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EFFECTS OF ADIPOCYTE DEFICIENCY OF ANGIOTENSIN TYPE 1A
RECEPTORS IN MODELS OF OBESITY AND HYPERCHOLESTEROLEMIA

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the Graduate School,
Center for Nutritional Sciences,
at the University of Kentucky

By

Kelly Anne Putnam

Lexington, Kentucky

Director: Dr. Lisa Cassis, Professor of Nutritional Sciences

Lexington, Kentucky

2012

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ABSTRACT OF DISSERTATION

EFFECTS OF ADIPOCYTE DEFICIENCY OF ANGIOTENSIN TYPE 1A RECEPTORS IN MODELS OF OBESITY AND HYPERCHOLESTEROLEMIA

Adipocytes express angiotensin II (AngII) receptors; however the direct effects of AngII at the adipocyte remain unclear. Knockout mouse models of renin-angiotensin system components exhibit reduced body weight, reduced adiposity, improved glucose tolerance, and improved blood pressure when fed high fat diets, which may be due to reduced action of AngII through the AT1aR in adipocytes. Additionally, hypercholesterolemic AT1aR deficient mice are protected from AngII-induced increases in atherosclerosis and abdominal aortic aneurysm (AAA) formation. We hypothesized that deficiency of AT1aR in adipocytes would reduce the development of obesity, obesity-induced disorders, and vascular diseases. To test this hypothesis, we created a mouse model of adipocyte AT1aR deficiency using the Cre/LoxP system. Adipocyte-AT1aR deficiency confers no protection from the development of obesity or obesity-associated parameters. However, low fat fed adipocyte-AT1aR deficient mice exhibit remarkable adipocyte hypertrophy and reductions in adipocyte differentiation. These results demonstrate that AngII is a stimulus for adipocyte

differentiation and adipocyte hypertrophy alone is insufficient to initiate obesity-associated disorders. In hypercholesterolemic mice, adipocyte AT1aR deficiency conferred no protection from diet or AngII-induced vascular diseases. Overall these studies suggest the primary role of adipocyte AT1aRs is to promote adipocyte differentiation and the development of small adipocytes.

Keywords: Angiotensin II, adipocytes, obesity, atherosclerosis, abdominal aortic aneurysm

Kelly Anne Putnam

7-10-2012

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SECTION I. BACKGROUND

1.1.1 The renin-angiotensin system (RAS)

The renin-angiotensin system (RAS) is an endocrine system that regulates blood pressure through the actions of angiotensin peptides acting through various angiotensin receptors. Angiotensin peptides are cleavage products of a precursor protein, angiotensinogen (AGT). AGT is a glycosylated protein secreted primarily from liver, though important secondary sources include adipose and brain tissues. The aspartyl protease renin is produced in juxtaglomerular cells and cleaves 10 amino acids from the N-terminus of AGT to form the inactive peptide, angiotensin I (AngI). Subsequent cleavage of two amino acids from the C-terminus of AngI by the metalloproteinase angiotensin converting enzyme (ACE) produces the predominant peptide of this system, angiotensin II (AngII) (Figures 1.1 and 1.2A). Catabolism of AngII by aminopeptidases produces smaller angiotensin peptides such as angiotensin III or IV and importantly, the C-terminal phenylalanine can be removed by angiotensin converting enzyme 2 (ACE2) to form angiotensin-(1-7) (Ang(1-7)), which acts through the Mas receptor to oppose the effects of AngII.

1.1.2 Angiotensin receptors

1.1.2.1 Types of angiotensin receptors and their tissue distributions

In humans, the actions of AngII are mediated by two G-protein coupled receptors, angiotensin type 1 (AT1R) and angiotensin type 2 (AT2R) receptors. Rodents, however, have two subtypes of the AT1R, the AT1aR and AT1bR,

which are 94% homologous (Hein 1998). Prior to the identification of the rodent subtypes, the human AT1R was found to share 95% homology with rat AT1R (Bergsma 1992). The AT1R in humans and the AT1aR in rodents are expressed with similar tissue distributions, including kidney, adrenal gland, vasculature, epicardium, liver, lung, circumventricular organs of the brain, ovaries/testes, and adipose tissues (44, 70). In contrast, expression of AT1bR is limited to adrenal gland, anterior pituitary gland, testes, hypothalamus, and resistance vessels (36, 52, 294). Knockout models provide another indication that the rodent AT1aR is most similar to the AT1R of humans because AT1aR deficient mice demonstrate reduced blood pressure in most studies, consistent with pharmacological blockade of AT1R in humans, whereas AT1bR deficient mice do not demonstrate this pathology (52, 119). AT1bRs have been implicated in contractile responses to AngII in mesenteric resistance vessels of mice, supporting tissue-specific roles for AT1bRs (294). The other AngII receptor, AT2R, is 34% homologous compared to AT1R. Although AT2R is highly expressed during fetal development (98), more restricted expression in adults has been demonstrated in brain, adrenal gland, liver, kidney, myocardium, endothelium, uterus/ovary, and adipose with upregulation of AT2R during some pathological states (61, 269).

1.1.2.2 Structure and signaling of angiotensin receptors

All AngII receptors are classic seven transmembrane G-protein coupled receptors. The AT1R comprises 359 amino acids (Figure 1.2B) and has a molecular mass of 41 kDa, though glycosylation at four extracellular residues increases the mass to 65 kDa (124). Binding of AngII to AT1R requires

interactions between the tyrosine residue of AngII and the Asn¹¹¹ residue of the third transmembrane domain as well as between the phenylalanine of AngII and His²⁵⁶ of the sixth transmembrane domain (186). These transmembrane residues of the AT1R are part of a transmembrane binding pocket present in several GPCRs and the aromaticity of the Tyr and Phe residues of AngII may facilitate these interactions. Multiple G protein-dependent signaling pathways can be activated by AngII through the AT1R (Figure 1.3A). The AT1R initially couples to G_{q/11} to activate phospholipase C-β (PLC- β), an enzyme that cleaves phosphatidylinositol (PIP₂) to diacylglycerol and inositol triphosphate (IP₃). IP₃ then stimulates intracellular calcium (Ca²⁺) release, which is a key initiating event in contraction of muscle cells. While this pathway is well-characterized, AT1R signaling pathways vary by cell type. In vascular smooth muscle cells, AngII-stimulated AT1Rs can activate phospholipase C-γ through tyrosine phosphorylation by intermediate tyrosine kinases, as the AT1R has no tyrosine kinase activity of its own; tyrosine kinases activated by AT1Rs include c-Src, focal adhesion kinase, Ca²⁺-dependent kinases, Janus kinases, and receptor tyrosine kinases (24, 67, 263). Activation of tyrosine kinases may play a role in transactivation of the epidermal growth factor receptor through AngII-induced phosphorylation of ADAM17, an enzyme that cleaves epidermal growth factor from its heparin binding (110, 168). AngII may also transactivate other growth factor receptors, such as platelet-derived growth factor or insulin-like growth factor receptors, though these mechanisms are less clear (72, 250). In adrenal, liver, kidney, and pituitary gland cells AT1R can activate G_{i/o} proteins that inhibit

adenylyl cyclase and in rat portal vein myocytes AT1R can activate $G_{12/13}$ proteins to activate L-type calcium channels (67, 156).

The AT1R can also activate signaling pathways independent of G-protein coupling (Figure 1.3B). Interestingly, the AT1R is subject to biased agonism, whereby these non-G-protein signaling pathways can be preferentially activated by analogues of AngII such as [SII] (AngII with substituted Sarcosine¹, Isoleucine⁴, Isoleucine⁸) (11, 139). These G-protein independent pathways involve the recruitment of β -arrestin as a result of phosphorylation by GPCR kinases (GRKs) and result in receptor internalization and activation of Extracellular signal regulated kinases 1/2 (ERK 1/2). In addition to ligand-activated signaling through G-protein coupled or G-protein independent pathways, AT1Rs are also capable of ligand-independent activation, such as by mechanical stress. Following up on initial reports that AT1R antagonists could only partially attenuate mechanical-stress induced activation (282), more recent studies have confirmed that AT1Rs undergo conformational changes during mechanical stress that initiate ERK activation and result in cardiac hypertrophy independent of AngII activation of the receptor, and administration of candesartan reduces these effects, suggesting the conformation of candesartan-bound AT1Rs is unable to respond to mechanical stress (166, 298).

The AT2R is 34% homologous with the AT1R and some of the structural differences include additional cysteine residues in the N-terminal region and the third extracellular loop which form stabilizing disulfide bonds in the AT1R but are not present in the AT2R (190). The AT2R binds to $G_{i\alpha 2}$ or $G_{i\alpha 3}$ (292) and

activates various phosphatases, including serine/phosphatase 2A (PP2A) and MAPK phosphatase (MKP-1), through which it can functionally antagonize signaling pathways activated by the AT1R (67). In cardiac myocytes, AT2R activation can result in the release of arachidonic acid via stimulation of phospholipase A2 (152) and it has also been reported in Ob1771 adipocytes that activation of the receptor mediates the release of prostacyclin (PGI₂), a downstream product of arachidonic acid metabolism (61).

1.1.2.3 Regulation of angiotensin receptor function at the plasma membrane

Similar to many plasma membrane receptors, GPCRs undergo internalization and subsequent degradation or recycling, which in the case of GPCRs is agonist-dependent. In the case of AT1Rs, internalization is downstream of β -arrestin signaling pathways which are G-protein independent but requires receptor phosphorylation at the C-terminal tail by G-protein coupled receptor kinases (GRKs) (192, 260). In vascular smooth muscle cells, AT1Rs are associated with caveolae within 5 minutes of AngII stimulation and data suggest AT1R internalization may in part be due to caveolae-mediated endocytosis (118). Interestingly, the AT2R is a rare example of a GPCR that does not undergo receptor internalization (265).

In recent years, the possibility of GPCR dimerization or oligomerization has surfaced as a potential modulator of receptor signaling and several studies suggest that AT1R signaling may be affected by homo- or hetero-oligomerization (252). Oligomerization may play a role in directing the propensity of AT1R for G-protein activation or ERK activation (106). Interestingly, inhibition of one AT1R

within an homo-oligomer is sufficient to inhibit signaling of other receptors within the complex, providing more evidence that receptor conformations can be altered by formation of oligomers (136). Aggregation of AT1Rs can also occur at the cell surface in response to environmental stimuli, including oxidative stress. Image correlation spectroscopy (ICS) has revealed that AT1R oligomerization at the cell surface increases in response to incubation with AngII, with further increases upon treatment with H₂O₂, and oligomerization is associated with enhanced AT1R-induced increases in intracellular Ca²⁺ (249). Potential pathological relevance of AT1R homodimerization was demonstrated in hypertensive patients with increased atherogenic risk factors who had increased levels of crosslinked AT1R homodimers in monocytes capable of enhanced signaling (2). Potentiation of AT1R signaling can also occur through heterodimerization with other receptors, such as the cannabinoid type 1 (CB₁R), β-adrenergic (β-AR), and bradykinin 2 (B₂R) receptors (3, 20, 219). AT1R/B₂R heterodimers have been implicated in the development of preeclampsia with studies demonstrating increased levels of heterodimers that protect AT1R from oxidative stress-induced inactivation (1). Oligomerization and heterodimerization present interesting means through which AT1R signaling can be augmented, especially in response to oxidative stress, with pathophysiological consequences and may lead to additional therapeutic strategies.

Another mode of regulation of AT1R activity is through its distribution in membrane microdomains within the plasma membrane. A recent study fluorescently labeled lipid raft and non-raft domains and examined the

redistribution and signaling capabilities of wild-type and mutant receptors upon ligand-binding. Under resting conditions, AT1aRs were found to localize in lipid raft domains, however AngII stimulation caused movement to non-raft domains through G_q protein activation in HEK-293 cells (18).

Regulatory proteins can also bind to AT1R at the cell surface to direct AT1aR internalization, such as angiotensin type 1 receptor-associated protein (ATRAP) and the rat type 1 angiotensin II receptor associated protein 1 (ARAP1). In mouse cardiomyocytes, ATRAP binds to an intracellular region of the receptor and remains with the receptor as it is internalized after stimulation with AngII and co-expression studies indicate that ATRAP plays an active role in decreasing AT1aRs at the cell surface.(255) ARAP1, on the other hand, binds to a similar region of the AT1aR but serves to enhance receptor recycling to the cell surface in HEK-293 cells (99). Dysregulation of these regulatory proteins in disease states may serve to potentiate AT1aR signaling and may present new therapeutic targets.

1.1.2.4 Regulation of angiotensin receptor expression

Regulation of AT1aR expression has been examined primarily in the context of vascular smooth muscle cells (VSMCs). Negative regulators of AT1R expression include AngII itself, estrogen, reactive oxygen species (ROS), high-density lipoprotein (HDL), and TGF-β1 (149, 180, 184, 272, 293). Pathogenic stimuli such as hypercholesterolemia and high salt diets are positive regulators of AT1R expression, which may contribute to the role of the RAS in diseases such as atherosclerosis or hypertension (181-182, 185).

Posttranscriptional regulation of AT1R mRNA can occur through stabilization or destabilization of AT1R mRNA. The AngII-mediated decay of AT1R mRNA is dependent on the AUUUUA hexamer within the 3' UTR which results in a stem-loop structure required for the recruitment of the exosome complex containing mRNA binding proteins that accelerate mRNA decay (23). Proteins such as nuclear riboprotein S1-1 and p100 protein stabilize while phosphorylated calreticulin destabilizes AT1R mRNA (173-174, 196). Interestingly, insulin has also been identified as having AT1R mRNA-stabilizing properties and enhanced AT1R expression induced by hyperinsulinemia may contribute to the role of the RAS in the metabolic syndrome (183).

1.2.1 Adipose RAS

Components of the RAS required to synthesize and respond to AngII are expressed in adipocytes. AGT expression was first discovered in peri-aortic adipose tissue and subsequently confirmed in other adipose depots, including both white and brown adipose tissue of rodents (39, 46, 94, 226) and white adipocytes of humans (76). In fact, adipocytes are an important extra-hepatic source of angiotensinogen, with 68% of the mRNA abundance observed in liver (154). The expression and activity of renin and ACE in adipocytes is less clear. While some groups have failed to detect renin mRNA transcripts in brown or white adipocytes (76, 235), others have detected transcripts (97, 137) and renin activity (230, 235). Similarly, reports of ACE expression and activity range from undetectable (59) to low (131, 198). The fact that AngII is detectable in the media of cultured adipocytes after 6 days of media changes suggests that while

renin and ACE activity may be low in these cells, it is sufficient for processing AGT to measurable levels of AngII (230). In whole adipose tissue *in vivo*, the ability of other cell types such as preadipocytes, endothelial cells, and macrophages to produce these components may contribute to the local production of AngII. Additionally, other RAS components, such as prorenin receptor and ACE2, are located in adipocytes and may regulate AngII production (89, 100).

1.2.2 Adipose angiotensin receptors

Adipocytes have the ability to respond to either locally or systemically produced AngII because they express angiotensin receptors. Initial ligand-binding assays by Crandall, et al. and Cassis, et al. demonstrated the presence of AT1R on rat adipocytes using the selective AT1R antagonist, losartan, to successfully compete for AngII binding (44, 59). Crandall, et al. observed AngII binding adipose AT1Rs with an approximate affinity of 1 nM and receptor density was greatest in epididymal, followed by retroperitoneal and mesenteric adipose depots when corrected for differences in cell volume (59). Cassis, et al. similarly observed AT1R affinity ranging from 0.3-0.6 nM with epididymal fat containing the greatest number of receptor sites and interscapular brown adipose containing the least (44). Both groups found that the selective AT2R antagonist, PD123319, was unable to effectively compete radiolabelled AngII binding, suggesting low expression levels of this receptor. Other groups have confirmed the expression of AT1Rs with low or undetectable levels of AT2Rs in adipocytes derived from rodents and humans (76, 97, 198). However, several groups have reported

AT2R-mediated effects of AngII on adipocytes using AT2R antagonists, so the functional relevance of AT2Rs cannot be ignored simply due to low expression levels (61, 130). Expression of AT1bR was not detectable in adipose tissue (36).

1.2.2.1 Regulation of adipocyte angiotensin receptors during differentiation

Expression levels of the AngII receptors change throughout the process of preadipocyte conversion to mature adipocytes, though there is controversy regarding the nature of these changes. One group has shown that AT1aR protein levels remain strongly expressed throughout the course of 3T3-L1 (murine embryonic fibroblast cell line capable differentiating to mature adipocytes) adipocyte differentiation while AT2R expression is lost after 12 days of differentiation (159). Similarly, our lab demonstrated that AT1aR mRNA abundance increased significantly after 5 days of differentiation of 3T3-L1 adipocytes and remained elevated through 10 days (100). In studies of human preadipocytes differentiated to mature adipocytes, results show transient AT2R expression with either low levels of AT1R (163, 284) or increased AT1R as cells became adipocytes (122). At least one study has shown increased protein expression of AT2R with differentiation of human preadipocytes, and interestingly, in this study AT1R mRNA abundance decreased throughout differentiation even though the protein levels were unchanged, suggesting reduced mRNA stability and that mRNA abundance may not be a reliable indicator of protein expression (228). However there is widespread controversy regarding the specificity of the antibodies of AT1Rs used in these studies for western blot analyses (212). Despite a few instances to the contrary, most

groups agree that the expression of RAS components to produce AngII are increased with adipocyte differentiation (122, 163), though the regulation of angiotensin receptors is more difficult to ascertain. Technical difficulties in measuring angiotensin receptors (e.g., specificity of antibodies), the species of adipocytes under study, or differences in expression levels resulting from experimental conditions (i.e., components included in cell culture media, duration of experiments, frequency of media changes, cellular production of AngII, etc) may contribute to divergence in AngII receptor subtypes on adipocytes.

1.2.2.2 Regulation of adipose angiotensin receptors with obesity

Changes in gene and protein expression during adipocyte differentiation *in vitro* may or may not reflect changes *in vivo* during states of over-nutrition, as the relative contributions of adipogenesis and adipocyte hypertrophy to the obesity phenotype are still unclear (15, 60). Early characterizations of AngII receptor expression in lean and obese Zucker rats revealed reductions in AT1R expression in liver but not in adipose depots (44). Another study using neonatal monosodium glutamate (MSG) injection in rats as a model of adult-onset adipocyte hypertrophy and insulin resistance demonstrated that AngII-AT1R binding was decreased significantly with obesity although protein expression increased markedly (199). While decreased ligand binding and increased protein levels may suggest a defect in receptor binding or internalization, few studies have followed up on these findings. In humans, a study comparing normal and overweight patients observed no differences in AT1R mRNA abundance between the different weight groups, but higher AT1R mRNA levels in visceral compared

to subcutaneous adipose tissue (91). Interestingly, another study examining AT1R expression among normotensive lean, obese normotensive, and obese hypertensive patients found that AT1R expression was similar in normotensive groups regardless of weight but was significantly increased in subcutaneous adipose of obese hypertensives (79). This association between adipose AT1R expression and hypertension could reflect a predisposition of persons with increased AT1R in adipose to become hypertensive or an upregulation of adipose AT1R in response to hypertension, and determining whether AT1R upregulation is a cause or consequence could have important implications in identifying at-risk populations or treating obesity-hypertension.

1.2.2.3 Regulation of adipose angiotensin receptors with age

Another important factor that may modulate adipose AT1R expression is the process of aging, especially because aging is associated with increased adiposity (256). One study demonstrated that adipose AT1R mRNA levels decreased significantly between 4 and 12 weeks of age in male Wistar rats (6). These data conflicts with a recent study demonstrating that AT1R mRNA abundance was similar between rats aged 9, 12, or 20 weeks but was markedly increased by 26 weeks of age (147). Interestingly, in this study protein levels of AT1R were not reflective of the mRNA abundance but were elevated after 12 weeks of age and maintained through 26 weeks of age.

1.2.2.4 Regulation of adipose angiotensin receptors by AngII

AT1R expression is downregulated in response to AngII incubation in VSMCs and in fact, most GPCRs exhibit reduced expression in response to

ligand-binding as a mechanism of self-regulation (149). In adipose tissue, however, studies from our laboratory have indicated the opposite, that AngII increased AT1aR expression (154). In this study, AT1aR mRNA levels were elevated in epididymal adipose of AT2R deficient mice fed standard mouse (chow) diet; however this increase was blocked by losartan. These data indicate that deficiency of AT2Rs per se did not change AT1aR expression rather that enhanced AT1aR signaling in the absence of AT2Rs resulted in increased AT1aR mRNA expression. Moreover, infusion of AngII markedly increased the expression of AT1aR mRNA in adipose tissue but not in liver, suggesting an adipose-specific positive feedback loop wherein AngII increases the expression of its own receptor. While adipose up-regulation by AngII may result from AngII actions at adipocyte AT1aRs, this study used whole adipose tissue lysates to isolate mRNA and it is possible that other cell types within adipose tissue contributed to this effect. In the setting of obesity, where the systemic RAS is thought to be elevated (57, 268), this positive feedback loop may be of particular importance and result in further elaboration of AngII's effects on adipocytes.

1.3 Evidence for a role of AngII in (patho)physiologic adipose tissue growth and function

1.3.1.1 The process of adipocyte differentiation

Adipocyte differentiation comprises two distinct phases (Figure 1.4), the first is the commitment to the adipocyte lineage wherein mesenchymal stem cells (MSCs) become preadipocytes and the second is the terminal differentiation of a preadipocyte into a mature adipocyte (60). In order for the commitment phase to

progress, inhibitory Wnt and TGF- β signaling pathways must be interrupted and stimulatory pathways involving zinc-finger protein 423 (ZFP423) and TCF7-like 1 (TCFL1) transcription factors must be turned on. Terminal differentiation begins with exogenous adipogenic stimuli that induce transient upregulation of CAAT/enhancer binding proteins (C/EBP β and δ) which then induce PPAR γ expression, which acts as a master regulator of differentiation through coordination with C/EBP α (280). PPAR γ is a nuclear receptor activated by polyunsaturated fatty acids and their derivatives that translocates to the nucleus, heterodimerizes with retinoid X receptor (RXR), and binds PPAR response elements (PPREs) in the DNA to recruit other co-activators to several target genes. PPAR γ and C/EBP α target genes include those for glycerophosphate dehydrogenase, fatty acid synthase, acetyl CoA carboxylase, malic enzyme, glucose transporters, insulin receptor, and fatty acid binding proteins (218). Other transcription factors involved in adipocyte differentiation include Krox20, Kruppel-like factors, Stat5A, and sterol-regulatory element-binding protein (SREBP)-1c (237). In fact, adipocyte determination and differentiation-dependent factor 1 (ADD1)/SREBP1 is another prominent regulator of differentiation, as dominant negative ADD1/SREBP1 attenuated differentiation of 3T3-L1 cells (140). Adipocyte differentiation can be inhibited by several growth factors, including FGF, PDGF, and EGF (including the membrane protein pref-1 which contains EGF-like repeats), which act by stimulating MAPKs that phosphorylate and inactivate PPAR γ (108, 239). Several other hormones and growth factors act as adipogenic stimuli, however, and traditional differentiation

cocktails used *in vitro* contain insulin, dexamethasone (glucocorticoid), insulin-like growth factor (IGF-1; present in fetal calf serum), isobutyl-1-methylxanthine (IMBX; cAMP-elevating agent), and more recently, a PPAR γ ligand (107).

Adipocyte differentiation can be regulated by nutritional status. Recent studies suggest that adipogenesis is inhibited in the setting of obesity (101, 116, 197). Specifically, lipid accumulation in differentiated human preadipocytes is inversely correlated to the subjects' BMI as well as the size of adipocytes obtained from collagenase digestion (116). Follow-up studies indicate that the mechanisms involved may include sustained inhibitory canonical WNT signaling pathways (101) or epigenetic changes resulting from hypertrophy-induced hypoxia (141). Correspondingly, the numbers of adipocyte precursor cells in adipose tissue increase 270% with high fat feeding, supporting the conclusion that adipocyte differentiation is impaired, not the availability of precursor cells (155). This suppression of adipocyte differentiation causes the remaining adipocytes to accumulate more lipid, thus contributing to adipocyte hypertrophy and its consequences, such as insulin resistance (13-14). While these results provide interesting new insights into mechanisms of adipocyte hypertrophy, they are seemingly incongruent with other reports of increased adipocyte cell numbers in obese subjects (80, 143, 243), demonstrating that more research is necessary to understand the dynamics of adipogenesis in obesity. Conversely, in the setting of nutrient deprivation, adipocyte differentiation may be enhanced. Calorie-restriction in rats for 2 or 25 months upregulates adipogenic markers, such as PPAR γ , C/EBP α , and adiponectin and consequently adipocyte size was

reduced in these rats (295). Other studies indicate that calorie-restriction of 3T3-L1 cells causes the cells to revert to a proliferative state (213) which may or may not be conducive to adipocyte differentiation, again indicating the need for further research into these mechanisms of adipogenesis.

1.3.1.2 Role of AngII in adipocyte differentiation

There are several methods that have been used to study the process of adipocyte differentiation, including preadipocyte differentiation *ex vivo*, clonal preadipocyte cell lines capable of adipocyte differentiation, isolated primary adipocytes, or adipose tissue explants. In the case of the RAS, these methods could be employed in the absence or presence of AngII and/or inhibitors of the RAS such as ACE inhibitors or angiotensin receptor blockers (ARBs). One caveat, however, regarding the use of ARBs to study adipose tissue development is that some ARBs have been found to stimulate PPAR γ , independently of AT1R blockade (22, 216, 231). Two independent reports were published in 2004 that certain ARBs have PPAR γ -agonizing properties independent of AT1R signaling (22, 231), which has been confirmed by subsequent studies in AT1aR^{-/-} mice (216). Telmisartan, irbesartan and candesartan consistently exert PPAR γ -agonism while eprosartan, valsartan, and losartan have little or no ability to do so (13, 22, 54, 78, 86, 123, 231, 245, 296). At least one study, however, has identified the EXP3179 metabolite of losartan as a partial PPAR γ agonist (232). These properties of ARBs must be considered when interpreting investigations of the role of AngII or the AT1R in the process of adipocyte differentiation.

Few studies have examined the effects of AngII on the initial commitment step in which MSCs commit to the adipocyte lineage (Table 1.1). One group incubated commercially available human mesenchymal stem cells with 3 cycles of adipocyte induction medium for 3 days followed by 3 days of adipocyte maintenance medium, including incubation with AngII alone or in combination with valsartan or PD123319 throughout the 18-day period and found that AngII decreased PPAR γ , FAS, and lipid accumulation (163). This anti-adipogenic effect was mediated through the AT2R, as valsartan further reduced the levels of PPAR γ and FAS, indicating a pro-adipogenic effect of AngII through the AT1R. It is difficult, however, to determine if AngII influenced the initial commitment step or the terminal differentiation step because the treatments were maintained through the entire differentiation period. In addition to influencing commitment to preadipocytes, AngII may also affect preadipocyte proliferation as it has been shown that AngII increases human preadipocyte proliferation through AT1R-mediated increases in cyclin D expression (58). AngII has also been reported to increase mature adipocyte cell numbers through the AT1R in serum-free conditions by increasing cell division, however it is unclear whether these results are relevant *in vivo* (225).

Several studies have used preadipocytes isolated from the stromal vascular fraction of whole adipose tissue digested with collagenase, with some investigators reporting an anti-adipogenic effect of AngII (85, 122, 229)(Table 1.1). Of these studies, only Janke, et al. determined the role of AT1R versus AT2R in their observed effects, demonstrating that irbesartan administration in

the absence of exogenous AngII increased lipid accumulation while the AT2R antagonist had no effect (122). However, as described above, irbesartan has AT1R-independent effects, complicating interpretation of these data. Mouse fibroblast-like embryonic cell lines (Ob1771, 3T3-L1) have been valuable tools employed to study AngII's role in adipocyte differentiation because they offer consistency in differentiation that is difficult to achieve from human-derived samples. However, these cell lines do not allow investigators to define the contributions of preadipocytes derived from different regional locations. One of the first studies in this field found that AngII reduced adipocyte differentiation in OB1771 cells through the AT2R (61). However, these results are also controversial because others found that exogenous (130) and endogenous (114) AngII increases lipid accumulation and markers of adipocyte differentiation. In these studies both AT1R and AT2R antagonists reduced FAS and GPDH enzyme activities (130) or lipid accumulation (114). Changes in adipocyte size and markers of adipocyte differentiation have also been studied *in vivo*, and these will be discussed later in terms of the role of AngII in the regulation of body weight.

1.3.2.1 The pathophysiology of obesity

The World Health Organization classifies people as obese with a body mass index (BMI) greater than 30 and overweight with a BMI of 25-29.9. The latest data from the National Health and Nutrition Examination Survey in 2009-2010 indicated that 35.7% of adults and approximately 17% of children nationwide are obese (187). As recently as 1990, no state had a prevalence of

obesity greater than 15%, whereas in 2010 all states had obesity rates above 20%, including 36 states above 25%, indicating the recent dramatic increases in obesity (187). Obesity is a component of the metabolic syndrome, which is a cluster of risk factors that greatly increase the risk for cardiovascular disease and includes elevated waist circumference, blood pressure, fasting blood glucose, triglycerides, and reduced HDL (8). Visceral obesity is the central component of the metabolic syndrome and is independently associated with each of the other factors (42). Despite the strong associations between obesity and disease, about 15% of obese individuals are unaffected by obesity-associated disorders and manage to possess “metabolically healthy” adipose tissue, which is thought to be due to a high number of small (non-hypertrophied, non-hypoxic, or non-inflamed) adipocytes (26).

While caloric intake in excess of energy requirements is the basis of obesity, it is a multi-factorial disease state that can be influenced by various genetic and environmental factors, including physical activity, regulation of satiety, addictive eating behaviors, hormonal imbalance, fetal programming, or even disturbances in intestinal microbiota. Regardless of the causes of obesity, changes in adipose tissue growth, function, and remodeling dictate whether an obese individual develops obesity-associated disorders. During the development of obesity, excess caloric intake results in increases in adipocyte size (hypertrophy), however as discussed previously, the contributions of precursor recruitment, commitment, proliferation, and differentiation are still under investigation (248). Obesity is associated with increased macrophage infiltration

into adipose tissue (277) resulting from inappropriate adipose tissue remodelling to accommodate larger adipocytes (248). Adipose tissue inflammation combined with ectopic lipid accumulation in non-adipose tissues connect obesity with other components of the metabolic syndrome to increase risk of cardiovascular disease (170).

1.3.2.2 Genetic evidence for a role of AngII in the development of obesity

Polymorphisms have been identified in the genes for AGT, ACE, and AT1R that result in either reduced or increased functions of these proteins, with possible implications in the development of obesity and related disorders. Three commonly studied mutations in the RAS include *M235T* AGT mutation, which results in higher plasma AGT levels (126), the *A1166C* AT1R mutation, which increases responsiveness of the AT1R (27), and the insertion/deletion (*I/D*) ACE mutation, in which *D/D* individuals have double the plasma ACE activity of *I/I* individuals (214). The Olivetti Prospective Heart Study of 959 Italian men examined associations of these three mutations with BMI, body fat distribution, and obesity-associated hypertension (246). This study found no association between the AGT or AT1R mutations with any of these parameters, however individuals with the *D/D* genotype in the ACE gene were significantly more likely to be overweight and have abdominal obesity. Other studies have also found significant associations between the D allele and increased BMI (275) and individuals with at least one *D* allele in the ACE gene were more likely to have at least one of the following; obesity, hypertension, hyperlipidemia, or diabetes mellitus (266). However, in the Health Aging and Body Composition Cohort

Study, the *I/I* genotype of the ACE mutation has also been positively associated with BMI despite resulting in lower serum ACE levels than the *D/D* genotype (146). In contrast to the Olivetti Prospective Heart Study, the Heritage Family study did find that mothers with the T allele of the *M235T* AGT mutation had significantly greater fat mass than mothers homozygous for the *M* allele (211). Interestingly, AGT secretion from isolated adipocytes was not affected by the individual's BMI, obesity, body fat, blood pressure, or AGT polymorphism, though adipocyte size was increased in individuals homozygous for the *T* allele (204). Several studies have failed to find any associations between RAS gene polymorphisms and obesity or BMI, possibly due to the population under study and the population size (84, 167, 267).

Metabolic studies on mice lacking one of the major components of the RAS (AGT, renin, ACE, AT1aR, AT2R) have demonstrated reductions in body weight and adiposity (Table 1.2), as well as improved insulin sensitivity. These results suggest that AngII may play a role in the development of adipose tissue and consequently the development of obesity and obesity-related insulin resistance. *AGT^{-/-}* mice exhibit reductions in body weight at weaning and throughout 46 weeks of age when maintained on either standard murine diet or when fed a high fat diet (65% fat) (160). Differences in body weight were due to lower body fat and hypotrophy of adipocytes, which were attributed to increased locomotor activity in *AGT^{-/-}* mice. Of note, however, these mice were of the ICR-CD1 strain (not the C57BL/6 typically used) and wild-type control mice were non-littermates, which may be of significance because wild-type ICR-CD1 mice were

resistant to diet-induced obesity. C57BL/6 mice lacking the *Ren1c* gene for renin also demonstrate lower body weights, both on standard murine diet and when fed a Western diet (42% fat) (253). Food intake, activity, and expression of adipogenic genes in epididymal adipose tissue were not regulated by renin deficiency under HF conditions; however, *Ren1c*^{-/-} mice had increased heat generation and decreased fat absorption, which may have contributed to the body weight phenotypes. Additionally, AngII infusion (1.5 ug/day) in *Ren1c*^{-/-} mice restored body weight and fat mass to levels of WT mice, implying that the effects of renin deficiency were due to reduced production and actions of AngII and not due to AngII-independent metabolic effects of renin. *ACE*^{-/-} mice fed standard murine diet displayed reduced body weight and fat mass with improved glucose tolerance; however, this study did not investigate the effects of ACE deficiency when mice were fed a high fat diet (125). Wildtype and *ACE*^{-/-} mice had similar locomotor activity but *ACE*^{-/-} mice exhibited increased total and resting energy expenditures, which was attributed to increased fatty acid metabolism in liver. These various models of reducing AngII synthesis indicate that AngII acts as a positive regulator of weight gain and adiposity; however, mechanisms for these effects are unclear and vary between models.

Mouse models of angiotensin receptor deficiencies have also been investigated for metabolic effects with less consistent results on body weight and adiposity. Initial studies by Kouyama, et al. showed *AT1aR*^{-/-} mice have similar body weights compared to littermate controls when fed standard murine diet; however, they are partially protected from high fat (60% kcal as fat) diet-induced

obesity and glucose intolerance (144). Food intake was not influenced by AT1aR deficiency, but body temperature and O₂ consumption were increased.

Adipocytes from high fat-fed AT1aR^{-/-} mice were modestly hypotrophic. Another group, Yamamoto, et al., also studied AT1aR^{-/-} mice fed standard murine diet (4.8 % fat) diet and observed increases in body weight beginning at 3 weeks of age and persisting through 24 weeks of age due to hyperphagia in AT1aR^{-/-} mice compared to controls (281). Consistent with results of Kouyama et al., in a pair-feeding study in which AT1aR^{-/-} mice were limited to the same food intake of WT controls, AT1aR^{-/-} mice exhibited reductions in body weight and fat mass (281).

Both *ad libitum* fed and pair-fed AT1aR^{-/-} had decreased levels of the anorexigenic peptide corticotrophin-releasing hormone (Crh) and luciferase assays confirmed regulation of hypothalamic Crh expression by AngII. High fat fed mice in this study, however, did not exhibit hyperphagia or differences in body weight. The reasons for the differences between these results and those of Kouyama, et al. are not entirely clear; however, Yamamoto, et al. describe different housing conditions as a possible confounder between studies.

Surprisingly, mice lacking AT2R have also been demonstrated to exhibit reduced body weight and fat mass following high fat feeding (289). Improvements in insulin resistance (calculated as a product of the area under plasma glucose and insulin curves as plotted from a glucose tolerance test) were observed in AT2R^{-/-} mice fed either a low fat or high fat diet. The results of Kouyama, et al. and Yvan-Charvet et al. are noteworthy because data suggests that AT1aR and

AT2Rs may have similar or overlapping functions in adipocytes, which is contrary to other cell types where AT1aR and AT2R signaling are in direct opposition (67).

Similar studies to those described above have been performed in AT1aR^{-/-} or AT2R^{-/-} mice on a hypercholesterolemic background, namely mice lacking apolipoprotein E (ApoE^{-/-}) or low density lipoprotein receptor (LDLR^{-/-}).

Hypercholesterolemia has been demonstrated to stimulate the RAS, increasing circulating AGT and angiotensin peptides (65) which may result in more pronounced effects of receptor deficiencies in a hypercholesterolemic setting.

AT1aR/ApoE double knockout mice had significantly reduced fat mass and adipocyte size compared to ApoE^{-/-} controls when fed standard murine diet (262).

Reductions in adipocyte size in double knockout mice were associated with increased adipogenesis as evidenced by increased PPAR γ , C/EBP α , and aP2 mRNA abundance, suggesting that AngII inhibits adipocyte differentiation

through the AT1aR in ApoE^{-/-} mice (262). In another study by the same group, there was no effect of AT2R deficiency in ApoE^{-/-} mice fed standard murine diet (121). However, with 4 weeks of high cholesterol diet (1.25% cholesterol)

AT2R/ApoE^{-/-} mice displayed increased fat mass, reduced adipocyte numbers, and reduced PPAR γ mRNA abundance, suggesting that AngII promotes adipogenesis through the AT2R in ApoE^{-/-} mice fed a high cholesterol diet (121).

It is unclear why hypercholesterolemia causes these receptors to oppose one another when they appear to have similar effects in C57BL/6 mice fed low or high fat diets.

1.3.2.3 Regulation of body weight by pharmacologic blockade of the RAS

Another model for investigating the role of angiotensin receptors in the development and function of adipose tissue involves administration of pharmacological antagonists to various animal models rather than genetic ablation of the receptors. Use of antagonists is more relevant to the treatment of human disease and an additional benefit is that the receptors are generally antagonized in adult mice, eliminating developmental artifacts that may contribute to adult phenotypes in murine knockout models. ACE inhibitors are one class of drug that have been extensively studied in animal models. Sprague-Dawley rats fed standard rat diet and administered perindopril in the drinking water either from birth through 10 weeks of age exhibited reduced fat mass (162, 278). Wistar rats fed either standard or a high fat diet (35% kcal as fat) containing enalapril also exhibited reduced fat mass, though food intake was also reduced in enalapril-treated rats (223). Mice fed a high fat diet (roughly 50% kcal as fat) for 12 weeks prior to captopril administration experienced dramatic weight loss when administered captopril that was maintained through 12 additional weeks of high fat feeding (206). In mice administered captopril, reductions in fat mass were attributed to increases in lipolysis and fatty acid oxidation both in liver and adipose tissues; however, food intake was also reduced in captopril-treated animals.

ARBs have also been extensively studied in regard to their ability to modulate adiposity, and given the previous discussion of the non-specific PPAR γ -agonism of certain ARBs, it is not surprising that several studies using

telmisartan, irbesartan, and candesartan in animal models have demonstrated reductions in body weight, fat mass, and adipocyte size concurrent with upregulation of PPAR γ and/or its target genes (12, 247, 296). The PPAR γ -stimulating properties of valsartan seem to be less clear because while one study found no evidence of PPAR γ -agonism (78), other studies using valsartan demonstrate reductions in fat mass and adipocyte size in Sprague-Dawley rats fed a high fat, high carbohydrate diet for 3 months (247) as well as upregulation of PPAR γ mRNA abundance in adipose tissue of diabetic KK-A y mice (262). A study using temocapril (ACE inhibitor) and olmesartan (not a PPAR γ ligand) in rats fed a fructose-rich diet, which is a non-obese model of insulin resistance, observed no changes in body or fat mass with either drug, but did see reductions in adipocyte size (88). Sprague-Dawley rats administered losartan (not a PPAR γ ligand) at 3 months of age for 2 weeks had attenuated weight gain, smaller adipose pads, and reduced cell size(59). Losartan administered to aged rats fed standard diet between the ages of 24 and 30 months failed to affect body weight or fat mass, which supports the lack of PPAR γ -agonism by losartan, (43). The results with non-PPAR γ -agonist ARBs suggest there may be an effect of AT1R blockade on adipose tissue growth; however, reductions in fat mass observed with valsartan, olmesartan, or losartan are modest and do not always translate to differences in body weight.

1.3.2.4 Regulation of body weight by AngII infusion (elevated RAS)

Deficiency of major RAS components consistently results in reduced body weight and adiposity, possibly due to reduced action of AngII at certain cell types.

However, models whereby AngII is increased by chronic infusion also demonstrate paradoxical reductions in body weight and adiposity. Initial studies in rats demonstrated that infusion of AngII (500ng/kg/min) to Sprague-Dawley rats for 14 days reduced body weight due to reductions in food intake while co-administration with losartan, but not hydralazine, could attenuate these effects (34). Cassis, et al. confirmed the blood pressure-independent and dose-dependent reductions of body weight caused by AngII infusion in rats (47). Further studies by this group demonstrated reductions in food intake in AngII-infused rats (77). Interestingly, intracerebroventricular (icv) infusion of AngII caused reductions in body weight similar to peripheral AngII infusion, and while food intake was reduced when AngII was infused icv, pair-fed rats also lost weight indicating that reduced food intake alone is not the sole mechanism for AngII regulation of body weight (68). This study and others (77) observed increased sympathetic nerve activity in brown and white adipocytes, which could increase energy expenditure and lipolysis, respectively, to contribute to AngII-induced weight loss. Lipolysis was quantified *in vivo* in AngII-infused Sprague-Dawley rats using a microdialysis technique to measure interstitial glycerol levels and results indicated that AngII stimulated lipolysis in white adipose tissue in an AT1aR-dependent manner (37). AngII-induced lipolysis in this study was also blocked by a nonselective β -adrenergic antagonist, suggesting interplay between presynaptic β -adrenoreceptors and AT1aRs.

The possible role of AngII to stimulate lipolysis has been examined in humans with conflicting studies demonstrating no effect (264), a weak stimulatory

effect (28-29) or an inhibitory effect of AngII on lipolysis (30-31, 95-96). In one study of healthy male subjects, both sub-pressor and pressor doses of AngII infusion failed to alter whole-body lipolysis rates, plasma free fatty acids, plasma insulin, or plasma norepinephrine concentrations (264). In contrast, subsequent studies utilized a microdialysis technique to study tissue-specific, rather than whole-body effects of AngII or ARB administration on blood flow (ethanol dilution technique) and lipolysis (glycerol accumulation). Initial studies by Boschmann, et al. demonstrated slight reductions of blood flow in response to AngII and a failure of AngII to change glycerol concentrations, though accumulation of glycerol was expected as a result of reduced blood flow. Thus, the authors concluded that a lack of change in glycerol levels in response to AngII suggests that AngII inhibited lipolysis (30-31). However, follow-up studies by this same group contradicted these results and revealed weak stimulation of adipose lipolysis by AngII (0.1 – 1.0 μ M) (28-29). Studies of the lipolytic effects of AngII by another group also found that doses of AngII that reduced blood flow inhibited lipolysis, while higher doses of AngII paradoxically stimulated lipolysis (95-96). A randomized, double-blind crossover study in which obese men were treated with either placebo or irbesartan for 10 days with a 2-4 week wash-out period between treatment phases found that blockade of endogenous AngII with irbesartan modestly reduced isoproterenol-induced glycerol production in adipose tissue, indicating an enhancement of lipolysis by AngII (28). While to this point most groups speculated that AngII acted on presynaptic AT1Rs on sympathetic nerve terminals innervating adipose tissue to modulate

norepinephrine production and thus influence β -adrenergic stimulation of lipolysis, Goossens, et al. performed a study in isolated adipocytes and found that AngII administration reduced glycerol production in an AT1R-dependent manner, implying a direct role for AngII at adipocyte AT1Rs (95). Unfortunately, differences in results from these studies examining AngII regulation of lipolysis have not been resolved, potentially influenced by direct versus indirect effects of AngII on lipolysis.

1.4 The role of AngII in obesity-induced hypertension

1.4.1 The association between obesity and hypertension

(73)[171]The Framingham Heart Study indicated that 78% of hypertension cases in males and 64% in females can be directly attributed to obesity, yet the mechanistic links between the obesity and hypertension remain unclear (90). There is also a linear relationship between BMI and hypertension risk, such that the odds ratio for the presence of hypertension is 1.7 for overweight individuals, 2.6 for those with a BMI of 30-34.9, 3.7 with a BMI of 35-39.9, and 4.8 with a BMI over 40 (179). While effective antihypertensive medications are available, the prevalence of resistant hypertension, referring to hypertension that persists when treated with more than 3 antihypertensive agents, is expected to increase as the population becomes heavier and older (38), highlighting the need for either more effective medications than those currently available or better identification of specific characteristics of patient populations governing their responsiveness to medications.(38)[174]

1.4.2 Mechanisms of obesity-induced hypertension

Postulated mechanisms linking obesity to hypertension include renal/hemodynamic changes, elevated levels of adipokines, insulin resistance, increased reactive oxygen species, enhanced sympathetic nervous activity, or activation of the RAS (71, 148, 178). Impaired kidney function brought on by the development of glomerulosclerosis or compression of the kidney by abdominal visceral fat could result in impaired pressure natriuresis and increased blood volumes seen in obese patients. While increased intra-abdominal pressure induced by artificial means (balloon) in dogs did result in increased blood pressure, the mechanisms and translatability of this study to human obesity are unclear (25). Insulin resistance is part of the metabolic syndrome and is frequently present in obese-hypertensive patients; however, a direct role for insulin or hyperinsulinemia to promote high blood pressure has not been demonstrated (9, 105). Factors produced by adipocytes, termed adipokines, represent an area of investigation that may identify mechanisms linking obesity to hypertension. Adipokines linked to the development of hypertension include resistin, adiponectin, leptin, and angiotensinogen (193, 208, 257, 286). Resistin is an adipose-derived vasoconstrictor involved in insulin resistance, though its role in non-diabetic hypertension is unclear (254). Adiponectin is a protective hormone associated with insulin sensitivity that can act as a vasodilator by upregulating eNOS production and reducing vascular reactive oxygen species (193), and obesity-associated hypoadiponectinemia may promote increases in blood pressure through ROS-induced endothelial dysfunction (148). Leptin is an

anorectic protein hormone secreted by adipocytes, which acts on the hypothalamus to suppress food intake and can also stimulate sympathetic nerve activity to organs implicated in control of the cardiovascular system. The role of leptin in obesity-hypertension is complex because while hyperleptinemia and resistance to the anorectic properties of leptin are characteristic features of obesity, the sympathetic stimulation by leptin appears to be maintained (209).

1.4.3 The RAS as a link between obesity and hypertension

While all of the above-mentioned mechanisms may contribute to obesity-induced hypertension, the RAS is another important candidate that has been suggested to contribute to obesity-induced hypertension since adipocytes express all components needed to synthesize and respond to AngII (48). The systemic RAS has been demonstrated to be activated in rodent and human obesity (32, 271), and inhibitors of the RAS are effective treatments to reduce blood pressure in obese rodents and humans (33, 142). In one study of 4 black populations, plasma AGT levels were highly correlated to BMI and interestingly, variation in BMI accounted for 90% of variation in AGT levels between populations (57). Another group extended these findings by demonstrating a significant correlation between plasma AGT levels and obesity (268). Adipose tissue distribution may play an important role in the relationship between BMI and plasma AGT levels since AGT mRNA abundance from visceral adipose tissue, but not subcutaneous, positively correlated with BMI (92). However, conflicting results have been obtained (283).

Data from animal models demonstrate that adipose-derived RAS components contribute to circulating levels of AngII and provide further evidence that the RAS is activated with obesity. Initial studies by Massiera, et al. demonstrated that AGT expression limited to adipocytes (AGT was overexpressed in adipocytes of AGT null mice) restores non-detectable plasma AGT levels of AGT null mice to 20-30% of that of wild-type mice (161). This was confirmed by our group using a model of adipocyte AGT deficiency in mice in which plasma AGT levels were reduced roughly 25% (21). In both studies by Massiera, et al. and Yiannikouris, et al., changes in AGT corresponded to changes in blood pressure, implicating adipose-derived AGT influences blood pressure. Our laboratory has demonstrated elevated concentrations of angiotensin peptides in plasma from rats (32) and C57Bl/6 mice made obese from consumption of a high fat diet (100). Moreover, obesity in both rodent models was associated with elevated blood pressure that was normalized by losartan, implicating the actions of AngII at the AT1R as a major contributor to obesity-induced hypertension (33).

1.5 The role of AngII and adipose in vascular diseases

1.5.1 The development, risk factors, and treatments of vascular disease in humans

Heart disease and cerebrovascular disease were among the top four leading causes of death in 2010 in the United States, leading to over 720,000 deaths combined (176). Atherosclerosis, an underlying cause of these conditions, is an inflammatory disease in which an initial endothelial insult results

in accumulation of cholesterol-rich macrophage foam cells in the sub-endothelial space of the aortic wall and develops into a plaque prone to thrombus (207). Atherosclerosis and arterial thrombosis can lead to restricted blood flow to the heart (coronary artery disease; CAD), peripheral artery disease (PAD), or cerebral ischemia (stroke) (115). Risk factors for atherosclerosis include components of the metabolic syndrome previously discussed (Section 1.2) as well as physical inactivity, smoking, poor diet, heavy drinking, stress, sleep apnea, age, and family history (115). Endothelial dysfunction, oxidation of LDL, inflammation, apoptosis, and coagulation are pivotal processes in the development of atherosclerosis, highlighting the participation of many different cell types ranging from vascular wall cells to immune cells to platelets and the need for cell-specific models to study each component of this disease.

Aortic aneurysms and aortic dissections were responsible for over 10,000 deaths in 2010, and are estimated to be present in approximately 1.1 million people (176, 261). AAAs are defined as a permanent dilation of the aorta in which the diameter is 50% greater than a normal, healthy infrarenal aorta, or at least three centimeters, and are characterized by thickening and inflammation of the adventitial layer of the aortic tissue (129). Risk factors for AAA formation include smoking, male gender, visceral obesity, and age > 65 years old (238). Experimentally induced AAAs, including those induced by infusion of AngII, exhibit elastin degradation within the medial layer of the aortic wall and macrophage infiltration into this region (224). The success of pharmacological RAS antagonists in reducing atherosclerosis (81, 135, 172, 217, 288) and

experimental AAAs (102, 169, 261) have initiated studies into the role of AngII as an inflammatory stimulus contributing to the development of these vascular pathologies.

1.5.2 The role of AngII and angiotensin receptors in vascular diseases

Several animal models reproduce findings in humans that ACE inhibitors reduce the severity of atherosclerosis (4, 50, 53, 145). Hypercholesterolemia significantly increases the production of angiotensin peptides in a process mediated by AT1aRs, though the exact mechanism is unknown (65). Our laboratories demonstrated that chronic infusions of AngII promoted atherosclerosis in LDLR^{-/-} and ApoE^{-/-} mice (62-63). During the course of these studies, AAAs were discovered in a significant number of mice and infusion of AngII has become a well-established model of AAA formation used by several investigators. Further experiments showed that AngII infusion promotes the formation of AAAs independent of increases in blood pressure, as norepinephrine infusion elevated blood pressure to the level of an AngII-infused mouse, but had no effect on the aortic diameter (45).

Mechanism(s) through which AngII promotes these vascular diseases are under intensive investigation. A consistent finding is that AngII-induced atherosclerosis and AAAs in mice are markedly reduced in mice with whole body AT1aR deficiency (49, 65, 276), indicating that both pathologies are AT1aR-mediated. In contrast, there is controversy regarding the role of AT2R in these AngII-mediated vascular diseases (64-65, 120, 128). In LDLR^{-/-} mice fed a high fat, cholesterol containing diet for 12 weeks, deletion of AT2R had no effect on

atherosclerosis (65). In contrast, another study found that AT2R deficiency in ApoE^{-/-} mice fed a high fat/cholesterol diet for 10 weeks augmented the development of atherosclerosis (120). These results suggest the AT2R is protective against atherosclerosis and thus enhanced activation of this receptor in AT1aR deficient mice contributes to reduced lesion formation, and accordingly, the effects of AT1aR antagonism are mitigated in AT2R deficient mice (120). Conflicting results were found using AT2R pharmacologic blockade with the compound PD123319 in chow-fed ApoE^{-/-} mice infused with AngII, where the compound either had no effect (128) or exacerbated atherosclerosis (64). The various study conditions and experimental designs used by these groups compromise the ability to interpret these results.

While data are in agreement with a pivotal role of AT1R in AngII-induced atherosclerosis and AAAs, the cell type(s) mediating these effects remain unknown. Bone marrow transplantation studies using AT1aR^{-/-} mice as donors and wild-type LDLR^{-/-} mice as recipients (effectively creating leukocyte-specific AT1aR^{-/-} mice) showed only a modest reduction in atherosclerosis and no effect on AAA formation, suggesting that leukocyte AT1aR are not the primary target of AngII to promote these pathologies (49). Similarly, deficiency of AT1aR on endothelial cells or smooth muscle cells also fails to affect the development of atherosclerosis or AAAs (unpublished observations), however endothelial AT1aRs do appear to mediate the development of ascending aortic aneurysms (212). Adipose tissue surrounds the length of the aorta in a way that no fascial layer separates adipocytes from the vascular wall. Adipocytes are gaining

interest as a source of inflammatory mediators and present another cell type through which AngII may induce atherosclerosis and AAAs.

1.5.3 Role of adipose in the development of atherosclerosis

Over the past several years, the idea of crosstalk between perivascular adipose tissue and blood vessels has given rise to what is known as the “outside to inside” signaling mechanism of atherosclerotic plaque formation. Investigators hypothesize that adipocytokines and chemokines released by adipose tissue can enhance various aspects of plaque development, such as endothelial dysfunction, vascular smooth muscle cell proliferation, and infiltration of inflammatory cells (273). Adipose tissue surrounds most blood vessels, including the coronaries and the aorta, and the distance between the two is less than 0.1 mm with no anatomical barrier to inhibit the diffusion of adipocytokines (109, 210). Early studies from our laboratory demonstrated that the presence of perivascular influenced the contractility of rat aorta to several vasoactive agents, including AngII (240). Since this initial report, several lines of evidence demonstrate that perivascular adipose-derived vasoactive factors influence the tone of blood vessels, demonstrating a functional link between these two tissues (151, 274).

The association between perivascular adipose tissue and atherosclerosis has gained increasing attention. In a segment analysis, pericoronary fat was associated with coronary atherosclerosis independent of overall epicardial adipose volume (158) and there is a higher incidence of plaques on the epicardial side of coronaries versus the myocardial side (205), suggesting a

causative role for epicardial adipose in plaque formation. Additional studies have revealed that pericardial fat volume, measured by cardiac computed tomography (CT), is associated with high-risk coronary lesions, independent of both BMI and the extent of plaque burden, supporting the conclusion that adipose has a local effect on the vessel (227). In fact, regions with low pericardial adipose had no high-risk coronary lesions (227).

More importantly, several studies have demonstrated that epicardial and perivascular adipose secrete more inflammatory mediators than other adipose depots (17, 51, 109, 165) and these proinflammatory mediators are increased with high fat feeding and obesity (51, 244). Specifically, secretion of the chemokine MCP-1, a macrophage chemoattractant with a pivotal role in the development of AngII-induced and human vascular diseases (117, 157), is upregulated 10-40 fold in perivascular adipose compared to perirenal and subcutaneous fat (51). Our lab has also demonstrated that MCP-1 secretion from abdominal peri-aortic fat is elevated in mice fed a high fat diet (203). Interestingly, another technique has recently been developed to study the role of adipose tissue in the development of atherosclerosis which involves transplantation of adipose depots between mice. Initial studies by Ohman, et al. demonstrated that the transplantation of visceral, but not subcutaneous, adipose tissue into the subcutaneous region of ApoE^{-/-} increases atherosclerotic lesion formation through elevation of systemic adipocytokines, in the absence of confounding factors such as high fat feeding, obesity, or diabetes (189). Subsequent studies by this group revealed that transplantation of visceral

epididymal adipose directly adjacent to the right common carotid artery dramatically increased atherosclerosis, though again subcutaneous adipose had no effect (188). These studies by Ohman, et al. provide further evidence that adipose tissue surrounding vessels can contribute to lesion development, possibly through “outside to inside” signaling mechanisms, though it remains to be determined if the AngII/AT1R axis activates these pathways.

1.5.4 Role of adipose in AAA formation

The Health in Men Study of more than 12,000 men found that both waist circumference and waist-to-hip ratio are positively associated with AAA size, after adjustment for other risk factors such as age, dyslipidemia, smoking, hypertension, coronary heart disease, and diabetes mellitus (93). Moreover, more severe aneurysms (those \geq 40mm compared to 30 mm) had an even greater association, suggesting that obesity may play a role in the severity as well as the development of AAA (93). Data from our laboratory corroborates findings that obesity promotes aneurysm formation (203) and furthermore that weight loss attenuates the adventitial expansion of formed AAAs in a model of AngII-induced AAAs in high-fat fed mice (202). Police, et al. demonstrated in both genetic (*ob/ob*) and high fat diet-induced obesity models that obesity increases AngII-induced AAA formation (203). Somewhat surprisingly, male C57BL/6 mice fed high fat diet for 4 months displayed similar aneurysm incidence to hypercholesterolemic LDLR^{-/-} mice. These results were attributed to the inflammatory nature of abdominal peri-aortic fat compared to thoracic abdominal fat, as AAAs develop specifically in the abdominal region where the

surrounding adipose secretes significantly more MCP-1. Additionally, macrophage accumulation was greater in the abdominal region of AngII-infused mice compared to saline-infused mice and the number of recruited macrophages to white adipose tissue was greater in mice that did develop AAAs compared to those that did not. These data suggest that in obese mice, AngII initiates macrophage infiltration into peri-aortic adipose surrounding regions associated with AAA formation. It is unclear, however, whether or not these effects are due to direct action of AngII at the adipocyte AT1aR.

Table 1.1 Summary of studies investigating the effects of AngII on adipocyte differentiation *in vitro*.

Publication	Adipocyte source	Effect of AngII treatment	Measurements	Receptors	Antagonist used	Additional information
Darimont 1994	Ob1771 (mouse)	Pro-adipogenic	GPDH activity	AT2R	PD123177	AngII elicits prostacyclin from mature adipocytes to promote differentiation of preadipocytes
Jones 1997	3T3-L1 (mouse)	Pro-adipogenic	TG, FAS and GPDH activity	AT2R*	P-186	*Losartan had similar effects to P-186
Crandall 1999	Human preadip.	Pro-adipogenic	Cyclin D expression	AT1R	Losartan	AngII increases cell cycle progression; PD123319 had no effect
Schling 2001	Human preadip.	Anti-adipogenic	GPDH activity	N/A	None	
Saint-Marc 2001	Rat preadip.	Pro-adipogenic	Number of GPDH-lipid positive cells	N/A	N/A	AngII effects blocked by aspirin and restored by additional prostacyclin treatment
Janke 2002	Human preadip.	Anti-adipogenic	Lipid content	AT1R	Irbesartan	PD123319 had no effect; neither antagonist was used in combination with AngII
Matsushita 2006	Human MSCs	Anti-adipogenic Pro-adipogenic	PPAR γ , FAS, Adiponectin	AT2R AT1R	PD123310 Valsartan	AngII+PD restored adipogenic markers to level of vehicle
Brucher 2007	Human preadip.	Anti-adipogenic	GPDH activity	AT1R	Losartan	Anti-adipogenic effect greater in preadipocytes from obese s

Table 1.1 (continued)

Publication	Adipocyte source	Effect of AngII treatment	Measurements	Receptors	Antagonist used	Additional information
Saiki 2008	3T3-L1 (mouse)	Anti-adipogenic	PPAR γ	AT1aR	Valsartan	AngII also transiently increase LPL activity
Sarzani 2008	Human preadip and adip	Pro-adipogenic	Cell number	AT1R	Valsartan	Cell number of mature and differentiated preadipocytes were measured in response to AngII
Fuentes 2010	Human preadip.	Anti-adipogenic	GPDH activity, PPAR γ	N/A	N/A	Mechanism involves ERK1/2 activation
Hung 2011	3T3-L1 (mouse)	N/A (Endogenous)	Lipid content	Both	Losartan PD123319	Losartan and PD reduced lipid content to the same extent

Table 1.1 Summary of genetic ablation models of RAS components reporting body weight regulation.

Publication	Model	Diet (weeks; beyond weaning)	Effect on body weight	Effect on fat mass	Effect on adipocyte size
Massiera 2001	AGT ^{-/-}	Chow High fat	Reduced Reduced	Reduced Reduced	N/A
Takahashi 2007	Renin (Ren1c ^{-/-})	Chow (8-20) Western (8-32)	Reduced Reduced	Reduced	Reduced
Jayasooriya 2008	ACE ^{-/-}	Chow	Reduced	Reduced	N/A
Kouyama 2005	AT1aR ^{-/-}	Chow (8) High fat (8)	No difference Reduced	No difference Reduced	No difference Reduced
Yamamoto 2011	AT1aR ^{-/-}	Chow (12) High fat (6)	Increased No difference	Increased N/A	Increased N/A
Daugherty 2004	AT1aR ^{-/-} x LDLR ^{-/-}	Western (12)	No difference	N/A	N/A
Tomono 2008	AT1aR ^{-/-} x ApoE ^{-/-}	Chow (20)	Increased	Reduced	Reduced
Yvan-Charvet 2005	AT2R ^{-/-}	Low fat (12) High fat (12)	No difference Reduced	No difference Reduced	Reduced Reduced
Iwai 2009	AT2R ^{-/-} x ApoE ^{-/-}	Chow (24) High Cholesterol (4)	No difference No difference	No difference Increased	No difference Increased

Table 1.2 Criteria for diagnosis of the metabolic syndrome from IDF and AHA/NHLBI Joint Scientific Statement

Defining parameter	Requisite Levels
Elevated waist circumference	Non-Europeans: men \geq 94 cm; women \geq 80 cm European origin: men \geq 102 cm; women \geq 88 cm
Elevated blood pressure*, mmHg	\geq 130/ \geq 85
Elevated fasting blood glucose*, mg/dL	\geq 110
Elevated triglycerides*, mg/dL	\geq 150
Reduced HDL cholesterol*, mg/dL	
Men	<40
Women	<50
<i>IDF, International Diabetes Federation; AHA, American Heart Association; NHLBI, National Heart, Lung, and Blood Institute. *Drug treatment for indicated parameter is an alternate indicator.</i>	

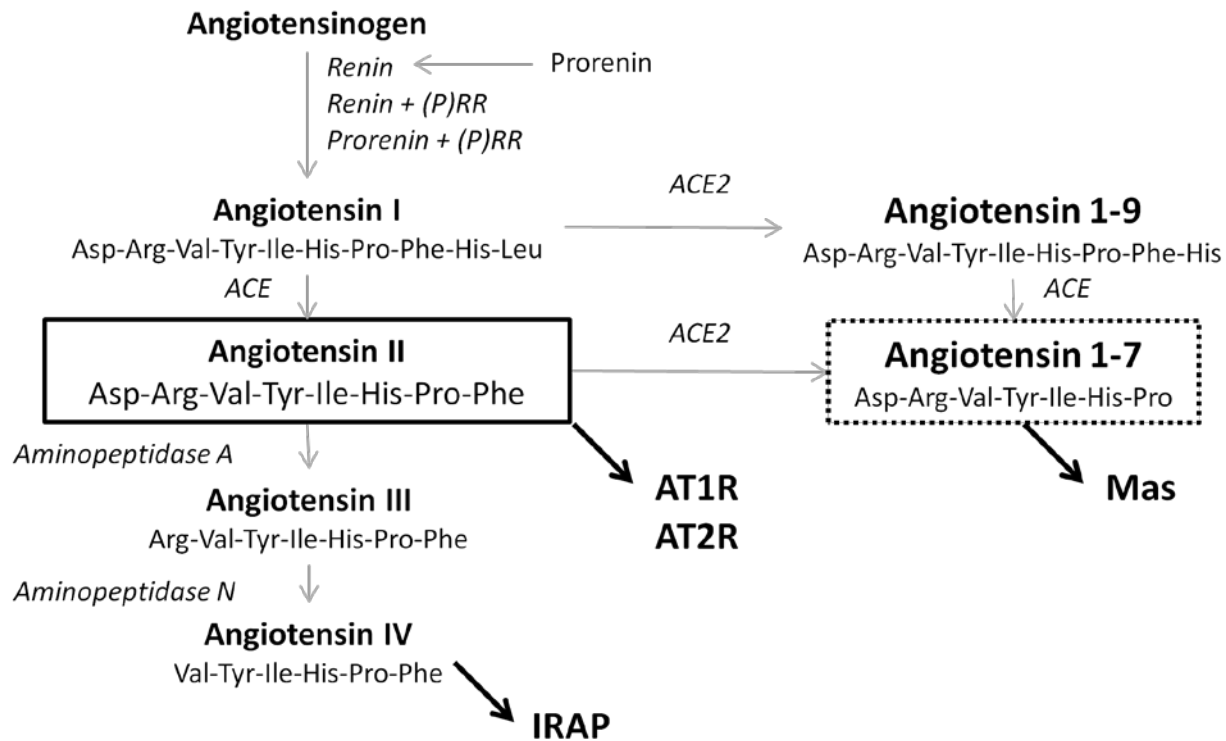


Figure 1.1 The renin-angiotensin system. AngII is formed from subsequent cleavage of angiotensinogen by renin and ACE. AngII can act through two receptors, AT1R or AT2R, while smaller fragments such as AngIV or Ang(1-7) can act through IRAP or the mas receptor, respectively.

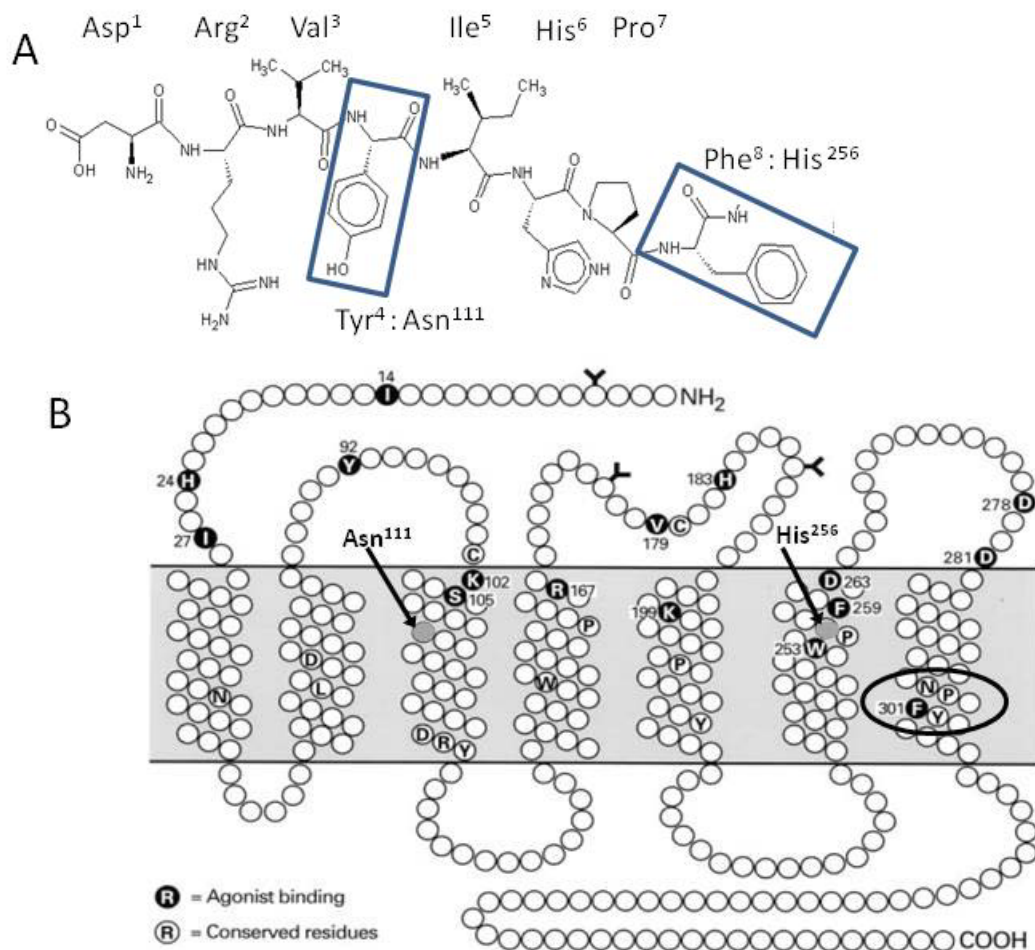


Figure 1.2 Structure of AngII and the AT1R. A, The linear amino acid structure of AngII. Boxed residues are involved in AT1R activation. B, Schematic of AT1R (adapted from de Gasparo, 2000). Labeled open circles are conserved residues, closed circles are involved in binding AngII, and the circled amino acids (298-302) are involved in G-protein activation. Receptor activation requires interactions between aromatic Tyr and Phe residues of AngII and the Asn¹¹¹ and His²⁵⁶ residues within the transmembrane binding pocket of the AT1R.

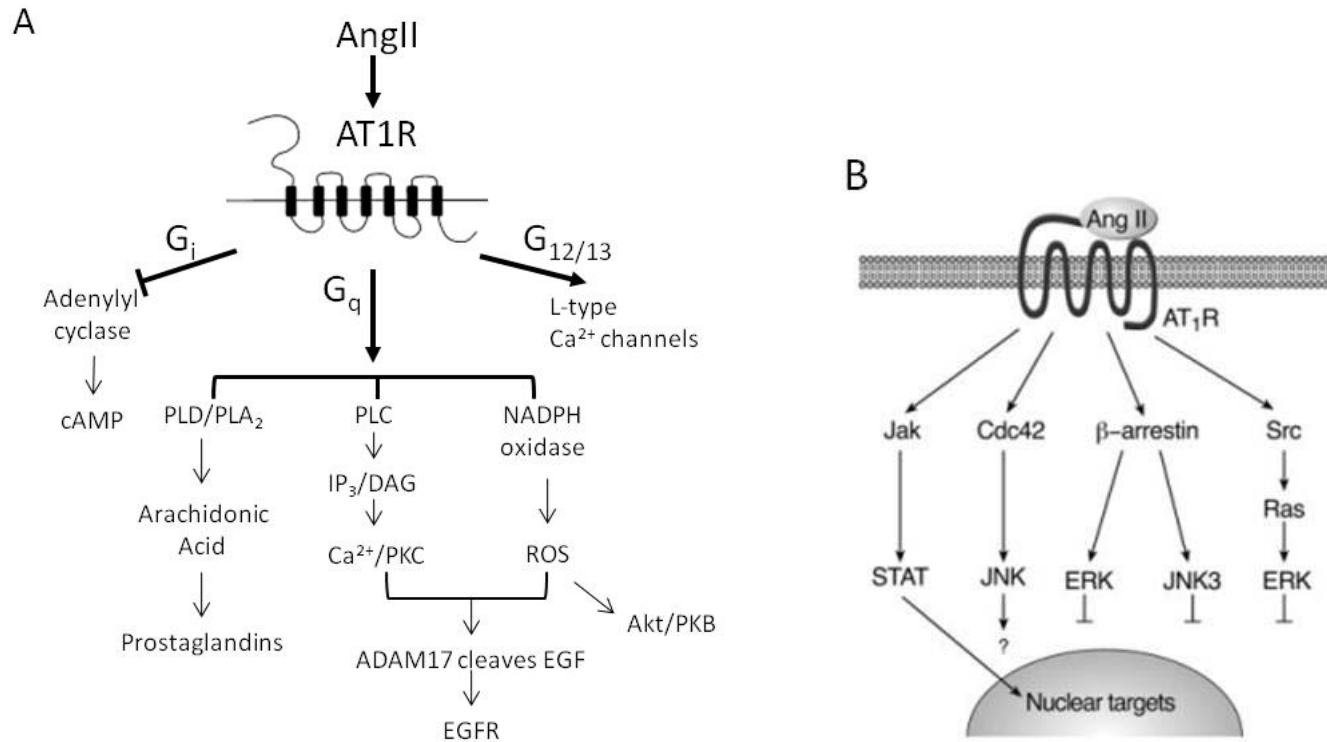
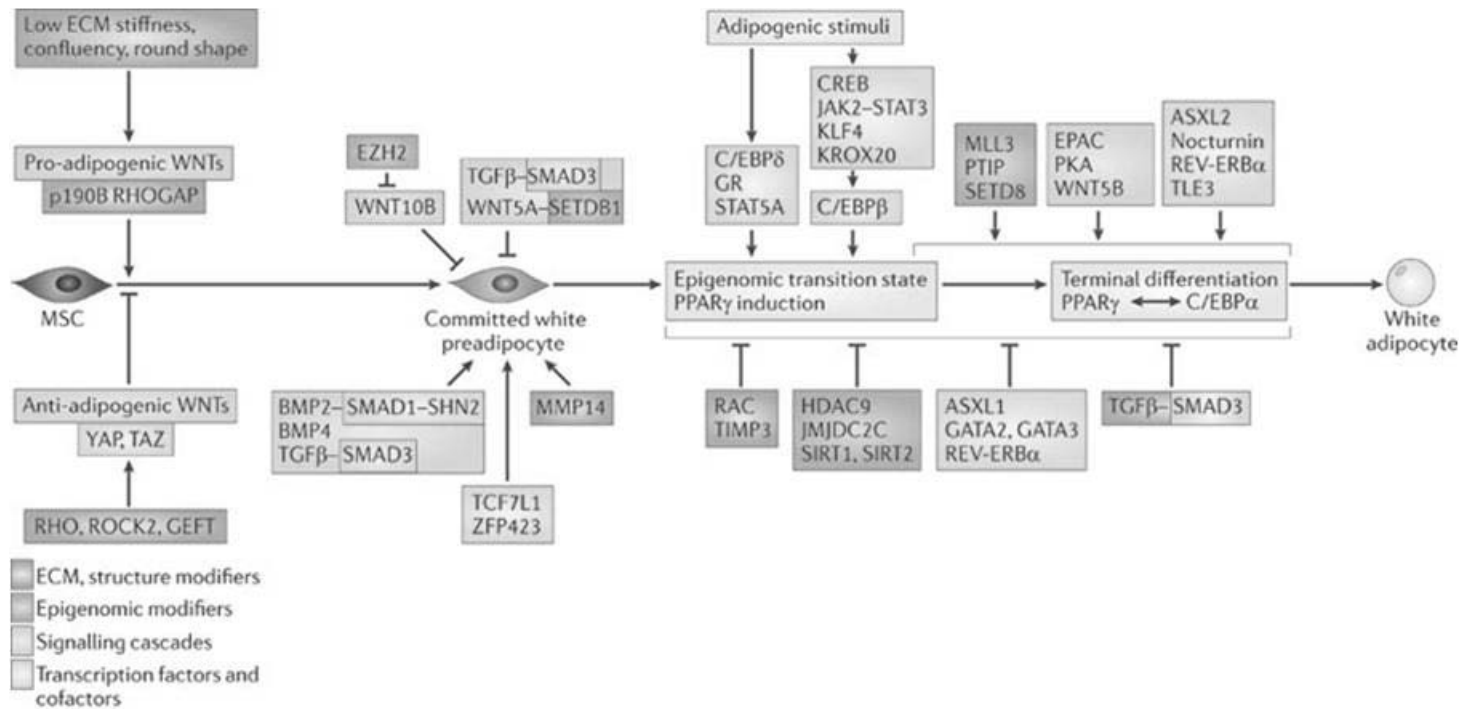


Figure 1.3 AT1R signaling pathways. A, G-protein dependent pathways, adapted from Dinh, et al. 2001. B, G-protein independent pathways, from Hunyady, et al. 2006.



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Figure 1.4 Regulation of adipocyte differentiation. Factors involved in the commitment of MSCs to the adipocyte lineage and the terminal differentiation of preadipocytes to mature adipocytes are shown; from Cristancho and Lazar 2011.

STATEMENT OF THE PROBLEM

The metabolic syndrome is a collection of risk factors that elevate an individual's risk for cardiovascular disease (Table 1.3). Obesity is a central component of the metabolic syndrome as well as an independent risk factor for cardiovascular disease. Understanding how obesity changes normal adipocyte physiology represents a major focus of current research aiming to ameliorate the burden of obesity-associated disease. Adipocytes express all major components of the renin-angiotensin system and are capable of both producing and responding to AngII, though the functional purpose of this system in adipose tissue is unclear. Adipose tissue is both a lipid storage and endocrine organ and AngII could potentially modulate several of its functions, such as regulation of adipogenesis, lipogenesis, lipolysis, insulin sensitivity, adipokine secretion, and production of other RAS components.

Investigations into the effects of AngII on adipocyte differentiation have yielded controversial results, leaving unanswered questions regarding whether AngII affects adipocyte differentiation and through which receptor subtype (Table 1.1). Further studies into the metabolic effects of AngII *in vivo* using murine models of whole-body angiotensinogen, renin, or ACE deficiency reveal that mice with reduced capacity to synthesize AngII exhibit protection from diet-induced obesity, improved glucose tolerance, and improved blood pressure when fed a high fat diet. Similar results were obtained from studies of AT1aR- or AT2R-deficient mice; however conflicting data have been reported. Paradoxically, chronic infusion of AngII into mice results in reduced body weight and adiposity,

similar to mice lacking major RAS components. In these studies, the mechanisms and tissues responsible for the observed effects remain unknown.

AngII also promotes vascular disease through the AT1aR, as mice deficient in this receptor are protected from hypercholesterolemia-induced atherosclerosis and AngII-induced abdominal aortic aneurysms (AAAs). Similar to the metabolic effects of AngII, however, it is unclear which tissues or cell types participate in these effects. Obesity is a well-established risk factor for cardiovascular disease and there is accumulating evidence for direct contributions of adipose tissue, specifically adipose surrounding blood vessels, in the development of these diseases. Our lab has demonstrated that obesity not only promotes AngII-induced AAA formation, but abdominal peri-aortic adipose tissue from the region susceptible to aneurysm formation secretes higher levels of chemokines compared to non-aneurysmal thoracic regions, implying there may be a direct effect of AngII on adipocytes to enhance AAA development.

The impact of adipocyte AT1aR activation on adipose tissue functions could have significant health consequences, especially in the context of obesity and its contribution to cardiovascular risk. The models used to study this receptor, however, fall short of identifying cell-specific mechanisms for the observed effects of AngII. To gain a better understanding of the physiologic and pathologic effects of AngII on adipocytes and their contributions to obesity and vascular disease, we created a model of adipocyte-AT1aR deficiency using the Cre/LoxP system. The overall hypothesis of this dissertation is that adipocyte AT1aRs mediate the development, function, and inflammatory profile of

adipocytes and thus contribute to the development of obesity-related disorders and vascular diseases. The following specific aims were designed to test this hypothesis:

Specific Aim 1: Determine the role of adipocyte AT1aRs on the development and function of adipose tissue.

- A.** Determine the effect of adipocyte-AT1aR deficiency on parameters of the metabolic syndrome in lean and obese mice.
- B.** Determine the effect of AngII and AT1aR deficiency on adipocyte differentiation using both 3T3-L1 and isolated stromal vascular cells from adipocyte-AT1aR deficient mice.

Specific Aim 2: Determine the role of the adipocyte AT1aR on the development of vascular disease.

- A.** Define temporal effects of high fat versus high fat/cholesterol diets on the systemic and adipose RAS in LDLR^{-/-} mice.
- B.** Determine the effect of adipocyte-AT1aR deficiency on the development of atherosclerosis in hypercholesterolemic LDLR^{-/-} mice fed Western diet for 3 months.
- C.** Determine the effect of adipocyte-AT1aR deficiency on atherosclerosis and AAAs induced by AngII infusion.

SECTION II. SPECIFIC AIM 1

Determine the role of the adipocyte AT1aR on the development and function of adipose tissue.

2.1 Summary

Adipocytes express angiotensin receptors, but the direct effects of angiotensin II (AngII) stimulating this cell type remain unclear. Adipocytes express angiotensin type 1a (AT1aR) and angiotensin type 2 receptors (AT2R), both of which have been implicated in obesity. In this study, we determined the effects of adipocyte AT1aR deficiency on adipocyte differentiation and the development of obesity in mice fed standard mouse diet, low (LF) or high fat (HF) diets. Mice expressing Cre recombinase under the control of the aP2 promoter were bred with AT1aR floxed mice to generate mice with adipocyte AT1aR deficiency (*AT1aR^{aP2}*). AT1aR mRNA abundance was reduced significantly in both white and brown adipose tissue from *AT1aR^{aP2}* mice compared to non-transgenic littermates (*AT1aR^{fl/fl}*). In mice fed standard mouse diet, there was no significant effect of adipocyte AT1aR deficiency on body weight, fat mass, or glucose tolerance in 12 month old mice. To stress the system, mice of each genotype were infused with saline or AngII for one month, after which modest adipocyte hypertrophy was observed in saline-infused *AT1aR^{aP2}* mice and this effect was significantly more pronounced in AngII-infused mice. To define the effect of adipocyte AT1aR on the development of obesity, mice of each genotype were fed LF or HF diets for 16 weeks. The mRNA abundance of other AngII receptors, AT2R or angiotensin type 1b (AT1bR), were unaffected by deficiency

of adipocyte AT1aRs. Additionally, angiotensinogen mRNA abundance in adipose tissue was not significantly affected by adipocyte AT1aR deficiency. Adipocyte AT1aR deficiency did not influence body weight, glucose tolerance, or blood pressure in mice fed either LF or HF diets. However, LF-fed *AT1aR^{aP2}* mice exhibited striking adipocyte hypertrophy even though total fat mass was not different between genotypes. Stromal vascular cells from *AT1aR^{aP2}* mice differentiated to a lesser extent to adipocytes compared to controls. Conversely, incubation of 3T3-L1 adipocytes with AngII increased Oil red O (ORO) staining and promoted mRNA abundance of PPAR γ that was antagonized by losartan. These results suggest that reductions in adipocyte differentiation in LF-fed *AT1aR^{aP2}* mice resulted in increased lipid storage and hypertrophy of remaining adipocytes. These results demonstrate that AngII regulates adipocyte differentiation and morphology through the adipocyte AT1aR in lean mice.

2.2 Introduction

The renin-angiotensin system (RAS) has well-established roles in fluid homeostasis, blood pressure regulation, and the development of various forms of cardiovascular diseases. Several cell types express components of the RAS (16) allowing for angiotensin II (AngII) to elicit endocrine, autocrine, and/or paracrine effects. Adipocytes express angiotensin receptors, with differences in angiotensin receptor subtype expression depending on the species and source of adipose tissue (44, 76, 297). In rodents, angiotensin type 1a receptors (AT1aR) and angiotensin type 2 receptors (AT2R) have been localized to adipocytes (59, 61), while angiotensin type 1b receptors (AT1bR) are not readily detectable in murine adipose tissue (36). The functional and/or pathophysiological role of angiotensin receptor subtypes in adipocytes is unclear.

One possible role of angiotensin receptors on adipocytes may be to regulate the production of angiotensinogen (AGT) from adipocytes, which are an important extrahepatic source of circulating AGT (21, 160-161). Increased AGT mRNA abundance in adipose tissue of AT2R deficient mice was reduced by an AT1R-antagonist, suggesting that AngII upregulates AGT expression through the AT1aR (154). Furthermore, AngII infusion dramatically increased AGT mRNA levels specifically in adipose tissue and not liver (154). It remains to be determined whether these results are due to direct effects of AngII at adipocyte AT1aRs or through indirect effects at other cell types.

Regulation of body weight through changes in fat mass represents another role of AngII, possibly through adipocyte receptors. Genetic ablation of

RAS components including angiotensinogen, renin, angiotensin converting enzyme (ACE), and AT1aR in mice results in reduced body weight due to reductions in fat mass when mice are fed standard or high fat (HF) diets (125, 144, 161, 253), although there are contrary data (65, 154). Even more perplexing, infusion of high doses of AngII can also result in weight loss and reduced adiposity (34, 37, 47, 68), making it difficult to define the role of AngII in the regulation of adipocyte growth and/or differentiation. These apparent contradictions have yet to be resolved and are complicated by direct versus indirect effects of AngII on adipose growth and development.

In studies aimed at examining direct effects of AngII to regulate adipocyte differentiation using clonal cell lines or *ex vivo* differentiation of preadipocytes, conflicting results have also been obtained. Several studies indicate that AngII enhances adipocyte differentiation and lipid accumulation (61, 130, 221), while others suggest that AngII inhibits adipocyte differentiation (85, 122, 220, 229). There are also conflicting reports regarding which of the AngII receptors are responsible for these effects, as investigators have reported that AngII stimulates adipogenesis through the AT1R or AT2R (61), while other studies have reported that AngII inhibits adipocyte differentiation (122). The use of AT1R antagonists with AT1R-independent effects on adipocyte differentiation (i.e., activation of peroxisome proliferator activated-receptor γ , PPAR γ (123, 231)) complicates interpretation of results from these studies.

Based on results from mouse models demonstrating that whole body deficiency of components of the RAS reduces body weight and fat mass, we

hypothesized that AngII promotes differentiation through direct effects at adipocyte AT1aR, which may have therapeutic implications in the development and/or treatment of obesity. To test this hypothesis, we generated mice with adipocyte deficiency of AT1aR. In HF-fed mice, deficiency of AT1aR in adipocytes had no effect on the development of obesity, glucose intolerance, and obesity-induced hypertension. Notably, LF-fed mice lacking AT1aR in adipocytes exhibited pronounced adipocyte hypertrophy, and stromal vascular cells (SVC) isolated from mice with adipocyte AT1aR deficiency exhibited reduced capacity to differentiate to adipocytes. These results suggest that AngII promotes adipocyte differentiation through direct effects at adipocyte AT1aR in lean mice.

2.3 Materials and Methods

2.3.1 Mice and diets

All experiments were conducted according to National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kentucky Institutional Animal Care and Use Committee. *AT1aR* floxed (*AT1aR^{fl/fl}*) mice (212) were crossed initially to FLPe mice (B6.SJL-Tg(ACTFLPe)9205Dym/J, The Jackson Laboratory, Bar Harbor, ME) to remove the neocassette and subsequently female *AT1aR^{fl/fl}* mice were bred to hemizygous transgenic male mice of Cre mice under control of an *aP2/promoter/enhancer.Cg-Tg (Fabp4-cre 1Rev/J*; The Jackson Laboratory, Bar Harbor, ME; Figure 2.1A). For all studies, male and female *AT1aR^{fl/fl}* littermates were used for comparison to mice with adipocyte *AT1aR* deficiency. Male mice of each genotype were fed standard murine diet (18% kcal from fat; Harlan Laboratories, Inc., Indianapolis, IN; Table 2.1) ad libitum through 12 months of age and subsequently infused with either saline or AngII for 1 month. In separate studies, male mice (8-10 weeks of age) of each genotype were fed either LF (10% kcal as fat; D12450B; Research Diets, New Brunswick, NJ) or HF diets (60% kcal as fat; D12492; Research Diets) ad libitum for 16 weeks with free access to water. *AT1aR^{aP2}* mice were bred to ROSA26 mice (B6.129S4-Gt(ROSA)26Sor<tm1Sor>/J, The Jackson Laboratory, Bar Harbor, ME) and β -galactosidase activity was measured to confirm lineage expression of Cre recombinase in adipose tissue. Briefly, adipose tissues collected from *aP2-Cre* null (*Cre 0/0*) and *aP2-Cre* positive (*Cre+ /0*) *AT1aR^{fl/+}* mice heterozygous for the

ROSA26 allele were sectioned and stained overnight with X-gal. Blue staining indicated successful removal of a stop codon from the β -galactosidase gene by Cre recombinase.

2.3.2 *In vivo* measurements

2.3.2.1 Standard murine diet with AngII infusion study

For mice fed standard mouse diet, body weights were recorded every other week beginning at 6 months of age. Body composition was measured at 6, 9, 12, and 13 months of age. Glucose tolerance test was performed as described above at 12 months of age. At 13 months of age, osmotic mini-pumps (Model 2004, DURECT Corporation, Cupertino, CA) filled with either saline or AngII (delivered at 1,000 ng/kg/min; Sigma, St. Louis, MO) were implanted subcutaneously under isoflurane anesthesia. Mice were sacrificed at the end of the 28-day infusion after a 4-hour fast. Blood pressure was measured by tail-cuff (Visitech Systems, Inc., Apex, NC) prior to and 3 weeks after pump implantation.

2.3.2.2 LF and HF diets study

Body weights were recorded weekly for all mice. The body composition of a subset of mice was analyzed by NMR spectroscopy (EchoMRI®) before mice began the LF or HF diets and after 14 weeks on diet. Glucose tolerance tests were performed after 8 and 15 weeks on diet. Mice were fasted 6 hours and blood glucose measurements were measured at 0 min (before injection of glucose solution) and at 15, 30, 60, and 120 minutes after i.p. injection of glucose (1 g/kg body weight). After 14 weeks on diet, insulin tolerance tests were

performed after a 4-hour fast. Insulin was administered at a dose of 0.5 U/kg body weight via i.p. injection and blood glucose was measured at 0 min (before injection of insulin) and at 30, 60, and 120 minutes after insulin injection. Blood pressure was measured by telemetry for 3-days. After 15 weeks on diet, telemetry implants (model TA11PA-C10, Data Sciences International, St. Paul, MN) were surgically inserted as described previously (100). After 16 weeks on diet, baseline blood pressures were recorded for 3 consecutive day and night periods. Mice were excluded if their mean pulse pressure was below 17 mmHg, as an indication of a poor signal from the telemeter to the receiver.

2.3.3 Plasma measurements

Mice were terminated after a 4 hour fast. Plasma renin concentrations were measured by incubating plasma (8 μ l) with exogenous angiotensinogen (25 nM) in the presence of ACE inhibitors and then angiotensin I (AngI) was quantified by radioimmunoassay (DiaSorin, Via Crescentino, Italy). Plasma insulin concentrations were quantified with the Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL) and plasma leptin concentrations were quantified with a Mouse Leptin ELISA kit (Millipore). Non-esterified fatty acids (NEFAs) were quantified with the NEFA-HR(2) kit (Wako Diagnostics, Richmond, VA). Plasma AGT concentrations were determined with the Mouse Total Angiotensinogen Assay Kit (Immuno-Biological Laboratories Co., Ltd., Japan).

2.3.4 Quantification of mRNA abundance

To quantify mRNA abundance, RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI). Reverse transcription was performed on RNA (0.4 µg) using qScript cDNA SuperMix as per manufacturer's instructions (Quanta Biosciences, Gaithersburg, MD). Real-time PCR was performed with PerfeCTa SYBR Green FastMix for iQ on 2ng of cDNA template (Quanta Biosciences, Gaithersburg, MD). A standard curve was generated from a series of ten-fold dilutions of cDNA with each real-time PCR plate and this was used to extrapolate the relative starting quantity of mRNA for the gene of interest from the given Ct values. Data are expressed as the ratio of the gene of interest starting quantity to that of 18S.

2.3.5 Differentiation of preadipocytes from stromal vascular cells (SVC).

Subcutaneous adipose tissue was dissected from the inguinal region, minced, and incubated in Basal Medium (OM-BM, Zenbio, Research Triangle Park, NC) supplemented with collagenase (1 mg/mL) and penicillin/streptomycin mixture (5%) for at least 1 hour with shaking at 37 °C as described previously (215). Two days after cells had achieved 100% confluency, media were changed to Differentiation Medium (OM-DM, Zenbio, Research Triangle Park, NC) and replaced every other day for 8 days. Cells were either harvested for RNA using TRIzol or fixed for Oil Red O (ORO) staining. For ORO measurements, cells were fixed in formalin (10%) and stained in filtered ORO (0.3%) solution for 30 minutes at room temperature. For quantification,

isopropanol (1 ml) was added to the plates to extract ORO stain, this solution was transferred to a microtiter plate, and absorbance was measured at 510 nm.

2.3.6 3T3-L1 adipocytes

3T3-L1 adipocytes were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM containing FBS (10%) and penicillin/streptomycin mixture (5%). Two days after cells were 100% confluent, differentiation of preadipocytes was initiated by administration of a cocktail containing insulin (0.1 μ M, Sigma-Aldrich, St. Louis, MO), dexamethasone (1 μ M; Sigma-Aldrich, St. Louis, MO), and isobutyl methyl xanthine (0.5 mM; Sigma-Aldrich, St. Louis, MO). Incubation with AngII (1 μ M, Sigma-Aldrich, St. Louis, MO) or losartan (1 μ M) was performed with fresh media containing drugs replaced every other day. After 6 days of differentiation cells were harvested for RNA isolation using TRIzol reagent (Invitrogen, Carlsbad, CA) or for quantification of ORO staining as described above.

2.3.7 Statistical analyses

Data were analyzed by ANOVA for comparisons between the 4 diet/genotype groups, as appropriate, using the Holm Sidak test for post-hoc analysis. When time was an additional variable, data were analyzed by repeated measures ANOVA. Data are represented as mean \pm SEM. If normality or equal variance tests failed, simple transforms were performed or the non-parametric Kruskal-Wallis test was used with Dunn's post-hoc analysis. Statistical significance was defined as $P < 0.05$.

2.4 Results

2.4.1 Generation and characterization of mice with adipocyte AT1aR deficiency

To confirm effective and specific deletion of exon 3 of AT1aR in adipocytes, AT1aR mRNA abundance was quantified in adipose tissues, liver, brain, heart, and kidney from mice fed standard laboratory diet (2 months of age). AT1aR mRNA abundances were not significantly different in liver, kidney, or brains from *AT1aR^{f/f}* compared to *AT1aR^{aP2}* mice (Figure 2.1B). In heart, AT1aR mRNA abundance was reduced modestly, but significantly, in *AT1aR^{aP2}* compared to *AT1aR^{f/f}* mice. In interscapular brown (BAT) and epididymal white (WAT) adipose tissue, AT1aR mRNA abundance was decreased significantly in *AT1aR^{aP2}* compared to *AT1aR^{f/f}* mice. Moreover, positive β -galactosidase staining was present in subcutaneous white and interscapular brown adipose tissues of Cre +/0 mice (Figure 2.1C).

Deficiency of AT1aR in adipocytes had no significant effect on body weight (Figure 2.2A), body composition (Figure 2.2B), or glucose tolerance (Figure 2.2C), quantified prior to infusions of saline or AngII in *AT1aR^{f/f}* and *AT1aR^{aP2}* mice. To stress the system, mice of each genotype were infused with saline or AngII for 1 month. Systolic blood pressure increased significantly in AngII-infused mice of each genotype, and was not significantly different between genotypes (Table 2.2). As anticipated in response to infusion of AngII, plasma renin concentrations decreased significantly in AngII-infused mice compared to saline-infused mice, with no differences between genotypes (Table 2.2). Infusion

of AngII had no significant effect on plasma NEFA concentrations, which were not significantly different between genotypes (Table 2.2). The mean adipocyte size in *AT1aR^{aP2}* mice infused with saline ($3890 \pm 157 \mu\text{m}^2$) was increased modestly, but not significantly, compared to *AT1aR^{fl/fl}* mice ($3365 \pm 117 \mu\text{m}^2$; $P = 0.052$, Figure 2.3G). Infusions of AngII resulted in a modest reduction in body weight and fat mass in mice of each genotype (Figure 2.3A,B), with no significant differences between genotypes. Interestingly, AngII infusion resulted in a striking reduction in adipocyte size in *AT1aR^{fl/fl}* mice ($2167 \pm 209 \mu\text{m}^2$; $P < 0.001$) compared to saline (Figure 2.3D versus 2.3C), but this effect was not apparent in *AT1aR^{aP2}* mice ($3514 \pm 183 \mu\text{m}^2$; Figure 2.3D). However, the increased adipocyte size in AngII-infused *AT1aR^{aP2}* mice compared to AngII-infused *AT1aR^{fl/fl}* controls ($P < 0.001$) was not associated with any differences in body weight, fat mass, or plasma NEFA levels (Table 2.2).

2.4.2 Adipocyte-AT1aR deficiency had no effect on the development of obesity or obesity-associated parameters

To define effects of adipocyte AT1aR deficiency on development of obesity, mice of each genotype were fed a LF or HF diet for 16 weeks. HF-fed mice of each genotype had significantly increased body weight and fat mass compared to LF-fed controls (Figure 2.4A, B). However, adipocyte AT1aR deficiency had no significant effect on body weight (Figure 2.4A) or fat/lean mass (Figure 2.4B) in either LF or HF-fed mice. While the mass of retroperitoneal (RPF) and epididymal (EF) adipose tissues were significantly increased by HF feeding, there was no significant effect of genotype in either diet group (Table

2.3). Glucose tolerance was significantly impaired in HF-fed mice of each genotype compared to LF-fed controls (Figure 2.4C,D). However, adipocyte AT1aR deficiency had no significant effect on glucose tolerance in either LF or HF-fed mice. Similarly, while HF feeding significantly impaired insulin tolerance tests in both genotypes, there was no significant difference in insulin tolerance between LF or HF *AT1aR^{aP2}* mice compared to *AT1aR^{fl/fl}* controls on respective diets (Figure 2.4E). Plasma insulin and leptin concentrations were increased significantly by HF-feeding in mice of each genotype, but there were no significant differences between genotypes in either diet group (Table 2.3). Plasma concentrations of non-esterified fatty acids (NEFAs) were not significantly influenced by diet or genotype (Table 2.3).

Systolic blood pressures (SBPs) during the day and night cycle were significantly increased in HF-fed mice of each genotype compared to LF-fed controls (Figure 2.4F; $P < 0.05$). However, deficiency of AT1aR in adipocytes had no significant effect on SBP in either LF or HF-fed *AT1aR^{aP2}* mice compared to *AT1aR^{fl/fl}* controls.

2.4.3 Adipocyte-AT1aR deficiency resulted in striking adipocyte hypertrophy in lean mice.

Since adipocytes also express AT2Rs, which have been proposed to contribute to obesity development in mice (289), we quantified mRNA abundance of AT2R and AT1bR in adipose tissue from mice of each genotype. In *AT1aR^{fl/fl}* controls, AT2R mRNA abundance was increased significantly in visceral adipose tissue from HF-fed mice compared to LF-fed controls (Figure 2.5A). However,

there was no significant effect of AT1aR deficiency in adipocytes on AT2R mRNA abundance in either visceral or subcutaneous adipose tissues of LF or HF-fed mice. Similarly, AT1b mRNA abundance was not significantly influenced by diet or genotype in visceral or subcutaneous adipose tissue (Figure 2.5B).

Quantification of AGT mRNA abundance in adipose depots revealed that AGT expression was suppressed by HF-feeding in both visceral and subcutaneous adipose depots (Figure 2.6A; $P < 0.05$), and adipocyte-AT1aR deficiency had no effect on AGT expression in liver or adipose. Similarly, there were no differences between genotypes in plasma AGT concentrations, although HF diet increased plasma AGT (Figure 2.6B; $P < 0.05$).

We quantified adipose morphology in LF or HF-fed mice of each genotype. In visceral retroperitoneal adipose tissue from LF-fed mice, adipocyte AT1aR deficiency resulted in a greater number of large adipocytes (Figure 2.7A-E). Mean adipocyte size in LF-fed $AT1aR^{aP2}$ mice ($2925 \pm 361 \mu\text{m}^2$) was increased significantly compared to adipocyte size in $AT1aR^{fl/fl}$ controls ($1328 \pm 146 \mu\text{m}^2$, $P < 0.05$; Figure 2.7E). Increases in adipocyte size in LF-fed $AT1aR^{aP2}$ mice resulted in a significant reduction in the number of adipocytes within a measurement frame compared to cell numbers in sections from LF-fed $AT1aR^{fl/fl}$ controls (Figure 2.7F). With HF feeding, the number of large adipocytes increased in both genotypes, but there were no significant differences between genotypes (Figure 2.7A-E). Mean adipocyte size increased in HF-fed mice of each genotype compared to LF-fed controls (HF, $AT1aR^{fl/fl}$, $2923 \pm 447 \mu\text{m}^2$; $AT1aR^{aP2}$, $3407 \pm 432 \mu\text{m}^2$; $P < 0.05$, Figure 2.7E), but the number of adipocytes

within a measurement frame was not different between genotypes (Figure 2.7F). Notably, the size of adipocytes from LF-fed *AT1aR^{aP2}* mice was similar to adipocyte sizes in HF-fed *AT1aR^{fl/fl}* controls. However, despite the increase in adipocyte size, adipose tissue mass was not significantly increased in LF-fed *AT1aR^{aP2}* mice compared to *AT1aR^{fl/fl}* controls (Table 2.3).

2.4.4 Deficiency of AT1aR in adipocytes decreased differentiation of SVCs to adipocytes, while AngII promoted differentiation of 3T3-L1 adipocytes

Increases in size of adipocytes from LF-fed mice with adipocyte AT1aR deficiency could result from reductions in lipolysis, alterations in lipid synthesis and/or uptake, or from decreased capacity of preadipocytes to differentiate to adipocytes. Plasma NEFA were not different between LF-fed mice of each genotype, suggesting that lipolysis was not influenced by adipocyte AT1aR deficiency. We examined the ability of preadipocytes within SVCs isolated from mice of each genotype to differentiate into mature adipocytes. AT1aR mRNA abundance was significantly decreased in adipocytes (day 8) differentiated from SVCs of *AT1aR^{aP2}* mice compared to *AT1aR^{fl/fl}* controls (*AT1aR^{fl/fl}*, 0.30 ± 0.02 ; *AT1aR^{aP2}*, 0.16 ± 0.01 AT1aR/18S RNA ratio; $P < 0.001$). Deficiency of AT1aR resulted in significantly reduced ORO staining on day 8 of differentiation compared to SVCs differentiated from *AT1aR^{fl/fl}* controls (Figure 2.8A; $P < 0.0001$). In addition, mRNA abundance of PPAR γ was significantly decreased in adipocytes differentiated from *AT1aR^{aP2}* mice compared to *AT1aR^{fl/fl}* controls (Figure 2.8B; $P < 0.05$). Reductions in ORO staining occurred in the absence of changes in mRNA abundance of either fatty acid synthase or CD36 (Figure

2.8C,D, respectively). Conversely, incubation of 3T3-L1 preadipocytes with AngII throughout the differentiation protocol significantly increased ORO staining and mRNA abundance of PPAR γ in mature adipocytes (Figure 2.8E,F, respectively; $P < 0.05$). AngII-mediated increases in PPAR γ mRNA abundance were abolished when 3T3-L1 adipocytes were incubated with the AT1R antagonist, losartan (Figure 2.8F). However, incubation of preadipocytes with losartan in the absence of AngII had no significant effect on PPAR γ mRNA abundance in differentiated adipocytes.

2.5 Discussion

Results from this study demonstrate that adipocyte AT1aR deficiency promotes the development of adipocyte hypertrophy in mice fed either standard mouse diet and infused with AngII or in mice fed a LF diet. Surprisingly, the phenotype of adipocyte AT1aR deficiency to increase adipocyte size was only apparent in lean mice (LF-fed or AngII-infused). The size of adipocytes in LF-fed mice *AT1aR^{aP2}* mice was similar to the size of adipocytes in non-transgenic littermate mice fed a HF diet. However, despite increases in adipocyte size in *AT1aR^{aP2}* mice, fat mass was similar in mice of each genotype fed standard mouse diet or the LF diet. Thus, adipocyte hypertrophy in lean *AT1aR^{aP2}* mice, in the absence of increases in total adipose tissue mass, was insufficient to promote differences in body weight, glucose tolerance or blood pressure. Preadipocytes from adipocyte-AT1aR deficient mice demonstrated reduced capacity to differentiate to adipocytes, suggesting that the increased adipocyte size in lean *AT1aR^{aP2}* mice occurred as a consequence of increased lipid storage in a reduced number of adipocytes. Conversely, incubation of 3T3-L1 adipocytes with AngII promoted ORO staining and PPAR γ expression in differentiating adipocytes, supporting a role for AngII to promote adipocyte differentiation. Surprisingly, the effects of adipocyte AT1aR deficiency to increase adipocyte cell size in LF-fed mice were not observed when mice were fed a HF diet. These results suggest that adipocyte AT1aR regulate adipocyte differentiation under lean, but not obese conditions.

Adipose tissue expresses several components of the RAS necessary to produce and respond to AngII. Results from this study confirm previous reports that murine adipocytes express both AT1aR and AT2R, with low levels of AT1bR (36, 61). Adipocyte AT1Rs have been suggested to regulate expression of RAS components in adipocytes, adipocyte differentiation or growth, or adipocyte glucose uptake or metabolism (61, 133, 154). Murine models of global genetic AT1aR deletion and systemic administration of angiotensin receptor blockers (ARBs) to rodents have been previously used to study effects of this receptor on the development of obesity (144, 175, 296). Specifically, whole body deficiency of angiotensinogen, renin, or ACE in mice fed standard mouse diet resulted in reduced body weight, fat mass, glucose intolerance, and decreased blood pressure (125, 161, 253). In addition, whole body deficiency of angiotensinogen, ACE, AT1aR or AT2R reduced the development of obesity in mice fed a HF diet (144, 161, 206). Conversely, previous results from our laboratories demonstrated that deficiency of AT1aR or AT2R in low density lipoprotein receptor deficient mice fed a HF diet had no effect on body weight (65). These conflicting data are confounded by an inability to define the cell type(s) responsible for effects of whole body deficiency of individual RAS components. To address the direct role of AngII effects at adipocyte AT1aR, we created mice with deficiency of AT1aR in adipocytes, and demonstrate a lack of effect on body weight or fat mass when mice are fed a LF or HF diet. These results demonstrate that previously observed effects of whole body AT1aR deficiency to decrease the development of obesity (144) result from reduced effects of AngII at

other cell types. Additionally, it is possible that improvements in glucose tolerance and blood pressure observed in mice with whole body AT1aR deficiency (144) are secondary to the previously observed leaner phenotype of these animals, as adipocyte AT1aR deficiency had no effect on these obesity-associated parameters.

Since whole body AT2R deficiency has also been reported to reduce the development of obesity (289), albeit with conflicting results (65) a possible explanation for the lack of effect of adipocyte-AT1aR deficiency on the development of obesity may be compensation by this receptor in adipocytes. In the present study, quantification of mRNA abundance of AT1bR and AT2R from whole adipose tissue lysates did not reveal transcriptional upregulation of either receptor to compensate for the loss of adipocyte AT1aRs. Thus, it is unlikely that effects of adipocyte AT1aR deficiency were masked by compensation through AngII effects at other receptor subtypes.

Adipose AGT production has been shown to be regulated by AngII through the AT1aR (154); however, the results of this study demonstrate that these effects are independent of direct actions at adipocyte AT1aRs. Consistent with other reports, HF diet reduced AGT mRNA abundance in both visceral and subcutaneous adipose tissues (191) while plasma AGT levels were actually increased (287). This apparent discrepancy is likely due to increased overall AGT production due to the dramatic increase in fat mass resulting from HF diet despite downregulation of gene transcription (283). Increased production of AGT by other tissues is also possible in the setting of obesity.

A surprising finding from this study was that *AT1aR^{aP2}* mice have large adipocytes when fed a LF diet or when fed standard diet and infused with AngII. Quantification of mean adipocyte size indicated that the magnitude of adipocyte hypertrophy in LF-fed *AT1aR^{aP2}* mice was similar to the hypertrophy observed in HF-fed control mice. Also, *AT1aR^{aP2}* mice exhibited adipocyte hypertrophy compared to *AT1aR^{f/f}* controls when infused with AngII. It is unclear why infusion of AngII was required to bring forth significant differences in adipocyte size in chow-fed mice, though this is likely related to the modest weight loss experienced by AngII-infused mice, suggesting the phenotypes of adipocyte-AT1aR deficient mice are most prominent when mice are leaner.

Despite these striking increases in adipocyte size, there were no differences in fat mass between genotypes on either chow or LF diets. The finding of similar fat masses in lean mice of each genotype, despite marked adipocyte hypertrophy in *AT1aR^{aP2}* mice, suggests that a smaller number of adipocytes in LF *AT1aR^{aP2}* mice accumulated more lipid to maintain adipose mass at a similar level between genotypes (13). In accordance with the lack of an effect on fat mass, there was no evidence of glucose intolerance, and no changes in plasma insulin or leptin concentrations in LF-fed *AT1aR^{aP2}* mice, signifying that in lean mice adipocyte hypertrophy alone is insufficient to alter levels of these proteins associated with an obese phenotype.

Adipocyte hypertrophy in lean mice with adipocyte AT1aR deficiency could result from several mechanisms, including reduced lipolysis, reduced numbers of adipocytes (with remaining adipocytes filling up with lipid), or increased lipid

synthesis or uptake in resident adipocytes. Since plasma concentrations of NEFA were not different in LF-fed mice of either genotype, lipolysis was most likely not influenced by AT1aR deficiency in adipocytes. The literature on AngII regulation of lipolysis is conflicting, with some reports demonstrating minimal effects of AngII on adipose tissue lipolysis in humans (264), as compared to reductions in lipolysis of human adipose tissue in response to AngII in normal weight (31) and obese subjects (95). In this study, mRNA abundance of fatty acid synthase and CD36 were not altered in AT1aR deficient adipocytes, suggesting that reduced synthesis and/or uptake of lipids most likely did not contribute to differences in Oil Red O staining. Others have reported that AngII induces fatty acid synthase expression in 3T3-L1 adipocytes, however it was unclear whether these effects were through AT1aR or AT2R (130) and little has been reported regarding regulation of CD36 by AngII in adipocytes.

Since results did not support changes in lipid uptake, mobilization and/or synthesis in adipose tissue from LF-fed mice with adipocyte AT1aR deficiency, we focused on adipocyte differentiation as a potential mechanism contributing to hypertrophy of remaining adipocytes. Indeed, preadipocytes isolated from mice lacking adipocyte AT1aR differentiated *ex vivo* had reduced lipid accumulation and PPAR γ mRNA abundance, indicating a reduced capacity for differentiation. Results from this study conflict with those of Kouyama, et al. who examined mouse embryonic fibroblasts (MEFs) isolated from wild type and whole body AT1aR deficient mice and found no difference in their ability to differentiate into adipocytes (144). Differences between results from these studies may reflect the

differences between culture systems (i.e., preadipocytes from the SVF compared to MEFs). In addition, results from this study demonstrate that previously observed protection against the development of obesity in whole body AT1aR deficient mice occurred independent of adipocyte AT1aR.

Notably, we observed no differences in adipocyte morphology in HF-fed *AT1aR^{aP2}* mice which may be due to the suppression of adipocyte differentiation that other groups have found occurs in the setting of obesity or prolonged HF feeding (101, 116, 127, 197), however conflicting reports highlight the complex dynamics of adipocyte cell numbers and turnover in obesity (15, 222, 243). Interestingly, some suggest that adipocyte hypertrophy in HF-fed humans and rodents is a result of reduced adipogenesis and that the remaining cells accumulate more lipid over time (13). Our results support this conclusion that reduced adipocyte differentiation results in adipocyte differentiation in lean mice. In HF-fed mice, however, the inhibition of adipocyte differentiation occurs as a result of an inability to suppress canonical anti-adipogenic WNT signaling (101). WNT signaling is an important determinant of mesenchymal stem cell commitment to the adipocyte lineage and appears to precede the regulatory effects of AngII on adipocyte differentiation since no changes in adipocyte size in HF-fed *AT1aR^{aP2}* mice were observed.

Our results demonstrate that AngII promotes differentiation of murine 3T3-L1 adipocytes in an AT1R-dependent manner. Several *in vitro* experiments using adipocyte cell lines have implicated a role for AngII in lipid accumulation and differentiation of adipocytes, with conflicting results. Initial studies from

Darimont, et al. demonstrated that AngII promoted adipocyte differentiation of Ob1771 mouse adipocytes by eliciting PGI₂ from mature adipocytes via AT2Rs (61). In another study, AngII increased glycerol-3-phosphate dehydrogenase (GPDH)-positive, lipid-containing cells in mouse adipose tissue explants, though the role of specific AngII receptors were not investigated (221). A pro-adipogenic effect of AngII was confirmed in 3T3-L1 and human adipocyte primary cells where AngII increased triglyceride content, fatty acid synthase activity, GPDH activity, and lipid accumulation, though in these studies both AT1R and AT2R antagonists abolished AngII-induced effects (114, 130). Other reports, however, indicate that AngII decreased lipid content as well as PPAR γ and fatty acid synthase in 3T3-L1 and isolated human adipocytes (85, 122, 220). While discrepancies in the literature may be due to the variety of culture systems, sources of adipocytes, differentiation cocktails, and experiment durations used by each group, this field of research is also complicated by the AT1R-independent stimulation of PPAR γ by some AT1R antagonists (22, 216, 231). For example, Wistar Kyoto rats administered candesartan exhibited increased expression of PPAR γ expression in adipose tissue and consequently had a larger number of small adipocytes (296). Similar results have been reported with telmisartan and irbesartan (123, 175, 242). It would be interesting in future studies to examine effects of AT1R antagonists with PPAR γ -stimulating properties in AT1aR deficient adipocytes to better define the AT1R-independent effect of this class of compounds.

In conclusion, adipocyte AT1aR deficiency had no effect on development of obesity or obesity-induced hypertension and dysregulated glucose homeostasis. However, in lean mice, deficiency of AT1aR in adipocytes promoted striking adipocyte hypertrophy, without the negative consequences typically associated with this phenotype. Mechanisms for effects of adipocyte AT1aR deficiency include reductions in differentiation of preadipocytes to mature adipocytes, resulting in increased lipid accumulation across a smaller number of adipocytes in LF-fed mice. Conversely, AngII promoted the differentiation of 3T3-L1 adipocytes through an AT1R-dependent mechanism. These results demonstrate that AngII acts at adipocyte AT1aR to regulate adipose tissue growth in lean mice, which may have implications in diseases associated with cachexia.

Table 2.1 Composition of diets.

	Standard chow	Low Fat	High Fat
Source	Harlan Teklad Global 18% Protein Rodent Diet, 2018	Research Diets, Inc. D12450B	Research Diets, Inc. D12492
Kcal/g	3.1	3.85	5.24
Protein (% of total kcal)	24	20	20
Carbohydrate (% of total kcal)	58	70	20
Fat (% of total kcal)	18	10	60
Fat sources	Soybean oil	Soybean oil (55% of fat kcal), lard (45% of fat kcal)	Lard (91% of fat kcal), soybean oil (9% of fat kcal)

Table 2.2 Effects of adipocyte AT1aR deficiency on fat mass and plasma measurements in mice fed standard murine diet.

	Saline		AngII	
	<i>AT1aR^{+/+}</i>	<i>AT1aR^{ap2}</i>	<i>AT1aR^{+/+}</i>	<i>AT1aR^{ap2}</i>
EF mass (g)	1.68 ± 0.4	1.72 ± 0.4	1.25 ± 0.2	1.344 ± 0.1
RPF mass (g)	0.40 ± 0.08	0.48 ± 0.08	0.40 ± 0.07	0.48 ± 0.04
Final systolic blood pressure (mmHg)	105 ± 2	104 ± 0.9	126 ± 5*	123 ± 5*
Plasma renin (AngI ng/mL)	2.00 ± 0.93	2.44 ± 0.70	0.084 ± 0.05*	0.30 ± 0.35*
Plasma NEFAs (mEq/L)	0.036 ± 0.003	0.04 ± 0.005	0.041 ± 0.003	0.037 ± 0.002

Values are means ± SEM (n = 4-5 per group). Effect of AngII, *P < 0.05.

Table 2.3 Effects of adipocyte AT1aR deficiency on fat mass and plasma measurements in LF- and HF-fed mice.

	LF		HF	
	<i>AT1aR^{fl/fl}</i>	<i>AT1aR^{aP2}</i>	<i>AT1aR^{fl/fl}</i>	<i>AT1aR^{aP2}</i>
EF mass (g)	1.01 ± 0.18	1.07 ± 0.21	1.66 ± 0.07*	1.54 ± 0.06*
RPF mass (g)	0.40 ± 0.09	0.39 ± 0.08	1.46 ± 0.06*	1.46 ± 0.13*
Plasma leptin (ng/mL)	11.8 ± 2.2	13.3 ± 2.8	56.0 ± 1.5*	49.5 ± 3.5*
Plasma insulin (ng/mL)	0.34 ± 0.04	0.55 ± 0.26	1.06 ± 0.19*	1.96 ± 0.42*
Plasma NEFAs (mEq/L)	1.38 ± 0.14	1.47 ± 0.12	1.51 ± .21	1.40 ± 0.27

Animals were fasted for 4 hours prior to sacrifice. Values are means ± SEM (n = 10-15 per group). Effect of diet, *P < 0.05.

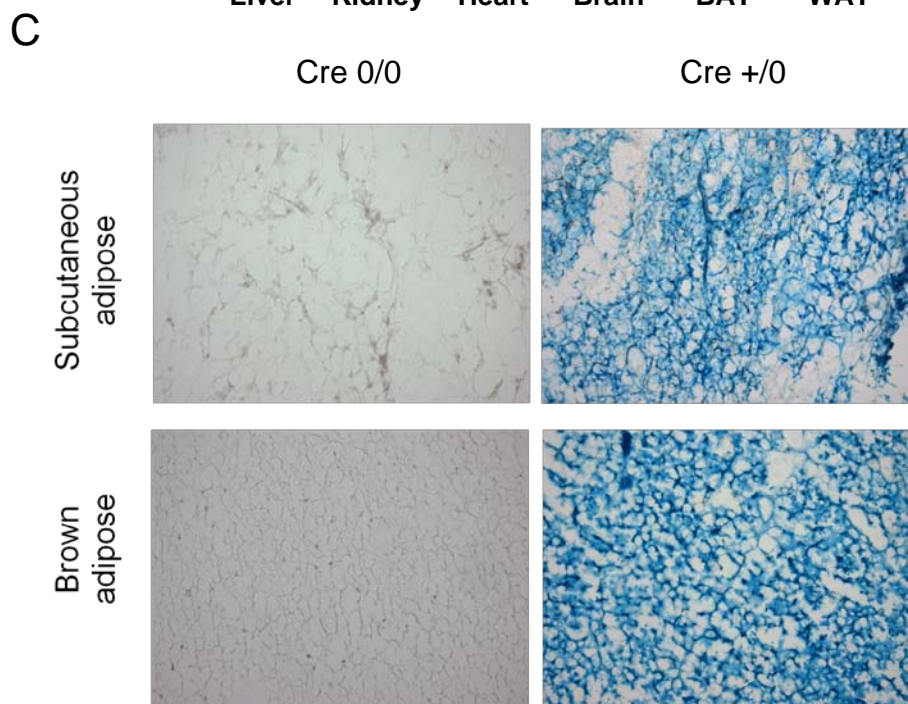
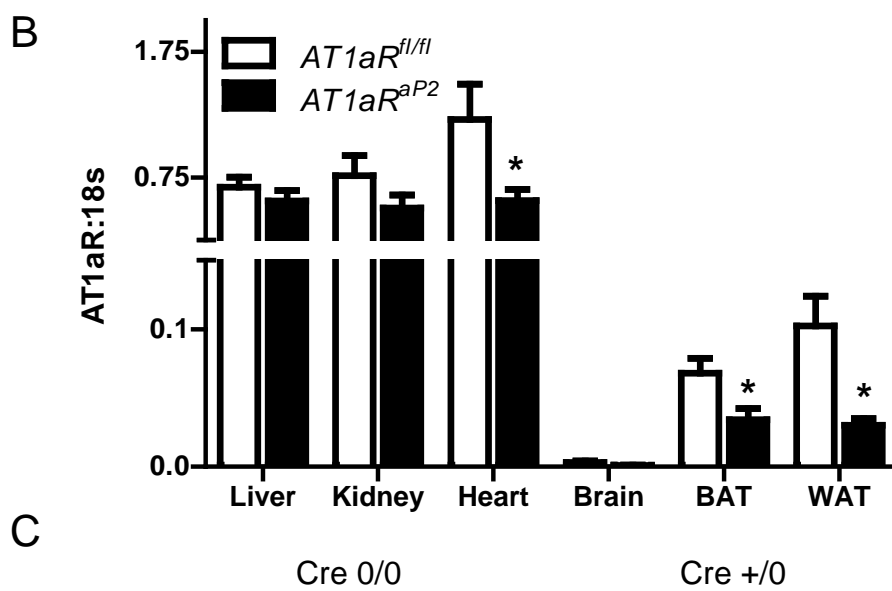
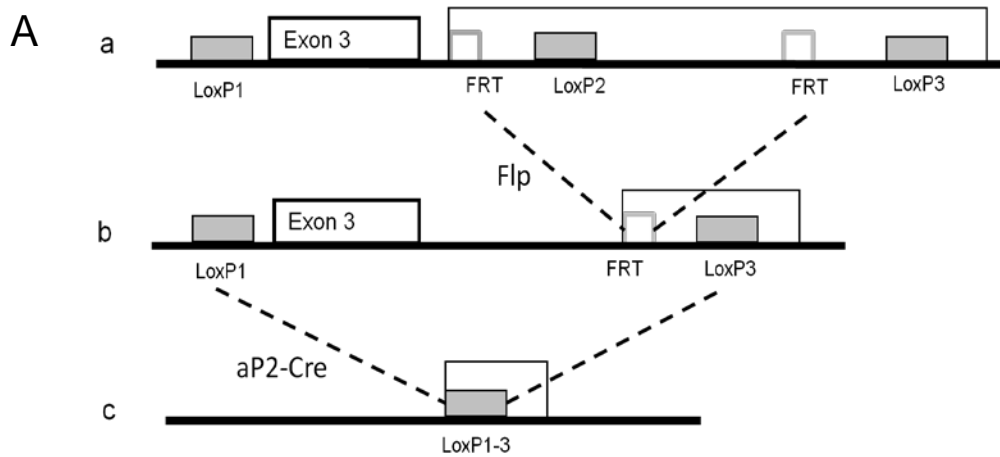


Figure 2.1 Development of mice with adipocyte deficiency of AT1aR. A, Mice with loxP sites flanking exon 3 of the AT1aR gene (a) were bred to mice expressing flippase (FLP) which recognizes the FRT sites to remove the neocassette (b). (c) $AT1aR^{fl/fl}$ mice were bred to transgenic mice expressing Cre recombinase driven by the aP2 promoter to generate adipocyte AT1aR deficient mice ($AT1aR^{aP2}$) and non-transgenic littermates ($AT1aR^{fl/fl}$). B, AT1aR adipocyte deficiency was confirmed in brown (BAT) and white adipose tissues (WAT, respectively). C, Cre recombinase activity was confirmed with X-Gal staining in BAT and subcutaneous adipose of $AT1aR^{aP2}$ bred to ROSA26 mice (Cre+/0) but not in mice lacking Cre recombinase (Cre 0/0). Data are represented as mean \pm SEM from n = 8-10 mice/group. *, P < 0.05 compared to $AT1aR^{fl/fl}$.

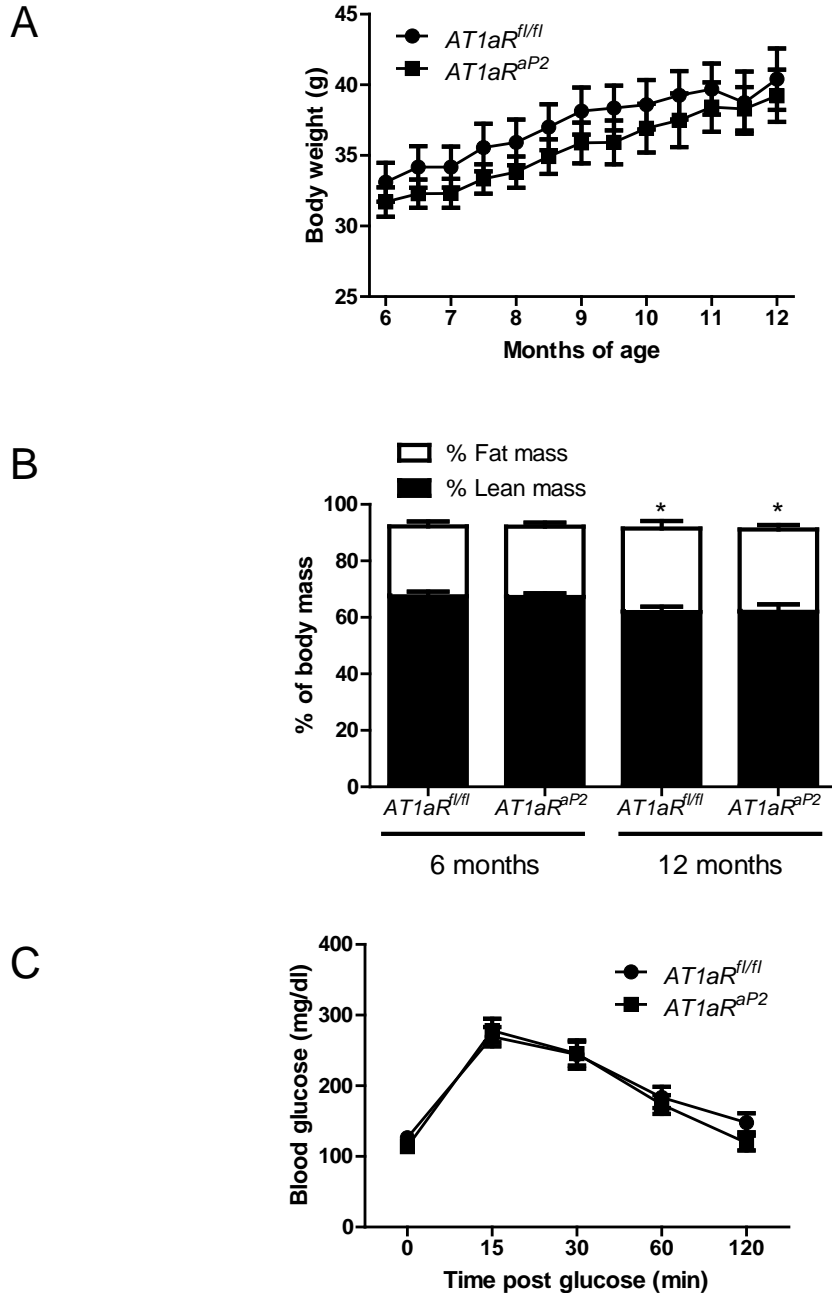
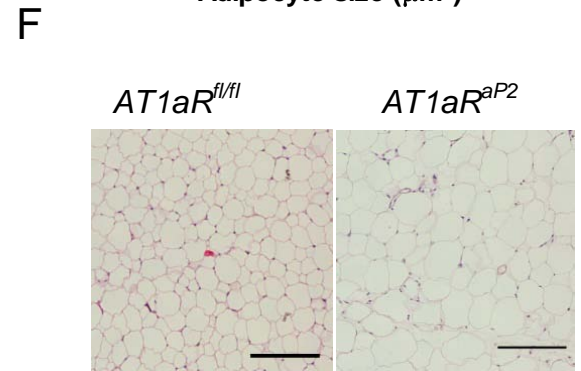
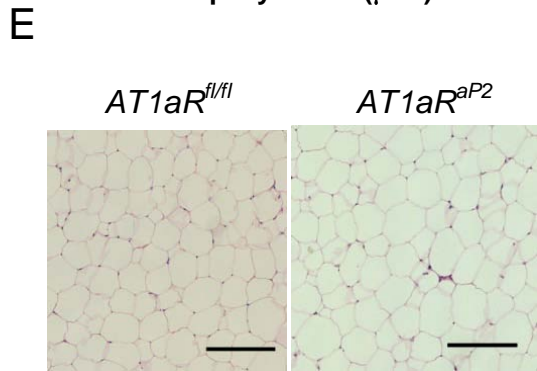
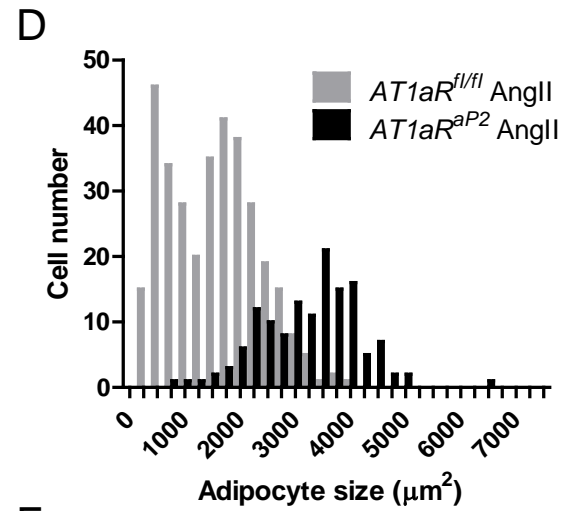
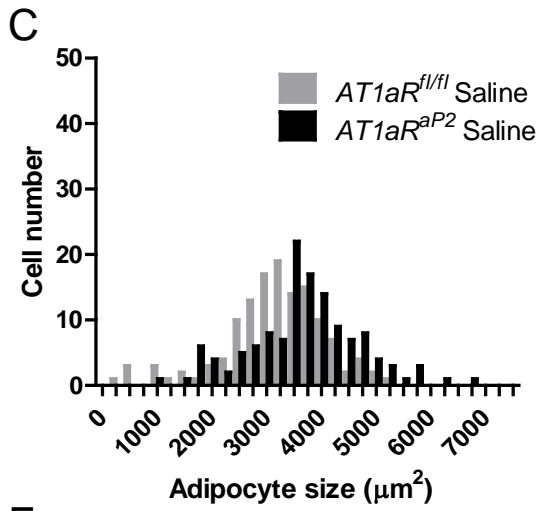
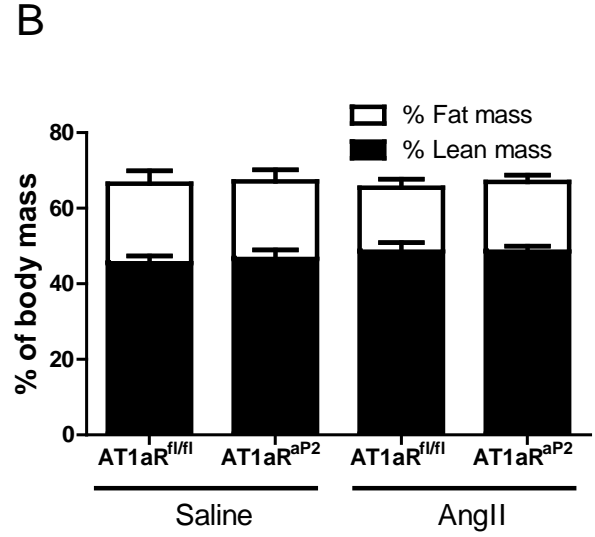
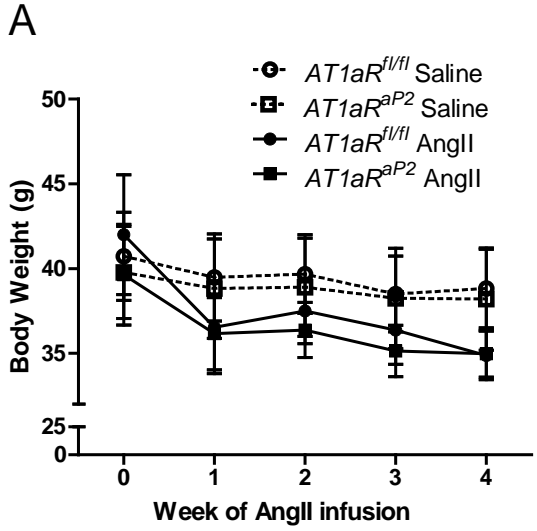


Figure 2.2 Adipocyte-AT1aR deficiency has no effect on body parameters of aged mice fed standard murine diet. A, Body weights were recorded every other week beginning at 6 months of age. B, Fat and lean mass were increased and decreased, respectively, at 12 months of age compared to 6 months of age, *, $P < 0.05$. C, Glucose tolerance test performed at 12 months of age. Data are represented as mean \pm SEM from 4-5 mice/group.



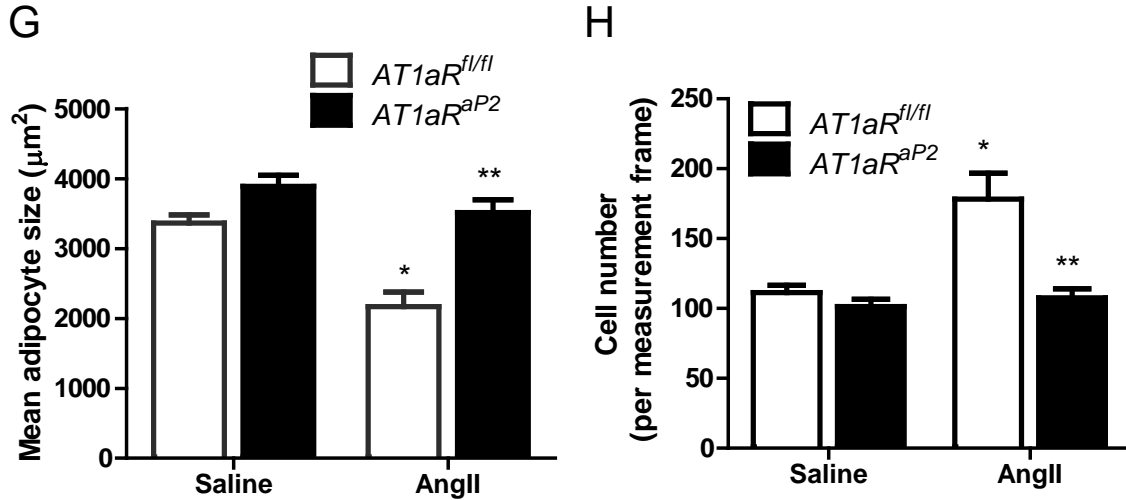


Figure 2.3 AngII infusion reduces adipocyte size in *AT1aR^{fl/fl}* but not

***AT1aR^{aP2}* mice fed standard diet.** A, AngII infusion for 4 weeks in mice fed standard murine diet causes modest, but not significant, weight loss. B, Neither AngII infusion nor adipocyte AT1aR deficiency affects % fat or lean mass in aged mice. C, Frequency distribution of saline- and (D) AngII-infused mice. E, Images of saline- and (F) AngII-infused H&E stained adipocytes (scale bar represent 200 μm). G, Saline-infused *AT1aR^{aP2}* mice exhibit modest adipocyte hypertrophy ($P = 0.052$) and AngII infusion reduces adipocyte size in *AT1aR^{fl/fl}* mice (*, $P < 0.001$ effect of treatment; **, $P < 0.001$ effect of genotype). Data are represented as mean \pm SEM from 3-5 mice/ group.

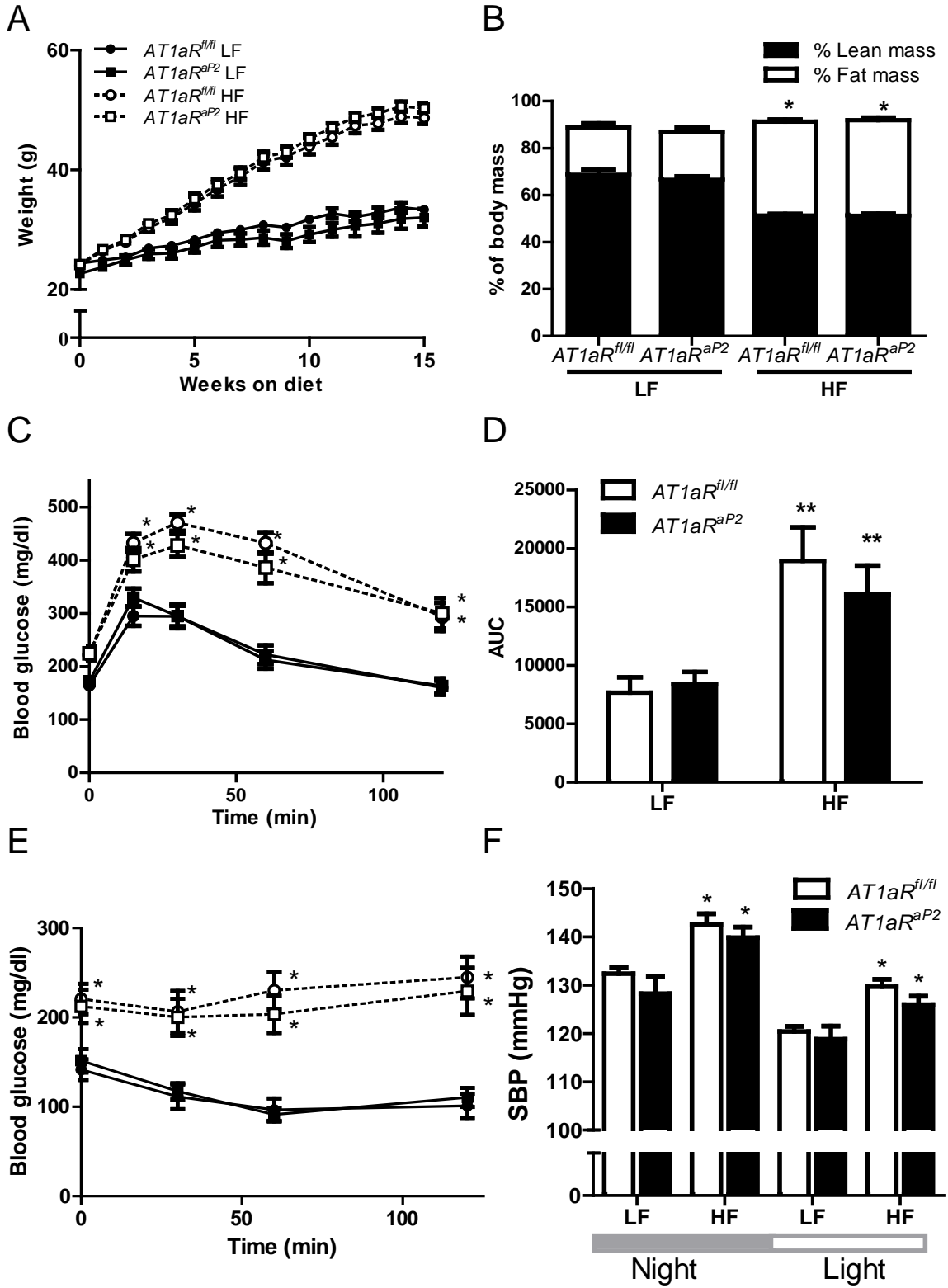


Figure 2.4 Deficiency of AT1aR in adipocytes had no effect on development of obesity or obesity-associated glucose intolerance. A, Body weight over 15 weeks of LF or HF feeding in mice of each genotype. Beginning at week 3, HF-fed mice of each genotype had significantly increased body weight compared to LF-fed controls. B, Fat and lean mass (% body weight) of *AT1aR^{fl/fl}* and *AT1aR^{aP2}* mice fed LF or HF diets for 16 weeks. C, Blood glucose concentrations at selected time points following a bolus of glucose (time 0) in *AT1aR^{fl/fl}* and *AT1aR^{aP2}* mice fed a LF or HF diet for 15 weeks. D, Area under the curve (AUC, arbitrary units) quantification of blood glucose concentrations shown in C. Data are represented as mean \pm SEM from n = 9-12 mice/group. *P < 0.05 or **P < 0.001 compared to LF within genotype.

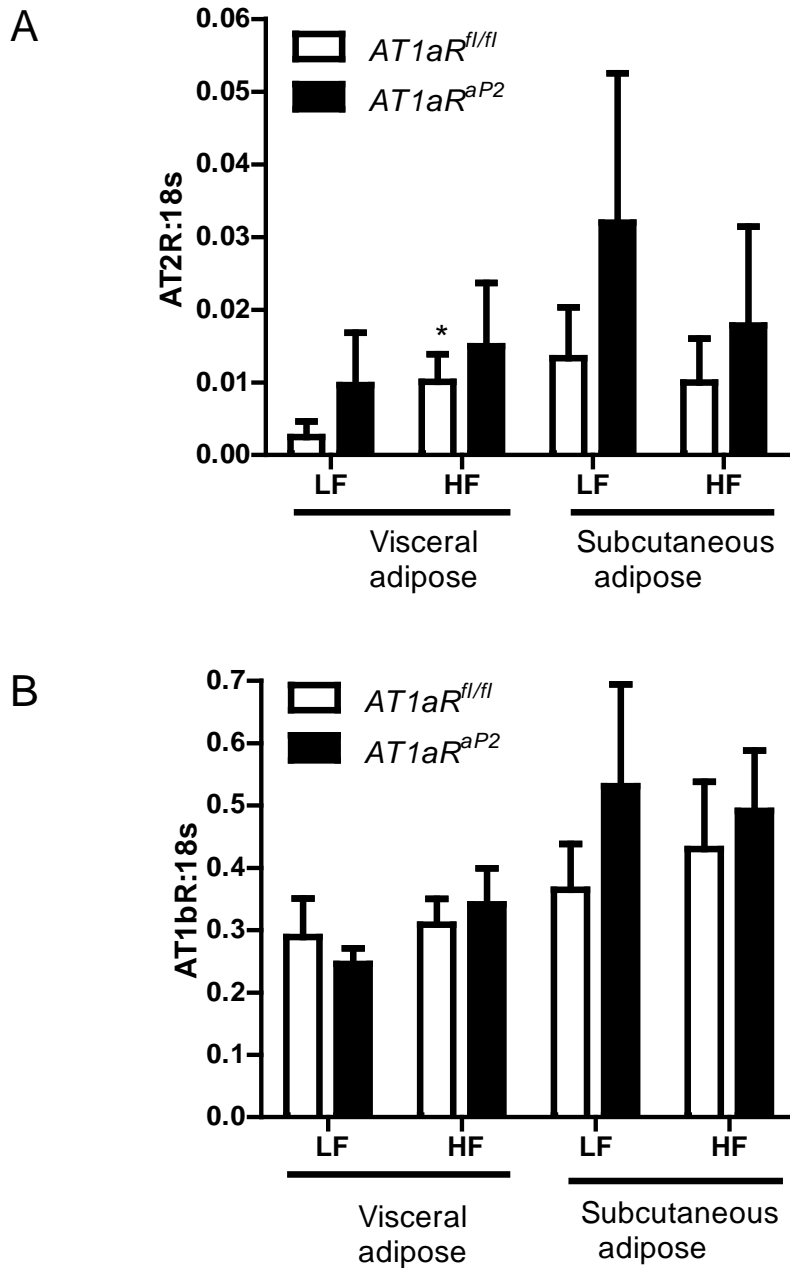


Figure 2.5 Deficiency of AT1aR in adipocytes has no effect on mRNA abundance of other angiotensin receptors in adipose tissues. mRNA abundance of AT2R (A) and AT1bR (B) was quantified in visceral and subcutaneous adipose tissues from LF and HF-fed *AT1aR^{fl/fl}* and *AT1aR^{aP2}* mice. Data are represented as mean ± SEM from n = 5-7 mice/group. *, P < 0.05 compared to LF within genotype and tissue.

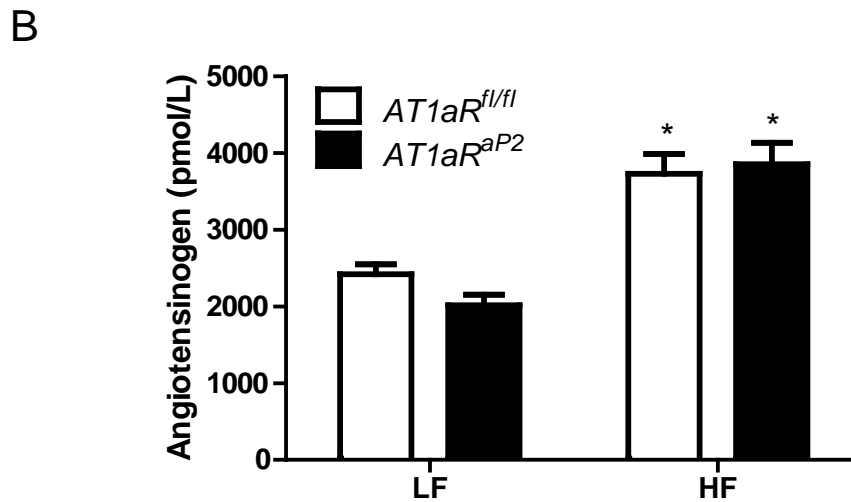
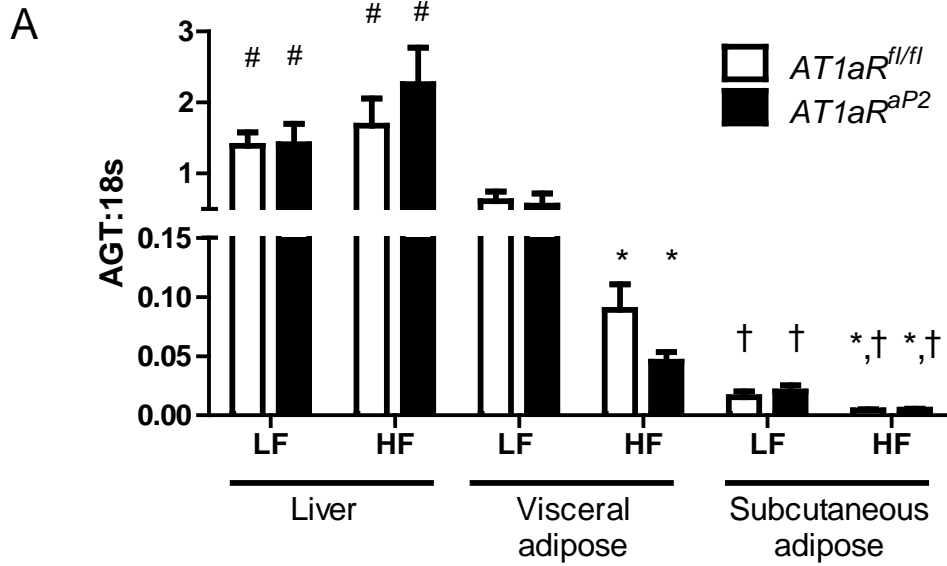


Figure 2.6 HF diet suppresses adipose AGT mRNA abundance and increases plasma AGT concentration. A, AGT mRNA abundance was quantified in liver, visceral and subcutaneous adipose tissues (#, $P < 0.05$ compared to either adipose depot; *, $P < 0.05$ compared to LF within adipose depot; †, $P < 0.05$ compared to visceral adipose or liver). B, Plasma AGT concentrations (*, $P < 0.05$ effect of diet). Data are represented as mean \pm SEM from $n = 4-10$ mice/group.

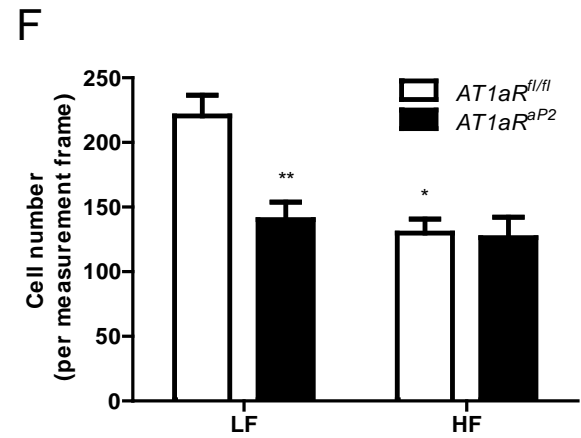
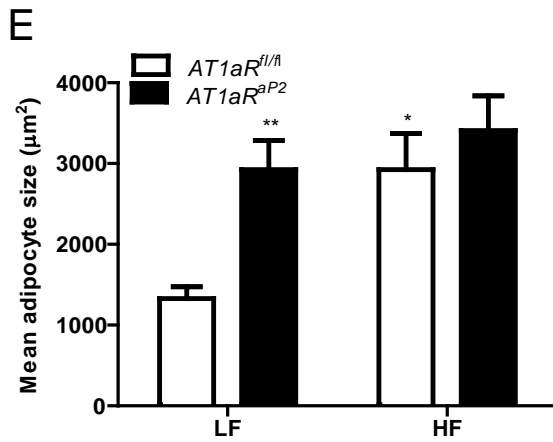
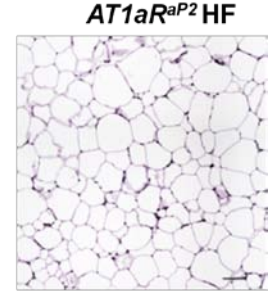
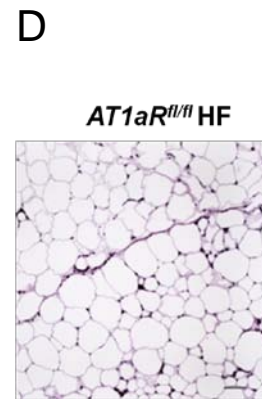
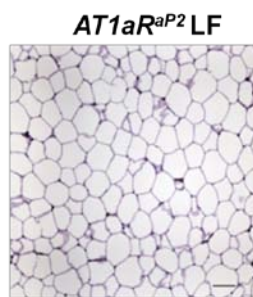
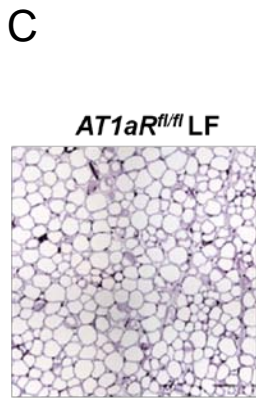
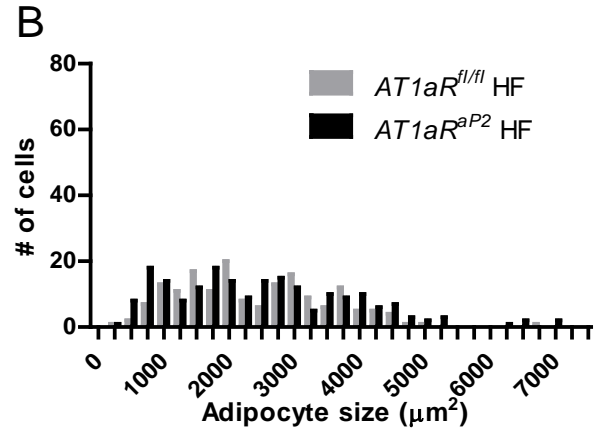
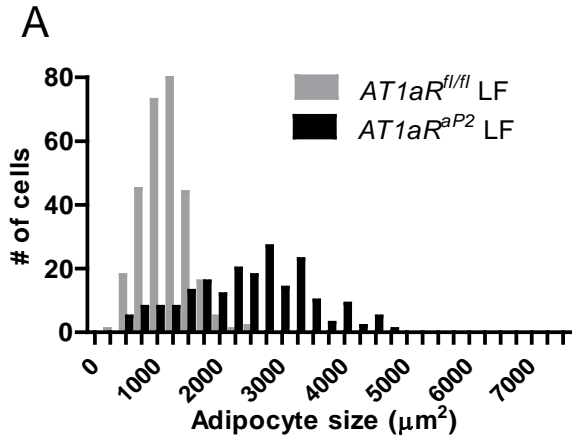


Figure 2.7 Adipocyte AT1aR deficiency resulted in striking adipocyte hypertrophy in LF-fed mice. Histograms of adipocyte number of selected areas quantified in adipose tissue sections using image analysis software (sections from n = 3 mice/group and 3 image fields per section) from LF (A) or HF-fed mice (B). Representative images of H&E stained sections of retroperitoneal visceral adipose tissue used for quantification of adipocyte sizes from LF (C) or HF-fed mice (D) of each genotype. Scale bar in lower right represents 200 μ m. E, Quantification of mean adipocyte area. F, Quantification of adipocyte number per measurement frame. Data are represented as mean \pm SEM from n = 9 mice/group. *P < 0.005 compared to LF within genotype. **P < 0.05 compared to *AT1aR^{fl/fl}* within diet group.

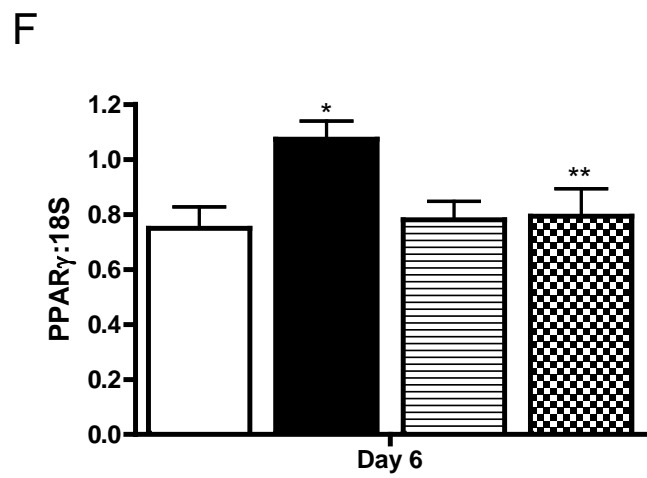
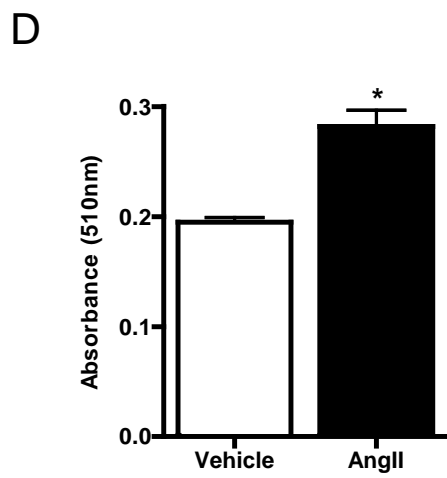
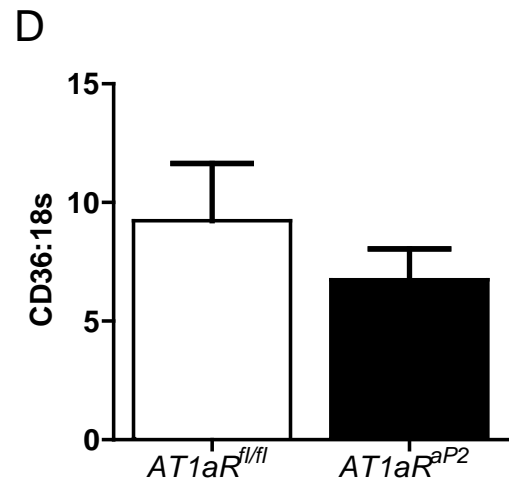
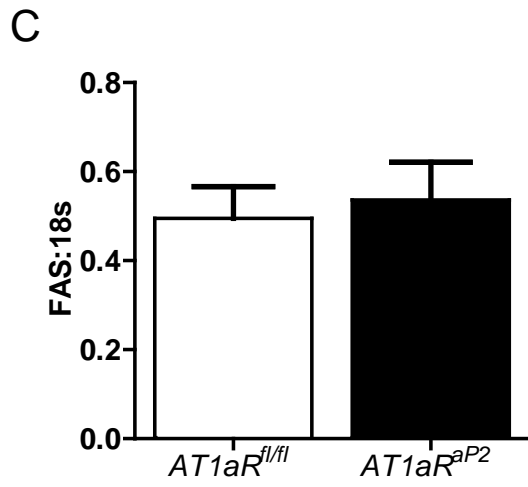
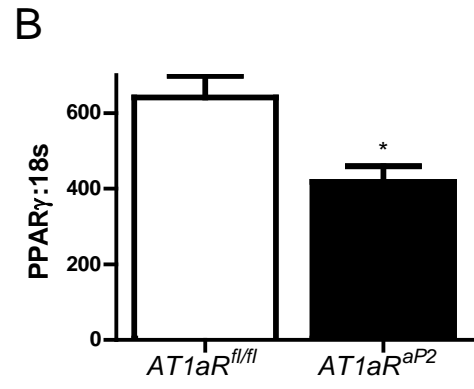
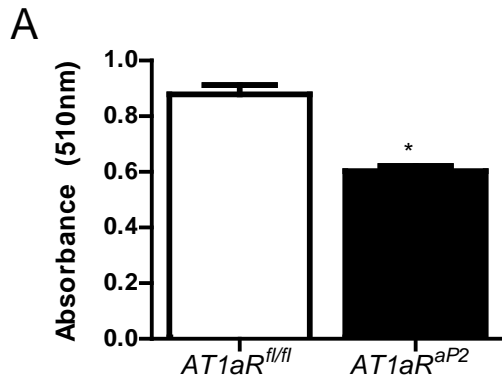


Figure 2.8 Deficiency of AT1aR reduced differentiation of SVC to adipocytes while AngII promotes differentiation of 3T3-L1 adipocytes. A, ORO staining (510 nm) of adipocytes differentiated from SVCs of *AT1aR^{fl/fl}* and *AT1aR^{aP2}* mice. Data are represented as mean \pm SEM from n = 3-5 samples/genotype. PPAR γ (B), fatty acid synthase (C), and CD36 (D) mRNA abundance in adipocytes (day 8) differentiated from SVCs of *AT1aR^{fl/fl}* and *AT1aR^{aP2}* mice. Data are mean \pm SEM from n = 8-11 samples/group. (E) ORO staining of 3T3-L1 adipocytes differentiated in the absence (vehicle) or presence of AngII (1 μ M). F, PPAR γ mRNA abundance in 3T3-L1 adipocytes differentiated in the absence or presence of AngII, losartan, or AngII + losartan (1 μ M of each compound). Data are represented as mean \pm SEM from n = 4-5 samples/group. A-C: *, P < 0.05 compared to *AT1aR^{fl/fl}*. F,G: *, P < 0.05 compared to vehicle; **, P < 0.05 compared to AngII.

SECTION III. SPECIFIC AIM 2

Determine the role of the adipocyte AT1aR on the development of vascular diseases.

3.1 Summary

While obesity is a well-established risk factor for cardiovascular diseases, few studies have investigated the possible involvement of adipose tissue surrounding blood vessels in the progression of this disease. The amount of epicardial or perivascular fat is positively correlated to the amount of atherosclerotic lesion area in the underlying vessels and perivascular fat has been shown to have a pro-inflammatory phenotype, though it remains unclear if perivascular adipose inflammation plays a causative role in lesion formation. AngII is known to promote various forms of vascular diseases, independent of blood pressure increases, though the cell type through which AngII acts remains unknown. We tested the hypothesis that AngII acts as a proinflammatory stimulus through the adipocyte AT1aR, and deficiency of this receptor in adipocytes is protective against the development of atherosclerosis and abdominal aortic aneurysms (AAAs). To understand how hypercholesterolemia affects the expression and production of RAS components, particularly in adipose tissue, LDLR^{-/-} mice were fed standard mouse diet (termed “chow”), Western, or high fat (HF) diets for 1 to 3 months. Body weights of HF- and Western-fed mice were significantly greater than those of chow-fed mice, though only Western diet induced hypercholesterolemia and hypertriglyceridemia. AT1aR mRNA abundance varied across adipose depots, but was markedly

suppressed in both thoracic and abdominal peri-aortic fat (PAF) depots after 3 months of Western or HF diets. In Western diet-fed mice previously demonstrated to exhibit an activated systemic renin-angiotensin system (RAS), plasma renin concentrations (PRC) were increased, while HF-fed mice exhibited elevations in plasma angiotensinogen (AGT) concentrations. $AT1aR^{fl/fl}$ x $LDLR^{-/-}$ mice and $AT1aR^{aP2}$ x $LDLR^{-/-}$ mice were fed Western diet for 3 months to examine the role of endogenous AngII acting at the adipocyte AT1aR in the development of atherosclerosis, though no differences between genotypes were observed in this study. A model of AngII infusion known to augment atherosclerosis and induce AAA formation in mice fed Western diet for 5 weeks was used to determine the role of the adipocyte AT1aR in exogenous AngII-induced vascular pathologies; however, there were no differences in lesion formation or AAA development between genotypes. Previous studies revealed that adipocyte-AT1aR deficiency resulted in adipocyte hypertrophy in lean but not obese mice, and interestingly, modest adipocyte hypertrophy was also noted in retroperitoneal adipose of AngII-infused $AT1aR^{aP2}$ x $LDLR^{-/-}$ mice. These effects were independent of adipose tissue lipolysis and similar to results in lean mice. Results from these studies demonstrate that adipocyte-AT1aR deficiency does not influence the development of atherosclerosis in $LDLR^{-/-}$ mice fed Western diet chronically or infused with AngII for 1 month during diet feeding. Also, deficiency of adipocyte AT1aRs did not alter the formation or severity of AngII-induced AAAs. Adipocyte hypertrophy was modest in $AT1aR^{aP2}$ x $LDLR^{-/-}$ mice compared

to lean mice in other studies, suggesting that the effect of adipocyte-AT1aR deficiency to reduce adipocyte differentiation is partially reduced by Western diet.

3.2 Introduction

Heart disease and stroke are among the leading causes of death worldwide with other forms of vascular disease not far behind (176). Understanding the mechanisms behind the initiation and progression of these diseases is essential for the development of effective therapeutic strategies. Pharmacologic blockade of the RAS through ACE inhibitors and ARBs reduces the risk of cardiovascular mortality (5), implying a causative role for AngII in these diseases. Murine models of hypercholesterolemia have been shown to stimulate the production of circulating angiotensin peptides and furthermore, infusion of AngII into hypercholesterolemic mice promotes the formation of atherosclerotic lesions (62) and induces aortic aneurysms (63). The exact mechanisms through which AngII promotes these vascular diseases are still being elucidated. Deletion of the AT1aR gene in LDLR^{-/-} and ApoE^{-/-} mice markedly reduces atherosclerotic lesion area and attenuates the formation of abdominal aortic aneurysms (AAAs) (65, 276). The AT2R, on the other hand, protects against these pathologies (64, 112, 120), though these results are controversial (65). Interestingly, although the AT1aR clearly has pathological effects, the cell type(s) mediating the increases in atherosclerosis and AAAs remain elusive (49, 87, 138, 212).

Adipocytes are an understudied contributor to vascular disease, though interest in perivascular adipose tissue has increased in the past few years (194, 251). Quantification of epicardial and perivascular adipose depots through various imaging techniques has allowed several groups to establish significant

positive correlations between the presence/amount of adipose tissue in epicardial, pericardial, or perivascular depots and the presence/severity of atherosclerotic lesions in corresponding blood vessels (56, 150, 158, 227, 285). A functional paracrine relationship between perivascular adipose and the inclusive vessel was first recognized with the discovery that perivascular adipose modulates vessel contractility through the release of vasoactive signaling molecules (151, 153, 240, 274). Furthermore, the proinflammatory phenotype of perivascular compared to visceral or subcutaneous adipose depots (51, 103, 109) suggests the influence of perivascular adipose extends beyond modulation of vessel contraction to impact the infiltration of immune cells to the vessel wall, a critical component of both lesion and aneurysm formation. Indeed, adipose transplant studies have provided direct evidence for the contribution of adipose tissue to the development of atherosclerosis (188-189). A large clinical trial of over 12,000 men has demonstrated an independent association between obesity and AAA formation (93). Correspondingly, obesity promoted the development of AngII-induced AAAs in normocholesterolemic mice fed a HF diet (203). Results from this mouse model indicate perivascular adipose tissue surrounding the abdominal region of aortas from obese mice secretes more chemokines than the thoracic region and thus may contribute to the inflammation and formation of aneurysms in this region (203). These studies suggest that AngII may act directly on perivascular adipocytes through the AT1aR to promote the formation of atherosclerotic lesions and AAAs. The following studies investigated the

possible role of the adipocyte-AT1aR on the development of atherosclerosis and AAA in hypercholesterolemic LDLR^{-/-} mice.

3.3 Materials and Methods

3.3.1 Mice and diets

All mice were fed respective diets ad libitum and given free access to water. Mice were group-housed and exposed to 12 hours of light and dark each day. Experimental protocols were approved by the University of Kentucky Institutional Care and Use Committee.

LDLR^{-/-} time course diet. Eight week old LDLR^{-/-} (Stock #; Jackson Laboratory, Bar Harbor, ME) were purchased and placed on either standard mouse diet (termed “chow”), Western diet (D ; Teklad) comprised of 42% kcal as fat and 0.15% cholesterol, or high fat (HF) diet (Research Diets) comprised of 60% kcal as fat (Table 3.1). Groups of mice were maintained on diet for either 1, 2, or 3 months (n = 8).

Diet-induced atherosclerosis. Mice with adipocyte-AT1aR deficiency (*AT1aR^{ap2}*) were bred to LDLR^{-/-} mice to generate *AT1aR^{ap2}* x LDLR^{-/-} and *AT1aR^{fl/fl}* x LDLR^{-/-} mice. At 8-10 weeks of age, all mice were fed Western diet for a total of 3 months.

AngII-induced AAA and atherosclerosis. *AT1aR^{ap2}* x LDLR^{-/-} and *AT1aR^{fl/fl}* x LDLR^{-/-} mice were fed Western diet for a total of 5 weeks beginning at 8-10 weeks of age. After the first week on diet, mice were implanted with Alzet 28-day osmotic mini-pumps (Model 2004, DURECT Corporation, Cupertino, CA) filled with either saline or AngII (Sigma, St. Louis, MO) delivered at a rate of 1,000 ng/kg/min. Mini-pumps were implanted subcutaneously under a low dose of ketamine/xylazine anesthesia and bupivacaine was used as a local anesthetic

over the stapled closure site. Anesthetized mice were exsanguinated at the end of the 28-day infusion after a 4-hour fast for plasma and tissue harvest.

3.3.2 *In vivo* measurements

Body composition measurements were performed monthly using NMR spectroscopy (EchoMRI). Blood pressure was measured non-invasively by the tail-cuff method (Visitech Systems, Inc., Apex, NC). Blood pressure measurements were performed at the end of each month during the LDLR^{-/-} time course diet study, during the week prior to and the third week during AngII infusion in the AngII-induced AAA study. Preliminary measurements were taken each day prior to data collection and the criteria for inclusion required at least 5 (out of 10) successful measurements per day with less than 30% standard deviation. For the AngII infusion study, ultrasound images of the abdominal aorta above the renal branches were obtained for quantification of lumen diameters at day 0, 14, and 28 of AngII infusion (Vevo 2100, VisualSonics, Toronto, Canada).

3.3.3 Plasma and serum measurements

Plasma renin concentrations were measured by incubating plasma (8 µl) with exogenous AGT (25 nM) in the presence of ACE inhibitors and then angiotensin I (AngI) production was quantified by radioimmunoassay (DiaSorin, Via Crescentino, Italy). Plasma AGT levels were measured with the Mouse Total Angiotensinogen Assay Kit (Immuno-Biological Laboratories Co., Gunma, Japan). Total cholesterol and triglyceride (LabAssay) levels were quantified with respective kits from Wako Diagnostics (Richmond, VA).

3.3.4 Assessment of AAAs in AngII-infused mice

A greater than 50% increase in lumen diameter, measured by ultrasound, was one parameter used to determine AAA incidence. Peri-aortic fat was carefully collected and placed into RNAlater so that cleaned aortic tissue could be placed in 10% w/v formalin. Maximal external suprarenal aortic diameters were obtained from cleaned aortas using NIS Elements imaging software. Presence and severity of AAA pathology were also graded by two individuals blinded to the genotype of the mice based on the Type I – Type IV grading system previously described (64). AAA pathology was only assessed in mice exhibiting at least 2 signs of successful AngII infusion (defective osmotic pumps can compromise effective AngII delivery), indicated by low plasma renin concentrations, increased blood pressure, or visual AAA pathology.

3.3.5 Quantification of atherosclerotic lesion area

After fixation in 10% formalin, aortas were cut longitudinally to expose the intimal surface and pinned for en face analysis. The arch and thoracic regions were determined (the arch area encompassed the ascending aorta down to 3 mm below the left subclavian artery; the thoracic area began at this point and extended 9 mm down the descending thoracic aorta) and lesion areas were expressed as a percent of the total area for each region. Lesion areas were calculated using NIS Elements software (Nikon Instruments, Inc., Tokyo, Japan).

3.3.6 Quantification of mRNA abundance

RNA was isolated using either Promega Total RNA Isolation System (Promega, Madison, WI) or Qiagen Lipid RNeasy kit (Qiagen, Valencia, CA). Reverse transcription reactions were set up with 0.4 µg RNA using qScript cDNA SuperMix and real-time PCR reactions were performed with PerfeCTa SYBR Green FastMix using 2 ng of cDNA template (Quanta Biosciences, Gaithersburg, MD). Standard curves were generated from ten-fold dilutions of concentrated cDNA from tissues known to express the gene of interest and this standard curve was used to calculate the starting quantity of template from the given Ct values. Data are expressed as the ratio of the gene of interest to the starting quantity of the housekeeping gene 18S.

3.3.7 Determination of adipocyte size

Sections of formalin (10% w/v) fixed pieces of retroperitoneal or subcutaneous adipose tissue were stained with hematoxylin and eosin (H&E). Images of slides were obtained at 10x magnification and using the “detect edges”, image threshold, and object count features of NIS Elements software (Nikon, Instruments, Inc., Tokyo, Japan), the area of each adipocyte within a 700 x 700 µm measurement frame was quantified. Three measurement frames on each slide and 3 slides per genotype group were analyzed for morphology.

3.3.8 Lipolysis assay

Epididymal adipose tissue explants (25 mg) were collected from saline-infused $AT1aR^{fl/fl}$ x $LDLR^{-/-}$ and $AT1aR^{aP2}$ x $LDLR^{-/-}$ mice fed Western diet for a

total of 5 weeks. Filtered Kreb's buffer supplemented with fatty-acid free bovine serum albumin (2% BSA) was used for washes and treatment dilutions. Explants were washed 3 times and incubated in a 48-well plate containing various treatments (0.5 mL) for a total of 5 hours, though "conditioned media" was sampled at 30, 90, and 180 minutes then frozen at -20°C. Treatments included 10 µM AngII (Sigma, St. Louis, MO), 1 µM losartan (Merck), 1 µM isoproterenol (Sigma, St. Louis, MO), or combinations of AngII with losartan or isoproterenol at these same concentrations (for combination treatments, explants were pre-treated in losartan or isoproterenol for 15 minutes prior to addition of AngII). At the end of the 5-hour incubation, glycerol concentrations were measured from "conditioned media" (15 µL) using a colorimetric assay (Cayman Chemical Co., Ann Arbor, MI). Explants were also collected for protein extraction and quantification using the Pierce®BCA Assay (Thermo Scientific, Rockford, IL). Data are expressed as the ratio of glycerol to protein concentrations.

3.3.9 Statistical analyses

Data from the LDLR^{-/-} time course study were analyzed by two way ANOVA to compare the different diet and duration groups. Student's *t*-tests were used for comparisons between the two genotypes, except in instances where treatment (saline or AngII infusion) was an additional factor, in which case two way ANOVA was used to compare the 4 treatment/genotype groups. Post-hoc analysis of two way ANOVA results used the Holm Sidak test. If normality or equal variance tests failed, simple transforms were performed or the non-parametric Kruskal-Wallis test was used with Dunn's post-hoc analysis. Data are

represented as mean \pm SEM and statistical significance was defined as $P < 0.05$.

3.4 Results

3.4.1 Diets modulate AT1aR expression in peri-aortic fat

Our first goal was to characterize changes in expression of RAS components in response to hypercholesterolemia, particularly in peri-aortic fat (PAF), to better understand how PAF may contribute to AngII-induced vascular diseases. Chow-fed LDLR^{-/-} mice gained little weight over the 3 month duration of the study (Figure 3.1A) In contrast, HF- and Western-fed mice exhibited significant elevations in body weight beginning at 2 and 3 weeks, respectively, and gained similar amounts of weight (Figure 3.1A). Fat mass increased in mice fed all diets after 3 months (Figure 3.1B, $P < 0.001$), though was significantly increased by Western and HF diets compared to chow ($P < 0.001$). Importantly, although Western- and HF-fed mice achieved similar levels of weight gain (Figure 3.1A), only Western-fed mice exhibited significant hypercholesterolemia (Figure 3.1C, $P < 0.05$) and triglyceridemia (Figure 3.1D, $P < 0.05$).

There were trends for age-dependent downregulation of AT1aR mRNA abundance in thoracic PAF, while age increased AT1aR mRNA abundance in abdominal peri-aortic fat of chow-fed mice (Figure 3.2A,B). However, these effects were not statistically significant. Interestingly, when mice were fed chow or HF diets, the age-dependent downregulation of AT1aR mRNA abundance in thoracic PAF was significant. AT1aR mRNA abundance was significantly decreased by the same extent in thoracic PAF of 2 or 3 month Western or HF diet-fed mice compared to 1 month (Figure 3.2A). In contrast, AT1aR mRNA abundance in abdominal PAF was strikingly reduced by both Western and HF

diets compared to mice fed chow after 3 months of respective diets (Figure 3.2B). Also, Western diet had a more robust effect to increase AT1aR mRNA abundance in abdominal PAF compared to HF-feeding at 1 and 2 months. In contrast to AT1aR expression, AGT mRNA abundance in PAF (Figure 3.2C,D) was not regulated by any of the diets at any time point. In liver, AGT mRNA abundance was modestly elevated by HF diet at 2 and 3 months, and was decreased by Western diet at 3 months (Figure 3.2F, $P < 0.05$).

Plasma renin and AGT concentrations were used as stable and reliable indicators of systemic RAS activation. Both hypercholesterolemia (65) and HF diets (100) have been shown to increase circulating angiotensin peptide levels, indicating an activated RAS. Hypercholesterolemia robustly increased plasma renin concentrations after 3 months of diet (Figure 3.3A, $P < 0.05$), while the HF diet increased circulating AGT levels after 3 months (Figure 3.3B, $P < 0.05$), indicating different mechanisms of RAS activation by these diets.

3.4.2 Adipocyte-AT1aR deficiency has no effect on diet-induced atherosclerosis

To determine the role for endogenous AngII acting at the adipocyte AT1aR in diet-induced atherosclerosis, $AT1aR^{fl/fl} \times LDLR^{-/-}$ and $AT1aR^{aP2} \times LDLR^{-/-}$ mice were fed Western diet for 3 months beginning at 8 weeks of age. No differences in body weight, percent fat mass, or percent lean mass were observed between the two groups (Figure 3.4A,B). Serum cholesterol and triglyceride levels were also not significantly different between AngII-infused $AT1aR^{fl/fl}$ and $AT1aR^{aP2}$ mice (Figure 3.4C,D). Atherosclerotic lesion areas were

quantified both on the arch and thoracic regions of the aorta. Adipocyte-AT1aR deficiency had no effect on the development of atherosclerosis in either region of the aorta (Figure 3.4E,F).

3.4.3 Adipocyte-AT1aR deficiency does not influence AngII-induced AAAs and atherosclerosis

LDLR^{-/-} mice fed Western diet for 1 week prior to and during subsequent 4 weeks of AngII infusion exhibit increases in atherosclerotic lesion development and AAA formation. To investigate the effect of adipocyte-AT1aR deficiency on these AngII-induced pathologies, we infused *AT1aR^{fl/fl}* x LDLR^{-/-} and *AT1aR^{aP2}* x LDLR^{-/-} mice with AngII for 1 month. Similar to observations in diet-induced atherosclerosis, there was no effect of adipocyte-AT1aR deficiency on percent lesion area in either the aortic arch or thoracic regions of AngII-infused mice (Figure 3.5A,B). Additionally, adipocyte AT1aR deficiency had no effect was on AngII-induced AAAs, as measured by abdominal aortic lumen and external diameters (Figure 3.5C,D).

3.4.4 Modest adipocyte hypertrophy is caused by adipocyte-AT1aR deficiency in retroperitoneal adipose

Adipocyte hypertrophy was observed in lean (LF-fed or AngII-infused chow fed) *AT1aR^{aP2}* mice, so we examined adipocyte morphology of AngII infused mice fed a Western diet. AngII-infused *AT1aR^{aP2}* x LDLR^{-/-} mice did exhibit adipocyte hypertrophy in retroperitoneal fat (RPF), though the effect was modest and differences in mean adipocyte size or cell number did not reach statistical significance (Figure 3.6 A-F). Body weight was not affected by

adipocyte-AT1aR deficiency in LDLR^{-/-} mice infused with saline or AngII infusion; however, AngII infusion did reduce body weight and adipose tissue masses to a greater extent in *AT1aR^{aP2}* x LDLR^{-/-} mice compared to *AT1aR^{fl/fl}* mice (Table 3.2). Adipose tissue lipolysis was examined as a potential mechanism contributing to adipocyte hypertrophy and increased fat mass, however glycerol production from adipose explants was unaffected by AngII, losartan, or by adipocyte-AT1aR deficiency (Figure 3.6G). However, isoproterenol significantly increase adipocyte lipolysis, which was not influenced by losartan or AngII (Figure 3.6G).

3.5 Discussion

AngII is known to promote various vascular pathologies through the AT1aR in hypercholesterolemic mice, though the cell types mediating these effects are unknown. We proposed that AngII acts through adipocyte AT1aRs to promote the development of vascular diseases. Despite investigating models of both endogenous and exogenously administered AngII, we found no role for the adipocyte AT1aR in the development of atherosclerosis or AAAs. Interestingly, adipocyte-AT1aR deficiency did result in modest adipocyte hypertrophy in AngII-infused mice. These effects were not due to changes in adipose tissue lipolysis and are possibly due to previously identified reductions of adipocyte differentiation in mice lacking adipocyte AT1aRs.

The effects of hypercholesterolemia and HF diet on mRNA abundance of AT1aR in adipose tissue were characterized to understand dietary manipulation of this receptor as a potential contributor to vascular diseases. In this study, even though both diets induced weight gain, only LDLR^{-/-} mice fed Western diet exhibited significant elevations in serum cholesterol and triglyceride levels. The purpose of using these diets was to determine whether hypercholesterolemia or high fat feeding was a better stimulus to activate the adipose RAS, however this study is limited by the inability to distinguish whether differential effects of these diets were due to the induction of hypercholesterolemia by Western diet, the different amounts of fat in the diets, or the different sources of dietary fat. Hypercholesterolemia has been previously reported to upregulate AT1aR expression in vascular tissues (181-182), though the effects on adipocytes are

unknown. In mice fed Western diet for 1 month, we also observed an increase in AT1aR mRNA abundance in abdominal, but not thoracic PAF compared to chow-fed controls. However, in contrast to previously observed effects of hypercholesterolemia to increase vascular wall cell AT1aR expression, in this study we observed a striking reduction in AT1aR mRNA abundance following 3 months of Western or HF diet feeding in thoracic and abdominal PAF. Effects of these diets to reduce AT1aR mRNA abundance were more pronounced in abdominal than thoracic PAF. This may relate to the types of adipocytes in adipose tissue surrounding these different aortic regions (203). Brown adipocytes are localized to thoracic PAF, while a mixture of white and brown adipocytes surround abdominal PAF. Differences in the type of adipocytes in these different aortic regions may have influenced regulation of AT1aR expression by Western and/or HF diets. However, since both diets resulted in a marked reduction in AT1aR mRNA abundance in PAF at 3 months, then differences in dietary composition or type of adipocytes most likely did not contribute to diet-induced decreases in AT1aR expression.

Circulating angiotensin peptides are increased in the setting of chronic Western feeding to LDLR^{-/-} (65) mice as well as in C57BL/6 mice fed a HF diet for 4 months (100). Results from our study indicate that RAS activation in these conditions occur through distinct mechanisms. Plasma renin concentrations were significantly elevated after consumption of the Western diet for 3 months. These results suggest that increased renin concentrations in plasma most likely contributed to previously observed elevations in plasma AngII concentrations in

Western diet-fed LDLR^{-/-} mice (65). However, a well known facet of the RAS in mice is the existence of negative feedback regulation of renin production in response to AngII. Thus, it is unclear why elevated plasma AngII concentrations in LDLR^{-/-} mice fed the Western Diet (65) did not result in negative feedback-mediated reductions in plasma renin concentrations. Indeed, following 1 month of Western or HF diet-feeding, mice exhibited reductions in plasma renin concentrations suggestive of increased levels of circulating AngII. Additional studies, including quantification of plasma AngII concentrations (this was not performed in this study due to insufficient volumes of mouse plasma) are required to determine mechanisms for high plasma renin concentrations in 3 month Western diet-fed LDLR^{-/-} mice. However, since whole body AT1aR deficiency markedly reduces atherosclerosis in LDLR^{-/-} mice (65), then it is unlikely that these mice exhibit low activity of the systemic RAS. In contrast to the Western diet, consumption of the HF diet increased plasma AGT levels, demonstrating an effect of this diet to increase the amount of precursor to promote the conversion to AngII. Indeed, chronic consumption of this HF diet in C57BL/6 mice is associated with robust elevations in circulating concentrations of AngII (100, 258). Although AGT mRNA abundance in adipose tissue was not regulated by any of the diets, increased fat mass with HF diet along with increased liver AGT expression are likely contributors to HF-induced elevations in circulating AngII. In contrast to previous findings (65), we did not observe elevations in plasma AGT concentrations in Western diet-fed LDLR^{-/-} mice. This discrepancy is most likely due to different methods for measuring plasma AGT

(Western blot versus ELISA in our study) or the different controls used (LDLR^{+/+} vs chow-fed LDLR^{-/-} mice in our study).

Chronic feeding of Western diet to LDLR^{-/-} over a period of 3 months results in the development of atherosclerotic lesions in the aorta and adipocyte-AT1aR deficiency was not protective against lesion formation in this model. While these findings may be due to the downregulation of AT1aR expression in PAF surrounding the thoracic region of the aorta observed in the LDLR^{-/-} time course study, this is unlikely because receptor expression was high through the first 2 months of diet feeding, which is the time frame during which the initial phases of lesion formation begin. Evidence for a direct role of adipocytes or adipocyte-derived factors in the development of atherosclerotic plaques continues to emerge as more groups characterize the proinflammatory phenotype of perivascular adipose (51, 109, 188). One approach to studying the direct influence of adipose tissue in atherosclerosis is the transplantation of non-perivascular fat directly adjacent to the carotid artery in ApoE^{-/-} mice susceptible to atherosclerosis (188-189). While results from these studies clearly demonstrate that inflammation in transplanted visceral adipose tissue serves as a causative factor in lesion formation, it remains unclear whether inflammation of endogenous perivascular adipose depots is sufficient to contribute to this disease. Our model of adipocyte-AT1aR deficiency has the advantage of examining the role of endogenous adipose tissue in vascular diseases. Adipocyte-AT1aR deficiency, however, did not have an effect on atherosclerosis in LDLR^{-/-} mice fed Western diet for 3 months, indicating that this receptor has no

role in adipose tissue inflammation. Other models of adipocyte-specific deficiency of proatherogenic adipocyte factors are required to delineate the role of adipocytes in atherosclerosis.

Infusion of AngII for 4 weeks in mice fed Western diet for a total of 5 weeks is a well-established model of AAA formation in LDLR^{-/-} mice (62). Deficiency of AT1aRs in adipocytes had no effect on the development of AngII-induced AAAs. Previous work from our lab found that HF diet and the development of obesity promotes AAA formation in normocholesterolemic C57BL/6 mice infused with AngII (203). Police, et al. demonstrated increased MCP-1 secretion from and macrophage infiltration in PAF surrounding the abdominal region of aorta where AAAs form, leading us to hypothesize that AngII may be acting at the adipocyte AT1aR to upregulate MCP-1 production, thus promoting macrophage infiltration and disease progression. Instead of using the obesity model from the work of Police, et al. (C57BL/6 mice fed HF diet for 4 months with AngII infusion during the final month of diet), we chose to use a mouse model of adipocyte-AT1aR deficiency in LDLR^{-/-} mice fed Western diet. This model allowed us to study the development of both atherosclerosis and AAAs, and compare our results to previous work investigating cell types involved in AngII-induced vascular pathologies which have been performed on hypercholesterolemic LDLR^{-/-} or ApoE^{-/-} mice. The lack of an effect of adipocyte-AT1aR deficiency on vascular disease in our model, however, suggests that the obesity or ApoE^{-/-} models may be more effective in elucidating the role of adipocyte AT1aRs. While our results demonstrate that 3 months of HF diet

tended to reduce PAF mRNA abundance of AT1aR and AGT in LDLR^{-/-} mice, this may not be the case in 4 month HF-fed C57BL/6 infused with AngII for 1 month. ApoE deficiency presents another potential model to study this receptor because hypercholesterolemia can be achieved in the absence of Western diet, avoiding possible diet-induced regulation of adipose RAS components. Future studies may make use of these models to further examine the role of adipocyte AT1aRs in the development of atherosclerosis and AAAs.

AngII infusion in several models has been reported to induce weight loss (34, 37, 77, 202) and our study confirms this in LDLR^{-/-} mice fed Western diet for 5 weeks. Some of these studies suggest that AngII may regulate adipose tissue lipolysis, however reports are conflicting. To determine if AngII regulates lipolysis through the adipocyte AT1aR, we measured glycerol production from epididymal adipose explants from saline-infused *AT1aR^{fl/fl}* x LDLR^{-/-} and *AT1aR^{aP2}* x LDLR^{-/-} mice in the presence or absence of AngII, losartan, or the β -adrenergic agonist isoproterenol. Incubation with AngII, losartan, or a combination of both had no effect on glycerol production in either genotype, demonstrating that AngII does not directly regulate lipolysis through the AT1aR. Glycerol production was dramatically increased when explants were exposed to isoproterenol independent of AngII co-incubation, suggesting that AngII does not modulate the lipolytic response to sympathetic stimulation. It is possible that AT1aR activation by AngII regulates the transcription of genes involved in lipolysis and the 5-hour incubation of explants throughout the lipolysis assay was insufficient for these changes to manifest and glycerol production to be affected.

Adipocyte-AT1aR deficiency was found to reduce adipocyte differentiation from isolated preadipocytes and cause striking adipocyte hypertrophy in lean but not obese (Chapter 2). These findings led us to quantify adipocyte size in AngII-infused mice fed Western diet, revealing modest but not significant adipocyte hypertrophy in RPF of *AT1aR^{aP2}* x *LDLR^{-/-}* mice. Interestingly, the effect of adipocyte-AT1aR deficiency to cause adipocyte hypertrophy seems to positively correlate with the leanness of the mice, with the most dramatic effects observed LF-fed mice and a lack of effect in obese mice. Obesity and HF-feeding are associated with inhibited adipocyte differentiation, suggesting that adipocyte differentiation is partially inhibited in Western fed mice (possibly due to the shorter study duration or slightly lower fat content of the diet compared to HF) resulting in modest adipocyte hypertrophy.

Overall the results from these studies confirm that adipocyte-AT1aR deficiency confers no protection against diet- or AngII-induced vascular diseases, suggesting that other cell types or synergistic effects between various cell types are responsible for the effects of AngII through the AT1aR. These studies confirm previous reports that a diet high in fat limits the regulation of adipocyte size by adipocyte-AT1aR deficiency.

Table 3.1 Composition of diets.

	Standard chow	Western	High Fat
Source	Harlan Teklad Global 18% Protein Rodent Diet, 2018	Harlan Teklad Custom Research Diet, 88137	Research Diets, Inc. D12492
Kcal/g	3.1	4.5	5.24
Protein (% of total kcal)	24	15.2	20
Carbohydrate (% of total kcal)	58	42.7	20
Fat (% of total kcal)	18	42	60
Fat sources	Soybean oil	Milkfat	Lard (91% of fat kcal), soybean oil (9% of fat kcal)
Cholesterol	--	0.2% (0.15% added, 0.5% from milkfat)	0.03%

Table 3.2 Body and tissue weights in saline or AngII-infused *AT1aR^{fl/fl}* x *LDLR^{-/-}* or *AT1aR^{aP2}* x *LDLR^{-/-}* mice.

	Saline		AngII	
	<i>AT1aR^{fl/fl}</i> x <i>LDLR^{-/-}</i>	<i>AT1aR^{aP2}</i> x <i>LDLR^{-/-}</i>	<i>AT1aR^{fl/fl}</i> x <i>LDLR^{-/-}</i>	<i>AT1aR^{aP2}</i> x <i>LDLR^{-/-}</i>
n	6	7	19	24
Final body weight (g)	33.2 ± 1.6	30.7 ± 0.9	28.3 ± 0.5*	29.4 ± 0.6
Liver (g)	1.66 ± 0.18	1.48 ± 0.06	1.41 ± 0.05	1.46 ± 0.05
Kidney (g)	0.42 ± 0.04	0.37 ± 0.03	0.31 ± 0.01*	0.031 ± 0.01*
Epididymal fat (g)	1.10 ± 0.17	1.14 ± 0.09	0.84 ± 0.07	1.02 ± 0.06
Retroperitoneal fat (g)	0.40 ± 0.07	0.41 ± 0.04	0.28 ± 0.03*	0.35 ± 0.02
Subcutaneous fat (g)	0.61 ± 0.10	0.63 ± 0.06	0.41 ± 0.03*	0.50 ± 0.04
BAT (g)	0.16 ± 0.02	0.17 ± 0.02	0.13 ± 0.01	0.15 ± 0.01

Data are mean ± SEM. *P < 0.05 compared to saline within genotype

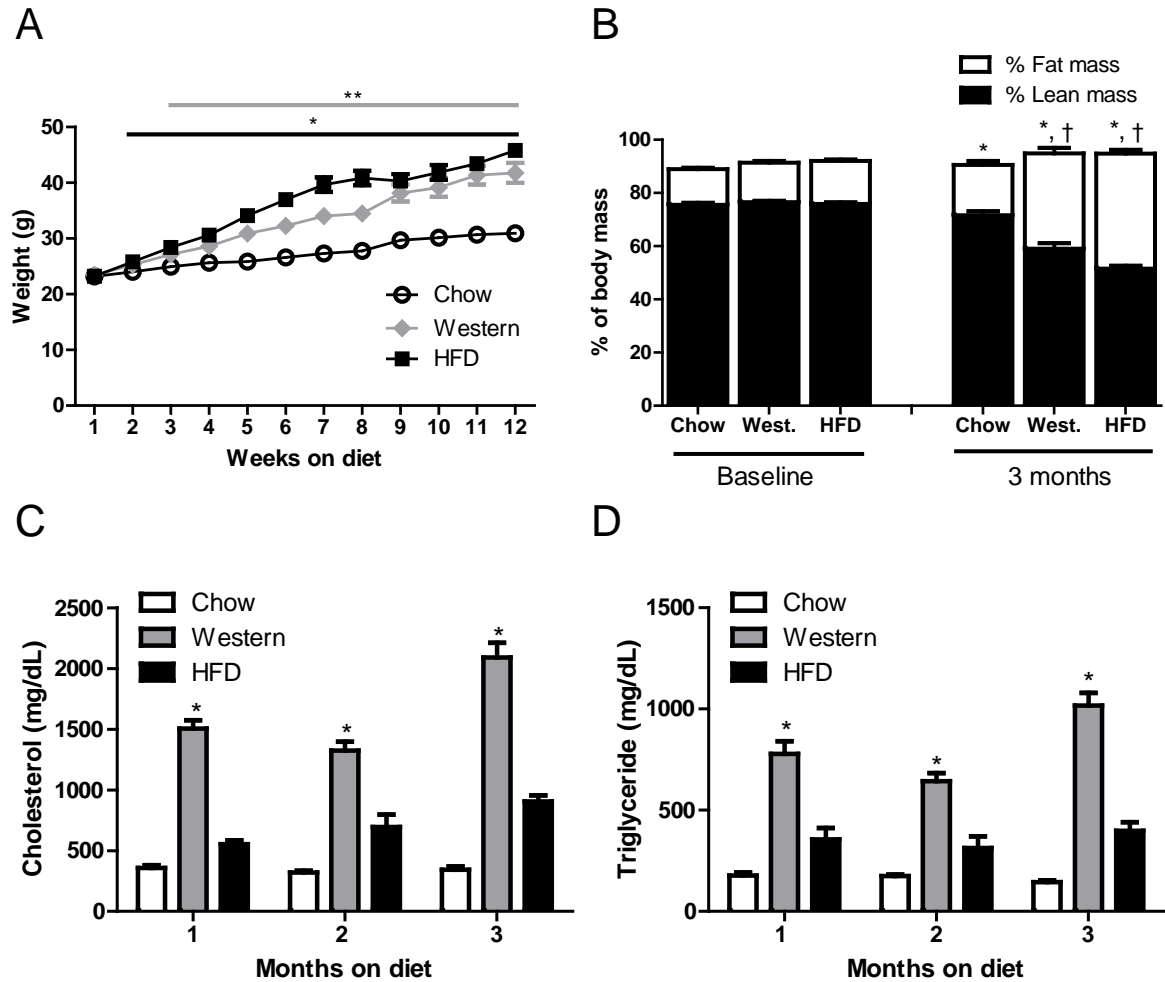
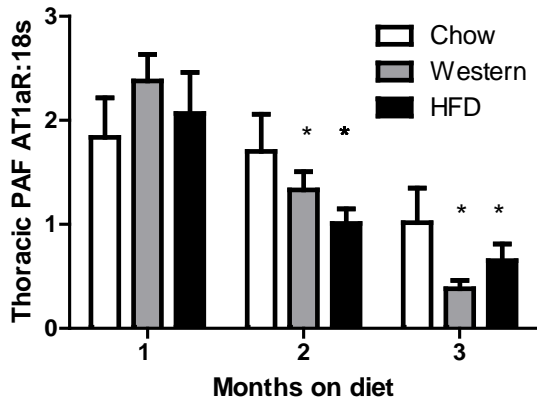
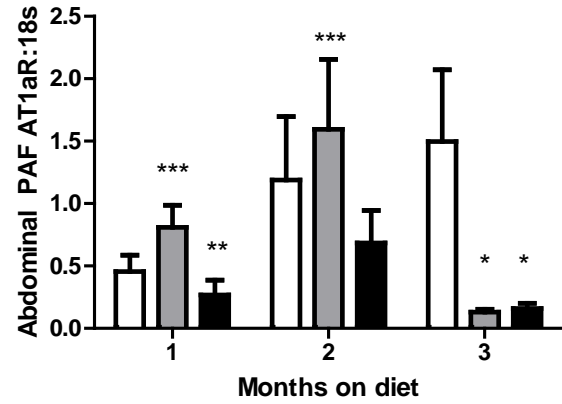


Figure 3.1 Characteristics of LDLR^{-/-} mice fed chow, Western, or HF diets for 1-3 months. (A) Body weights through the duration of the study. Compared to chow-fed mice, body weights of HF- and Western-fed mice were significantly higher beginning at week 2 (*, $P < 0.05$) and 3 (**, $P < 0.05$), respectively. (B) Mice of all groups had significantly increased % fat mass and reduced % lean mass after consumption of diets for 3 months (*, $P < 0.001$ compared to baseline) and mice fed HF or Western diet had greater fat mass/ reduced lean mass (†, $P < 0.001$ compared to chow after 3 months). Serum cholesterol (C) and triglycerides (D) were elevated in Western-fed mice compared to chow at each time point (*, $P < 0.05$). Data are represented as mean \pm SEM from 6-8 mice/group.

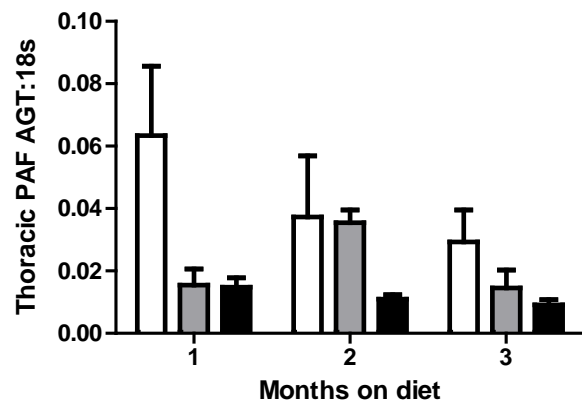
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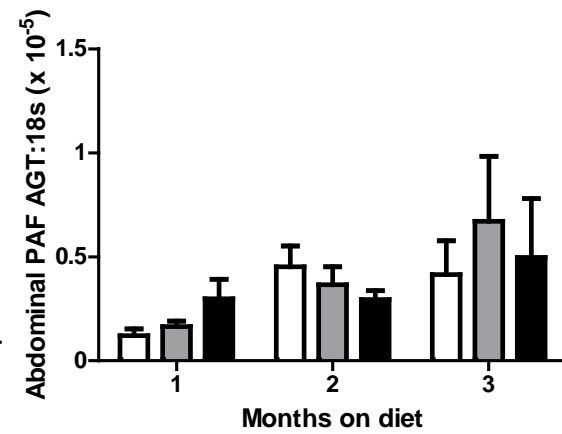
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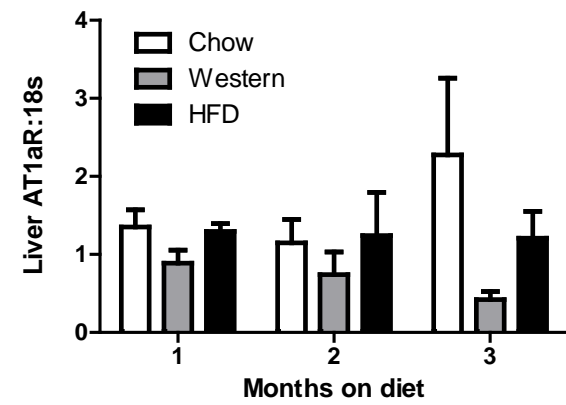
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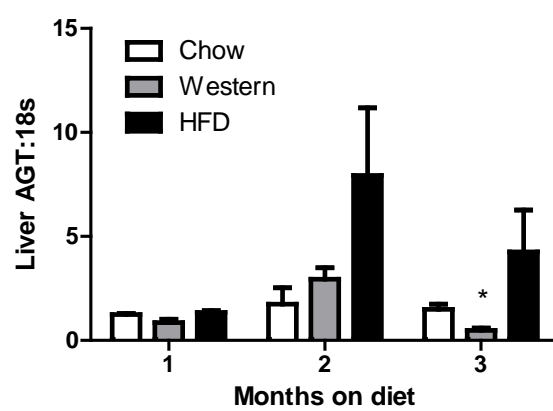


Figure 3.2 mRNA abundance of AT1aR and AGT in peri-aortic fat and liver.

AT1aR mRNA abundance was reduced in thoracic PAF (A) after 2 and 3 months of Western or HF diet consumption (*, $P < 0.05$ compared to 1 month within diet) AT1aR mRNA abundance in abdominal PAF(B) was suppressed in Western- and HF-fed mice (*, $P < 0.05$ compared to chow within time group; **, $P < 0.05$ compared to mice fed Western for 1 month; and ***, $P < 0.05$ compared to mice fed Western for 3 months). AGT mRNA abundance in thoracic (C) and abdominal PAF (D) were unaffected by diet. Liver AT1aR mRNA abundance (E) was unchanged, however AGT was modestly increased by HF diet and suppressed after 3 months of Western diet consumption (*, $P < 0.05$ compared to chow within month). Data are represented as mean \pm SEM from 6-8 mice/group.

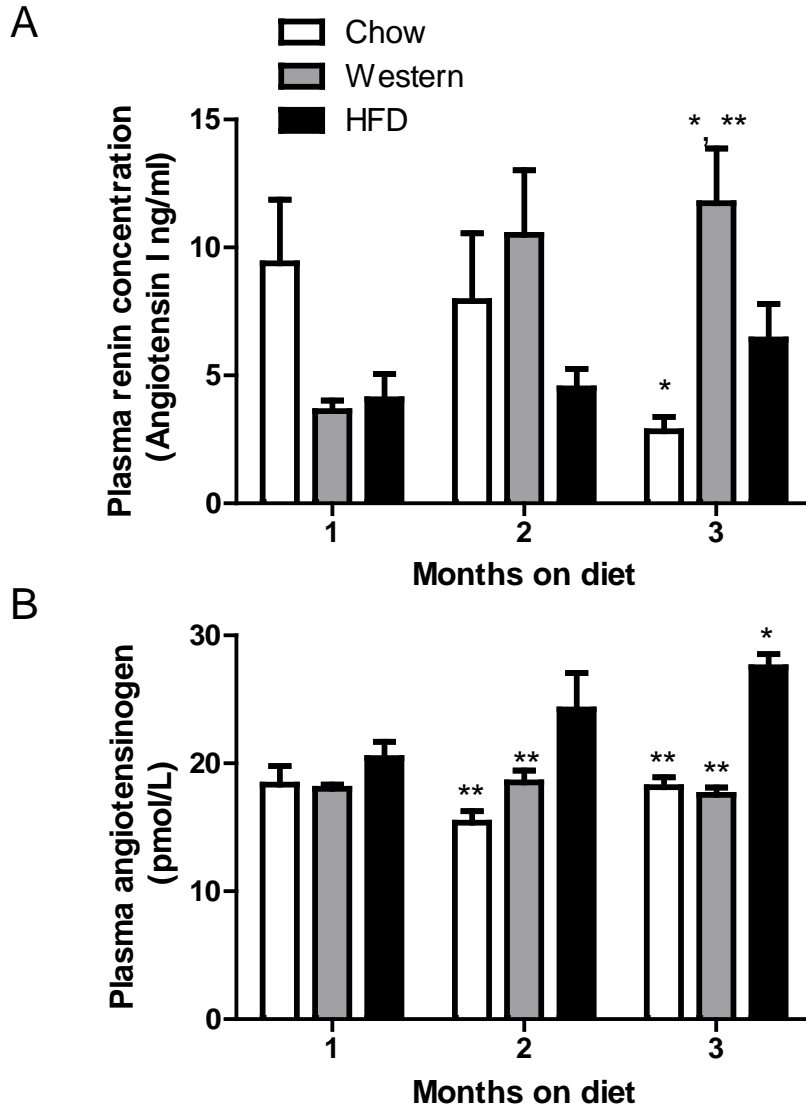


Figure 3.3 Plasma renin and AGT concentrations in $LDLR^{-/-}$ mice fed chow, Western or HF diets. A, Plasma renin concentrations were suppressed by chow diet and elevated by Western diet consumption after 3 months (*, $P < 0.05$ compared to 1 month within diet; **, $P < 0.05$ compared to chow within month). B, Plasma angiotensinogen levels were elevated by consumption of HF diets compared to chow or Western diets (**, $P < 0.001$) and compared to 1 month of HF diet consumption (*, $P < 0.05$). Data are represented as mean \pm SEM from 6-8 mice/group.

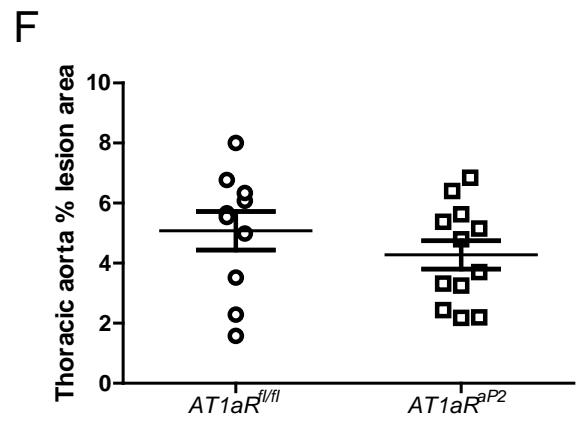
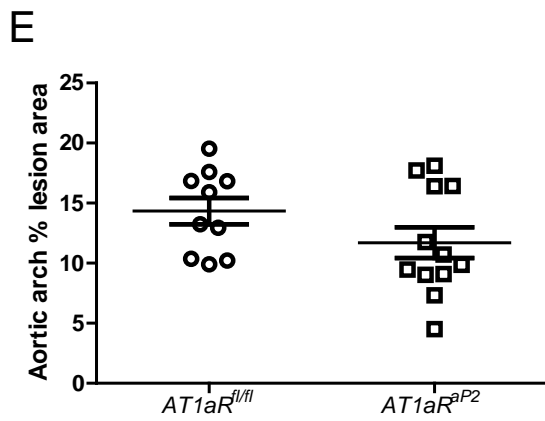
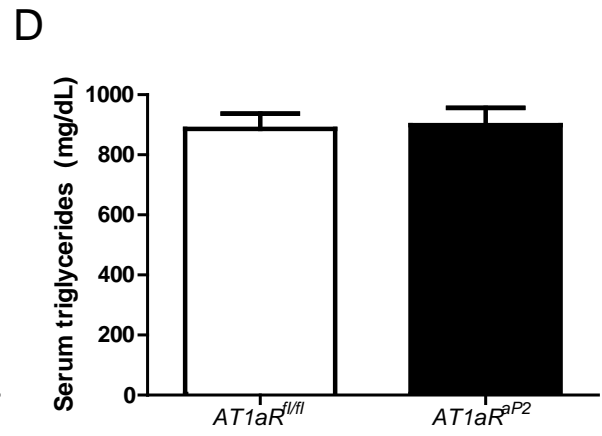
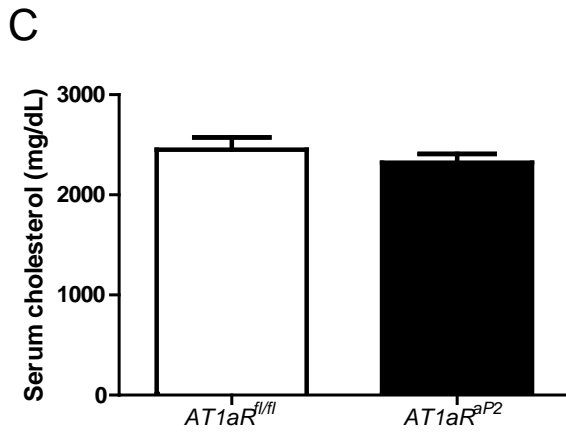
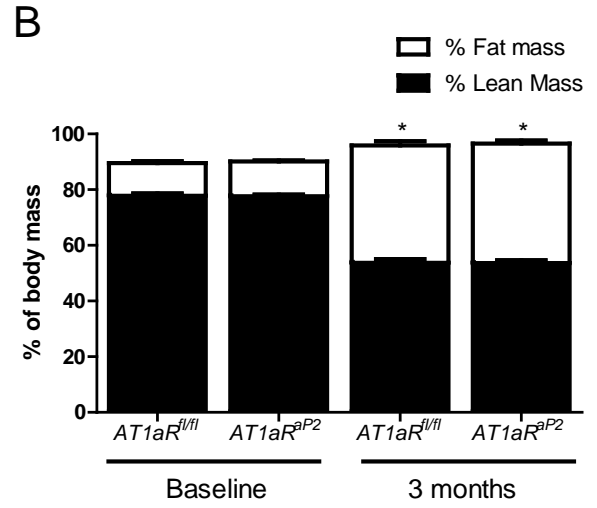
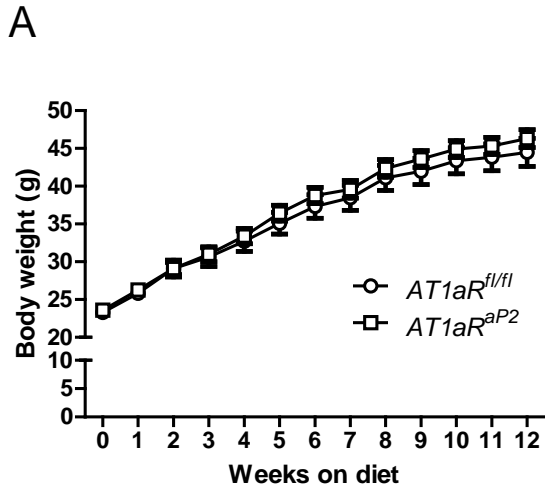


Figure 3.4 Adipocyte-AT1aR deficiency has no effect on diet-induced atherosclerosis in LDLR^{-/-} mice. A, Body weights of mice fed Western diet for 3 months. B, Percent fat mass was increased and lean mass decreased after 3 months of diet consumption (*, P < 0.05). Serum cholesterol (C) and triglycerides (D) were unaffected by adipocyte AT1aR deficiency. Lesion areas were calculated as a percentage of total area in both the aortic arch (E) and thoracic aorta (F). Data are represented as mean ± SEM from 10-12 mice/group.

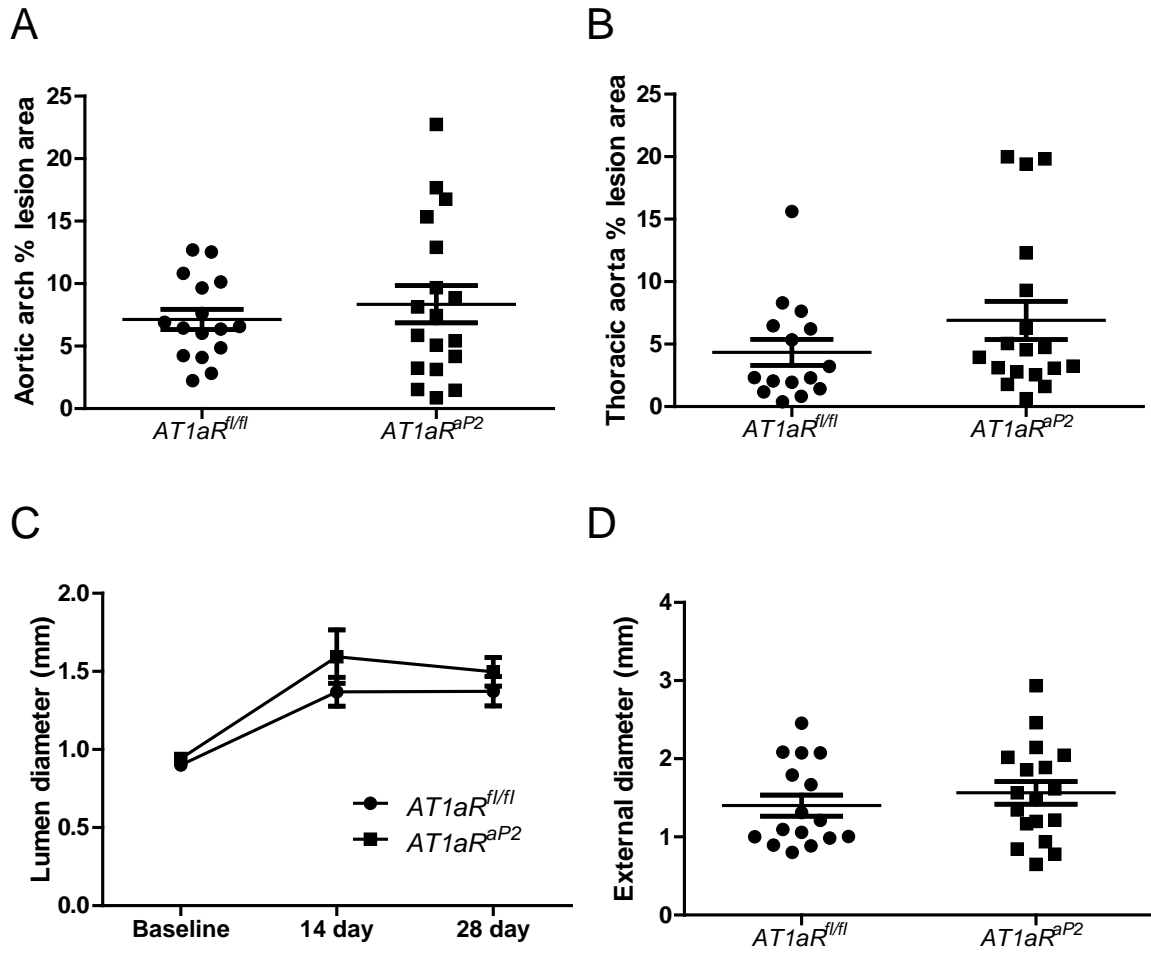
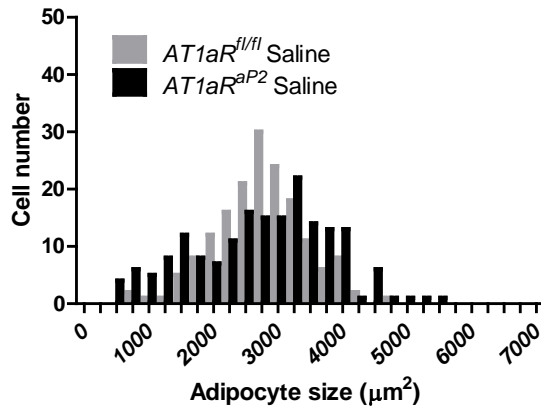
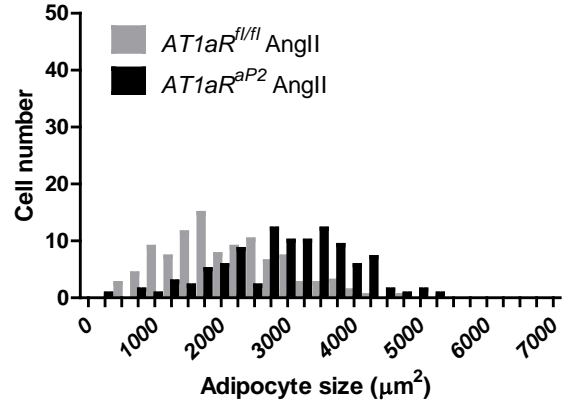


Figure 3.5 Adipocyte-AT1aR deficiency has no effect on the development of atherosclerosis or AAAs in AngII-infused mice. A, Lesion areas were calculated as a percentage of total area in both the aortic arch (A) and thoracic aorta (B). C, Lumen diameters were quantified from ultrasound images taken prior to, 14 days and 28 days into AngII infusion. D, Maximal external diameters were measured from aortas cleaned of peri-adventitial tissue.

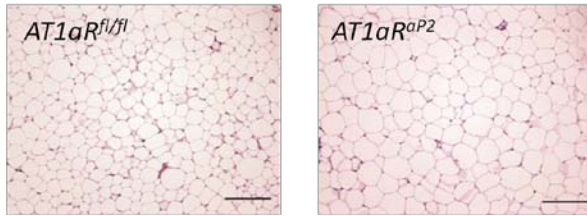
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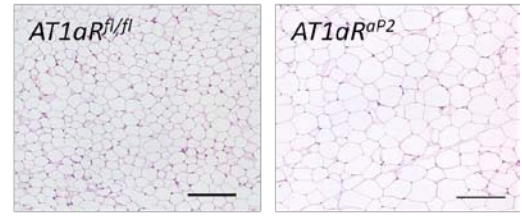
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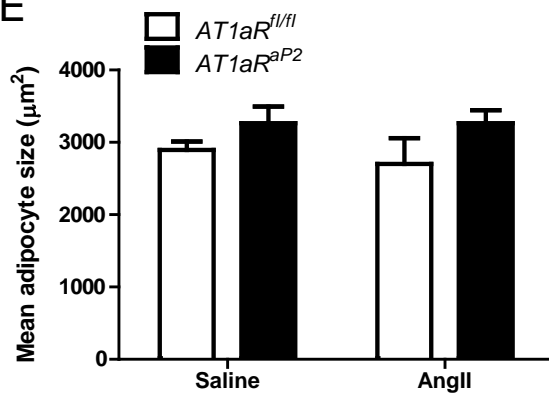
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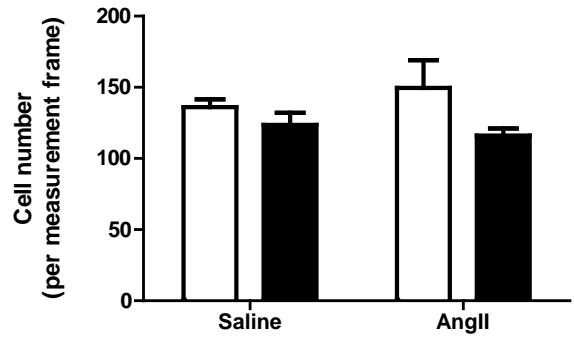
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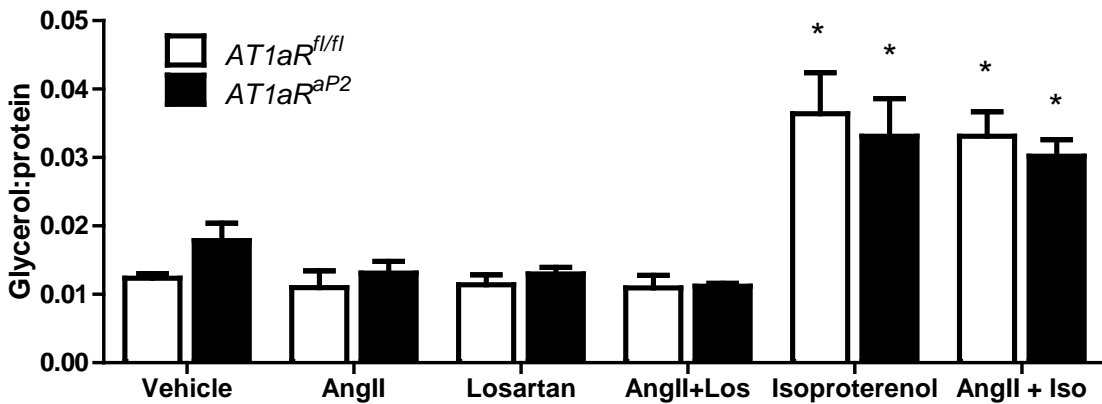


Figure 3.6 Adipocyte-AT1aR deficiency causes modest adipocyte hypertrophy in retroperitoneal fat (RPF) of LDLR^{-/-} mice. Frequency distributions of adipocyte size in RPF of mice infused with saline (A) or AngII (B). Representative images of H&E stained RPF from mice infused with saline (C) or AngII (D). Mean adipocyte size (E) and cell numbers per measurement frame (F) were unaffected by AngII infusion or adipocyte AT1aR deficiency. G, Production of glycerol from epididymal adipose explants was used to quantify lipolysis in response to incubation with AngII, losartan (Los), isoproterenol (Iso), as well as combinations of AngII with Los or Iso (*, P < 0.001, compared to vehicle).

SECTION IV: GENERAL DISCUSSION

4.1 Summary

The purpose of the studies described in this dissertation was to test the hypothesis that AngII regulates adipose tissue development, function, and inflammation through the adipocyte AT1aR. A mouse model of adipocyte-AT1aR deficiency was developed using the Cre/LoxP system to investigate these potential roles of the adipocyte-AT1aR in lean, obese, and hypercholesterolemic mice. *AT1aR^{aP2}* mice fed chow diet for one year exhibited no changes in body weight or fat mass. To stress these mice, we infused saline or AngII for 28 days, which led to modest weight loss in AngII infused mice of both genotypes. However fat mass and glucose tolerance were unchanged. Analysis of adipocyte morphology revealed modest adipocyte hypertrophy in saline-infused *AT1aR^{aP2}* mice. AngII infusion caused a reduction in adipocyte size in *AT1aR^{fl/fl}* controls, and, surprisingly, *AT1aR^{aP2}* mice were protected from this effect.

Adipocyte-AT1aR deficiency was also studied in mice fed LF or HF diets for a period of 16 weeks. Lack of AT1aRs had no effect on the body weight, fat mass, glucose intolerance, or blood pressure on either diet, however all mice fed HF diet had significant elevations in all of these parameters. Similar to AngII-infused mice fed chow diet, LF-fed *AT1aR^{aP2}* mice displayed striking adipocyte hypertrophy compared to *AT1aR^{fl/fl}* mice. This phenotype, however, was lacking in *AT1aR^{aP2}* HF-fed mice.

The adipocyte differentiation capacity of preadipocytes isolated from *AT1aR^{aP2}* mice was reduced, possibly due to downregulation of PPAR γ ,

providing a potential mechanism of adipocyte hypertrophy in which the remaining differentiated adipocytes in $AT1aR^{aP2}$ mice accrue more lipid over time. The positive regulation of adipocyte differentiation and PPAR γ mRNA abundance by AngII was also confirmed in 3T3-L1 cells.

The contribution of adipocyte-AT1aRs to vascular disease was also explored because while AngII is known to promote these diseases through the AT1aR, the cell type mediating these pathological effects remains unknown. Adipocyte-AT1aR deficiency in $LDLR^{-/-}$ mice fed Western diet for 3 months had no effect on the formation of atherosclerotic lesions in the arch or thoracic regions of the aorta. In a model of AngII-induced AAAs, adipocyte-AT1aR deficiency also had no effect on atherosclerosis or AAAs. Like LF-fed and AngII-infused chow-fed $AT1aR^{aP2}$ mice, adipocyte hypertrophy was also observed in $AT1aR^{aP2} \times LDLR^{-/-}$, however the effect was more modest which may relate to the effects of diets high in fat to suppress adipocyte differentiation. These changes in adipocyte size and fat mass occurred independent of any changes in lipolysis.

Overall, the results of these studies suggest that the primary role of the adipocyte AT1aR is to promote adipocyte differentiation. The *in vivo* manifestations of this effect appear to be a regulation of adipocyte size in lean, but not obese, mice. It is unclear why $AT1aR^{aP2} \times LDLR^{-/-}$ mice fed Western diet for 5 weeks with AngII infusion display moderate protection from AngII-induced reductions in fat mass, especially when the effects on adipocyte morphology are

modest, however future studies including measurements of food intake and energy expenditure may resolve this matter.

4.2 The interplay between the adipose RAS and metabolic syndrome: Insights from adipocyte-AT1aR deficiency

4.2.1 Regulation of the adipose RAS in obesity

AngII has been suggested to participate in a feed-forward loop to promote further AngII production by increasing the expression of AGT in liver (111) and adipose (154). A study from our group by Lu, et al. demonstrated that chronic AngII infusion increased mRNA abundance of AGT and AT1R in adipose tissue, however this regulation was absent in liver (154). Furthermore, increased AGT expression in adipose was attributed to AT1aR-mediated effects because losartan reduced increased expression in AT2R deficient mice. Obesity is associated with RAS activation and high levels of circulating AGT and AngII levels (100, 268), which may be due in part to increased AGT production by adipose tissue. It has been unclear, however, whether increased adipose AGT secretion in obesity is due to the AT1aR-mediated positive feed-forward loop in adipose tissue described by Lu, et al. To address this possibility, we measured plasma AGT levels in HF-fed *AT1aR^{fl/fl}* and *AT1aR^{aP2}* mice, and while adipocyte-AT1aR deficiency had no effect on plasma AGT concentrations, we did confirm that circulating AGT levels are increased with HF diet. AGT mRNA abundance was quantified in liver to determine if liver production of AGT was increased to compensate for possible reductions in adipose tissue, but liver and adipose AGT expression were unaffected by adipocyte-AT1aR deficiency. These results

indicate that AGT production in adipocytes is not regulated by a direct effect of AngII at adipocyte AT1aR. Increased circulating AGT despite reduced mRNA abundance in adipose is likely due to the dramatic increases in fat mass with obesity that result in overall increased levels of adipose-derived AGT.

4.2.2 Contribution of the adipose RAS to metabolic syndrome

Most of the obesity-related disorders associated with the metabolic syndrome (Table 1.3) were unaffected by adipocyte-AT1aR deficiency, including high blood pressure, glucose intolerance, and atherogenic dyslipidemias. Although blood pressure was elevated in mice fed a HF diet or infused with AngII, adipocyte-AT1aR deficiency did not result in lower blood pressures in chow, LF, HF, or Western-fed mice. Glucose tolerance was measured in chow-fed mice, LF-, and HF-fed mice without any changes in *AT1aR^{adP2}* mice compared to controls. Total cholesterol and triglyceride serum concentrations were not influenced by adipocyte-AT1aR deficiency in mice fed Western diet for 3 months or for 5 weeks with concurrent 28-day AngII infusion. Deficiency of adipocyte AT1aRs also did not impact weight gain in mice chronically fed HF or Western diets, however unexpected elevations in body weight and fat mass were observed in AngII-infused mice fed Western diet for 5 weeks. These results would indicate that adipocyte-AT1aR deficiency has no protective role in the development of the various elements of metabolic syndrome.

4.3 Limitations of the model of adipocyte-AT1aR deficiency

4.3.1 Non-specific reductions in AT1aR mRNA abundance

The model of adipocyte-AT1aR deficiency used in these studies was developed using the Cre/LoxP system, in which the only coding exon of the AT1aR gene, exon 3, was flanked by LoxP sites and mice were bred to transgenic mice expressing Cre recombinase driven by the aP2 promoter. The aP2 protein, also known as fatty-acid binding protein 4 (Fabp4), was originally described as adipocyte-specific and used by several groups to reduce gene expression in adipocytes. However, as more of these models have been made, it has become clear that the aP2 gene is also expressed in other tissues, such as cardiomyocytes. Indeed, mRNA abundance of AT1aR in *AT1aR^{aP2}* mice was reduced significantly in the heart. To better understand the non-specific expression of Cre recombinase driven by the aP2 promoter, we bred *AT1aR^{aP2}* mice to ROSA26 mice, which contain a stop codon flanked by LoxP sites within the β -galactosidase gene (241). In tissues where Cre recombinase is expressed, the stop codon is removed, active β -galactosidase is produced, and activity of this enzyme can be detected through X-gal staining as a marker of Cre recombinase expression. Several tissues were collected from *AT1aR^{fl/+},aP2* x *ROSA26^{+/-}* (Cre 0/+) mice for X-gal staining and compared to the robust staining in brown and white adipose depots, staining in heart tissue was present but faint. Moreover, quantification of heart weight to body weight ratios as a measure of cardiac hypertrophy, which is AT1aR-mediated (195), revealed no differences between *AT1aR^{fl/fl}* and *AT1aR^{aP2}* mice (data not shown). Additionally, although

AngII can have positive chronotropic and inotropic effects (171, 270), there were no reductions in heart rate (data not shown) or blood pressure in *AT1aR^{aP2}* mice. Therefore, these data suggest that the reductions in heart AT1aR mRNA abundance were not physiologically relevant.

4.3.2 Model validation

Validating models of cell-specific AT1aR deletion at the protein level is difficult due to the lack of a specific AT1aR antibody (212). AT1aR mRNA abundance was quantified in whole adipose tissue lysates, the adipocyte fraction of collagenase-digested adipose tissue, as well as in adipocytes differentiation from isolated stromal vascular cells, and residual AT1aR mRNA abundance was detectable in all of these adipocyte sources from *AT1aR^{aP2}* mice. This may reflect sub-optimal efficiency of Cre recombinase enzyme activity at the LoxP sites surrounding exon 3 of the AT1aR gene. Another possibility is that AT1aR deficiency regulates transcription factors controlling aP2 promoter activity. In adipocytes differentiated *ex vivo* from *AT1aR^{aP2}* mice, aP2 mRNA abundance was reduced ($p = 0.052$). If this regulation by AT1aR is at the aP2 promoter, then it suggests reduced aP2 promoter activity in response to AT1aR deficiency may subsequently reduce the expression of Cre recombinase, thus compromising the deletion of AT1aR. The use of another promoter to drive Cre recombinase activity may be one solution to this issue as well as the issue of non-specific expression described above. The adiponectin promoter has recently been suggested to be more efficient and specific than the aP2 promoter for

adipocyte-specific gene deletion; however studies from our lab using this promoter also show reductions in target gene expression in heart.

4.4 Insights into regulation of adipocyte size and fat mass by AngII

4.4.1 Differential consequences of adipocyte differentiation in LF- and HF-fed mice

The finding that adipocyte AT1aR deficiency regulates adipocyte differentiation and size *in vivo* could be through several potential mechanisms. AngII promotes adipocyte differentiation through the adipocyte AT1aR by upregulating PPAR γ expression, which is a major regulator of terminal adipocyte differentiation from committed preadipocytes (Figure 4.1A). When stromal vascular cells containing preadipocytes were isolated from subcutaneous adipose depots of *AT1aR^{fl/fl}* and *AT1aR^{aP2}* mice, preadipocytes from *AT1aR^{aP2}* mice had reduced lipid accumulation and mRNA abundance of PPAR γ . The *in vivo* consequences of reduced adipocyte differentiation are increased lipid accumulation and hypertrophy of remaining cells (13). Interestingly, this resultant adipocyte hypertrophy was observed in LF-fed, but not HF-fed, *AT1aR^{aP2}* without any adverse metabolic consequences.

The enhancement of adipocyte differentiation in response to AngII is arguably a good thing. Research by several groups indicates that formation of many small adipocytes is less of a health risk than the development of hypertrophied adipocytes, which can become inflamed and eventually lead to insulin resistance (248). One potential mechanism linking adipocyte hypertrophy to inflammation may be an insufficient angiogenic (40) or an increased fibrotic

(104) response to hypoxic conditions experienced by adipocytes, leading to cell death and macrophage infiltration (277). While our finding that mice lacking the adipocyte AT1aR experience adipocyte hypertrophy is consistent with a reduced capacity for differentiation, the lack of an inflammatory response to this striking level of adipocyte hypertrophy is puzzling. Conceivably, the inflammatory response to adipocyte hypertrophy may be mediated by the actions of AngII through the AT1aR, in which case deficiency of this receptor would cause hypertrophy on the one hand, but simultaneously protect from the inflammatory response. This scenario, however, seems unlikely because it would suggest that HF-fed *AT1aR^{ap2}* mice should be similarly protected from adipose inflammation and related insulin-resistance, however these mice show no improvements in glucose or insulin tolerance tests. Alternatively, our data suggests that lean mice were capable of accommodating larger adipocytes without resulting inflammation, possibly because the fat pads were still small, such that sufficient blood flow was maintained to avoid the hypoxic conditions that lead to adipocyte cell death. Our results indicate that adipocyte hypertrophy alone is insufficient to drive the development of obesity-related insulin resistance in otherwise lean mice.

The lack of an effect of adipocyte-AT1aR deficiency on adipocyte size, fat mass, and body weight in HF-fed was initially surprising. However, further reading of the literature revealed that adipocyte differentiation may be suppressed in the setting of obesity (13, 116, 177, 197). Specifically, the ability of isolated human preadipocytes to successfully differentiate is inversely

correlated with BMI (116), waist circumference or percent body fat (197), as well as adipocyte size (referring to the size of adipocytes collected after collagenase digestion)(101, 116). In fact, some suggest the initiating event in the development of inflamed adipose tissue is the inhibition of adipogenesis and this inability to recruit new adipocytes causes the adipocyte hypertrophy that leads to obesity-related disorders (14, 101). These groups distinguish between “hyperplastic obesity” (i.e., many small adipocytes) and “hypertrophic obesity” (fewer but larger adipocytes) in which adipocyte size, not necessarily BMI, determines the metabolic health of adipose tissue. For instance, one study found that women with “hyperplastic obesity” have significantly lower plasma insulin and HOMA-IR levels compared to women with similar BMI characterized as having “hypertrophic obesity” (13). Conversely, lean individuals with a genetic predisposition for type 2 diabetes have significantly greater adipocyte size compared to lean individuals with no predisposition for type 2 diabetes (14). The mechanism through which adipogenesis is inhibited in obesity appears to be a defect in the differentiation process and not an inability to recruit stem cells because the number of precursor cells actually increases with obesity (116, 155). Inhibited differentiation may be due to an inability to suppress canonical anti-adipogenic WNT signaling pathways because incubation with a WNT inhibitor, DKK1, restores differentiation of preadipocytes from obese individuals to 80% that of normal weight subjects (101). With all of this in mind, the reason for a lack of effect of adipocyte-AT1aR deficiency in HF-fed *AT1aR^{ΔP2}* mice may be the general suppression of adipocyte differentiation already occurring in HF-fed mice.

These changes in WNT signaling must occur upstream of the effects of AT1aR on PPAR γ in the signaling cascade determining adipocyte differentiation since adipocyte-AT1aR deficiency did not affect HF-induced downregulation of differentiation.

4.4.2 Lipolysis

Lipolysis presents another potential mechanism through which AngII may regulate adipocyte size. Lipolysis is the hydrolysis of triglycerides, the primary form of lipid storage within adipocytes, into free fatty-acids and glycerol. If AngII stimulated lipolysis, this mobilization of fatty-acids from adipocytes would limit adipocyte size and deficiency of adipocyte AT1aRs would have more lipid accumulation, which is consistent with our findings. However, analysis of lipolysis from adipose tissue explants determined that AngII did not directly stimulate lipolysis. If anything, there was a modest increase in basal glycerol production from *AT1aR^{ap2}* explants, which would conflict with the *in vivo* adipocyte size quantifications of hypertrophy in these mice. An inhibitory effect of AngII on lipolysis is consistent with several studies in the literature (31, 96), including one specifically showing that this effect in adipocytes is AT1R-mediated (95). However, it is still possible that AngII may participate in long-term regulation of lipolytic enzymes that neither our lipolysis explants assay nor the microdialysis with AngII infusion experiments of others are designed to elucidate. Future work should determine the phosphorylation status and enzyme activity of hormone-sensitive lipase and adipose triglyceride lipase with a longer duration of AngII incubation to determine if AngII regulates lipolysis through this mechanism.

4.5 Future directions

4.5.1 Other mechanisms through which AngII may regulate adipocyte differentiation and size through the AT1aR

4.5.1.1 Adipocyte aldosterone secretion

It has recently been reported that adipocytes are capable of producing not only mineralocorticoid-releasing factors (74), but also aldosterone itself (35). Briones, et al. reported that stimulation of either 3T3-L1 or isolated adipocytes with AngII increases aldosterone secretion as well as aldosterone synthase expression (35). Interestingly, this group also found that incubation of 3T3-L1 preadipocytes with a specific inhibitor of aldosterone synthase decreased adipocyte differentiation significantly (35). Therefore, AngII may stimulate adipocyte aldosterone production which then acts in an autocrine manner at adipocyte mineralocorticoid receptors to promote adipocyte differentiation (41).

4.5.1.2 Conversion of white to brown adipocytes

White adipocytes are capable of taking on the phenotypic characteristics of brown adipocytes, such as increased mitochondrial biogenesis and thermogenesis, through a process commonly referred to as transdifferentiation (279). This process is primarily mediated by the actions of PRDM16 to increase the expression of PGC-1 α , UCP-1, Cidea, and other proteins that are hallmarks of brown adipocytes (233-234) and can be induced by cold exposure (19). The ability of white adipocytes to transdifferentiate presents an interesting mechanism through which adipocyte size may be regulated. The accumulation of brown-like

adipocytes in traditionally white adipose depots would likely mobilize fatty acids from lipid droplets for oxidation and thermogenesis, reducing lipid accumulation in white adipocytes. Conversely, reducing transdifferentiation may result in enlarged adipocytes due to increased lipid accumulation. In our studies, UCP-1 expression was reduced significantly in subcutaneous adipose of *AT1aR^{aP2}* mice fed LF diet (data not shown), and reduced transdifferentiation is consistent with the adipocyte hypertrophy observed in these mice. However, data collected from calorimetry chambers during the final week of diet did not reveal any changes in oxygen consumption (data not shown) nor was body temperature affected by adipocyte-AT1aR deficiency in any of the models in which it was measured (chow-fed and Western-fed mice infused with saline/AngII). Nonetheless, other groups are currently investigating the role of adipocyte AT2Rs in the process of differentiation and have demonstrated that administration of an AT2R agonist increases UCP-1 expression in 3T3-L1 adipocytes (data presented at Gordon Research Conference 2012 by Justin Grobe; as yet unpublished). Further investigation is required to determine if white adipocyte transdifferentiation to brown adipocytes plays a role in the adipocyte hypertrophy and increased fat mass observed in our models of adipocyte-AT1aR deficiency.

4.5.2 Further exploration of the adipose RAS

4.5.2.1 Overactivation of adipose RAS

An interesting experiment would be to overexpress the AT1aR in adipocytes and study the effects *in vivo*. Because the function of adipocyte AT1aRs has been poorly understood, overexpressing the receptor to

supraphysiological levels would amplify the effects of AngII and perhaps clarify what pathways are modulated in adipocytes. Establishing the signaling pathways and physiological processes affected by AngII would guide future experiments with adipocyte-AT1aR deficient mice. According to our studies, AngII promotes adipocyte differentiation through the AT1aR, so overexpression of this receptor may further increase the number of small adipocytes, though other outcomes are also possible. These effects may also translate into changes in fat mass, glucose tolerance, and blood pressure in lean, obese, and hyperlipidemic mice. Adipocyte-AT1aR deficiency led to increased fat mass in AngII-infused mice fed Western diet, so overexpression of adipocyte AT1aRs may result in weight loss in this model. No differences in adipocyte size or adipose weights were observed in HF-fed *AT1aR^{AP2}* mice, possibly due to HF diet-induced impairment of adipocyte differentiation, but AT1aR overexpression might restore differentiation in these mice. However, the ability of AT1aR overexpression to restore adipocyte differentiation in HF-fed mice would depend on the exact nature of the suppression of differentiation and whether or not AT1aR signaling can overcome the epigenetic changes brought on by uninhibited canonical WNT signaling (101).

Studies of adipocyte-AGT overexpression have demonstrated modest increases in adiposity associated with increased glucose intolerance and adipose inflammation in lean mice (134, 291). These effects were notably absent in HF-fed mice, similar to our findings with adipocyte-AT1aR deficiency (134). Although the effects of adipocyte-AGT overexpression on adipose tissue were normalized

by AT2R deficiency in the AGT-overexpression model (291), there may be a role for enhanced AT1aR activation in AT2R knockout mice and it is also possible that AT2R deficiency in non-adipocyte cells contributed to these effects. Overexpression of AT1aRs in adipocytes would clarify whether any of the effects of AGT overexpression were through direct effects of AngII at the adipocyte AT1aR, or conversely, adipocyte AGT overexpression could be studied in the context of adipocyte AT1aR deficiency.

Another method of possibly increasing the activity of the local RAS in adipose tissue would be to make a mouse model of adipocyte-specific ACE2 deficiency. ACE2 is expressed in adipose tissue and activity of ACE2 in adipose is blunted after 4 months of HF diet, possibly contributing to the increased circulating levels of angiotensin peptides observed with HF feeding (100). Whole-body ACE2 deficiency has been shown to increase atherosclerosis (259). It might be particularly interesting to observe the effects of adipocyte-ACE2 deficiency in models of vascular disease to see if reductions in adipocyte-ACE2 contribute to the effect observed in whole-body knockouts. Reduced catabolism of AngII by ACE2 might locally increase AngII levels in perivascular adipose tissue, enhancing the effects of AngII on adipose tissue macrophages and nearby vascular wall cells.

4.5.2.2 Exploring the role of adipocyte AT2Rs

In addition to AT1aRs, adipocytes also express AT2Rs. Previous studies regarding the role of both receptors in the process of adipocyte differentiation have been mixed and inconclusive (61, 85, 220-221, 225). It would be

interesting to compare adipocyte deficiency of AT1aRs to that of AT2Rs and AT1aR/AT2R double knockouts to further delineate the effects that AngII can have on adipocytes. Complications of these studies include the possibilities that deficiency of one receptor may change the expression of the other receptors or enhance AngII signaling through the remaining receptor. *AT1aR^{aP2}* mice in our studies did not show any transcriptional regulation of AT2Rs in response to AT1aR deficiency in adipocytes; however the possibility of enhanced signaling remains. The development of mice with adipocyte AT1aR and AT2R deficiency would help clarify this point.

In *AT1aR^{aP2}* mice we observed a reduction in differentiation. If there is spillover of AngII to enhance AT2R signaling in this mice, there are 3 possibilities: AT2Rs have no effect on differentiation; AT2Rs inhibit differentiation; or AT2Rs weakly stimulate differentiation but deficiency of AT1aRs has a more powerful effect to reduce differentiation. *AT1aR/AT2R^{aP2}* mice would help distinguish between these possibilities. If the double adipocyte “knockouts” are phenotypically similar to *AT1aR^{aP2}* mice then AT2Rs likely have no effect. If AngII promotes differentiation through the AT1aR and inhibits it through the AT2R, then *AT1aR^{aP2}* mice would display the greatest reduction in differentiation and *AT1aR/AT2R* double “knockouts” would be similar to floxed controls. Conversely, if AngII promotes differentiation through both receptors, then *AT1aR/AT2R* mice would exhibit reduced adipocyte differentiation to a greater extent than that of *AT1aR^{aP2}* mice. While floxed AT2R mice are not currently available, these would be the most useful tool to understanding the

possible roles of AT2Rs in adipocytes because the alternative options, using whole-body AT2R knockout mice or treating *AT1aR^{aP2}* mice with a pharmacologic AT2R antagonist may have indirect effects through other tissues.

4.5.3 Dietary manipulations

In our studies, adipocyte-AT1aR deficiency was studied under several conditions including aged mice fed chow diet (with or without AngII infusion), lean mice, obese mice, hypercholesterolemic mice chronically fed Western diet, and hypercholesterolemic mice fed Western diet for 1 month (with or without AngII infusion). The most striking effects on adipocyte size was observed in LF-fed mice and in chow-fed mice infused with AngII (Figure 4.1B), suggesting that the effects of adipocyte-AT1aR deficiency may be most relevant in lean mice with high AngII levels and future studies may employ the following models to achieve these conditions.

4.5.3.1 Low salt diet

To maintain blood pressure under low-salt conditions, increased renin secretion increases AngII production, which then stimulates aldosterone secretion to induce sodium and water retention. Feeding *AT1aR^{aP2}* mice a low salt diet represents a unique model where normal weight mice have a physiologically activated RAS, in contrast to AngII infusion which is an artificial means of increasing systemic AngII levels and has the added complication of potential weight loss. A low-salt diet with similar fat content as normal chow may provide the necessary conditions for the maximum reduction of adipocyte

differentiation or other effects of adipocyte-AT1aR deficiency, which may then result in altered adipose tissue function.

A few studies have investigated the effects of high and low salt diets on adipose tissue. Lima, et al. found that Wistar rats fed a high salt diet for 6 weeks have increased white adipose tissue mass compared to normal and low salt groups (83). Interestingly, lipolysis assays performed on an adipocyte suspension found that basal and isoproterenol-induced lipolysis was stimulated in rats fed a high salt diet (83). This is consistent with an inhibitory effect of AngII on lipolysis, because AngII levels are suppressed in high salt conditions so the inhibition of lipolysis would be curtailed. Interestingly, a human study examining changes in adipose RAS components with low and high sodium diets found that expression of AGT, AT1R, and renin in subcutaneous adipose tissue were unchanged but ACE expression was actually upregulated with a high sodium diet (75). These results suggest that circulating and tissue RAS components may be subject to differential regulation by sodium intake and adipose tissue itself can be directly influenced by sodium status. Experiments using our model of adipocyte-AT1aR deficiency would determine if the described changes in white adipose tissue due to sodium intake are due to direct effects of AngII at the adipocyte AT1aR.

4.5.3.2 High carbohydrate diet

The LF and HF diets used in our studies are matched for protein content (20% kcal as protein) and are comprised of varying amounts of the same ingredients. The HF diet contains roughly 12 times more lard than the LF to

increase the fat content and consequently, the LF diet contains 3.5 times more carbohydrates, which makes the LF diet arguably a high carbohydrate diet (70% kcal from carbohydrates). From our studies, LF diet was the greatest stimulus for adipocyte hypertrophy in *AT1aR^{AP2}* mice, which may suggest a role for adipocyte AT1aRs in the response of adipose tissue to a high carbohydrate diet and other high carbohydrate diets may provide interesting models to further elucidate the roles this receptor. AT1aR mRNA abundance in epididymal adipose tissue of rats was increased 50% by 14 days of high fructose diet (66% kcal from fructose) and increased 300% by 3 weeks of a high fructose, high salt diet; however adipose tissue mass or morphology was not characterized in this study (92). High sucrose diets also stimulate AT1aR expression specifically in rat epididymal adipose tissue but not in renal tissue (55). If this drastic upregulation of receptor expression is in adipocytes and not other cell types, then studying adipocyte-AT1aR deficiency under either of these dietary conditions may reveal further roles of adipocyte AT1aRs.

4.5.3.3 Fasting or caloric restriction

Because the most dramatic effects of adipocyte-AT1aR deficiency on adipocyte hypertrophy were observed in lean mice, the purpose of adipocyte AT1aR may be to protect adipocyte differentiation in lean times. Therefore, adipocyte-AT1aR deficient mice subjected to either acute fasting or chronic caloric restriction might lose more weight under these conditions compared to control mice, wherein AngII can temper the loss of adipose mass by promoting differentiation and/or possibly inhibiting lipolysis. One study examining the

effects of an overnight fast in AT2R knockout mice found that adipose AT1aR mRNA abundance doubled in response to fasting (290). Furthermore, AT2R knockout mice administered valsartan did indeed experience further reductions in adipocyte size and plasma NEFAs in response to fasting, supporting the idea of a protective role for AngII/AT1aR signaling in adipose under these conditions (290). While this study was performed with an acute overnight fast, the protective role of AngII on adipose tissue may persist in the setting of prolonged undernutrition, such as caloric restriction. The elevated fat mass observed in our studies of hyperlipidemic *AT1aR^{aP2}* mice infused with AngII suggest that this theoretical protective role of AngII may be specific to lean mice, and becomes dysregulated in hypercholesterolemic mice fed Western diet. Alternatively, if *AT1aR^{aP2}* mice exposed to fasting or caloric restriction are also protected from the resulting reductions in body weight or fat mass, it would suggest that AngII acts through the adipocyte AT1aR to limit adipose tissue growth in some manner, though more studies would be required to determine if this is due to the established pro-differentiation effects of AngII.

4.6 Clinical Implications

The primary role of AngII acting through adipocyte AT1aRs is to promote adipocyte differentiation, suggesting that AngII would have similar effects in humans as pharmacologic PPAR γ ligands, by increasing the number of small, healthy adipocytes and potentially increasing insulin sensitivity. This would imply that treatment with AT1R antagonists would result in adipocyte hypertrophy and potentially increased fat mass, as observed in *AT1aR^{aP2}* mice studied here.

However, clinical trials to date have found little evidence that ARBs influence body weight of patients taking them, especially when considering ARBs lacking PPAR γ -agonizing properties that may represent AT1R-dependent effects more closely, such as losartan or valsartan (113, 132, 164, 236). While two recent studies have found that losartan may reduce adiposity, measured by ultrasound (82) or waist circumference (7), the majority of studies have failed to find any effect of losartan on fat mass. These clinical trials primarily include individuals with various combinations of metabolic risk factors in their study populations and thus limit their focus to overweight or obese patients. Our data would suggest that the lack of an effect of ARB treatment on body weight may relate to the epigenetic suppression of adipocyte differentiation in the setting of obesity, which limits the effect of blocking the AT1aR on adipose tissue.

Adipocyte AT1Rs may play a more significant role in lean people, especially those with an activated RAS. These conditions are met in patients with chronic heart failure and certain cancers known to have high circulating levels of AngII and experience weight loss (69). Data from rodent models confirm that chronic high doses of AngII cause weight loss (34, 37, 47, 68). Weight loss and cachexia in chronic heart failure patients is associated with a worse prognosis, which is the basis of the “obesity paradox” whereby obese patients fare better (66). Indeed, clinical trials indicate that administration of candesartan (201) or enalapril (10), though not losartan (200), mitigate weight loss in patients suffering from heart failure. These cachectic effects of AngII have been attributed to increased sympathetic nervous activity (77) or muscle atrophy

due to reduced insulin-like growth factor-1 (IGF-1) (34), however, reduced adiposity is a prominent feature of this phenotype and few have suggested a direct effect of AngII at adipocyte AT1Rs as a cause of weight loss. Reduced adiposity may be due to a stimulatory effect of AngII on lipolysis (37, 68), however other animal studies indicate that AngII has, if anything, an anti-lipolytic effect on adipocytes (31, 96) and our own data show no effect of AngII on lipolysis. Alternatively, AngII may not contribute to weight loss through the adipocyte AT1Rs, but may instead have a compensatory role to protect against too much weight loss and preserve the adipose depot by stimulating adipocyte differentiation. In this case, the success of RAS blockade depends on the magnitude of pro-cachectic effects in some tissues weighed against the anti-cachectic effects at adipocytes.

4.7 Concluding remarks

Overall these studies suggest the primary role of AT1aRs on adipocytes is to promote adipocyte differentiation and increase or maintain the number of small adipocytes. The consequences of AngII acting through adipocyte AT1aRs are most prominent in lean conditions and are absent in the setting of obesity. Future studies should identify the mechanisms through which AngII regulates adipocyte differentiation and explore non-obese models of RAS activation to fully understand the clinical significance of these findings. Additionally, the impact of ARB administration on body weight and fat mass in patients suffering from cachexia should be studied in greater detail.

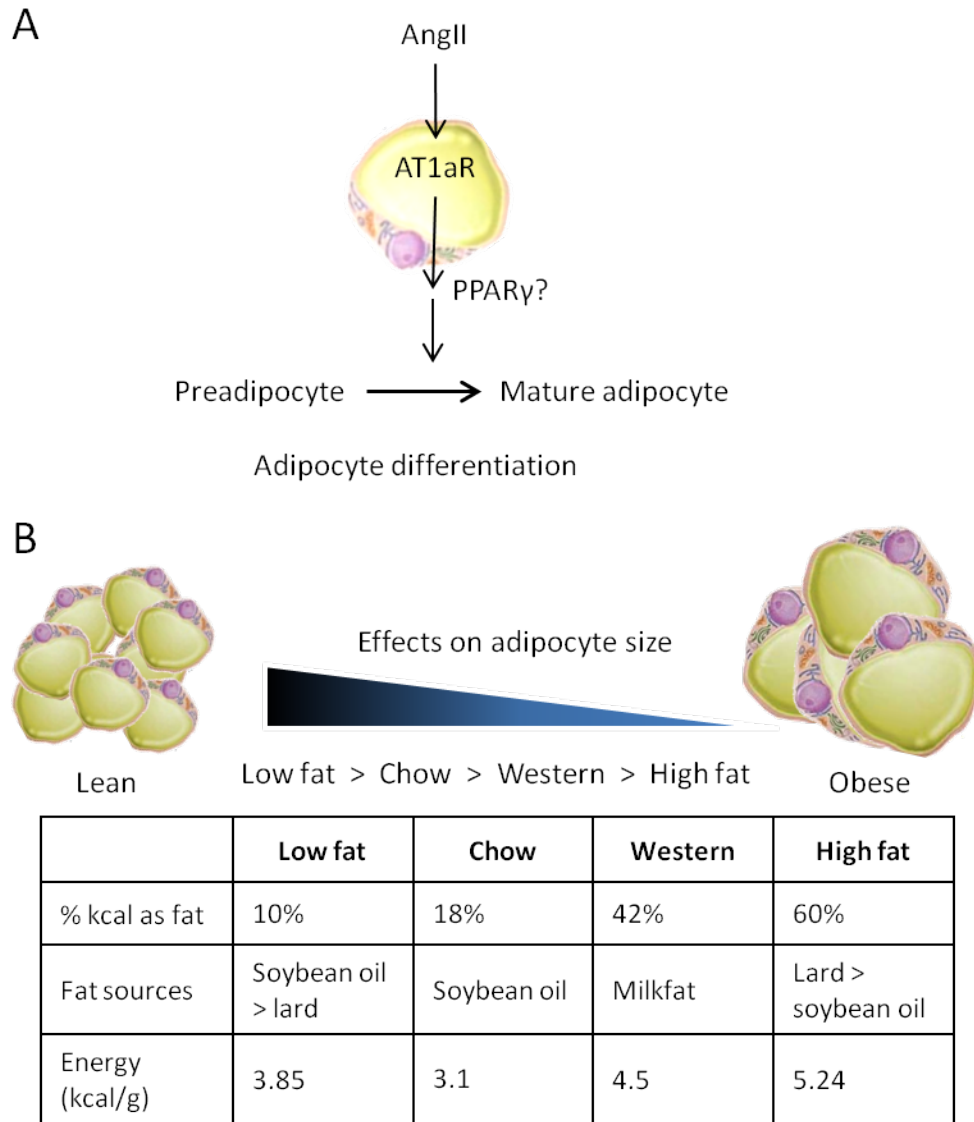


Figure 4.1 Role of adipocyte AT1aR in adipocyte differentiation. A, AngII promotes adipocyte differentiation and the formation of small adipocytes through adipocyte AT1aRs. B, The effect of adipocyte AT1aR deficiency to promote adipocyte hypertrophy was greatest in lean mice and there was no effect in obese mice, which may be due to the amount or source of fat in the diets used across the various models.

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VITA

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Educational History

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University of Kentucky, Lexington, KY

PhD Candidate

Graduate Center for Nutritional Sciences

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University of Cincinnati, Cincinnati, OH

Bachelor of Science, *Summa cum laude*

Major: Biological Sciences

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Research Experience

2008 – Present

University of Kentucky, Lexington, KY

Investigating the role of the local renin-angiotensin system of adipose tissue in the development of cardiovascular disease.

(Mentor: Lisa Cassis, PhD; Graduate Center of Nutritional Sciences, University of Kentucky)

2007 – 2008

Genome Research Institute, Cincinnati, OH

Studied cholesterol metabolism and the roles of lipoprotein receptors in the pathology of obesity as a student researcher.

(Mentor: David Hui, PhD; Department of Pathology, University of Cincinnati)

2007 – 2008

University of Cincinnati, Cincinnati, OH

Studied microsatellite markers of endemic Hawaiian shrubs as a model system for various principles of population genetics as a student researcher.

(Mentor: Theresa Culley, PhD; Department of Biological Sciences, University of Cincinnati)

Summer 2006

Children's Hospital Research Foundation, Cincinnati, OH

Studied the role of lysosomal acid lipase in the progression of Wolman's disease.

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Teaching Experience

Spring 2011 – **Bluegrass Community and Technology College,**
Spring 2012 Lexington, KY
Lab instructor for Anatomy and Physiology course

Honors and Awards

October 2010 3rd place, SAHA Cardiovascular Research Day Poster Competition,
Lexington, KY

October 2009 American Heart Association fundraising award, Lexington Heart
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August 2009 Stipend support to attend American Heart Association
Hypertension Summer School, Portland, OR

Summer 2006 Charlotte R. Schmidlapp Scholars Award, supporting the Career
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Spring 2006 President's Volunteer Service Award

Research Support

May 2011 - present American Heart Association Predoctoral Fellowship stipend support, GRA Spring 2011 (AHA 11PRE6760002)

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Memberships

2008 – Present American Heart Association

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Publications

Putnam K, Bharadwaj K, Yiannikouris F, Daugherty A, and Cassis L. Deficiency of angiotensin type 1a receptors in adipocytes reduces differentiation and promotes hypertrophy of adipocytes in lean mice. (Manuscript under review Endocrinology, May 2012).

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Abstracts

Putnam K, Yiannikouris F, Lewis E, Karounos M, Daugherty A, and Cassis L. Deficiency of angiotensin type 1a receptors in adipocytes increases fat mass in angiotensin II-infused LDLR-deficient mice but has no effect on vascular pathologies. Arteriosclerosis, Thrombosis, and Vascular Biology, Chicago, IL. April 2012.

Putnam K, Yiannikouris F, Lewis E, Karounos M, Daugherty A, and Cassis L. Deficiency of angiotensin type 1a receptor in adipocytes increases fat mass in male LDLR deficient mice infused with angiotensin II. Gordon Research Seminar and Conference, Ventura, CA. February 2012.

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Putnam K, Yiannikouris F, Lewis E, Karounos M, Daugherty A, and Cassis L. Effects of adipocyte angiotensin type 1a receptor (AT1aR) deficiency in male C57BL/6 mice fed low or high fat diets for 16 weeks. Gill Heart Institute Cardiovascular Research Day, Lexington, KY. October 2010.

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Weight loss in obese C57BL/6 mice limits adventitial expansion of chronic AngII-induced abdominal aortic aneurysms. *Arteriosclerosis, Thrombosis, and Vascular Biology*, Washington D.C. April 2009.

Putnam K, Perez-Tilve D, Basford J, Konanah E, Tschop M, and Hofmann S.

Overexpression of Apolipoprotein AI improves glucose homeostasis *in vivo*.

Keystone Symposia Obesity: Novel Aspects of the Regulation of Body Weight (J4). Fairmont Banff Springs, Alberta. January 2009.

Oral Presentations

Putnam K. The role of adipocyte-derived RAS components in obesity-induced hypertension. Gordon Research Seminar on Angiotensin, Ventura, CA. February 2012.

Putnam K. The Effects of AT1aR Deficiency in Adipocytes: Does the adipocyte AT1aR contribute to obesity-associated hypertension? Gill Heart Institute Cardiovascular Research Day, Lexington, KY. October 2010.

Putnam K. The Adipocyte Renin-Angiotensin System: What does Angiotensin II do to Adipocytes? Gill Heart Institute Cardiovascular Research Day, Lexington, KY. October 2009.