12-2009

Calcitriol and the Renin Angiotensin System, and Adipose Tissue Inflammation

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To the Graduate Council:

I am submitting herewith a thesis written by Christina Marie Caserio entitled "Calcitriol and the Renin Angiotensin System, and Adipose Tissue Inflammation." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Michael Zemel, Major Professor

We have read this thesis and recommend its acceptance:

Jay Whelan, Jang Han Kim

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Calcitriol, the Renin Angiotensin System, and Adipose Tissue Inflammation

A Thesis

Presented for the Masters of Science Degree

The Unviersity of Tennessee, Knoxville

Christina Marie Caserio

December 2009

Dedication

This thesis is dedicated in loving memory to my grandparents,

James Caserio and Jolanda Caserio

Acknowledgements

I wish to thank all those who helped me complete my Master of Science degree in Nutritional Science.

I would like to thank Dr. Zemel for his guidance and introduction to calcitriol and adipose tissue metabolism. I would like to thank Dr. Whelan and Dr. Kim for serving on my research committee. I would like to thank Dr. Sun for her valuable instruction in laboratory techniques and interpretation of data. I would also like to thank Beth Wilson for her statistical support.

Lastly, I would like to thank my family, whose encouragement and support made this work possible.

Abstract

Adipose tissue is well recognized as an endocrine organ and a source of proinflammatory molecules [1]. We recently demonstrated calcitriol stimulates adipocte reactive oxygen species (ROS) production and inflammatory stress (IS), while dietary calcium suppression of calcitriol exerted the opposite effect. These effects are mediated, in part, by calcitriol modulation of intracellular calcium (Ca^{2+}) signaling and mitochondrial potential. However, adipocytes contain a functional RAS, and angiotensin II (ANGII) modulates ROS and IS. Accordingly, we investigated the role of ANGII in mediating calcitriol effects. Calcitriol (1 nM) stimulated NOX4 expression and ROS production in 3T3-L1 adipocytes by 67% ($p<0.01$), while these effects were reversed by ACE inhibition (enalipril) or antagonism of either ANGII type 1 receptor (AT1R) or ANGII type 2 receptor (AT2R), antagonism. Similarly, ANG (0-.1-1.0 nM) stimulated NOX4 expression ($p<0.03$) and this effect was reversed by AT1R or AT2R antagonism. Calcitriol and ANGII both suppressed adiponectin expression ($p<0.04$) and increased IL6 and MCP-1 expression ~2 fold ($p<0.03$), and these effects were reversed with enalapril or AT2R, but not AT1R, antagonism. These data demonstrate that calcitriol modulation of adipocyte ROS production and IS is modulated, in part, by the adipocyte RAS.

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Abbreviations

ACC2	Acetyl-coA carboxylase-2
ACE	angiotensin converting enzyme
ADD	adipocyte determination and differentiation
AGEs	advanced glycated end products
AGT	Angiotensinogen
ANGII	angiotensin II
AP	activator protein
aP2	adipocyte lipid binding protein
ARBs	ANGII receptor blockers
ATP	adenosine triphosphate
BAT	brown adipose tissue
BMI	body mass index
Ca^{2+}	intracellular calcium
cAMP	cyclic adenosine monophosphate
CD14	macrophage surface specific protein
C/EBP	CCAAT/enhancer binding protein
CREB	cAMP response element binding protein
CYP27A1	25-hydroxylase
CYP27B1	1-α-hydroxylase
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle medium
ERK1/2	extracellular signal regulated kinase1/2
FAS	fatty acid synthase
FBS	fetal bovine serum
GLUT	glucose transporter
GPCRs	G protein coupled receptors
GRK	G-protein coupled receptor kinase
GST-p65	Glutathione-S-transferase p65
HIF	hypoxia inducible factor
IBMX	Iso-butyl-1-methyl-xanthine
ICAM	intracellular adhesion molecule
IGF	insulin like growth factor
IKB	I-kappa beta
IKKB	IKB kinase beta
IKK	IKB kinase
IL	Interleukin
IR	insulin receptor
IRS	insulin receptor substrate
JNK	c-jun N terminal kinase

LPL	lipoprotein lipase
M1	classically activated macrophages
M2	alternatively activated macrophage
MAPK	mitogen activated protein kinase
MCP	monocyte chemoattractant protein
M-CSF	macrophage colony stimulating factor
MEK	Ras/mitogen activated kinase
MIF	macrophage inhibitory factor
mVDR	membrane VDR
NADPH	nicotinamide adenine dinucleotide phosphate
NEFA	non-esterified free fatty acids
NIK	NFKB inducing kinase
NFKB	nuclear factor kappa beta
NO	nitric oxide
NOS	nitric oxide synthase
NOX4	NADPH Oxidase isoform
nVDR	nuclear VDR
OD	optical density
PAI	plaminogen activator inhibitor
PCR	polymerase chain reaction
PD	PD123177 AT2R chemical inhibitor
PKC	protein kinase C
PI3-K	phosphoinositide 3- kinase
PPAR	peroxisome proliferator-activated receptor
PS	penicillin/streptomycin
PTH	parathyroid hormone
RANKL	receptor activator for NFKB ligand
RAS	renin angiotensin system
RAW264.7	clonal murine monocyte/macrophage cell line
ROS	reactive oxygen species
RSK	ribosomal S6 kinase
RXR	retinoid-X-receptor
SD	standard deviation
SOD	superoxide dismutase
SPARMS	Selective PPAR modulators
SREBP	sterol regulatory element binding protein
T2DM	type 2 diabetes mellitus
TLR	toll-like receptor
TM	telmisartan AT1R chemical inhibitor
TNF	tumor necrosis factor
TZDs	Thiazolidinediones
UCP	uncoupling protein

VDR	vitamin D receptor
VEGF	vascular endothelial growth factor
WAT	white adipose tissue
$1,25D_3$ MARRS	membrane associated rapid response steroid binding protein
11βHSD-1	11-β-hydroxysteroid dehydrogenase type 1
24OHase	25-hydroxyvitamin D_3-24-hydroxylase

Chapter I

Introduction and Background Information

The Obesity Epidemic

Obesity is a multifactorial disease that increases the risk for type 2 diabetes mellitus, cardiovascular disease, osteoarthritis, chronic obstructive pulmonary disease and development of numerous forms of cancer [2]. Obesity has reached a pandemic status, becoming a top public health concern due to the increased morbidity and mortality associated with obesity [2]. According to the International Obesity Task Force, more than 1.1 billion adults worldwide are overweight and 312 million adults are considered obese [3]. Both developed and developing countries are confronted with an obesity epidemic with the emergence of multifactorial cluster of metabolic diseases [3]. While the etiology of obesity is complex, the root cause is quite simple, energy excess. Although genetics may influence an individuals susceptibility towards obesity, the increased prevalence of obesity is most likely attributed to environmental factors [2].

Environmental factors that influence increased caloric consumption include increased portion sizes, increased consumption of high sugar beverages, refined carbohydrates, restaurant dining, and a sedentary lifestyle [2]. Weight gain reflects a long term positive energy balance resulting from energy consumption being greater than energy expenditure [4]. Decreased energy expenditure has been an issue of concern dating back to the 1950's when President Eisenhower initiated the council on fitness and health to promote the importance of physical activity as a component to a healthy lifestyle [5]. However, decreased physical activity has remained a problem and has been compounded by the popularity of sedentary activities such as television viewing, increase usage of computers and the convenience of time and energy saving transportation

methods [4]. Numerous studies investigating the impact of physical activity on body weight have uniformly concluded that body fat increases proportionally with a decrease in the level of physical activity [6-9]. The major driving forces of obesity are environmental factors that shift the energy balance creating an increase in energy input and a decrease in energy output [4].

The International Obesity Task Force of the World Health Organization developed an index for classifying degrees of obesity based on the body mass index (BMI) [10]. BMI=(weight [kg]/height [m]²). Using BMI as an index, grade 1 consists of a BMI of 25-29.9 kg/m² and is defined as overweight [10]. Grade 2 is classified by a BMI of 30-39.9 kg/m² and is called obese [10]. Grade 3 consists of a BMI of greater than or equal to 40 kg/m² and is defined as morbid obesity [10]. In the United States it has been estimated greater than 60% of adults are categorized as overweight or obese [4]. In order to gain control of this epidemic, it is critical to clarify the mechanisms responsible for modulating lipid metabolism and thereby provide therapeutic anti-obesity strategies.

Function of Adipose Tissue

Due to the prevalence of obesity, adipose tissue has gained scientific interest in the biology of adipose tissue lipid metabolism and its affects on systemic metabolism and physiology [11-13]. Two types of adipose tissue exist, white and brown adipose tissue with dichotomous physiological function [14]. White adipose tissue (WAT) is found in all vertebrates and functions as a storage depot for times of energy excess [14]. In contrast, brown adipose tissue (BAT) functions in energy dissipation instead of storage and is essential for thermal homeostasis of hibernating mammals [15]. The classical function of WAT was perceived to be a passive storage site for excess fatty acids after ingestion of food [12, 16, 17]. However, later research emerged showing that adipose

tissue is an endocrine organ that synthesizes and secretes a plethora of adipokines in addition to fatty acids [11, 12, 17-19].

Adipokines have a wide range of physiological and metabolic effects contributing to energy balance, insulin sensitivity, vascular hemodynamics, lipid metabolism, angiogenesis, blood pressure regulation, and inflammation [20]. In mammals the predominate form of adipose tissue is WAT [1]. WAT is composed of a heterogeneous mixture of cell types including mature adipocytes, pre-adipocytes, endothelial cells, vascular smooth muscle cells, leukocytes, monocytes and macrophages [16, 21]. WAT demonstrates substantial fluidity in its ability to increase or decrease its mass over a 10-fold range to accommodate lipid storage [20]. During lipogenesis, enzymes responsible for import of fatty acids, glucose, and intra-adipocyte lipid synthesis stimulate accumulation of triglyceride in lipid droplets [22]. Lipid droplets coalesce together distending the size of the adipocyte up to 120 μm in diameter [22]. The tendency for enlarged adipocytes and increased number are directly regulated by circulating hormones and nutrients[22]. The process of overfilling lipid droplets, as a consequence of obesity, has been demonstrated to modulate adipocyte metabolism, resulting in altered function [23-25].

Excess fatty acids can be secreted from hypertrophic adipocytes, potentially impairing the functional activity of peripheral tissues promoting the development of obesity-induced morbidities [26]. A pattern of body fat distribution localized to the abdominal region is highly correlated with a significant increase in risk factor for the development of obesity associated metabolic complications and pathologies [27-29]. In the 1950's, the French physician, Jean Vague, was the first to recognize the direct correlation between body fat distribution and metabolic morbidities [28, 30]. Vague identified that android (upper body) obesity was highly associated in patients presenting with diabetes, gout and atherosclerosis and less evident in patients with gynoid (lower body) obesity [28, 30]. Later studies confirmed Vague's initial clinical observations

demonstrating the significance of the anatomical distribution of adipose tissue to development of metabolic disease [27, 29, 31-36].

WAT is a critical regulator in buffering the uptake of dietary fat acids from circulation [16, 19]. This buffering action is maintained by inhibiting the release of non-esterified fatty acids into circulation while increasing the removal of triglycerides from circulation [16, 37]. In the obese state, the buffering capacity for lipid storage is diminished due to overfilling of lipids [1]. Consequently a redirection in the storage of fatty acids in non-adipose tissues may occur, resulting in ectopic lipid accumulation [11, 16, 38, 39]. Deposition of lipids within the cytoplasm of non-adipose tissue cells, such as skeletal muscle and liver has been demonstrated to impair cell signaling and tissue function [21, 39]. The occurrence of insulin resistance in obese subjects has been demonstrated to be a consequence of hypertrophic fat cells [40, 41]. Hypertrophic adipocytes have a tendency to be insulin resistant corresponding to a diminished clearance of plasma triglycerides and increased leakage of free fatty acids [41]. In contrast, a lack of adipose tissue, known as lipodystrophy, has also been shown to lead to ectopic lipid accumulation, insulin resistance, and development of type 2 diabetes mellitus (T2DM)[42, 43].

Genetically modified fatless mice, A-ZIP/F-1 have extreme insulin resistance and excessive ectopic lipid accumulation in skeletal muscle and liver[43]. Surgical transplantation of adipose tissue from healthy donor mice into lipodystrophic mice reduced hyperglycemia, normalized insulin levels, and improved insulin sensitivity [43]. High levels of circulating free fatty acids induce accumulation of intracellular lipids in non-adipose tissues increasing peripheral insulin resistance [44]. In addition, excess circulating fatty acids may also cause pancreatic dysfunction and possibly apoptosis of pancreatic beta cells, resulting in a diminished insulin supply [45]. Consequently, these conditions enhance insulin resistance, glucose intolerance, hyperlipidemia, and hyperinsulinemia [46]. These findings demonstrate that impairment in the

storage of fatty acids may promote ectopic fat accumulation impairing metabolism. Therefore, maintaining the buffering function of adipose tissue is critical for the prevention of obesity-induced metabolic disturbances.

Effects of Obesity on Adipose Tissue

Multiple cellular processes, including adipoycte hypertrophy, recruitment of adipocyte precursors, adipocyte differentiation, and neovascularization are all coordinated in the development of obesity [47]. Recent evidence has emerged demonstrating that cellular inflammation is a primary mediator of obesity associated adipose tissue dysfunction [48]. Obesity is associated with an up-regulation in inflammatory signaling pathways such as c-jun N terminal kinase (JNK) and nuclear factor kappa beta (NFKB) [49]. Stimulation of inflammatory pathways significantly increase expression of downstream inflammatory cytokines, including tumor necrosis factor-α (TNF)-α, interleukin-6 (IL)-6, and monocyte chemoattractant protein-1 (MCP)-1 [50-52]. Furthermore, a comparison of tissues from obese people and lean people demonstrated that obese subjects had an increase in classically activated macrophages (M1) which are associated with secreting inflammatory cytokines TNF-α and IL-6 [17].

Local pro-inflammatory paracrine loop has been suggested to result from adipoycte secreted free fatty acids and macrophage production of TNF-α [17, 53]. As free fatty acids are released from enlarged adipocytes, the free fatty acids bind to macrophage-toll-like receptor-4 (TLR)-4 which leads to the activation of NFKB resulting in an increased production of TNF-α [17, 53]. The up-regulation of TNF-α then leads to activation of numerous other pro-inflammatory molecules including; intracellular adhesion molecule-1 (ICAM)-1, IL-6, and MCP-1 [17, 53, 54]. In addition to an up-regulation of pro-inflammatory molecules, enlarged adipocytes have reduced levels of the

anti-inflammatory molecule, adiponectin [17, 54, 55]. Adiponectin is an important adipokine in the regulation of metabolism by reducing hepatic gluconeogenesis and increasing lipid oxidation in muscle [56]. Reduced levels of adiponectin have been suggested to augment the vicious paracrine cycle between pro-inflammatory state of macrophages and adipocytes [17, 53, 55].

The excessive abundance of adipose tissue requires an increase in nutrients and oxygen for the transport of fatty acids. Consequently, an expansion of the microvasculature is required and is an intricate part in the development of obesity [17]. Hypertrophic adipocytes are also associated with local adipocyte hypoxia as the diffusion limit of oxygen (~100 μM) is unable to keep up with the rate of growth of the adipocyte [12, 17, 53]. Hypoperfusion activates the expression of hypoxia-inducible transcription factors resulting in the expression of angiogenic factors including vascular endothelial growth factor (VEGF), angiopoietins, and plasminogen activator inhibitor-1 (PAI)-1 [54]. The up-regulation of angiogenic factors inhibits adiponectin gene transcription, as observed in obese, insulin resistant mice [17]. Adiponectin functions to enhance insulin sensitivity via reducing hepatic glucose production and augmenting fatty acid oxidation in muscle and liver [17, 55]. Consequently, a reduction in the expression of adiponectin increases insulin resistance augmenting the vicious pro-inflammatory paracrine loop between adipoyctes and macrophages [17].

Vitamin D: A multifunctional Hormone

Synthesis and Metabolism of Vitamin D

Vitamin D is a fat soluble pro-hormone found in two major forms, ergocalciferol D_2 and cholecalciferol D_3 [57]. Ergocalciferol D_2 is synthesized from irradiation of ergosterol occurring in plankton and has only 1/3 the biological activity as vitamin D_3 in humans [58]. Cholecalciferol vitamin D_3 is the natural form of vitamin D that can be endogenously synthesized by the skin given appropriate wavelengths of UV light [58]. Vitamin D_3 is produced from 7-dehydrocholesterol, a derivative of cholesterol, and then photolyzed by UV light to produce pre-vitamin D_3 [58]. Pre-vitamin D_3 spontaneously isomerizes into biologically inert vitamin D_3 [58]. Whether vitamin D_3 is obtained from the diet or endogenously synthesized by the skin it must be converted into its biologically active form via a two step hydroxylation process [58].

The first hydroxylation occurs in the liver by the enzyme 25-hydroxylase (CYP27A1) to produce the metabolically inactive 25-hydroxycholecalciferol (calcidiol) [58]. A second hydroxylation occurs in the kidney by the enzyme 1-α-hydroxylase (CYP27B1) to produce two dihydroxylated metabolites the primary hormone 1,25-dihydroxycholecalciferol (calcitriol) and the candidate hormone 24R,25$(OH)_2$ D_3 [58]. Plasma vitamin D levels are measured using the primary circulating metabolite, 25-hydroxycholecalciferol (calcidiol) [59]. Calcidiol has a longer half life than calcitriol suggesting that its concentration is more stable and a better predictor of plasma vitamin D status [59]. The average plasma concentration of calcidiol in a healthy individual averages around 25-125 nM and while circulatory levels of calcitriol range from 0.1 nM to 10 nM [60] [59]. Calcitriol is released into circulation bound to the carrier protein, vitamin D

binding protein and transported to target organs [58]. Binding of calcitriol to a nuclear receptor or plasma membrane receptor of target cells generates the biological responses of calcitriol [58].

Classical Function of Calcitriol: Bone Mineralization

The classic physiological function of calcitriol is mineralization of the skeleton by increasing plasma calcium levels [58]. Extracellular calcium concentration is strictly maintained at 5 mM while intracellular concentration is significantly lower around 0.05-10 μM [61 62, 63]. Calcitriol stimulates plasma calcium levels via three distinct mechanisms [58]. First calctiriol induces active intestinal absorption of both calcium and phosphate [58]. Second, in response to hypocalcemic plasma levels, calcium sensitive proteins on parathyroid cells stimulate secretion of parathyroid hormone (PTH) [58]. In a concerted mechanism, PTH and calcitriol activate osteoblasts generating receptor activator nuclear factor kappa beta ligand (RANKL) a critical factor in bone metabolism [58]. RANKL activates osteoclasts resulting in calcium mobilization from bone, raising serum calcium levels [58].

PTH also activates CYP27B1 in the distal renal tubule stimulating the synthesis of calcitriol [58]. Calcitriol and PTH synergistically stimulate the reabsorption of the 1% of the last filtered load of calcium [58]. The increase in plasma calcium levels beyond the set point of the calcium sensing system suppresses PTH production completing the negative feedback loop [58]. If plasma calcium levels become hypercalcemic, the "C" cells of the thyroid gland synthesize the peptide calcitonin [58]. Calcitonin blocks mobilization of calcium from bone and calcium reabsorption from the kidney [58]. Under normocalcemic conditions calcitonin up-regulates renal CYP27B1 for synthesis of calcitriol [58].

A critical function of calcitriol is maintaining the parathyroid status in normal individuals [58]. Calcitriol suppresses the preproparathyroid gene and thereby inhibits proliferation of parathyroid gland cells regulating PTH production [58]. Degradation of the vitamin D metabolites is induced by the enzyme, 25-hydroxyvitamin D_3-24-hydroxylase (24OHase) to produce either $24,25(OH)_2D_3$ or $1,24,25(OH)_3D_3$ [64]. Following hydroxylation at carbon number 24, the metabolites are further degraded and excreted as calcitroic acid or 23-carboxyl derivates [64]. This degradation pathway is directly monitored by levels of calictriol which stimulates expression of 24OHase, thereby inhibiting excessive production of the hormone [64].

Structure of Calcitriol

The molecular structure of calcitriol is similar to that of the classic steroid hormones estradiol, cortisol and aldosterone [57]. Structurally they are related as they share the same root ring structure cyclopentanoperhydrophenanthrene [57]. However, calcitriol differs in that it is a secosteroid with one root ring structure broken [57]. Steroid hormones function as chemical messengers transmitting signals to produce genomic and rapid responses [65]. The presence of cognate vitamin D receptors in target tissues and organs enables calcitriol to generate its biological effects [65]. In 1969, the discovery of the nuclear vitamin D receptor spurred extensive research into the physiological function of the vitamin D endocrine system [65].

The ligand activated transcriptional effects of calcitriol occur upon binding of calcitriol to the nuclear vitamin D receptor (nVDR) inducing heterodimerization of nVDR with the retinoid X receptor (RXR) [65]. Calcitriol induced heterodimerization of nVDR-RXR is the functionally active transcription factor in calcitriol targeted gene activation [64]. The heterodimer DNA binding to the vitamin D response elements results in a concomitant recruitment of numerous co-activator

proteins initiating formation of multi-protein complexes that together with basal transcriptional machinery and histone modifiers stimulate transcription of vitamin D responsive elements of well over 200 genes [64-66].

In the late 1980's, evidence emerged demonstrating that calcitriol could quickly, within seconds generate a multitude of cellular responses via a plasma membrane receptor [65]. This membrane receptor was later identified as the membrane associated rapid response steroid binding protein (1,25D_3-MARRS) [65]. Activation of the membrane vitamin D receptor (mVDR) is associated with signal transduction pathways leading to stimulation of calcium channels with an influx of intracellular calcium [67]. Evidence of the non-genomic actions of calcitriol in various cell types include; rapid transcellular movement of calcium across chick enterocytes, rapid calcium mobilization from internal stores of osteoblasts and mitogen activated protein kinase (MAPK) stimulated calcium release from sarcoplasmic reticulum of skeletal muscle cells [67-71]. In addition to location on the plasma membrane, the mVDR has also been demonstrated on the endoplasmic reticulum [72].

Treatment with calcitriol stimulated a redistribution of the 1,25D_3-MARRS from both the plasma membrane and endoplasmic reticulum to the nVDR [72]. It has been suggested that the traditional nVDR may also have non-transcriptional functions [73]. Localization studies demonstrate that calcitriol stimulates a rapid nuclear translocation of 1,25D_3-MARRS to the plasma membrane [73]. This finding suggests that the 1,25D_3-MARRS may therefore be a recycling of the nVDR to the plasma membrane [74]. Additional evidence indicating the recycling of the nVDR was demonstrated by Zanello et al. who suggested that the membrane initiated events of calcitriol are linked to a functional nVDR [75]. Zanello et al. found that gene knockout of the nVDR in mice inhibits the calcitriol mediated activation of calcium channels on osteoblasts [75].

This finding demonstrates that the calcitriol mediated activity of calcium channels is fundamentally linked to the nVDR[75].

Nonskeletal Functions of Calcitriol

The demonstration of nVDR and mVDR in tissues and cells not associated with bone-mineral homeostasis indicated additional roles for vitamin D in endocrine function [76-79]. Substantial evidence demonstrates that calcitriol alters cellular differentiation and metabolism impacting various disease states [76, 78, 79]. Adipose tissue is a target organ for the calcitrophic hormones, calcitriol and PTH [60, 80-87 88, 89]. Substantial evidence has demonstrated a dysregulation in vitamin D metabolism related to obesity[90, 91]. Decreased levels of serum calcidiol and increased levels of PTH have been reported in obese subjects [92-99]. Intriguingly, elevated levels of calcitriol serum concentrations have been confirmed in obese subjects compared to non-obese subject controls [92, 100, 101]. It has been suggested that up-regulated PTH levels in obesity stimulate CYP27B1, possibly accounting for increased calcitriol levels with obesity [102]. Furthermore, it has been proposed that increased calcitriol levels in obese subjects is a negative feedback regulator on the synthesis of calcidiol in the liver accounting for reduced levels of calcidiol associated with obesity [102].

Elevated PTH and calcitriol levels have both been indicated to increase intra-adipocyte calcium levels altering adipocyte metabolism [89, 103]. Alteration of intracellular calcium levels play a critical function in modulating adipocyte metabolism in favor of adipogenesis [87]. Stimulation of receptor or voltage mediated calcium channels in adipocytes alters key lipid metabolizing enzymes enhancing lipogenesis and suppressing lipolysis [86, 104-106]. Influx of intracellular calcium increases expression and activity of a key lipogenic enzyme, fatty acid synthase (FAS), enhancing

triglyceride content of adipocytes and suppressing lipolysis [104, 106, 107]. Addition of a calcium channel antagonist blocked these effects demonstrating that modulation of intracellular calcium is a key factor in adipoycte metabolism [104, 105]. Calcitriol (5 nM) has been shown to stimulate an influx of intracellular calcium, resulting in upregulation of FAS expression and activity and inhibition of lipolysis [86]. This calcitriol mediated effect was repeatable using a vitamin D membrane agonist, 1α,25-$(OH)_2$-lumisterol$_3$, and blocked by a vitamin D membrane antagonist, 1β,25-dihydroxyvitamin D_3 [86]. These findings demonstrate that calcitriol stimulates an influx of intracellular calcium modulating adipoycte lipid metabolism via the 1,25 D_3 MARRS [86].

PTH stimulates an increase in intra-adipocyte intracellular calcium associated with reduced sensitivity of insulin stimulated glucose uptake in adipocytes [108]. Adipocytes and muscle cells are the primary targets of insulin-stimulated glucose uptake and are key determinants of overall insulin sensitivity [109]. Insulin is a critical regulator of systemic energy homeostasis, controlling the storage, mobilization and utilization of free fatty acids and glucose [110]. A coordinated orchestration of a cascade of phosphorylation/dephosphorylation signaling events are responsible for the action of insulin stimulated glucose uptake by adipocytes [109]. The initial events of insulin signaling include tyrosine phosphorylation of the insulin receptor (IR), tyrosine phosphorylation of the insulin receptor substrates (IRS)-1 and (IRS)-2 and activation of phosphatidylinositol 3-kinase (PI3-K) [109, 111].

PI3- K induces the translocation of the primary insulin responsive glucose transporter- 4 (GLUT)-4 to the plasma membrane for insulin stimulated uptake of glucose [109, 111]. High levels of intra-adipocyte calcium have been shown to inhibit the translocation of GLUT-4 from the intracellular vesicles to the plasma membrane resulting in insulin-resistant adipocytes [112]. High intra-adipocyte levels of calcium have been suggested to alter the phosphatase activity involved in the dephosphorylation of GLUT-4, reducing intrinsic activity of GLUT-4 and impairing

response of adipocytes to insulin [113]. In vitro induced insulin response has been demonstrated to be abolished by the addition of a calcium channel antagonist, nitrendipine, as a result of decreasing intracellular calcium concentration [114]. Dietary calcium supplementation has also been demonstrated to reduce intracellular calcium in adipocytes [89, 115-117]. This anti-obesity effect of high dietary calcium supplementation is mediated, in part, by suppressing calcitriol and possibly PTH induced influxes of intracellular calcium (Ca^{2+}) [89, 115-117]. Thus, it remains possible that the calcitrophic hormones may play a pivotal role in the regulation of adipocyte metabolism relative to the pathogenesis of obesity [89, 104, 106].

Calcitirol Modulates Adipose Tissue Metabolism

Adipocytes are derived from fibroblastlike cells during normal mammalian development and under various pathological conditions [118, 119]. A shift in adipocyte gene expression patterns results in morphological alterations in adipocyte shape and an increase in lipid accumulation stimulate adipocyte differentiation [120]. Adipoycte differentiation requires three transcriptional factors, CCAAT/enhancer binding proteins (C/EBP), peroxisome proliferator-activated receptor-γ (PPAR)-γ, and adipocyte determination and differentiation factor-1 (ADD)-1/sterol regulatory element binding protein- 1c (SREBP)-1c [118, 120, 121]. Cross regulatory and auto-regulatory mechanisms drive and maintain C/EBP and PPAR- γ protein expression, inducing expression of various other genes responsible for differentiation of adipocytes [109, 120, 121]. Differentiation of preadipocytes into adipocytes can be induced by insulin like growth factor- 1, (IGF)-1 glucocorticoids, and agents that increase cyclic adenosine monophosphate (cAMP) levels [122, 123]. The process of differentiation is characterized by re-entry into the cell cycle and expression of a cascade of transcription factors regulating adipogenesis [124].

C/EBP-β is a key transcription factor in preadipocyte differentiation and is rapidly increased following induction of differentiation [125]. Transcriptional activation of C/EBP-β is regulated via phosphorylation of the two cAMP response element binding protein (CREB) sites [126]. Following phosphorylation, acquisition of DNA-binding by C/EBP-β occurs initiates the entry of preadipocytes into the G_1-S cell check-point, a growth-arrested stage, stimulating terminal differentiation of preadipocytes into mature adipocytes [127]. Phosphorylated C/EBP-β induces additional adipogenic transcription activators including C/EBP-α, PPAR-γ, ADD-1/SREBP-1c leading to the expression of enzymes responsible for stimulating fatty acid synthesis, binding, uptake, and storage [124].

Evidence for the role of calcitriol modulation in adipocyte metabolism has been conflicting [60, 80, 88, 128-131]. Research suggests that intracellular calcitriol levels have dual effects on adipocyte differentiation [60, 88, 128]. Calcitriol has been demonstrated to inhibit adipocyte differentiation based on reduced activity of glycerophosphate dehydrogenase, suppression of triglyceride accumulation, and inhibitory effect on PPAR-γ, a transcription factor required for differentiation of adipocytes [132-134]. The molecular mechanisms by which calcitriol inhibits adipogenesis have been suggested to antagonize the C/EBP-β signaling pathway [128, 129]. Specifically, calcitriol has been demonstrated to inhibit the expression of C/EBP-β mRNA and the levels of C/EBP-β protein present during the induction of differentiation [128]. Consequently, reduced levels of C/EBP-β are incapable of inducing C/EBP-α and PPAR-γ, two critical transcriptional activators required for expression of adipogenesis related genes [128, 129]. Moreover, very high doses of calcitriol (100 nM) have demonstrated anti-proliferative activity inducing pro-apoptotic factors initiating adipocyte apoptosis [60].

In contrast, physiological doses of calcitriol (0.1-10 nM) has been demonstrated to induce a cascade of events that trigger adipocyte proliferation and differentiation [60, 130, 135-137].

Lipoprotien lipase, (LPL) is a key enzyme involved in lipogenesis and differentiation of adipocytes [136]. Treatment of 3T3-L1 adipocytes with calcitriol resulted in a 3-fold increase in LPL mRNA expression and LPL activity compared to control [136]. Calcitriol has also been reported to stimulate an increase in expression of adipocyte lipid binding protein (aP2), a specific adipocyte marker of differentiation [135, 136]. Up-regulation of aP2 mRNA expression was associated with increased induction of adipocytic cells in rat bone marrow stromal cell cultures [135]. In addition, calcitriol treated adipoyctes morphologically appeared rounder with increased number of adipoycte foci compared to control [136, 138].

Dace et al. demonstrated that physiological levels of calcitriol are capable of inducing terminal differentiation in Ob 17 cells, a clonal cell line established from epidymal fat pad of the obese mice, C57BL/6J [135]. The adipogenic effect of calcitirol was evident from the finding that no additional factors were required to stimulate differentiation in Ob 17 cells [135]. Dace et al. concluded that calcitriol induced differentiation is mediated through the VDR due to increased expression of the VDR mRNA during the differentiation process [135]. The impact of VDR signaling on adipose tissue metabolism is evident from the finding that ablation of the VDR in mice resulted in significant atrophy of adipose tissue mass [139]. In addition, mice lacking CYP27B1, also demonstrate a reduction in adipose tissue mass [131]. These findings suggest that calcitriol-VDR signaling plays a pivotal role in modulating adipocyte lipid metabolism regulating adiposity.

Energy Metabolism

Production of Cellular Energy

The cellular organelle of energy in adipocytes, as with any cell, is the mitochondria [41, 140]. In vitro studies have demonstrated that adipose tissue metabolism is regulated by the energy charge of the adipocyte[141, 142 143]. Mitochondrial efficiency, also known as the coupling efficiency, refers to the proportion of calories burned and oxygen consumed coupled to the synthesis of adenosine triphosphate (ATP) [144]. Coupling efficiency can be approximated in cells by using oligomycin, a specific antagonist for ATP synthase [144]. At high coupling efficiency there is an increased conversion rate of calories consumed to production of ATP [144]. Increased production of ATP can be used for cellular processes or stored for later use in the form of fat [144]. In contrast at low coupling efficiency, there is a reduced ATP synthesis and increased conversion of energy into heat [144].

As electrons are reduced to water and oxygen via the mitochondrial electron transport chain, protons are pumped from the mitochondrial matrix into the inner mitochondrial membrane [142, 144]. The pumping of protons into the inner mitochondrial membrane generates an energy potential and protonmotive force [142, 144]. This source of potential energy is harvested when protons are driven back into the mitochondrial matrix passing through ATP synthase [142, 144]. A high coupling efficiency is achieved when the majority of protons from the inner membrane are passed back into the mitochondrial matrix generating ATP [144]. However, the coupling efficiency can be reduced by leakage of protons back across the inner membrane through alternative proton conductance pathways not associated with the synthesis of ATP [144]. Inducible proton leak utilizes

uncoupling proteins (UCP), located in the inner mitochondrial membrane, that dissipate the proton gradient generated by the respiratory chain [144].

Uncoupling Proteins

The classic uncoupling protein, UCP-1 primarily functions in brown adipose tissue of rodents and is involved in cold-induced thermogenesis [144, 145]. UCP-1 homologues (UCPs 2-5) have been found in non-thermogenic mammalian tissues, prompting the re-examination of their function beyond thermogenesis [144, 146]. UCP-2 is expressed in white adipose tissue and is 59% homologous to UCP-1 [147]. UCP-3 is primarily expressed in skeletal muscle and was found to be 57% homologous to UCP-1 and 73% homologous to UCP-2 [148]. UCP-4 and UCP-5 are highly expressed in the central nervous system and as a result less emphasis has been focused on them with regards to altering metabolism [144]. In addition UCPs partially uncouple electron transport from ATP synthase, reducing the rate of the mitochondrial respiratory chain and thus diminishing production of reactive oxygen species (ROS), possibly guarding against oxidative stress [149].

Accumulating evidence suggests that UCPs are key targets in regulating energy metabolism [60, 80, 85]. In vitro studies have indicated that mitochondrial uncoupling may contribute to the control of lipid metabolism and adiposity due to a reduction in ATP production [40, 41]. Synthesis of UCP from the fat specific promoter of the aP2 gene in C57BL/6J transgenic mice serve as a useful model for investigating the role of mitochondria in WAT metabolism [14]. Diet or genetic induced obesity in aP2-UCP1-1 transgenic mice were found to have a reduction in adiposity associated with ectopic expression of UCP-1 [41]. Analysis of transgenic aP2-UCP-1 adipocytes indicated a decreased intracellular charge to be the driving force for the alteration in lipid metabolism and protection from obesity [41]. Therefore, mild uncoupling of mitochondrial

respiration from oxidative phosphorylation may provide a therapeutic target for counteracting obesity-induced oxidative stress [144, 150].

Investigation into the mechanism of UCPs and decreased adiposity was examined by overexpressing UCP-1 in adipose tissue of aP2-UCP-1 transgenic mice [41]. Results indicate that the mitochondrial electron transport chain has three coupling sites that require at least one molecule of UCP-1 to achieve a reduction in the mitochondrial membrane potential [145]. Flow cytometry experiments using fluorescent dye showed that the mitochondrial membrane potential of adipocytes from aP2-UCP-1 transgenic mice was decreased compared to wild-type controls [145]. In addition, augmented expression of UCP-1 generated a significant reduction in accumulation of lipids in adipose tissue under diet-inducing obesity [145]. This phenotypic anti-obesity effect suggests that transgenic modification of adipose tissue by uncoupling may affect lipid metabolism by altering lipid metabolizing enzymes [41]. Indeed, ectopic expression of UCP-1 in adipose tissue demonstrated an augmentation in LPL, indicating that respiratory uncoupling increased LPL-stimulated clearance of triglycerides [41]. Furthermore, transgenic mice were found to have reduced plasma triglyceride levels compared to matched wild type controls [41, 151]. These findings suggest that uncoupling respiration in adipocytes may provide beneficiary control over lipid metabolism [41].

Overexpression of UCP-1 was also associated with stimulating mitochondrial biogenesis and increasing mitochondrial content [141]. Electron microscopy revealed changes in mitochondrial morphology with ectopic expression of UCP-1 in adipocytes, indicating that UCP-1 induces mitochondrial biogenesis [141]. These mitochondrial characteristics included a thick peripheral rim surrounding the adipcoytes with inclusion of larger sized mitochondria that were oval shaped with a high degree of cristae per mitochondria [141]. In contrast, mitochondria from control group had a thin peripheral rim surrounding the adipocyte, elongated mitochondria with sparse

distribution of cristae [141]. Structural changes in mitochondria associated with overexpression of UCP-1 in adipocytes demonstrate an intermediate phenotype between brown and white adipocytes [141, 152]. These findings indicate that white adipocytes can acquire some brown adipocytes characteristics and thereby augment energy expenditure. UCP-2 was first identified when UCP-1 knock out mice failed to become obese resulting in a search for a UCP-1 homolog [153]. UCP-2 is upregulated in WAT in response to high fat feeding of mice [147]. UCP-2 and UCP-3 mediate the transport of fatty acids and oxidation during situations of fatty acid oversupply [154-156]. These findings suggest a key role of UCP-2 in energy metabolism and protection from development of obesity.

Calcitriol Modulates Uncoupling Protein Expression

Calcitriol has been demonstrated to act via the adipocyte nVDR to inhibit the expression of UCP-2 [60, 80]. Treatment of human adipocytes with 1 nM calcitriol resulted in a 50% reduction in UCP-2 mRNA and protein expression [80]. Anti-sense nuclear oligodeoxynucleotide knockout of the nVDR prevented his effect, confirming that calcitriol inhibits UCP-2 expression via the nVDR [80]. Thus, suppressing calcitriol levels and upregulating UCP-2 expression may inhibit lipogenesis and thereby regulate adipocyte lipid metabolism [80]. Increasing dietary calcium suppresses circulating calcitriol levels resulting in increased expression of UCP-2 and a reduction in metabolic efficiency and adiposity in mouse models of obesity [117, 157, 158].

Glucocorticoids Modulate Adipose Tissue Metabolism

The Role of Glucocorticoids in the Development of Obesity

Adipose tissue function can be modulated by changes in intracellular cortisol levels [159]. The function of glucocorticoids in fat accumulation is demonstrated in Cushing's syndrome, in which hypercortisolemia results in a significant increase intraadominal fat accumulation [160]. Cortisol is a potent stimulator of adipocyte differentiation [161]. In the presence of insulin, the addition of cortisol resulted in a 70-fold increase in the number of developing adipocytes, indicating a role for glucocorticoids in the development of hyperplastic obesity [161]. A critical determinant of triglyceride accumulation and adipocyte expansion is the activity level of LPL [162]. LPL catalyzes the hydrolysis of circulating triglcyerides into free fatty acids for re-esterification and storage in adipocytes [162]. In the presence of insulin, cortisol enhances lipid accumulation by activating LPL and inhibiting lipolysis [163]. The expression of glucocorticoid receptors have been demonstrated to be increased in visceral versus subcutaneous adipocytes, resulting in a heightened response to glucocorticoid stimulation [164].

Excess visceral adipose tissue has been identified as the most powerful predictor for the development of the metabolic syndrome [165]. Visceral adipocytes are notorious for increased fatty acid turnover and lipolysis compared to subcutaneous adipocytes and are less responsive to antilipolytic effects of insulin [166]. The increased supply of free fatty acids may be directed to the liver via the portal circulatory system, interfering with insulin signaling and increasing hepatic insulin resistance [165]. Consequently, insulin resistant hepatocytes, a contributor to metabolic syndrome, may result in increased glucose output by the liver inducing hyperglycemia [167]. In addition, gluconeogensis may be up-regulated as a result of the increased supply of glycerol from

the high oxidation rate of fatty acids in visceral adipose tissue [165]. Gluteofemoral adipose tissue releases stored fatty acids primarily under circumstances of metabolic glycogen depletion in which fatty acids are released and rapidly oxidized to meet the requirement as a fuel source [168]. In contrast, visceral adipose tissue releases excessive amounts of fatty acids even when glycogen availability is high and insulin levels are reduced, resulting in inhibition of free fatty acid oxidation and increased intramuscular accumulation of lipids, potentially creating metabolic dysfunction [168].

A key factor in the development of visceral obesity is glucocorticoid level [159]. Visceral adipocytes have increased expression of glucocorticoid receptor levels compared to subcutaneous adipocytes [169]. Moreover, 11-β-hydroxysteroid dehydrogenase type-1 (11βHSD-1) expression is significantly higher in visceral adipose tissue, with only negligible amounts detected in subcutaneous adipose tissue [170]. 11βHSD-1 regenerates active cortisol from inactive cortisone [159]. 11βHSD-1 is located in the endoplasmic reticulum and exhibits bidirectional functioning as a dehydrogenase in the conversion of cortisol to cortisone and a reductase in the conversion of cortisone to cortisol [171]. However, in adipose tissue 11βHSD-1 functions exclusively as a reductase, producing active cortisol [171].

Activity of adipose tissue 11βHSD-1 is tightly controlled by various growth factors, cytokines and pharmacological agents [172-177]. In human adipose tissue IGF-1 inhibits activity of 11βHSD-1 suppressing cortisol levels [173]. In contrast, the cytokines TNF-α and IL-1β stimulate enzyme activity and increase local production of cortisol [172]. Moreover, the product of 11βHSD-1, cortisol, promotes a positive feedback mechanism on intracellular availability of 11βHSD-1, magnifying the effects of cortisol [159, 170]. Pharmacological agents such as thiazolidinediones (TZDs) for the treatment of diabetes and protease inhibitors used in the management of human immunodeficiency virus infected patients inhibit activity of 11βHSD-1 [174-177]. Intriguingly, both

of these medications dichotomously modulate location of adipose tissue fat depots [174, 175, 177, 178]. Protease inhibitors increase fat deposition centrally while TZDs decrease visceral adiposity but increase subcutaneous adiposity [174-176, 178].

Increased levels of local glucocorticoid production has been reported in adipose tissue and skeletal muscle in human models of obesity and metabolic syndrome [179-183]. Numerous studies have demonstrated that the expression of 11βHSD-1 enzyme activity is significantly up-regulated in adipose tissues from obese humans and rats [180, 181, 184, 185]. These findings indicate a direct role for local regeneration of cortisol and amplification of glucorticoid production in obesity and the metabolic syndrome [180, 181, 184, 185]. The metabolic function of 11βHSD-1 in mice was examined using gene knockout and transgenic overexpression [159, 186-189]. 11βHSD-1 null mice demonstrated an insulin sensitive response with phenotypic resistance to the development of visceral adiposity even when challenged with a high fat diet [187-189]. In contrast, transgenic mice overexpressing 11βHSD-1 in adipose tissue expressed a stimulatory effect in enzyme activity with phenotypic visceral adiposity and insulin resistance, comparable to that observed in the metabolic syndrome [159, 186].

Calcitriol Up-regulates Glucocorticoid Activity in Adipose Tissue

Calcitriol has been demonstrated to directly up-regulate the expression and activity of 11βHSD-1 in human adipocytes and to increase the production and release of cortisol from adipocytes [190]. Cortisol itself enhances activity of 11βHSD-1, creating a positive feedback mechanism on glucocorticoid activity [170, 190-192]. Long term treatment of 48 hours with the murine precursor of active glucocorticoid, 11β-dehydrocorticosterone, stimulated expression of the nVDR in 3T3-L1 cells [88], and the combination of calcitriol and 11β-dehydrocorticosterone

further enhanced expression of the nVDR [88]. Therefore, it has been suggested that inhibition of 11βHSD-1 would down-regulate expression of nVDR, suppressing calcitriol stimulated activity of glucocorticoid activity [88]. Indeed, siRNA knockdown of 11βHSD-1 suppressed nVDR expression [88]. However, calcitriol still stimulated production of corticosterone in 11βHSD-1 siRNA treated adipocytes, suggesting that calcitriol may modulate glucocorticoid release by a nVDR-independent mechanism [88].

Although calcitriol stimulated 11βHSD-1 expression is nVDR mediated, calcitriol stimulated cortisol release is mediated through the rapid non-genomic action of 1,25D_3 MARRS [190]. Calcitriol mediated calcium signaling augmented cortisol release, an effect that was also achieved by treatment of adipoyctes with lumisterol, a 1,25D_3 MARRS agonist, calcitriol, calcium channel depolarizer and L-type calcium channel agonist [88 190]. Thus, calcitriol increases local glucocorticoid activity in visceral adipose tissue by both nVDR and MARRS-mediated mechanisms [190]. It has been established that calcitriol regulates glucocorticoid activity by a positive feedback mechanism [88]. Calcitriol stimulates increased activity of 11βHSD-1 which increases corticosterone levels and up-regulates expression of nVDR [88]. An increase in levels of nVDR enhances ligand binding ability to the nVDR increasing glucocorticoid activity [88]. Diets high in calcium have been indicated to suppress calcitriol levels attenuating intra-adipocyte calcium levels [87, 190]. Increasing dietary calcium may antagonize activity of 11βHSD-1 reducing local cortisol production regulating visceral adiposity [87, 190].

The Renin Angiotensin System

Adipose RAS and Adipose Tissue Dysfunction

Dysfunctional adipose tissue is associated with an up-regulation in the secretion of bioactive molecules such as angiotensinogen (AGT), pro-inflammatory cytokines, and reactive oxygen species [193]. It has been proposed that dysfunctional adipose tissue may lead to activation of the sympathetic nervous system stimulating the renin angiotensin system (RAS), promoting vasoconstriction and influencing the development of obesity-induced hypertension [193, 194]. Classically RAS functions in salt and extracellular fluid homeostasis for blood pressure regulation [195]. Activation of the classic RAS components angiotensinogen, renin and angiotensin converting enzyme result in production of the bioactive peptide hormone angiotensin II (ANGII) [195]. The primary effect of activation of the classic RAS is vasoconstriction, which is achieved via binding of ANGII, an octapeptide, to its receptor [195]. The normal physiological concentration of ANGII in plasma is around 10 pM [196]. It should be noted that local production of ANGII may result in higher intracellular concentrations compared to circulation [197]. For example, in the rat kidney, renal interstitial fluid concentrations of ANGII have been demonstrated to be around 3.76 nM, about 30 fold higher than circulating levels [197].

In addition to the classic RAS, local production of all RAS components have been confirmed in a multitude of tissues indicating the existence of a functional tissue RAS [198 199, 200 201]. Mature adipocytes produce and secrete vasoactive factors that enhance adipogenesis and may contribute to obesity-induced hypertension [202, 203]. Mature adipocytes express all molecules, enzymes, and receptors that comprise a functional RAS [11, 202, 203]. The main effector molecule of RAS, ANGII, is a vasoactive and proatherogenic peptide that stimulates production and release of prostacyclin,

which has been suggested to stimulate differentiation of preadipocytes into mature adipocytes [11, 203, 204]. It has been proposed that ANGII enhances activity of glycerol-3-phosphate dehydrogenase, a marker of adipocyte formation, in preadipocytes as a response to prostacyclin release from adipocytes [205]. In addition, ANGII was demonstrated to stimulate adipocye lipid accumulation by up-regulating expression and activity FAS and glycerol-3-phosphate dehydrogenase in both murine and human adipocytes [206]. These findings suggest that ANGII coordinately stimulates genes responsible for adipoycte expansion contributing to an increase in adipose tissue mass.

Research discrepancies demonstrating an anti-adipogenic role of ANGII has created ambiguity as to the true effect of ANGII on adipocyte differentiation [207-209]. ANGII has been demonstrated to block in vitro recruitment of undifferentiated cells from visceral adipocytes during adipocyte differentiation as evident by a reduction in glycerol-3-phosphate dehydrogenase activity [210]. In addition, this effect of ANGII was found to be greater in visceral adipocytes from obese subjects than subcutaneous adipocytes [210]. In accordance with this finding, other studies have reported that visceral adipose tissue significantly expresses higher levels of RAS transcripts compared to subcutaneous adipose tissue [211-213]. It has been postulated that an up-regulation in visceral ANGII production may inhibit the number of newly differentiated adipocytes contributing to the development of hypertrophic adipocytes creating a link between ANGII generation and visceral fat dysfunction [210].

The increased production of ANGII resulting in a decreased recruitment of newly differentiated adipocytes indicates the presence of a paracrine negative feedback loop [203]. Accordingly, the selective over-expression of AGT in murine adipose tissue resulted in adipoycte hypertrophy, suggesting that the effects of ANGII are trophic rather than adipogenic [13]. In addition, the over-expression of adipose AGT in mice was demonstrated to augment circulating

levels of AGT resulting in hypertension compared to wild-type mice [13]. Up-regulation in the expression of adipose AGT may also influence an elevation in systemic levels of AGT, demonstrating that cross talk may exist between adipose tissue RAS and the systemic RAS [37]. ANGII inhibits nitric oxide (NO), resulting in a diminished vasodilator capacity in the vessel wall, modulating vascular tone and promoting hypertension [204]. Adipocytes from obese subjects produce and secrete increased levels of AGT and ANGII which have been found to elevate systemic blood pressure levels [17]. These findings support a role for the adipose tissue RAS to activate systemic RAS contributing to obesity-induced hypertension.

The promoter region of the ANGII gene contains two glucocorticoid response-elements, and glucocorticoids have been demonstrated to induce expression of AGT in murine adipocytes [214-216]. Glucocorticoid stimulated expression of ANGII mRNA was associated with an increase in ANGII synthesis and secretion from adipocytes [216]. Calcitriol stimulates activity of 11βHSD-1 enhancing cortisol production in adipose tissue indicating a regulatory function of calcitriol in glucocorticoid activity [190]. Transgenic over-expression of 11βHSD-1 in mice was found to stimulate the systemic RAS system contributing to a significant rise in blood pressure [186]. In addition, transgenic over-expressing of AGT specific to adipose tissue also produced hypertensive effects [13].

Dysregulation of Adipocytokine Production

ANGII has been demonstrated to promote inflammation through upregulation of inflammatory cytokines or through increased oxidative stress [217, 218]. Tsuchiya et al. was the first to demonstrate that exogenous infusion of ANGII in rats resulted in increased expression of MCP-1 in adipose tissues [217]. Inflammation in adipose tissue has been suggested to be a link between obesity and the development of inflammatory-associated disease states of diabetes and

cardiovascular disease [56, 202, 219]. Obesity promotes a dysregulation in the expression and production of adipokines, resulting in an up-regulation of inflammatory cytokine expression [220].

Hypertertrophic adipocytes are associated with an increase in the expression of inflammatory adipocytokines TNF-α and IL-6 [56, 220]. It has been suggested that obesity-induced up-regulation of inflammatory cytokines may result in dysfunctional adipose tissue, leading to oxidative stress in accumulated fat [221]. ANGII has also been demonstrated to stimulate oxidative stress by binding to the AT1R resulting in activation the membrane bound, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a major contributor to ROS production [222]. Obesity induced oxidative stress has been demonstrated to result in a dysregulation in production of local adipocytokines as a consequence of increased activation of NADPH oxidase and a reduction in cellular antioxidant system [83, 221].

The AngII Receptor

Due to the vast physiological and pathological effects of the AT1R, pharmaceutical research has placed immense importance on determining the molecular mechanism essential for AT1R activation [223]. Current AT1R research has yet to fully identify the exact mechanistic approach but has proposed that AT1R stimulation occurs via G-protein dependent and G-protein independent signaling events [224]. The AT1R is comprised of 359 amino acids and belongs to class A family of G-protein coupled receptors (GPCRs) better known as seven transmembrane 7(TM) receptors due to the molecular organization composed of 7TM helical structures [225]. Research with mutated receptors and modified ligand binding has demonstrated that the ANGII binding site involves the interaction of amino acid residues in the TM helical segments of TM3, TM5, and TM6 enabling ligand binding and receptor activation[226, 227].

Although the precise mechanistic mode of activation has not been fully elucidated, a "global toggle switch" has been suggested to characterize the general and conserved features of the conformational transformations between the inactive and activated state of 7TM receptors [228]. According to this model, receptor activation requires an inward movement of the upper part of the extracellular segments of the TM helices with an oppositional movement of the lower cytoplasmic part of the helical bundle [229],[230],[231]. Research suggests that it is the separation of the cytoplasmic parts of TM3 and TM6 and the outward movement of TM6 away from the central axis of the helical bundle that makes the intracellular loops flexible enough to present epitopes for binding of G- proteins [232]. This restructuring initiates G- protein coupling as well as uncovering sites that can be modified via phosphorylation on the intracellular domains [233],[234].

In the classical model of AT1R activation via G- protein coupling, ANGII binds the AT1R stimulating the binding of regulatory G proteins resulting in second messenger signaling activating phospholipase A2, C and D which stimulates the primary transduction pathway, inositol triphosphate (the frequently used indicator of AT1R activation) and calcium signaling[225],[235]. The activated AT1R is phosphorylated by the G- protein coupled receptor kinase (GRK) or second messengers such as PKC[225],[235]. Second messenger signaling promotes beta-arrestin recruitment which physically uncouples G -protein from the receptor and terminates further G- protein activity [235]. The recruitment of beta-arrestins function as scaffolding mediating receptor internalization via clathrin coated pits initiating a second phase of activation defined as G- protein independent, beta-arrestin dependent[235]. Although receptor desensitization is considered the primary role of beta-arrestins for altering signal transduction pathways, several studies demonstrate that beta-arrestins are capable of initiating signaling independent of G protein activation [236],[237],[238].

The carboxy terminal tail of the AT1R favors binding and stabilization of beta-arrestins as it contains epitopes that enable direct binding and activation of beta-arrestins [239]. Exposing sites for beta-arrestin binding on the carboxy terminal tail of the AT1R requires conformational changes of the helical bundle in favor of G -protein independent signaling[235],[236]. Research demonstrates that separation of the cytoplasmic parts of TM3 and TM6 are required for G-protien activation of 7TM receptor, while inhibiting this movement abolished G- protein coupling [229],[240]. Mutational studies using zinc binding sites in which zinc cross links with TM3 and TM6, did not interfere with GRK phosphorylation and beta-arrestin recruitment [240],[241].

In addition, ANGII binding to AT1R has been demonstrated to induce beta-arrestin dependent signaling with receptor internalization and stimulation of ERK1/2 without inositol triphosphate generation [224]. Research has suggested that receptor conformations with high affinity for beta-arrestins would favor G -protein independent signaling while simultaneously inhibiting G -protein dependent signaling [242]. In addition, it has been proposed that beta-arrestin degradation of second messengers may promote a switch from G- protein dependent to G -protein independent signaling [225]. Moreover, pre-assembled beta-arrestin scaffolding proteins interacting with the AT1R can prevent the receptor from adopting various conformations and as a result this constraint on conformation would discriminantly regulate activity of signaling pathways[243].

ERK1/2 is the most well known example of beta-arrestin activated signaling pathway [238],[242],[244]. In cells overexpressig AT1R, G-protein activation occurred rapidly within two minutes and stimulated nuclear and cytoplasmic localization of activated ERK1/2 [245]. In addition, G-protein stimulated nuclear ERK1/2 localization promoted transcriptional activity of growth response 1 [245]. Alternatively, beta-arrestin had delayed and prolonged pattern of AT1R activation with only cytoplasmic localization [245]. Accordingly, G-protein and beta-arrestin demonstrate differential regulation of ERK1/2 signaling down stream of AT1R activation [245].

These findings suggest that modulation of AT1R activation by G-protein dependent or G-protein independent mechanisms can functional select signaling pathways and potenitate regulating cellular consequences.

Inflammation in Obese Adipose Tissue

Stimulators of Reactive Oxygen Species Production

Furukawa et al. demonstrated that increased ROS production in the adipose tissue of obese mice was responsible for modulating adipocytokine gene expression and enhancing systemic oxidative stress [221]. It was recently confirmed that the adipose tissue of mice on a high fat diet demonstrated an up-regulation in activity of the NADPH oxidase complex, which contributes to adipose tissue ROS production [246]. Calcium signaling is required for activation of ROS generating enzymes[247]. Influx of calcium through voltage-gated channels has been demonstrated to activate protein kinase C (PKC) contributing to the assembly of NADPH oxidase complex [83, 247]. Furthermore, numerous ROS-generating enzyme activities are calcium dependent such as myeloperoxidase which contains a calcium binding site that is critical for activation [247].

It has been suggested that ROS stimulates intracellular calcium levels and likewise high intracellular calcium levels favors production of ROS creating a positive feedback mechanism [83]. This suggestion was confirmed by the ability of the calcium channel blocker, nifedipine, to coordinately suppress intracellular calcium influxes simultaneously decreasing ROS formation [83]. Calcitriol has been reported to stimulate intracellular calcium levels, via $1,25D_3$ MARRS, enhancing ROS production and up-regulating gene expression of NADPH oxidase in 3T3-L1 adipocytes, whereas nifedipine inhibited these effects [83]. Calcitriol has also been suggested to act as a pro-oxidant, down-regulating cellular anti-oxidant defense systems [248]. In addition, ANGII

has been reported to enhance ROS production via influx of intracellular calcium, elevating mitochondrial calcium uptake resulting in an increase in mitochondrial ROS production [249, 250].

ROS are byproducts of normal physiological respiration and mitochondria contribute a significant proportion of ROS generation to total cellular ROS [83, 247]. ROS have been suggested to function as signaling molecules stimulating in vitro cellular proliferation and growth [83, 251]. In adipocytes, a low does of hydrogen peroxide was found to increase cell DNA synthesis and inducing adipocyte proliferation while the addition of the anti-oxidant, α+/- tocopherol, antagonized these effects [83]. In addition, the mitochondrial uncoupling inhibitor, guanosine 5'-diphosphate, was found to augment the hydrogen peroxide proliferative effects while nifedipine, a calcium channel blocker antagonized the proliferative effects of hydrogen peroxide [83]. ROS production is regulated in part by the mitochondrial potential, as a slight decrease in the mitochondrial potential of 10 mV was sufficient to abolish 70% of mitochondrial ROS formation [252]. In addition, mild activation of UCP via transfection was also found to diminish the mitochondrial potential and suppress ROS production [83]. Calcitriol has been shown to increase adipoycte ROS production by inhibiting mitochondrial uncoupling while overexpression of UCP-2 attenuated this effect [80, 83].

It is well accepted that ROS are key factors involved in oxidative damage to cellular structure and dysregulation of redox-sensitive signaling pathways. A moderate increases in ROS can act as intracellular signaling molecules to regulate protein kinase activity and transcription factor activity production to stimulate mammalian cellular growth and proliferation [251]. ROS modulate cellular signal transducers of MAPK such as the extracellular signal regulated kinase ½ (ERK ½) that activates the downstream transcriptional effecter complex activator protein-1 (AP)-1 dimer consisting of the transcription factors c-fos and c-jun [253, 254]. Calcitriol contributes to ROS production by increasing cytosolic calcium levels and inhibiting mitochondrial uncoupling

[83]. Calcitriol has been found to alter cellular signaling by stimulating PKC leading to activation of MAPK signaling pathways to induce AP-1 dimer complex enhancing gene expression to positively modulate cellular proliferation [83, 253, 255].

In addition, calcitriol was demonstrated to stimulate adipocyte proliferation as indicated by an increase in expression of cyclin a, required for entry into the synthesis stage of the cell cycle [83]. ANGII also activates MAPK signal transduction pathways of ERK ½ and JNK to positively modulate cellular proliferation [256]. Furthermore, ANGII has been demonstrated to reduce the number of preadipocytes in the growth arrested phase of the cell cycle and increase the proportion of 3T3-L1 cells in the synthesis phase of the cell cycle [257]. Calcitriol and ANGII independently alter signaling transduction pathways to increase ROS that function as second messengers in the modulation of MAPK pathways that activate transcription factors responsible for cellular proliferation [255, 256, 258].

Macrophage Accumulation and Adipose Tissue Inflammation

A critical component to the inflammatory state of obese adipose tissue is the presence of activated macrophages [259]. Macrophages are leukocytes which are derived from bone marrow and mature and enter the circulation as monocytes [260, 261]. The circulating monocytes are then recruited into various tissues where they reside and differentiate into macrophages to function in phagocytic and microbial cellular activities [260, 261]. Macrophages are recruited from bone marrow and infiltrated into adipose tissue via the residential adipose tissue MCP-1 [259]. Adipose tissue of obese individuals exhibit an increased infiltration of macrophages [262 259]. Genetic analysis of gene transcripts from mouse adipose tissue revealed that 30% of 1,304 transcripts that were

highly correlated with body mass in genetic and diet-induced models of obese mice compared to lean control [56].

Annotation of the transcripts indicated a characteristic upregulation of genes that are associated with enhancing the expression of macrophage transcripts in the models of obesity [56]. This finding suggests that a positive correlation exists between the infiltration of macrophages with increasing adiposity [56]. Infiltrated macrophages recruit haemopoietic cells to areas of local inflammatory sites to mediate the innate and adaptive immune responses [260]. Macrophages are classically activated by interferon gamma, or in combination with lipopolysaccharide to produce the the classically activated macrophage, M1 [263]. M1 macrophages release numerous cytokines such as TNF-α and IL-6 which in turn activate inflammatory cell signaling pathways to produce acute phase inflammatory molecules such as C-reactive protein and PAI-1[56, 260]. In contrast, an alternative pathway of macrophage activation occurs by the cytokines IL-4 and IL-13 which lead to activation of the alternatively activated macrophage, M2 [264]. The M2 phenotype has been suggested to function in reducing the inflammatory effects of M1 macrophages and to participate in repair processes [264].

The significant contrast in properties of macrophage polarization states are due to the regulation of arginase and nitric oxide synthase (NOS), which both require the substrate L-arginine [265, 266]. Competition between NOS and arginase for the substrate L-arginine results in significantly contrasting effects[266]. Arginase requires L-arginine as a substrate for the production of L-ornithine, a precursor for proline, required for collagen synthesis [265, 266]. NOS utilizes L-arginine for the production of NO, a ROS, that increases inflammation [265, 266]. In addition, ANGII antagonizes NO resulting in promotion of macrophage adhesions and increasing monocyte/macrophage accumulation in the vessel walls promoting endothelial dysfunction and oxidative stress [204]. Comparison of adipose tissue macrophages from obese and lean mice

revealed that arginase activity is augmented in lean mice due to an increase in M2 phenotype [266]. This finding led to the suggestion that maintaining a positive balance in favor of arginase may function to downregulate the production of ROS via NOS thus reducing inflammatory effects and preventing adipocyte dysregulation [266]. Furthermore, gene expression analysis of adipose tissue macrophages between lean and obese mice found that lean mice have an increased expression of the cytokine, IL-10 [266]. IL-10 is a critical mediator for insulin sensitivity which has been indicated to attenuate the proinflammatory effects of TNF-α [266].

The chronic low-grade inflammatory state associated with obesity is a result of infiltration and activation of M1 macrophages in adipose tissue [261]. Obesity favors a shift in macrophage phenotype towards the "classically" activated M1 macrophage [266]. M1 activated macrophages produce proinflammatory cytokines (TNF-α, IL-6, IL-12) and increase reactive oxygen species production such as nitric oxide [266]. It has been suggested that factors such as MCP-1, TNF-α, and saturated fatty acids induce the expression of TLR-4, leading to an alteration in circulating monocytes in favor of the proinflammatory phenotype [266, 267]. Therefore, the "classically activated", M1 macrophage, has been characterized as the primary producer of inflammatory molecules that have been detected in the pathogenesis of obesity [262]. Augmented production of MCP-1 from hypertrophied adipocytes enhances the release of free fatty acids, promoting inflammatory modulation in the adipoycte metabolism [53]. Takahashi et al. observed that obese mice have a 7.2 fold increase in the inflammatory expression of MCP-1 compared to normal mice [268]. More importantly, Takahashi et al. also demonstrated that a positive correlation in the upregulation of MCP-1 protein levels was associated with a concomitant increase in the CD11b-positive monocyte/macrophage in adipose tissue of obese mice [268].

Recent evidence confirmed that consumption of high fat diets results in a phenotypic switching of adipose tissue macrophages resulting in an alteration in the cytokine expression of

murine adipose tissue macrophages from M2 to M1, as a result of the decreased expression of IL-10 and increased expression of TNF-α [263]. Weisberg et al. analyzed the expression profile of macrophage and nonmacrophage cell populations isolated from adipose tissue and found that adipose tissue derived macrophages are the primary source of adipose tissue TNF-α and MCP-1 [56]. Weisberg et al. concluded that an increase in quantity of adipose tissue macrophages activates inflammatory pathways in the adipose tissue of obese individuals [56]. Furthermore, macrophages appear to inhibit adipocyte differentiation, potentially influencing adipocyte hypertrophy, adipokines secretion, and ectopic lipid accumulation [262].

Lumeng et al. demonstrated that the phenotypic switching of macrophages in obese adipose tissue is due to spatial differences in macrophage subtypes [269]. Resident adipose tissue macrophages from lean C57Bl/6 mice expressed markers associated with M2 phenotype localized to interstitial spaces between adipocytes[269]. The "alternatively" activated macrophage, M2, has been suggested to function as a tumor-associated macrophage and in parasitic infections with the potential to regulate tissue repair and inhibit inflammation [266]. Diet induced obesity in C57Bl/6 mice resulted in a high concentration of M1 gene expression localized to clusters of necrotic adipocytes with a significant reduction in markers for M2 gene expression [269]. This differences in macrophage subtypes demonstrates that there is an obesity induced switch in activation of adipose tissue macrophages with a concomitant recruitment of inflammatory M1 macrophage subtype from the circulation to surround local necrotic adipocytes in obese adipose tissue [269].

Resident macrophages in adipose tissue are surrounded by adipocytes that are continually releasing free fatty acids that have the potential to activate adipose tissue macrophages [270]. The excessive production of free fatty acids via lipolysis have the ability to activate inflammatory pathways primarily through the JNK cascade signaling system and increased I-kappa-B-kinase-beta (IKKB) in adipose tissue [270]. Nguyen et al. demonstrated that clonal murine

monoctye/macrophage cells (RAW264.7) treated with a mixture of saturated and unsaturated free fatty acids invoke a proinflammatory response by activation of JNK and IKKB signaling [270]. The proinflammatory effects of free fatty acids were found to be time and concentration dependent [270]. At approximately 5 minutes after treatment of RAW264.7 cells with 500 μM free fatty acids, JNK and IKKB activation was confirmed by western blot analysis for components of the JNK and IKKB signaling pathway [270]. Furthermore, 500 μM of free fatty acids at approximately 3 hours induced activation of JNK pathway-mediated proinflammatory genes including IL-1β, IL-6, MCP-1 and TNF-α [270].

Xu et al. demonstrated that the up-regulation of macrophages in white adipose tissue in the obese mouse model initiates an inflammatory response and interferes with insulin signaling, resulting in systemic insulin resistance [219]. Xu et al. hypothesized that the excessive release of free fatty acids from adipoyctes into systemic circulation impairs insulin signaling pathways in muscle and liver [219]. Coculturing of differentiated 3T3-L1 adipocytes and RAW264.7 macrophage cells resulted in a dramatic increase in expression of TNF-α and a concomitant inhibition in the expression of the anti-inflammatory cytokine adiponectin [53]. It has been suggested that a paracrine loop exists between adipocytes and macrophages, resulting in a vicious cycle that enhances inflammation in obese adipose tissue [53, 271].

A large scale gene expression analysis between the common features shared by preadipocytes, adipocytes, and macrophages revealed that the preadipcoyte profile is more intimately related to the macrophage than the adipocyte [272]. Moreover, given appropriate environmental conditions, preadipocytes can display cellular plasticity with the potential to efficiently be converted into macrophages [272]. Charriere et al. indicated that direct contact between preadipocytes and macrophages appears to be required for phenotypic conversion between two distinct cell lineages [272]. Accordingly, preadipocyte cellular plasticity may be a

contributor to the enhanced macrophage activity and inflammatory state of obese adipose tissue [219, 272].

Calcitriol Modulates Macrophage Activity

Calcitriol has been found to induce adipocyte-macrophage cross-talk, resulting in modulation of adipose cytokine production [81]. Calcitriol was found to enhance the expression of two macrophagic factors in differentiated adipocytes; macrophage inhibitory factor (MIF) and macrophage surface-specific protein (CD14) [81]. This effect was inhibited by the addition of the calcium channel blocker, nifedipine, indicating an intracellular calcium dependent mechanism in the calcitirol mediated macrophagic activity [81]. In addition, calcitriol increased expression of macrophage colony stimulating factor (M-CSF), macrophage inflammatory protein, IL-6, TNF-α and also MCP-1 [81]. These effects were attenuated with the addition of either a calcium channel blocker, (nifedipine), or a mitochondrial uncoupler (dintrophenol) [81].

Furthermore, co-culturing of RAW264.7 macrophages and 3T3-L1 adipocytes significantly up-regulated expression and production of inflammatory cytokines compared to individual cultures [81]. These findings indicate a key role of calcitriol in increasing adipoycte inflammation by modulating the interaction between adipocytes and macrophages [81]. It has previously been demonstrated that increasing dietary calcium attenuates obesity-induced oxidative stress by suppressing inflammatory markers [81]. The principle factor responsible for this effect is a suppression of calcitriol resulting in regulation of calcium signaling and mitochondrial uncoupling leading to a reduction in the inflammatory status of adipose tissue [81].

Pharmacological Inhibition of RAS

Additional Benefits

Recent studies have indicated that anti-hypertensive treatment via pharmacological blockade of the RAS guards against the development of T2DM in high risk patients with or without hypertension [273-282]. AT1R blockade has been demonstrated to enhance insulin sensitivity and thereby reduce the incidence of T2DM in hypertensive patients [283]. Moreover, elevated levels of plasma concentrations of ANGII have been reported in diet induced obese, hypertensive rats [284]. In contrast, the administration of AT1R blockers such as telmisartan to diet induced obese mice resulted in a reduction of weight gain and a decrease in plasma levels of glucose, insulin, and triglycerides compared to non-treated, control mice [285]. Various mechanisms have been proposed to explain how the inhibition of RAS provides these therapeutic metabolic effects independent of the primary hypotensive effects. These include enhanced muscle glucose uptake, protection of beta cell function, remodeling of adipose tissue structure, suppression of NADPH oxidase activity, increased activation of insulin signaling, and an up-regulation in expression of PPAR-γ by selective subclasses of pharmacological AT1R blockers [285-291]. Collectively, these observations have stimulated a significant amount of interest to be placed on ANGII receptor blockers (ARBs) as potential therapeutic agents for the management of obesity related metabolic complications.

Improvement in Metabolic Parameters

Clinical studies have indicated that ANGII inhibition by subclasses of ARBs significantly reduce the incidence of T2DM when compared to other anti-hypertensive medications [273, 274, 292-

[295]. In addition, several studies collectively indicate that sub-types of ARBs including, telmisartan, irbesartan, and losartan, stimulate the nuclear hormone receptor, PPAR-γ [285, 296, 297]. PPAR-γ is considered a master regulator for adipocyte differentiation[118, 298, 299]. The strength of PPAR-γ stimulation by ARBs (in descending order) is telmisartan, irbesartan and losartan [297, 300]. The additional subtypes of ARBs, valsartan, candesartan, olmesartan, and eprosartan have little or no effect on PPAR-γ activity [285, 300]. However, clinical studies indicate that these ARBs do provide metabolic improvements regardless of their effects on PPAR-γ [293, 295, 296, 301-303]. Moreover, the ARBs, losartan, telmisartan, and irbesartan have been shown to up-regulate plasma levels of adiponectin [280, 285, 292, 293, 296]. Adiponectin levels are suppressed with increasing adiposity and are positively correlated with insulin sensitivity [292, 293, 304-306].

PPAR-γ has been demonstrated to be a key factor responsible for increased expression levels of adiponectin [307]. It is generally accepted that the clinical use of pioglitazone results in increased serum adiponectin secondary to transcriptional regulation mediated by PPAR-γ [307]. However pre-treatment with a PPAR-γ inhibitor under basal conditions had no effect on the level of adiponectin expression, indicating that basal transcriptional activity of adiponectin may occur independently of PPAR-γ stimulation [308]. Intriguingly, investigation into the mechanism responsible for the associated increase in adiponectin levels via ARB treatment has also proposed both PPAR-γ dependent and PPAR-γ independent regulatory mechanisms [296, 308].

Special Attributes of Telmisartan

Telmisartan has been demonstrated to be the most potent PPAR-γ agonist [285, 297, 309]. Treatment with telmisartan increased PPAR-γ expression, and this effect was not antagonized by a PPAR-γ inhibitor [285, 308, 310]. This finding suggests that telmisartan's effect on adiponectin

expression may be independent of PPAR-γ stimulation [308]. Investigation into this unique effect of telmisartan identified specific biological features applicable only to telmisartan, and not the additional ARBs, and traditional inducers of PPAR-γ, TZDs [285, 297, 300, 311]. These unique properties are a result of the molecular structure of telmisartan which is significantly different from the structures of other ARBs [312]. Molecular modeling studies have identified distinct features of the structure of telmisartan which include, a carboxyl substituent in place of the tetrazole group, the heterocyclic substituent of the benzimidazole moiety is absent, and the imididazole moiety is lacking a carboxyl group [312]. It is these structural differences that have been proposed to provide telmisartan with specific attributes unrelated to the additional ARBs [300, 312, 313].

In vitro studies comparing cellular diffusion levels of telmisartan to losartan reported that the intracellular concentration of telmisartan was 10 fold higher than the concentration in the culture medium while losartan was undetectable intracellularly compared to culture medium [314]. This effect of telmisartan was suggested to be due to its higher lipophilicity and higher membrane permability compared to losartan [315]. As a result, these properties have been suggested to endow telmisartan with a unique fully penetrable cellular structure non-existent to the other ARBs [309, 314, 315]. Molecular characterization of telmisartan within the LBD of PPAR-γ has indicated a unique orientation which produces a highly stabilized complex [316]. Crystallography studies indicate that the binding mode of telmisartan in the receptor pocket is stabilized by hydrophobic interactions between helixes H3 and H7 and by hydrogen bonding with an amide proton [285, 316]. These conformational arrangements within the receptor pocket do not appear to exist with the additional ARBs or traditional TZDs [285, 316]. These structural differences between telmisartan and the other ARBs may explain the underlying differences in the selective PPAR modulators (SPARMs) by ARBs [297].

In comparison to TZDs, telmisartan was found to act as a partial agonist of PPAR-γ, resulting in differential modulation of PPAR target gene expression patterns compared to a full agonist of PPAR-γ such as, rosiglitazone [285]. This differential regulation is evident by investigation of the gene expression pattern of acetyl-CoA carboxylase-2, (ACC2), a primary regulator of muscle fatty acid metabolism [285]. Treatment of murine muscle myotubes with telmisartan resulted in a down-regulation of ACC2 gene expression while treatment with rosiglitazone, irbesartan, and valsartan had no effect [285]. Reducing ACC2 expression has been suggested to promote fatty acid oxidation in muscle with a reduction in adiposity, possibly explaining the observed in vivo weight loss via treatment with telmisartan [317]. As a result this raises significant interest in the ability of SPPARMs to regulate carbohydrate and lipid gene expression without activating expression of genes associated with weight gain, fluid retention and additional adverse effects associated with the treatment of traditional PPAR-γ modulators [285, 318, 319].

Irbesartan Prevents Degradation of Adiponectin

Irbesartan appears to function as another SPPARM which increases adiponectin levels, [296]. Mechanistically irbesartan has been suggested to prevent cellular adiponectin protein depletion while significantly augmenting the half life of adiponectin dependently on PPAR-γ activity [296]. However, irbesartan had no effect on mRNA adiponectin levels [296]. Instead, this appears to be a result of abolishment of the ubiquitin-proteasome pathway, upstream of the 26S-proteasome complex [296]. The 26S-proteosome is a multi-subunit enzyme complex located in the nucleus and cytoplasm of the cells which plays a key role in the degradation of proteins [320]. The 26S-proteosome utilizes a unique substrate identification mechanism which results in covalent modification of substrates which are then tagged with the well recognized protein, ubiquitin [320].

In vitro treatment of adipocytes with peptide aldehyde proteasome inhibitors prevented adiponectin protein depletion as effectively as treatment with irbesartan [296]. As a result, cellular adiponectin levels are stabilized and enhanced via treatment with irbesartan (10 μmol/L). In addition to stimulation of PPAR-γ, irbesartan also was found to induce the PPAR-γ target gene, aP2 [296]. Collectively, these findings suggest that the PPAR-γ inducing effects of the ARB, irbesartan, protects against degradation of adiponectin protein at the post-transcriptional level [296].

To investigate PPAR-γ activating and non-PPAR-γ activating ARBs on adiponectin level in vivo, obese Zucker fa/fa fatty rats were administered Irbesartan (50 mg/kg) orally for 21 days or given an oral saline solution for duration of treatment [296]. Metabolic comparison at day 0 and day 21 of treatment showed that the irbesartan group had a 36% decrease in fasting insulin levels and a significant reduction in serum triglyceride levels, demonstrating a marked improvement in metabolic parameters compared to saline (control) group [296]. Serum adiponectin levels of the irbesartan treated group remained stable through duration of treatment period while the control group had a 20% reduction in serum adiponectin levels at end of treatment period [296]. These findings suggest that treatment with irbesartan provides protection against the degradation of serum adiponectin levels.

Irbesartan has been demonstrated to activate the insulin sensitizing nuclear transcription factor PPAR-γ, independent of inhibition of AT1R [285, 300]. This finding is supported by in vitro studies demonstrating that pharmacological antagonism of PPAR-γ suppresses irbesartan's effects on adiponectin expression [296]. Moreover, obese rats treated with irbesartan showed an improvement in insulin sensitivity associated with a stabilization in serum adiponectin levels [296]. These findings suggest that irbesartan maintains adiponectin levels via stimulation of PPAR-γ[296]. In contrast non PPAR-γ activating ARBs such as eposartan failed to have an effect on adiponectin expression [296].

Remodeling of Adipose Tissue

The remodeling of adipose tissue mediated by TZDs is associated with newly formed small clusters of adipocytes, an increase in the expression of key lipogenic enzymes with a increased response to the effects of insulin [321]. Despite the quantitative increase in adipose tissue mass by treatment with TZDs, there is an associated improvement in the metabolic state [321]. In contrast, in vivo treatment with telmisartan has demonstrated an insulin sensitizing effect without the simultaneous stimulation in adiposity [322-324]. The insulin sensitizing effects of specific ARBs have been suggested to result from up-regulation in the expression of insulin sensitizing adipocytokines [296, 324-326]. The associated insulin sensitizing effects of adiponectin have been attributed to the stimulation of AMP-kinase, leading to an enhancement in glucose disposal and fatty acid oxidation, reducing tissue triglyceride accumulation [327].

The apparent decrease in adipose tissue mass with telmisartan results from enhanced expression of adipose tissue UCP-1 [324]. This is in agreement with a prior study reporting an up-regulation in adipose UCP-1 expression via AT1R knock-out mice [328]. Moreover, the telmsiartan increase in UCP-1 expression was accompanied with an increase in oxygen consumption and a decrease in respiratory quotient compared to control [324]. This evidence indicates the ability of specific ARBs such as, telmisartan, to be regulators of adipose tissue metabolism [324].

Up-regulation of UCP-1 expression via telmisartan increases oxygen consumption and decreases the respiratory quotient indicates a switch from carbohydrate to lipid as a metabolic fuel source [324]. This metabolic switch has been proposed to be responsible for the phenotypic decrease in body adiposity observed with treatment of diet induced obese mice with the ARB, telmisartan [324]. The apparent decrease in adiposity while retaining PPAR-γ modulating activity

associated with selective ARBs such as telmisartan and irebesartan demonstrates the efficacy of using ARBs as therapeutic treatment agents for obesity associated metabolic disorders [296, 324-326].

Negate Effects of Obesity Induced Hypoxia

Increasing adipose tissue mass is significantly correlated with alterations in the endocrine and metabolic functions of adipose tissue [56]. Specifically, adipose tissue hypoxia has been recognized as a key consequence of obesity [329]. In obesity, adipocytes become hypertrophic, resulting in a significant increase in diameter of up to 140-180 μM [330]. The capacity for adipocyte hypertrophy is limited by the 100 μM diffusion limit of oxygen and consequently oxygen availability does not meet oxygen demand during obesity [331, 332]. Previous reports have demonstrated that adipocyte hypoxia stimulates adipocytokine dysregulation secondary to up-regulation in oxidative stress induced dependent posttranscriptional mechanisms [329]. The effects of hypoxia are mediated by the transcription of hypoxia inducible factor (HIF) responsible for stimulating expression of hypoxia sensitive markers, such as GLUT-1 [333].

Hypoxia up-regulates the expression of inflammatory adipocytokines such as IL-6 and MCP-1 and down-regulates expression of anti-inflammatory adipocytokines [334, 335]. Moreover, obesity stimulates an increase in HIF which has been suggested to negatively regulate insulin signaling, contributing to insulin resistant adipocytes [336]. HIF expression has been shown to stimulate insulin resistance whereas HIF inhibition via HIF siRNA restored insulin stimulation of the IR [336]. The inhibitory effects of hypoxia were found to occur at the step of insulin receptor tyrosine autophosphorylation [336]. Chronic up-regulation in HIF has been reported to also stimulate infiltration of macrophages, leading to a further dysregulation in adipocytokine expression [329, 337].

HIF proteins are stimulated not only by hypoxia but also by various growth factors and inflammatory cytokines such as ANGII [336, 338, 339]. A primary adaptive change associated with chronic hypoxia is angiogenesis and ANGII is a potent angiogenic factor [338, 340, 341]. Mounting evidence indicates that therapeutic intervention of obesity induced hypoxia may be achieved via targeting the vasculature [342, 343]. ARBs have been reported to reduce the expression of HIF with a concomitant attenuation in the expression of inflammatory cytokines and abrogation of the angiogenic response to chronic hypoxia [338, 341, 344, 345].

Antioxidant Effect

It is well accepted that activation of the AT1R by ANGII is associated with induction of ROS formation mediated by the AT1R [346-349]. In vitro studies have demonstrated a time and dose dependent induction of superoxide production in response to treatment with ANGII [350-353]. The molecular source of ANGII induced superoxide production has been attributed to an up-regulation in the expression of NADPH oxidase subunits [349, 354-357]. Treatment with NADPH oxidase inhibitors attenuated the dysregulation of adipocytokine expression suggesting that the redox state of adipose tissue is a powerful regulator of local oxidative stress [221].

Several studies have indicated that the AT1R inhibitor, valsartan, acts as an anti-oxidant [358, 359]. This anti-oxidant activity of valsartan has been suggested to be a result of its molecular structure [359]. The presence of phenolic rings or conjugated double bonds has been suggested to be the determinant for the scavenging properties of anti-oxidant like molecules [360]. Valsartan is composed of several aromatic rings which have been suggested to provide the anti-oxidant effect associated with its usage [358, 359]. Additional studies suggest alternative mechanisms for the observed anti-oxidant effect of ARBs [314, 350, 361-365]. Telmisartan was found to directly suppress

reactive oxygen species production without up-regulating intracellular anti-oxidant molecules [314]. Further investigation into the anti-oxidant ability of telmisartan revealed a non-receptor-mediated anti-oxidant effect of telmisartan, but not losartan, in AT1R knockout mouse mesangial cells [314]. These findings indicate that telmisartan's anti-oxidant properties occur in both receptor-independent and receptor-dependent fashion [314].

A major advantage of in vitro studies using ARBs is that the results observed can not be mediated by any additional cellular intermediates making clear the in vitro effects of ARB agents. In vivo studies with hypertensive patients have been reported to have increased ROS production accompanied by an up-regulation in superoxide dismutase (SOD) activity [358]. This finding indicates that increased expression of endogenous anti-oxidant enzymes may signify cellular defense mechanisms for combating oxidative stress [358]. Interestingly, administration of 180 mg daily of valsartan for three months to hypertensive patients resulted in a 2 fold decrease in SOD activity and protein expression when compared to pre-treatment measurements [358]. This finding is consistent with other findings demonstrating an antioxidant effect associated with pharmaceutical blockage of AT1R [359, 366].

In vivo, ARBs have the ability to target all three significant sources of oxidative stress such as; suppression of glycated proteins, oxidative metabolism, and chelation of transition metals respectively [367, 368]. ARBs are the only hypotensive agent demonstrated to inhibit advanced glycation [368]. Valsartan and additional ARBs have been shown to successfully suppress the inflammatory action of advanced glycated end products (AGEs) providing an additional defense against oxidative damage [366]. Mechanistically it has been suggested that AT1R inhibitors reduce glycated proteins by chelating transition metals and inhibiting oxidative steps such as carbon centered and hydroxyl radicals at the pre and post stages of the Amadori reaction [366, 369]. These effects of AT1R inhibitors result in a reduction of reactive carbonyl precursors generated by auto-

oxidation of carbohydrates or lipid peroxidation [370]. The ability of AT1R inhibitors to chelate transition metals holds great therapeutic potential in combating cross linking of proteins and formation of AGEs [371].

Highly potent sources of ROS are generated by the metal catalyzed glucose autooxidation and oxidation of glycated residues resulting in the cross link of proteins, formation of AGEs and incurring insults to cellular structures and tissue functions [371]. The ability of AT1R antagonists to chelate transition metals has been reported to down-regulate the cross-linking of proteins demonstrating an alternative, off label approach for the use of AT1R antagonists [368]. Iron deposition stimulates the fenton reaction and hydroxyl radical generation exacerbating oxidative stress [372]. Chronic infusion of ANGII into rats has been reported to accelerate the deposition of iron and infiltration of inflammatory cells [373, 374]. All ARB agents have been shown to share the same characteristic of transition metal chelation [367, 369, 375].

Pancreatic Beta Cell Function and Insulin Sensitivity

The pancreas also appears to have a complete functional RAS which is up-regulated as a result of hyperglycemia and oxidative stress [288, 290, 376-380]. Moreover, exogenously administered ANGII has been demonstrated to directly inhibit insulin release as a result of decreasing islet blood flow and suppressing (pro) insulin biosynthesis [381]. Stimulation of the pancreatic RAS mediated by hyperglycemia was demonstrated to induce pancreatic stellate cells responsible for contributing to pancreatic inflammation, fibrosis, insulin resistance, and pancreatic oxidative stress [380]. Pancreatic oxidative stress via RAS mediated ROS production is a key factor initiating beta cell dysfunction [382]. Moreover, beta cell dysfunction is considered the primary metabolic defect in T2DM [383].

Reducing the decline and eventual failure of beta cells is a critical step in preventing the development of diabetes [384]. Therapeutic and prophylactic treatments with ARBs have been shown to block NADPH oxidase activity attenuating oxidative modification of pancreatic proteins [382]. A decrease in pancreatic oxidative damage, via treatment with ARBs, has been shown to improve beta cell function and glucose tolerance in part by increased islet blood flow [378]. Collectively, these findings indicate that antagonism of the RAS may play a significant role in the protection and preservation of pancreatic beta cell structure and function [380].

Obesity induced hypertrophic adipocytes has been indicated to be a key factor in the development of adipose tissue dysfunction [385, 386]. Adipocyte hypertrophy is associated with a significant increase in the rate of lipolysis suggesting that there is an increase in circulatory levels of non-esterified fatty acids (NEFA) in obese individuals [387, 388]. The increased rate of lipolysis due to adipocyte hypertrophy has been proposed to be the driving force of whole body insulin resistance [388]. AT1R blockade was shown to decrease plasma levels of NEFA in obese mice, stimulate formation of smaller, more insulin sensitive adipocytes, and ameliorate adipocytokine dysfunction [286].

Administration of AT1R pharmacological inhibitors in insulin resistant, hypertensive rats and hypertensive human subjects results in a therapeutic improvement in insulin sensitivity [389-391]. It has been suggested that the determining factor in the attenuation of skeletal muscle glucose transport by AT1R antagonism is mediated in part by an up-regulation in GLUT-4 protein expression [288, 390]. Accordingly, AT1R blockade with irbesartan was shown to increase the translocation of GLUT-4 to the plasma membrane, augmenting whole body insulin sensitivity in obese Zucker rats [390]. This effect of irbesartan was suggested to be a result of abrogating ANGII induced suppression of insulin signaling [288].

ANGII has been shown to impair insulin signaling by suppressing IRS-1 tyrosine phosporylation and activation of PI3-K, thus diminishing the insulin signaling cascade system [291]. This effect of ANGII was attenuated by addition of saralasin, a specific inhibitor of ANGII [291]. Selective AT1R blockade via treatment with valsartan reportedly increased insulin sensitivity and glucose uptake in skeletal muscle of T2DM mice, thereby increasing GLUT-4 translocation to the plasma membrane [287]. Valsartan treatment intensified insulin induced tyrosine phosphorylation of IRS-1, enhancing PI3-kinase activity, and GLUT-4 translocation to the plasma membrane [287].

Research demonstrates that TNF-α suppresses insulin signaling leading to a reduction in GLUT-4 translocation to the plasma membrane [392]. Moreover, ANGII is considered to be a primary factor upregulating skeletal muscle TNF-α [393]. Accordingly, AT1R blockade with valsartan reportedly decreased TNF-α expression in skeletal muscle of diabetic mice [287]. These findings indicate that suppressing production of TNF-α via ARBs in skeletal muscle improves insulin sensitivity [287]. In addition, it has been demonstrated that ROS play a critical role in the development of insulin resistance and ANGII is a well known stimulator of ROS production [394]. ANGII activates NADPH oxidase to produce superoxide, which leads to phosphorylation of IRS-1, resulting in inhibition of IRS-1 activity and down-regulation of GLUT-4 translocation [395]. Administration of valsartan was found to significantly decrease production of superoxide in skeletal muscle of diabetic mice [287]. These findings suggest that tissue superoxide production is modulated by AT1R activation and inhibition also enhances insulin sensitivity by decreasing ROS production [287]. Collectively, these findings indicate that AT1R blockade has potential to ameliorate inhibition of insulin signaling thereby improve insulin signaling.

Down-Regulate Inflammatory Signaling

ANGII exerts pro-inflammatory effects in a multitude of cell types including endothelial cells, vascular smooth muscle cells, and also adipocytes [396-400]. A critical component linking ANGII and inflammation is the transcription factor, NFKB [396]. NFKB is a redox sensitive nuclear transcription factor, composed of two protein subunits, p65 and p50 [401]. NFKB plays a key role in mediating the inflammatory effects of ANGII and increased expression of inflammatory cytokines including, TNF-α, IL-6, IL-1, adhesion molecules, and chemokines [353, 396]. In the cytoplasm, NFKB is bound to an inhibitory protein, IKB, resulting in the inactive conformation of NFKB [402]. The IKB proteins consist of three functional proteins, IKB-α, IKB-β, and IKB-ε residing in the cytoplasm of un-stimulated cells and coupled to NFKB in the inactive conformation [403]. The regulatory factor of NFKB activation is the phosphorylation of IKB proteins by the IKK complex [403]. The IKK complex is comprised of two homologous kinase subunits, IKK-α and IKK-β and the regulatory subunit IKK-γ [404].

Inducers of NFKB activation include the pro-inflammatory cytokines and chemokine families such as, TNF-α, IL-1, IL-6, ROS and ANGII [405]. These inducers of the NFKB pathway stimulate the IKK complex to phosphorylate IKB proteins resulting in the ubiquitylation and proteosomal degradation [403]. The degradation of IKB proteins transforms NFKB into its activated form resulting in the translocation of NFKB to the nucleus [403]. In the nucleus, NFKB binds to promoters of target genes including IKB-α which terminates transcriptional activity by binding to NFKB in the cytoplasm [403, 406]. The signaling pathway of NFKB has proven to be quite complex as post-translational modifications of NFKB enable this transcription factor to both stimulate and inhibit the expression of target genes [403].

Previous research has demonstrated that infusion of ANGII in rats up-regulated renal and VSMC NFKB binding activity [407-409]. Accordingly, ANGII mediated activation of NFKB

stimulated inflammatory cell infiltration and tubule-interstitial inflammatory responses [407, 410]. In cultured adipocytes, ANGII has been demonstrated to also up-regulate expression of pro-inflammatory mediators, IL-6, IL-8, and PAI-1, mediated by activation of NFKB signaling pathway [400, 411]. Antagonism of redox sensitive NFKB mediated inflammation has been shown to be effective by treatment with the ARB, candesartan [412]. Attenuation of NFKB signaling, by candesartan, was suggested to result in suppressing TNF chemokine expression and maintain redox homeostasis in cultured renal tubular epithelial cells [412]. This decrease in oxidative stress was suggested to be a result of the anti-oxidant effect of candesartan [412]. This effect was dose dependent, and a dose five times the standard therapeutic dose significantly reduced renal inflammation and blocked NFKB activity in spontaneously hypertensive rats [412]. Moreover, an ultra dose, fifteen times the therapeutic standard, intensified this renal anti-oxidant effect [412].

ANGII was demonstrated to up-regulate MCP-1 mRNA expression in rat pre-adipocytes via AT1R mediated and NFKB dependent pathway [217]. This effect was attenuated by the ARB, valsartan, and also by a NFKB inhibitor [217]. Immunocytochemical studies revealed that ANGII induces translocation of the NFKB subunit p65 from the cytoplasm to the nucleus, characterized as the hallmark of NFKB activation [217]. MCP-1 expression is associated with a reduction in insulin stimulated glucose up-take and decreased expression of adipogenic genes such as PPAR-γ, aP2, adipsin, and LPL [413]. Mechanistically these findings show that ANGII induces NFKB activation, resulting in transcription of inflammatory cytokines such as, MCP-1 [217, 413].

The up-stream signaling pathways regulating NFKB activation have been well characterized and consist of the canonical (classical) and noncanonical (alternative) pathways [405, 414]. The classical NFKB pathway is the primary regulator of NFKB activation, with the associated up-regulation of inflammatory cytokines, chemokines, and growth factors and is well characterized for the inflammatory process of NFKB in cells and tissues [415, 416]. A key feature

required for stimulation of the canonical pathway is activation of the IKK complex [414]. In the classical pathway, inducers of NFKB bind to their cell surface receptors along with attachment of adaptor proteins to the cytoplasmic domains of the inducers [405]. This binding pattern stimulates recruitment and activation of the catalytic subunits IKK-α and IKK-β and the regulatory subunit IKK-γ [405]. Activated IKK phosphorylates IKB at its serine residues which are then ubiquinated resulting in degradation of the inhibitory protein IKB [405]. Removal and degradation of IKB releases NFKB allowing for translocation to the nucleus and regulation of target genes [405]. In contrast, while not fully elucidated, the alternative pathway responds to B-cell related signaling playing an important role in premature B-cell survival and lymphoid organ development [417].

Interestingly, the alternative pathway acts independent of IKB but rather an IKK complex consisting of only two IKK-α subunits are recruited and no regulatory subunit IKK-γ [417]. This IKK complex is then activated via phosphorylation by an up-stream NFKB inducing kinase (NIK) releasing NFKB [417]. NFKB activation by ANGII is not well characterized, but it is suggested to require AT1R activation and G protein coupled receptors (GPCRs), following a series of complex cellular mechanisms [396, 399, 418]. Binding of ANGII to the AT1R results in a cascade of signaling events starting with activation of phospholipase C (PLC)/Ca^{2+}/PKC signaling, transactivation of epidermal growth factor receptors and activation of receptor tyrosine kinase consisting of ERK1/2 and p38 MAPK [419-421]. Activation of the AT1R signaling cascades recruits adaptor proteins for the activation of IKK complex, phosphorylating IKB followed by proteosomal degradation of IKB [422-424].

Studies indicate that ANGII stimulation of NFKB occurs by both canonical and noncanconical signaling pathways [422, 424]. Moreover, research suggests that ANGII can induce cross-talk between both pathways achieving maximal stimulation of NFKB [422, 425]. This noncanonical cellular signaling pathway has been proposed to involve Ras/mitogen activate

kinase-1 (MEK-1)/ ERK1/2/ribosomal S6 kinase (RSK) resulting in phosphorylation and activation of p65 subunit of NFKB [422]. This signaling pathway is supported by the finding that addition of MEK-1 inhibitor to ANGII treated VSMC suppressed transcriptional activity of NFKB [422]. In addition, ANGII was found to up-regulate RSK resulting in direct phosphorylation of p65 and activation of NFKB [422]. This RSK mediated phosphorylation of p65 was further investigated by an in vitro assay using recombinant glutathione-S-transferase p65 (GST-p65) [422]. Immunopurified RSK from ANGII treated VSMC resulted in phosphorylation of recombinant GST-p65 [422]. These findings confirm that ANGII induces activation of NFKB by a noncanonical signaling pathway that is MEK-1 dependent leading to RSK mediated phosphorylation of p65 [422].

ANGII has been demonstrated to stimulate the expression and release of the inflammatory cytokines, IL-6 and IL-8 in cultured adipocytes [400]. This inflammatory effect was demonstrated to be due to activation of the AT1R and stimulation of the NFKB pathway [400]. Treatment with the ARB, candesartan and the NFKB inhibitor suppressed expression of IL-6 and IL-8 [400]. To examine ANGII stimulatory effects of NFKB in adipocytes a western blot analysis was performed [400]. Approximately five minutes post-incubation with ANGII (10^{-5}M) resulted in a two fold increase in phosphorylation of NFKB p65 subunit [400]. An electromobility shift assay was used on nuclear extracts from cells treated with or without ANGII to determine if ANGII translocates NFKB to the nucleus [400]. As expected, ANGII catalyzed the translocation of NFKB to the nucleus in adipocytes [400]. This activity of NFKB mediated by ANGII was abolished by addition of the ARB, candesartan, while the AT2R blocker, PD123319 had no effect [400]. These findings demonstrate that adipose AT1R activation acts via NFKB signaling pathway to contribute to an inflammatory response [400].

Additional research also confirmed a key role for the activation NFKB in mediating ANGII induced inflammatory effects in adipocytes [217]. ANGII was shown to positively effect the

mRNA and protein expression of MCP-1 in rodent preadipocytes via an AT1R mechanism [217]. MCP-1 expression was also up-regulated via exogenously administered ANGII in vivo to rodents [217]. This finding is in accordance with current research supporting the notion that inflammatory adipocytokines such as ANGII and MCP-1, secreted from obese adipose tissue promotes and exacerbates cardiovascular disease by favoring an atherogenic effect via NFKB dependent mechanism in cardiovascular cells [426-428].

ANGII induced up-regulation of MCP-1 expression in adipocytes was abolished by the addition of a NFKB blocker, a direct inhibitor of IKB-α phosphorylation [217]. Moreover, luciferase assay using rat MCP-1 promoter constructs indicated two NFKB binding sites in the MCP-1 enhancer region required for the ANGII stimulated MCP-1 transcription [217]. Elevated expression of adipose tissue MCP-1 is positively associated with macrophage infiltration and intensification of the inflammatory response in adipocytes [429]. Treatment of diet induced obese rats with ARBs potently inhibited the expression of MCP-1 and down-regulated the level of macrophage infiltration leading to an overall reduction in systemic inflammation [430]. Accordingly, pharmacological RAS inhibition by ARBs may serve as therapeutic targets to suppress the molecular mechanisms underlying the inflammatory changes in obese adipose tissue and thereby attenuate obesity induced diseases [400, 430, 431].

Problem Statement

The RAS via ANGII generates inflammatory and oxidative stress altering the redox status of adipose tissue. Calcitriol has also been demonstrated to alter the redox status of adipose tissue, contributing to oxidative stress. However, data are lacking to determine whether or not calcitriol up-regulates the adipocyte RAS to generate local inflammatory cytokine expression in adipose tissue. This research aims to investigate; the role of RAS inhibition in the modulation of inflammatory cytokine expression in adipocytes, determine role of calcitirol modulation of the adipoycte RAS in the regulation of ROS and inflammatory cytokine expression, and evaluate discrepancies in inflammatory cytokine expression from the pharmaceutical ARBs using siRNA to knockdown expression of AT1R.

Chapter II

Research Paper

The Adipocyte Renin Angiotensin System Mediates the Effects of Calcitriol on Oxidative Stress and Cytokine Production in Cultured 3T3-L1 Cells

Introduction

Adipose tissue is a multifunctional endocrine organ that plays a central role in modulating metabolic function and inflammation [19, 38, 432]. Excess adipose tissue, specifically in the visceral region, is a critical contributor to systemic oxidative stress and metabolic dysfunction [82, 220, 433]. The recognition of adipose tissue as an endocrine organ suggests that adipose tissue derived biological molecules may be a key factor in the metabolic disturbances associated with obesity [37]. Adipocytes synthesize and secrete a variety of inflammatory and anti-inflammatory cytokines (adipocytokines) [11, 219, 434, 435]. Obesity is associated with a dysregulation in adipocytokine production which is characterized by increased expression of inflammatory cytokines such as TNF-α, IL-6, and IL-8 and a decrease in the anti-inflammatory cytokines such as adiponectin and IL-15 [82, 221, 346, 436-439].

Adipose tissue possesses all components of a functional RAS including local production of the main effector molecule ANGII [11, 440]. Traditionally the RAS was identified as a critical regulator of blood pressure, electrolyte balance, and a critical factor of hypertension [441, 442]. Obesity is frequently associated with hypertension and the discovery of an active adipose tissue RAS suggests the possibility that locally formed ANGII may contribute to the pathophysiology of obesity providing a link connecting obesity with the development of hypertension [37, 190, 443]. Locally formed ANGII has been demonstrated to modulate adipocyte lipid metabolism by up-

regulating key lipogenic enzymes, FAS and glycerol-3-phosphate dehydrogenase, stimulating preadipocyte differentiation and accumulation of lipids [206].

Obesity is recognized as a low grade inflammatory state associated with macrophage infiltration in adipose tissue and an increase in circulating levels of inflammatory molecules [262, 432, 444]. Increasing adiposity is accompanied with an up-regulation in adipocyte monocyte chemotactic factors that facilitate macrophage infiltration and local oxidative stress in adipose tissue [56, 219, 445-448]. Increased oxidative stress has been demonstrated in obese verses lean individuals and it has been suggested that pro-inflammatory chemokines, cytokines, and hormones released from adipose tissue are significant mediators of systemic oxidative stress in obesity [445, 447, 449, 450]. ANGII increases oxidative stress in adipose tissue by up-regulating expression of a key ROS generating enzyme, NADPH oxidase (NOX4) and stimulating superoxide release [11, 202, 221, 451]. ANGII alteration of adipose tissue redox state increases expression of pro-inflammatory adipocytokines, exacerbating the inflammatory state of adipose tissue [219, 449].

We previously have demonstrated that dietary calcium modulates adipocyte metabolism by inhibiting lipogenesis, stimulating lipolysis and thermogenesis, and increasing adipocyte apoptosis [82, 103, 116]. We have also shown that oxidative stress due to diet induced-obesity can be inhibited by increasing dietary calcium and decreasing adiposity [84]. These effects are accomplished by suppressing both the genomic and non-genomic actions of calcitriol [80, 83, 86, 88, 116]. Adipocyte intracellular calcium signaling is a key factor in the modulation of adipocyte metabolism [86]. Increasing intradipocyte calcium stimulates expression of key lipogenic enzymes and suppresses lipolysis [63, 452]. We previously demonstrated calcitriol induced calcium influx in adipocytes, resulting in increased expression of lipogenic enzymes and expansion of adipocyte triglycerides [86].

Calcitriol also acts via the adipocyte nVDR to suppress expression of UCP-2 [80]. UCPs diminishes the mitochondrial proton gradient resulting in a thermogenic effect and a potential role in energy metabolism [149]. We previously showed calcitriol to suppress expression of adipocyte UCP-2 [80]. We also demonstrated that calcitriol increases ROS production and that this effect is mediated by both an increase in calcium influx and a decrease in UCP-2 expression [82, 83, 88]. Furthermore, we previously demonstrated that calcitriol directly stimulates local inflammation by modulating the interaction between adipocytes and macrophages to synergistically up-regulate production of inflammatory cytokines [81]. In the present study we investigated the role of the adipocyte RAS in mediating these effects of calcitriol in cultured 3T3-L1cells. We hypothesize that calcitriol directly up-regulates the adipose tissue RAS and that calcitriol stimulation of inflammatory cytokine expression is partially mediated by activating the adipocyte RAS.

Methods

Cell Culture and Differentiation of 3T3-L1

3T3-L1 preadipocytes were obtained from American Type Culture Collection (Manassas, VA, USA). Preadipocytes were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) and incubated at 37°C with 5% CO_2 in air. Confluent preadipocytes were induced to differentiate with standard differentiation cocktail containing: DMEM, 10% FBS, 1% PS, 1µM dexamethasone, 0.5 mM iso-butyl-1-methyl-xanthine (IBMX). Preadipocytes remained in differentiation medium for three days and then transferred to adipocyte medium; cells were re-fed every two days until 90% of cells were fully differentiated and lipid-filled.

RNA Extraction

RNA extraction from 3T3-L1 cells was performed using Total Cellular RNA Isolation Kit (Ambion Inc., Austin, TX), according to manufacturer's guidelines. The RNA concentration was determined by measuring the optical density (OD) at 260. All samples were diluted to 20 ng total RNA/µL in diethylpyrocarbonate (DEPC) water.

Quantitative Real Time Polymerase Chain Reaction

18S, IL6, NOX4, MCP-1 and adiponectin mRNA levels were quantitatively determined using a Smart Cycler Real –Time Polymerase Chain Reaction (PCR) System (Cephied, Sunnyvale, CA, USA) with a TaqMan 1000 Core Reagent Kit (Applied Biosystems, Branchburg,

NJ, USA). The primer and probe sets were supplied by Applied Biosystems TaqMan Assays-on Demand Gene Expression primer and probe set collection. 3T3-L1 adipocyte total RNA was serial-diluted in the range of 1.5625-25 ng and used to construct a linear standard curve. Total RNAs for samples were also diluted in this range and then calculated for quantification of mRNA of unknown samples from the standard curve. This method evaluates the cycle threshold changes for each target gene and reports the quantification of each target in arbitrary units. Accurate quantification of the arbitrary units of the target gene of interest are normalized as ratios to 18S arbitrary units. Quantitative real-time PCR for standards and unknown samples were performed in accordance to the instructions of the Smart Cycler System and TaqMan Real Time PCR Core Kit supplied by Applied Biosystems.

Gene Silencing with Small Interfering RNA

Small interfering RNA (siRNA) targeted against Angiotensin II type 1 receptor (AT1R) mRNA was purchased from Applied Biosystems (Foster City, CA USA). The two sequences CCUCGAUGGUAAUAAAUGUtt (sense) and ACAUUUAUUACCAUCGAtt (antisense) were simultaneously transfected into differentiated 3T3-L1 cells according to manufacturer's instructions (catalog # AM16708). Non-targeting siRNA provided by the manufacturer was used as a negative control to test non-specific effects on gene expression (catalog # AM4611). Differentiated 3T3-L1 cells were transfected using siPORT NeoFX transfection agent for 48 hours in 6 well plates with 5 nmole/well/siRNA for a totoal siRNA concentration of 10 nM siRNA/well. At 48 hours post-transfection cells were treated with angiotensin II (1 nM), angiotensin II (100 nM), or calcitriol (10 nM) for an additional 48 hours. RNA analysis and Real-Time PCR were used to measure knockdown of the AT1R.

Chemicals

Angiotensinn II, calcitriol, P5749 S-(+)-PD 123177 (PD), telmisartan, dexamethasone, PS, FBS, and IBMX were all obtained from Sigma (St. Louis, MO USA). The pre-designed siRNA AT1R, negative control, and siPORT NeoFX transfection agent was supplied by Applied Biosystems.

Statistical Analysis

Data were evaluated for statistical significance by analysis of variance to determine if there were pair wise differences between means of cytokine ratio. Significantly different group means were then separated by the least significant difference test by using SPSS (SPSS Inc., Chicago, IL). The alpha level of 0.05 was used to determine statistical significance. All data presented are expressed as mean ± standard deviation (SD).

Results

Effects of Calcitriol

Treatment with calcitriol for 48 hours significantly up-regulated the expression of the inflammatory cytokines IL-6, MCP-1, and NOX4 (Figure 1A, 1B, 1C) compared to control group. This effect was significantly inhibited by the addition of the Angiotensin II type 2 receptor (AT2R) inhibitor (PD) for all three cytokines compared to Calcitriol group. The addition of the angiotensin converting enzyme (ACE) inhibitor (enalipril) significantly attenuted the effects of calcitriol on MCP-1 and NOX4 expression. The addition of the Angiotensin II type 1 receptor (AT1R) inhibitor (telmisartan) significantly blocked the expression of NOX4 compared to calcitriol group. Calcitriol suppressed the expression of the anti-inflammatory cytokine adiponectin compared to control. Adiponectin expression was recovered by the addition of AT2R inhibitor PD and the ACE inhibitor enalipril compared to calcitriol group.

Effect of RAS

ANGII significantly up-regulated the expression of IL-6 and MCP-1 (Figure 2A and 2B). This effect was reversed by the AT2R inhibitor PD. The addition of the AT1R inhibitor telmisartan also significantly suppressed the expression of IL-6 compared to ANGII group (Figure 2A). Adiponectin expression was significantly up-regulated by the addition of both the AT1R and AT2R inhibitors compared to ANGII treated cells, while the AT2R antagonist also significantly increased the expression of adiponectin compared to control group (Figure 2C).

Effect of AT1R Knockdown

Treatment with ANGII increased expression of AT1R (Figure 3A). AT1R siRNA treatment resulted in AT1R knockdown of 68% and resulted in significant inhibition of MCP-1 and NOX4 expression (Figure 3B and 3C). AT1R knockdown significantly decreased expression of AT1R, MCP-1, and NOX4 (Figure 4A, 4B, 4C),and attenuated calcitriol stimulation of MCP-1 and NOX4 expression (Figure 4B and 4C).

Discussion

Data from this study demonstrates that calcitriol mediated oxidative and inflammatory stress results, in part, from calcitriol modulation of the adipocyte RAS. Human adipose tissue contains all the functional components of an active RAS, and ANGII stimulates oxidative and inflammatory stress [37, 213, 443, 453-459]. Our data confirm that ANGII up-regulates expression of IL-6 and MCP-1, while antagonism of either subtype 1 or subtype 2 ANGII receptor attenuated suppressed IL-6 expression. Antagonism of the AT2R with PD suppressed MCP-1 expression. These findings are congruent with other data demonstrating that ANGII directly affects adipocytokine expression, resulting in an up-regulation in the inflammatory cytokines MCP-1 and IL-6 [328, 400]. ANGII also stimulates production of ROS by up-regulating expression of NADPH oxidase cytosolic proteins required for the activation of NOX4 [460-462]. In addition, ANGII has been demonstrated to affect adipose tissue metabolism by up-regulating expression of key lipogenic enzymes in 3T3-L1 and human adipocytes while suppressing lipolysis [206, 463]. An inverse relationship has been reported between the expression of adipose tissue derived ANGII converting enzymes and degree of insulin sensitivity and inhibition ameliorates RAS induced oxidative stress [208, 431, 464-467].

We also have demonstrated that calcitriol alone stimulates a similar pattern of up-regulation of the inflammatory cytokines IL-6, MCP-1, and NOX4 and suppresses expression of the anti-inflammatory cytokine adiponectin. These effects were partially reversed by the AT2R inhibitor, PD, while the addition of the AT1R inhibitor, telmisartan, had no effect. Adipose tissue oxidative stress suppresses adiponectin levels, and decreased adiponectin levels have been reported in obese and diabetic subjects [306, 468-470]. A possible explanation for this hypoadiponectinemia may be a consequence of local oxidative stress inhibiting adiponectin gene transcription and rapidly degrading adiponectin mRNA, resulting in reduced adiponectin levels

[468]. Previous work from our laboratory has shown calcitriol to induce oxidative stress and increase lipid accumulation in adipocytes mediated by modulating calcium signaling as well as mitochondrial uncoupling [80, 86].

Our incongruent results between AT1R and AT2R inhibitors on cytokine expression are puzzling, as AT1R has been reported to remain stable during adipogenesis while the AT2R was reported to be suppressed and eventually undetectable in mature adipocytes [471]. However, other results demonstrate expression of AT2R in mature adipocytes [206, 472-475]. Prostacyclin has been shown to stimulate maturation of adipocytes mediated by the AT2R, confirming the presence of this receptor in late differentiated adipocytes [475, 476]. This finding was affirmed by data showing that ANGII increased production of prostacylin 4-6 fold higher in mature differentiated adipoyctes compared to preadipocytes [475]. The effect of prostacyclin was abolished by the addition of the AT2R antagonist, PD, while the AT1R antagonist, losartan had no effect [475]. In addition, radioligand binding studies also demonstrated that the high affinity ANGII bindings sites in mature adipocytes were of the AT2R subtype [206]. Both ANGII and the AT2R antagonist, PD were reported to compete for radiolabeled ANGII binding while the AT1R antagonist (losartan) had no effect [206].

A key factor influencing the effects of ANGII is the intracellular location of the ANGII receptors [471]. Intracellular distribution studies using green fluorescent fusion protein for AT1R revealed nuclear localization of the AT1R directly after treatment of cells with ANGII [477]. This internalization of the ANGII bound receptors down-regulates the number of ANGII receptors available for binding at the plasma membrane affecting the quantity of receptors at the plasma membrane [478]. The type of ANGII receptor and quantity of receptor subtype present at the plasma membrane appears to be a critical factor in determining the effect on adipose tissue [206, 477, 479, 480].

Increased ANGII levels have been positively correlated with increased adiposity and in expression of inflammatory cytokines [206, 212, 213, 328, 474]. These findings in accordance with our ANGII results provide support for the role of ANGII in modulating cytokine expression and increasing inflammatory stress in adipose tissue. However, our findings with the ANGII pharmacological antagonists were inconclusive with regard to which ANGII receptor specificity. These incongruent results may be attributed to the non-specific actions of the chemical ANGII receptor antagonists, as these have been indicated to produce non-ANGII receptor related events in various cell types [206, 312, 480-482]. There appears to be a small population of non-angiotensin receptor related binding sites that bind with high affinity for the chemical ANGII receptor antagonists [483, 484]. The binding of the chemical antagonists to these non-angiotensin receptor binding sites may modify the true pharmocophore effects of the chemical receptor antagonists for the ANGII subtype receptors.

It is well recognized that the inflammatory effects of ANGII are mediated by the pleiotropic activation of the transcription factor, NFKB, stimulating expression of inflammatory gene products in various cell types [485-488]. In human preadipocytes ANGII has been demonstrated to degrade IKB an inhibitor of NFKB, phosphorylating the p65 subunit of NF-KB with translocation to the nucleus mediated by AT1R [400]. These effects of ANGII were reversed by the addition of a NFKB inhibitor (BAY 117082) [400]. In addition, an antagonist for AT1R abolished NFKB activity, demonstrating that ANGII is a stimulator of NFKB signaling in adipocytes [268, 485]. These findings indicate that in adipocytes, the inflammatory effects of ANGII are mediated, in part, by stimulation of NFKB signaling pathway [217, 400]. In newly differentiated adipocytes, the majority of ANGII effects are mediated by the AT1R; however the AT2R is also expressed in adipocytes [400, 479]. The exact functional role of AT2R is unclear, as some reports indicate that AT2R antagonizes the effects of AT1R [485, 489, 490]. However, other reports suggest that AT2R

participates in inflammatory events directly mediated by activating NFKB signaling pathway [399, 491-495].

It has been proposed that chronic activation of RAS may result in altering the function of AT2R in favor of mediating inflammation and oxidative stress [496, 497]. These findings provide framework for potential overlap in common signaling pathways shared by the two receptor subtypes resulting in modulation in the effects of ANGII [400]. ANGII induces oxidative stress by activating NADPH oxidase resulting in stimulation of the redox sensitive NFKB pathway [49]. The ANGII induced activity of NADPH oxidase has been demonstrated to be down-regulated by the addition of both chemical antagonists forAT1R and AT2R and with the antioxidant tempol [498].

Since chemical antagonism of the ANGII receptor resulted in inconclusive results on cytokine expression we used small interfering RNA to specifically knock down expression of the AT1R and then re-examine adipocytokine expression mediated by ANGII and calcitriol. The AT1R knockdown markedly inhibited expression of MCP-1 and NOX4 stimulated by treatment with ANGII. This finding is consistent with our earlier observation that Telmisartan inhibited the effects of NOX4 and MCP-1, and demonstrate that the AT1R is responsible for mediating some of the inflammatory effects of calcitriol.

Conclusions

Based on these findings RAS via AT1R has been demonstrated to mediate the expression of some of the inflammatory cytokines induced by calcitriol. Although chemical antagonism of the AT1R led to inconclusive results, the siRNA knockdown of AT1R did provide evidence for supporting the role of AT1R in mediating some of the inflammatory effects of calcitriol. Accordingly, strategies designed to down-regulate adipose tissue levels of calcitriol by consuming a diet high in calcium could play a role in attenuating adipose tissue inflammation associated with obesity.

Future Research

In the present study, ANGII via AT1R was demonstrated to mediate the mRNA expression of some inflammatory cytokines induced by calcitriol. Future research is needed to investigate if the protein levels of these inflammatory cytokines are also up-regulated. If so, this would provide a complete framework demonstrating that AT1R mediates an inflammatory response induced by calcitriol stimulation at each level of gene transcription. In addition, the co- treatment of adipocytes with ARBs and also with siRNA of AT1R needs to examine simultaneous dual antagonism of the AT1R by pharmacological antagonism and direct knockdown of AT1R and the effects on inflammatory cytokine expression.

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Appendix

Figures

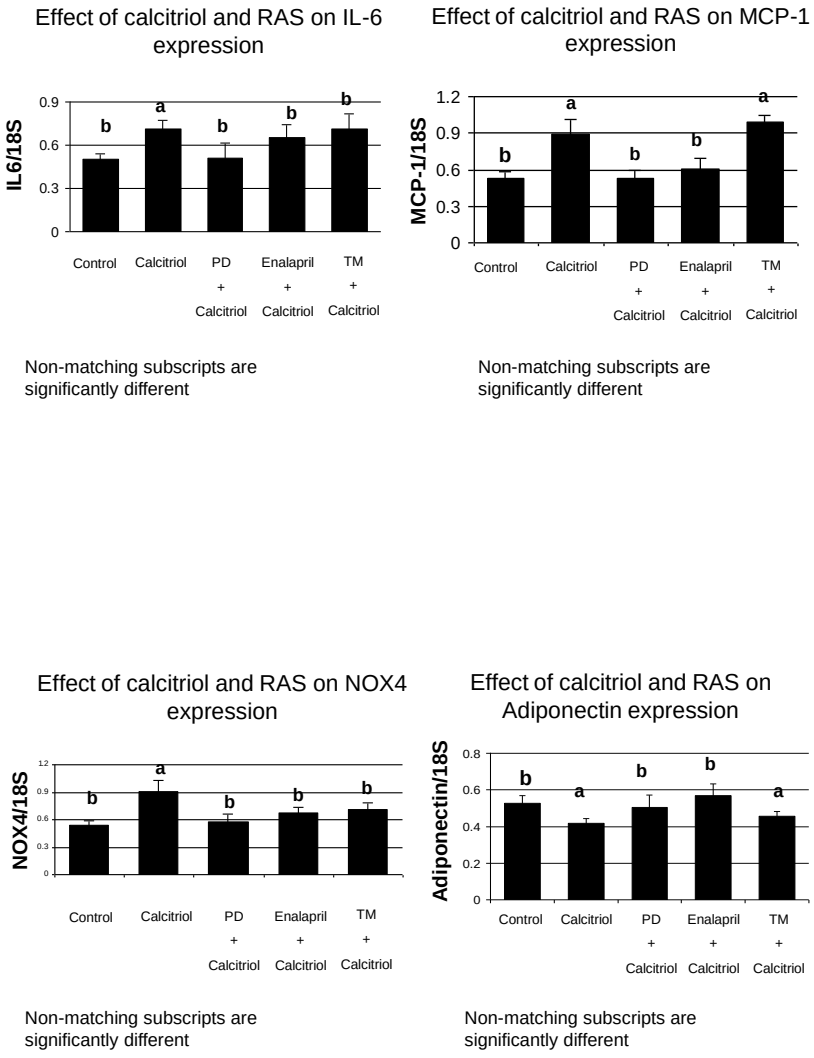
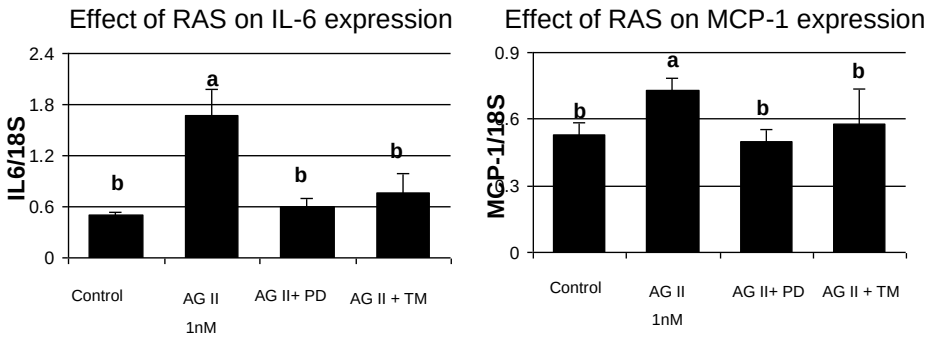


Figure 1. Effects of calcitriol and RAS on: IL-6 and 18S expression ratio (A), MCP-1 and 18S expression ratio (B), NOX4 and 18S expression ratio (C), Adiponectin and 18S expression ratio (D) with differentiated 3T3-L1 adipocytes.



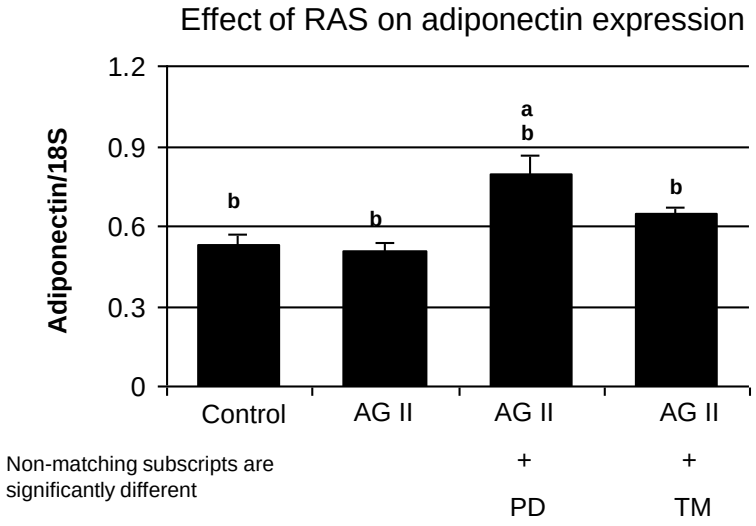
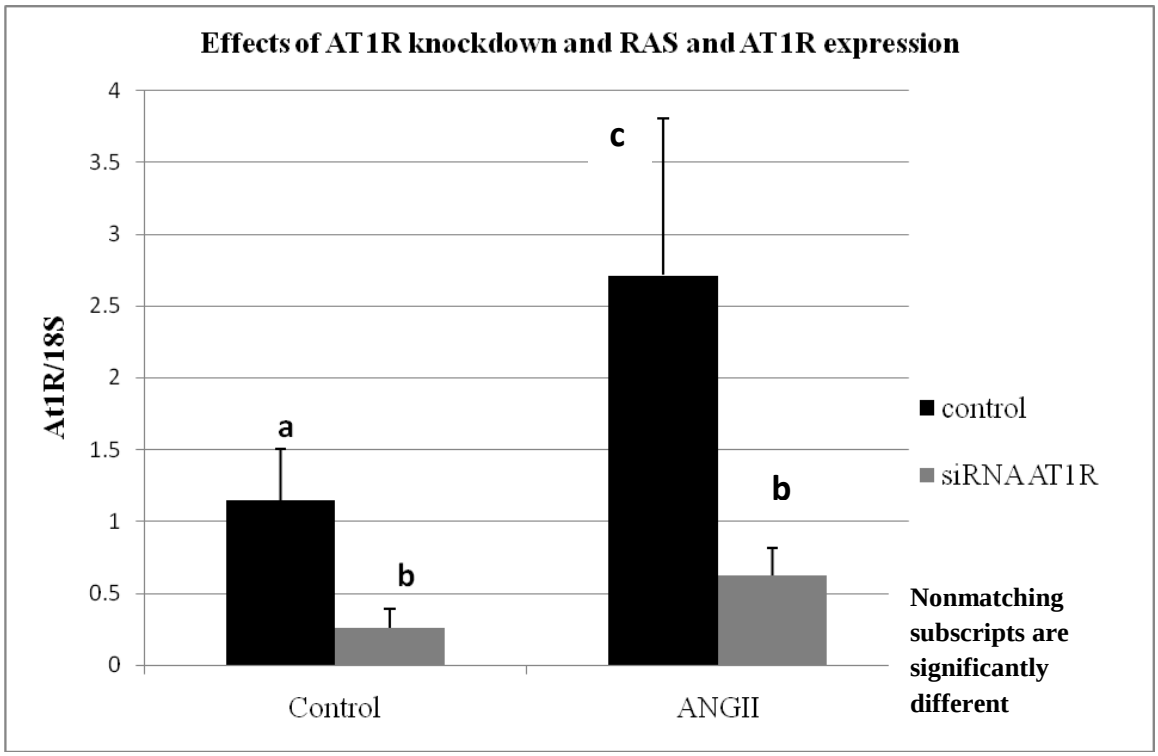


Figure 2. Effects of RAS on IL-6 and 18S expression ratio (A), MCP-1 and 18S expression ratio (B), Adiponectin and 18S expression ratio (C) in differentiated 3T3-L1 adipocytes.



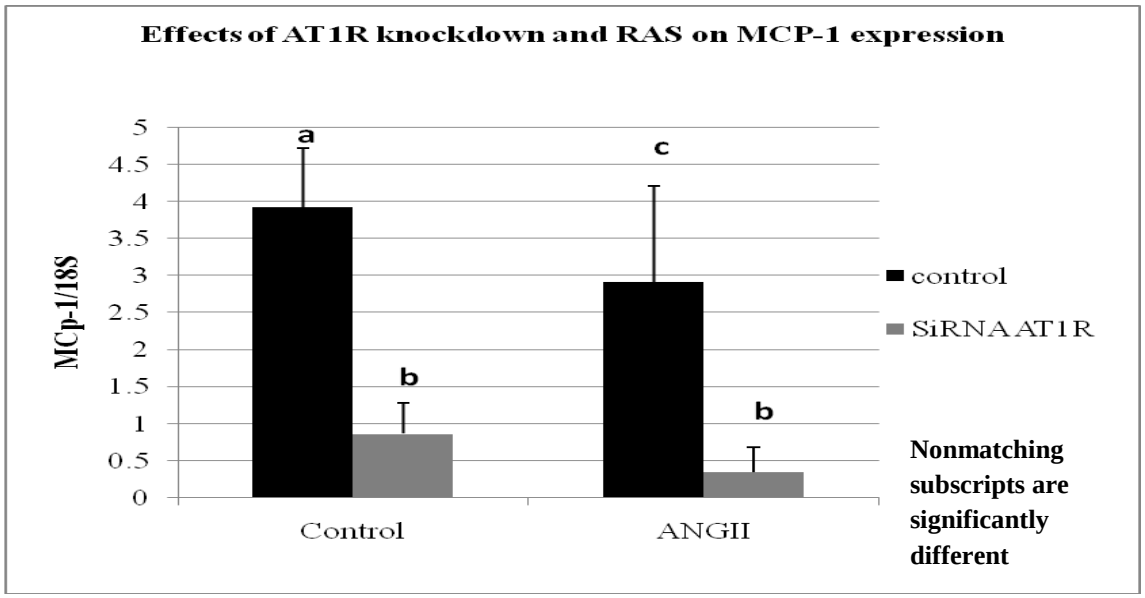


Figure 3(A and B). Effects of AT1R small interfering RNA knockdown and RAS on AT1R and 18S expression ratio (A) and MCP-1 and 18S expression ratio (B) in differentiated 3T3-L1 adipocytes.

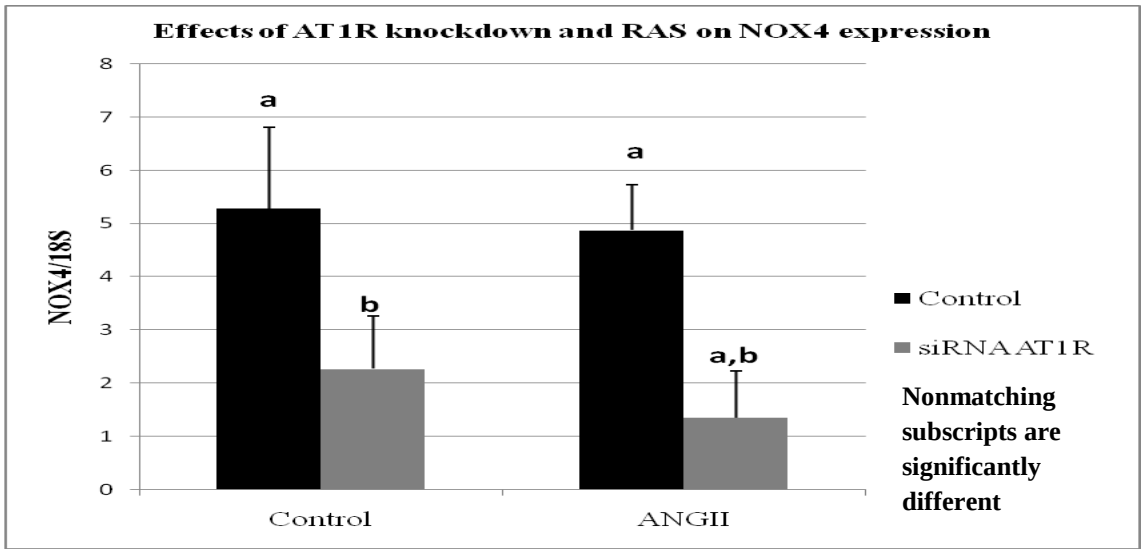


Figure 3C. Effects of AT1R small interfering RNA knockdown and RAS on NOX-4 and 18S expression ratio in differentiated 3T3-L1 adipocytes.

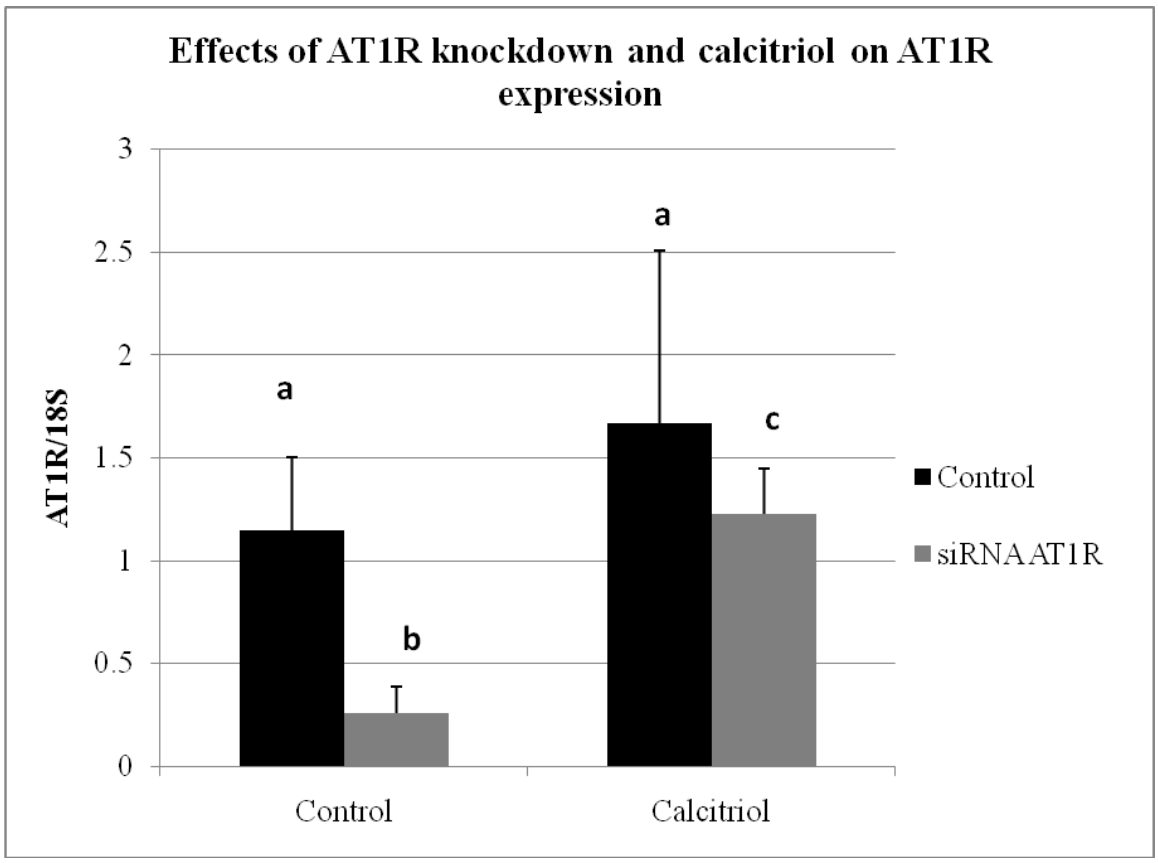


Figure 4A. Effects of AT1R small interfering RNA on AT1R and 18S expression ratio in differentiated 3T3-L1 adipocytes.

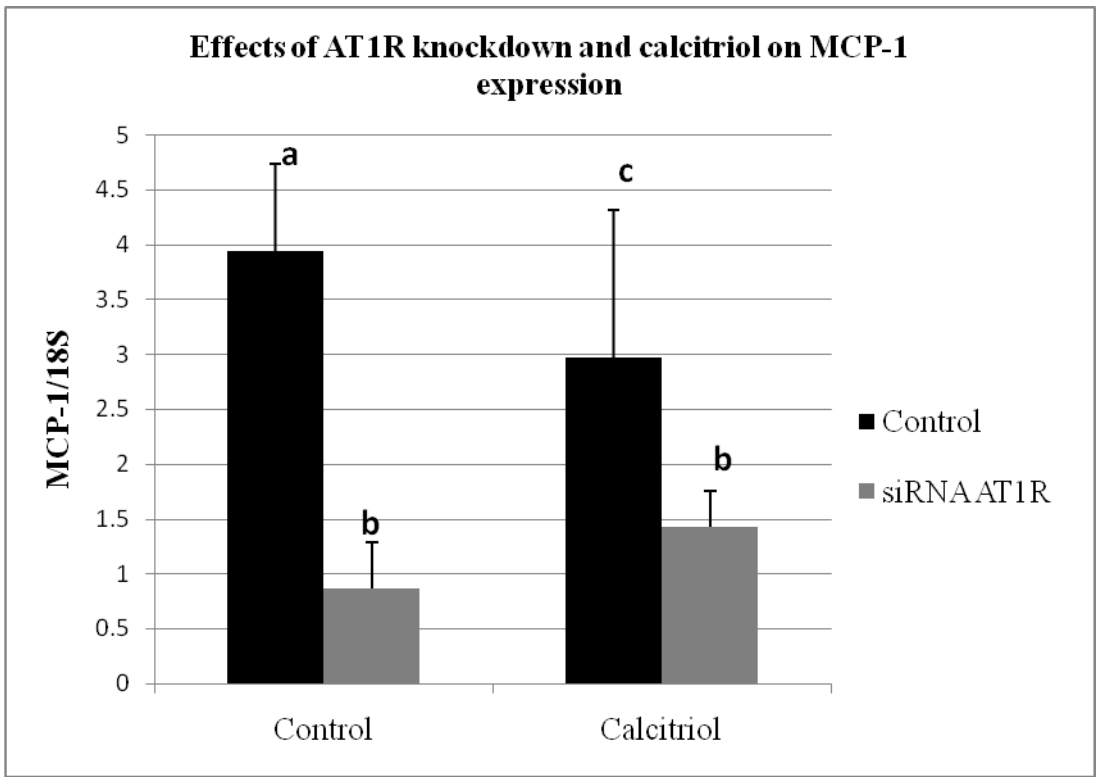


Figure 4B. Effects of AT1R small interfering RNA on MCP-1 and 18S expression ratio in differentiated 3T3-L1 adipocytes.

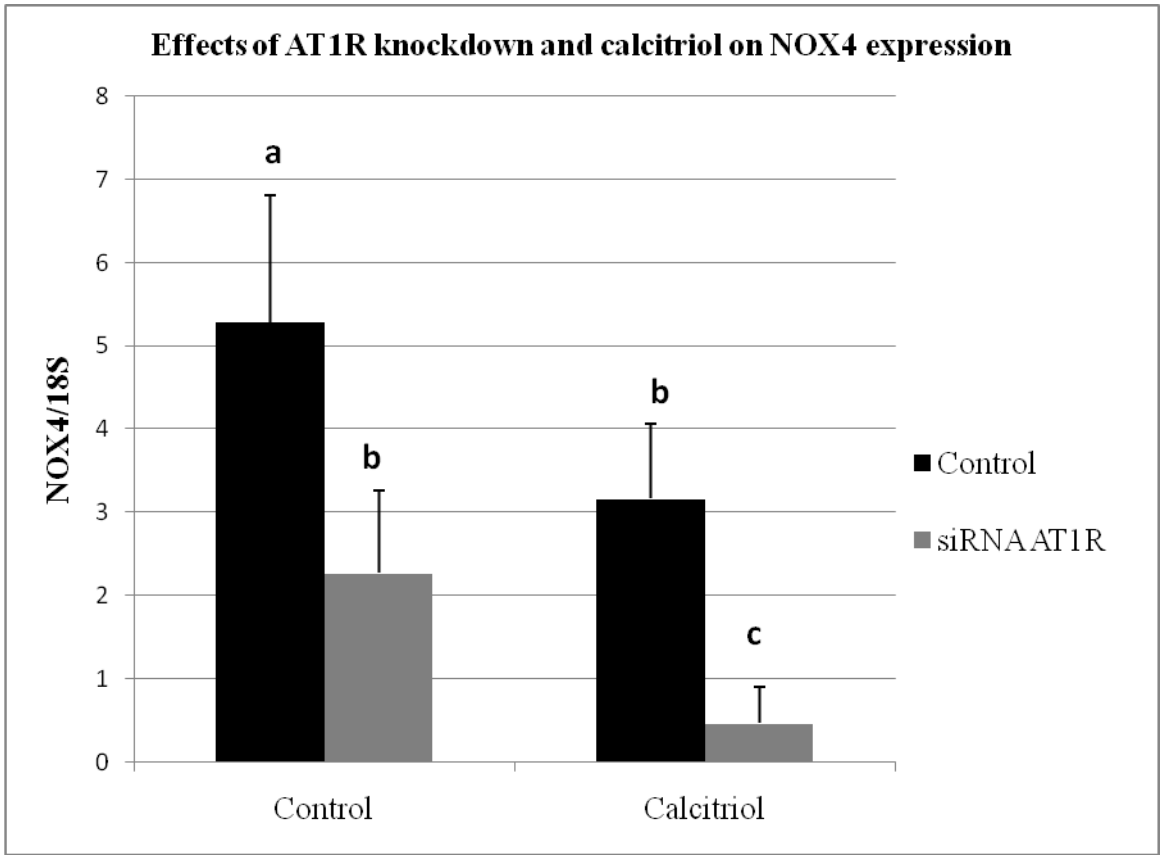


Figure 4C. Effects of AT1R small interfering RNA on NOX-4 and 18S expression ratio in differentiated 3T3-L1 adipocytes.

Vita

Christina Marie Caserio was born in Pitsburgh, PA on Octorber 23, 1980. She was raised in Hendersonville, NC and went to grade school and junior high school at Immaculata in Hendersonville. She graduated from Hendersonville High School in 1999. From there, she went to the University of Tennessee, Knoxville and received a B.S. in psychology in 2005 and a M.S. in Nutrition in 2009. Christina is currently pursuing research in the field of biochemistry.