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ABSTRACT OF DISSERTATION

Manisha Gupte

The Graduate School

University of Kentucky

2011

ROLE OF ANGIOTENSIN CONVERTING ENZYME 2 (ACE2) IN OBESITY-ASSOCIATED HYPERTENSION

ABSTRACT OF DISSERTATION

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

By
Manisha Gupte

Lexington, Kentucky

Director: Dr. Lisa Cassis, Chair and Professor of Graduate Center
for Nutritional Sciences

Lexington, Kentucky

2011

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ABSTRACT OF DISSERTATION

ROLE OF ANGIOTENSIN CONVERTING ENZYME 2 (ACE2) IN OBESITY-ASSOCIATED HYPERTENSION

The purpose of this research was to determine whether adipocytes express ACE2 and its role in obesity-associated hypertension with diet-induced obesity.

To determine if ACE2 was expressed in adipose tissue and its regulation in the setting of diet-induced obesity, we fed male mice either a low fat (LF) or high fat (HF) diet acutely (1 week) or chronically (4 months). We demonstrated that ACE2 was regulated specifically in adipose tissue with consumption of a HF diet. However, with chronic HF feeding adipose ACE2 was dysregulated resulting in activation of the systemic RAS and increased blood pressure.

To determine the role of ACE2 in obesity-associated hypertension, we used ACE2 deficient male and female mice. Wild type and ACE2 deficient mice were chronically fed either a LF or HF diet. Metabolic parameters were measured during the entire course of the study and blood pressure was measured by telemetry at the end of the study. Results from these studies demonstrate that HF diet promotes obesity-associated hypertension in male mice which is further augmented with ACE2 deficiency. Further, ACE2 deficiency resulted in marked glucose intolerance suggesting that stimulation of ACE2 may blunt the progression of obesity-associated diabetes.

In contrast to the males, females are protected against obesity-associated hypertension. However, this protection in the females is lost with ovariectomy and ACE2 deficiency. These results suggest that female sex hormones protect the females against obesity-associated hypertension by regulating ACE2.

To define mechanisms for HF diet-induced regulation of ACE2 in adipose tissue we examined various fatty acids for their ability to regulate ACE2 mRNA abundance in 3T3-L1 adipocytes. We revealed that omega-3 fatty acids, known regulators of PPARγ, increased ACE2 mRNA abundance in adipocytes. Therefore, we examined *in vitro* and *in vivo* regulation of ACE2 in 3T3-L1 cells and adipose tissue by PPARγ receptor ligands (TZDs). Results demonstrate

regulatory effects of PPARγ to promote ACE2 gene transcription. These effects were associated with changes in glucose tolerance.

Taken together, these results demonstrate that adipose ACE2 plays a protective role against obesity-associated hypertension in male and female mice and is regulated by natural and synthetic ligands of PPARγ.

KEYWORDS: Obesity, Diet, Hypertension, PPARγ, ACE2

Manisha Gupte

1-25-2011

ROLE OF ANGIOTENSIN CONVERTING ENZYME 2 (ACE2) IN OBESITY-ASSOCIATED HYPERTENSION

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DISSERTATION

Manisha Gupte

The Graduate School

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2011

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DISSERTATION

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Lexington, Kentucky

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2011

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To my loving dad

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Table of Contents

ACKNOWLEDGEMENTS iii
LIST OF TABLES ix
LIST OF FIGURES x
Section I. BACKGROUND 1
1.1. Obesity 1
1.2. Obesity hypertension 2
1.3. Causes of obesity-induced hypertension 2
1.4. Classical RAS 6
1.5. Angiotensin Converting Enzyme 2 (ACE2) 12
1.6. ACE2 regulation 17
1.6.1. ACE2 shedding 17
1.6.2. Regulation of ACE2 by hormones 19
1.7. ACE2 knock out models 20
1.8. ACE2 in hypertension 22
1.9. ACE2 in diabetes 24
1.10. Role of the RAS in obesity-induced hypertension 25
1.11. The adipose renin-angiotensin system 28
1.12. Role of the adipose RAS in obesity-induced hypertension 30
1.13. Obesity-hypertension: influence of gender 32
1 A. STATEMENT OF THE PROBLEM 45
Section II. SPECIFIC AIM 1 A 48
2.1. Summary 48
2.2. Introduction 50
2.3. Methods 53
2.3.1. Animals and diets 53
2.3.2. Measurement of blood pressure 53
2.3.3. 3T3-L1 adipocytes 54
2.3.4. mRNA quantification 55
2.3.5. Measurement of ACE2 enzymatic activity 56

2.3.6. Measurement of ACE2 protein 57
2.3.7. Measurement of angiotensin peptides 58
2.3.8. Statistical analysis 58
2.4. Results.. 59
2.4.1. Expression of ACE2 in 3T3-L1 Murine Adipocytes and
in Mouse Adipose Tissue.. 59
2.4.2. The Temporal Effect of HF Feeding on Adipose ACE2 Expression
and Enzymatic Activity.. 59
2.5. Discussion 62
SectionIII. SPECIFIC AIM 1 B 81
3.1. Summary 81
3.2. Introduction.. 84
3.3. Methods... 86
3.3.1. 3T3-L1 adipocytes 86
3.3.1.1. Incubation of 3T3-L1 adipocytes with fatty acids 86
3.3.1.2. TZD (Rosi) treatment.. 87
3.3.2. Chromatin immuno precipitation (ChIP) assay 87
3.3.3. Measurement of ACE2 protein 88
3.3.4. Animals... 88
3.3.5. Drugs.. 89
3.3.6. Measurement of blood pressure 89
3.3.7. Statistical analysis 89
3.4. Results.. 91
3.4.1. n-3 fatty acids and Rosi stimulate ACE2 m RNA, protein abundance
and enzyme activity in 3T3-L1 adipocytes 91
3.4.2. In vivo Rosi administration to C57BL/6 male mice increases adipose
ACE2 mRNA abundance and activity 92
3.4.3. Rosi administration reduces blood pressure and glucose tolerance:
effects of ACE2 deficiency... 92
3.5. Discussion 94
SectionIV. SPECIFIC AIM 2 A 112

4.1. Summary 112
4.2. Introduction.. 114
4.3. Methods.. 117
4.3.1. Animals and diet 117
4.3.2. Glucose and insulin tolerance tests 117
4.3.3. Measurement of food intake and physical activity 118
4.3.4. Measurement of plasma parameters 118
4.3.5. Measurement of blood pressure 119
4.3.6. Statistical analysis 119
4.4. Results.. 120
4.4.1. Metabolic characteristics of HF feeding in *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ Mice . 120
4.4.2. HF-fed *Ace2*$^{-/y}$ mice exhibit an activated systemic RAS 121
4.4.3. ACE2 deficiency markedly promotes obesity hypertension 122
4.5. Discussion 123
SectionV. SPECIFIC AIM 2 B.. 135
5.1. Summary 135
5.2. Introduction.. 138
5.3. Methods.. 141
5.3.1. Animals and diets 141
5.3.2. Glucose tolerance tests 141
5.3.3. Measurement of plasma parameters 142
5.3.4. Measurement of blood pressure 142
5.3.5. Echocardiography methods 142
5.3.6. Statistical analysis 142
5.4. Results.. 144
5.4.1. Metabolic characteristics of *Ace2*$^{+/+}$ and $^{-/-}$ female mice fed LF or HF diets.. .144
5.4.2. Systemic RAS in LF and HF fed *Ace2*$^{+/+}$ and $^{-/-}$ Mice 145
5.4.3. Blood pressure in LF and HF fed *Ace2*$^{+/+}$ and $^{-/-}$ Mice 145
5.5. Discussion 147
SectionV1. GENERAL DISCUSION 160

6.1. Summary 160
6.2. Insights from diet-induced regulation of ACE2....................................... 165
6.3. Insights from *Ace2*$^{+/y, -/y}$ (male) and *Ace2*$^{+/+, -/-}$(female) mice: Role in body weight, fat and lean mass regulation.. 167
6.4. Insights from *Ace2*$^{+/y, -/y}$ (male) and *Ace2*$^{+/+, -/-}$(female) mice: Role in glucose homeostasis 170
6.4.1. Effect of HF diet 170
6.4.2. Role of ACE2 deficiency 171
6.5. Insights from *Ace2*$^{+/y, -/y}$ (male) and *Ace2*$^{+/+, -/-}$(female) mice: Role in obesity-induced hypertension 173
6.6. Insights from Ovx *Ace2*$^{-/-}$ (female) mice: Role in cardiac function........... 178
6.7. Future directions 179
6.7.1. Role of specific fatty acids in obesity-induced hypertension 179
6.7.2. Role of AngII in metabolic parameters.. 180
6.7.3. Role of AngII or Ang-(1-7) in glucose metabolism 180
6.8. Clinical implications 181
6.9. Concluding Remarks 182
REFERENCES
VITA

LIST OF TABLES

Table 1.1. Key measurements for assessing weight and health risk 35

Table 1.2. Putative mechanisms of obesity-related hypertension....................... 36

Table 1.3. Characteristics of *Ace2*$^{-/y}$ models 37

Table 1.4. Prevalence of Hypertension in age matched men and women.......... 38

Table 2.1. Composition of diets used in the study 67

Table 2.2. Characteristics of LF and HF-fed C57BL/6 mice 68

Table 3.1. Body weight (g) in *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ treated with Vehicle or Rosi for 21 days... 99

Table 3.2. mRNA expression of genes of the RAS in Vehicle or Rosi treated *Ace2*$^{+/y}$ Mice 100

Table 3.3. Systolic blood pressure (mmHg) in *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice treated with Vehicle or Rosi.. 101

Table 3.4. Area under curve (AUC), Plasma Ang-(1-7) and Plasma insulin levels in *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice treated with Vehicle or Rosi 102

Table 4.1. Characteristics of *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet............... 127

Table 4.2. 24h Diastolic Pressure, Pulse Pressure and Heart Rate of *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet 128

Table 5.1. Characteristics of *Ace2*$^{+/+}$ and $^{-/-}$ mice fed a LF or HF diet and effects of ovariectomy (Ovx) 152

Table 5.2. Hemodynamic characteristics of *Ace2*$^{+/+}$ and $^{-/-}$ female mice fed a LF or HF diet and effects of ovariectomy (Ovx) 153

Table 5.3. Cardiac function in HF-fed *Ace2*$^{-/-}$ female mice with and without ovariectomy (Ovx) 154

LIST OF FIGURES

Figure 1.1. Classical RAS 39
Figure 1.2. Updated RAS, with biological active peptides highlighted in blue..... 40
Figure 1.3. Action of AngII on tissues associated with cardiovascular functions 41
Figure 1.4. Effects of angiotensin peptides and renin/prorenin mediated by their corresponding receptors 42
Figure 1.5. Comparison of gene structure of ACE and its homologue ACE2 43
Figure 1.6. Actions of Ang-(1-7) on tissues associated with cardiovascular functions 44
Figure 2.1. A .Relative expression of PPARγ in differentiating 3T3-L1 adipocytes 69
Figure 2.1. B. Relative expression of ACE2 in differentiating 3T3-L1 adipocytes69
Figure 2.1. C. Relative expression of Mas in differentiating 3T3-L1 adipocytes . 70
Figure 2.1. D. Relative expression of AT1a receptor in differentiating 3T3-L1 adipocytes 70
Figure 2.2. A. The effect of 1week and 4 months of HF feeding on ACE2 activity in epididymal adipose tissue 71
2.2. B. The effect of 1week and 4 months of HF feeding on ACE2 activity in subcutaneous adipose tissue 71
Figure 2.3. A .ACE2 protein in epididymal adipose tissues at 1 week of LF and HF feeding 72
2.3. B. ACE2 protein in epididymal adipose tissues at 4 months of LF and HF feeding........ 72.
Figure 2.4. A. Plasma angiotensin peptide concentrations at 1 week of LF and HF-feeding 73
Figure 2.4. B. Plasma angiotensin peptide concentrations at 4 months of LF and HF-feeding........ 73

Figure 2.5. A. ADAM-17 mRNA expression in differentiating 3T3-L1 adipocytes during the course of differentiation 74
Figure 2.5. B. ACE2 activity decreased from day 8 to 10 of differentiation 74
Figure 2.5. C. Effect of ADAM-17 inhibition on ACE2 activity 74
Figure 2.5. D. ADAM-17 mRNA expression in adipose tissue from 4 month HF-fed mice 75
Figure 2.6. A. Systolic blood pressure of 4 month LF and HF-fed mice 76
Figure 2.6. B. Diastolic blood pressure of 4 month LF and HF-fed mice 76
Figure 2.7. A. Left, HPLC chromatogram for resolution of angiotensin 1-7 (Ang-(1-7); retention time = 6.6 min) and angiotensinII (AngII; retention time = 13.6 min), Right, Effect of protease inhibitors on Ang-(1-7) generation. 77
Figure 2.7. B. Left, ACE2 activity increases as adipose membrane protein increases , Right, Dependence of [^{125}I]Ang-(1-7) product formation from varying concentrations of [^{125}I]AngII substrate 77
Figure 2.7. C. ACE2 activity is markedly decreased in EF membranes from *Ace2$^{-/y}$* mice compared to control (C57BL/6) 77
Figure 2.8. Inhibitors used in ACE2 activity assay 79
Figure 2.9. Proposed model for aim 1 80
Figure 3.1. A. ACE2 mRNA in 3T3-L1 adipocytes treated with specific fatty acids(100µm) for 24 hours 103
Figure 3.1. B. ACE2 activity in 3T3-L1 adipocytes treated with specific fatty acids (100µm) for 24 hours 103
Figure 3.2. A. ACE2 mRNA (Rosi) in 3T3-L1 adipocytes treated with TZD (0.01-1µm) for 24 hours 104
Figure 3.2. B. ACE2 activity (Rosi) in 3T3-L1 adipocytes treated with TZD (0.01-1µm) for 24 hours 104
Figure 3.2. C. ACE2 protein (Pio) in 3T3-L1 adipocytes treated with TZD (0.01-1µm) for 24 hours. 105
Figure 3.3. Chip assay..................... 106

Figure 3.4. A. ACE2 m RNA (EF) in *Ace2*$^{+/y}$ mice treated with Vehicle or Rosi 107
Figure 3.4. B. ACE2 activity (EF) in *Ace2*$^{+/y}$ mice treated with Vehicle or Rosi. 107
Figure 3.4. C. ACE2 activity (Kidney) in *Ace2*$^{+/y}$ mice treated with Vehicle or Rosi 107
Figure 3.4. D. Plasma Ang-(1-7) concentrations in *Ace2*$^{+/y}$ mice treated with Vehicle or Rosi 108
Figure 3.5. A. AUC for glucose tolerance test after 21 days of Rosi treatment in *Ace2*$^{+/y}$ mice 109
Figure 3.5. B. Systolic blood pressure at baseline and after Rosi treatment in *Ace2*$^{+/y}$ mice 109
3.6. A. AUC for Glucose tolerance test after 21 days of Rosi treatment in *Ace2*$^{-/y}$ mice .... .110
Figure 3.6.B. Systolic blood pressure at baseline and after Rosi treatment in *Ace2*$^{-/y}$ mice 110
Figure 3.7. Plasma insulin concentrations in *Ace2*$^{+/y}$ and $^{-/y}$ Vehicle or Rosi treated mice 111
Figure 4.1. A. Body weight at 16 weeks in *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet 129
Figure 4.1.B. Physical activity at 16 weeks in *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet.. 129
Figure 4.1.C. Energy intake at 16 weeks in *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet.. 129
Figure 4.1.D. Oxygen consumption at 16 weeks in *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet.. 129
Figure 4.2. A. Glucose tolerance tests in *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet. 130
Figure 4.2. B. Area under the curve (AUC) for glucose tolerance tests performed in A 130

Figure 4.2. C. Plasma insulin concentrations at study endpoint in mice from each group 130
Figure 4.2. D. Insulin tolerance tests in *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a HF diet 130
Figure 4.3. A. Fasting blood glucose in *Ace2*$^{+/y}$ and $^{-/y}$ male mice (8 weeks of age) fed standard diet 131
Figure 4.3. B. Insulin tolerance tests in *Ace2*$^{+/y}$ and $^{-/y}$ male mice (8 weeks of age) fed standard diet. 131
Figure 4.4. A. Plasma concentrations of AngII in HF-fed *Ace2*$^{+/y}$ and $^{-/y}$ mice .. 132
Figure 4.4. B. Plasma concentrations of Ang-(1-7) in LF and HF-fed *Ace2*$^{+/y}$ and $^{-/y}$ mice 132
Figure 4.5. A. Systolic arterial pressures (24 hours of recording) in LF and HF fed *Ace2*$^{+/y}$ and $^{-/y}$ mice 133
Figure 4.5. B. Systolic arterial pressures (24 hours of recording) in LF and HF fed *Ace2*$^{+/y}$ and $^{-/y}$ mice before (baseline) and after 3 days of losartan administration 133
Figure 4.6. Working model of obesity-induced hypertension in *Ace2*$^{+/y}$ male mice 134
Figure 5.1. A. Body weight progression in *Ace2*$^{+/+}$ and $^{-/-}$ female mice fed a LF or HF diet 155
Figure 5.1. B. Body weight at week 16 in *Ace2*$^{+/+}$ and $^{-/-}$ female mice fed a LF or HF diet 155
Figure 5.2. A. Area under the curve (AUC) for glucose tolerance tests 156
Figure 5.2. B. Plasma insulin concentrations at study endpoint in mice from each group 156
Figure 5.3. A. Plasma rennin concentrations in *Ace2*$^{+/+}$ and $^{-/-}$ female mice 157
Figure 5.3. B. Plasma Ang-(1-7) concentrations in *Ace2*$^{+/+}$ and $^{-/-}$ female mice 157
Figure 5.4. A. Effects of ACE2 deficiency and Ovx on 24 h systolic arterial pressure in *Ace2*$^{+/+}$ and $^{-/-}$ mice 158
Figure 5.4. B. Effects of ACE2 deficiency and Ovx on 24 h diastolic arterial pressure in *Ace2*$^{+/+}$ and $^{-/-}$ mice. 158

Figure 5.5. Working model of obesity-induced hypertension in *Ace2*$^{+/+}$ female mice 159
Figure 6.1. Role of ACE2 in Obesity-Associated Hypertension 183
Figure 6.2. Pancreas from 8 week old male *Ace2*$^{+/y}$ and $^{-/y}$ stained for insulin . 184

Section I. BACKGROUND

1.1.Obesity.

The prevalence of obesity is rising in the United States with 30% of the population obese with a body mass index (BMI) ≥ 30 (Flegal et al. 2010; BMI = weight in kg/height in m^2; NHLBI, 1998). Alarmingly, the percentage of American men with a BMI of ≥ 30 in the last ten years went from 28% to 32% whereas the percentage of women with BMI ≥ 30 only rose by 1% (34 to 35%) (NHANES 2007-2008) (Flegal et al. 2010). Additionaly, obesity affects 17% of the children in the age group of 2-19 years (Whitaker et al. 1997; Serdula et al. 1993). A recent report in the Journal of American Medical Association indicated that 9.5% of infants and toddlers were at or above the 95th percentile of the weight-for-recumbent-length growth charts and 17% of children and adolescents were at or above the 95th percentile of BMI for age (Odgen et al. 2010). According to the National Heart, Blood and Lung Institute, three parameters that need to be assessed as risk factors for obesity and associated disease are BMI, waist to hip ratio (WHR) and risk factors for diseases associated with obesity (Table 1.1). These marked increases in the prevalence of obesity are associated with significant elevations in cardiovascular pathologies associated with obesity, including hypertension, type 2 diabetes, stroke, heart disease and certain types of cancers.

1.2. Obesity hypertension.

Despite a doubling of obesity prevalence over the last two decades, the number of deaths from heart diseases has declined in the past fifty years as a result of improved health care and awareness (Cutler et al. 2008). In contrast, the prevalence of hypertension has risen in the last ten years (24 to 29%), with up to one fourth of the increase attributed to an increased BMI (Cutler at al. 2008).

Longitudinal studies in humans have demonstrated BMI as an independent risk factor for hypertension after controlling for associated risk factors such as age, sex, ethnicity, gender and smoking (Dyer et al. 1990; Huang et al. 1998; Brown et al. 2000). Importantly, recent studies suggest that 60-70% of hypertension in adults is attributed to obesity with a 3.5-fold increase in likelihood of developing hypertension in the setting of obesity (20% increases in body weight) (Kotchen. 2010). Thus, obesity is closely linked to the development of hypertension.

1.3. Causes of obesity-induced hypertension.

Obesity has been demonstrated to raise pressure by increased renal sodium reabsorption, increased intravascular volume, impaired natriuresis, impaired endothelial function and also physical compression of kidneys, especially with visceral obesity (da Silva et al. 2009). Different factors involving multiple systems are indicated to play a role in obesity-related hypertension (Table 1.2 Kotchen. 2010). However, the key systems that have been implicated

in obesity-induced hypertension are an activated sympathetic nervous system (SNS) and a stimulated renin angiotensin system (RAS) (da Silva et al. 2009).

Numerous studies in animals and humans demonstrate an important role for the SNS in the eitiology of obesity-hypertension. High fat (HF) diet (normal diet supplemented with cooked beef fat) for 5 weeks in dogs resulted in significant increases in body weight (~12 kg) along with an increase in mean arterial pressure (MAP; ~ 17 mmHg) (Hall et al. 1993a). Additionally, dogs with diet-induced obesity exhibited an increase in extracellular fluid volume and sodium retention (Hall et al.1993a). Administration of α- and β-adrenergic blockers (prazosin and propranolol) lowered blood pressure to a much greater extent in obese hypertensive dogs compared to lean dogs (21 ± 4 in obese versus 10 ± 1 mmHg in lean dogs (Hall et al.1993a). Furthermore, male Sprague Dawley (SD) rats administered a HF diet for 6 weeks exhibited a significant increase in body weight and MAP (113 ± 2 versus 94 ± 8 mmHg; Nagae et al.2009). Administration of a neuronal acetylcholine receptor antagonist reduced MAP to a greater magnitude in HF-fed rats (LF: -46+/-3.5; HF: -62+/-1.5 mmHg), indicating increased sympathetic activity with diet-induced obesity (Nagae et al. 2009). In another study male SD rats fed a moderately high fat (MHF) diet for 13 weeks exhibited a significant increase in MAP (~11mmHg) in obesity- prone (OP) rats compared to the low fat (LF) controls. Interestingly, administration of muscimol, a GABA(A) receptor agonist in the rostral ventrolateral medulla resulted in a greater reduction in MAP in OP rats compared to controls, thus identifying a specific region in the central nervous system contributing to

increased pressure with obesity (Stocker et al. 2007) . Similar to the previous findings Chen et al. demonstrated a reduction in lumbar sympathetic nerve activity (LSNA) (35%) and MAP (-14mmHg) in HF-fed rats with muscimol administration in the paraventricular nucleus, indicative of an activated SNS with diet-induced obesity (Chen et al. 2010).

Leptin has been implicated as an adipokine contributing to increased sympathetic nerve activity (SNA) with obesity. Female rats fed either a LF or HF diet for 8 weeks were injected intracerebroventrically (i.c.v) with leptin and LSNA, heart rate and MAP were measured (Lu et al. 1998). In a separate group of animals, HF diet for 8 weeks was followed by a restricted diet (50% less calories from fat) for 3 weeks. LSNA and MAP were increased with leptin injection in LF or rats administered a restricted diet. In contrast, obese rats exhibited a reduction in LSNA and MAP (Lu et al. 1998). However, since obesity is associated with leptin resistance it is unclear if the resistance to a blood pressure increase in obese rats was a result of decreased response or increased resistance to leptin with obesity (Hall et al. 2001). In contrast, leptin administration (intraperitoneally or i.c.v) to HF-fed mice resulted in reduced metabolic effects indicating leptin resistance (Rahmouni et al. 2005). HF-fed mice demonstrated a blunted response to lumbar and adipose tissue SNA; however, renal SNA was preserved with obesity. Importantly, obese mice exhibited a significant increase in MAP (~10mmHg) with leptin, indicating leptin-mediated increases in SNA and blood pressure are preserved in obese mice (Rahmouni et al. 2005). Rabbits fed a HF diet for 4 weeks exhibited a 3-fold increase in visceral fat, MAP (8%), heart rate

(26%), plasma norepinephrine (87%) and renal sympathetic activity (48%) compared to controls (Prior et al. 2010). Leptin administration intracerebrally resulted in comparable elevations in blood pressure between HF-fed and control animals. In contrast, renal SNA was significantly higher in HF-fed rabbits, despite evidence of leptin resistance to metabolic effects as demonstrated by reduced expression of c-Fos (expressed in appetite and sympathetic actions of leptin) (Prior et al. 2010). These authors implicated marked selective leptin resistance to metabolic, but not cardiovascular effects of leptin with obesity.

Similar to animals, studies in humans also indicate a role for an activated SNS in obesity-hypertension. Muscle sympathetic activity was increased by 2-fold in normotensive adults with weight gain (Grassi et al. 1995). Weight loss attenuated this increase (Grassi et al.1998). An activated SNS in obesity was further supported by a study administering an α_1 receptor blocker (doxazosin) or β_1 receptor blocker (atenolol) to obese and lean hypertensive adults (Wofford et al. 2001). Administration of an adrenergic receptor antagonist resulted in a greater reduction in blood pressure in obese (16mmHg) compared to lean adults (8mmHg). Body fat distribution has also been demonstrated as an important predictor of SNS activation (Alvarez et al. 2002; Alvarez et al. 2004). SNA was unaltered in obese and non-obese subjects with comparable visceral obesity. In contrast, an increased visceral adiposity was associated with elevated SNA and blood pressure (Alvarez et al. 2002; Alvarez et al. 2004). Increased systolic blood pressure (SBP) and muscle sympathetic activity measured by microneurographic methods has been demonstrated in normal healthy adults even with modest diet-

induced weight gain (Gentile et al. 2007). These results from animal and human studies demonstrate an important role of SNS in the eitiology of obesity-hypertension. Similar to SNS, studies in animals as well as humans demonstrate a strong association between RAS and obesity-hypertension, which will be discussed in section 1.10.

1.4. Classical RAS.

The discovery of the first enzyme of the RAS dates back to 1896-1897, when a Finnish physiologist and his Swedish student isolated a compound from extracts of rabbit kidney cortex that was able to raise blood pressure, which they called renin (Tigerstedt et al. 1898; Fyhrquist. et al. 2008). Almost 35 years later in 1934 Henry Goldblatt demonstrated chronic hypertension in dogs whose renal arteries were clamped using silver clips (Goldblatt et al. 1934). Following this Braun et al. (1940) as well as Page and Helmer (1940) also using similar techniques demonstrated a pressor compound from kidneys which they called angiotensin (Page and Helmer et al.1940; Braun et al. 1940; Braun et al.1958; Benigni et al. 2010). By the early 1970's major components of the classical RAS were identified such as angiotensinogen , renin, angiotensin converting enzyme (ACE), angiotensin receptor subtype 1 (AT1) and angiotensin receptor subtype 2 (AT2) (Figure 1.1). Additionally, in the last two decades studies from different groups have identified new components of the RAS, constantly altering the dynamics of the RAS (Figure 1.2).

The classical RAS begins with angiotensinogen, the only known precursor of angiotensin (Ang) peptides. Angiotensinogen, a 58kD protein consisting of 452 amino-acids (Kageyama et al. 1984) is synthesized primarily by the liver, which secretes this protein in the systemic circulation to be acted upon by other enzymes (Dévény et al. 1968). However, studies demonstrate a role for other tissues and cells to synthesize angiotensinogen locally and secreted into the systemic circulation, which includes adipocytes (Cassis et al. 1988). Studies in rodents in the last few years demonstrated an important role of angiotensinogen in blood pressure regulation. One of the earlier studies to demonstrate the role of angiotensinogen in blood pressure regulation was by Tanimoto et al. where mice lacking angiotensinogen exhibited 20 mmHg lower MAP compared to wild type controls (Tanimoto et al.1994). Importantly, studies in rats and mice demonstrated a dose-dependent increase in MAP with increasing doses of infused angiotensinogen. Administration of 0.8, 1.2, and 2.9 mg/kg angiotensinogen increased MAP of rats by 8 ± 0.4, 19.3 ± 2.1, and 32 ± 2.4 mmHg, respectively (Klett et al. 2001). Similarly, Cholewa et al. demonstrated an increase in MAP of mice that were infused with angiotensinogen. Intravenous infusion of angiotensinogen at a dose of 5nmol/kg/min resulted in significant increases in plasma angiotensinII (AngII) levels (34+/-6 vs 288+/-109 pg/ml) along with an increase in arterial pressure (119 +/- 5 to 141 +/- 3 mmHg, $p<0.05$) (Cholewa et al. 2005).

Angiotensinogen is converted to angiotensinI (AngI) by renin, an aspartyl protease secreted from the juxtaglomerular (JG) cells of the kidney by the

conversion of pro renin to renin. Three factors play an important role in stimulating renin secretion, i.e., a reduction in renal perfusion pressure, activation of sympathetic nerve activity and decreased sodium delivery to the macula densa (Lorenz et al.1990; Data et al. 1978; Gaal et al. 1979).

Besides the JG cells of the kidney, renin is localized to brain (Inagami et al.1980), blood vessels (Mizuno et al. 1984), testis (Pandey et al. 1984) macrophages (Lu et al. 2008) and adipose tissue (Karlsson et al. 1998). An area which has recently been of interest is the newly identified human renin receptor which binds renin and pro renin known as renin(pro) receptor, expressed in heart, brain, placenta, liver, kidney (Nguyen et al. 2002) and adipose tissue (Galvez-Prieto et al. 2008).

AngiotensinI (AngI), a biologically inactive peptide of this system is converted by ACE to AngII. ACE, identified in 1956, is a dipeptidyl carboxypepetidase which converts AngI to AngII (Skeggs et al. 1956). Two forms of ACE produced from a single gene by alternate promoters have been identified (Soubrier et al. 1988, Ehlers et al. 1989), somatic ACE which is primarily expressed in endothelial cells of lung (Ryan et al. 1976), kidney (Wallace et al. 1978), intestine (Bruneval et al. 1986), placenta (Sim et al. 1984) and adipose tissue (Crandall et al. 1992; Karlsson et al. 1998) and germinal ACE, found exclusively in testis (Velletri et al. 1985). Importantly, besides synthesizing AngII, ACE also degrades bradykinin (Ryan et al.1975), a vasodilator peptide, giving ACE inhibitors another mechanism of action as antihypertensive drugs (Turner et al. 2002). Besides AngI and bradykinin, ACE also hydrolyzes substance P

(Couture et al. 1981), cholecystokinin (Dubreuil et al. 1989) and luteinizing hormone-releasing hormone (LHRH) (Skidgel et al. 1985).

Undoubtedly, the role of AngII has been well established in blood pressure regulation, vasoconstriction and sodium absorption via its action on different tissues involved in fluid volume homestasis (Figure 1.3). In the vasculature AngII has been demonstrated to elicit vasoconstriction in vascular smooth muscle cells (Chiu et el. 1991), exhibit mitogenic effects causing vascular smooth muscle cell growth (Lyall et al. 1988), proliferation (Stouffer et al. 1992) and hypertrophy (Chiu et al. 1991). AngII also increases blood pressure via stimulation of sympathetic neurotransmission where even at subpressor levels AngII has been demonstrated to enhance vasopressor responses to norepinephrine (Reams et al. 1987), or facilitate norepinephrine release (Hughes et al. 1971). Besides increasing norepinephrine release during sympathetic stimulation, AngII can also inhibit norepinephrine reuptake (Campbell et al. 1979) and to stimulate catecholamine release from the chromaffin cells of the adrenal medulla (Peach et al. 1974). In the kidney AngII has been demonstrated to increase pressure via efferent arteriole vasoconstriction (Plante et al. 1988). A well known effect of AngII in the kidney is to facilitate sodium reabsorption in the distal collecting tubule via aldosterone release from the adrenal cortex (Chui et al. 1974; Carpenter et al. 1961). In the the heart AngII elicits a positive inotropic and chronotropic effect (Baker et al. 1992; Li et al. 1996) and regulates cardiac growth (Schorb et al. 1993). AngII mediates its effects in the central nervous system where it has been demonstrated to mediate drinking and pressor

response via its actions in the subfornical organ of the brain (Gutman et al. 1988) and through mediating vasopressin release (Mouw et al. 1971; Keil et al. 1975).

AngII mediates its effects via binding to two distinct G-protein coupled receptors (GPCR) i.e angiotensin receptor type1 (AT1R) and angiotensin receptor type 2 (AT2R) (Lin et al.1970; Chiu et al.1989). Most of the known classical effects of AngII such as vasoconstriction (Touyz et al.1997), growth and differentiation (Huang et al.1996; Touyz et al.1997), tubular reabsorption (Navar et al.1999) and aldosterone synthesis and secretion (Balla et al.1991) are mediated via AT1R, while AT2R counterbalances majority of the effects mediated via AT1R (Figure 1.4). While humans possess only one AT1R subtype, rodents possess two subtypes, termed AT1a receptors (AT1aR) and AT1b receptors (AT1bR), with 95% sequence homology (Inagami et al. 1993). AT1aR are expressed in adult kidney, heart, adrenal gland, lung , brain, adipose tissue, and testis while AT1bR are expressed in the brain, testis, anterior pituitary gland and adrenal zona glomerulosa (Burson et al. 1994; Oliverio et al. 2000). One of the first studies to demonstrate the role of AT1aR in blood pressure regulation was by Ito et al. where deletion of the gene encoding AT1aR reduced blood pressure by ~ 24mmHg compared to wild type mice (Ito et al. 1995). In contrast, targeted deletion of AT1bR did not alter blood pressure or plasma aldosterone levels (Chen et al. 1997). These results indicated AT1aR as the major receptor involved in blood pressure regulation in mice. However, AT1aR deficient mice exhibit a modest vasoconstrictor response to AngII, which is blunted by an AT1 receptor antagonist, suggesting a role of AT1bR in blood pressure regulation. Additionally,

vasoconstrictor responses to AngII were totally lost in AT1aR/AT1bR double knock out mice (Oliverio et al. 2000). In contrast to AT1aR-/- mice that exhibit lower blood pressure, deletion of AT2R did not alter blood pressure; however, AT2R deficient mice exhibited an increased vasopressor response to AngII (Hein et al. 1995).

Angiotensin (2-8) (AngIII), a biologically active peptide of this system is generated from AngII by aminopepetidase A (Vaughan et al.1974; Kugler et al.1982). Some of the important functions of AngIII are similar to AngII i.e facilitating vasoconstriction (Satoh et al. 1981), vasopressin (Yamaguchi et al.1979), and aldosterone release (Campbell et al.1974) mediated via ATI receptor. Infusion of AngII intravenously or directly in renal artery elicited a more potent vasoconstrictor response in anaesthesized dogs compared to AngIII, demonstrating AngII as a more potent vasoconstrictor peptide of this system compared to AngIII (Satoh et al. 1981). In contrast, in a recent study administration of either AngII or AngIII i.v in conscious dogs elicited comparable effects on blood pressure, plasma renin activity, and aldosterone release, which were inhibited by an AT1R antagonist (Gammelgaard et al. 2006). However, AngIII was cleared metabolically five times faster compared to AngII.

AngIII is rapidly converted by aminopeptidase N to Angiotensin (3-8) (AngIV), which elicits its action via the AT4 receptor also called IRAP (insulin-regulated amino peptidase receptor) (Albiston et al. 2001; Keller et al.1995). AngIV has been demonstrated to increase expression of plasminogen activator inhibitor -1 (PAI-1) in bovine aortic endothelial cells in a time and dose dependent

manner (Kerins et al. 1995). In line with this finding a recent study demonstrated an increase in PAI-1 expression in endothelial cells stimulated with AngII or AngIV (Numaguchi et al. 2009). In contrast, PAI-1 expression is reduced in endothelial cells from IRAP$^{-/-}$ mice. Reduced occlusive thrombosis and increased fbrinolysis is observed in IRAP$^{-/-}$ mice compared to wild type in a carotid artery ligation model indicating a role for this peptide in thrombosis and fibrinolysis (Numaguchi et al. 2009). Additionally, this peptide has been implicated in memory and learning (Wright et al. 2008).

1.5. Angiotensin Converting Enzyme 2 (ACE2).

Almost 50 years after the discovery of ACE two independent groups using unique molecular strategies identified a homologue of ACE known as ACE2 (Tipnis et al. 2000; Donoghue et al. 2000). The discovery of this enzyme was exciting as it was shown to convert AngII, a potent vasoconstrictor peptide of the RAS to angiotensin-(1-7) (Ang-(1-7)), a vasodilator peptide. This enzyme was discovered by one group in a human lymphoma cDNA library when screening for zinc metalopepetidases, and these investigators called it ACEH (Angiotensin Converting Enzyme Homologue) (Tipnis et al. 2000). Simultaneously, another group also identified this enzyme, which they called ACE2, from 5' sequence of a human heart failure ventricle cDNA library (Donoghue et al. 2000). On its initial discovery its expression was thought to be limited to heart, kidney and testis, hence the enzyme was implicated in cardiovascular and kidney diseases (Tipnis et al. 2000; Donoghue et al. 2000).

ACE2, a 40kb gene located on chromosome Xp22 consists of 18 exons with the zinc binding motif (HEMGH) which contains the catalytic site of this enzyme located on exon 9 (Turner et al. 2002). The full length cDNA encodes 805 amino acids with an N terminal which harbors the signal sequence a typical feature of zinc peptidases and a hydrophobic C terminal which serves as a membrane anchor (Turner et al. 2002) (Figure 1.5). Overall, ACE2 has 40% identity at the protein level to the catalytic domain of ACE (Turner et al. 2002) and the C terminal of ACE2 shares homology with collectrin, a protein expressed in the kidney (Zhang et al. 2001). Unlike mammalian ACE gene which generates two isoforms, only a single ACE2 protein is formed (Hamming et al. 2007; Warner et al. 2004). The ACE2 protein has seven potential N-linked glycosylation sites with a molecular weight of 120kDa. Deglycosyation of the protein reduces its size to 85kDa (Turner et al. 2002). ACE2 is proteolytically active at a pH of 6.5 and is activated by monovalent anions like chloride and fluoride but not bromide (Vickers et al. 2002). ACE2 activity is inhibited by EDTA, but is not affected by classical ACE inhibitors such as captopril, enalapril and lisinopril (Tipnis et al. 2000). Though initial studies demonstrated expression of this enzyme limited to heart, kidney and testis later studies in humans and animals demonstrate a more widespread distribution of ACE2 mRNA, protein and activity in rodents and humans (Gembardt et al. 2005; Hamming et al. 2007).

Interestingly, ACE2 serves as receptor for the SARS-CoV pathogen that caused a respiratory illness in 2003, called severe-acute respiratory syndrome as demonstrated by in vitro as well as in vivo studies (Li et al. 2003).

ACE2 is a monocarboxypeptidase cleaving a single amino acid from the C- terminal of the substrate (Turner et al. 2002). In the RAS, ACE2 hydrolyzes AngI and AngII to generate Ang (1-9) and Ang-(1-7), respectively. Studies on enzyme kinetics demonstrated a 400-fold higher catalytic efficiency to hydrolyze AngII as a substrate compared to AngI (Vickers et al. 2002). Besides AngII and AngI, ACE2 also hydrolyzes des-Arg-Bradykinin, neurotensin 1-8, kinetensin and apelin-13 (Vickers et al. 2002).

Ang-(1-7), a heptapeptide product of ACE2 generated from AngI and AngII in canine brain was demonstrated to possess biological activity such as vasopressin release from the brain. In contrast, unlike AngII, Ang-(1-7) does not stimulate vasoconstriction, thirst or aldosterone release (Ferrario et al. 1988; Santos et al. 1988) (Figure 1.6). Importantly, Ang-(1-7) infusion in spontaneously hypertensive rats (SHR) elicited significant reductions in plasma vasopressin levels and increased urinary prostaglandins, diuresis and natriuresis (Benter et al. 1995). In addition, SBP was reduced significantly in response to Ang-(1-7), indicating a blood pressure lowering effect of this peptide. Using canine coronary artery rings, investigators demonstrated that Ang-(1-7) induced vasodilation was mediated via nitric oxide and bradykinins as pretreatment with a nitric oxide synthase inhibitor (L-NAME) completely abolished the vasodilator response (Brosnihan et al. 1996). In addition, a bradykinin receptor antagonist blocked Ang-(1-7)-mediated vasodilation by 75%. A non-selective AngII receptor antagonist blocked the vasodilator response to Ang-(1-7) by 80%; however, selective AT1 and AT2 receptor antagonists were ineffective. These results

suggested that Ang-(1-7) elicits vasodilation via endothelium-dependent nitric oxide involving involving bradykinin receptors (Brosnihan et al. 1996).

ACE2/Ang-(1-7) has been recently highlighted as the counter balancing arm of the RAS to the ACE/AngII axis. Though the earlier studies described above demonstrated a functional role of Ang-(1-7) as far back as 1988, it was the discovery of ACE2 in 2000 that stimulated renewed interest in Ang-(1-7) as a peptide influencing cardiovascular function.

Ang-(1-7) mediates its effects via a distinct GPCR which was identified in 1988 as a Mas oncogene. However, at that time the ligand for this receptor was yet unknown (Jackson et al. 1988). In 2003, Ang-(1-7) was demonstrated as an endogenous ligand for Mas (Santos et al. 2003), expressed predominantly in testis, forebrain, kidney and heart (Bunnemann et al. 1990; Alenina et al. 2002).

Unlike AngII, Ang-(1-7) has been demonstrated to have vasodilatory, antiproliferative and antitrophic properties (Figure 1.6). Cardiac dysfunction and remodeling were attenuated in transgenic-DOCA-SD rats with increased Ang-(1-7) in the plasma via Ang-(1-7) fusion protein (Santiago et al. 2010). Ang-(1-7) fusion protein resulted in a reduction in pressure and increased cardiac Ang-(1-7) levels almost 3-fold in rats and attenuated cardiac dysfunction and prefibrotic lesions (Santiago et al. 2010). Ang-(1-7) has also been demonstrated to be important for endothelial function. Mas receptor knock out mice on C5BL/6 and FVBN backgrounds exhibit diminished vasodilation in response to acetylcholine and alteration in nitric oxide synthesis and reactive oxygen species production (Rabelo et al. 2008; Xu et al. 2008). Additionally, SD rats expressing Ang-(1-7)

fusion protein exhibit lower body weight, fat mass, triglycerides and cholesterol (Santos et al. 2010). Moreover, these animals exhibit increased insulin sensitivity, glucose tolerance and improved glucose uptake in adipocytes. Insulin signaling in adipose tissue is improved as demonstrated by increased phosphorylation of PI3K/ AKT along with increased plasma adiponectin levels (Santos et al. 2010). These results are in agreement with a previous report by the same group using Mas receptor deficient mice on an FVB/N background (Santos et al. 2008a). The Mas receptor deficient mice exhibited increased adipose mass, glucose intolerance, decreased insulin sensitivity and elevated plasma lipids compared to controls. Mas receptor deficiency resulted in increased angiotensinogen expression in adipose tissue along with a 10 mmHg increase in blood pressure. Insulin stimulated glucose uptake was blunted in mice with Mas receptor deficiency, as evidenced by decreased expression of GLUT4 in adipose tissue (Santos et al. 2008a). In contrast, there are studies which demonstrated either no effect of Mas deficiency on blood pressure (Walther et al. 2000) or increased pressure in Mas deficient mice (de Moura et al. 2010).

These studies together highlight the importance of ACE2 in numerous pathologies given its important function in the RAS, i.e catabolising AngII to generate Ang-(1-7), peptides shown to exhibit opposite functions within the same system.

1.6. ACE2 regulation.

1.6.1. ACE2 shedding.

ACE2 is a type I integral membrane protein, where the ectodomain which contains the active site of this enzyme faces the extracellular surface and hydrolyzes the circulating peptides (Hamming et al. 2007). Shedding of the ectodomain of enzymes has been an important phenomenon in regulation and expression of these enzymes in normal physiological conditions or in different pathologies (Lambert et al. 2005). Ectodomain shedding has been observed in a number of membrane proteins such as tumor necrosis factor-α (TNFα), ACE, amyloid precursor protein (APP), and β site amyloid cleaving enzyme (BACE) (Black et al. 1997; Hundahausen et al. 2003; Beldent et al. 1993; Hussain et al.2003; Palecanda et al. 1992; Arribas et al. 1995; Parkin et al. 2004). Ectodomain shedding of enzymes can occur under normal conditions or stimulation via phorbol esters such as phorbol -12-myristate-13-acetate (PMA). Proteins that have been implicated in PMA mediated shedding are mostly from the ADAM (a disintegrin and metalloproteinase) family (Arribas et al. 2002) which constitutes a number of members such as ADAM-9, ADAM-10, ADAM-12, ADAM-17. ADAM-17, also known as TACE (TNF-α converting enzyme) since it was first identified to shed TNF-α, is the best characterized protein to mediate shedding of membrane bound proteins on stimulation by PMA. To test if ACE2 also undergoes shedding, ACE2 protein was expressed in two stable cell lines, HEK293 and CHO cells (Lambert et al. 2005). HEK-ACE2 exhibited a 120kDa band typical of mature ACE2 protein. Western blot from media of these cells

revealed two bands at ~105 and 95 kDa. However, on stimulation with PMA they saw an intense band of ~105kDa. An antibody targeting the cytoplasmic domain did not detect ACE2 in the media with or without stimulation by PMA. Furthermore, the shed form of ACE2 was demonstrated to possess catalytic activity. Thus, these results indicate that ACE2 ectodomain can be shed via phorbol ester stimulation and this shed form is enzymatically active. Further, using different inhibitors and siRNA, they were able to identify ADAM-17 as the protease mediating ectodomain shedding of ACE2 (Lambert et al. 2005).

ADAM-17 has been demonstrated to be upregulated in vivo by high fat diets as well as in adipocytes on stimulation with fatty acids, insulin or glucose. ADAM-17 expression was increased in 3T3F442A adipocytes stimulated with palmitic acid, lipolysaccharide, high glucose, and high insulin (Fiorentino et al. 2010). Similarly, C57BL/6 fed a high fat diet for 20 weeks exhibited increased ADAM-17 activity in adipose depots (Fiorentino et al. 2010).C57BL/6 129Svj mice fed a high fat (42%) diet for 15 weeks exhibited an increased expression of ADAM-17 in subcutaneous adipose tissue compared to the mice that were fed standard diet (Voros et al. 2003). In line with these studies C57BL/6 male mice chronically fed a high fat (60%) for 4 months demonstrated a significant elevation in ADAM-17 expression in adipose depots (Gupte et al. 2008). These results demonstrated that the ACE2 shedding enzyme ADAM-17 is increased in adipose tissue upon HF feeding.

1.6.2. Regulation of ACE2 by hormones.

Some of the components of the RAS such as angiotensinogen, ACE and AT1R have been shown to be regulated by gonadal hormones such as estrogen
(Dzau et al.1982; Gallagher et al.1999; Nickenig et al.1998; Krishnamurthi et al. 1999). In a study by Brosnihan et al using ApoE-/- mice with or without estrogen receptor α (ERα), administration of 17β estradiol to ovariectomized mice resulted in down regulation of ACE2 mRNA in kidneys of mice that possess the ERα receptor (Brosnihan et al. 2008). However, this effect was lost in ERα knockout mice suggesting a role of estrogen via ERα receptor in ACE2 regulation (Brosnihan et al. 2008). Another study demonstrated upregulation of ACE2 activity and proteins in rat kidneys by 17β estradiol in a model of renal wrap hypertension. ACE2 activity, protein and mRNA were reduced in ovariectomized Sprague Dawley rats administered a high salt (4%) diet for 6 weeks (Ji et al. 2008). However estrogen replacement prevented these effects and protected the rats against renal hypertensive disease (Ji et al. 2008). A study examined the expression of Ang-(1-7) and ACE2 staining in either virgin or 19 day pregnant Sprague Dawley rats (Brosnihan et al. 2003). At 19 days of pregnancy they demonstrated increased ACE2 and Ang(1-7) immunostaining in the inner cortex and outer medulla of the kidneys of pregnant rats. Estrogen levels are elevated in pregnancy; hence it is possible that in these animals the increased ACE2 in kidneys may have resulted from increased estrogen (Brosnihan et al. 2003).

1.7. ACE2 knock out models.

To study the role of this enzyme in various pathologies, three different investigators have generated ACE2 deficient mice targeting specific exons, resulting in loss of functional protein and activity. The first group (Crackower et al. 2002) targeted deletion of portions of exon 7-9, as exon 9 contains the catalytic site of ACE2, resulting in loss of ACE2 protein and mRNA. ACE mRNA in heart and kidney was unaltered in ACE2 deficient mice. ACE2 deficient mice did not exhibit any abnormalities and were fertile. ACE2 deficiency did not result in alterations in blood pressure at 3 months in male or female mice. However, ACE2 deficiency resulted in a cardiac phenotype in male mice which progressed with age, including wall thinning of the left ventricle and increased diameters of cardiac chambers. In spite of the mild chamber dilation there was no evidence of cardiac myopathy or hypertrophy. At 6 months of age the ACE2 deficient mice exhibited reduced blood pressures resulting from severe cardiac function. Plasma, kidney and heart AngII levels were elevated (Crackower et al. 2002).

Another line of ACE2 deficient mice were generated by deleting exon 3 of the mouse ACE2 gene (Yamamoto et al. 2006) and used to define the role of ACE2 in pressure overload. At baseline cardiac function as well as plasma and cardiac AngII levels were similar in ACE2 deficient and wild type mice. However, pressure overload after transverse aortic constriction (TAC) resulted in cardiac hypertrophy and decreased contractility in ACE2 deficient mice, and heart failure

related deaths were higher in ACE2 deficient mice. Ventricular and plasma AngII levels were elevated with ACE2 deficiency. Cardiac hypertrophy and contractility in ACE2 deficient mice were reversed by administration of AT1 receptor antagonist (Yamamoto et al. 2006).

The third colony of ACE2 deficient mice was generated by Gurley et al, (Gurley et al. 2006) by replacing the zinc-binding motif which contains the active site of this enzyme with a neocassette. In the initial cohort of mice on a mixed background (129SvEv x C57BL/6) SBP was 7mmHg higher in ACE2 deficient mice compared to wild type. On a C57BL/6 background, blood pressure measurements exhibited a modest but significant elevation of ~7mmHg in ACE2 deficient mice but there was no difference in blood pressure of ACE2 deficient mice on a 129SvEv background. Plasma AngII levels were not increased by ACE2 deficiency; however, acute infusion of AngII resulted in a 3-fold increase in plasma AngII in ACE2 deficient mice compared with wild type. Further, chronic infusion of AngII for 14 days resulted in more pronounced elevations in blood pressure in ACE2 deficient mice (+64mmHg) compared to wild type (+36mmHg). Plasma and kidney levels of AngII were significantly elevated in ACE2 deficient mice, indicating an important role for this enzyme in AngII catabolism and AngII mediated hypertension. In this model, cardiac function was not altered by ACE2 deficiency (Gurley et al. 2006). Mechanisms for differences in effects of ACE2 deficiency on blood pressure and cardiac function between these models are unclear (Table.1.3).

Since ACE2 is located on the X chromosome, in the present dissertation ACE2 deficient male mice are denoted as *Ace2*$^{-/y}$ and ACE2 deficient females are denoted as *Ace2*$^{-/-}$.

1.8. ACE2 in hypertension.

Since its initial discovery in 2000, the role of ACE2 in different models of hypertension has been studied given its ability to convert AngII, a vasoconstrictor peptide of this system to Ang-(1-7), a vasodilator peptide. In the preceding section, effects of ACE2 deficiency on blood pressure control were discussed. In this section we highlight other data indicating a role for ACE2 in blood pressure control. In a recent study by Wysocki et al (2010), administration of recombinant ACE2 reversed AngII-induced increases in blood pressure in ACE2 deficient mice. Further, recombinant ACE2 reduced plasma AngII and increased plasma Ang-(1-7) in wild type and ACE2 deficient mice. Administration of a selective Ang-(1-7) receptor antagonist had no effect on the blood pressure reduction from recombinant ACE2; however, an ACE2 inhibitor blunted reductions in blood pressure. These results indicated that decreased systemic AngII from recombinant ACE2 protected mice against AngII-mediated hypertension (Wysocki et al. 2010). A recent study by Zhong et al (2010) also demonstrated reductions in AngII-induced elevations in blood pressure in mice that were infused with recombinant ACE2 (Zhong et al. 2010).

Several researchers have demonstrated a role for ACE2 in hypertension in different animal models by overexpressing the enzyme in different tissues.

Using lentiviral gene therapy, Diez-Freire et al (2006) demonstrated that intracardiac injection of lentiviral vector containing murine ACE2 to 5 day old SHR rats reduced blood pressure when rats were 4 months of age. In contrast, there was no effect of lentiviral ACE2 on blood pressure in WKY rats (Diez et al. 2006). The same group overexpressed ACE2 in the rostral ventrolateral medulla of SHR and demonstrated a role for ACE2 in central regulation of blood pressure (Yamazato et al. 2007). To determine the role of vascular smooth muscle ACE2 in blood pressure regulation, transgenic expression of human ACE2 using an SM22 promoter in SHRSP rats attenuated the vasoconstrictive response to AngII and reduced blood pressure (Rentzsch et al. 2008). Similarly, Xia et al (2009) over-expressed human ACE2 in the brain and reversed AngII-induced increases in blood pressure. In this study administration of an Ang-(1-7) receptor antagonist reversed reductions in blood pressure from ACE2 overexpression (Xia et al. 2009). In another study the same group using adenoviral vectors expressed human ACE2 in subfornical organ (SFO) and demonstrated a reduction in AngII-mediated pressor and drinking responses (Feng et al. 2008). Taken together, these studies indicate a role for ACE2 in different models of hypertension.

An unresolved issue in studies manipulating ACE2 is whether effects result from changes in Ang-(1-7) or AngII. In ACE2 deficient mice infused with AngII, plasma (3-fold) and kidney (6-fold) levels of AngII increased compared to wild type mice (Gurley et al. 2006). In mice infused with recombinant human ACE2, AngII-induced elevations in blood pressure were reduced and administration of an Ang-(1-7) antagonist had no effect, indicating the importance

of ACE2 to lower AngII rather than increase Ang-(1-7) to blunt the effects of AngII-mediated hypertension (Wysocki et al. 2010).

Several lines of evidence support ACE2 polymorphisms in humans with hypertension. One of the initial studies in randomly selected Chinese patients with essential hypertension showed that a single nucleotide polymorphism (SNP) in the fourth base pair of the third Intron of the ACE2 gene may be associated with essential hypertension and cardiac incompetence (Liu et al. 2005). A single A/G polymorphism of the ACE2 gene was associated with hypertension in patients with the metabolic syndrome (Zhong et al. 2006). An interesting study measured macrophage ACE2 activity in normotensive, pre-hypertensive and hypertensive subjects. Interestingly ACE2 activity was increased in macrophages of pre-hypertensive subjects suggesting a protective role of ACE2 to counteract the increased levels of AngII (Keidar et al. 2007). Collectively, these studies in animals and humans demonstrate a role for ACE2 in hypertension.

1.9. ACE2 in diabetes.

One of the first studies to indicate a role for ACE2 in diabetes examined ACE and ACE2 mRNA and protein expression in kidneys of 24 week streptozocin diabetic SD rats (Tikellis et al. 2003). ACE2 protein and mRNA were downregulated in the renal tubules and glomerulus of diabetic rats, and administration of an ACE inhibitor rescued the downregulation of ACE2. In an interesting study examining ACE and ACE2 mRNA, protein and activity in

kidneys from type 1 (streptozotocin) and type 2 (db/db) mouse models of diabetes, Wysocki et al (2006) demonstrated in both models that ACE2 protein and activity were significantly lower in diabetic mice. Interestingly, these changes were restricted to ACE2 protein and not reflected by changes in ACE2 mRNA, indicating post translational modification of ACE2 in pathology and cautioning against making interpretations based solely on mRNA data (Wysocki et al. 2006).

In addition to regulating kidney AngII levels and function, studies have suggested a role for ACE2 in glucose homeostasis and type 2 diabetes. ACE2 immunostaining is evident in the endocrine and exocrine pancreas of the mouse (Niu et al. 2008). In addition, ACE2 deficient mice have impaired first phase insulin secretion and impaired glucose tolerance which progresses with age (Niu et al. 2008). However, insulin sensitivity was not influenced by ACE2 deficiency. A recent study by Bindom et al demonstrated a pivotal role for pancreatic ACE2 over-expression in regulation of glucose homeostasis in db/db mice (Bindom et al. 2010). Using recombinant human ACE2 (hACE2) to drive expression of ACE2 in the pancreas of db/db mice, glucose tolerance improved and pancreatic insulin content increased. ACE2 expression in pancreas increased β-cell proliferation and reduced apoptosis in db/db mice (Bindom et al. 2010).

1.10. Role of the RAS in obesity-induced hypertension.

An activated RAS is one of the key systems implicated in the etiology of obesity-hypertension, especially since all the components of the RAS

are expressed in adipose tissue. Excess weight gain has been demonstrated to increase renal sodium reabsorption which shifts the pressure natruresis curve to the right, resulting in increased blood pressure in obese subjects to maintain sodium balance (Hall et al. 1999). Obese subjects have been demonstrated to exhibit an increased plasma renin activity in spite of an increased sodium retention and extracellular volume leading to further sodium reabsorption and elevation in arterial blood pressure (Hall et al. 1999). In contrast, reduction in body weight paralleled reduction in plasma renin activity and blood pressure in obese subjects (Hall et al. 1999; Tuck et al. 1981). A study by Cooper et al (1997) demonstrated a positive correlation between BMI and an activated systemic RAS. Serum ACE and angiotensinogen levels were significantly elevated in individuals with BMI >30 (Cooper et al.1997). Weight loss of 6+/-3% of initial body weight in obese subjects resulted in reductions in blood pressure (systolic and diastolic), plasma renin activity and systemic ACE activity (23%) (Harp et al. 2002). Similarly, in another study weight loss resulted in a significant reduction in plasma renin activity and aldosterone levels and blood pressure ([systolic (~9mmHg) and diastolic (~7mmHg]) in obese subjects (BMI of 32.9+/-4.3kg/m^2) (Ho et al. 2007). An activated RAS along with a beneficial effect of weight loss was also demonstrated by Engeli et al (2005) in post menopausal obese and lean subjects. Plasma ACE, angiotensinogen, aldosterone and renin activity were higher in obese compared to lean subjects. Adipose angiotensinogen was reduced with obesity. In contrast a 5% weight loss resulted in a reduction of the systemic RAS components such as angiotensinogen, renin,

aldosterone and ACE activity. Importantly, they saw a ~7mmHg reduction in ambulatory systolic pressure with a 5% reduction in body weight (Engeli et al. 2005). These results indicate that the systemic RAS in humans is activated with obesity, and reductions in body weight blunt the activated RAS and lower blood pressure.

In spite of the high prevalence of obesity-induced hypertension, not many studies in humans are available that have tested the efficacy of drugs related to the RAS in treatment of resistant hypertension associated with obesity. The TROPHY (treatment in obese patients with hypertension) trial was one of the first randomized double blinded trials that compared the effects of either an ACE inhibitor (lisinopril) or diuretic (hydrochlorothiazide) for treatment of hypertension and associated metabolic parameters such as lipid and glucose profile in obesity-associated hypertensive subjects. TROPHY was a 12 week study with 232 enrolled subjects with average BMI of 32. While neither of the drugs completely blunted hypertension, obese patients exhibited a 40% reduction in pressure with lisinopril and 33 % with a diuretic. Other parameters such as blood glucose and lipid levels were not significantly altered. Thus, at the end of the trial the authors recommended a monotherapy with either ACE inhibitor or diuretic as the initial treatment for subjects with obesity-induced hypertension (Jansen et al. 2010). In the CROSS (Candesartan Role on Obesity and on Sympathetic System) study with 127 obese subjects (BMI of 32 kg/m^2), antihypertensive effects of an AT1 receptor antagonist were compared to hydrochlorothiazide. Though both drugs decreased blood pressure (80%), the AT1R antagonist, candesartan, improved

insulin sensitivity and modestly lowered sympathetic nerve activity (Grassi et al. 2003; Jansen et al. 2010). In a study in 489 obese subjects, blood pressure lowering effects of the direct renin inhibitor, aliskiren, were compared with amlodipine (calcium channel blocker) or irbesartan (AT1receptor antagonist) in patients who had not responded to an initial 4 week therapy with hydrochlorthiazide (Jordan et al. 2007). Results demonstrated that combination treatment with aliskiren was effective in obese patients with hypertension who fail to achieve blood pressure control with a first-line diuretic. Taken together, these studies demonstrate an important role for RAS in obesity-induced hypertension.

1.11. The adipose renin-angiotensin system.

Though traditionally the RAS is a circulating endocrine system, numerous studies in the last two decades support the presence of a local RAS in numerous tissues including adipocytes. Cassis et al (1988) demonstrated the expression of angiotensinogen mRNA and protein in brown (perivascular adipose tissue surrounding rat aorta) and white adipose tissue (epididymal adipose tissue) from rats (Cassis et al. 1988). Similarly, Campbell et al demonstrated angiotensinogen expression in rat periatrial and periaortic brown adipocytes (Campbell et al. 1987). Gomez et al contrasted developmental expression of angiotensinogen in different tissues in rats during maturation and demonstrated adipose tissue (brown fat) as the primary source of angiotensinogen in the fetus rather than the liver (Gomez et al.1988). In 3T3-L1 cells, angiotensinogen expression has been shown to be increased with differentiation as the cells

acquired an adipocyte phenotype (Saye et al. 1989). Additionally, angiotensinogen in adipocytes is regulated hormonally and nutritionally by insulin, β adrenergic agonists (Jones et al. 1997), glucocorticoids (Aubert et al. 1997), fatty acids (Safonova et al. 1997) and androgen (Serazin-Leroy et al. 2000). One of the earlier studies to indicate nutritional regulation of adipose angiotensinogen was by Frederich et al (1992) where angiotensinogen expression in adipocytes from SD rats was reduced on fasting (14.6+/-2.3%) and increased on refeeding (228+/- 53%). Importantly, SBP was reduced by ~25mmHg with fasting and increased by ~15 mmHg on refeeding, indicating a role for adipose derived angiotensinogen in blood pressure homeostasis (Frederich et al. 1992). Additionally, AngII, which has been demonstrated to be elevated with diet-induced obesity, has also been demonstrated to increase adipose angiotensinogen (Lu et al. 2007). Additionally glucose and fructose recently were demonstrated to increase angiotensinogen expression in mature 3T3-L1 adipocytes (Carvalho et al. 2010), indicating nutrional regulation of angiotensinogen in adipocytes.

Shenoy et al (1997) also demonstrated renin-like activity in rat interscapular brown adipose tissue; however, renin mRNA was undetectable by polymerase chain reaction in brown and white adipose tissue (Shenoy et al. 1997). In contrast, renin as well as renin binding protein is expressed in mature and preadipocytes from human adipose tissue (Karlsson et al. 1998, Schling et al. 1999). In addition to angiotensinogen, each component of the RAS has been localized to adipose tissue (for review see Thatcher et al. 2009). The following

section will overview changes in the adipose RAS in the setting of obesity hypertension.

1.12. Role of the adipose RAS in obesity-induced hypertension.

A number of studies in animals and humans have looked at the regulation of different RAS components in the setting of diet-induced obesity, with the majority of these studies focused on the role of adipose angiotensinogen in obesity-hypertension. Frederich et al (1992) demonstrated increased release of angiotensinogen from explants of adipose tissue from genetically obese mice (ob/ob) indicating a role of obesity in regulating adipocyte angiotensinogen expression (Frederich et al. 1992). In contrast, using obese Zucker rats, Jones at al (1997) demonstrated a reduction in angiotensinogen expression in adipose tissue of obese compared to lean rats (Jones et al. 1997). In contrast to the previous finding Hainault at al (2002) demonstrated an increase in adipose angiotensinogen expression and release in Zucker rats with obesity (Hainault et al. 2002).

One of the earlier studies to demonstrate a role for the RAS in obesity – hypertension in mice was by Ortlepp et al (2002). In HF-fed mice administered an ACE inhibitor (captopril) or AT1R antagonist (Irbesartan), MAP was reduced by 47 and 50%, respectively, indicating a role for an activated RAS in obesity-hypertension in mice (Ortlepp et al. 2002). Similarly, Rahmouni et al. (2004) also demonstrated increased angiotensinogen expression in omental, reproductive and peri renal adipose tissue in HF-fed mice. Unfortunately, blood pressure was

not measured in this study (Rahmouni et al. 2004). Boustany et al (2004) fed rats a HF diet for 11 weeks, at which time adipose angiotensinogen expression and plasma AngII concentrations were increased and blood pressure was elevated (Boustany et al. 2004). Elevated pressures in obese hypertensive rats were abolished by an AT1R antagonist (Boustany et al. 2005). Transgenic mice either expressing angiotensinogen only in adipose tissue (TG-KO) or over expressing angiotensinogen in adipose tissue (TG-WT) exhibited increased plasma angiotensinogen levels and rescued the hypotension of angiotensinogen deficient mice (Massiera et al. 2001a). Of note, over-expression of angiotensinogen in adipose tissue of wild type mice increased plasma angiotensinogen concentrations and elevated blood pressure, implicating adipose derived angiotensinogen in the etiology of obesity-hypertension (Massiera et al. 2001a). Spontaneously hypertensive rats fed a HF diet (45%) for 12 weeks resulted in significant elevations in body weight, SBP and glucose intolerance (Chung et al. 2010). Morover, HF diet resulted in increased expression of all components of the RAS such as, AngII, renin, ACE and angiotensinogen expression in kidney, and administration of candersartan or tempol reversed these effects.

Studies in humans looking at the different RAS components with obesity have provided contradictory results. Gorzelniak et al (2002) examined expression of different components of the RAS in subcutaneous adipose tissue from lean, obese normotensive and obese hypertensive subjects. Adipose angiotensinogen was decreased in obese normotensive and hypertensive subjects. In contrast

expression of renin, ACE and AT1 receptor was significantly increased in adipose tissue from obese hypertensive subjects (Gorzelniak et al. 2002).In contrast Van Harmelem et al (2000) demonstrated a 2-fold increase in adipose angiotensinogen with obesity (Van Harmelem et al. 2000). Faloia et al (2002) reported no differences in parameters of the circulating RAS with obesity. However, they reported a significant increase in AT1 receptor expression in adipose tissue from obese subjects. Additionally, studies in humans also demonstrate a differential expression of RAS components in visceral (VAT) versus subcutaneous (SAT) adipose tissue (Faloia et al. 2002). Giacchetti et al (2002) looked at the expression of angiotensinogen, renin, ACE, ATIR, AT2R in VAT and SAT from either lean or obese normotensive subjects. AT1R expression was higher in VAT compared to SAT. In contrast ACE expression was not different in the two depots. Renin and AT2R were undetected in either depot (Giacchetti et al. 2002).

1. 13. Obesity-hypertension: influence of gender.

Though women develop hypertension, the prevalence of obesity-associated hypertension is lower in pre-menopausal women compared to age matched men inspite of a higher BMI in females (Table 1.4). A number of studies implicate a role for sex hormones as mediators of sex differences in hypertension. In contrast, there is a paucity of studies defining mechanisms for differences in obesity-hypertension in females compared to males.

One of the earliest reports to demonstrate the role of estrogen in lowering blood pressure was by Brosnihan et al (1997), where administration of 17β-estradiol to transgenic (mRen2)27-positive [Tg(+)] and negative [Tg(-)] rats resulted in significant reductions in blood pressure in normotensive and hypertensive rats. Estrogen administration increased plasma Ang-(1-7) levels and reduced plasma AngII levels in Tg(+) rats. Additionally, estrogen reduced ACE activity in plasma, kidney and aortas of Tg(+) rats (Brosnihan et al. 1997). The same group then demonstrated that reduced ACE mRNA expression in kidney cortex, medulla, lung and aorta of ovariectomized Sprague-Dawley rats could be reversed upon administration of 17β-estradiol (Gallagher et al. 1999). Investigators have contrasted AngII-induced hypertension between male and female rats to begin to understand mechanisms for sex differences. Infusion of AngII to male SHR rats resulted in greater blood pressure elevations compared to females (Sullivan et al. 2010). Though plasma AngII levels were comparable between male and female rats infused with AngII, female SHR exhibited elevated plasma Ang-(1-7) levels compared to males before and after AngII infusion. In renal cortex, expression of AT1R and AT2R were higher in males compared to females; in contrast, females exhibited increased Mas receptor expression. Administration of a Mas receptor antagonist to AngII-infused rats eliminated gender differences in blood pressure. The authors concluded that Ang-(1-7) is a mediator of sex differences in AngII-induced hypertension (Sullivan et al. 2010).

Studies have provided similar results in mice, where administration of AngII resulted in a greater increase in blood pressure in males compared to

females (Xue et al. 2005). Gonadectomy reduced blood pressure in male mice, but increased pressure in females (~23mmHg), indicating that female sex hormones render protection against AngII-mediated hypertension (Xue at al. 2005). In a rat model of renal wrap hypertension, renal glomerulosclerosis was higher in intact males compared to females (Ji et al. 2005). Gonadectomy decreased the pathology in males; however, this protection was lost in females. In gonadectomized females, estrogen replacement reversed renal pathology. Follow-up studies by this group examined estrogen regulation of ACE2 in renal wrap hypertension (Ji et al. 2008). In ovariectomized female rats, ACE2 activity in renal cortex was decreased compared to sham-operated controls, and these effects were reversed by estrogen administration. Infusion of Ang-(1-7) prevented effects of ovariectomy on renal pathology in female rats, but effects were unrelated to blood pressure (Ji et al. 2008). These investigators suggested positive regulation of kidney ACE2 by estrogen. In summary, while a variety of studies demonstrate sex differences in the RAS that relate to blood pressure control, including studies supporting a role for ACE2, no studies have defined mechanisms of sex differences in obesity-hypertension.

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Table 1.1. Key measurements for assessing weight and health risk. (Source: National Heart,Blood and Lung Institute, 2010)

A. Body Mass Index (BMI)

	BMI
Underweight	Below 18.5
Normal	18.5-24.9
Overweight	25.0-29.9
Obesity	30 and above

BMI = weight in kg/height in meter ^{2}

B. Waist circumference: Higher waist circumference increases risk for heart disease and type 2 diabetes

Men	>35 inches
Women	> 40 inches

C. Risk factors for Obesity-Associated Diseases

- High blood pressure (hypertension)
- High LDL cholesterol ("bad" cholesterol)
- Low HDL cholesterol ("good" cholesterol)
- High triglycerides
- High blood glucose (sugar)
- Family history of premature heart disease
- Physical inactivity
- Cigarette smoking

Table 1.2 Putative mechanisms of obesity-related hypertension.

Primary mechanism	**Possible underlying mechanism**
Sodium retention	Antinatriuretic effect of insulin Increased renal SNS activity Increased aldosterone Increased cortisol activity Anatomic renal compression
Increased Sympathetic Nervous System activity	Insulin resistance Renin-angiotensin Leptin/adipokines Obstructive sleep apnea B adrenergic receptor polymorphisms Psychological stress
Increased circulating renin-angiotensin	Increased renal sympathetic nerve system
Increased adipose renin-angiotensin	
Impaired vascular endothelial function	Insulin resistance
Other vascular mechanisms	Insulin resistance Altered vascular ion transport

(Kotchen. 2010)

Table 1.3 Characteristics of *Ace2*$^{-/y}$ models.

	Crackower et al	**Yamamoto et al**	**Gurley et al**		
Genetic Background	Mixed	Mixed	Mixed	129/SvEv	C57BL/6
Cardiac systolic function	Impaired	Normal	Normal	Normal	Normal
Heart weight	Normal	Normal	Normal	Normal	Normal
Blood pressure	Decreased	Normal	Variable	Normal	Increased
Plasma angiotensin II	Increased	Normal	Normal	n.d	n.d
Cardiac angiotensin II	Increase	Normal	n.d	n.d	n.d
Renal angiotensin II	Increase	n.d	n.d	n.d	n.d

(Gurley et al. 2008)

Table 1.4. Prevalence of Hypertension in age matched men and women.

Age	Men (%)	Women (%)
20-34	9.2	2.2
35-44	21.1	12.6
45-54	36.2	36.2
55-64	50.2	54.4
65-74	64.1	70.8
75 and older	65.0	80.2
All	31.8	30.3

Source: National Center for Health Statistics. *Health, United States, 2008*

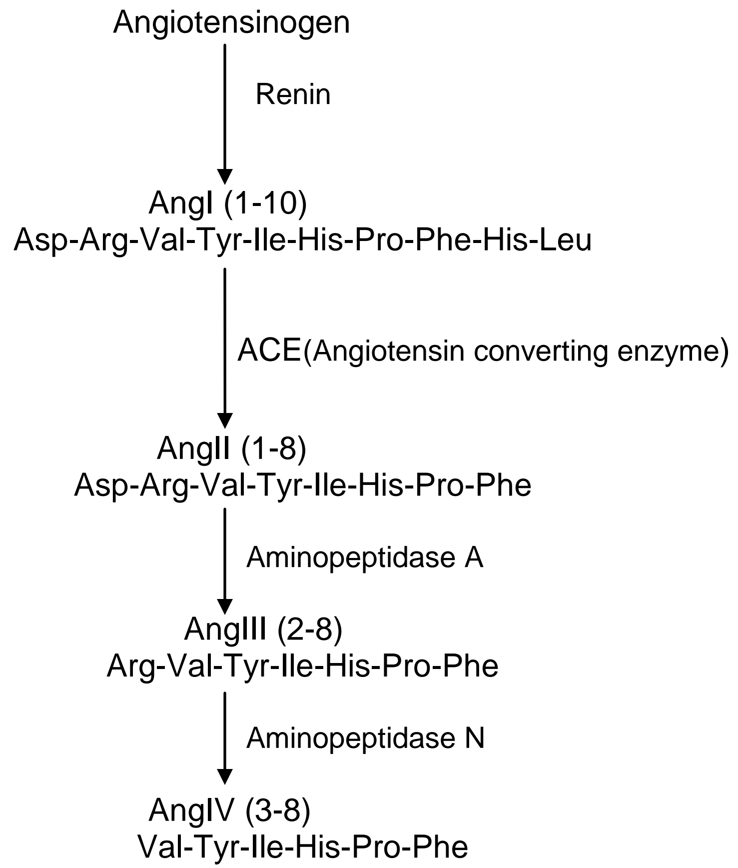

Figure 1.1. Classical RAS.

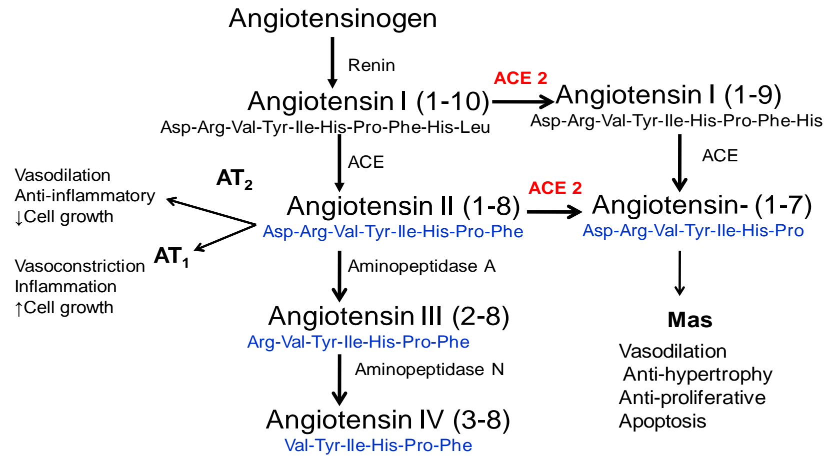

Figure 1.2. Updated RAS, with biological active peptides highlighted in blue.

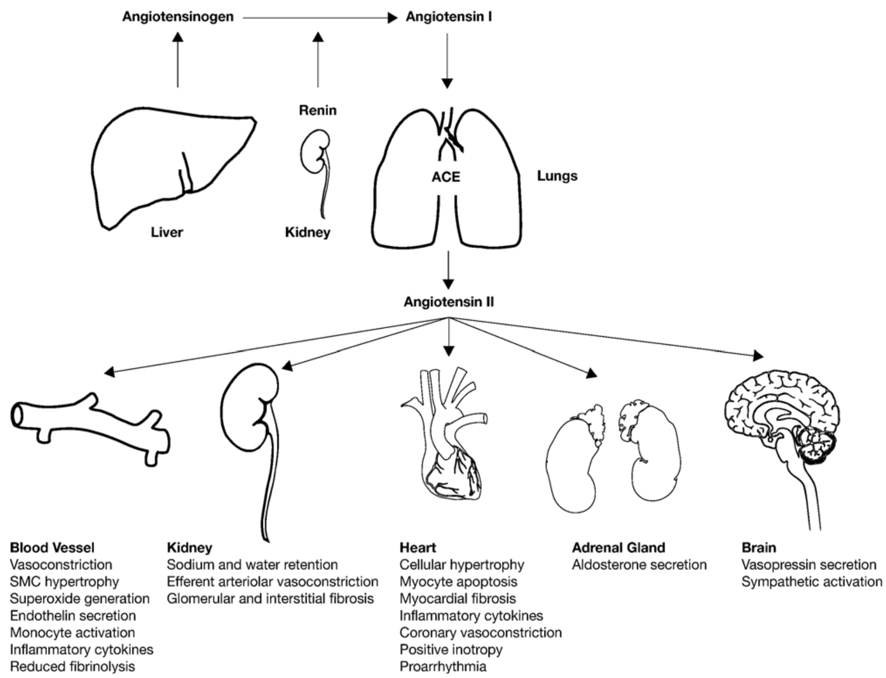

Figure 1.3. Action of AngII on tissues associated with cardiovascular functions.

(Cohn et al. 2006)

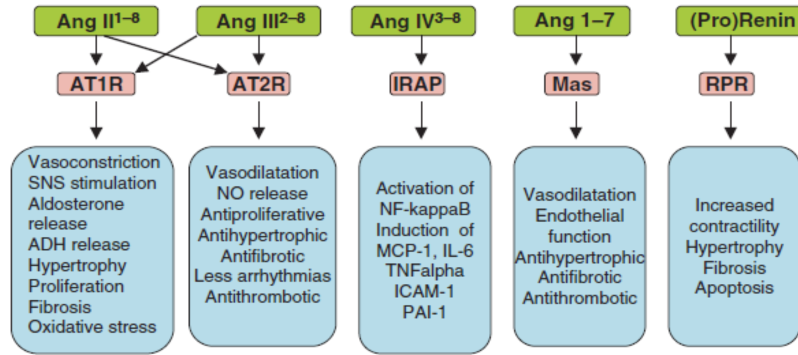

Figure 1.4. Effects of angiotensin peptides and renin/prorenin mediated by their corresponding receptors. (Fyhrquist et al. 2008)

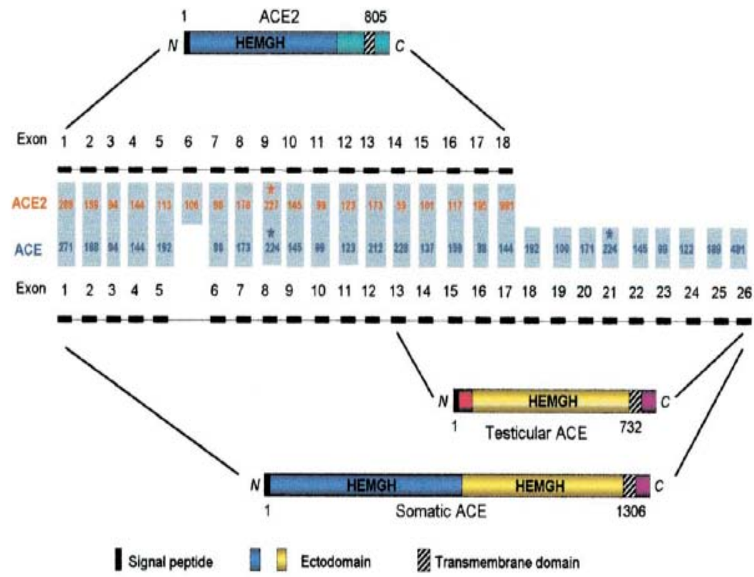

Figure 1.5. Comparison of gene structure of ACE and its homologue ACE2.

(Warner et al. 2004)

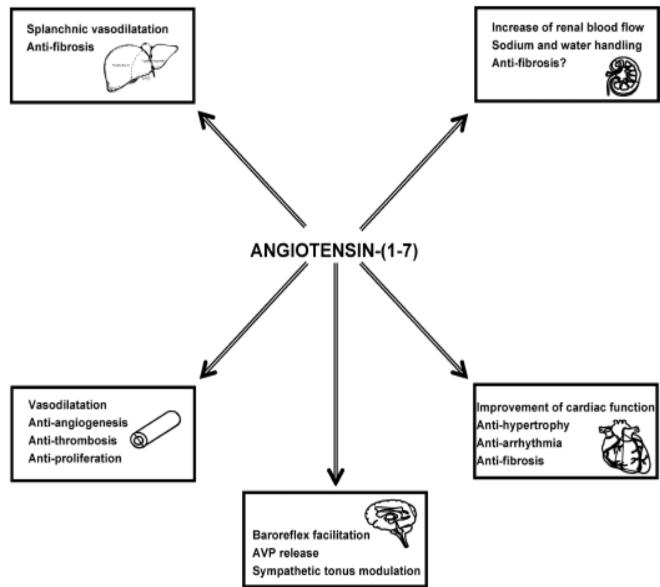

Figure 1.6. Actions of Ang-(1-7) on tissues associated with cardiovascular function. (Santos et al. 2008)

1 A. STATEMENT OF THE PROBLEM

Western countries are experiencing an epidemic of obesity and face increasing rates of related complications such as diabetes mellitus, elevated lipid levels and hypertension. A variety of mechanisms have been proposed to link obesity to the high prevalence of hypertension, including activation of the RAS. However, mechanisms for activation of the RAS in the development of obesity-hypertension are undefined. Moreover, the role of the RAS in obesity-related hypertension in females is undefined.

The mono-carboxypeptidase, ACE2, is a homolog of ACE that cleaves the potent vasoconstrictor AngII as a substrate for the production of the vasodilator peptide, Ang-(1-7). Through these dual mechanisms (e.g., reducing AngII and elevating Ang-(1-7)), ACE2 is capable of blunting activation of the RAS. In experimental models of hypertension involving AngII, ACE2 blunts blood pressure responses to AngII, demonstrating it can protect against AngII-related hypertension. However, the role of ACE2 in the development of obesity-related hypertension has not been previously defined. Moreover, since studies suggest differences in the ACE2/Ang-(1-7) axis between males and females in AngII-induced models of hypertension, it is conceivable that differential regulation of ACE2 between males and females contributes to sex differences in obesity-hypertension.

Adipocytes express several components of the RAS, which have been suggested as a link between obesity and hypertension. Nutritional regulation of

RAS components, including angiotensinogen, has been demonstrated in the setting of diet-induced obesity in a manner consistent with the development of obesity-related hypertension. To date, a few studies have examined nutritional regulation of ACE2 in the context of sodium intake; however, no studies have focused specifically on nutritional regulation of ACE2 in adipose tissue in response to HF feeding. However, fatty acids, enriched in HF diets, are well known regulators of gene expression and adipocyte hypertrophy from obesity has been demonstrated to activate the ACE2 shedding protein, ADAM-17. Nutritional regulation of adipose ACE2 would be predicted to influence adipocyte-derived synthesis and secretion of angiotensinogen and/or AngII, potentially contributing to the development of obesity-hypertension. **Thus, the overall hypothesis of this dissertation is that nutritional regulation of ACE2 in the setting of diet-induced obesity protects males and females against obesity-related hypertension.** We examined the following Specific Aims to test this hypothesis:

Specific Aim 1: Determine effects of HF feeding on adipose ACE2 and the systemic RAS in the development of obesity-related hypertension.

A. Define effects of acute and chronic HF feeding on adipose ACE2 expression (mRNA, protein, activity) in relation to activation of the systemic RAS and the development of hypertension.

B. Define mechanisms for HF-regulation of adipose ACE2, focusing on ADAM-17-mediated shedding from hypertrophied adipocytes, and fatty acid and/or PPARγ regulation of ACE2 mRNA express

Specific Aim 2: Determine effects of ACE2 deficiency on the development of obesity-induced diabetes and hypertension.

A. Define effects of ACE2 deficiency in male C57BL/6 mice on the development of obesity, diabetes and hypertension.

B. Define effects of ovariectomy on the development of obesity-induced diabetes and hypertension in C57BL/6 control and ACE2 deficient female mice.

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Section II. SPECIFIC AIM 1 A

Determine expression and regulation of ACE2 in adipose tissue with diet induced obesity.

2.1. Summary.

Adipose tissue expresses components of the renin-angiotensin system (RAS). Angiotensin Converting Enzyme 2 (ACE2), a recently described component of the RAS, catabolizes the vasoconstrictor peptide angiotensinII (AngII) to form the vasodilator angiotensin-(1-7) (Ang-(1-7)). We examined whether adipocytes express ACE2 and its regulation by high fat feeding. ACE2 mRNA expression increased during differentiation of 3T3-L1 adipocytes. Male C57BL/6 mice were fed low (LF) or high fat (HF) diets for 1 week or 4 months. At 1 week of HF feeding, C57BL/6 male mice fed a HF-diet exhibited a significant increase in body weight and adipose depots. ACE2 activity and protein levels were increased specifically in adipose tissue of 1 week HF-fed mice, and systemic angiotensin peptide concentrations and blood pressure were not altered. These results suggest that initial increases in ACE2 activity in response to short-term HF feeding may protect mice against an activated adipose and systemic RAS.

In contrast, at 4 months of HF feeding ACE2 activity and protein in adipose tissue were reduced compared to LF-fed controls. Increased expression

of ADAM-17, a protease responsible for ACE2 shedding from cell membranes, was evident in adipose tissue from 4 month HF-fed mice, and plasma ACE2 activity increased. In vitro studies using 3T3-L1 adipocytes demonstrated that lipid-laden mature adipocytes exhibited reductions in cell-associated ACE2 activity, and an ADAM-17 inhibitor decreased levels of ACE2 activity released into media from incubated adipocytes. These results suggest enhanced ACE2 shedding from hypertrophied adipocytes with chronic HF feeding. Importantly, despite modest increases in plasma ACE2 activity in 4 month HF-fed mice, obese mice exhibited marked increases in plasma angiotensin peptide concentrations (LF: 2,141 $\pm$ 253; HF: 6,829 $\pm$ 1,075 pg/ml) and elevated blood pressure.

These results demonstrate that adipocytes express ACE2 that is dysregulated in HF-fed mice with and activated systemic RAS and elevated blood pressure compared to LF controls.

2.2. Introduction.

In 2000, a new component of the RAS was described as a monocarboxypeptidase homolog of ACE, identified as ACE2 (Donoghue et al. 2000, Tipnis et al. 2000). ACE2 exhibits catalytic activity for both angiotensinI (AngI) and angiotensinII (AngII); however, its catalytic efficiency for AngII is approximately 400-fold greater than AngI (Vickers et al. 2002). The product of ACE2 cleavage of AngII is Ang-(1-7), a peptide of the RAS which exhibits several effects to decrease blood pressure (Ferrario et al. 2005, Ferreira et al. 2007). The ability of ACE2 to catabolize AngII to Ang-(1-7) has been suggested as the counterbalancing arm of the RAS in blood pressure control, limiting the effects of AngII and promoting effects of Ang-(1-7) (Ferrario et al. 2005). Supporting this hypothesis, recent studies demonstrated that deficiency of ACE2 on a C57BL/6 background results in a modest increase in blood pressure, and a markedly elevated response to acute and chronic AngII (Gurley et al. 2006). Additionally, using recombinant human ACE2 a recent study demonstrated reductions in blood pressure in C57BL/6 and ACE2 deficient mice infused with AngII, indicating an important role of this enzyme in AngII mediated hypertension (Wysocki et al. 2010).However, the role of ACE2 in obesity-induced hypertension is yet unknown. This is particularly important as systemic AngII concentrations are elevated in obesity-induced hypertension (Boustany et al. 2004).

The tissue distribution of ACE2 was originally thought to be restricted to kidney and heart; however, further studies demonstrated relatively wide-spread distribution of ACE2 mRNA and enzymatic activity in rodents (Gembardt et al. 2005). Interestingly, a comprehensive analysis of ACE2 mRNA and protein in tissues from rats and mice demonstrated ACE2 expression in adipose tissue (Gembardt et al. 2005). The physiological relevance of ACE2 expression in kidney, heart and brain has been investigated, but the role of ACE2 in other tissues, including adipose tissue, is not as well understood. Regulatory mechanisms for ACE2 include differential glycosylation, shedding from the cell membrane (Lambert et al. 2005; Tipnis et al. 2000), and tissue-specific regulation through inhibition of AngII synthesis or activity (Ferrario et al. 2005). Recent studies demonstrated that ADAM-17, the metallopeptidase that cleaves TNF-α from cell membranes, can mediate shedding of ACE2 (Lambert et al. 2005). However, the role of ADAM-17-mediated ACE2 shedding in diseases associated with an activated RAS is unknown.

Adipocytes express components of the RAS, including angiotensinogen (Campbell et al. 1987, Cassis et al.1988), renin-like activity (Shenoy et al. 1997), ACE (Saye et al.1993; Schling et al.1999) and angiotensin receptors (type 1 and type 2)(Cassis et al. 1996; Mallow et al. 2000). Interestingly, adipocyte expression of angiotensinogen has been demonstrated to exhibit nutritional regulation by fatty acids and by HFfeeding (Engeli et al. 2005; Gorzelniak et al.

2002; Safonova et al. 1997). Moreover, angiotensinogen expression in adipose tissue, similar to the liver, is positively regulated by AngII (Lu et al. 2007). In addition to components of the RAS, adipocytes express the ACE2 shedding metallopeptidase ADAM-17, which has been suggested to contribute to enhanced release of TNF-α from adipocytes with obesity (Hotamisligil et al. 1995; Hotamisligil et al. 1993). In this study, we investigated whether adipocytes express ACE2, and defined mechanisms for regulation of adipose ACE2 nutritionally by HF feeding.

2.3. Methods.

2.3.1. Animals and diets.

Male, C57BL/6 mice (2 months of age; The Jackson Laboratory, Bar Harbor, MA) were fed a LF (10% kcal as fat; D12450B; Research Diets, Inc, New Brunswick, NJ; n=10 mice per group) or a HF diet (60% kcal as fat; D12492, Research Diets, Inc, New Brunswick, NJ; n=10 mice per group) for 1 week or 4 months. Diets were matched in protein content (20% kcal) and provided energy at 3.85 or 5.25 kcal/gm (LF and HF, respectively) (Table. 2.1). Diets were provided to mice *ad libitum*, and body weight recorded weekly. All experiments involving mice conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kentucky Institutional Animal Care and Use Committee.

2.3.2. Measurement of blood pressure.

Systolic blood pressure was measured by tail cuff using the Visitech 2000 system for mice fed the LF or HF diet for 1 week. Measurements were obtained 4 days/week beginning 1 week prior to initiation of diet and through day 7 (Henriques et al. 2004). Criteria for inclusion of measurements from individual mice were 5 out of 10 successful measurements with a standard deviation < 50. For mice fed the LF or HF diets for 4 months, radiotelemetry was used to measure blood pressure. At month 4, mice were anesthetized (isoflurane), the

left carotid artery was isolated, and the telemeter catheter was inserted into the artery and advanced to reach the aortic arch. The telemetry implants (model TA11PA-C10, Data Sciences International, St. Paul, MN) were placed in a subcutaneous pocket on the right flank. Mice were allowed to recover for 1 week before recordings began (3 consecutive days at 24 hours/day). The telemeter signal was processed using a model RPC-1 receiver, a 20 channel data-exchange matrix, APR-1 ambient pressure monitor, and Dataquest ART 2.3 acquisition system (Data Sciences International). The system was programmed to acquire data for 10 seconds every minute and to calculate 10-min averages of the mean, systolic and diastolic blood pressure.

2.3.3. 3T3-L1 adipocytes.

3T3-L1 cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Cells were grown to confluence, and then differentiation was induced with a cocktail of insulin (0.1 μM, Sigma, St. Louis, MO), dexamethasone (1 μM; Sigma, St. Louis, MO) and isobutyl methyl xanthine (0.5 mM; Sigma, St. Louis). Cells were collected for mRNA analysis every day beginning 1 day prior to addition of cocktail through day 10. In separate experiments (n = 3), 3T3-L1 adipocytes (day 10) were incubated with vehicle or GM6001 (10 μM) for 1 hour prior to measurement of ACE2 activity in media

2.3.4. mRNA quantification.

Total RNA was extracted from cells (3T3-L1) and tissues (epididymal fat, EF; subcutaneous fat, SubQ; retroperitoneal fat, RPF) using Trizol reagent (Invitrogen, CA). RNA (0.4 μg) was reverse transcribed for 1 hr at 55°C with the following components: random decamers, 10X reverse transcription buffer, deoxynucleotide triphosphate mix, ribonuclease inhibitor and reverse transcriptase (RETROscript; Ambion, TX). Relative quantification of gene expression was performed with an iCycler (BioRad, CA) using the SYBR Green PCR core reagent (Applied Biosystems, CA). The reaction mix consisted of: SYBR Green mix (1X), $MgCl_2$ (3 mM), dNTP mix (1.25 mM), fluorescein (0.01 μM), primers (0.5 μM) and AmpliTaq gold (2.5 units). The real-time PCR conditions were: 5 min at 94°C, 40 cycles for 1 min at 94°C, 1 min at the annealing temperature, 1 min at 72°C, and a final elongation step for 10 min at 72°C. 18S rRNA was used as the endogenous control for normalization. The abundance of each mRNA transcript was measured using a standard curve method. Briefly, cDNA (10^{-7} to 10^{-3} starting concentration, total of 5 concentrations) from a tissue source known to express each gene of interest was amplified with unknowns. Software on MyiQ Single-Color Real Time PCR Detection System (Bio-Rad, CA) plotted the Ct value for each DNA standard against the starting quantity (SQ) of cDNA (R^2 = 0.98 - 1.00), and extrapolated unknowns from the standard curve. cDNA template (SQ) for each gene was normalized to 18S RNA (SQ obtained using the standard curve method) to

control for starting amount of DNA, and data are expressed as the ratio of gene/18S RNA.

2.3.5. Measurement of ACE2 enzymatic activity.

ACE2 activity was quantified in 3T3-L1 adipocytes, tissues and plasma by determining the conversion of [^{125}I]AngII to [^{125}I]Ang-(1-7) (Ferrario et al. 2005a). Tissues were homogenized in a Tris buffer (100 mM) containing NaCl (0.3 M), $ZnCl_2$ (10 µM), and Z pro-prolinal (10 µM). Following centrifugation (30,000g for 20 min, 4°C), pellets were reconstituted in the above buffer containing 0.5% triton-X and incubated overnight at 4°C. Samples were centrifuged, and the supernatant used for measurement of ACE2 enzyme activity. Membrane (0.05 - 0.2 mg protein) was added to tubes with buffer (Tris, 100 mM; total volume 250 µl) containing the following inhibitors: thiorphan (0.1 mM), phoshoramidon (0.1 mM), bestatin (100 µM), pepstatin (100 µM), and captopril (10 µM). Initial experiments optimized the ACE2 activity assay for membrane protein, inhibitor cocktail, substrate concentration and validated loss of ACE2 activity in adipose membranes from *Ace2*$^{-/y}$ mice (Fig.2.7 B, 2.7 C, 2.8). [^{125}I]AngII (specific activity 2,200 Ci/mmol; 2 x 10^6 cpm equivalent to 414 fmoles) was incubated with samples for 30 minutes, and the reaction stopped by the addition of 1% phosphoric acid. Samples were centrifuged, filtered, and injected onto a Beckman HPLC system for resolution of [^{125}I[AngII from [^{125}I]Ang-(1-7). Reverse phase HPLC was used to resolve angiotensins with a linear gradient varying from 15% to 33% acetonitrile (0.5 ml/min). The mobile phase was 25 mM

$NaPO_4$, with a retention time of 6.6 min for [^{125}I]Ang-(1-7) and 13.6 min for [^{125}I]AngII (Fig. 2.7 A). HPLC fractions (1 min) were collected and radioactivity quantified by gamma counting. ACE2 activity is expressed as fmol/mg protein/min, based on the specific activity of [^{125}I]AngII (2175 Ci/mmol). Protein was measured using the BCA Assay (Pierce Chemicals, Rockford, IL).

2.3.6. Measurement of ACE2 protein.

Tissues were homogenized on ice in M-PER reagent (Pierce, Rockford, IL), sonicated (2 min), and lysates were pelleted by centrifugation. Protein (25 μg) from adipose tissue (EF) was electrophoresed on a 7.5% SDS-PAGE gel under reducing conditions. Proteins were blotted onto PVDF membrane (GE Healthcare, Piscataway, NJ), blocked overnight (5% nonfat milk, 4°C) and incubated with anti-goat ACE2 antibody (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour (22°C). Goat IgG conjugated horseradish peroxidase antibody (1:5000 dilution, Santa Cruz) was used for chemiluminescent detection. Blots were stripped and re-probed with anti-mouse β-actin antibody (1:1000 dilution, clone AC-15, Sigma, St. Louis, MO) for normalization of protein loading. Controls included incubation with a blocking peptide for ACE2 (1:1 peptide/primary antibody; SC-21834, Santa Cruz, CA), which eliminated all immunoreactivity from ACE2 primary antibody. Images were collected on a Kodak Image Station 440CF and analyzed using Kodak 1D analysis system software (Version 3.6.4, New Haven, CT).

2.3.7. Measurement of angiotensin peptides.

Angiotensin peptides were measured in mouse plasma (150 µl) using reverse phase HPLC followed by radioimmunoassay as previously described (Daugherty et al. 2004).

2.3.8. Statistical analysis.

Data are expressed as mean $\pm$ SEM. Data were tested for normality and equal variance. For *in vitro* studies examining mRNA expression during adipocyte differentiation, data were analyzed by 1-way ANOVA. For *in vivo* studies, data were analyzed by 2-way ANOVA, with time of diet feeding and diet as between group factors. For post-hoc analysis, data were analyzed using Tukey's test, with significance at $P < 0.05$.

2.4. Results.

2.4.1. Expression of ACE2 in 3T3-L1 Murine Adipocytes and in Mouse Adipose Tissue.

ACE2 mRNA expression in differentiating murine 3T3-L1 adipocytes was contrasted to other components of the RAS and to PPARγ as an index of adipocyte differentiation (Fig. 2.1). PPARγ mRNA abundance increased beginning on day 3 compared to undifferentiated (Undiff) preadipocytes (Fig.2.1 A, $P < 0.05$). ACE2 mRNA expression increased on day 5 (Fig. 2.1 B, $P < 0.05$). Similarly, expression of Mas and AT1a receptors also increased during the course of adipocyte differentiation (Fig. 2.1 C, D; $P < 0.05$).

2.4.2. The Temporal Effect of HF Feeding on Adipose ACE2 Expression and Enzymatic activity.

We determined whether ACE2 was nutritionally regulated by short-term and chronic HF feeding. Body weight and EF mass were modestly increased in HF mice at 1 week (Table 2.2, $P < 0.05$ compared to LF). Blood glucose concentrations were increased at 1 week of HF-feeding (Table 2.2, $P < 0.05$ compared to LF). At 1 week of HF feeding, expression of angiotensinogen was increased in adipose tissue, but not liver, of HF-fed mice (Table 2.2). ACE2 activity (EF, Fig. 2.2 A, SubQ, Fig. 2.2 B) and protein (EF, Fig. 2.3 A) were increased in adipose tissue of HF compared to LF-fed mice. Plasma ACE2 activity was modestly, but not significantly, increased by 1 week of HF feeding (LF, 0.004 ± 0.001; HF, 0.009 ± 0.001 fmol/ml/min; $P > 0.05$). Moreover, plasma

concentrations of angiotensin peptides (Fig. 2. 4 A) and systolic blood pressure (SBP: LF, 120 $\pm$ 5; HF, 120 $\pm$ 4 mmHg; $P > 0.05$) were not altered by 1 week of HF feeding.

At 4 months of HF feeding, body weight and adipose mass were markedly increased compared to LF (Table 2.2, $P < 0.05$). Moreover, blood glucose concentrations were increased in HF-fed mice (Table 2.2, $P < 0.05$ compared to LF). Expression of angiotensinogen in adipose tissue increased with age in both LF and HF-fed mice (Table 2.2, $P < 0.05$, 1 week compared to 4 months within diet group). Moreover, liver and adipose angiotensinogen mRNA expression was increased in 4 month HF compared to LF mice (Table 2.2, $P < 0.05$). Surprisingly, with 4 months of chronic high fat feeding ACE2 activity (Fig. 2.2 A, B) and protein (Fig. 2.3 B) were reduced in adipose tissue of HF-fed mice. Since ADAM-17 has been demonstrated to shed ACE2 from cell membranes (Lambert et al. 2005), we measured ADAM-17 mRNA expression (Fig. 2.5 A) and ACE2 enzymatic activity in 3T3-L1 adipocytes at day 8 and 10 of differentiation (Fig. 2.5 B). These time points were chosen to represent early-stage (8 days) and lipid-laden mature adipocytes (10 days). ADAM-17 mRNA expression increased over this time course (Fig. 2.5 A) and was associated with reductions in membrane ACE2 activity (Fig. 2.5 B). ACE2 activity was also detected in the media of 3T3-L1 adipocytes, and levels were reduced by an ADAM-17 inhibitor (Fig. 2.5 C). Adipose tissue from 4 month HF-fed mice exhibited an increase in ADAM-17 mRNA expression (Fig. 2.5 D; $P < 0.05$), and plasma ACE2 activity increased (LF, 1.8 $\pm$ 0.3; HF, 3.1 $\pm$ 0.1 fmol/mg/min, $P < 0.05$). Despite modest

increases in plasma ACE2 activity, plasma concentrations of AngII, AngIV and AngI were greater in HF compared to LF mice (Fig. 2.4 B). Moreover, systolic and diastolic pressures were significantly increased in both the light and dark cycle of 4 month HF-fed compared to LF mice (Fig. 2.6 A, B, $P < 0.05$), contributing to an elevation in mean arterial pressure.

2.5. Discussion.

This study examined whether adipocytes express ACE2 and its regulation by HF feeding. Results demonstrate expression of ACE2 mRNA, protein and enzymatic activity in 3T3-L1 adipocytes and in mouse adipose tissue, and regulation of adipose ACE2 during HF feeding. With short-term HF-feeding of C57BL/6 mice, ACE2 protein expression and enzymatic activity were stimulated in adipose tissue, and blood pressure was not altered. With chronic HF feeding, ACE2 protein and enzymatic activity in adipose tissue was reduced. Expression of ADAM-17, a protease which can shed ACE2 from membranes, increased with differentiation of 3T3-L1 adipocytes and in adipose tissues from chronic HF-fed mice. With chronic HF feeding, expression of angiotensinogen increased in both adipose tissue and liver, plasma concentrations of angiotensin peptides were markedly elevated and HF-fed mice exhibited higher blood pressure compared to LF-fed controls. These results demonstrate that adipocytes express ACE2, and that ACE2 is nutritionally regulated by HF feeding.

The monocarboxypeptidase ACE2 was identified by 5' sequencing of a human heart ventricle or lymphoma cDNA library (Donoghue et al. 2000; Tipnis et al. 2000). Expression of ACE2 was originally suggested to be restricted to human heart, kidney and testis (Donoghue et al. 2000); however, recent studies have demonstrated more widespread distribution of ACE2 (Gembardt et al. 2005). In a comparison of tissues from mice and rats, Gembardt et al.

(Gembardt et al. 2005) reported ACE2 expression in adipose tissue, and recent studies extend ACE2 expression to both brown and white adipose tissue from rats (Galvez-Prieto et al. 2008). Similarly, ACE2 mRNA was detected in human adipose tissue, with greater ACE2 expression in visceral compared to subcutaneous adipose tissue (Li et al. 2007; Zhang et al. 2006). However, the relative expression of ACE2 in adipocyte versus non-adipocyte fractions of adipose tissue has not been defined. 3T3-L1 cells are a fibroblast-like cell line from the Swiss mouse embryo which differentiates to mature white adipocytes upon exposure to a differentiating cocktail (Green et al. 1975). This system has been used previously to define adipocyte expression of RAS components, including angiotensinogen (Saye et al. 1989) and AT1 receptors (Mallow et al. 2000). Our results confirm previous findings of AT1 receptor expression in differentiating 3T3-L1 adipocytes (Mallow et al. 2000), and extend these findings by demonstrating that mRNA expression of ACE2 and the Mas receptor increases upon differentiation of preadipocyte to adipocyte (Hotamisligil et al. 1995; Hotamisligil et al. 1993). The functional relevance of Mas receptor expression on adipocytes was not defined in the current study. Interestingly, recent studies demonstrate that Mas receptor deficient mice exhibit a phenotype characteristic of the metabolic syndrome, with increased abdominal adipose mass, dyslipidemia, hyper-insulinemia and leptinemia, and glucose intolerance (Santos et al. 2008a). Moreover, in agreement with our findings in 3T3-L1 adipocytes, Mas receptor expression was detected in mouse adipose tissue (Santos et al. 2008a). Additionally, recent studies demonstrate improvements in

lipid and glucose homeostasis such as improved glucose tolerance, increased insulin stimulated glucose uptake in adipocytes, improved insulin sensitivity, lower plasma cholesterol and triglycerides in transgenic rats expressing Ang-(1-7) producing fusion protein (Santos et al. 2010). A number of studies recently in Mas deficient mice demonstrate a role of this peptide in blood pressure homeostasis. However, the results are varying with no difference (Walther et al. 2000) or increased blood pressure (de Moura et al. 2010) with Ang-(1-7). Altered expression of RAS components in adipose tissue has been linked to obesity-related hypertension in experimental models and humans (Boustany et al. 2004; Cassis et al. 1996; Engeli et al. 2005; Giacchetti et al. 2000; Massiera et al. 2001a; Rahmouni et al. 2004; Van Harmelen et al. 2000). In rats with diet-induced obesity and hypertension, expression of angiotensinogen in visceral adipose tissue increased, and was associated with elevated concentrations of angiotensin peptides (Boustany et al. 2004). Administration of an AT1 receptor antagonist to rats with obesity-hypertension decreased blood pressure (Boustany et al. 2005). Similarly, in mice over-expressing human angiotensinogen under the control of its own promoter, a HF diet resulted in an increase in angiotensinogen expression in visceral adipose tissue (Rahmouni et al. 2004). However, blood pressure was not examined. In human obesity-hypertension, plasma concentrations of angiotensinogen, renin, aldosterone and ACE were increased compared to lean controls, and weight loss resulted in reductions in these RAS components (Engeli et al. 2005). Our results extend previous findings by demonstrating that while short term HF feeding does not activate the systemic

RAS, chronic HF-feeding is associated with a stimulated RAS (angiotensinogen and AngII) at a time when blood pressure is increased.

Since other RAS components in adipocytes exhibit nutritional regulation (Bertile et al. 2004; Einstein et al. 2005; Frederich et al. 1992; Jones et al. 1997; Le Lay et al. 2001; Safonova et al. 1997), we examined effects of HF feeding on adipose ACE2 expression. Results from this study are the first to demonstrate that short term HF feeding increases ACE2 expression and enzymatic activity in adipose tissue. A recent study in rats also demonstrated increased expression of ACE2 and Ang-(1-7) levels in adipose tissue of animals administered a high sucrose diet (Coelho et al. 2010), supporting nutritional regulation of this enzyme in adipose tissue.

The human ACE2 protein is a type I integral membrane glycoprotein (Donoghue et al. 2000; Tipnis et al. 2000), but the enzyme can be shed from the cell surface through proteolytic cleavage of its extracellular domain by tumor necrosis factor-α convertase (ADAM-17) (Lambert et al. 2005). Mechanisms for regulation of ACE2 at the mRNA or protein level have not been well described; however, differential glycosylation of the enzyme and shedding from the cell surface have been suggested to regulate cellular ACE2 activity (Lambert et al. 2005; Tipnis et al. 2000). Shedding of ACE2 may impact the RAS, as well as the infectivity of the SARS coronavirus since ACE2 serves as the virus receptor (Li et al. 2003). Moreover, phorbol ester-induced shedding of ACE2 in HEK293 cells overexpressing the enzyme was suggested to result in the release of a soluble

form of catalytically active enzyme (Lambert et al. 2005). Our results demonstrate that ACE2 activity can be detected in media from adipocytes and activity manipulated by an ADAM-17 inhibitor. These results suggest that elevated ADAM-17 mRNA expression in adipose from chronic HF-fed mice may contribute to shedding of ACE2 from adipocyte membranes. These findings are in agreement with previous results demonstrating that adipocyte hypertrophy with obesity results in elevated expression and release of another adipocytokine shed by ADAM-17, TNF-α (Hotamisligil et al. 1995; Hotamisligil et al. 1993). Further studies are required to determine if ADAM-17 mediates enhanced shedding of ACE2 from adipocytes with chronic HF feeding, and the implication of these findings on the systemic RAS.

In conclusion results from this study suggest that ACE2 protects male mice against obesity-induced hypertension. However, with chronic high fat feeding this effect is overwhelmed with loss of ACE2 activity resulting in an activated systemic RAS and obesity-induced hypertension.

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Table 2.1. Composition of diets used in the study.

	D12450B (10%)	**D12492 (60%)**
Fat	10	60
Protein	20	20
Carbohydrates	70	20
Total (kcal/g)	3.85	5.2
Fat Sources	**Total %**	**Total %**
Lard	45	91
Soyabean oil	55	9
Total fat (g)/ kg	45	270
Kcal from fat /kg diet	405	2430

Table 2.2. Characteristics of LF and HF-fed C57BL/6 mice.

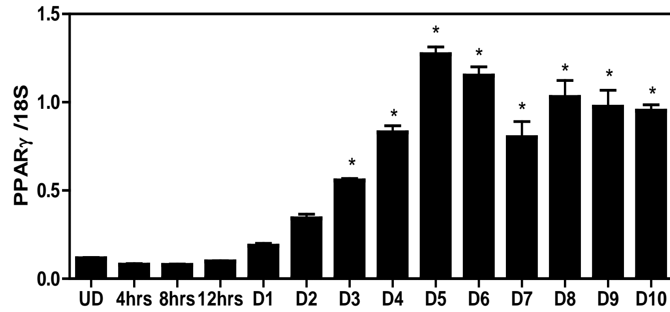
	LF		HF	
	1 week	4 months	1 week	4 months
Body weight (g)	25.2 ± 0.9	29.9 ± 0.3	27.8 ±0.5*	48.0 ± 1.6*
Blood Glucose (mg/dl)	130 ± 8	132 ± 5	155 ± 8*	161 ± 7*
EF (gm)	0.43 ± 0.03	0.62 ± 0.1	0.74 ± 0.06*	2.19 ± 0.15*
Liver angiotensinogen mRNA	0.85 ± 0.33	0.84 ± 0.09	0.90 ± 0.24	1.62 ± 0.14*,**
EF angiotensinogen mRNA	0.11 ± 0.01	0.25 ± 0.01	0.42 ± 0.08*	0.58 ± 0.01*,**

EF, epididymal fat.

*, P < 0.05 compared to LF within time point.

**, P < 0.05 compared to 1 week within diet group

A)



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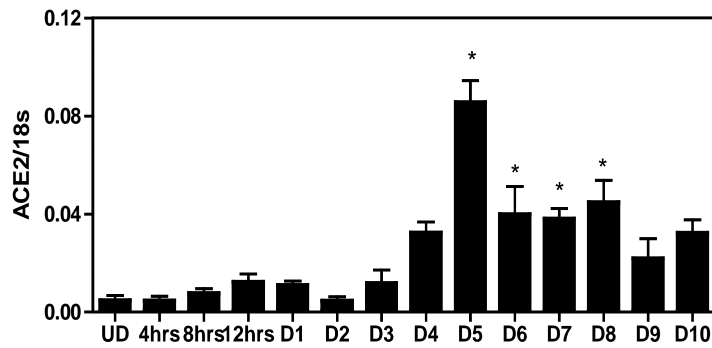


Fig. 2.1. A**.** Relative expression of PPARγ in differentiating 3T3-L1 adipocytes, B. Relative expression of ACE2 in differentiating 3T3-L1 adipocytes (n=4 for each time point). *, significantly different from UD (Undiff), $P < 0.05$.

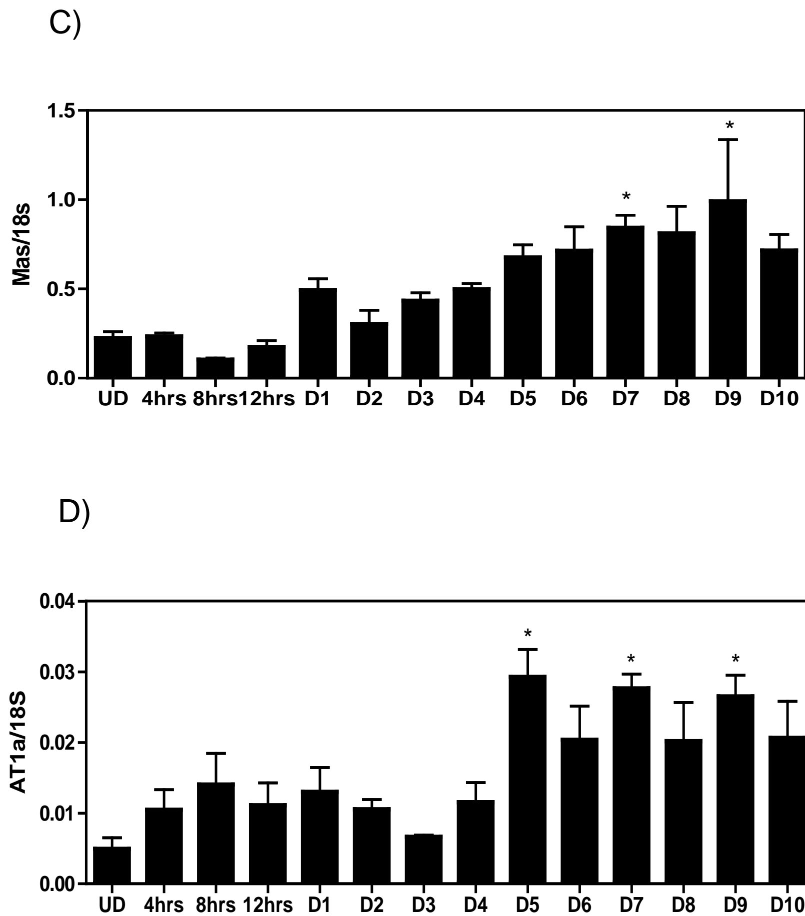
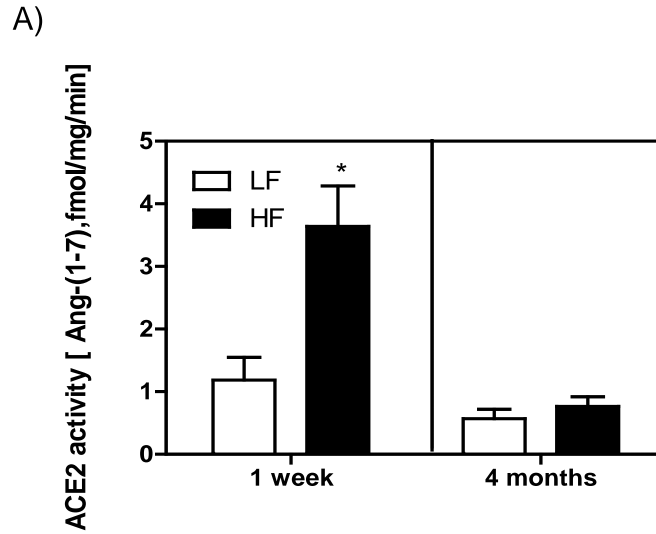


Fig. 2.1. C. Relative expression of Mas in differentiating 3T3-L1 adipocytes, D. Relative expression of AT1a receptor in differentiating 3T3-L1 adipocytes (n=4 for each time point). *, significantly different from UD (Undiff), $P < 0.05$.

A)



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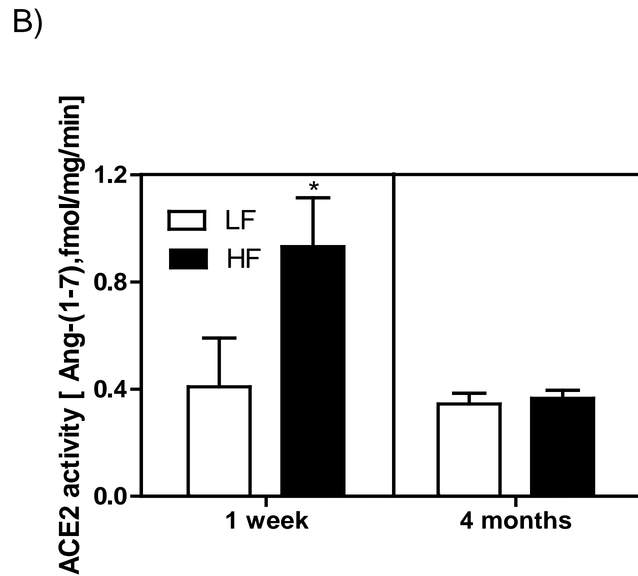
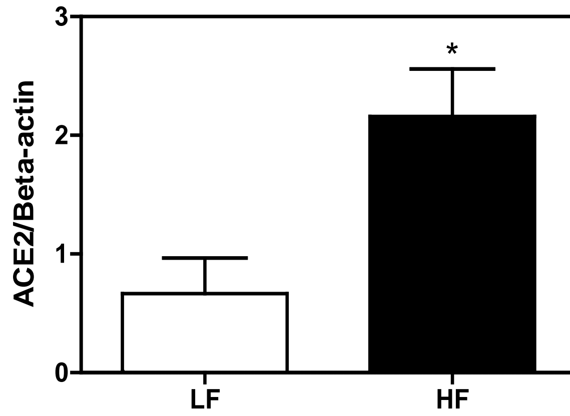


Fig.2.2. A. The effect of 1week and 4 months of HF feeding on ACE2 activity in epididymal adipose tissue, B. The effect of 1week and 4 months of HF feeding on ACE2 activity in subcutaneous adipose tissue. Data are mean $\pm$ SEM from n = 5 mice/time point/diet. *, significantly different from LF.

A)



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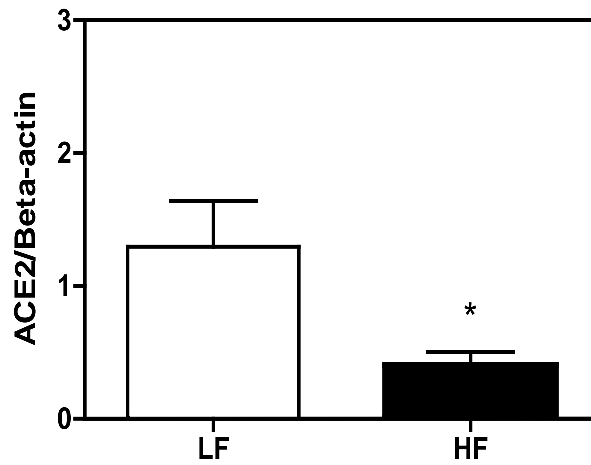
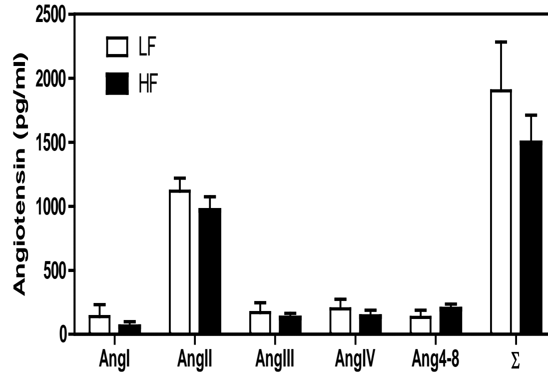


Fig.2.3. A. ACE2 protein in epididymal adipose tissues at 1 week of LF and HF feedin, B. ACE2 protein in epididymal adipose tissues at 4 months of LF and HF feeding. Data are mean $\pm$ SEM from n = 5 mice/time point/diet. *, significantly different from LF, $P < 0.05$.

A)



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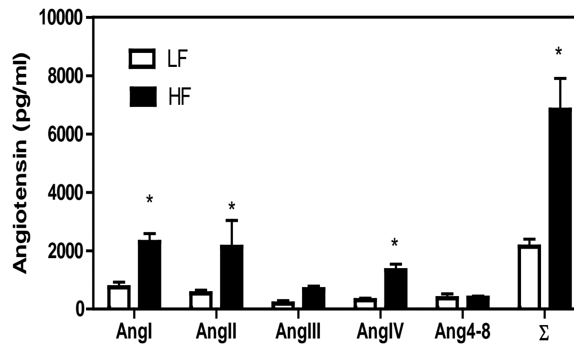
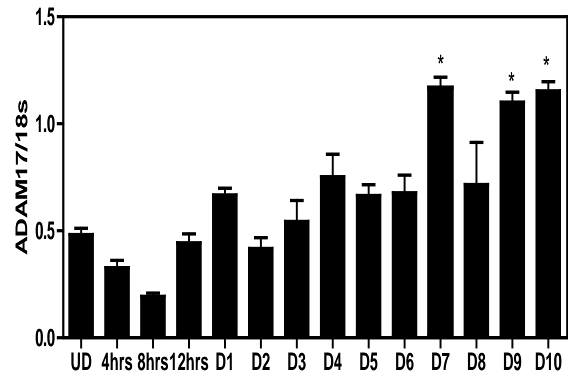


Fig. 2.4. A.Plasma angiotensin peptide concentrations at 1 week of LF and HF-feeding, B. Plasma angiotensin peptide concentrations at 4 months of LF and HF-feeding. Data are mean ± SEM from n = 10 mice/time point/diet. *, significantly different from LF, $P < 0.05$.

A)



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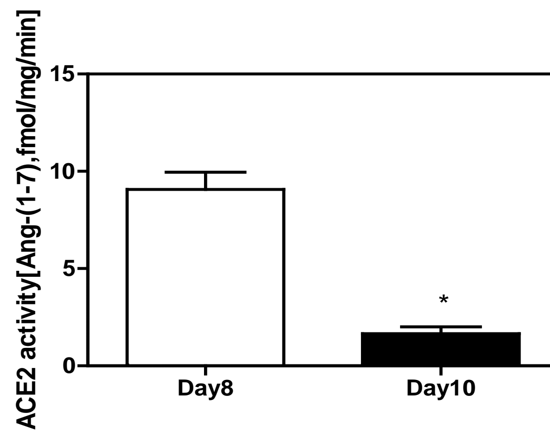
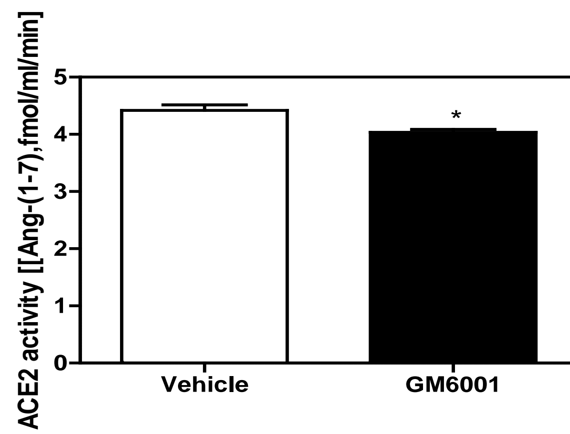


C)



D)

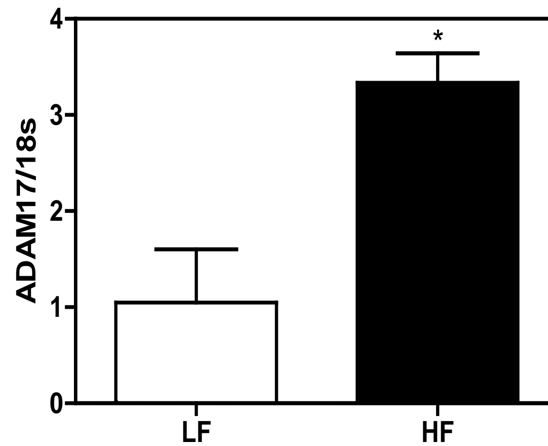
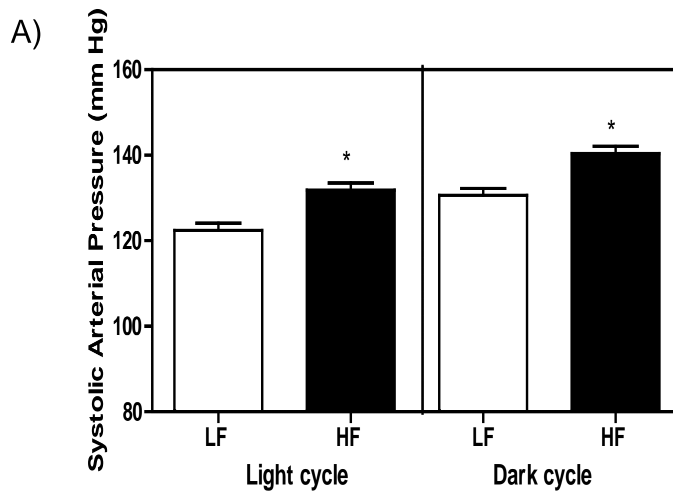


Fig. 2.5. A. ADAM-17 mRNA expression in differentiating 3T3-L1 adipocytes during the course of differentiation, n=4 for each time point. *,significantly different from Undiff, P < 0.05, B. ACE2 activity decreased from day 8 to 10 of differentiation, C. Effect of ADAM-17 inhibition on ACE2 activity, (n = 3), D. ADAM-17 mRNA expression in adipose tissue from 4 month HF-fed mice. Data are mean ± SEM from n = 5 mice/group. *, significantly different from LF, P < 0.05.

A)



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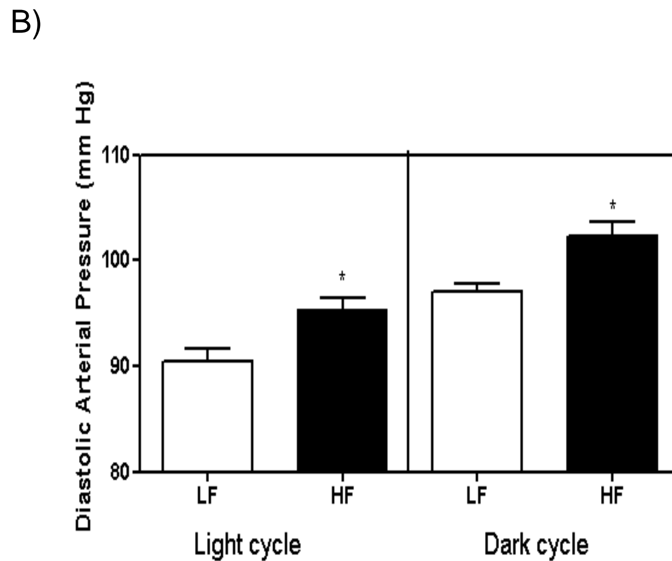
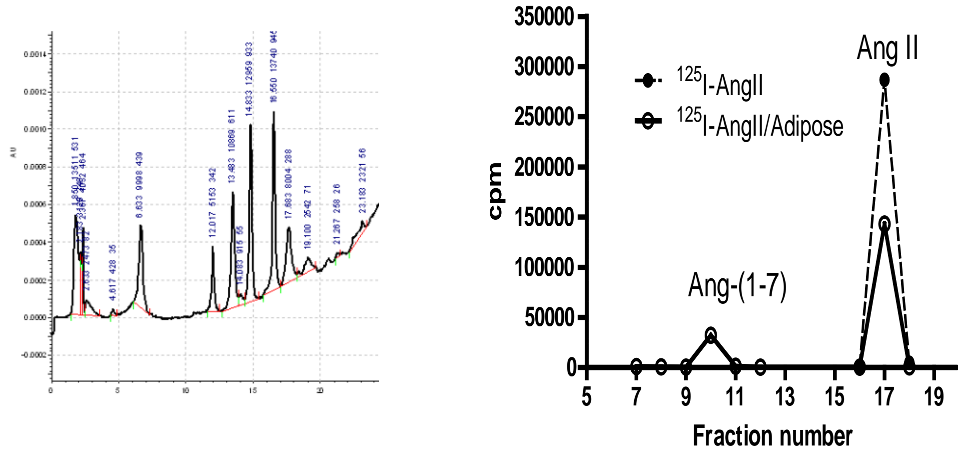


Fig. 2.6. A. Systolic blood pressure of 4 month LF and HF-fed mice, B. Diastolic blood pressure of 4 month LF and HF-fed mice. Blood pressure was measured using radiotelemetry during the light and dark cycle in the final week of month 4. Systolic (SBP) and Diastolic (DBP) blood pressure increased in 4 month HF compared to LF mice. Data are mean ± SEM from n = 5 mice/group. *, significantly different from LF, $P < 0.05$.

A)



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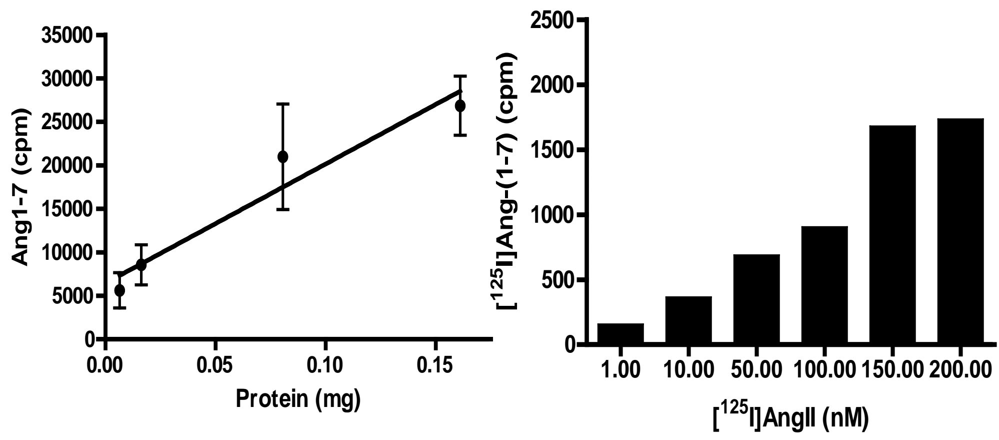

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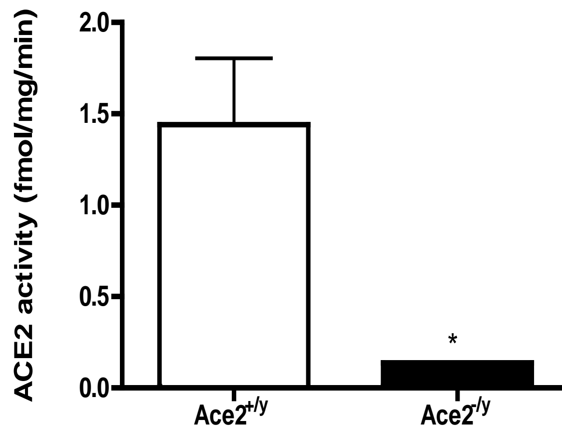

Fig. 2.7. A. Left, HPLC chromatogram for resolution of angiotensin 1-7 (Ang-(1-7); retention time = 6.6 min) and angiotensinII (AngII; retention time = 13.6 min). Right, Effect of protease inhibitors on Ang-(1-7) generation. When [^{125}I]AngII is injected onto the HPLC in the absence of adipose membrane ([^{125}I]AngII), only one peak of radioactivity is demonstrated in HPLC fractions. In the presence of adipose membrane ([^{125}I]AngII/adipose), radioactivity is detected in the Ang-(1-7) fraction, but not in other HPLC fractions. Radioactivity appears in HPLC fractions with a 3 minute lag time from the retention time of individual peptides. B. Left, ACE2 activity increases as adipose membrane protein increases. A membrane protein concentration of 0.05 mg was chosen for studies. Right, Dependence of [^{125}I]Ang-(1-7) product formation from varying concentrations of [^{125}I]AngII substrate. Measurements were performed at a saturating concentration of [^{125}I]AngII substrate (200 nM). Moreover, under these experimental conditions, less than 5% of [^{125}I]AngII substrate was hydrolyzed to [^{125}I]Ang-(1-7), to assure that substrate concentrations were not limiting. C. ACE2 activity is markedly decreased in EF membranes from *Ace2*$^{-/y}$ mice compared to control (C57BL/6).

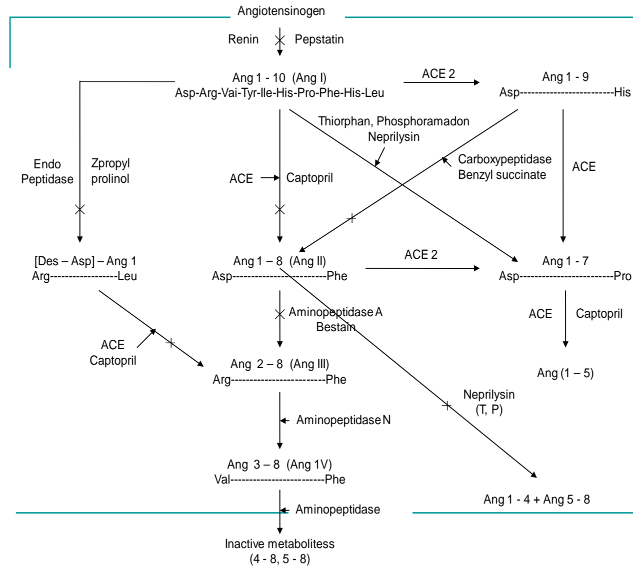

Fig. 2.8. Inhibitors used in ACE2 activity assay.

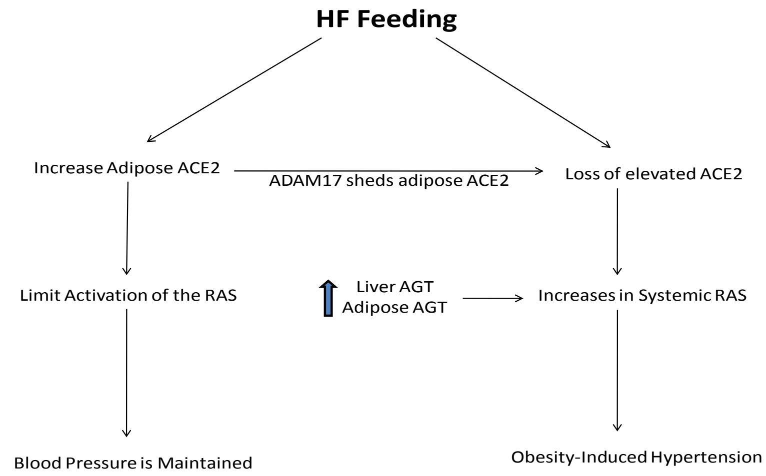

Fig. 2.9. Proposed model for Aim 1.

Section III. SPECIFIC AIM 1 B

Define mechanisms for regulation of adipose ACE2, focusing on fatty acids and peroxisome proliferator activated receptor gamma (PPARγ) ligands (thiazolidinediones; TZDs).

3.1. Summary.

Administration of a high fat (HF) diet to C57BL/6 (*Ace2*$^{+/y}$) male mice increased adipose ACE2 mRNA expression, suggesting regulation of ACE2 expression in adipose tissue by dietary lipids. In addition, expression of PPARγ preceded expression of ACE2 in differentiating 3T3-L1 adipocytes. Specific fatty acids serve as ligands of PPARγ. Therefore, we sought to determine if natural (fatty acids) or synthetic ligands (TZDs) of PPARγ regulate ACE2 expression and activity in 3T3-L1 mature adipocytes. Additionally, since TZDs have been demonstrated to reduce blood pressure and improve insulin sensitivity, we focused on *in vivo* regulation of ACE2 in adipose tissue by TZDs as a potential mechanism for the blood pressure lowering and insulin sensitizing effects of these compounds.

Fatty acids of the n-3 family, specifically docosahexaenoic acid (DHA), increased ACE2 mRNA expression in differentiated 3T3-L1 adipocytes. In contrast, fatty acids of the n-6 family had no effect on adipocyte ACE2 expression or activity. Since n-3 fatty acids are ligand activators of PPARγ, we defined effects of TZDs on ACE2 expression *in vitro* and *in vivo*. Synthetic

PPARγ ligands, including rosiglitazone (Rosi) and pioglitazone (Pio), increased ACE2 mRNA, activity and protein in a concentration-dependent manner in differentiated 3T3-L1 adipocytes. To determine if PPARγ interacts with PPREs on the ACE2 promoter, we employed ChIP assays using differentiated 3T3-L1 cells incubated with vehicle or Rosi. Chromatin immunoprecipitation with a PPARγ antibody followed by PCR amplification using primers designed against a putative PPRE site within the ACE2 promoter revealed binding to DNA from adipocytes incubated with Rosi.

To determine if PPARγ activation regulated ACE2 *in vivo*, eight week old *Ace2*$^{+/y}$ male mice were administered vehicle or Rosi (p.o.) at a daily dose of 25 mg/kg body weight for 21 days. ACE2 expression and activity were increased in adipose tissue of mice administered Rosi. In contrast, Rosi had no effect on ACE2 expression in kidney and heart, two prominent tissue sources of ACE2. Plasma concentrations of angiotensin-(1-7) (Ang-(1-7)), a catabolic product of ACE2, were increased in mice administered Rosi. Administration of Rosi significantly improved glucose tolerance and reduced systolic blood pressures (SBP) in *Ace2*$^{+/y}$ mice compared to vehicle-treated controls. Improvements in glucose tolerance in Rosi-administered mice were accompanied by reductions in plasma insulin concentrations.

We then sought to determine if the reductions in blood pressure and improved glucose tolerance observed in *Ace2*$^{+/y}$ mice administered Rosi were mediated via ACE2. Administration of Rosi to *Ace2*$^{-/y}$ mice reduced SBP,

indicating that Rosi-induced reductions in blood pressure were not mediated via ACE2. In contrast, glucose tolerance was reduced, rather than improved, by Rosi administration in *Ace2*$^{-/y}$ mice. Moreover, administration of Rosi had no effect on plasma insulin concentrations in *Ace2*$^{-/y}$ mice.

Results from this study indicate that PPARγ ligands stimulate ACE2 mRNA expression, activity and protein *in vitro* and *in vivo*. Blood pressure lowering effects of TZDs were not ACE2 mediated. In contrast, improvements in glucose tolerance by Rosi administration were lost in *Ace2*$^{-/y}$ mice, and associated with suppressed plasma insulin concentrations.

3.2. Introduction.

Previous studies in our laboratory demonstrated that ACE2 mRNA expression in adipose tissue is transiently increased in response to consumption of a high fat (HF) diet (Gupte et al. 2008), suggesting nutritional regulation of adipose ACE2 expression. The high fat (HF) diet (D12492, Research Diets) used in previous studies consists of a mixture of saturated, monounsaturated and polyunsaturated fatty acids. A well described effect of fatty acids is regulation of expression of various genes by acting as ligands for specific transcription factors. Thus, it is conceivable that specific fatty acids contributed to increased ACE2 mRNA abundance in adipose tissue of HF-fed mice.

PPARs are ligand-activated transcription factors with three known isoforms (PPARα, PPARβ/δ and PPARγ; Kliewer et al.1994), all expressed in tissues associated with cardiovascular function and glucose homeostasis. The most widely studied isoform of the PPAR family, PPARγ, is expressed at a high level in adipose tissue, with lower levels of expression in liver, kidney, skeletal muscle, monocytes and macrophages (Roszer et al. 2010). PPARγ has been demonstrated to play a pivotal role in adipocyte differentiation (Brun et al. 1997). In type 2 diabetics, PPARγ ligands improve glucose homeostasis (Picard et al. 2002), an effect thought to involve increased differentiation of new, insulin sensitive, adipocytes. Well known natural ligands of PPARγ include long chain fatty acids and eicosanoids such as arachidonic acid, docosahexanoic acid, and eicosapentaenoic acid (Kliewer et al. 1997). Similarly, thiazolidinediones (TZDs),

such as rosglitazone (Rosi) and pioglitazone (Pio), are well known synthetic ligands of PPARγ that have been widely used to improve insulin sensitivity in type 2 diabetics (Olefsky et al. 2000).

In addition to their insulin sensitizing effects, a number of studies in rodents and in humans and rodents demonstrate blood pressure lowering effects of TZDs (Sarafidis et al. 2006; de Oliveira Silva-Junior et al. 2009; Blasi et al. 2009; for review see Ketsawatsomkron et al. 2010), as well as natural fatty acid ligands of PPARγ (e.g., docosahexaenoic acid; Grynberg et al. 2005). In this study, we sought to identify mechanisms for regulation of adipocyte ACE2 by HF feeding, focusing on natural fatty acid as well as synthetic ligands of PPARγ. Our results demonstrate that natural and synthetic ligands of PPARγ stimulate ACE2 mRNA abundance, activity and protein expression in adipocytes. Since ACE2 catabolizes AngII to the vasodilator peptide angiotensin-(1-7) (Ang-(1-7)), and since activation of PPARγ has been demonstrated to lower blood pressure, we defined blood pressure lowering effects of a synthetic TZD in *Ace2*$^{+/y}$ and $^{-/y}$ mice. Moreover, since previous studies demonstrated hyperglycemia and impaired glucose tolerance in *Ace2*$^{-/y}$ mice (Bindom et al. 2009; Niu et al. 2008), we defined effects of ACE2 deficiency on insulin sensitizing effects of TZDs.

3.3. Methods.

3.3.1. 3T3-L1 adipocytes.

3.3.1.1. Incubation of 3T3-L1 adipocytes with fatty acids.

3T3-L1 cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Cells were grown to confluence, and then differentiation was induced with a cocktail of insulin (0.1 μM, Sigma, St. Louis, MO), dexamethasone (1 μM; Sigma, St. Louis, MO) and isobutyl methyl xanthine (0.5 mM; Sigma, St. Louis). On day 8 of differentiation 3T3-L1 adipocytes were incubated with 100μm of various fatty acids (palmitic acid (PA), stearic acid (SA), docosahexaenoic acid (DHA), linoleic acid (LA)) for 24 hours and cells were harvested for quantification of ACE2 mRNA abundance and enzymatic activity (described in section 2.3.4 and 2.3.5).

Preparation of fatty acid-enriched media: Stock solutions of high purity (>99%) fatty acids (Nu-Chek,MN) were prepared in hexane. NaOH (6 M, or 30x molarity of fatty acid) was used for saponification to convert the fatty acids into a water-soluble form. The desired amount of fatty acid was aliquoted, mixed with 6 M NaOH, and dried under high purity nitrogen gas. The residue was dissolved in experimental medium containing albumin. The final ratio of fatty acids to albumin was 4:1. Then, the pH was adjusted to 7.4 with HCL and the medium was sterilized through a syringe-driven filter unit (Toborok et al. 2002).

3.3.1.2. TZD (Rosi) treatment.

3T3-L1 cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Cells were grown to confluence, and then differentiation was induced with a cocktail of insulin (0.1 μM, Sigma, St. Louis, MO), dexamethasone (1 μM; Sigma, St. Louis, MO) and isobutyl methyl xanthine (0.5 mM; Sigma, St. Louis). On day 8 of differentiation cells were stimulated with varying concentrations of Rosi (0.01-1μm) for 24 hours. Cells were then harvested for quantification of ACE2 mRNA abundance and/or enzymatic activity (described in section 2.3.4 and 2.3.5).

3.3.2. Chromatin immuno precipitation (ChIP) assay.

ChIP assay was performed using an EZ-Chip assay kit (Upstate) according to the manufacturer's instruction. Mature adipocytes (day 8) were stimulated with Rosi (1uM) for 24 hours. Cells were harvested, and soluble chromatin was prepared. Chromatin was immunoprecipitated using an antibody (4μg) directed against PPARγ (catalog number SC 7273; Santacruz Biotechnology). Final DNA product was PCR-amplified using the following primer pairs that cover the PPRE consensus sequences between -1367 and -1355 in the ACE2 promoter: forward, 5'-TGGAATTATTTTCAGGCTTGG-3'; reverse, 5'-CTCATGGGCCTGCTTGATTA -3'.

3.3.3. Measurement of ACE2 protein.

3T3-L1 cells were grown to confluence, and then differentiation was induced with a cocktail as described in section 3.3.1.1. On day 8 of differentiation cells were stimulated with varying concentrations of Pio (0.01-1μm) for 24 hours. At the end of 24 hours cells were harvested on ice in M-PER reagent (Pierce, Rockford, IL), sonicated (2 min), and lysates were pelleted by centrifugation. Protein (25 μg) from 3T3-L1 adipocytes was electrophoresed on a 7.5% SDS-PAGE gel under reducing conditions. Proteins were blotted onto PVDF membrane (GE Healthcare, Piscataway, NJ), blocked overnight (5% nonfat milk, 4°C) and incubated with anti-goat ACE2 antibody (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour (22°C). Goat IgG conjugated horseradish peroxidase antibody (1:5000 dilution, Santa Cruz) was used for chemiluminescent detection. Blots were stripped and re-probed with anti-mouse β-actin antibody (1:1000 dilution, clone AC-15, Sigma, St. Louis, MO) for normalization of protein loading. Images were collected on a Kodak Image Station 440CF and analyzed using Kodak 1D analysis system software (Version 3.6.4, New Haven, CT).

3.3.4. Animals.

Male *Ace2*$^{+/y}$ mice (8 weeks of age; The Jackson Laboratory, Bar Harbor, MA; n=10 per group) or *Ace2*$^{-/y}$ mice (8 weeks of age; backcrossed 10 times onto a C57BL/6 background) originally obtained from Dr. Thomas Coffman (Duke University, NC; n=10 per group) were fed normal laboratory diet

ad libitum. All experiments involving mice conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kentucky Institutional Animal Care and Use Committee.

3.3.5. Drugs.

Ace2$^{+/y}$ or $^{-/y}$ were administered vehicle or Rosi (25 mg/kg body weight, by gavage; Glaxo Smith Kline) daily for 21 days. Body weight was measured daily to calculate dose and to monitor daily dosages.

3.3.6. Measurement of blood pressure.

Systolic blood pressure (SBP) was measured by tail cuff using the Visitech 2000 system in all mice for one week prior to starting the drug and during the last five days of the study. Criteria for inclusion of measurements from individual mice were 5 out of 10 successful measurements with a standard deviation < 50 (Henriques et al. 2004).

3.3.7.Statistical analysis.

Data are expressed as mean $\pm$ SEM. For *in vitro* studies examining mRNA expression and activity with different treatments, data were analyzed by 1-way ANOVA with Tukey's test for post-hoc comparisons. For *in vivo* studies, non-paired t-test was performed to determine statistical significance of ACE2 expression, activity, plasma Ang-(1-7) concentrations. Differences between SBP at baseline and after treatments were analyzed by 1-way ANOVA with Tukey's test for post-hoc comparisons. For body weight and plasma insulin

concentrations, glucose tolerance we used a 2-way anova with genotype (*Ace2*$^{+/y}$ vs $^{-/y}$) and treatment (Vehicle vs Rosi), with significance at $P < 0.05$.

3.4. Results.

3.4.1. n-3 fatty acids and Rosi stimulate ACE2 m RNA, protein abundance and enzyme activity in 3T3-L1 adipocytes.

We incubated 3T3-L1 adipocytes with various fatty acids that are either enriched in the HF diet, or ligand activators of PPARγ. Saturated fatty acids (SA, PA), as well as an n-6 polyunsaturated fatty acid (LA) had no effect on ACE2 mRNA abundance or enzymatic activity (Fig. 3.1 A). In contrast, incubation with the n-3 fatty acid DHA resulted in increased ACE2 mRNA abundance in 3T3-L1 adipocytes (Fig. 3.1 A). Similarly, synthetic ligands of PPARγ such as Rosi and Pio significantly increased ACE2 mRNA abundance, protein expression and enzymatic activity in 3T3-L1 mature adipocytes. Rosi increased ACE2 mRNA and activity at concentrations of 0.01 and 0.1 μM (Fig. 3. 2 A, B). However, higher concentrations (1 μM) of Rosi had no effect on ACE2 mRNA abundance or activity. In contrast Pio increased ACE2 protein in a concentration dependent manner (0.01-1 μM) (Fig. 3.2 C).

To determine if stimulation of PPARγ by synthetic ligands results in binding at putative PPRE sites on the ACE2 promoter, we employed ChIP assays. After incubation of differentiated 3T3-L1 adipocytes with Rosi and chromatin immunoprecipitation with a PPARγ antibody, primers spanning a putative PPRE within the ACE2 promoter amplified a product of the appropriate size (Fig. 3.3).

3.4.2. In vivo Rosi administration to C57BL/6 male mice increases adipose ACE2 mRNA abundance and activity.

Administration of Rosi for 21 days had no effect on body weight (Table 3.1). In EF, mRNA abundance of ACE2 was increased in response to Rosi (Table 3.2; Fig.3.4 A). In contrast, ACE2 mRNA abundance was not altered in kidney or heart (Table 3.2). We also examined other components of the renin-angiotensin system (RAS), including renin in kidney, angiotensin type 1a receptor (AT1aR) in adipose, and angiotensinogen mRNA abundance in liver or adipose (Table 3.2).

To confirm that Rosi-mediated increases in ACE2 mRNA abundance resulted in increased functional protein, we measured ACE2 enzymatic activity in adipose and non-adipose tissue sources. Administration of Rosi increased ACE2 enzymatic activity in adipose tissue (Fig. 3.4 B), but had no effect on ACE2 activity in kidney (Fig. 3.4 C). Rosi-induced stimulation of ACE2 activity in adipose tissue was associated with increased plasma concentrations of the ACE2 product, Ang-(1-7) (Table 3.4, Fig.3.4 D).

3.4.3. Rosi administration reduces blood pressure and glucose tolerance: effects of ACE2 deficiency.

After 21 days of Rosi treatment glucose tolerance (area under the curve; AUC) was improved in *Ace2*$^{+/y}$ mice (Table 3.4, Fig.3.5 A). Additionally, we did not see any difference in blood pressure in vehicle-treated *Ace2*$^{+/y}$ mice. However, Rosi administration resulted in a significant reduction in SBP in *Ace2*$^{+/y}$ mice (Table 3.3, Fig. 3.5 B). We then administered vehicle or Rosi

to *Ace2*$^{-/y}$ mice using the same protocol. Administration of Rosi to *Ace2*$^{-/y}$ mice had no effect on body weight (Table. 3.1). Similar to findings in *Ace2*$^{+/y}$ males, Rosi administration reduced SBP in *Ace2*$^{-/y}$ mice (Table 3.3, Fig. 3.6 B). In contrast, rather than reduce AUC indicating improved glucose tolerance, administration of Rosi to *Ace2*$^{-/y}$ mice worsened glucose tolerance (Table 3.4, Fig. 3.6. A). In addition, Rosi administration had no effect on plasma insulin concentrations in *Ace2*$^{-/y}$ mice (Table 3.4, Fig. 3.7). Moreover, plasma insulin concentrations were lower in *Ace2*$^{-/y}$ compared to $^{+/y}$ mice administered vehicle (Table 3.4, Fig. 3.7).

3.5. Discussion.

Results from the present study demonstrate that natural fatty acid ligands, as well as synthetic ligands of PPARγ, increase ACE2 expression in adipocytes. Chromatin immunoprecipitation demonstrated binding of PPARγ to PPREs on the ACE2 promoter in adipocytes. Administration of Rosi to *Ace2*$^{+/y}$ mice resulted in increased ACE2 mRNA and activity in adipose tissue, but not in other tissues expressing a high level of ACE2. Moreover, Rosi administration elevated systemic concentrations of the ACE2 product, Ang-(1-7). Interestingly, blood pressure lowering effects of Rosi remained evident in ACE2 deficient mice, suggesting other mechanisms contribute to this effect. In contrast, ACE2 deficient mice no longer exhibited improved glucose tolerance in response to Rosi. These results suggest that stimulation of ACE2 expression by PPARγ in target tissues, such as adipose tissue, may contribute to improved glucose homeostasis following TZD administration. Additional studies are necessary to identify mechanisms of PPARγ/ACE2 interactions in the regulation of glucose homeostasis.

A number of dietary fatty acids are known to regulate various proteins by influencing gene transcription, mRNA processing, or post-translational modification of proteins (Madsen et al. 2005; Clarke et al. 2004). In vitro studies suggest that several fatty acids can bind to and activate PPARγ (Sauma et al. 2006; Kliewer et al. 1997). Interestingly, a recent study demonstrated increased

expression of renin in CaLu-6 cells stimulated with oleic acid (Todorov et al. 2007), indicating a role of fatty acids to regulate genes of the RAS.

This is of interest as ingestion of diets rich in polyunsaturated fatty acids(PUFAs) are beneficial for cardiac health with a reduction in cardiovascular morbidity and mortality (Sauma et al. 2006). Importantly, heart healthy fatty acids from the n-3 family such as DHA and EPA are well known natural ligands of PPARγ (Kliewer et al. 1997). Since in our previous study the expression of PPARγ preceded the expression of ACE2 in 3T3-L1 adipocytes, we incubated mature adipocytes with specific fatty acids. Natural ligands of PPARγ, such as DHA, increased ACE2 mRNA expression in adipocytes. In contrast, saturated fatty acids as well as an n-6 fatty acid had no effect on ACE2 expression in adipocytes. A recent study examined effects of specific fatty acids on ACE2 expression in porcine adipocytes (Tseng et al. 2010). In contrast to our findings, incubation of adipocytes with DHA reduced porcine ACE2 expression. Mechanisms for differences in results are unclear, but may relate to species of adipocytes (porcine vs murine in Tseng et al. 2010 vs this study, respectively), duration of incubation (48 hours vs 24 hours), dose (50 vs 100μM), and incubation conditions (1% albumin vs 4:1 fatty acid/albumin ratio).

In addition to DHA as a regulator of ACE2, in this study we demonstrated that two different synthetic ligands of PPARγ increased ACE2 mRNA abundance, protein and enzymatic activity in adipocytes. Results from ChIP assays support binding of PPARγ to PPREs on the ACE2 promoter. To determine if PPARγ

regulation of ACE2 occurs in vivo, we administered Rosi to mice and demonstrated increased expression of ACE2 in adipose tissue, but not in heart of kidney. Regulation of ACE2 in adipose, but not non-adipose tissues, may relate to differences in expression levels of PPARγ between these tissues. Stimulated ACE2 expression by Rosi administration in adipose tissue was associated with increased plasma concentrations of the ACE2 product, Ang-(1-7), suggesting that adipose ACE2 stimulation was sufficient to influence the systemic RAS. However, elevations in systemic Ang-(1-7) concentrations did not appear to influence blood pressure, since blood pressure lowering effects of Rosi persisted in ACE2 deficient mice. The contribution of Ang-(1-7) to blood pressure control is unresolved. Studies in Mas receptor deficient mice have shown either no change in blood pressure (Walther et al. 2000) or increased blood pressure (de Moura et al. 2010). In transgenic rats engineered with a fusion protein to chronically drive elevated production of Ang-(1-7) (2.5-fold higher plasma levels), blood pressure was not altered (Botelho-Santos et al. 2007). Collectively, these results support the observation that Rosi-induced elevations in systemic Ang-(1-7) concentrations did not contribute to blood pressure lowering effects of this compound, as demonstrated by continued reductions in blood pressure following Rosi administration to ACE2 deficient mice.

An interesting finding from this study was that effects of Rosi to improve glucose tolerance were reversed in ACE2 deficient mice. Recent studies support a potential role for ACE2/Ang-(1-7) in glycemic control. Mas receptor deficient mice exhibit glucose intolerance and reduced insulin sensitivity including

reductions in glucose uptake by adipocytes and decreased GLUT4 expression in adipose tissue (Santos et al. 2008a). Conversely, in transgenic rats over-expressing Ang-(1-7) glucose tolerance, insulin sensitivity and insulin-stimulated glucose uptake were enhanced (Santos et al. 2010). Recent studies using db/db diabetic mice demonstrated that adenoviral over-expression of ACE2 in pancreas improved fasting glycemia and glucose tolerance and increased islet insulin content, and that these effects could be blocked by a Mas receptor antagonist (Bindom et al. 2010). Our results demonstrate that ACE2 deficient mice have reduced plasma concentrations of insulin compared to wild type mice. In wild type mice, Rosi administration reduced plasma insulin concentrations and enhanced glucose tolerance. In contrast, ACE2 deficient mice did not respond to Rosi to improve glucose tolerance and lower circulating insulin concentrations. Future studies should examine whether altered glucose homeostasis in ACE2 deficient mice administered Rosi result from defects in insulin synthesis and secretion, or altered insulin responsiveness. Interestingly, PPARγ is expressed in the pancreas and has been suggested to preserve beta-cell function (Kanda et al. 2010; Gupta et al. 2008).

In conclusion, results from this study support regulation of ACE2 in adipocytes by natural and synthetic PPARγ ligands *in vitro* and *in vivo*. Deficiency of ACE2 did not influence blood pressure lowering effects of Rosi, but reversed Rosi-induced improvements in glucose tolerance.

These results suggest that stimulation of ACE2 by PPARγ agonism may contribute to beneficial effects of these agents on glucose homeostasis.

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Table 3.1. Body weight (g) in *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ treated with Vehicle or Rosi for 21 days.

	Ace2$^{+/y}$		*Ace2*$^{-/y}$	
	Vehicle	Rosi	Vehicle	Rosi
Day 1	25 ± 0.4	24 ± 0.4	25 ± 0.5	25 ± 0.5
Day 21	26 ± 0.5	25 ± 0.4	24 ± 0.4	24 ± 0.4

Data are mean ± SEM from n = 10 mice/group

Table 3.2. mRNA expression of genes of the RAS in Vehicle or Rosi treated *Ace2*$^{+/y}$ Mice.

	Vehicle	Rosi
PPAR-EF	0.6±0.0	0.8±0.1
ap2-EF	1.7±0.0	3.2±0.0*
ACE2-Kidney	11.4±1.1	10.4±1.5
ACE2-Heart	0.4±0.0	0.6±0.1
ACE2-EF	0.007±0.0	0.02±0.0*
AT1-EF	0.5±0.0	0.5±0.0
Renin –Kidney	2.0±0.3	2.4±0.5
AO-Liver	0.8±0.1	1.4±0.1
AO-EF	0.5±0.0	0.3±0.0

EF, epididymal fat.

Data are mean $\pm$ SEM from n = 5 mice/group

*, $P < 0.05$ compared to Vehicle

Table 3.3. Systolic blood pressure (mmHg) in *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice treated with Vehicle or Rosi.

	Ace2$^{+/y}$		*Ace2*$^{-/y}$	
	Vehicle	Rosi	Vehicle	Rosi
Baseline	121 ± 1	126 ± 6	124 ± 3	124 ± 6
Final	118 ± 2	109 ± 4*	123 ± 1	109 ± 1*

Data are mean ± SEM from n = 8-10 mice/group, *, $P < 0.05$ compared to vehicle

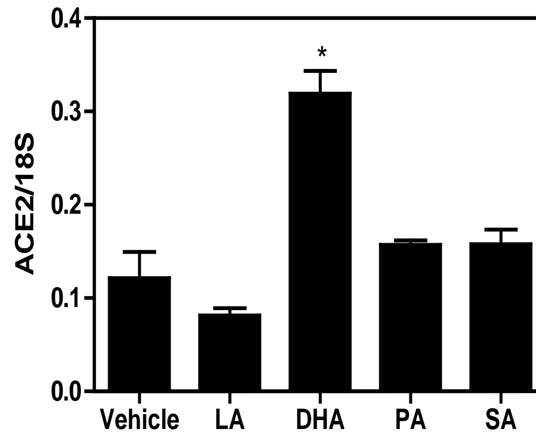
Table 3.4. Area under curve (AUC), Plasma Ang-(1-7) and Plasma insulin levels in *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice treated with Vehicle or Rosi.

	Ace2$^{+/y}$		*Ace2*$^{-/y}$	
	Vehicle	Rosi	Vehicle	Rosi
AUC	14495±1140	4053±663*	6645±596**	11033±876*,**
Plasma Ang-(1-7)(ng/ml)	0.13±0.0	0.19±0.0*	0.12±0.0	0.11±0.0
Plasma Insulin (ng/ml)	0.68±0.0	0.49±0.0*	0.38±0.0**	0.34±0.0

*, $P < 0.05$ compared to Vehicle

**, $P < 0.05$ compared to *Ace2*$^{+/y}$

A)



B)

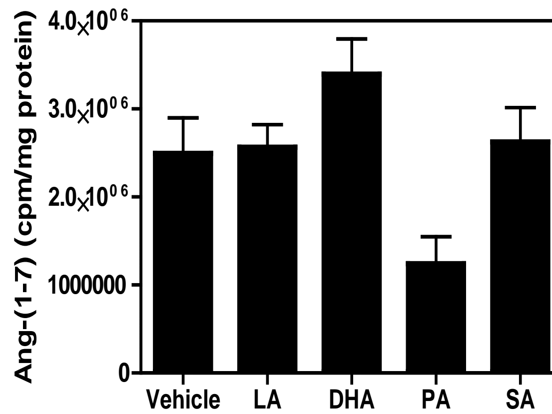
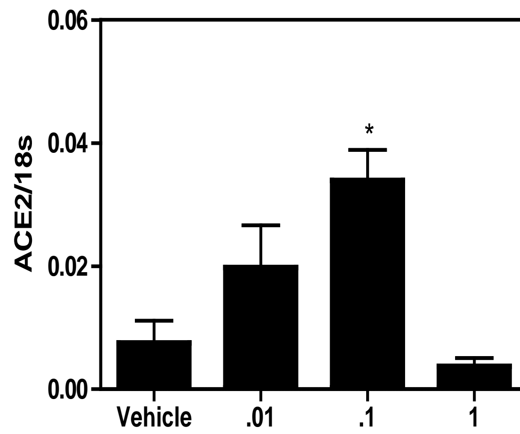


Fig .3.1. A. ACE2 mRNA in 3T3-L1 adipocytes treated with specific fatty acids (100μm) for 24 hours, B. ACE2 activity in 3T3-L1 adipocytes treated with specific fatty acids (100μm) for 24 hours. Data are mean ± SEM from n =3- 4 /group. *, P < 0.05 compared to Vehicle.

A)



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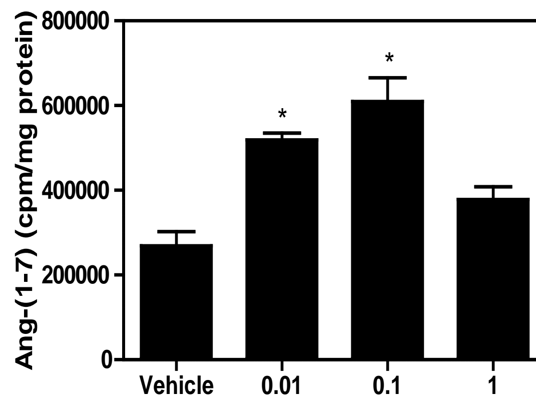


C)

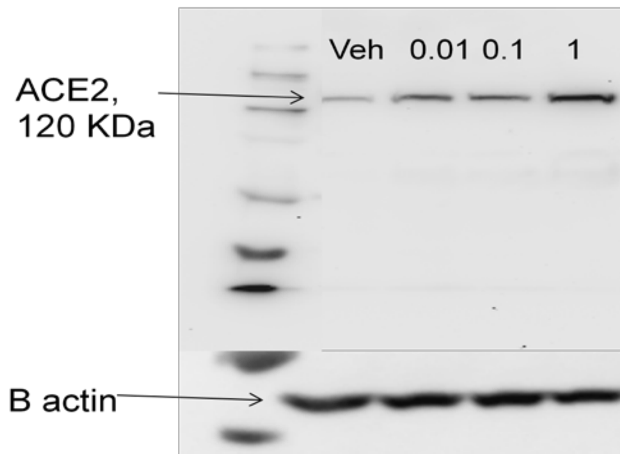


Fig .3.2. A. ACE2 mRNA (Rosi) in 3T3-L1 adipocytes treated with TZD (0.01-1μm) for 24 hours, B. ACE2 activity (Rosi) in 3T3-L1 adipocytes treated with TZD (0.01-1μm) for 24 hours C. ACE2 protein (Pio) in 3T3-L1 adipocytes treated with TZD (0.01-1μm) for 24 hours. Data are mean $\pm$ SEM from n = 3-4 /group, *, $P < 0.05$ compared to Vehicle.

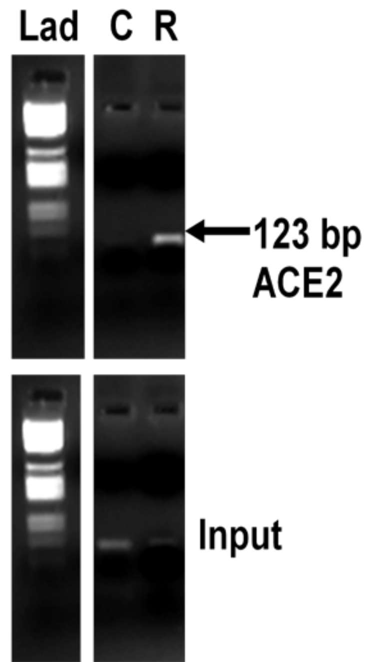
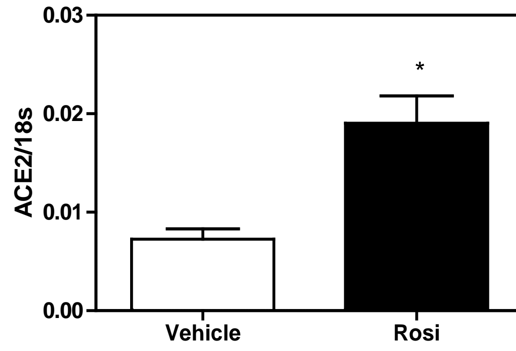


Fig .3.3. ChIP assay. 3T3-L1 cells stimulated with 1µm Rosi for 24 hours.

A)



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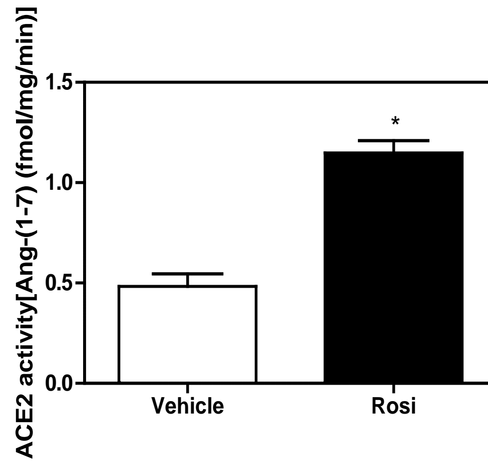
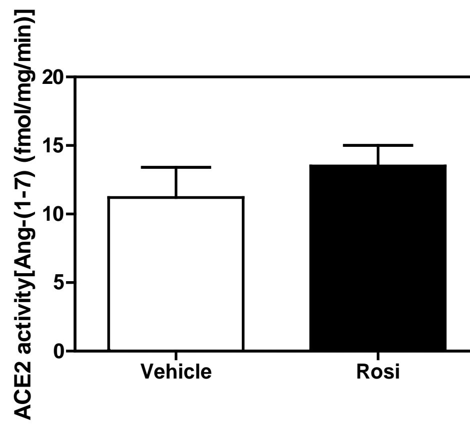


C)



D)

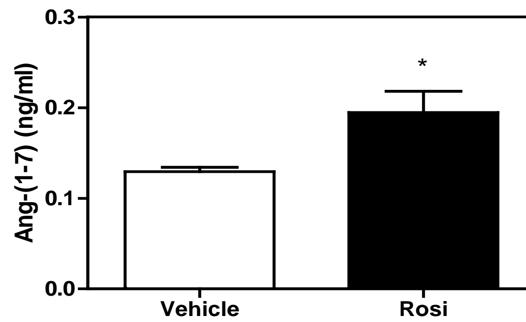
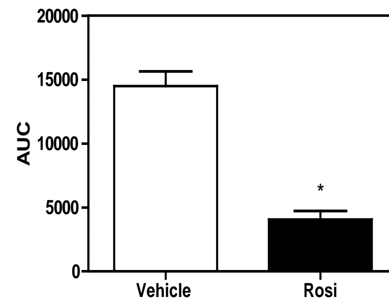


Fig .3.4. A. ACE2 m RNA (EF) in *Ace2*$^{+/y}$ mice treated with Vehicle or Rosi, B. ACE2 activity (EF) in *Ace2*$^{+/y}$ mice treated with Vehicle or Rosi, C. ACE2 activity (Kidney) in *Ace2*$^{+/y}$ mice treated with Vehicle or Rosi, D. Plasma Ang-(1-7) concentrations in *Ace2*$^{+/y}$ mice treated with Vehicle or Rosi. Data are mean $\pm$ SEM from n = 5 mice /group. *, $P < 0.05$ compared to Vehicle.

A)



B)

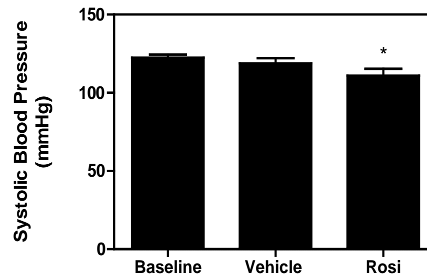


Fig .3.5. A. AUC for glucose tolerance test after 21 days of Rosi treatment in *Ace2*$^{+/y}$ mice (n = 5-8 mice /group), B. Systolic blood pressure at baseline and after Rosi treatment in *Ace2*$^{+/y}$ mice (n= 8-10 mice/group). *, P < 0.05 compared to baseline.

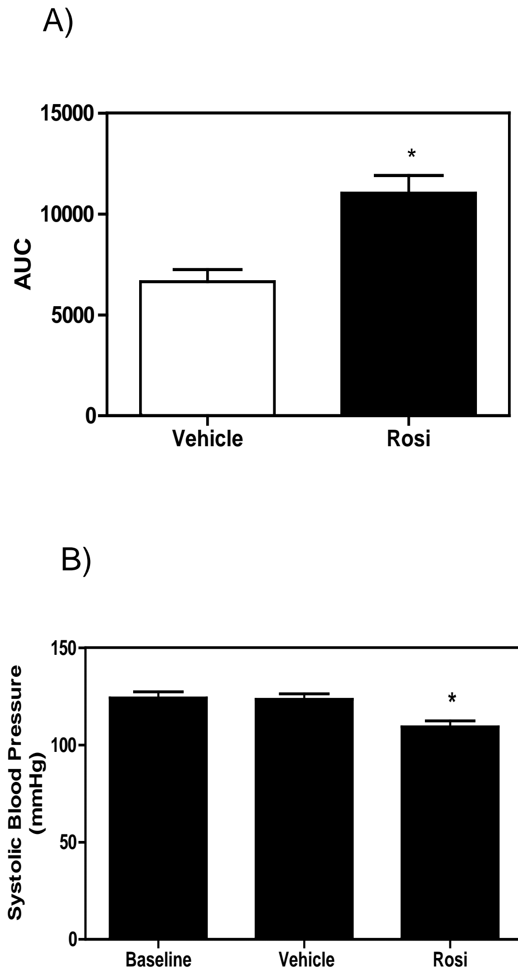


Fig .3.6. A. AUC for Glucose tolerance test after 21 days of Rosi treatment in *Ace2*$^{-/y}$ mice (n = 5-8 mice /group), B. Systolic blood pressure at baseline and after Rosi treatment in *Ace2*$^{-/y}$ mice (n= 8-10 mice/group) *, P < 0.05 compared to baseline.

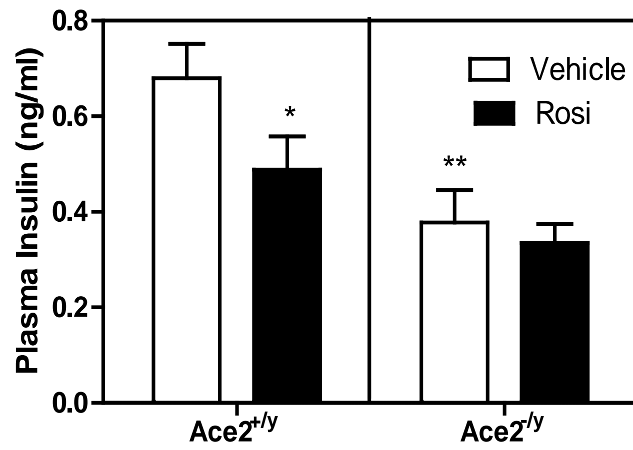


Fig .3.7. Plasma insulin concentrations in *Ace2*$^{+/y}$ and $^{-/y}$ Vehicle or Rosi treated mice (n = 5-7 mice/group). *, $P < 0.05$ compared to vehicle; **, $P < 0.05$ compared to *Ace2*$^{+/y}$.

Section IV. SPECIFIC AIM 2 A

Determine the effects of ACE2 deficiency on the development of obesity and diabetes and hypertension in male C57BL/6 mice.

4.1. Summary.

Previous studies demonstrated ACE2 expression in adipose tissue. ACE2 gene expression in adipose tissue was stimulated by high fat (HF) feeding. However, chronic obesity was associated with dysfunctional adipose ACE2 activity, elevated systemic angiotensinII (AngII), and hypertension. These results suggested that ACE2 may protect against obesity-hypertension. Therefore, in this study we examined effects of ACE2 deficiency in mice on the development of obesity-induced hypertension. Moreover, since diet-induced obesity is associated with impaired glucose homeostasis, we measured effects of ACE2 deficiency on glucose and insulin tolerance. Male C57BL/6 *Ace2*$^{+/y}$ or $^{-/y}$ mice were fed a low fat (LF, 10% kcal as fat) or HF diet (60% kcal as fat) for 16 weeks. Body weight increased with HF feeding in *Ace2*$^{+/y}$ and $^{-/y}$ mice; however, body weight was modestly lower in *Ace2*$^{-/y}$ compared to $^{+/y}$ mice. *Ace2*$^{-/y}$ mice exhibited glucose intolerance compared to controls even when fed a LF diet. With HF feeding, both genotypes exhibited glucose intolerance. However, HF-fed *Ace2*$^{-/y}$ mice had lower plasma insulin concentrations compared to HF-fed controls and were more insulin sensitive. Importantly, ACE2 deficiency promoted increased plasma AngII concentrations in HF-fed *Ace2*$^{-/y}$ mice compared to HF-fed controls. HF feeding

reduced plasma concentrations of Ang-(1-7) in *Ace2*$^{+/y}$ mice compared to LF controls. Systolic blood pressure (SBP) was increased in HF-fed *Ace2*$^{+/y}$ and $^{-/y}$ mice compared to LF-fed controls. Importantly, SBP was increased in HF-fed *Ace2*$^{-/y}$ mice compared to HF-fed *Ace2*$^{+/y}$ mice. We administered losartan to determine if increases in SBP in HF-fed *Ace2*$^{-/y}$ mice were AngII/AT1 receptor-mediated. Administration of losartan eliminated differences in SBP between HF-fed *Ace2*$^{+/y}$ and $^{-/y}$ mice.

These results demonstrate that ACE2 deficiency promotes obesity-induced hypertension.

4.2. Introduction.

Obesity is an epidemic worldwide leading to an increase in cardiovascular pathologies such as hypertension (Kotsis et al. 2010). Early reports in the Framingham Study demonstrated that 78% of essential hypertension in men can be directly attributed to obesity (Garrison et al. 1987). Given the unabating rise in obesity prevalence, it is clear that obesity-related hypertension will increase in the future. Despite a strong association between obesity and hypertension, mechanisms linking the two diseases are not fully understood.

Hemodynamic characteristics of obese hypertensive subjects include an increase in intravascular volume, increased cardiac output, endothelial dysfunction and abnormal kidney function (Reisin et al.1978; Carroll et al. 1995; Davy et al. 2004). Candidate systems contributing to these hemodynamic changes in obese hypertensives include activation of the sympathetic nervous system (Hall et al. 1997; O'Dea K et al. 1982) and the renin-angiotensin system (RAS) (Coopert et al.1998; Cooper et al. 1997). Clinical studies comparing obese to lean subjects demonstrated a positive correlation between body mass index (BMI) and plasma concentrations of angiotensinogen (Cooper et al.1998; Cooper et al. 1997; Umemeura et al. 1997). Several studies have demonstrated activation of the RAS in experimental models of diet-induced obesity (Boustany et al. 2004; Boustany et al. 2005; Rahmouni et al. 2004; Gupte et al. 2008). However, mechanisms for an activated RAS in obesity-hypertension are unclear.

In 2000, two independent groups using unique molecular strategies identified a homologue of angiotensin converting enzyme (ACE) known as ACE2 (Tipnis et al. 2000; Donoghue et al. 2000). The discovery of ACE2 was particularly exciting since it was shown to convert angiotensinII (AngII), a vasoconstrictor peptide, to angiotensin-(1-7) (Ang-(1-7)), a vasodilator peptide (Vickers et al. 2002). Studies in male mice with ACE2 deficiency have demonstrated a significant increase in systolic blood pressure with chronic AngII infusion, indicating an important role for this enzyme in AngII degradation (Gurley et al. 2006). Based on its ability to blunt an activated RAS, ACE2 has been suggested as a potential target for activation to decrease blood pressure.

Previous studies demonstrated that ACE2 is expressed in rodent adipose tissue (Gupte et al. 2008; Gembardt et al. 2005; Galvez-Prieto et al. 2008). Moreover, high fat (HF) feeding in mice initially resulted in an increase in ACE2 gene expression and activity in adipose tissue, suggesting a protective role for ACE2 against obesity-induced hypertension (Gupte et al. 2008). However, with chronic HF feeding, stimulated ACE2 activity in adipose tissue was lost, and obese mice exhibited an activated systemic RAS and hypertension. These results demonstrated that ACE2 is nutritionally regulated by HF feeding in adipose tissue and suggest that this regulation contributes to activation of the systemic RAS and the development of obesity-related hypertension. To determine if ACE2 protects against obesity-hypertension, we tested the hypothesis that deficiency of ACE2 promotes obesity-induced hypertension. Moreover, since diet-induced obesity is associated with impaired glucose

homeostasis, we defined effects of ACE2 deficiency on glucose and insulin tolerance.

Results from this study demonstrate that deficiency of ACE2 markedly promotes obesity-induced hypertension.

4.3. Methods.

4.3.1. Animals and diet.

Male *Ace2*$^{+/y}$ and $^{-/y}$ mice (8 weeks of age; backcrossed 10 times onto a C57BL/6 background)(Gurley et al. 2006) were fed a low fat (LF;10% kcal as fat; D12450B; Research Diets, Inc, New Brunswick, NJ ; n=20) or HF diet (60% kcal as fat; D12492, Research Diets, Inc, New Brunswick, NJ ; n=20) for sixteen weeks. Diets were matched in protein content (20% kcal) and provided energy at 3.85 or 5.25 kcal/gm (LF and HF, respectively). Diets were provided to mice *ad libitum*. All experiments involving mice conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kentucky Institutional Animal Care and Use Committee.

4.3.2. Glucose and insulin tolerance tests.

Fasting (6 hr) blood glucose concentrations were measured using a One Touch glucometer. Glucose tolerance was measured in fasted mice injected with glucose intraperitoneally at a dose of 2 g/kg body weight. Blood glucose concentrations were measured at times 0, 15, 30, 60, 90 and 120 minutes after the injection. Insulin sensitivity was measured in fasted mice injected (i.p.) with insulin (0.5 U/kg in 0.9% saline). Blood glucose concentrations were measured at times 0, 15, 30, 60, 90 and 120 minutes.

4.3.3. Measurement of food intake and physical activity.

Mice (n = 5/genotype/diet group) were housed individually in TSE Lab Master System (TSE Systems, Inc., Chesterfield,MO) for measurement of food intake, indirect calorimetry and physical activity every 30 minutes over 3 days. Mice were acclimated to the test cages for 1 day prior to measurements. Activity (counts in the X plane/30 minutes) was averaged over the 3 days of recording. Energy intake was calculated as food intake (per day averaged over 3 days) times the energy density of LF or HF diets. Energy expenditure was measured as oxygen consumption (VO_2: $Flow_{ml}$ x (V1 + V2)/(N_2 Ref x 100) (V1=N_2 Ref * dO_2, V2=O_2 Ref *(dO_2-dCo_2) and was normalized to mouse lean mass measured using a PIXI-mus Densitometer (GE Lunar Corp., Madison, WI) at the end of the recording session.

4.3.4. Measurement of plasma parameters.

Plasma insulin concentrations (Rat/Mouse Insulin ELISA Kit, Millipore, MA) or Ang-(1-7) concentrations (Peninsula laboratories, LLC Bachem group, CA, USA) were quantified using commercial kits. Plasma AngII concentrations were measured as previously described (Daugherty et al. 2004). Due to the large volume of plasma required for accurate quantification of concentrations of AngII (150 µl) or Ang-(1-7) (100µl) in mice, we measured concentrations of Ang-(1-7) in plasma from all mice, but quantified plasma AngII concentrations only in HF-fed groups ($Ace2^{+/y}$ and $^{-/y}$) since previous studies from

our laboratory demonstrated increased plasma AngII concentrations in HF compared to LF-fed C57BL/6 mice (Gupte et al. 2008).

4.3.5. Measurement of blood pressure.

Blood pressure was measured by radiotelemetry during week 16 according to previously described method (Gupte et al. 2008). Briefly, mice were implanted with carotid artery catheters during week 15 of LF or HF feeding, allowed one week to recover, and then pressure was recorded continuously (5 minute sampling) for 3 days. After baseline recordings of blood pressure, mice (n = 5/group) were administered losartan in the drinking water (10 mg/kg body weight) and recordings continued for 3 days.

4.3.6. Statistical analysis.

Data are expressed as mean ± SEM. All data were analyzed using SigmaStat for equal variance. For data with equal variance, two- way ANOVA was used to analyze end-point measures followed by Tukey's test for post-hoc analysis. Significance was accepted at a $P<0.05$.

4.4. Results.

4.4.1. Metabolic characteristics of HF feeding in *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ Mice.

In LF-fed mice, body weights were not different between *Ace2*$^{+/y}$ and $^{-/y}$ mice (Figure 4.1 A). Both *Ace2*$^{+/y}$ and $^{-/y}$ mice exhibited increased body weights (Fig. 4.1 A) when fed a HF diet. However, HF-fed *Ace2*$^{-/y}$ mice had lower body weights compared to *Ace2*$^{+/y}$ controls (Fig. 4.1 A; $P<0.05$). To define mechanisms for differences in body weight between HF-fed *Ace2*$^{+/y}$ and $^{-/y}$ mice, in the last week of the study a subset of mice from each group were placed individually in sealed chambers for measurement of physical activity, food intake, and energy expenditure. Physical activity was reduced by HF feeding to a similar extent in both *Ace2*$^{+/y}$ and $^{-/y}$ mice (Fig. 4.1 B). Similarly, energy intake was increased by a similar extent from HF-feeding in *Ace2*$^{+/y}$ and $^{-/y}$ mice (Fig. 4.1 C). In contrast, energy expenditure was increased by HF feeding in *Ace2*$^{-/y}$, but not in *Ace2*$^{+/y}$ mice (Fig.4.1 D; $P<0.05$).

Previous investigators have reported hyperglycemia in *Ace2*$^{-/y}$ mice fed a standard diet (Bindom et al. 2009). Moreover, HF feeding in mice promotes glucose intolerance and type 2 diabetes. Thus, we examined effects of ACE2 deficiency on glucose and insulin tolerance in HF-fed mice. At 8 weeks of age, *Ace2*$^{-/y}$ mice fed standard diet exhibited elevated fasting blood glucose compared to controls (*Ace2*$^{+/y}$, 127 $\pm$ 3; *Ace2*$^{-/y}$, 138 $\pm$ 3 mg/dl; $P<0.05$; Fig. 4.3 A). Moreover, at 16 weeks of LF feeding, *Ace2*$^{-/y}$ mice were glucose intolerant compared to LF-fed controls (Fig.4.2 A, B; $P<0.05$). After 16 weeks of HF

feeding, both *Ace2*$^{+/y}$ and $^{-/y}$ mice exhibited markedly impaired glucose tolerance (Fig. 4.2 A, B). Non-fasted insulin concentrations in control *Ace2*$^{+/y}$ mice were robustly increased by HF feeding (LF-fed, 0.6 ± 0.1; HF, 2.9 ± 0.8 ng/ml; P<0.01; Fig. 4.2 C). In contrast, *Ace2*$^{-/y}$ mice did not exhibit an increase in plasma insulin concentrations with HF feeding (LF-fed, 0.5 ± 0.0; HF-fed, 0.8 ± 0.1 ng/ml; Fig. 4.2 C). To further examine glucose homeostasis in *Ace2*$^{-/y}$ mice, we performed insulin tolerance tests. Age-matched (8 weeks) *Ace2*$^{-/y}$ mice fed standard diet were more sensitive to insulin to reduce blood glucose concentrations compared to *Ace2*$^{+/y}$ controls (Fig. 4.3 B). With HF feeding, control *Ace2*$^{+/y}$ mice did not respond to insulin to lower blood glucose concentrations (Fig. 4.2 D). In contrast, HF-fed *Ace2*$^{-/y}$ mice remained relatively more insulin sensitive compared to controls (Fig. 4. 2 D).

4.4.2. HF-fed *Ace2*$^{-/y}$ mice exhibit an activated systemic RAS.

We previously reported that HF feeding resulted in elevated systemic concentrations of AngII in male mice (Gupte et al. 2008). Deficiency of ACE2 resulted in a further increase in plasma concentrations of AngII with HF-feeding (*Ace2*$^{+/y}$, 2.2 ± 0.2; *Ace2*$^{-/y}$, 3.2 ± 0.1 ng/ml, P<0.05; Fig.4.4 A). Plasma concentrations of the catabolic product of ACE2, Ang-(1-7), were decreased by HF feeding in *Ace2*$^{+/y}$ controls (LF, 0.45 ± 0.1; HF, 0.12 ± 0.02 ng/ml , P<0.05 ; Fig.4.4 B). As anticipated, ACE2 deficient mice exhibited low concentrations of plasma Ang-(1-7).

4.4.3. ACE2 deficiency markedly promotes obesity hypertension.

There was no effect of HF feeding or of ACE2 genotype on pulse pressures or heart rates (Table 4.2). In *Ace2*$^{+/y}$ mice, HF feeding had no effect on diastolic or mean arterial pressure (Table 4.2). As previously reported (Gupte et al. 2008), HF feeding resulted in an increase in SBP (24 hours of recording) of *Ace2*$^{+/y}$ controls (LF-fed, 124 ± 1; HF-fed, 135 ± 2 mmHg; p=0.05; Fig. 4.5 A). Deficiency of ACE2 had no effect on SBP (Fig. 4.5 A), diastolic or mean arterial pressure in LF-fed mice (Table 4.2). Similar to wild type controls, HF feeding increased SBP (24 hours of recording) of *Ace2*$^{-/y}$ controls (LF-fed, 128 ± 2; HF-fed, 145 ± 5 mmHg; P<0.05; Fig. 4.5 A). Importantly, with HF feeding, *Ace2*$^{-/y}$ mice exhibited elevations in SBPs compared to HF-fed *Ace2*$^{+/y}$ mice (*Ace2*$^{+/y}$,135 ± 2; *Ace2*$^{-/y}$, 145 ± 5 mmHg; P<0.05; Fig. 4.5 A). To determine if elevated blood pressures in *Ace2*$^{-/y}$ mice were AngII/AT1 receptor-mediated, we administered losartan to LF and HF-fed *Ace2*$^{+/y}$ and $^{-/y}$ mice. Administration of losartan to HF-fed *Ace2*$^{+/y}$ and $^{-/y}$ mice normalized elevated systolic blood pressures, but had no effect in other groups (Fig. 4.5 B).

4.5. Discussion.

In the present study we demonstrate for the first time that ACE2 deficiency markedly promotes obesity-induced hypertension. Interestingly, ACE2 deficient mice exhibited impaired glucose tolerance, lower plasma insulin concentrations, and relatively greater insulin sensitivity when challenged with a HF diet. The systemic RAS, as indicated by plasma concentrations of AngII, was further activated in ACE2 deficient mice fed a HF diet. Of note, ACE2 deficiency markedly promoted the development of systolic hypertension in obese mice, with normalization of blood pressures upon treatment with losartan. These results demonstrate a pivotal role for ACE2 in obesity-induced hypertension.

In the present study ACE2 deficient mice that exhibit significant elevations in systemic AngII concentrations had lower body weights when fed a HF diet. Previous studies demonstrated a dose-dependent reduction in body weight and lean mass in rats and/or mice infused with AngII (Cassis et al. 1998a; English et al. 1999; Brink et al. 1996; Brink et al. 2001). Further studies suggested that AngII-induced reductions in body weight resulted from elevated energy expenditure (Brink et al. 1996). In this study, elevated plasma concentrations of AngII in HF-fed ACE2 deficient mice may have contributed to modest reductions in lean mass and elevated energy expenditure. However, modest reductions in body weight in HF-fed ACE2 deficient mice did not provide protection against obesity-induced hypertension.

An interesting finding from the present study was impaired glucose tolerance in ACE2 deficient mice. These results are in agreement with previous findings demonstrating progressively impaired glucose tolerance in aging ACE2 deficient mice (Niu et al. 2008). Moreover, previous investigators demonstrated that ACE2 deficiency resulted in a selective decrease in first phase insulin secretion in *Ace2*$^{-/y}$ mice (Niu et al. 2008). Recent studies demonstrated that adenoviral expression of ACE2 in pancreas of *db/db* mice reduced hyperglycemia and glucose intolerance, and improved insulin secretion and β-cell function (Bindom et al. 2010). These results suggested that ACE2 favorably modulates insulin production upon targeted over-expression in the pancreas. Our results are the first to demonstrate that deficiency of endogenous ACE2 impairs glucose homeostasis in HF diet-induced type 2 diabetes. In contrast to wild type mice that exhibited hyperinsulinemia with impaired insulin and glucose tolerance, ACE2 deficient mice were glucose intolerant, remained responsive to insulin, and did not exhibit hyperinsulinemia. However, blunted hyperinsulinemia with HF feeding in ACE2 deficient mice did not protect mice against glucose intolerance. Similar to recent results (Bindom et al. 2010), our data suggest a protective role for endogenous ACE2 in β-cell insulin production and/or secretion. However, since our studies were performed in mice with whole body ACE2 deficiency, it is unclear whether blunted hyperinsulinemia from HF feeding in ACE2 deficient mice results from direct or indirect effects of ACE2 at the pancreas. Taken together, results suggest a novel role for ACE2 in the regulation of glucose homeostasis in type 2 diabetes.

An important finding of the present study was the ability of ACE2 deficiency to markedly promote systolic hypertension in HF-fed mice. Plasma concentrations of Ang-(1-7) were reduced in obese mice, suggesting that reductions in this peptide contribute to obesity-hypertension. However, HF-fed *Ace2*$^{-/y}$ mice exhibited similar plasma Ang-(1-7) concentrations as HF-fed controls, but had greater blood pressures, suggesting that reduced concentrations of Ang-(1-7) alone are insufficient to promote obesity-hypertension. Previous studies from our laboratory demonstrated that mice with diet-induced obesity and hypertension exhibited an increase in plasma concentrations of AngII (Gupte et al. 2008). Of note, in this study plasma AngII concentrations were further augmented by HF feeding in ACE2 deficient mice. Based on results demonstrating amelioration of obesity-hypertension upon losartan administration, augmented obesity-induced hypertension in ACE2 deficient mice most likely resulted from increased plasma concentrations of AngII acting at AT1 receptors in cardiovascular target organs.

In conclusion, results from this study demonstrate that ACE2 deficiency augments obesity-hypertension in mice. Elevated concentrations of plasma AngII, and amelioration of augmented hypertension with losartan, suggest that effects of ACE2 deficiency to promote obesity-hypertension are AngII/AT1 receptor-mediated. Interestingly, glucose and insulin homeostasis were impaired in ACE2 deficient mice challenged with a HF diet. Results from this study suggest that ACE2 plays a protective role against the development of obesity-associated diabetes and hypertension. Therapeutic targeting of ACE2 activation,

and/or nutritional methods to modulate ACE2 expression in pivotal sites, like adipose tissue, may serve as a novel strategy for treating obesity-associated diseases.

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Table 4.1. Characteristics of *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet.

	Ace2$^{+/y}$ LF	Ace2$^{+/y}$ HF	Ace2$^{-/y}$ LF	Ace2$^{-/y}$ HF
Lean mass (g)	20.2 ± 0.6	25.0 ± 0.3 *	19.6 ± 0.3	23.1 ± 0.4 *,†
Fat mass (g)	6.5 ± 1.3	18.60 ± 0.9 *	3.3 ± 0.3†	18.6 ± 1.2 *
RPF (%body weight)	1.0 ± 0.1	2.7 ± 0.2*	0.7 ± 0.0	2.5 ± 0.2*
Kidney(%bodyweight)	1.2 ± 0.0	0.8 ±. 0 *	1.2 ± 0.0	0.8 ± 0.0 *

RFP, retroperitoneal fat.

Data are mean ± SEM from n = 20/group.

*, P<0.05 compared to LF within genotype.

†,P<0.05 compared to *Ace2*$^{+/y}$ within diet.

Table 4.2. 24h Diastolic Pressure, Pulse Pressure and Heart Rate of *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet.

	Ace2$^{+/y}$ LF	*Ace2*$^{+/y}$ HF	*Ace2*$^{-/y}$ LF	*Ace2*$^{-/y}$ HF
Diastolic Pressure (mmHg)	94 ± 2.3	100.0 ± 2.2	93.1 ± 1.0	111.0 ± 6.1*
Mean Pressure (mmHg)	107.9 ± 1.8	117.5 ± 1.2	106.9 ± 2.1	125.8 ± 4.7*
Pulse Pressure (mmHg)	30.5 ± 2.5	34.7 ± 3.0	34.6 ± 1.6	34.1 ± 2.3
Heart Rate (beats per minute)	585.8 ± 5.0	602.5 ± 16.0	607.5 ± 9.0	603.2 ± 7.8

Data are mean ± SEM from n = 8-10 mice/group.

*, $P < 0.05$ LF vs HF within genotype.

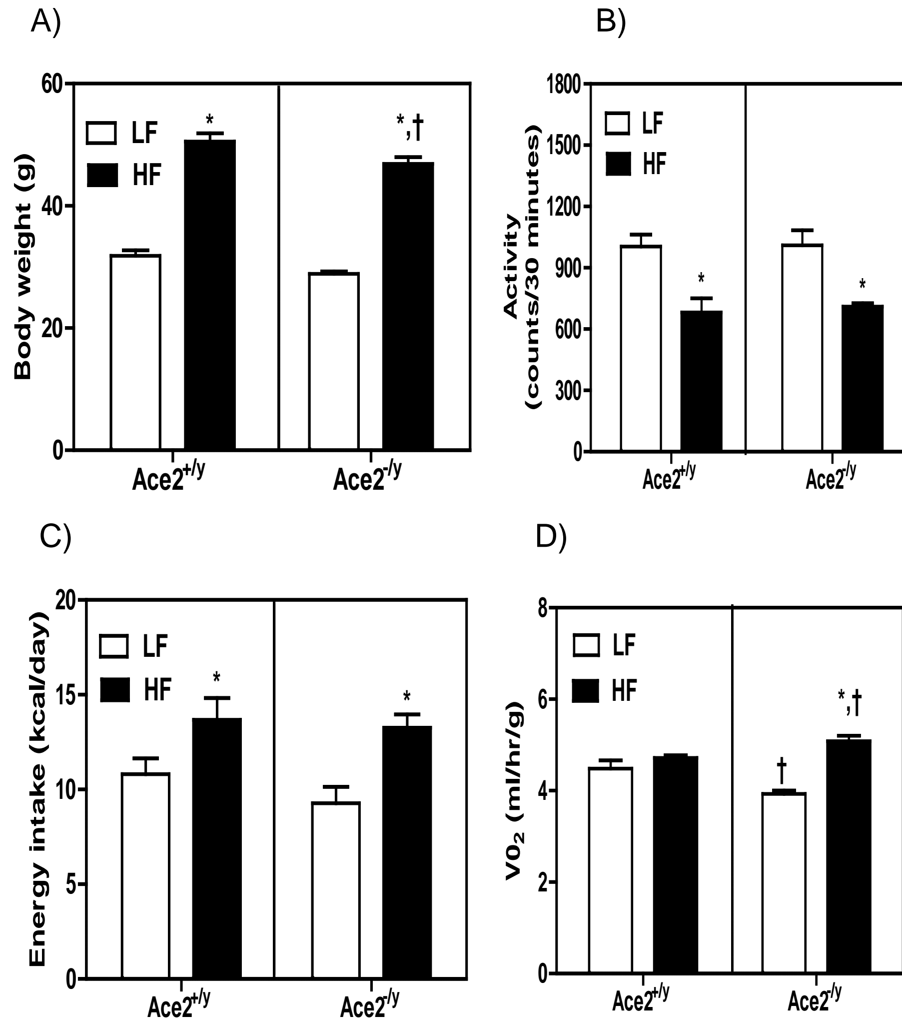


Fig. 4.1. A. Body weight at 16 weeks in *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet, B. Physical activity at 16 weeks in *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet, C. Energy intake at 16 weeks in *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet, D. Oxygen consumption at 16 weeks in *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet. Data are mean $\pm$ SEM from n = 20 mice/group (A) or n = 5 mice/group (B-D). *, significantly different from LF within genotype, $P<0.05$; †, significantly different from *Ace2*$^{+/y}$ within diet, $P<0.05$.

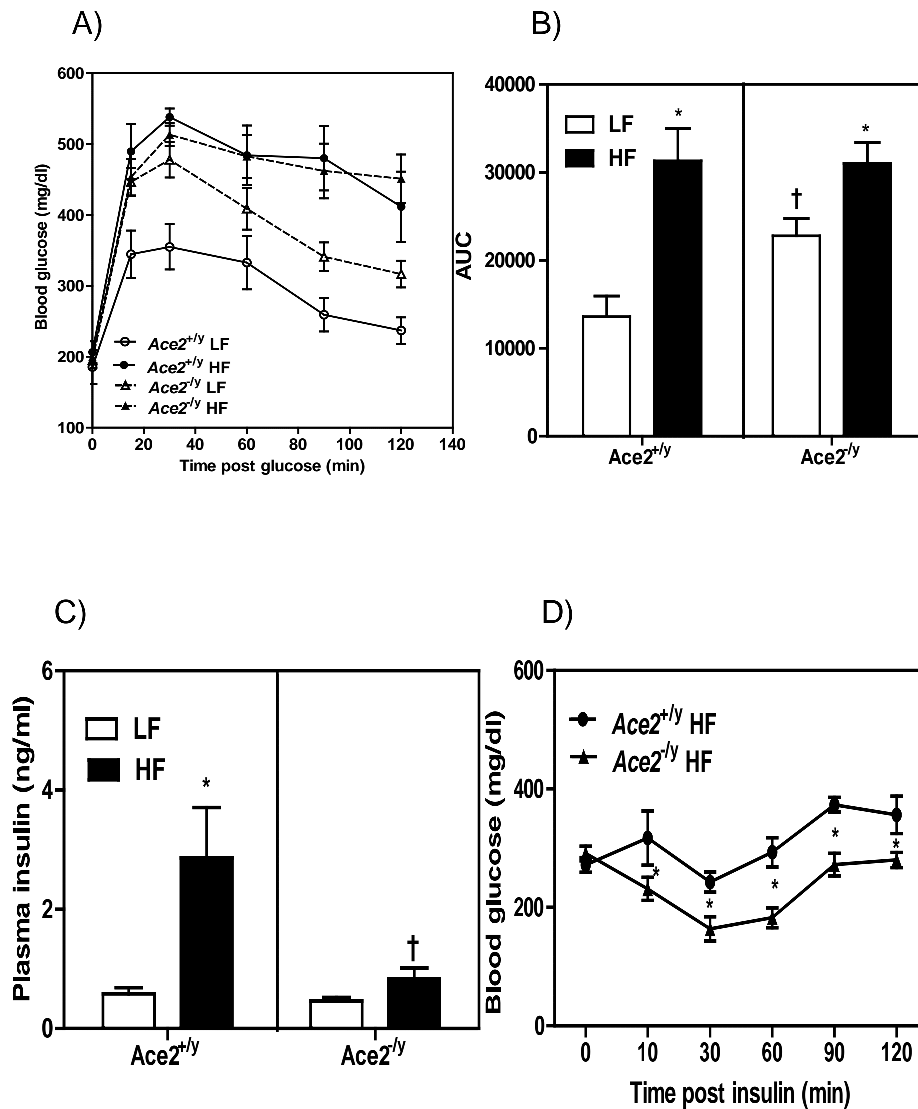
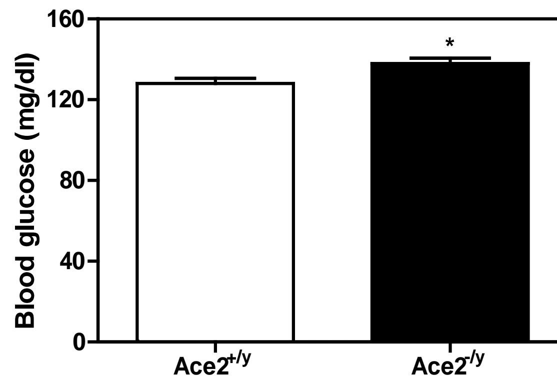


Fig. 4.2. A. Glucose tolerance tests in *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a LF or HF diet. B. Area under the curve (AUC) for glucose tolerance tests performed in A. C. Plasma insulin concentrations at study endpoint in mice from each group. D. Insulin tolerance tests in *Ace2*$^{+/y}$ and $^{-/y}$ mice fed a HF diet. Data are mean ± SEM from n = 8 mice/group. *, significantly different from LF within genotype, P<0.05; † , significantly different from *Ace2*$^{+/y}$ within diet, P<0.05.

A)



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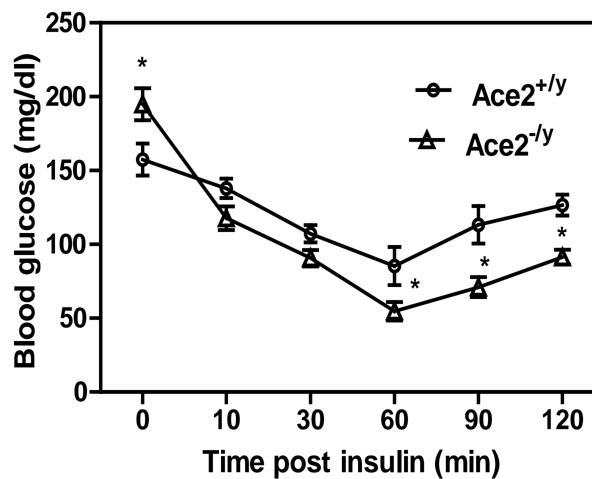

Fig. 4.3. A. Fasting blood glucose in *Ace2*$^{+/y}$ and $^{-/y}$ male mice (8 weeks of age) fed standard diet, B. Insulin tolerance tests in *Ace2*$^{+/y}$ and $^{-/y}$ male mice (8 weeks of age) fed standard diet. Data are mean $\pm$ SEM from n = 20 mice/group (A) or n = 5 mice/group (B).*, P<0.05 compared to *Ace2*$^{+/y}$.

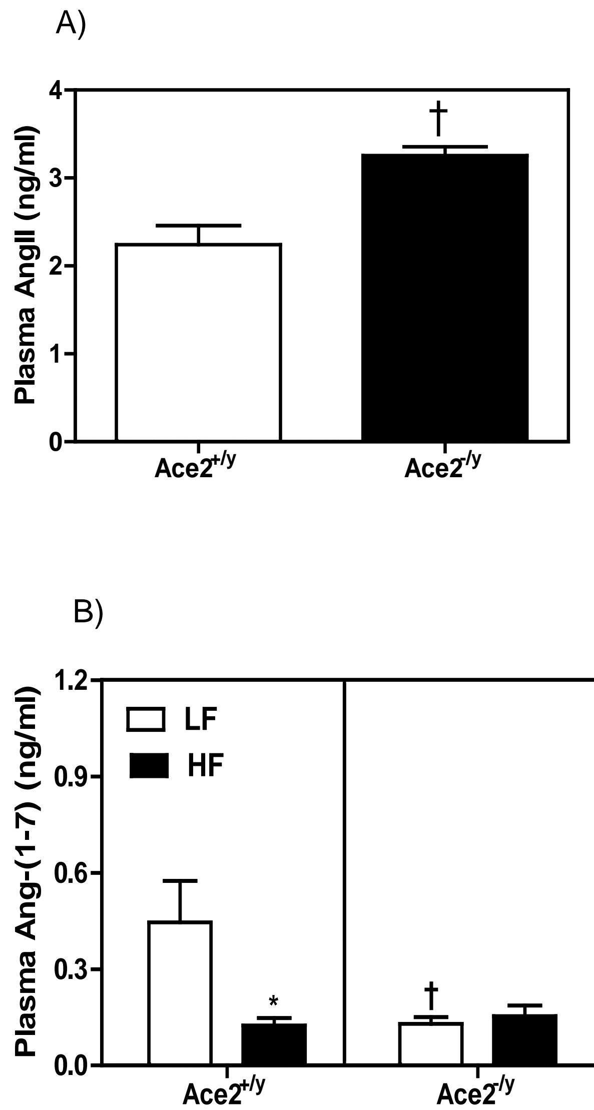

Fig .4.4. A. Plasma concentrations of AngII in HF-fed *Ace2*$^{+/y}$ and $^{-/y}$ mice. B. Plasma concentrations of Ang-(1-7) in LF and HF-fed *Ace2*$^{+/y}$ and $^{-/y}$ mice. Data are mean ± SEM from n = 8-10 mice/group. *, significantly different from LF within genotype, $P<0.05$; † , significantly different from *Ace2*$^{+/y}$ within diet, $P<0.05$.

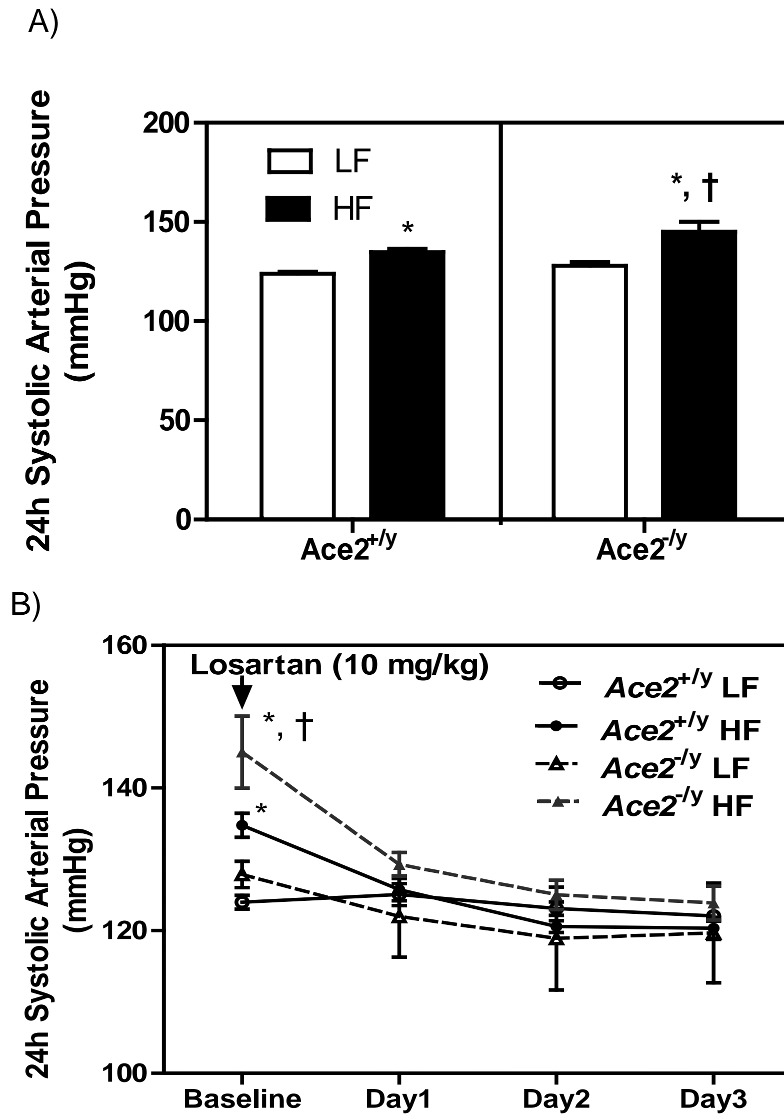

Fig. 4.5. A. Systolic arterial pressures (24 hours of recording) in LF and HF fed *Ace2*$^{+/y}$ and $^{-/y}$ mice, B. Systolic arterial pressures (24 hours of recording) in LF and HF fed *Ace2*$^{+/y}$ and $^{-/y}$ mice before (baseline) and after 3 days of losartan administration. Data are mean $\pm$ SEM from n = 5 mice/group. *, significantly different from LF within genotype, P<0.05; †, significantly different from *Ace2*$^{+/y}$ within diet, P<0.05.

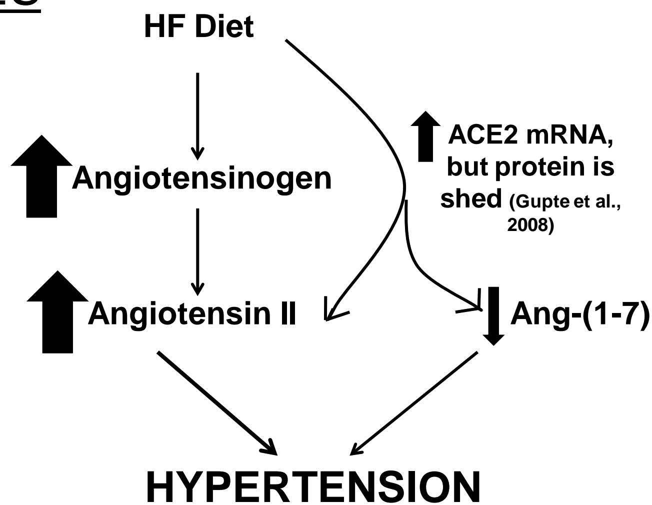

Fig. 4.6. Working model of obesity-induced hypertension in *Ace2*$^{+/y}$ male mice.

Section V. SPECIFIC AIM 2 B

Determine the effects of ACE2 deficiency on the development of obesity and hypertension in female C57BL/6 mice.

5.1. Summary.

Previous studies in males demonstrated that initial increases in adipose ACE2 expression with high fat (HF) feeding were followed by dysregulation of ACE2 during chronic obesity associated with increased systemic angiotensinII (AngII), lower angiotensin-(1-7) (Ang-(1-7) and hypertension. These results suggested that ACE2 initially protects males against obesity-hypertension, but that with chronic obesity protective effects of ACE2 are lost and mice exhibit obesity-hypertension. In this study we defined effects of ACE2 deficiency on the development of obesity-induced hypertension in females. Since studies in humans demonstrate a lower incidence of obesity-hypertension in pre-menopausal women compared with age-matched men, we hypothesized that females would be protected against obesity-hypertension. Moreover, previous investigators demonstrated that ACE2 activity and protein in kidney were down regulated by ovariectomy (Ovx) of female rats, suggesting that estradiol is a positive modulator of ACE2. Thus, in this study we defined effects ACE2 deficiency in the absence or presence of Ovx on the development of obesity-induced diabetes and hypertension. Female C57BL/6 *Ace2*$^{+/+}$ or $^{-/-}$ mice were fed a low fat (LF, 10% kcal as fat) or a HF diet (60% kcal as fat) for 16 weeks. A

subset of HF-fed *Ace2*$^{+/+}$ or $^{-/-}$ females underwent sham or Ovx surgery prior to beginning the diet. Body weight increased with HF feeding in *Ace2*$^{+/+}$ and $^{-/-}$ females; however, HF-fed *Ace2*$^{-/-}$ females had lower body weights than controls. In addition, Ovx significantly increased body weights in HF-fed *Ace2*$^{+/+}$ and $^{-/-}$ mice; however, differences in body weights between *Ace2*$^{-/-}$ and $^{+/+}$ mice remained. With HF feeding, both genotypes exhibited glucose intolerance, which was further augmented by Ovx. HF- feeding reduced plasma renin concentrations in *Ace2*$^{+/+}$ and $^{-/-}$ mice compared to controls. Importantly, Ovx further decreased plasma renin concentrations in *Ace2*$^{+/+}$ and $^{-/-}$ mice. Plasma concentrations of the catabolic product of ACE2, Ang-(1-7), were elevated in female *Ace2*$^{+/+}$ mice fed a HF diet; however, this effect was lost with Ovx and was not present in female *Ace2*$^{-/-}$ mice. Systolic (SBP) (*Ace2*$^{+/+}$, LF: 117±3, HF: 122±2 mmHg, $P>0.05$) and diastolic blood pressures (DBP) (*Ace2*$^{+/+}$, LF: 90±2, HF: 92±2 mmHg , $P>0.05$) were not influenced by HF feeding in *Ace2*$^{+/+}$ mice fed a HF diet compared to LF-fed controls. In contrast, *Ace2*$^{-/-}$ females fed a HF-diet exhibited a significant increase in SBP (*Ace2*$^{-/-}$, LF: 126±1, HF: 134±2 mmHg, $P<0.05$) and DBP (*Ace2*$^{-/-}$, LF: 95±2, HF: 101±2 mmHg) compared to LF-fed controls. Ovx significantly increased SBP in HF-fed *Ace2*$^{+/+}$ mice compared to LF controls to a level comparable to HF-fed *Ace2*$^{-/-}$ mice (*Ace2*$^{+/+}$ Ovx: 134± 3.3, *Ace2*$^{-/-}$ sham: 134±2 mmHg, $P>0.05$). In contrast, SBP and DBP were reduced in HF-fed Ovx *Ace2*$^{-/-}$ females compared to sham-operated *Ace2*$^{-/-}$ females. To define mechanisms for reductions in blood pressure in Ovx *Ace2*$^{-/-}$ females fed a HF diet, we used ultrasound and demonstrated contractile dysfunction as

indicated by reduction in ejection fraction and fractional shortening. These results suggest that ACE2 protects females against obesity-induced hypertension through an estradiol-dependent mechanism. Reductions in cardiac function in Ovx *Ace2*$^{-/-}$ mice fed a HF diet may arise from marked activation of the systemic renin-angiotensin system.

5.2. Introduction.

The prevalence of obesity is rising at an epidemic rate in the United States. With obesity, there is a marked increase in cardiovascular pathologies such as hypertension, coronary artery disease, stroke, type 2 diabetes and certain types of cancer. A recent report by the National Health and Nutrition Examination Survey estimates 32% of men and 36% of women exhibit a BMI of ≥ 30 (K Flegal et al. 2010). Though females develop hypertension during their lifetime, for adults under 45 years of age the prevalence of hypertension is higher in males whereas after 65 years the prevalence is higher in females (National Center for Health Statistics. Health, United States, 2008). Given that obesity is more prevalent in females compared to males, it is likely that sex hormones, namely estradiol in females, protects premenopausal women against obesity-hypertension in spite of a higher body mass index (BMI).

Sex hormones have been demonstrated to regulate components of the renin-angiotensin system (RAS; Yanes et al. 2010; Fischer et al. 2002). In general, estrogen has been suggested to blunt expression of each component of the RAS, with the exception of angiotensinogen, while androgen has been reported to stimulate RAS components (Fischer et al.2002). These sex-mediated differences have been suggested to contribute to a greater blood pressure response to chronically infused angiotensinII (AngII) in male compared to female mice (Xue et al. 2007; Xue et al. 2005), or in the greater blood pressure of male compared to female spontaneously hypertensive rats (SHR; Reckelhoff et al. 2000; Radin et al. 2002).

Angiotensin Converting Enzyme 2 (ACE2) converts AngII, a vasoconstrictor peptide, to angiotensin (1-7) (Ang-(1-7)), a vasodilator peptide. Through these effects, ACE2 has been suggested to blunt the RAS and protect against hypertension induced by infusion of AngII (Gurley et al. 2006), or from diet-induced obesity (Gupte et al. 2008). A recent study in SHR suggested that the catabolic product of ACE2, Ang-(1-7), mediates differences in blood pressure between male and female rats infused with AngII (Sullivan et al. 2010). In addition, ovariectomy (Ovx) of females rats reduced kidney ACE2 expression and increased renal hypertension in a rat renal ablation model, and these effects were reversed when Ovx rats were administered estrogen (Ji et al. 2008). Collectively, these results suggested that estrogen-mediated regulation of ACE2 may contribute to differences in blood pressure between males and females.

In spite of a strong association between obesity and hypertension, the mechanistic link between the two pathologies is yet unclear. Consumption of a high fat (HF) diet, coupled with a sedentary lifestyle, has been suggested as an environmental factor contributing largely to the increased prevalence of obesity. In male and female rats fed a cafeteria diet containing 49% fat, despite a similar increase in fat pad weight, only male mice exhibited an increased blood pressure (Plut et al. 2002). Previous results from our laboratory demonstrated that diet-induced obesity in male rats was associated with an activated RAS and hypertension, which was abolished by administration of an AT1 receptor antagonist (Boustany et al. 2004; Boustany et al. 2005). In addition, we demonstrated that obesity-induced hypertension and an activated systemic RAS

in male mice fed a HF diet were accompanied by diet-induced dysregulation of ACE2 in adipose tissue (Gupte et al. 2008). These results support a role for the RAS, and specifically ACE2, in the development of obesity-related hypertension. Unfortunately, very few studies have focused on sex differences in the RAS as a potential contributor to differences in susceptibility between males and females to obesity-induced hypertension.

The purpose of the present study was to define (1) if females are protected against the development of obesity-related hypertension, (2) if ACE2 deficiency confers obesity-hypertension in female mice, (3) if Ovx promotes obesity-hypertension in female mice, and (4) if Ovx augments obesity-hypertension in ACE2 deficient females. The hypothesis of these studies is that sex hormones protect female mice against the development of obesity-hypertension through an ACE2-dependent mechanism.

5.3. Methods.

5.3.1. Animals and diets.

Female *Ace2*$^{+/+}$ (C57BL/6, 2 months of age; The Jackson Laboratory, Bar Harbor, MA) and $^{-/-}$ mice (2 months of age; backcrossed 10 times onto a C57BL/6 background ; Gurley et al. 2006) were fed a low fat (LF;10% kcal as fat; D12450B; Research Diets, Inc, New Brunswick, NJ; n=20) or HF diet (60% kcal as fat; D12492, Research Diets, Inc, New Brunswick, NJ; n=30) for sixteen weeks. Diets were matched in protein content (20% kcal) and provided energy at 3.85 or 5.25 kcal/gm (LF and HF, respectively). Diets were provided to mice *ad libitum*. A subset of *Ace2*$^{+/+}$ and $^{-/-}$ mice (n=10) underwent either sham surgery or ovariectomy (Ovx) and were fed a HF-diet for 16 weeks. All experiments involving mice conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Kentucky Institutional Animal Care and Use Committee.

5.3.2. Glucose tolerance tests.

Fasting (6 hr) blood glucose concentrations were measured using a One Touch glucometer. Glucose tolerance was measured in fasted mice (6 hours) injected with glucose intraperitoneally at a dose of 2 g/kg body weight. Blood glucose concentrations were measured at times 0, 15, 30, 60, 90 and 120 minutes after the injection.

5.3.3. Measurement of plasma parameters.

Ang-(1-7) concentrations (Peninsula laboratories, LLC Bachem group, CA, USA) were quantified using commercial kits. Plasma renin concentrations were measured as described previously (Cassis et al. 2004).

5.3.4. Measurement of blood pressure.

Blood pressure was measured by radiotelemetry during week 16 according to previously described methods (Gupte et al. 2008). Briefly, mice were implanted with carotid artery catheters during week 15 of LF or HF feeding, allowed one week to recover, and then pressure was recorded continuously (5 minute sampling) for 3 days.

5.3.5. Echocardiography methods.

Mice were placed under isoflurane gas and measurements of the heart were taken with a VisualSonics 660 ultrasound using a RMV 707 30MHz probe. Images of the left ventricle were acquired in short axis parasternal view at the level of the papillary muscle in M-mode. Measurements were made in B-mode and analysis was done through Vevo 660 Analytic Software.

5.3.6. Statistical analysis.

Data are expressed as mean ± SEM. All data were analyzed using Sigma Stat for equal variance. For data with equal variance, two- way

ANOVA was used to analyze end-point measures followed by Tukey's test for post-hoc analysis. Significance was accepted at a $P<0.05$.

5.4. Results.

5.4.1. Metabolic characteristics of *Ace2*$^{+/+}$ and $^{-/-}$ female mice fed LF or HF diets.

Ace2$^{+/+}$ mice exhibited significant increases in body weights when fed a HF diet which were further increased by Ovx compared to LF-fed controls (Fig. 5.1 A, B). Both HF-diet and Ovx significantly increased fat as well as lean mass in *Ace2*$^{+/+}$ mice. In spite of a significant increase in body weights of HF-fed *Ace2*$^{-/-}$ female mice, ACE2 deficient mice exhibited significantly lower body weights, fat and lean mass compared to HF-fed controls (Table 5.1). Additionally, differences between *Ace*2$^{-/-}$ and $^{+/+}$ females remained with Ovx (Table 5.1).

Previous investigators have reported hyperglycemia in *Ace2*$^{-/-}$ mice fed a standard diet (Bindom et al. 2009). Moreover, HF feeding in male mice promotes glucose intolerance and type 2 diabetes. Thus, we examined effects of ACE2 deficiency and Ovx on glucose tolerance in female HF-fed mice. At 8 weeks of age, *Ace2*$^{-/-}$ female mice fed standard diet exhibited elevated fasting blood glucose compared to controls (*Ace2*$^{+/+}$,115 $\pm$ 4; *Ace2*$^{-/-}$, 134 $\pm$ 4 mg/dl; $P<0.05$). Moreover, at 16 weeks of HF feeding, *Ace2*$^{+/+}$ *and* $^{-/-}$ mice were glucose intolerant compared to LF-fed controls (Fig. 5.2). However, glucose intolerance was blunted in HF-fed *Ace2*$^{-/-}$ females with lower body weights compared to controls. Ovx resulted in further impairment of glucose tolerance in HF-fed *Ace2*$^{+/+}$, but not $^{-/-}$ females (Fig. 5.2 A). HF fed *Ace2*$^{+/+}$ and Ovx *Ace2*$^{+/+}$ mice exhibited significant increases in plasma insulin concentrations and glucose intolerance, however these effects were blunted in *Ace2*$^{-/-}$ mice (Fig. 5.2 B).

5.4.2. Systemic RAS in LF and HF fed *Ace2*$^{+/+}$ and $^{-/-}$ Mice.

We measured concentrations of renin in plasma as a relatively stable inverse predictor of plasma concentrations of the labile peptide, AngII. HF-diet significantly decreased plasma renin concentrations in *Ace2*$^{+/+}$ mice, which were reduced further in HF-fed Ovx females (*Ace2*$^{+/+}$, LF: 12.7 ± 1.4; HF: 5.9 ± 1.2; HF/Ovx: 1.1 ± 0.2 pg/ml/30 min, $P<0.05$, Fig. 4.3 A). In *Ace2*$^{-/-}$ females, reductions in plasma renin concentrations by HF feeding were augmented (*Ace2*$^{-/-}$, LF: 9.2 ± 1.3; HF: 3.1 ± 0.4; HF/Ovx: 1.4 ± 0.4 pg/ml/30 min, $P<0.05$, Fig. 5.3 A). Plasma concentrations of Ang-(1-7) were increased by HF feeding in *Ace2*$^{+/+}$ females. However, this effect was absent in HF-fed Ovx *Ace2*$^{+/+}$ females (*Ace2*$^{+/+}$, LF: 0.21 ± 0.0; HF: 0.32 ± 0.0; HF/Ovx: 0.21 ± 0.0 ng/ml). As anticipated, HF-feeding did not promote increases in plasma concentrations of Ang-(1-7) in *Ace2*$^{-/-}$ females (Fig 5.3 B). Of note, deficiency of ACE2 in LF-fed female mice did not reduce plasma concentrations of Ang-(1-7).

5.4.3. Blood pressure in LF and HF fed *Ace2*$^{+/+}$ and $^{-/-}$ Mice.

Obese HF-fed *Ace2*$^{+/+}$ mice did not exhibit an increase in systolic (SBP), diastolic (DBP) or mean (MAP) arterial pressure compared to LF-fed controls (Fig. 5.4 A, Table 5.2). In contrast, ovariectomized HF-fed *Ace2*$^{+/+}$ females exhibited significant elevations in SBP, DBP and MAP compared to LF-fed controls (Fig.5.4 A, Table 5.2). In addition, *Ace2*$^{-/-}$ fed a HF diet exhibited significant elevations in SBP compared to LF-fed *Ace2*$^{-/-}$ mice and compared to HF-fed *Ace2*$^{+/+}$ controls. Surprisingly, in HF-fed *Ace2*$^{-/-}$ females, SBP and DBP

were significantly reduced by Ovx compared to non-ovariectomized females (Fig. 5.4 A). Since previous studies have demonstrated a reduction in SBP in 6 month old male and female ACE2 deficient mice due to severe contractile dysfunction (Crackower et al. 2002), we examined heart function in *Ace2*$^{-/-}$ females. In *Ace2*$^{-/-}$ females that were ovariectomized, ejection fraction and fractional shortening were reduced compared to sham-operated controls (Table 5.3).

5.5. Discussion.

Our results demonstrate that female mice are protected against obesity-induced hypertension. While C57BL/6 female mice exhibited marked development of obesity from chronic HF feeding including the development of obesity-induced diabetes, they were totally resistant to obesity-induced hypertension. Resistance to obesity-induced hypertension in female mice was associated with an increase in plasma concentrations of the vasodilator peptide Ang-(1-7), and marked reductions in plasma renin concentrations indicative of elevated systemic AngII. Of note, ovariectomy of *Ace2*$^{+/+}$ females conferred obesity-hypertension. Moreover, elevations in plasma Ang-(1-7) concentrations were abolished by ovariectomy in HF-fed *Ace2*$^{+/+}$ female mice exhibiting obesity-hypertension, suggesting that elevated plasma levels of Ang-(1-7) protected females against obesity-hypertension. In support, ACE2 deficient female mice fed a HF diet exhibited obesity-hypertension at a level similar to that observed in Ovx-*Ace2*$^{+/+}$ controls. Moreover, the ability of ovariectomy to confer obesity-hypertension was lost in *Ace2*$^{-/-}$ females. On the contrary, ovariectomized *Ace2*$^{-/-}$ females exhibited reduced blood pressures and cardiac dysfunction compared to HF-fed sham controls. These results demonstrate a pivotal role for sex hormones and ACE2 in protection against obesity-hypertension in female mice.

Several studies have demonstrated that sex hormones regulate various components of the RAS important in blood pressure control, collectively favoring a stimulated RAS in males compared to females. Specifically, estradiol has been demonstrated to decrease tissue levels of AngII (Senanayake et al. 1998; Wu et

al. 2003), ACE expression and activity (Brosnihan et al. 1997; Seltzer et al. 1992), and AT1 receptor expression (Rogers et al. 2007). Conversely, estradiol promotes expression of angiotensinogen (Feldmer et al.1991; Clauser et al. 1989), making it difficult to predict the overall effects of estradiol on the RAS and blood pressure control. However, chronic infusion of AngII resulted in a greater blood pressure increase in male compared to female mice (Xue et al. 2005). Further studies demonstrated that administration of an estrogen receptor (ER) antagonist to female mice, or use of female mice with ERα deficiency, augmented the blood pressure response to AngII (Xue et al. 2007). In a recent study in male and female SHR though the magnitude of increase in blood pressure with AngII infusion was higher in males compared to females, plasma and renal concentrations of AngII were not different between the sexes (Sullivan et al. 2010). Importantly, female rats exhibited significantly higher plasma concentrations of Ang-(1-7) levels at baseline as well as after AngII infusion, and increased expression of the Mas receptor. Administration of a Mas receptor antagonist eliminated differences in blood pressure between the two sexes. These results demonstrated a protective role of Ang-(1-7) in AngII-induced hypertension in females (Sullivan et al. 2010). While considerable studies support sex differences in the RAS that may contribute to different susceptibilities to hypertension between males and females, our studies are the first to define mechanisms for sex differences in the context of obesity-related hypertension.

Results from this study demonstrate that female mice are protected against the development of obesity-hypertension, but that ovariectomy of females

can confer obesity-induced hypertension. Previous investigators have demonstrated that ovariectomy augments blood pressure elevations of female mice infused with AngII (Xue et al. 2005, 2007). In this study, plasma concentrations of renin were reduced in HF-fed females, indicative of increased concentrations of plasma AngII. However, HF-fed females did not exhibit hypertension despite marked obesity, potentially resulting from HF-induced elevations in plasma Ang-(1-7). In support, previous studies in male mice fed a HF diet and exhibiting obesity-hypertension demonstrated reductions in plasma concentrations of Ang-(1-7) (Gupte et al. 2010), suggesting that elevations in concentrations of this vasodilatory peptide in females may protect against obesity-hypertension. Indeed, ACE2 deficient females exhibiting obesity-hypertension in this study did not exhibit an increase in plasma concentrations of Ang-(1-7). Mechanisms for increased plasma Ang-(1-7) concentrations in HF-fed females are unknown; however, it is conceivable that HF diet-induced increases in adipose ACE2 expression (Gupte et al. 2008) are maintained by estradiol in female mice.

A recent study demonstrated a protective role for ACE2 in kidneys of female rats in a renal wrap model of hypertension (Ji et al. 2008). In ovariectomized female rats, ACE2 activity and protein in renal cortex were reduced. However, repletion with 17-β estradiol in ovariectomized female rats normalized renal ACE2 activity and protein and protected the rats against renal hypertensive disease (Ji et al. 2008). In the present study, ovariectomy of female mice conferred obesity-hypertension, and eliminated HF diet-induced increases

in plasma concentrations of the ACE2 vasodilator product, Ang-(1-7). If ovariectomy and ACE2 deficiency exerted distinct mechanisms to confer obesity-hypertension in females, we would have anticipated additive effects of these parameters on obesity-related hypertension. Rather, we observed a reduction in blood pressures of HF-fed *Ace2*$^{-/-}$ females with ovariectomy, suggesting that ACE2 and ovariectomy promote obesity-hypertension in female mice through a similar mechanism.

An interesting finding of the present study was the observed reductions in blood pressure of HF-fed Ovx *Ace2*$^{-/-}$ mice. To define mechanisms for reduced pressures, we examined cardiac function and demonstrated reduced ejection fraction and fractional shortening in ovariectomized HF-fed ACE2 deficient females. One of the first studies using ACE2 deficient mice demonstrated severe reductions in cardiac contractility in 6 month old male and female *Ace2*$^{-/-}$ mice (Crackower et al. 2002). Reductions in cardiac output from elevated cardiac and systemic AngII concentrations were demonstrated to contribute to decreased SBP in 6 month old ACE2 deficient male mice. Several additional studies have followed up on this finding and demonstrated a role for ACE2 in the regulation of heart function (Nakamura et al. 2008, Zhong et al. 2010). In the present study we demonstrate a similar phenotype of cardiac dysfunction associated with reduced SBPs in ovariectomized ACE2 deficient female mice with chronic obesity. Chronic activation of the systemic RAS, as evidenced by low plasma renin concentrations, coupled with a loss of stimulated plasma concentrations of Ang-

(1-7) in ovariectomized ACE2 deficient females may have contributed to cardiac dysfunction and the observed decrease in blood pressure.

In conclusion, these results demonstrate that female mice are protected against obesity-hypertension, but not diabetes. Ovariectomy, or ACE2 deficiency, confers obesity-induced hypertension in females. Moreover, a lack of additive effects of ovariectomy and ACE2 deficiency on blood pressure control in HF-fed female mice suggests similar mechanisms for protection by estradiol and ACE2 against obesity-hypertension in females. These results suggest that female mice are protected against obesity-hypertension through estradiol regulation of ACE2.

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Table 5.1. Characteristics of *Ace2*$^{+/+}$ and $^{-/-}$ mice fed a LF or HF diet and effects of ovariectomy (Ovx).

	LF		HF		HF/Ovx	
	Ace2$^{+/+}$	*Ace2*$^{-/-}$	*Ace2*$^{+/+}$	*Ace2*$^{-/-}$	*Ace2*$^{+/+}$	*Ace2*$^{-/-}$
Body weight (g)	22 ±0.4	22 ±0.3	39 ± 1.2*	34 ± 0.9*,**	49 ± 2.0†	40 ± 2.0$^{†,\wedge}$
Fat mass (g)	2.2±0.2	2.6±0.4	13 ± 1.0*	10 ± 0.8*,**	17 ± 1.5†	12 ± 1.7$^{\wedge}$
Lean mass (g)	15 ±0.7	14 ±0.1	17 ± 0.3*	16 ± 0.5*,**	19 ± 0.8†	15 ± 0.6$^{\wedge}$
RPF (% body weight)	0.6±0.0	0.9±0.0	3.6 ± 0.2*	2.9 ± 0.4*	3.8 ± 0.2	2.3 ± 0.4
Kidney (% body weight)	1.2±0.0	1.2±0.0	0.8 ± 0.0*	1.0 ± 0.0*,**	0.8 ± 0.0	0.9 ± 0.0
Heart (% body weight)	0.5±0.0	0.5±0.0	0.4 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.4 ± 0.0

RPF, retroperitoneal fat.

Data are mean ± SEM from n = 10-15 mice/group.

*, P<0.05 compared to LF within genotype

**, P<0.05 compared to genotype within diet

†, P<0.05 HF vs HF/Ovx within genotype

^ , P< 0.05 compared to *Ace2*$^{+/+}$ within HF/Ovx

Table 5.2. Hemodynamic characteristics of *Ace2*$^{+/+}$ and $^{-/-}$ female mice fed a LF or HF diet and effects of ovariectomy (Ovx).

	LF		HF		HF/Ovx	
	Ace2$^{+/+}$	*Ace2*$^{-/-}$	*Ace2*$^{+/+}$	*Ace2*$^{-/-}$	*Ace2*$^{+/+}$	*Ace2*$^{-/-}$
SBP(mmHg)	117 ± 3	126±1**	122 ± 2	134 ± 2*,**	134±3.3†	124 ± 3$^{\dagger,\wedge}$
DBP(mmHg)	90 ± 3	95 ± 2	92 ± 2	101 ± 2**	102 ± 3†	88 ± 3$^{\dagger,\wedge}$
MAP(mmHg)	102 ± 3	110 ± 1	106 ± 2	115 ± 2**	116 ± 3†	106 ± 2$^{\dagger,\wedge}$
Heart Rate (bpm)	595±21	618 ±8*	639 ± 7	638 ± 5.3	620 ±9.4	647 ± 6
Pulse Pressure	23 ± 3	32 ±2**	29 ± 2	33 ± 2	32 ± 2	36 ± 3
Activity	7 ± 0	7 ± 1	8 ± 1	6 ± 1	5 ± 0	4 ± 0

SBP, Systolic blood pressure; DBP, Diastolic blood pressure; MAP, Mean arterial pressure

Data are mean ± SEM from n = 10-15 mice/group. *, P<0.05 compared to LF within genotype; **, P<0.05 compared to genotype within diet; †, P<0.05 HF vs HF/Ovx within genotype; ^ , P< 0.05 compared to *Ace2*$^{+/+}$ within HF/Ovx

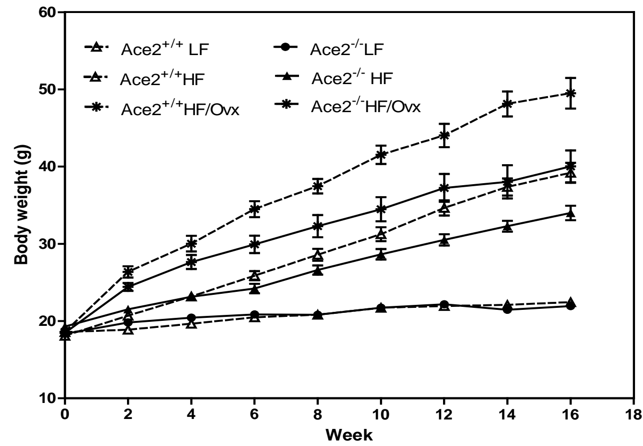
Table 5.3. Cardiac function in HF-fed *Ace2*$^{-/-}$ female mice with and without ovariectomy (Ovx).

	Ace2$^{-/-}$ HF	*Ace2*$^{-/-}$ HF/Ovx
Heart rate (b.p.m)	656.8 ± 3.5	656.5 ± 7.4
IVS s	1.09 ± 0.03	1.07 ± 0.07
IVS d	0.81 ± 0.03	0.78 ± 0.04
LVID s	2.58 ± 0.05	2.49 ± 0.17
LVID d	3.74 ± 0.08	3.35 ± 0.24
LVPW s	1.28 ± 0.10	1.18 ± 0.09
LVPW d	0.91 ± 0.10	1.08 ± 0.07
%EF	59.56 ± 0.76	51.61 ± 1.8 *
%FS	31.05 ± 0.55	25.63 ± 1.1*

Data are mean ± SEM from n = 5 mice/group.

*, P<0.05 compared to HF.

A)



B)

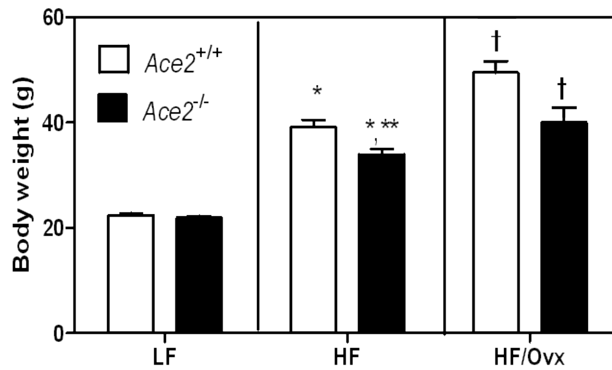
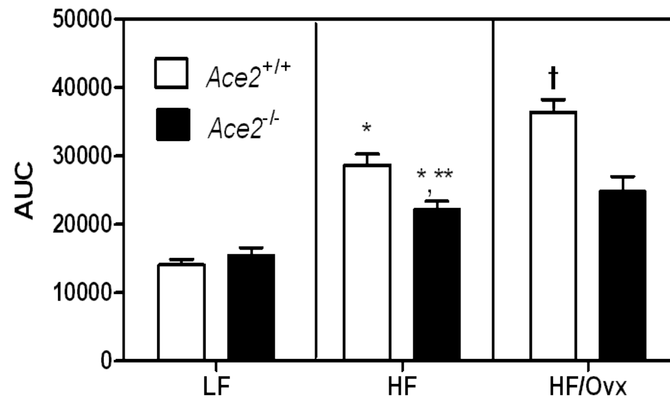


Fig. 5.1. A. Body weight progression in *Ace2*$^{+/+}$ and $^{-/-}$ female mice fed a LF or HF diet. B. Body weight at week 16 in *Ace2*$^{+/+}$ and $^{-/-}$ female mice fed a LF or HF diet. (n = 10-15 mice/group). *, significantly different from LF within genotype; **, significantly different from *Ace2*$^{+/+}$ within diet; †, significantly different from HF vs HF/Ovx , P<0.05.

A)



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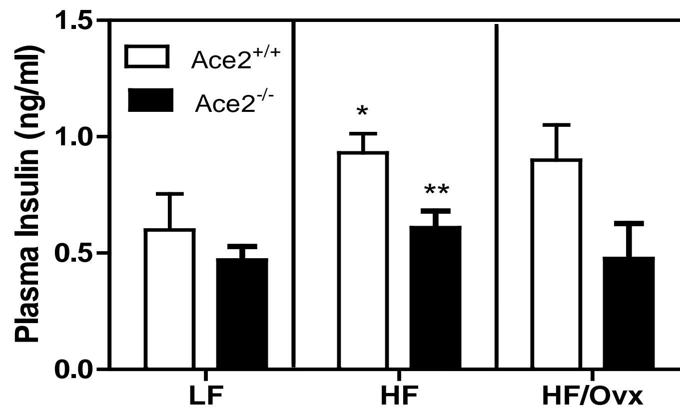
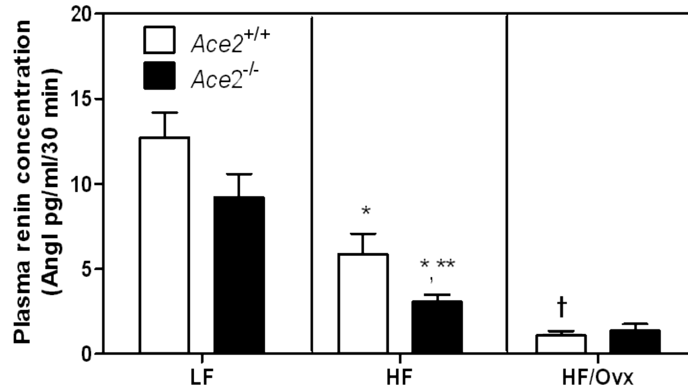


Fig. 5.2. A. Area under the curve (AUC) for glucose tolerance tests B. Plasma insulin concentrations at study endpoint in mice from each group. Data are mean $\pm$ SEM from (n = 8-10 mice/group). *, significantly different from LF within genotype; **, significantly different from *Ace2*$^{+/+}$ within diet; †, significantly different from HF vs HF/Ovx , P<0.05.

A)



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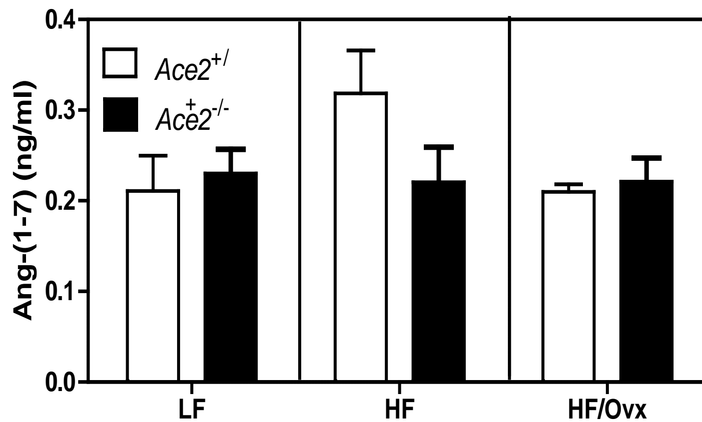

Fig .5.3. A. Plasma rennin concentrations in *Ace2*$^{+/+}$ and $^{-/-}$ female mice, B. Plasma Ang-(1-7) concentrations in *Ace2*$^{+/+}$ and $^{-/-}$ female mice. Data are mean $\pm$ SEM from n = 8-10 mice/group. *, significantly different from LF within genotype; **, significantly different from Ace2$^{+/+}$ within diet; †, significantly different from HF vs HF/Ovx , $P<0.05$.

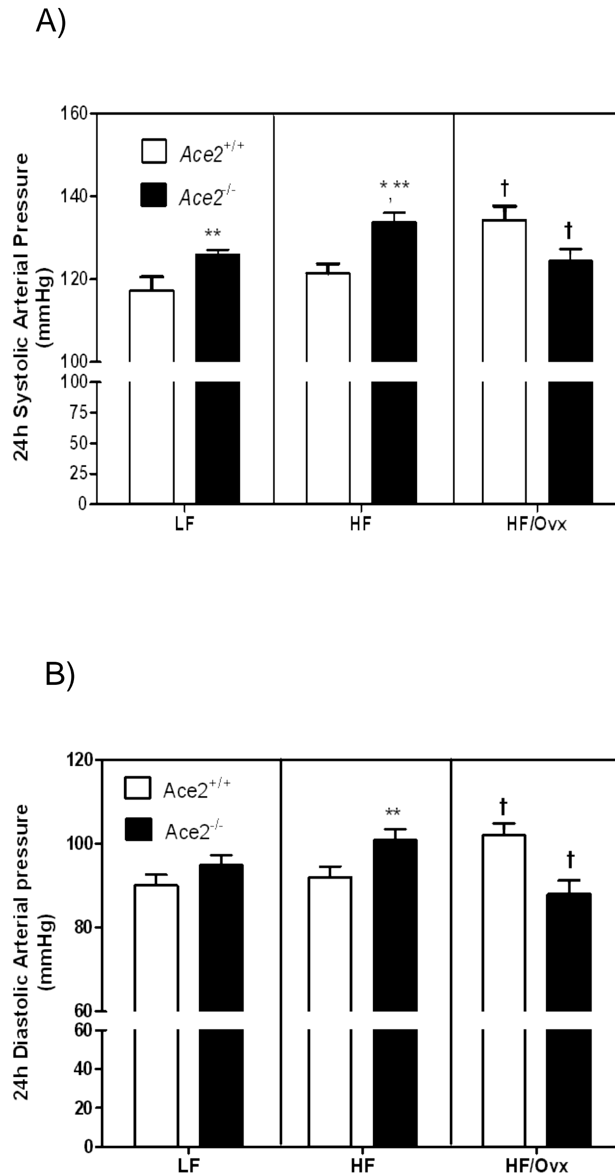

Fig. 5.4. A. Effects of ACE2 deficiency and Ovx on 24 h systolic arterial pressure in *Ace2*$^{+/+}$ and $^{-/-}$ mice, B. Effects of ACE2 deficiency and Ovx on 24 h diastolic arterial pressure in *Ace2*$^{+/+}$ and $^{-/-}$ mice. Measurements taken during week 16; n = 8-10 mice/group) *, significantly different from LF within genotype; **, significantly different from Ace2$^{+/+}$ within diet; † , significantly different from HF vs HF/Ovx , $P<0.05$.

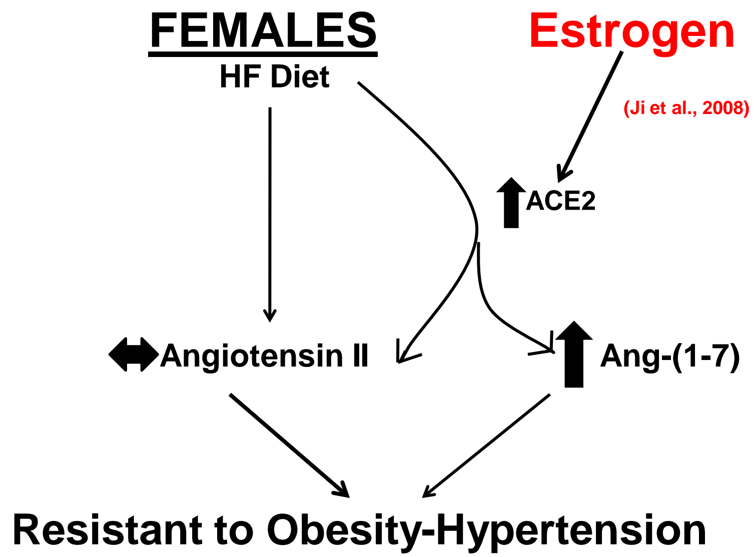

Fig. 5.5. Working model of obesity-induced hypertension in *Ace2*$^{+/+}$ female mice.

Section VI. GENERAL DISCUSSION

6.1. Summary.

The purpose of this research was to determine the role and regulation of ACE2 in obesity-induced hypertension. Initially, we started with 3T3-L1 cells, which are a mouse embryonic fibroblast-like cell line from Swiss mouse embryos which differentiate to mature white adipocytes upon exposure to a differentiating cocktail (Green et al. 1975). Using this cell system we determined expression of ACE2 during the course of differentiation. Importantly, ACE2 expression increased with differentiation when the cell acquired a mature adipocyte phenotype. Additionally, expression of PPARγ preceded expression of ACE2 during the course of differentiation. We also demonstrated expression of the AT1aR, which has been reported previously during differentiation. Mas, a receptor for Ang-(1-7) was also expressed in adipocytes during the course of differentiation.

These preliminary experiments in an adipocyte cell system demonstrated to us that besides the classical components of the RAS necessary for synthesis and responsiveness to AngII, components of the RAS related to AngII catabolism (e.g., ACE2) and responsiveness to additional angiotensin peptides such as Ang-(1-7) (e.g., Mas receptor) are also expressed in adipocytes and their expression is increased during the course of differentiation.

Since some of the components of the RAS such as angiotensinogen have been demonstrated to be regulated nutritionally, we then went *in vivo* to

determine ACE2 regulation in adipose and non-adipose tissues by HF-feeding. We wanted to determine the temporal effect of HF feeding on adipose ACE2 expression and activity. Hence we fed C57BL/6 male mice either a LF or a HF diet for a short duration (I week) or chronically (4 months). Even at 1 week HF-fed mice exhibited an increase in body weight (~17% increase from baseline) compared to the LF (~5% increase from baseline) controls. Importantly HF- fed mice exhibited a 1.7-fold increase in adipose mass compared to the LF. This increase in body weight and fat mass also resulted in a significant increase in fasting blood glucose. Thus, even at 1 week the HF-fed mice exhibited some features of the metabolic syndrome, namely obesity and hyperglycemia. HF diet and obesity have been demonstrated to activate the adipose RAS. At 1 week along with an increased adiposity we saw an increase in adipose angiotensinogen, indicating an activated adipose RAS. Surprisingly with increased body weight and activated adipose RAS, we saw a significant elevation in ACE2 activity and protein specifically in the adipose tissue of the HF-fed mice. Plasma angiotensin peptides were unaltered in spite of marked obesity. Importantly, we did not see any difference in blood pressure between the LF and HF fed mice at 1 week of HF feeding.

In contrast, at 4 months, HF-fed mice exhibited a 4-fold (~100% increase from baseline) increase in body weight compared to the LF (~25% increase from baseline) controls. Adipose angiotensinogen was elevated further with HF feeding and the mice exhibited significant elevations in plasma angiotensin peptides. In contrast to data from 1 week of HF feeding, at 4 months we saw a

reduction in ACE2 activity and protein specifically in the adipose tissue of HF-fed mice. HF-fed mice exhibited a significant elevation of blood pressure in the light as well as the dark cycle, thus exhibiting obesity- induced hypertension.

The ectodomain of ACE2 which contains the active site of this enzyme has been demonstrated to undergo shedding via ADAM-17, a protease shown to be upregulated with HF feeding (Lambert et al. 2005, Voros et al. 2003). ADAM-17 expression increased in 3T3-L1 adipocytes at day 10 of differentiation when adipocytes were lipid loaded, and this coincided with a reduction in ACE2 activity in the cells. We then incubated the cells on day 10 with an ADAM-17 inhibitor and demonstrated a modest but significant reduction in ACE2 activity in the media. We demonstrated a significant increase in ADAM-17 expression in adipose depots of HF-fed mice compared to the LF controls. These experiments suggest that chronic obesity results in loss of ACE2 activity in adipose tissue as a result of increased ADAM-17 which sheds the ectodomain of ACE2 containing the catalytic site of this enzyme. Collectively these results indicate that ACE2 initially protects against an activated RAS with obesity. However with chronic obesity ACE2 activity in adipose tissue is lost, the systemic RAS is activated and the mice exhibit obesity-induced hypertension.

Since HF diet increased ACE2 expression and activity specifically in adipose tissue and expression of PPARγ preceded the expression of ACE2 during the course of differentiation, we focused on natural (fatty acids) and synthetic ligands (TZDs) of PPARγ as regulators of ACE2 expression in adipocytes. In this experiment we used fatty acids that were part of the diet used

in the previous study. Additionally we also included examination of DHA in the present experiment, given its protective effects against cardiovascular diseases including hypertension. Additionally, we also incorporated synthetic ligands of PPARγ i.e TZDs such as Rosi and Pio.

DHA increased ACE2 expression in 3T3-L1 adipocytes. Importantly, we demonstrated a concentration-dependent increase in ACE2 mRNA, protein and activity with Rosi as well as Pio. We then administered either Rosi or vehicle to 8 week old *Ace2*$^{+/y}$ male mice. Similar to the *in vitro* results Rosi administration significantly increased ACE2 mRNA and activity in adipose tissue. Administration of Rosi significantly improved glucose tolerance and reduced blood pressure in *Ace2*$^{+/y}$ mice compared to vehicle-treated controls. Improvements in glucose tolerance in Rosi-administered mice were accompanied by reductions in plasma insulin concentrations.

To determine if the reductions in blood pressure in *Ace2*$^{+/y}$ male mice administered Rosi were mediated via ACE2, we administered Rosi to *Ace2*$^{-/y}$ male mice. We saw a similar reduction in blood pressure in *Ace2*$^{-/y}$ male mice as demonstrated in wild type *Ace2*$^{+/y}$ mice. In contrast, glucose tolerance was reduced, rather than improved, by Rosi administration in *Ace2*$^{-/y}$ male mice. Moreover, administration of Rosi had no effect on plasma insulin concentrations in *Ace2*$^{-/y}$ mice. These results indicate that the reduction in blood pressure with Rosi is not mediated via ACE2. In contrast, improvements in glucose tolerance by Rosi administration were lost in *Ace2*$^{-/y}$ mice, and associated with suppressed plasma insulin concentrations.

In our first *in vivo* experiment we demonstrated that loss of ACE2 with chronic HF-diet elevated blood pressure in male C57BL/6 mice. To follow up on this finding we used ACE2 deficient mice and induced obesity with chronic HF diet. Moreover, since ACE2 is an X linked gene and not many studies in mice have looked at the effect of chronic HF-diet on obesity and hypertension in females, we investigated the effects of HF-diet and obesity in ACE2 deficient male (*Ace2*$^{-/y}$) as well as female (*Ace2*$^{-/-}$) mice, to uncover potential roles of sex hormones in regulating this enzyme in the setting of obesity. *Ace2*$^{-/y}$ male and *Ace2*$^{-/-}$ female mice fed a HF-diet exhibited a lower body weight compared to the HF fed controls. However, we did not see any differences in body weight with LF diet and ACE2 deficiency in either gender. *Ace2*$^{-/y}$ male mice exhibited marked glucose intolerance with LF diet, with HF feeding both *Ace2*$^{+/y}$ and $^{-/y}$ male mice exhibited glucose intolerance. In contrast, we did not see any difference in glucose tolerance between the *Ace2*$^{+/+}$ and $^{-/-}$ female mice fed a LF diet. However, with HF feeding in contrast to males *Ace2*$^{+/+}$ female mice exhibited marked glucose intolerance compared to $^{-/-}$ female mice. Ovx exacerbated glucose intolerance in *Ace2*$^{+/+}$ and $^{-/-}$ female mice. Interestingly, *Ace2*$^{-/y}$ male mice fed a HF- diet exhibited a significant increase in blood pressure compared to *Ace2*$^{+/y}$ male mice. In contrast we did not see any difference in blood pressure between *Ace2*$^{-/y}$ and *Ace*2$^{+/y}$ male mice fed a LF diet.

Female *Ace2*$^{+/+}$ mice exhibited resistance to obesity-induced hypertension in spite of the development of pronounced obesity. In contrast, *Ace2*$^{-/-}$ female mice exhibited a marked increase in blood pressure with HF feeding. This

increase in blood pressure with ACE2 deficiency indicates that ACE2 protects the females against obesity-induced hypertension.

Several studies have demonstrated that sex hormones regulate various components of the RAS, collectively favoring a stimulated RAS in males compared to females. Specifically, estradiol has been demonstrated to regulate tissue levels and activity of ACE2 (Ji et al. 2008). Hence we ovariectomized *Ace2*$^{+/+}$ female mice to evaluate the role of sex hormones in the differences in blood pressure observed between the two genders. Ovariectomy conferred a hypertensive phenotype in HF-fed *Ace2*$^{+/+}$ female mice as they demonstrated an increase in blood pressure similar to HF-fed *Ace2*$^{-/-}$ female mice. These results suggest that *Ace2*$^{+/+}$ female mice are protected against obesity-induced hypertension via estrogen regulation of ACE2. In contrast, ovariectomized *Ace2*$^{-/-}$ female mice demonstrated a reduction in blood pressure. Along with a reduction in blood pressure ovariectomized *Ace2*$^{-/-}$ female mice demonstrated a reduction in cardiac function as indicated via a reduction in ejection fraction and fractional shortening compared to non ovariectomized *Ace2*$^{-/-}$ female mice.

Results from these studies demonstrate a protective role of ACE2 against obesity-induced hypertension and cardiac function.

6.2. Insights from diet-induced regulation of ACE2.

Since results from the present study indicate that a HF-diet stimulates ACE2 specifically in adipose tissue, an important unanswered question arises.

What in the HF diet stimulated ACE2 in adipose tissue? Excess food intake with reduced energy expenditure still remains a major cause of obesity. Since dietary fat contains more calories than protein and carbohydrates, it is generally recommended that dietary fat content should be decreased in the treatment of obesity (Madsen et al. 2005). However, it is the composition of the diet that is very important, as not all fat is bad (Grynberg et al. 2005). Our diets are composed of a mixture of fatty acids, i.e saturated as well as unsaturated fatty acids, which are further divided into mono (MUFA) and polyunsaturated fatty acids (PUFA). PUFAs are further divided into fatty acids belonging to the n-3 or n-6 family. Importantly, PUFAs are essential fatty acids as the regular diet does not provide enough precursors for their synthesis *in vivo* (Maurice Shills et al. 2006). These fatty acids are extremely important for vital functions of the body including blood pressure homeostasis (Grynberg et al. 2005). Additionally, the majority of the essential fatty acids have been demonstrated to activate PPARγ and thus regulate transcription of several genes including those of the RAS (Kliewer et al.1997).

Since some of the components of the RAS can be nutritionally regulated via fatty acids, we also wanted to determine if ACE2 can be regulated nutritionally. In the present study we used two diets, HF (60% calories from fat) and LF (10% calories from fat) composed of different fatty acids such as palmitic (PA), stearic (SA), oleic (OA), linoleic (LA) and arachidonic (AA) acid. We acknowledge that the fat content of the mouse diet was higher than the average fat composition of the human diet; however, this diet was necessary to create a

model to study obesity-hypertension especially given the fact that mice are resistant to hypertension from AngII infusion (Cassis et al. 2004) and exhibit modest but significant increases in blood pressure from HF feeding (Gupte et al. 2008).

We demonstrated an increase in ACE2 activity and protein with 1 week of HF feeding specifically in adipose tissue which has been demonstrated to abundantly express PPARγ. Thus, we hypothesized that fatty acids in the diet activated PPARγ in adipose tissue to stimulate ACE2 expression and activity locally to catabolize AngII and thus protect mice against obesity-induced hypertension. However, incubating 3T3-L 1 cells with individual fatty acids representative of those in the HF diet did not stimulate ACE2 activity *in vitro*. It is possible that a combination of fatty acids which mimics those in the diet used in the present study could elicit the *in vivo* response. Additionally, other factors such as differentiation, concentration of fatty acid used, and duration of incubation may also have affected the results.

6.3. Insights from *Ace2*$^{+/y}$, $^{-/y}$ (male) and *Ace2*$^{+/+, -/-}$(female) mice: Role in body weight, fat and lean mass regulation.

In the present study *Ace2*$^{-/y}$ male and *Ace2*$^{-/-}$ female mice were fed a LF or HF diet for 16 weeks and body weight progression was compared to the control. *Ace2*$^{-/y}$ male mice exhibited a modest resistance to weight gain with HF-diet. Importantly, lower body weights in *Ace2*$^{-/y}$ males did not arise from a lower fat mass; however, *Ace2*$^{-/y}$ male mice exhibited a lower lean mass (15%)

compared to *Ace2*$^{+/y}$ male mice. Our data suggests that this difference in body weight in males was not a result of decreased calorie intake or increased physical activity as we did not see any differences between the genotypes on either diet. Interestingly, previous studies from our lab have demonstrated a dose-dependent reduction in body weight in rats with AngII infusion that was independent of blood pressure (Cassis et al.1998a).This reduction in body weight was attributed to increased energy expenditure with chronic AngII exposure (Cassis et al. 2002). Earlier, studies from our lab have also shown that elevations in systemic AngII can lead to sympathetic activation (Cassis et al.1998b) to tissues involved in metabolism and result in increased energy expenditure peripherally in tissues such as adipose or skeletal muscle. Interestingly, in the present study *Ace2*$^{-/y}$ male mice fed a HF diet exhibited increased energy expenditure compared to *Ace2*$^{+/y}$ male mice. Thus, it is plausible that increased AngII concentrations due to ACE2 deficiency may have contributed to lower body weights in *Ace2*$^{-/y}$ male mice fed a HF diet.

Similar to males, *Ace2*$^{-/-}$ females exhibited a lower body weight compared to *Ace2*$^{+/+}$ female mice fed a HF diet. Even though body weights were similar in LF-fed *Ace2*$^{+/+}$ and $^{-/-}$ females, with HF feeding *Ace2*$^{-/-}$ female mice exhibited a lower fat mass (~25%) and lean mass (~38%) compared to *Ace2*$^{+/+}$ female mice. Mechanisms for reductions in fat and lean mass in HF-fed *Ace2*$^{-/-}$ females were not identified in the present study. However, it is conceivable that elevated systemic AngII concentrations raised energy expenditure and lowered body weight to a greater degree in female compared to male ACE2 deficient mice.

However, preliminary data suggests that energy expenditure is similar in HF-fed *Ace2*$^{+/+}$ and $^{-/-}$ mice. Recent studies suggest restricted fetal growth in offspring from *Ace2*$^{-/-}$ females (Brosnihan et al. 2010), suggesting that developmental effects of ACE2 deficiency could blunt expanded fat and lean mass in *Ace2*$^{-/-}$ females in response to HF feeding. Indeed, we routinely breed heterozygote *Ace2*$^{+/-}$ females to *Ace2*$^{-/y}$ males for generation of experimental mice because we have noted smaller offspring and fetal death from breeding homozygous ACE2 deficient females. Increases in fat mass and body weight have been demonstrated in several species following ovariectomy (Zoth et al. 2010; Anderson et al. 2009). In this study, ovariectomy increased the development of obesity in both genotypes fed HF diets. Thus, the ability of ovariectomy to increase body weight and fat mass appears to be independent of ACE2 deficiency. However, even though ACE2 deficient females gained weight from ovariectomy, their weight gain was lower than wild type females. Alarmingly, ovariectomized *Ace2*$^{-/-}$ female mice exhibited lower fat mass (~30%) and lean mass (~67%) compared to ovariectomized *Ace2*$^{+/+}$ female mice. Reductions in cardiac function in ovariectomized ACE2 deficient females may have contributed to these alarming reductions in fat and lean mass, similar to cardiac cachexia seen in patients with chronic heart failure (Piepoli et al. 2006).

6.4. Insights from *Ace2*$^{+/y,\ -/y}$ (male) and *Ace2*$^{+/+,\ -/-}$(female) mice: Role in glucose homeostasis.

6.4.1. Effect of HF diet.

HF fed mice exhibited marked glucose intolerance compared to the LF fed mice across both genders. Results from our study indicate fat mass as an important determinant of glucose intolerance. Our findings in HF-fed males and females are in agreement with previous studies in humans, demonstrating positive correlations between fat mass and insulin resistance in postmenopausal women (Van Pelt et al. 2002) and men and women (Wu et al.1998; Ito et al. 2003). Thus, diet-induced obesity in mice is similar to humans in terms of the relationship between body weight and the development of diabetes. Importantly, the WT HF ovariectomized females which gained the highest fat mass (~14 fold increase) exhibited the highest glucose intolerance. To illustrate the relationship between body weight and glucose intolerance in our studies, we ranked mice across all groups and genders for glucose intolerance as follows: WT HF, ovariectomized females> WT HF-fed males>Ace2-/y HF-fed males>Ace2-/- ovariectomized HF-fed females>WT HF-fed females. This ranking of glucose intolerance precisely parallels rankings of body weight across these groups. Thus, increased adiposity with HF diet appears to be an important determinant of glucose tolerance.

6.4.2. Role of ACE2 deficiency.

In the present study we demonstrated glucose intolerance in *Ace2*$^{-/y}$ males fed a LF diet. Additionally, non fasted plasma insulin levels are lower in ACE2 deficient animals even in the face of marked glucose intolerance, which is a consistent finding in all our studies. A complete RAS has been identified in the pancreas (Tikellis et al. 2006). Additionally, a review of studies in animals and humans highlights the role of AngII systemically as well as locally in the development of insulin resistance and its implication for diabetes (Olivares et al. 2009). A recent study demonstrated that administration of an AT1R antagonist rendered protection against the inflammatory and metabolic consequences of HF diet in pancreas and adipose tissue in mice fed a western diet for 12 weeks (Cole et al. 2010). The AT1R antagonist improved glucose tolerance, fasting blood glucose and glucose stimulated insulin release in mice fed a high fat diet. In addition, mice administered the AT1R antagonist were demonstrated to have enhanced mitochondrial function, reduced β-cell hyperplasia and increased insulin content (Cole et al.2010). To date, no studies have examined the role of endogenous ACE2 expression in β cell function. However, our findings suggest that elevated AngII levels either systemically or locally in the pancreas of ACE2 deficient fed the HF diet contributed to glucose intolerance possibly from decreased insulin synthesis and/or secretion from the pancreas. To test this hypothesis we attempted to stain pancreas from 8 week old *Ace2*$^{+/y}$ and $^{-/y}$ deficient male mice with insulin. Immunostaining of *Ace2*$^{+/y}$ and $^{-/}$ mice pancreas

for insulin indicated less staining in *Ace2*$^{-/y}$ mice as compared to $^{+/y}$ mice (Figure 6.2). Unfortunately, we were unable to quantify insulin content in the pancreas.

It is well known that diet-induced obesity results in glucose intolerance in experimental models and humans through insulin resistance primarily at skeletal muscle, adipose tissue, and liver. In our studies, HF-fed ACE2 deficient male mice were glucose intolerant, did not increase systemic insulin concentrations with HF feeding, and had better insulin tolerance compared to wild type controls. Despite the potential for different mechanisms contributing to glucose intolerance between ACE2 deficient males compared to wild type, both groups most likely developed some degree of insulin resistance. Several lines of evidence suggest that AngII can induce insulin resistance at skeletal muscle and adipose tissue (for review see Henriksen, 2007). Thus, elevations in systemic or local AngII in HF-fed ACE2 deficient mice may have altered metabolic pathways of insulin signaling i.e (PI3K/AKT).

Another possible mechanism for glucose intolerance in ACE2 deficient mice is lower systemic concentrations of Ang-(1-7), as demonstrated in HF fed mice. Recent studies demonstrated a beneficial effect of Ang-(1-7) on glucose homeostasis using an Ang-(1-7) producing fusion protein to increase insulin stimulated glucose uptake in adipocyes in rats (Santos et al. 2010). Additionally, Ang-(1-7) fusion protein reduced adipose AO expression and increased expression of proteins of the insulin metabolic pathway (PI3K/AKT) (Santos et al. 2010). Thus, imbalances between systemic and/or local concentrations of

AngII/Ang-(1-7) may have contributed to the observed effects of ACE2 deficiency on glucose homeostasis in the setting of diet-induced obesity.

It is also conceivable that a crosstalk between the adipose tissue and pancreas may also have resulted in the above dysfunctions. Using co-culture systems Zhao et al. demonstrate that 3T3-L1 adipocytes induce dysfunction of insulin producing β cells (Zhao et al. 2007).

6.5. Insights from *Ace2*$^{+/y}$, $^{-/y}$ (male) and *Ace2*$^{+/+,\ -/-}$(female) mice: Role in obesity-induced hypertension.

Previous studies demonstrated that ACE2 deficient mice exhibit increased blood pressures in response to AngII infusion (Gurley et al. 2006). Plasma and kidney levels of AngII were increased in AngII-infused mice with ACE2 deficiency, supporting local and systemic activation of the RAS (Gurley et al. 2006). Our results suggest that initial activation of ACE2 in adipose tissue may protect male mice against obesity-induced hypertension. However, with chronic HF feeding ACE2 activity in adipose tissue was lost and the mice exhibited a significant increase in SBP and DBP compared to LF controls. Chronic HF feeding resulted in loss of ACE2 activity via shedding of the catalytic domain of ACE2 mediated via ADAM-17, which is upregulated with chronic obesity. Importantly, plasma Ang II levels were elevated in obese mice exhibiting lower ACE2 activity in adipose tissue. These results indicated that increased AngII resulting from reduced ACE2 activity contributed to obesity-induced hypertension in HF fed C57BL/6 male mice.

Since increases in blood pressure coincided with a reduction in ACE2 activity in adipose tissue we hypothesized that ACE2 protects the mice against obesity-induced hypertension. Following up on this finding we determined the effect of ACE2 deficiency on obesity-induced hypertension in ACE2 deficient male and female mice. Importantly, *Ace2*$^{-/y}$ male mice exhibited significant elevations in SBP compared to HF-fed *Ace2*$^{+/y}$ mice. Since ACE2 has been demonstrated to catabolize AngII to generate Ang-(1-7), we predicted that ACE2 deficiency would increase systemic concentrations of AngII. This is particularly important in diet-induced obesity where plasma AngII levels have been demonstrated to be increased significantly with HF feeding and obesity (Boustany et al. 2004; Gupte et al. 2008). In the present study *Ace2*$^{-/y}$ mice fed a HF diet exhibited significant elevations in plasma AngII levels compared to *Ace2*$^{+/y}$ mice. This suggests that the increase in blood pressure observed in HF fed *Ace2*$^{-/y}$ resulted from increased plasma AngII levels as a result of ACE2 deficiency. Further evidence supporting a primary role for AngII as a mediator of blood pressure elevations in HF-fed *Ace2*$^{-/y}$ males comes from data using losartan. Losartan administration reversed elevations in blood pressure from HF-feeding in *Ace2*$^{-/y}$ males. Thus, in the males it appears that increased systemic AngII in obese ACE2 deficient mice contributed to the etiology of obesity-induced hypertension. Since ACE2 deficiency can also result in lowered plasma Ang-(1-7), it is conceivable that the increases in pressure seen in *Ace2*$^{-/y}$ male mice could have resulted from lower Ang-(1-7) levels. This is especially important as HF-fed wild type mice exhibited lower concentrations of plasma Ang-(1-7).

However, our data does not support a prominent role for Ang-(1-7) in blood pressure differences with obesity and ACE2 deficiency, as we saw similar plasma Ang-(1-7) concentrations in HF fed *Ace2*$^{+/y}$ and $^{-/y}$ male mice, even though blood pressure was different between these groups.

In contrast to males, HF fed females were protected against obesity-induced hypertension. However, with ACE2 deficiency this protection against obesity-induced hypertension was lost and the female mice also exhibited increased SBP as well as DBP. To address mechanisms for differences between males and females in their susceptibility to obesity-hypertension in relation to ACE2 deficiency and the RAS, we provide the following discussion. We compared plasma concentrations of Ang-(1-7), a catabolic product of ACE2, in HF fed male and female mice. Interestingly, *Ace2*$^{+/+}$ female mice fed a HF diet exhibited significantly higher plasma Ang-(1-7) concentrations compared to *Ace2*$^{+/y}$ HF male (female : 0.32 +/-0.0, male : 0.12+/-0.0 ng/ml, p=0.001). This is an interesting finding, as it indicates that unlike males where ACE2 activity is lost and Ang-(1-7) levels are lowered with chronic obesity, ACE2 activity is preserved in females which exhibit higher plasma Ang-(1-7) levels in response to HF feeding. Thus, it is likely that increased Ang-(1-7) concentrations, presumably through an ACE2-dependent mechanism, protected the females against obesity-induced hypertension.

It is unclear why HF feeding would lower plasma Ang-(1-7) concentrations in males, but raise them in females. Moreover, our data using losartan suggests that plasma concentrations of AngII, and not Ang-(1-7), primarily mediate

enhanced obesity-hypertension in ACE2 deficient males. Moreover, since mouse groups with similar plasma concentrations of Ang-(1-7) (HF-fed *Ace2*$^{-/y}$ and $^{+/y}$) exhibited disparate blood pressures, these results do not support reductions in plasma Ang-(1-7) concentrations in HF-fed male mice as a contributor to augmented obesity hypertension in ACE2 deficient males. In contrast, female mice exhibited elevated plasma concentrations of Ang-(1-7) and were resistant to obesity-hypertension, a phenomenan reversed by ACE2 deficiency. These results suggest that females respond to plasma concentrations of Ang-(1-7) to regulate blood pressure differently than males. Unfortunately, there are no studies comparing expression levels of Mas receptors in tissues from male compared to female mice under control conditions or in response to HF feeding. Additional studies are required to elucidate mechanisms for differences in blood pressure responses to plasma concentrations of Ang-(1-7) between obese male and female mice.

A second interesting aspect of comparisons of male to female mice in their responses to HF feeding and the development of obesity is the role of female sex hormones through studies using ovariectomy. Previous studies in rats demonstrated that estrogen positively modulates ACE2 protein in rat kidney (Ji et al. 2008), supporting our observation that female mice exhibit higher plasma concentrations of Ang-(1-7) compared to males. Our results demonstrate that HF feeding in females increases plasma concentrations of Ang-(1-7), and that these effects are lost in ovariectomized females. These results suggest an interaction between HF feeding and female sex hormones. Results from ACE2 deficient

females demonstrated that HF diet-induced increases in plasma concentrations of Ang-(1-7) are ACE2-mediated. It is conceivable that estrogen stimulated ACE2 expression in female mice, allowing for HF diet-induced increases in production of Ang-(1-7) and protection against obesity-hypertension. Recent studies demonstrated expression of the Mas receptor in human ovaries (Reis et al. 2010). In addition, Ang-(1-7) production and Mas expression were stimulated by gonadotropin (Pereira et al. 2009). Thus, either increased concentrations of Ang-(1-7) or alternatively an increased sensitivity to Ang-(1-7) via increased expression of Mas may have contributed to this protection. Additional studies are needed to directly define effects of estrogen on ACE2 mRNA in the context of obesity hypertension.

An important indicator of an activated systemic RAS is plasma renin concentrations. In mice a lower plasma renin concentration indicates increased AngII concentrations because of an endocrine negative feedback loop of AngII at kidney AT1R. HF-fed *Ace2*$^{+/+}$ female mice exhibited lower plasma renin concentrations, indicative of increased plasma AngII levels compared to *Ace2*$^{+/+}$ mice fed a LF diet. This indicates that similar to males plasma AngII concentrations are elevated in females with HF diet and chronic obesity. However, in spite of an activated RAS, females were protected against obesity-induced hypertension. As described above, elevations in plasma Ang-(1-7) concentrations most likely mediated protection against obesity-hypertension in females, as ACE2 deficiency eliminated these elevations and promoted obesity-hypertension. Plasma renin concentrations were further lowered by ovariectomy

in *Ace2*$^{+/+}$ and $^{-/-}$ female mice, suggesting that ovariectomy elevated plasma AngII concentrations to promote obesity-hypertension. The lack of an additive effect of ACE2 deficiency and ovariectomy on blood pressure in female mice suggests that ACE2 and estrogen most likely induced obesity-related hypertension through similar mechanisms.

6.6. Insights from Ovx *Ace2*$^{-/-}$ (female) mice: Role in cardiac function.

In the present study we hypothesized that *Ace2*$^{+/+}$ females are resistant to obesity-induced hypertension via an ACE2-estrogen mediated mechanism. To test this hypothesis we ovariectomized *Ace2*$^{+/+}$ and $^{-/-}$ female mice. Surprisingly, we found that ovariectomy decreased systolic and diastolic blood pressures in *Ace2*$^{-/-}$ females. The first publication examining ACE2 deficiency in mice demonstrated a reduction in blood pressure in 6 month old *Ace2*$^{-/y}$ male mice as a result of cardiac dysfunction, indicating an important role for ACE2 in cardiac function (Crackower et al. 2002). Similar to their findings, in our studies we demonstrate reductions in blood pressure of ovariectomized *Ace2*$^{-/-}$ mice, coinciding with reduced ejection fraction and fractional shortening. These results indicate that in contrast to the pathology of hypertension where ACE2 and estrogen deficiency mediate their effects via similar mechanisms, they appear to function via distinct mechanisms in the pathology of heart failure as this pathology is absent in non ovariectomized *Ace2*$^{-/-}$ females. Cardiac dysfunction in ovariectomized *Ace2*$^{-/-}$ females may have resulted from increased AngII levels systemically as well as locally in the heart as a result of combined

ACE2 and estrogen deficiency. Since increased AngII levels locally in the heart have been demonstrated to promote cardiac hypertrophy and fibrosis (Baker et al. 1992, Zhong et al. 2010), it is likely that cardiac remodeling due to increased AngII adversely influenced cardiac function. Additionally, this could also be aggravated via AngII-mediated increases in sympathetic activation to the heart, as previous studies from our lab have demonstrated a role for AngII to increase sympathetic activation in ventricles of rats experiencing pressure overload (Akers et al. 2004).

6.7. Future directions.

There are many new questions that arise from this research that should be addressed in the future.

6.7.1 Role of specific fatty acids in obesity-induced hypertension.

In the present study we demonstrated increased ACE2 expression via DHA, an n-3 fatty acid well known for its cardioprotective effects. This would be an important area to be investigated as an effective dietary intervention against obesity-induced hypertension. An interesting study would be to administer diets enriched in specific fatty acids (DHA) and contrast these with the different parameters measured in the present study. Alternatively, a group could be fed a regular HF (used in the study) and then switched to a diet containing n-3 fatty acids to study the effects of this fatty acid to reverse the effects of diet-induced obesity.

6.7.2 Role of AngII in metabolic parameters.

In the present study we demonstrated lower body weights in HF-fed ACE2 deficient mice. We implicate increased AngII as a result of ACE2 deficiency for these differences. Moreover since studies in animals deleting other components of the RAS such as AO (Massiera et al. 2001) also demonstrated resistance to obesity with HF feeding the exact role of AngII in diet-induced obesity is unclear. In the present study we demonstrated increased energy expenditure in male HF-fed mice with ACE2 deficiency. Previous studies from our lab have demonstrated an increased sympathetic activity via increased systemic AngII (Cassis et al.1998 b) to tissues involved in metabolism. Hence to understand mechanisms for differences in body weight, an important experiment would be to administer either an AT1 receptor antagonist or a β receptor antagonist to wild type and ACE2 deficient mice during the progression of obesity. If the differences in body weight are blunted via AT1 receptor antagonist then we can implicate the RAS as a mediator of differences in body weight. In contrast, if the body weight differences are blunted by a β receptor antagonist, then an activated SNS via AngII is implicated in these differences.

6.7.3 Role of AngII or Ang-(1-7) in glucose metabolism.

Since studies implicated a role for increased AngII as well as lowered Ang-(1-7) levels for perturbations in glucose homeostasis, to dissect specific roles of these peptides an important study would be to infuse *Ace2*$^{+/y}$ and

$^{-/}$ fed either a LF or HF diet with AngII or Ang-(1-7) chronically and monitor different parameters associated with glucose homeostasis. Alternatively, as results from the present study suggest a role for the pancreatic RAS in effects of ACE2 deficiency on glucose homeostasis, it would be interesting to define effects of HF feeding on the pancreatic RAS. Moreover, available mouse models in our laboratory, namely AO or AT1aR floxed models, could be used with pancreatic β-cell promoters to target deficiency of these RAS components to islet cells.

6.8. Clinical implications.

Increased ACE2 has been demonstrated to render protection in numerous cardiovascular pathologies including obesity-induced hypertension as demonstrated by the present research. Additionally, we demonstrated that ACE2 can be stimulated via specific fatty acids. This can be facilitated by incorporating fatty acids in the diet which could potentially tonically stimulate ACE2 activity. Additionally, intake of fatty acids that increase ADAM-17 expression resulting in loss of ACE2 activity should be limited to sustain ACE2 activity.

A study in SHR demonstrated a beneficial effect of an ACE2 activator (xanthenone and resorcinolnaphthalein) to reduce blood pressure in a dose dependent manner (Hernández-Prada et al. 2008). In the present study we demonstrated that ACE2 was stimulated by TZDs, some of which are effectively used in the treatment of type 2 diabetes (Olfesky et al. 2000). Since majority of patients with type 2 diabetes are also obese and hypertensive, it is possible that addition of a TZD to these patients may provide potential benefit through

stimulation of ACE2. Additionally, studies have also tested the efficacy of ACE2 gene transfer in tissues associated with cardiovascular functions such as heart (Diez-Freire et al. 2006) and smooth muscle (Rentzsch et al. 2008) and demonstrated beneficial effects on cardiac function and blood pressure. Since results from our laboratory implicate that adipose RAS in the etiology of obesity-induced hypertension, it would be interesting to increase ACE2 in adipose tissue directly via gene transfer as an alternative therapy against obesity-induced hypertension. Additionally, in females Ang-(1-7) appears to play an important role in protecting against obesity-induced hypertension. Studies in animals have demonstrated a beneficial role of an Ang-(1-7) producing fusion protein, which results in increased levels of Ang-(1-7) over lifetime. This would be particularly important for females as our results indicate that levels of this peptide fall with ovariectomy. Thus, in post menopausal women who exhibit a higher prevalence of obesity and hypertension compared to pre-menopausal women, we suggest testing this therapy.

6.9. Concluding Remarks.

In conclusion, findings of this dissertation demonstrate that PPARγ ligands promote ACE2 expression and activity in adipose tissue. Additionally, results from these studies demonstrate a pivotal role of ACE2 in male as well in female mice in the development of obesity-associated cardiovascular pathologies such as hypertension, diabetes and cardiac functions (Figure 6.1).

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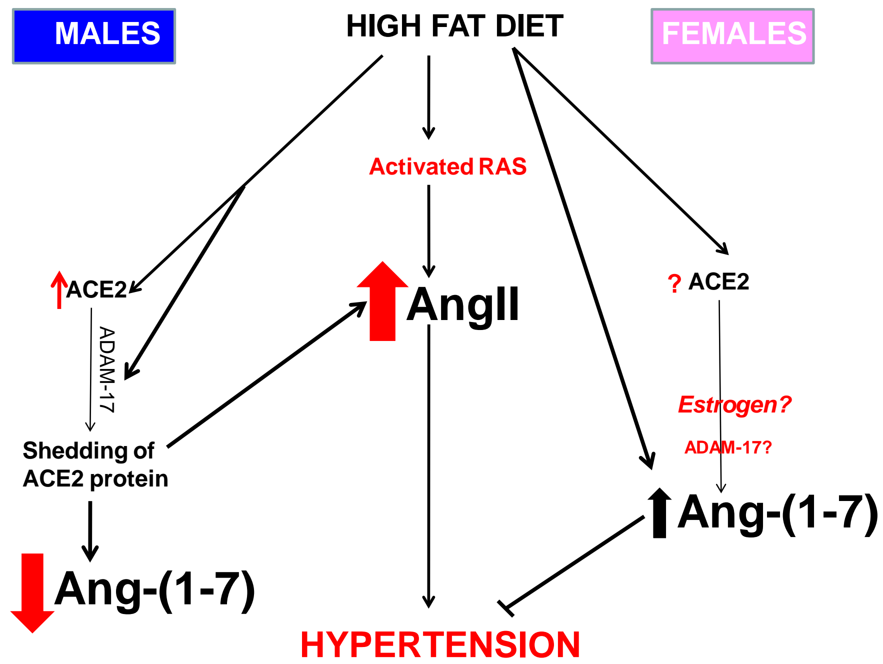

Figure. 6.1 Role of ACE2 in Obesity-Associated Hypertension.

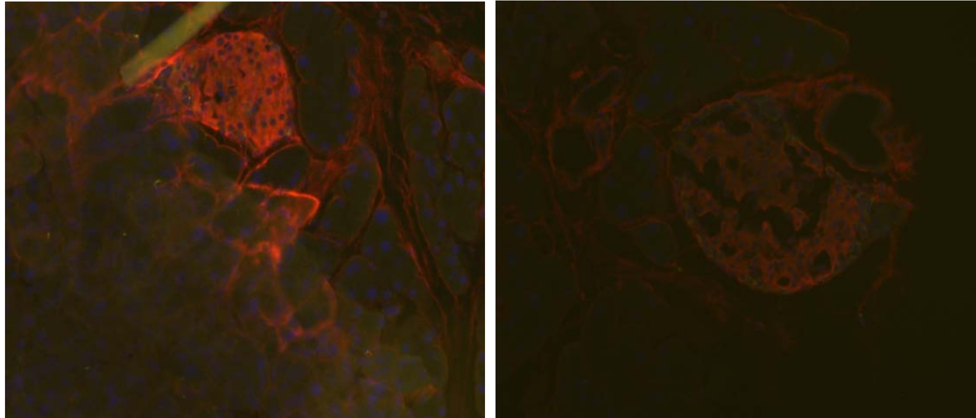

Figure. 6.2 Pancreas from 8 week old male *Ace2*$^{+/y}$ and $^{-/y}$ stained for insulin.

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