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Angiotensin II Increases Leptin Secretion in Adipocytes via a Prostaglandin-Independent Mechanism

Suyeon Kim
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To the Graduate Council:

I am submitting herewith a thesis written by Suyeon Kim entitled "Angiotensin II Increases Leptin Secretion in Adipocytes via a Prostaglandin-Independent Mechanism." 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:

Jay Whelan, James Bailey

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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Associate Vice Chancellor and
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ANGIOTENSIN II INCREASES LEPTIN SECRETION IN ADIPOCYTES VIA A PROSTAGLANDIN-INDEPENDENT MECHANISM

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Suyeon Kim
August 2000

DEDICATION

This thesis is dedicated to

my parents

Mr. KunTae Kim and Mrs. JoJa Kang

sister

JiYeon Kim

for their ongoing support and encouragement throughout my life.

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ABSTRACT

Adipose tissue functions as a secretory organ. Adipocyte-derived AII and prostaglandins are involved in promotion of adipocyte hyperplasia through enhancement of preadipocyte differentiation as well as adipocyte hypertrophy. AII was proposed to regulate adipocyte differentiation in part via prostacyclin. The peptide hormone leptin encoded by the *ob* gene is produced primarily by adipocytes and is believed to be the signal indicating the level of adiposity. We have previously demonstrated that AII increases *ob* gene transcription in adipocytes. In this study, we have examined mechanisms of AII effect on leptin secretion in adipocytes. AII at physiological concentrations significantly increased leptin secretion in human adipose tissue and 3T3-L1 adipocytes in a dose and time-response manner. Consistently, 6-keto $PGF_{1\alpha}$ (a stable degradation product of PGI_2) and PGE_2 secretions were also significantly stimulated by physiological doses of AII. Inhibitors of prostaglandin biosynthesis, namely indomethacin and aspirin significantly blocked basal as well as AII-induced prostaglandin synthesis. However, inhibition of prostaglandin synthesis by these inhibitors did not modify AII-induced leptin secretion. Consequently, these findings demonstrate that AII regulation of leptin secretion from adipocytes is independent of prostaglandin.

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LIST OF ABBREVIATIONS

AII	Angiotensin II
ACC	Acetyl CoA carboxylase
ADD	Adipocyte determination differentiation dependent factor
AGRP	Agouti-related protein
AGT	Angiotensinogen
ASP	Acylation stimulation protein
aP2	Adipocyte-specific fatty acid binding protein
ARC	Arcuate nucleus
C/EBP	CCAAT/enhancer binding protein
COX	Cyclooxygenase
CRH	Corticortrophin-releasing hormone
DHET	Dehydroxyeicosatrienoic acid
EET	Epoxyeicosatrienoic acid
FAS	Fatty acid synthase
GAL	Galanin
GDPH	Glycerol-3-phosphate dehydrogenase
HETE	Hydroxyeicosatetraenoic acid
HPETE	Hydroperoxyeicosatetraenoic acid
IL-6	Interleukin-6
JAK	*janus* kinase

LH	Lateral hypothalamus
LPL	Lipoprotein lipase
MCH	Melanin-concentrating hormone
NEFA	Non-esterified fatty acids
NSAIDs	Nonsteroidal anti-inflammatory drugs
NPY	Neuropeptide Y
PPARγ	Peroxisome proliferator-activated receptor γ
PEPCK	Phospho*enol*pyruvate carboxykinase
PGHS	Prostaglandin endoperoxide synthase
PGs	Prostaglandins
PGH_2	Prostaglandin H_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI_2	Prostacyclin
POMC	Pro-opiomelanocortins
PVN	Paraventricular nucleus
RAS	Renin Angiotensin System
SOCS	Suppressor of cytokine signaling
STAT	Signal transduction and activation of transcription
SREBP	Sterol regulatory element binding protein
TNFα	Tumor necrosis factor α
TXA_2	Thromboxane A_2
VMH	Ventromedial hypothalamus

PART I

INTRODUCTION

Obesity is a complex multifactorial disease characterized by interactions of genetic predisposition with physiological and behavioral alterations and it is associated with a number of pathological disorders including cardiovascular disease, non-insulin-dependent diabetes, and hypertension. Several studies have recently investigated the role of fat cells in the development of human obesity. Beyond its role in energy storage, adipose tissue also participates actively in energy regulation and lipid homeostasis via production of biologically active compounds. Adipose tissue has been shown to be a potential site for the local synthesis of AII that is the main effector peptide of the RAS (Renin Angiotensin System) and plays an important role in the regulation of vascular homeostasis. Recent studies on the role of AII in adipocytes have illustrated that AII increases fat mass and lipid storage as well as transcription of *ob* gene, a marker of adiposity. The *ob* gene product leptin binds to its receptors in the hypothalamus and plays an important role in the regulation of food intake and energy metabolism in rodents and humans. Adipocytes also secrete prostaglandin, which has been shown to mediate AII effect on adipocyte hyperplasia. Therefore, the purpose of this study is to investigate the effect of AII on lepin secretion as well as to determine the role of prostaglandins as possible mediators of this effect. This investigation may offer means to develop pharmacological and therapeutic strategies for treatment and prevention of obesity.

RESEARCH OBJECTIVES

The objective of this research is to determine whether AII regulates leptin secretion in adipocytes via a prostaglandin-mediated mechanism. This objective will be examined by addressing the following specific aims.

1) Determine AII effect on leptin secretion in human adipose tissue as well as 3T3-L1 adipose cells.
2) Determine AII effect on prostaglandin secretion in 3T3-L1 adipose cells.
3) Investigate the effect of prostaglandin biosynthesis on AII regulation of leptin secretion in 3T3-L1 adipose cells.

PART II

REVIEW OF THE LITERATURE

I. ADIPOSE TISSUE: AN ENDOCRINE AND PARACRINE ORGAN

A. Adipose tissue: a secretory organ

Adipocytes are highly specialized cells that play critical roles in energy regulation as well as homeostasis. Their primary role is to store energy in the form of triglycerides during periods of nutritional abundance and to release it in the form of free fatty acids at time of nutritional deprivation (1). Along with their active role in regulating energy balance, adipocytes behave as endocrine as well as paracrine/autocrine cells via producing and releasing many different bioactive compounds (2). Secretory products of adipocytes including leptin, angiotensinogen (AGT), prostaglandins (PGs), cytokines such as tumor necrosis factor α (TNF-α) and interleukin (IL-6), non-esterified fatty acids (NEFA), acylation stimulating protein (ASP) and others are involved in immunological responses, cardiovascular function and appetite regulation (4-11). Thus, adipose tissue plays more dynamic roles than previously recognized in physiological mechanisms including the autoregulation of adipocyte growth and development. However, abnormal quantitative increases in mass of adipose tissue associated with obesity give rise to quantitative and qualitative alterations in adipose tissue endocrine responses, leading to maladaptive effects predisposing to metabolic disease (2).

B. Adipocyte differentiation

Adipose tissue growth is accompanied by an increase in adipocyte size (hypertrophy) and the formation of new adipocytes from precursor cells (hyperplasia) (113). Adipocyte differentiation is a complex process characterized by many changes in cell morphology, hormone sensitivity and expression of adipose-specific genes controlling lipogenesis and lipolysis (14-16, 28). Many hormones, growth factors, cytokines and arachidonate metabolites modulate adipogenic processes (17-19, 21, 23). Among these hormones, glucocorticoids

increase differentiation of preadipocytes by transcriptional repression of *pref-1*, which is an epidermal growth factor-like domain-containing transmembrane protein with anti-adipogenic function that is highly expressed in 3T3-L1 preadipocytes (178). The cytokine TNF-α and $PGF_{2\alpha}$ exert inhibitory effects on adipocyte differentiation (20-21). In contrast, differentiation of preadipocyte cell lines such as 3T3-L1 is induced by continuous exposure of pharmacological doses of insulin and physiologic doses of insulin-like growth factor 1 (22-23). Moreover, angiotensin II (AII) and arachidonic acid metabolites such as PGI_2 promote the conversion of fibroblasts to functionally mature adipocytes in Ob1771 adipose cells (24-25). In addition, dietary fatty acids induce differentiation of these cells by activating peroxisome proliferator-activated receptor γ (PPARγ) (26).

Two classes of transcription factors, CCAAT/enhancer binding protein family (C/EBP family) and PPAR γ play significant roles as positive mediators of adipogenesis (27-28, 41). PPARγ is one member of the PPAR subfamily of ligand-dependent nuclear hormone receptors and its gene generates two isoforms PPARγ1 and γ2 through alternative splicing (29). The functional domains of PPARγ are composed of five regions: the transactivation function domain, DNA binding domain, receptor dimerization function domain and ligand binding domain (30). While PPARγ1 is expressed at low levels in several tissues such as large intestine, kidney and liver, PPARγ2 is abundantly and specifically expressed in adipocytes (31). PPARγ plays a central role in regulating fat-specific gene expression such as an adipocyte-specific fatty acid binding protein (aP2), phosphoenolpyruvate carboxykinase (PEPCK), lipoprotein lipase (LPL), glucose transporters (GLUT4) and leptin by binding to DNA sequences termed PPAR response elements as a heterodimer with retinoid X receptor

(RXRα) (32-34). Further, PPARγ is activated by various synthetic and naturally-occurring substances including antidiabetic thiazolidinediones, polyunsaturated fatty acids, 15-deoxy-delta prostaglandin J_2 and lipoxygenase products such as 8-(S)-hydroxyeicosatetraenoic acid (HETE), leading to adipocyte differentiation (19, 35-38,130). Recently, non-steroidal anti-inflammatory drugs (NSAIDs) classified as cyclooxgenase blockers, such as indomethacin and ibuprofen, also have been shown to act as PPARγ agonists (39). However, the identities of endogenous ligands for PPARγ and characteristics of their production in vivo have not been established.

The C/EBP family also plays an important role as an adipogenic factor, in part by modulating the expression and activity of PPAR γ. Hormonal induction of C/EBP β and δ stimulates PPARγ expression (40). C/EBPα, which may induce continued expression of PPARγ, binds to the promoters of fat cell genes leading to maintenance of the fully differentiated state (41-42).

II. THE ROLE OF ANGIOTENSIN II IN ADIPOCYTE GROWTH AND DEVELOPMENT

A. Classic Renin-Angiotensin system (RAS)

The renin-angiotensin system (RAS) plays a vital role in the regulation of blood pressure as well as fluid and electrolyte balance (45). The RAS consists of several components, which include angiotensinogen (AGT), renin, angiotensin-converting enzyme (ACE), and AII receptors. The RAS produces the vasoactive peptide hormone AII. The components of classic RAS are synthesized in the liver, kidney and endothelium (43). In addition, local synthesis of components of the RAS (nonclassic RAS) has been found, mostly in organs involved in

cardiovascular regulation including the heart, peripheral blood vessels, brain, adrenals as well as adipocytes (46-47, 51, 57).

B. Renin-Angiotensin system (RAS) in adipose tissue

AGT expression has been identified in murine adipose cell lines and rodent adipose tissue (48-49). Human adipose tissue also expresses AGT mRNA, ACE mRNA, and the renin gene (50-51, 68). In brown adipose tissue from rodents, local production of AGT and renin activities have been described (52-53). AII receptor type 1 (AT1) is expressed in adipose cells from rats as well as in human preadipocytes and adipocytes, and AII receptor type 2 (AT2) is expressed in murine, human and mouse adipose cells (54-56, 58-59). Additionally, transcripts for cathepsin D and cathepsin G, enzyme components of the nonrenin-angiotensin system (NRAS), are detectable in human adipose tissue by polymerase chain reaction (PCR) (60). Collectively, these findings strongly suggest that adipose tissue is an important peripheral site containing regulatory components of the local RAS, providing insights into the significant function of adipocyte-derived AII in adipocyte metabolism and cellularity.

C. Effect of AII on adipogenesis

AII, which is the primary effector peptide of RAS is metabolized rapidly by a number of peptidases and thus has a relatively short half-life of approximately 15s (61). In spite of its short half-life, AII functions as a modulator of vascular tone (45). In addition to its role in vasoconstriction, early investigations on the role of peripheral RAS have illustrated that AII has hypertrophic and hyperplastic activities *in vitro* leading to growth and development of nonclassical RAS in tissues such as brain and fetal tissue (63-64). This suggests the possibility that the AGT produced in adipose tissue itself may influence adipogenesis and play a role in the pathogenesis of obesity. In light of a potential physiological role for AII in regulating

adiposity, AGT expression is differentiation-dependent (12). Consistently, Harp and DiGirolamo has illustrated a positive relationship between expression of AGT mRNA and protein, the only known precursor to AII, and relative rates of adipocyte growth in rats (65). Further, under conditions of genetic obesity *(ob/ob and db/db)*, AGT synthesis by adipocytes is increased compared with lean controls, suggesting a potential link between obesity and functional local RAS in adipose tissue (67). Consistent with this concept, AGT expression in adipocytes is reduced in states of fasting and enhanced in overfeeding and AGT expression is stimulated by a high fat diet concomitant with enlargement of fat mass (66-67). In addition, while β-adrenergic receptor agonist decreases AGT expression in 3T3-L1 adipose cells, insulin increases AGT expression (68). However, decreased AT mRNA and secretion of AII by low concentrations of insulin were observed in cultured Ob1771 and 3T3-F442A adipose cells (13). Similarly, glucocorticoids upregulate AGT gene expression in Ob1771 cells in a dose-dependent manner, in turn leading to growth of adipose tissue (69). Epidemiological studies have shown a tight correlation between accumulation of intra-abdominal fat and hypertension (70-71, 44). A significant correlation between plasma AGT level, blood pressure and the *ob* gene product leptin, which is proposed as an endogenous marker of adiposity was reported (72). However, the contribution of adipose-derived AII to pathophysiological hypertension is not well understood. Taken together, these findings suggest that adipose tissue hypertrophy is accompanied by an increased activity of the tissue RAS. Along with its function in adipose tissue development, AII may also contribute to the association between hypertension and obesity.

D. Effect AII on function of brown adipose tissue

A previous study has demonstrated that rat brown adipose tissue (BAT), which plays a critical role in cold-induced thermogenesis, is capable of producing AII (176). The thermogenic function of BAT is mediated primarily by norepinephrine (NE) released from sympathetic nerves densely innervating this tissue (82). Accordingly, recent research has reported the potential role of AII in BAT function. The study of Cassis has demonstrated that production of endogenous AII is increased in rat intrascapular BAT (ISBAT) during cold acclimation and AII-induced [^{3}H] NE release is enhanced from ISBAT in response to cold, suggesting a potential role of AII in stimulating thermogenesis in BAT (73). In subsequent studies, decreased content of AII in ISBAT of young obese Zucker rats was observed and AII-mediated neuronal uptake of [^{3}H] NE in ISBAT was also decreased in both young and adult obese rats (74). This finding suggests that alterations of AII production and function in the obese animal model appear to decrease energy expenditure associated with BAT thermogenesis, consequently contributing to development of obesity.

E. Mechanisms of AII effects on adipose tissue

AII exerts its action via at least two distinct receptors subclassified as AT1 and AT2 (Table 1) (75). The recent study has demonstrated that treatment of losartan, an AT1 receptor specific antagonist, significantly inhibits the age-dependent increase in rat adipocyte size (76). Further, the studies of Darimont *et al* have illustrated by using a coculture of preadipocytes and adipocytes, that AII promotes adipocyte differentiation by stimulating release of prostacyclin (PGI_2) from mature fat cells, which has an autocrine adipogenic effect *in vitro* through an AT_2-receptor mediated mechanism (77). In addition, AII enhances expressions of PGHS-1 (prostaglandin endoperoxide synthase) and PGHS-2 mRNA in mature adipocytes, thus

Table 1. Characteristics of Angiotensin II receptor subtypes

	Receptors	
	AT1	AT2
Selective antagonists	Losartan (DuP 753) SK&F 108566 L-158809	PD123177 PD123319 WL19 (PD121981) CGP 42112A
G-protein coupled Signal transduction	YES (Gi, Gq) ↑ IP_3 and $[Ca^{2+}]$ ↓ cAMP	YES ↓ cGMP ↓ Phosphotyrosin Phosphatase
Species	Human Bovine Rat (AT_{1A}, AT_{1B}) Mouse	Human 3T3-L1 adipose cells Ob1771 adipose cells Rat Mouse

promoting prostaglandin (PG) production (78). Interestingly, AII regulates lipogenesis by increasing fatty acid synthase (FAS) and glycerol-3-phosphate dehydrogenase (GPDH) activities as well as triglyceride storage via an AT_2-dependent mechanism, concurrent with elevated transcription rate of *ob* gene in 3T3-L1 and human adipose cells (79). More recently, studies *in vivo* have shown that PD123139 (an AT2 receptor antagonist)-treated mice display significant decreased leptin content in adipose tissue as well as decreased epididymal fat pad mass, compared to controls (Kim et al 1999). Collectively, these findings suggest that adipocyte-derived AII regulates secretion of leptin, which is positively correlated with adipose mass, via possibly a PG-mediated mechanism that controls growth and development of adipocytes.

III.LEPTIN REGULATION OF ENERGY HOMEOSTASIS

A. Obese(ob) gene and leptin : discovery and structure

The important role of *ob* gene in energy balance was originally demonstrated by the Coleman's parabiosis (cross-circulation) experiments with *ob/ob*, *db/db* and lean mice (81). This study indicated that a circulating factor present in the blood of wild type lean or *db/db* obese mice reversed the effects of *ob* mutation and furthermore the *db/db* mice lacked ability to respond to this factor. *Ob/ob* and *db/db* are mutant mice that have single gene mutations residing on mouse chromosomes 6 (*ob*) and chromosomes 4 (*db*) respectively. Both mice exhibit a reduced basal metabolic rate, hyperphagia and massive obesity (81). Importantly, the positional cloning of this gene by Friedman's group in 1994 led to the discovery of its gene protein product named leptin. Consistent with early parabiosis studies, it was demonstrated that *ob/ob* mice produce a truncated and inactive form of leptin hormone due to a premature stop

codon in the gene (4). Leptin is synthesized as a 167 amino acid protein primarily in adipose tissue. Small amounts of leptin are produced by muscle, placenta and stomach and released into the circulation after cleavage of a 21 amino acid signal peptide (83-85). It circulates in mouse and human plasma either as free or bound to other circulatory proteins such as several hormone receptors or soluble leptin receptors (179). Circulating leptin is transported to the cerebrospinal fluid where it is available to bind and activate specific receptors on the hypothalamus to regulate energy balance via interaction with its receptors (86, 91-92,104). In addition, Zhang *et al* have observed that leptin folds into a cytokine-like structure, which includes 4 α-helices, 2 β-sheets, and a single disulfide bond between cysteines 96 and 146 (88). Consistent with leptin protein structure, the receptor for leptin protein is a single membrane-spanning protein with structural and functional homology to the class I cytokine receptor family (98).

B. Leptin receptors

The leptin receptor was cloned from mouse choroid plexus and the gene encoding the leptin receptor was genetically mapped to the same 5-centimorgan interval on mouse chromosome 4 containing the *db* locus (90, 98). Spontaneous mutations in the leptin receptor gene in *db/db* mice and *fa/fa* rats produce defective leptin receptors, consequently these rodents develop severe obesity with resistance to endogenous and exogenous leptin (93-95). The leptin receptor (Ob-R) is a member of the cytokine receptor family. Receptors of this class lack intrinsic tyrosine kinase activity and are activated by ligand-induced receptor homo-or heterodimerization and utilize *janus* kinases (JAK) and signal transducers and activators of transcription (STAT) family proteins to modulate transcription of target genes (96-97). Multiple splice variants of Ob-R mRNAs encoding proteins with identical extracellular

domains but different length intracellular domains have been detected. The short splice variants, Ob-R (a, c and d) encode a receptor with a single transmembrane domain, a cytoplasmic region of variable length and diminished signaling capabilities (98). One of the shorter splice variants, Ob-Re encodes a soluble form lacking a transmembrane domain. Ob-Rb encodes a long form of leptin receptor with a long intracytoplasmic domain of 302 amino acids containing several motifs known to be important for protein-protein interactions and signal transducing activity, including both JAK interaction and STAT docking sites (96, 99-100). In mammals, the short forms of the leptin receptor are expressed ubiquitously and found in high amounts in the lungs, kidneys and uterus. In addition, it is highly expressed in the choroid plexus, suggesting the possibility that this isoform mediates translocation of leptin across the blood-brain barrier (177). The long form is found at high levels in the arcuate nucleus (ARC) within the hypothalamus known to be important for body weight regulation, with lesser amounts observed in peripheral tissues including liver, heart, skeletal muscle, pancreatic β cells and brown and white adipose tissue (98, 102-103). This isoform of the receptor is proposed to mediate leptin's effects on body weight homeostasis by decreasing food intake and increasing energy expenditure

Transient co-transfection studies have revealed that the long form of receptor protein is capable of activating STAT proteins in response to ligand binding (100). Similarly, STAT3 and STAT5 have been stimulated in COS cells by expressing Ob-R (115). *In vivo*, STAT3 activation in the hypothalamus has been detected when exogenous recombinant leptin is administered (105). In addition, leptin induced a dose-and time-dependent increase in mitogen-activated protein kinase (MAPK) in C3H10T1/2 cells in parallel with increased cell number and JAK-2 has also been activated via long form leptin receptor upon leptin stimulation

(96,106). Recently, receptor chimera studies have indicated that the long form of the receptor signals via homo-oligomers (107). JAKs associated with this receptor become activated, autophosphorylated and phosphorylate STAT proteins, which then dimerize and translocate to the nucleus to activate transcription of target gene for weight-reducing effects of leptin (108,115). Furthermore, recent study has demonstrated a leptin inducible inhibitor of leptin signal transduction, SOCS-3 (suppressor of cytokine signaling) family blocks leptin-induced activation of STAT3 in cells expressing the long form of the leptin receptor. This suggests a potential mechanism for leptin resistance related to obesity (180). Cumulatively, these findings suggest that the long form of leptin receptor plays a role as a signal transducing receptor in the leptin signaling cascade. Lack of the intracellular long form of leptin in *db/db* mice is due to a premature stop codon resulting from G to T substitution. Thus, receptors cloned from *db/db* mice are shown to be unable to activate STAT protein (94, 97). In *fa/fa* obese Zucker rats, a missense point mutation (A→C) results in Gln to Pro substitution in the extracellular ligand binding domain of Ob-R, producing partial deficiency of Ob-R function (95). Consequently, the *fa*-type receptors exhibit a reduced leptin-binding affinity and signal transduction.

C. Regulation of leptin expression and leptin secretion

Leptin research has shown that environmental and hormonal factors contribute to leptin gene expression and secretion. Leptin expression is influenced by the degree of energy restriction. In rodents and humans, leptin mRNA and plasma levels are greatly decreased after fasting and increased by refeeding and overfeeding (109-112). This suggests that function of leptin is involved in controlling of energy balance. Recent studies have demonstrated that changes in leptin concentration in response to diet composition such as the high fat diet (60 energy % fat, for 7 days) correlated with changes in insulin concentration, implying that leptin

production is positively regulated by insulin (114). Consistent with the proposed insulin regulation of leptin, insulin administration increases *ob* mRNA expression as well as leptin secretion in rat adipocytes (117). However, other studies have reported no effect of insulin on *ob* expression and leptin secretion (80,116). These conflicting reports have been suggested to result from the use of different culture model (118). Glucocorticoid stimulation of leptin gene expression and circulating leptin levels has been shown both *in vivo* and *in vitro* studies (80, 116, 118-119). In contrast, catecholamines, by increasing intracellular cAMP levels via β_2 and β_3 adrenoreceptors, suppress *ob* mRNA expression and leptin production (120). Furthermore, several lines of evidence have indicated that plasma leptin levels and *ob* gene expression vary in proportion to the degree of adiposity in lean and obese animals and humans, indicating a leptin role as an "adipostat" signal (121-123, 125). Additionally, the studies of Masuzaki *et al* have reported that *ob* mRNA levels are higher in the subcutaneous adipose tissue compared to the omental, retroperitoneal, and mesenteric adipose tissue in the same individual. This suggests regional differences in leptin production rates (124).

Recently, C/EBPα, which is a transcription factor regulating adipocyte differentiation, has been identified as a transactivator of the leptin promoter through binding to a consensus C/EBP-binding site in the proximal leptin gene promoter (126). Treatment with the antidiabetic drug thiazolidinedione, a ligand for PPARγ, decreases expression of the endogenous leptin gene in rodent and 3T3-L1 adipocytes, suggesting that PPARγ is involved in the transcriptional regulation of the *ob* gene (127). Studies of Hollenberg *et al* have revealed that PPARγ2 mediates downregulation of the leptin promoter through inhibition of C/EBPα-activated transcription (128). Additionally, adipocyte determination differentiation dependent

factor1/sterol regulatory element binding protein1 (ADD1/SREBP1), which is regulated by nutritional status and insulin level, has been shown to be involved in the transcriptional regulation of the leptin gene promoter (129). These findings suggest that adipocyte differentiation and growth is critical for modulating the *ob* gene expression and leptin production.

D. Biological function of leptin in energy balance

Several studies have demonstrated that the hypothalamus is a potential site for the action of leptin on body weight regulation (86). Injection of leptin into the lateral ventricle of *ob/ob* mice (characterized by hyperphagia, increased adiposity, hyperglycemia and insulin resistance) induces suppression of food intake and body fat (87). Similarly, after leptin was administered intracerebroventricularly (ICV) reduced food intake and body weight were observed in diet-induced obese (DIO) and *ob/ob* mice as well as lean mice (132-133). Leptin appears to act in maintaining energy homeostasis through the long form of the receptor in the hypothalamus. In addition, chemical lesions of the ventromedial hypothalamus (VMH) where leptin receptors are expressed cause overeating and severe obesity (134). Leptin was suggested to inhibit food intake by down-regulating NPY. In agreement with this hypothesis, pre-pro-neuropeptide Y (NPY) mRNA (a potent stimulator of food intake) in the arcuate nucleus (ARC) of the hypothalamus was overexpressed in the genetically obese (*ob/ob*) and *db/db* mice as well as *fa/fa* rats (89,101). Leptin administration significantly lowered NPY mRNA levels in the ARC of *ob/ob* mice (101). Thus, impaired leptin signaling may stimulate NPY production and release, suggesting that leptin exerts its central effects through NPY. Furthermore, the ICV NPY infusion induces increases in leptin production by rat adipose tissue (132). In addition, the direct microinjection of NPY into the paraventricular nucleus (PVN) inhibited the

hypophagic and thermogenic effects of centrally (ARC) administered leptin, suggesting that leptin produced by white adipose tissue and the NPY in the ARC-PVN axis may interact in a homeostatic loop to regulate body fat mass (62,136). However, studies in NPY knockout mice have shown that the obesity phenotype in NPY-null *ob/ob* mice is only partially alleviated, implying that additional neuropeptides may be involved in the development of *ob/ob* phenotype (137).

In addition to NPY, other neuropeptide are also regulated by leptin. Administration of leptin increases corticotrophin-releasing hormone (CRH) mRNA levels in the PVN (143). CRH administration reduces food intake and stimulates the metabolic rate in genetically obese animals and lean controls through increasing sympathetically mediated energy expenditure (135). Leptin inhibits the orexigenic effects of galanin (GAL) and melanin-concentrating hormone (MCH) and decreases GAL mRNA as well as MCH expression in the hypothalamus (139). When administered centrally, MCH increases food intake in a dose-dependent manner and lowers plasma glucocorticoid levels through a mechanism involving adrenocorticotropic hormone (ACTH) (140). Since the MCH and leptin receptor are coexpressed in the lateral hypothalamus (LH), feeding evoked by MCH is possibly suppressed by a direct effect of leptin signaling (141). Pro-opiomelanocortins (POMC) is produced in a subset of neurons in the ARC where it is co-localized with leptin receptor (131). POMC levels in the ARC are lower in *ob/ob* mice than in wild type mice and leptin increases POMC to nearly normal levels in leptin-deficient mice, suggesting POMC neurons may mediate weight-reducing effects of leptin (142). Moreover, the studies of Boston *et al* have demonstrated a relationship between the leptin and POMC signaling pathways. The lethal yellow (A^y/a) mouse that has a defect in POMC signaling in the brain develops obesity. The leptin-deficient lethal yellow mice (A^y/a

ob/ob, double mutant) showed a significant restoration of leptin sensitivity after leptin administration (144). This observation suggests that leptin functions to regulate feeding behavior independent of POMC neurons. The specific product of POMC, α-melanocyte-stimulating hormone (α-MSH) inhibits feeding in mice and is an agonist of melanocortin-3 (MC3R) and MC4R, whose mutation is associated with development of human obesity (146-149). The inhibitory effect of α-MSH on feeding is completely blocked by administration of the MC-R antagonist SHU9119 (150). Additionally, agouti and agouti-related protein (AGRP) are endogenous antagonists of melanocortin receptor and overexpression of AGRP results in mice that are hyperphagic, hyperinsulinemic and obese (151-152). Leptin injection causes reduction of AGRP mRNA in wild type as well as in leptin deficient mice (153).

In addition to control effect of leptin, peripheral effects of leptin on body weight have also been reported (145, 156). Leptin directly inhibits triglyceride and free fatty acid formation in both adipocytes and nonadipocytes (e.g. liver, pancreas and skeletal muscle) by reducing acetyl-CoA carboxylase (ACC) activity and increasing lipid oxidation.

Cumulatively, leptin modulates not only neuropeptide/neuromodulator levels in most hypothalamic feeding centers through direct or possible indirect manners but also peripheral lipid metabolism to regulate energy balance and adiposity.

E. Leptin and obesity

Obesity is most likely a polygenic disease characterized by interactions between genetic and environmental factor (154). The most common complications of obesity include insulin resistance, type II diabetes mellitus and hypertension. The epidemic of obesity probably results from increasingly sedentary lifestyles and easy availability of palatable, high fat foods. Recently, candidate genes such as agouti, tubby, leptin and leptin receptor associated with

obesity have been identified and also mutations of genes which favor excess body fat have been characterized in these animal models (162). The vast majority of obese individuals exhibit elevated circulating leptin levels and appear to be leptin resistant similar to the syndrome of *db/db* obese animal models, suggesting that human *ob* mutations are extremely rare (122,155). Leptin resistance in human obesity has been suggested to be associated with impaired transport of leptin across the blood-brain barrier in the hypothalamus and impaired leptin signal pathways (100,155). Only a few mutations in the *ob* gene and receptors have been associated with human obesity. A homozygous frame-shift mutation involving the deletion of a single guanine nucleotide in codon 133 of the gene for leptin was found in two cousins born from extended family (157). Both children exhibit marked hyperphagia, excessive weight gains in early life and severe obesity. Another leptin missense mutation was found in a highly consanguineous extended Turkish pedigree to be associated with low plasma leptin, hyperphagia, hypogonadism, hyperglycemia and morbid obesity (158). Moreover, a homozygous mutation in the human leptin receptor gene results in a truncated leptin receptor lacking both transmembrane and intracellular domains and is associated with early-onset morbid obesity, reduced growth hormone secretion as well as no pubertal development (160). More recently, cohort studies have shown that daily subcutaneous recombinant leptin injection in both lean and obese subjects reduced body weight and fat mass in a dose-response manner, including in some obese subjects with elevated endogenous serum leptin concentration (161). Also, injection of recombinant leptin has induced a sustained reduction in weight, predominantly as a result of a loss of fat in a nine-year-old patient with congenital leptin deficiency (163). Consequently, leptin as well as its agonists and antagonists are emerging as novel therapeutic targets in effective drug development and strategies in treatment of obesity.

IV. EFFECTS OF ARACHIDONATE METABOLITES ON ADIPOGENESIS

A. Arachidonate metabolites: pathways of biosynthesis

Among foods commonly consumed in the American diet, meat (chicken thigh) and eggs are good sources of arachidonate (181). The formation of arachidonate (20:4 n-6) is a process involving the elongation and desaturation of dietary linoleic acid (18:2 n-6) or direct consumption of arachidonate. Arachidonate is found in cells with relatively abundant amounts but almost exclusively incorporated at the *sn*-2 position of membrane phospholipid (164). Arachidonate from phospholipids is released by the action of hormone-regulated phospholipases (165). Free, arachidonate is processed by cyclooxygenases (COXs), lipoxygenases or cytochrome p450 oxygenases to the various physiologically important eicosanoids (Fig.1). Two COX isoforms have been identified and are referred to as COX-1 and COX-2. Both convert arachidonate to prostaglandin G_2 (PGG_2). PGG_2 is subsequently reduced to prostaglandin H_2 (PGH_2) by the peroxidase activity and then PGH_2 is transformed by a range of enzymes and nonenzymatic mechanisms into biologically active prostanoid products such as PGE_2, PGI_2, $PGF_{2\alpha}$, PGD_2 and TXA_2 (166). The COX-1 activity is constitutively present in most cell types at a constant level, whereas COX-2 is rapidly induced only after exposure of cells to hormones, growth factors, mitogenic and inflammatory stimuli, cytokines such as interleukin-1 or 2 and tumor promoters (168). COX-1 is responsible for the biosynthesis of PGs in the gastric mucosa and in the kidney, implying a role for COX-1 in gastric and renal homeostasis (169). In contrast, COX-2 induces production of PGs at inflammatory sites and during pain and thus COX-2-derived PGs play a role as inflammatory

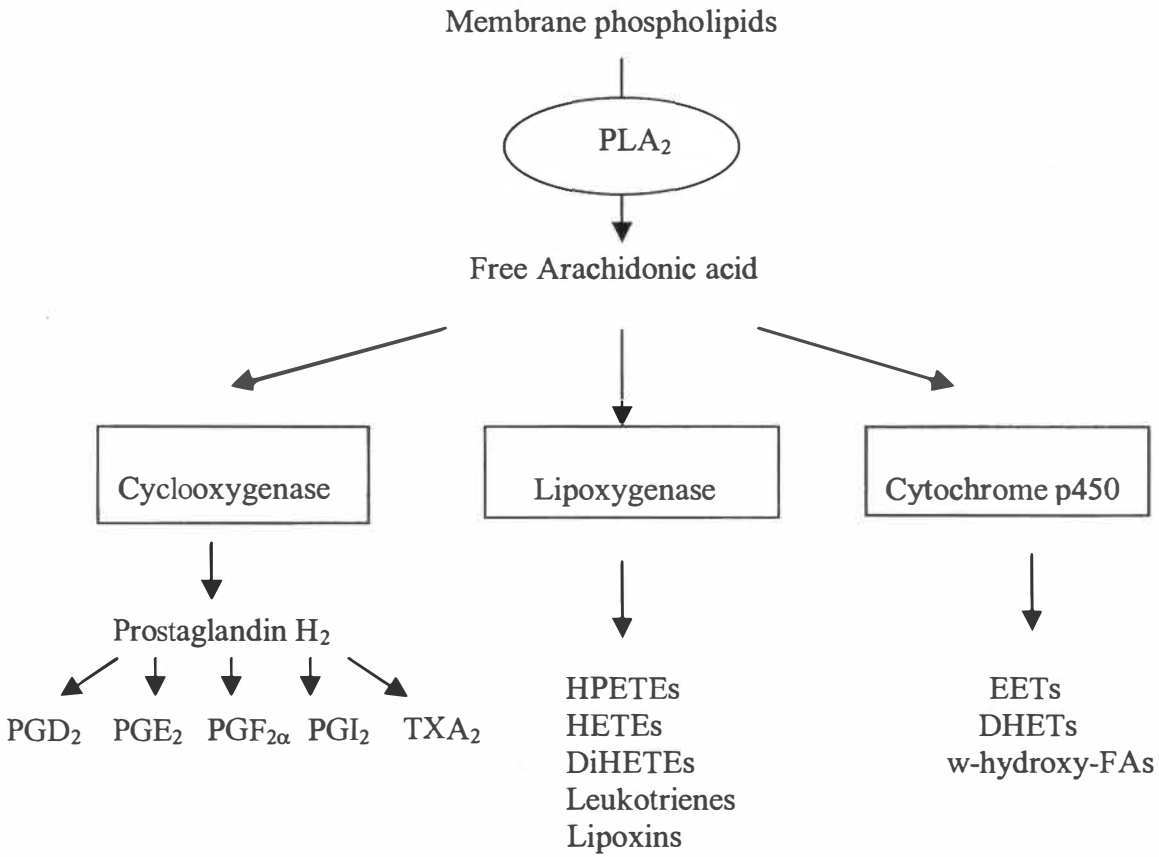

Fig 1. Arachidonate metabolites metabolism

mediators in the injured tissue (169). Additionally, COX-2 expression is markedly enhanced in various cancer cells, indicating that this enzyme is also involved in carcinogenesis (169). Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and indomethacin inhibit both COX-1 and COX-2 to different extents and compete with arachidonate for binding to the COX active site (171). These compounds exert effective anti-inflammatory actions but also unwanted side effects such as ulcerogenesis (169). NSAIDs can be classified into three groups based on their binding kinetics with the COXs. Class I compounds are simple competitive inhibitors which include ibuprofen and piroxicam. Class II compounds include indomethacin, flurbiprofen and meclofenamate, which bind to the COXs leading to an initial *E*I (enzyme and inhibitor) complex, but this *E*I complex slowly rearranges to an *E*I* complex from which the inhibitors dissociate very slowly (minutes to hours). Thus, these are known as time-dependent and reversible inhibitors. Aspirin is one of the class III inhibitors, which are competitive, time-dependent and irreversibly and covalently modify COXs. It acetylates a serine residue positioned in the arachidonate-binding channel, thereby blocking the binding of the fatty acid substrate to the active site for its oxygenation and also exhibits greater potency (10 to 100 times) against COX-1 than against COX-2 (172).

B. Role of arachidonate metabolites in adipogenesis

Arachidonate and its oxidized derivatives have been known as important mediators of many physiological and pathophysiological processes. Furthermore, several studies have demonstrated that arachidonate metabolites modulate positively or negatively differentiation and maturation of adipose tissue (37). Prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) have been described as two major PGs produced from rodent and human adipocytes (6, 159). In addition, low levels of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) released from adipocytes have been reported

(6). Recently, the studies of Vassaux *et al* have shown that PGE_2 negatively modulates cAMP production and thus lipolysis in rat and human adipocytes via interaction with its specific receptors (25). This suggests that the antilipolytic effect of PGE_2 contributes to hypertrophic development of adipose tissue (25). Conversely, PGI_2 and carbaprostacyclin ($cPGI_2$), its stable analogue, are proposed to act as adipogenic-hyperplastic effectors. Differentiation of Ob1771 preadipocytes is stimulated by $cPGI_2$. This effect is mediated by increased cAMP production and free intracellular calcium release, leading to an elevation of fat cell number from predominant preadipocytes (3,170). More recently, studies by Hertz have demonstrated that $cPGI_2$ activates the three known mammalian PPARs (α,δ and γ), indicating that its ability to promote differentiation may be mediated by PPARs (175). Another PG, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2) is an important ligand for PPARγ, an adipogenic transcription factor, that induces adipogenesis of cultured fibroblasts (35). In addition, prostaglandin D_2 (PGD_2) derivatives have been reported to be efficacious activators of both PPARα and PPARγ (130). In contrast, $PGF_{2\alpha}$ has been shown to inhibit the differentiation of 3T3-L1 and rat preadipocytes (173). In murine 3T3-L1 cells, inhibition of differentiation by $PGF_{2\alpha}$ is mediated by specific binding to a FP receptor that activates phospholipase C, resulting in the formation of inositol phosphate and mobilization of intracellular calcium. Moreover, FP receptor stimulation causes activation of calcium/calmodulin-dependent protein kinase (CaMK) and an increase in DNA synthesis without cell proliferation. Addition of CaMK inhibitor KN-62 blocks the inhibitory effects of $PGF_{2\alpha}$ in the presence of the FP receptor agonist. In combination, this suggests that CaMK and augmentation of DNA synthesis are responsible for $PGF_{2\alpha}$-mediated inhibition of differentiation (20,138,167). Further, 8-(S)-

hydroxyeicosatetraenoic acid (HETE), a product of the lipoxygenase pathway, has been reported as an activator of PPARγ2 leading to differentiation of 3T3-L1 preadipocytes (19). Interestingly, recent research has investigated effects of inhibitors of PG biosynthesis on adipocyte differentiation. The studies of Lehmann *et al* have addressed the mechanism of stimulation of differentiation by indomethacin. At low concentrations, indomethacin exhibits its inhibitory effects on COXs, but at high concentrations, it directly binds and activates PPARα and γ and thus induces the differentiation of C3H10T1/2 fibroblasts, suggesting that PPARγ plays an important role as a mediator in indomethacin-induced adipogenesis (39). Furthermore, the role of indomethacin in *ob* gene expression, a known marker of terminal differentiation, has been examined by using differentiating and fully differentiated 3T3-L1 adipocytes. Indomethacin exerts inhibitory effects on leptin secretion at the concentration at which it activates PPARγ in differentiating and fully differentiated adipocytes, implying that indomethacin-induced PPARγ activation may be involved in initial-stage rather than the later-stage of differentiation (174). Consequently, arachidonate metabolites produced locally by adipocytes participate in adipocyte growth and development via interactions with not only their cell surface receptors but also nuclear receptors and thus may act as important paracrine/autocrine effectors in adipogenesis.

In summary, adipose tissue secretes various bioactive molecules including AII, leptin and prostaglandins. Available data indicate that AII increases triglyceride content and *ob* gene transcription in adipocytes. In addition, AII modulates adipocyte differentiation via a prostaglandin-dependent mechanism. The objective of this thesis is to investigate whether AII modulates leptin secretion via a prostaglandin-dependent mechanism.

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PART III

ANGIOTENISN II INCREASES LEPTIN SECRETION IN ADIPOCYTES VIA A PROSTAGLANDIN-INDEPENDENT MECHANISM

This manuscript is being prepared for publication with co-authors Whelan, J. and Moustaid-Moussa, N in the Journal of Nutrition

I. ABSTRACT

We previously reported that AII increases fatty acid synthesis and triglyceride storage in adipose tissue. Several studies have demonstrated that triglyceride stores or adiposity correlate significantly with the amount of circulating leptin. AII has been shown to increase adipocyte differentiation and growth by promoting prostaglandin production in OB1771 cell line. The purpose of this study was to determine whether AII regulates leptin secretion via a prostaglandin-dependent mechanism in adipocytes. Physiological doses of AII significantly increased leptin secretion by 3T3-L1 adipocytes and human adipose tissue in a dose-and time-dependent manner. Elevation of prostaglandin secretions was elicited at physiological concentrations of AII ($p<0.05$). Secretions of 6-keto $PGF_{1\alpha}$, a stable derivative of PGI_2, and prostaglandin E_2 were significantly induced by physiological concentrations of AII in a time-response fashion ($p<0.05$). Inhibition of prostaglandin synthesis by indomethacin and aspirin significantly suppressed basal as well as AII-induced prostaglandin levels, but did not significantly affect basal and AII-induced leptin secretions. In conclusion, regulation of leptin secretion by AII in adipocytes is not modulated by a prostaglandin-dependent mechanism.

II. INTRODUCTION

Angiotensin II (AII) is well known as a vasoactive peptide hormone that regulates blood pressure as well as fluid and electrolyte balance (1). AII is classically synthesized by two enzymatic steps in which renin and angiotensin converting enzyme (ACE) cleave the precursor angiotensinogen produced by the liver (1). Local synthesis of RAS has been found in liver, kidney and various tissues including adipose tissue (2). In brain and

kidney, RAS has been shown to control morphological development (29, 34). Adipose tissue represents the potentially largest sources of the angiotensinogen (AGT), especially in obese patients (5-6, 31,13). Angiotensinogen (AGT) mRNA expression in mouse adipose tissue is reduced by fasting and enhanced by overfeeding (6). AGT gene expressed in human adipose tissue is regulated nutritionally as well as hormonally, providing important insights into the function of adipocyte-derived AII in adipocyte metabolism (9). Data from cultured murine adipocytes indicate an adipogenic role of AII in adipocyte growth and development (4,12,14). Increased AGT protein synthesis in adipocytes of genetic obesity models (*ob/ob* and *db/db*) suggests a potential link between obesity development and functional local AII in adipose tissue (6). Epidemiological studies show a tight correlation between accumulation of intra-abdominal fat and hypertension (10). Cumulatively, these data suggest that adipocyte hypertrophy is accompanied by an elevated activity of RAS. In addition to its function in adipose tissue growth, AII might contribute to obesity as well as obesity-associated hypertension, although the contribution of adipocyte-derived AII to pathophysiological hypertension is largely unknown.

Leptin is the protein product of *ob* gene cloned by Friedman's group in 1994 and is primarily expressed in adipose tissue (8). Leptin circulates in the blood and acts on the central neural network that regulates weight and energy (17,19). Defects in the *ob* gene as well as leptin receptor genes cause obesity in *ob/ob* and *db/db* mice and humans (rare mutations) (8,16, 21). Most human obesity is positively correlated with elevated serum concentrations of leptin as well as decreased sensitivity to leptin, inducing leptin resistance (20-21, 28). Leptin administration leads to decreased food consumption and

increased energy expenditure, both of which result in loss of adipose mass in lean, obese and diet-induced obese (DIO) models, indicating a weight-reducing and anti-obesity effect of leptin (18, 27). Investigations into the mechanisms of leptin (a marker of adiposity) regulation by AII may provide new insights into the development of therapeutic approaches to treat obesity.

Previous studies from our lab have demonstrated that AII activates *ob* gene expression (12). I*n vivo* data have shown that mice treated with an AT_2 receptor antagonist, PD123139 display significantly decreased leptin content of adipose tissue concomitant with decreased epididymal fat pad mass (Kim *et al* 1999). These results provides additional evidence that AII functions as an adipogenic factor in adipocyte metabolism and support potential mechanisms for the effect of AII on adiposity.

Mechanistically, AII has been shown to enhance adipocyte differentiation by AT_2-dependent generation of prostacyclin (14). Studies by Borglum *et al* (15) have demonstrated that AII increased expression of PGHS-1 and PGHS-2 mRNA in mature adipocytes leading to prostaglandin production (15). Prostacyclin and prostaglandin E_2 have been shown to exert their roles as paracrine or autocrine adipogenic effectors in adipocyte growth and development (7, 11, 24). However, the mechanism of AII regulation of adiposity remains under investigation.

We hypothesized that AII might regulate leptin in adipocytes via a prostaglandin-mediated mechanism. We first analyzed dose and time effects of AII on adiposity and prostaglandin secretions in adipocytes, using leptin as an adiposity marker. The effects of prostaglandin biosynthesis inhibition by indomethacin and aspirin on AII regulation of leptin in adipocytes were investigated. We confirmed the inhibitory effect of

prostaglandin by indomethacin is independent of regulation of leptin secretion by AII in adipocytes. This study may provide evidence of a potential mechanism of AII regulation of adiposity as well as clues for AII signaling pathway in adipocyte metabolism.

III.RESEARCH DESIGN AND METHODS

A. Experimental design (Fig.1)

Experiment 1: we have demonstrated that dose and time-response study of leptin secretion to AII treatment in human adipose tissue and 3T3-L1 adipose cells. We conducted dose and time-response analyses of 6-keto $PGF_{1\alpha}$ and PGE_2 secretions in response to AII in 3T3-L1 adipose cells only.

Human adipose tissue: Human adipose tissue was cultured in 6-well (35mm) plates in DMEM supplemented with 1% FBS then serum sustained for 24h prior to treatment with serum-free media (1% BSA) containing 10nM AII or 10nM insulin used as a positive control for 48h. Leptin content in culture media was then assayed using RIA. In the time-response studies, human adipose tissue was incubated with 10nM AII or without AII for the indicated time intervals (3, 6, 12, 24 and 48h). The culture media were collected at the indicated time points to measure leptin level. Data were expressed as leptin (ng/ml) per g of tissue.

3T3-L1 adipocytes: Optimal responses of secreted leptin and cyclooxygenase-derived eicosanoids (6-keto $PGF_{1\alpha}$ and PGE_2) to AII treatment were established by determining an optimal dose-response (1pM-1μM) and time-dependence (0, 3, 6, 12, 24, 36, 48, 72 and 96 h) curve. 3T3-L1 adipose cells were cultured with serum-free media overnight in 100mm culture dishes or 6-well (35 mm) plates and then treated with AII (Sigma

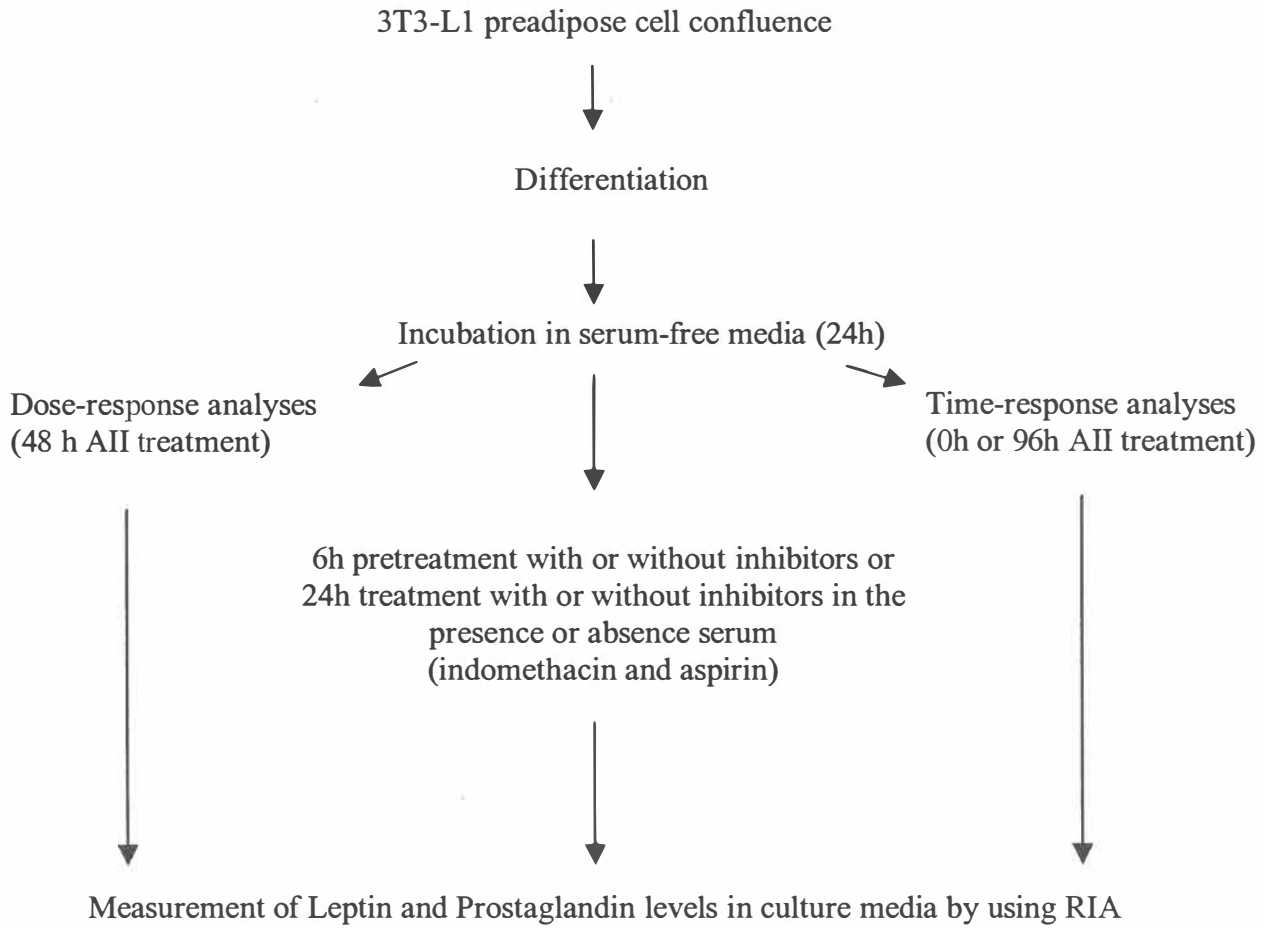

Fig 1. Experimental protocol using 3T3-L1 adipose cells

Chemical Co., St. Louis, MO) in serum-free media. In dose-response studies, cells were incubated for 48h with or without AII. After 48h of treatment culture media were collected to measure leptin and prostaglandin levels. To analyze time-response, culture media of 3T3-L1 adipose cells treated with 10nM AII or without AII were used to measure the levels of leptin, 6-keto $PGF_{1\alpha}$ and PGE_2 at the indicated time points. Cell extracts were prepared for cellular protein contents. The levels of leptin and eicosanoids were normalized to dish or cellular protein levels.

Experiment 2: we investigated the effect of prostaglandin biosynthesis inhibition by indomethacin and aspirin on AII-induced prostaglandin and leptin secretions in 3T3-L1 adipocytes. Indomethacin and aspirin used in this study inhibit both COX-1 and COX-2. Indomethacin is a time-dependent and reversible inhibitor whereas aspirin is time-dependent and irreversible (22). Inhibition of cyclooxygenase was conducted before AII treatment. Adipose cells were grown in 6-well plates and were preincubated with serum-free media in the absence or presence of various concentrations (1, 10, 50 or 100 μM) of indomethacin and aspirin dissolved in appropriate solvent (0.2 % ethanol of final conc.) for 6 h. After pretreatment with inhibitors, cells were treated with 10nM AII or vehicle alone and maintained for 24h to investigate prostaglandin-mediated AII regulation of leptin secretion. In separate experiments, cells were simultaneously treated with indomethacin and AII. Cultured media were collected for determining leptin and eicosanoid levels and cell extracts were prepared for cellular protein contents. The levels of leptin and eicosanoids were normalized to dish or cellular protein contents.

Experiment 3: we examined the effect of indomethacin on AII regulation of leptin and 6-keto $PGF_{1\alpha}$ secretions in the presence or absence of serum.

Cells were incubated in either 10% FBS or 1% BSA. AII (10nM), indomethacin (10μM), or AII plus indomethacin treatments were carried out in both media for 24h and controls were treated with vehicle only. The levels of leptin and 6-keto $PGF_{1\alpha}$ secreted from both media were measured by using RIA. The levels of leptin and eicosanoids were normalized to cellular protein levels.

B. Methods

1. Culture of human adipose tissue

Subcutaneous abdominal adipose tissue was taken from one subject, 26 yr of age, undergoing elective cosmetic surgery. A sample was obtained in compliance with a protocol approved by the Institutional Review Board for Human subjects and by the Committee for Research Protocols at the University of Tennessee, Knoxville. This subject was non-diabetic and non-obese with normal body mass index (BMI: 24.3 m/kg^2) and had no known metabolic abnormalities. Tissue was minced into 0.1-0.2 g pieces and washed several times with Hank's medium to remove blood clots. Tissue fragments (~1 g/well) were primary cultured in DMEM supplemented with 1% FBS media, antibiotics (penicillin-100U/ml, streptomycin-100μg/ml and gentamicin-50μg/ml) for two days. Human adipose tissue was incubated with serum-free media containing 1% BSA for 24h prior to treatment.

2. Culture of 3T3-L1 adipose cells

3T3-L1 adipose cell lines are derived from mouse embryo (33). Cells were purchased from the American Type Culture Collection (ATCC) and were grown in 100 mm dishes or 6-well (35 mm) plates and cultured in DMEM supplemented with 10% FBS and 1%

penicillin/streptomycin (regular media); 3T3-L1 cells (~200,000 cells/100 mm dish; 50,000 cells/35mm dish) were plated at 0 days and grown for 3-4 days (confluence). At confluence, the media were supplemented with 250nM dexamethasone and 0.5mM isobutyl methylxanthine (MIX) for 72 h, after which cells were cultured with regular media (29). Differentiation was considered to be complete at 5-7 days after postconfluence. All the studies were conducted in differentiated cells (~80-95%). Prior to any treatment with AII or inhibitors, cells were incubated for 24h in serum free media containing 1% BSA.

3. Quantitation of leptin in the media

Leptin secreted in the media was determined by radioimmunoassay (RIA) that is a subclass of competitive protein binding assay in which radiolabeled antigen (hormone) complete with unlabeled antigen for sites on its antibody. The measurement of the amount of radioactivity determines the amount of antigen present (30). The mouse leptin RIA kit used in this study were purchased from Linco Research (St Charles, MO). 100 µl of media collected was used and all samples were measured in duplicate. Leptin level was corrected to mg of cellular protein contents or dish (well) for 3T3-L1 adipocytes and g of tissue for human adipose tissue.

4. Prostaglandin assay

Culture media were treated with cold acid methanol solution (MeOH: Formic acid, 9:1(v/v), pH 3.5). Prostaglandins were isolated by solid-phase extraction using an octadecyl C_{18} cartridge (Burdick & Jackson, Muskegon, MI) and eluted with 100 % methanol. The methanol was evaporated under an atmosphere of nitrogen and the extracts were resuspended with 250µl of 0.1M PBSG (Phosphate Buffered Saline (0.9 %) with

Gelatin (0.1%), pH7.4). 100 μl of resuspended solution was used for measuring contents of each prostaglandin. The amount of PGE_2 and 6-keto $PGF_{1\alpha}$ was assayed by RIA using antiserum obtained from PerSeptive Diagnostics, Inc (Cambridge, MA). All standards were purchased from Cayman Chemical (Ann Arbor, MI), and [^{3}H] PGE_2 and [^{3}H] 6-keto $PGF_{1\alpha}$ were obtained from New England Nuclear (Boston, MA). All experiments were repeated twice. Data are expressed as pg of PGE_2 and 6-keto $PGF_{1\alpha}$ per dish (well) or mg of cellular protein contents.

5. Protein assay

The cellular protein contents were measured by Bradford method (32) that is a dye-binding assay. The acidic blue dye binds to primarily basic and aromatic amino acid residues, especially arginine. A differential color change of the dye in response to various protein concentrations is spectrophotometrically measured. Adipocytes were harvested by scraping into 500μl of 0.5M sucrose (pH 7.4), 1mM dithioreitol, 1mM EDTA and 100mM phenylmethylsulfonyl fluoride (PMSF). Cell homogenates were sonicated for 5 sec and after 1h of ultracentrifugation (12,000 x g) at 4 °C supernatants were taken to assay cellular protein contents. 25 μl of cell extracts was used and all samples were assayed in duplicate.

6. Statistics

Data first were analyzed by one-way analysis of variance (ANOVA). When significant F ratios were obtained, differences among different concentrations of AII were analyzed by Bonferroni test for multiple comparison (SAS, Cary, N.C.). The significant differences between means with the same variances were evaluated by

independent student's *t* test. All values were expressed as mean ± SEM. Values of $P<0.05$ were considered statistically significant.

IV. Results

Effect of AII on leptin secretion in human adipose tissue

Previous studies showed that physiological concentration of AII upregulates *ob* gene transcription in human adipocytes (12). We first investigated the effect of AII on leptin secretion using culture of human adipose tissue. In good agreement with the AII effect on *ob* gene transcription, both AII and insulin (used as a positive control) significantly increased leptin secretion (Fig. 2). When human adipose tissue was exposed to AII (10nM) for various lengths of time, leptin secretion progressively increased from 3h to 24h (Fig. 3). At 24 h, leptin secretion was stimulated by approximately 3.5-fold compared to controls ($p<0.001$). Similarly, AII exhibited significant stimulation of leptin secretion at 48h compared to controls. However, the decrease in AII-induced leptin level between 24 h and 48h may result from leptin protein degradation. These results indicate that physiological concentration of AII increases leptin secretion after 24h in human adipose tissue.

Effect of AII on leptin and prostaglandin secretions in 3T3-L1 adipocytes

We investigated the dose and time-response effects of AII on leptin and prostaglandin secretions in 3T3-L1 adipose cells.

Leptin: Consistent with the increase in transcription rate of *ob* gene upon AII treatment (12), AII at concentrations of 1 and 10nM significantly increased leptin

secretion by 3T3-L1 adipose cells at 48h by 24 ± 9.8% and 57± 6.7%, respectively (Fig. 4). Significant effect of AII on leptin secretion was observed at 12, 24, 36 and 48h by 10nM of AII treatment (Fig. 7) when leptin secretion was increased by approximately 3-fold over control levels ($p<0.01$).

Protstaglandins: Similarly, prostaglandin secretions were significantly stimulated by physiological doses of AII (Fig. 5 and 6). Significant effect of AII on 6-keto $PGF_{1\alpha}$ secretion (Fig. 5) was present at 48h of treatment with 1nM and 10nM AII ($p<0.05$). In concordance, 3T3-L1 adipose cells cultured with 1nM and 10nM AII also increased PGE_2 secretion (Fig. 6) at 48h ($p<0.05$). AII treatment (10nM) markedly stimulated 6-keto $PGF_{1\alpha}$ secretion from 3T3-L1 adipose cells within 24h and this stimulatory effect by AII was sustained for an additional 72h (Fig. 8). AII significantly elevated PGE_2 secretion within 24h and this level was maintained through 96h (Fig. 9). Consistent with significant induction of prostaglandin secretions by AII (10nM) after 24 h, AII at concentration of 1nM significantly enhanced secretions of both prostaglandins after 24h (data not shown).

AII at physiological concentrations increased leptin and prostaglandin secretions from 3T3-L1 adipose cells in a time-dependent manner. These results are consistent with the previously reported induction of *ob* and PGHS gene expressions by AII in murine adipose cell lines (12,15) and with induction of 6-keto $PGF_{1\alpha}$ secretion by the presence of AII stimulus (14).

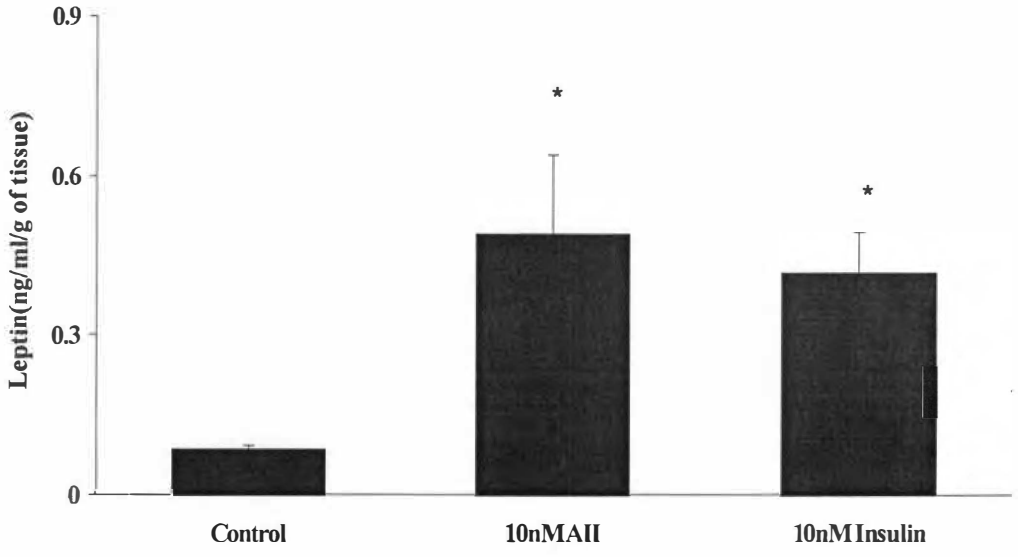

Fig. 2 Effect of AII on leptin secretion in human adipose tissue. Isolated human adipose tissue was cultured in DMEM supplemented with 1% FBS for 2 days and maintained serum-free media for 24h before treatment. Cells were treated for 48h with serum-free media supplemented with 10nM AII, 10nM insulin and control adipose tissue was treated with serum-free media containing 1% BSA. Leptin concentration in the culture media was measured by radioimmunoassay (RIA). Data shown are expressed as mean ± SEM (n=3). Statistical differences from the corresponding control-treated and AII or insulin-treated values are shown as $p<0.05$(*).

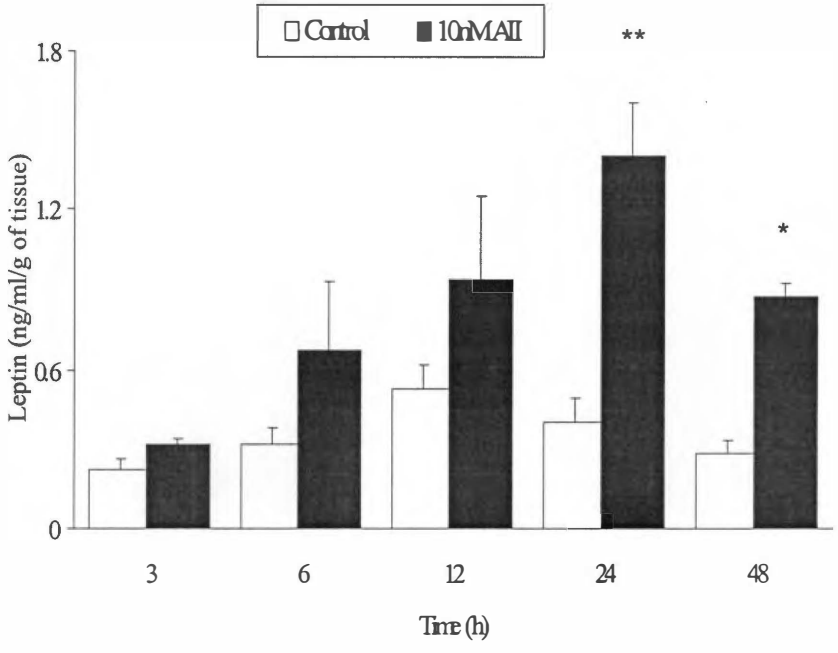

Fig. 3 Time-response effect of AII on leptin secretion in human adipose tissue. Isolated human adipose tissue was maintained in DMEM supplemented with 1% FBS for 2 days and incubated with serum-free media for 24h before treatment. Adipose tissue was treated for 48h with serum-free media supplemented with 10nM AII or without AII as control. The culture media were collected at 3, 6, 12, 24, and 48h and leptin concentration in the culture media was measured by RIA. Data shown are expressed as mean ± SEM (n=3). Statistical differences from the corresponding control-treated and AII-treated values at the indicated time points are shown as $p<0.01$(*) and $p<0.001$(**).

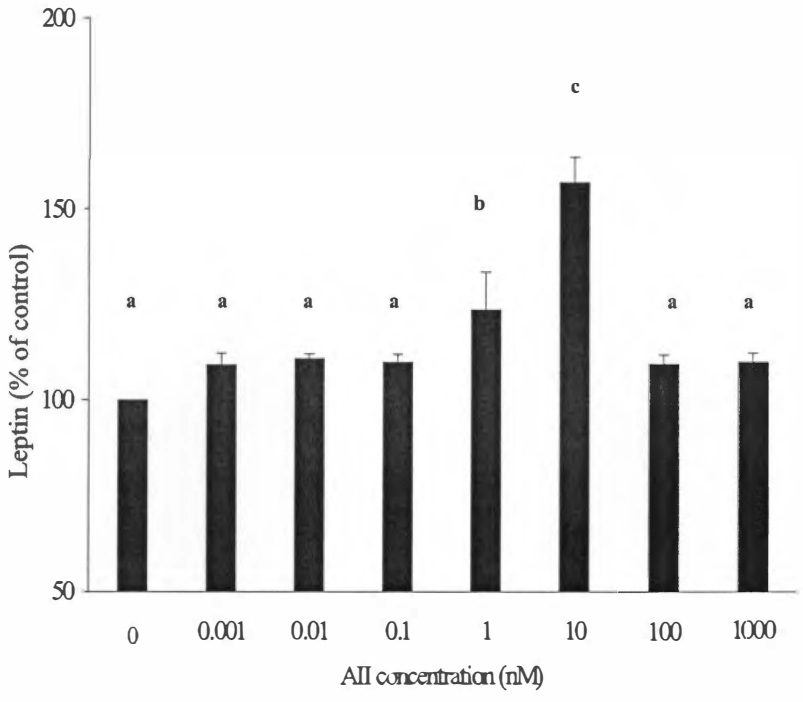

Fig. 4 Dose effect of AII on leptin secretion in 3T3-L1 adipose cells. Fully differentiated adipocytes were incubated with serum-free media containing graded concentrations of AII (1pM-1μM) or without AII as control for 48h. Leptin secreted in medium was measured by using RIA at the specified time. Results are expressed as mean ± SEM (n=9). Data shown are analyzed by ANOVA and Bonferroni multiple-comparison procedure. The values with different letters are significantly different at $p<0.05$.

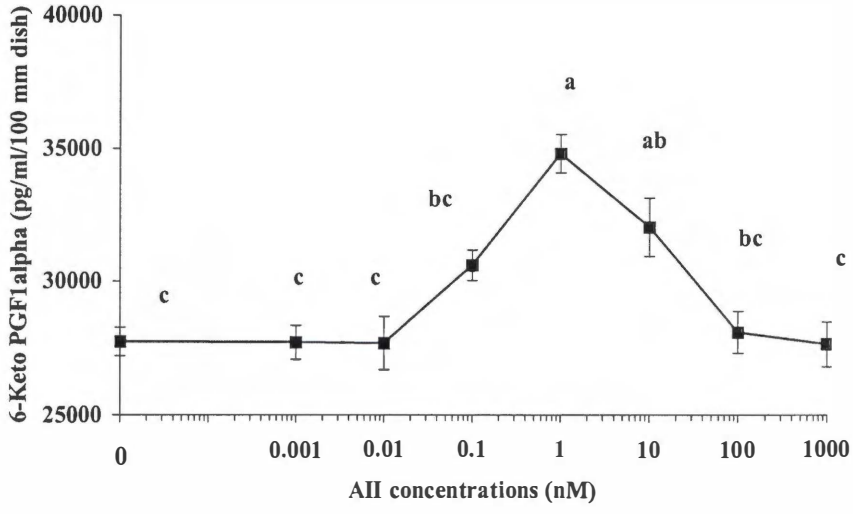

Fig. 5 Dose effect of AII on 6-keto $PGF_{1\alpha}$ secretion in 3T3-L1 adipose cells. Differentiated 3T3-L1 adipocytes were incubated with serum-free media supplemented with increasing concentrations (1pM-1μM) of AII or without AII for 48 h. The amount of 6-keto $PGF_{1\alpha}$, the stable degradation product of PGI_2, in the culture media was measured by RIA. Data are expressed as mean ± SEM (n=3). Results are analyzed by ANOVA and Bonferroni multiple comparison procedure. The values with different letters are significantly different at $p<0.05$.

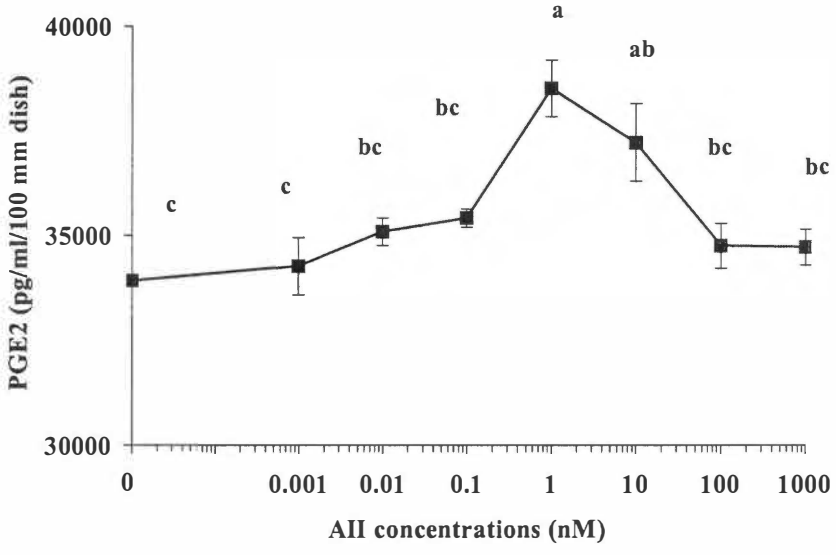

Fig. 6 Dose effect of AII on PGE_2 secretion in 3T3-L1 adipose cells. Differentiated 3T3-L1 adipocytes were incubated with serum-free media supplemented with increasing concentrations (1pM-1μM) of AII or without AII for 48h. PGE_2 concentration in the culture media was measured by RIA. Data are expressed as mean ± SEM (n=3). Results are analyzed by ANOVA and Bonferroni multiple comparison procedure. The values with different letters are significantly different at $p<0.05$.

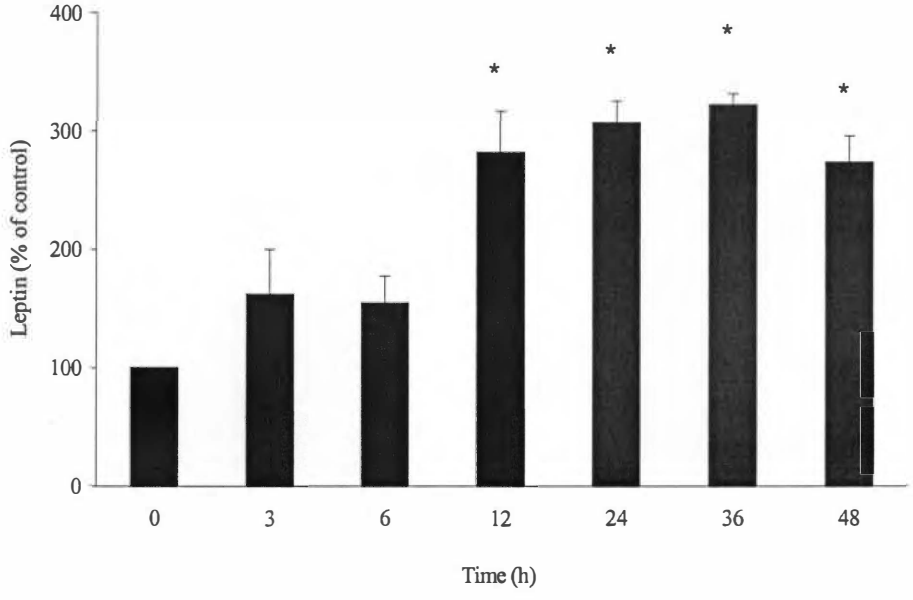

Fig. 7 Time-response effect of AII on leptin secretion in 3T3-L1 adipose cells. Differentiated adipose cells were incubated for 24h with serum-free media supplemented with 10nM AII and culture media were collected at 0, 3, 6, 12, 24, 36 and 48h. The amount of leptin secreted in the culture media was assessed by RIA. Data shown are expressed as mean ± SEM (n=3). Statistical differences from the basal concentration of leptin at time zero for 3T3-L1 adipose cells are shown as $p<0.01$(*).

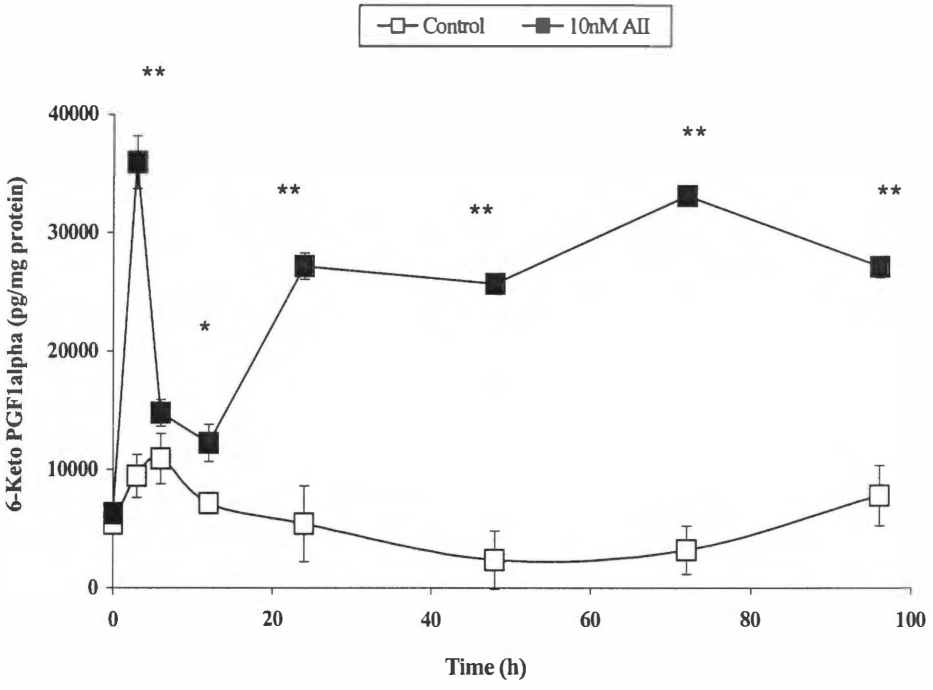

Fig. 8 Time-response effect of AII on 6-keto $PGF_{1\alpha}$ secretion in 3T3-L1 adipose cells. Differentiated adipose cells were incubated for 96h with serum-free media containing 10nM AII or without AII and the culture media were collected at 0, 3, 6, 12, 24, 48, 72 and 96h. The amount of 6-keto $PGF_{1\alpha}$, a stable derivative of PGI_2, in the culture media was assayed by RIA and normalized to milligram of cellular protein values. Data shown are expressed as mean ± SEM (n=5-6). Statistical differences between control-treated and AII-treated values at the indicated time points are shown as $p<0.01$(*) and $p<0.0001$(**).

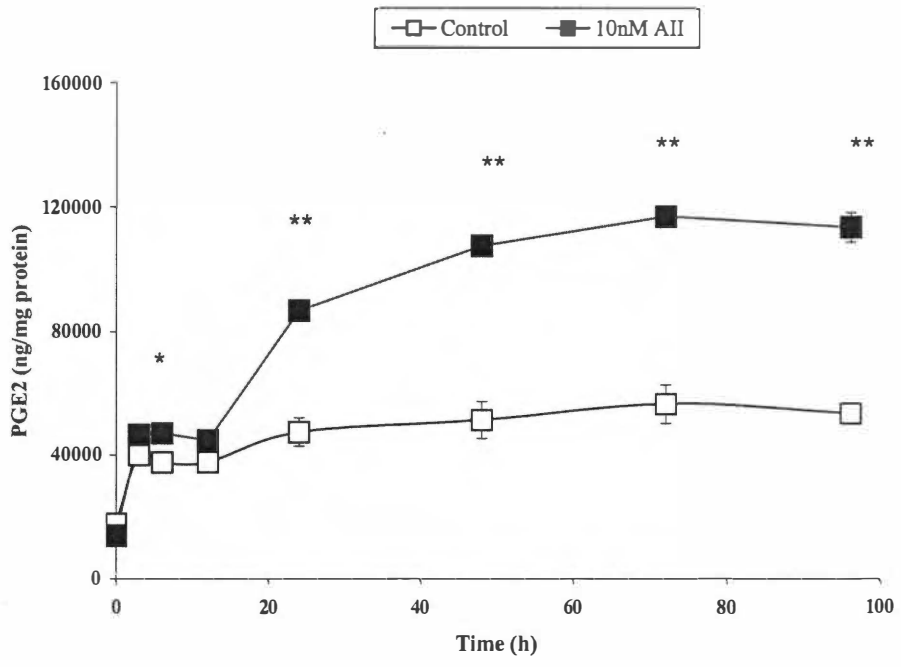

Fig. 9 Time-response effect of AII on PGE_2 secretion in 3T3-L1 adipose cells. Differentiated adipose cells were maintained in serum-free media with 10nM AII or without AII for 96h and cultured media were collected at 0, 3, 6, 12, 24, 48, 72 and 96h. PGE_2 concentration in the culture media was measured by RIA and corrected to milligram of cellular proteins. Data shown are expressed as mean ± SEM (n=5-6). Statistical differences between the corresponding control-treated and AII-treated values at the indicated each time points are shown as $p<0.05$ (*) and $p<0.001$(**).

Effect of indomethacin and aspirin on AII-induced prostaglandin and leptin secretions in 3T3-L1 adipocytes

To further investigate the mechanisms through which AII increases leptin secretion in adipocytes, effect of prostaglandin synthesis inhibition by indomethacin and aspirin on AII-induced prostaglandin and leptin secretions was conducted.

Prostaglandins: As anticipated, pretreatment (6h prior to 10nM AII of treatment) of indomethacin (Fig. 10) and aspirin (Fig. 11) at concentrations of 1, 10, 50 and 100μM significantly inhibited both basal and AII-induced 6-keto $PGF_{1\alpha}$ secretions in a dose-response manner ($p<0.05$). The concentration of 10μM of indomethacin maximally reduced 6-keto $PGF_{1\alpha}$ synthesis in both vehicle and AII-treated cells by approximately 43% and 58%, respectively (Fig. 10). Similarly, aspirin at a concentration of 10μM maximally inhibited basal and AII-induced 6-keto $PGF_{1\alpha}$ levels by ~45 and 65%, respectively (Fig. 11). These similar reductions (~35-60%) in basal and AII-induced 6-keto $PGF_{1\alpha}$ levels were also noted when either inhibitor was added concomitantly with AII for 24h (data not shown). However, both inhibitors decreased basal and AII-induced PGE_2 secretions to a lesser extent (~10-30% reduction) compared to those observed with 6-keto $PGF_{1\alpha}$ (Fig. 12 and 13). This differential inhibition of 6-keto $PGF_{1\alpha}$ *versus* PGE_2 by indomethacin and aspirin was unexpected.

Leptin: The effects of maximal inhibitory concentration (10μM) of indomethacin (Fig. 14) and aspirin (Fig. 15) on basal as well as AII-stimulated leptin secretions were evaluated. Inhibition of AII-induced prostaglandin synthesis by 6h of pretreatment with 10μM indomethacin did not modify basal or AII-induced leptin secretion (Fig. 14).

Similarly, aspirin did not alter basal or AII-induced leptin level. Consistent with these results, the inhibitory effect of indomethacin and aspirin when added simultaneously with AII for 24h did not affect basal or AII-increased leptin secretion (Fig. 15).

Effect of indomethacin on AII-induced prostaglandin and leptin secretions from 3T3-L1 adipocytes in the presence or absence of serum

To confirm the effect of indomethacin on AII-induced leptin secretion, we used 10% fetal bovine serum (FBS) as a positive control and 1% bovine serum albumin (BSA) of culture media supplemented with AII (10nM), indomethacin (10μM), and AII (10nM) plus indomethacin (10μM). FBS was used to elicit maximal induction of prostaglandins as previous studies have demonstrated that FBS stimulates prostaglandin synthesis in adipocytes (37).

Prostaglandins: We have examined the inhibitory effect of 6-keto $PGF_{1\alpha}$ secretion by indomethacin. An inhibitory effect of indomethacin (10μM) for 24h on basal and AII-induced 6-keto $PGF_{1\alpha}$ secretions in both media (Fig 16). Indomethacin treatment significantly reduced basal and AII-induced 6-keto $PGF_{1\alpha}$ secretions by approximately 66% and 79%, respectively in 10% FBS media. Similarly, indomethacin significantly blocked basal and AII-induced 6-keto $PGF_{1\alpha}$ secretions in 1% BSA media. Accordingly, we confirmed an inhibitory effect of indomethacin on basal and AII-induced 6-keto $PGF_{1\alpha}$ secretions from 3T3-L1 adipose cells in both the presence and absence of serum. Interestingly, compared to 1% BSA media, higher inhibitory effect of indomethacin on basal and AII-induced 6-keto $PGF_{1\alpha}$ levels were observed in 10% FBS media at 24h. A possible explanation for lower basal and AII-induced 6-keto $PGF_{1\alpha}$ secretions in 10%

FBS media by indomethacin is the high cellular protein contents in FBS-treated cells, resulting in lower prostaglandin/protein level in FBS-treated cells *versus* BSA-treated cells; these data also were normalized per well (Appendix, Fig.1). Along this possibility, we have examined the levels of 6-keto $PGF_{1\alpha}$ and PGE_2 in the presence or absence of serum with or without 3T3-L1 adipose cells; these data were corrected to well or total cellular proteins (Appendix, Fig 3-5). It seems clear that lower basal and AII-induced 6-keto $PGF_{1\alpha}$ levels in FBS media is due to increased total cellular protein levels in the presence of serum.

Leptin: As seen with prostaglandin inhibitor pretreatment results, indomethacin treatment (Fig. 17) did not affect the basal and AII-induced leptin secretions in 10 % FBS media. Similarly, in the 1% BSA media, inhibition of prostaglandin synthesis by indomethacin did not modify basal and AII-induced leptin secretions. Additionally, compared to those in BSA-treated cells, similar results of lower basal and AII-induced leptin levels were obtained in FBS-treated cells; these data also were normalized on a per well basis (Appendix, Fig. 2).

V. Discussion & Conclusion

In this study, we investigated the biochemical mechanisms by which AII regulates leptin secretion in adipocytes. Previous investigations have reported that AII may function as an adipogenic factor in adipocyte growth and development through interaction with its receptors (4, 12,14). Further, we have demonstrated that AII increases leptin expression in cultured adipocytes (12). These cumulative findings suggest a strong link between AII and

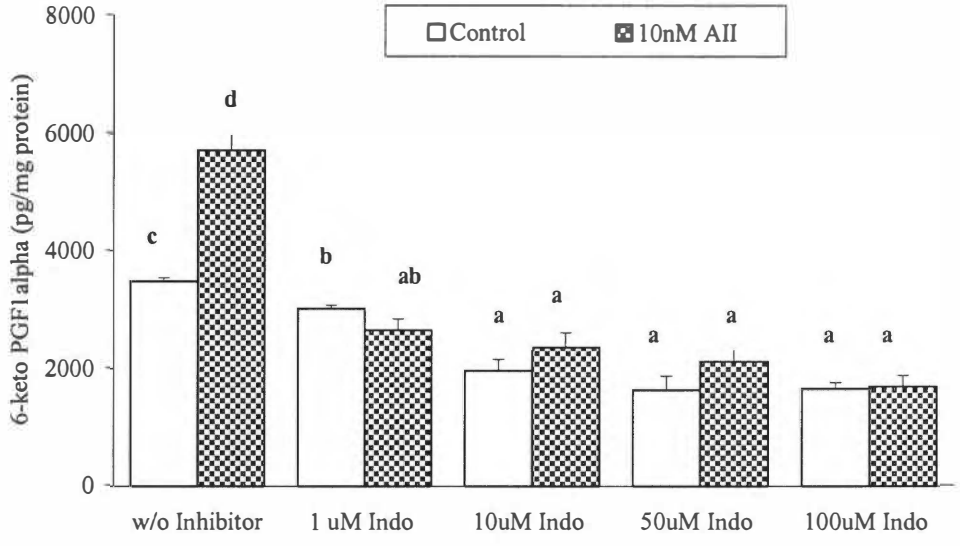

Fig. 10 Effect of indomethacin in AII-induced 6-keto $PGF_{1\alpha}$ secretion in 3T3-L1 adipose cells. Differentiated adipose cells were preincubated with serum-free media supplemented with vehicle (0.2 % ethanol), 1μM, 10μM, 50μM or 100μM indomethacin, an inhibitor of prostaglandin synthesis, for 6h. After pretreatment cells were incubated for 24h with serum-free media supplemented with 10nM AII or vehicle alone. Treated cells were harvested in 0.5M sucrose buffer and the culture media were collected. The amount of 6-keto $PGF_{1\alpha}$ secreted in the culture media was assayed by RIA and normalized to milligram of total cellular proteins. Results are expressed as mean ± SEM (n=5-6). Statistical differences between vehicle or indomethacin-treated values and AII or AII plus indomethacin -treated values are shown; between vehicle-treated and indomethacin-treated values; between AII-treated and AII plus indomethacin-treated values. The values with different letters are significantly different at $p<0.05$.

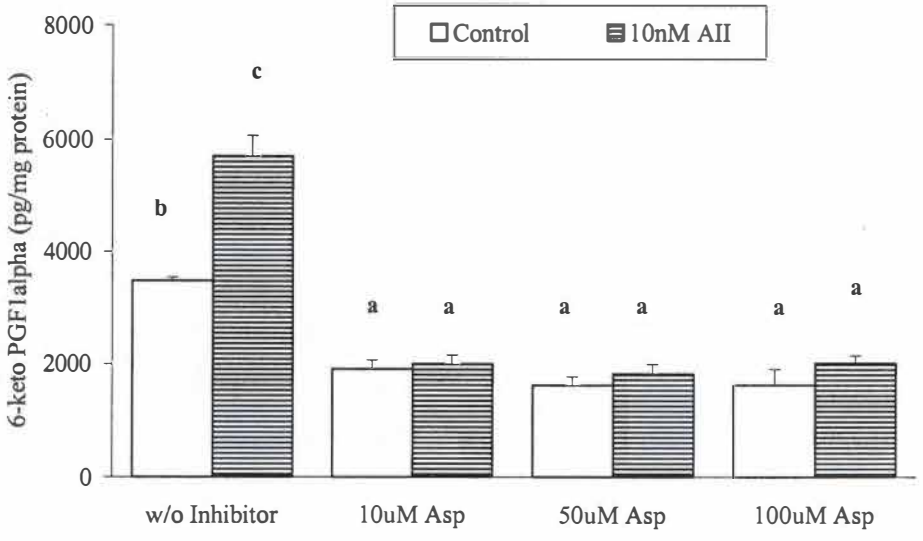

Fig. 11 Effect of aspirin on AII-induced 6-keto $PGF_{1\alpha}$ secretion in 3T3-L1 adipose cells. Differentiated adipose cells were pretreated with serum-free media supplemented with vehicle (0.2 % ethanol), 10μM, 50μM or 100μM aspirin for 6h and then incubated for 24h with 10nM AII or vehicle alone. Treated cells were harvested in 0.5M sucrose buffer and the culture media were collected. 6-keto $PGF_{1\alpha}$ level secreted in the culture media was assayed by RIA and normalized to milligram of total cellular proteins. Results are expressed as mean ± SEM (n=5-6). Statistical differences between vehicle or aspirin-treated values and AII or AII plus aspirin-treated values are shown; between vehicle-treated and aspirin-treated values; between AII-treated and AII plus aspirin-treated values. The values with different letters are significantly different at $p<0.05$.

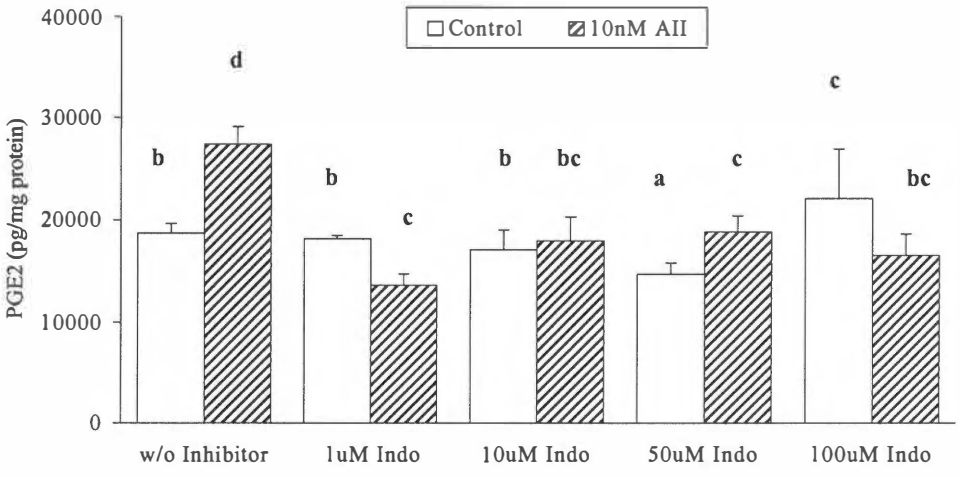

Fig. 12 Effect of indomethacin on AII-induced PGE_2 secretion in 3T3-L1 adipose cells. Differentiated adipose cells were pretreated with serum-free media in the presence of vehicle (0.2% ethanol), 1μM, 10μM, 50μM or 100μM indomethacin for 6h. After pretreatment cells were cultured for 24h with serum-free media supplemented with 10nM AII or vehicle alone. Treated cells were harvested in 0.5M sucrose buffer and the culture media were collected. The amount of PGE_2 secreted in culture media was measured by RIA and corrected to milligram of total cellular proteins. Results are expressed as mean ± SEM (n=5-6). Statistical differences between vehicle or indomethacin-treated values and AII or AII plus indomethacin-treated values are shown; between vehicle-treated and indomethacin-treated values; between AII-treated and AII plus indomethacin-treated values. The values with different letters are significantly different at $p<0.05$.

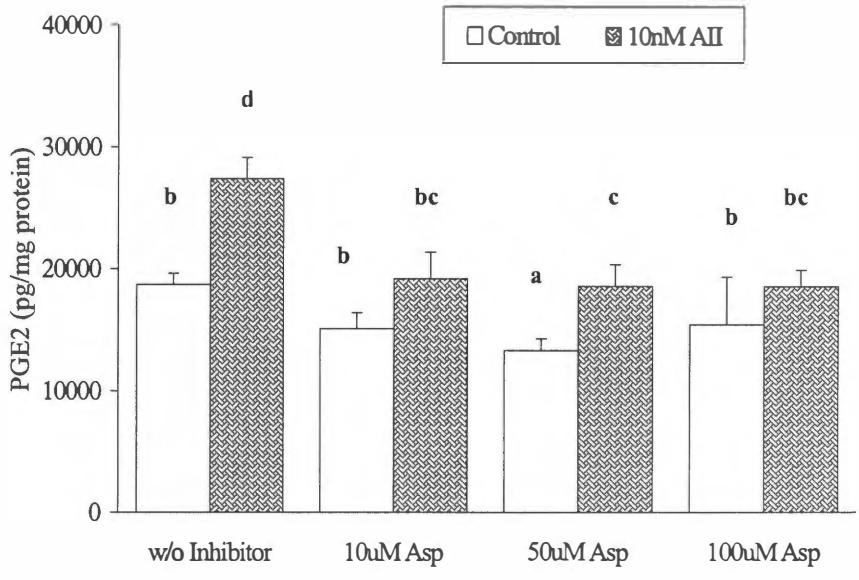

Fig. 13 Effect of aspirin on AII-induced PGE_2 secretion in 3T3-L1 adipose cells. Fully differentiated adipose cells were pretreated with serum-free media containing vehicle (0.2% ethanol), 10μM, 50μM or 100μM aspirin for 6h and then incubated for 24h with 10nM AII or vehicle alone. Treated cells were harvested in 0.5M sucrose buffer and culture media were collected after 24-h treatment. The concentration of PGE_2 secreted in the culture media was measured by RIA and normalized to milligram of cellular proteins. Results are expressed as mean ± SEM (n=5-6). Statistical differences between vehicle or aspirin-treated values and AII or AII plus aspirin-treated values are shown; between vehicle-treated and aspirin-treated values; between AII-treated and AII plus aspirin-treated values. The values with different letters are significantly different at $p<0.05$.

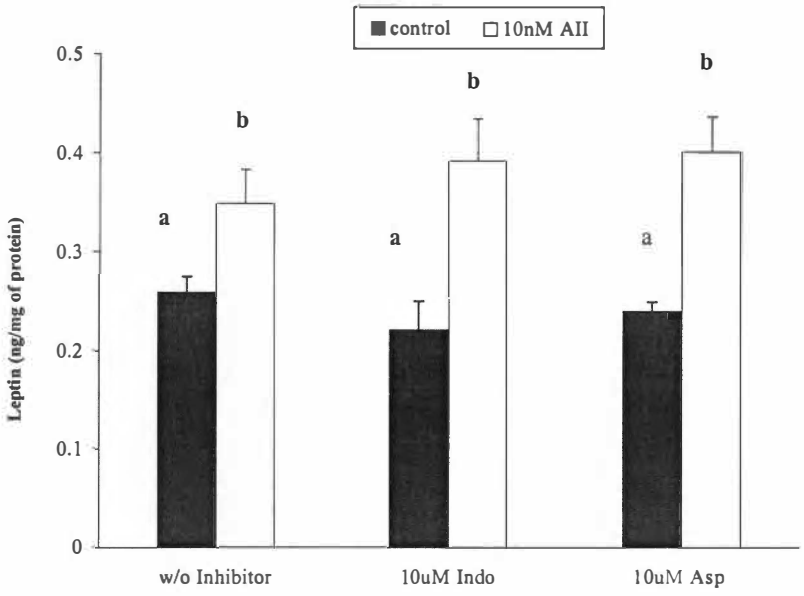

Fig. 14 Effect of indomethacin & aspirin pretreatment on AII-induced leptin secretion in 3T3-L1 adipose cells. Fully differentiated adipose cells were pretreated with serum-free media (1% BSA) supplemented with vehicle (0.2 % ethanol), 10μM indomethacin or 10μM aspirin for 6h and then maintained for 24h with vehicle alone or 10nM AII. After treatment, treated cells were harvested in 0.5M sucrose buffer and the culture media were collected. The amount of leptin secreted in the culture media was measured by RIA and normalized to cellular protein values. Results are expressed as mean ± SEM (n=5-6). Statistical differences between vehicle or indomethacin or aspirin –treated values and AII or AII plus indomethacin or AII plus aspirin-treated values are shown; between vehicle-treated and indomethacin or aspirin-treated values; between AII-treated and AII plus indomethacin or aspirin-treated values. The values with different letters are significantly different at $p<0.05$.

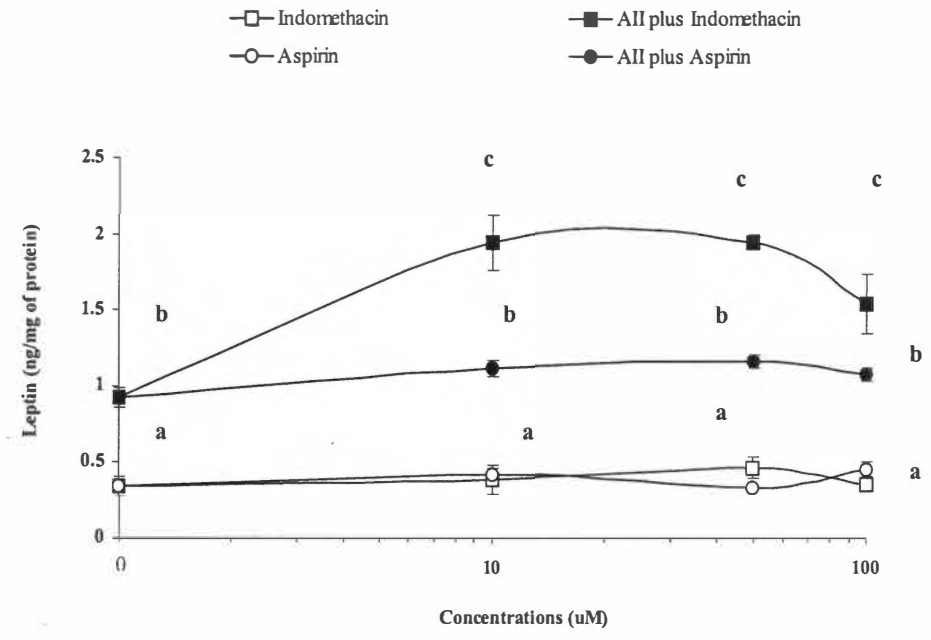

Fig. 15 Effect of indomethacin & aspirin on AII-induced leptin secretion in 3T3-L1 adipose cells. Fully differentiated adipose cells were treated with serum-free media (1% BSA) supplemented with vehicle (0.2 % ethanol), 10nM AII, indomethacin (10, 50, 100μM), aspirin (10, 50, 100μM), or AII plus indomethacin or aspirin for 24h. After treatment, treated cells were harvested in 0.5M sucrose buffer and the culture media were collected. Leptin level secreted in the culture media was measured by RIA and normalized to cellular protein values. Results are expressed as mean ± SEM (n=5-6). Statistical differences between vehicle or indomethacin or aspirin-treated values and AII or AII plus indomethacin or AII plus aspirin-treated values are shown; between vehicle-treated and indomethacin or aspirin-treated values; between AII-treated and AII plus indomethacin or aspirin-treated values. The values with different letters are significantly different at $p<0.05$.

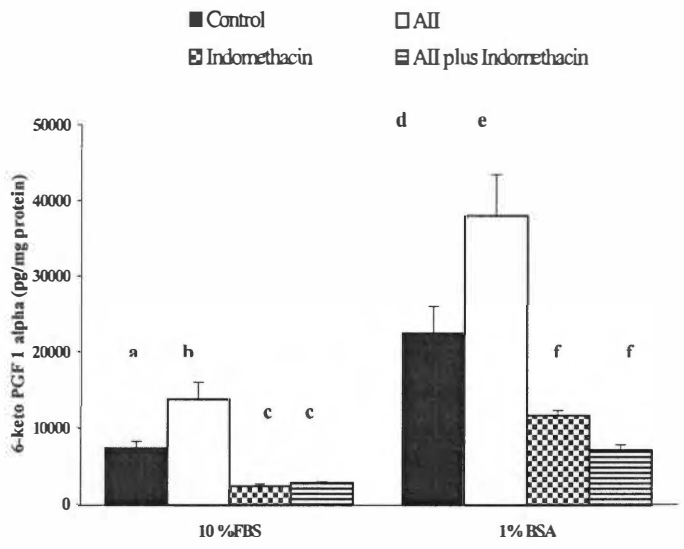

Fig. 16 Effect of indomethacin on AII-induced 6-keto PGF $_{1\alpha}$ secretion by 3T3-L1 adipose cells in the presence or absence of serum. Fully differentiated adipose cells were cultured in 10% FBS or 1% BSA media before treatment. Cells were incubated with both media supplemented with vehicle (0.2% ethanol), 10nM AII, 10μM indomethacin or AII (10nM) plus indomethacin (10μ M) for 24h. Treated cells were harvested in 0.5M sucrose buffer and the culture media were collected. The amount of 6-keto PGF $_{1\alpha}$ was measured in both media by RIA and normalized to milligram of total protein values. Results are expressed as mean ± SEM (n=5). Statistical differences between vehicle or indomethacin-treated values and AII or AII plus indomethacin-treated values are shown; between vehicle-treated and indomethacin-treated values; between AII-treated and AII plus indomethacin-treated values. The values with different letters are significantly different at $p<0.05$.

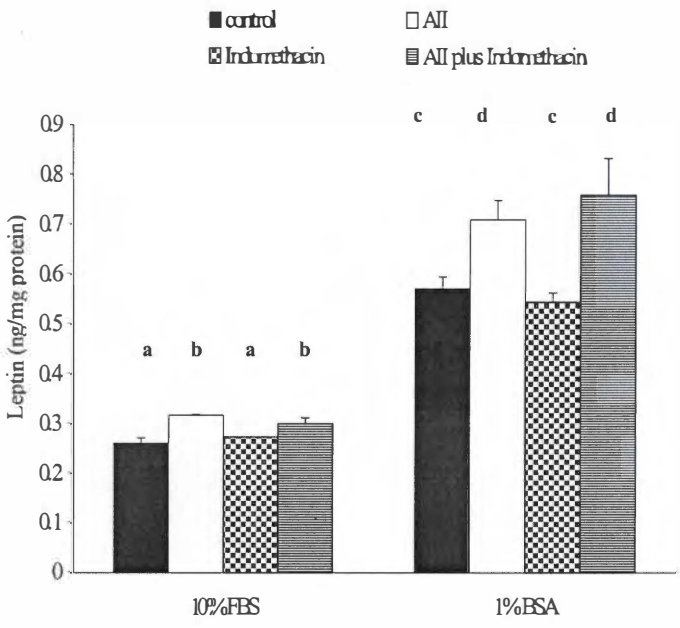

Fig. 17 Effect of indomethacin on AII-induced leptin secretion by 3T3-L1 adipose cells in the presence or absence of serum. Fully differentiated adipose cells were cultured with 10% FBS or 1% BSA media before treatment. Cells were incubated with both media containing vehicle (0.2% ethanol) as control, 10nM AII, 10μM indomethacin, or AII (10nM) plus indomethacin (10μM) for 24h. Treated cells were harvested in 0.5M sucrose buffer and the culture media were collected. Leptin level was measured in both media by RIA and normalized to cellular protein values. Results are expressed as mean ± SEM (n=5). Statistical differences between vehicle or indomethacin-treated values and AII or AII plus indomethacin -treated values are shown; between vehicle-treated and indomethacin-treated values; between AII-treated and AII plus indomethacin -treated values. The values with different letters are significantly different at $p<0.05$.

leptin in the adipogenic functions of AII. Our results clearly demonstrate that AII increases leptin secretion from human and 3T3-L1 adipose cells in a time-responsive manner. Dose response analysis shows that AII at physiological concentrations stimulates leptin secretion from human and 3T3-L1 adipose cells. Accordingly, our results support that AII may play a role as a regulator of leptin in adipocyte metabolism. To date, the signaling mechanisms by which AII increases leptin secretion and promotes adiposity remain to be elucidated. Previous study by Darimont has suggested that prostacyclin synthesized by mature adipocytes functions as a mediator of AII action on adipocyte growth (14). Additionally, preliminary research has shown that PGE_2 and PGI_2 mainly produced from rodent and human adipocytes function as hypertrophic as well as hyperplastic effectors in adipocyte differentiation and development through paracrine/autocrine effects (11, 14, 24-25). Recent research has demonstrated that PGE_2 functions tumor vascularization thus leading to tumor growth, suggesting that prostaglandin may act as an important paracrine effector in angiogenesis of adipocyte (3).

Consistent with previous reports (14-15), our study demonstrates that AII at physiological concentrations produces time-dependent stimulation of 6-keto $PGF_{1\alpha}$ and PGE_2 secretions in adipose cells. These results suggest that these prostaglandins may be potential candidates for mediating the effect of AII on leptin secretion. Accordingly, we investigated whether AII enhances leptin stimulation via a prostaglandin-mediated mechanism. As expected, both inhibitors significantly decreased basal and AII-induced 6-keto $PGF_{1\alpha}$ secretions from adipose cells. However, both indomethacin and aspirin showed less effective inhibitory effects on basal and AII-induced PGE_2 secretions from 3T3-L1 adipose cells. The slight blockades of basal and AII-induced PGE_2 secretions by

both inhibitors may possibly be explained by alternative or additional pathway (COX-3) of prostaglandin biosynthesis in adipocytes (36).

In the subsequent experiments, cyclooxygenase inhibition by indomethacin and aspirin did not modify basal or AII-induced leptin releases from 3T3-L1 adipose cells. Results from this study clearly demonstrate that AII stimulates leptin secretion but that this effect is not mediated via prostaglandin. In addition, coupled with inhibitor pretreatment, data from the presence and absence serum experiments also show that AII regulates leptin secretion in adipocytes via a prostaglandin-independent mechanism.

Additionally, indomethacin may play a critical role as a ligand of adipogenic transcription factor, PPARγ, rather than as an inhibitor in prostaglandin biosynthesis. Recent research has shown that indomethacin at high concentrations (μM range) binds and activates PPARγ, thus inducing adipogenesis, but at low concentrations (nM range), it exhibits an inhibitory effect on prostaglandin synthesis (26). More recently, a study by Sinha *et al* has illustrated that indomethacin displays an inhibitory effect on leptin secretion at concentration at which it activates PPARγ in adipocytes (23). In the present study, high concentrations of indomethacin which induced reduction of prostaglandin secretion did not modify leptin secretion, suggesting that it may predominately affect COX inhibition *versus* PPARγ activation under the present experiment conditions. Also, indomethacin plus AII treatment increases leptin secretion in adipocytes due to AII effect on leptin secretion and results also observed with aspirin.

In summary, our data indicate that AII increases leptin secretion from adipocytes via a prostaglandin-independent mechanism, suggesting that alternative or additional

mechanisms are involved in AII-induced leptin secretion. In addition to stimulation of FAS and triglyceride synthesis by AII, this hormone also increases leptin secretion. This study provides therefore a potential mechanism by which AII may regulate adiposity. This understanding of the RAS and AII paracrine effects in adipocytes may provide insights into the development of new therapeutic approaches to prevent obesity and its associated disorders.

Further work is required to characterize the mechanism of AII regulation of the *ob* gene transcription and identify mechanism of AII signaling in adipocytes leading to regulation of fatty acid synthesis and leptin secretion.

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APPENDIX

Previous research has shown that calcium ionophore A23187 and serum elicit a release of arachidonic acid, leading to an increase in prostaglandin formation. In this regard, we used fetal bovine serum (FBS, 10%) as a positive control to stimulate prostaglandin synthesis. As unexpected, we observed higher inhibitory effect of indomethacin on basal and AII-induced 6-keto $PGF_{1\alpha}$ levels in FBS-treated cells. One possibility is that the high cellular protein contents in FBS media may contribute to lower basal and AII-induced 6-keto $PGF_{1\alpha}$ levels in FBS-treated cells compared to those in bovine serum albumin (BSA, 1%)-treated cells. To test this possibility, we measured the levels of 6-keto $PGF_{1\alpha}$ and PGE_2 in the presence or absence of serum with or without 3T3-L1 adipose cells; all data were also corrected to well or total cellular protein contents. These results clearly demonstrate that the lower 6-keto $PGF_{1\alpha}$ and PGE_2 levels in FBS stimulation is due to increased total cellular protein levels in the presence of serum.

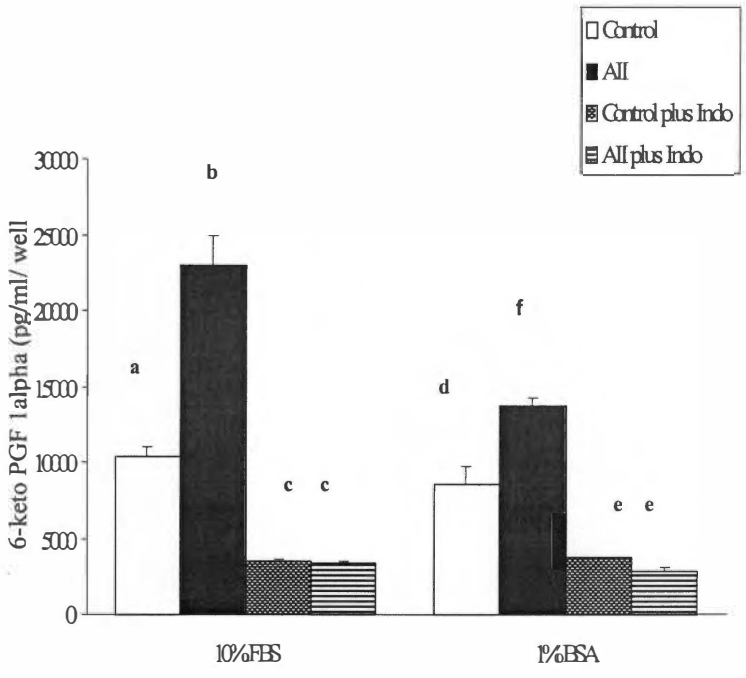

Fig. 1 Effect of indomethacin on AII-induced 6-keto $PGF_{1\alpha}$ secretion by 3T3-L1 adipose cells in the presence or absence of serum. Fully differentiated adipose cells were cultured in 10% FBS or 1% BSA media before treatment. Cells were incubated with both media containing vehicle (0.2% ethanol), 10nM AII, 10μM indomethacin or AII (10nM) plus indomethacin (10μM) for 24 h. Treated cells were harvested in 0.5M sucrose buffer and the culture media were collected. The amount of 6-keto $PGF_{1\alpha}$ was measured in both media by RIA and normalized to well. Results are expressed as mean ± SEM (n=5). Statistical differences between vehicle or indomethacin-treated values and AII or AII plus indomethacin-treated values are shown; between vehicle-treated and indomethacin-treated values; between AII-treated and AII plus indomethacin-treated values. The values with different letters are significantly different at $p<0.05$.

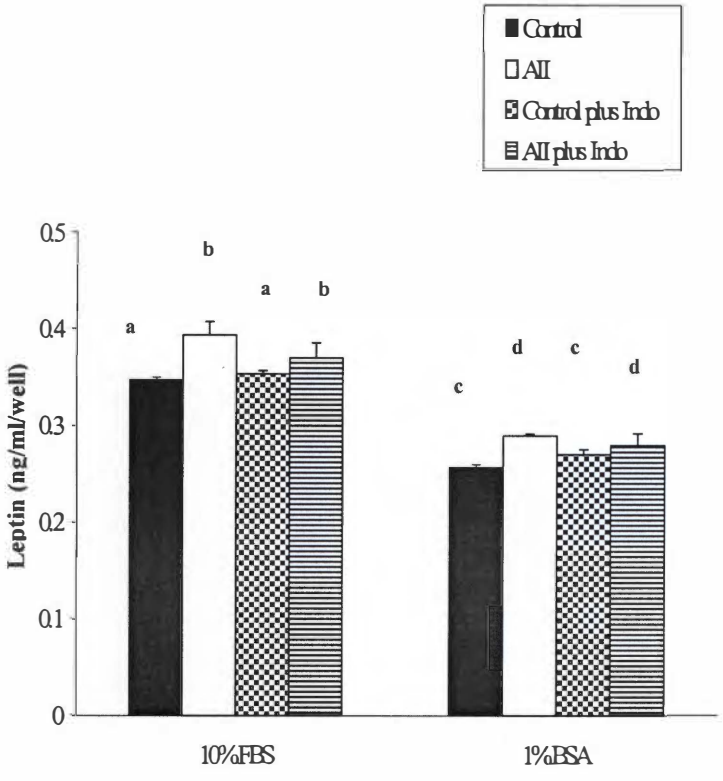

Fig. 2 Effect of indomethacin on AII-induced leptin secretion by 3T3-L1 adipose cells in the presence or absence of serum. Fully differentiated adipose cells were cultured with 10% FBS or 1% BSA media before treatment. Cells were incubated with both media containing vehicle (0.2% ethanol) as control, 10nM AII, 10μM indomethacin, or AII (10nM) plus indomethacin (10μM) for 24 h. Treated cells were harvested in 0.5M sucrose buffer and the culture media were collected. Leptin level was measured in both media by RIA and normalized to well. Results are expressed as mean ± SEM (n=5). Statistical differences between vehicle or indomethacin-treated values and AII or AII plus indomethain-treated values are shown; between vehicle-treated and indomethacin-treated values; between AII-treated and AII plus indomethacin-treated values. The values with different letters are significantly different each other at $p<0.05$.

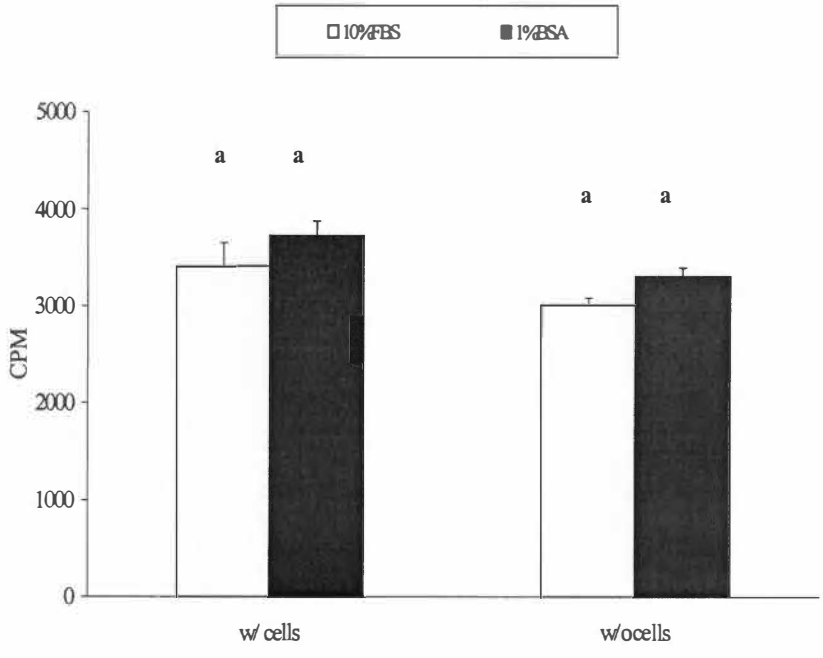

Fig. 3 Measurement of exogenous 6-keto $PGF_{1\alpha}$ level in the presence or absence of serum. Differentiated adipose cells were cultured in DMEM containing 10% FBS or 1% BSA and incubated for 24 h with [^{3}H] 6-keto $PGF_{1\alpha}$, which was directly added in both media with or without cells. After 24-h treatment culture media were collected and prostaglandins were isolated by solid-phase extraction using an octadecyl C_{18} cartridge and eluted with 100% methanol (2ml) twice. The radioactivity of eluted prostaglandins (2ml) was measured using β counter. No significant differences between 10% FBS-treated and 1% BSA-treated values with or without cells were shown.

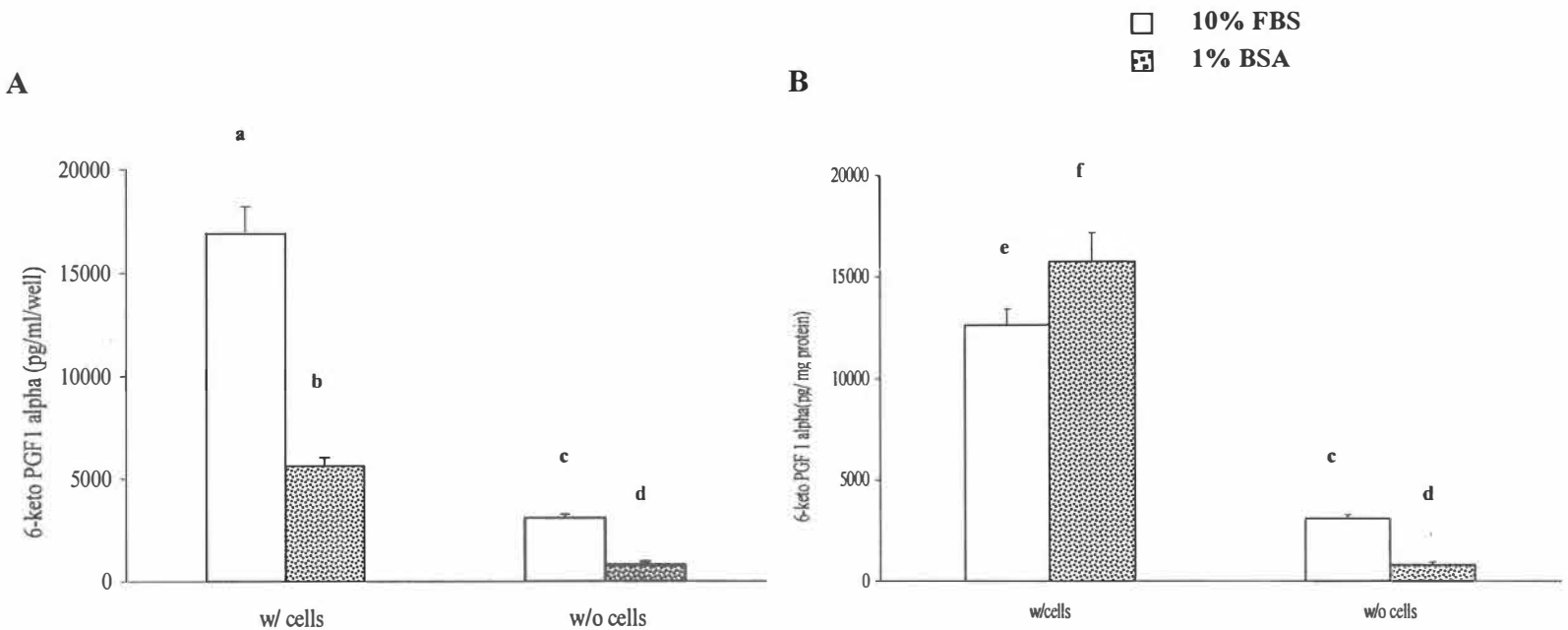

Fig. 4 Measurement of 6-keto $PGF_{1\alpha}$ level in the presence or absence of serum with or without 3T3-L1 adipose cells. Differentiated adipose cells were incubated for 24 h in DMEM containing 10% FBS or 1% BSA. After 24-h culture cells were harvested in 0.5M sucrose buffer and the culture media were collected. The amount of 6-keto $PGF_{1\alpha}$ was measured in both media by RIA and normalized per well (A) or milligram of total cellular proteins (B). Data are expressed as mean ± SEM (n=6). Statistical differences between 10% FBS and 1% BSA-treated values. The values with different letters are significantly different each other at $p<0.05$.

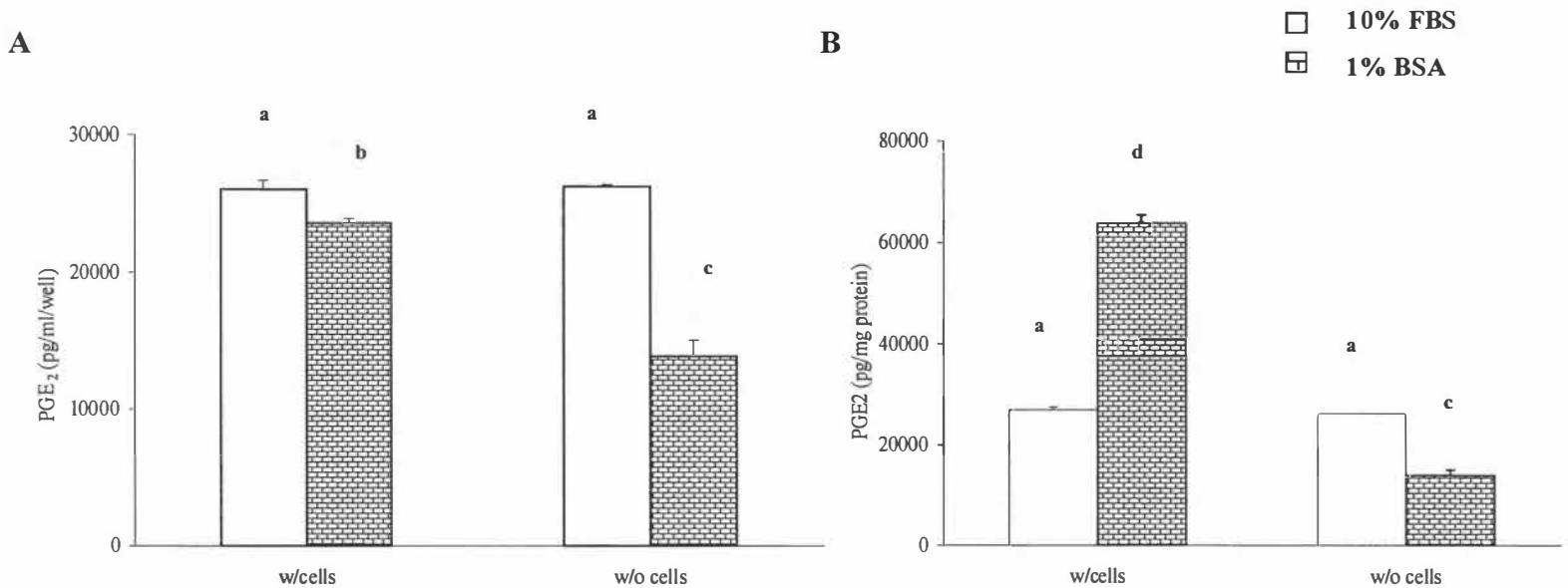

Fig. 5 Measurement of PGE_2 level in the presence or absence of serum with or without 3T3-L1 adipose cells. Differentiated adipose cells were incubated for 24 h in DMEM containing 10% FBS or 1% BSA. After 24-h culture cells were harvested in 0.5mM sucrose buffer and the culture media were collected. PGE_2 concentration was measured in both media by RIA and normalized per well (A) or milligram of total cellular proteins (B). Data are expressed as mean ± SEM (n=6). Statistical differences between 10% FBS and 1% BSA-treated values. The values with different letters are significantly different at $p<0.05$.

VITA

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