Inhibition of renin angiotensin system improves leptin and insulin sensitivity, but causes severe anemia due to hypothyroidism

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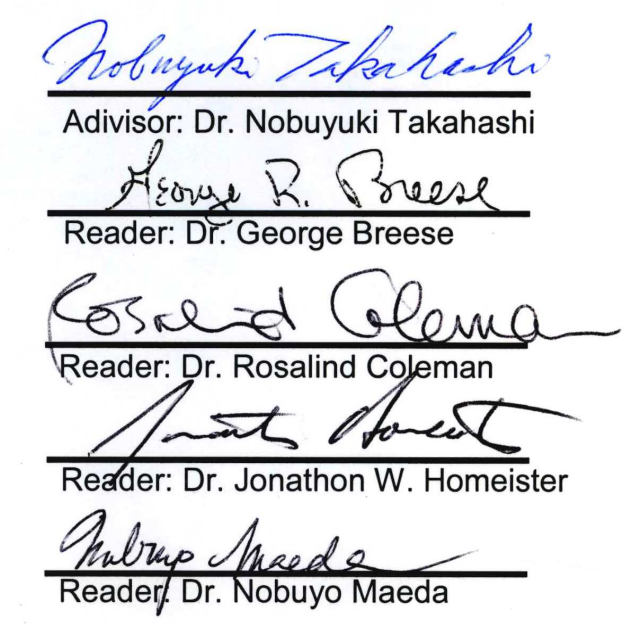

ABSTRACT

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Inhibition of renin angiotensin system improves leptin and insulin sensitivity, but causes severe anemia due to hypothyroidism

(Under the direction of Nobuyuki Takahashi)

An overactive Renin-Angiotensin System (RAS) is associated with the metabolic syndrome, and inhibiting RAS causes severe anemia. Our previous study has demonstrated that mice lacking renin (*Ren1c*) are lean and insulin sensitive. However, to our surprise the *Ren1c-/-* mice have low circulating total thyroid hormones, and their plasma catecholamine levels were similar to those of wild type (WT) mice. Although angiotensin II (Ang II) is suggested to regulate blood pressure, and insulin and glucose homeostasis, the precise mechanisms of its contribution to the metabolic syndrome are not completely understood.

Obesity is often associated with leptin resistance, while leptin sensitivity is a potential therapeutic strategy to treat obesity. To establish the role of renin in the leptin sensitivity, and to investigate whether improved leptin sensitivity is responsible for lean and insulin sensitive phenotype of the *Ren1c-/-* mice, I generated mice lack of both renin and leptin (*Ren1c-/-; ob/ob)*. The *Ren1c-/-; ob/ob* mice have lower body weight compared to the *ob/ob* mice at 3 months old despite similar food intake and

fat weight to body weight ratio. They also have improved leptin sensitivity, demonstrated after leptin administration by larger decrease in body weight, body fat, and food intake in the *Ren1c-/-; ob/ob* mice relative to the *ob/ob* mice. In addition, the smaller body weight of *Ren1c-/-; ob/ob* mice are due to increased fecal fat excretion and fatty acid oxidation in the liver. Moreover, The *Ren1c-/-;ob/ob* mice are more insulin sensitive than the *ob/ob* mice as are the *Ren1*c-/- mice compared to wild type mice. Improved leptin and insulin sensitivity by the absence of renin was recapitulated by a type 1 Ang II receptor (AT1R) blocker losartan. Inhibiting AT1R improves leptin and insulin sensitivity, and may be useful in treating insulin resistance and leptin resistance in human obesity and diabetes.

I next investigated whether the *Ren1*c-/- mice really have hypothyroidism, and if they have whether hypothyroidism contributes to anemia caused by inhibiting RAS. I provide evidence that the *Ren1*c-/- mice have tertiary hypothyroidism due to low expression of the hypothalamic TRH levels. Tertiary hypothyroidism of the *Ren1*c-/- mice causes impaired erythropoiesis in bone marrow and results in severe anemia, while exogenous thyroid hormone (T3) corrects it. Inhibition of AT1R or of Ang converting enzyme in WT mice recapitulated tertiary hypothyroidism and anemia in mice lacking renin. Although Ang II is suggested to stimulate erythropoiesis, my results demonstrated that hypothyroidism is the predominant cause of anemia induced by inhibiting RAS, and that without correcting hypothyroidism Ang II is not sufficient to correct anemia caused by RAS inhibition. Thyroid hormone is a potential therapeutic strategy for anemia of patients with chronic renal failure, who are treated with inhibitors for RAS.

DEDICATION

For my lovely family.

A special thanks to my loving parents, who have been full of support and never left my side. Although my mother passed away after I entered the pathology program, she is always in my mind and I would never forget the encouragement she gave me.

My brother gave me the greatest support. I will always miss my young brother, Chih-En. He gave me the bravery to challenge PhD degree.

To my loving wife, Kui-Ling, and daughter, Lauren. They are my inspiration and motivation. They give me strong supports to face different challenges, and the reasons I am the happiest person in the world.

I will always appreciate all they have done and made this possible.

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LIST OF ABBREVIATIONS

ACE	angiotensin-converting enzyme
ACOX1	acyl-CoA oxidase 1
AGT	angiotensinogen
AgRP	agouti-related peptide
Ang II	angiotensin II
α-MSH	α-Melanocyte Stimulating Hormone
ARBs	ang II receptor blockers
ARC	arcuate nucleus
AT1R	angII type 1 receptor
AT2R	ang II type 2 receptor
BAT	brown adipose tissue
BFU-E	burst-forming units-erythroid
BP	blood pressure
CAKUT	congenital anomalies of the kidney and urinary tract
CFU-E	erythroid colony-forming units
CPT-1	carnitine palmitoyl transferase-1
DIO	diet-induced obesity
Dio2	type II deiodinase
DN	diabetic nephropathy
EPO	erythropoietin

FAS	fatty acid synthase
G6P	glucose-6-phosphatase
GPAT1	glycerol-3-phosphate acyltransferase
Hb	hematoglobin
HCT	hematocrit,
HPT	hypothalamic-pituitary-thyroid
ITT	insulin tolerance test
JAK2	Janus kinase 2
LRb	leptin receptor
MCH	mean corpuscular hemoglobin
MCV	mean corpuscular volume
MMI	methimazole
NPY	neuropeptide Y
OGTT	oral glucose tolerance test
POMC	pro-opiomelanocortin
PEPECK	phosphoenolpyruvate carboxykinase
PPARγ	peroxisome proliferator-activated receptor-gamma
PPARα	peroxisome proliferator-activated receptor-alpha
PRR	(pro)renin receptor
PTP1B	tyrosine phosphatase protein-tyrosine phosphatase 1B
PTT	pyruvate challenge test
PTU	propylthiouracil
PVN	paraventricular nucleus

RAS	renin angiotensin system
RQ	respiratory quotient
SNS	sympathetic nervous system
SOCS3	suppressor of cytokine signaling-3
Stat3	signal transducer and activator of transcription 3
TG	triglyceride
TH	thyroid hormone
TRH	thyrotropin-releasing hormone
TSH	thyroid-stiumating hormone
UCP1	uncoupling protein
WAT	white adipose tissue

Chapter 1: General introduction

Background and Significance

The metabolic syndrome

The metabolic syndrome is a widely accepted concept that identifies the centrally obese patients with increased risk of cardiovascular diseases and diabetes. The syndrome has a rising prevalence worldwide, which related largely to increasing obesity and sedentary lifestyles (1). In the US approximately 34% of adults meet the criteria for the metabolic syndrome, and the syndrome is 3 times more prevalent in males and females 40-59 years of age compared to those 20-39 years of age (2). Patients with the metabolic syndrome have twice the risk of developing cardiovascular disease over the next 5 to 10 years relative to individuals without the syndrome (1). The syndrome confers 5-fold risk for type 2 diabetes. The metabolic syndrome is clinically characterized by several inter-related symptoms including obesity, dyslipidemia, high blood pressure, insulin resistance, impaired glucose tolerance or diabetes. Each of these is a risk factor of cardiovascular disease and diabetes, but combinations of them greatly increase the risk of cardiovascular diseases (3). However, there were many definitions of the metabolic syndrome, which led to confusion regarding how to identify patients with the syndrome (4, 5), leading to a confusion in clinical practice. The main difference was that The International Diabetes Federation (IDF) had a threshold value for waist circumference as obligatory, whereas American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) had it one of the factors but not obligatory. Accordingly, IDF and AHA/NHLBI, jointed by the World Heart Federation, International Atherosclerosis

Society, and International Association for the Study of Obesity recently developed one unified definition (1) (**Table 1.1**): there should be no obligatory component, but waist measurement would continue to be a useful screening tool. Three abnormal findings out of 5 would qualify a person for the metabolic syndrome. A single set of cut points would be used for all components except waist circumference. Yet because the relation between waist circumference and cardiovascular disease and diabetes risk differs globally, the definition for waist circumference remains unsettled. However, the molecular mechanism of the pathogenesis of the metabolic syndrome is not well understood.

The Renin-Angiotensin System and the metabolic syndrome

The renin angiotensin system (RAS) is one of the most important systems that regulate cardiovascular and fluid homeostasis (**Fig. 1.1**). Angiotensins can also act as neurotransmitters, regulating blood pressure (BP), memory, cognition and stress in the brain (6-8). The RAS recently has been implicated in the metabolic syndrome. Ang II induces adipogenesis (differentiation into adipocytes) (9, 10) and lipogenesis (triglyceride storage in adipocytes) *in vitro* (11). The effects of Ang II on adipose tissue are mediated by Ang II type 1 and type 2 receptors(12). However, the *in vivo* role of RAS in the metabolic syndrome has been unclear.

Renin, released from the kidney juxtaglomerular (JG) cells, is a rate-limiting enzyme in angiotensin II (Ang II) production. Renin cleaves the liver- and adipose-tissue-derived angiotensinogen (AGT) to form inactive angiotensin I

Table 1.1 Criteria for clinical diagnosis of the metabolic syndrome.

	Categorical cutpoints
Increased waist circumference*	Population-specific and country-specific definitions
Increased triglycerides (drug treatment for elevated TG is alternate indicator†)	≥150 mg/dL (1.7mmol/L)
Reduced HDL cholesterol (drug treatment for reduced HDL cholesterol is alternate indicator†)	<40 mg/dL (1.0 mmol/L) in men <50 mg/dL (1.3 mmol/L) in women
Increased blood pressure (antihypertensive drug treatment in patient with history of hypertension is alternate indicator)	Systolic ≥130 and/or diastolic ≥85 mm Hg
Increased fasting glucose‡ (drug treatment of increased glucose is alternate indicator)	> 100 mg/dL (5.5 mmol/L)

*It is recommended that the IDF cutpoints be used for non-Europeans and either the IDF or AHA/NHLBI cutpoints used for people of European origin until more data are available. †Most commonly used drugs for increased triglycerides and reduced HDL cholesterol are fibrates and nicotinic acid. A patient on one of these drugs can be presumed to have high triglycerides and low HDL. Use of high-dose ω-3 fatty acids presumes high triglycerides. ‡Most patients with type 2 diabetes will have the metabolic syndrome by the proposed criteria.

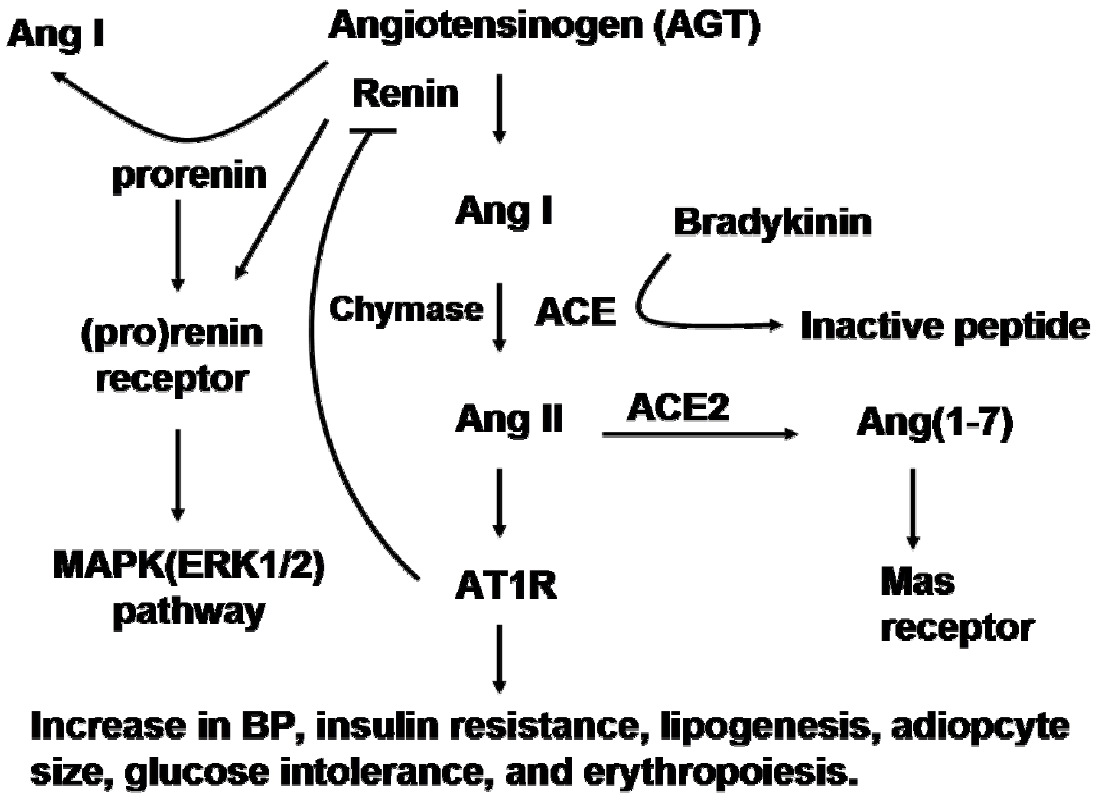

Figure 1.1 The Renin-angiotensin system (RAS). Angiotensin II effects are predominately mediated by angII type 1 receptor (AT1R). In addition to the classical RAS, ACE2 and (pro)renin receptor are new players of this system.

(Ang I) (13). The angiotensin-converting enzyme (ACE), which is mainly expressed in the endothelium, then removes two C-terminal amino acids and generates active peptide Ang II. Interestingly, the human *ACE* insertion/deletion (ID) polymorphism does not affect BP despite the powerful antihypertensive effects of ACE inhibitors (reviewed in (14, 15)), although mild increase in ACE causes exacerbation of diabetic nephropathy (see the section on ACE). The majority of the biological effects of Ang II, including BP elevation, are mediated by the type 1 Ang II receptor (AT1R). An Ang II negative-feedback mechanism inhibits the expression and secretion of renin. Treatments of hypertension often involve the inhibition of RAS with Ang receptor blockers (ARB), ACE inhibitors, or renin inhibitors, all of which disrupt this negative-feedback mechanism and increase renin levels.

In addition to these classical RAS components, several new molecules have been discovered in recent years. A homolog of ACE, ACE2, was discovered and shown to convert Ang II to Ang(1-7) (16). Santos et al. discovered that the receptor for Ang(1-7) is the Mas proto-oncogene, and that this ACE2–Ang(1–7)–Mas pathway counteracts the effects of Ang II (17). Furthermore, Nguyen et al. discovered the (pro)renin receptor (PRR) in 2002 (18, 19). This receptor is expressed in many tissues including brain, adipose tissue, endothelial cells, vascular smooth muscle cells, and kidney. PRR binds and activates prorenin. This newly discovered RAS component likely impacts local Ang II effects in cardiovascular diseases and the metabolic syndrome. However, the detail mechanism of how RAS modify the metabolic phenotype is not known.

Renin in humans and in mice

Renin is an important enzyme for regulation of BP and cardiovascular homeostasis. Cleavage of AGT to Ang I by renin is a rate-limiting step of Ang II production (20). Renin is secreted from juxtaglomerular cells, which are modified smooth muscle cells of the afferent arterioles in the kidney. In Spanish populations, individuals with the GG genotype at the rs5707 intron 4 polymorphism of renin have significantly higher BP compared to those with the TT or TG genotype (21). Individuals with GG genotype of the missense mutation in exon 9 (G1051A) have higher plasma renin activity, and this polymorphism may be involved in the etiology of hypertension (22).

Renin is encoded by a single gene in humans, rats, and some strains of laboratory mice such as C57BL/6, BALB/c, and C3 (**Fig.1. 2**). However, some laboratory mouse strains (eg. 129, DBA/2J) have 2 renin genes (*Ren2* and *Ren1d*) in tandem on the same chromosome due to natural duplication of the gene. The regulation of expression and tissue specificity of *Ren1d* and *Ren*2 are different (23), which complicates renin studies. Mice lacking either *Ren1d* or *Ren*2 exhibited no change in BP or any other apparent phenotype (24, 25), although homozygous mice with *Ren1d* replaced with GFP had lower BP (26).

Since C57BL/6 mice, like humans, have only one renin gene, *Ren1c*, and are susceptible to diet-induced obesity, they are more suitable for research involving changes in renin expression and the metabolic syndrome (23). Because of the difficulty in generating mice by gene targeting with C57BL/6 embryonic

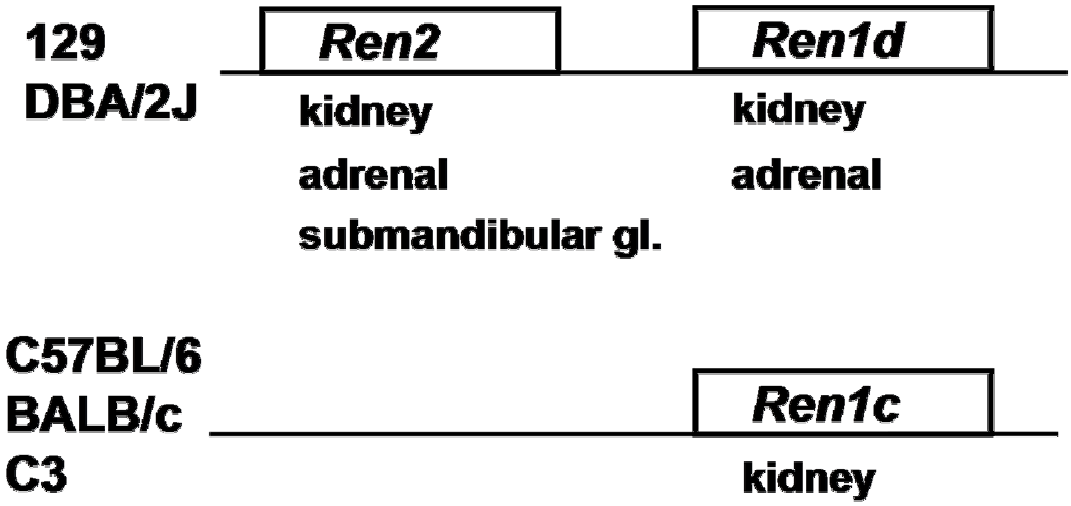

Figure 1. 2 Mouse renin genes. Renin is encoded by a single gene in human, rats and some of strains of laboratory mice such as C57bl/6, BALB/c, and C3. However, some laboratory mouse strains (eg. 129, DBA/2J) have 2 renin genes (Ren2 and Ren1d) in tandem on the same chromosome through natural duplication. Tissue expression of these genes is different.

stem cells, Yanai et al. disrupted the *Ren1c* gene using TT2 ES cells derived from an F1 hybrid between C57BL/6 and CBA (27). The authors demonstrated that these homozygous mutant mice had undetectable levels of plasma renin activity and plasma Ang I. In addition, the BP of these animals was lower than wild type by 20-30 mm Hg. The knockouts also had increased urine and drinking water volume and hydronephrosis, as observed in the *Agt*-/- mice. Abnormal granular cell layers in the hippocampus, as observed in the *Agt-/-* mice(28), were absent in mice lacking renin(27).

We have independently generated mice lacking *Ren1c* using embryonic stem cells from C57BL/6N mice (20). Our *Ren1c-/-* mice have low BP and undetectable levels of plasma renin, Ang I, and Ang II (20). Similar to the *Ace-/-* mice, our *Ren1c-/-* mice are anemic, as reflected by their low hematocrit (32 ± 3% vs. 45 ± 5 % in wild-type litter mates). Administration of Ang II (200 ng/kg/min) using an osmotic minipump restored BP levels in the *Ren1c*-/- mice to wild-type levels, but preexisting damage to the kidney medulla by hydronephrosis prevented restoration of the ability to concentrate urine. Heterozygous *Ren1c*+/- mice are normal with wild type levels of BP, and their kidney renin mRNA expression and plasma renin concentration are indistinguishable from those of wild type control mice (20).

Metabolic phenotype of mice lacking renin

We have recently investigated the role of renin in the metabolic syndrome and demonstrated that the *Ren1c*-/- mice have less than 50% of wild-type adipose tissue weight, which is consistent with the reduced fat volume of the knockouts analyzed by MRI. These mice were resistant to diet-induced obesity, although there were no changes in food intake (29). The *Ren1c*-/- mice had smaller adipocytes in all adipose tissue tested, including white adipose tissue (WAT) and brown adipose tissue (BAT), and reduced hepatic triglyceride. Based on energy balance, the lean phenotype seen in these mice should be due to either increased energy expenditure and/or increased fecal energy loss (**Fig. 1. 3**). Indeed, the null mice displayed gastrointestinal loss of dietary fat as indicated by a higher steatocrit and a lower daily fat absorption (**Table 1.2**).

Indirect calorimetry showed that, although physical activity was the same in the *Ren1c*-/- and wild-type mice, the *Renc1-/-* mice displayed increased heat generation and lipid combustion, as indicated by their low respiratory quotient (RQ). These mice require a smaller amount of insulin to maintain the same or lower plasma glucose levels relative to wild types. These results suggest a role for renin in the pathogenesis of diet-induced obesity and insulin resistance. Inhibition of renin may provide a good therapeutic target in the metabolic syndrome.

Other investigators have demonstrated that mice lacking *Agt* or *Ace* also have low BP, lean body type, and resistance to diet-induced obesity. Interestingly, however, these mice are not as lean as mice lacking renin (**Table1. 2**).

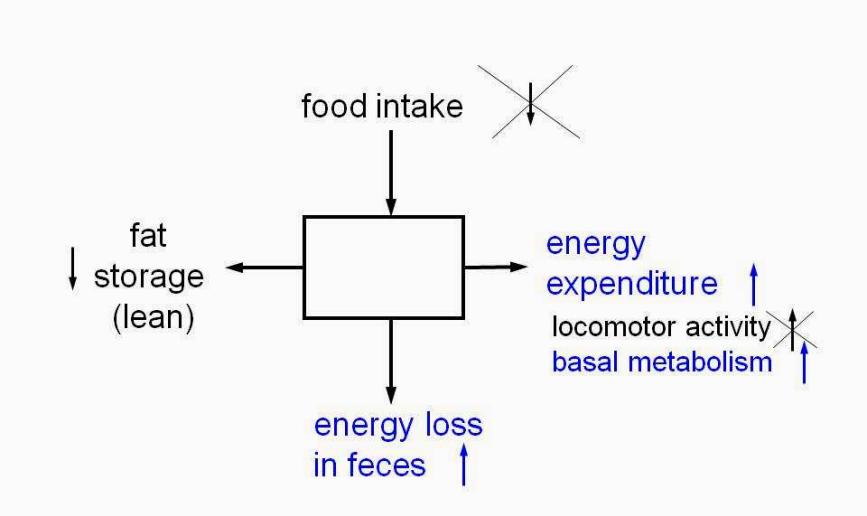

Figure 1.3 The energy flow of *Ren1c-/-* mice. Mice lacking renin are lean and resistant o diet-induced obesity due to increased energy expenditure and increased fecal energy loss.

Table 1. 2 Metabolic phenotype of mice lacking renin, AGT and ACE.

	% body fat (%WT)	food intake	fecal fat	Locomotor activity	heat generation	RQ	UCP1	renin
Ren1c-/-	45%	NS	High	NS	High	Low	High	absent
Agt-/-	76%	NS	NS	High	NS		NS	High
Ace-/-	53%	NS	NS	NS	NS	NS	NS	High

RQ: respiratory quotient, UCP1: uncoupling protein 1, NS, not significantly different from wild type controls.

The *Ren1c*-/- mice also display a unique increase in fecal fat excretion, heat generation, uncoupling protein (UCP1) expression in brown adipose tissue (BAT), and a reduced respiratory quotient (RQ), which were not observed in the *Agt*-/- and the *Ace*-/- mice (**Table 1.2**). Because BAT is required for most non-shivering thermogenesis in rodents, these data suggest that the *Ren1c*-/- mice generate more heat by combusting fat in BAT. The *Agt*-/-, *Ace*-/- and *Ren1c-/-* mice have no detectable levels of Ang II, but different from the *Ren1c-/-* mice, both *Agt*-/- and *Ace*-/- mice have very high levels of renin due to disruption of a negative feedback mechanism. Accordingly, the lack of Ang-independent effects of renin likely contributes to decreased adiposity by decreasing dietary fat absorption and increasing heat generation.

(Pro)renin receptor

The (pro)renin receptor (PRR) is a single transmembrane protein of 350 amino acids. This specific renin receptor, discovered by Nguyen and colleagues in 2002, has dual functions (**Fig. 1.4**): (i) increases catalytic activity of the PRR-bound prorenin to produce Ang I (30); and (ii) activates MAP kinases independently of Ang II (31). When prorenin binds PRR, a conformational change occurs in the prorenin molecule conferring full enzymatic activity and the ability to produce Ang I without undergoing proteolytic cleavage (32). Prorenin makes up 70-90% of total circulating renin (33), but it will not be converted to renin (34), which is synthesized only in the kidney. The binding of prorenin and renin to the cell surface in tissues is of pivotal importance in the

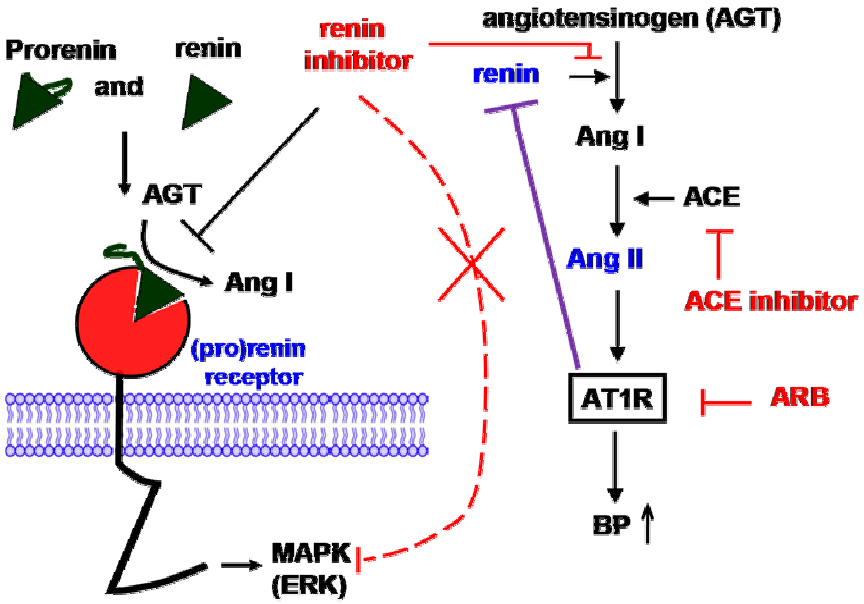

Figure 1.4 Classical RAS pathway and novel effects of (pro)renin mediated by a (pro)renin receptor. Prorenin and renin effects *via* PRR include Ang II-dependent and independent actions. Ang II- independent actions involve increased catalytic activity of the PRR-bound prorenin to generate Ang I from AGT. Ang II-indepent action involves intracellular signaling that triggers activation of an extracellular signal related protein kinase (ERK1/2) pathway.

physiology of local RAS in organs, since it provides a mechanism to generate Ang II locally in excess of the Ang II that is produced in plasma. Nguyen and colleagues also demonstrated that the binding of renin to its receptor increases the conversion rate of AGT to Ang I by four-fold (31). In addition, both renin and prorenin bind to the PRR with similar affinity and activate the MAP kinase pathway (31, 33, 35).

Ang-independent effects of renin or prorenin on adiposity, human renin transgenic (*hREN Tg)* mice weigh twice as much as wild-type mice at 60 weeks of age and display normal BP (36). BP and plasma Ang II levels in *hREN Tg* mice are indistinguishable from wild-type mice, while double transgenic mice harboring both *hREN Tg* and *hAGT Tg,* have high BP and Ang II levels (37), suggesting that human renin does not generate Ang I from mouse AGT. Indeed, when incubated with plasma from our *Ren1c*-/- mice, which contains high levels of mouse AGT, exogenous recombinant human renin ($10^{-12\sim-8}$ M) does not cause Ang I production even at a concentration 10,000 times higher than normal physiological levels of mouse renin, although both human and mouse renin equally activates ERK1/2 in mouse vascular smooth muscle cells (**Fig. 1.5**)**.** Thus, the obesity phenotype observed in human renin transgenic mice underscores the importance of an Ang-independent role of renin in regulating body weight possibly via (pro)renin receptor.

Abnormal signaling from the (pro)renin receptor is involved in cardiac fibrosis (38), nephrosclerosis (39), and microvascular complications (40). Binding of PRR to its receptor in renal glomerular mesangial cells and cardiomyocytes activates ERK and p38, respectively, independent of Ang II (31, 41). The (pro)renin receptor is highly conserved in human, mouse, rat, and other species (33), and our data show that human renin and

mouse renin are equally effective at activating MAPK in mouse cells (**Fig. 1. 5**). A renin inhibitor, aliskiren, inhibits Ang I synthesis and is widely used for treatment of hypertension (42). Ichihara and colleagues proposed using a 10-amino acid sequence of the prorenin prosegment, termed “handle region peptide (HRP)”, and demonstrated that HRP blocks binding of prorenin to PRR (40). These authors showed that subcutaneous administration of HRP to streptozotocin-induced diabetic rats decreased renal content of Ang I and Ang II, and inhibition of diabetic nephropathy (40). However, Feldt et al. were not able to block prorenin and renin-induced ERK1/2 activation by HRP or aliskiren (35). It is possible that HRP efficacy *in vivo* depends on an undefined mechanism rather than competitive antagonism for the PRR.

Ang II receptors

Ang II has two receptors, the Ang II type I receptor (AT1R) and the Ang II type II receptor (AT2R). Both receptors are G-protein-coupled receptors with seven transmembrane domains (43). The AT1R and AT2R subtypes have similar Ang II-binding properties but different genomic structure and localization, as well as tissue-specific expression and regulation.

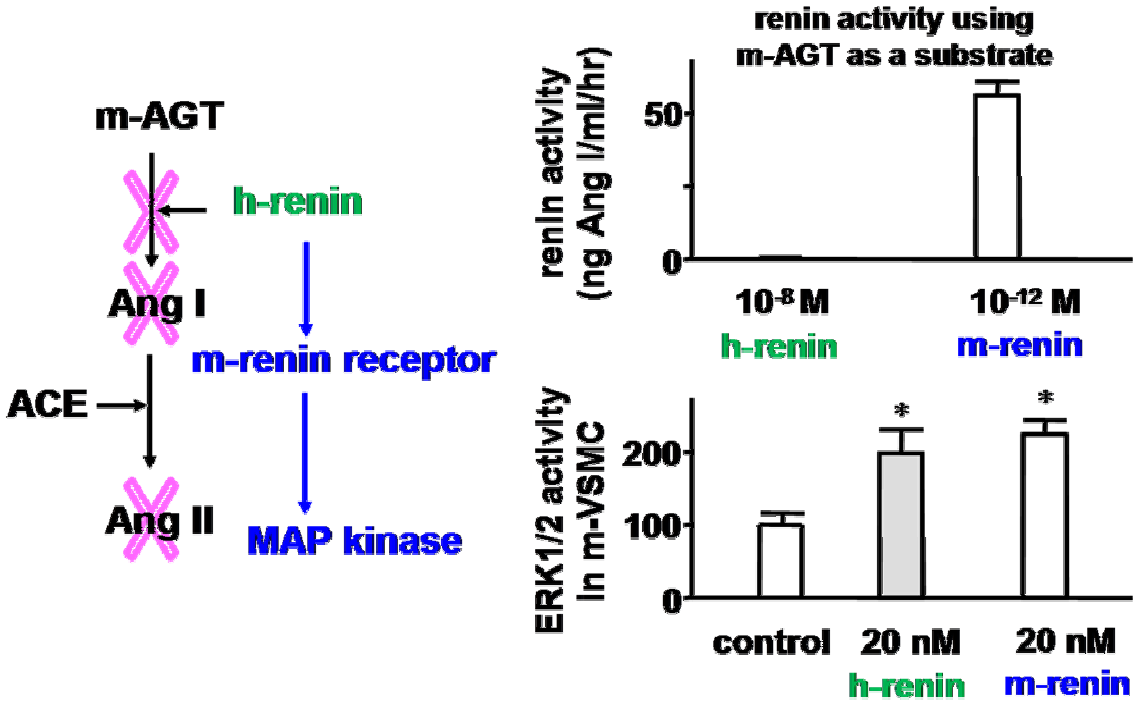

Figure 1.5 Human (h-) renin does not generate Ang I from mouse (m-) AGT. Human renin does not generate Ang I from mouse AGT. However, h-renin activates MAPK in mouse vascular smooth muscle cells possibly via m-(pro)renin receptor. *p<0.05 vs. control.

The AT1R is responsible for the classical actions of Ang II such as vasoconstriction, aldosterone release from the adrenal zona glomerulosa, salt retention in the renal tubules, and stimulation of the sympathetic nervous system. AT2R plays an important role in the growth, differentiation, and regeneration of neuronal tissue (44, 45).

The A1166C polymorphism in the 3'UTR of AT1R is strongly associated with the incidence of essential hypertension and increased coronary artery vasoconstriction (46), cardiac hypertrophy (47), and diabetic nephropathy (48), and predicts the development of the metabolic syndrome (49, 50). Humans have only one AT1R, but mice have two AT1R subtypes: AT1aR and AT1bR (*Agtr1a* and *Agtr1b*) (51). Their signaling mechanisms are almost identical but the regulation of their expression differs. AT1aR is dominant in most tissues relevant to the cardiovascular system, while AT1bR is only expressed in pituitary glands, testes, and adrenal gland (52, 53). The type 2 receptor is highly and widely expressed during fetal development; but in adults, its expression is confined to the adrenal medulla, uterus, ovary, vascular endothelium and certain areas of the brain. AT2R appears to counterbalance some of the effects of AT1R by inducing vasodilatation, growth arrest and apoptosis (**Fig. 1. 6**). Deletion of AT1aR causes hypotension and an increase in renin which leads to a profound increase in Ang II. Interestingly, *Agtr1b-/-* mice exhibit normal BP and pressor response to Ang II (54), whereas mice lacking both AT1aR and AT1bR have low BP similar to *Agt-/-* mice (55).

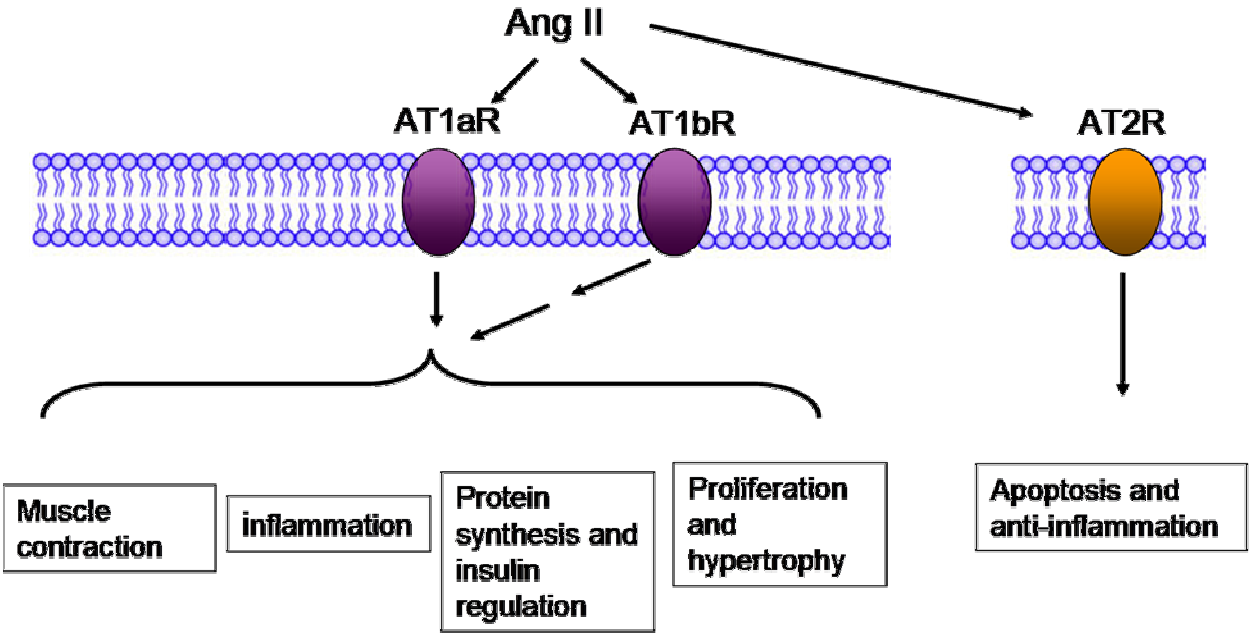

Figure 1.6 Ang II receptors. Type 1 receptor (AT1aR and AT1bR in mice) mediates the majority of Ang II effects, and regulates muscle contraction, inflammation, insulin levels and cell proliferation, while AT2R regulates apoptosis.

The dual null *Agtr1a-/-; Agtr1b-/-* mice also develop abnormal renal phenotypes identical to those observed in the *Agt-/-*, *Ace-/-,* and *Ren1c-/-* mice. In contrast, mice lacking the AT2R gene do not exhibit these phenotypes. BP in the *Agtr2*-/- mice is approximately 3-17 mmHg higher than in wild-type mice (56).

When the *Agtr2*-/- mice were treated with deoxycorticosterone acetate (DOCA) and salt, it became obvious that they are salt sensitive (56). The *Agtr2*-/- mice show congenital anomalies of the kidney and urinary tract (CAKUT) as observed in humans, namely lack of interstitial fibrosis at birth, with some hypoplastic, cystic, and/or dysplastic parenchyma (57). They also identified a single nucleotide transition (A-1332G) in intron 1 of the *AGTR2* gene in patients with CAKUT, which decreases the amount of *AGTR2* mRNA expression.

Kouyama et al. have shown that the *Agtr1a-/-* mice are lean, resistant to diet-induced obesity, with improved insulin sensitivity (58). These authors also found that these mutant mice have increased sympathetic activity and energy expenditure, probably due to the activation of AT1bR. These observations suggest that *Agtr1a-/-* mice are protected from some components of the metabolic syndrome. In addition, blocking AT1R with losartan activates the insulin-mediated IRS1/PI3/GLUT4 cascade in skeletal muscle and white adipose tissue, leading to improved glucose tolerance and insulin sensitivity (59). AT2R is expressed in the heart, kidney, brain, uterus, and adipose tissue (60). AT2R expression in adipose tissue is low, but AT2R seems to mediate Ang II stimulation of adipose tissue development (61). AT2R can induce production and release of prostacyclin from adipocytes, which in turn stimulates differentiation of preadipocytes (62). Mice lacking *Agtr2* have increased glucose uptake

in adipose tissues (62). Laurent et al. have shown that the *Agtr2*$^{y/-}$ mice (AT2R gene is on the X chromosome) have normal adiposity but display small adipocytes at an increased number (63). These authors found that mice lacking *Agtr2* have increased lipid oxidation, which is caused by increased expression of fatty acid translocase, uncoupling protein-3, peroxisome proliferation activated receptor (α, δ), and carnitine palmitoyl transferase-1 (CPT-1). In agreement with their lean phenotype, the *Agtr2*$^{y/-}$ mice have decreased food intake and increased total energy expenditure. These studies indicate that inhibition of AT1R and AT2R improves insulin sensitivity and causes fat loss, which is in agreement with the involvement of Ang II in the insulin signaling pathway and in the control of adipose tissue metabolism.

Pharmacological inhibition of RAS

Blocking RAS with ACE inhibitors or Ang receptor blockers (ARB) has become a crucial element of cardiovascular and renal medicine. Clinical studies indicate that ARBs and ACE inhibitors prevent the onset of diabetes and improve insulin sensitivity (64, 65). In mice, blocking AT1R improves insulin sensitivity and other diabetes symptoms due to increased glucose uptake in skeletal muscle and white adipose tissue (66). This is due to enhanced insulin signaling and GLUT4 translocation to the plasma membrane (66). Treatment with ARBs (losartan, or candesartan) does not reverse diabetes, but it effectively improves glucose tolerance and protects β-cell function by attenuating oxidative stress, islet fibrosis, sparse blood supply, and disruption of ultrastructure in a dose-dependent and BP-independent manner (67). In addition, ARBs may protect the pancreas in type II diabetic animal models with enhanced insulin

secretion (68). Furthermore, in diabetic models, some ARBs induce adipocyte differentiation by increasing peroxisome proliferator-activated receptor-gamma (PPARγ) in adipose tissue (69). Accordingly, ARBs could be a novel form of treatment for the metabolic syndrome and associated pathological disorders.

Moreover, aliskiren is a novel renin inhibitor for the treatment of hypertension (70). This renin inhibitor has protective effects on endothelial function and atherosclerotic changes. Lu et al. showed that aliskiren resulted in striking reductions of atherosclerotic lesion size in both the aortic arch and the root in fat-fed LDL receptor–deficient (*Ldlr*$^{-/-}$) mice (71). Furthermore, cotreatment with aliskiren and an ARB has additive protective effects (72). Thus, aliskiren may also be a promising protective drug for patients with hypertension and diabetes (73).

Leptin resistance and the Renin Angiotensin System

Obesity associated with leptin resistance results in elevated blood leptin levels. Leptin is a 16 kDa polypeptide hormone secreted by adipose tissue which conveys information about energy storage and availability to the hypothalamus. The hypothalamus is a critical area that senses and integrates various neuronal, hormonal and nutrient-related signals. (74). Leptin signals in the hypothalamus by binding to leptin receptor (LRb), and activating the associated tyrosine kinase JAK2 (Janus kinase 2) phosphorylates residues Tyr985 and Tyr1138 of LRb, and recruits, and activates the transcription factor STAT3 (signal transducer and activator of transcription 3) within the LRb cytoplasmic tail(75). Reduced activation of STAT3 phosphorylation in response to leptin is evident in the arcuate nucleus of the hypothalamus and induces leptin

resistance and diet-induced obesity (DIO) *via* SOCS3 that has assessed DIO and leptin resistance (76). Increased hypothalamic SOCS3 levels are associated with leptin resistance including hyperplasia, hyperleptinemia and accumulation of triglyceride in the liver and skeletal muscle (76). Mice with haploinsufficiency of SOCS3, or those with neuronal deletion of SOCS3 have demonstrated improved leptin and insulin sensitivity, and resistance to diet-induced obesity (77, 78). The efficacy of leptin therapy in obese humans, however, has been in a large frustration due to the development of leptin resistance. Thus, targeting molecules required for the development of leptin resistance is a potentially powerful therapeutic approach for anti-obesity.

Moreover, leptin resistance is a hallmark and key determinant of obesity, and leads to an accumulation of triglyceride (TG) not only in adipose tissues but also in the liver, skeletal muscle, and other peripheral tissues- leading to obesity, insulin resistance, and non-alcoholic steatohepatitis (NASH). Enhanced leptin sensitivity is associated with improved glucose homeostasis (79). Leptin activates JAK2, a cytoplasmic tyrosine kinase that binds to LRb and activates STAT3 transcription factor. STAT3 induces suppressor of cytokine signaling-3 (SOCS3) expression, which inhibits leptin signaling by JAK2 inactivation (80). In addition, Ang II increases SOCS3 *via* JAK-STAT3 pathway in the hypothalamus, and heart, while inhibition of Ang II by ARB losartan causes decreased leptin signals (**Fig 1.7**) (81). However, relationship between Ang II and leptin remains unclear. Accordingly, I investigated whether Ang II plays an important role in the regulation of food intake and leptin sensitivity *via* JAK2/STAT3 pathway.

Leptin and hypothalamic neuropeptides: POMC, NPY and AgRP

Leptin can regulate food intake and energy expenditure by modulating orexigenic and anorexigenic signaling pathway in the hypothalamus **(Fig 1.7)**. Binding of leptin to LRb decreases food intake and increases energy expenditure. LRb is highly expressed in two groups of neurons in the arcuate nucleus (ARC) of the hypothalamus: one group expresses either orexigenic neuropeptide Y /Agouti-related protein (NPY/AgRP), and the other group expresses anorexigenic pro-opiomelanocortin POMC (82). Stimulating POMC neurons by leptin results in inhibiting NPY/AgRP neurons and decreasing food intake, body weight, and AMPK activity as well as increasing energy expenditure and expression of thyrotropin releasing hormone (TRH) (83) **(Fig 1.7)**. POMC neurons have been considered as the primary cell type for mediating leptin effect through alpha-melanocyte-stimulating hormone (α-MSH), which is a cleavage product of POMC. α-MSH belongs to a melanocortin group including ACTH, α-MSH, β-MSH and γ-MSH(84). Anorexigenic effect of lepitn is mediated by binding of α-MSH to MC4 receptor (MC4R) resulting in suppression of NPY/AgRP neuron, increased energy expenditure, decreased food intake and TRH synthesis (85). Thus, POMC increases metabolic rate and energy expenditure by stimulating hypothalamic TRH synthesis(86).

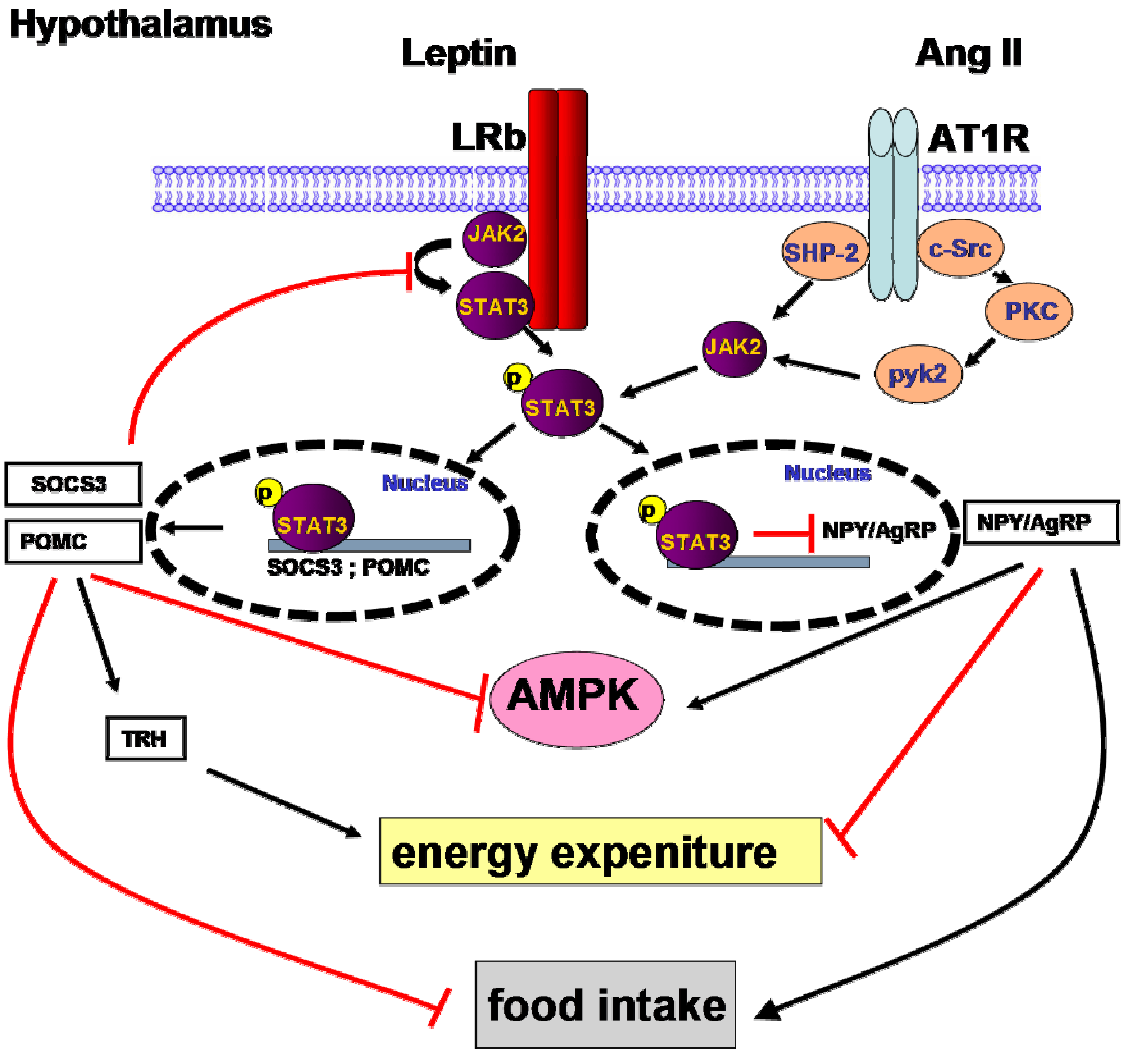

Figure 1.7 Leptin signaling pathway regulates AMPK activity, food intake and energy expenditure via anorexigenic, and orexigenic neuropeptides in the hypothalamus. Leptin inhibits AMPK activity and food take and increases energy expenditure directly or indirectly *via* STAT3 phosphorylation, and increases the expression of anorexigenic neuropeptides POMC, and decreases the expression of orexigenic neuropeptides AgRP and NPY.

Moreover, several studies have shown that α-MSH via melanocortin 4 receptor (MC4R) increases TRH synthesis in the paraventricular hypothalamic nucleus (PVN) (85). Leptin can increase metabolic rate and energy expenditure by activating POMC neurons, which in turn increases synthesis of hypothalamic TRH, leading to elevated thyroid hormones (83). Accordingly, fasting reduces plasma leptin level, which leads to increased food intake and diminished metabolic rate and energy expenditure by decreasing thyroid hormones(85), whereas ablation of POMC neurons leads to profound obesity(84) and reduced circulating thyroid hormone levels due to decreased hypothalamic TRH gene expression(87). In addition, leptin improves glucose tolerance, and increases glucose transporter and nitric oxide expression by PI3K signaling pathway(88). Thus, leptin sensitivity could provide an avenue for fighting diabetes and obesity. However, whether RAS modulates the expression or function of POMC, NPY, and AgRP in the hypothalamus is not well known.

A central role for neuronal AMP-activated protein kinase (AMPK) for food intake and energy expenditure

AMP-activated protein kinase (AMPK) plays an important role in the regulation of cellular energy metabolism by phosphorylation of key enzymes in the metabolic pathways, such as acetyl–coenzyme A carboxylase (ACC)(89). In mammalian, AMPK is a trimeric enzyme including a catalytic α subunit (63 kDa) and non-catalytic β and γ subunits. Multiple isoforms of each mammalian enzyme exist (α1, α2, β1, β2, γ1, γ2, γ3), each encoded by a different gene (90). The β subunits have a molecular mass in 38

kDa (β1) and 34 kDa (β2), whereas the three γ isoforms have molecular masses of 37 kDa (γ1), 63 kDa (γ2) and 55 kDa (γ3).(91) AMPK complexes containing α2 (the first catalytic isoform to be cloned) (89) is predominately expressed in the hypothalamus, skeletal and cardiac muscle, whereas equal levels of α1 and α2 complexes are present in the liver.

AMPK functions have been extensively investigated in the hypothalamus, liver, and skeletal muscles(89). In the hypothalamus, AMPK activity is altered by hormonal and metabolic signals and mediates the feeding response to leptin and insulin **(Fig 1.7 and 1.8)**. Hypothalamic AMPK signaling plays an important role in the control of food intake (92, 93), particularly in the arcuate nucleus (ARC) integrates peripheral nutritional and feeding signals. When energy intake exceeds expenditure, the expression of orexigenic neuropeptides, such as AgRP and NPY, decreases. Conversely, the expression of anorexigenic neuropeptides, such as POMC, increases. Opposite changes occur when energy expenditure exceeds energy intake **(Fig 1.8)**. Mice lacking AMPK function in POMC neurons became obese because they reduced their energy expenditure and increased their food intake after fasting (94). By contrast, mice lacking AMPK function in AgRP neurons showed age-dependent decrease in body weight (94) and the anorexigenic signals (POMC) inhibit AMPK activity in orexigenic AgRP/NPY neurons and increases energy expenditure (93). This suggests that leptin indirectly inhibits hypothalamic paraventricular nucleus AMPK activity via α-melanocyte–stimulating hormone (α-MSH) release and activation of melanocortin 3/4 receptors (MC3/4Rs) (93).

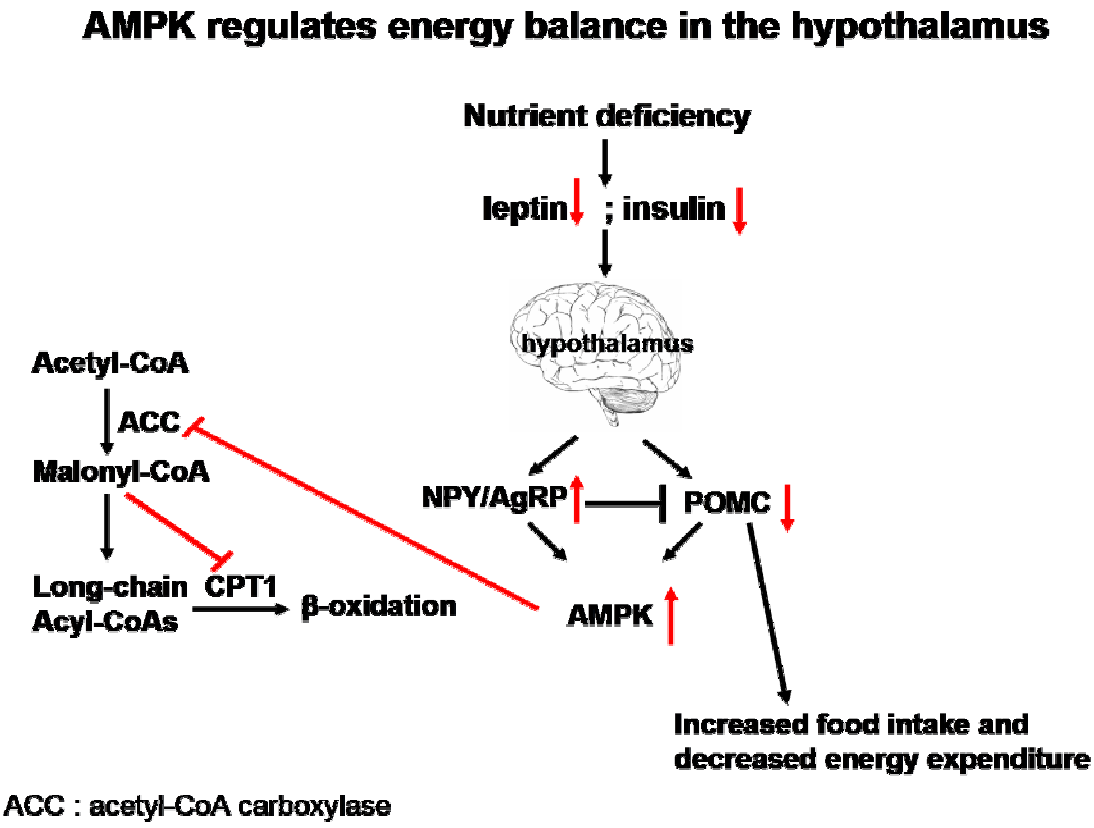

Figure 1.8 AMPK mediates leptin and insulin to regulate food intake and fatty acid oxidation in the hypothalamus. Increase orexigenic neuropeptides AgRP and NPY, and decrease the expression of anorexigenic neuropeptides POMC by AMPK, which also regulates fatty acid oxidation *via* acetyl-CoA carboxylase (ACC).

Moreover, in the peripheral tissues, AMPK acts as a counter-regulatory mechanism that switches off ATP-consuming processes while switching on catabolic processes that produce ATP and restore the AMP: ATP ratio. AMPK stimulates pathways, which increase energy production(95)(glucose transport, and fatty acid oxidation) and switches off pathways, which consume energy (lipogenesis, protein synthesis, and gluconeogenesis)(95). Adiponectin and leptin as well as hypoglycemic drugs activate AMPK in the liver and skeletal muscle. However, whether RAS modulates AMPK activity is not fully understood.

Uncoupling protein-1 (UCP-1) and energy expenditure

Mitochondrial UCP-1 is a 32 kDa transmembrane protein which discharges the proton gradient for oxidative phosphorylation(96). UCP-1 is a major regulator of heat generation, allowing substrate oxidation with a low rate ATP production. It is predominately expressed in the brown adipose tissue (BAT), which is responsible for cold adaptation/acclimation, increases fatty acid oxidation and electron transport chain activity resulting in heat production by this tissue. This adaptive process is termed non-shivering thermogenesis(97).

In addition, the activation of UCP1 is accompanied by a dramatic increase in lipolysis and mobilization of lipids in white adipose tissue depots. In mice, increased content of UCP1 in adipose tissue is strongly linked to protection against diet-induced obesity(98), which is consistent with UCP-1 knockout mice. Ablation of UCP-1 in mice induces obesity with decreased oxygen consumption and energy expenditure at

thermoneutrality (at 30℃)(99) . Therefore, UCP-1 protein plays an important role in diet-induced thermogenesis and can be a target molecule for treatment of obesity.

UCP1 and RAS

BAT UCP-1 is required for most non-shivering thermogenesis to combat obesity(96) and the *Agt*-/-, *Ace*-/- , *Agt1a*-/- and *Ren1c*-/- mice are resistant to diet-induced obesity(58, 100-102). However, *Agt*-/-, *Ace*-/- and *Agt1a*-/- mice have BAT UCP-1 expression comparable to WT mice, whereas the *Ren1c*-/- mice have an increase of BAT UCP-1 expression and generate more heat by combusting fat in BAT. Although the *Agt*-/-, *Ace*-/- and *Ren1c*-/- mice have no detectable levels of Ang II, different from the *Ren1c*-/- mice(102), both *Agt*-/- and *Ace*-/- mice have very high levels of renin due to disruption of a negative feedback mechanism. Accordingly, lack of Ang-independent effects of renin likely contributes to decreased adiposity by increasing heat generation.

Thyroid hormone and RAS

UCP1 is predominately regulated by sympathetic nervous system (SNS) and thyroid hormone (TH) (96). TH plays a critical role in development, growth, and cellular metabolism. TH production is controlled by a complex mechanism of positive and negative regulation. Hypothalamic TRH stimulates TSH secretion from the anterior pituitary, which in turn promotes TH synthesis and release from the thyroid gland. The synthesis of TRH and TSH gene expression is inhibited at the transcriptional level by TH, which also inhibits posttranslational modification and release of TSH **(Fig 1.9).**

Although opposing TRH and TH inputs regulate the hypothalamic-pituitary-thyroid axis (HPT), TH negative feedback at the hypothalamus plays a critical regulator of TH levels (103).

Recently, the role for TRH in TH feedback of the HPT axis has been demonstrated. Mice lacking either TRH (*TRH-/-*), the β-isoforms of TH receptors (*TRβ-/-*), or both (double KO) were generated (104). As previously reported, *TRβ-/-* mice have significantly increased TH, TSH and TRH levels compared with wild-type mice. In contrast, double KO mice had reduced TH, TSH and TRH levels compared with control animals. Unexpectedly, hypothyroid double KO mice also failed to increase plasma TSH levels, and pituitary TSH immunostaining was markedly reduced compared with all other hypothyroid mouse genotypes (104). This impaired TSH response, however, was not due to a reduced number of pituitary thyrotrophs because thyrotroph cell number, as assessed by TSH-immunopositive cell number, was restored after chronic TRH treatment (105). Thus, the TRH neuron is absolutely required for both TSH and TH synthesis and appears to be the locus of the set-point in the HPT axis.

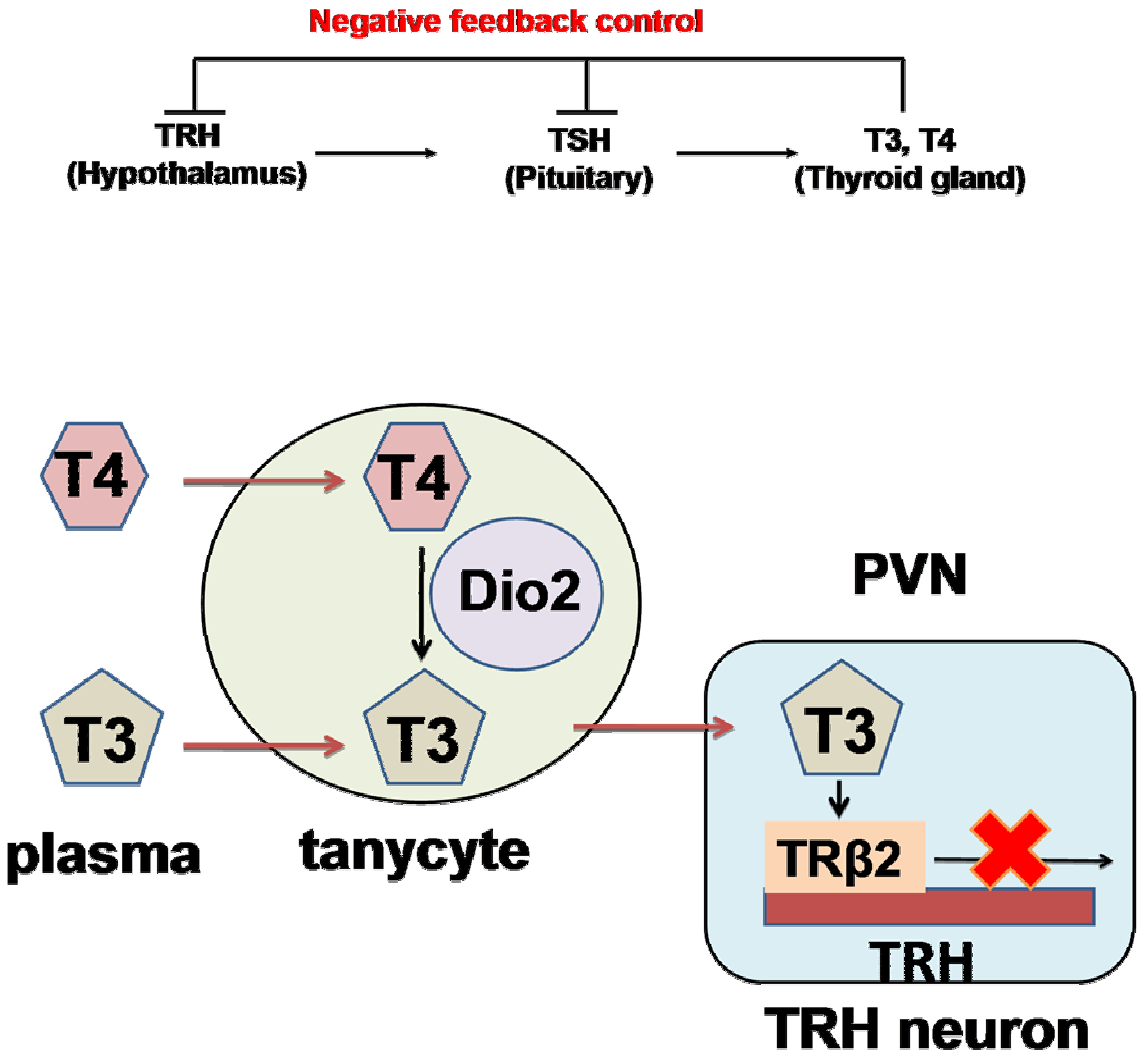

Figure 1.9 Feedback regulation of thyroid hormone. Hypothalamic TRH is regulated by TH levels via type 2 iodothyronine deiodinase (Dio2), which converts T4 to a more potent T3 in tanycytes. After increased local T3 in TRH neurons, T3 inhibits the prepro-TRH transcription and processing of pro-TRH into the mature TRH peptide through TRβ2.

Moreover, hypothalamic TRH gene expression is regulated by TH levels and production through a negative feedback mechanism: TRH expression is high when TH levels are low, and TRH expression is suppressed when TH levels are increased. This mechanism is dependent on type II deiodinase (Dio2), which converts T4 to a more potent T3 in tanycytes (103). After T3 enters TRH neurons in the paraventricular nucleus (PVN), regulation occurs at two levels: inhibition of expression of the prepro-TRH transcription and processing of pro-TRH into the mature TRH peptide. The regulation of TRH gene expression by T3 occurs mostly through TRβ2, presumably via a direct mechanism (106). Another possibility is that T3 acts in the signaling pathway of TRH gene expression *via* other hypothalamic nuclei because TRβ2 is also expressed in the arcuate and ventromedial nuclei, and both nuclei can alter TRH expression (107).

Leptin is one of the important regulators in hypothalamic TRH expression and thyroid hormones homeostasis(108). Leptin stimulates hypothalamic TRH gene expression *via* JAK2-STAT3 signaling pathway in arcuate nucleus neurons, where orexigenic *NPY/AgRP* and anorexigenic *POMC* signals integrate. Increased leptin levels cause elevated POMC signals, which in turn promotes increased TRH expression in PVN, while reduced leptin levels cause increased NPY/AgRP signals, which inhibits TRH expression. In addition, AngII increases the suppressor of cytokine signaling (SOCS3) through JAK2/STAT3 signaling pathway, and angiotensin II type 1 receptor (AT1R) is able to signal through the intracellular phosphorylation, which depends on its association with Janus kinases (JAKs) and signal transducer and activator of transcription (STAT) factors(109). The AT1R has a preferential effect on JAK2 activation and results in STAT3 phosphorylation (110). Several studies have shown that TRH

expression is directly regulated by the effect of leptin on PVN *via* JAK2/STAT3 signaling pathway. However, whether angiotensin II regulates thyroid hormones homeostasis and hypothalamic TRH expression through JAK2/STAT3 pathway remain uncertain. Here, I investigated whether inhibition of RAS affects thyroid hormone function.

RAS and erythropoiesis

RAS regulates erythropoiesis(111), but its mechanism is not well known. Several studies have shown that Ang II stimulates proliferation of erythroid progenitor cells via AT1 receptors *in vitro*, whereas the inhibition of Ang II decreased burst-forming units-erythroid (BFU-E) colony formation (112). In addition, the *Ace-/-* mice have severe anemia and administration of Ang II corrects it (113). Interestingly, anemic *Ace-/-* mice have higher plasma erythropoietin (EPO) levels than WT mice (113), although Tsukuba hypertensive mice (114) and patients administered with Ang II have been shown to increase plasma EPO levels. However, the precise mechanism of Ang II on erythropoiesis needs further investigation.

Hypothyroidism and anemia

Hypothyroidism is a common endocrine disorder caused by low circulating thyroid hormone. It usually is a primary hypothyroidism in which the thyroid gland is the primary site of causing this disorder. It can also be secondary or tertiary hypothyroidism —that is, lack of thyroid hormone secretion due to inadequate secretion of either TSH from the pituitary gland or TRH from the hypothalamus (115), respectively. The patient's presentation may vary from asymptomatic to, rarely, coma with multisystem organ

failure (myxedema coma). However, the common sign seen in hypothyroidism is anemia (116), which the red blood cells count or hemoglobin is less than normal. Anemia is caused by essentially through two basic pathways. One is caused by a decrease in production of red blood cell or hemoglobin, while another is caused by a loss or destruction of blood. In infants, the liver and spleen are the main organs to produce red blood cells, whereas bone marrow is a major place to produce red blood cells in adult. Thyroid hormone has been suggested to regulate erythropoiesis in the bone marrow by stimulating burst-forming units-erythroid (BFU-E) colony formation (117, 118), while EPO or the erythropoietin receptor (EPOR is not required for committing erythroid BFU-E and CFU-E progenitor cells(119). CD 71 is a transferrin receptor, which is predominately expressed in erythroid BFU-E and CFU-E progenitor cells, whereas ter119 erythroid marker is expressed in late stage erythroid progenitor cells **(Fig 1.10)**. A number of studies have shown that thyroid hormone regulates erythropoiesis in bone marrow(120), and mice lacking thyroid hormone α receptor (*TR α-/-*) have impaired erythropoiesis (121). Interestingly, however, patients who have chronic kidney diseases (CKD) and renal transplantation who are treated with Ang I converting enzyme inhibitor (ACEI) (122, 123) or Ang II type 1 blocker (ARB) have severe anemia (124), and mice lacking Ang I converting enzyme display decreased red blood cells, hemoglobin and hematocrit, whereas plasma erythropoietin was elevated (113). Angiotensin II directly stimulates erythropoiesis, but Ang II indirectly stimulates erythropoiesis as well. However, whether thyroid hormone is responsible for mediating Ang II-induced erythropoiesis is not known.

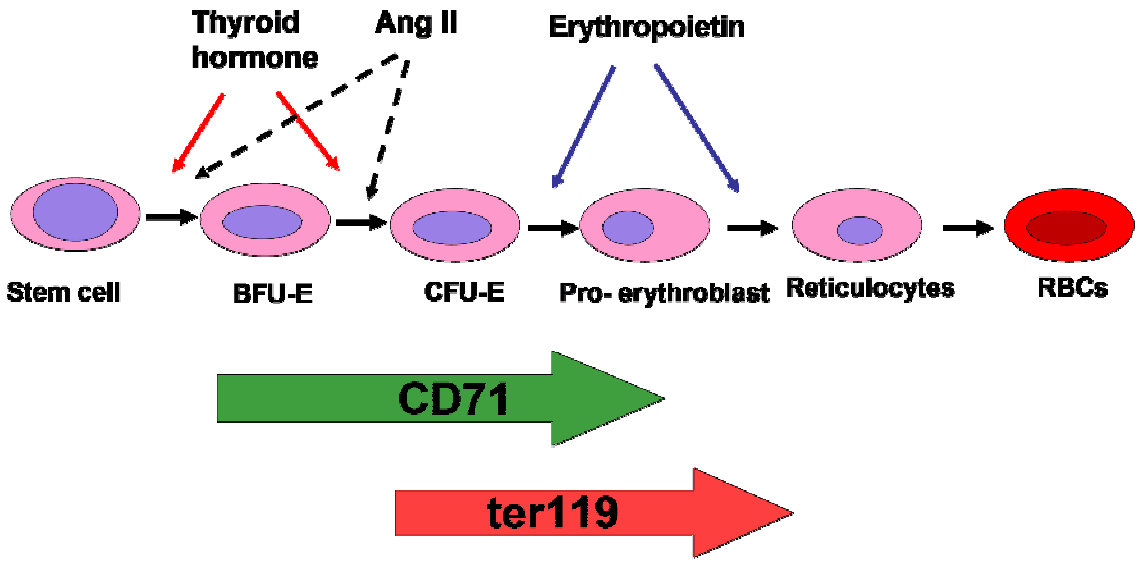

Figure 1.10 Erythroid differentiation. CD71 is expressed in early stage erythroid progenitor cells, whereas ter119 is expressed in late stage erythroid progenitor cells.

Significance

The overall goal of the research demonstrated in this dissertation is to unveil the complexity of the RAS in leptin sensitivity, thyroid function, and erythropoiesis. Although the importance of RAS in the metabolic syndrome is generally acknowledged, whether Ang II modulates leptin sensitivity is not addressed. Understanding how renin and Ang II relate to fat metabolism could help in designing effective treatments of obesity and the metabolic syndrome.

In addition, patients who have chronic kidney diseases and renal transplantation treated with ACEI or ARBs have severe anemia, suggesting Ang II plays an important role in erythropoiesis. To clarify how Ang II regulates erythropoiesis, the *Ren1c-/-* mice provide important information. The results of my study provide important insight into the treatment of obesity with RAS inhibitors and treatment of hypothyroidism and anemia caused by RAS inhibition.

Chapter 2:

Inhibition of angiotensin II-JAK2-STAT3 pathway improves leptin and insulin sensitivity

SUMMARY

Obesity is often associated with leptin resistance, and improving leptin sensitivity will be a valuable tool for treatment of obesity. We have recently demonstrated that the mice lacking renin (*Ren1c*-/-) are lean and insulin sensitive, which is associated with increased energy expenditure and fecal fat excretion. These mice have decreased tissue expression of suppressor of cytokine signaling 3 (SOCS3), a molecule that contributes to leptin resistance. The aim of the present study is to investigate whether inhibiting the renin angiotensin system (RAS) improves leptin sensitivity. An increase in phosphorylation of STAT3 and a decrease in AMPK activity of the hypothalamus after leptin administration were larger in the *Ren1c*-/- mice than in WT mice. The expression of hypothalamic POMC was lower, and that of NPY and AgRP was higher in the *Ren1c*-/- mice compared to WT mice. These changes were recapitulated by administration of a type 1 Ang II receptor blocker losartan to WT mice, and Ang II reversed these changes whereas human renin did not. Although a larger response to leptin of the *Ren1c*-/- mice is consistent with improved leptin sensitivity, it could simply be due to lower plasma leptin levels of the knockouts compared to WT as a result of lean phenotype of these mice. To test this possibility I generated mice lacking both renin and leptin (*Ren1c*-/-;*ob/ob*). Although the *Ren1c*-/-;*ob/ob* mice have 10% lower body weight than the *ob/ob* mice, administration of leptin (100μg/kg/day for 2 weeks) decreased the % body fat more in the *Ren1c*-/-;*ob/ob* mice than in the *ob/ob* mice. A decrease in body fat of the *Ren1c*-/-;*ob/ob* mice is associated with a larger decrease in food intake and food efficiency, a larger increase in heat generation and increased fecal fat excretion compared to the *ob/ob* mice. The *Ren1c*-/-;*ob/ob* mice also showed decreased

triglyceride content in the liver and the skeletal muscle. The hypothalamus of the *Ren1c*-/- and the *Ren1c*-/-;*ob/ob* mice have decreased SOCS3 and phosphorylation of STAT3, and increased AMPK activity relative to WT or *ob/ob* mice. Compared to the ob/ob mice, the *Ren1c*-/-;*ob/ob* mice showed an increase in response to exogenous leptin, which resulted in a larger changes in phosphorylation of STAT3 and AMPK activity with a smaller elevation of hypothalamic SOCS3 gene expression. Changes in the expression of hypothalamic POMC, NPY, and AgRP after leptin administration were significantly larger in the *Ren1c*-/-; *ob/ob* mice than in the *ob/ob* mice. The *Ren1c*-/-;*ob/ob* mice have improved glucose tolerance, insulin tolerance and lower gluconeogenesis compared to the *ob/ob* mice. Administration of leptin further improved glucose tolerance of all animals but minimized the difference between the two groups of mice. Improved leptin and insulin sensitivity by the absence of renin was recapitulated by losartan. Together, my results demonstrate that inhibiting Ang II-AT1R pathway improves leptin and insulin sensitivity, and may be useful in treating insulin resistance and leptin resistance in human obesity and diabetes.

INTROUCTION

An overactive renin-angiotensin system (RAS) induces insulin resistance and obesity (125, 126). Mice lacking renin (*Ren1c-/-*) are lean and insulin sensitive(102). Although obesity is often associated with leptin resistance (127), it is not well known whether inhibition of RAS improves leptin sensitivity, and whether improved leptin sensitivity contributes to the lean and insulin sensitive phenotype of *Ren1c-/-* mice. Leptin resistance is often induced by hyperleptinemia (127) , and low plasma leptin levels increase leptin sensitivity via decreased expression of the suppressor of cytokine signaling-3 (SOCS3), which is a negative-feedback regulator of leptin signaling JAK2-STAT3 pathway (79). Improving leptin sensitivity is thought to be useful for treatment of obesity (75); however, the target molecules for leptin sensitivity are not well identified. Our previous work has shown that mice lacking renin (*Ren1c*) have low blood pressure and undetectable plasma angiotensin II (Ang II). These mice are lean and insulin sensitive without changes in physical activity and food intake (102). Remarkably, their plasma leptin levels were only approximately 40% of those of wild type (WT) mice. Mice lacking angiotensinogen (AGT), Angiotensin converting enzyme (ACE), type 1 Angiotensin receptor (AT1R), or type 2 Angiotensin receptor (AT2R) are also lean, and have low plasma leptin levels (29, 58, 126, 128). The decreased Ang II level is likely responsible for reduced plasma leptin levels when RAS is inhibited. Interestingly, it was recently shown that Ang II and leptin share the JAK2 -STAT3 signaling pathway (81, 129-131) that regulates physiological homeostasis such as thyroid hormone levels, blood pressure and fluid homeostasis. Therefore, I hypothesize that inhibition of Ang II improves leptin sensitivity via the attenuation of leptin-JAK2-STAT3 pathway.

Leptin is an important hormone that regulates energy balance via JAK2-STAT3 signaling pathway. Leptin is mainly expressed in adipose tissue and research hypothalamus via circulation, and binds to the leptin receptor (LRb) resulting in activation of the receptor-associated tyrosine kinase JAK2, which in turn phosphorylates STAT3 within the LRb cytoplasmic tail (75). Through activation of JAK2-STAT3 signaling pathway, leptin can regulate appetite and thyroid hormone homeostasis, and increase energy expenditure (74). However, an overactive JAK2-STAT3 signaling pathway causes a negative feedback inhibition. Thus, the elevated hypothalamic SOCS3 levels lead to leptin resistance resulting in hyperphasia, hyperleptinemia and accumulation of triglyceride in liver and skeletal muscle (76). In contrast, mice with haploinsufficiency of SOCS3, or those with neuronal deletion of SOCS3 have shown to improve leptin and insulin sensitivity (77, 78). Furthermore, blockade of Ang II action by AT1R blockers result in decreased JAK2-STAT3 signals leading to in lower levels of SOCS3 expression in the hypothalamus, astrocytes, heart, and vascular smooth muscle (81, 129-131). Accordingly, attenuation of leptin signaling by inhibition of Ang II is likely to improve leptin sensitivity. Moreover, the arcuate nucleus (ARC) of the hypothalamus is a critical area that senses and integrates various hormones and nutrient-related signaling to orexigenic and anorexigenic neurons. LRb is highly expressed in these neuron, which express orexigenic neuropeptide Y and Agouti-related protein (NPY/AgRP) or anorexigenic pro-opiomelanocortin (POMC) (132). Phosphorylation of STAT3 by leptin also regulates expression of NPY, AgRP and POMC in the hypothalamus (132). Elevated phosphorylation of STAT3 levels by leptin

increases the expression of POMC, which in turn inhibits the expression of NPY/AgRP (133). Thus, leptin stimulates POMC neurons, and inhibits NPY/AgRP neurons resulting in decreased food intake and body weight (BW). Furthermore, hypothalamic AMPK is an important sensor of energy balance, which is inhibited by leptin via anorexigenic POMC (92, 93). Stimulating AMPK by various means including adiponectin, metformin, and exercise has shown a great benefit in obesity and diabetes. The usefulness of leptin therapy in obesity, however, has not been proven because it can lead to the development of leptin resistance. Thus, indentifiying signaling pathway and mechanism to improve leptin sensitivity is critical a potentially powerful therapeutic approach for treating obesity.

Here I demonstrate that mice lacking both renin and leptin have improved leptin and insulin sensitivity, which leads to reduced triglyceride (TG) accumulation and increased fatty acid oxidation in liver and skeletal muscle. These studies directly point to a novel role for Ang II in leptin sensitivity and energy balance via JAK2-STAT3 signaling pathway. Inhibition of Ang II combined with leptin administration provides a potentially powerful therapeutic approach to obesity.

MATERIAL AND METHODS

Mice: The generation of *Ren1c*–/– mice (C57BL/6 genetic background) (20) were described previously. *ob*/+ mice on a C57BL/6J genetic background were purchased from the Jackson Laboratory (Bar Harbor, Maine) (134). All mice were housed in standard cages on a 12-hour light/dark cycle, allowed free access to normal chow and water, and handled in accordance with the National Institutes of Health guidelines for the use and care of experimental animals, as approved by the IACUC of UNC-CH.

Plasma leptin adiponectin measurement: Plasma levels of leptin, and adiponectin were determined using ELISA kits (cat number: 90040, Crystal Chem Inc. for leptin; cat number: K1002-1; B-Bridge International Inc. for adiponectin).

MRI: Mouse body composition was evaluated using EchoMRI-100 (Echo Medical Systems Houston, TX) according to manufacturer's instruction.

Energy balance study using indirect calorimetry: Mice were placed in a chamber individually of an Oxymax system (Sophisticated Life Science Research Instrument) for 24 hours, and readings were taken next 24 hrs after acclimation. Measurements of food and water intake, O_2 consumption, CO_2 production, and respiratory exchange ratio (RER) were obtained. Data were collected and analyzed with the Oxymax Windows V2.3 software. Measurement and settle times were set at 60 and 120 seconds, respectively.

Liver TG measurement and plasma AST, and ALT: About 50 mg of excised frozen liver tissue was extracted in 1 ml of a chloroform-methanol mixture (2:1). After re-dissolving the lipid pellet with 60 µl of tert-butanol and 40 µl of Triton X-114-methanol (2:1) mixture, the triglyceride was measured by triglyceride kit (cat number: 2151-015, StanBio) . Plasma AST and ALT were measured by automatic chemical analyzer (Johnson&Johnson's VT250).

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Ang II, human renin, and losartan challenge *in vivo*: Mice were administered Ang II (7.5 µg/kg/day, Sigma) or human renin (0.1µg/kg/day; cat number: 10006217, Cayman Chemical) with intraperitoneal i.p. Alzet miniosmotic pumps (cat number: 10190-08, Durect Corp., Cupertino, CA) for 2weeks. The 3-month-old male C57BL/6 WT mice or *ob/ob* mice were fed regular chow with or without 0.45 g/l of losartan (Merck & Co., Inc.) in drinking water for 2 weeks (29).

Oral glucose tolerance test (OGTT), insulin tolerance test (ITT), and pyruvate challenge test (PTT). 3-month old male mice were used by OGTT, ITT and PTT. For OGTT, mice were fasted overnight and glucose was administered by gavage at a dose of 2 mg glucose/g BW. At 0, 15, 30, 60, and 120 minutes after glucose administration blood was drawn retro-orbitally, and plasma glucose and insulin levels were measured by a colorimetric assay (Wako) and Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem Inc), respectively. For ITT, mice were fasted overnight and blood glucose level was measured before (0) and after 15, 30, 60, and 120 min after intraperitoneal injection of insulin at a dose of 0.75 U insulin/kg BW (Eli Lily, Indianapolis, IN) (135). For PTT,

mice were deprived of food for 16 hours were injected intraperitoneally with pyruvate dissolved in saline (2 g/kg). Blood glucose level was measured at 0, 15, 30, 60, and 120 min.

Acid Steatocrit: Acid steatocrit was measured as described previously (29). In short, 0.05 g of powdered specimen was mixed in 200 ml of 1 N perchloric acid. One drop of 0.5% oil red O was added and mixed. Specimens were placed in non-heparinized capillary tubes and spun. Steatocrit was calculated as 100 x length of fatty layer/(length of solid layer + length of fatty layer).

Leptin sensitivity: The 3-month old male mice were tested for leptin response after 2 weeks, and 4 weeks on a normal chow. The *Ren1c-/-;ob/ob and ob/ob* mice were infused with 5µg of recombinant murine leptin (100µg/kg/day, R&D Systems, Minneapolis, MN) by Alzet miniosmotic pumps (cat number: 10190-08, Durect Corp., Cupertino, CA) for 2weeks (79). BW, food intake and feces were measured before and after injection (136).

Quantitative RT-PCR: mRNA levels were quantified with the Mastercycler® ep realplex S (Eppendorf, Westbury, NY) as described previously (29). Relative gene expression levels, expressed as a percentage of WT, were determined with hypoxanthine phosphoribosyltransferase (*Hprt*) as an internal standard.

Phospho-STAT3, AMPK activity and alpha-MSH: For measurements of STAT3 phosphorlaton and isoform-specific AMPK activity, hypothalamus, liver and skeletal muscle tissues were lysed in ice-cold RIPA buffer (150 mM NaCl, 1% sodium desoxycholate, 0.1% SDS, 1% Triton X-100, and 50 mM Tris, pH 7.2) containing 1 mM PMSF, 5 µg/ml aprotinin, and 5µg/ml leupeptin). STAT3 phosphorylaton was determined by mouse phosp-STAT3 ELISA kit (cat number: DYC4607-2**,** R&D systems***)***. AMPK activity was measured by cyclex AMPK α activity assay kit (cat number: CY-1182, MBL international) (137). Alpha-MSH was measured by ELISA kit ***(***cat number: EK-043-01**,** Phoenix Pharmaceuticals, INC).

Data management/Statistical analyses: All values were expressed as mean ± SEM. Data were analyzed by Student's *t* test or analysis of variance (ANOVA). A multiple comparison Tukey-Kramer post-hoc test was performed to evaluate effects between genotypes and dietary treatment with JMP software version 8 (SAS Institute Inc., Cary, NC).

RESULTS

Lack of Ang II decreases SOCS3 expression.

Mice lacking renin (*Ren1c-/-*) are lean and insulin sensitive (102), but we do not know what precise mechanism causes lean phenotype and improves insulin sensitivity of *Ren1c-/-* mice. Because leptin resistance is asscoiated with obesity and inuslin resistance (127), improving leptin senstivity is likely to contribute lean phenotype of the *Ren1c-/-* mice. Because elevated SOCS3 levels lead to leptin resistance, I first investigated whether the lean phenotype of the *Ren1c*-/- mice have low expression of SOCS3 levels in white adipose tissue (WAT), liver, hypothalamus and skeletal muscle. I found that levels of SOCS3 in these tissues were 40~50% of that in WT mice (**Fig. 2.1A**). Since the *Ren1c-/-* mice lack renin and Ang II, I next tested whether the decrease in SOCS3 expression is due to lack of Ang II or lack of Ang-independent effect of renin. When the *Ren1c-/-* mice were treated with a low dose of Ang II (7.5 g/kg/day), that dose did not affect blood pressure, but it restored SOCS3 expression to WT levels (**Fig. 2.1B**). In contrast, infusion of human renin (hRen) did not increase SOCS3 expression of the *Ren1c-/-* mice. Furthermore, WT mice treated with losartan (0.45g/l) showed a low expression of SOCS3 levels in the hypothalamus (**Fig. 2.1B**), indicating that the inhibition of Ang II effects decreases SOCS3 expression in the hypothalamus.

Blockade of the AT1R enhances leptin response by exogenous leptin in the hypothalamus.

Because SCOS3 is a negative regulator of leptin signaling pathway, decreased SOCS3 in the *Ren1c-/-* mice and WT mice treated with losartan is likely to improve

leptin sensitivity and enhance leptin signals by exogenous leptin. To test this I examined activation of JAK2-STAT3 signaling pathway and found that the hypothalamus of the *Ren1c*-/- mice and WT mice treated with losartan had lower levels of STAT3 phosphorylation (**Fig. 2.1C**). In contrast, the levels of STAT3 phosphorylation were significantly increased in the *Ren1c*-/- mice and WT mice treated with losartan after infusion of exogenous leptin (100μg/kg/day) (**Fig. 2.1C**), indicating that inhibition of Ang II enhaced leptin signals after infusion of exogenous leptin.

Moreover, leptin is able to regulate energy balance in the hypothalamus via increased expression of POMC and decreased expression of NPY and AgRP as well as inhibiting AMPKα activity. To investigate whether inhibition of Ang II alters energy balance in the hypothalamus via JAK2-STAT3 signaling pathway, I measured the expression of POMC, NPY and AgRP and AMPKα activity in the hypothalamus. I found that *Ren1c*-/- mice and WT mice treated with losartan had 5-fold lower POMC expression and 3-fold higher expression of NPY and AgRP in the hypothalamus relative to untreated WT mice (**Figs. 2.1E-G**). After Ang II (7.5 g/kg/day) infusion, the levels of expression of POMC, NPY and AgRP were similar to WT levels (**Fig. 2.1E-G**). In contrast, infusion of human renin did not change these gene expression levels (**Fig. 2.1E-G**). In addition, both *Ren1c*-/- mice and WT mice treated with losartan have higher basal AMPKα activity and a larger decrease of AMPKα after leptin administration (**Fig. 2.1D**). These data show that blockade of Ang II effects via the AT1R reduces basal leptin effects and enhances the response to exogenous leptin.

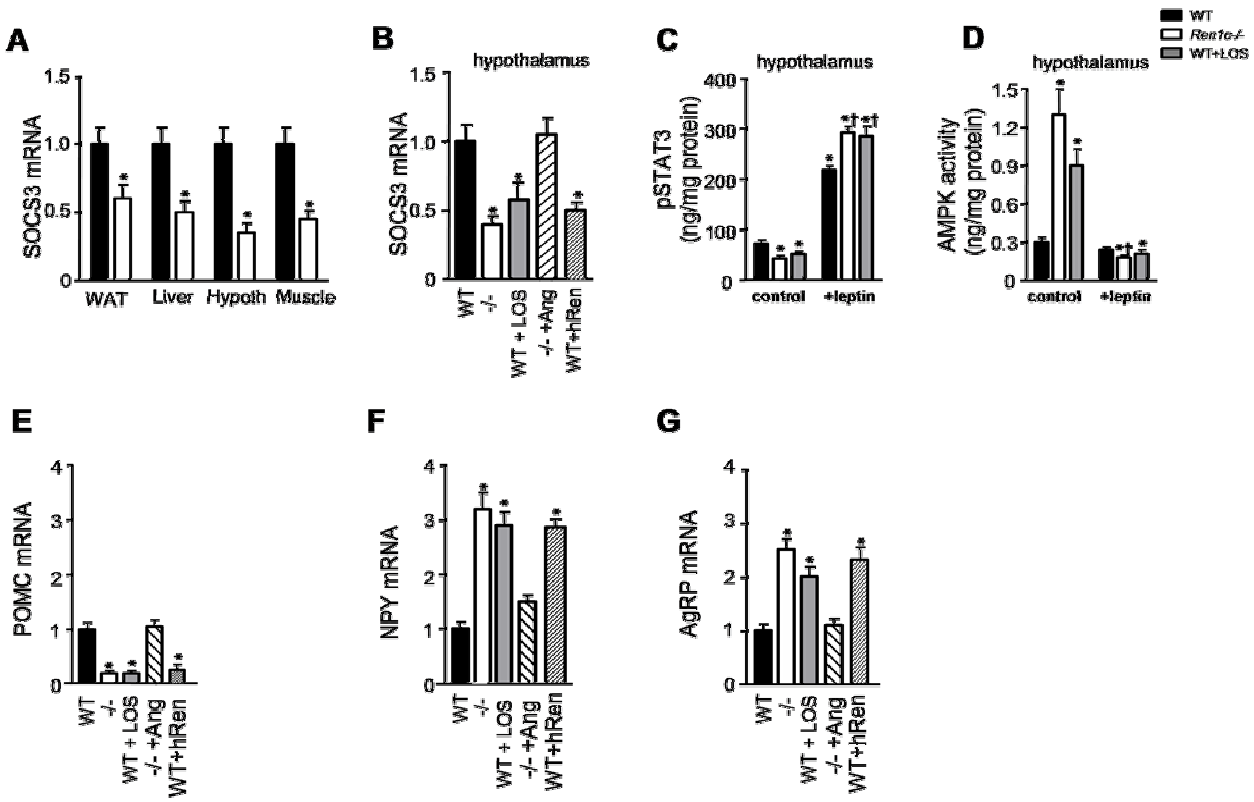

Figure 2.1 Lack of renin decreases leptin signaling pathway. A. SOCS3 expression levels of the white adipose tissue (WAT), liver, hypothalamus and skeletal muscle in the *Ren1c-/-* and WT mice. B. Hypothalamic SOCS3 expression in the *Ren1c-/-* mice, WT treated with losartan (0.45g/L) for 2 weeks, and *Ren1c-/-* mice administered with angiotensin II (7.5μg/kg/day) or human renin (0.1μg/kg/day) for 2 weeks. C, D. phospho-STAT3 levels and AMPKα activity of the hypothalamus in the *Ren1c-/-* mice, WT mice treated with losartan (0.45g/L) for one month with leptin (0.1mg/kg/day) or saline (control) administration. E-F. Hypothalamic neuropeptides POMC, NPY, and AgRP expression levels in the *Ren1c-/-* mice, WT mice treated with losartan and WT mice. All values are expressed as means ± S.E.M. n>7 *p< 0.05 vs. WT mice, and $^{\dagger}p$ < 0.05 vs. WT with leptin. Hypoth: hypothalamus, -/-: *Ren1c-/-* mice; ang: Ang II; hRen: human renin. Black bar: WT mice; white bar: *Ren1c-/-* mice and gray bar: WT mice treated with losartan.

Blockade of the AT1R augments anti-obesity effect of exogenous leptin.

I have shown that blockade of the AT1R improves the response to exogenous leptin in the *Ren1c-/-* mice, but this change could simply be because the basal levels of leptin are low in the *Ren1c*-/- mice and WT mice treated with losartan. Moreover, the response to leptin shown above in **Fig. 2.1** was from leptin administration i.p. for 45 min. Therefore, I do not know whether chronic blockade of Ang II would enhance the response to leptin. To investigate this, I generated mice lacking both renin and leptin (*Ren1c-/-;ob/ob*).

Interestingly, the *Ren1c-/-;ob/ob* mice had lower BW than the 3-month old *ob/ob* mice (50.4 ± 1.2 g vs. 56.5 ± 1.3 g in *ob/ob* mice, $p<0.001$) (**Figs 2.2A, B**) and significantly lower body fat and lean mass (**Figs 2.2C, D**). However, the ratio of fat/BW did not differ (0.57 ± 0.09 vs. 0.59 ± 0.07 in *ob/ob* mice, $p<0.5$) (**Fig 2.2E**).

To investigate whether blockade of the AT1R improves leptin sensitivity, I administered murine leptin (100μg/kg/day) to the *Ren1c-/-;ob/ob* mice, the *ob/ob* mice treated with losartan, and the *ob/ob* mice by using osmotic minipumps for 2 weeks. I measured food intake, BW, and body composition before and after leptin administration. I found that the *Ren1c-/-;ob/ob* mice and the *ob/ob* mice treated with losartan lost more BW compared to *ob/ob* mice (9.5 ± 0.5; 8.4 ± 0.6 vs. 5.1 ± 0.2 g in *ob/ob* mice, $p<0.03$), body fat mass, and lean mass, and obsevered decreased fat/BW ratio (**Figs 2.3 A-D**) and daily food intake relative to the *ob/ob* mice after leptin infusion (4.8 ± 0.5, 5.3 ± 0.3 vs. 6.1 ± 0.8 g in *ob/ob* mice).

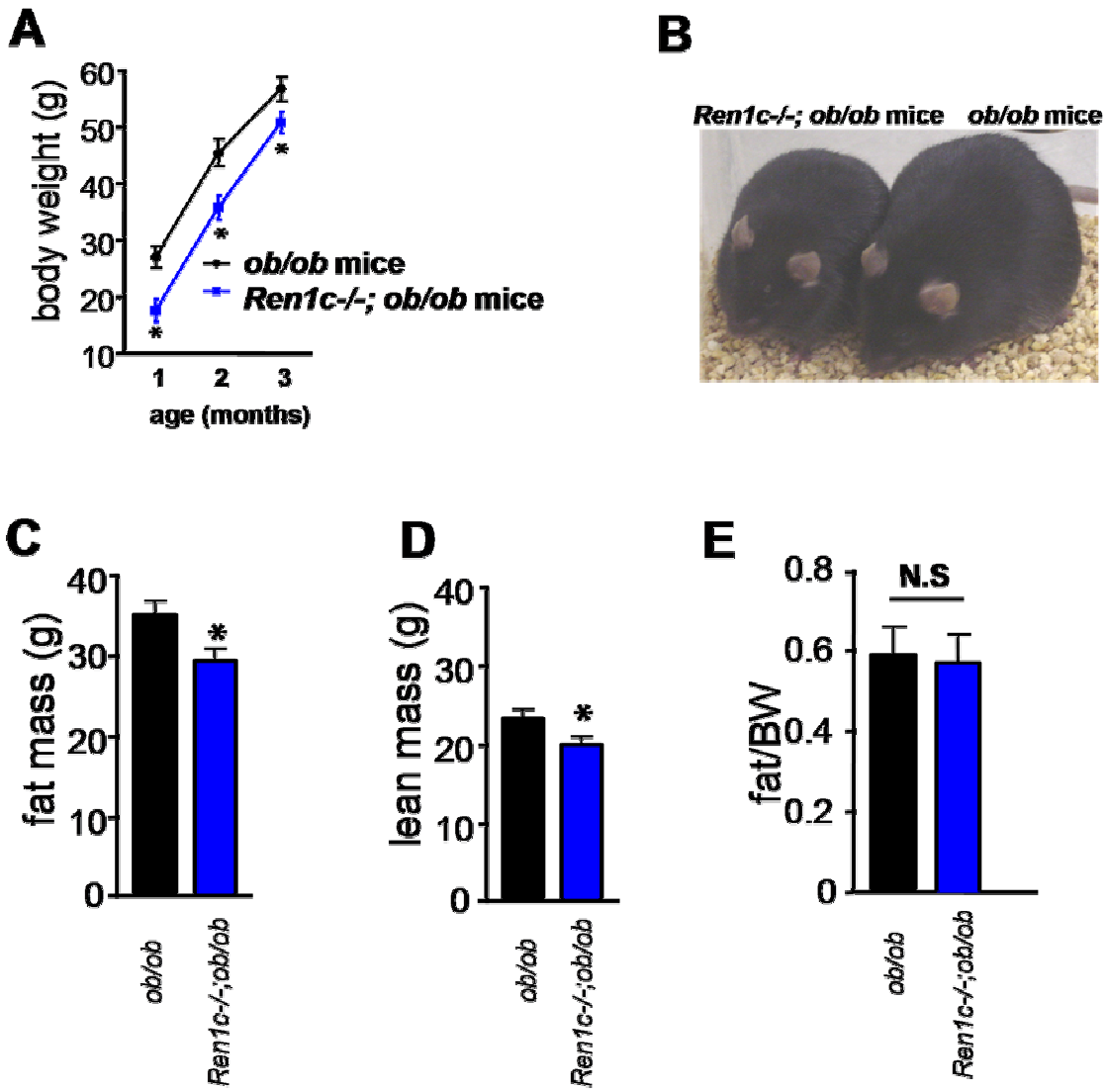

Fig 2. 2 Growth curve, and body composition of the *Ren1c-/-;ob/ob* and *ob/ob* mice. *A*, Growth curves of the *Ren1c-/-;ob/ob* and *ob/ob* mice from 1 to 3 months age(**n** ≥10) is shown. B. 3 month-old *Ren1c-/-;ob/ob* and *ob/ob* mice C-E. fat, lean mass and fat/BW by MRI in the *Ren1c-/-;ob/ob* and *ob/ob* mice. Black bar: *ob/ob* mice; blue bar: *Ren1c-/-; ob/ob* mice. All values are expressed as means ± S.E.M. n≥8 *p< 0.05 vs. *ob/ob* mice.

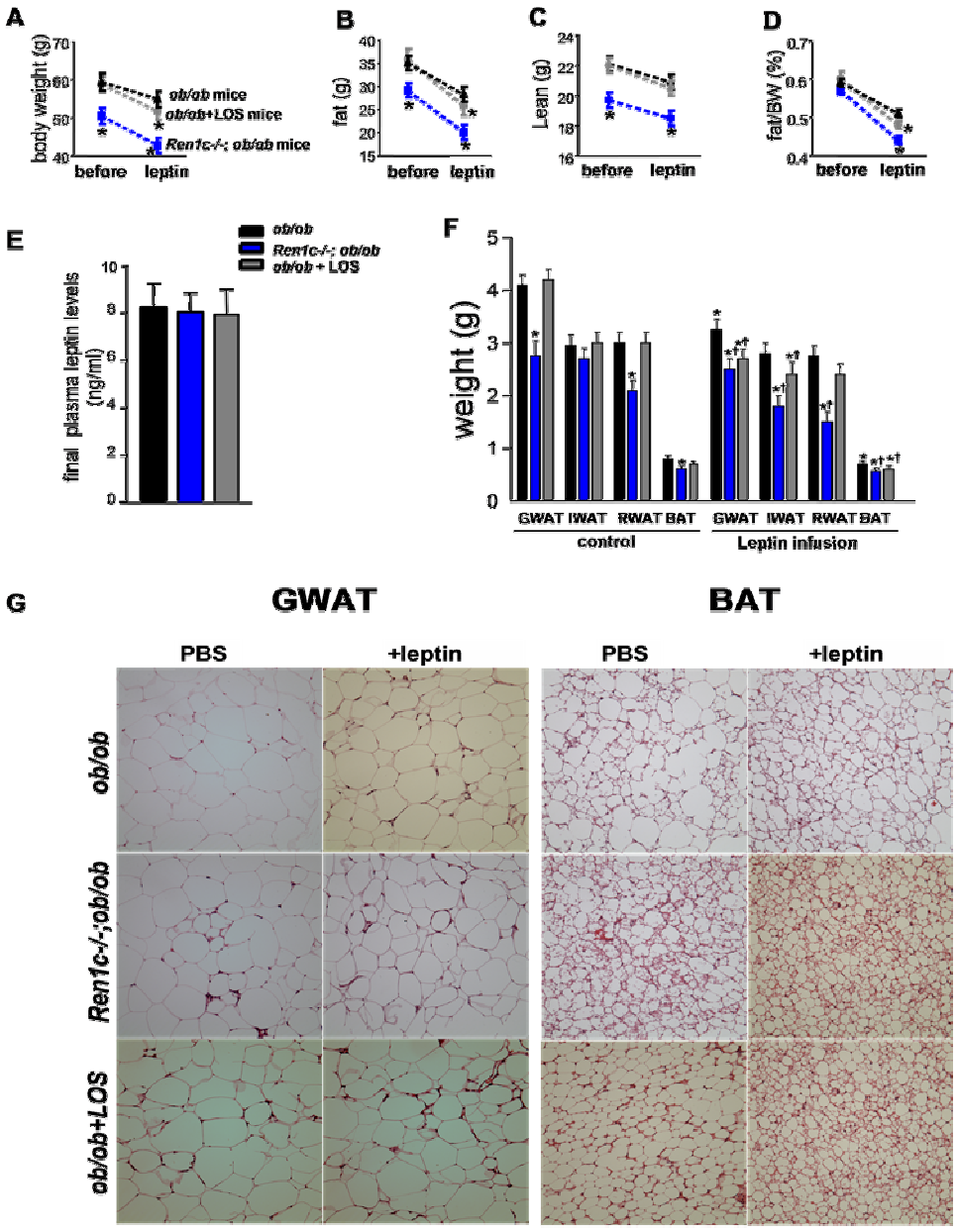

Figure 2.3 The *Ren1c-/-; ob/ob* mice lose more body weight and fat after administration of exogenous leptin compared to *ob/ob* mice. A-D. Body weight, fat mass, lean mass, and fat/BW before and after administration of exogenous leptin (100μg/kg/day) for 2 weeks. E. Plasma leptin levels after administration of exogenous leptin for two weeks, F,G. Fat distribution and histology of GWAT and BAT before and after administration of exogenous leptin for 2 weeks. All mice were 3 months old and values are expressed as means ± S.E.M. ***n*** ≥8 *p< 0.05 vs. *ob/ob* mice; $^{\dagger}p$< 0.05 *ob/ob* mice with leptin. Black bar: *ob/ob* mice; blue bar: *Ren1c-/-; ob/ob* mice, and gray bar: *ob/ob* mice treated with losartan (0.45g/L) for one months. White adipose tissues (WAT) include gonada (GWAT), retroperitoneal. (RWAT), inguinal (IWAT), and brown adipose tissue (BAT)

After leptin infusion, plasma leptin levels did not differ among the three groups (**Fig 2.3E**). I also noted that *Ren1c-/-;ob/ob* mice had lower fat distribution in gonadal (GWAT), retroperitoneal. (RWAT), inguinal (IWAT) white adipose tissues, and brown adipose tissue (BAT), and particularly in GWAT (**Figs 2.3F, G**). After leptin infusion, the *Ren1c-/-;ob/ob* mice had markedly decreased fat mass compared to *ob/ob* mice (**Figs 2.3F, G**). These data indicate that inhibition of Ang II improves leptin sensitivity.

Mechanisms of lower BW and improved leptin sensitivity of the *Ren1c-/-;ob/ob* mice.

There are several mechanisms including food intake, absorption, utilization and storage of fat, and basal metabolic rate that lead to lower body weight. Next, I investigated the mechanisms of lower BW and body fat and a larger weight loss response to leptin of the *Ren1c-/-;ob/ob* mice compared to the *ob/ob* mice. Food intake of the *Ren1c-/-;ob/ob* mice was similar to that of the *ob/ob* mice, but their feed efficiency (ΔBW/food intake) was lower than that of the *ob/ob* mice (**Figs 2.4A, B, C**). Although the *Ren1c-/-;ob/ob* mice had significantly lower respiratory exchange ratio (RER) in comparison with *ob/ob* mice (**Fig 2.4D**), their heat generation and rectal temperature were indistinguishable from WT mice (**Figs 2.4E, and F**). Because UCP-1 is responsible for thermogenesis and uncouples mitochondrial electron transport from ATP synthesis to dissipate energy as heat, I measured the brown adipose tissue (BAT) UCP-1 gene expression among the three groups and found that BAT UCP-1 was not different between *Ren1c-/-;ob/ob* mice and *ob/ob* mice without fasting (**Fig 2.4G**), indicating that heat generation is not a major contributor for lowering BW in the *Ren1c-/-;ob/ob* mice.

Inestestingly, I also found that fecal fat excretion, plasma adiopnectin and β-hydroxybutyrate (ketone body) (**Fig 2.4 H, K, and L**), were higher in the *Ren1c-/-;ob/ob* mice and *ob/ob* mice treated with losartan compared to *ob/ob* mice, implicating that impairment of fat aborption and increased fatty acid oxidation causes lower body weight of the *Ren1c-/-;ob/ob* mice.

After leptin infusion for 2 weeks, a decrease in food intake (4.8 ± 0.5 vs. 6.1 ± 0.8 g in *ob/ob* mice) and feed efficiency, were significantly larger in the *Ren1c-/-;ob/ob* mice than that of *ob/ob* mice (**Figs 2.4 A, B, and C**). An increase of heat generation, rectal temperature, and BAT UCP1 expression were larger in the *Ren1c-/-;ob/ob* mice, but the decrease of RER was similar (**Figs 2.4C-G**). Moreover, an increase of fecal fat excretion was larger in the *Ren1c-/-;ob/ob* mice than in *ob/ob* mice (13.3± 0.5 % vs. 8.5± 0.4 %, $p<0.01$, **Fig 2.4H**), which was consistent with the expression of pancreatic lipase and colipase. Both enzymes were markedly decreased (**Figs 2.4 I, and J**). The effects of the *Ren1c-/-;ob/ob* mice on these parameters was recapitulated by *ob/ob* mice treated with losartan. These results show that inhibition of Ang II combined with chronic leptin infusion increases loss of body weight due to a decrease in food intake and an increase in heat generation.

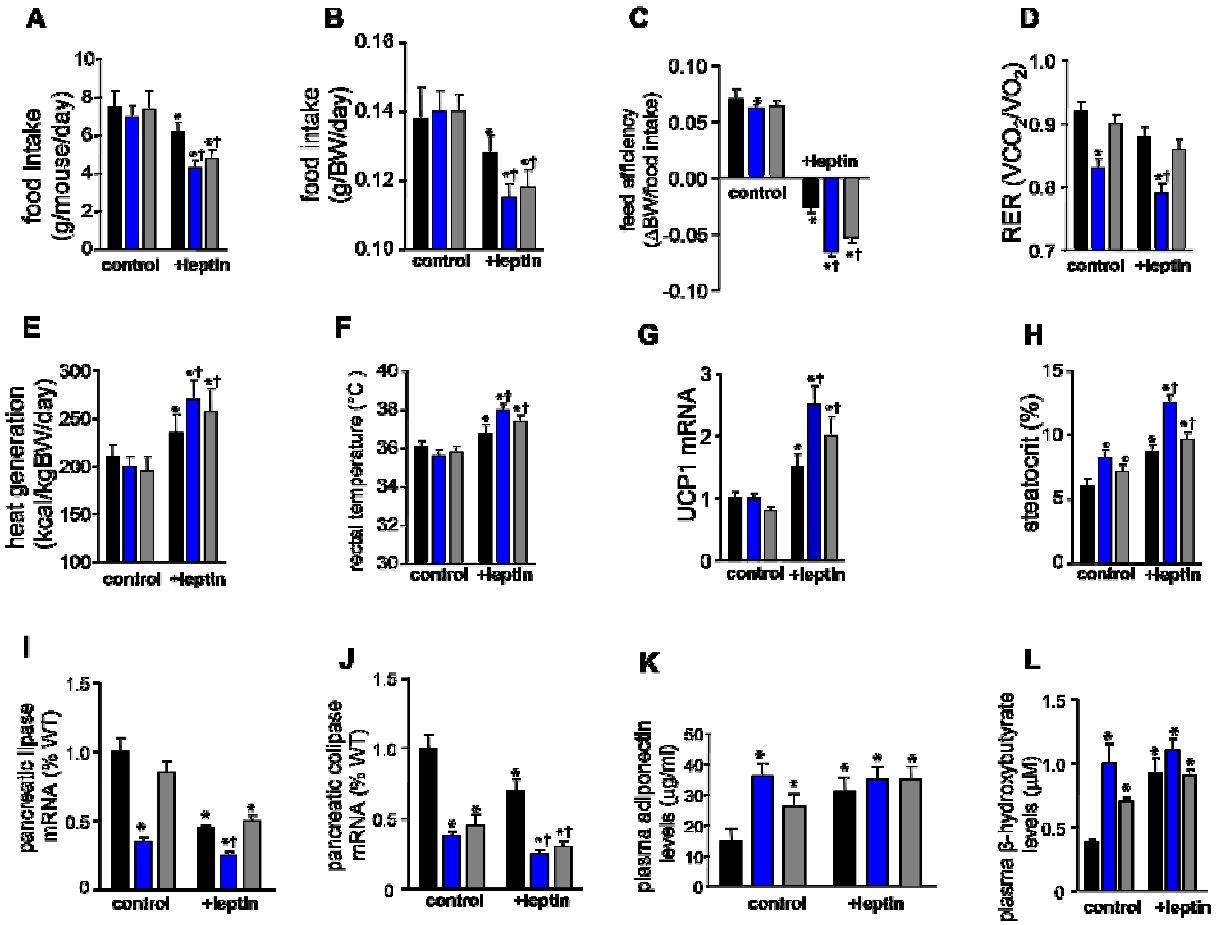

Fig 2.4 Energy balance and plasma adiponectin of the *Ren1c-/-;ob/ob* decreased food intake and increased fecal fat excretion. A, B. Daily food intake. C. feed efficiency. D. Respiratory exchange ratio (RER), E. Heat generation. F. Rectal temperature. G. BAT UCP1 gene expression. H. Fecal fat, and I, J. Expression of pancreatic lipase and colipase in three groups. K, L. Plasma adiponectin and β-hydroxybutyrate levels in the *ob/ob* mice, *Ren1c-/-;ob/ob* mice, and *ob/ob* mice treated with losartan (0.45g/L) for one months with leptin infusion (100μg/kg/day) for two weeks. All mice at 3 months old and values are expressed as means ± S.E.M. ***n*** ≥8 *p< 0.05 vs. *ob/ob* mice, ; †p< 0.05 *ob/ob* mice with leptin. Black bar: *ob/ob* mice; blue bar: *Ren1c-/-;ob/ob* mice; grey bar: *ob/ob* mice treated with losartan.

Increased dietary fat wasting in the *Ren1c-/-;ob/ob* mice

Because the expression of pancreatic lipase and colipase was lower in the *Ren1c-/-;ob/ob* mice and in the *ob/ob* mice treated with losartan compared to *ob/ob* mice (**Fig. 2.4 I, and J**), I investigated dietary fat absorption in the *Ren1c-/-;ob/ob* mice and the effect of leptin on these enzymes. Although the food intake of *Ren1c-/-;ob/ob* mice was indistinguishable from *ob/ob* mice, the amount of lipid in the feces was higher in the *Ren1c-/-;ob/ob* mice and leptin synergistically increased it. The estimated dietary fat absorption (fat calories of daily food intake - fat content from daily feces) of the *Ren1c-/-;ob/ob* mice was lower than that of *ob/ob* mice (6.4 ± 0.7 kcal/day In *Ren1c-/-;ob/ob* mice vs. 7.8 ± 0.7 kcal/day in *ob/ob* mice at 3 months old, $p<0.001$), and leptin further inhibited fat absorption in all three groups (3.5 ± 0.3 kcal/day In *Ren1c-/-;ob/ob* mice, 4.2 ± 0.3 kcal/day in *ob/ob* mice with losartan vs. 5.5 ± 0.4 kcal/day in *ob/ob* mice) (**Figs. 2.5A-C**), indicating that dietary fat wasting of *Ren1c-/-;ob/ob* mice most likely contributes to their reduced BW and fat mass.

Leptin signaling and expression of neuropeptides in the hypothalamus of the *Ren1c-/-;ob/ob* mice

Because the expression of neuropeptides in the hypothalamus are associated with food intake, I next tested whether changes in leptin signals and neuropeptides in the hypothalamus support the food intake data by inhibition of Ang II and by leptin.

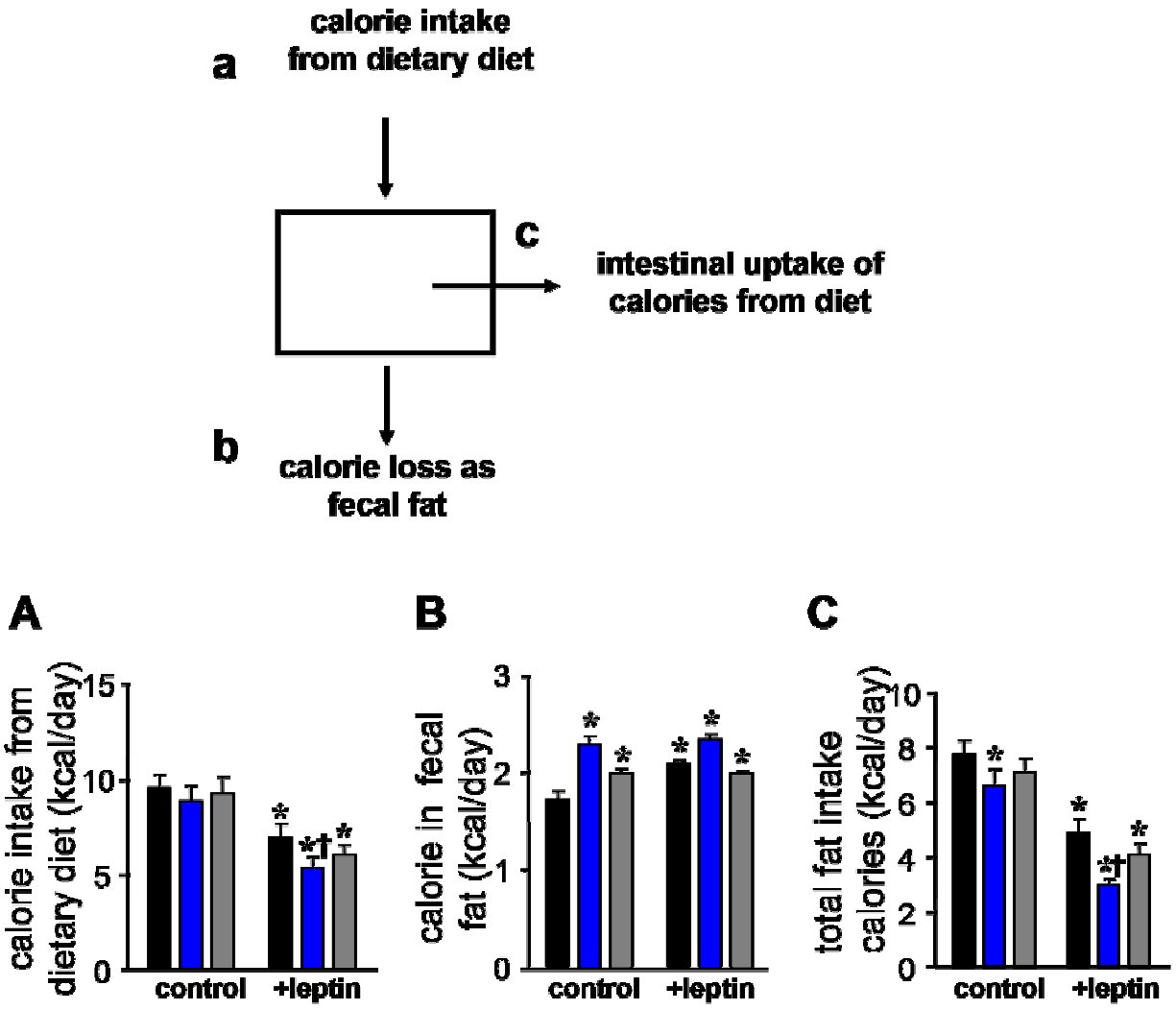

Figure 2.5 Inhibition of Ang II-AT1R pathway causes increased fecal fat excretion and decreased total dietary fat intake and leptin further decreased fat absorption. A . Calorie intake from dietary fat, B. calorie loss as fecal fat, and C. intestinal uptake of calories from fat before and after administration of exogenous leptin (100μg/kg/day) for 2 weeks. All mice were 3 months old and values are expressed as means ± S.E.M. ***n*** ≥8 **p*< 0.05 vs. *ob/ob* mice; †*p*< 0.05 *ob/ob* mice with leptin. Black bar: *ob/ob* mice; blue bar: *Ren1c-/-; ob/ob* mice, and gray bar: *ob/ob* mice treated with losartan (0.45g/L) for one month.

Similar to the *Ren1c-/-* mice compared to WT mice, the *Ren1c-/-;ob/ob* mice had lower SOCS3 and phosphorylated STAT3 but higher AMPK activity in the hypothalamus compared to the *ob/ob* mice (**Figs. 2.6A-C**). Differences in the expression levels of POMC, NPY and AgRP in the hypothalamus of the *Ren1c-/-;ob/ob* mice compared to the *ob/ob* mice are very small in comparison with that between the *Ren1c-/-* mice and WT mice (**Figs. 2.6D-F, and Figs. 2.1E-G**).

After leptin infusion, the *Ren1c-/-;ob/ob* mice and the *ob/ob* mice treated with losartan had reduced SOCS3 levels, and enhanced increase in STAT3 phosphorlyation and enhanced decrease in AMPKα activity (**Figs. 2.6A-C**). Inhibition of Ang II effects via AT1R and leptin synergistically increased POMC expression and decreased expression of NPY and AgRP in the hypothalamus (**Figs. 2.6D-F**). Because the alpha-melanocyte-stimulating hormone (alpha-MSH) is an agonist for the melanocortin receptors and this peptide is processed from POMC, I also investigated that alpha-MSH level among the three groups and found that the *Ren1c-/-;ob/ob* mice and *ob/ob* mice treated with losartan had lower alpha-MSH levels compared to *ob/ob* mice in the hypothalamus, while inhibition of Ang II effect via AT1R and leptin synergistically increased alpha-MSH levels (**Figs. 2.6G**). These results suggest that inhibition of Ang II effects via AT1R improves leptin sensitivity in the hypothalamus and contributes to reduction of food intake and weight loss.

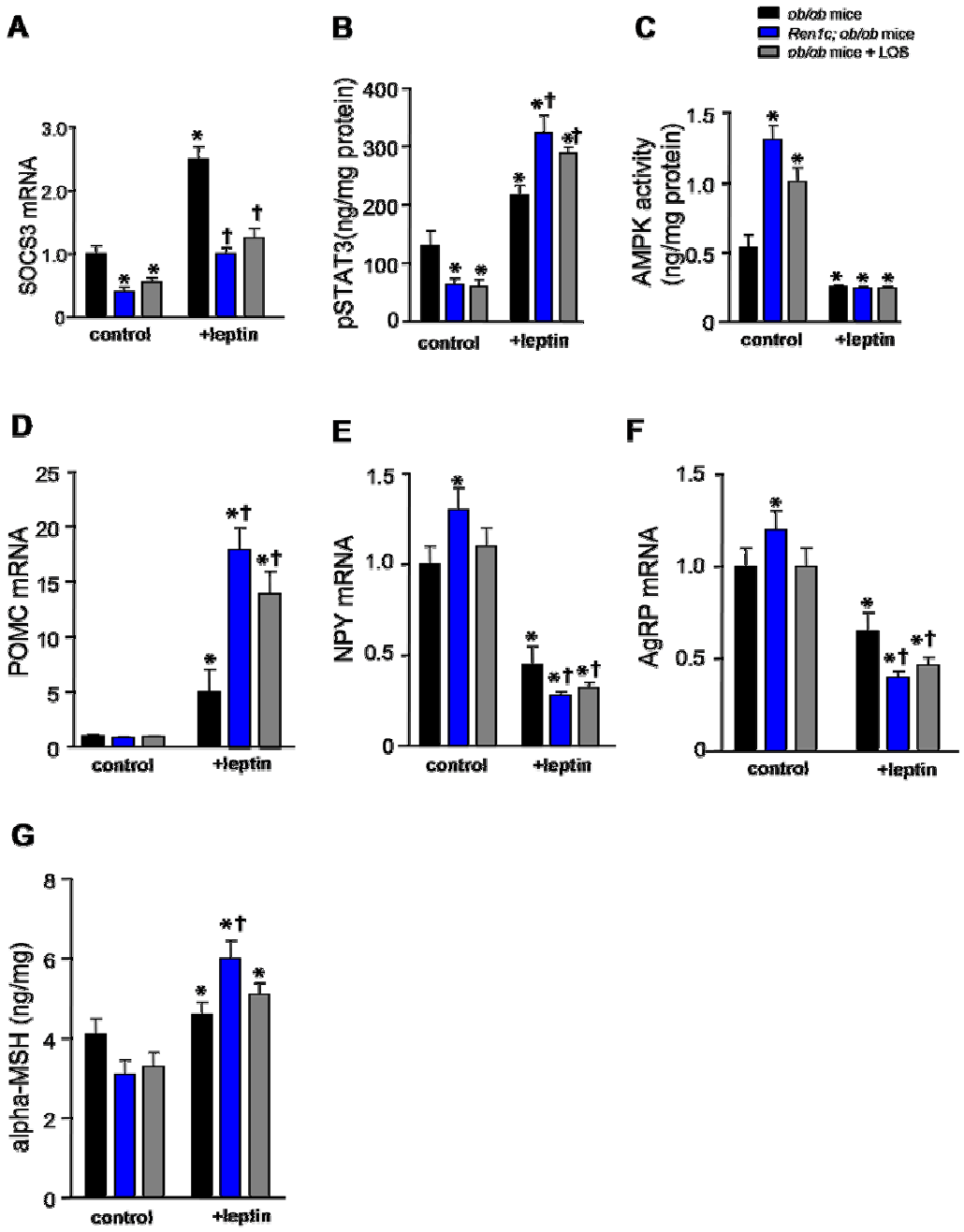

Figure 2.6 The *Ren1c-/-;ob/ob* mice show increased phospho-STAT3 and decreased AMPKα activity after administration of leptin. A. Hypothalamic SOCS3 levels in 3-month old mice. B, C phospho-STAT3 and AMPKα levels in the hypothalamus with PBS (control) or leptin infusion (100μg/kg/day). D-F. Expression of neuropeptides POMC, NPY and AgRP with PBS and leptin infusion, and G. hypothalamic alpha-MSH levels. All values are expressed as means ± S.E.M. n=6 *p< 0.05 vs. *ob/ob* mice, and †p< 0.05 vs. *ob/ob* mice with leptin infusion. Black bar: *ob/ob* mice; blue bar: *Ren1c-/-;ob/ob* mice, and gray bar: *ob/ob* mice treated with losartan (0.45g/L) for 2 weeks.

Blockade of the AT1R augments anti-non-alcoholic liver disease (NAFLD) effect and fatty acid oxidation by exogenous leptin.

Since increased plasma β-hydroxybutyrate is related to fatty acid oxidation in the liver and skeletal muscle, I measured levels of triglycerides, fatty acid oxidation, and fatty acid synthesis and their relative genes expression levels in the liver and skeletal muscle. Indeed, the *Ren1c-/-;ob/ob* mice had lower triglyceride levels in the liver and skeletal muscle, and inhibition of AT1R and administration of leptin decreased liver and skeletal muscle triglyceride content (**Figs 2.7C, D and Fig 2.8A**). I also found that plasma ALT and AST levels and liver weight of the *Ren1c-/-; ob/ob* mice were significantly lower than those of the *ob/ob* mice (ALT: 88.7 ± 14 U/L vs. 287.4 ± 54 U/L in *ob/ob;* AST: 120 ± 8 vs. 245 ± 17 U/L in *ob/ob;* liver weight: 1.32 ± 0.3 vs. 3.2 ± 0.5 g in *ob/ob*), and inhibition of AT1R and leptin synergistically decreased ALT and AST levels in the liver (**Figs 2.7 A, B**). Moreover, expression of fatty acid oxidation related genes-PPARα, ACOX and CPT1-in liver and skeletal muscle were increased in the *Ren1c-/-; ob/ob* mice relative to the *ob/ob* mice (**Figs 2.7 E-G and Figs 2.8 B-D**). In contrast, expression of fatty acid synthesis related genes- fatty acid synthase (FAS) and glycerol-3-phosphate acyltransferase (GPAT1)- were markedly decreased in the *Ren1c-/-; ob/ob* mice relative to the *ob/ob* mice (**Figs 2.7 H, I and Figs 2.8 E, F**). This finding indicates that increased fatty acid oxidation in the liver and skeletal muscle of the *Ren1c-/-; ob/ob* mice results in increased plasma ketonbodies, which is consistent with reduced RER. I conclude that inhibition of AT1R and administration of leptin increased fatty acid oxidation in the liver and skeletal muscle, and could contribute to fat utilization in the *Ren1c-/-; ob/ob* mice.

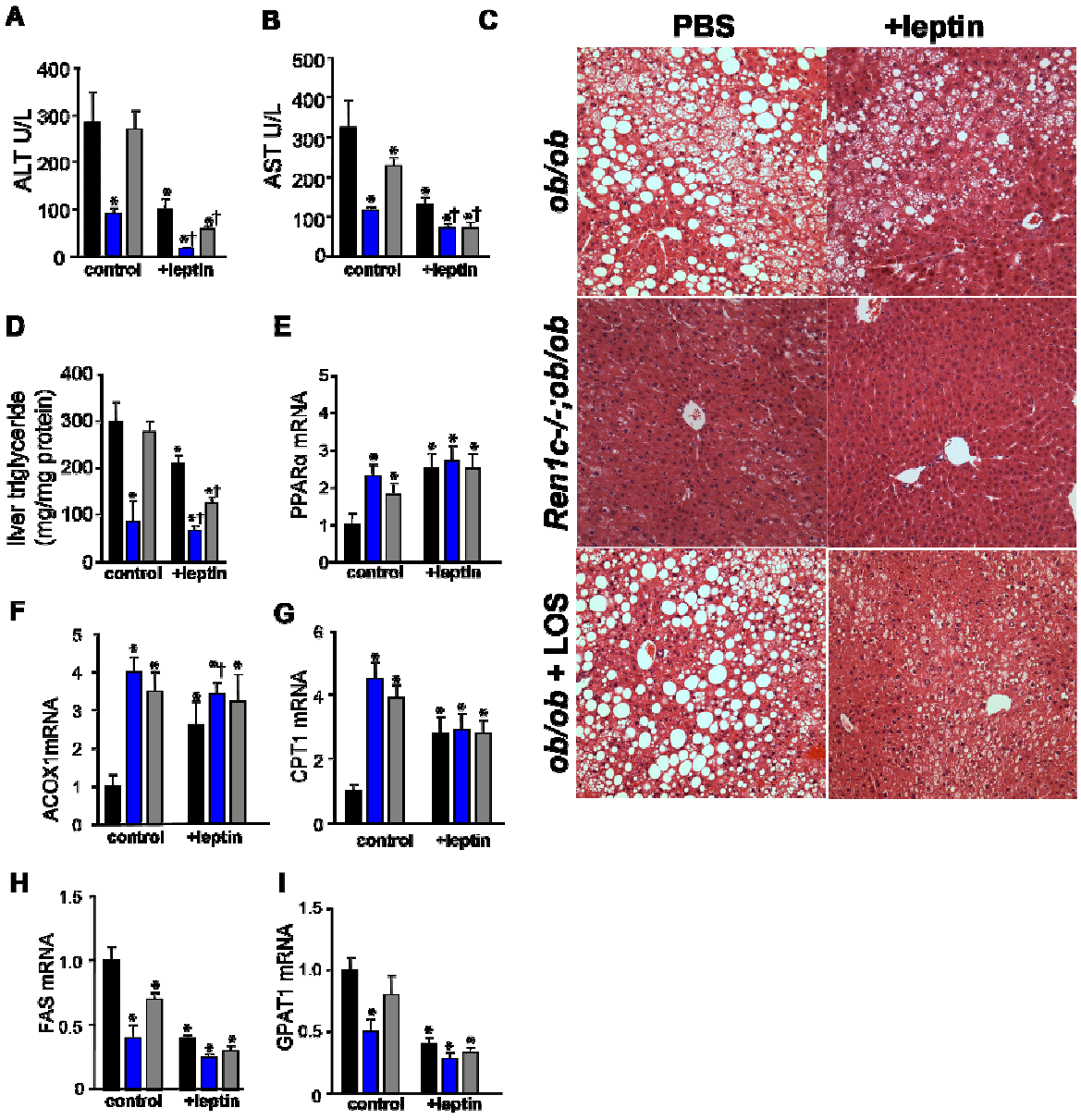

Figure 2.7 Inhibition of Ang II effects protects against non-alcoholic liver disease and enhance fatty acid oxidation. A, B. Liver function (AST and ALT) in the *ob/ob* mice, *Ren1c-/-;ob/ob* mice, and *ob/ob* mice treated with losartan. C. Liver histology of the *ob/ob* mice, *Ren1c-/-;ob/ob* mice, and *ob/ob* mice treated with losartan. D. Liver triglyceride contents of the *ob/ob* mice, *Ren1c-/-; ob/ob* mice, and *ob/ob* mice treated with losartan with PBS (control) or leptin (100μg/kg/day) for 2 weeks. E-G mRNA levels of liver PPARα, acyl-coenzyme A oxidase 1 (ACOX1), and carnitine palmitoyltransferase I (CPT1). H,I. mRNA levels of fatty acid synthase (FAS) and glycerol-3-phosphate acyltransferase (GPAT1). Data were obtained from 3-month-old male mice. All values are expressed as means ± S.E.M. ***n***=7 **p*< 0.05 vs. *ob/ob* mice; †*p*< 0.05 *ob/ob* mice with leptin. Black bar: *ob/ob* mice; blue bar: *Ren1c-/-;ob/ob* mice, gray bar: *ob/ob* mice treated with losartan(0.45g/L) for one month.

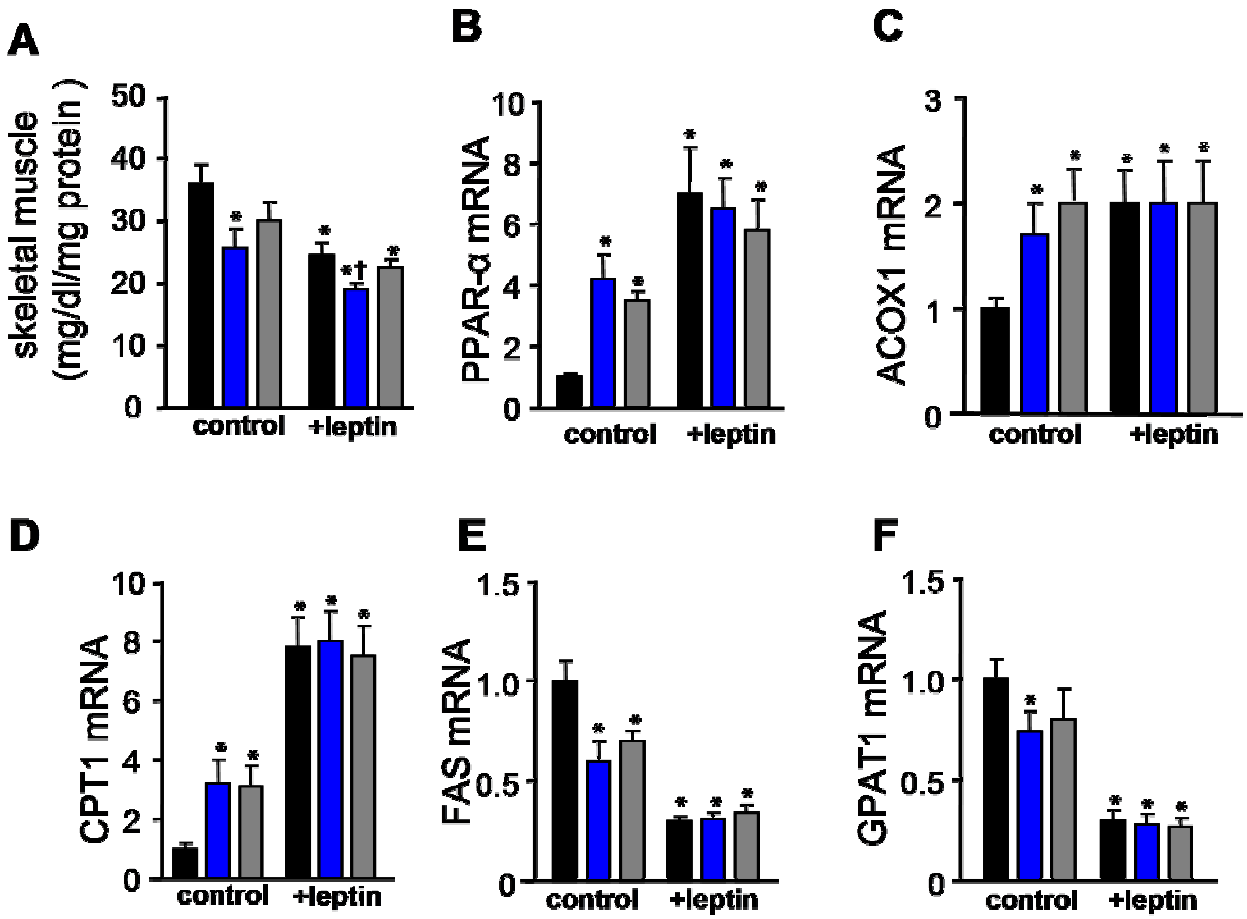

Figure 2.8 Inhibition of Ang II effects causes increased fatty acid oxidation related gene expression in skeletal muscle. A. skeletal muscle triglyceride content of the *ob/ob* mice, *Ren1c-/-; ob/ob* mice, and *ob/ob* mice treated with losartan with PBS (control) or leptin (100μg/kg/day) for 2 weeks. B-D skeletal muscle PPARα, acyl-coenzyme A oxidase 1 (ACOX1), and carnitine palmitoyltransferase I (CPT1) mRNA levels and E, F. fatty acid synthase (FAS) and glycerol-3-phosphate acyltransferase (GPAT1) at 3-month old male mice. All values are expressed as means ± S.E.M. ***n***=7 **p*< 0.05 vs. *ob/ob* mice; †*p*< 0.05 *ob/ob* mice with leptin. Black bar: *ob/ob* mice; blue bar: *Ren1c-/-;ob/ob* mice, gray bar: *ob/ob* mice treated with losartan(0.45g/L) for one month.

Inhibition of Ang II in *ob/ob* mice improves insulin sensitivity.

Leptin and AT1R inhibition improve insulin sensitivity (125). I next investigated whether improved insulin sensitivity by inhibition of RAS is independent of leptin or is due to improved leptin sensitivity. The *Ren1c-/-;ob/ob* mice and the *ob/ob* mice with losartan treatment had lower plasma level of glucose and insulin (**Figs 2.9 A, B**), and improved glucose tolerance and insulin tolerance compared to the *ob/ob* mice (**Figs 2.9 C-E**). They also had reduced gluconeogenesis evaluated by pyruvate tolerance test (**Figs 2.9 F**), which is consistent with reduced expression levels of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6P) in the liver (**Figs 2.9 G, and H**). After leptin infusion for 2 weeks, all groups had improved glucose, insulin and pyruvate tolerance, but the difference between the *Ren1c-/-;ob/ob* mice and the *ob/ob* mice became smaller as compared to before leptin infusion (**Figs 2.9 C-F**).

Because adiponectin has demonstrated improved insulin sensitivity and plasma adiponectin levels of the *Ren1c-/-; ob/ob* mice were three times higher than those of the *ob/ob* mice (**Figs 2.4 K**) when leptin did not further plasma adiponectin levels of the *Ren1c-/-; ob/ob* mice, but it increased them in the *ob/ob* mice and the *ob/ob* mice treated with losartan, and the plasma levels of adiponectin became similar among the three groups of mice (**Fig 2.4K**). Accordingly, the adiponectin results are consistent with the glucose and insulin tolerance after leptin infusion.

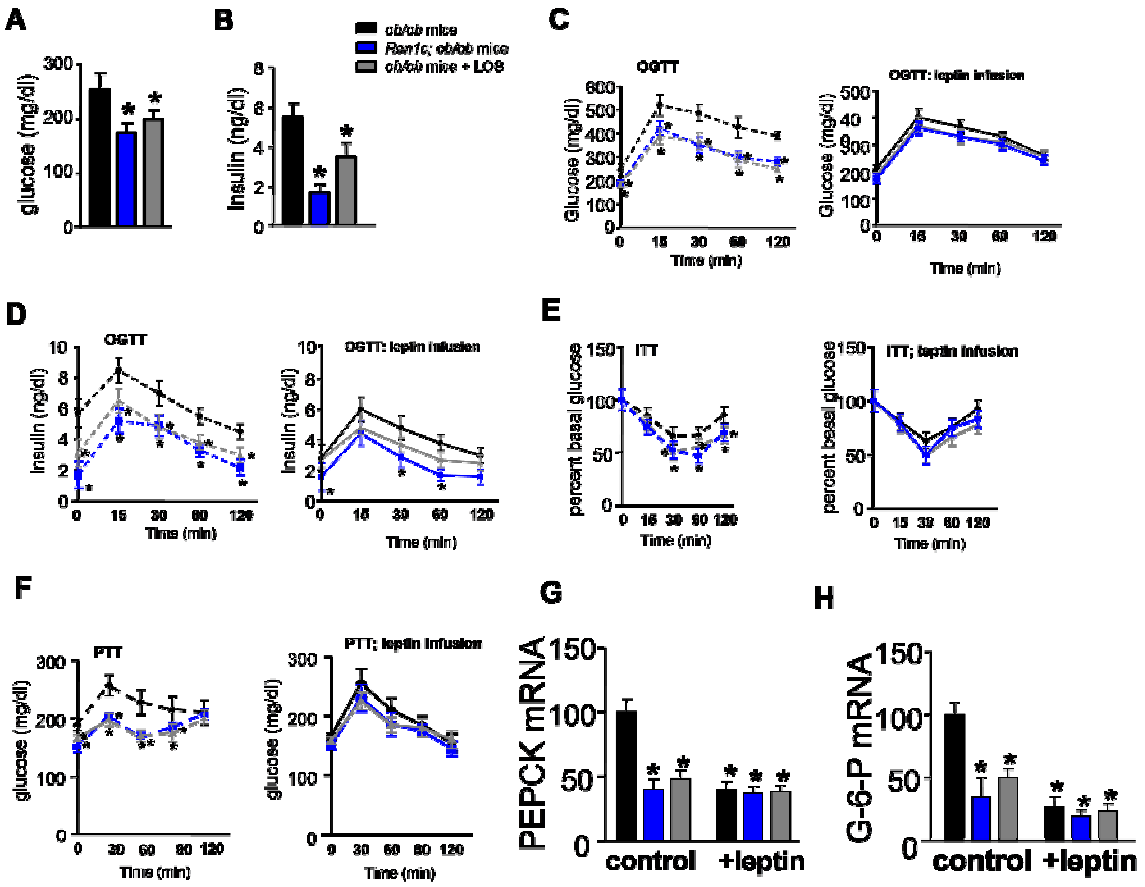

Figure 2.9 Inhibition of Ang II Improves glucose tolerance and insulin tolerance. A, B. Plasma glucose and insulin levels. C, D. Oral glucose tolerance test (OGTT) for the *ob/ob* mice, *Ren1c-/-; ob/ob* mice, and *ob/ob* mice treated with losartan with PBS (control) or leptin (100µg/kg/day) for 2 weeks. E. Insulin-tolerance test (ITT) for the *ob/ob* mice, *Ren1c-/-; ob/ob* mice, and *ob/ob* mice treated with losartan with PBS (control) or leptin (100µg/kg/day) for 2 weeks). F. pyruvate challenge test (PTT) for the *ob/ob* mice, *Ren1c-/-; ob/ob* mice, and *ob/ob* mice treated with losartan with PBS (control) or leptin (100µg/kg/day) for 2 weeks, and G, H. liver gluconeogenesis gene expression, PEPCK and G-6P. All mice at 3 months old and values are expressed as means ± S.E.M. ***n*** ≥8 *p< 0.05 vs. *ob/ob* mice. Black bar: *ob/ob* mice; blue bar: *Ren1c-/-; ob/ob* mice, and gray bar: *ob/ob* mice treated with losartan (0.45g/L) for one month.

I conclude that inhibition of Ang II effects or leptin infusion improves glucose tolerance and insulin sensitivity possibly due to increased plasma adiponectin **(Fig 2.10).**

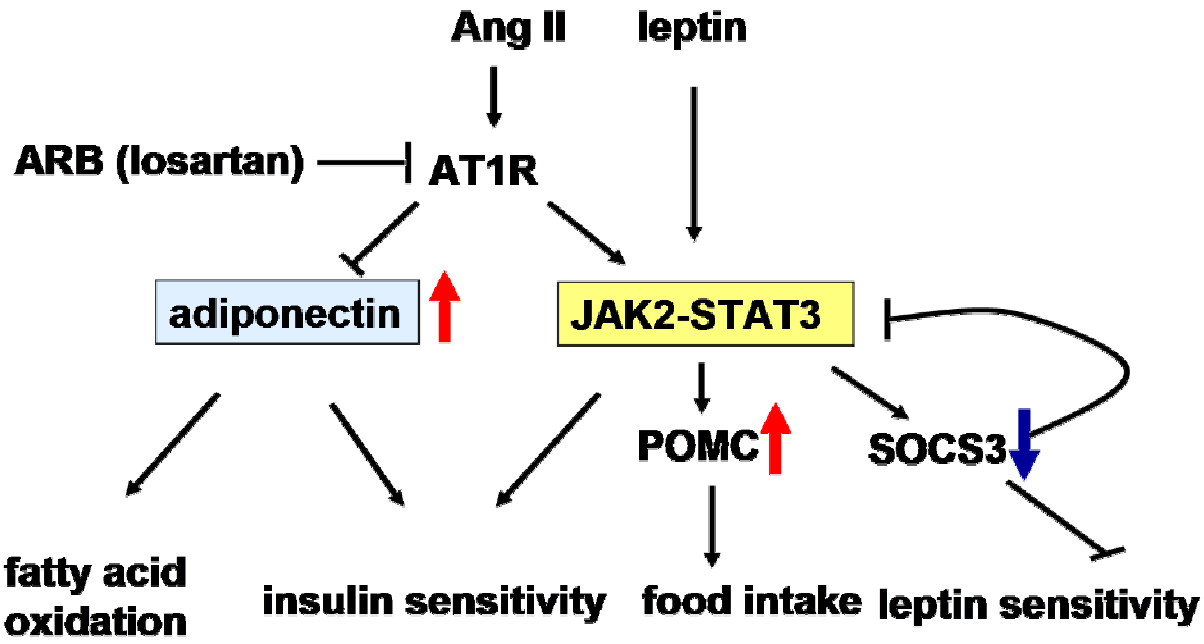

Figure 2.10 Proposed mechanism of how inhibition of Ang II effects improves leptin and insulin sensitivities and enhances fatty acid oxidation.

DISCUSSIONS

In this study I have demonstrated that inhibiting Ang II improves leptin and insulin sensitivity. Previously we reported that food intake (g/mouse/day) of the *Ren1c-/-* mice was similar to that of WT mice (29). Because the body weight of the *Ren1c-/-* mice was smaller than that of WT mice, when food intake is expressed as food intake (g)/body weight (g)/day, the values of the *Ren1c-/-* mice were larger than those of WT mice (0.15 ± 0.01 g/body weight/day vs. 0.09 ± 0.01 g/body weight/day in WT, $p<0.01$), showing that absence of renin increases food intake. Therefore the lean phenotype of the *Ren1c-/-* mice is due to some mechanisms that overcome the increase in food intake.

Absence of renin decreased plasma leptin levels(102), which is consistent with reduced SOCS3 expression and STAT3 phosphorylation, and increased AMPK activity in the hypothalamus as well as decreased expression of POMC, increased expression of NPY and AgRP in the hypothalamus (**Figs. 2.1 A-G**). AMPK works as an energy gauge in the hypothalamus and a number of factors including adiponectin, insulin, glucose and leptin levels are involved in regulating hypothalamic AMPK activity(95). Increased AMPKα activity in the *Ren1c-/-* mice is probably due to reduced plasma insulin and leptin concentrations together with increased plasma adiponectin levels. Intracerebroventricular administration of insulin and leptin inhibits hypothalamic AMPK activity and food intake (138). Changes in the expression of these neuropeptides are consistent with increased food intake (g/body weight/day) of the *Ren1c-/-* mice compared to WT mice, and are due to lack of Ang II, and not to a decrease in renin per se, because hypothalamic expression of POMC, NPY, and AgRP in the *Ren1c-/-;ob/ob* mice was similar to that of the *ob/ob* mice treated with losartan (**Figs. 2.6 D-F**).

The *Ren1c-/-;ob/ob* mice had lower body fat and weight than the *ob/ob* mice. However, because the *Ren1c-/-;ob/ob* mice also had reduced lean body mass, they had fat/BW similar to that of the *ob/ob* mice (**Figs. 2.2 A-E**). The food intake of the *Ren1c-/-;ob/ob* mice (g/body weight/day) was similar to that of the *ob/ob* mice, and the heat generation of the *Ren1c-/-;ob/ob* mice did not differ from the *ob/ob* mice. Absence of the difference in food intake is consistent with similar levels of expression of POMC, NPY, and AgRP between the *Ren1c-/-;ob/ob* mice and the *ob/ob* mice (**Figs. 2. 6 D-F**). Accordingly, lower body weight of the *Ren1c-/-;ob/ob* mice relative to *ob/ob* mice is mainly due to an increased fecal fat loss, which is likely due to reduced pancreatic lipase and colipase in the absence of renin as we previously demonstrated (**Figs. 2.4 H-J**)(29).

After leptin infusion, the *Ren1c-/-;ob/ob* mice and the *ob/ob* mice treated with losartan had a larger anti-obesity and anti-non-alcoholic steatohepatitis effect than *ob/ob* mice, demonstrating improved leptin sensitivity when RAS is inhibited. These effects are likely the results of a combination of reduction in food intake, increase in heat generation, fecal fat excretion and fatty acid oxidation **(Figs. 2.4 A, E, H and L**). Suppression by exogenous leptin of food intake of the *Ren1c-/-; ob/ob* mice and of the *ob/ob* mice treated with losartan is probably due to increased levels of hypothalamic POMC expression. Indeed, mice lacking SOCS3 in POMC expressing cells have increased leptin sensitivity and decreased BW and food intake due to elevated levels of hypothalamic POMC expression in the hypothalamus (79). Delivery of POMC peptide into the hypothalamus of obese Zucker (fa/fa) rats increased energy expenditure and augmented thermogenesis in brown adipose tissue (139), whereas POMC null mice

have decreased energy expenditure (140). In addition, leptin increases energy expenditure and thermogenesis by activating the sympathetic nervous system (SNS)(141), and by increasing thyroid hormone synthesis via increasing TRH through activating JAK2-STAT3 pathway.

The *Ren1c-/-;ob/ob* mice had increased fecal fat excretion relative to the *ob/ob* mice. Interestingly, leptin infusion markedly increased fecal fat excretion in the *Ren1c-/-;ob/ob* mice (**Fig. 2.4 H**). This result is consistent with the finding that leptin directly inhibited both secretion and intracellular activity of pancreatic lipase(142). Because the *Ren1c-/-;ob/ob* mice had only 25% expression of pancreatic lipase and colipase as WT mice (**Figs. 2.4 I, and J**), administration of leptin further decreased pancreatic lipase, leading to more fecal fat loss and smaller BW change.

Compared to WT and *ob/*ob mice the *Ren1c-/-* mice and the *Ren1c-/-;ob/ob* mice have lower plasma glucose concentrations and insulin levels by OGTT, have a larger decrease in plasma glucose levels by ITT, and their gluconeogenesis was less by PTT (**Figs. 2.9 A-F**). Increased plasma adiponectin likely contributes to these results because adiponectin improves insulin sensitivity (**Fig. 2.4 K**)(143), and because Ang II decreases adiponectin production in 3T3-L1 adipocytes and losartan inhibits it (Li et al., our unpublished observation). Treatment of WT mice and the *ob/ob* mice with losartan have significantly increased plasma adiponectin concentrations, and they had improved glucose tolerance and insulin sensitivity, and had reduced gluconeogenesis similar to those of the *Ren1c-/-* mice and the *Ren1c-/-;ob/ob* mice (**Figs. 2.9 G, and H**), indicating the contribution of Ang II-dependent mechanism(s). Interestingly, chronic leptin administration improved insulin sensitivity in the *ob/ob* mice, but not in the *Ren1c-/-*

;ob/ob mice and the *ob/ob* mice treated with losartan (**Figs. 2.9 C- E**). This may be because chronic leptin administration increased plasma adiponectin levels in the *ob/ob* mice, but not in the *Ren1c-/-;ob/ob* mice and the *ob/ob* mice treated with losartan, simply because adiponectin production reached maximum levels by inhibiting RAS, and further stimulation by leptin did not increase adiponectin any more (**Fig. 2.4 K**).

In summary, I have shown that inhibition of RAS improves leptin sensitivity and insulin sensitivity, and augments anti-obesity and anti-diabetic effects of leptin. Inhibition of RAS increased fecal fat excretion and fatty acid oxidation independently of leptin (**Figs. 2.10**). Inhibition of Ang II effects may provide a novel molecular approach for treatment of obesity.

Chapter 3: Absence of angiotensin II causes anemia due to tertiary hypothyroidism

SUMMARY

Inhibiting the renin angiotensin system (RAS) causes anemia. However, its mechanism is not fully understood. Here we provide evidence that inhibiting angiotensin II (Ang II) effects via its type 1 receptor causes tertiary hypothyroidism by directly decreasing TRH and by feedback inhibition of TRH by increased hypothalamic iodothyronine deiodinase Dio2 and thus T3 in the hypothalamus. Moreover, inhibiting RAS in mice causes severe normochromic, normocytic anemia with normal reticulocyte index and elevated erythropoietin, which is corrected by administration of Ang II or T3. However, Ang II did not correct anemia in wild type mice that were made hypothyroid by methimazole. Interestingly, *Ren1c-/-* mice treated with high dose erythropoietin did not correct their anemia. In contrast, inhibiting RAS caused markedly decreased BFU-E and CFU-E colonies, which was corrected by thyroid hormone. I conclude that hypothyroidism is the predominant cause of anemia caused by inhibiting RAS.

INTROUCTION

As discussed in chapter 2, JAK2-STAT3 signaling pathway plays an important role in leptin sensitivity. Because *Ren1c-/-* mice and inhibition of RAS by losartan or enalapril cause decreased phosphorylation of STAT3, which is associated with reduced expression of TRH in the hypothalamus and results in hypothyroidism, I investigated the role and mechanism of Ang II on the thyroid hormone pathway in the chapter 3. Mice lacking renin (*Ren1c*) have low BP, are lean, and have increased energy expenditure and improved insulin sensitivity (102). Given that their increased energy expenditure is associated with elevated thermogenesis, we expected an activation of sympathetic nervous system and/or an increase in thyroid hormones. However, plasma norepinephrine concentration and norepinephrine production in brown adipose tissue of the *Ren1c-/-* mice are similar to those of WT mice (29). Surprisingly, plasma levels of total T3 and T4 were approximately 50% of those of WT mice(29). However, we were not able to find clear clinical findings in the *Ren1c-/-* mice that were consistent with hypothyroidism such as growth inhibition (shorter body length), lower rectal temperature, and a decrease in α-myosin, and an increase in□□-myosin heavy chain (MHC) expression in the heart. The histology of the thyroid gland was also indistinguishable between the *Ren1c-/-* and WT mice. Although thyroid hormones activate RAS (144, 145), whether RAS conversely affect thyroid hormones is not known. Reduced total T3 and T4 might simply reflect the reduced thyroid hormones bound to thyroxine binding globulin (TBG) and might not necessarily mean that free active forms of thyroid hormones in the plasma of the *Ren1c-/-* mice are decreased.

If an inhibition of RAS really causes hypothyroidism, the cause of hypothyroidism could be low TRH, low TSH, and/or low thyroid hormone synthesis itself. When renin or (pro)renin bind to a recently found (pro)renin receptor, they activate MAP kinases independently of Ang II (30). Thus it is possible that hypothyroidism in the *Ren1c-/-* mice is mediated either by the absence of Ang II or by lack of Ang II-independent direct effect of renin to activate MAP kinases. Accordingly, in the first half of this study I tested whether and how inhibiting RAS causes hypothyroidism.

It is well established that inhibiting RAS causes anemia. A number of clinical reports have shown that RAS is associated with erythropoiesis(146, 147). Patients with chronic kidney diseases treated with Ang converting enzyme (ACE) inhibitors (122, 123) or angiotensin receptor blockers (ARB) develop severe anemia (124), which is consistent with severe anemia in mice lacking ACE (113). The *Ace-/-* mice have decreased red blood cell number, hemoglobin (Hgb) and hematocrit (Hct) (113). Administration of Ang II to *Ace*-/- mice corrected anemia (113). However, the mechanisms of how decreased Ang II effects causes anemia is not well known. Moreover, several studies have demonstrated that administration of Ang II stimulates proliferation of erythroid progenitor cells *in vitro* (112) and increases erythropoietin (EPO) levels (122, 148), a potent humoral stimulator of erythropoiesis. In addition, Tsukuba hypertensive mice that harbor both human renin transgene and human angiotensinogen transgene have high BP and erythrocytosis (114). Interestingly, transplanting bone marrow of AT1aR-/- mice to Tsukuba hypertensive mice did not correct erythrocytosis, showing that AT1aR is dispensable for erythropoiesis in the bone marrow (114). This

study suggests indirect effect(s) of Ang II mediate erythropoiesis. However, the mechanism of how RAS affects erythropoiesis is not fully understood.

Moreover, anemia is often seen in patients who have hypothyroidism (149). Mice lacking thyroid hormone receptor α (*TR α-/-)* are anemic due to impaired erythropoiesis in the bone marrow (120, 121). Because thyroid hormone is an important regulator of erythropoiesis in the bone marrow by stimulating burst-forming units-erythroid (BFU-E) colony formation (117, 118), in the second half of this study I tested the hypothesis that inhibition of RAS causes anemia through inhibiting thyroid hormone pathway. My data demonstrate that inhibiting RAS causes tertiary hypothyroidism, and that hypothyroidism is the primary cause of anemia induced by inhibiting RAS.

MATERIAL AND METHODS

Mice: Three to four-month-old male WT and *Ren1c-/-* mice(20) on C57BL/6 genetic background were housed in standard cages on a 12-hour light/dark cycle, allowed free access to normal chow and water, and handled in accordance with the National Institutes of Health guidelines for the use and care of experimental animals, as approved by the IACUC of UNC-CH.

Histology of thyroid gland: Thyroid glands were removed from mice and put on 4% paraformaldehyde (PFA) for 24 hours and sent them to UNC histology core facility.

Rectal temperature: Rectal temperature of mice was measured using a precalibrated thermistor probe YSI 4600 (Yellow Springs Instrument Co., Yellow Springs, Ohio, USA). Mice were fasting for 4 hours first and then exposure at 4 ℃ for 2 hours to measure rectal temperature every 30 minutes.

BP: BP was measured using a computerized tail-cuff method(150).

Measurement of hypothalamic T3 and plasma free T3 and free T4 and alpha-MSH: HypothalamicT3 was extracted from the hypothalamus by adding methanol 95%

containing PTU 10^{-4} M. Tissues were homogenized and centrifuged at 13 000 rpm and the pellets re-suspended twice using methanol solution. The supernatants were evaporated to dryness and resuspended in GAB buffer (0.2 M glycine/0.13 M acetate with 0.02% BSA). T3 was determined by radioimmunoassay system (RIA). Samples and standard curve were incubated at 4 ℃ with poly clonal antibody against T3 (Fitzgerald Industries International, Concord, MA) in RIA buffer GAB. Three days later, 10 000 cpm of radiolabeled ^{125}I T3 (Specific activity 2200 Ci/mmol; Perkin–Elmer Life Sciences, Boston MA) was added to each tube. After two days of incubation, a rabbit gamma globulin (Jackson ImmunoResearch Laboratories) diluted in 0.1 M EDTA and 16% polyethylene glycol with goat ant-rabbit IgG (Antibodies Incorporated, Davis CA) in GA buffer (0.2 M glycine/0.13 M acetate) was added to precipitate the antibody–T3 complex. After centrifugation the precipitates were counted in a γ-counter. T3 is expressed in picogram per milligram of weight tissue. Plasma free T3 and T4 levels were measured with ELISA kit (catalog number: 25-FT3HU-E01, and 25-FT4HU-E01; ALPCO). TSH was measured with ELISA kit (catalog number: SY45201, IBL International GmbH). Hypothalamic α-MSH was measured by mouse α-MSH EIA kit (catalog number: EK-043-01, Phoenix Pharmaceuticals, Inc.).

pSTAT3: STAT3 phosphorylation levels in hypothalamus were measured by ELISA kit (Catalog Number KCB4607 R&D Systems). The results were repeated by western blot method.

Administration of angiotensin II, human renin, human EPO, thyroid hormone, losartan, enalapril, and methimazole challenge *in vivo*: Losartan (Merck & Co., Inc.) 0.45 g/l or enalapril (Sigma) 0.3g/l or methimazole (Sigma) 2g/l were given in the drinking water for 2 months. Angiotensin II (7.5 µg//kg/day), human renin (0.1µg/kg/day) or human EPO (5000IU/kg/ three times/per week, Cell Science) or triiodothyronine T3 (5µg/kg/day, or 300ng/kg/day) were administered i.p. using osmotic minipumps (Durect Corp) for 2weeks.

N1 Cell Culture: Mouse hypothalamic cell line (N1 cells) was from Cellutions Biosystems Company. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum in 5% CO_2. Serum was deprived for 24 hrs before testing the effects of Ang II or human renin.

Hypothalamic tanycytes primary cell culture. Primary cultures were established from mice fetal day 17 hypothalamus as described (151). In brief, pregnant females were anaesthetized with isoflurane, the embryos removed individually, and the hypothalamus was excised under a stereoscopic microscope and placed in Hank's solution. After the removal of embryos, pregnant females were killed under anesthesia. Tissues were enzymatically dissociated with trypsin (Sigma, Missouri, USA). Cells were plated on dishes coated with 1.5 mg/mL poly D-lysine (Sigma) for RT-PCR. The medium used was Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (10%), vitamins, glucose, glutamine and insulin. The losartan (10 nmol/L)

and AG-490 (10 nmol/L) were added to cell medium for 24 hrs for measuring TRH mRNA (152).

Dio2 activity Dio2 activity was measured as previously described (153, 154) by the release of ^{125}I from 2 nM [^{125}I] T4 (NEN Life Science Products Inc., Boston) in the presence of 20 mM DTT and 1 mM PTU in dissected tissues. All activity values were corrected by protein concentration in each sample.

Hematological indices: Total blood of 20μl were drawn for determination of RBC number, hemoglobin, hematocrit, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH). All analyses were performed by Heska Veterinary CBC DIFF machine (Heska).

Reticulocyte stain and reticulocyte index: Blood and reticulocyte staining solution, New Methylene Blue (sigma), were mixed together for 5 minutes and marked blood smear for counting reticulocyte number under light microscope 1000x magnificent**.** Ribosomal RNA is precipitated by the cationic dye. Corrected reticulocyte count index was determined by reticulocyte % x corrected factor/ RBC volume. Corrected factor is dependent on HCT levels. HCT: 40-45% is 1, 35-39 is 1.5 and 25-34% is 2.

Plasma volume, blood volume, and red blood cell volume

Plasma volume (PV) was determined by Evans blue dye dilution. As previously described (155), 20 μl of 0.4% solution of Evans blue dye (sigma) injected into tail vein. Blood samples (l0 μl) were collected every 10 minutes until 30 minutes after injection. Plasma was diluted 1:100 in water and Evans blue dye was used as standard to measured concentration at 620 nm. Extrapolation of the regression line to 0 to 30 minutes, and allowed 0 minute as total injection concentration. Blood volume (BV) is estimated by PV divided by 1-HCT, red blood cell volume is BV-PV.

Plasma EPO: 5 μl of blood were drawn and collected on ice in tubes. Plasma was frozen immediately after blood collection and stored at -80ºC until were performed. EPO was measured using a ELISA kit (R& D systems).

Bone marrow: mice bone marrow suspensions were from the femurs. After removing femurs, using No.18 needle flashed femur and collected bone marrow cells in DMEM medium. Adjust the cell density of each suspension to 1×10^6cells/ml for flow cytometry.

Fluorescence-activated cell sorting (FACS): Single cell suspensions obtained from the bone marrow were labeled with antibodies PE-TER119, and FITC-CD71 (Pharmingen, San Diego) for 30 min. Cells were analyzed on Cytek FACS machine (Becton Dickinson) using Summit software. For CD71 and TER119 percentages were

dependent on markers intensity of FACS results multiple with bone marrow cells number and then divided by WT mice. Total FACS cells were 150,000 cells/each sample.

CFU-E and BFU-E colony assay: For evaluation of burst-forming unit erythroid (BFU-E) and colony-forming unit erythroid (CFU-E), bone marrow cells were seeded at various concentrations (1×10^5 cells/well) into semisolid medium MethoCultM334 (Stem Cell Technologies) in 12 well dishes. For evaluation of CFU-Es, small compact colonies were counted 48 hours later, and for BFU-Es 6 days later. The cells stained for the presence of hemoglobin with benzidine, and counted with brown color positive cells.

RNA analysis: Total RNA was extracted from brain tissues by a monophasic solution of phenol and guanidine isothiocyanate (TRIzol Reagent, Life Technologies, Invitrogen, Carlsbad, California, USA) (20). mRNA levels were quantified with the Mastercycler® ep realplex S (Eppendorf, Westbury, NY) (29). Relative gene expression levels were determined with hypoxanthine phosphoribosyltransferase (*Hprt*) as an internal standard, and expressed as a percentage of WT.

Data management/Statistical analyses: All values were expressed as mean ± SEM. Data were analyzed by Student's *t* test or analysis of variance (ANOVA). Tukey-Klamer post-hoc test was performed with JMP software version 8 (SAS Institute Inc., Cary, NC).

RESULTS

The *Ren1c-/-* mice have tertiary hypothyroidism.

Because reduced total T3 and T4 in the *Ren1c-/-* mice might simply reflect the reduced thyroid hormones bound to thyroxine binding globulin (TBG) and might not necessarily mean hypothyroidism, I first measured free forms of thyroid hormones in the plasma of the *Ren1c-/-* mice. The *Ren1c-/-* mice showed reduced levels of free T3 and T4 compared with WT (fT3: 1.3 ± 0.2 pg/ml vs. 2.2 ± 0.2 pg/ml, $p< 0.01$; fT4: 0.4 ± 0.02 ng/dl vs. 1.1 ± 0.04 ng/dl , $p< 0.01$) (**Figs. 3.1A,and B; Table 3.1**). Plasma TSH and gene expression of TRH in the hypothalamus of the *Ren1c-/-* mice were also approximately half of WT mice (**Figs. 3.1C, and D**). Decreased fT3, fT4, TSH, and TRH were all recapitulated by administering WT mice with an ARB losartan or an ACE inhibitor enalapril (**Figs. 3.1A-D**), indicating that inhibition of Ang II effects via AT1R decreases circulating thyroid hormone.

Core body temperature decreases in hypothyroidism. However, the basal rectal temperature was indistinguishable between the *Ren1c-/-* and WT mice when we measured it without fasting the animals (not shown). Interestingly, after 4 hrs of fasting, it was lower in the *Ren1c-/-* mice compared to WT mice, although it did not reach statistical significance. When the animals were then kept at 4℃, the rectal temperature of the *Ren1c-/-* mice decreased rapidly compared to WT mice (**Fig. 3.1E**). Cold intolerance of the *Ren1c-/-* mice was also recapitulated by WT mice treated with losartan or enalapril (**Fig. 3.1E**).

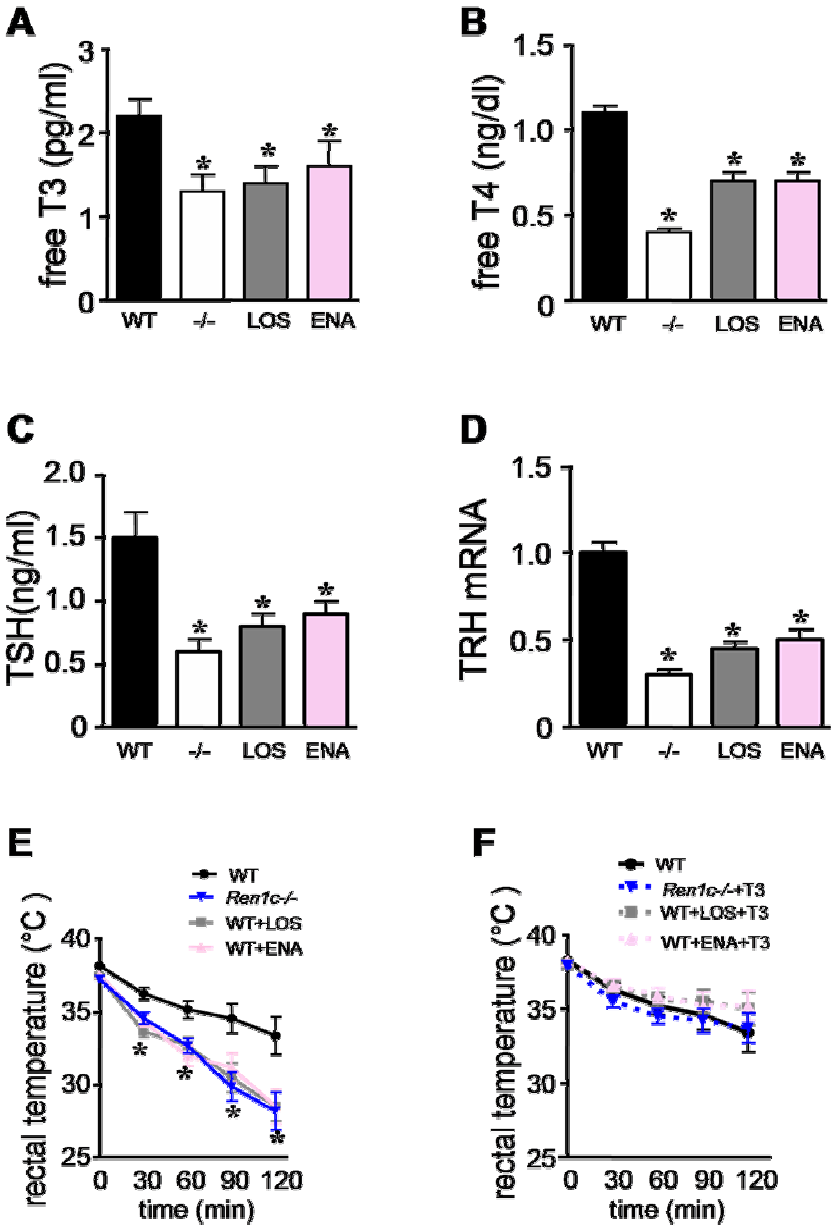

Figure 3.1 The effects of inhibition of renin angiotensin system on fT3, fT4, TSH, and TRH and cold tolerance. A, B. plasma free thyroid hormones (fT3 and fT4), C. TSH levels, and D. hypothalamic TRH expression levels. E. Rectal tempature after cold exposure (4℃) of the *Ren1c-/- mice,* WT mice and mice treated with losatan(0.45g/L drinking water) or enalarpil (0.3g/L drinking water) for two weeks. F. Rectal tempature after cold exposure of the same groups of mice as show E. treated with these mice treated with thyroid hormone (T3, 5μg/kg/day) for two weeks. All values are expressed as means ± S.E.M. ***n*** ≥8 *p< 0.05 vs. WT mice.

Reduction in rectal temperature of the *Ren1c-/-* mice was restored by administration of thyroid hormone (T3; 5μg/kg i.p.) using osmotic minipumps for 2 weeks (37.9 ± 0.7℃ vs. 37.8℃ ± 0.5 in WT) (**Fig 3.1F**).

To determine whether low levels of thyroid hormones and TSH are caused by reduced TRH, I administered TRH (5.0 μg/kg, i.p.) into the *Ren1c-/-* mice, which restored their thyroid hormones and TSH to the levels of WT (**Figs. 3.2A-C**). Hypothyroidism recapitulated by an ARB or an ACE inhibitor was corrected by administering TRH (**Figs. 3.2A-C**). I conclude that inhibition of Ang II effects via AT1R causes tertiary hypothyroidism,

Ang II corrects hypothyroidism of the *Ren1c-/-* mice by directly increasing TRH via AT1R-JAK-STAT pathway.

I next asked relative contribution of Ang II and Ang-independent effect of renin on tertiary hypothyroidism of the *Ren1c-/-* mice. Given that human renin does not cleave mouse angiotensinogen to produce Ang I (156), but both human and mouse renin are capable of activating MAPK in mouse cells, I took advantage of this property and administered human renin to the *Ren1c-/-* mice to investigate the Ang-independent effects of renin. Ang II but not human renin restored TRH, TSH, and fT3 to the WT levels (**Figs. 3.3A-D**), demonstrating that Ang II but not renin per se stimulates hypothalamic-pituitary-thyroid axis.

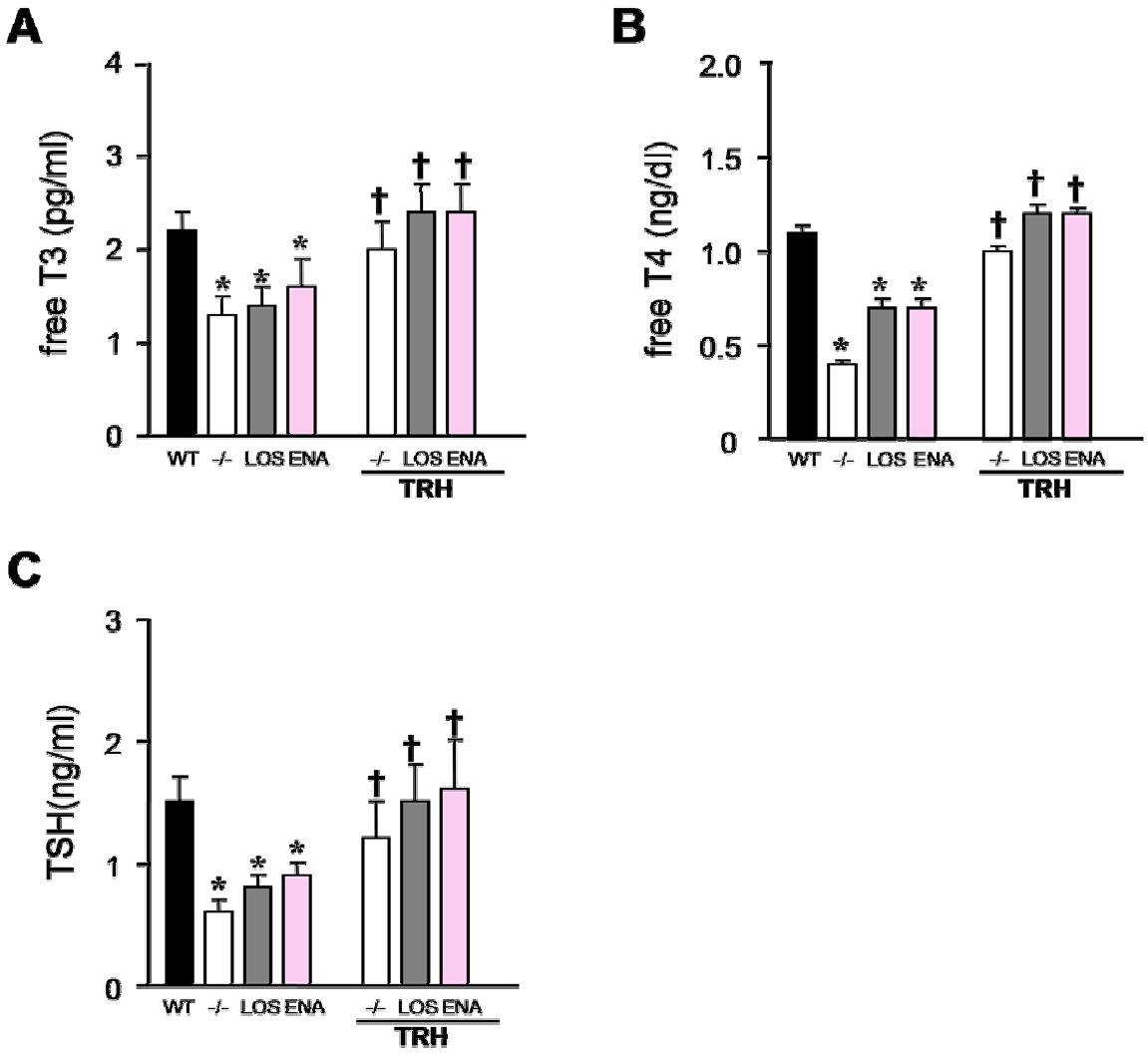

Figure 3.2 TRH corrects teritary hypothyroidism. A. plasma free T3, B. plasma free T4. C. plasma TSH levels. All values are expressed as means ± S.E.M. n ≥8 *p< 0.05 vs. WT mice. -/-: *Ren1c-/-* , LOS: WT mice treated with losartan(0.45g/L drinking water) for 4 weeks or ENA, WT mice treated with enalapril (0.3g/L drinking water) for 4 weeks.$^{\dagger}p$< 0.05 vs. without treating TRH.

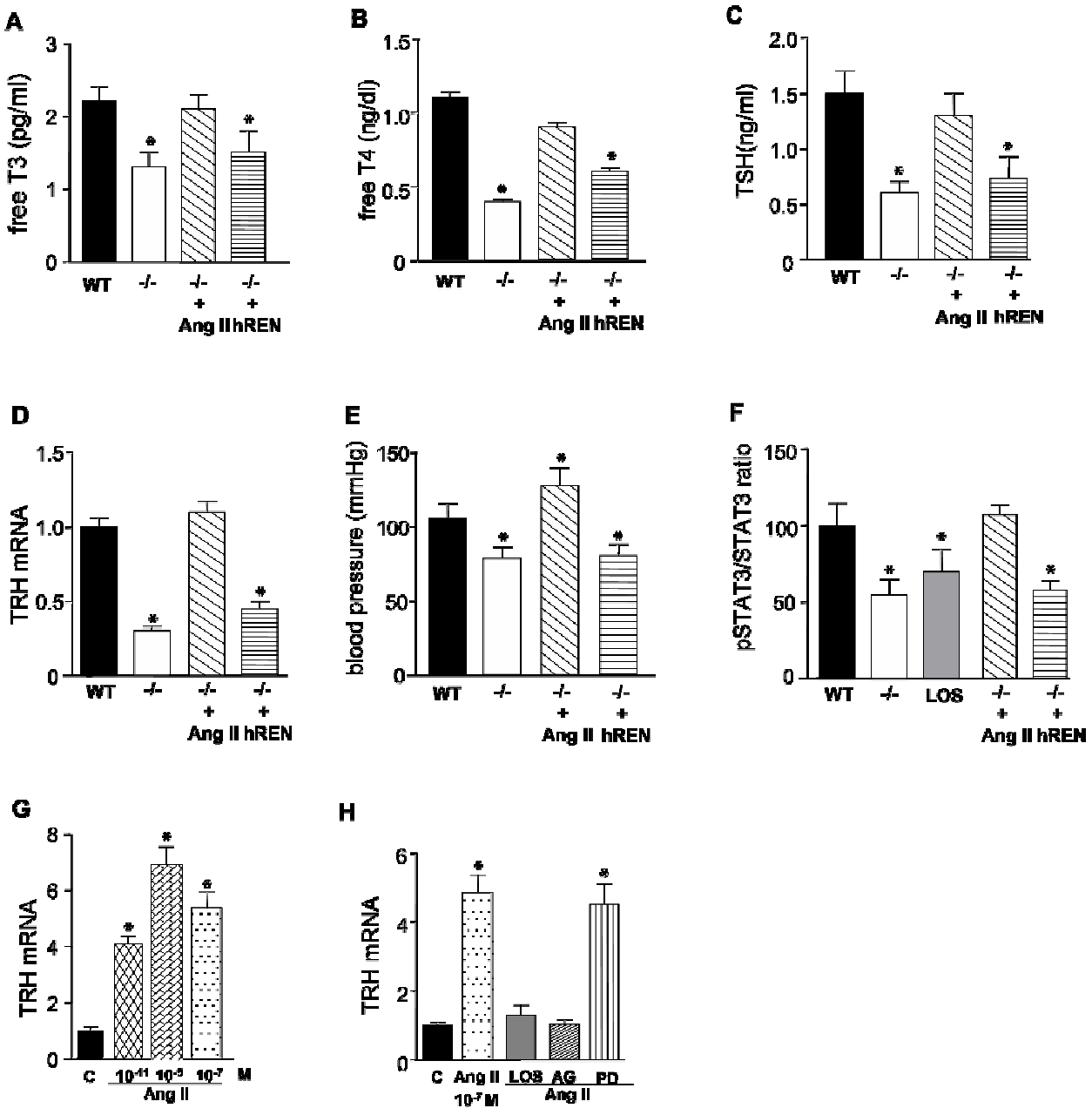

Figure 3.3 Reduced Ang II causes tertiary hypothyroidism and Ang II corrects it via AT1R. A-D Ang II effect and renin effect on plasma free T3, T4 and TSH and hypothalamic TRH gene expression. E. Ang II effect on blood pressure. F. STAT3 phosphorylation levels in the hypothalamus. G. Ang II effect in N1 cells. H. Ang II increases hypothalamic TRH via Ang II type1 receptor and Jak2. All values are expressed as means ± S.E.M. ***n*** ≥8 *$p < 0.05$ vs. WT mice or N1 cell without treatment. Ang II: angiotensin II(7.5μg/kg/day) for 2 weeks, and hREN:human renin (0.1μg/kg/day) for 2 weeks. -/-: *Ren1c-/-* mice, LOS: WT mice treated with losartan (0.45/L in drinking water) or N1 cells treated with losartan (10 nmol/L), AG: N1 cells treated with AG490 (10 nmol/L), and PD: N1 cells treated with PD123319 (10 nmol/L).

It is known that AT1R activates JAK2 and phosphorylates STAT3 (110, 157), and leptin increases hypothalamic TRH expression via JAK2-STAT3 pathway (158). Phosphorylated STAT3 binds to TRH promoter and increases its expression (83, 158). Consistent with this previous finding, hypothalamic phosphorylated STAT3 levels of the *Ren1c-/-* mice and of WT mice treated with losartan were significantly lower than those of WT control mice (**Fig. 3.3F**), which supports the idea that Ang II increases hypothalamic TRH expression by activating JAK-STAT3 pathway. In addition, the *Ren1c-/-* mice have 40% WT levels of leptin, which is comparable to that of heterozygous leptin deficient mice. However, TRH, TSH and free forms of thyroid hormones of heterozygous leptin deficient *ob*/+ mice did not differ from those of WT mice (**Figs. 3.4 A, B**), indicating reduced leptin in the *Ren1c-/-* mice does not contribute to hypothyroidism of these mice.

The *Ren1c-/-* mice, WT mice with losartan or enalapril all have similar levels of low BP, which might contribute to hypothyroidism. However a calcium channel blocker amlodipine, or an inhibitor of NaK2Cl cotransporter, furosemide, that decrease BP to the levels of the *Ren1c-/-* mice did not affect fT3 and fT4 (**Figs 3.4C-E**), showing that hypotension of the *Ren1c-/-* mice does not alter the hypothalamic-pituitary-thyroid axis. To test whether Ang II directly increases TRH, I treated mouse hypothalamic neuronal cell line N1 with Ang II ($10^{-11,}$ 10^{-9}, and 10^{-7} M). Ang II increased TRH gene expression in N1 cells (**Fig. 3.3G**), which was abolished by an AT1R antagonist losartan, but not by an AT2R antagonist PD123319 (**Fig. 3.3H**), showing that Ang II increases TRH by activating AT1R. Consistent with *in vivo* data (**Fig.3.3.F**) a JAK/STAT3 inhibitor (151), AG490 abolished the increase in TRH by Ang II (**Fig. 3.3H**).

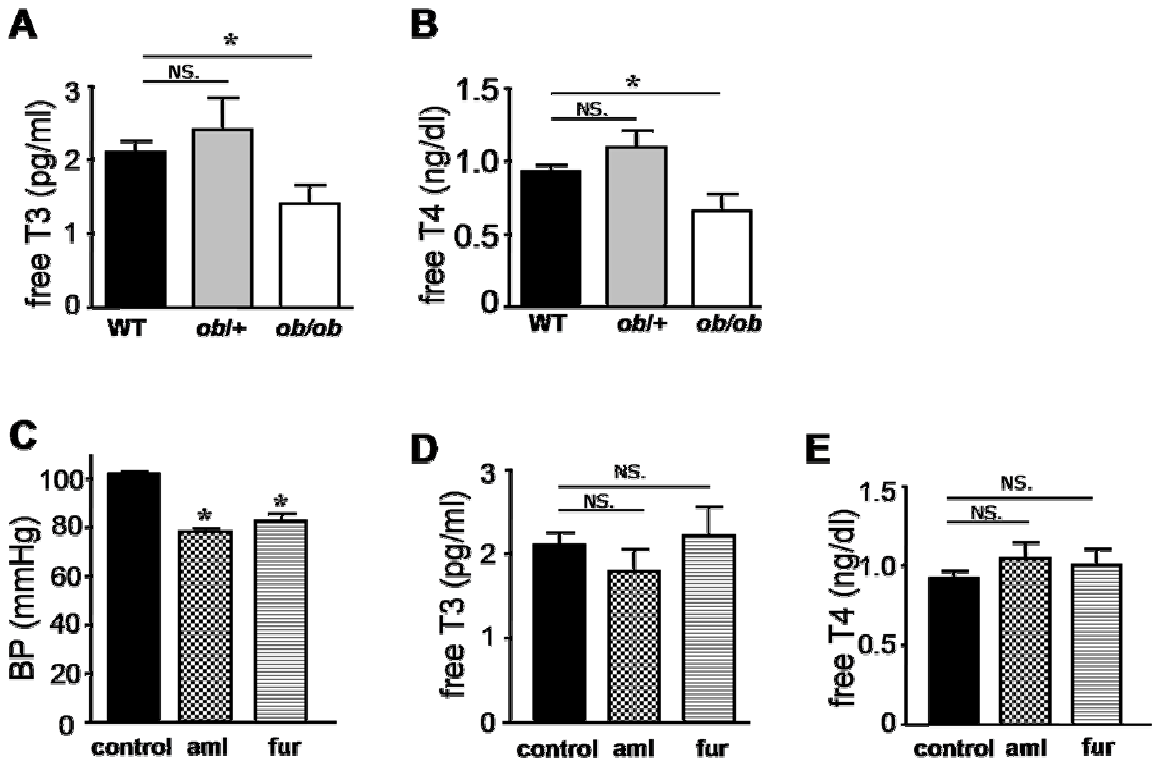

Figure3.4 Hypothyroidism is not due to reduction in blood pressure. A, B. thyroid hormones (free T3 and T4) in *ob*+/- and *ob/ob* mice C-E. Tail cuff blood pressure and thyroid hormones (free T3 and T4) in WT mice treated with amlodipine (0.1µg/L) and furosemide (0.1µg/L) for 2 weeks. All values are expressed as means ± S.E.M. n=7 *p< 0.05 vs. WT mice.

I conclude that reduced activation of AT1R causes tertiary hypothyroidism by directly decreasing TRH expression in the hypothalamus via JAK2-STAT3 pathway.

Inhibition of RAS-AT1R directly increases Dio2, which increases hypothalamic T3 and decreases TRH.

Another possible mechanism of inhibiting hypothalamic TRH expression is its feedback inhibition by T3. Approximately 80% of hypothalamic T3 is derived from tanycytes, special ependymal cells located in the floor of the 3rd ventricle. Tanycytes take up T4 from plasma and convert it to T3 by type II deiodinase (Dio2) (159-161). T3 travels into the hypothalamus via tanycyte processes extending deep into the hypothalamus. Although plasma levels of T3 and T4 are lower in the *Ren1c-/-* mice, it is possible that hypothalamic Dio2 and thus hypothalamic T3 are elevated in these animals, leading to suppression of TRH expression in the hypothalamus. Indeed the levels of hypothalamic T3, Dio2 gene expression and Dio2 activity of the *Ren1c-/-* mice were significantly higher than those of WT mice, and were recapitulated by losartan (**Fig. 3.5A-C**).

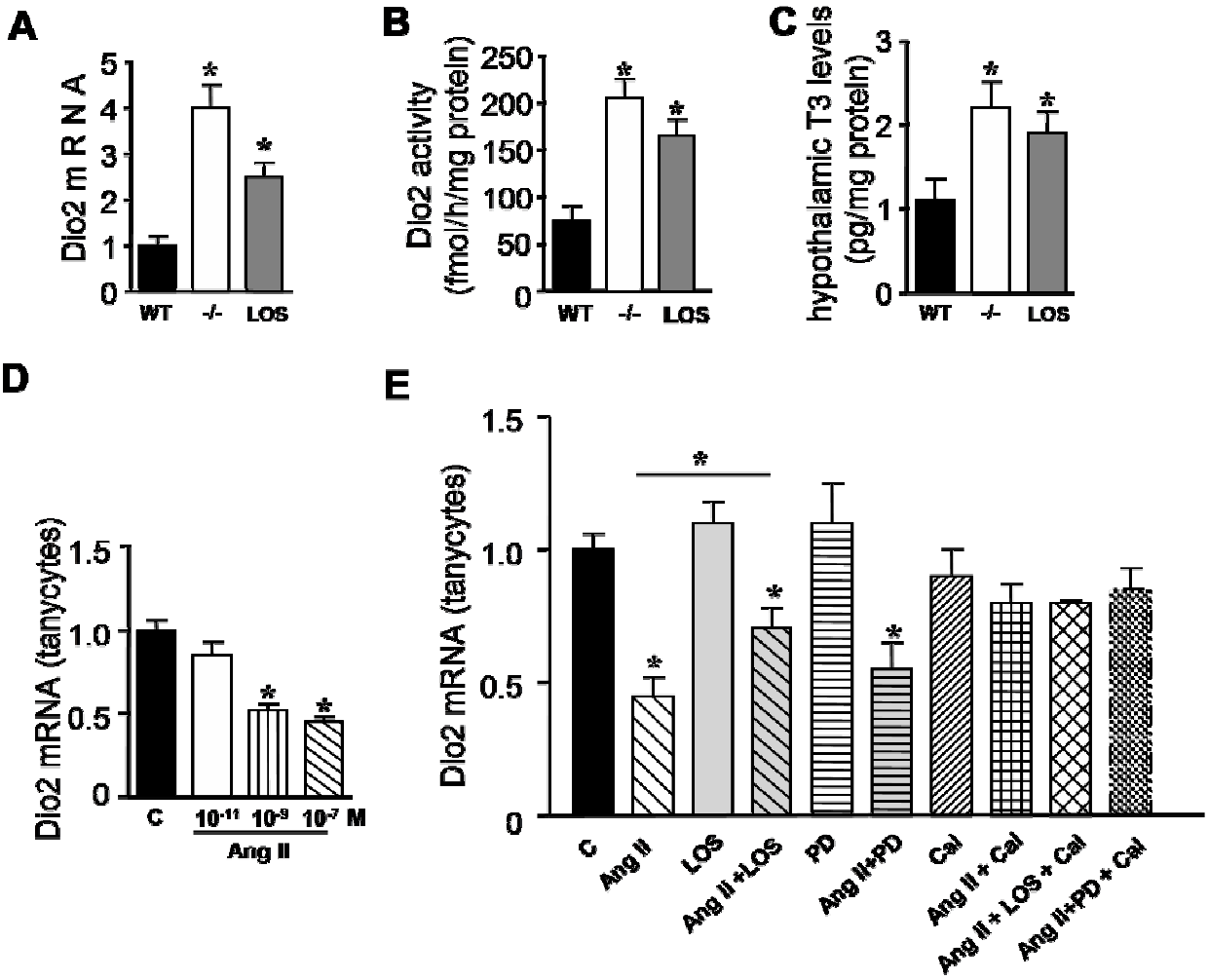

Figure 3.5 Inhibition of Ang II increased Dio2 expression and T3 levels in the hypothalamus. A. Dio2 gene expression levels and B. Dio2 activity in the hypothalamus. C. Hypothalamic T3 levels. D, E. Ang II effect on Dio2 expression and its mechanism in tanycytes. Cal: calphostin C (10 μM), PKC inhibitor. All values are expressed as means ± S.E.M. n=7 *$p < 0.05$ vs. WT mice or tanycytes without treatment. -/-: *Ren1c-/-* mice, LOS: WT mice treated with losartan (0.45 g/L) for 4 weeks or N1 cells treated with losartan (10 nmol/L), and PD: N1 cells treated with PD123319 (10 nmol/L).

Moreover, Ang II significantly decreased Dio2 expression in primary culture of tanycytes, which was inhibited by losartan (10 nmol/L), but not by a type 2 Ang II receptor blocker, PD123319 (10 nmol/L) (**Fig. 3.5D, E**). A PKC inhibitor calphostin C (10 nmol/L) abolished the reduction of Dio2 by Ang II (**Fig. 3.5F)**, indicating that Ang II via AT1R-PKC directly suppressed Dio 2 expression in the tanycytes. I conclude that inhibiting RAS causes tertiary hypothyroidism by directly inhibiting Ang II-AT1R-Jak2-STAT3-TRH pathway in hypothalamic TRH producing cells and by eliminating the suppression of Dio2-T3 via AT1R, leading to feedback inhibition of TRH expression by increased hypothalamic T3.

Hypothyroidism is responsible for anemia caused by the inhibition of RAS-AT1R.

Consistent with well documented anemia caused by RAS inhibition (113), the *Ren1c-/-* mice had severe normocytic normochromic anemia (**Figs. 3.6A-E; Table 3.2**). Because RBC volume per body weight of the *Ren1c-/-* mice was 35% smaller than that of WT mice (**Fig 3.6H**), their low RBC number, hemoglobin and hematocrit were not simply due to dilution of the blood by increased plasma volume, but they truly had anemia. The *Ren1c-/-* mice had normal reticulocyte index in their peripheral blood (**Figs. 3.6 F, and G; Table 3.2**) and normal plasma levels of iron and transferrin (**Table 3.2**), excluding hemolytic anemia and iron deficient anemia and suggesting impaired erythropoiesis in their bone marrow.

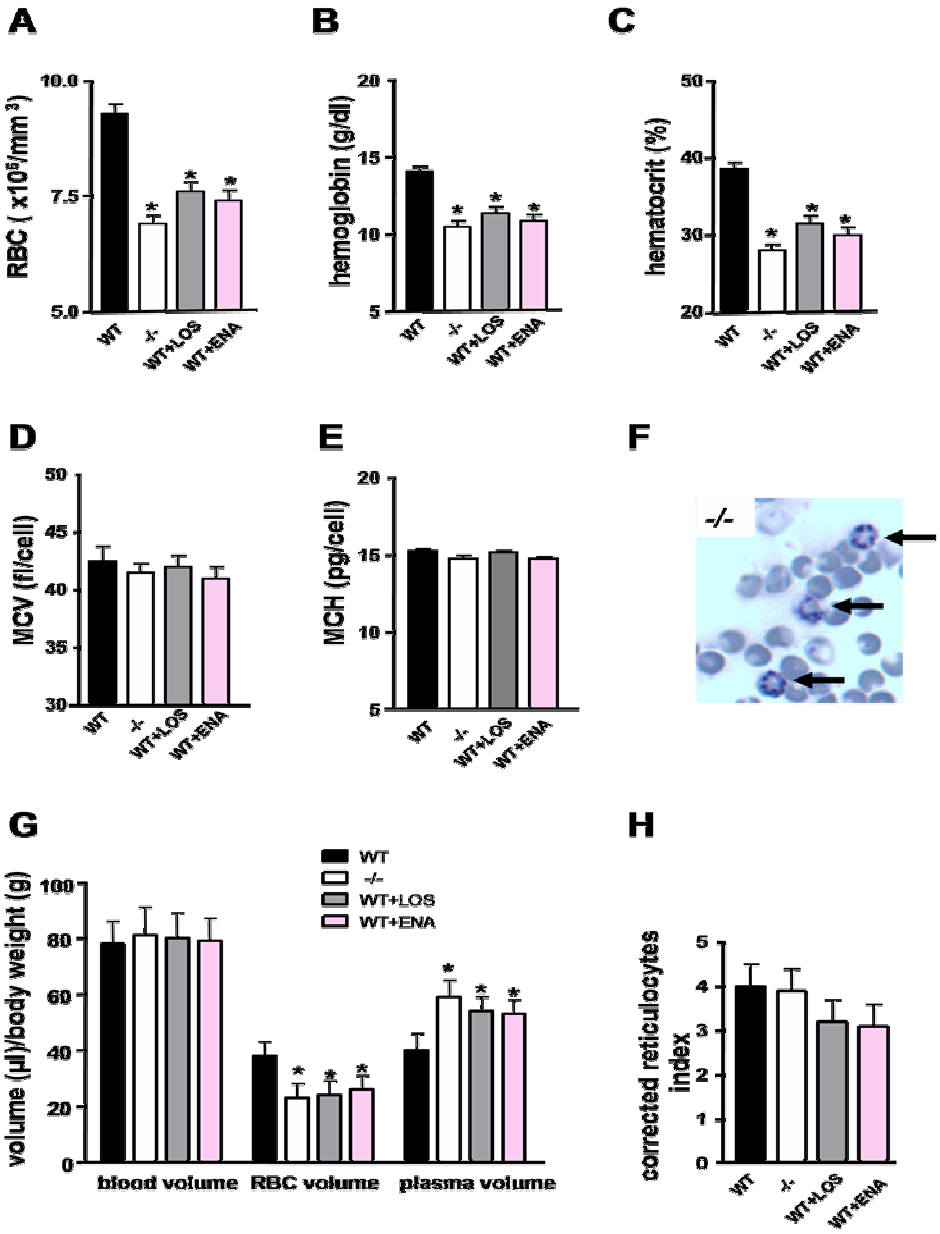

Figure 3.6 Inhibition of Ang II does not decrease blood volume, but increased plasma volume and decreased red blood cell volume. A. red blood cell (RBC) number, B. hemoglobin (Hgb), C. hematocrit (Hct), D. mean corpuscular volume (MCV), E. mean corpuscular hemoglobin (MCH), and F, G. reticulocytes in among *Ren1c-/-* mice, WT mice, WT mice treated with losartan or enalapril. H. blood volume, plasma volume and red blood cell volume in All values are expressed as means ± S.E.M. ***n*** ≥8 **p*< 0.05 vs. WT mice.. Black bar: WT mice; white bar: *Ren1c-/-* mice, gray bar: WT mice treated with losartan (0.45g/L drinking water), and pink bar: mice treated with enalapril (0.3g/L drinking water).

Additionally, WT mice treated with losartan or enalapril for two months also had severe anemia comparable to the *Ren1c-/-* mice (**Fig. 3.6A-E; Table 3.2**), showing that the inhibition of AT1R causes anemia.

As expected administration of Ang II corrected anemia of the *Ren1c-/-* mice (**Figs. 3.7A-E**). Because a number of studies have shown that thyroid hormone can stimulate erythropoiesis in early stages of erythroid progenitor cells (117, 118, 120, 121), and because inhibition of RAS causes hypothyroidism as I demonstrated in Chapter 2, I next investigated whether low circulating thyroid hormones of the *Ren1c-/-* mice are responsible for their severe anemia. Administration of T3 (5μg/kg/day) by osmotic minipump to the *Ren1c-/-* mice for two weeks restored their RBC number, Hct, and Hgb to the levels comparable to those of WT mice (**Figs3.7A-E**; **Table 3.2**) without increasing plasma Ang II levels, which were undetectable and not different from zero as in the *Ren1c-/-* mice (not shown), showing that thyroid hormone corrects anemia in the *Ren1c-/-* mice independently of Ang II. Administration of T3 also corrected anemia of WT mice treated with losartan or enalapril (**Figs. 3.7A-E**; **Table 3.2**), indicating hypothyroidism is responsible for anemia caused by inhibiting AT1R. However, after administration of 5μg/kg/day T3 the *Ren1c-/-* mice, and WT mice with losartan or enalapril had two-fold levels of plasma T3 compared to WT mice (~4.0 pg/ml vs. 2.2 pg/ml in WT mice; **Table 3.2**). Erythropoietin is an important stimulator for erythropoiesis and is elevated in the *Ren1c-/-* mice and WT mice treated with losartan or enalapril. Interestingly administration of 5 μg of T3 increased plasma EPO levels twice as high as those of WT mice despite the correction of anemia (**Table 3.2**). This

could be because high dose T3 (5µg/kg/day) elevates plasma EPO via Hypoxia induced factor (HIF)(162).

Interestingly, administration of low dose of T3 (300ng/kg/day) for 2 weeks corrected anemia and elevated plasma EPO levels of the *Ren1c-/-* mice and WT mice with losartan or enalapril to the levels similar to WT control mice, and the effects of 5µg/kg/day and 300ng/kg/day T3 to correct anemia caused by inhibiting RAS were similar (**Fig. 3.7, Table 3.2**). Low dose T3 increased plasma T3 levels of the *Ren1c-/-* mice to the levels indistinguishable from those of WT control mice (**Table 3.2**).

Importantly, although Ang II is known to increase EPO(147), high dose EPO (5000 IU) did not correct anemia of the *Ren1c-/-* mice (**Figs. 3.7 A-E; table 3.2**), while the same dose of EPO increased RBC number, hemoglobin and hematocrit in WT mice (**Figs. 3.7 A-E; table 3.2**), showing that anemia of the *Ren1c-/-* mice is resistant to EPO. To address the contribution of direct effect on erythropoiesis of Ang II independent of thyroid hormone, I treated WT mice with a thyroid hormone synthesis blocker methimazole (MMI) together with an iodine uptake blocker sodium perchlorate ($NaClO_4$) for 2 months, and tested the effect of Ang II on anemia of these animals. WT mice treated with MMI and $NaClO_4$ had undetectable levels of circulating thyroid hormones, and microcytic hypochromic anemia, which was corrected by administration of T3 (5µg/kg/day) (**Table 3.2**). Administration of Ang II to these animals did not correct anemia caused by MMI, although the same dose of Ang II corrected anemia of the *Ren1c-/-* mice (**Table 3.2**).

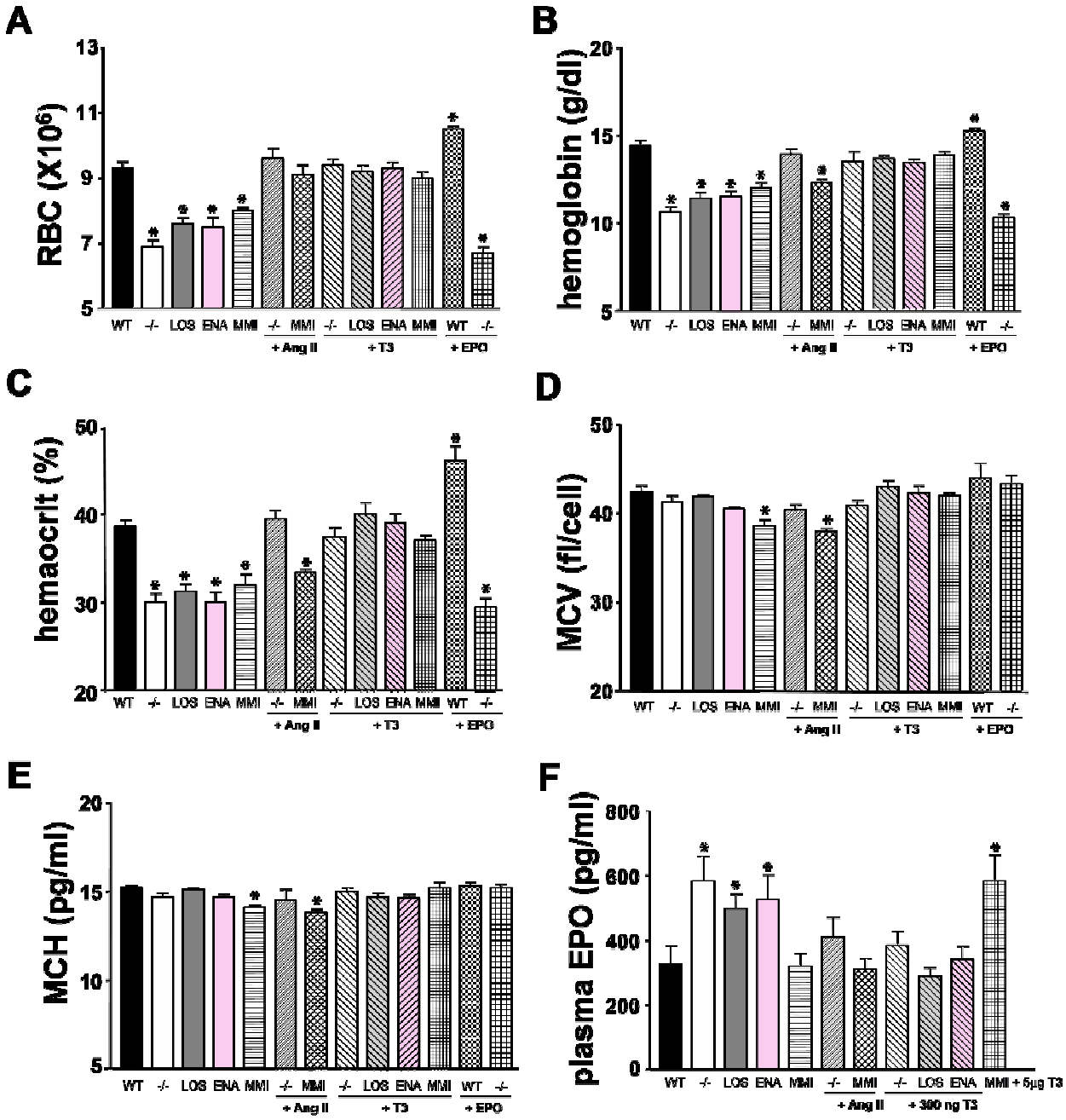

Figure 3. 7 Inhibition of Ang II causes anemia, while T3 restores it. A. RBC number, B. hematoglbin (Hgb), C. hematocrit (HCT), D. MCV, E. MCH, and F, G. plasma EPO levels in *Ren1c-/-* mice, WT mice, WT mice treated with losartan or enalapril with T3 (5µg/kg/day) or Ang II (7.5µg/kg/day) or human EPO (5000 U/ kg/day) for two weeks. *Ren1c-/-* mice also treated with low dose T3 (300 ng/kg/day) for 2 weeks. All values are expressed as means ± S.E.M. n=8 *p< 0.05 vs. WT mice.

In addition, although MMI+$NaClO_4$ caused hypothyroidism and anemia, plasma EPO levels stayed similar to the levels of WT control mice, indicating that thyroid hormone-independent effect of Ang II on anemia caused by RAS inhibition is small and insufficient to correct the anemia.

I conclude that inhibiting RAS causes normocytic normochromic anemia that low dose thyroid hormone is sufficient to correct this anemia, and that direct effects of Ang II independent of thyroid hormone are insufficient to correct this anemia.

Inhibition of RAS-AT1R impairs early stage erythropoiesis in the bone marrow.

Because the bone marrow is the main organ for erythropoiesis in adults, I counted the number of cells in the bone marrow. The bone marrow of the *Ren1c-/-* mice had 35% lower cell number than that of WT mice, and that of WT mice treated with losartan or enalapril had 25% lower cell number than WT mice (**Fig 3.8A**). Moreover, the numbers of their burst-forming unit erythroid (BFU-E) and colony-forming unit erythroid (CFU-E) colonies obtained from the culture of the bone marrow cells of the *Ren1c-/-* mice were lower than those of WT mice, and administration of T3 corrected the numbers of these colonies to WT control levels (**Figs. 3.8B-C**). Inhibition of thyroid hormone synthesis by MMI and NaClO4 decreased the numbers of bone marrow cells and of BFU-E and CFU-E colonies, which was corrected by T3 but not by Ang II or human EPO, indicating that thyroid hormone is required but Ang II is dispensable for early stage of erythropoiesis in the bone marrow.

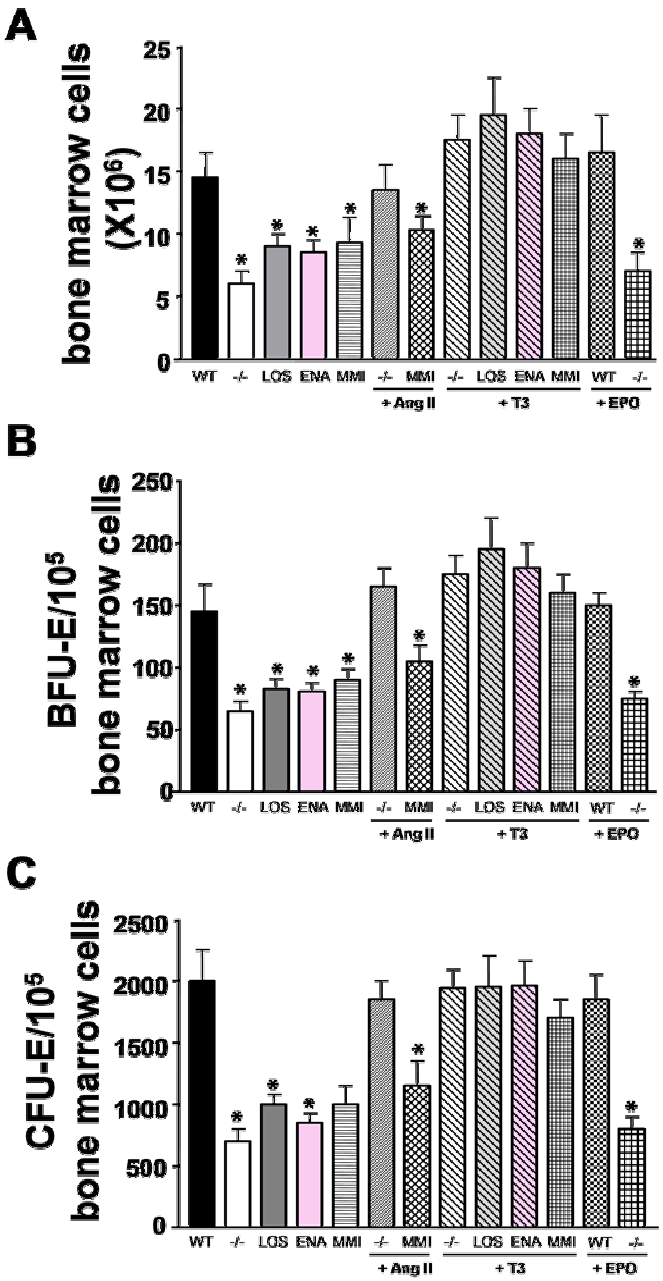

Figure 3.8 Inhibition of Ang II causes decreased early stage of erythroid progenitor cells in bone marrow. A. Cell number count in the bone marrow from both femurs of each mouse. B. blast forming unit-erythroid (BFU-E), and C. Colony forming unit-erythroid (CFU-E) cell number in the *Ren1c-/-* mice, WT mice, WT mice treated with losartan or enalapril. All values are expressed as means ± S.E.M. n=7 *p< 0.05 vs. WT mice. -/-: *Ren1c-/-* mice, LOS: WT mice treated with losartan (0.45g/L in drinking water) for 2 months, ENA: mice treated with enalapril (0.3g/L in drinking water) for two months, and MMI: WT mice treated with methimazole (2g/L in drinking water) for two months.

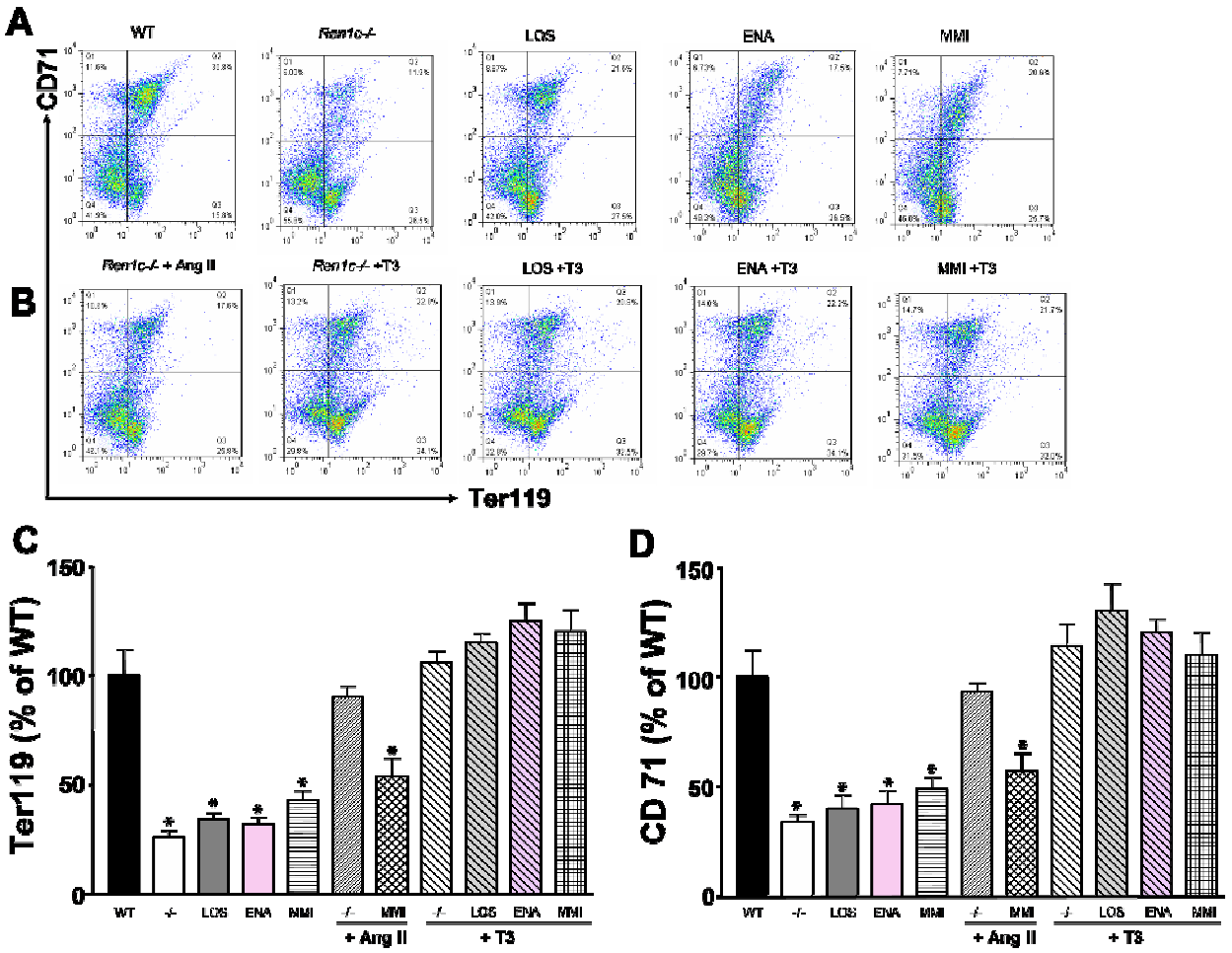

Figure 3.9 Inhibition of Ang II decreases erythroid progenitor cells positive for surface markers CD71 and ter119, and T3 corrects it. A. FACS of bone marrow cells in the *Ren1c-/-* mice, WT mice, WT mice treated with losartan or enalapril. B FACS of bone marrow cells after administration of T3 (5µg/kg/day) for two weeks. C. percentage of CD 71, and D. ter119 without or with administration of T3 in the *Ren1c-/-* mice, WT mice, WT mice treated with losartan or enalapril. All values are expressed as means ± S.E.M. n=5 *p< 0.05 vs. WT mice. -/-: *Ren1c-/-* mice, LOS: WT mice treated with losartan (0.45g/L in drinking water) for 2 months, ENA: mice treated with enalapril (0.3g/L in drinking water) for two months, and MMI: WT mice treated with methimazole (2g/L in drinking water) for two months.

To investigate which stage of erythroblast differentiation is affected by thyroid hormone, I used flow cytometry and labeled CD71 antibody to early stage of labeled and ter119 to late stage of erythroblast. Interestingly, the *Ren1c-/-* mice and WT mice treated with losartan or enalapril had significantly reduced number of CD71 and ter119 positive bone marrow cells, indicating decreased early stage erythroblasts in these mice compared to WT control mice (**Fig. 3.9A, 3.9C, 3.9D**).

Administration of T3 increased CD71 and ter11 positive cells in the bone marrow of the *Ren1c-/-* mice and of WT mice treated with losartan or enalapril to the levels of WT control mice, showing that T3 increased the number of early stage of erythroblast (**Fig. 3.9B-D**). Inhibition of thyroid hormone synthesis decreased the numbers of CD71 and ter119 positive cells in the bone marrow, which was corrected by T3 but not by Ang II. Although the spleen can contribute to erythropoiesis in anemia, the weight, histology (red pulp and white pulp), and cell number in the spleen was indistinguishable from those of WT mice (not shown).

I conclude that hypothyroidism is the predominant cause of anemia induced by inhibiting RAS, and that thyroid hormone is required but Ang II is dispensable for early stage of erythropoiesis in the bone marrow (**Fig. 3.10)**.

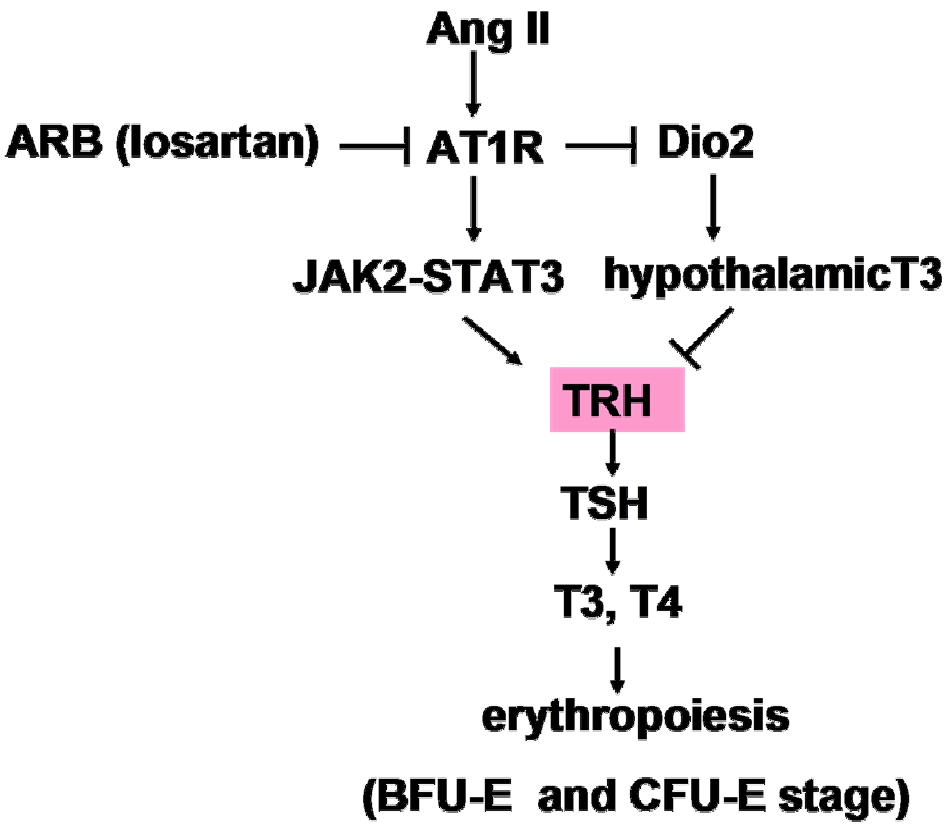

Figure 3.10 The mechanism of inhibition of Ang II causes anemia through inhibiting thyroid hormone pathway.

Table 3.1: Characteristics of 4-month-old male mice plasma thyroid hormones & TSH levels

Groups	fT3 (pg/ml)	fT4 (ng/dl)	TSH levels (ng/ml)
Wild type (n=12)	2.2 ± 0.2	1.10 ± 0.04	1.5 ± 0.2
Ren1c-/- (n=10)	1.3 ± 0.2 *****	0.42 ± 0.02 *****	0.6 ± 0.1 *****
WT+LOS (n=7)	1.4 ± 0.2 *****	0.71 ± 0.05 *****	0.9 ± 0.1 *****
WT+ENA (n=7)	1.6 ± 0.3 *****	0.80 ± 0.06 *****	0.9 ± 0.2 *****
Ren1c-/-+Ang II (n=5)	2.1 ± 0.2	0.95 ± 0.03	1.3 ± 0.2
Ren1c-/-+ hREN(n=5)	1.5 ± 0.3 *****	0.62 ± 0.02 *****	0.8 ± 0.2 *****
Ren1c-/-+TRH (n=7)	2.0 ± 0.3	0.91 ± 0.02 *****	1.2 ± 0.3
WT+LOS +TRH (n=7)	2.4 ± 0.3	1.22 ± 0.05	1.5 ± 0.3
WT+ENA +TRH (n=7	2.4 ± 0.3	1.20 ± 0.04	1.6 ± 0.4
WT+MMI/NaClO4 (n=8)	ND	ND	ND
WT+MMI/NaClO4+Ang II(n=6)	ND	ND	ND

Data are given as mean ±SEM. fT3: free thyronine, fT4: free thyroxine, TSH: thyroid-stimulating hormone**,** Ang II: angiotensin II (7.5μg/kg/day) for 2 weeks, hREN: human renin (0.1μg/kg/day) for 2 weeks. LOS: WT mice treated with losartan(0.45g/L in drinking water) for 4 weeks, ENA: mice treated with enalapril (0.3g/L in drinking water) for 4 weeks. TRH : mice treated with TRH (5μg/kg)for one hour. *****$p < 0.05$ vs. wild type. ND: non-detectable, the free T3 and T4 value under 0.05 pg/ml and 0.05 ng/dl.

Table 3.2: Hematologic indices of 4-month old male mice

Groups	RBC (10^6/mm^3)	Hgb (g/dl)	Hct (%)	MCV (fl/cell)	MCH (pg/cell)	plasma iron (µg/dl)	transferrin (mg/ml)	free T3 (ng/ml)	Plasma EPO (pg/ml)
Wild type (n=12)	9.3 ± 0.2	14.4 ± 0.3	38.6 ± 0.7	42.4 ± 0.8	15.2 ± 0.1	120 ± 12	3.0 ± 0.2	2.3 ± 0.1	343 ± 42
Ren1c-/- (n=12)	6.9 ± 0.2*****	10.5 ± 0.3*****	29.6 ± 1.0*****	41.3 ± 0.7	14.7 ± 0.2	110 ± 15	3.4 ± 0.4	1.3 ± 0.2*****	583 ± 70*****
WT+LOS (n=10)	7.6 ± 0.2*****	11.5 ± 0.3*****	31.3 ± 0.8*****	41.9 ± 0.2	15.1 ± 0.1	114 ± 4.0	3.0 ± 0.1	1.4 ± 0.2*****	450 ± 42*****
WT+ENA (n=10)	7.5 ± 0.3*****	11.4 ± 0.3*****	30.0 ± 1.2*****	40.4 ± 0.2	14.7 ± 0.1	116 ± 7.0	3.0 ± 0.2	1.6 ± 0.3*****	526 ± 80*****
MMI (n=10)	8.0 ± 0.1*****	12.0 ± 0.3*****	32.2 ± 1.2*****	38.6 ± 0.7*****	14.1 ± 0.1*****	150 ± 6.0*****	2.9 ± 0.2	ND	280 ± 38
Ren1c-/-+ HT3 (n=10)	9.4 ± 0.3	13.4 ± 0.5	36.3 ± 1.2*****	40.9 ± 0.5	15.0 ± 0.2	-	-	3.9 ± 0.6*****	1032 ± 182*****
WT+LOS + HT3 (n=8)	9.2 ± 0.2	13.5 ± 0.2	40.0 ± 1.2	43.0 ± 0.7	14.7 ± 0.2	-	-	4.0 ± 0.5*****	765 ± 50*****
WT+ENA + HT3(n=8)	9.3 ± 0.1	13.4 ± 0.1	39.0 ± 0.9	42.3 ± 0.8	14.6 ± 0.2	-	-	3.9 ± 0.5*****	1035 ± 170*****
Ren1c-/-+ LT3 (n=6)	9.0 ± 0.3	13.4 ± 0.3	37.1 ± 1.0	40.0 ± 0.4	14.5 ± 0.1			2.2 ± 0.2	384 ± 40
WT+LOS + LT3 (n=6)	9.3 ± 0.2	13.8 ± 0.4	37.2 ± 1.5	40.4 ± 0.2	14.9 ± 0.3			2.4 ± 0.3	285 ± 26
WT+ENA + LT3(n=6)	9.2 ± 0.1	13.7 ± 0.4	37.8 ± 1.2	41.0 ± 0.7	14.7 ± 0.2			2.4 ± 0.2	343 ± 40
Ren1c-/-+ AII (n=7)	9.1 ± 0.3	13.9 ± 0.3	39.4 ± 1.0	40.4 ± 0.6	14.5 ± 0.5	-	-	2.1 ± 0.2	410 ± 45
MMI+ AII (n=7)	9.2 ± 0.1	12.8 ± 0.1*****	33.4 ± 0.3*****	38.1 ± 0.7*****	13.8 ± 0.2*****	-	-	ND	312 ± 31
MMI+ T3 (n=7)	9.0 ± 0.2	13.8 ± 0.2	36.9 ± 0.6	42.4 ± 0.3	15.4 ± 0.1	122 ± 10	3.1 ± 0.2	3.3 ± 0.7*****	545 ± 80*****
WT+EPO (n=7)	10.5 ± 0.2*****	16.1 ± 0.1*****	46.3 ± 1.6*****	44.0 ± 2.0	15.3 ± 0.2	-	-	2.4 ± 0.1	-
Ren1c-/-+EPO (n=7)	6.8 ± 0.2	10.3 ± 0.2	29.3 ± 1	43.3 ± 1.2	15.2 ± 0.1	-	-	1.1 ± 0.3*****	-

Data are given as mean ±SEM. Hgb: Hemoglobin, Hct: hematocrit, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Hemoglobin. LOS: WT mice treated with losartan(0.45g/L in drinking water) for 2 months, ENA: mice treated with enalapril (0.3g/L in drinking water) for two months, and MMI: WT mice treated with methimazole (2g/L in drinking water) for two months; HT3: high dose of T3 (5µg/kg/day), and LT3: low dose of T3 (300 ng/kg/day) for 2 weeks, Ang II: angiotensin II (7.5µg/kg/day) for 2 weeks, LOS: WT mice treated with losartan(0.45g/L in drinking water) for 4 weeks, ENA: mice treated with enalapril (0.3g/L in drinking water) for 4 weeks. EPO: mice treated with EPO (5000 U/kg/day; i.p) for two weeks. *****$p < 0.05$ vs. wild type. ND: non-detectable, the free T3 and T4 value under 0.05 pg/ml and 0.05 ng/dl.

DISCUSSIONS

In this study I have shown that inhibiting RAS causes tertiary hypothyroidism by reducing the effect of Ang II via AT1R to directly stimulate TRH via JAK2-STAT3 pathway, and by increasing Dio2 and T3 in the hypothalamus and augmenting feedback inhibition of TRH. I also demonstrated that hypothyroidism is the predominant cause of anemia induced by inhibiting RAS, and that thyroid hormone is required but Ang II is dispensable for early stage of erythropoiesis in the bone marrow. Although inhibitors of RAS such as ARB, ACE inhibitors and renin inhibitors are widely prescribed for the treatment of hypertension, hypothyroidism is not known as their side effect. We believe that there are a lot of patients with hypothyroidism due to treatment with RAS inhibitors, but many of them are probably overlooked. This is simply because RAS-inhibitor-induced hypothyroidism is difficult to detect by at least two reasons: 1) The doctors are screening hypothyroidism by expecting an increase in TSH. However, in tertiary hypothyroidism caused by RAS inhibition TSH and TRH decrease. 2) Low body temperature is considered to be the most sensitive method to detect hypothyroidism, but it is difficult to detect unless the measurement is carried out after fasting as Dr. Broda Barness suggested (163). This is probably because fasting decreases leptin(164) resulting in decreased TRH expression, which probably makes it easier to detect the changes in body temperature. It is therefore important to measure body temperature as a first thing in the morning in the bed before any activity. The values taken at outpatient clinic may not be sensitive enough to detect low body temperature caused by hypothyroidism.

Patients with hypothyroidism reported to have various types of anemia including iron deficient anemia and macrocytic anemia. Our data show that inhibiting RAS causes normocytic, normochromic anemia without decreasing reticulocyte index, plasma iron and transferrin (**Fig. 3.6A-H; Table 3.2**). However, WT mice that were made profoundly hypothyroid by MMI and $NaClO_4$ have microcytic and hypochromic anemia, which was corrected by administration of T3 (**Fig. 3.7A-G; Table 3.2**). These data suggest that mild hypothyroidism caused by inhibiting RAS leads to normocytic normochromic anemia, but if thyroid hormones decrease further patients likely develop microcytic hypochromic anemia. Various kinds of anemia in patients with hypothyroidism could be due to the severity of hypothyroidism and to concomitant conditions other than hypothyroidism.

We used high doses of losartan and enalapril that decrease BP to the levels of the *Ren1c-/-* mice, which clearly manifested hypothyroidism and anemia. However, because it is not widely known that RAS inhibition causes anemia despite these drugs are widely used for the treatment of hypertension, anemia caused by RAS inhibition does not seem to be clinically relevant in the majority of hypertensive individuals who do not have other complications. However, if RAS is inhibited in patients with subclinical or overt hypothyroidism, hypothyroidism is likely exacerbated and the patients may develop anemia with relatively low doses of RAS. Although Ang II is known to increase EPO (147), the *Ren1c-/-* mice, and WT mice administered with losartan or enalapril all showed increased EPO probably in response to anemia because correcting anemia using T3 reduced EPO back to WT control levels (**Fig. 3.7F,G**). Administration of EPO increased Hct of WT mice but not in the *Ren1c-/-* mice, indicating the *Ren1c-/-* mice are

EPO resistant. Therefore, in patients with chronic kidney disease who have reduced capability of producing EPO and often have high BP, inhibiting RAS is expected to easily cause clinically relevant anemia. Because T3 corrects RAS-inhibition-induced anemia independently of Ang II, using low dose thyroid hormone to correct hypothyroidism as an adjunct therapy will be useful to reduce the dose of EPO for the treatment of anemia, to correct EPO resistance, to minimize the side effects of EPO, and to improve anemia and long-term prognosis and quality of life of patients with chronic renal failure. However, we should be cautious not to administer too much thyroid hormone to correct hypothyroidism caused by RAS inhibition, because hyperthyroidism increases EPO and possibly its side effects.

Chapter 4: Summary, Perspectives, and Concluding Remarks

SUMMARY AND CONCLUSION

An overactive RAS is suggested to worsen the metabolic syndrome, and an inhibition of RAS causes severe anemia. Our previous study has demonstrated that mice lacking renin (*Ren1c-/-)* are lean and insulin sensitive. To our surprise, the *Ren1c-/-* mice have low circulating total thyroid hormones, and their plasma catecholamine levels were similar to those of WT mice. Although Ang II is suggested to contribute the metabolic syndrome, the precise mechanisms are not completely understood. Obesity is often associated with leptin resistance, and improving leptin sensitivity is a potential therapeutic strategy to treat obesity. This dissertation has investigated whether inhibiting RAS improves leptin sensitivity, and whether and how inhibiting RAS causes hypothyroidism and anemia.

The main approach in Chapter 2 to investigate the effects of inhibiting RAS on leptin and insulin sensitivity is the generation and use of mice lacking both renin and leptin (*Ren1c-/-;ob/ob)*. Because lower plasma leptin levels of the *Ren1c-/-* mice can improve leptin sensitivity, this strategy allows me to test leptin sensitivity with the same basal levels of leptin. I also investigated the role of Ang II and Ang II-independent of renin effects on lean phenotype and insulin sensitivity of the *Ren1c-/-* mice by administering the *Ren1c-/-* mice with Ang II or human renin, and blockade of AT1R of WT mice by losartan. In Chapter 3, I investigated why the *Ren1c-/-* mice have low circulating total thyroid hormones and severe anemia. To achieve this goal, I used the *Ren1c-/-* mice, and WT mice administered with an AT1R blocker losartan, or an Ang converting enzyme inhibitor enalapril, as well as anti-thyroid drug, methimazole.

Leptin sensitivity of the *Ren1c-/-* mice

The *Ren1c-/-;ob/ob* mice had smaller body weight and fat mass than the *ob/ob* mice despite their fat mass/BW ratio was indistinguishable from that of the *ob/ob* mice. This phenotype is associated with increased fecal fat excretion and fatty acid oxidation. Compared to the *ob/ob* mice, the *Ren1c-/-;ob/ob* mice, and the *ob/ob* mice treated with losartan showed greater loss of body weight, and fat mass by administration of exogenous leptin due to suppression of food intake, which is consistent with changes in the levels of hypothalamic expression of POMC, NPY, and AgRP, and low expression of SOCS3 in the hypothalamus. Indeed, mice lacking SOCS3 in POMC expressing cells have increased leptin sensitivity and decreased BW and food intake due to elevated levels of hypothalamic POMC expression in the hypothalamus (79). Thus, inhibition of RAS improves leptin sensitivity and provides a novel target to treat obesity.

Interestingly, fecal fat excretion markedly increased by leptin infusion in the *Ren1c-/-;ob/ob* mice. This result is consistent with the finding that leptin directly inhibits both secretion and intracellular activity of pancreatic lipase(142). Because the *Ren1c-/-;ob/ob* mice had only 25% WT expression of pancreatic lipase and colipase, administration of leptin further decreased pancreatic lipase, leading to more fecal fat loss and smaller BW.

The *Ren1c-/-;ob/ob* mice had increased fatty acid oxidation. This is consistent with decreased RER in the indirect calorimetry, and increased expressions of PPAR-α, ACOX1, and CPT-1 in the liver. CPT-I is the rate limiting step in fatty acid oxidation and regulated by AMPK(165). AMPKα activity of the *Ren1c-/-;ob/ob* mice was markedly

increased in the liver. A number of studies have showed that AMPKα plays an important role in the regulation of fatty acid oxidation *via* CPT1 and improves non-alcoholic fatty liver disease (NFLD)(166). This is also consistent with reduced liver triglyceride content and ALT and AST levels of the *Ren1c-/-;ob/ob* mice. Increased plasma adiponectin of the *Ren1c-/-;ob/ob* mice likely contributes to these results because adiponectin increased fatty acid oxidation via AMPK(167), and because Ang II decreases adiponectin production in 3T3-L1 adipocytes and losartan inhibits it (Li et al., our unpublished observation). WT or the *ob/ob* mice treated with losartan had significantly increased plasma adiponectin concentrations. Accordingly, Ang II plays an important role in fatty acid oxidation.

Insulin sensitivity of the *Ren1c-/-* mice.

The *Ren1c-/-* mice and the *Ren1c-/-;ob/ob* mice have low plasma glucose concentrations and insulin levels by OGTT, have larger decrease in plasma glucose levels by ITT, and their gluconeogenesis was less by PTT compared with WT mice and *ob/ob* mice, respectively. Increased plasma adiponectin likely contributes to these results because adiponectin improves insulin sensitivity (143), and because WT or the *ob/ob* mice treated with losartan had significantly increased plasma adiponectin concentrations. They had improved glucose tolerance and insulin sensitivity, and had reduced gluconeogenesis similar to those of the *Ren1c-/-* mice and the *Ren1c-/-;ob/ob* mice, suggesting the contribution of the mechanism(s) independent of leptin. Interestingly, chronic leptin administration improved insulin sensitivity in the *ob/ob* mice, but not in the *Ren1c-/-;ob/ob* mice and the *ob/ob* mice treated with losartan. This may

be because chronic leptin administration increased plasma adiponectin levels in the *ob/ob* mice, but not in the *Ren1c-/-;ob/ob* mice and the *ob/ob* mice treated with losartan, simply because adiponectin production reached maximum levels by inhibiting RAS, and further stimulation by leptin does not increase adiponectin anymore. Accordingly, increased fatty acid oxidation and suppression of gluconeogenesis when RAS is inhibited is likely due to increased plasma adiponectin.

Hypothyroidism of the *Ren1c-/-* mice.

Mice lacking renin have low circulating thyroid hormones. In this dissertation I have shown that inhibiting Ang II-AT1R causes tertiary hypothyroidism by reducing the effect of Ang II to directly stimulate TRH via JAK2-STAT3 pathway. Both Ang II and leptin enhance JAK2-STAT3 signaling pathway, which in turn increases hypothalamic expression of TRH. Although the *Ren1c*-/- mice have low plasma leptin levels comparable to the *ob*+/- mice, the TRH levels in the hypothalamus of the *ob*+/- mice were indistinguishable from those of WT mice, indicating that the reduced leptin levels in the *Ren1c*-/- mice are unlikely the cause of decreasing hypothalamic TRH levels. Moreover, the *Ren1c*-/- mice and WT mice treated with losartan had increased Dio2 and T3 in the hypothalamus causing decreased hypothalamic expression of TRH, which results in hypothyroidism.

Anemia of the *Ren1c-/-* mice due to hypothyroidism

Anemia often occurs in hypothyroidism. I demonstrated that hypothyroidism caused by RAS inhibition causes anemia. Although Ang II and thyroid hormone have

shown to stimulate BFU-E and CFU-E, the precise mechanism is not known. In chapter 3 I have shown that inhibition of Ang II causes tertiary hypothyroidism. Interestingly, I also found that the *Ren1c-/-* mice have severe anemia. To investigate whether the *Ren1c-/-* mice cause impaired erythropoiesis by low circulating thyroid hormone, I administered low dose T3 (300ng/kg/day) or high dose T3 (5ug/kg/day) to the *Ren1c-/-* mice for two weeks. Anemia of the *Ren1c-/-* mice was corrected by both low and high doses of T3. Plasma thyroid hormone levels of the *Ren1c-/-* mice treated with low dose T3 were similar to those of WT mice. These results were replicated by losartan and enalapril. This result demonstrates that inhibition of AT1R activation causes anemia *via* hypothyroidism. To address the contribution of the direct Ang II effect on erythropoiesis, I administered Ang II to MMI-induced hypothyroid mice. Although their RBC number was indistinguishable from that of WT mice, Hgb and Hct were still lower than those of WT mice. In contrast, the *Ren1c-/-* mice treated with Ang II corrected anemia. Similar to thyroid hormone receptor TRα-/- mice, the *Ren1c-/-* mice have impaired erythropoiesis in the bone marrow. Taken together, thyroid hormone plays an important role in the erythropoiesis and provides a novel therapeutic strategy to treat anemia caused by RAS inhibition.

PERSPECTIVES AND CONCLUDING REMARKS

In this dissertation I have investigated the role of Ang II in leptin sensitivity, thyroid function, and erythropoiesis. Although Ang II plays an important role in the regulation of the metabolic syndrome and inhibition of RAS causes anemia, several points deserve further research.

In chapter 2, I have demonstrated that the *Ren1c-/-* mice have increased fatty acid oxidation and fecal fat excretion. Although increased fecal fat excretion is due to decreased expressions of pancreatic lipase and colipase, the role of Ang II in the regulation of pancreatic lipase and colipase levels is still not completely understood. Adiponectin likely plays an important role in fatty acid oxidation and insulin sensitivity of the *Ren1c-/-;ob/ob* mice. Increased fatty acid oxidation and suppression of gluconeogenesis of the *Ren1c-/-;ob/ob* mice is likely due to increased liver AMPKα activity, However, whether lean phenotype of the *Ren1c-/-* mice is due to increased adiponectin is still not understood.

In chapter 3, I have shown that the inhibition of RAS causes tertiary hypothyroidism. I believe there are a lot of patients with hypothyroidism due to treatment with RAS inhibitors. Many of them are probably overlooked. This is simply because detecting RAS-inhibitor-induced hypothyroidism is difficult to detect by at least two reasons: 1) many doctors are screening hypothyroidism by expecting an increase in TSH, which does not happen by RAS inhibition, because it causes tertiary hypothyroidism, and thus TSH becomes low instead of high. 2) Low body temperature is considered to be a most sensitive method to detect hypothyroidism, but it is difficult to detect unless the measurement is carried out after fasting as Dr. Broda Barness suggested (163). I first measured rectal temperature of mice without fasting, and was not able to detect significant difference between the *Ren1c-/-* mice and WT control mice. However, when I measured rectal temperatures of leptin deficient *Ren1c-/-;ob/ob* mice, I was able to easily detect lower rectal temperatures of the *Ren1c-/-;ob/ob* mice compared to those of the *ob/ob* mice without fasting. The reduction of rectal

temperature by cold exposure was bigger in the *Ren1c-/-;ob/ob* mice than in the *ob/ob* mice. Thus, it is important to measure body temperature in human individuals after fasting at rest in the morning.

I have also shown that hypothyroidism is the predominant cause of anemia induced by inhibiting RAS, and that without correcting hypothyroidism Ang II is not sufficient to correct anemia caused by RAS inhibition. Administration of EPO increased Hct of WT mice but not in the *Ren1c-/-* mice, indicating the *Ren1c-/-* mice are EPO resistant. Therefore, in patients with chronic kidney disease who have reduced capability of producing EPO and often have high BP, inhibiting RAS is expected to easily cause clinically relevant anemia. Because T3 corrects RAS-inhibition-induced anemia independently of Ang II, using low dose thyroid hormone to correct hypothyroidism as an adjunct therapy will be useful to reduce the dose of EPO for the treatment of anemia, to correct EPO resistance, to minimize the side effects of EPO, and to improve anemia and long-term prognosis and quality of life of patients with chronic renal failure.

Overall, in these studies, I have demonstrated that the lack or decrease in Ang II or its effects increase fatty acid oxidation and fecal fat excretion, and improve leptin and insulin sensitivity. My results provide scientific basis for inhibiting RAS as a treatment of diabetes and obesity. Although inhibition of RAS has great benefit in diabetes and obesity, it causes tertiary hypothyroidism and anemia. Thyroid hormone is a potential therapeutic strategy for anemia of patients with chronic renal failure, who are treated with inhibitors for RAS. However, caution should be paid to hypothyroidism when using high dose of inhibitors of RAS.

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