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Differential effects of angiotensin II type 2 receptor antagonism in mice models of obesity

Melissa Meredith Derfus
University of Tennessee

To the Graduate Council:

I am submitting herewith a thesis written by Melissa Meredith Derfus entitled "Differential effects of angiotensin II type 2 receptor antagonism in mice models of obesity." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Naima Moustaid-Moussa, Major Professor

We have read this thesis and recommend its acceptance:

Gary Truett, Jay Whelan

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

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

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DIFFERENTIAL EFFECTS OF ANGIOTENSIN II TYPE 2 RECEPTOR ANTAGONISM IN MICE MODELS OF OBESITY

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Melissa Meredith Derfus

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DEDICATION

This thesis is dedicated to my parents, Daniel J. and Marcy H. Derfus,

who taught me the importance of education.

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ABSTRACT

Angiotensin II (ang II) is a vasoactive hormone derived from the renin angiotensin system (RAS), which regulates blood pressure and fluid balance in the body. Ang II effects are mediated via two major receptors: type 1 (AT_1) and type 2 (AT_2). Adipocytes contain a local RAS in which ang II upregulates adipogenesis, fatty acid and triglyceride synthesis primarily mediated via the AT_2 receptor in cultured adipocytes. Preliminary studies from our lab tested the importance of AT_2 receptors in vivo and reported a decrease in adiposity by AT_2 antagonism in the lean, but not the genetically obese *db/db* mouse. To further explore these effects, we used another genetic model of obesity (*ob/ob*) and diet-induced obese (DIO) mice and treated them for 2-3 weeks with the AT_2 receptor antagonist, PD 123,319. Body weight, fat pad weight and plasma glucose, leptin and insulin levels and fatty acid synthase (FAS) and glycerol-3-phosphate dehydrogenase (GPDH) activity were measured. Consistent with previous findings in lean mice, the AT_2 antagonist decreased abdominal fat pad weight in *ob/ob* mice and accelerated weight loss in DIO mice. Also, correlated with these effects, AT_2 blockade decreased FAS activity in *ob/ob* mice and lowered blood glucose levels in DIO mice. No significant changes were seen in the other parameters that were measured. In combination with recently published data, this research further supports the role of the AT_2 receptor in modulating ang II effects on adipocyte metabolism. Defining this role is crucial in determining and preventing the contribution of adipocyte-derived RAS to systemic disorders such as obesity-related hypertension.

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LIST OF ABREVIATIONS

Ang	Angiotensin
AT_1	Angiotensin II type 1 receptor
AT_2	Angiotensin II type 2 receptor
ACE	Angiotensin Converting Enzyme
Agt	Angiotensinogen
BMI	Body mass index
BSA	Bovine serum albumin
DIO1	Diet-induced obesity, study #1
DIO2	Diet-induced obesity, study #2
FAS	Fatty acid synthase
FBS	Fetal bovine serum
GPDH	Glycerol-3-phosphate dehydrogenase
GTP	Guanosine triphosphate
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
PBS	Phosphate buffered saline
PD	PD 123,319, AT_2 antagonist
PGI_2	Prostacyclin
RAS	Renin angiotensin system
RNA	Ribonucleic acid

CHAPTER I

INTRODUCTION

Sixty-one percent of the adult population in the United States is overweight or obese (1). This growing number has major public health implications. There are many chronic health conditions associated with obesity, such as type 2 diabetes, cardiovascular disease and hypertension (2,3). The increasing prevalence of these and other obesity-related health risks emphasizes the need for understanding the pathogenesis of obesity. This will allow us to better prevent and treat obesity as a condition and prevent these associated diseases.

Obesity is a disease characterized by excessive accumulation of adipose tissue in the body (4). While adipose tissue was once thought of as only a storage depot for triglycerides, it is now known to also function as a paracrine and endocrine organ (5). The paracrine function of adipocytes includes regulation of adipose tissue metabolism and gene expression (6). The endocrine function of adipose tissue includes systemic effects on blood pressure and kidney homeostasis.

The renin-angiotensin system (RAS) is the major regulator of blood pressure, fluid and electrolyte balance (7). A local RAS has been identified in adipose tissue (8). The purpose of this study is to determine how the end product of RAS, angiotensin II, functions in adipocytes by identifying the receptors mediating its effects in vivo.

CHAPTER II

LITERATURE REVIEW

Obesity and Hypertension

Obesity

The combined prevalence of overweight and obesity among men and women between the ages of 20 and 80 is 61 percent (1,9). Not only is the majority of our adults overweight or obese, but the number of children and adolescents with obesity and type II diabetes is also increasing (1).

There are several ways to estimate body fat. One of the easiest, most affordable and least invasive ways is body mass index (BMI). The National Institutes of Health recommend physicians use BMI when assessing overweight and obesity (Table 1) (2). BMI is defined as weight (in kg) divided by height in meters squared (in m^2).

Table 1. Classification of Overweight and Obesity by BMI.

	Obesity Class	**BMI (kg/m^2)**
Underweight		< 18.5
Normal		18.5 - 24.9
Overweight		25.0 - 29.9
Obesity	I	30.0 - 34.9
	II	35.0 - 39.9
Extreme Obesity	III	≥ 40

Health professionals agree that an increase in body weight of twenty percent or more is considered a health hazard (10). Health risks of overweight and obesity include (1-3):

- Hypertension
- Type 2 diabetes
- Coronary heart disease
- Stroke
- Dyslipidemia
- Gallbladder disease
- Osteoarthritis
- Sleep apnea and respiratory problems
- Certain types of cancers

Hypertension, hypercholesterolemia (blood cholesterol over 250 mg/dL) and diabetes (blood glucose over 126 mg/dL) are more than twice as prevalent in overweight vs. non-overweight individuals (10).

Obesity is a complex and multi-factorial disorder. Genetics, lifestyle and endocrine disorders all contribute to the epidemic of obesity (1,4,11). The importance of heredity was shown in a study by Stunkard et al. (12) that found the BMI of adopted children strongly correlated with the BMIs of their biological family members and did not correlate with the BMIs of their adoptive family. Studies such as this one and others by Bouchard et al. (13,14) demonstrate the importance of genetics in the pathogenesis

and etiology of obesity. Researchers are presently working to link the obese phenotype to the responsible genes.

Lifestyle is a major determinant of obesity, the two main factors being food intake and physical activity (15). Western lifestyle has changed the energy balance for many Americans by increasing caloric intake and decreasing physical activity. In many cases these environmental factors will exacerbate an existing genetic disposition towards obesity (2).

Obesity due to genetic defects is very rare, but genetic syndromes such as Prader-Willi, Cohen, Borjeson-Forssman-Lehmann that have been identified to cause obesity as well as other endocrine dysfunctions (16,17). Prader-Willi syndrome, for example, is caused by the deletion of multiple genes in close proximity on chromosome fifteen (18,19). Among other symptoms, a hypothalamic dysfunction causes extreme hyperphagia (19). This disordered eating is compounded by decreased energy utilization, making these individuals morbidly obese (20).

While obesity mutations are rare in humans, many animal models of obesity have been developed based on single gene mutations. Table 2 gives a summary of some major obesity genes and their effects (11,21-23).

Two mouse mutations commonly used in research are the *db/db* and *ob/ob*. The *db/db* mouse has a mutation in the gene coding for the leptin receptor while the *ob/ob* mouse is leptin-deficient (21). Both cause similar phenotypes in mice (23). These animals have severe juvenile-onset obesity and exhibit reduced basal metabolic rate,

Table 2. Genetic mutations resulting in obesity.

Mutation	Gene product	Rodent chromosome	Human chromosome	effects of mutation
agouti yellow (A^y)	agouti signaling protein	2	20	ubiquitous over-expression of agouti
obese (*ob*)	leptin	6	7	deficiency of leptin
diabetes (*db*)	leptin receptor	4	1	defect in leptin signal
fat (*fat*)	carboxypeptidase E	8	4	processing of prehormone
tubby (*tub*)	insulin signaling protein	7	11	undefined

hyperphagia, hyperinsulinemia, insulin resistance, glucose intolerance and diabetes (11,22,23).

Hypertension

Hypertension is defined as high blood pressure that remains elevated over time (Table 3) (24,25). It is caused by narrowing of the arteries, greater than normal amount of blood or the heart beating faster and more forcefully than normal (24). Hypertension increases stress on the heart and contributes to atheriosclerosis and increases the risk of heart disease and stroke.

Approximately 50 million adult Americans have high blood pressure and it is more common in African Americans and in the elderly (> 60 years of age) (24).

Table 3. Classification of Blood Pressure for Adults (≥18 years of age).

	Systolic and/or Diastolic (mm Hg)
Optimal	<120 and <80
Normal	<130 and <85
High-normal	130-139 or 85-89
Hypertension	
Stage 1	140-159 or 90-99
Stage 2	160-179 or 100-109
Stage 3	≥180 or ≥110

Individuals who have a high-normal blood pressure are also at risk for hypertension (26). Others at risk for hypertension are people who are overweight or have a family history of high blood pressure (24,25).

Hypertension rates are correlated among family members (27), however the disease is also multi-factorial. It appears to be polygenic and influenced by the environment (28).

Health risks associated with hypertension include heart disease, stroke, congestive heart failure, kidney disease and blindness (24,25). Hypertension can be prevented or controlled by the following lifestyle modifications (24,25):

- Maintaining a healthy weight
- Following a healthy eating pattern
- Being physically active
- Reducing salt and sodium in the diet

- Maintaining adequate intakes of potassium, calcium and magnesium
- Limiting alcohol intake
- Quitting smoking

Another option for controlling hypertension is pharmacological interventions, which will be discussed in more detail in later sections.

Obesity-related hypertension

Hypertension is the most common co-morbidity associated with obesity (29). Thirty-six percent of overweight (BMI >25) individuals are hypertensive and over sixty percent of obese (BMI >35) individuals are hypertensive (30).

Researchers have found a close correlation between increased BMI and increased rates of hypertension (2,25,31). The prevalence of hypertension in overweight individuals is 2.9 times higher than in non-overweight individuals (10). When an individual is diagnosed with hypertension, one of the first recommendations is maintaining a healthy weight, or weight reduction if the individual is overweight (24,25,32,33).

Adipose tissue physiology

Adipose tissue is a specialized form of loose connective tissue containing undifferentiated fibroblasts (preadipocytes), adipocytes, vascular and endothelial cells (5,34). Fat is stored in the form of triglycerides in the adipocytes. Adipose tissue pads

and protects organs, insulates the body and stores energy in the form of triglycerides for use in times of metabolic need (6,10,35). Adipose tissue mass can be increased in two ways, either by hypertrophy, expanding the size of the adipocyte by storing more triglycerides, or by hyperplasia, increasing the number of adipocytes (36).

Adipose tissue plays a more complex role in whole body homeostasis than just energy regulation. Recent research shows that adipose tissue is not only a storage site for excess energy, but also an endocrine, paracrine/autocrine organ (6,35).

Distribution of fat depots is also an important indicator of disease risk. Different depots of tissue vary in their level of metabolic activity and metabolic risks (4). Upper-body obesity, known as android obesity, is associated with increased rates of heart disease, hypertension and type 2 diabetes (4). Excess abdominal fat has a stronger correlation with coronary heart disease than does thigh or gluteal fat pads (36). Individuals with lower-body obesity, gynoid obesity, usually have a lower health risks, however it is often harder to lose this weight (4). These differences could be due to the composition, amount of blood flow and the metabolic role of each tissue depot, as well as differences in response to nutritional and hormonal stimuli (4).

Renin-Angiotensin System (RAS)

Systemic RAS

The Renin-Angiotensin System (RAS) is comprised of two enzymatic reactions that convert angiotensinogen (agt) into angiotensin II (ang II) (Figure 1) (7). Agt is the only precursor for ang II. RAS is one of the main regulators of blood pressure, plasma

volume, sympathetic nervous activity and thirst responses (37). The end product, ang II is a vasoactive hormone that is able to act systemically as well as locally.

Agt is a glycoprotein synthesized by the liver. The liver only stores small quantities; therefore it is made and released as needed. Renin is a protease synthesized and stored by the juxtaglomerular apparatus in the afferent arterole of the kidney. Renin cleaves agt into angiotensin I (ang I), an inactive intermediate in the pathway. Angiotensin Converting Enzyme (ACE) is a dipeptidyl peptidase that cleaves the carboxyl-terminal end of ang I and forms the active octapeptide, ang II. There are several other enzymes that are able to convert agt to ang II (tissue plasminogen activator, cathepsin G, tonin) and ang I to ang II (chymostatin-sensitive angiotensin II-generating enzyme, cathepsin G, chymase) (38). The clinical significance of these alternate pathways is unknown.

Decreased blood pressure and sodium concentration stimulates renin release and increases ang II production (7). Ang II effects are mediated by specific receptors throughout the body and uses different signaling mechanisms (39).

RAS-related hypertension

The association between obesity and hypertension has been established for a long time, but the mechanisms behind this relationship are not completely understood (40). The RAS pathway is a major determinant in hypertension.

Ang II increases sodium concentration, blood volume and blood pressure. This is accomplished by the actions of different signaling mechanisms (Figure 1) and at several sites in the body.

In the kidney, ang II contracts glomular mesangial cells and smooth muscle in the efferent arterioles of the kidney to decrease filtration (41). In the pituitary, ang II increases the secretion of vasopressin, which is a vasoconstrictor and anti-diuretic (41). In the brain, ang II increases salt appetite and thirst (41). Ang II increases heart rate, constricting blood vessels and smooth muscle cells (41). Ang II acts on the glomerulosa cells of the adrenal cortex that release aldosterone and signal the reabsorption of sodium and water in the kidneys (41). The net effect of all of these actions is increased blood pressure.

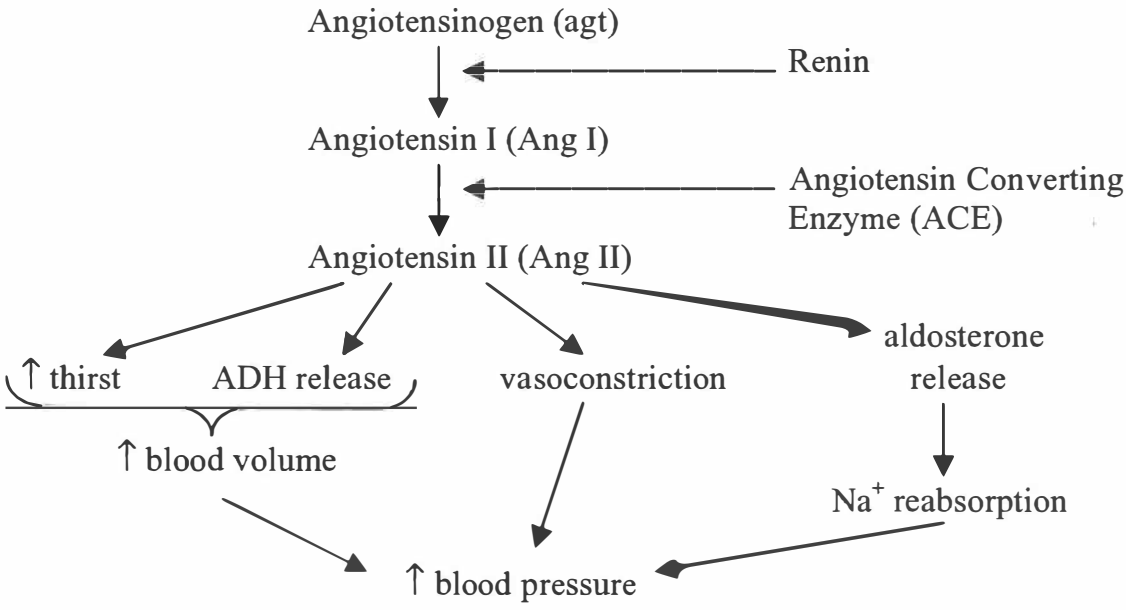


Figure 1. RAS and its biological effects.

Two of the commonly prescribed blood pressure medications function by blocking the RAS. One inhibits ACE and blocks the conversion of ang I to ang II. The other blocks an ang II receptor, preventing ang II from acting on target cells. Treatment with both ACE inhibitors (such as captopril) and AT_1 receptor antagonists (such as losartan) have been clinically shown to significantly reduce blood pressure (42-46).

Ang II Receptors

Ang II acts through two main receptors: angiotensin II type 1 (AT_1) and type 2 (AT_2) receptors. Although several others have been reported, they are less studied (39,47). Both receptors are G-protein coupled and have seven hydrophobic transmembrane segments (37). The receptors have unique signaling and physiological functions as well as different sites of expression (37,48). There is a 34 percent homology between the amino acid sequences of the two receptors (49,50). The receptors are able to be distinguished from each other according to their inhibition by specific receptor antagonists (Figure 2). AT_1 receptors are selectively blocked by biphenylimidazoles such as losartan, and AT_2 receptors are blocked by tetrahydroimidazopyridines such as PD 123,319 (PD), also called P-186 (51-54).

The AT_1 receptor is expressed in somatic and brain tissues, and located primarily in the adrenal glands, vascular smooth muscle cells, kidney and heart (52). The biological actions of ang II discussed earlier such as vasoconstriction, aldosterone secretion, osmoregulation and sodium balance are mediated though the AT_1 receptor (37,47,52,54,55).

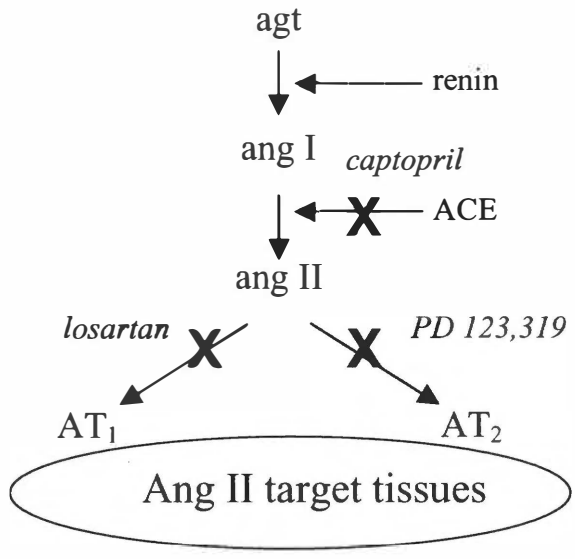


Figure 2. Antagonism of RAS.

The AT_1 receptor has two isoforms in the rat, AT_{1A} and AT_{1B}, which share 94% homology (56,57). The AT_{1A} isoform is found predominantly in the kidney, liver, lung and vascular smooth muscle, whereas the AT_{1B} isoform is found predominantly in adrenal and anterior pituitary glands (54). Through selective receptor inactivation in knockout mice, researchers have determined that the AT_{1A} receptor is responsible for the systemic blood pressure effects of ang II (58-60).

Ang II binding at AT_1 receptors in blood vessels causes vasoconstriction and increases blood pressure (61,62). In addition, the AT1 receptor mediates the growth and proliferation of cardiac myocytes, causing cardiac hypertrophy independent of blood pressure (54). All of these effects contribute to the pathology of hypertension.

The AT_1 receptor has 5 different signaling mechanisms: activation of phospholipase A_2 (PLA_2), phospholipase C (PLC), phospholipase D (PLD), L-type calcium (Ca^{2+}) channels and inhibition of adenylate cyclase (AC) (reviewed in (54)). Activation of PLC results in the formation of inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2). IP_3 increases intracellular Ca^{2+} and DAG activates protein kinase C (PKC), with the final effect of both being vasoconstriction. The inhibition of AC also causes vasoconstriction by decreasing levels of adenosine 3',5'-monophosphate (cAMP), a vasodilator. Opening Ca^{2+} channels also signals vasoconstriction and aldosterone production and release. The activation of PLA_2 releases arachidonic acid, which produces prostaglandins.

Until recently there was little known about the AT_2 receptor. It was originally distinguished from the AT_1 subtype by its resistance to sulfhydryl-reducing agents and GTP and the inhibition of T-type (cytosolic) Ca^{2+} channel currents (63-65). Unlike AT_1, which is associated with many tissues, the AT_2 receptor is predominantly found in fetal tissues, developing brain, adrenal glands and ovarian follicles (49,64,66,67). The expression of the AT_2 receptor in these tissues suggests a role in growth and development. The AT_2 receptor is expressed almost exclusively in fetal tissues, but then declines dramatically after birth (68,69). Expression of the AT_2 receptor is upregulated during times of injury, such as wound healing and vascular remodeling (52).

In general, the cardiovascular effects of the AT_2 receptor seem to be functionally opposite to those of AT_1 (70). For example, AT_2 receptor knockout mice exhibit increased blood pressure. A possible explanation is that the AT_2 receptor regulates

Table 4. Summary of the main functions of AT_1 and AT_2 receptors.

AT_1	AT_2
somatic and brain tissues	fetal tissue development
fluid-electrolyte balance	cell proliferation
blood pressure regulation	cell differentiaion and development
vasoconstriction	angiogenesis
aldosterone synthesis and secretion	wound healing
renal sodium reabsorption	tissue regeneration
vasopressin secretion	apoptosis
cardiac hypertrophy and contractility	vasodilation?

vasodilation (71,72). However, when the AT_1 receptor is blocked, the AT_2 receptor is able to regulate vasoconstriction (37). Table 4 is a summary of the main functions of the AT_1 and AT_2 receptors (37,52,54,73,74)

The AT_2 receptor is G-protein coupled, however it does not appear to function by the typical signaling pathway (39). Ang II binding at the AT_2 receptor inhibits guanylate cyclase (GC) activity (75). This decrease in GC causes an AT_2 –mediated decrease in cGMP levels (65).

Adipose Tissue RAS

In addition to the classical system described earlier, there are several peripheral tissues that contain their own local RAS (76). Adipose tissue is one of these tissues, having all of the components necessary to produce ang II locally (77,78). The products of adipose tissue RAS not only act directly on adipose tissue, but also are able to contribute

systemically to blood pressure regulation and kidney filtration (78,79). This suggests that this adipose tissue RAS plays an important role in obesity and obesity-related hypertension.

Regulation of RAS

As the precursor to the RAS pathway, availability of agt is a major regulator of the amount of ang II that is produced. Agt is produced in adipose tissue (77,78,80) and contributes to circulating agt levels (81). Proportionally elevated levels of agt are seen with increased BMI (82,83), implying that agt production is directly related to amount of adipose tissue. Adipose tissue is able to indirectly regulate agt circulating levels in part through estrogen (81). Adipocytes produce estrogen which is able to bind hepatic receptors to increase agt expression (81).

Agt mRNA is nutritionally regulated in adipose tissue. Both agt mRNA and protein are decreased during fasting and increased with refeeding (80). Ang II, fatty acids, adrenal glucocorticoids and insulin increase adipocyte-derived agt (7,84-86) Agt mRNA is expressed in a differentiation-dependent manner (87). Stimulators of adipocyte differentiation as well as lipogenic factors such as isobutylmethylxanthine, dexamethasone, ethynylestradiol and T3 also increase agt mRNA levels (87,88). Conflicting results have been reported regarding the effects of obesity on agt expression (89). Expression of agt mRNA is decreased in obese adult A^{vy} and fatty rats, but increased in *ob/ob* and *db/db* mice and in young fatty rats (80,86,90). However, further

studies are needed to determine if these differences are possibly due to the developmental stage, mutation or sex (89,91).

Effects of RAS

Endocrine effects

Adipocyte agt contributes to circulating levels of agt (79,80,92) and affects systemic RAS functions. Massiera et al. reported increased fat mass and high blood pressure in transgenic animals that over-express agt in adipose tissue (79). While knockout mice exhibit low blood pressure, decreased adiposity and renal abnormalities, re-expressing agt in these mice normalized fat mass as well as restored blood pressure and renal functions of these animals (79). Recent studies from our lab confirmed these changes in adiposity in the knockout and transgenic versus the control mice (93). This is a prime example of the endocrine capacity of adipose tissue, which also establishes a direct relationship between adipose tissue mass and hypertensive effects; hence, obesity-related hypertension.

Paracrine effects

In adipose tissue, ang II stimulates adipocyte growth and differentiation (94,95). By using receptor antagonists, studies from our lab and others identified the AT_2 receptor as the predominant receptor expressed in adipose tissue (94,96). Furthermore, the effects of ang II on adipocyte lipogenesis were shown to be mediated by the AT_2 receptor (96).

Adipose tissue hyperplasia is regulated by ang II in a paracrine manner: ang II stimulates the release of prostacyclin (PGI_2) by mature adipocytes (94,97). The PGI_2 produced stimulates the differentiation of preadipocytes into adipocytes in ob1771 cells (Figure 3) (94). Administering the AT_2 receptor antagonist blocked both the production and lipogenic effects of PGI_2 demonstrating that both of these actions are mediated by the AT_2 receptor (94).

Hypertrophic growth of the adipocyte is also regulated via the AT_2 receptor. The activities of both fatty acid synthase (FAS) and glycerol-3-phosphate dehydrogenase (GPDH) are increased when ang II binds at the AT_2 receptor (96). The net effect of increasing these enzyme activities is triglyceride synthesis (Figure 3). Inhibition of the AT_2 receptor by PD prevents these effects (96).

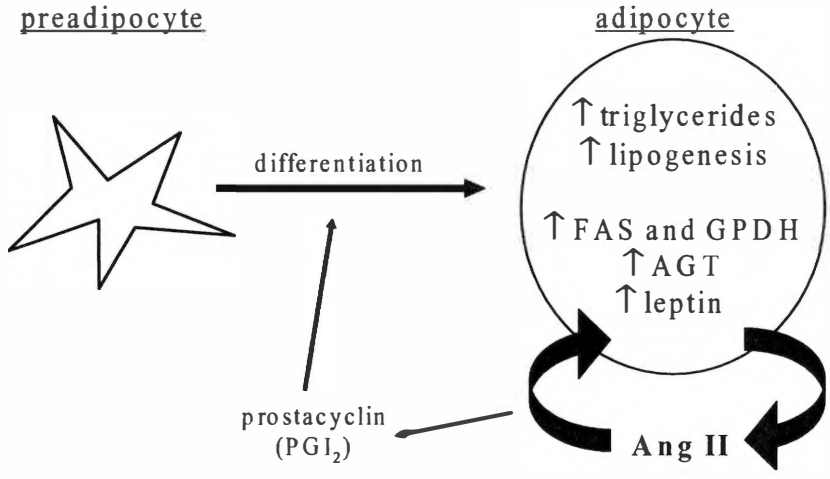


Figure 3. Summary of ang II effects in adipose tissue via the AT_2 receptor.

In tissues that express both AT_1 and AT_2 receptors, the AT_2 receptor has the ability to function as an antagonist by binding directly to the AT_1 receptor and inhibiting its activation (98).

Unfortunately the data on ang II receptors in adipose tissue is still sparse and sometimes controversial. This is possibly due to variance in receptor expression and activity depending on species, developmental stage and nutritional and hormonal states. Table 5 is an overview of results thus far.

In summary, adipose tissue produces ang II as well as other lipogenic factors, which in turn increase the amount of adipose tissue through both hypertrophy and hyperplasia. This is a vicious circle that ultimately leads to high blood pressure (Figure 4). Adipose tissue affects blood pressure via adipose tissue derived agt (AT_1 signaling) that enters the bloodstream (79) and also via the paracrine effects of ang II (AT_2 signaling).

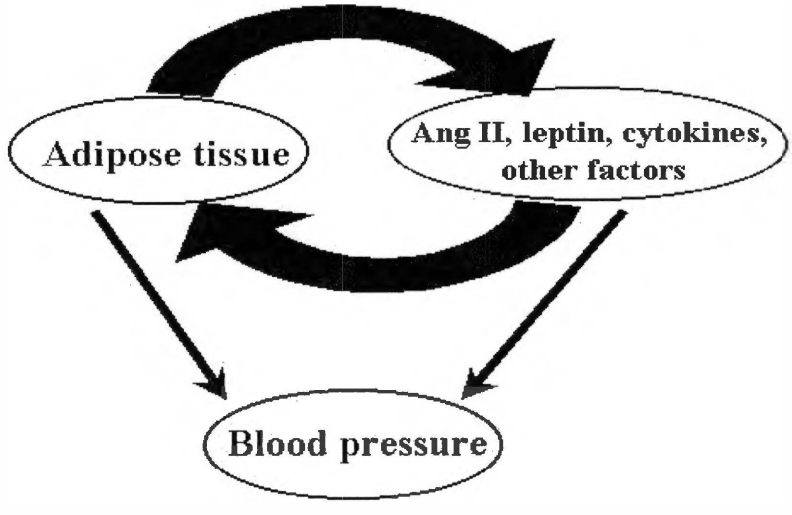


Figure 4. Summary of endocrine and paracrine effects of adipose tissue on blood pressure.

Table 5. Current research on ang II receptor expression in adipose tissue.

receptor expressed	animal species	location of receptor	type of adipose tissue	preadipocyte or adipocyte	function	reference
AT1	3T3-L1 cell line			preadipocyte & adipocyte	?	(99)
	rat	adipocyte membrane	epididymal	adipocyte	?	(100)
		adipocyte membrane	epididymal, mesenteric, retroperitonal	adipocyte	?	(101)
		adipocyte membrane	brown and white adipose tissue	adipocyte	?	(102)
				adipocyte	regulate adipocyte size	(103)
	rat (obese)	adipocyte membrane	epididymal	adipocyte	age-dependent hypertrophy	(104) *
	human	whole adipose tissue and cultured adipocytes	subcutaneous, omental and mammary	adipocyte	?	(105)
	human (obese)	whole adipose tissue	subcutaneous upper abdominal	preadipocyte	increase in cyclin D1	(95)
		adipocyte membrane	omental or subcutaneous	adipocyte	?	(101)
AT2	3T3-L1 cell line			adipocyte	up-regulation of FAS and GPDH	(96)
				preadipocyte	?	(99)
	Ob1771 cell line			preadipocyte	differentiation	(94)
				adipocyte	increase in PGI2 production	(94)
	human		primary culture	adipocyte	?	(96)†

* This study showed a decrease in amount of AT_1 receptor mRNA; however there were increased levels of AT_1 receptor protein. † Also unpublished data from our lab.

CHAPTER III

EXPERIMENTAL SECTION:

DIFFERENTIAL EFFECTS OF ANGIOTENSIN II TYPE 2 RECEPTOR ANTAGONISM ON ADIPOSITY IN MICE MODELS OF OBESITY

Introduction

With the discovery of a local RAS in adipose tissue, the importance of understanding ang II effects becomes crucial in researching obesity-related hypertension and other disorders. It is now well established that ang II increases adipocyte differentiation (94) and lipogenic enzymes (96) in adipocytes and that this occurs via the AT_2 receptor. However, these findings have all been performed using in vitro studies. The purpose of this research is to test these effects in vivo.

Preliminary studies from our lab explored the effects of ang II receptor antagonists in lean, normal C57BL/6J mice (106). The animals lost weight and had smaller fat pad weight and lower leptin levels when treated with AT_2 antagonist PD vs. vehicle. This experiment was repeated in a *db/db* obese mouse, to test the weight reducing effect of PD in a genetically obese mice (106,107). Surprisingly, AT_2 antagonism in these animals increased body weight and leptin levels. Both of these studies were confirmed by primary culture of adipocytes from these mice where treatment with the AT_2 antagonist in adipocytes from lean mice reduced leptin secretion

compared to vehicle and ang II treatment (106,107). Treatment with the AT_2 antagonist did not have an effect in the *db/db* mice (106,107). The *db/db* mice have severe diabetes, kidney disease and obesity, which may have played a role in these unexpected results. In addition, the *db/db* mouse is on a different genetic background than C57BL/6J, which may be an additional confounding variable.

Research Objectives

Given the above-described studies from our lab, and the conflicting effect of PD in lean vs. obese *db/db* mice, the goal of this thesis project was to further investigate the effects of AT_2 receptor antagonist in other mouse models of obesity. Specifically, we propose the following aims:

1. Determine the effects of the ang II receptor antagonist in another genetic model of obesity, the leptin deficient *ob/ob* mice. The antagonist was administered in vivo to these mice or used to treat primary adipocytes isolated from these animals. Due to limited availability of *ob/ob* mice, PD was the only drug used in this study.

2. Determine the effects of ang II antagonism in a non-genetic model of obesity, high-fat diet induced obesity (DIO). This model more closely mimics human obesity. After obesity is induced, mice are treated with the antagonist and switched to a chow diet (DIO1) or kept on a high-fat diet (DIO2). In addition, adipocytes from these obese

mice were cultured in vitro to investigate the direct effect of the antagonist. In addition to the AT_2 antagonist, the AT_1 antagonist, losartan, and ACE inhibitor, captopril were used in all DIO experiments as additional means to block the action or production of ang II.

Experimental Design and Approach

Animals

ob/ob

Twenty-four 6-week-old male *ob/ob* mice (C57BL/6J background) were purchased from Jackson Labs (Bar Harbor, ME). The animals were maintained on a chow diet throughout the experiment. After a one-week acclimation period, mice were divided into two groups, control (n=12) and treatment (n=12) group. The control group was injected with phosphate buffered saline (PBS). The treatment group was injected with PD 123,319 ditrifluoroacetate (PD) (Sigma, St. Louis, MO) (15 mg/kg body weight) dissolved in PBS. The animals were given subcutaneous injections daily for 21 days and were sacrificed on day 22.

DIO1: Treatment of chow-fed mice

Twenty-nine 6-week-old male C57BL/6J mice were purchased from Jackson Labs (Bar Harbor, ME). The animals were fed a high fat diet (45 kcal% fat) (D12451,

Research Diets, New Brunswick, NJ) for 10 weeks to induce obesity (See appendix A-2 for diet composition). At the start of injections, the animals were switched to chow diet.

The animals were divided into four groups: control (n=7), AT_1 antagonist (n=7), AT_2 antagonist (n=8) and ACE inhibitor (n=7). The control group was injected with PBS. The AT_1 antagonist group was injected with losartan (Merck, Whitehouse Station, NJ) (15 mg/kg body weight). The AT_2 antagonist group was injected with PD (15 mg/kg body weight). The ACE inhibitor group was injected with captopril (Sigma, St. Louis, MO) (0.625 mg/kg body weight). All drugs were dissolved in PBS. The animals were given subcutaneous injections daily for 19 days and were sacrificed on day 20.

DIO2: Treatment of high-fat-fed mice

Forty 6-week-old C57BL/6J mice were purchased from Jackson Labs (Bar Harbor, ME). The animals were fed a high fat diet (45 kcal% fat) (D12451, Research Diets, New Brunswick, NJ) for 12 weeks to induce obesity (See appendix A-2 for diet composition). The animals remained on the high-fat diet throughout the course of the experiment.

The animals were divided into four groups: control (n=10), AT_1 antagonist (n=10), AT_2 antagonist (n=10) and ACE inhibitor (n=10). The dosage of losartan for the AT_1 antagonist group was increased to 30 mg/kg body weight. The dosage of PD for the AT_2 antagonist group was also increased to 30 mg/kg body weight.

The dosage of captopril for the ACE inhibitor group was increased to 1.25 mg/kg body weight. The drugs were dissolved in PBS and were given daily by subcutaneous injection.

Due to the large number of animals, half the animals in each group were sacrificed after 21 days of treatment and the other half after 22 days. The animals sacrificed on day 21 were injected for 20 days and the animals sacrificed on day 22 were injected for 21 days.

An overview of the experimental design can be found in Figure 5.

A dose response in lean and DIO mice is ideal to determine the most effective dose of PD. The dose chosen here took into the account the cost and availability of PD, and the previous findings from our lab and others (106-110).

Based on published studies, the doses of both losartan and PD used in rodents range from 5 to 50 mg/kg body weight (108-110). The dosages were chosen to be within this range and were based on previous findings from our lab using lean mice where 15 mg/kg body weight was sufficient to notice significant decrease in body weight and leptin (106,107).

The prescribed dose of captopril for humans is 25 to 50 mg/day (111). Based on a 70 kg person, this is 0.357 to 0.714 mg/kg body weight. The lower dosage of captopril used in the DIO1 study (0.625 mg/kg body weight) is within this range and comparable to what is prescribed. The DIO2 dosage (1.25 mg/kg body weight) is above the normal range prescribed.

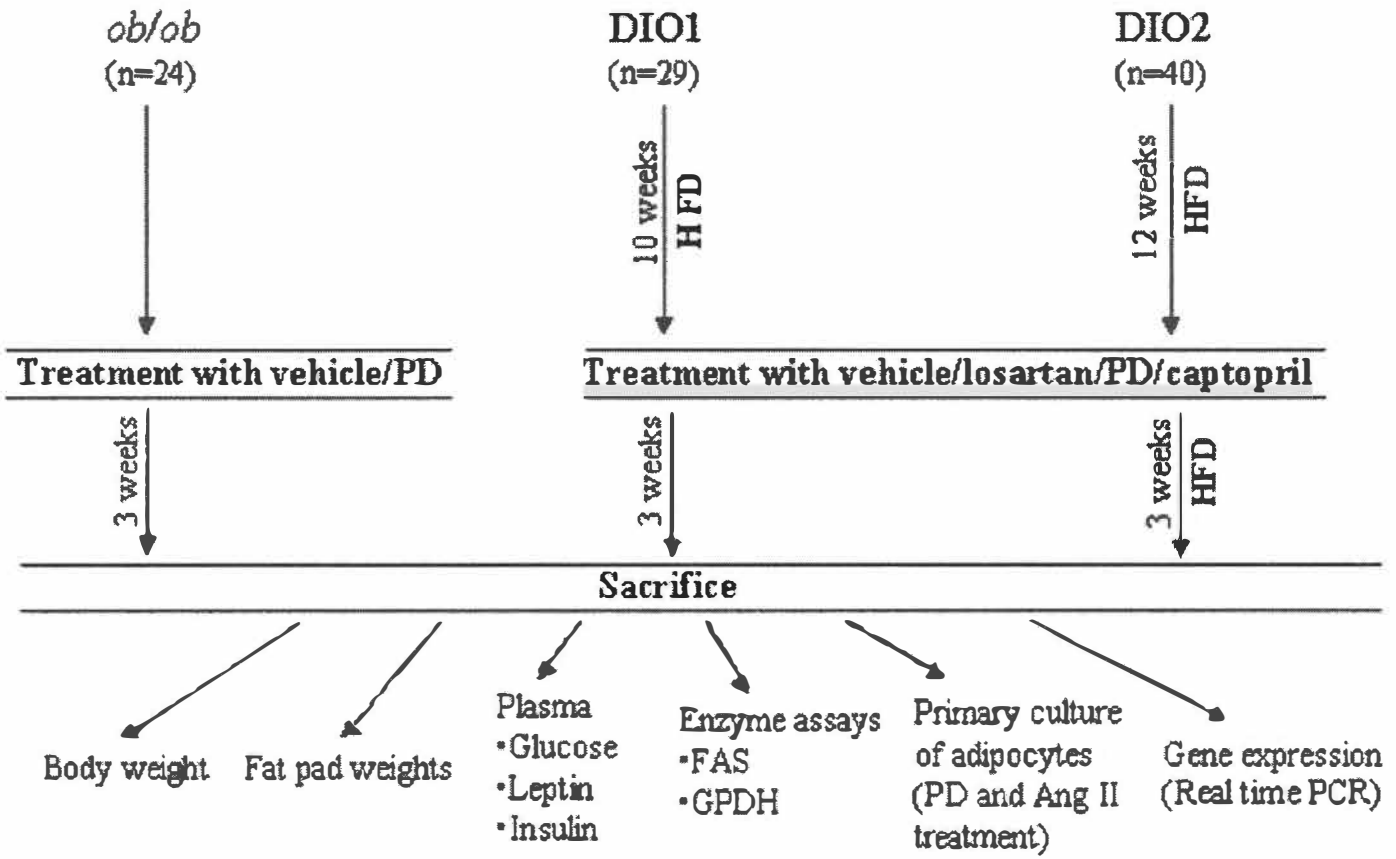


Figure 5. Overview of experimental design of animals studies. The animals were fed chow diet unless otherwise specified. DIO, diet-induced obesity; HFD, high fat diet

In all of the above studies the animals were given free access to food and water. The environment was temperature controlled and on a 12 hour light/dark cycle. The animals were randomized into groups such that the average weight in each group was comparable. The animals were weighed twice a week and the amounts injected were recalculated accordingly. The food was weighed daily. In cases when there were two or more animals per cage, the food weight was averaged for each animal. Unless specified, the animals were maintained on chow diet (8640, Harlan, Madison, WI) (See appendix A-1 for diet composition).

All animals were fasted for 10 hours prior to sacrifice. On the day of sacrifice, the animals were exsanguinated while under isofluorine anesthesia. Blood was collected by cardiac puncture. The following tissues were harvested: liver, kidney, epididymal fat pad, abdominal fat (perirenal and mesenteric fat pads), heart and brain. The liver and adipose tissue samples were weighed at the time of collection. All tissues were snap frozen in liquid nitrogen for further analysis.

Primary culture of adipocytes

DIO mice were sacrificed by the aforementioned procedure. Primary culture of adipocytes was performed as previously described (112). The adipose tissue was removed and washed twice with Hanks. The tissue was cut into small pieces and divided into culture dishes (100 mm or 35mm/6 well plates). The tissue was incubated with DMEM media supplemented with 1% BSA, 0.1% FBS, 1% penicillin/streptomycin and 0.5% gentamicin for 48 hours. The media was removed after 2 days and replaced with

new media containing the following treatments: PBS, ang II (10nM), PD (100μM) and both ang II and PD. After two days of incubation, the media was removed and frozen. When applicable, fresh media along with the treatment was added and incubated another day before both tissue and media were collected. The doses of ang II and PD used were submaximal and were based on previous dose-response studies in our lab.

Glucose

Fasting blood glucose readings were taken at the time of sacrifice. The whole blood was measured using a ONE TOUCH Profile glucose meter (LifeScan, Milpitas, CA).

Leptin and Insulin Radioimmunoassay

Leptin and insulin concentrations were determined by radioimmunoassay (RIA). The leptin was measured using a mouse leptin RIA kit from Linco Research (St. Charles, MO). The leptin concentration was measured using 50 μL of plasma for both DIO in vivo studies and 100 μL of media from the in vitro studies. The insulin was measured using a rat insulin RIA kit from Linco Research (St. Charles, MO). Insulin concentrations were measured using 50 μL of plasma from both of the DIO in vivo studies.

RIA kits measure hormone concentrations by quantifying the amount of binding to a specific antibody. This is done by allowing the hormone and antibody to bind, and then introducing a radiolabeled hormone. In this case the proteins were labeled with ^{125}I. The radio-labeled hormone will bind to open sites on the antibody where the sample has

not bound. The radioactivity is measured on a gamma counter and there is an inverse relationship between the amount of radioactivity present (in counts per minute) and hormone concentration. This relationship is graphed forming a standard curve using standard concentrations provided with the kit. The sample values are extrapolated based on this standard curve.

FAS and GPDH assays

The epididymal fat pads were homogenized in 250 mM sucrose buffer containing protease inhibitor, 0.1 mM phenylmethanesulfonyl-fluoride (PMSF) and centrifuged to collect the cytosolic fraction. The activities of FAS and GPDH were determined in cytosolic extracts by measuring the rate of oxidation of NADPH (for FAS assay) and NADH (for GPDH assay) (113). The oxidation rate was measured spectrophotometrically at 340nm. These numbers were corrected for protein concentration.

Protein assay

Protein concentration was determined by the Bradford assay (114). The dye used for this assay, Coomassie blue (Bio-Rad), is a red-brown that absorbs light at 470nm. When the dye binds to protein, the color changes to blue and the absorption wavelength shifts to 595nm.

Each sample was done in duplicate with 10 µL sample and 200 µL of dye being combined in a 96-well plate. After a 5 minute incubation, the plate was read at 590nm.

A standard curve was created using dilutions of bovine serum albumin (BSA) at known concentrations. The sample concentrations were extrapolated based on this standard curve.

RNA isolation

RNA was extracted from abdominal fat pads by homogenizing adipose tissue in guanidine isothiocyanate (GTC) (Invitrogen, Carlsbad, CA) containing 7% β-mercaptoethanol and purified using the cesium chloride density gradient method (115). The RNA was further purified using phenol/chloroform (Ambion, Austin, TX) followed by ethanol precipitation. RNA was resuspended in 0.1 TE and stored at -80°C until further analysis.

Real time PCR

Expression of FAS mRNA was determined using real time PCR (116). This probe-based PCR product detection was used to quantify RNA concentration from a standard curve that was obtained using total RNA.

Real time PCR was used because it is more sensitive that a Northern blot and can quickly and accurately quantify concentrations of RNA. During the PCR reaction, the probe binds specifically to the target sequence. The probe has a fluorescent reporter dye and quencher dye that, when in close proximity, reduces the fluorescence of the reporter. The probe is cleaved by Taq polymerase at the 5' end when the primer is extended. This

cleavage separates the reporter from the quencher, which increases the signal of the reporter.

The primers and probe were ordered from Sigma Genosys (The Woodlands, TX). The sequence of the forward primer is 5' CCCAGAGGCTTGTGCTGACT 3'. The sequence of the reverse primer is 5' CGAATGTGCTTGGCTTGGT 3'. The sequence of the probe is 5' (TET)CCGATCTGGAATCCGCACCGG(TAMRA) 3'. The SmartCycler software (Cepheid, Sunnyvale, CA) was programmed as indicated in Table 6.

Statistical analysis

Statistical analysis was done in SPSS (SPSS for Windows, Rel. 11.0.1.2001. Chicago: SPSS Inc.) The data was analyzed using either a one-way analysis of variance (ANOVA) or t-test. Tukey's HSD post-hoc analysis was used to determine differences between groups if the ANOVA was significant (117). A 95% confidence interval was used in determining significance in all tests. All data is expressed as mean ± SD.

Table 6. Cycles of real time PCR.

stage	temperature	seconds	number of cycles
1	48°C	1,800	1
2	95°C	600	1
3	95°C	15	40
	60°C	60	

Results

Effects of ang II antagonism in ob/ob mice

Treatment with PD lowered abdominal fat pad weights ($p<0.001$) and decreased FAS activity ($p<0.03$) in *ob/ob* mice (Figure 6). There was no difference in FAS mRNA concentration with treatment of PD. It is worth noting that gene expression was measured in epididymal fat pads, which was not responsive to PD. There was no change in body weight at the end of treatment, amount of weight gained over the course of treatment, food intake per day, blood glucose, epididymal fat pad weight or GPDH activity in PD-treated mice vs. control (Table 7 and Figure 7).

Effects of ang II antagonism in DIO1 mice maintained on chow diet

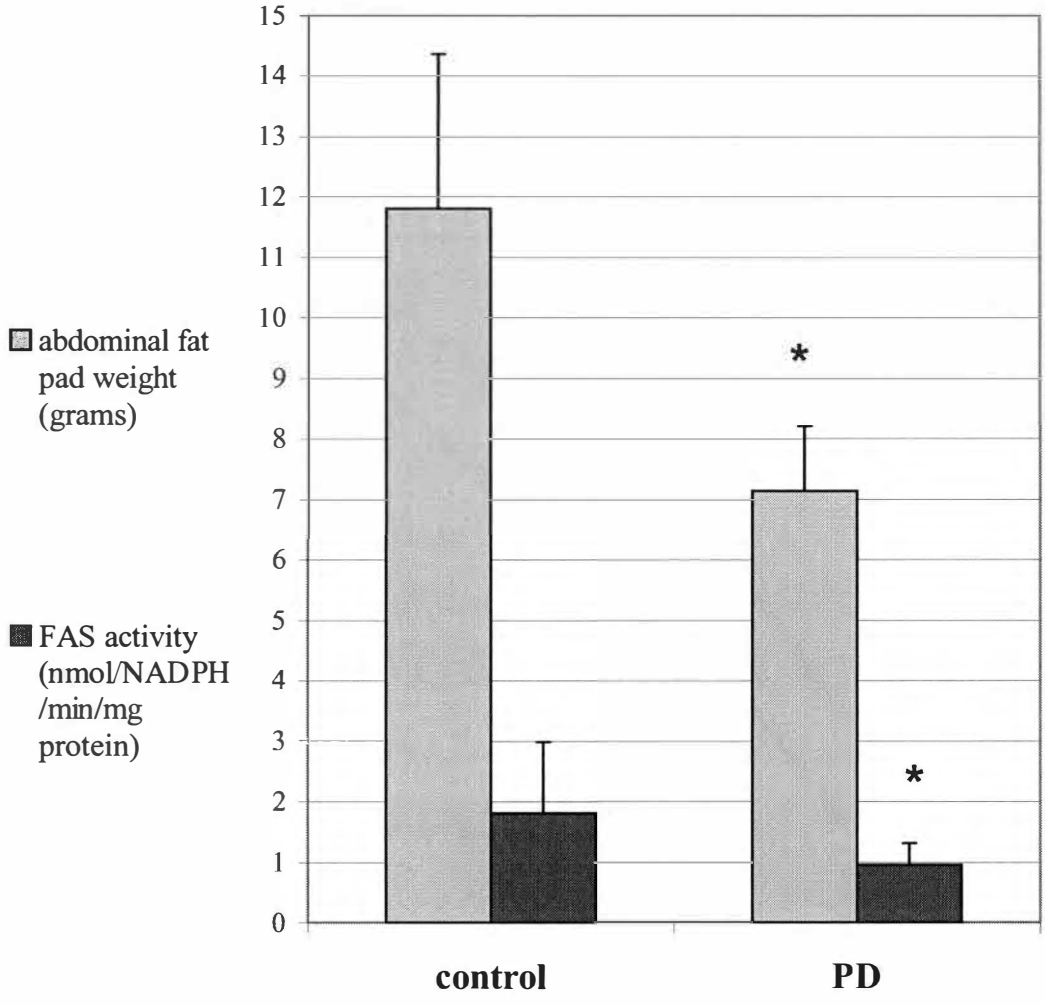
Treatment with PD decreased blood glucose levels in the DIO1 mice compared to control mice ($p<0.03$) (Table 8). Captopril also decreased blood glucose but this effect did not reach significance ($p=0.075$). In addition, at the end of treatment, there was no difference in body weight, amount of weight lost over the course of treatment, food intake per day, fat pad weights, leptin levels, insulin levels, FAS or GPDH activity between PD and vehicle groups (Figure 8).

Effects of ang II antagonism in DIO2 mice maintained on high-fat diet

DIO2 mice treated with PD lost more weight over the entire treatment period than all other groups ($p<0.01$) (Figure 9). Food intake was higher in mice treated with

Table 7. Effects of ang II antagonism in *ob/ob* mice.

	control (n=12)	PD (n=12)
body weight (g) at the end of treatment	55.1 ± 2.8	55.5 ± 2.7
weight gain (g) over course of treatment	4.1 ± 1.1	3.6 ± 1.2
food intake/day (g)	5.8 ± 0.4	5.9 ± 0.3
blood glucose (mg/dL)	266 ± 69	266 ± 61
epididymal fat pad (g)	4.5 ± 0.6	4.2 ± 0.4
GPDH activity (nmol NADH/min/mg protein) measured in epididymal fat pad	4.52 ± 2.64	5.38 ± 1.08
FAS mRNA concentration (ng/μL) measured in abdominal fat pad	14.98 ± 1.09	18.19 ± 1.04



* significantly different from respective controls ($p<0.05$)

Figure 6. Effects of AT_2 receptor antagonism on abdominal fat pad weight and FAS activity in *ob/ob* mice.

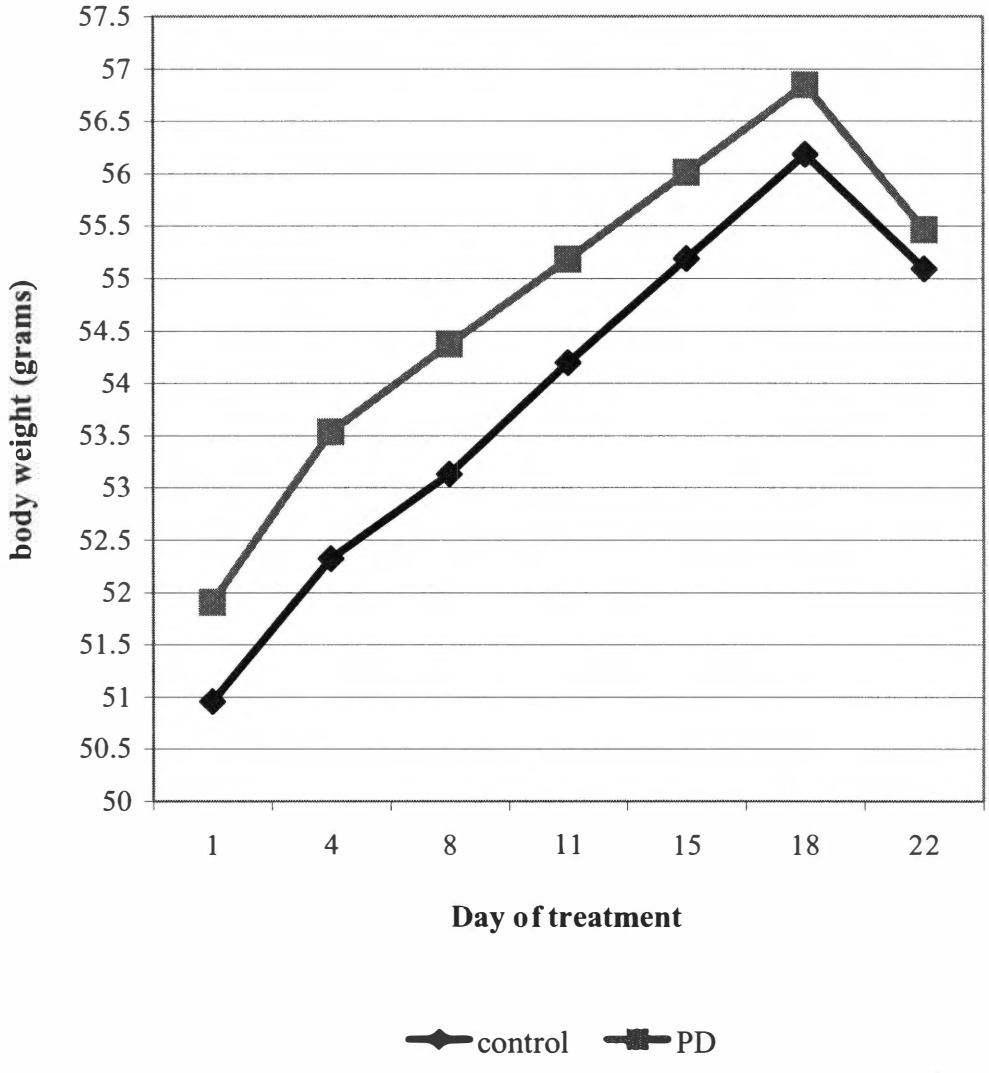


Figure 7. Change in body weight of *ob/ob* mice over course of treatment.

Table 8. Effects of ang II antagonists in DIO1 mice.

	control (n=7)	losartan (n=7)	PD (n=8)	captopril (n=7)
body weight (g) at the end of treatment	31.7 ± 2.0	31.5 ± 2.1	32.4 ± 2.1	31.8 ± 2.0
weight loss (g) over the course of treatment	3.0 ± 1.7	3.0 ± 2.2	2.7 ± 2.1	3.1 ± 1.8
food intake/day (g)	4.3 ± 0.5	4.4 ± 0.5	4.5 ± 0.6	4.6 ± 0.5
blood glucose (mg/dL)	205 ± 28[b]	186 ± 116[a,b]	167 ± 23[a]	173 ± 26[a,b]
epididymal fat pad (g)	0.8 ± 0.3	0.7 ± 0.4	0.8 ± 0.3	0.8 ± 0.3
abdominal fat pad (g)	0.7 ± 0.4	0.6 ± 0.3	0.7 ± 0.3	0.6 ± 0.3
leptin (ng/mL)	4.91 ± 2.05	3.27 ± 0.66	5.37 ± 2.03	3.91 ± 1.32
insulin (ng/mL)	0.90 ± 0.52	0.66 ± 0.19	0.81 ± 0.34	0.58 ± 0.21
FAS activity (nmol NADPH/min/mg protein) measured in epididymal fat pad	2.06 ± 0.87	1.29 ± 0.70	1.27 ± 0.85	1.30 ± 0.55
GPDH activity (nmol NADH/min/mg protein) measured in epididymal fat pad	3.60 ± 0.75	5.27 ± 4.04	3.02 ± 1.45	3.63 ± 1.15

Values with the different letters are significantly different ($p<0.05$). Values with the same letter are not significantly different.

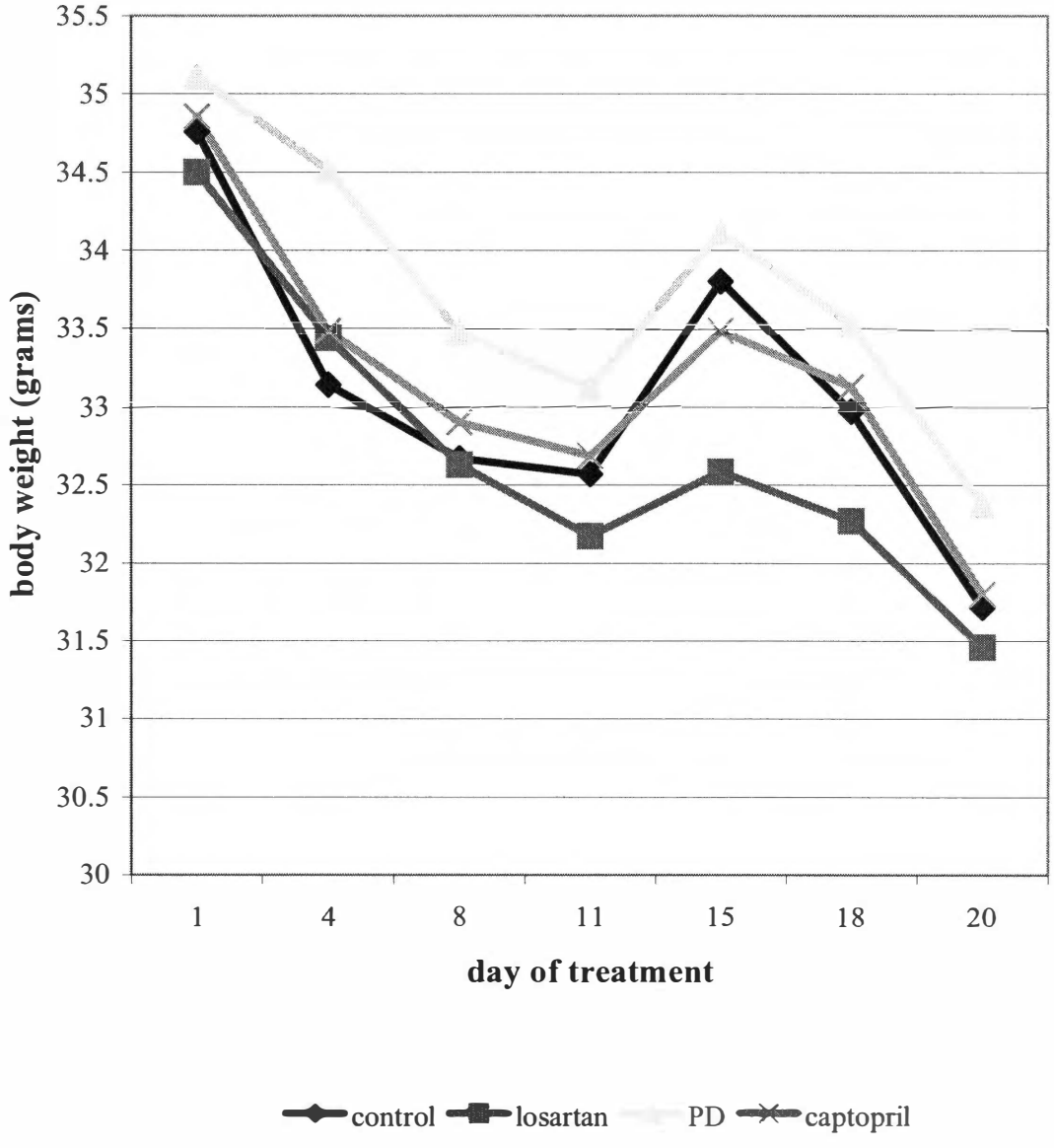


Figure 8. Change in body weight of DIO1 mice over course of treatment.

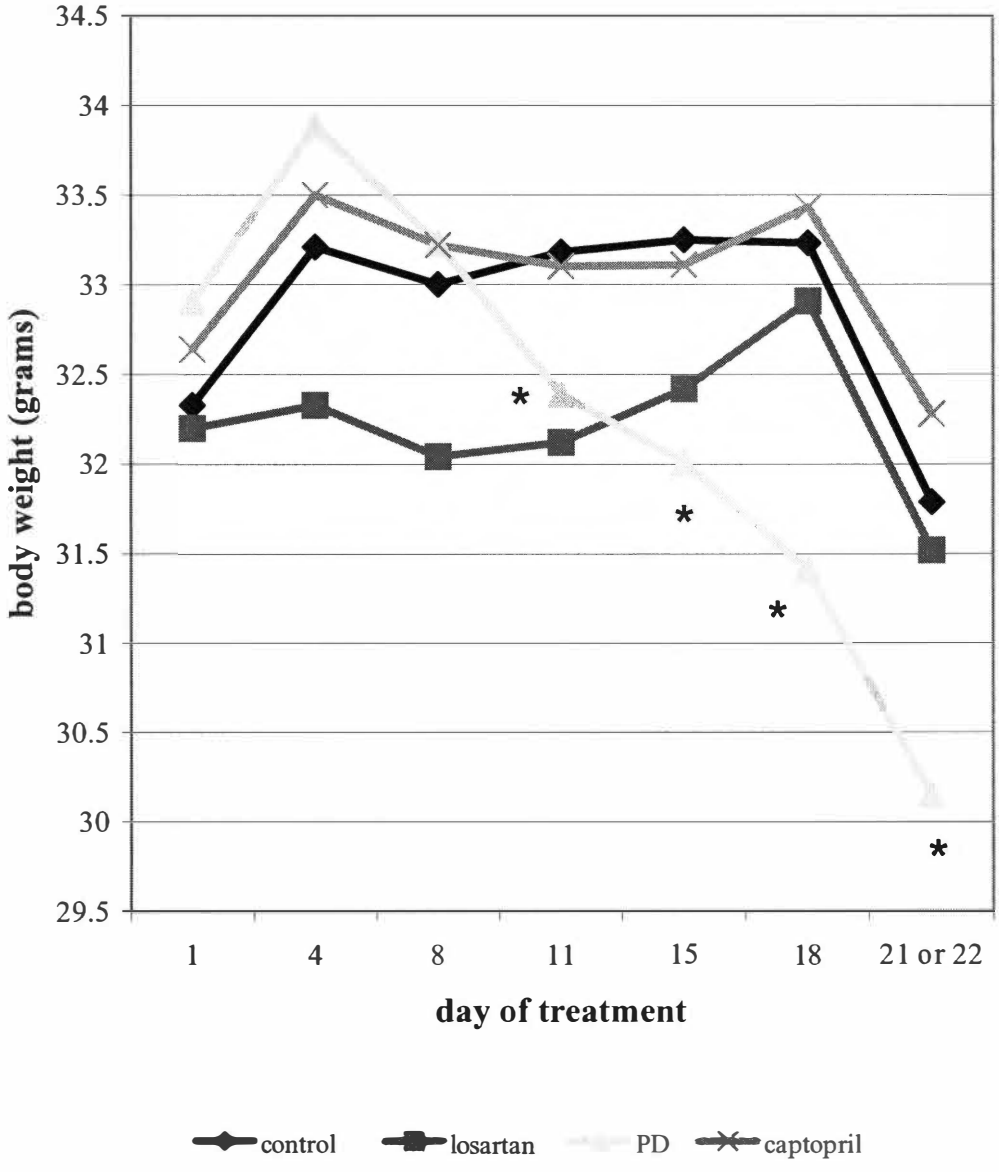


Figure 9. Change in body weight of DIO2 mice over course of treatment. *Mice injected with PD lost more weight than vehicle-treated mice ($p<0.03$ at day 11, $p< 0.002$ at day 15, $p<0.01$ at day 18 and 21/22).

losartan vs. PD ($p<0.01$), and was also higher in losartan (approached significance, $p=0.056$) compared to vehicle-treated mice (Figure 10). At the end of treatment, there was no difference in body weight, blood glucose, fat pad weights, leptin, insulin, FAS or GPDH activity between any of the groups of mice (Table 9).

Effects of ang II and PD in primary adipocytes

When primary adipocytes from DIO mice were treated with ang II there was no change in leptin secretion (Table 10). There was also no effect with PD treatment alone or with ang II and PD in combination.

Discussion

Our lab has previously shown that blocking the AT_2 receptor decreases fatty acid and triglyceride synthesis in cultured 3T3-L1 adipocytes (96). In addition, Darimont et al. demonstrated that the AT_2 receptor mediated ang II-induced PGI_2 release and adipocyte differentiation in cultured ob 1771 adipocytes (94). In agreement with these findings, pilot studies from our lab demonstrated that the AT_2 receptor antagonist, PD123,319, decreased weight gain, fat pad weight and plasma leptin in lean C57BL/6J mice, while in *db/db* mice, PD increased weight gain and plasma leptin levels (106,107). The observations indicate that the AT_2 receptor mediates ang II effects on adipose tissue metabolism.

Table 9. Effects of ang II antagonism in DIO2 mice.

	control (n=10)	losartan (n=10)	PD (n=10)	captopril (n=10)
blood glucose (mg/dL)	192 ± 54	225 ± 35	203 ± 37	199 ± 35
epididymal fat pad (g)	1.1 ± 0.5	0.9 ± 0.4	0.8 ± 0.4	1.2 ± 0.7
abdominal fat pad (g)	0.9 ± 0.4	0.5 ± 0.3	0.6 ± 0.4	0.9 ± 0.6
leptin (ng/mL)	8.28 ± 5.72	4.36 ± 2.15	5.76 ± 4.88	9.38 ± 7.14
insulin (ng/mL)	0.65 ± 0.80	1.00 ± 1.17	0.60 ± 0.75	0.61 ± 0.36
FAS activity (nmol NADPH/min/mg protein) measured in epididymal fat pad	2.14 ± 0.63	2.01 ± 0.80	1.85 ± 0.66	2.31 ± 0.91
GPDH activity (nmol NADH/min/mg protein) measured in epididymal fat pad	3.74 ± 1.84	2.64 ± 1.64	2.42 ± 1.82	2.02 ± 1.47

Table 10. Effects of ang II and PD on leptin secreted from adipocytes of DIO mice.

	control	ang II	PD	ang II + PD
leptin (ng/mL)	5.65 ± 3.72	5.44 ± 3.45	4.60 ± 2.84	5.83 ± 3.54

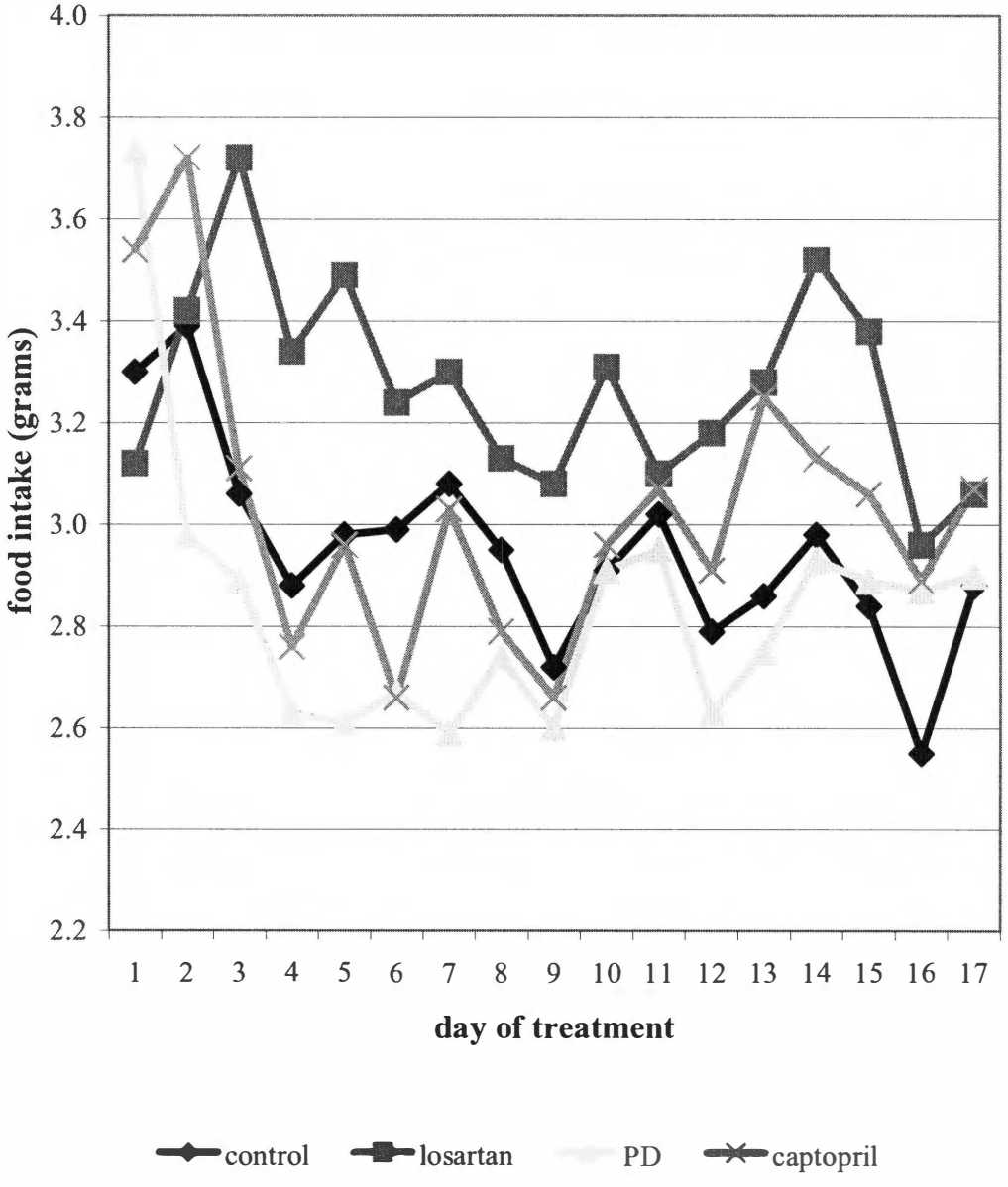


Figure 10. Effects of ang II antagonists on food intake in DIO2 mice.

In this study, consistent with findings from Jones et al (96) in cultured adipocytes, we found that FAS activity in epididymal fat pad decreased with PD treatment in *ob/ob* mice, however, fat pad weight was unaffected. Gene expression was quantified using mRNA isolated from the abdominal fat pads, which were smaller with PD treatment; however, FAS mRNA content in this tissue was not affected by PD. The entire epididymal fat pad was used for enzyme activity assays; therefore we were unable to measure gene expression to determine if the decrease in activity correlates with change in FAS mRNA concentration.

The decrease in blood glucose with PD treatment in the DIO1 study is supported by recent studies demonstrating increased insulin sensitivity and decreased hyperinsulinemia with ang II antagonism (118-120). However there was no change in insulin levels at the time of sacrifice in our mice. Furthermore, there was no change in blood glucose or insulin levels in mice from DIO2 study.

Treatment with PD did not affect weight loss in the DIO1 mice, but did cause weight loss in the DIO2 mice. This could be due to the increased dosage of PD (30 mg/kg body weight) in the second study, or possibly the effect of PD was masked in the first study by the weight loss associated with changing the high fat diet to the chow diet at the start of treatment. In all studies, regardless if the mice were gaining or losing weight, the body weight of the mice decreased over the last interval of measurement independent of treatment (Figures 7, 8, 9).

The only obese animals that exhibited a significant reduction in body weight were the DIO2 mice maintained on a high fat diet during AT_2 antagonist treatment. In the

DIO2 study, mice treated with losartan ate more, however, the body weight at the end of treatment is not different from other groups. This could be due to increased activity in these mice as blocking ang II production in agt knockout mice has been shown to increase locomotor activity in these mice (121). Although not measured in this study, we observed more aggression and activity in all drug-treated animals. In future studies it would be more informative to measure metabolic activity of these mice.

Several studies have determined that ang II increases leptin secretion in adipocytes in culture (93,106,107). Previous studies in our lab have reported that treatment with PD decreased leptin secretion below the control levels in lean mice (106,107). In the in vitro study performed here, PD had no effect on leptin secretion from adipocytes of DIO mice. The former studies used 3T3-L1 and human adipocytes (93) while in our current studies we used primary culture of mouse adipocytes.

In part, our findings support the results from the lean mice study in that some metabolic parameters were decreased in *ob/ob* and DIO mice upon treatment with PD; however, not all parameters were consistently affected. However, none of the obese models in this study have replicated the increase in adiposity and leptin in the *db/db* mice. The background strain of the *db/db* mouse is C57BLKS/J and is different from the C57BL/6J background of the lean, *ob/ob* and DIO mice. The unexpected effect of PD in *db/db* mice may be due to the background strain, genetic mutation, diabetes or kidney disease in these animals.

In using antagonists to block RAS, the expected result, based primarily on in vitro studies, is decreased fat pad mass via decreased adipocyte metabolism (95,96). In

addition, insulin secretion would be lowered and thus decrease its effect on agt expression (86), further depressing this system. There is some evidence of these actions in the in vivo studies. However, there was no overall or consistent effect of ang II antagonism in the different models of mice obesity used in this and previous studies by our lab. It is possible that statistical significance may have been achieved in more parameters if the sample size had been larger for each group. However significant and dramatic changes were previously seen in lean mice with only 6 to 8 mice in each group, using the lower dosage of the drugs. It is possible that in an obese mouse there is an increased variability in the effects of ang II antagonism and possibly requires a larger dosage to see an effect. Alternatively, the responsiveness of obese mice may reflect the intrinsic properties of the adipocyte, the level of obesity, diabetes, insulin and leptin resistance in these models, which may counteract the weight reducing effect of PD. Just as many obese mice are resistant to insulin or leptin, it is possible that obese mice are resistant to the effects of ang II in adipose tissue.

Summary and Conclusions

The goal of these studies was to confirm in vivo the weight reducing effects of RAS blockade. The overall results demonstrate that RAS inhibition does decrease some symptoms of the metabolic disorder of obesity. We did see a decrease in weight and FAS activity in *ob/ob* mice and weight and blood glucose levels in DIO mice, however the results were inconsistent in the different mouse models of obesity and between normally

coordinated parameters (such as glucose and insulin). Additional studies are needed to confirm the effects of PD in obese mice. However, our findings in *ob/ob* and DIO mice are consistent with a potential role of the AT_2 receptor in adipocyte metabolism and energy regulation.

The inconsistencies in responsiveness in the above metabolic parameters measured in lean vs. obese mice demonstrate the complexity and multi-factorial nature of the system being studied. Variability among individuals is inherent to an in vivo study and metabolic alterations associated with obesity are additional confounding factors.

The importance of this research is very significant, however, as RAS has many links to the metabolic syndrome of obesity and related disorders. This thesis mainly focused on blockade of the adipocyte RAS and the potential of improving both obesity and hypertension, which are closely linked.

In conclusion, blocking ang II reduces hypertension in two ways (figure 11): by decreasing systemic effects of RAS and by inhibiting the excess contribution of adipose tissue to ang II production.

In many cases of hypertension where obesity is the cause, these findings constitute a preliminary step to develop treatments for obesity-related disorders that more specifically target the AT_2 receptor instead of blocking the entire RAS. This approach would reduce the disruption in systemic functions by targeting adipose tissue. This would be especially important in cases of obesity without hypertension that did not require a systemic blockage of RAS. However, further studies are warranted to ascertain the effects of ang II receptors in mice before clinical trials can be undertaken.

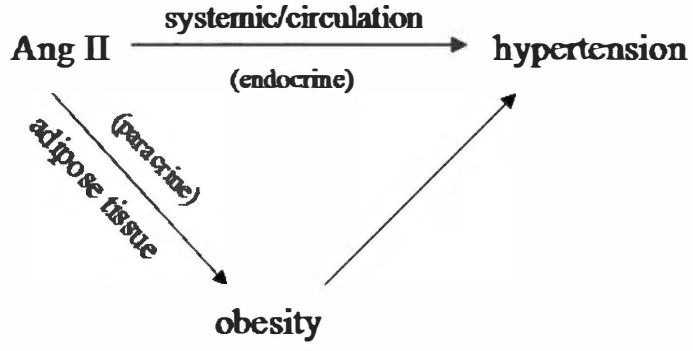


Figure 11. Contribution of adipocyte ang II to obesity and hypertension.

Finally, as with any pharmacological treatment of obesity, AT_2 receptor antagonism if proven effective in vivo, should be used in conjunction with behavioral therapy including diet and physical activity to reduce weight and adiposity.

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APPENDIX

Table A-1. Composition of chow diet. Harlan Teklad 22/5 Rodent Diet (W) 8640. www.teklad.com/rodent/standard/8640.htm

Guaranteed Analysis

Crude Protein	22.0% min.
Crude Fat	5.0% min.
Crude Fiber	4.5% max.

Average Nutrient Composition

Protein	22.58%
Fat	5.23%
Fiber	3.94%
Ash	7.06%
Nitrogen-Free Extract	51.19%
Gross Energy	3.82 kcal/g
Digestible Energy	3.38 kcal/g
Metabolizable Energy	3.11 kcal/g
Linoleic Acid	3.32 kcal/g

Amino Acids

Arginine	1.65%
Methionine	0.37%
Cystine	0.37%
Histidine	0.52
Isoleucine	1.17
Leucine	1.88%
Lysine	1.29%
Phenylalanine +Tyrosine	2.04%
Threonine	0.93%
Tryptophane	0.29%
Valine	1.17%

Minerals

Calcium	1.13%
Phosphorus	0.94%
Sodium	0.40%
Chlorine	0.67%
Potassium	1.00%
Magnesium	0.24%
Iron	348.75 mg/kg
Manganese	104.19 mg/kg
Zinc	78.84 mg/kg
Copper	24.07 mg/kg
Iodine	2.69 mg/kg
Cobalt	0.72 mg/kg
Selenium	0.26 mg/kg

Vitamins

Vitamin A	15.93 IU/g
Vitamin D_3	2.99 IU/g
Vitamin E	109.54 IU/kg
Choline	2.39 mg/g
Niacin	65.61 mg/kg
Pantothenic Acid	22.51 mg/kg
Pyridoxine	14.45 mg/kg
Riboflavin	8.56 mg/kg
Thiamine	32.62 mg/kg
Menadione	5.22 mg/kg
Folic Acid	3.19 mg/kg
Biotin	0.42 mg/kg
Vitamin B_{12}	54.60 mg/kg
Vitamin C	0.00 mg/kg

Table A-2. Composition of high-fat diet. Research Diets Rodent Diet D12451. www.researchdiets.com

	gm	kcal
Protein	24%	20%
Carbohydrate	41%	35%
Fat	24%	45%
kcal/gm	4.7	

Ingredient		
Casein, 80 Mesh	200	800
L-Cystine	3	12
Corn Starch	72.8	291
Maltodextrin 10	100	400
Sucrose	172.8	691
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard	177.5	1598
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate, 1 H_2O	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Red Dye #40	0.05	0

VITA

Melissa Meredith Derfus was born in Conroe, Texas on August 27, 1977. After living several different places around the world, the Derfus family settled in Largo, Florida. Melissa graduated from the International Baccalaureate Program at St. Petersburg Senior High School in St. Petersburg, Florida in 1995. She then went on to receive a B.S. in Microbiology and Cell Science from the University of Florida in 1999.

After completing her M.S. in Nutrition Science from the University of Tennessee, Melissa will begin dental school at the University of Florida in Gainesville, Florida.