Type Culture Collection, Rockville, MD) were grown and differentiated as described previously (7). In brief, cells were grown to confluence in standard DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum. At confluency, cells were induced to differentiate by the addition of dexamethasone (250nM) and isobutylmethyxanthine (0.5mM) to standard medium for 72h. Cells were maintained for additional 3 days in standard medium, then changed to serum-free medium (SFM containing 1% (v/v) BSA) for 12h followed by treatment with Ang II (10nM), insulin (10nM) and various inhibitors of insulin signaling molecules as indicated in the figure legends. All treatments were performed in SFM to test individual and direct hormone effects. FAS enzyme activity was measured after 24h and normalized to protein content.

3. Transient Transfections

3T3-L1 adipocytes were transfected with FAS-luciferase gene fusion constructs (2μg/well) containing two direct repeats of sequences spanning the E box, -70 to -54 (CAGCCCATGTGGCGTGG) and pGL2-control plasmid (2μg/well) using the calcium phosphate-DNA co-precipitation method (Gibco BRL, Bethesda, MD) as described previously (8). Transfection efficiency was monitored by cotransfection of β-galactosidase expression vector pSV-βgal (0.2μg/well) (Promega). The differentiated transiently transfected adipocytes were maintained overnight in SFM. Before adding 10nM of Ang II or 10nM of insulin, cells were pretreated with SFM supplemented with vehicle (0.1% Mg_2SO; DMSO), 50μM LY294002 (PI-3K inhibitor) or 100μM PD98059 (MAPK Kinase inhibitor) for 1h and a second batch of inhibitors was added after 12h of Ang II or insulin treatment. Luciferase activity was assayed

after 24h of transfection in transient transfection experiments.

4. FAS Enzyme Activity Assay

Cells were harvested by scraping into 250ul of 0.25M sucrose buffer (pH 7.4) containing 0.1mM phenylmethysulfonyl fluoride (PMSF), 1mM dithiothreitol and 1mM EDTA. Cell homogenates were sonicated for 5 sec and ultracentrifuged (12,000 x g) for 30 mins at 4 °C. FAS enzyme activity in crude cytosolic extracts of adipocytes was measured spectrophotometrically by monitoring the oxidation rate of NADPH at 340 nm. The enzyme activity was normalized to per mg protein as previously described (8).

5. Reporter Gene Assays

For luciferase assays, the cells were lysed in 100mM potassium phosphate (pH 7.8)/0.2% (w/v) Triton X-100/1mM dithiothreitol. The cytosolic extracts were used to measure luciferase as well as galactosidase activities with a luminometer (PerkinElmer, Boston, MA) and a kit for luciferase assay (Tropix). Luciferase activity was normalized to protein content measured with the Bradford assay (9).

6. Immunoprecipitation

After various stimulation times (5mins, 10mins, 30mins, 1h), cells were lysed in ice-cold lysis buffer (20mM Tri-HCl, pH 7.4, 100mM NaCl, 1mM EDTA, 1mM EGTA, 2mM Na_3VO_4, 20mM $Na_2P_2O_7$, 1mM NaF, 1% Trition X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1mM PMSF, 5% (v/v) protease inhibitor cocktail (Sigma) and 1% Nonidet P-40) at 4 °C for 30 mins. The cell lysates were centrifuged at 10,000 x g for 30mins at 4 °C to remove cell debris and nuclei. Protein was determined in the cleared supernatant using the Bradford assay reagent. For immunoprecipitation, equal amounts of protein (~1000 μg) were incubated with appropriate

antibodies overnight with gentle agitation at 4 °C, followed by addition of 50ul protein A/G-Sepharose beads into immune complexes and further incubation at 4 °C for 5h on a rotating device. The immunoprecipitates were gentle washed five times in cold lysis buffer and solubilized in 50ul of 2 X SDS/PAGE sample buffer and then boiled for 5mins. The soluble supernatants were further analyzed by SDS/PAGE. The antibodies and their concentrations used in immunoprecipitation were anti-phosphotyrosine antibody (5μg/ml) and anti-IRS antibody (5μg/ml).

7. Immunoblot Analysis

Cells were prepared as described above for the immunoprecipitation method. For immunoblotting, protein sample (25~50μg) were resolved by 8-10 % SDS/PAGE and electronically transferred onto nitrocellulose membrane. The membranes were blocked with Tris-buffered saline-0.05% Tween 20 (TBST) containing 5% non fat dry milk for overnight at 4 °C or BSA for 3hrs at 23 °C. The blots were then incubated with an appropriate dilution of primary antibody, washed six times in TBST and further incubated with the appropriate secondary antibody conjugated to horseradish peroxidase and then washed another six times in TBST. Bound peroxidase was visualized by a high sensitive chemiluminescence system (ICN Biomedicals, Inc., Costa Mesa, CA). The bands obtained in the western blots were scanned and quantified using Zero-Dscan software (Scanalytics Inc., Fairfax, VA). The antibodies and their concentrations used in immunoblotting are phosphospecific anti-ERK1/2 (20ng/ml), anti-IR antibody (2μg/ml), anti-IRS antibody (2μg/ml), anti-p85α antibody (5μl/ml), phosphospecific-anti-Akt/PKB antibody (0.4μg/ml) and anti-Akt/PKB antibody (0.4μg/ml).

8. Statistical Analysis

Data comparison was performed using one-way or two-way ANOVA and presented as mean ± SEM. If F-ratio was significant, further comparisons were made using the Bonferroni test. Values of $p<0.05$ were considered significantly different.

D. RESULTS

1. PI3-K mediates Ang II-induced FAS enzyme and promoter activities

Ang II has previously been shown to act via an insulin-like mechanism in the regulation of FAS activity, mRNA level and rate of gene transcription in adipocytes (5). Recently, it has been shown that PI-3K and Akt/PKB play roles as mediators in insulin-induced FAS promoter activation in adipose cells (6). To define potential signaling mechanisms responsible for the Ang II-induced FAS transcription in adipocytes, we first examined the effects of inhibitors for Ang II and insulin signal transduction pathways on Ang II-stimulated FAS enzyme and promoter activities. As shown in Fig 1& 2, Ang II and insulin significantly induced FAS enzyme and promoter activities in non-transfected 3T3-L1 and transiently transfected adipose cells ($p<0.05$), consistent with previous data from our lab (5). A PI3-K inhibitor, LY 294002, significantly suppressed Ang II-dependent increases in FAS enzyme and promoter activities ($p<0.05$). In contrast, the responsiveness to Ang II was essentially unaffected or increased by PD 98059, an inhibitor of MAPK Kinase (MEK). Consistent with previous data, with this MEK inhibitor treatment, insulin-stimulated FAS enzyme and promoter activities were also enhanced (6). In accordance with previous results, insulin responsiveness was significantly decreased by 50μM of LY 294002 treatment ($p<0.05$) (6). In addition, no interactions between both hormones and

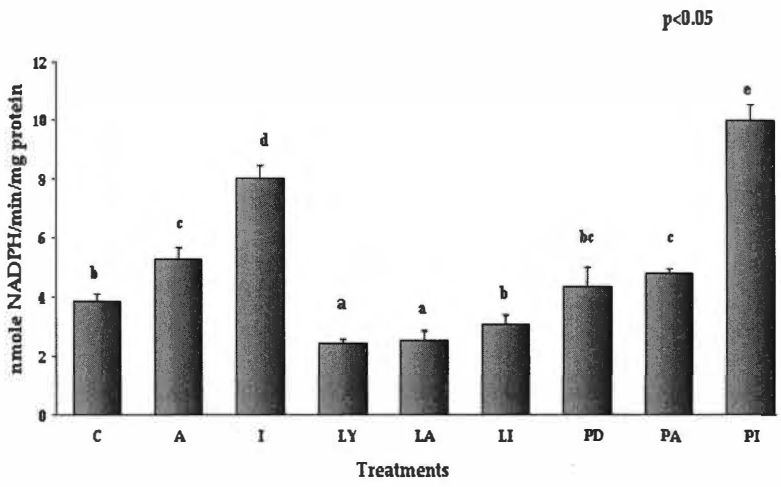

Fig. 1 ***Ang II stimulation of FAS enzyme activity is dependent on PI3K.*** Differentiated adipocytes were pretreated with or without vehicle (0.1% DMSO), 50μM LY 294002 (PI3K inhibitor) or 100μM PD 98059 (MEK inhibitor) for lh and stimulated with or without 10nM Ang II or 10nM insulin for 24h. A second aliquot of the inhibitors was added after 12h treatment with Ang II or insulin. FAS activity was measured after 24h and normalized to protein content. [a,b,c,d] Values with different letters are significantly different ($p<0.05$). Results are representative of four separate experiments (n=6). Control, C; Ang II, A; Insulin, I; LY 294002, LY; LY 294002 plus Ang II plus, LA; LY 294002 plus insulin, LI; PD98059, PD; PD98059 plus Ang II, PA; PD98059 plus insulin, PI

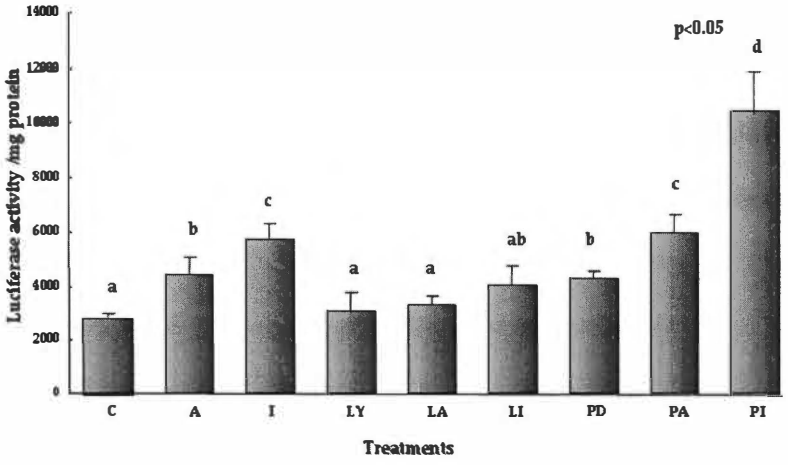

Fig. 2 ***Ang II stimulation of FAS promoter activity is dependent on PI3K.*** Adipocytes were transfected with FAS-luciferase constructs containing E box, -70 to +67 of FAS promoter or empty vector. The differentiated transfected adipocytes were pretreated with or without vehicle (0.1% DMSO), LY 294002(50μM) or PD98059 (100μM) for 1h and stimulated with or without 10nM Ang II or 10nM insulin for 24h. A second aliquot of the inhibitors was added after 12h treatment with Ang II or insulin. Luciferase activity was normalized to protein. ^{a,b,c,d} Values with different letters are significantly different ($p<0.05$). Results are representative of four separate experiments (n=6). Control, C; Ang II, A; Insulin, I; LY 294002, LY; LY 294002 plus Ang II plus, LA; LY 294002 plus insulin, LI; PD98059, PD; PD98059 plus Ang II, PA; PD98059 plus insulin, PI

inhibitors were observed. This result suggests involvement of PI3-K not MAPK in both hormone-induced FAS transcription in adipose cells. To confirm the above results, we examined the effect of Ang II stimulation on dual phosphorylation levels of MAPK (ERK1/2) at different time points, which reflects the activation of MEK and indirectly MAPK cascade. As expected, insulin treatment led to a marked increase in the dual phosphorylation of ERK1/2 by about 4-fold at 5 min, compared with control (Fig 3, upper panel) (10). Conversely, Ang II did not modify basal activities of ERK1/2 for 10 min of incubation and then blunted basal ERK1/2 phosphorylation after 30 min (Fig 3, upper panel), suggesting that MAPK pathway is not likely to be involved in FAS transcription activation in response to Ang II. The amount of total cytosolic ERK1/2 was similar (Fig 3, lower panel).

2. Ang II stimulates phosphorylation of IRβ, IRS-1 and p85α association with IRS-1

To further elucidate possible mechanisms of crosstalk between Ang II and insulin receptor signaling in adipocytes, we studied the effect of Ang II on tyrosine phosphorylation of major upstream mediators of PI3-K in the process of insulin signaling. As expected, 10nM insulin treatment for 5 min caused rapid tyrosine phosphorylation of IRβ (Fig 4, upper panel) and IRS-1 (Fig 4, lower panel). Compared with the response to insulin, IRβ (Fig 4, upper panel) and IRS-1 (Fig 4, lower panel) activations were delayed in the presence of Ang II. These stimulatory effects by Ang II on IRβ were peaked at 10 min but reduced by 30 and 60 min of incubation, representing approximately 2-fold and 3-fold decreases compared with those produced by 10 min Ang II stimulation, respectively ($p<0.05$). Similar to Ang II-induced IRβ activation, IRS-1 phosphorylation was observed at 10 min Ang II treatment and this phosphorylation also was reduced by longer treatment of Ang II. The tyrosine-phosphorylated IRS-1 is essential for its

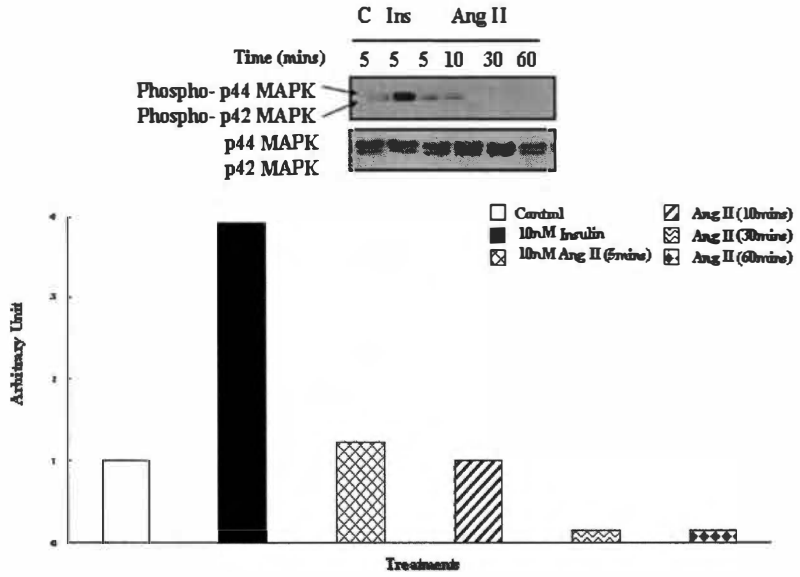

Fig. 3 ***The inhibitory effect of Ang II on p42/44 MAPK phosphorylation.*** Fully differentiated adipocytes were stimulated with or without 10nM Ang II or 10nM insulin as a positive control for the indicated times. The cell lysates were analyzed by immunoblotting with anti-phospho-p42/44 MAPK antibody (Thr^{183} /Tyr^{185}) and anti-p42/44 MAPK antibody for loading control. A representative autoradiograph of phosphorylated ERK1/2 and bar graph of quantitation of two experiments are shown.

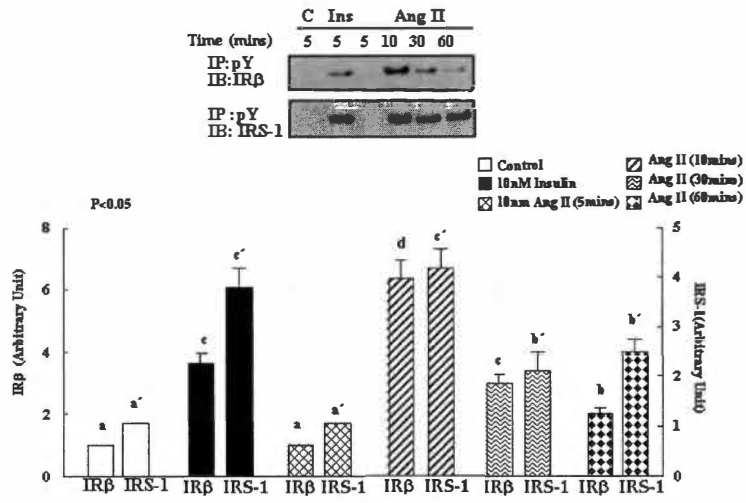

Fig. 4 ***Ang II-induced phosphorylation of insulin receptor β (IRβ) or insulin receptor substrate-1 (IRS-1).*** Fully differentiated adipocytes were stimulated with or without 10nM Ang II or 10nM insulin (positive control) for the indicated times. The cell lysates were immunoprecipitated with antiphophotyrosine antibody and immunoblotted with anti-IRβ subunit antibody or anti-IRS-1 antibody. A representative autoradiograph of phosphorylated IRβ and IRS-1 and bar graph of quantitation (mean ± SEM) from three separate experiments are shown. [a,b,c,d] Values with different letters are significantly different ($p<0.05$).

association with tyrosine phosphorylated IRS-1 as an indicator of PI3-K activation. As shown in binding to SH2 domains of the p85 regulatory subunit of PI3-K and its activation of PI3-K enzymatic activity (10). We next investigated the effect of Ang II on p85α

Fig 5 (upper panel), a low level of IRS-1/p85α association was present in the basal state and this was markedly stimulated by insulin (5min of incubation) and Ang II. The maximal increase of Ang II-induced IRS-1/p85 docking was observed at 10min but the effect was diminished after 30 min of incubation. The amount of IRS-1 immunoprecipitated was similar (Fig 5, lower panel). This implies that PI3-K is a signaling molecule involved in Ang II action in 3T3-L1 adipocytes

3. Ang II stimulates serine phosphorylation of Akt

Akt (protein kinase B, PKB) is a major downstream target of PI3-K in the insulin receptor signaling pathway. Akt activation is associated with insulin-stimulated glucose transport, glycogen synthesis and expression of genes such as FAS (6, 10). Specifically, a recent study has shown that Akt activation was involved in insulin-stimulated ADD1/SREBP1c gene expression in the liver (11). To further investigate whether Akt mediates the effect of Ang II in adipocytes, we assessed serine phosphorylation of Akt in the absence or presence of Ang II. As shown in Fig 6 (upper panel), insulin and Ang II induced significant increases in serine phosphorylation of Akt by about 3-fold and 2.8-fold, respectively, in adipose cells, compared with basal values ($p<0.05$). The longer stimulation of 3T3-L1 adipose cells by Ang II showed slight decrease in Akt activation. The amount of total cytosolic Akt was similar (Fig 6, lower panel). This demonstration indicates that PI3-K-Akt pathway mediates Ang II signaling to the lipogenic response in adipocytes.

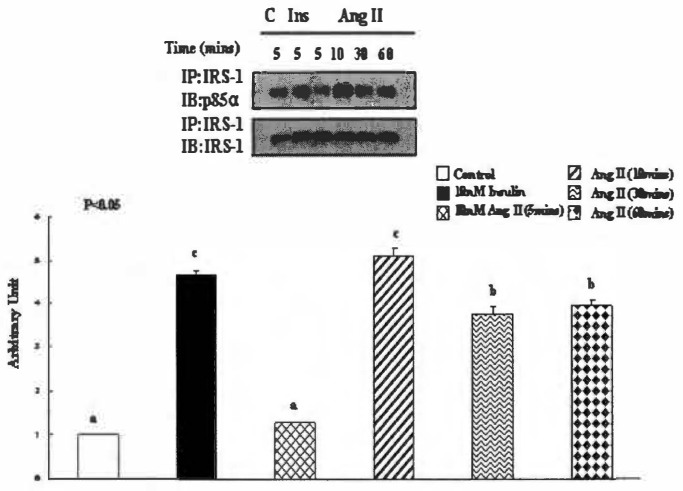

Fig. 5 ***Ang II-induced p85α association with tyrosine-phosphorylated insulin receptor substrate-1 (IRS-1).*** Fully differentiated adipocytes were stimulated with or without 10nM Ang II or 10nM insulin as a positive control for the indicated times. The cell lysates were immunoprecipitated with anti-IRS-1 antibody and immunoblotted with anti-p85α antibody or anti-IRS-1 antibody as loading control. A representative autoradiograph of IRS-1/p85α association and cytosolic IRS-1 and bar graph of quantitation (mean ± SEM) from three separate experiments are shown. [a,b,c,d] Values with different letters are significantly different ($p<0.05$).

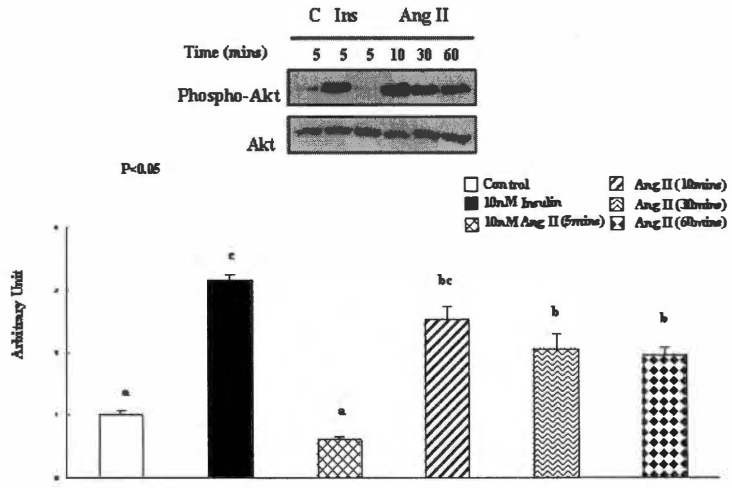

Fig. 6 ***Ang II-induced Ser^{473} phosphorylation of Akt/PKB.*** Fully differentiated adipocytes were stimulated with or without 10nM Ang II or 10nM insulin (positive control) for the indicated times. The cell lysates were analyzed by immunoblotting with anti-phospho-Ser^{473}-Akt/PKB antibody or anti-Akt/PKB antibody as a loading control. A representative autoradiograph of phosphorylated Akt/PKB and Akt/PKB and bar graph of quantitation (mean ± SEM) from three separate experiments are shown. ^{a,b,c,d} Values with different letters are significantly different ($p<0.05$).

4. Ang II receptors mediates Ang II-induced insulin singling molecules

Since Ang II has been shown to increase FAS activity in an AT2-dependent manner (2), we determined whether Ang II-induced phosphorylation events are receptor-mediated. Fig 7 shows that pharmacological inhibitor of AT1 receptor activity, losartan, completely blocked stimulatory effect of Ang II on IRS-1 (Fig 7, upper panel) and Akt activation (Fig 7, lower panel) but PD-186, an AT2 receptor antagonist only partially altered this Ang II action, indicating that Ang II-stimulated IRS-1 and Akt activations are primarily mediated via AT1 receptors and partly through AT2 receptors.

E. DISCUSSION

We have previously reported Ang II exerts several biological effects in adipocytes including lipogenic actions and prostaglandin secretion (5). We showed that Ang II increased activity and mRNA level and gene transcription of FAS, a key lipogenic enzyme (5). Furthermore, we have recently defined the Ang II-responsive element (E box motif) to the proximal promoter region and have demonstrated that transcription factor ADD1 is functionally required for Ang II regulation of the FAS gene (5). Likewise, the well-known lipogenic hormone insulin increases triglyceride accumulation via FAS gene activation in adipocytes. Both hormones did not synergize in the context of stimulation of the FAS gene (5, 6). With respect to signaling mechanisms for insulin-mediated gene regulatory effects, a considerable number of studies have found that insulin positively regulates the expression of specific genes such as FAS, GLUT1, glucose kinase (GK) and SREBP1c via activation of the PI3-K/Akt branch of insulin signaling pathways in insulin-responsive tissues (6, 12-14). However, the Ang II signaling mechanisms

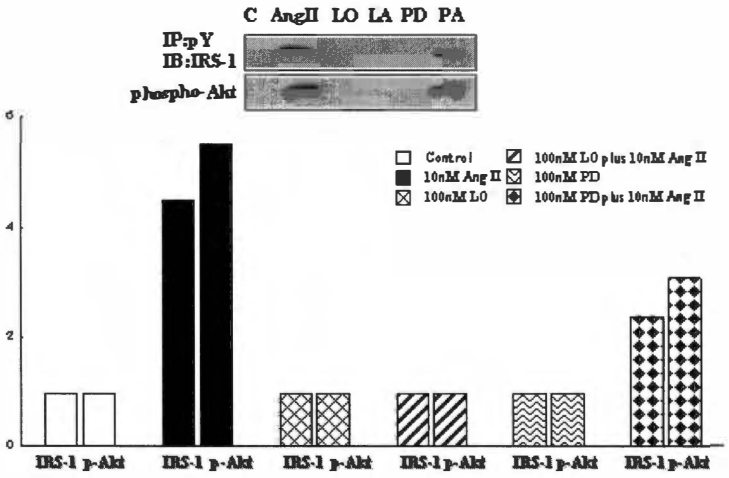

Fig. 7 ***Angiotensin receptor inhibitors inhibit Ang II-induced IRS-1 and Akt/PKB activations.***
Fully differentiated adipocytes were pretreated with vehicle or 100nM losartan (AT1 receptor antagonist) or 100nM PD-186 (AT2 receptor antagonist) for 1h and stimulated for 10min in the absence or presence Ang II. Immunoprecipitates (IP) of anti-phosphotyrosine antibody were analyzed by immunoblotting with anti-IRS-1 antibody or total cell lysates were immunoblotted with anti-phospho-Ser^{473}-Akt/PKB. An autoradiograph of phosphorylated IRS-1 and Akt/PKB and bar graph of quantitation of two experiments are shown. Control, C; Ang II; Losartan, LO; Losartan plus Ang II plus, LA; PD-186, PD; PD-186 plus Ang II, PA.

which mediate Ang II action in adipocytes are largely unknown. In the present study, we explored Ang II signaling adipocytes, focusing on the potential intracellular interactions between Ang II and insulin signaling that may lead to regulation of adipocyte metabolism. The results from PI-3K and MAPK inhibitor experiments suggest that the Ang II effect on FAS gene and enzyme activities was virtually unaffected or enhanced by the MEK inhibitor, PD98059, suggesting that Ang II-stimulatory effect on FAS gene does not appear to require activation of the MAPK cascade. By contrast, data from LY294002 clearly implies the involvement of the PI-3K signaling pathway in Ang II regulation of FAS gene. Furthermore, the collective results from immunoblotting analysis for IRβ, IRS-1, IRS-1/p85α association and Akt indicate that Ang II signaling in adipose cells is linked to PI-3K cascade via functionally positive crosstalk with important mediators involved in key intermediate steps within insulin signaling pathways. Accordingly, this study provides the first evidence that the Ang II-activated IRβ/PI-3K/Akt pathway is responsible for Ang II regulation of FAS gene expression in adipocytes.

In both *in vivo* and *in vitro* studies, Ang II has shown to positively or negatively interact with insulin signaling pathways at multiple levels via AT1 and AT2 receptors in Ang II-responsive tissues such as the vasculature (15-20). These intracellular interactions of Ang II with insulin-mediated pathways have been strongly implicated in pathophysiologic states, *e.g.* insulin resistance in hypertension, cardiovascular diseases such as atherosclerosis and cardiac myopathy in type 2 diabetes (15-21). In terms of molecular mechanisms of Ang II-induced peripheral insulin resistance, Ang II has been demonstrated to interfere with insulin signaling pathways though inactivation of insulin signaling molecules in Ang II-responsive tissues such as vascular smooth muscle cells and cardiac myocytes (16, 17, 21), although the precise mechanism remains

elusive. Further, fat insulin receptor knockout mouse models have shown that insulin signaling interruption in adipocytes is associated with decreased fat mass and altered adipocyte size rather than insulin resistance, indicating that insulin signaling in adipose cells is not critical for the maintenance of euglycemia but is required for triglyceride storage in adipocytes (22).

Here, our data show that like insulin, acute Ang II treatment is sufficient to promote IRβ subunit and IRS-1 kinase activities. These activations may be dependent on activity of JAK2 which was shown to serve as an important linker protein involved in mediating AT1 receptor-mediated IRS-1 and IRS-2 phosphorylation by Ang II in cardiac tissues (19, 23). As a consequence of IRS-1 activation, Ang II-induced IRS-1/PI-3K association is significantly increased. This, in turn, stimulates serine phosphorylation of Akt. Thus, Ang II exhibits its lipogenic actions by enhancing insulin signaling pathways in adipocytes, suggesting that the mechanism underlying Ang II-induced lipogenesis in adipocytes differs from that previously associated with peripheral insulin resistance. In PI-3K-dependent Akt activation, arachidonic acid (AA)/ reactive oxygen species (ROS) pathways have currently been identified to play important roles in Akt activation in Ang II signaling pathway of mesangial cells (24). In the present study, it cannot be ruled out that both PI-3K and other factors (*e.g.* ROS) could also contribute to Ang II-stimulated Akt activation in adipocytes. Insulin-activated Akt has been shown to mediate several biological actions including stimulation of glucose transport, glycogen synthesis and lipogenesis in insulin-responsive tissues (6, 10) whereas the exact role of PI-3K and Akt in Ang II signaling pathways is still unknown. Recently, Akt was shown to phosphorylate several transcriptional activators of the forkhead family including AFX and FKHR (25-27). Further, insulin-induced ADD1/SERBP1c expression was mediated through Akt activation in the liver (11). Taken

together, despite the fact that further targets of this signaling pathway remain unknown, it is reasonable to speculate that Akt activation induced by positive interaction of Ang II with insulin signaling might participate in SERBP1c gene expression via phosphorylation of transcriptional activators involved in transcriptional regulation of SEREP1c. Our previous data, showing that Ang II shows direct effects on ADD1 gene expression in a glucose-dependent manner, support this hypothesis. Furthermore, the transcription factor SERBP1c has been shown to be regulated by phosphorylation/dephosphorylation mechanisms (13). Accordingly, it is plausible that ADD1/SERBP1c activation by Ang II-mediated Akt binds to Ang II-responsive element of the FAS promoter region and thus enhances transcriptional activity of FAS and FAS enzyme activity, consequently contributing to accumulation of triglyceride storage in adipocytes.

AT1 and AT2 receptors have been shown to exert counteracting effects on cellular growth and differentiation in a variety of cell types (1). In our previous study, both receptors showed similar functions in lipogenesis of adipocytes by preventing Ang II induction of FAS activity, however, only AT2 antagonist was able to potently inhibit Ang II binding to 3T3-L1 adipocytes. In this study, the AT2 receptor was partially involved in this Ang II regulation of insulin signaling. This is inconsistent with our previous demonstration that AT2 receptor mainly mediates Ang II-stimulated FAS enzyme activity and gene expression (2). Previously, we observed significant attenuation of FAS activity by inhibition of AT1 receptor eliciting lower binding affinity to this cell line, implying that AT1 receptor also may play a role as a critical mediator in this Ang II-mediated FAS gene regulation in adipocytes. Other numerous studies on the regulation of Ang II receptor expression and Ang II receptor-mediated physiological effects in murine and human adipocytes have shown inconclusive results (28-30). Additionally, another

report showed that activated AT2 receptor has recently negatively interacts with insulin signaling pathway in cardiac tissue thereby inhibiting insulin-induced cell growth and proliferation (15, 20), although the exact signaling pathways and the functional roles of AT2 receptor remain unclear. These observed contradictory data could possibly be due to different species and different cell lines and culture conditions used.

In addition to the function of Ang II in lipogenesis, another potential role of adipose Ang II in obesity-associated insulin resistance was recently proposed (28). The inhibitory effect of Ang II on human preadipocyte recruitment ultimately induces redistribution of fat accumulation to nonadipose tissues such as liver and muscle, thereby resulting in insulin resistance (28). Our present study provides another possible mechanism for insulin resistance in obesity. Adipose Ang II appears to develop insulin resistance arising from fat mass increase though positively modulating insulin signaling mechanism in adipocytes. One possible mechanism is that Ang II stimulates lipogenesis through the transactivation of insulin signaling molecules that further promotes adipocyte hypertrophy, thereby leading to enlargement of adipocyte mass, a characteristic feature of obesity. As a consequence, the secretion of cytokine tumor nuclear factor α (TNFα), free fatty acids (FFA) and resistin which are important linkers between obesity and insulin resistance is elevated, thereby contributing to the development of insulin resistance and increased risk of diabetes in obesity. Thus, adipose Ang II may play a lipogenic role in adipocyte physiology and hence be a major candidate linking obesity and insulin resistance.

In conclusion, our findings indicates that Ang II regulates fat cell metabolism by increasing lipogenic activity via activation of insulin signaling transduction; primarily linked to activation of the IRβ/PI3-K/Akt cascade in adipocytes. This effect appears to be mediated through

both Ang II receptor subtypes. Additional studies are warranted to determine the mechanisms linking Ang II binding to its receptors to activation of insulin receptor and signaling molecules.

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PART VI

IN VIVO ENDOCRINE EFFECTS OF ADIPOSE ANGIOTENSINOGEN ON ADIPOSE AND RENAL TISSUES

The manuscript is being prepared for publication with co-authors Jones Voy, B. and Moustaid-Moussa, N. for the Journal of Hypertension

A. ABSTRACT

Using knockout and transgenic strategies, recent studies provided evidence for the contribution of adipose angiotensinogen (agt) to regulation of fat mass, systemic blood pressure and kidney homeostasis. However, mechanisms that account for the physiological effects of adipose RAS are poorly defined. In the current study, we have used agt knockout ($agt^{-/-}$, KO) mice and transgenic (Tg) mice harboring adipose tissue-specific expression of rat agt (Tg-KO) or rat agt overexpression in adipose tissue (Tg-WT). We examined changes in adipose tissue metabolism and further dissected endocrine effects of adipose agt on regulation of RAS components of adipose tissue and kidney in these mice. Total body weight, epididymal fat pad weight, and circulating leptin and insulin levels were significantly decreased ($p<0.05$) in KO mice compared to wild type (WT) mice. Higher fat pad weight and plasma leptin and insulin levels were observed in Tg-WT mice compared to WT mice. In particular, agt knockout mice had significantly increased plasma adiponectin (an insulin sensitivity marker) and decreased circulating resistin (an insulin resistance marker) levels whereas mice carrying the other three genotypes (WT, Tg-KO and Tg-WT) showed comparable circulating adiponectin and resistin levels. Subsequently, western blot analysis of total kidney protein extracts demonstrated that markedly elevated agt and AT1 receptor protein levels were observed in Tg-WT mice compared with WT animals, implying that adipose agt may regulate systemic blood pressure at least partly mediating the stimulation of renal RAS. Moreover, oligonucleotide microarray analysis illustrated that targeted reexpression of agt only in adipose tissue was able to restore altered expression of

genes associated with renal function and development seen in KO mice in an endocrine manner. Taken together, the results from this study provide possible mechanisms responsible for the endocrine effects of adipose agt. These effects appear to be mediated in part via activation of renal RAS and correction in altered expression of genes associated with renal function or development in combination with hypertrophic actions on adipose tissue.

B. INTRODUCTION

The renin-angiotensinogen system (RAS) plays a central role in blood pressure regulation and fluid-electrolyte homeostasis and may also contribute to essential hypertension (1). In addition to its vascular effects, the RAS has been shown to be an important regulator in renal growth, development and function (1). Angiotensin II (Ang II), the major effector of the RAS, is produced by the successive enzymatic cleavage of the hepatic glycoprotein agt by kidney-derived renin and lung-derived angiotensin-converting enzyme (ACE). Ang II exerts its actions by binding to G-protein coupled angiotensin receptors, AT1 and AT2 (2). Most well-documented biological actions of Ang II are primarily mediated by AT1 receptors. In addition to this circulating RAS, accumulated evidence from studies of angiotensin's physiological properties has suggested that distinct local tissue RAS with different regulatory mechanisms from endocrine RAS may exist and function in the brain, heart, adrenal gland, kidney, vessel wall and adipose tissue (3). Previous studies showed that a variety of stimuli including blood pressure, sodium intake, inflammation, and sympathetic nerve activity modulate the expression of the local RAS mRNA and proteins in physiological and pathophysiological conditions (4). Of note, adipose tissue in rodents and humans possess physiologically active RAS components (2). In adipocytes, Ang II has been implicated in induction of differentiation of preadipocytes into adipocytes via an

adipogenic factor, prostacyclin, and key genes for lipogenesis via a paracrine/autocrine mechanism (5, 6). Currently, increasing evidence has suggested that alterations in adipocyte production of RAS components contribute to metabolic disorders including obesity, obesity-associated hypertension and diabetes (2). Several studies have reported RAS hyperactivity in obese rodents and humans (7, 8). Additionally, subcutaneous and omental adipose agt expression have been shown to be positively associated with increased body mass index (BMI) and waist-to-hip ratio (9, 10). A very recent agt transgenic study also presented that high fat-induced obesity was highly related to increased expression of both mAGT and hAGT genes in visceral adipose depots such as omental, reproductive and perirenal fat (11). Other RAS components, renin and AT1 receptor genes in adipose tissue were upregulated exclusively in obese hypertensive subjects and angiotensin converting enzyme (ACE) was increased in obesity, with highest expression levels in obese hypertensive women (12). Of note, recent studies with agt knockout and transgenic mice expressing agt selectively in adipose tissue have suggested a significant role for adipose agt in obesity-associated hypertension. Agt inactivation was associated with the resistance to high fat diet-induced obesity, reduced adipose tissue mass, increased locomotor activity, profound hypotension and renal abnormalities (13, 14). Adipose tissue-specific restoration of agt partially rescued these phenotypes seen in KO mice whereas moderate overexpression of agt in adipose tissue led to a significant increase in fat pad mass and a doubling in FAS activity. Interestingly, Tg-WT mice exhibited systemic hypertension coupled with elevated plasma agt levels, suggesting that adipocytes are a significant source of circulating agt (14, 15). In addition, the growing body of evidence from experimental, epidemiological and genetic studies have also addressed a potential role for adipose Ang II in obesity-related hypertension (16-19).

Accordingly, in this study, we examined changes in adipose tissue metabolism in these

agt knockout and transgenic mice and metabolic parameters. Further, we determined endocrine effects of adipose agt on expression of RAS components in adipose and renal tissues as well as global expression profile of genes associated with renal regulation in these mice exhibiting distinct phenotypes with respect to adiposity, insulin sensitivity, systemic blood pressure and renal function/morphology.

C. MATERIALS AND METHODS

1. Experimental Animals

The agt gene was inactivated in the mouse as previously described (15, 20). Transgenic mice (aP2-agt$^{+/-}$) were generated by using a transgenic construct containing adipocyte P2 (aP2) gene promoter, intron, agt cDNA and a polyadenylation site (15). Mice expressing agt only in adipose tissue (Tg-KO) were generated by crossing transgenic mice with agt-deficient mice (agt$^{-/-}$, KO), whereas mice overexpressing agt selectively in adipocytes were generated by crossing transgenic mice with wild type mice (agt $^{+/+}$, WT) (15). WT (ICR-CD1 strain, Harlen, Gannat, France), KO, Tg-KO, Tg-WT male mice provided by Dr. Gerard Ailhaud (University of Nice, France) were housed 4-6 per cage and fed a chow diet (Research diets, 20 Jules Lane New Brunswick, NJ) and water *ad libitum* in a 12:12 h light: dark cycle at constant temperature (22 °C). Body weight was measured and mice were sacrificed at 28 weeks for body composition analysis. Individual adipose depots (perirenal and inguinal depots) were removed along with liver and kidney; the epididymal fat pad was weighed. Mice were bred at the CNRS 6543, Centre de Biochimie at Nice, France, and shipped to the Department of Nutrition at the University of Tennessee, Knoxville. All experiments described were conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee.

2. Plasma Measurements

Following an overnight fast, blood was collected by cardiac puncture for glucose, insulin, leptin, adiponectin and resistin assays. The plasma was separated by centrifugation at 12,000 rpm for 20 min and stored at -80 °C. Plasma leptin and insulin levels were measured by RIA using mouse leptin and rat insulin RIA kits, respectively, obtained from Linco Research (St. Charles, MO). Blood glucose concentrations were measured using a glucose analyzer (LIFESCAN Co, MI). Plasma adiponectin and resistin levels were analyzed by enzyme immunoassay kits obtained from B-Bridge International, Inc. (San Jose, CA) and Phoenix Pharmaceuticals, Inc. (Belmont, CA), respectively.

3. Western Blot Analysis

Proteins were extracted from epididymal fat depot and total kidneys of WT, KO, Tg-KO and Tg-WT mice after homogenization with protease inhibitor cocktail and quantitated by the method of Bradford et al (21). Total kidney protein extracts (50 μg) and epididymal fat pad protein extracts (~25 μg) were electrophoretically separated by SDS/PAGE on 8-10% running gel, respectively. Proteins were subsequently transferred to nitrocellulose membranes in transfer buffer (20% methanol, 12mM Tri base, 96mM glycine, at pH 8.3), blocked overnight and incubated with primary polyclonal antibody Angiotensin I/II detecting agt (52 kDa), AT1 (47 kDa) and AT2 (46 kDa) receptors (Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:100, 1:200, 1:200 dilution, respectively for 3h at room temperature. Signals were detected using enhanced chemiluminescence (ICN Biomedicals, Inc., Costa Mesa, CA). Duplicate gels were prepared and stained with 0.1% Coomassie blue R250 and then distained in 7% acetic acid-5% methanol to visualize protein bands for total protein quantification and confirmation of equal protein loading. The bands obtained in the western blots were scanned using Digital Imagining

and Analysis Systems (Zero-Dscan software, Scanalytics Inc., Fairfax, VA). In addition to protein analysis in these mice, Affymetrix Microarray was conducted to investigate gene expression changes of RAS components as well as global gene expression profile in adipose tissue and kidney of these knockout and transgenic mice (see Appendix section)

4. Statistical Analysis

All data are expressed as the mean ± SEM. ANOVA was used to determined between-group differences in measured parameters. The values were examined by the Bonferroni test for multiple comparisons (SAS, Carry, NC) to compare differences among group means after a significant F-test and statistical significance was defined as $p<0.05$.

D. RESULTS

1. Effect of Adipose agt on Fat Pad Weight and Metabolic Parameters

The four groups of mice were fed a chow diet from weaning up to 28 weeks of age. The effects of adipose agt on body weight and fat pad mass are shown in Table 1. Consistent with previous results (14, 15), total body weight and epididymal fat pad mass of agt knockout mice (KO) were significantly lower than those of wild type (WT) mice. Compared to KO mice, Tg-KO mice reexpressing agt only in adipose tissue showed a slight increase in total body weight and adipose tissue weight of epididymal fat. Although Tg-WT mice overexpressing agt in adipose tissue exhibited almost normal body weight, an approximate 1.5-fold increase in the weight of epididymal fat pads was observed compared with WT mice ($p<0.05$). This observation directly supports that adipose agt plays a hypertrophic role in adipose tissue metabolism (5).

We evaluated effects of adipose agt on plasma leptin and insulin levels, as these hormones have been known to be involved in regulation of energy homeostasis (22). As shown in

Table 1. ***Body weight, fat pad weight and blood parameters in 28-wk-old WT, KO, Tg- KO and Tg-WT mice***

	WT (n=10)	KO (n=8)	Tg-KO (n=9)	Tg-WT (n=9)
Body weight (g)	50 ± 2.4^{b}	35 ± 0.9^{a}	38 ± 1.09^{a}	52 ± 2.8^{b}
Epididymal fat (g)	$1.1 \pm 0.18^{b'}$	$0.4 \pm 0.02^{a'}$	$0.5 \pm 0.18^{a'}$	$1.6 \pm 0.15^{c'}$
Leptin (ng/mL)	$5.7 \pm 0.1^{b''}$	$4.0 \pm 0.05^{a''}$	$5.5 \pm 0.06^{b''}$	$8.3 \pm 0.1^{c''}$
Insulin (ng/mL)	$1.2 \pm 0.03^{b*}$	$0.6 \pm 0.03^{a*}$	$0.8 \pm 0.03^{a*}$	$2.3 \pm 0.1^{c*}$
Resistin (ng/mL)	$43.4 \pm 0.7^{b**}$	$8.7 \pm 1.3^{a**}$	$42.0 \pm 1.6^{b**}$	$44.3 \pm 0.1^{b**}$
Adiponectin (ng/mL)	$1.1 \pm 0.17^{a***}$	$1.9 \pm 0.1^{b***}$	$1.1 \pm 0.2^{a***}$	$1.1 \pm 0.2^{a***}$

Values with different letters are significantly different ($p<0.05$). Values with the same letter are not significantly different. Results are the mean ± SEM, n, number of animals.

Table1, the plasma level of leptin was significantly decreased in KO mice compared to that in WT mice, in contrast with previous data showing no difference in plasma leptin concentrations between KO and WT animals (14). KO mice elicited a significant decrease in the plasma level of leptin, although KO and Tg-KO mice showed comparable fat pad mass. In contrast with previous observations in 6-week old Tg-WT mice, a significant increase (about 1.5-fold) was found in the level of leptin in 28-week old Tg-WT mice compared with that in WT animals. The level of insulin was similar in KO and Tg-KO mice while Tg-WT mice showed higher insulin levels (about 1.9-fold) compared with WT mice, suggesting that agt overexpression in adipose tissue could contribute to insulin resistance associated with a parallel increase in fat mass. Subsequently, we examined potential roles of adipose agt in obesity-associated insulin resistance by measuring circulating levels of adiponectin and resistin, which are adipocyte-derived plasma hormones (23). It has recently been documented that adiponectin has anti-insulin resistance and anti-atherogenic properties while resistin is highly associated with insulin resistance (24-27). No statistically significant difference among three genotypes (WT, Tg-KO and Tg-WT) was observed with respect to plasma adiponectin and resistin levels although Tg-WT mice seemed to exhibit insulin-resistance. Strikingly, a reversed ratio of these hormones was found in the KO mice; a significant (approx.1.9-fold) increase in the level of adiponectin and a substantial decrease (approx 4.8-fold) in the level of resistin were observed in agt knockout mice compared with those in WT mice. Similar plasma concentrations of glucose were also found in these four mouse genotypes (data not shown).

2. Effects of Adipose agt on the Regulation of RAS Components in Adipose and Renal Tissues

Since agt inactivation was associated with reduced adiposity and lower blood pressure whereas targeted overexpression of agt in adipose tissue led to the development of hypertension along with increased adiposity (14, 15), we further analyzed endocrine effects of adipose agt on the regulation of RAS proteins in adipose and renal tissues. Fig. 1 shows a representative western blot of adipose agt, AT1 and AT2 receptor proteins from these four mouse genotypes. As expected, the adipose agt protein was not detected in agt knockout mice. Total agt protein levels were similar between WT and Tg-KO mice selectively expressing agt in adipose tissue. Tg-WT mice exhibited a moderate increase in adipose agt protein expression compared with WT mice, confirming previous northern blot analysis in these mice (15). The KO mice had approximately 1.7-fold and 1.8-fold higher levels of AT1 and AT2 receptor proteins in adipose tissue than WT mice, respectively, whereas WT, Tg-KO and Tg-WT mice exhibited comparable adipose level of AT1 receptor protein. A 1.4-fold increase in AT2 receptor protein expression was observed in Tg-KO mice as compared to that of WT mice. By contrast, a considerable decrease in AT2 receptor protein expression was found in Tg-WT mice compared to that of WT mice.

In the total kidney (Fig. 2), the agt protein was expressed in WT mice but not in KO and Tg-KO mice as previously reported. Further, marked increases in agt and AT1 receptor protein expression were found in Tg-WT mice overexpressing agt in adipose tissue with resultant increases in systemic agt concentration (22-44% higher than that of WT mice) ($p<0.05$). This overexpression agt in kidney was tissue-specific, compared to hepatic agt expression in these transgenic animals (data not shown). Tg-KO mice targeted reexpression of agt only in adipose tissue exhibited a partial restoration of circulating agt levels (20-30% of agt levels of WT mice)

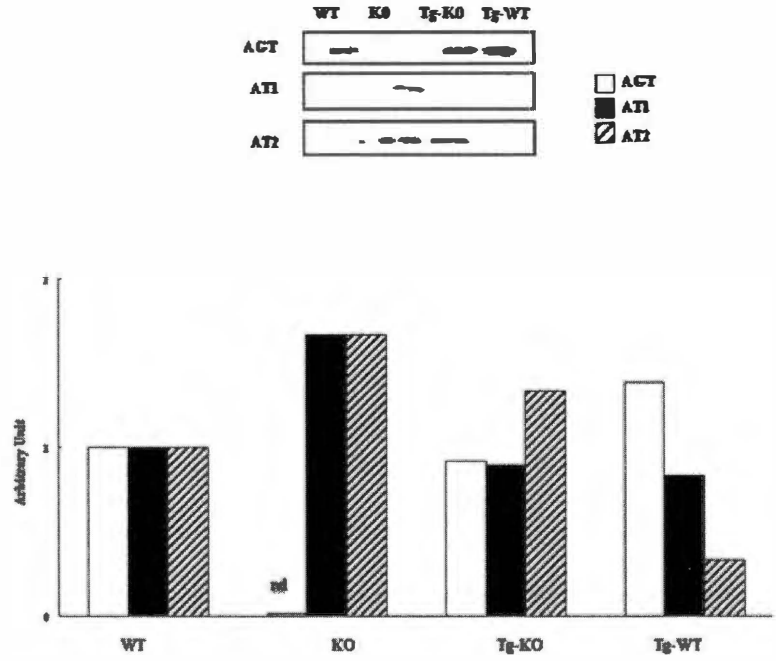

Fig. 1 ***Protein expression of agt, AT1 & AT2 in adipose tissue of WT, KO, Tg-KO and Tg-WT mice.*** Representative immunoblots of agt, AT1 and AT2 proteins are shown in the upper, middle and lower panels, respectively. Bars depict means of the quantitated agt, AT1 and AT2 bands, independently obtained in duplicate. nd, not detectable.

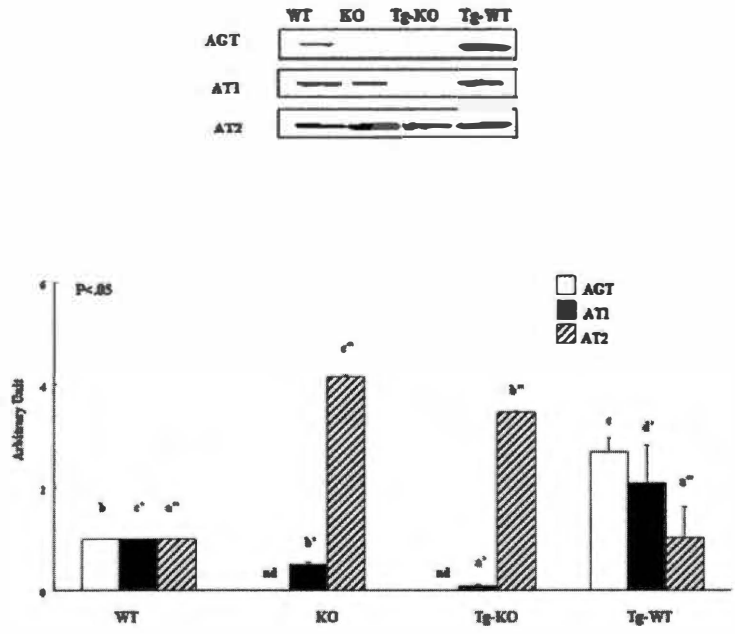

Fig. 2 ***Protein expression of agt, AT1 & AT2 in kidney of WT, KO, Tg-KO and Tg-WT mice.***
Representative immunoblots of agt, AT1 and AT2 proteins are shown in the upper, middle and lower panels, respectively. Bars depict mean ± SEM of the quantitated agt, AT1 and AT2 protein bands, independently obtained in triplicate. Values with different letters are significantly different ($p<0.05$). nd, not detectable.

and had significantly lower protein expression of AT1 receptor than did KO mice. Compared with WT mice, a decreased AT1 protein expression was observed in KO mice ($p<0.05$). Tg-WT mice showed similar AT2 receptor protein expression to that of WT mice whereas KO and Tg-KO mice displayed ~8-fold and ~7.6-fold increases in AT2 protein levels compared to those of WT mice, respectively.

E. DISCUSSION

The present study has addressed potential mechanisms by which adipose agt influences systemic blood pressure and renal regulation in an endocrine manner, using agt knockout and transgenic mice. First, we examined changes in adipose tissue metabolism and metabolic parameters. Agt deficient mice elicited markedly diminished fat pad mass and body weight gain along with significant decreased circulating insulin and leptin levels compared with WT mice. Clearly, Tg-WT mice overexpressing adipose agt were hyperinsulinemic and hyperleptinemic with a significant increase in fat pad mass compared to WT mice despite the fact that Tg-KO mice reexpressing agt only in adipose tissue exhibited comparable fat pad weight to KO mice. These results support previous *in vivo and in vitro* findings showing a hypertrophic function of Ang II in adipocytes through activation of key lipogenic enzymes, FAS and GPDH (5, 13, 15). In contrast with our findings, it was reported that transgenic mice (20 wk of age) ubiquitously overexpressing agt in multiple tissues including adipose tissue, liver, heart and kidney showed comparable plasma leptin and insulin levels to WT mice (11).

Of significance, KO mice displayed markedly increased circulating adiponectin and decreased plasma resistin concentrations while the other three genotypes showed comparable plasma levels of these proteins. Both adiponectin and resistin are recently discovered adipocyte-

derived proteins and their plasma levels have been shown to be highly associated with insulin sensitivity and resistance (23-26). Thus, these protein levels seen in KO mice may reflect the increased insulin sensitivity. This observation supports the previously defined inhibitory roles of Ang II in insulin signaling transduction in vascular tissue (28, 29). This result is also in agreement with a recent clinical report demonstrating that inhibition of the RAS improves insulin sensitivity in subjects with essential hypertension, with parallel elevation in plasma adiponectin concentrations (30).

Using western and oligonucleotide microarray analyses (see Appendix), the current study extends previous findings and specifically demonstrates adipose agt regulation of adipose and renal RAS components in these agt knockout and transgenic mice. Ang II has been shown to exert various effects on the expression of major component genes of the RAS by positive and negative feedback mechanisms (31-33). In addition, several studies of regulation of the RAS components in experimentally and genetically hypertensive animals as well as hypotensive animals have reported significant abnormalities of RAS gene expression in some tissues (34-36). In the present study, compared with WT mice, we showed higher AT1 and AT2 receptor protein levels in adipose tissue of KO mice which have no detectable plasma agt and lower blood pressure. These results support the previous well-documented feedback inhibition of Ang II on angiotensin receptors (34). Thus, complete loss of Ang II-mediated negative feedback caused by genetic deficiency of Ang II endogenous production in agt knockout mice may be involved, in part in upregulation of both receptors in adipose tissue (31-34). In the kidney, western and microarray analyses of KO mice demonstrated markedly increased renin and ACE mRNA levels, as previously described in the kidney of agt knockout mice (31, 34). Similar to the regulation of adipose AT2 receptor, increased total kidney AT2 receptor protein and mRNA expression were

detected in these null mice. In contrast with AT2 receptor, we found a significant repressed level of total kidney AT1 receptor protein. One possible explanation for altered renal RAS regulation in agt deficient mice may be remarkable renal defects at the function and structure levels including marked vascular hypertrophy, atrophic changes in the tubules and papilla, increased urine output and decreased urine osmolality, as previously reported in the kidney of agt null mutant mice (37, 38). Thus, these pathological changes could participate in altered gene and protein expression in renin, ACE, AT1 and AT2 in the kidney in agt knockout mice. In particular, Taumra et al proposed that abnormal renal structure such as reduced renal medullas seen in agt deficient mice may contribute to increased mRNA level of ACE which is mainly produced by the renal cortex (34). Another possibility is that the higher renin, ACE, and AT2 expression in the kidney of agt knockout mice compared to WT mice may be in part due to complete disruption of the negative feedback of Ang II on renin, ACE, and AT2-expressing cells in the kidney of agt deficient mice, as seen in adipose tissue of KO mice (31-33). Alternatively, alterations of hormone levels, including vasopressin and aldosterone which are known to modulate AT1 and ACE gene expression and signaling pathways as well as compensatory mechanisms induced by agt gene ablation, could change regulation of RAS genes and proteins in a tissue-specific manner (39). Microarray data demonstrating altered expression of genes associated with transcription regulation and signal transduction cascades in the kidney of KO mice provided additional evidence for altered renal gene regulation in these mice. Moreover, our result of reduced renal AT1 expression seen in agt knockout mice is in contrast to a previous report showing upregulation of renal AT1 mRNA and protein expression in KO mice, raising the possibility that differences in RAS regulation in agt null mice may also be associated with genetic background used (34, 40).

In contrast with the observations in KO mice, Tg-WT mice overexpressing agt in adipose tissue displayed prominent systemic hypertension along with circulating agt level increased by 22-44 % compared with WT mice (15). These observations, along with several reports demonstrating that introduction of human agt and renin genes or rat agt into different tissues of agt knockout mice using tissue or cell specific promoters induced higher plasma Ang II levels, support that increased circulating agt level is a causative factor in the development of systemic hypertension (40-44). In the present study, the western blot analysis of Tg-WT mice showed slightly decreased adipose AT1 receptor protein expression with markedly reduced adipose AT2 receptor protein level compared with WT mice. In contrast with KO mice, the repressive protein levels of both receptors in Tg-WT mice suggest that the complete restoration of circulating agt and predictably Ang II stimulates a negative regulation of expression of both receptor genes in the adipose tissue (34). A marked increase in agt and AT1 receptor protein levels was evident in the kidney of Tg-WT mice. However, similar hepatic agt and slightly increased AT1 protein contents were noted in Tg-WT mice compared with WT mice (data not shown). Our results from this study suggest that adipose agt overexpression induces overproduction of agt in the circulation, which enhances renal RAS activity in a tissue-specific manner and further activated renal RAS increases intrarenal Ang II level, thereby contributing to the development of hypertension in Tg-WT mice. Our results support previous studies demonstrating that specific targeting of RAS overexpression in the kidney was able to elevate systemic blood pressure independent of endocrine RAS, suggesting that intrarenally-derived Ang II is a potent regulator of systemic blood pressure (43, 45, 46). Consistent with this, double transgenic mice (R^+/A^+) overexpressing human renin and agt in the renal proximal tubule exhibited increased arterial pressure(47). Recently, studies on Ang II-induced hypertensive rats have emphasized that

elevated intrarenal Ang II levels play a pivotal role in the development and maintenance of hypertension (48, 49). In Ang II-induced hypertension (80ng/min, 2 wk), chronically elevated circulating Ang II was actively taken up through an AT1 receptor-mediated process and thus augmented intrarenal Ang II. This, in turn, exerts a sustained influence on tubular reabsorption and subsequently induces hypertension without any increase in the plasma levels of Ang II (45, 50). Of importance, the enhanced intrarenal Ang II has been shown to exert a positive feedback action to augment intrarenal levels of agt mRNA and protein, which likely contributes to the progressive increases in intrarenal endogenous production of Ang II in hypertensive states (48, 49). Thus, it is conceivable that specific targeting of agt overexpression in adipose tissue leads to increased circulating agt concentrations, which are potentially responsible for elevated systemic Ang II production. This, in turn, is taken up by the kidney via an AT1 receptor-mediated endocytosis and subsequently enhances intrarenal Ang II. Renal agt mRNA and protein levels in Tg-WT mice in this study may be stimulated by elevated intrarenal Ang II via a positive feedback mechanism, thereby increasing Ang II-mediated tubular transport and consequently causing systemic hypertension in these transgenic mice. This is also supported by microarray results of Tg-WT mice showing significantly upregulated expression of genes related to ion exchange and ion channels which are involved in tubular sodium reabsorption and renal volume expansion, thereby resulting in systemic increases in arterial pressure compared with WT mice. Additionally, since insulin increases agt expression and production in vascular smooth muscle and adipose tissue, our data do not exclude the plausibility that elevated renal agt expression in Tg-WT showing hyperinsulinemia may result from the additive effects of insulin and potential overproduction of intrarenal Ang II (51, 52). Moreover, significantly increased renal AT1 protein level was evident in Tg-WT mice compared with WT mice, which was in contrast to the

observations from studies of Ang II-induced hypertension animals which showed a common phenotype, namely hypertension, with Tg-WT mice (48). In genetic hypertension models, tissue-specific activation of AT1-mediated hypertension mechanisms has been reported (3, 40, 53-55). Further, Ang II-induced tubular sodium transporter activation through increased renal AT1 receptor number and function was observed in obese zucker rats which are characteristically hypertensive and hyperinsulinemia, similar to those seen in Tg-WT mice although results on renal AT1 receptor mRNA and protein expression in hypertensive animal models remain elusive (53). Additionally, microarray data of Tg-WT mice exhibited marked upregulation of renal H^+-ATPase transports and potassium channels, which have been shown to be stimulated by AT1 receptor mediation. Taken together with microarray results (see Appendix), this result suggest that potential overproduction of renal Ang II increases renal AT1 receptor activity and subsequently stimulates AT1-mediated ion transport and channel activation, thereby elevating blood pressure in Tg-WT mice. In addition, in Ang II-induced hypertension, markedly suppressed renal renin mRNA and content but increased renal ACE mRNA levels, activity and binding were recently reported. It has been suggested that this differential regulation of RAS components may help maintain enhanced endogenous Ang II formation in the kidney. In our study, increased renal renin gene expression and comparable renal ACE mRNA level were observed in Tg-WT mice compared with WT mice despite the fact that both Ang II-infused rats and Tg-WT mice displayed significantly increased renal agt protein levels and systemic hypertension. It is possible that the adipose agt ectopic expression-induced compensation in Tg-WT mice and other regulatory mechanisms may be involved in these differences, in part. In renal AT2 receptor expression of Tg-WT mice, in spite of a significant increase in renal AT2 mRNA levels, comparable protein content was observed. Along with renal AT2 function, little is known about regulation of the renal AT2

gene in hypertensive models. Our results was consistent with previous study showing that renal AT2 receptor protein expression was not altered in hypertension models (34). Altogether, blood pressure effects of adipose agt overexpression observed in Tg-WT mice appears to be in part mediated by elevated systemic agt and activation of renal RAS, providing potential mechanisms responsible for obesity-associated hypertension. It is important to point out that with respect with blood pressure mechanisms, adipose RAS differs from kidney or brain RAS; kidney or brain-induced blood pressure regulation is independent of endocrine RAS (45, 55). We also cannot preclude the possibility that blood pressure effects of adipose agt overexpression may also result from the combined effects of intrarenal Ang II, activation of other local RAS such as brain and systemic RAS.

Massiera et al showed that Tg-KO mice reexpressing agt only in adipose tissue exhibit 1.5-fold fat mass increase and have intermediate blood pressure. Notably, targeted agt expression on adipose tissue was able to reverse renal structural abnormalities and dysfunction as well as altered blood pressure seen in KO mice with partial restoration of systemic agt concentrations at 20%-30% of those of WT mice (15). In Tg-KO mice, maintained AT1 and increased AT2 protein levels were observed in adipose tissue compared with WT mice. On the basis of previous studies, this observation may be in part due to partial restoration of the negative feedback effect of locally produced Ang II in adipose tissue of Tg-KO mice. Of note, in comparison with Tg-WT mice, Tg-KO mice displayed significantly decreased renal AT1 protein levels despite the fact that sole reexpression of agt in adipose tissue was sufficient to correct altered systemic blood pressure seen in KO mice. This finding reflects that adipose agt influence of blood pressure in these animals may be at least partly mediated via partial restoration of systemic agt levels rather than stimulation of renal AT1-mediated hypertensive effects observed in Tg-WT mice although exact

mechanisms need further investigation. Further, increased renal AT2 gene and protein levels as well as increased renal renin mRNA and ACE mRNA were observed in these mice compared with WT mice. Of note, altered renin gene expression in KO mice was nearly normalized by reexpression agt only in adipose tissue. In addition, microarray results also presented evidence that adipose tissue-specific agt restoration can partially normalize altered expression of genes associated with renal function and development in KO mice. These results support previous studies showing that reexpression of adipose agt was capable of correcting renal abnormalities such as hydronephtosis (14). Evidence from other genetic models of hypertension has demonstrated that brain-specific restoration of human Ang II, multi-tissue restoration of human agt or systemic expression of human agt and renin genes in numerous tissue except targeted restoration of agt only in the kidney prevent renal anomalies observed in agt-deficient mice, suggesting that extra-renal tissue RAS activation is required for renal development and function (40-42, 45, 46). Taken together, our results support that adipose RAS play pivotal roles in renal function and development in an endocrine manner.

In summary, using agt knockout and transgenic models, we confirmed the positive association of systemic blood pressure with increased fat mass. Further, we provided potential mechanisms responsible for endocrine effects of adipose agt using knockout and transgenic animal models. Adipose agt overexpression potentiates argumentation of systemic Ang II which further activated renal RAS in a tissue-specific manner, thereby contributing to the development of hypertension in Tg-WT mice whereas adipose tissue-specific restoration of agt may affect renal function and development through restoring altered expression of genes associated with renal regulation. Collectively, adipose RAS plays pivotal roles in renal and adipose function and potentially development as well as obesity-associated hypertension via an endocrine mechanism.

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PART VI1

SUMMARY AND CONCLUSION

The findings of this dissertation provide new insights into possible mechanisms responsible for paracrine and endocrine actions of Ang II in modulating adiposity and hypertension associated with obesity. Previously, the components of the RAS were shown to be expressed in the adipose tissue and appear to be upregulated in obesity which is associated with enhanced plasma agt levels. Genetic and biochemical studies demonstrate functional roles of local adipose RAS in adipocyte differentiation and possibly in body-fat accumulation. Further, precious data from our lab indicate a pivotal role of Ang II in lipid metabolism in adipose tissue. However, no studies have evaluated the molecular or signaling mechanisms mediating Ang II regulation of adipocyte gene expression. We demonstrated that Ang II plays a paracrine role in regulation of adipocyte metabolism by upregulating a lipogenic gene, namely FAS in a glucose and ADD1/SERBP1c-dependent manner. This effect of Ang II in adipocytes was mediated by its receptor-mediated crosstalk with insulin signaling molecules. These results illustrate the involvement of adipose RAS in the development of obesity. Using agt knockout and transgenic mouse models, our *in vivo* findings confirmed the lipogenic effect of adipose agt on adipose tissue metabolism and also demonstrated endocrine actions of adipocyte agt on kidney function.

Obesity is an increasingly prevalent condition associated with a large number of comorbid diseases, one of the most important of which is obesity-related hypertension. Weight gain was shown to be a significant factor in elevating arterial pressure in many essential hypertensive patients although precise mechanisms for obesity-induced hypertension remain elusive. Taken together with clinical studies showing upregulation of RAS genes in adipose tissue of obese hypertensive subjects, genetic manipulation approaches of RAS have provided the evidence for a possible role of adipose RAS in altering systemic blood pressure; an associated elevation in blood pressure combined with increased fat mass in the transgenic mice

overexpressing agt in adipose tissue. Our data further demonstrate that reexpression of adipose agt reverses the pattern of expression of genes associated with blood pressure regulation and renal function in agt knockout mice and support important endocrine functions of adipose RAS. Importantly, our findings indicate that adipose agt contributes to the development of hypertension in obesity by activation of local renal RAS in Tg-WT mice (Fig 1).

On the other hand, obesity predisposes to insulin resistance and further type 2 diabetes. There has been some evidence to support a potential link of agt polymorphism to insulin resistance. In line with this, clinical trial studies show that inhibition of RAS improves insulin sensitivity. Our result demonstrating that adipose agt inactivation improves insulin sensitivity supports this notion. This implicates a possible role of adipose RAS in the development of insulin sensitivity and diabetes. To further ascertain and confirm the above paracrine and endocrine actions of the adipose agt, it would be important to engineer mouse models where agt is specifically inactivated in adipose tissue (such as using cre-lox recombinase tissue specific systems) and determine the impact of the inactivation on adiposity, blood pressure and insulin sensitivity.

The significance of this research is to help to develop treatments of obesity and hypertension in obese patients. Accordingly, blockade of RAS components (*e.g.* antagonists of AT1 receptor and ACE) might be an effective therapeutic approach to prevent the development of obesity and obesity-associated metabolic alternations including insulin resistance and diabetes. In addition, this present study unraveling the mechanisms by which weight gain raises blood pressure provides a better understanding of human essential hypertension. Further studies are needed to more fully elucidate the quantitative importance of these mechanisms that influence the risk of hypertension in obese subjects.

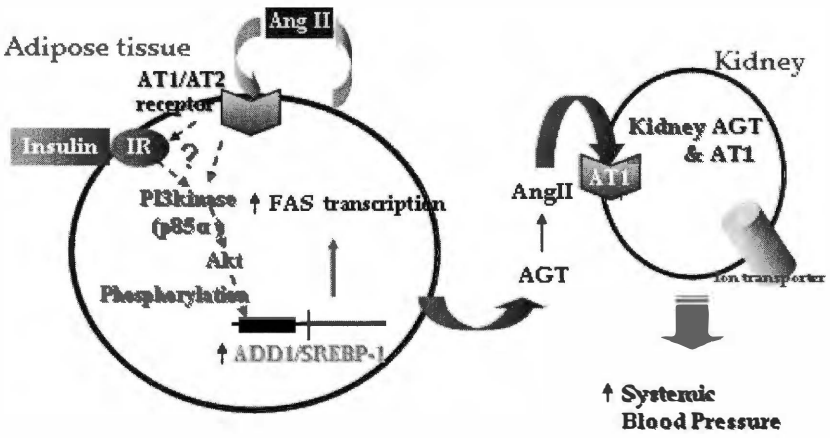

Fig. 1 ***Mechanisms of paracrine and endocrine actions of Ang II.*** In paracrine effects of Ang II, Ang II binds to AT1/AT2 receptors and increases ADD1 and FAS gene expression via activation of PI-3K-Akt signaling pathways. In endocrine effects of Ang II, adipose agt is secreted into the circulation, thus increasing systemic agt levels as well as acts on kidney and elevates renal agt and AT1 expression, activating renal RAS and thus increasing systemic blood pressure

Appendix

A. GeneChip Microarray assay and Data Analysis

Total RNA from kidneys of KO and Tg-KO mice maintained on a Chow Diet (CD) was isolated by the cesium chloride density gradient method and further purified by phenol chloroform extraction. Microarray analysis was conducted using the Affymetrix Murine Genome U74v2 set that contains probe sets interrogating approximately 36,000 full-length mouse genes and EST clusters and was performed at the Genome Explorations Inc. in Memphis, TN. Kidneys from four to six KO or Tg-KO mice were used and pooled into 2 sets of each genotype (Fig 1). Total renal RNA (~20μg) from each of these genotypes was reverse-transcribed and the phycoerythrin dye was incorporated to the second strand. cRNA was synthesized from cDNA and biotinylated and hybridized to a set of Murine genome GeneChips. Following hybridization and washing, the overall fluorescence intensity across each chip was scaled to a target intensity of 1500 using Affymetrix GeneChip Microarray Suite 5.0 software, and pairwise comparisons of mRNA levels were performed. Each experiment was performed twice in each genotype and a minimum expression ratio of 2-fold differences was used as the selection criteria for the differentially expressed genes between the KO (2 sets) and Tg-KO mice (2 sets). The fold changes in gene expression are presented as the mean of four datasets which are associated with renal function and blood pressure regulation.

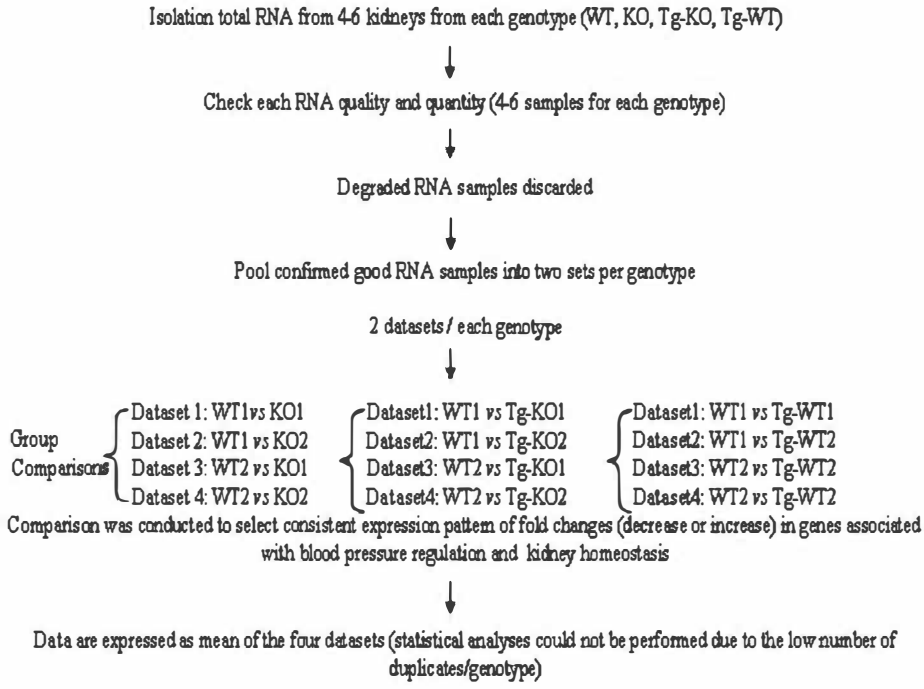

Fig. 1 ***Flow chart for GeneChip microarray experiments and data analysis.***

B. GeneChip Microarray assay and Data Analysis

Total RNA from kidneys of WT, KO, Tg-KO and Tg-WT mice maintained on a chow diet was isolated by the cesium chloride density gradient method and further purified by phenol chloroform extraction. Microarray analysis was conducted using the GeneChip® Mouse Expression Set 430A that contains probe sets interrogating approximately 14,484 full-length mouse genes and EST clusters and was performed at the Genome Exploration Inc. in Memphis, TN. Kidneys from four to six WT, KO, Tg-KO or Tg-WT mice were used and pooled into 2 sets each genotype (Fig 1). Total renal RNA (~20μg) from each of these genotypes was reverse-transcribed and the phycoerythrin dye was incorporated to the second strand. cRNA was synthesized from cDNA and hybridized to a set of Murine genome GeneChips. Following hybridization and washing, the overall fluorescence intensity across each chip was scaled to a target intensity of 1500 using Affymetrix GeneChip Microarray Suite 5.0 software. Each experiment was performed twice in each genotype and a minimum expression ratio of 2-fold differences was used as the selection criteria for the differentially expressed genes between WT mice (2 sets) and KO (2 sets), Tg-KO mice (2 sets) and Tg-WT mice (2 sets). The fold changes in gene expression were presented as the mean for the four datasets in each comparison which are associated with renal function and blood pressure regulation.

Microarray data of four genotype mice were analyzed using GeneSifter.Net™ (VizX Labs LLC, Seattle, WA, www.vizxlabs.com). Only genes with a fold change greater than 2 that passed spot quality filtering were included in the analysis. The resultant gene list was further analyzed by partition clustering using PAM (partitioning around medioids) to separate genes into similar expression profiles. Gene ontology reports (Biological Process, Cellular Component, and Molecular Function) were also generated using GeneSifter.Net™. The three distinct clusters were

selected to analyze endocrine effects of adipose agt in renal gene regulation; 1) the cluster is composed of genes which are downregulated by agt inactivation but corrected by agt reexpression in only adipose tissue 2) the cluster is composed of genes which were upregulated in the KO mice but normalized toward wild-type levels by reexpression of agt in only adipose tissue 3) the cluster reveals genes which were upregulated by overexpression of agt in adipose tissue compared with WT mice. Microarray data for these knockout and transgenic mice are explained by the following section.

1. Effects of Adipose agt on mRNA Expression Levels of Renal RAS Components

Using affymetrix microarray analysis, we also examined the effects of adipose agt on gene expression of renal RAS. A minimum differential expression ratio of 2-fold was used as selection criterion for differentially expressed genes between the KO, Tg-KO, Tg-WT and WT mice, respectively (Table 1). In comparison with expression pattern of renal agt protein, Tg-WT mice showed an increased agt gene expression by approximate 1.6-fold compared with WT mice, although this value was not statistically significant (data not shown). Consistent with previous studies demonstrating abnormalities of RAS component gene expression in hypotensive agt null mutant mice (1-3), KO mice had significantly higher renal renin, ACE and AT2 mRNA expression levels than WT mice. Tg-KO mice reexpressing adipose agt exhibited similar expression levels of ACE and AT2 genes compared with KO mice, but higher mRNA levels of renin, ACE and AT2 than WT mice. In Tg-WT mice, renin and AT2 receptor gene expression were significantly increased compared with WT mice but ACE expression was similar to that of WT mice.

Table 1. ***Gene expression of RAS components in the renal tissue, showing ≥ 2-fold differences in levels between 28 week-old KO, Tg-KO, Tg-WT and WT mice, respectively***

Gene	*Accession Number*	*Fold Changes in Expression*		
		KO *vs* WT	Tg-KO *vs* WT	Tg-WT *vs* WT
Renin 1 structural	NM 031192	↑ (20.8)	↑ (2.3)	↑ (2.5)
Angiotensin converting enzyme	M55333	↑ (2.6)	↑ (2.5)	↔ (1.1)
Angiotensin II, type 2 receptor	MN 007429	↑ (2.2)	↑ (2.6)	↑ (4.6)

↑ : Increase, ↓ : Decrease, ( ): Fold changes expressed as Mean

2. Effects of Adipose agt on Global Renal Gene Expression

We and others previously demonstrated that transgenic replacement of adipose agt was able to correct renal structural abnormalities and altered expression of genes associated with blood pressure regulation and renal function (4, 5). To further define endocrine effects of adipose agt on the global expression profile of renal genes involved in renal function, development and blood pressure homeostasis, expression analysis was first performed with total RNA isolated from renal tissue of these four mouse genotypes using oligonucleotide microarray analysis. Resulting data were analyzed by GeneSifter.Net™ software including cluster analysis, which groups genes with similar expression pattern and gene ontology, which is then used to assign the regulated genes into functional categories. Three distinct clusters were selected to elucidate the endocrine influence of adipose agt on renal gene regulation in these mice.

The results from cluster analysis showed that two clusters were composed of twenty-one genes that were dysregulated threefold or more in agt null mice, which are specifically corrected toward wild-type levels by restoration of adipose agt (Fig 2 & 3). Fig 2 shows that the

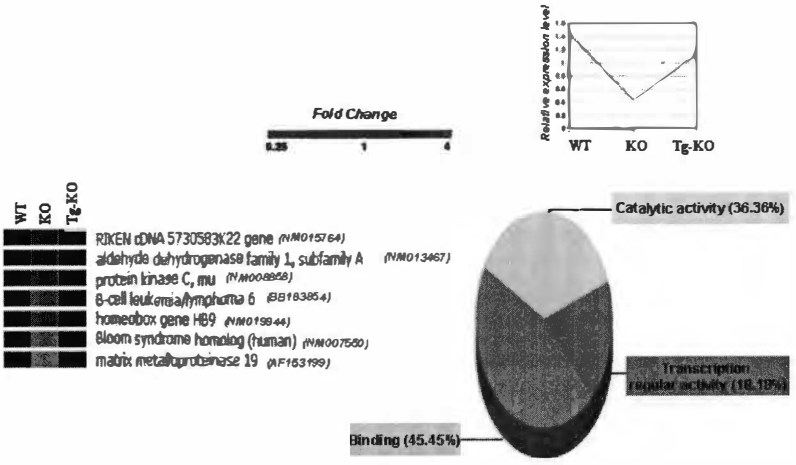

Fig. 2 ***The cluster of genes specifically suppressed in KO renal tissue and functional classification of the downregulated genes.*** We investigated adipose agt effects on the kidney gene expression profile using oligonucleotide microarray analysis. The resulting data were further analyzed by GeneSifter.Net™ software including cluster analysis and gene ontology which is used to assign the regulated genes into functional categories. The results showed that genes suppressed in agt knockout renal tissue were normalized by sole reexpression of agt in adipose tissue. These genes are involved in binding and catalytic processes or transcription regulation. Gene names are given along with Genebank accession numbers

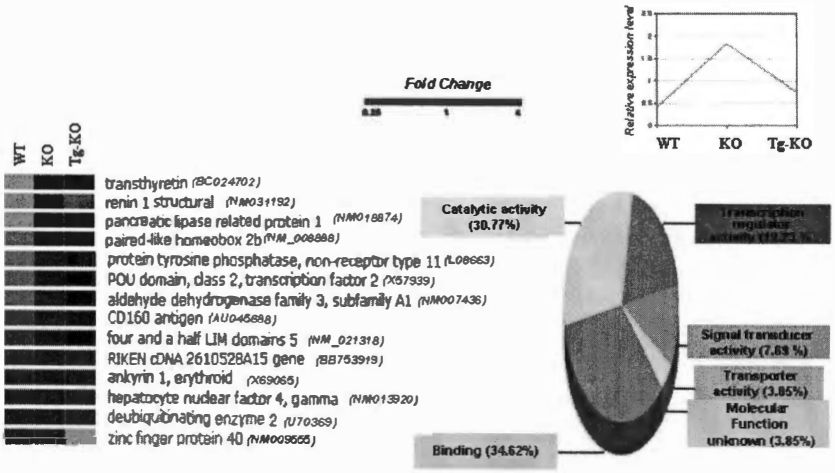

Fig. 3 ***The cluster of genes specifically upregulated in KO renal tissue and functional classification of the upregulated genes.*** We investigated adipose agt effects on the kidney gene expression profile using oligonucleotide microarray analysis. The resulting data were further analyzed by GeneSifter.Net™ software including cluster analysis and gene ontology, which is used to assign the regulated genes into functional categories. The results showed that genes upregulated in renal tissue of KO mice were normalized by sole reexpression of agt in adipose tissue. These genes are involved in binding and catalytic processes, transcription regulation, signal transduction cascade or ion transport. Gene names are given along with Genebank accession numbers.

seven genes that were downregulated 3.5-fold in agt knockout mice were corrected in Tg-KO mice to levels approaching those in WT mice. As anticipated, alterations in expression of Ang II signaling molecules were observed in agt knockout mice. Particularly, expression of protein kinase C, which was shown to play a role as an important mediator in Ang II-induced hypertrophic action associated with vascular remodeling, was significantly decreased in KO mice characterized by renal structural and vascular abnormalities (*e.g.* hypoplastic renal papilla) but adipose agt reexpression restored this expression level to that of wild-type mice (6). This supports the previous study demonstrating that Ang II plays a critical role in renal function and development. Of these 7 genes with known functions, the majority corresponded to genes coding proteins involved either in binding (45.4 %), catalytic activity (36.3%) or transcriptional regulation (18.1%). Fourteen of the 21 genes were upregulated approximately 3-fold in KO mice and significantly corrected toward wild-type expression levels by reexpression of adipose agt (Fig 3). Similar to results of previous studies, a substantial increase in the levels of renin mRNA in the kidney was found in agt deficient mice (1, 3). This altered renin expression was partially normalized in Tg-KO mice. Of interest, upregulation of hepatic nuclear factor 4 (HNF 4) gene, which was demonstrated to be one of the transcription factors involved in the regulation of agt gene transcription, was observed in KO mice but targeted restoration of agt only in adipose tissue in these mice returned this altered expression to the level observed in WT (7, 8). The expression of other transcription factors like POU domain, class 2, transcription factor 2 and zinc finger protein 40 was also significantly increased by agt inactivation and their altered expression levels were corrected by targeted adipose agt reexpression. Expression in genes associated with development such as paired-like homeobox 2b and protein tyrosine phosphatase, non-receptor was increased in agt null mice but these levels was nearly equivalent to those of

WT mice by restoration of adipose agt. Gene ontology reports revealed that this cluster contained genes which are involved in transcription regulation (19.23 %), signal transduction (7.69%), binding (34.6%), catalytic activity (30.7 %) or transport (3.85%). Another cluster consisted of genes that were upregulated approximately 5-fold by overexpression of adipose agt compared with those of WT mice. Table 2 presents forty-five out of eighty-eight genes, which have known functions. An analysis of the genes in this cluster demonstrated that adipose agt overexpression specifically increased the expression of genes responsible for ion transport and channel, signal transduction, transcription regulation, cell growth and apoptosis in renal tissue of Tg-WT mice. Specifically, Tg-WT mice displayed upregulation of renal ion channel and exchanger genes like H^+-ATPase and K channel which was known to be involved in blood pressure homeostasis (9, 10). Consistent with previous findings that Ang II stimulates the expression and production of plasminogen activator inhibitor-1 (PAI-1; serine proteinase inhibitor, clade B, member 2) in vascular and adipose tissues, thereby emphasizing its potential prothrombotic effects, a significant increase in PAI-1 gene expression was also observed in renal tissue of Tg-WT mice (11-13). This supports additional functional roles for Ang II in hypertension-associated renal disease progression (6).

Table 2. ***Effects of adipose agt overexpression on renal regulation*** We investigated adipose agt effects on the kidney gene expression profile using oligonucleotide microarray analysis. The resulting data were further analyzed by GeneSifter.Net™ software including cluster analysis and gene ontology, which is used to assign the regulated genes into functional categories. The presented genes are upregulated in renal tissue of Tg-WT mice compared with WT mice and associated with renal function and blood pressure regulation. Gene names are given along with Genebank accession numbers

Function	*Accession Number*	*Gene*
Metabolic function	AI195046	RIKEN cDNA 2410027J01 gene
	M30774	Thymidylate synthase
	NM010455	Homeo box A7
	AB032770	Myeloid/lymphoid or mixed lineage-leukemia translocation to 7 homolog
	BB284199	Thioredoxin reductase 1
	NM023894	Placenta specific homeobox 2
	BB166592	Topoisomerase (DNA) II beta
	NM009548	Zinc finger protein 179
	NM009990	Cytoplasmic linker 2
	BC018323	D site albumin promoter binding protein
	BC014718	Deoxyribonuclease I
	AW741575	Histone 1, H1c
	NM028094	RIKEN cDNA 2010321J07 gene
	NM009171	Serine hydroxymethyl transferase 1(soluble)
	AK017701	Karyopherin (importin) beta 3
	AK008023	Protease, serine, 32
	AK017573	Mitochondrial translation optimization 1 homolog
	NM011111	Serine (or cysteine) proteinase inhibitor, clade B, member 2
	BE553261	RIKEN cDNA 2810457M08 gene

Table 2. *Continued*

Function	*Accession Number*	*Gene*
Metabolic function	AA543265	Heat shock protein A
	AI327006	Cytochrome P450, family 4, subfamily a, polypeptide 14
	BM117916	Xeroderma pigmentosum, complementation group A
	NM008792	Proprotein convertase subtilisin/kexin type 2
Cell growth (Skeleton and adhesion)	BB19683	Sarcoglycan, gamma (dystrophin-associated glycoprotein)
	BB538661	RIKEN cDNA E130012P22 gene
	NM007605	Capping protein alpha 3
	AV341395	Calsyntenin 3
Transport	AV238793	Apoptosis, caspase activation inhibitor
	NM008218	Hemoglobin alpha, adult chain 1
	AK004157	ATPase, H+ transporting, V1 subunit C, isoforms 2
	NM011395	Solute carrier family 22 (organic cation transporter), member3
	BC018335	Solute carrier family 15 (H+/peptide transporter), member 1
	AV309591	ATP-binding cassette, sub-family F (GCN20), member 1
	NM023782	Solute carrier organic anion transporter family, member 1a6
	BC001995	ATPase, H+ transporting, lysosomal V 0 subunit a isoforms 1
	AF319542	Potassium channel, subfamily K, member 5
	NM0019640	Phosphotidylinositol transfer protein, beta
Signal transducer and transcription regulator	NM007557	Bone morphogenetic protein 7
	NM020490	Leukotriene B4 receptor 2
	BC011215	Histocompatibility 2, Q region locus 10
	BE852312	RIKEN cDNA 1700001C14 gene
	NM009491	Vomeronasal 2, receptor 16
	NM008883	Plexin A3

Table 2. *Continued*

Function	*Accession Number*	*Gene*
Signal transducer and transcription regulator	NM007429	Angiotensin II receptor type 2
	NM009548	Zinc finger protein 179
	NM011746	Makorin, ring finger protein, 3
	BB024536	Growth hormone releasing hormone receptor
Stress response	AA543265	Heat shock protein, A
	AF349142	Immunoglobin heavy chain 4 (serum IgG1)
Apoptosis	AV238793	Apoptosis, caspase activation inhibitor
	NM007544	BH3 interacting domain death agonist
	BC 014718	Deoxyribonuclease I

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VITA

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