THE ROLE OF RENIN IN THE ADIPOSE TISSUE RENIN-ANGIOTENSIN SYSTEM

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Dedication

This work is dedicated in its entirety to Aaron James Fowler.

May 31st, 1988 – October 28th 2005

We do not learn by inference and deduction

and the application of mathematics to philosophy,

but by direct intercourse and sympathy.

- Henry David Thoreau

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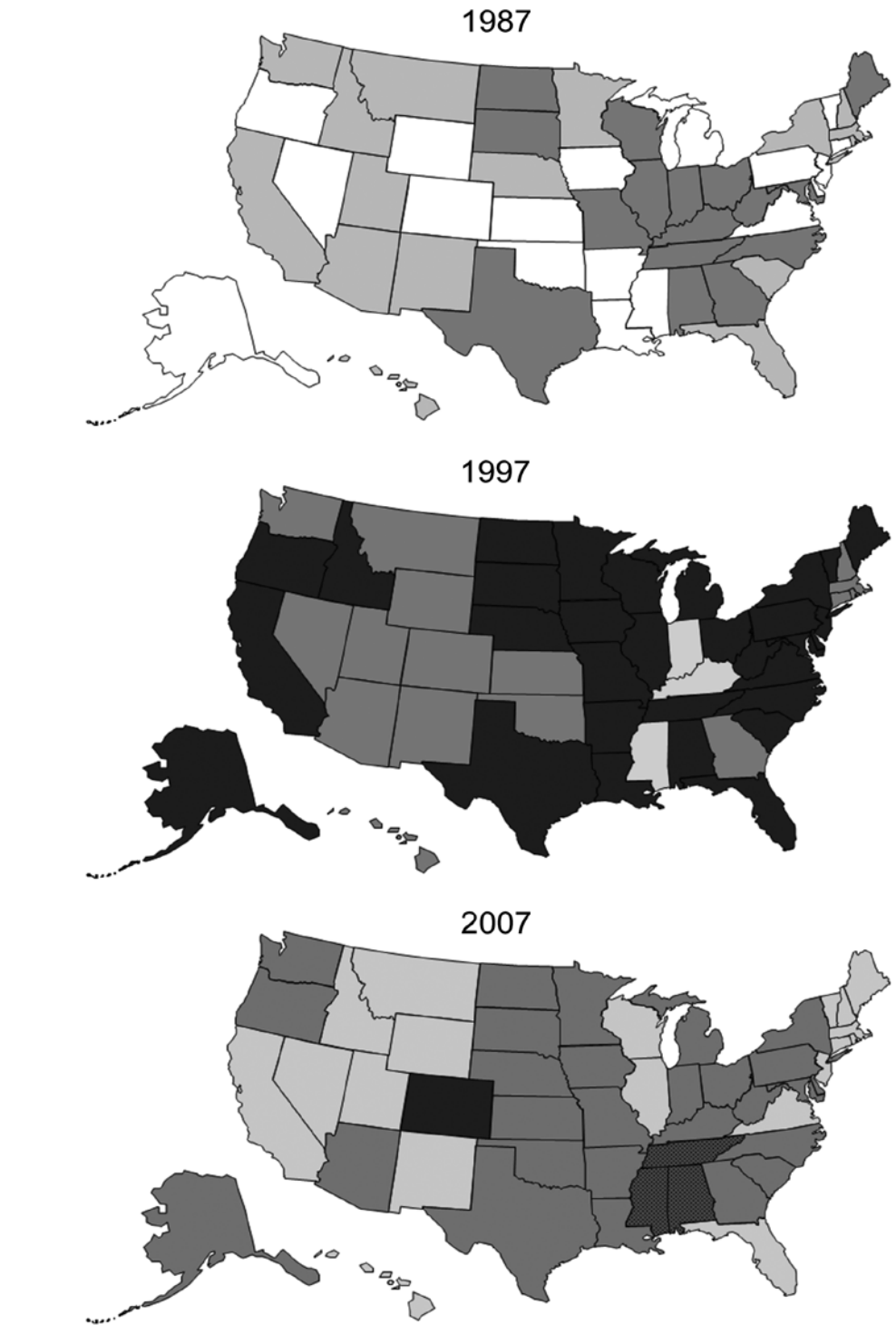
Chapter 1

Introduction and Dissertation Objectives

Obesity has been increasing at a staggering rate over the past few decades resulting in significant costs to both society and the individual. The prevalence of obesity, defined as a body mass index (BMI) greater than 30, more than doubled over the last 25 years (Figure 1) (42). This equates to over one in four individuals afflicted with obesity and its associated morbidity and mortality. To put these numbers in perspective, a male of average height at an ideal BMI of 22.5 (155 lbs) would have to gain over 50 lbs to be classified as obese while an average woman must gain an equally dramatic 44 lbs; an increase in body weight of over 33%. The obesity burden comes at a price far greater than pounds for the obese patient, as obesity is associated with an increased risk of hypertension, type II diabetes, coronary artery disease, stroke, and cancer. These increased risks translate into an economic burden for society as the average health care cost for the obese patient has been reported to increase as much as 54% over that of a normal weight individual (10; 46). Overall, the percentage of health care dollars allocated toward obesity associated health care approached 10% of all health care spending in 1998 (10).

Hypertension is a common and devastating complication associated with obesity (45). The Framingham Heart Study, a cardiovascular study which began in 1948 and is currently on its third generation of participants, has shown that as much as 78% of hypertension was attributed to obesity while increases in population attributable risk for cardiovascular disease rose as high as 23% (14; 61). The association between obesity and hypertension is poorly

Figure 1. Obesity Trends in the United States. Prevelance of obesity percentage on a state by state basis as a function of BMI, defined as a BMI > 30. Data obtained from data CDC's Behavioral Risk Factor Surveillance System (BRFSS).



Source: Behavior Risk Factor Surveillance System CDC

understood, complex and multifactorial. From a basic physiological perspective, adipose tissue growth is tightly coupled to angiogenesis and microcirculation development, however, large increases in adipose tissue depots can significantly increase peripheral vascular resistance thereby increasing afterload and contributing to hypertension (7). If adipose tissue expansion was reliably associated with new vessel synthesis and recruitment of new adipocytes for lipid storage, elevated PVR would not be observed. However, since increased body mass in always associated with increases in blood volume and cardiac output, in the case of hypertrophied adipose tissue in the obese individual much of the weight gain (if not all in the morbidly obese patient), is from individual adipocyte hypertrophy in lieu of adipose tissue bed expansion. This results in increased cardiac output and blood volume in the face of a tissue vascular supply that is no longer significantly expanding, hence an overall increased in peripheral vascular resistance. Some products secreted by adipocytes (adipokines) such as leptin, adiponectin and angiotensinogen (AGT) have been proposed to participate in the development of hypertension. Plasma leptin, which regulates metabolism and food intake, has been positively correlated with increases in coronary artery disease and is proposed to have functions in vasorelaxation and vessel oxidative stress (9; 58). Furthermore, hypoadiponectinemia has been linked to both endothelial cell dysfunction and hypertension (5). Adipose derived AGT, a component of the renin angiotensin system (RAS), has been shown to contribute to circulating levels of AGT and may increase blood pressure by further increasing peripheral vascular

resistance in addition to influencing hypertension associated maladaptive cardiac hypertrophy (37; 65). Angiotensin II has also been shown to exacerbate hypoadiponectinemia associated with obesity as well as enhance insulin sensitivity (5; 18; 63).

Similar to the interplay between hypertension and obesity, the link between obesity and insulin resistance/type II diabetes has not been fully elucidated. Many factors have been proposed linking insulin resistance to obesity including oxidative stress, sub-acute chronic inflammation and adipokine dysregulation. Inflammatory factors produced by adipose tissue such as TNFα and MCP-1 are increased in obesity and are proposed to work via a feed forward mechanism to upregulate additional inflammatory mediators which worsen insulin resistance. In addition, insulin resistance upregulates the renin-angiotensin system (32) and angiotensin II (Ang II) administration has been shown to further increase insulin resistance through a proposed mechanism of oxidative stress (63). Hyperlipidemia and increased circulating free fatty acids associated with adipocyte hypertrophy also affect distant tissues such as muscle and liver, decreasing the insulin sensitivity of these target tissues and further exacerbating insulin resistance and inflammation associated with obesity.

Many facets of biology contribute to the various complications associated with obesity. While the list is vast and continues to grow, one potential factor affecting multiple aspects of the interplay between obesity and its associated morbidity and mortality are components of the renin angiotensin system (RAS).

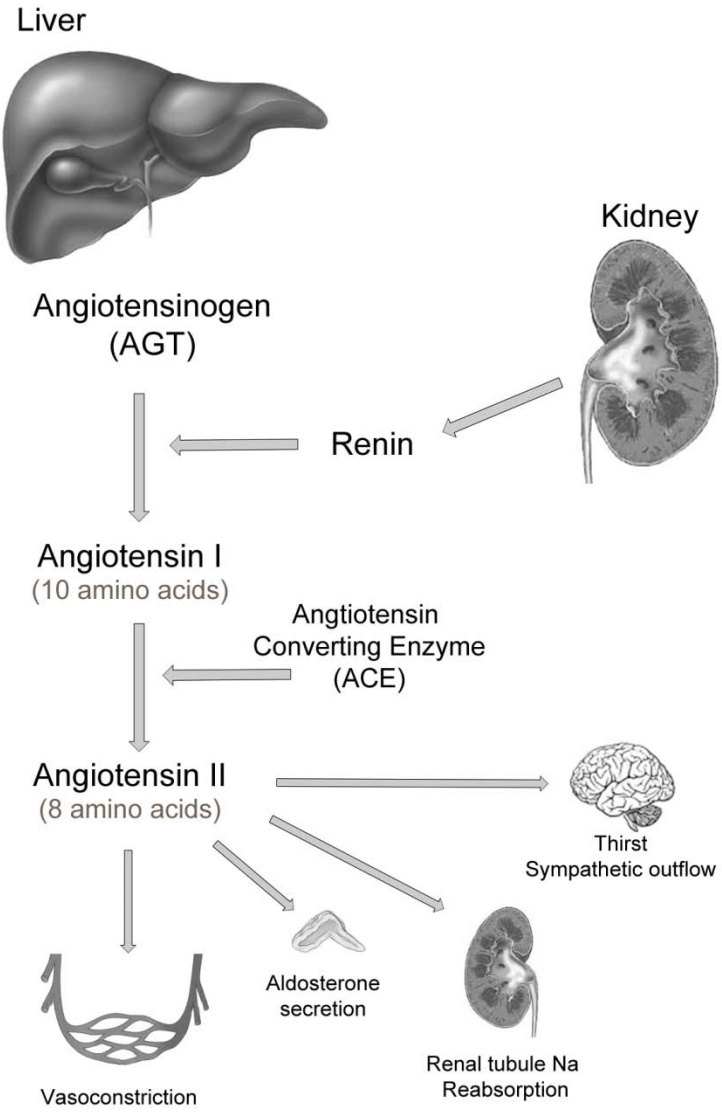
The renin angiotensin system (RAS) was first investigated in 1898 when the pressor effects of a crude renal extract was witnessed upon intravenous injection by Tigerstedt and Bergman (32). What was unknown at the time was that the active ingredient in the crude kidney extract was renin, an enzyme capable of generating large amounts of a vasoactive peptide through the cleavage of plasma AGT. Over the following century, thousands of articles have been published characterizing and describing the systemic actions of the RAS. Until relatively recently, however, the RAS was considered to have a nearly exclusive role in the regulation of blood pressure and blood volume regulation.

During adult life, the primary function of the systemic RAS is blood pressure homeostasis. The RAS is primarily controlled through renin release from the specialized kidney juxtaglomerular cell. Decreases in blood pressure, renal tubule sodium delivery, or Ang II levels and increased sympathetic stimulation cause renal renin to be secreted into the plasma (Figure 2). Renin then acts on liver derived plasma angiotensinogen (AGT) to produce angiotensin I (Ang I) which is further cleaved by endothelial angiotensin converting enzyme (ACE) to form the 8 amino acid peptide angiotensin II (Ang II), the biologically active component of the RAS. Renin concentration is the primary rate-limiting step in this plasma cascade (16), although changes in plasma AGT levels can also influence the generation of angiotensins. In adults, functional consequences are primarily mediated through Ang II binding to the angiotensin II type I

receptor, AT_1R, which acts within the kidney, brain, adrenal glands, and vasculature to mediate increased blood volume and total peripheral resistance, thereby maintaining blood pressure (47).

The significance of the RAS during fetal development has also been investigated. Two major types of Ang II receptors exist. Ang II type II (AT_2R) receptors are expressed at high levels during fetal life and as development progresses Ang II type I (AT_1R) receptors begin to predominate (35). AT_1R and AT_2R are distinct receptor subtypes which often display opposing functions (59). In addition to differential receptor expression, the involvement of multiple aspects of the RAS during nephrogenesis and collagen deposition during cardiac development have been described (31; 35). Interestingly, the necessity of the RAS during fetal development appears to be species specific. Initial evaluation of various RAS component null mice show various phenotypic changes, such as renal artery hyperplasia and resistance to diet induced obesity, however; regardless of what RAS component is lacking, mice are able to survive at birth (29; 38; 56; 67). In humans however, this is not the case as inactivating mutants of either renin, AGT or the AT_1R are incompatible with life. Inactivating defects of any of the RAS components are classified as an autosomal recessive syndrome coined ‘renal tubular dysgenesis’ or RTD. RTD consists of profound abnormalities of renal tubular development resulting in anuria and perinatal death (6). It should be noted that many of the fetal

Figure 2. Classical Renin Angiotensin System (RAS). Liver derived plasma angiotensinogen is cleaved by kidney derived plasma renin to produce the 10 aminon acid peptide angiotensin I. Angiotensin I is further processed through additional removal of 2 amino acids by the ubiquitously expressed endothelial enzyme ACE to produce angiotensin II (Ang II). Plamsa derived Ang II exerts its systemic effects primarily by action on angiotensin II type I receptors in the brain, kidney, adrenal gland and vasculature to control pressure and electrolyte homeostasis.


Liver
Kidney
Angiotensinogen
(AGT)
Renin
Angiotensin I
(10 amino acids)
Angtiotensin
Converting Enzyme
(ACE)
Angiotensin II
(8 amino acids)
Thirst
Sympathetic outflow
Aldosterone
secretion
Renal tubule Na
Reabsorption
Vasoconstriction

abnormalities are hypothesized to be a result of kidney dysfunction and subsequent fluid retention and not a direct effect of loss of Ang II action on the affected organ (other than the kidney) (6). In addition, ACE inhibitors and ARBs are contraindicated during pregnancy, especially the second and third trimesters, due to birth defects. Apart from the systemic and developmental functions of the RAS, local RAS systems have been describe in a variety of other organ systems including the brain, heart, adrenal glands, testis and adipose tissue (2; 11). One of the most recent tissues in which a local RAS has been investigated is adipose tissue. In 1989 Saye *et al.* detected AGT mRNA in differentiating 3T3-L1 adipose cells which was further described as steroid responsive (52). At this time, neither renin nor any other RAS components had been reported in adipose tissue. Following this observation, synthesis of Ang II by adipose tissue and its link to adiposity was described (12; 17). Further investigation lead to the discovery of additional regulatory molecules capable of adipose derived AGT induction such as insulin, fatty acids, β-adrenergic stimulation and Ang II (22; 33; 49). AGT deficient mice show a decrease in fat mass with smaller adipocyte cell size and do not gain weight in response to a high fat diet, suggesting AGT involvement in adipocyte metabolism and adipose triglyceride storage (38). In 2006, human adipose tissue AGT gene expression as well as the enzymes necessary for Ang II generation was reported (24). Increased RAS activation in diet induced obesity models coupled with laboratory/clinical studies reporting weight loss as a side effect of RAS

modifying drugs as a means for blood pressure control have further implicated a role for a local RAS in obesity related pathologies (3; 24; 40).

While AGT expression and regulation have been extensively described in adipose tissue (12; 22; 38), the enzyme necessary for its conversion to Ang I, renin, has not been extensively investigated. In fact, many conflicting reports exist regarding the synthesis and secretion of renin by adipose tissue. Renin enzymatic levels have not been consistently detected in adipose tissue and while renin mRNA expression within adipose tissue has been demonstrated (24; 44) it has not been a constant observation (13; 13). One previous study of interscapular brown adipose tissue homogenates from rats with bilateral nephrectomy report tissue renin enzymatic activity, noting that residual adipose vascular smooth muscle or vessel wall associated plasma derived renin could not be excluded as a potential contaminating source (54). However, the method of renin activity employed in this study utilized a renin enzymatic assay that has since been shown to have significant cathepsin D contamination, a lysosomal enzyme capable of AGT cleavage, thereby complicating interpretation and extrapolation of such data (25). Nevertheless, renin appears to be an integral part in some parts of adipose biology. Mice lacking renin are resistant to diet induced obesity similar to their AGT null counterparts (56). In addition, when inserted into mice, the human renin gene showed tissue specific regulation including adipose tissue expression (55). Since renin action on AGT is the rate-limiting step in the production of plasma Ang II, the ultimate RAS

effector, local expression and regulated release from adipose tissue may be an important regulatory mechanism for production of local Ang II. Further research is necessary to demonstrate the adipocyte as a source of renin synthesis and secretion.

In addition AGT and renin, mRNA expression of all other components necessary for a complete local adipose RAS have been detected including angiotensin converting enzyme (ACE) and Ang II receptors (24; 36; 53). While some debate remains as to the origin of adipose tissue Ang II, many observations concerning Ang II have been extensively investigated in adipose tissue biology including adipose tissue growth, differentiation, metabolism, and inflammation (1; 8; 20; 21). For example, Ang II can increase expression of AGT in adipose tissue through its interaction with the AT_1R receptor (33). Ang II has been shown to induce lipolysis in 3T3-L1 adipocytes and human adipose cells while other reports suggest an inhibitory function (15; 21). Furthermore, AT_1R blockade in adipose tissue was shown to reduce the production of reactive oxygen species and improve some effects associated with adipokine dysregulation including adiponectin expression and reduced TNF-α and MCP-1 expression (30). In addition to oxidative stress induction, Ang II is capable of increasing toll-like receptor 4 in mesangial cells as well as inducing MCP-1 expression in rat preadipocytes via an NFκB dependent pathway further implicating a role for the RAS as a proinflammatory mediator in adipose tissue (57; 62). Ang II has been shown to increase insulin sensitivity both in vivo and

in vitro, however the overall biological effects of Ang II on insulin sensitivity remain controversial and some reports suggest that early effects attributed to Ang II were in part due to the PPAR-gamma mediated actions of early RAS system modifying compounds (23; 41). Leptin release from adipocytes has also been shown to be, in part, mediated by Ang II levels (4). Finally, many reports implicate Ang II in the regulation of adipocyte differentiation, although any precise role remains unclear (8; 20; 39; 51).

In addition to adipocytes, other adipose tissue cellular components may also play a critical role in local adipose tissue RAS dynamics. An active RAS has been described in macrophages related to primarily to atherosclerotic plaque development associated with hyperlipidemia and hypertension (34). Furthermore, macrophages accumulation is associated with the development of obesity and some reports suggest that macrophage infiltration precedes systemic hyperinsulinemia (60; 64). Similar to the adipocyte, mRNA has been detected for all components necessary for a local RAS in macrophages including renin, AGT, ACE, and angiotensin receptors (27; 34; 50). Furthermore, oxidative stress, which is known to be increased in obesity, upregulates macrophage angiotensin receptor expression, further exacerbating Ang II mediated effects (27). In addition to the RAS interactions with resident adipose tissue macrophages, subsequent TNFα production associated with increased macrophage infiltration plays an important role in the pathogenesis of obesity induced inflammation. This indicates a dynamic adipose tissue RAS

involved in adipocyte and macrophage cytokine production within adipose tissue.

Another potential interplay between adipocyte/macrophage produced RAS components and Ang II action is vascular expansion of adipose tissue. Vascular development is an integral part of adipose tissue expansion (7). Adipose tissue derived Ang II may act on adipose tissue endothelial cells to induce angiogenesis through Ang II mediated induction of VEGF, analogous to that observed in the retina, kidney and cardiovascular systems (19; 28; 43; 48). This may serve to increase adipose tissue angiogenesis and in part relieve the tissue hypoxia associated with obesity (66).

The objectives of this thesis dissertation are to evaluate a local RAS in adipose tissue through two primary experimental models. First, the independence of a local RAS in adipose tissue apart from the classical plasma RAS is assessed in a Sprague Dawley rat model. Plasma RAS components were experimentally varied and the influence of plasma RAS component concentrations on that of adipose tissue was evaluated. Second, cultured 3T3-L1 adipocytes were evaluated for regulated expression and release of renin enzymatic activity. Taken together, the finding in this dissertation provide strong evidence in support of a local adipose tissue RAS. Furthermore, a local adipose RAS may be modulated through the regulated release of renin from mature adipocytes.

While some tissues reported to contain an independent, local RAS system are privileged against potential communication with the classical plasma RAS by the presence of a blood-organ barrier such as the brain and testis, others are subject to capillary beds that have varied levels of permeability. Such organ systems (e.g. adipose tissue) are not necessarily independent from plasma fluctuation in RAS components, such as renin, AGT, or plasma derived Ang II. Plasma derived RAS components can change rapidly associated with blood pressure and plasma sodium homeostasis. Since many functions have been proposed for a local adipose tissue RAS such as differentiation or inflammatory mediation, one would assume that variations in plasma RAS components would not necessarily translate into concomitant adipose tissue RAS component changes. In order to test the independence of a local adipose RAS system from that of plasma, male Sprague Dawley rats were surgically and pharmacologically treated to either acutely or chronically alter levels of two major plasma RAS components, renin and AGT. Plasma renin and AGT alterations were then compared to adipose tissue renin and AGT levels after removal of contaminating blood RAS components from adipose tissue via saline perfusion. Lack of perfusion could have produced false positive associations due to plasma RAS component contamination of adipose tissue. Perfused cardiac tissue was also analyzed as a positive control since previous publications have shown plasma spillover into the interstitial compartment as a primary source for cardiac RAS components (25; 26). The resulting publication from this study is in described in Chapter 2 of the thesis.

Many reports have analyzed the expression of AGT and Ang II receptor expression in adipose tissue(1; 2; 22; 67), however, few have addressed the ability of adipose tissue to synthesis and secrete renin, the rate limiting step in the production of Ang II in plasma. To evaluate the role of expression, synthesis and secretion of renin by adipocytes, I performed four primary studies. 3T3-L1 fibroblast cells were assessed for intra/extracellular renin enzymatic activity and renin expression via real time PCR during 3T3-L1 adipocyte differentiation to ascertain if the commonly used 3T3-L1 adipocyte culture system could be used as a model system for adipose tissue renin secretion investigation. Furthermore, mature, cultured adipocytes were evaluated for renin expression and subjected to various treatments including CNP, lipopolysaccharide, Isoproterenol, prostaglandin E2, IL-6, Angiotensin II, Insulin, hydrogen peroxide, palmitate, oleate, methylisobutylxanthine, forskolin and TNFα in order to analyze potential molecules which may regulate renin secretion. Regulated renin release from mature adipocytes was further characterized for time and dose dependent induction of renin message and protein. Lastly, C57BL/6J male mice on a high fat diet were compared to lean counterparts for the adipose tissue expression of renin message. The resulting publication from this study is in described in Chapter 2 of the thesis.

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Chapter 2

Renin dynamics in adipose tissue:

Adipose tissue control of local renin concentrations

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ABSTRACT

The renin angiotensin system (RAS) has been implicated in a variety of adipose tissue functions including tissue growth, differentiation, metabolism, and inflammation. While expression of all components necessary for a locally derived adipose tissue RAS have been demonstrated within adipose tissue, independence of local adipose RAS component concentrations from corresponding plasma RAS fluctuations has not been addressed. To analyze this, we varied *in vivo* rat plasma concentrations of two RAS components, renin and angiotensinogen (AGT), to determine the influence of their plasma concentrations on adipose and cardiac tissue levels in both perfused (plasma removed) and nonperfused samples. Variation of plasma RAS components was accomplished by 4 treatment groups: Normal, DOCA-salt, Bilateral nephrectomy, and Losartan. Adipose and cardiac tissue AGT concentrations correlated positively with plasma values. Perfusion of adipose tissue decreased AGT concentrations by 11.1% indicating that adipose tissue AGT was in equilibrium with plasma. Cardiac tissue renin levels positively correlated with plasma renin concentration for all treatments. In contrast, adipose tissue renin levels did not correlate with plasma renin, with the exception of extremely high plasma renin concentrations achieved in the Losartan treated group. These results suggest that adipose tissue may control its own local renin concentration independently of plasma renin as a potential mechanism for maintaining a functional local adipose RAS.

INTRODUCTION

The renin angiotensin system (RAS) has classically been considered a blood pressure and blood volume regulation system. Decreases in blood pressure, renal tubule sodium delivery, or angiotensin II levels and increased sympathetic stimulation cause renal renin to be secreted into the plasma. Renin then acts on plasma angiotensinogen (AGT) to produce angiotensin I (Ang I) which is further cleaved by angiotensin converting enzyme (ACE) to form the 8 amino acid peptide angiotensin II (Ang II), the biologically active component of the RAS. Renin concentration is the primary rate-limiting step in this plasma cascade (7), although changes in plasma AGT levels can also influence the generation of angiotensins. Functional consequences are primarily mediated through Ang II binding to the angiotensin II type Ia receptor, AT_1R, which acts within the kidney, brain, adrenal glands, and vasculature to mediate increased blood volume and total peripheral resistance, thereby maintaining blood pressure (23).

Recently, increasing evidence has implicated a locally derived RAS within adipose tissue as an important regulator of adipose tissue growth, differentiation, metabolism, and inflammation (2; 4; 10; 11). Current literature indicates mRNA expression of all the components necessary for a complete local RAS in adipocytes including angiotensin II receptors, angiotensinogen, renin, and angiotensin converting enzyme (13; 20; 22; 26). Angiotensin II has been shown to induce lipolysis in 3T3-L1 adipocytes and human adipose cells

(11). In addition, Ang II can increase expression of AGT in adipose tissue through its interaction with the angiotensin II type Ia receptor (19). Furthermore, angiotensin II Type I receptor blockade in adipose tissue was shown to reduce the production of reactive oxygen species and improve some effects associated with adipokine dysregulation including adiponectin expression and reduced TNF-α and MCP-1 expression (18). Many reports implicate Ang II in the regulation of adipocyte differentiation, although any precise role remains unclear (4; 10; 22; 25).

Given recent research concerning the effects that locally produced Ang II is proposed to have within adipose tissue, the independence of adipose tissue RAS components from that of plasma RAS components has not been adequately addressed. Circulating RAS components primarily change with respect to salt and water balance as well as blood pressure and not with situations involving adipocyte metabolism or differentiation. Local generation of Ang II near adipose cell membranes should be, at least in part, a function of adipose interstitial fluid renin and AGT concentrations. Plasma derived renin and AGT are present in the interstitial fluid (ISF) of many tissues including the heart (9). Although adipose capillaries are not highly fenestrated, adipose tissue ISF albumin concentration has been shown to be 15% of plasma (5) and albumin permeability across adipose capillaries was found to increase with sympathetic stimulation and histamine release (24). Since renin and AGT are smaller than albumin, circulating renin and AGT could have access to adipose

tissue. We therefore measured renin and AGT in adipose tissue and compared their levels to corresponding plasma levels. If plasma renin or AGT is able to move across adipose capillary walls, then plasma renin or AGT changes could result in concomitant adipose tissue RAS changes. If so, a local adipose RAS would not necessarily control local adipose growth, differentiation, metabolism, and inflammation independently of the plasma RAS.

Previous reports have measured the plasma contribution of RAS components in cardiac tissue (9; 15; 16). Analogous to that of adipose tissue, all components necessary for a local RAS within cardiac tissue have also been detected (30). Studies varying plasma concentrations of renin and AGT have generally shown that left ventricular cardiac renin and AGT concentrations are dependent on plasma concentrations of renin and AGT (9; 15; 16). Therefore, we also measured cardiac renin and AGT to compare the known changes of renin and AGT in cardiac tissue to the possible changes in adipose tissue during alterations of plasma renin and AGT.

In light of the many recent implications for the function of Ang II within adipose tissue, insight into the potential influence of plasma RAS components to that of adipose tissue will prove useful for elucidation of the control and functional significance of a local adipose RAS. In order to address this, rat plasma concentrations of renin and AGT were pharmacologically and surgically varied to examine how plasma fluctuations influence adipose tissue RAS component concentrations. The comparison was also extended to cardiac tissue. We

tested the hypothesis that variations in the plasma RAS would be reflected in adipose tissue, as previously reported for cardiac tissue (9; 14; 16).

Manipulation of plasma renin and AGT was done with treatments previously shown to alter their plasma levels. 48 hour-bilateral nephrectomy (BNX) removes the primary plasma renin source resulting in decreased plasma renin and increased AGT (15). Long-term deoxycorticosterone acetate (DOCA) treatment causes plasma renin to fall. AGT plasma levels also fall (16) due to reduced chronic hepatic production of AGT secondary to reduced Ang II levels acting at hepatic AT1 receptors that normally stimulate AGT secretion (27). Losartan treatment inhibits negative feedback of renal renin secretion causing increasing plasma renin which then hydrolyzes plasma AGT and reduces plasma AGT. Hepatic production of AGT via Ang II stimulation of hepatic AT1 receptors is again reduced.

Our data indicate that adipose tissue AGT concentrations correlate positively with plasma AGT. However, in our study, visceral white adipose tissue renin was found to be independent of plasma renin except at extremely high levels. This is in striking opposition to cardiac tissue where plasma and cardiac renin were found to be positively correlated throughout the observed plasma renin range. A local adipose RAS, therefore, may be capable of functioning independently of the plasma RAS via independent control of renin levels.

MATERIALS AND METHODS

Animals: Procedures involving animals were approved by the Institution of animal care and use committee at the University of Minnesota. Male Sprague Dawley rats were purchased from Charles River Laboratories at 251-275 grams. After approximately 10 days of acclimation, rats were randomly divided into normal, deoxycorticosterone acetate (DOCA), bilateral nephrectomy (BNX) or Losartan treatment groups. All animals had free access to standard rat chow and water unless otherwise noted.

DOCA Treatment: 100mg deoxycorticosterone acetate (DOCA, Sigma) imbedded in a silastic pellet was subcutaneously implanted via a flank incision. In addition, unilateral nephrectomy was performed on the opposing flank for each animal according to previously published laboratory protocol (16). Animals (n= 9) were maintained with 1% NaCl in their drinking water and normal chow for 3 weeks. DOCA animals gained 46.4 ± 5.6 grams in 3 weeks.

Losartan Treatment: Losartan, (Merck Research Laboratories) was administered in the drinking water at a concentration of 0.4- 0.5mg/mL to result in a daily consumption of 30-40 mg/kg/day. Losartan treatment (n= 11) was maintained for 3 weeks prior to sacrifice. Losartan animals gained 73.7 ± 4.7 grams in 3 weeks.

Bilateral Nephrectomy: Both kidneys were removed from each animal by separate flank incisions under pentobarbital anesthesia (50mg/Kg

intraperitoneal injection). Ten mL normal saline was added to the intra-abdominal cavity at the completion of surgery to maintain adequate hydration. Animals (n= 9) were maintained for 48 hours with minimal food and free access to water as previously published (14). Weight change was not measured over the course of 48 hours.

Normal Treatment: Rats (n=12) were given no special treatment and were maintained with free access to food and water. Normal animals gained 82.6 ± 7.9 grams over approximately 3 weeks.

Tissue collection and Perfusion: Animals were euthanized with 100% CO_2 in a chamber able to accommodate the entire cage so as to reduce stress and subsequent sympathetically mediated renal renin secretion. When breathing had ceased and the animal was unresponsive, the chest cavity was quickly opened to expose the heart. A right or left visceral fat pad was randomly isolated, clamped and removed from each animal (non-perfused visceral adipose tissue). Blood was drawn from the right ventricle into a syringe containing 5% EDTA, final 0.1%, immediately chilled, centrifuged and the plasma frozen at -70°C for plasma analysis of RAS components. After blood collection, the left ventricular wall was pierced at its apex with a blunted 18 gauge needle and a solution of 0.9% saline at 37°C was perfused at a pressure of 90mm Hg through the heart for approx 10 seconds to flush the coronary circulation. The catheter was then advanced into the ascending aorta and perfusion was continued for an additional 45-60 seconds. Liver tissue and the

testicular vein were monitored for blanching to ensure complete visceral adipose tissue perfusion. Upon completion of perfusion, the remaining visceral adipose fat pad, a retroperitoneal adipose tissue sample (peri-renal fat), and left ventricular cardiac tissue (perfused tissues) were collected from each animal. Specifically, the visceral adipose tissue was a combination of omental and reproductive adipose tissue. Care was taken to remove both the testis and seminal vesicles from isolated white visceral adipose tissue. Tissues were diced into 3-4 mm sections, frozen and stored at -70°C until extract preparation.

Extract Preparation: Visceral and retroperitoneal adipose tissue was homogenized at a ratio of 1 gram tissue to 1 mL ice cold protease inhibitor buffer (PIB) consisting of 0.15 M sodium phosphate buffer (pH 7.45), 1 % BSA, 0.05% NaN3 and serine-,metallo-,and thiol-protease inhibitors (15 mM EDTA, 2.3 mM Captopril, 2 mM 8-hydroxyquinoline, 10 mM sodium tetrathionate, 20 mM benzamidine, 10 mM N-ethylmaleimide, 3 mM PEFABLOC SC (AEBSF), 20 µM leupeptin, and 450 µM aprotinin)**.** Cardiac extracts were homogenized at 1 gram to 5 mL PIB. Briefly, PIB was added to frozen tissue in a 7 mL dounce glass homogenizer. Samples were homogenized on ice to a uniform consistency. Homogenized samples were frozen at -70°C, thawed and centrifuged at 100,000 x g for 1 hour. The aqueous phase, containing soluble RAS components was removed and frozen at -70°C for subsequent assay of renin and angiotensinogen levels.

Renin Concentration Measurement: Adipose extract, plasma, or cardiac extracts were diluted in PIB to yield similar renin concentration measurements in each assay tube. 100µL assay tubes containing diluted samples, 50% PIB, 25% renin-free rat angiotensinogen (obtained from 48 hour bilaterally nephrectomized rats as previously described (16), and 25% sodium phosphate buffer (0.15 M, pH 7.45, 2.5% BSA) were incubated at 37°C for 0, 3, and 6 hours in duplicate. During the 37°C incubation Ang I was generated by cleavage of AGT. Ang I initially present in the sample, 0 hr assay tube, is subtracted from the 3 and 6 hour time points to yield a linear generation of Ang I/ mL plasma or gram tissue/ hour. Ang I measurement is described below. As previously described, angiotensinogen levels in the assay are at saturating conditions such that Ang I generation over time is directly proportional to renin concentration (14-16).

Angiotensinogen Measurement: Adipose extract, plasma, and cardiac extracts were diluted in PIB to yield similar AGT concentrations. 100µL assay tubes containing diluted samples and 50% PIB, 49% sodium phosphate buffer (0.15 M, pH 7.44, 2.5% BSA) and 1% semipurified rat renin source, obtained from perfused, homogenized and ultracentrifuged rat renal cortex (14), were incubated at 37°C for 0, 4 and 6 hour time points. During incubation at 37°C in the presence of excess renin, all AGT is cleaved to produce Ang I in a 1:1 molar ratio. Two time points are taken to ensure the Ang I generation has completed. AGT data is expressed as an average of both the 4 and 6 hour time points

corrected for any baseline measurement detected at time 0. Since renin is in significant excess and is able to convert nearly 100% of the angiotensinogen (AGT) to Ang I within the first hour, 4 and 6 hour time points are similar (Ang I degradation over 6 hours is negligible). Ang I is measured by RIA as described below and the angiotensinogen concentration was back calculated and expressed as nmol angiotensinogen/ mL plasma or gram tissue.

Measurement of Angiotensin I: Ang I generated in both the renin and angiotensinogen assays was measured by radioimmunoassay as previously described (14). Briefly, addition of I^{125} labeled Ang I and primary antibody directed against Ang I to each Ang I sample was followed by overnight incubation at 4°C. Secondary antibody (anti-rabbit IgG, Sigma) in a suspension of 4% polyethylene glycol (MW = 8,000) with 0.5% rice starch was then added and incubated for 10 minutes at 4°C. Samples were centrifuged for 20 min at 2,000 x g to precipitate Antibody bound Ang I and the precipitates are counted in a gamma counter. Sample concentration of Ang I was calculated by comparing radioactive counts against control standards containing known Ang I concentrations.

Statistics*:* Differences in plasma renin and AGT were determined by one-way ANOVA. Renin values were log transformed to pass the normality test. Individual differences between groups were further assessed by the Holm-Sidak all pairwise multiple comparison test after significance was obtained from the ANOVA procedure. Renin and AGT differences between visceral adipose,

perfused visceral adipose, and perfused retroperitoneal adipose tissues across all 4 treatment groups was assessed by two-way ANOVA. If renin or AGT data did not pass a normality test, they were log transformed. Individual differences between groups were further assessed by the Holm-Sidak all pairwise multiple comparison tests after a significant ANOVA result. Overall differences across all treatments for angiotensinogen in visceral and perfused visceral adipose tissue were assessed by the Wilcoxen Signed Ranks test. Cardiac extract renin and angiotensinogen differences between treatment groups, as well as weight gain differences were assessed by one-way ANOVA. Cardiac extract data for renin and angiotensinogen analysis were natural log and arcsin square root transformed respectively, to pass the normality test. Linear regression was performed between tissue and plasma renin or AGT. Statistical significance was set at $P < 0.05$.

RESULTS

Alteration of plasma renin concentration. To address the influence of plasma renin concentration on adipose and cardiac tissue renin levels, we surgically and pharmacologically altered the plasma levels of renin and measured their influence on adipose tissue renin concentrations. Plasma renin levels ranged from undetectable to over 600 ng Ang I generated/mL/hr. All treatment groups yielded significant changes in plasma renin concentration versus normal animals (Figure 1A) and each treatment group had a significantly different mean plasma renin concentration than all other treatment groups except BNX versus DOCA. Losartan treatment yielded average renin plasma concentrations that were greater than 20 fold that of normal rats while DOCA and 24 hour BNX treated animals had nearly undetectably low plasma renin levels (Figure 1A).

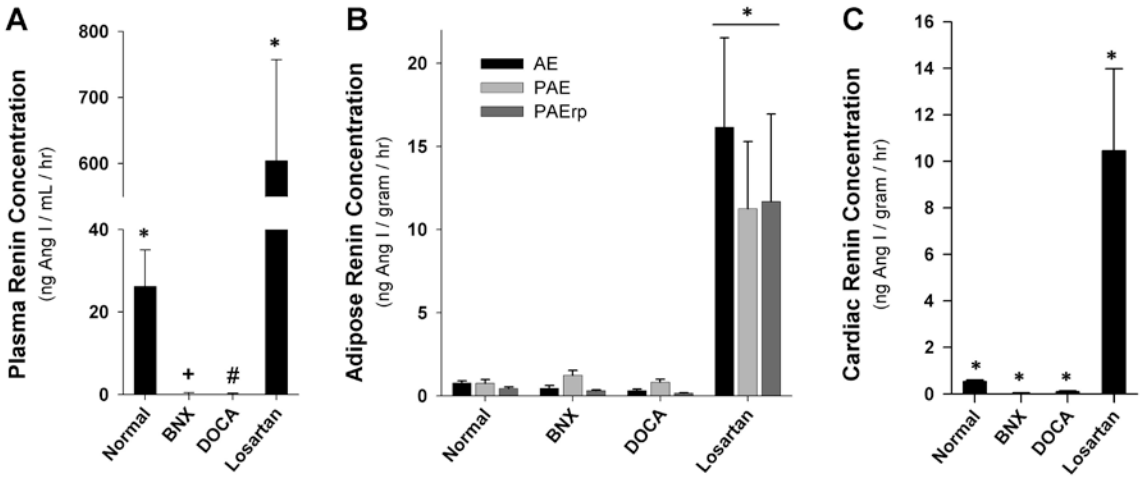
Tissue renin concentrations. Adipose tissue renin concentrations were not statistically different between normal, BNX and DOCA treated animals (Figure 1B). In addition, no statistical differences were observed between visceral adipose tissue (AE), perfused visceral adipose tissue (PAE) or perfused retroperitoneal adipose tissue (PAErp) within any treatment group (Figure 1B). Normal treatment adipose tissues contained renin levels that were not different than BNX or DOCA treatment despite approximately 100 fold greater plasma renin values in the normal treatment group (Figure 1A,B). Losartan treated animals had significantly higher adipose tissue renin concentrations; approximately 10 to 20 times that of normal, BNX and DOCA animals (Figure

Figure 1. Plasma, adipose tissue and cardiac renin concentrations. (A) Plasma renin concentrations among treatment groups. Normal (n = 11), BNX (n = 9), DOCA (n = 9) and Losartan (n = 11). Renin activity (ng angiotensin I/mL/hr) is directly proportional to renin concentration. (B) Renin concentrations in adipose extract (AE), perfused adipose extract (PAE) and retroperitoneal, perfused adipose extract (PAErp) among treatment groups. (C) Left ventricular cardiac extract renin concentrations among treatment groups. PAErp n = 5; n is between 9 and 12 for all other groups.

$*$ $P < 0.05$ versus all other treatment groups.

+ $P < 0.05$ versus all other groups except DOCA.

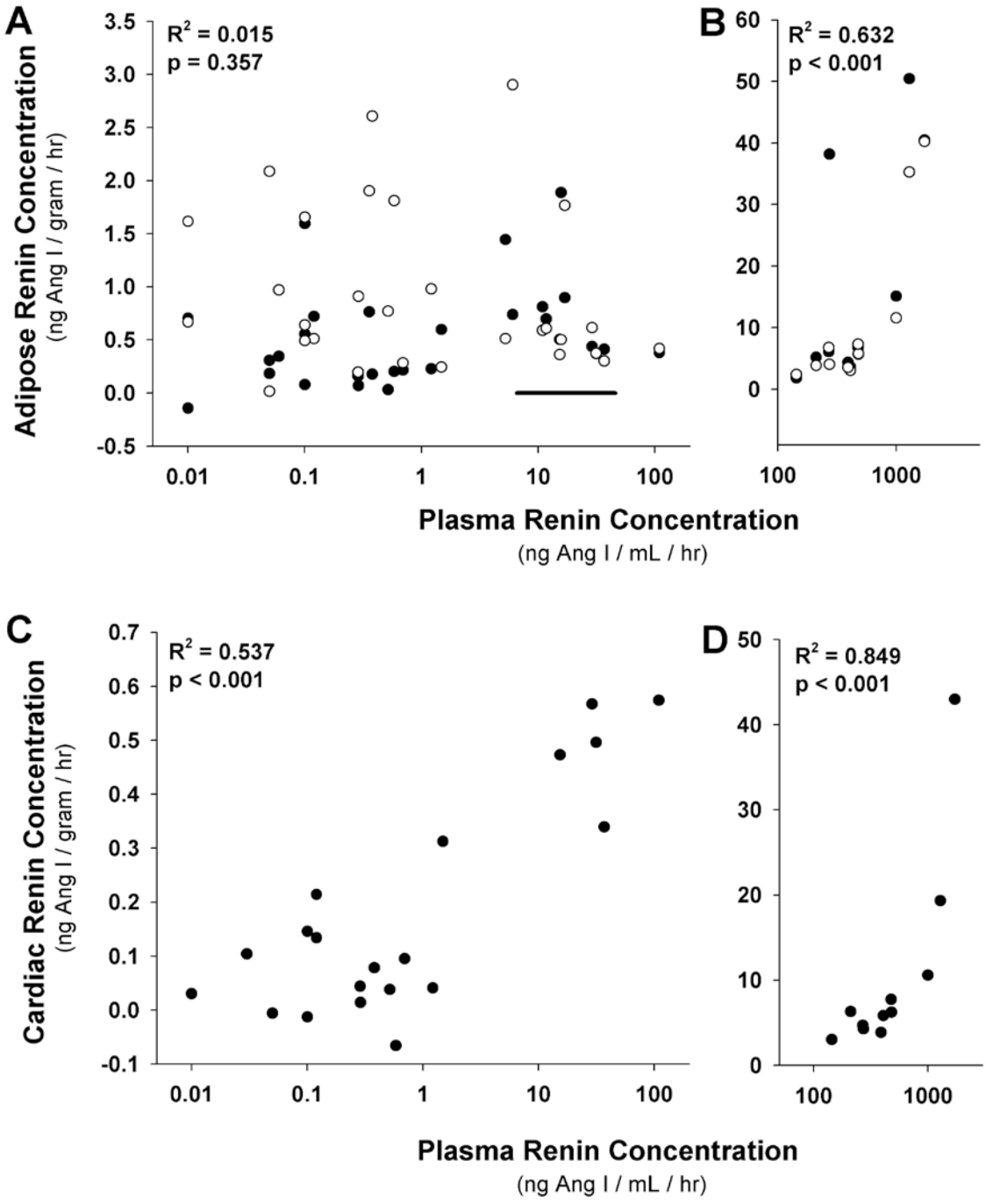
# $P < 0.05$ versus all other groups except BNX.


A
Plasma Renin Concentration
(ng Ang I / mL / hr)
800
700
600
40
20
0
*
+
#
*
Normal
BNX
DOCA
Losartan
B
Adipose Renin Concentration
(ng Ang I / gram / hr)
20
15
10
5
0
AE
PAE
PAErp
*
Normal
BNX
DOCA
Losartan
C
Cardiac Renin Concentration
(ng Ang I / gram / hr)
16
14
12
10
8
6
4
2
0
*
*
*
*
Normal
BNX
DOCA
Losartan

1B). Left ventricular cardiac extract renin concentrations were statistically different between all treatment groups (Figure 1C). Cardiac extract renin concentrations ranged from undetectable to over 10 ng angiotensin I/gram/hr.

Adipose tissue renin versus plasma renin. If plasma renin concentrations are reflected in proportional adipose tissue changes, then the significance of an independently controlled local RAS within adipose tissue is unclear. However, as shown in figure 2B, only the highest plasma renin values (Losartan treatment group) were able to significantly change adipose renin values. Plasma renin concentrations did not correlate with visceral adipose renin among combined Normal, BNX and DOCA treated animals (Figure 2A, $R^2 = 0.015$, $p = 0.357$, combined perfused and nonperfused). When analyzed on an individual treatment basis, only Losartan treatment yielded a statistically significant positive correlation between visceral adipose tissue renin and plasma renin (Figure 2B, $R^2 = 0.632$, $p < 0.001$, combined perfused and nonperfused data). There was no apparent change in adipose tissue renin between plasma renin values of 0.01 to 100 ng Ang I/ml/hr (Figure 2A). The 95% confidence interval for normal treated animals was 6.5 to 45.9 ng Ang I/ml/hr (solid bar, Figure 2A). There was no consistent relationship between nonperfused (AE) and perfused (PAE) visceral adipose renin. *Cardiac tissue renin levels positively correlate with changes in plasma renin levels*. In contrast to adipose tissue, left ventricular cardiac tissue renin concentrations were directly correlated to plasma renin.

Figure 2. Plasma renin vs. visceral adipose tissue and cardiac tissue renin. (A) Normal (n = 11), BNX (n = 9) and DOCA (n = 9) treated adipose renin vs. plasma renin. Closed circles, non-perfused visceral adipose extract (AE). Open circles, perfused visceral adipose tissue (PAE). Solid bar represents the 95% confidence interval for plasma renin concentration in Normal animals. (B) Losartan (n = 11) treated adipose extract renin vs. plasma renin. Closed circles, non-perfused visceral adipose extract (AE). Open circles, perfused visceral adipose tissue (PAE). (C) Normal (n = 5), BNX (n = 6) and DOCA (n = 9) treated perfused cardiac renin vs. plasma renin. (D) Losartan (n = 11) treated cardiac renin vs. plasma renin.


A
B
C
D
Adipose Renin Concentration
(ng Ang I / gram / hr)
Cardiac Renin Concentration
(ng Ang I / gram / hr)
R² = 0.015
p = 0.357
R² = 0.632
p < 0.001
R² = 0.537
p < 0.001
R² = 0.849
p < 0.001
Plasma Renin Concentration
(ng Ang I / mL / hr)
Plasma Renin Concentration
(ng Ang I / mL / hr)

Cardiac tissue renin concentrations significantly correlated with changes in plasma renin across Normal, BNX, DOCA (Figure 2C, $R^2 = 0.537$, $p<0.001$) and Losartan treatment (Figure 2D, $R^2 = 0.849$, $p<0.001$). This is consistent with previously published cardiac and plasma renin associations (9).

Alteration of plasma angiotensinogen concentration. Since adipose tissue was able to maintain a near constant renin level in the face of large changes in plasma renin concentration, we extended this comparison to another RAS component, angiotensinogen (AGT). We assessed the levels of plasma, adipose and cardiac tissue AGT to determine if they were coordinately or independently regulated. Plasma AGT levels varied over a 5 fold range for all treatment groups combined (Figure 3A). BNX treatment plasma AGT was significantly different than DOCA and Losartan treatments. Losartan treatment was significantly different from normal treatment (Figure 3A).

Tissue angiotensinogen (AGT) concentrations. Mean adipose tissue AGT levels ranged from 0.02 nmoles/gram tissue to 0.3 nmoles/gram tissue (Figures 3B). Perfused retroperitoneal adipose tissue AGT levels were reduced compared to visceral adipose tissue and perfused visceral adipose tissue in normal and Losartan treated animals (Figure 3B). No statistical difference was seen between any adipose depot within the BNX and DOCA treated animals. Unlike renin, each treatment produced a significant difference in adipose tissue

Figure 3. Plasma, adipose tissue and cardiac angiotensinogen (AGT) concentrations. (A) Plasma AGT concentrations among treatment groups; Normal (n = 11), BNX (n = 9), DOCA (n = 9) and Losartan (n = 11). AGT concentration was back calculated from angiotensinogen conversion to angiotensin I (1: 1 molar ratio) and expressed as nmol angiotensinogen/ mL plasma or gram tissue. (B) Adipose tissue angiotensinogen (AGT) concentrations. Adipose tissue, depot specific, extract angiotensinogen concentrations within treatment groups. Visceral Adipose Extract (AE), perfused visceral adipose extract (PAE), perfused retroperitoneal adipose extract (PAErp). PAErp n = 5; n is between 9 and 12 for all other groups. Data analyzed by two-way ANOVA. (C) Perfused (PAE) versus nonperfused (AE) visceral adipose extracts from all treatment groups combined (n=41). Data assessed by Wilcoxen Signed Rank test. (D) Left ventricular cardiac AGT concentrations among treatment groups.

** P < 0.05 versus DOCA and Losartan.

++ P < 0.05 versus Normal.

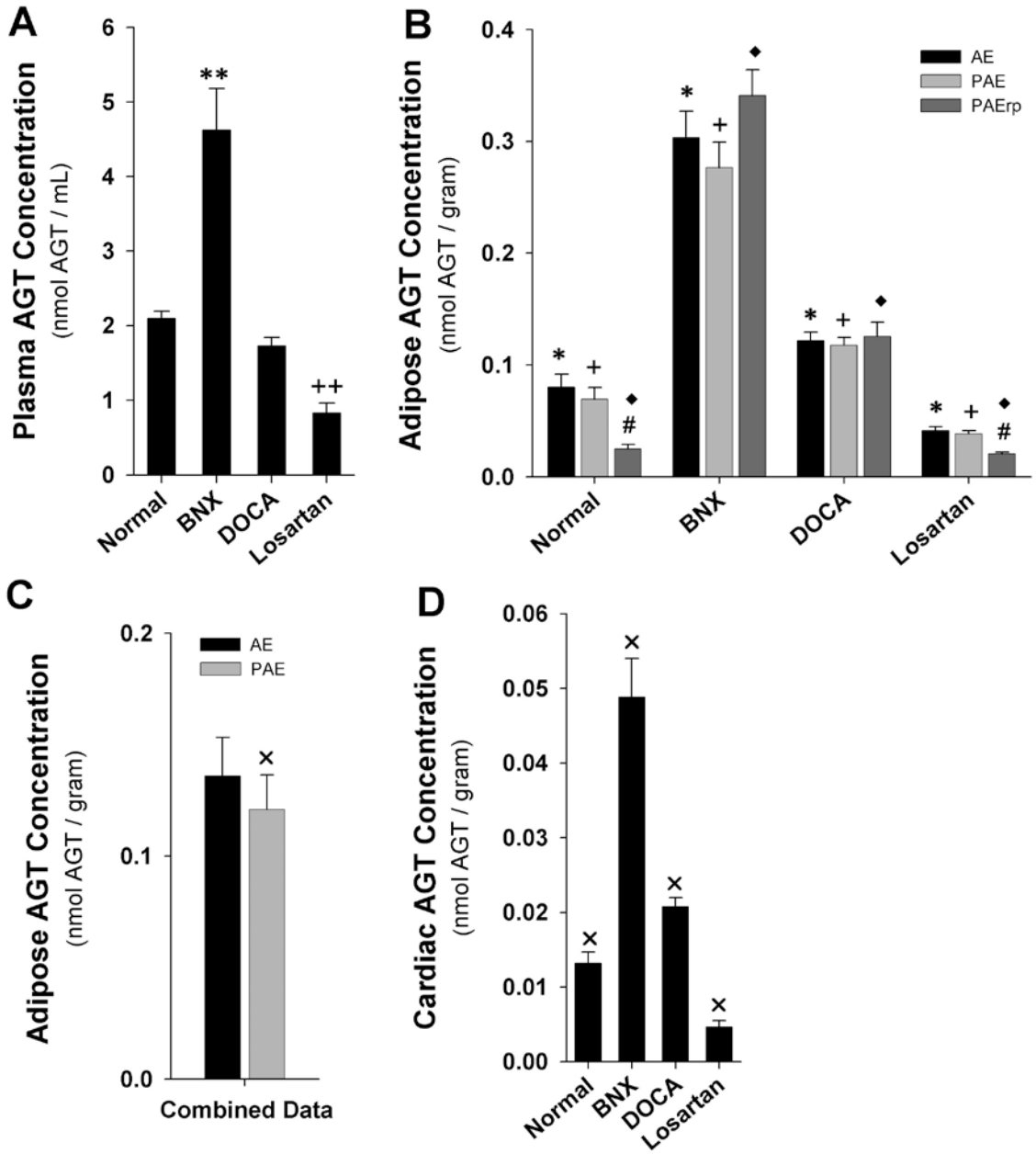
* P < 0.05 versus all other AE treatment groups

+ P < 0.05 versus all other PAE treatment groups

♦ P < 0.05 PAErp versus all other treatment groups except Normal vs. Losartan

# P < 0.05 versus AE and PAE within treatment group

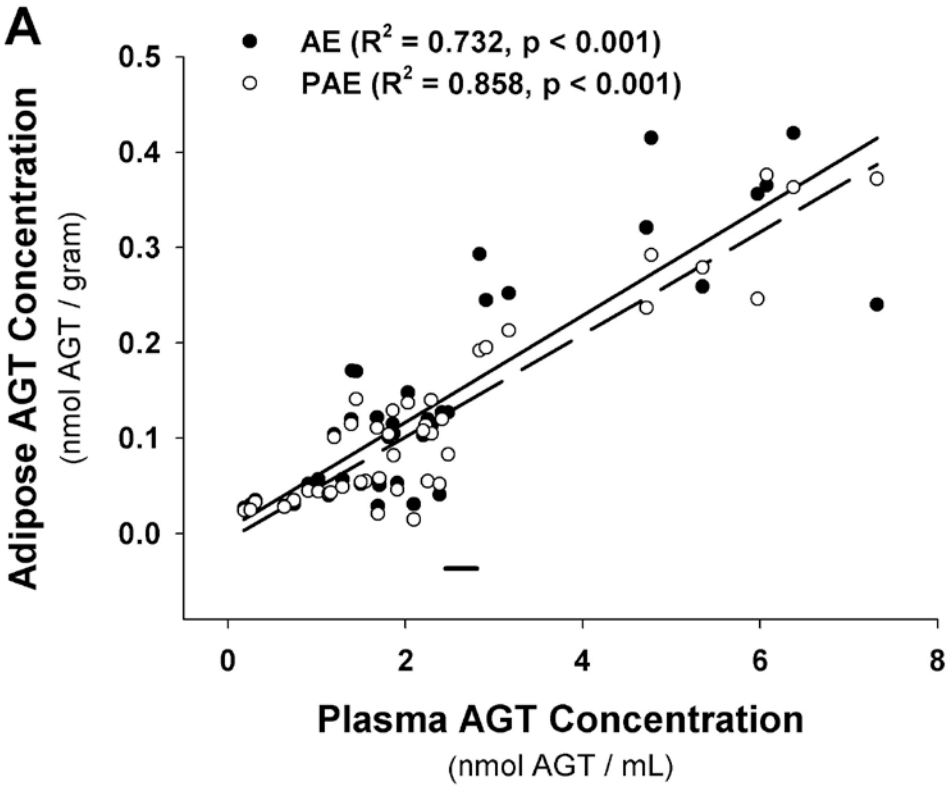
x P < 0.05 versuss all other treatment groups

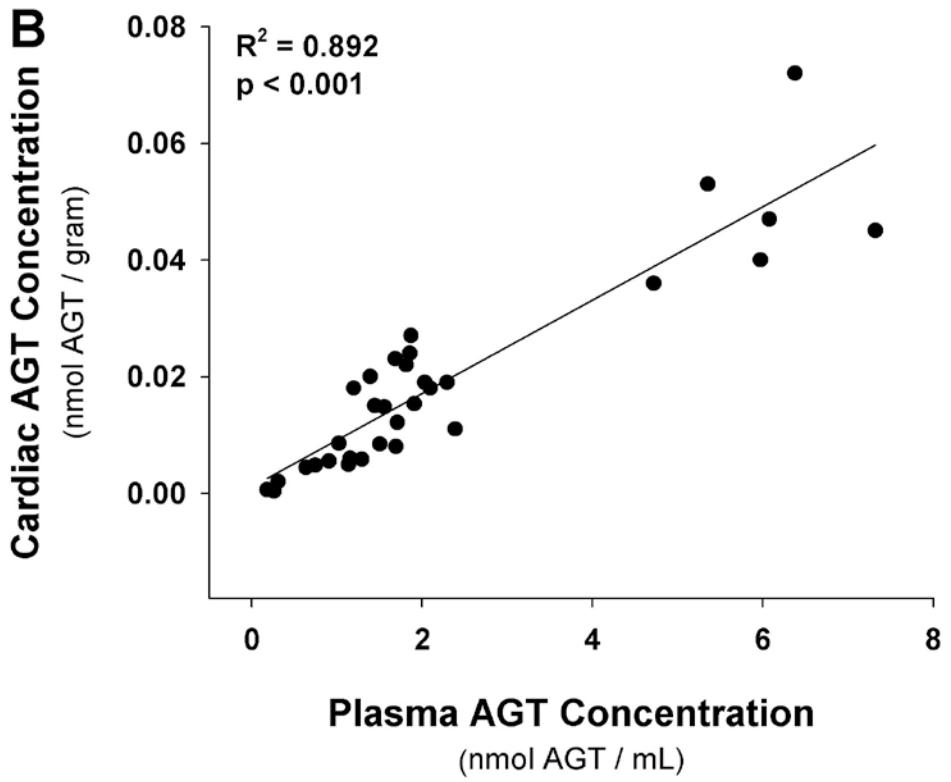

A
Plasma AGT Concentration
(nmol AGT / mL)
6
5
4
3
2
1
0
**
++
Normal
BNX
DOCA
Losartan
B
Adipose AGT Concentration
(nmol AGT / gram)
0.4
0.3
0.2
0.1
0.0
AE
PAE
PAErp
*
+
◆
#
Normal
BNX
DOCA
Losartan
C
Adipose AGT Concentration
(nmol AGT / gram)
0.2
0.1
0.0
AE
PAE
×
Combined Data
D
Cardiac AGT Concentration
(nmol AGT / gram)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
×
Normal
BNX
DOCA
Losartan

angiotensinogen for visceral adipose tissue, perfused visceral adipose tissue and perfused retroperitoneal adipose tissue (Figure 4B); except for normal versus Losartan treatment only within perfused retroperitoneal adipose tissue. Adipose tissue perfusion displayed a consistent trend of reduced angiotensinogen levels in the perfused visceral adipose tissue compared to non-perfused visceral adipose tissue, although this lacked statistical significance within each treatment (Figure 3B). However, combined data for visceral adipose tissue versus perfused visceral adipose tissue across all treatment groups revealed a significant decrease in tissue AGT levels upon tissue perfusion of 11.1 %, $p = 0.01$, $n = 41$ (Figure 3C). Left ventricular cardiac AGT concentrations ranged from 0.005 nmol (Losartan) to 0.05 nmoles (BNX) (Figure 3D). Cardiac extract AGT concentrations differed between all treatment groups, $p < 0.05$ (Figure 3D).

Adipose tissue angiotensinogen versus plasma angiotensinogen. Unlike renin, visceral adipose tissue AGT levels positively correlated with plasma AGT concentrations across all treatment groups for both perfused ($R^2=0.858$, $p<0.001$) and nonperfused visceral adipose tissue ($R^2=0.732$, $p<0.001$) (Figure 4A). The 95% confidence interval for plasma AGT in normal treated animals was 1.9 to 2.3 nmoles AGT/ml (solid bar, Figure 4A). Plasma AGT levels (per ml) tended to be approximately 20 fold greater than adipose tissue AGT (per gram) across all treatment groups.

Figure 4. Plasma AGT vs. visceral adipose tissue and cardiac renin. (A) Normal (n = 11), BNX (n = 9), DOCA (n = 9) and Losartan (n = 11) treated adipose AGT vs. plasma AGT. Closed circles, non-perfused visceral adipose extract (AE). Open circles, perfused visceral adipose tissue (PAE). Solid line, AE, dashed line, PAE. Solid bar represents the 95% confidence interval for plasma AGT concentration in Normal animals. (B) Normal (n = 5), BNX (n = 6) and DOCA (n = 9) and Losartan (n = 11) treated cardiac AGT vs. plasma AGT.


A
AE (R^2 = 0.732, p < 0.001)
PAE (R^2 = 0.858, p < 0.001)
Adipose AGT Concentration
(nmol AGT / gram)
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
Plasma AGT Concentration
(nmol AGT / mL)


B
R^2 = 0.892
p < 0.001
Cardiac AGT Concentration
(nmol AGT / gram)
0.08
0.06
0.04
0.02
0.00
0
2
4
6
8
Plasma AGT Concentration
(nmol AGT / mL)

Cardiac tissue angiotensinogen levels positively correlate with changes in plasma angiotensinogen levels. Cardiac tissue AGT levels positively correlated with plasma AGT concentrations across all treatment groups (Figure 4B, $R^2 = 0.892$, $p < 0.001$). Hence, cardiac AGT (Figure 4B) as well as adipose tissue AGT levels (Figure 4A) varied directly with plasma levels over all four treatment groups. BNX treatment resulted in the highest levels of AGT within plasma, perfused visceral adipose tissue and perfused cardiac tissue groups while Losartan treatment resulted in the lowest.

DISCUSSION

The relationship between local adipose tissue RAS components and plasma RAS variation has not been previously addressed. The results of the present experiments demonstrated that adipose tissue renin was largely independent of circulating renin while adipose tissue AGT was not independent of plasma AGT.

Since plasma renin can be highly variable in order to control blood pressure and Na^+ homeostasis, adipose renin levels involved with local adipocyte biology would likely require independence from plasma renin fluctuations. To analyze this, we varied plasma renin (and angiotensinogen levels) using four treatment groups (Figure 1A , 3A) and subsequently measured the associated changes in adipose tissue RAS components. This study does not address whether RAS components produced by adipose tissue can contribute to circulating RAS levels as has been shown for AGT, but not for angiotensin II (8)**.** Instead, this study examines whether adipose tissue can maintain renin and AGT concentrations independent of plasma fluctuations.

Local generation of Ang II near adipose cell membrane Ang II receptors should be, at least in part, a function of adipose interstitial fluid renin and AGT concentrations. Since renin and AGT are smaller than albumin, which has previously been shown to be present in adipose interstitial fluid (ISF) (5), and since plasma derived renin and AGT have been shown to be present in the ISF

of many tissues including the heart (9), we compared renin and AGT levels in plasma, adipose tissue, and cardiac tissue.

Adipose tissue was perfused to remove plasma renin and AGT circulating within the adipose tissue plasma compartment. Adipose plasma could have provided a false positive correlation between plasma and adipose RAS components. Perfused adipose tissue is therefore composed of ISF and cells. Since the adipose plasma space is approximately 10% (by weight) of adipose tissue (24), if RAS components in adipose tissue are in equilibrium with plasma, a 10% decline in renin and AGT concentrations within the perfused visceral adipose tissue compared to non-perfused visceral adipose tissue should have been observed. This was true for AGT (Figure 3B,3C) but not for renin (Figure 1B).

Previous investigations have shown that after bilateral nephrectomy, plasma and cardiac renin levels decrease dramatically (14; 15). This has led to the suggestion that most cardiac renin is derived from plasma renin of renal origin (9; 14). In this report, we initially used a similar strategy to decrease plasma renin levels; however, visceral and retroperitoneal adipose renin did not fall after bilateral nephrectomy (BNX) compared to normal treatment (Figure 1B, 2A) despite a large, highly significant decease in plasma renin (Figures 1A) and cardiac renin (Figure 1C). Previous studies of interscapular brown adipose tissue homogenates from rats with bilateral nephrectomy reported similar findings, noting that residual adipose vascular smooth muscle or vessel wall associated plasma derived renin could not be excluded as a potential

explanation (28). However, this previous study employed an acidic renin enzymatic assay with angiotensinogen substrate. Our renin enzymatic assay using angiotensinogen substrate was performed at pH 7.45, and we have previously shown (14) that when the assay is performed at pH 5.5, both renin and cathepsin D contribute to Ang I generation such that bilateral nephrectomy artifactually appears to cause no change in cardiac renin activity. Hence an alkaline enzymatic incubation is required to help exclude cathepsin D activity from the renin assay. The rat renin assay in this study does not measure rat cathepsin D (14).

Since the initial bilateral nephrectomy treatment was only 48 hours long, and in an attempt to remove residual sources of bound plasma derived renin activity, we also employed a three week DOCA-salt treatment group where plasma renin was reduced to near zero for a much longer period of time. A possible study limitation was that DOCA animals gained significantly less weight then Normals. However, as with bilateral nephrectomy, visceral and retroperitoneal adipose tissue renin concentration did not fall compared to normal treatment (Figures 1B, 2A) despite long-term near zero plasma renin (Figure 1A) and cardiac renin (Figure 1C). Hence, unlike cardiac tissue, local adipose tissue renin can be independent of decreases in plasma renin. Since all components of the RAS have been previously identified in adipose tissues (13; 20; 22; 26), it seems likely that a local adipose RAS can operate independently of the plasma RAS at least during situations involving normal and low plasma renin states.

Many reports have been published on the role of AGT levels in obesity, obesity related hypertension, adipokine expression and adipocyte metabolism (2; 3; 6; 21). Previously published reports have demonstrated that AGT protein expression restricted to adipose tissue is able to gain access to the plasma compartment and reconstitute a low level of plasma AGT (30%) compared to wild type mice (21). In addition, AGT overexpression increased fat mass and adipocyte hypertrophy while AGT KO mice displayed decreased fat mass and adipocyte size (17). AGT levels, therefore, are capable of affecting multiple aspects of adipocyte function. However, to the best of our knowledge, no studies have addressed the relationship between circulating plasma AGT levels and adipose tissue AGT levels. We have demonstrated that adipose tissue AGT levels correlate positively with changes in plasma AGT levels (Figure 4A). Furthermore, tissue perfusion shows a significant decline in adipose tissue AGT levels of 11.1%, P = 0.01 (Figure 3C). As indicated previously, an estimate of adipose tissue plasma compartment volume is approximately 10% of adipose tissue weight. It is possible; therefore, that plasma AGT is in equilibrium with the adipose tissue ISF. This observation is consistent with liver derived AGT from the plasma reaching equilibrium with adipose AGT and/or adipose derived AGT equilibrating with plasma. However, the observation is not consistent with independent control of AGT levels between plasma and adipose tissue. Plasma AGT (per ml) was approximately 20 fold greater than perfused adipose tissue AGT (per gram) (Figure 5). This is consistent with many possibilities with respect to adipose AGT distribution and the present study does not address the

source or the cell versus interstitial distribution of AGT. However, if the adipose ISF compartment was approximately 5% of the intracellular compartment by weight, a 20 fold greater plasma level than adipose tissue (ISF plus cells) would not be unreasonable if plasma and adipose ISF AGT were in diffusion equilibrium. The ISF of adipose tissue has been reported to be in the range of 3% -15% of tissue weight (23).

Renin action on AGT is the initiation step for ultimate Ang II synthesis and the overall effects of the RAS. Previous studies have detected renin mRNA within adipose tissue (22). If a local RAS system is functioning within adipose tissue it is likely that renin is a necessary component involved in Ang II generation. We have shown renin enzymatic activity within perfused adipose tissue (Figure 1B). Interestingly, unlike AGT, renin levels do not correlate with those of plasma across most treatment groups. In fact, the only instance in which adipose tissue renin levels began to reflect those of plasma renin levels was in the Losartan treated group when plasma renin averaged nearly 25 fold greater than normal.

Bilateral nephrectomy, DOCA, and Losartan treatments are well documented to alter plasma levels of renin and AGT (15; 16). It is also possible that these treatments altered adipose expression of renin and AGT. However, the fact that adipose renin was independent of plasma renin over a wide range of circulating renin levels suggest that adipose renin can be expressed independently of circulating renin, This does not appear to be true for AGT. If

adipose renin levels help determine the rate of adipose angiotensin II generation, then an adipose RAS can exist independently of the circulating RAS regardless of AGT dynamics.

How adipose tissue is able to maintain renin concentration near normal levels in the face of chronically reduced plasma renin levels is unclear. It may be that renin cannot easily cross the adipose capillary wall even though larger molecules such as albumin and AGT apparently have adipose capillary permeability. In this case, adipose renin levels may be due to local adipocyte (or other cellular components) synthesis. Perhaps an adipocyte membrane renin binding protein can maintain renin activity levels when plasma renin is low by activating prorenin. Renin binding protein activation of prorenin has been previously hypothesized (12) and prorenin receptor has been detected in human adipose tissue (1). Alternatively, adipose renin that ultimately catalyzes Ang II formation may primarily be in the intracellular compartment and therefore not influenced by changes in a relatively small extracellular renin pool. An intracellular (intacrine) role of Ang II has recently been shown to be activated in cardiac fibroblasts by high glucose levels (29). When Losartan treatment significantly increases plasma renin, some plasma renin may then cross the capillary wall and contribute to the previously existing adipose derived renin activity or adipose tissue angiotensin II receptor blockade may result in upregulation of adipose renin expression as in kidney tissue, although no upregulation was seen during bilateral nephrectomy. Regardless, adipose

tissue control of local renin concentrations may be a potential mechanism maintaining a functional local RAS independent of plasma fluctuations.

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GRANTS

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Chapter 3

Regulated Renin Release from 3T3-L1 Adipocytes

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ABSTRACT

Whereas adipose tissue possesses a local renin-angiotensin system, the synthesis and regulated release of renin has not been addressed. To that end we utilized differentiating 3T3-L1 cells and analyzed renin expression and secretion. Renin mRNA expression and protein enzymatic activity were not detectable in preadipocytes. However, upon differentiation, renin mRNA and both intracellular and extracellular renin activity were up regulated. In differentiated adipocytes, forskolin treatment resulted in a 28-fold increase in renin mRNA while TNFα treatment decreased renin mRNA 4-fold. IL-6, insulin, and angiotensin II (Ang II) were without effect. In contrast, forskolin and TNFα each increased renin protein secretion by 12- and 7-fold, respectively. Although both forskolin and TNFα induce lipolysis in adipocytes, fatty acids, prostaglandin E2 or lipopolysaccharide had no effect on renin mRNA or secretion. To evaluate mechanism(s) by which forskolin and/or TNFα are able to regulate renin secretion, a general lipase inhibitor (E600) and PKA inhibitor (H89) were used. Both inhibitors attenuated forskolin induced renin release while having no effect on TNFα regulated secretion. In contrast, E600 potentiated forskolin-stimulated renin mRNA levels while H89 had no effect. Neither inhibitor had any influence on TNFα regulation of renin mRNA. Relative to lean controls, renin expression was reduced 78% in the epididymal adipose tissue of obese male C57Bl/6J mice, consistent with TNFα-mediated down regulation of renin mRNA in the culture system. In conclusion, the expression

and secretion of renin are regulated under a complex series of hormonal and metabolic determinants in mature 3T3-L1 adipocytes.

INTRODUCTION

A local renin angiotensin system (RAS) has been recently described in adipose tissue (1; 18; 20; 25; 29; 32; 35; 37; 44) where its functional significance has been linked to a number of processes including metabolism, differentiation, oxidative stress and inflammation (1; 11; 19; 25; 37). All the necessary components of the RAS including renin, angiotensin converting enzyme, and angiotensinogen (AGT) (20; 35) are expressed in adipose tissue, although the specific cell type(s) responsible have not been completely defined. While the role of the local adipose RAS is complex, it is clear that Ang II, the terminal product of the catalytic cascade system, has many effects within adipose tissue. Indeed, antagonism of the RAS using pharmacologic or molecular means improves insulin sensitivity (15) and decreases inflammation. Moreover, angiotensin II increases lipogenesis in cultured human and murine cells (19) and AGT knock out mice display decreased weight gain and decreased adipocyte cell size (30).

Angiotensinogen mRNA expression, protein synthesis and regulation within adipose tissue has been extensively investigated, particularly with regard to the issue of fat-derived AGT contribution to circulating levels (29). Renin enzymatic levels, however, have not been consistently detected in adipose tissue and while renin expression within adipose tissue has been demonstrated (20; 35) it has not been a consistent observation (9). Nevertheless, renin appears to be an integral part in some parts of adipose biology. Mice lacking renin are

resistant to diet induced obesity (44). In addition, when inserted into mice, the human renin gene showed tissue specific regulation including adipose tissue expression (40). Since renin action on AGT is the rate-limiting step in the production of Ang II, the ultimate RAS effector, local expression and regulated release from adipose tissue may be an important regulatory mechanism for production of local Ang II.

Given the wide disparity of conclusions concerning adipose renin biology, we utilized the 3T3-L1 system to systematically investigate renin expression and enzymatic activity. During the course of this study, we made the novel observation that forskolin and TNFα function as major regulators of renin synthesis and secretion and report herein findings that characterize a linkage between pro-inflammatory cytokines and the local RAS system.

MATERIALS AND METHODS

Cell Culture. 3T3-L1 preadipocytes, maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum were differentiated to adipocytes as previously described (41). Briefly, cells were treated with 174 μM insulin, 0.5 mM dexamethasone, and 0.25 μM methylisobutylxanthine in DMEM supplemented with 10% fetal bovine serum at two days post confluence (designated "day 0") and again with insulin at day 2. Insulin was withdrawn at day 4 and cells were maintained in DMEM 10% FBS until mature (day 6-8). Unless otherwise indicated, experiments were conducted with day 8 adipocytes.

Animals. C57BL/6J male mice were maintained on a high fat (HF) or normal chow (LF) diet (F3282, BioServ Inc), approximately 35% and 3.5% fat by weight, respectively, at weaning and maintained for an additional 7-10 weeks. High fat feeding of C57Bl/6J mice results in diet-induced obesity and insulin resistance (42). Animal protocols were approved by The University of Minnesota Institutional Animal Care and Use Committee.

Protein Isolation. To isolate intracellular protein for renin activity measurement, cells in culture were rinsed with 1X PBS to remove residual media. Ice cold protease inhibitor buffer (PIB) consisting of 0.15 M sodium phosphate buffer (pH 7.45), 1 % BSA, 0.05% NaN3 and serine-,metallo-,and thiol-protease inhibitors (15 mM EDTA, 2.3 mM Captopril, 2 mM 8-hydroxyquinoline, 10 mM sodium tetrathionate, 20 mM benzamidine, 10 mM N-ethylmaleimide, 3 mM

PEFABLOC (Sigma), 20 µM leupeptin, and 450 µM aprotinin), was added directly to culture plate and cells were scraped off the plate. Cell suspension was homogenized with a 1 mL dounce glass homogenizer and centrifuged at 100,000 x g. to remove cellular debris. Supernatant was collected and frozen at -70° C for renin activity assay.

Renin Concentration Measurement: 50 µL of sample (either soluble adipose protein or culture medium), 25 µL renin-free rat AGT (obtained from 48 hour bilaterally nephrectomized rats as previously described (23)), and 25 µL sodium phosphate buffer (0.15 M, pH 7.45, 2.5% BSA) were incubated at 37° C for 0, 0.5, and 1 hour in duplicate to generate angiotensin I (Ang I). As previously described, AGT levels in the assay are at near-saturating conditions such that Ang I generation over time is directly proportional to renin concentration (21-23). Ang I was subsequently quantified by radioimmunoassay as previously described (21). Briefly, I^{125} labeled Ang I was mixed with a polyclonal primary antibody directed toward Ang I (27) and incubated at 4° C overnight. Secondary antibody (anti-rabbit IgG, Sigma-Aldrich) in a mixture of 4% polyethylene glycol (MW = 8,000) with 0.5% rice starch was added for 10 minutes at 4° C to precipitate primary antibody-Ang I complex. Samples were centrifuged for 20 minutes at 2,000 x g and the supernatant was discarded. Radioactivity in the pellets was measured and compared to known Ang I standards to calculate the initial renin concentration.

Shallow Gradient Isoelectric Focusing of Renin and Cathepsin D. Shallow gradient isoelectric focusing was performed as previously described (21). Gels were frozen, cut into 65 slices and incubated in PIB overnight at 4° C to elute the separated proteins. Fifty µL of the extracted protein from each gel slice was assayed for renin activity at pH 7.45 as described. Cathepsin D activity was measured from 10 uL of the extracted protein at pH 4.1 using porcine tetradecapeptide (TDP) as a substrate as previously described (21).

RNA Isolation and real time reverse transcriptase polymerase chain reaction (qRT- PCR). Cultured adipocytes and adipose tissue were harvested in Trizol (Invitrogen) for total RNA isolation according to manufacturers protocol and DNase treated (Ambion) prior to cDNA synthesis. cDNA synthesis was performed using iScript cDNA synthesis kit (Bio-Rad). 5ng cDNA in duplicate was diluted to 20 µL total volume in iQ SYBR green supermix (Bio-Rad) for qRT-PCR analysis of renin and transcription initiation factor IIE (TFIIE) message levels. qRT-PCR was performed using a Biorad My iQ iCycler with subsequent data acquisition and analysis with Biorad iQ5 software. Real time RT-PCR primers to renin (forward TGAAGAAGGCTGTGCGGTAGT, reverse TCCCAGGTCAAAGGAAATGTC) and TFII-E (forward CCAGGCTTTAGGG

GACCAGATAC, reverse CATCCATTGACTCCACAGTGACAC) were designed using MacVector Software. All qRT-PCR data was normalized to TFIIE.

Statistical analysis. Renin enzymatic activity during 3T3-L1 adipose conversion was analyzed by Kruskal-Wallis One Way Analysis of Variance on Ranks. Differences between groups were further analyzed via the Student-Newman-Keuls method for all pairwise multiple comparisons. n is between 3 and 6 for each sample. Inducible renin release from cultured preadipocytes and adipocytes was analyzed by Kruskal-Wallis one way analysis of variance on ranks followed by Dunn's Method for Multiple Comparisons versus Control Group, data is representative of multiple experiments, n = 3 for each treatment. Renin release dose-response curves were analyzed via sigmoidal dose response curve calculation of simple ligand binding for calculation of $K_{0.5}$. n = 6 for each data point. Statstical analysis of TNFα and forskolin treatment of Day 8 3T3-L1 adipocytes on renin release, intracellular renin levels, and renin mRNA expression were analyzed by Two Way ANOVA followed by the Holm-Sidak all pairwise multiple comparison procedure. Culture media renin activity data for both TNFα and forskolin treatment was square root transformed to pass normality test. Statistical analysis was performed on the delta ct values for renin message data. n = 6 per data point. Attenuation of regulated renin release enzyme activity measurements were analyzed by Kruskal-Wallis One Way Analysis of Variance on Ranks followed by the Student-Newman-Keuls method for all pairwise multiple comparisons. Relative renin expression data was analyzed by One Way ANOVA followed by Holm-Sidak all pairwise multiple comparison procedure to analyze differences between groups. n = 4 per

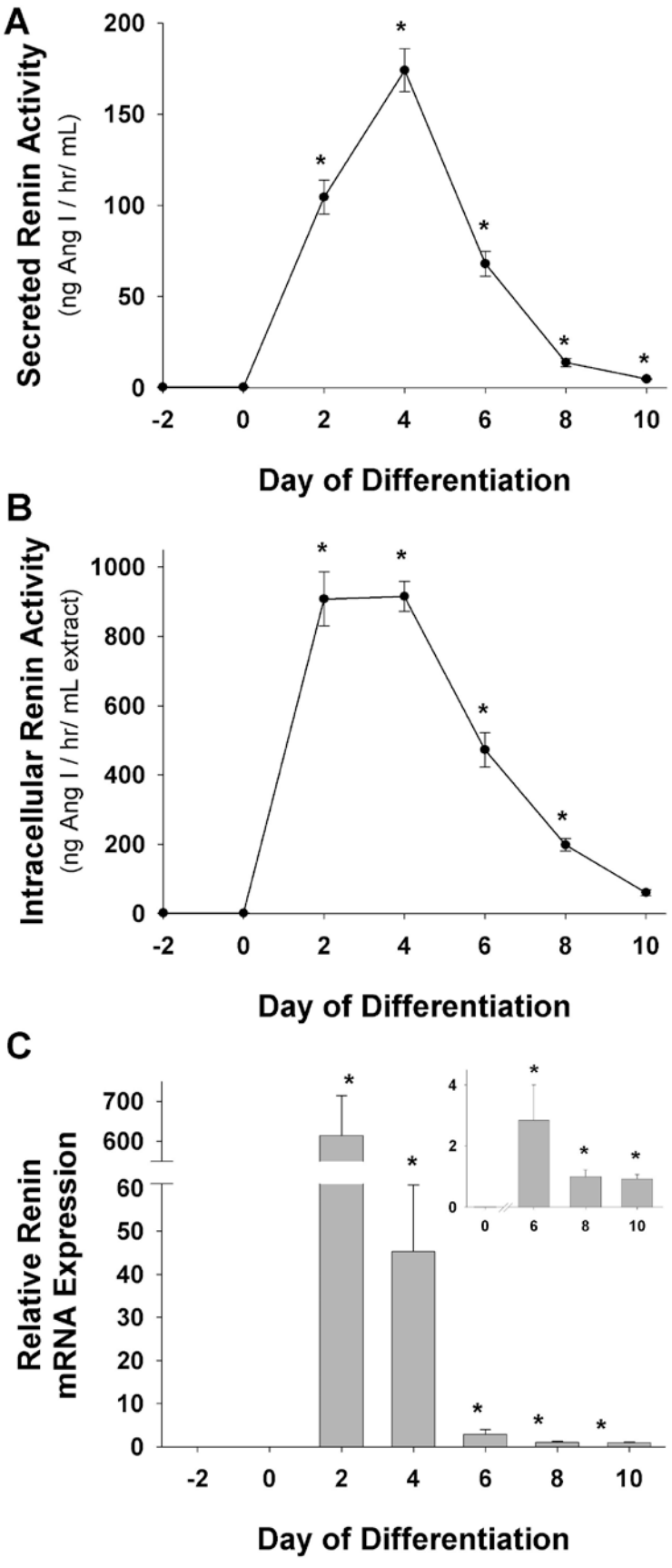
treatment, $p < 0.05$ is significant. The expression of renin in epididymal adipose tissue was analyzed by Students t-test.

RESULTS

The renin angiotensin system has been implicated in a variety of adipose tissue functions and the significance of adipose derived renin to either a local or systemic RAS is controversial. Some reports have detected renin protein enzymatic activity from adipose tissue in addition to renin mRNA (35) while others have failed to detect renin mRNA expression (9). To address the role of renin enzyme production and release from adipocytes, the 3T3-L1 culture system was investigated for secreted (medium) and intracellular renin protein enzymatic activity and message (Figure 1). Renin message and protein activity was undetectable in preadipocytes (Figure 1, day -2 and day 0) however, during adipose conversion, renin mRNA levels increased dramatically (day 2-4) with significant increases in intracellular renin (over 1000-fold, $p < 0.05$) and media renin (over 500 fold, $p < 0.05$) (Figure 1). Renin mRNA, intracellular renin and secreted renin all declined in abundance over the next 3-5 days to new steady state levels markedly above those in preadipocytes. Intracellular renin activity remained significantly elevated above preadipocyte levels (nearly 300 fold) in day 8 fully mature adipocytes (Figure 1B). Treatment of preadipocytes with insulin, dexamethasone, or MIX individually displayed no significant increases in renin message level or protein activity above untreated preadipocytes (data not shown).

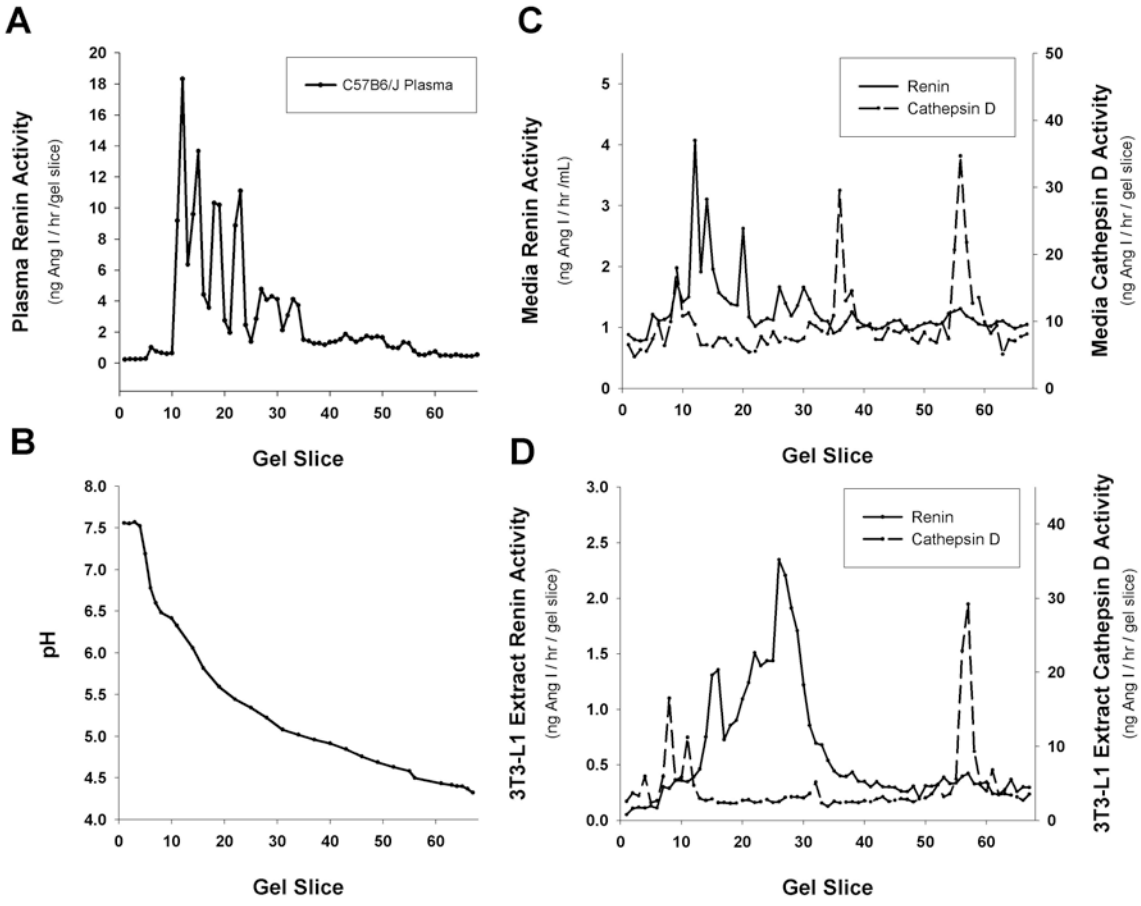
While AGT is the only known substrate for renin, other enzymes are able to cleave AGT to Ang I and thus potentially obscure measurement of renin activity

Figure 1: Renin enzymatic activity during 3T3-L1 adipose conversion. 3T3-L1 preadipocytes were maintained and differentiated as described in Materials and Methods. Media, cells and total RNA were collected every 48 hours and assayed for secreted or intracellular renin activity via enzymatic radioimmunoassay and renin message via qRT-PCR. (A) Secreted (medium) renin enzymatic activity (B) Intracellular renin enzyme activity from cultured adipocytes cell extracts (C) Relative renin mRNA levels during adipose conversion normalized to TFIIE. Data is representative of 3 separate experiments for each plot. n = 3 - 6 for each sample. Data was analyzed by Kruskal-Wallis one way analysis of variance on ranks followed by Student-Newman-Keuls method for all pairwise multiple comparison procedures. * $p < 0.05$ vs. Day 0 (preadipocytes)


A
Secreted Renin Activity
(ng Ang I / hr/ mL)
200
150
100
50
0
-2
0
2
4
6
8
10
Day of Differentiation
B
Intracellular Renin Activity
(ng Ang I / hr/ mL extract)
1000
800
600
400
200
0
Day of Differentiation
C
Relative Renin
mRNA Expression
700
600
60
50
40
30
20
10
0
Day of Differentiation

in the 3T3-L1 cells and media. Although serine-,metallo-,and thiol-proteases are inhibited in the renin assay, aspartyl-proteases are not inhibited. Since cathepsin D is an aspartyl-protease with ~40% sequence similarity to renin and is capable of cleaving AGT to Ang I, we demonstrated that the renin activity measured in 3T3-L1 cells is derived from renin and not cathepsin D. We utilized shallow gradient isoelectric focusing to separate the two enzymes followed by enzyme assay. In this assay, the activity profile as a function of gel slice (pH) represents a diagnostic fingerprint for distinguishing various enzyme activities. Since C57BL/6J mice only have the single *ren1* gene, a C57BL/6J mouse plasma focusing profile is shown as a positive control for murine ren1 (Figure 2A) and demonstrates that renin glycoforms (22) focus as a cluster of activities between pH 5.0 and 6.5 (Figure 2B). Renin activity in the media from 3T3-L1 adipocytes (Figure 2C) focused with a similar isoelectric point activity profile as does mouse plasma renin. In contrast, the cathepsin D activity profile was distinct from that shown by renin and focused with a distinctive pattern of activities at pH 6.5, pH 5.0 and at pH 4.5. Since the renin activity profile does not overlap with that from cathepsin D (Figure 2C), the 3T3-L1 renin concentration measurements are independent of cathepsin D activity as has been previously reported in rat and human (21). Similarly, cell extract renin activity profile (Figure 2D) focused with the same pH range as did C57BL/6J plasma renin while the cathepsin D activity profile focused outside the pH range of C57BL/6J plasma renin, media renin, and cell extract renin.

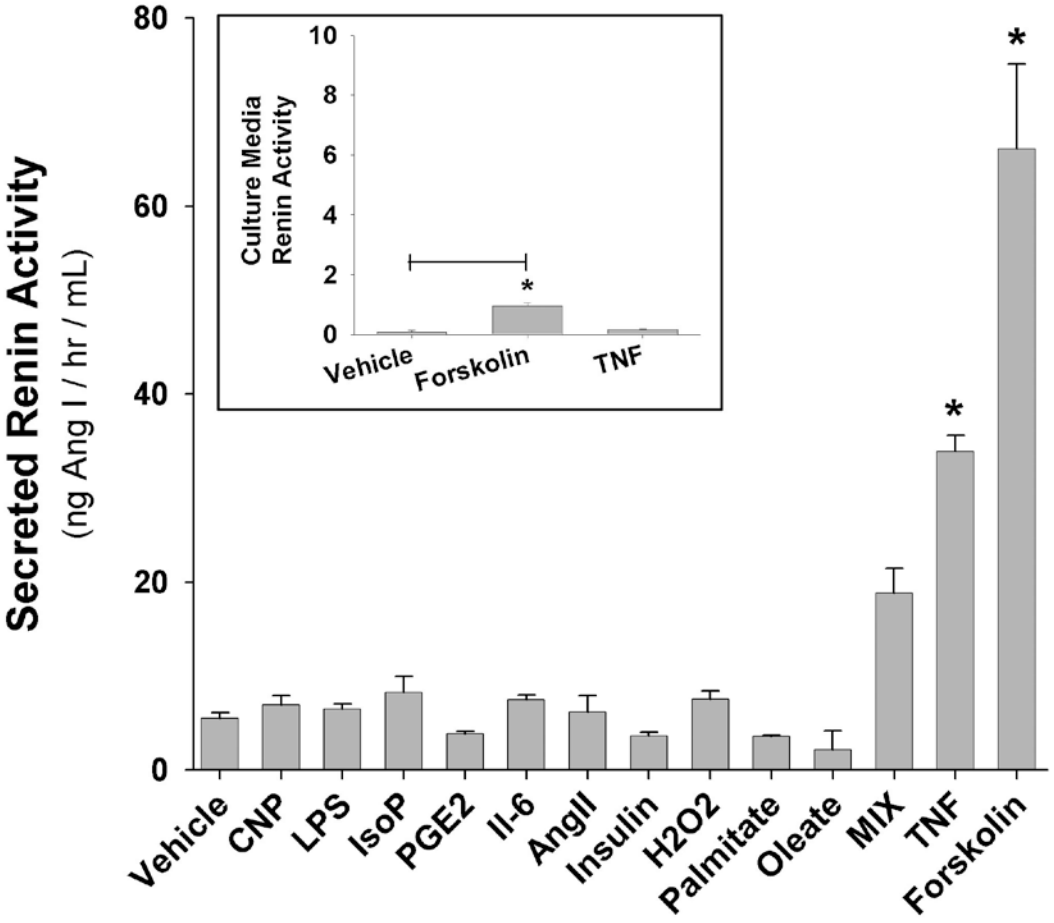
Figure 2: Shallow gradient isoelectric focusing profiles of mouse and 3T3-L1 renin and 3T3-L1 cathepsin D. (A) Focusing profile of C57BL/6J mouse plasma renin activity. (B) Representative pH gradient from individual gel slices. (C) Renin and cathepsin D focusing profiles in 3T3-L1 day 8 culture media. (D) Renin and cathepsin D focusing profiles in 3T3-L1 day 8 cellular extracts. Data is representative of at least 3 experiments.


A
Plasma Renin Activity
(ng Ang I / hr /gel slice)
C57B6/J Plasma
Gel Slice
B
pH
Gel Slice
C
Media Renin Activity
(ng Ang I / hr /mL)
Renin
Cathepsin D
Media Cathepsin D Activity
(ng Ang I / hr / gel slice)
Gel Slice
D
3T3-L1 Extract Renin Activity
(ng Ang I / hr / gel slice)
Renin
Cathepsin D
3T3-L1 Extract Cathepsin D Activity
(ng Ang I / hr / gel slice)
Gel Slice

The wide disparity of reports on the mRNA or secreted levels of renin suggested that the control of renin production and release may be a regulated process. To test this hypothesis, day 8 adipocytes were treated for 24 hours with a variety of potential regulatory molecules followed by media analysis of renin enzymatic activity. Treatment of adipocytes with 1 μM CNP, 1μg/mL lipopolysaccharide, 10 μM Isoproternol, 100 ng/mL IL-6, 100 nM prostaglandin E2, $1x10^{-5}$ M Ang II, 100 μM H_2O_2, 500 μM oleate, 500 μM palmitate (each fatty acid delivered on 100 μM bovine serum albumin) or 100 nM Insulin were each ineffective in altering media renin levels above vehicle controls (Figure 3). While not statistically significant methylisobutylxanthine did slightly increase media renin levels suggesting that some component of the cAMP and/or lipolysis system may be regulatory. Pursuing this concept further, 100 μM forskolin and 1 nM TNFα, two agents known to increase fat cell triglyceride hydrolysis, dramatically increased renin enzymatic activity in the culture media of day 8 adipocytes by 12.6 and 7.5 fold, respectively ($p<0.05$) (Figure 3).

Although adipose conversion in culture is routinely greater than 85% (measured by macroscopic lipid accumulation), it can experimentally vary leading to differing levels of residual preadipocytes. While preadipocytes did not show detectable renin activity or message (Figure 1, day 0), it may be possible for preadipocytes to synthesis or secrete renin upon 24 hour TNFα or forskolin stimulation. We therefore treated preadipocytes with TNFα and forskolin to measure their effects on culture media renin activity. TNFα was unable to able

Figure 3: Inducible renin release from cultured adipocytes. Day 8 cultured 3T3-L1 adipocytes were treated for 24 hours with various compounds to stimulate renin release. Vehicle bar is representative for each individual treatment as vehicle treatment was not statistically different than no treatment for any compound, 1 μM CNP, 1 μg/mL lipopolysaccharide (LPS), 10 μM Isoproterenol (IsoP), 100 nM prostaglandin E2 (PGE2), 100 ng/mL IL-6, $1x10^{-5}$ M Angiotensin II (Ang II), 100 nM Insulin, 100 μM hydrogen peroxide (H_2O_2), 100 μM palmitate, 100 μM oleate (fatty acids delivered on 100 μM bovine serum albumin), 0.115 mg/mL methylisobutylxanthine (MIX), 100 μM forskolin and 1 nM TNFα. * $p < 0.05$ vs. vehicle control. n = 4 – 6 for each sample. **Insert:** Effect of 100 μM forskolin and 1 nM TNFα on culture medium renin activity of cultured preadipocytes. * $p < 0.05$ vs. vehicle control. n = 6.


Secreted Renin Activity
(ng Ang I / hr / mL)
80
60
40
20
0
Vehicle
CNP
LPS
IsoP
PGE2
Il-6
AngII
Insulin
H2O2
Palmitate
Oleate
MIX
TNF
Forskolin
*
*
*
Culture Media
Renin Activity
10
8
6
4
2
0
Vehicle
Forskolin
TNF

to increase culture media renin enzymatic activity above that of untreated preadipocytes (both were nearly undetectable) (Figure 3, insert). Forskolin treatment did show a significant increase in culture media enzymatic activity versus untreated preadipocytes; however, absolute renin activity was negligible compared to forskolin treated mature adipocytes (approximately 100 fold less) (Figure 3, insert). Therefore residual preadipocytes in the day 8 adipocyte population do not significantly contribute to renin activity.

To further characterize the effects of forskolin and TNFα on renin release from cultured adipocytes, we evaluated the concentration dependence for each compound (Figure 4). Day 8 adipocytes were treated with a range of concentrations of either forskolin or TNFα for 24 hours and culture media renin activity was assayed. TNFα displayed a maximal culture media renin activity of approx 35 ng Ang I/hr/mL at 1nM, with a $K_{0.5}$ of 270 pM (Figure 4B). Forskolin treatment displayed a more robust increase in media renin activity than TNFα of 66 ng Ang I/hr/mL at a dose of 100 μM with a $K_{0.5}$ of 38 μM (Figure 4A).

Forskolin treatment displayed a time dependent increase in media renin activity with a maximum induction of ~13-fold at 24 hours (Figure 5A) as well as a decrease in intracellular renin protein levels of 42%, $p < 0.05$, at 24 hours relative to vehicle (Figure 5B). qRT-PCR analysis of renin message during forskolin stimulation revealed a significant increase in renin message of 28 fold ($p < 0.05$), after 16 hours treatment and then declined to a 9.5 fold increase after 24 hours

Figure 4: Renin release dose-response curves. Day 8 3T3-L1 adipocytes were treated for 24 hours with forskolin or TNFα at increasing concentrations to induce renin release into culture media. (A) Forskolin dose-response curve (B) TNFα dose-response curve. n = 6 for each data point.

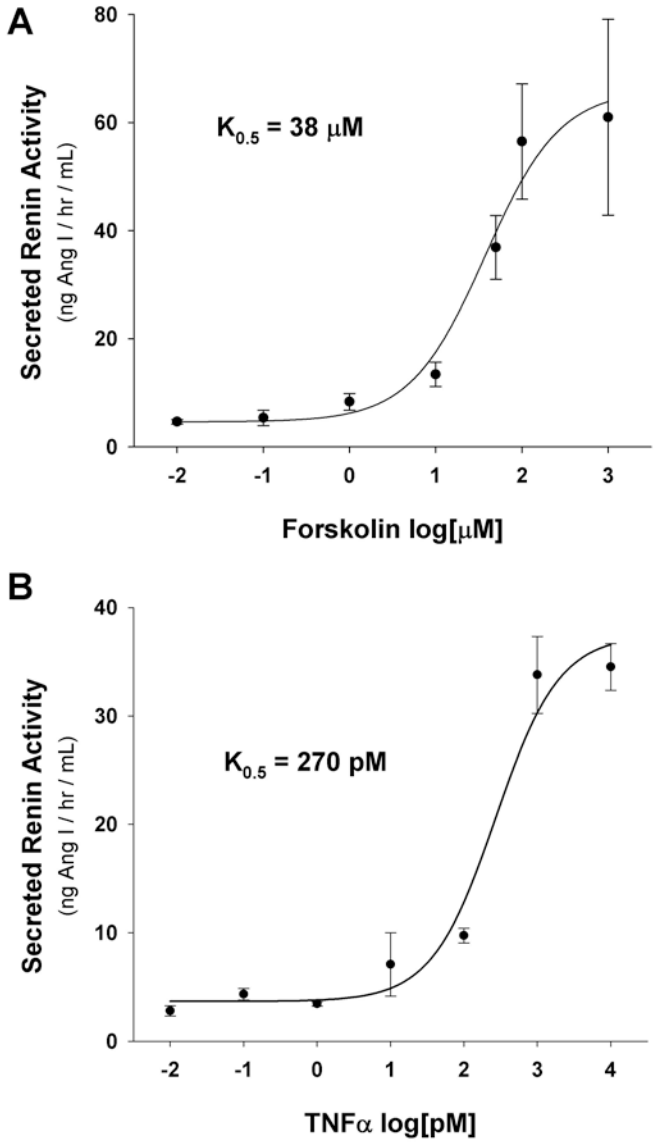
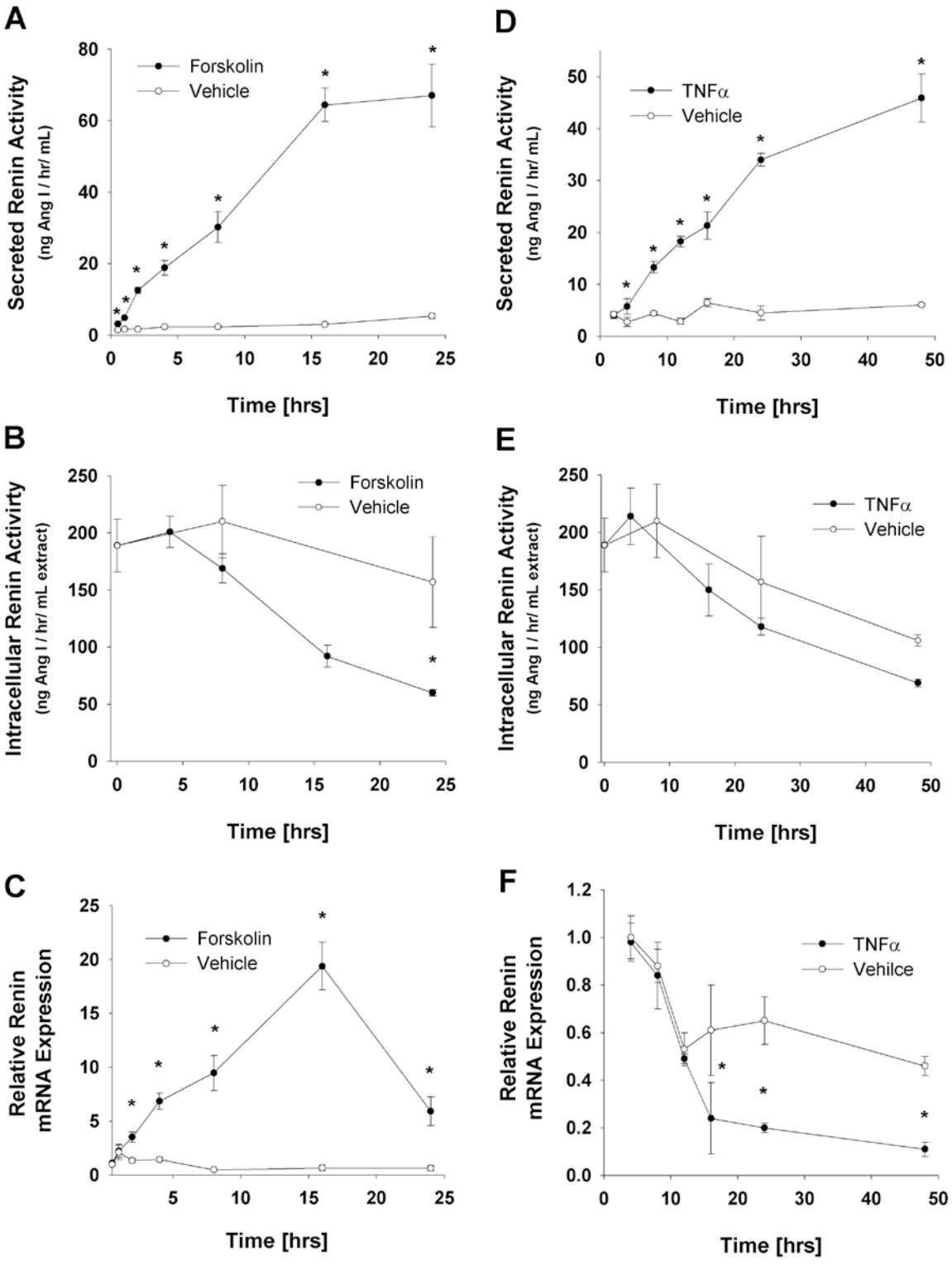

A
Secreted Renin Activity
(ng Ang I / hr / mL)
$K_{0.5}$ = 38 μM
Forskolin log[μM]
B
Secreted Renin Activity
(ng Ang I / hr / mL)
$K_{0.5}$ = 270 pM
TNFα log[pM]

Figure 5: Effect of forskolin and TNFα treatment on renin enzymatic activity and message levels in 3T3-L1 cultured adipocytes. Day 8 3T3-L1 adipocytes were treated with either 100 μM forskolin or 1 nM TNFα for indicated periods of time and medium renin enzymatic activity, intracellular renin enzymatic activity, and renin mRNA levels were analyzed. (A) Forskolin regulated secreted renin enzymatic activity. (B) Forskolin regulated intracellular renin enzymatic activity. (C) qRT-PCR analysis of renin message levels normalized to TFIIE following forskolin treatment. (D) TNFα regulated secreted renin enzymatic activity. (E) TNFα regulated intracellular renin enzymatic activity. (F) qRT-PCR analysis of renin message levels normalized to TFIIE following TNFα treatment. Statistical analysis via two way ANOVA followed by Holm-Sidak all pairwise multiple comparison procedure to analyze differences between groups. For all panels (A-F) * signifies $p < 0.05$ vs. same time point vehicle control. Data is representative of multiple experiments. n = 6 per data point.


A
Secreted Renin Activity
(ng Ang I / hr/ mL)
Forskolin
Vehicle
Time [hrs]
D
Secreted Renin Activity
(ng Ang I / hr/ mL)
TNFα
Vehicle
Time [hrs]
B
Intracellular Renin Activirty
(ng Ang I / hr/ mL extract)
Forskolin
Vehicle
Time [hrs]
E
Intracellular Renin Activity
(ng Ang I / hr/ mL extract)
TNFα
Vehicle
Time [hrs]
C
Relative Renin
mRNA Expression
Forskolin
Vehicle
Time [hrs]
F
Relative Renin
mRNA Expression
TNFα
Vehilce
Time [hrs]

(Figure 5C). In parallel studies, TNFα treatment increased media levels of renin by 7.4 fold ($p < 0.05$) at 48 hours (Figure 5D). While forskolin displayed significant increases (2.1 fold, $p < 0.05$) in media renin activity at 30 min, TNFα induction of renin media activity was significantly delayed and did not increase above vehicle control until 4 hrs treatment (Figure 5D). In addition, while intracellular levels did trend toward a decrease in the TNFα treated samples (Figure 5E), no statistical difference was observed, perhaps due to the decline in intracellular renin levels between day 8 and 10 adipocytes (Figure 1B). In contrast to forskolin, TNFα treatment resulted in a 4.2 fold decrease ($p < 0.05$) in renin message by 16 hrs that continued through the furthest time point evaluated of 48 hours (Figure 5C).

Given the ability of forskolin and TNFα to regulate adipocyte lipolysis, albeit by different mechanisms, we utilized pharmacologic inhibition as an experimental tool to probe the mechanism of regulated renin release. Firstly, since forskolin activates adenylyl cyclase, H89 a protein kinase A inhibitor, was used to disrupt cAMP activation and subsequent intracellular signaling. Moreover, some reports have suggested a role of cAMP mediated intracellular signaling with TNFα stimulation (26; 36; 48). Secondly, E600, a general lipase inhibitor was used to block the production of fatty acids from triacylglycerol hydrolysis. H89 decreased forskolin induced renin release by 59% ($p <0.05$) at 24 hours treatment while E600 completely attenuated rennin secretion Figure 6A). Neither inhibitor had any effect on TNFα induced renin release (Figure 6B).

Renin message levels were also analyzed after 24-hour treatment with or without E600 and H89. 24 hour forskolin treatment increased renin expression above vehicle control by 3.5 fold (Figure 6C). Whereas H89 had no effect on forskolin induced induction of renin message (Figure 6C), E600 in contrast increased renin expression nearly 40 fold above vehicle control (Figure 6C). 24 hour TNFα treatment decreased renin expression by greater than 60% (Figure 6D) while TNFα plus H89 or E600 treatment did not significantly affect the reduction in renin message (Figure 6D). E600 and H89 alone had no effect on renin expression with 24 hour treatment (Figure 6C and 6D).

Obesity and insulin resistance are associated with increased basal inflammation as well as sympathetic drive (14). It is likely therefore that obesity may modulate renin message levels in the pathological obese/insulin resistant state. To address the potential interaction of obesity and insulin resistance on renin message levels, obese insulin resistant C57BL/6J mice maintained on a high fat diet (42), were compared to normal chow (LF) fed mice. Twelve week old male mice on a high fat diet (n=9) displayed a 2.5 fold increase in epididymal white adipose tissue (EWAT) as a percentage of total body mass over normal chow counterparts (n=9) (Figure 7A). Total body weight was also significantly increased 1.43 fold in the HF mice, $p < 0.001$ (data not shown). qRT-PCR analysis revealed a 78.1% decrease ($p<0.01$) in the epididymal white adipose tissue (EWAT) renin expression of obese male mice compared to normal chow controls (Figure 7B).

Figure 6: Attenuation of regulated renin release. Day 8 3T3-L1 adipocytes were treated for 1 hour with either 10 μM H89 (PKA inhibitor) or 100 μM E600 (general lipase inhibitor) followed by 20 hour incubation with 100 μM forskolin or 1 nM TNFα in the presence of inhibitor. H89 and E600 alone were preincubated for 1 hour and changed to H89 and E600 for additional 24 hours. (A) Secreted renin enzymatic activity during treatment. (B) Secreted renin enzymatic activity during the indicated treatment. (C) Relative renin mRNA expression normalized to TFIIE during the indicated treatment. (D) Relative renin mRNA expression normalized to TFIIE during the indicated treatment. For each panel, * $p < 0.05$ vs TNF or forskolin treatment, # $p < 0.05$ vs Vehicle.

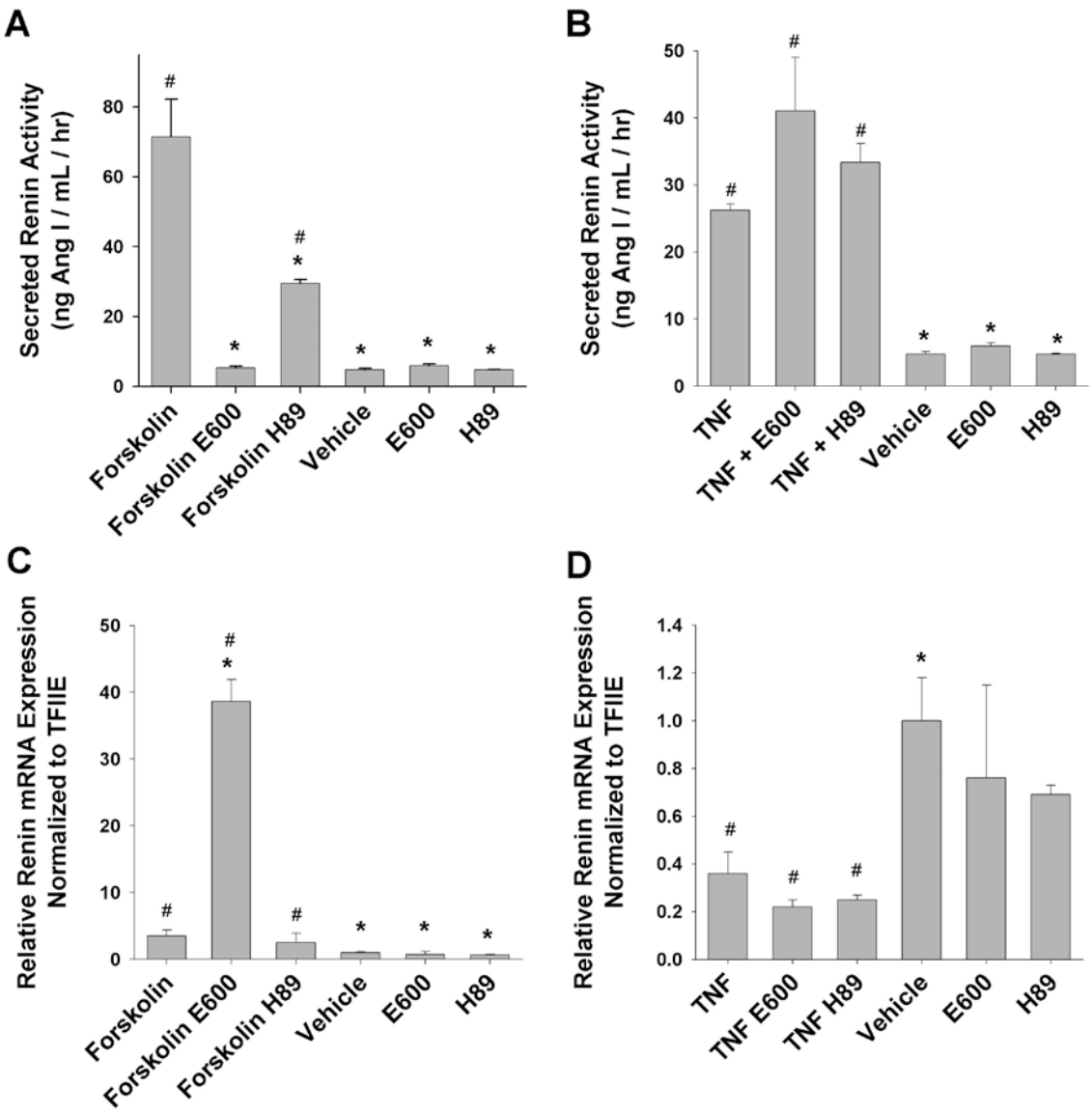
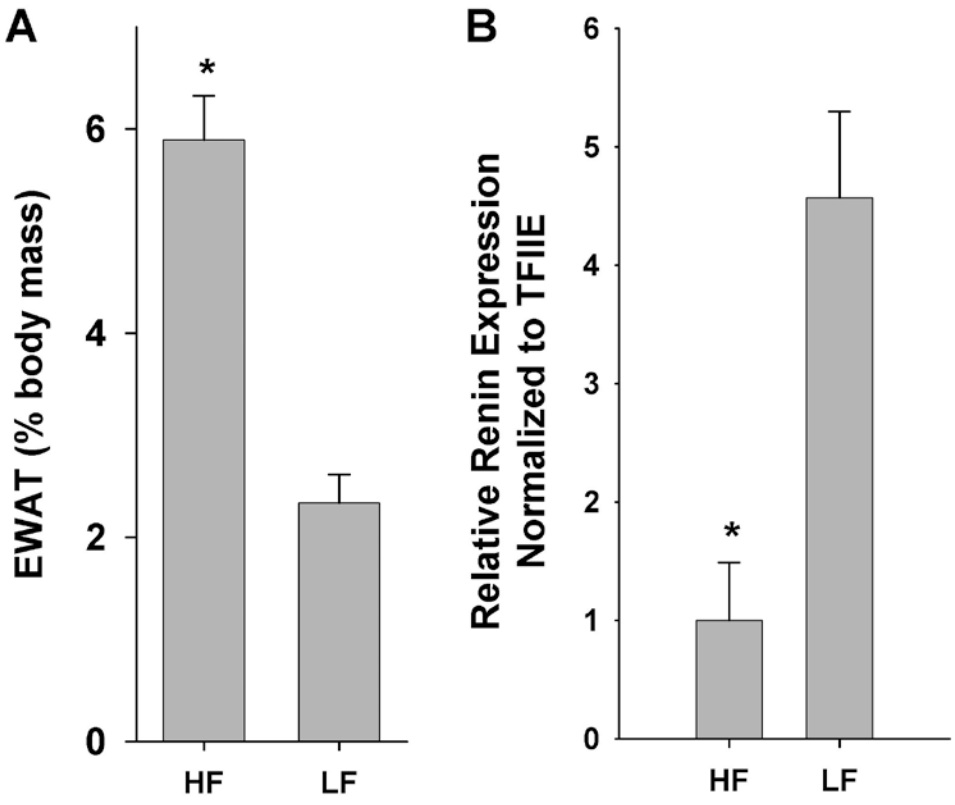

A
Secreted Renin Activity
(ng Ang I / mL / hr)
0
20
40
60
80
Forskolin
Forskolin E600
Forskolin H89
Vehicle
E600
H89
B
Secreted Renin Activity
(ng Ang I / mL / hr)
0
10
20
30
40
50
TNF
TNF + E600
TNF + H89
Vehicle
E600
H89
C
Relative Renin mRNA Expression
Normalized to TFIIE
0
10
20
30
40
50
Forskolin
Forskolin E600
Forskolin H89
Vehicle
E600
H89
D
Relative Renin mRNA Expression
Normalized to TFIIE
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
TNF
TNF E600
TNF H89
Vehicle
E600
H89

Figure 7: Obesity/insulin resistance effects on epididymal adipose tissue renin expression. Epididymal adipose tissue depots were harvested from 12 week old male C57BL/6J mice maintained on either a high fat (HF) or normal chow (LF) diet. (A) Epididymal adipose tissue mass expressed as percentage total body weight. (B) qRT-PCR analysis of relative renin expression normalized to TFIIE. * $p < 0.001$.


A
EWAT (% body mass)
0
2
4
6
*
HF
LF
B
Relative Renin Expression
Normalized to TFIIE
0
1
2
3
4
5
6
*
HF
LF

DISCUSSION

A variety of studies support an interaction between a local adipose renin-angiotensin system and obesity, oxidative stress, insulin resistance and inflammation (13; 19; 25; 32; 37; 39; 44). Considerable effort has been directed toward adipose tissue derived AGT effects within adipose tissue as well as its contribution to the systemic RAS (1; 29; 30). Previous reports have shown that AGT null mice have hypotrophic adipose tissue (30) while over expression of AGT is able to attenuate adipose tissue dysfunction as well as partially reconstitute plasma AGT levels (29; 30). Moreover, adipose tissue expresses Ang II receptors, implying a local signaling function of AGT (5) through Ang II production. Despite the widespread interest in adipose tissue RAS function, little data exist with regard to the role of renin (9; 10; 20; 35; 38). Herein we report that renin is synthesized and secreted by cultured 3T3-L1 cells in a regulated manner.

To assess renin expression, we utilized the differentiating 3T3-L1 cell culture system. While 3T3-L1 adipocytes do not faithfully mirror all the functions of adipocytes in vivo, they do represent a convenient, reproducible cell culture based system for analysis of adipose genes and protein (3). Using the 3T3-L1 system we found that renin message and enzymatic expression are greatly increased during adipocyte differentiation (Figure 1). Renin expression was maximal on ~day 4 and declined by day 8-10 to a new steady state level. The biphasic nature of adipose renin expression during differentiation is shared with

many other adipocyte proteins such as the adipocyte fatty acid binding protein (aP2), pyruvate carboxylase and fatty acid synthase (3; 6) commonly referred to as “late” adipose markers. Despite the decline in renin expression late in the differentiation program, renin expression and activity in adipocytes is much greater than in undifferentiated preadipocytes (Figure 1).

Macrophage infiltration and subsequent TNFα production play an important role in the pathogenesis of obesity-induced inflammation (2; 14; 31; 33; 43). Adipose tissue in both diet-induced and molecular genetic models of obesity has significant increases in macrophages and that adipose tissue TNFα production is due primarily to macrophage synthesis (47). In addition, renin expression has been demonstrated in rat peritoneal macrophage cells (17), suggesting that adipose tissue renin may be derived from macrophages. However, we were unable to measure renin mRNA expression in RAW264.7 macrophage cells by qRT-PCR and renin enzymatic activity was undetectable in cell extracts or culture media (data not shown). These results suggest that in adipose tissue, the adipocyte is a primary locus for renin expression.

To ensure that the renin activity measured in the 3T3-L1 system was due to renin protein and not to a similar enzyme, we evaluated the potential contamination of cathepsin D. Early literature measuring tissue renin enzymatic activity was often performed at an acidic pH allowing for the lysosomal enzyme acitivity of cathepsin D to contribute to perceived renin activity (21). Previously published reports in the rat and human have demonstrated that a neutral pH

assay buffer is able to effectively distinguish between renin and cathepsin D activity. Here we demonstrate that the assay for murine renin is independent of contaminating cathepsin D activity. This was shown by the diagnostic non-overlapping isoelectric focusing activity profiles for murine renin and cathepsin D. In addition, since the renin detected in media and cellular extracts from 3T3-L1 cells has the same focusing profile as C57Bl/6J mouse plasma renin, and it is distinct from that exhibited by cathepsin D, it is highly unlikely that other contaminating proteases are interfering with the renin measurement. Consistent with this, plasma from a C57Bl/6J mouse that had undergone a bilateral nephrectomy exhibited a 96% reduction in renin activity (data not shown). In addition, unlike other species, some mice including those from which the 3T3-L1 cell system was developed, possess 2 renin genes, *ren1* and *ren2*. Ren2 has previously shown to be non-glycosylated and will display a single peak of activity in an isoelectric focusing gel while the differentially glycosylated ren1 will display multiple peaks. C57Bl/6J mice possess only as single renin gene, ren1, which was measured as a positive control for ren1 profiling (Figure 2A). Here we show that ren1 activity is expressed in our 3T3-L1 system as evidenced by multiple renin activity peaks (Figure 2) but is clearly distinct from the cathepsin D profile. It is also possible that ren2 is synthesized as a relatively minor component.

The renin angiontensin system has been implicated in a variety of adipose tissue functions (25; 29; 30; 37; 39). We therefore evaluated a variety of

regulatory factors thought to be involved in obesity associated pathologies or previously linked to kidney regulation of renin synthesis/secretion to assess which, if any, may regulate renin synthesis and/or secretion from differentiated adipocytes (Figure 3). Since cAMP and cGMP are known to regulate kidney renin we treated cells with CNP to induce cGMP synthesis as well as forskolin and MIX to increase cAMP production. In addition, LPS and TNFα are important regulators of inflammation associated with pathological obesity, while isoproterenol treatment stimulates β-adrenergic receptors activated in adipose tissue. Furthermore, we treated cells with hydrogen peroxide to increase oxidative stress as well as prostaglandins and Ang II; known regulators of kidney renin levels (34). Of these treatment groups forskolin and TNFα were identified as potent agonists of renin release from mature adipocytes (Figure 3). Both compounds displayed sigmoid activity plots suggesting a single binding event. Forskolin displayed a $K_{0.5}$ of 38 uM and TNFα a $K_{0.5}$ of 270 pM, both of which are consistent with the induction of other regulated processes in cultured adipocytes (Figure 4) (7; 24; 28).

The kinetics of renin secretion in the culture medium from intracellular stores and concomitant changes in renin mRNA expression in response to either forskolin or TNFα followed distinctly different progressions. Forskolin induced a rapid release of renin into the culture medium and was accompanied by a decrease in intracellular renin and increase in renin mRNA expression (Figure 5). In contrast, TNFα treatment resulted in a relatively slow appearance of

renin activity in the medium and was accompanied by a decrease in renin mRNA expression. As such, while both forskolin and TNFα lead to increased renin secretion, their differing kinetics and opposite effects on renin mRNA expression imply distinctly different regulatory processes.

While little is known about the regulation of renin release from adipocytes, it is interesting that increased cAMP strongly stimulates adipocyte renin release analogous to kidney juxtaglomerular cells (34). TNFα has also been shown to decrease renin expression in juxtaglomerular cells primarily by transcriptional inhibition and TNFα -/- mice have 3 fold higher basal renin mRNA expression (45) Furthermore, modulation of cAMP activity via PKA inhibition (H89) supports cAMP mediated renin release in forskolin treated adipocytes while in contrast the lack of H89 effect in TNFα treated adipocytes (Figure 6) suggests that renin release may be cAMP independent. Since forskolin and TNFα both induce lipolysis in adipocytes, albeit by different mechanisms, a potential linkage to renin release may be related to production of free fatty acids. However, fatty acids themselves (oleate or palmitate) were ineffective in regulating renin release (Figure 3). Interestingly, E600 (a pan-specific triglyceride hydrolase inhibitor) completely attenuated forskolin-induced renin release while having no effect on TNFα mediated renin release (Figure 6). As such, it is unclear at this time what mechanism(s) are involved in the E600 inhibition of renin release. Further experiments may elucidate the signaling

mechanisms by which cAMP and TNFα induce renin release from cultured adipocytes.

In pathological obesity, adipose tissue is associated with increased inflammatory cytokine burden (2; 14; 31; 33; 43). TNFα has previously been shown to increase AGT and Ang II in cultured adipocytes (12) and in this report, we extend the effects of TNFα to increased secretion of renin. Concomitant with increased renin secretion was a 4.2 fold ($p<0.05$) decrease in renin message in TNFα treated cultured adipocytes. A decrease in renin message was also observed in high fat fed obese, insulin resistant mice compared to normal chow-fed control animals. Moreover, suppression of renin expression by chronic TNFα exposure may decrease the effectiveness of other renin stimulating processes. While the mechanistic basis of TNFα-mediated renin secretion and suppression of message remains unclear, one explanation is in an obese insulin resistant state, chronically elevated TNFα levels alter the normal role of a local adipose RAS system though decreased renin expression and in part, contribute to obesity associated pathology.

One intriguing hypothesis regarding the role of a local RAS system in adipose tissue is participation in adipose tissue expansion and differentiation. Adipose tissue is highly vascularized and is dependent on neovascularization (possibly by vascular endothelial growth factor and/or Ang II) for expansion (4; 8; 46). Consistent with this, Ang II receptor blockers decrease the angiogenic effects of

Ang II (16). Therefore, TNFα or cAMP mediated induction of renin release may be involved in the production of Ang II and induction of adipose angiogenesis during normal adipose tissue expansion. If the RAS is involved in normal adipose tissue expansion, as evidenced by decreased adipose tissue mass in AGT -/-, ACE -/- and renin -/- mice (18; 30; 44), then pathological decreases in renin expression and subsequent decreased production of Ang II may be in part responsible for failure of normal adipose expansion in the obese state. Modulation of the RAS, specifically at the rate-limiting step (renin action on AGT), may be a potential therapeutic option to limit further adipose tissue expansion. Future studies will focus on the mechanism of renin secretion and role(s) for the local RAS system in adipose biology.

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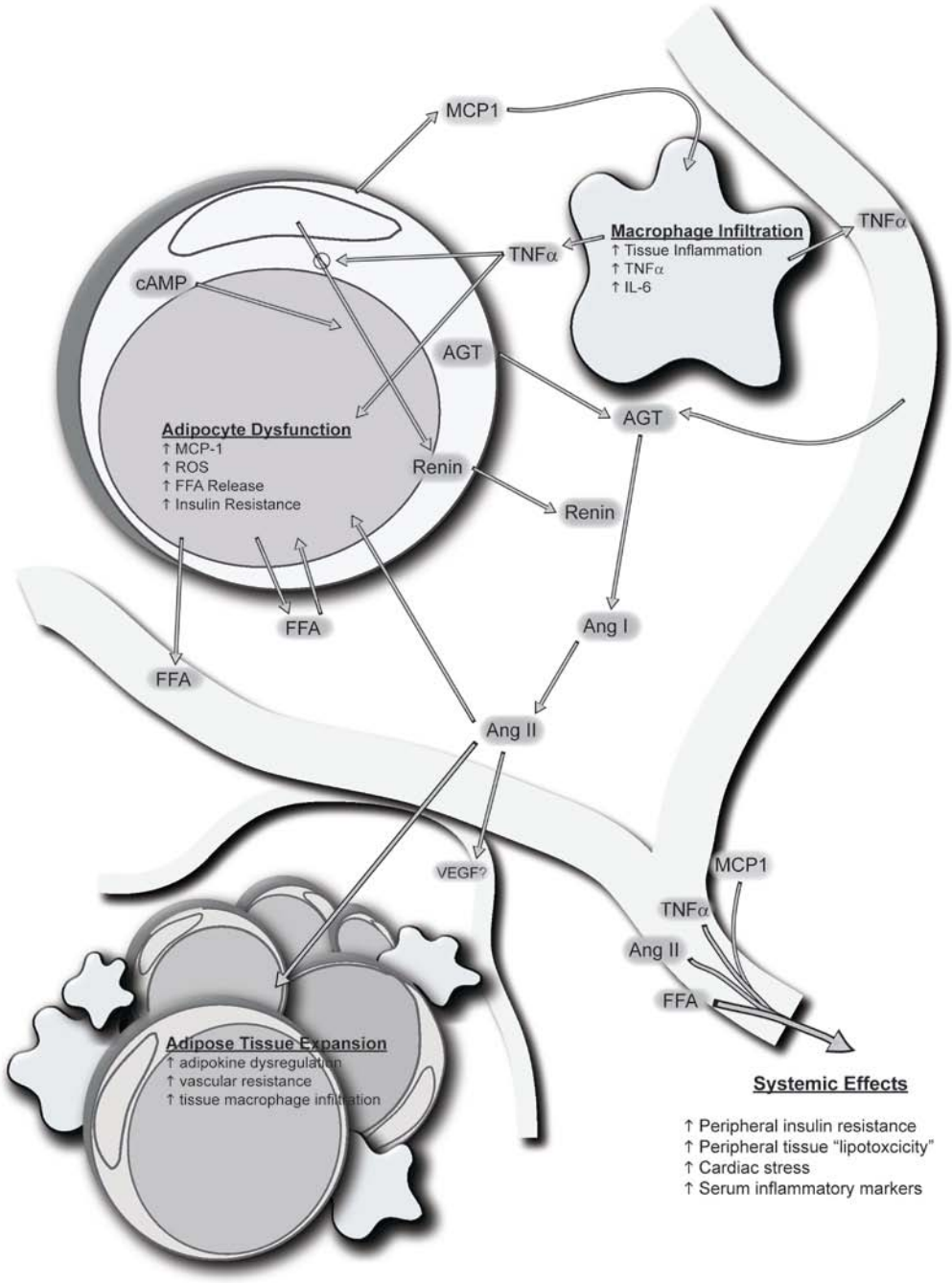
Chapter 4

Discussion and Future Directions

Obesity pathology is complex and multifactorial. While multiple treatments have been developed, the prevalence of obesity in the United States has climbed to over 30%. No single treatment, with the exception of diet and exercise, has proven radically effective in the treatment of obesity. The recent observation of a local RAS in adipose tissue provides additional treatment options that may ameliorate some of the negative outcomes of obesity (9; 35; 39). Here we have shown for the first time independent regulation of adipose tissue renin levels as a potential mechanism for modulating a local adipose RAS (13). In addition, in vitro analysis of renin secretion from cultured adipocytes has further defined regulated renin release from adipocytes as cAMP and TNF inducible (12). Further study and understanding of the adipose tissue RAS may prove useful for the application of current and/or new RAS drugs as treatment options for obesity associated pathologies.

Much remains to be discovered concerning the role of a local RAS in adipose tissue. Many functions have been proposed for a local adipose RAS including metabolism, differentiation, oxidative stress and inflammation (1; 16; 21; 25; 37). Evidence is mounting as to the significance and magnitude by which a local adipose RAS may modulate such processes (1; 12; 20; 25; 44; 46). One basic feed forward process by which locally produced adipose Ang II may be involved in adipose biology is illustrated in Figure 1 and further described here (Figure 1). This model of adipose tissue RAS functions aims to incorporate

Figure1. Local Adipose Renin Angiotensin System (RAS): Adipocyte hypertrophy, dysfunction and tissue expansion. Adipose tissue expansion during obesity is associated with macrophage infiltration, adipokine/cytokine deregulation and adipocyte dysfunction. Adipokines/cytokines dysfunction have adipose tissue effects including increased macrophage infiltration, exacerbation of adipocyte dysfunction as well as systemic effects resulting from inadequate or inappropriate adipose tissue expansion in the face of obesity.


MCP1
TNFα
Macrophage Infiltration
↑ Tissue Inflammation
↑ TNFα
↑ IL-6
TNFα
cAMP
AGT
AGT
Adipocyte Dysfunction
↑ MCP-1
↑ ROS
↑ FFA Release
↑ Insulin Resistance
Renin
Renin
FFA
Ang I
FFA
Ang II
VEGF?
MCP1
TNFα
Ang II
FFA
Adipose Tissue Expansion
↑ adipokine dysregulation
↑ vascular resistance
↑ tissue macrophage infiltration
Systemic Effects
↑ Peripheral insulin resistance
↑ Peripheral tissue "lipotoxcicity"
↑ Cardiac stress
↑ Serum inflammatory markers

recently published observations outlined in Chapters 2 and 3. As adipose tissue begins to accumulate lipid as a product of increased caloric intake relative to expenditure, preadipocytes are recruited as adipose tissue is expanded to meet lipid storage demand (38). As outlined in Chapter 3 of this thesis, differentiating adipocytes synthesis and secrete renin which may indirectly induce angiogenesis, potentially via endothelial VEGF induction, and adipose differentiation thereby supporting normal adipose tissue expansion (12; 41). Eventually, increased demand for lipid storage begins to burden adipose tissue depots as preadipocyte expansion into new adipocytes slows or stops resulting in increased existing individual adipocyte cellular hypertrophy (26; 32; 38). Increased FFA and tissue macrophage infiltration, mediated in part by adipocyte MCP1 release, begin to negatively affect insulin sensitivity and inflammation within adipose tissue (10; 16; 21; 25; 34). While the mechanism remains entirely unclear, our observations show both in vitro and in vivo declines in renin message associated with obesity and increased TNFα (13). Decrease in renin expression may account for a reduction in inducible adipose tissue renin activity thereby contributing the lack of vascular expansion and preadipocytes recruitment observed in obese, dysfunctional adipose tissue characterized by large, lipid laden adipocytes with high macrophage content and comparatively few vessels compared to *healthy* adipose tissue (6; 11; 42). Increased inflammation and sympathetic drive may serve to release what little renin remains within a dysfunctional adipocyte. Ang II production within dysfunctional adipose tissue may further retard adipose tissue function in an

autocrine and paracrine fashion (15; 25; 40). Peritoneal macrophages have been shown to increase Ang II receptor expression during oxidative stress while additional research has postulated a role for Ang II in macrophage associated inflammatory cytokine production (23; 25). In addition, macrophage accumulation in adipose tissue is further associated with insulin resistance ,vascular endothelial dysfunction, and impaired adipogenesis (2; 26). In the kidney, Ang II stimulates intracellular ROS production by activation of NADPH oxidase while simultaneously increasing ROS production in the mitochondria (36). These observations may extrapolate to local adipose tissue RAS functions and contribute to adipose tissue dysfunction. While much remains to be elucidated, regulated renin synthesis, secretion and expression from adipose tissue may prove to be an important regulatory step in the generation of local Ang II, thereby controlling the many effects Ang II is proposed to have within adipose tissue including adipose conversion, angiogenesis, lipolysis and inflammation.

In lieu of the staggering prevalence of obesity it is not surprising that adipose tissue expansion is often viewed in a negative light with a majority of basic research focused on reducing adipose tissue accumulation to ameliorate the morbidity and mortality associated with obesity. However, some researchers have significant interest in just the opposite. The popularity of fat grafts for soft tissue augmentation of facial contours has been increasing recently in the plastics and reconstructive surgery arena (14; 17; 30). The attractiveness of fat

grafting can be easily appreciated as it is inexpensive, natural, has minimal immune reactions and has the potential to be long-lasting. Unlike in obesity research, the major issue in fat grafting is not focused on a decrease in adipose tissue, rather the prevention of fat tissue resorption or conversion to fibrous tissue is the primary goal (14; 30). The paramount factor thought to contribute to fat graft survival is early and adequate vascularization of the transplanted tissue (3; 22). Some studies have demonstrated that pre-ischemia treatment of adipose tissue is capable of significant increases in VEGF levels (47). In addition, fat grafts to highly vascularized recipient areas have increased survival rates (3). This is not surprising since angiogenesis is thought to precede adipose tissue expansion (4; 7). In addition, adipose tissue has been demonstrated to have a low tolerance for tissue ischemia further complicating adipose graft survival (22). While many studies have attempted to increase the survival rate and longevity of autologously transplanted fat uncertainty remains as to the clinical outcome associated with fat transfer.

From a conceptual standpoint it is not unreasonable to propose a link between adipose angiogenesis and a local adipose RAS. Ang II is known to modulate angiogenesis in a variety of organ systems including the retina, kidney, and cardiovascular systems (18; 24; 31). Knock out animal studies of all major RAS components have shown resistance to weight gain and hypotrophy of adipose tissue (including the individual adipocyte) in response to a high fat diet (20; 28; 44; 46). It is possible that the decreased development of adipose tissue is in

part due to the lack of locally produced Ang II (a result of decreased renin production in the dysfunctional adipocyte) stimulus on the developing vasculature preceding adipogenesis. Our data have shown that renin is synthesized and secreted from adipose tissue in a regulated fashion. In addition, factors that may be elevated during adipose tissue grafting or normal tissue expansion such as TNF, associated with inflammation, or sympathetic stimuli have been demonstrated to increase the release of cultured adipocyte renin. It is therefore possible that RAS modulation may impact fat graft survival.

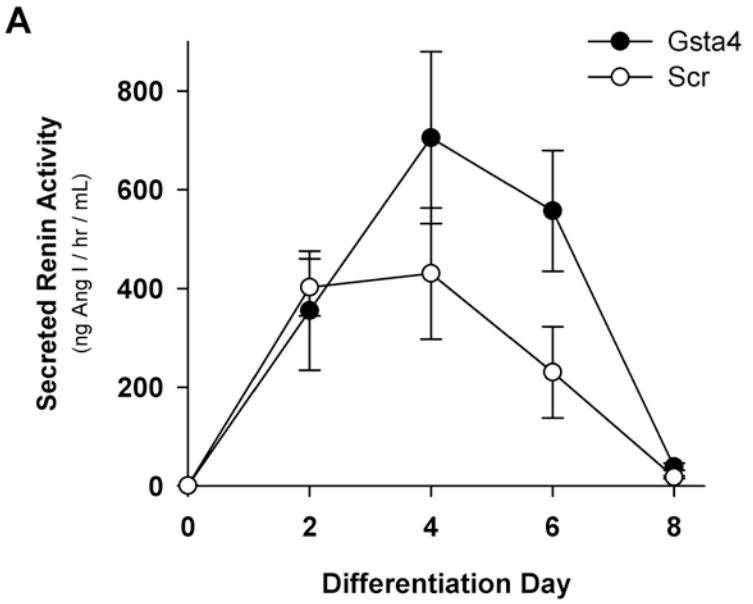
Tissue hypoxia related to both adipose tissue expansion and transplant is also associated with adipocyte dysfunction. Tissue hypoxia and increased TNFα have been shown to increase mitochondrial reactive oxygen species and these mitochondrial changes are further hypothesized to participate in the cellular changes associated with hypoxic insult (45). To this end, I have evaluated glutathione s-transferase α4 (GSTA4) 3T3-L1 adipocyte siRNA knock down cells for regulated renin release. GSTA4 is an antioxidant present within mitochondria. Decreased GSTA4 in cultured adipocytes has recently been shown to increase mitochondrial ROS (Cutis J. et al. manuscript submitted), potentially recapitulating part of the cellular response to hypoxic injury. Preliminary results indicate increased secreted renin protein enzymatic activity during cellular differentiation of GSTA4 kd adipocytes (Figure 2A). Increases in tissue Ang II, secondary to increased renin release, may aid adipocyte and vascular development during hypoxia associated adipose tissue expansion.

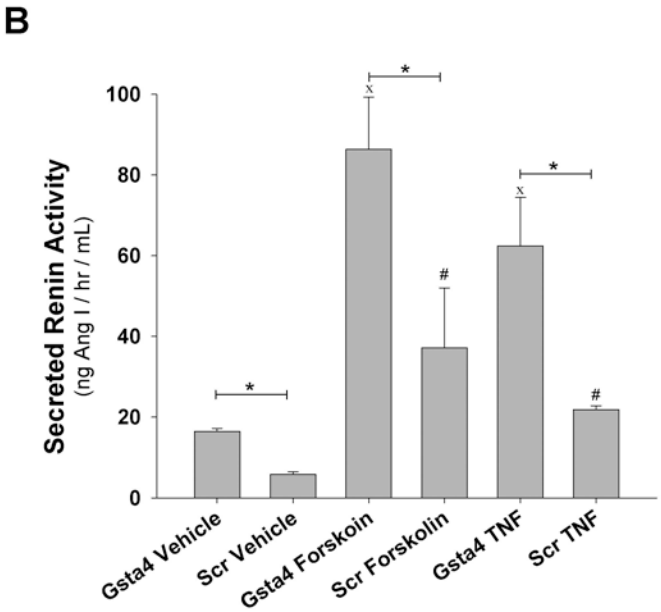
Figure 2: GSTA4 shRNA knock down 3T3-L1 adipocyte renin activity. (A) Secreted (media) 3T3-L1 cultured adipocyte renin enzymatic activity. Cells were differentiated as previously described (see chapter 3, materials and methods). Media was replaced every 48 hours. Renin enzymatic activity was quantified at each interval for both Scrambled and GSTA4 kd cells via by radioimmunoassay as previously described. n = 3 per sample. (B) Day 8 mature 3T3-L1 adipocytes were treated with TNFα or forskolin for 18 hours and secreted renin activity was measured. n = 3 per sample. Data analyzed by One-Way ANOVA followed by Holm-Sidak all pairwise multiple comparison procedure to detect differences between groups.

X $p < 0.05$ vs. GSTA4 vehicle treatment

# $p < 0.05$ vs. Scr vehicle treatment

* $p < 0.05$ vs. within same treatment group


A
Gsta4
Scr
Secreted Renin Activity
(ng Ang I / hr / mL)
800
600
400
200
0
0
2
4
6
8
Differentiation Day


B
Secreted Renin Activity
(ng Ang I / hr / mL)
100
80
60
40
20
0
*
*
*
#
#
Gsta4 Vehicle
Scr Vehicle
Gsta4 Forskoin
Scr Forskolin
Gsta4 TNF
Scr TNF

Further experiments on day 8 fully differentiated GSTA4 knock down adipocytes confirm that mature GSTA4 kd adipocytes display a 3 fold increase in basal renin release (Figure 2B). Forskolin and TNF treatment result in a statistically significant increase in renin release from cultured adipocytes compared to scrambled knock down control (Figure 2B) suggesting an increase RAS response associated with mitochondrial dysfunction. This is an interesting result in that it provides further evidence for the dynamic regulation of renin secretion by the adipocyte. In addition, it provides some evidence for the interaction between one factor associated with tissue hypoxia, increased mitochondrial ROS, and increased renin release. This adds depth to the hypothesis that a local RAS system may, in part, be responsible for vascular development in hypoxic adipose tissue.

FUTURE DIRECTIONS

Four experimental systems would aid in delineation of renin in relation to adipose tissue function. Generation and characterization of an adipose specific renin knock out mouse would further define functions of locally produced renin within adipose tissue. Assessment of RAS involvement in adipose tissue graft survival and vascular formation in adipose tissue bed expansion should also be explored. Additionally, the mechanism of action for TNFα induced renin release should be further investigated. Finally the role of prorenin as it relates to local adipose RAS system activity and Ang II generation should be investigated. Specific details regarding each future direction are discussed below.

Independent adipose tissue regulation of renin observed in Fowler *et al* (12) provides strong evidence implicating renin as a rate limiting factor in adipose tissue specific Ang II generation. However, it is possible that other mechanism may account for the observation of adipose tissue renin activity in the absence of kidney derived plasma renin. While our in vitro data suggest that adipocytes, not macrophages, are a primary source of active renin, other cell types are present within perfused adipose tissue extract which may contribute to renin activity in vivo. Furthermore, cultured cells, while often recapitulating many in vivo functions, do not always accurately reflect in vivo processes. To this end, an adipose specific renin knock out mouse should be generated to assess many of these concerns. An adipose specific renin knock out would further verify the contribution which adipocytes contribute to adipose tissue

renin. Specifically, if an adipose specific renin knock out mouse were bilaterally nephrectomized, thereby removing any detectable source of plasma renin, adipose tissue crude homogenate could be measured for renin activity. In this instance, absence of activity, correlated with previous observations (Chapter 2, Figure 1), would further implicate adipocytes as a primary source of adipose tissue renin; while continued renin activity would strongly suggest an additional cell type capable of contributing to adipose tissue renin activity.

As outlined previously, the recent emergence of fat grafting for cosmetic repair is problematic. An adipose specific renin knock out mouse would also prove useful in ascertaining the role of a local RAS system in angiogenesis and adipose tissue survival during fat transfer. An adipose specific renin knock out mouse would yield a source of adipocytes incapable of synthesizing and secreting their own renin. This would prove useful in analyzing the contribution of adipocyte derived renin activity as it pertains to adipose tissue survival during grafting. In addition, wild type, renin producing cells can be transplanted to the adipose tissue k/o mouse to compare the development of vasculature and adipose survival against that or renin -/- adipocytes. This would provide further information concerning the local role of renin in adipose tissue. Currently, a rat renin -/- exists that could also be used as a source of renin free adipocytes. However, since renin is important in other cellular processes, a whole body knock out is not an ideal host for many experiments designed to test the significance of adipose derived renin apart from other sources.

We have demonstrated a time and dose dependent affect of TNFα treatment on renin release from cultured day 8 3T3-L1 adipocytes. TNFα induced renin release is independent from cAMP and lipolysis mediated mechanisms as measured by PKA (H89) and lipolysis (E600) inhibition (Chapter 3, Figure 6). Despite these early attempts to attenuate TNF induced renin release, the mechanism of action of TNFα induced renin release remains unknown. TNFα signals through various intracellular mediators including ERK, JNK, NF-kB and p38. Preliminary attempts were made to inhibit each of these signaling molecules to ascertain which, if any, were involved in the propagation of TNFα mediated renin release by the addition of U0126, SP600125, SN50 and SD106 respectively (5; 27; 43). 18 hour treatments of each inhibitor, calculated from previous publications (5; 27; 43), were unable to attenuate TNFα induced renin release (Figure 3). Verification of successful inhibition of each signaling molecule via western blot phosphorylated protein analysis was attempted, however, only U0126, showed convincing evidence of successful inhibition as measured by attenuation of p44/p42 phosphorylation (Figure 4). It is therefore unlikely that MEK/ERK are primary mediators of TNFα induced renin release. Antibody difficulty prevented definitive conclusions of effective inhibition of the other 3 inhibitors of TNFα signaling. It is unknown at this time if JNK, NF-kB and/or p38 are involved in TNFα mediated renin release. Further experimentation toward defining the intracellular signaling pathway(s) involved in TNFα mediated renin release should be conducted. Additional inhibitors

Figure 3. Inhibition of TNFα inducible renin release from day 8 3T3-L1 adipocytes. Day 8 3T3-L1 cells were pretreated with either (A) 25 mM U0126 (MEK/ERK inhibitor) (B) 100 µg/mL SN50 (NF-κB inhibitor) (C) 10 µM SP600125 (JNK inhibitor) or (D) 50µM SD-169 (p38 inhibitor) for 30 minutes, except vehicle and TNF alone treated groups. Media was changed and cells were treated as indicated for an additional 18 hours. Secreted media renin activity was measured as previously indicated. n = 3 per samlple. Data analyzed by One-Way ANOVA followed by Holm-Sidak all pairwise multiple comparison procedure to detect differences between groups. No statistical difference was detected between TNF and TNF + inhibitor for any group.

* $p < 0.05$ both vehicle and inhibitor alone.

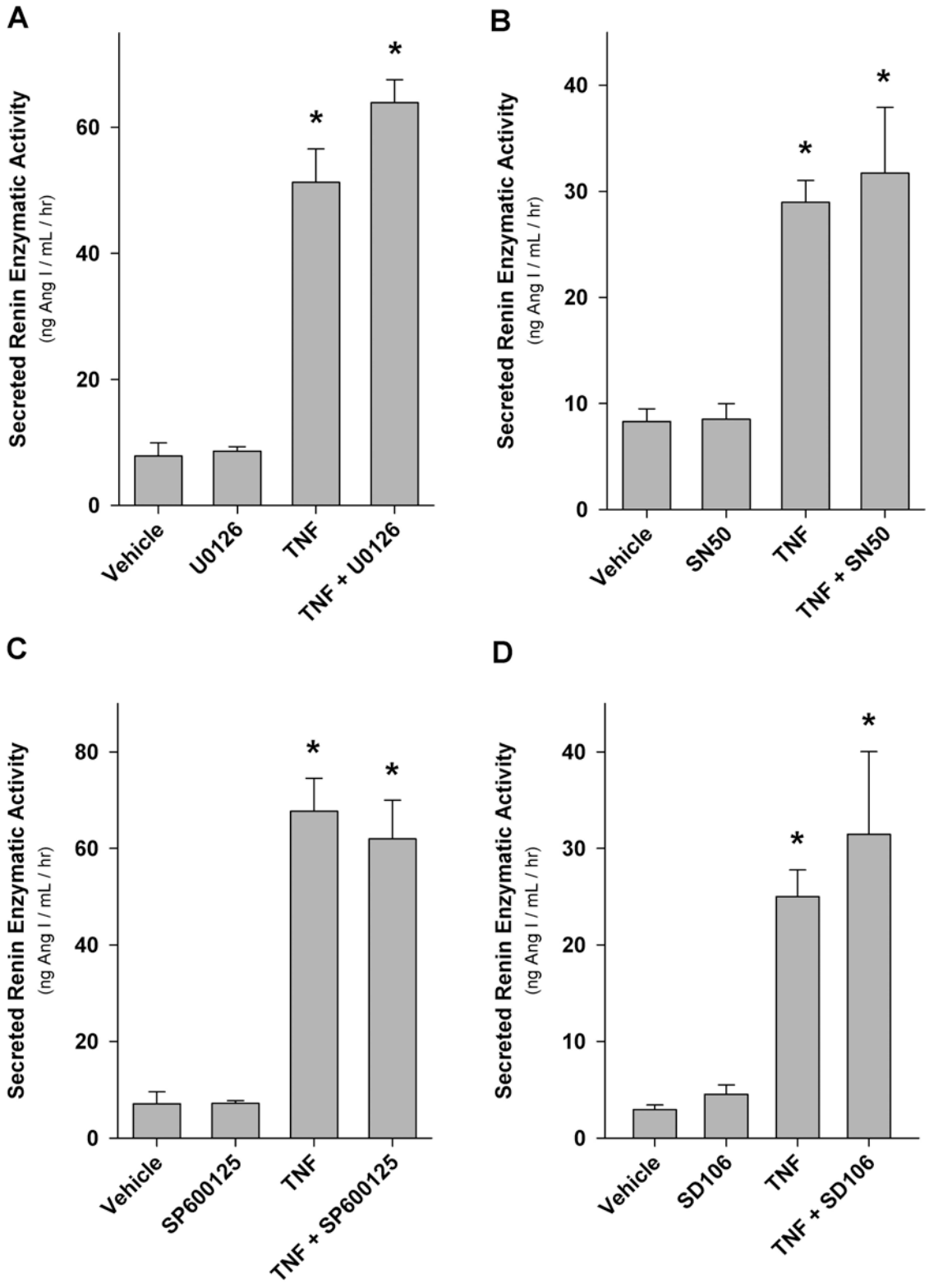
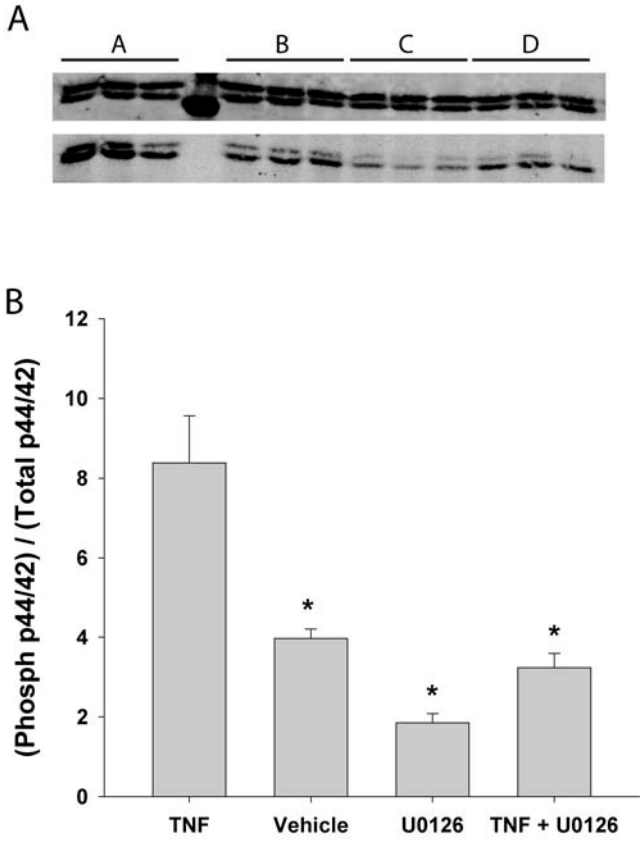

A
Secreted Renin Enzymatic Activity
(ng Ang I / mL / hr)
0
20
40
60
*
*
Vehicle
U0126
TNF
TNF + U0126
B
Secreted Renin Enzymatic Activity
(ng Ang I / mL / hr)
0
10
20
30
40
*
*
Vehicle
SN50
TNF
TNF + SN50
C
Secreted Renin Enzymatic Activity
(ng Ang I / mL / hr)
0
20
40
60
80
*
*
Vehicle
SP600125
TNF
TNF + SP600125
D
Secreted Renin Enzymatic Activity
(ng Ang I / mL / hr)
0
10
20
30
40
*
*
Vehicle
SD106
TNF
TNF + SD106

Figure 4. TNFa signaling transduction inhibition. TNFα signal transduction through MEK was inhibited by the potent and selective MEK inhibitor U0126, BIOMOL. Day 8 3T3-L1 adipocytes were pre-treated with U0126 for 30 min (except controls). Cells were further treated as indicated for 18 hours. Cells were homogenized in RIPA buffer as previously described. 50 µg of each sample was loaded to an 8% SDS PAGE gel. Western blot analysis was performed with a primary antibody directed against p44/42 or phospho p44/42 (Cell Signaling). Li-cor Odyssey infrared imaging system detection. A. Western blot of total p44/42 (upper) and phosphorylated p44/42 (lower) Group A) TNFα B) Vehicle C) U0126 D) TNFα + U0126. B. Phopho p44/42/p44/42 Licor image intensity analysis. * $p < 0.05$ vs TNFα alone. Statistical analysis via One-way ANOVA followed by Holm-Sidak all pairwise analysis to detect differences between groups.

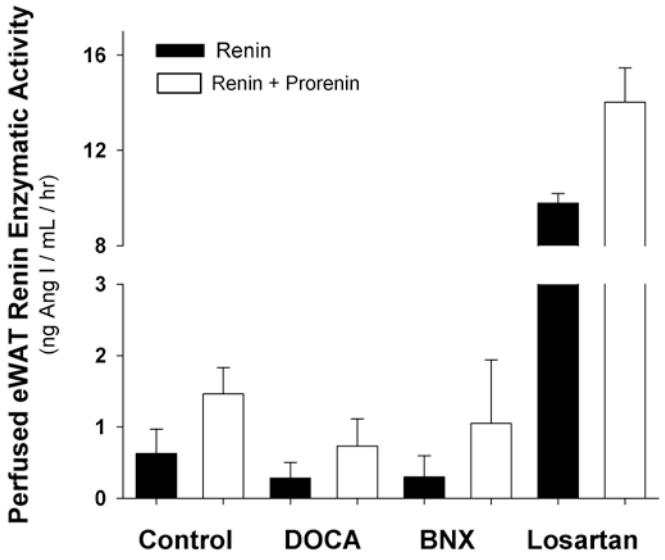

A
A
B
C
D
B
(Phosph p44/42) / (Total p44/42)
12
10
8
6
4
2
0
*
*
*
TNF
Vehicle
U0126
TNF + U0126

and/or further analysis of the effectiveness of previous inhibitory may prove useful toward this aim.

Active renin is synthesized as an inactive zymogen, prorenin, which is further processed intracellularly into mature renin that is released into plasma by the highly specialized kidney juxtaglomerular cell (8; 33). Prorenin is also present in plasma as a result of kidney release and to a lesser extent, release from extrarenal tissue such as brain, testis, ovaries and adrenal glands (8). To the best of my knowledge, prorenin has not been addressed in adipose tissue and it is entirely unknown if prorenin is released from adipocytes. Although no known function was thought to exist for inactive pro-renin, a prorenin binding protein has been recently hypothesized to bind prorenin and active renin (8). Tissue prorenin receptor binding may sequester prorenin/renin such that observed tissue renin activity could potentially be, at least in part, plasma derived. Some reports suggest that prorenin binding non-proteolytically activates prorenin providing an additional source of Ang I generation (19; 29). Our preliminary laboratory data suggest that prorenin is present in adipose tissue homogenates (Figure 5). While prorenin is not measured in the classical renin assay, it is possible to proteolytically activate prorenin (removing the inhibitory peptide sequence) rendering it active. Once activated, both renin and prorenin can be measured and the prorenin contribution to renin activity can be calculated by deducting non-activated renin activity of an equivalent sample. Here we show preliminary data from previous published samples (see chapter 2, Figure 1) in

which prorenin has been activated (Figure 5). Since this is the first time prorenin activity has been measured in adipose tissue homogenates, further characterization of prorenin activity from adipose tissue and cultured adipocytes as well as analysis of prorenin binding protein expression in adipose tissue as it pertains to increased in renin activity will prove useful in fully elucidating the importance of a local adipose tissue RAS.

Figure 5. Prorenin concentration in epididymal white adipose tissue (eWAT) extracts. Control, DOCA, BNX and Losartan treated rats eWAT was homogenized and renin activity was measured as previously described in Chapter 2, Materials and Methods. Renin activity of homogenates was measured via radioimmunoassay and is displayed as solid black bars. Open bars represent renin + prorenin activity. Homogenate prorenin was proteolytically activated and combined renin and prorenin was measured. Prorenin activity is therefore the difference between renin activity and renin + prorenin activity. n = 2 per sample. Preliminary data: Statistics not performed per insufficient n.


Perfused eWAT Renin Enzymatic Activity
(ng Ang I / mL / hr)
Renin
Renin + Prorenin
16
12
8
3
2
1
0
Control
DOCA
BNX
Losartan

REFERENCE LIST

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