Role of Angiotensin II Type 2 Receptor in Regulation of Lipid Metabolism and Adiposity

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Doctor of Philosophy

By

Sourashish Nag, M.Pharm. (Pharmacology)

December 2013

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Lipid Metabolism and Adiposity

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Abstracts and Recognitions

Abstracts

Nag S, Khan MA, Hussain T. Activation of angiotensin type 2 receptor rescues high-fat diet-induced adipocyte size in young male mice. FASEB J, **April 2012**.

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Recognitions

- **JOGUE Junior Scientist Award in Nutrition** - Association of Scientists of Indian Origin in America (2013) Boston, MA.
- **Certificate of Excellence** - Texas Obesity Research Center (2012) Houston, TX.
- **1st Place** - Young Scientist Award (Obesity Research), American Society for Nutrition (2012) San Diego, CA.
- **3rd Place** - Graduate Scholars Symposium, Auburn University (2012) Auburn, AL.

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"There are only two ways to live your life. One is as though nothing is a miracle. The other is as though everything is a miracle." -- Albert Einstein

I believe miracles happen, however I don't know whether it is all miracles which pave the journeys in life. Indeed it is miracle that we find some hands suddenly help lift the slipping weight and the life seems beautiful and easy. My journey of life and graduate school are no exceptions.

At the outset, I am indebted to merciful, blissful, and beneficent divine mother "Ma Kali" for miraculously showering blessings over me and providing me with the strength to keep going on.

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Sourashish Nag

The lines from the poem by *Alicia Yeargin*…….

"Why oh why is America obese?
Every mother, ever daughter, every uncle, every niece,
They are so large and porcine.
The more they eat and the more they dine,
Just expanding their own waistline.
Where oh where are the smart people of today?
It's a shame intelligence was thrown away,
By the media and cinema and Hollywood.
No longer do teachers teach what they should.
I would make a difference in this world, if only I could."

Hope to see a different world, a world free from obesity.

This thesis is dedicated to the people who suffer from obesity

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ABSTRACT

Obesity is becoming increasingly common in the United States and worldwide. Abundant supplies of cheap foods and sedentary life styles are predisposing human beings to obesity which is an underlying risk factor for metabolic syndrome and cardiovascular diseases. Adipose tissue is the obligatory site of fat accumulation which causes obesity. Moreover, adipose tissue expresses local renin angiotensin system (RAS) which has been implicated in obesity. Recent studies using angiotensin type 2 receptor (AT2R) knockout mice suggested that AT2R contributes to adiposity and obesity in male mice while prevents adiposity and obesity in female mice. On the other hand, deficiency of AT2R in Apolipoprotein E knockout mice (a model of atherosclerosis) exaggerated adiposity in male mice suggesting that AT2R prevents adiposity in this animal model. Thus, role of AT2R on adiposity in knockout studies was in-conclusive and also indicated a gender specific role of AT2R. Furthermore, the studies showed that the relative expression of AT2R deceases after birth through adolescence and adulthood. Also, there are reports suggesting that estrogen (E2) positively regulates AT2R expression and AT2R affects levels of urinary E2 which is known regulator of adiposity.

Hence, we designed the present study to investigate the effect of pharmacological activation of AT2R on adiposity and to study whether the said effect is reduced at adolescence. Male C57BL/6 mice (5 and 12 weeks old) were pretreated with the AT2R agonist C21 (0.3 mg/kg, daily i.p.) for 4 days. Thereafter the animals were placed on normal chow diet (ND) or high-fat diet (HFD) with concurrent drug treatment

for the next 10 days. The HFD significantly increased epididymal WAT (eWAT) weight, adipocyte size, plasma free fatty acid (FFA) and triglyceride (TAG) in these mice. The pharmacological activation of AT2R reduced the HFD-induced increase in eWAT weight, adipocyte size, plasma FFA and TAG. Thus, we observed that the pharmacological activation of AT2R reduced the HFD-induced adiposity and plasma parameters in male mice, both in young and adults.

To explore the effects of C21 on lipid metabolism we investigated the adipose lipid metabolism (lipogenic and lipolytic) regulators in the eWAT of 5 weeks old male mice only. We observed that HFD increased the protein expressions (measured by western blot) of eWAT lipogenic regulators, namely adipose fatty acid binding protein (FABP4) and fatty acid synthase (FASN). On the other hand, HFD caused a decrease in the protein expression of eWAT adipose triglyceride lipase (ATGL) and an increase in hormone-sensitive lipase (HSL) protein expression 5 weeks old male mice. Interestingly, C21 treatment altered HFD-induced changes in lipogenic and lipolytic regulators. To explore further whether C21 directly impacts eWAT lipogenic/lipolytic regulators or impacts them due to its effect on fatty acid uptake in adipocytes, we performed an in vitro study using isolated primary epididymal adipocytes from 5 weeks old male mice. We observed that C21 acting on epididymal adipocyte AT2R via a NOS/GC/PKG-dependent pathway reduced fatty acid transport in adipocytes. Thus, pharmacological activation of AT2R reduced the fatty acid transport in adipocytes and possibly in consequence prevented HFD-induced adiposity in the male mice.

Our studies with AT2R knock out female mice demonstrated that genetic deletion of AT2R causes an increase in adiposity with a parallel decrease in urinary E2 in female mice. Thus to explore whether the pharmacological activation of AT2R using C21 prevents adiposity in female mice and whether the E2 plays a role in C21-mediated effect on adiposity, female C57BL/6 ovary-intact (Ovi) and ovariectomized (Ovx) mice were used. These mice were treated with C21 (0.3 mg/kg, daily i.p.) with(out) E2 (5 µg/day) supplementation and placed on HFD for 10 days. We observed that the C21 treatment prevented the HFD-induced adiposity and plasma parameters, namely FFA, TAG and insulin in female mice independent of E2. In the liver, C21 treatment increased the CPT1A expression (an index of β-oxidation) and prevented liver weight increase under HFD. Interestingly, we also observed that C21 treatment increased the physical activity of female mice. Thus, this study demonstrated that pharmacological activation of AT2R increased physical activity of the female mice and prevented the HFD-induced adiposity in these mice.

Overall these studies demonstrate that the pharmacological activation of AT2R reduces fatty acid uptake in adipocytes. This reduction in fatty acid uptake possibly prevents HFD-induced changes in lipid metabolism, adiposity and plasma parameters in mice. Moreover, pharmacological activation of AT2R also increases physical activity which may increase utilization of the fatty acid via oxidation and thus remove fatty acid from the circulation. In light of these studies, we propose that AT2R activation may serve as a potential therapeutic target to control obesity which in turn protects against obesity-associated metabolic disorders.

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LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
Ang II	Angiotensin II
ANOVA	Analysis of variance
ATGL	Adipose triglyceride lipase
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
C21	Compound 21
cGMP	cyclic guanosine monophosphate
CPT1	Carnitine palmitoyltransferase 1
DAG	Diacylglycerol
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular-signal-regulated kinases 1 and 2
FABP4	Fatty acid binding protein 4
FA	Fatty acid
FASN	Fatty acid synthase

FATP	Fatty acid transport protein
FFA	Free fatty acid
GC	Guanylyl cyclase
GPCR	G-protein coupled receptor
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HFD	High-fat diet
HRP	Horse radish peroxidase
HSL	Hormone sensitive lipase
i.p.	Intra-peritonial
kD	Kilo Dalton
kg	Kilogram
MAG	Monoacylglycerol
MAL	Monoacylglycerol lipase
MAPK	Mitogen activated protein kinases
mRNA	messenger Ribonucleic acid
µM	Micromolar

mM	Millimolar
nM	Nanomolar
ND	Normal chow diet
NO	Nitric oxide
NOS	Nitric oxide synthase
PI3K	Phosphoinositol-3-kinase
PKG	Protein kinase G
RAS	Renin angiotensin system
ROS	Reactive oxygen species
SEM	Standard error of the mean
TAG	Triacyl glycerol / triglyceride
TNFα	Tumor necrosis factor alpha
WAT	White adipose tissue

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1: INTRODUCTION AND STATEMENT OF PROBLEM

Obesity, characterized by increase of body weight and adiposity, is a major risk for the development of dyslipidemia, atherosclerosis, insulin resistance, diabetes and hypertension (Barrett-Connor, 1985; Bonadonna *et al.*, 1990; Elmarakby and Imig, 2010; Hubert *et al.*, 1983; Mathieu *et al.*, 2010). The incidence of obesity has been increasing worldwide and is now considered a global epidemic, irrespective of age or sex. According to the international guidelines, the lifestyle modification (decreased caloric intake and increased physical exercise) is primary part of body weight management (Fodor *et al.*, 2009). However, the long-term effects of diet and exercise on weight have been mostly disappointing (Jansson *et al.*, 2013). Further strategies for body weight management include pharmacological intervention and bariatric surgery (Bloom *et al.*, 2008). Most anti-obesity drugs are associated with serious side effects (Rodgers *et al.*, 2012). Thus, there is a pressing need for the discovery of newer anti-obesity drugs to combat this global epidemic of obesity.

Under normal physiological condition, as we eat food, the surplus or extra energy from lipid is stored in adipocytes as triglycerides (TAG) for future use (Gregoire *et al.*, 1998). As and when the energy demand increases, the stored TAGs are broken down to free fatty acids. However, due to excess feeding or decreased energy expenditure as the body weight increases, the increased accumulation of TAG causes increase in adipocyte size. Larger adipocytes have impaired lipid metabolism which in the long-term leads to obesity-associated disorders namely dyslipidemia, atherosclerosis, insulin resistance, diabetes and hypertension (Bjorntorp, 1971).

Although renin angiotensin system (RAS) is mostly known for its role in cardiovascular function and blood pressure regulation, it has been recently implicated to have role in lipid metabolism and adiposity (Yvan-Charvet and Quignard-Boulange, 2011; Kalupahana and Moustaid-Moussa, 2012). Adipocytes are known to have all the components of the RAS. In high-fat diet (HFD)-induced obesity, increased local formation of angiotensin II (Ang II, the major peptide hormone of RAS) in adipose tissue is observed. Ang II is thus considered as a trophic factor for the adipose tissue growth. It acts via angiotensin type 1 and 2 receptors (AT1R and AT2R). The physiologic effects of these receptors generally are believed to be antagonistic (Hakam and Hussain, 2005; Jones *et al.*, 2012). While the blocking of AT1R decreases adipocyte size in male mice (Putnam *et al.*, 2012), the role of AT2R in adiposity is not yet established. Recent studies demonstrated that the genetic deletion of AT2R resulted in a decrease in adiposity in male mice (Yvan-Charvet *et al.*, 2005; Samuel *et al.*, 2013) while leading to an increase in adiposity in female mice under high-fat diet (HFD) conditions (Samuel *et al.*, 2013). On the contrary, deficiency of AT2R in Apolipoprotein E knockout mice (a model of atherosclerosis) increased adipose tissue weight in male mice (Iwai *et al.*, 2009). Thus, the role of AT2R on adiposity in knockout studies was in-conclusive and also indicated a gender biased role of AT2R. Furthermore, estrogen, the female sex steroid hormone, is known to regulate the lipid metabolism, adiposity and increase the AT2R expression (D'Eon *et al.*, 2005; Armando *et al.*, 2002). Interestingly, we observed that the AT2R-KO female mice were having decreased urinary estrogen under HFD condition (Samuel *et al.*, 2013).

Therefore, we designed the current study i) to investigate the effect of the AT2R agonist (C21) on adiposity and lipid metabolism using young and adult male mice. Also as the relative expression of AT2R decreases after birth through adolescence and adulthood, we investigated ii) if the pharmacological stimulation of AT2R using C21 impacts adiposity and lipid metabolism in adult male mice as well. Finally, we investigated iii) whether estrogen plays a role in AT2R agonist-induced effect on adiposity and lipid metabolism in female mice.

2: REVIEW OF LITERATURE

2.1: Obesity – a burden on society

According to the World Health Organization (WHO), obesity is a condition in which excessive or abnormal body fat accumulates that poses a risk on health. A crude index to measure obesity is the body mass index (BMI) which is calculated by dividing a person's weight (kilograms) by the square of his/her height (meters). A person is classified as overweight when his/her BMI ≥ 25 kg/m^2 and obese when BMI ≥ 30 kg/m^2. Of the worldwide deaths, overweight and obesity are the fifth foremost risk factors.

Globally, obesity has almost doubled since the year 1980 according to WHO. They estimated that more than 1.4 billion adults worldwide were overweight in the year 2008. Out of this population, over 200 million men and nearly 300 million women were classified in the obese category. In year 2011, more than 40 million children (below five years of age) were overweight. According to Centers for Disease Control and Prevention (CDC), more than one-third of US adult population is obese. In the US, childhood obesity has been reported to have affected nearly 12.5 million children and adolescents (Ogden *et al.*, 2010), who in the future are going to add up to the adult obese population. According to CDC, annual medical costs associated with obesity were estimated at 147 billion US dollars in the year 2008. Moreover, the obesity trend in US has been ever increasing and it is forecasted that by the year 2030, 51% of the US population will be obese (Finkelstein *et al.*, 2012).

Obesity has been closely linked to the increased risk for metabolic syndrome (Galic *et al.*, 2010). The term "metabolic syndrome" was coined by Haller in the year

1977 for the associations of obesity, diabetes mellitus, hyperuricemia, hyperlipoproteinemia and hepatic steatosis (fatty liver) to describe the additive effects of the risk factors on atherosclerosis. Nowadays, the term "metabolic syndrome" is used to define a collection of abnormalities associated with the increased risk for the onset of type 2 diabetes and atherosclerosis associated vascular diseases namely stroke (Morley and Sinclair, 2009). In addition, recent reports indicate that obesity is associated with chronic low grade inflammation (Emanuela *et al.*, 2012).

In general, obesity is associated with insulin resistance leading to type 2 diabetes. Diabetes is associated with two terms “insulin sensitivity” and “insulin resistance” which are often confusing (Gregoire *et al.*, 1998; Kahn, 1978). The term “insulin sensitivity” is defined as the tissue responsiveness to insulin, i.e., how successfully the insulin receptors function to affect the glucose clearance. In optimal insulin sensitivity, after a high sugary meal, insulin sharply increases, pushing glucose into the tissues quickly and then dissipates. In poor insulin sensitivity, insulin's elevation is sustained because of inability to force glucose into tissues. On the other hand, the term “insulin resistance” is defined as a pathophysiological state in which insulin-sensitive tissues exhibit an abnormally low response to normal to high levels of circulating insulin leading to frank hyperglycemia and type 2 diabetes. It may exist whenever normal levels of insulin produce a biologic response lesser than normal physiologic condition. The insulin resistant states may be divided into those:

- due to decrease in sensitivity to insulin (a right ward shift in the dose-response curve),
- due to decrease in the ceiling response to insulin, and

- that are combinations of decrease in sensitivity (disorders due to alterations before the interaction of insulin with its receptor) and decrease in responsiveness (disorders due to alterations at the intracellular steps of insulin effect).

2.2: Adiposity – the concept

Obesity is caused by increased adiposity or white adipose tissue (WAT) weight (Gregoire *et al.*, 1998). In the abdominal region of mice, there are various types of WAT namely inguinal WAT (subcutaneous WAT found in the groin region), retroperitoneal WAT (visceral WAT found behind kidneys), mesenteric WAT (visceral WAT found in linings of intestines) and gonadal WAT (visceral WAT found surrounding the gonads or reproductive organs) (Sackmann-Sala *et al.*, 2012). The gonadal WAT is the most vulnerable types of WAT associated with adiposity and obesity (Foster *et al.*, 2013). The gonadal WATs is termed as epididymal WAT (eWAT) in males and as parametrial WAT (pWAT) in females. The eWAT surrounds the testis and epididymis while the pWAT surrounds the uterus and ovaries.

The adipose tissue in recent years has been recognized as highly active metabolic and endocrine organ (Kershaw and Flier, 2004; Hajer *et al.*, 2008) besides being the largest source of fuel depot of the body (Galic *et al.*, 2010). They secrete cytokines called as adipokines which have specialized functions in metabolism (Galic *et al.*, 2010). These adipocyte-derived secreted proteins are capable of setting cross-talk with distant organs like brain, liver, skeletal muscles, etc. Following are the some important adipokines:

A. **Leptin**. Leptin is a mediator of adipose-to-brain cross-talk with regard to the nutrient status. It enters the brain via endocytosis and reduces food intake, increases energy expenditure and insulin sensitivity, restores euglycemia / normoglycemia and reproductive function and increases fatty acid oxidation in skeletal muscle (Campfield *et al.*, 1995; Halaas *et al.*, 1995; Pelleymounter *et al.*, 1995). Although leptin level is positively related to adipose size and mass (Lonnqvist *et al.*, 1997), serum leptin levels per gram of adipose tissue are increased in obesity leading to a leptin-resistant state (Fried *et al.*, 2000). Recent reports suggest that skeletal muscle also secrete leptin (Wolsk *et al.*, 2012; Raschke and Eckel, 2013).

B. **Adiponectin**. Adiponectin is secreted exclusively by adipose tissue (Hu *et al.*, 1996). It is an anti-inflammatory adipokine which improves the insulin sensitivity in genetic and diet-induced obesity (Yamauchi *et al.*, 2001). In concert, adiponectin has been reported to increase fatty acid oxidation and glucose uptake in adipose tissue (Wu *et al.*, 2003a) and skeletal muscle (Yamauchi *et al.*, 2002) and to suppress hepatic glucose output (Combs *et al.*, 2001) via AMPK signaling. Thus, between the plasma adiponectin and fat mass, a negative correlation exists (Hu *et al.*, 1996).

C. **Tumor necrosis factor α (TNFα):** TNFα is one of the major pro-inflammatory adipokine (Aldhahi and Hamdy, 2003). A positive correlation exists between the adipose TNFα and BMI (Bullo *et al.*, 2002). TNFα is known to impair insulin signaling in hepatocytes and adipocytes via inhibition of insulin receptor substrate (IRS) signaling through activation of serine

kinases namely c-Jun-N-terminal kinase (JNK) or inhibitor of NF-κB kinase (IKK) or enhanced expression of suppressor of cytokine signaling 3 (SOCS3) (Hotamisligil *et al.*, 1993; Steinberg *et al.*, 2006a). Also, it reduces fatty acid oxidation in skeletal muscle via induction of protein phosphatase 2C and/or suppression of AMPK (Steinberg *et al.*, 2006b). Skeletal muscles also secrete TNFα (Pedersen and Febbraio, 2012).

Adipocyte number (adipocyte hyperplasia) stays considerable stable in human adults (Spalding *et al.*, 2008). Mostly adipocyte size increase (adipocyte hypertrophy) has been reported to be associated with human obesity and obesity-induced disorders (Bjorntorp, 1971). Even the plasma insulin positively correlates with the adipocyte size but not with body fat content under conditions of unaltered diet and physical activity. In fact, body fat content tends to show a negative correlation with adipocyte number in obesity. However only in severe obesity, adipocyte number may contribute to obesity.

A major function of white adipocytes is to act as a warehouse for storage of triglycerides or triacylglycerol (TAG) (Gregoire *et al.*, 1998). Under normal physiological condition, as food is eaten, the surplus or extra energy from lipids is stored in adipocytes as TAG for future use. As and when the energy demand increases, the stored TAGs are broken down to release free fatty acids for energy (Ahmadian *et al.*, 2007) or heat (Matthias *et al.*, 2000) generation. However, due to excess feeding or decreased energy expenditure as the body weight increases, the increased accumulation of TAG causes increase in adipocyte size. The larger adipocytes have impaired lipid metabolism (Engfeldt and Arner, 1988) which leads to obesity-associated disorders like type 2

diabetes, atherosclerosis, hypertension, obesity-induced renal damage, arthritis, gall-bladder disease, certain forms of cancer (Bray and Bellanger, 2006).

2.3: Lipid Metabolism – as we understand it today.

Adipocyte size is regulated by fatty acid uptake and release which involve a number of lipogenic and lipolytic regulators. The metabolism of lipids includes the process of building-up of lipid known as lipogenesis and the process of break-down of lipid known as lipolysis. These processes of lipogenesis and lipolysis are essential for the homeostasis.

In the postprandial phase, the fatty acids are first transported inside the adipocytes by fatty acid transport proteins (FATPs) (Schaffer and Lodish, 1994). Once inside the aqueous cytosol, fatty acids are bound to the fatty acid binding proteins like FABP4 for cytosolic transportation (Thompson *et al.*, 2009). These fatty acids along with the de novo synthesized fatty acids are packed and stored as TAG in the lipid droplets of adipocytes.

The de-novo fatty acid synthesis requires conversion of acetyl-CoA to malonyl-CoA which is catalyzed by a multi-domain enzyme, acetyl-CoA carboxylase (ACC). The fatty acid synthase (FASN) catalyzes the synthesis of palmitate utilizing malonyl-CoA. Thus both lipogenic enzymes, ACC and FASN facilitate the fatty acid biosynthesis (Wakil and Abu-Elheiga, 2009).

With the energy demand, the TAGs are first broken-down to diacylglycerols (DAGs) mainly by ATGL (Ahmadian *et al.*, 2007; Zechner *et al.*, 2009). The DAGs are further catabolized to produce monoacylglycerols (MAGs). Finally, MAGs are cleaved by

monoglyceride lipase (MGL) to release the glycerol and free fatty acids (FFAs). Once liberated, FFAs are shunted towards the hepatocytes/skeletal myocytes for break-down and energy (ATP) generation via a process called as β-oxidation which occurs in mitochondria. The enzyme, carnitine palmitoyl transferase (CPT), facilitates this shunting process to mitochondria and thus is used as an index for the β-oxidation (Wakil and Abu-Elheiga, 2009).

Although WAT is the predominant type of fat tissues, another type is brown adipose tissue (BAT) in mammals (Armani *et al.*, 2010). As described above, WAT serves as a warehouse for storage of TAG while the BAT produces heat via the mitochondrial uncoupling of fatty acid oxidation. The BAT activates the heat production in the need of extra heat for physiological homeostasis, for instance, during entry into a febrile state post-natally and during arousal from hibernation (Cannon and Nedergaard, 2004).

The physiologic activation of the sympathetic nervous system (SNS) centrally triggers the release of norepinephrine (NE). The NE acting on β-adrenergic receptors of WAT causes browning of WAT. Simultaneously, browning of WAT is also stimulated by NE-mediated release of irisin (a myokine) and naturietic peptide (a hormone) from skeletal and cardiac muscle, respectively (Kozak and Young, 2012). In the mitochondria of BAT, fatty acids activate the uncoupling protein 1 (UCP1) which serves as a proton translocator. The activated UCP1 causes shunting of the proton away from the ATP synthase and translocates the protons inside the mitochondria and this process generates the heat (Richard and Picard, 2011).

2.3.1: The Lipid Metabolism Enzymes/Proteins

A. **Fatty Acid Transport Protein**. Fatty acid transport proteins (FATPs) are the transmembrane proteins which facilitate the uptake of long-chain (Carbon:13-21) and very long chain fatty acids (Carbon:>22) (Schaffer and Lodish, 1994; Steinberg *et al.*, 1999) and also their activation via ligation to CoA in cells (Fisher and Gertow, 2005). The amino acid sequence is highly conserved among FATP family members (Hirsch *et al.*, 1998). The six members of the FATP family (FATP1-6) have been identified in human beings (Stahl, 2004) and the different FATPs are expressed in different tissues with varied levels.

Adipocytes express both FATP1 and FATP4. However in adipocytes, the major fatty acid transporter is FATP1 (Stahl *et al.*, 2001) which is an insulin-sensitive fatty acid transporter and is involved in diet-induced obesity (Wu *et al.*, 2006). Overexpression of murine FATP1 in human embryonic kidney 293 (HEK-293) cells has been shown to cause increase in long-chain fatty acid (LCFA) uptake and TAG accumulation (Hatch *et al.*, 2002). While knocking out of FATP1 in mouse, decreases muscle acyl-CoA levels and increases insulin sensitivity (Kim *et al.*, 2004). Also, the FATP4 expressions in human adipose tissue positively correlate with obesity and insulin resistance (Gertow *et al.*, 2004).

Skeletal muscle also expresses mainly FATP1 which possibly increases the rate of LCFA transport and channelizes the lipids to oxidation in skeletal muscle (Holloway *et al.*, 2011). However, the FATP1 overabundance in skeletal muscle neither cause

intramuscular lipid accumulation nor predispose animals to diet-induced insulin resistance.

Liver expresses both FATP2 and FATP5. In liver, the major fatty acid transporter is FATP5 which has been reported to be involved in the lipid homeostasis process (Doege *et al.*, 2006). The FATP2 in liver possibly is involved in de novo synthesis of bile salts (Mihalik *et al.*, 2002).

Heart predominantly expresses FATP6. The FATP6 is involved in cardiac fatty acid uptake and a polymorphism associated with FATP6 has been reported to protect from characteristics of the metabolic syndrome and cardiovascular (CV) disorders (Auinger *et al.*, 2012).

The FATPs are regulated by diet and hormones (Stahl, 2004). The high-fat diet causes increase in the FATP expression (Ouali *et al.*, 2000). Insulin has been shown to cause translocation of FATP1 from intracellular compartment to the plasma membrane to affect the LCFA transportation (Stahl *et al.*, 2002). Also reports suggest that there is a positive regulation of mouse FATP by ligands that activate the adipogenic transcription factor, peroxisome proliferator-activated receptor-γ (PPAR-γ) (Martin *et al.*, 1997). This indicates a positive association between FATPs and adiposity.

B. **Fatty Acid Binding Protein**. Fatty acid binding proteins (FABPs) are the family of cytosolic carrier proteins for fatty acids and facilitate the intra-cellular transfer of fatty acids (Weisiger, 2002). The members of FABP family range from FABP1-12. The adipose-FABPs (AFABP) or aP2 or FABP4 are expressed in cytosol of mature adipocytes (Storch and McDermott, 2009). The FABP4 knockout mice are protected from the onset of dyslipidemia, fatty liver disease, hyperglycemia and insulin resistance

in both genetic and diet-induced obesity (Maeda *et al.*, 2005; Uysal *et al.*, 2000). Also the circulating FABP4 has been suggested as an indicator of the development of metabolic syndromes in human subjects (Xu *et al.*, 2007).

C. **Fatty Acid Synthase**. Although nearly all tissues in the human body have some level of fatty acid synthase (FASN), it is highly expressed in tissues like adipose, liver and lactating mammary glands (Jayakumar *et al.*, 1995). FASN is a multi-enzyme complex that catalyzes the 7 different biochemical reactions starting with acetyl-CoA and malonyl-CoA and utilizing 4 NADPHs and 7 ATPs to ultimately synthesize the palmitate. Then the elongase catalyzes the biochemical reactions to lengthen palmitate and produces the long-chain saturated fatty acids.

The FASN knockout mice study reveals that it is essential in embryonic development: most of the $Fasn^{-/-}$ mutant embryos died prior to implantation while the $Fasn^{+/-}$ embryos expires at different stages of their development (Chirala *et al.*, 2003). The FASN is transcriptionally regulated and is sensitive to hormonal and nutritional regulation (Sul and Wang, 1998). The mRNA expression of FASN is regulated by sterols (Bennett *et al.*, 1995). In the FASN promoter region that is required for the sterol regulation, there are two binding sites for sterol regulatory element-binding protein 1 (SREBP1). One of them is situated close to a binding site for the transcription factor Sp1. The growth hormone and prolactin reduces the mRNA and protein levels of FASN in 3T3-L1 cells (Hogan and Stephens, 2005). The prolactin represses the expression of FASN through STAT5A binding to the −908 to −893 sites. In adipocytes, expression of FASN is also inhibited by leptin.

D. **Adipose Tissue Triglyceride Lipase**. The recently discovered adipose tissue triglyceride lipase (ATGL) is predominantly expressed in adipocytes (Zimmermann *et al.*, 2004). It is the first and rate-limiting lipolytic enzyme in the TAG catabolism cascade (Haemmerle *et al.*, 2006). The ATGL catabolizes TAG to diacylglycerol (DAG). The mRNA expressions of ATGL is regulated by feeding/fasting (Villena *et al.*, 2004) as well as hormones and cytokines (Kralisch *et al.*, 2005; Kershaw *et al.*, 2006). Insulin, isoproterenol and TNFα have been reported to downregulate ATGL. The ATGL KO mice have severely increased adipose mass and adipose size (Haemmerle *et al.*, 2006). Also, the expression of ATGL is decreased in obesity models (Villena *et al.*, 2004; Jocken *et al.*, 2007).

E. **Hormone-Sensitive Lipase.** Hormone-sensitive lipase (HSL) is the second lipolytic enzyme in the TAG catabolism cascade and catabolizes DAG to monoacylglycerol (MAG). Although HSL is predominantly expressed in adipose tissue, it is also found in adrenal, ovary, testis, macrophages, skeletal and cardiac muscles (Holm *et al.*, 1988; Kraemer *et al.*, 1993). The term "hormone-sensitive lipase" was designated because epinephrine triggers HSL to cause increased release of FFA by adipocytes in fasting-state (Kraemer and Shen, 2002). Other hormones namely adrenocorticotropic hormone (ACTH) and glucagon can also stimulate such responses while insulin inhibits HSL. The hormonal stimulation of HSL is affected via cyclic AMP dependent protein kinase (PKA) which phosphorylates the HSL (Yeaman, 1990). The HSL KO mice have reduced fat deposition, gain body weight and adipocyte size increase (Sekiya *et al.*, 2004). Often, HSL expression has been correlated with the pathogenesis of obesity, type 2 diabetes and metabolic syndromes (Girard and Lafontan, 2008; Jocken *et al.*, 2007).

F. **Carnitine Palmitoyl Transferase I**. Carnitine palmitoyl transferase I (CPT1) is an outer mitochondrial membrane enzyme which is responsible for the formation of fatty acyl carnitine by catalyzing the transfer of acyl group of fatty acyl-CoA (Jogl *et al.*, 2004; Bonnefont *et al.*, 2004). The fatty acyl carnitine then are transported across the inner mitochondrial membrane via carnitine translocase (CAT) and finally the inner mitochondrial membrane enzyme CPT2 converts the fatty acylcarnitine back to fatty acyl-CoA which enters the fatty acid β-oxidation pathway inside mitochondria. The CPT1 is inhibited by malonyl CoA, the precursor for the de novo synthesis of fatty acids (Rasmussen *et al.*, 2002). Thus CPT1 catalyzes the rate-limiting step of mitochondrial fatty-acid β-oxidation pathway for energy (ATP) generation and used as bio-marker of β-oxidation (van der Leij *et al.*, 2000). The following three isoforms of CPT1 have been identified:

- CPT1A (known as the liver isoform) is expressed throughout the body organs except for skeletal muscles and adipose tissue. This isoform is linked to carnitine palmitoyl transferase I deficiency (Ogawa *et al.*, 2002) which is a rare metabolic disorder where the body is prevented from converting certain LCFAs into energy. Overexpression of CPT1A via gene therapy has been shown to protect against obesity, hepatic steatosis, diabetes, and insulin resistance in mice (Orellana-Gavalda *et al.*, 2011).
- CPT1B (also called as the muscle isoform) is found in skeletal muscles, adipose tissue and heart. Although inhibition of CPT1 activity has been suggested to exert protective effects against cardiac hypertrophy and heart failure, CPT1B

knocking out in mice has been observed to cause lipotoxicity in heart under pathological stress (He *et al.*, 2012).

- CPT1C (known as the brain isoform) is expressed mainly in brain and testes. This isoform was discovered in 2002. Recent study suggests CPT1C may not play a significant role in fatty acid oxidation but can play a role in neuronal oxidative metabolism (Lee and Wolfgang, 2012).

2.4: Renin Angiotensin System (RAS) components – role in regulation of obesity

2.4.1: General aspects of RAS

The renin-angiotensin system (RAS) and its major components were identified in early 1970s. Classically, RAS was proposed to be comprised of circulating components namely angiotensinogen (Agt) produced by liver, renin by kidneys and angiotensin-converting enzyme (ACE) by lungs. First, the renin acting on Agt produces angiotensin I (Ang I) which is then cleaved to angiotensin II (Ang II) via ACE (Campbell, 1987) (Fig. 1). The Ang II, the effector peptide, acting mainly via angiotensin II type 1 receptor (AT1R) is known to contribute to the pathogenesis of hypertension. The ACE-inhibitors (ACE-I), by inhibiting the production of Ang II in heart and blood vessels, control the hypertension (Ondetti *et al.*, 1977). Thus, RAS was considered to have only endocrine function.

However, it was observed lately that the tissues locally produce RAS in most organs besides the circulating RAS (Kumar *et al.*, 2007). The adrenal glands, brain and adipose RAS are reported to work independently of circulating RAS. Although the classical RAS is known to play an important role in cardiovascular biology - electrolyte

homeostasis, sympathetic nervous system, blood pressure regulation, recently adipose RAS has been implicated in adipocyte biology, adiposity and metabolic disorders (Kalupahana and Moustaid-Moussa, 2012). Moreover, human obesity is mostly reported to be positively associated with systemic as well as local adipose RAS. Also it was observed adipose tissue expresses almost all the important components of the RAS (Yvan-Charvet and Quignard-Boulange, 2011). Possibly, the adipose RAS locally affects the cell proliferation, growth, protein synthesis, etc. and thus impacts adipose biology locally. This led to the belief that RAS not only have endocrine but also important paracrine and intracrine functions. Although all the RAS components have not been studied in context to adiposity and obesity, however their involvement in other pathological or CV disorders can give us an insight of their role in pathological conditions.

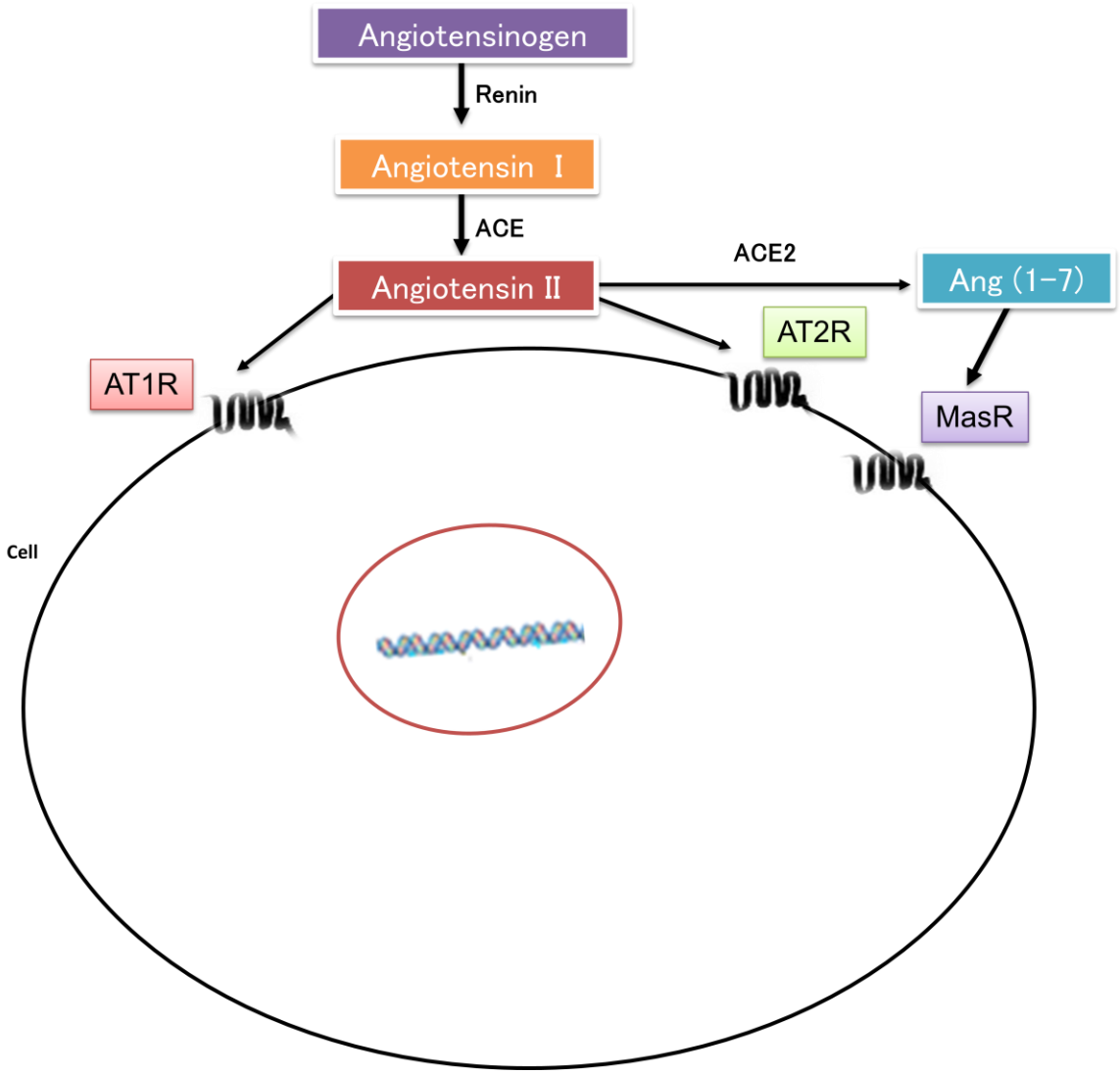


Fig. 1: Major components of renin angiotensin system (RAS). AT1R – angiotensin type 1 receptor, AT2R – angiotensin type 2 receptor, ACE – angiotensin converting enzyme, ACE2 – angiotensin converting enzyme 2, Ang(1-7) – angiotensin (1-7), MasR – mas receptor.

2.4.2: Angiotensinogen in adiposity

Angiotensinogen (Agt), the precursor peptide of RAS, is released into the circulation mainly by the liver. However, adipose tissue is another important source of plasma Agt (upto 30% in obesity) besides liver (Massiera *et al.*, 2001). Although human adipose Agt has been reported to have positive or negative or no association with obesity (Kalupahana and Moustaid-Moussa, 2012), overexpression of Agt in adipose tissue has been reported to be positively associated with protein expression of both renal AT1R and Agt (Kim *et al.*, 2006). Moreover, the knocking out of Agt gene has been observed to have smaller adipocyte size. These findings indicate that Agt may contribute to the progression of adiposity.

2.4.3: Renin – Renin/Prorenin Receptor in adiposity

Renin secretion is the main rate limiting step (Atlas, 2007) and activated renin acting on Agt produces the Ang I. The interaction of prorenin with renin/prorenin receptor (R/PR) allow prorenin to become fully active and increases renin catalytic activity as well as activates MAP kinases p44/p42 or extracellular regulated kinases 1/2 (ERK1/2) leading to activation of genes like TGF-β, collagen, etc. These factors then increases hypertrophy and fibrosis in cardiac and kidney tissues (Nguyen, 2007). In accordance, human plasma and adipose renin has been reported to have positive association with obesity (Kalupahana and Moustaid-Moussa, 2012). Moreover, visceral adipose tissue is shown to express R/PR more than the subcutaneous adipose tissue (Achard *et al.*, 2007), possibly explaining the deleterious effects of visceral adipose tissue in obesity. Also, the knocking out of renin has been shown to have smaller adipocytes (Kalupahana

and Moustaid-Moussa, 2012). Although, these findings indicate that renin may be contributing to adiposity, but the failure of attempt to block RAS completely using renin inhibitor (aliskiren) in patients with type 2 diabetes having high risk for cardiovascular and renal problems (Cleland *et al.*, 2013) has revealed recently that not all the components of RAS contribute to obesity and most likely RAS has components that help prevent obesity as well.

2.4.4: Angiotensin-Converting Enzyme (RAS enzyme) and Chymase (Non-RAS enzyme) in adiposity and other pathological conditions

Angiotensin-converting enzyme (ACE) converts Ang I into Ang II. Although plasma ACE has positive association with adiposity (Kalupahana and Moustaid-Moussa, 2012) and ACE inhibitors (ACE-I) has been used to treat CV disorders, ACE-I has been in found to be ineffective in many pathological condition (Miyazaki *et al.*, 2006). This indicates that non-RAS pathways may be involved in at least some pathological conditions.

Chymase (a non-RAS enzyme), released by mucosal mast cells cleaves angiotensin I at the same site as ACE to convert Ang I into Ang II (Fyhrquist and Saijonmaa, 2008). It is enzymatically inactive in normal physiological condition and generates Ang II only in damaged (or atherosclerotic) condition. Thus, it possibly plays a role in at least certain pathophysiological conditions. Moreover as human adipose tissue expresses non-RAS enzymes to bypass the renin-ACE axis (Karlsson *et al.*, 1998), the Agt generation might be key step of RAS regulation in adipose RAS at least in certain pathophysiological conditions.

2.4.5: Angiotensin II receptors

Angiotensin II (Ang II), the main effector peptide of RAS, is known to mediate its actions mainly via two receptor subtypes – type 1 (AT1R) and type 2 (AT2R). The characterization of the Ang II receptor subtypes was made possible by the advent of selective Ang II receptor antagonists, namely losartan (AT1R selective) and PD123,319 (AT2R selective) (Timmermans *et al.*, 1993; Mukoyama *et al.*, 1993). Although Ang II binds with similar affinity to both AT1R and AT2R (de Gasparo *et al.*, 1995) and both the receptor belong to the 7-transmembrane class of G-protein coupled receptors (GPCRs) (de Gasparo *et al.*, 1995; Griendling *et al.*, 1996), the AT2R is substantially different from AT1R in many aspects. The AT2R shares only 34% sequence homology with the AT1R (Mukoyama *et al.*, 1993) and unlike AT1R, the agonist binding to AT2R has been shown to cause no internalization or desensitization of AT2R (Thomas *et al.*, 1996; Hein *et al.*, 1997), possibly because the third intracellular loop of AT2R is short and does not offer enough Ser phosphorylation sites for G protein-coupled receptor kinase (GRK).

The AT1R is expressed in the heart, vasculature, adipose tissue, kidney, central nervous system (CNS) while the AT2R is expressed in the adrenal gland, adipose tissue heart, brain, kidney (Allen *et al.*, 1990; Allen *et al.*, 1999*;* Yvan-Charvet and Quignard-Boulange, 2011). Unlike the AT1R, the expression of AT2R rapidly decline after birth (Lazard *et al.*, 1994; Shanmugam and Sandberg, 1996). It has also been reported that the relative expression of AT2R decreases through adolescence and diminishes in adult life (Sampson *et al.*, 2012; Ozono *et al.*, 1997). Moreover, female mice have been observed to express greater renal AT2R than male mice and the AT1R/AT2R ratio in

kidneys has been reported to be less in females compared to males (Baiardi *et al.*, 2005). Under pathophysiological conditions namely obesity, heart failure, myocardial infarction, vascular injury, renal failure, the AT2R expression is upregulated (Ha *et al.*, 2011; Hakam and Hussain, 2005; Tsutsumi *et al.*, 1998; Kimura *et al.*, 1992; Nio *et al.*, 1995; Viswanathan *et al.*, 1994). Interestingly, the up-regulation of AT1R is associated with enhanced AT2R expression in pathophysiological conditions. However, inhibition of AT1R by ARBs (valsartan and irbesartan) has also been shown to upregulate AT2R expression.

2.4.6: Physiological roles of angiotensin receptors

The Ang II acting via AT1R causes vasoconstriction, cell growth, proliferation, fibrosis, aldosterone and vasopressin secretion, production of superoxides, lowering of plasma adiponectin, inflammation, oxidative stress and anti-natriuresis (Tallam and Jandhyala, 2001; Kanaide *et al.*, 2003; Carey, 2007). The Ang II acting via AT2R causes opposite functions namely vasodilation, cell growth inhibition, natriuresis, production of nitric oxide (NO) and inhibition of fibrosis. However, AT2R has been shown to cause apoptosis, nuclear factor-kappa B (NF-κB) transduction and chemokine induction which may have impact on cellular or tissue integrity.

Interestingly, ARB treatment also increases plasma Ang II levels and possibly causes increased stimulation of AT2R. Several lines of evidence suggest that AT2R is a functional antagonist of AT1R and the signaling pathway associated with AT1R can be blocked by AT2R activation.

Ang II acting via AT1R activates phospholipase C (PLC) to produce IP3 from PIP2, thus mobilizing the release of $[Ca^{2+}]i$ to cause vasoconstriction (Kanaide *et al.*, 2003). Also Ang II acting via AT1R activates NADPH which leads to production of reactive oxygen species (ROS) (Peters *et al.*, 2000). The ROS is known to cause vasoconstriction. On the other hand, AT2R and bradykinin (BK) receptor (B2R) hetero-dimerization causes phosphorylation of c-Jun terminal kinase, phosphotyrosine phosphatase, inhibitory protein κBα and activating transcription factor 2 (Carey, 2007) (Carrillo-Sepulveda *et al.*, 2013). Also the AT2-bradykinin receptors heterodimer causes dephosphorylation of p38 and p42/44 mitogen-activated protein kinase and signal transducer inhibitor of transcription 3 and increases production of NO and cyclic guanosine monophosphate (cGMP). The NO neutralizes ROS and causes vasodilatation. Thus, Ang II mediates opposite effects via AT1R and AT2R on vessels.

In the kidney, Ang II acting on AT1R mediates anti-natriuresis (inhibition of excretion of sodium in urine) and the release of vasopressin and aldosterone contributing to increase in blood pressure (BP) (Tallam and Jandhyala, 2001). In contrast, Ang II acting via AT2R has been shown to promote natriuresis (Hakam and Hussain, 2005). Losartan (antihypertensive, AT1R blocker) has also been shown to increase cAMP generation (Li *et al.*, 2012) indicating that AT1R decreases cAMP to increase BP while AT2R activation (Ali *et al.*, 2013) decreases BP. Possibly, AT2R activates cGMP which in turn decreases PDE3 activity to oppose the AT1R mediated BP increase via decreased cAMP.

2.4.7: Role of angiotensin receptors in inflammation and insulin resistance

ACE-I (enalapril) and ARB (losartan) have been shown to prolong the life spans of rats possibly by inhibition of ROS formation and reduction of oxidative stress (Basso *et al.*, 2007). In concert to this, Ang II acting via AT1R activates NADPH causing production of ROS that activates and causes nuclear translocation of NF-κB (Chan and Leung, 2007). The NF-κB triggers production of TNFα and IL-6, leading to inflammation. In contrast, AT2R stimulation has been reported to reduce pro-inflammatory cytokines namely TNFα and IL-6 and increase anti-inflammatory cytokines namely IL-10 (Sabuhi *et al.*, 2011; Dhande *et al.*, 2013); thus AT2R counters AT1R-induced inflammation.

Ang II infusion chronically acting via AT1R induces insulin resistance. As mentioned above, Ang II acting on AT1R affects generation of TNFα and IL-6. The TNFα acting via c-Jun-N-terminal kinase (JNK) and IL-6 acting via JAK/STAT/SOCS3 (suppressor of cytokine signaling 3) inhibits the tyrosine phosphorylation of IRS-1 leading to deactivation of insulin signaling and inhibition of GLUT4 translocation to cell membrane culminating in reduced glucose entry into cell (Taniguchi *et al.*, 2006). Thus, Ang II acting on AT1R impairs glucose transport via inhibition of insulin signaling and in addition decreases anti-inflammatory adipokine adiponectin which is an insulin sensitizer. On the other hand, AT2R stimulation has been shown to ameliorate insulin resistance via PPARγ activation (Ohshima *et al.*, 2012).

2.4.8: Role of angiotensin receptors in cell differentiation and growth

Blocking of AT1R promotes differentiation of preadipocytes by increasing the expression and activation of PPAR-γ (Jing *et al.*, 2013) (Koh *et al.*, 2013). On the other hand, blocking of AT2R inhibits differentiation of preadipocytes with a decrease in expression of PPAR-γ and also AT2R activation has been reported to activate PPAR-γ (Ohshima *et al.*, 2012). Thus, Ang II mediates opposite effects on cell differentiation via AT1R and AT2R.

AT1R mediates triiodothyronine (T_3)-induced activation of PI3K/Akt/GSK3-β (Glycogen synthase kinase-3β)/mTOR (*mammalian target of rapamycin*) signaling pathway to cause hypertrophy in cardiomyocytes (Diniz *et al.*, 2009). While Ang II via AT2R either activates phosphotyrosine phosphatases (PTP) to deactivate STAT (tyrosine dephosphorylation of STATs) or activates protein phosphatase 2A (PP2A) to oppose ERK-mediated activation of STAT (serine dephosphorylation of STATs) or activates SHP-1 to deactivate PI3K/AkT to oppose hypertrophy. Also, the overexpression of AT2R attenuates the cardiac hypertrophy (Metcalfe *et al.*, 2004). Thus, AT1R and AT2R mediate opposing effects on cardiac hypertrophy.

In adipose tissue, ARB (candesartan) treatment has been shown to reduce adipocyte size (Putnam *et al.*, 2012) indicating AT1R causes hypertrophy of adipocytes. However, role of AT2R in regulation of adipocyte size has not been explored.

2.4.9: Inter-RAS regulatory role of angiotensin II

Losartan treatment increases plasma renin activity (Wang *et al.*, 1997) indicating AT1R negatively regulates renin. Although AT2R stimulation reduces the AT1R function and/or expression, the renin expression is not impacted (Ali *et al.*, 2013). Possibly, there is also a regulatory interplay between AT2R and R/PR, since both share the adapter protein PLZF (promyelocytic leukaemia zinc finger) (Horiuchi *et al.*, 2012) and this interaction possibly inhibits the detrimental cardiovascular effect of R/PR activation.

Ang II acting via AT1R has been shown to up-regulate ACE and down-regulate ACE2 via the AT1R-ERK/p38 MAPK pathway (Koka *et al.*, 2008) shifting a balance between Ang II and Ang (1-7) production. On the other hand, stimulation of AT2R increases ACE2/Ang(1-7)/MasR (beneficial axis of RAS) and decreases ACE/AT1R (detrimental axis of RAS) (Nag *et al.*, 2012; Ali *et al.*, 2013) shifting ACE/ACE2 ratio in the direction opposite to AT1R. Thus, AT2R play a beneficial role via ACE2.

2.4.10: Angiotensin-converting enzyme 2 / Angiotensin (1-7) / Mas receptor axis of RAS in adiposity and other pathological conditions

The ACE2, a carboxypeptidase enzyme, is abundant in kidney, heart, hypothalamic region of brain, adipose, aortic wall and testis (Fyhrquist and Saijonmaa, 2008). Regulation of ACE2 under various pathological or dietary conditions is still not very clear. Chronic HFD results in adipose ACE2 dysregulation and increases blood pressure in rodents (Gupte *et al.*, 2008). Recent studies suggest that adipose ACE2 exerts a protective role against obesity-associated hypertension via PPARγ activation

(Gupte *et al.*, 2008) and endogenous ACE2 plays a role in maintaining insulin sensitivity and glucose homeostasis in metabolic syndrome (Takeda *et al.*, 2013). Moreover it is suggested that stimulation of ACE2 has beneficial effects in obesity-associated hypertension (Gupte *et al.*, 2012).

Angiotensin 1-7 (Ang 1-7), another peptide in RAS, is produced by the cleavage of Ang II by ACE2 (Fyhrquist and Saijonmaa, 2008). ACE2 can also cleave Ang I to Angiotensin 1-9 (Ang 1-9) which is further cleaved to Ang 1-7 via ACE (RAS enzyme) or neutral endopeptidase (non-RAS enzyme). Also, neutral endopeptidases are capable of generating Ang (1-7) directly. The Ang (1–7) binding to the MasR exerts actions opposing to those of Ang II via AT1R namely vasodilation, antiproliferative, antifibrotic, antihypertrophic, promotes endothelial function and antithrombotic.

The activation of MasR via Src homology 2-containing protein-tyrosine phosphatase-1 (SHP-1) inhibits MAP kinases activation by AT1R (Mercure *et al.*, 2008). MasR also has been reported to hetero-oligomerize with AT1R to inhibit the actions of Ang II via AT1R. Moreover, Ang (1–7) acting on MasR has been shown to attenuate Ang II-mediated inhibition of insulin signaling by the phosphorylation of insulin signaling mediators (Akt, GSK-3β and AS160 (Akt substrate) in liver, skeletal muscle and adipose tissue (Munoz *et al.*, 2012). Furthermore, the deficiency of MasR increases WAT weight, plasma insulin, total serum cholesterol, TG, adipose Agt indicating a potential beneficial role of the MasR on adiposity (Santos *et al.*, 2008). These findings suggest that the ACE2/Ang (1-7)/MasR axis of RAS is beneficial for preventing obesity and metabolic syndrome.

2.5: Estrogen – role in regulation of obesity, metabolic syndrome and RAS

Although the female steroid hormone, estrogen (17,β-estradiol / E2) is mainly produced by ovaries and plays a central role in reproduction, E2 is also produced locally by the other organs namely adipose tissues, bones, blood vessels, smooth muscle cells and brain and is involved in various metabolic and physiological functions involving a variety of organs (Simpson, 2003). Recent findings indicated that the E2 plays an important role in energy homeostasis (Mauvais-Jarvis, 2011)

E2 acts via two types of estrogen receptors (ER) namely ERα and ERβ. ER signaling for reproductive functions are mostly mediated by genomic pathways (involving transcription factors like Fos/Jun) while the energy metabolism functions are mediated by mainly non-genomic pathways (involving STAT and PI3K) (Liu and Mauvais-Jarvis, 2010; D'Eon *et al.*, 2005).

2.5.1: Estrogen and brain

The anti-obesity effects of (E2) are thought to be largely mediated by the central nervous system (Mauvais-Jarvis, 2011). In central nervous system (CNS), E2 acting possibly on ERα in the nucleus of the solitary tract (NTS) and/or arcuate nucleus (ARC) – proopiomelanocortin (POMC) neurons reduces food intake (Gao *et al.*, 2007; Xu *et al.*, 2011) while in the ventromedial hypothalamus (VMH)-steroidogenic factor 1 (SF-1) neurons increases energy expenditure to prevent adiposity or obesity (Musatov *et al.*, 2007). Also, E2 has been shown to increase the ability of centrally administered leptin to cause anorexia or suppress food intake in rats (Clegg *et al.*, 2006). At the molecular

level, E2 possibly by a leptin-induced STAT pathway in NTS-POMC neurons regulates food intake (Gao *et al.*, 2007) and by the PI3K pathway and/or modulating leptin sensitivity in VMH-SF-1 neuron affects the increase in the energy expenditure (Musatov *et al.*, 2007). Although E2 acting via the ERβ in the brain has also been reported to reduce food intake and increase energy expenditure to prevent obesity, the molecular mechanisms involved are not clear yet (Mauvais-Jarvis, 2011).

2.5.2: Effect of estrogen on adipose tissue

As mentioned above, E2 is involved in various metabolic and physiological functions in white adipose tissue (WAT), skeletal muscle, liver, pancreatic β cells, etc. In white adipose tissue (WAT), E2 acting on ERα has been shown to reduce total WAT mass, adipogenesis, lipogenesis, increase lipolysis (D'Eon *et al.*, 2005) and promote subcutaneous WAT distribution via central (Clegg *et al.*, 2006) as well as peripheral mechanisms (Cooke and Naaz, 2004). Also E2 acting via ERβ reduces WAT mass, lipogenesis by downregulation of acetyl-coA carboxylase (ACC) and fatty acid synthase (FASN) (Mauvais-Jarvis, 2011) and adipogenesis, possibly by negatively regulating the PPAR-γ or PPAR-γ coactivator 1α (PGC-1α) in WAT (Foryst-Ludwig *et al.*, 2008). Interestingly, the increased energy expenditure by E2 is also at least in part mediated by increased thermogenic uncoupling protein-1 (UCP-1) in brown adipose tissue (BAT) via ERβ (Yepuru *et al.*, 2010).

Recent findings suggest a cross-talk between adipose and kidney via E2. Lipocalin 2, a recently recognized adipokine, has been shown to be essential for chronic kidney disease progression in both rodents and humans (Viau *et al.* , 2010). Lipocalin 2

possibly regulates E2, ERα and adiposity (Fried and Greenberg, 2012). Moreover, a deficiency of lipocalin 2 possibly via reduced aromatase (enzyme that synthesizes E2 from testosterone in WAT but not ovary) leads to decreases in serum E2 and also decreases estrogen receptor α (ERα), lipid metabolism regulators - PPARγ, liver-x-receptor beta (LXRβ), low density lipoprotein (LDL) receptor protein expression in gonadal adipose tissue.

2.5.3: Effect of estrogen on lipid metabolism in skeletal muscle and liver

In skeletal muscles, E2 via ERα improves insulin sensitivity and increases GLUT4 expression (Barros *et al.*, 2009). In contrast, in the absence of ERα signaling, E2 acting on ERβ in skeletal muscle reduces insulin sensitivity and GLUT4 expression to promote insulin resistance (Barros *et al.*, 2009). These findings suggest that E2 mediates opposing functions via ERα and ERβ in skeletal muscles.

Unlike skeletal muscles, E2 seems to mediate analogous functions via ERα and ERβ in liver. In liver, E2 acting via ERα increases the insulin sensitivity, reduces the hepatic glucose production (HGP) and possibly also reduces the lipogenesis while acting via ERβ, E2 has been shown to reduce TAG accumulation (Bryzgalova *et al.*, 2006; Bryzgalova *et al.* , 2008; Yepuru *et al.*, 2010).

2.5.4: Effect of estrogen on lipid metabolism in pancreatic β cells

Although the molecular mechanism(s) involved in the regulation of lipid metabolism by E2 has not been studied in WAT; it has been studied in pancreatic β cells. In pancreatic β cells, ERα activation via tyrosine-protein kinase Src activates

STAT3 (phosphorylation of STAT3) which by inhibiting the liver X receptor-β / LXRβ suppresses the sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate response element binding protein (ChREBP). The activation of this signaling pathway causes a decrease in FASN and prevents lipogenesis / lipotoxicity (Tiano and Mauvais-Jarvis, 2012). Furthermore, ERα activation by activating AMP-activated protein kinase directly suppresses SREBP1c to decrease FASN / lipogenesis in β cells. In concert, E2 acting on ERβ increases β-cell survival and possibly increases glucose-stimulated insulin secretion (GSIS) (Liu *et al.*, 2009). Thus, E2 seems to mediate analogous functions via ERα and ERβ in pancreatic β cells.

2.5.5: Effect of estrogen on RAS regulation

Both clinical and animal studies have shown that E2 provides protective effects by directly altering RAS (Maric-Bilkan and Manigrasso, 2012; Xue *et al.*, 2013). The induction of angiotensinogen mRNA levels has been reported in liver, kidney and aorta by E2 treatment in ovariectomized rats (Gordon *et al.*, 1992). E2 treatment decreases AT1R mRNA translation (Wu *et al.*, 2003b) and E2 and AT1R blocker synergistically attenuate the vascular remodeling, possibly via inhibition of ERK1/2 and STAT (Liu *et al.*, 2002). Renal AT2R expression is upregulated by E2 treatment (Armando *et al.*, 2002). Our AT2R-KO study also indicates that AT2R prevents adiposity and lowers E2 in female mice under HFD (Samuel *et al.*, 2013). The beneficial cardiovascular effects of E2 have been attributed at least in part to downregulation of ACE (Gallagher *et al.*, 1999) while E2-mediated protection from hypertensive renal disease has been suggested by E2-induced upregulation of renal ACE2 and Ang-(1-7) (Ji *et al.*, 2008).

More recently, central E2 effects have been suggested to protect against the development of hypertension by downregulating the ACE/Ang II/AT1R axis and also possibly by upregulating the ACE2/Ang (1-7)/MasR axis and AT2R via inhibition of ROS production (Xue *et al.*, 2013). Thus, E2 positively regulates the ACE2/Ang (1-7)/MasR axis and AT2R (beneficial axis of RAS) whereas negatively regulates the ACE/AT1R (detrimental axis of RAS).

2.6: Animal models of obesity

The forms of obesity can be classified as monogenic (result from modification in a single gene) and polygenic (result from modification of more than one gene) (Liu *et al.*, 2003). Although obesity has been studied using both monogenic and polygenic animal models of obesity, human obesity is mostly polygenic. Further, genetically engineered animals (mice overexpressing corticotrophin releasing factor, glucose transporter subtype 4, melanin concentrating hormone and mice lacking beta-3 adrenergic receptor, serotonin 5-HT-2c receptor, neuropeptide-Y 1 receptor) and the surgically modified (surgical lesions of ventromedial hypothalamus, paraventricular nucleus, arcuate nucleus and ovariectomy in rodents) animal models of obesity are available nowadays.

A. Monogenic animal models of obesity are as the followings:

i) As animals with a deficiency of leptin or defect in a leptin receptor develop an obese phenotype, several models have been developed and used, namely db/db mice with mutated leptin receptor (Trayhurn and Fuller, 1980), ob/ob mice with leptin deficiency (Ingalls *et al.*, 1950), obese Zucker rats with mutation in the leptin receptor (the recessive fa/fa) (Bray, 1977) and Wistar Kyoto fatty rat (WDF

rat) created by crossing obese Zucker rats with Wistar-Kyoto (WKY) rats (Figlewicz *et al.*, 1986).

ii) Several animal models have been developed by knocking out downstream targets of the brain leptin receptor, namely POMC knockout mice (Yaswen *et al.*, 1999), MC4R knockout mice (Huszar *et al.*, 1997) and Carboxypeptidase E (CPE) mutation mice (Naggert *et al.*, 1995).

B. Polygenic animal models of obesity are as the followings:

i) The New Zealand obese (NZO) mouse with a number of adipogenic gene variants develops obesity (Joost, 2010).

ii) Age-related obesity develops in C57BL/6 mice and rhesus macaque monkeys (late-onset obesity or LOO models) (Becskei *et al.*, 2009; Schwartz *et al.*, 1993).

iii) Diet-induced obesity (DIO) is observed in rats on a high-energy diet (Levin *et al.*, 1997), in many rat strains on cafeteria (western human fast-food) diet (Agius *et al.*, 1980) and in drosophila and rodents (including C57BL/6 mice) on a high-fat diet (HFD) (Diop and Bodmer, 2012; Zaragoza and Felber, 1970; Lemonnier *et al.*, 1971; Schreyer *et al.*, 1998).

The feeding of HFD induces adiposity, obesity and metabolic disorders in rodents resembling the metabolic syndrome in human beings (Ji *et al.*, 2005; Buettner *et al.*, 2007). Not only does HFD-induced obesity in animals resemble the phenotype of human obesity but also HFD affects polygenic regulators (like insulin, leptin) and signaling pathways in brain areas (like hypothalamic POMC) involved in human obesity (Lin *et al.*, 2000). Thus, the use of HFD-induced

animal models of obesity is a powerful approach to study adiposity and obesity. In this thesis, all the studies were carried out using HFD-induced obese mice.

3: MATERIALS AND METHODS

3.1 Animals and experimental protocols

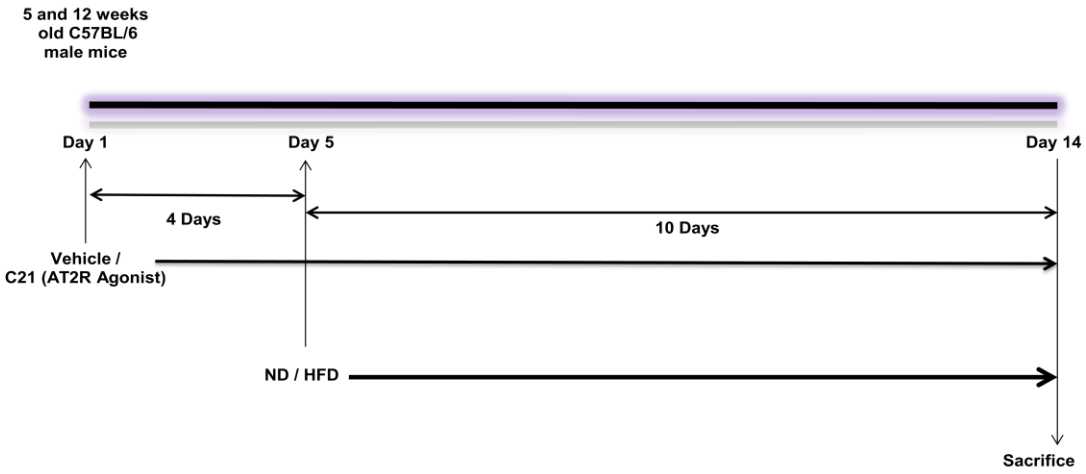
3.1.1 Animal: Drug treatment protocols

Male C57BL/6 (5 week old), male C57BL/6 (12 week old), female ovary-intact C57BL/6 (8 week old), bilateral-ovariectomized C57BL/6 (8 week old) and ovary-intact C57BL/6 (5 week old) mice were obtained from Harlan (Indianapolis, IN). The mice were housed at the University of Houston's animal care facility with free access to food and water and maintained under a 12-hr light/dark cycle in compliance with federal guidelines for animal care. The Institutional Animal Care and Use Committee approved the animal experimental protocols at the University of Houston. The following experimental protocols were used:

a) *Male C57BL/6 (5 and 12 week old) mice*: Male C57BL6 mice (5 and 12 week old) were pretreated with 'Compound 21' (C21) at a dose of 0.3 mg/kg, daily i.p. for 4 days (Fig. 2A and B). Thereafter the mice were placed on either on normal diet (ND) or high-fat diet (HFD) with concurrent drug treatment for the next 10 days. At the end of 14 days, animals were euthanized in non-fasting state by cervical dislocation under anesthesia. Plasma was collected and stored at -80^{o}C until further use. Epididymal white adipose tissue (eWAT) pads were removed, patted dry, weighed and a part of the tissue was preserved in buffered formalin for histology. The remaining part of the eWAT was frozen and stored at -80^{o}C for biochemical measurements.

b) *Female C57BL/6 (8 week old) mice*: Female mice reach sexual maturity by 8 weeks of age. Thus, sham-treated ovary-intact (Ovi) and ovariectomized (Ovx) C57BL6 mice (8 week old) were pretreated with 'Compound 21' (C21) at a dose of 0.3 mg/kg, daily i.p. for 4 days (Fig. 3A and B). Also, Ovx mice were supplemented with 17,β-estradiol / E2 (5 µg/day) pellets alone and with C21 (0.3 mg/kg, daily i.p.) for 4 days. Thereafter the mice were placed on either on normal diet (ND) or high-fat diet (HFD) with the concurrent hormone/drugs treatment for the next 10 days. Urine was collected after placing the individual mice in a metabolic cage for 5 days before sacrificing the animals. At the end of 14 days, animals were sacrificed in a non-fasting state by cervical dislocation under anesthesia. Plasma was collected and stored at -80^{o}C until further use. Parametrial white adipose tissue (pWAT) pads were removed, patted dry, weighed and a part of the tissue was preserved in buffered formalin for histology. The remaining part of the eWAT was frozen and stored at -80^{o}C for biochemical measurements.

(A)



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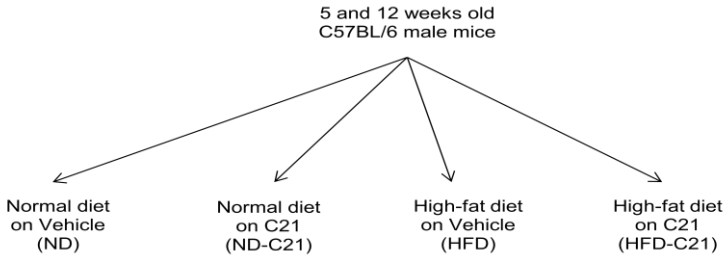
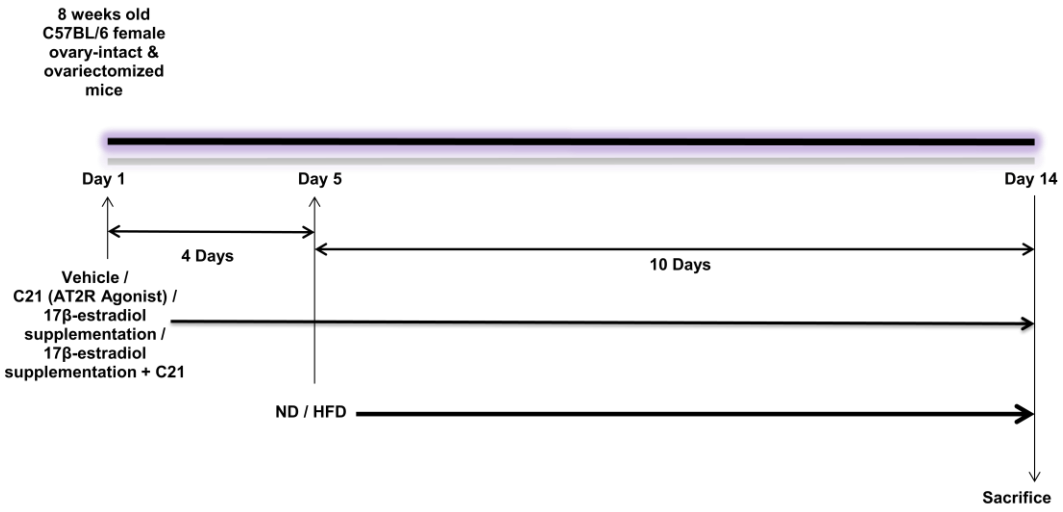


Fig. 2: (A) High-fat diet and C21 treatment protocol and (B) Animal groups using 5 and 12 week old male C57BL/6 mice.

(A)



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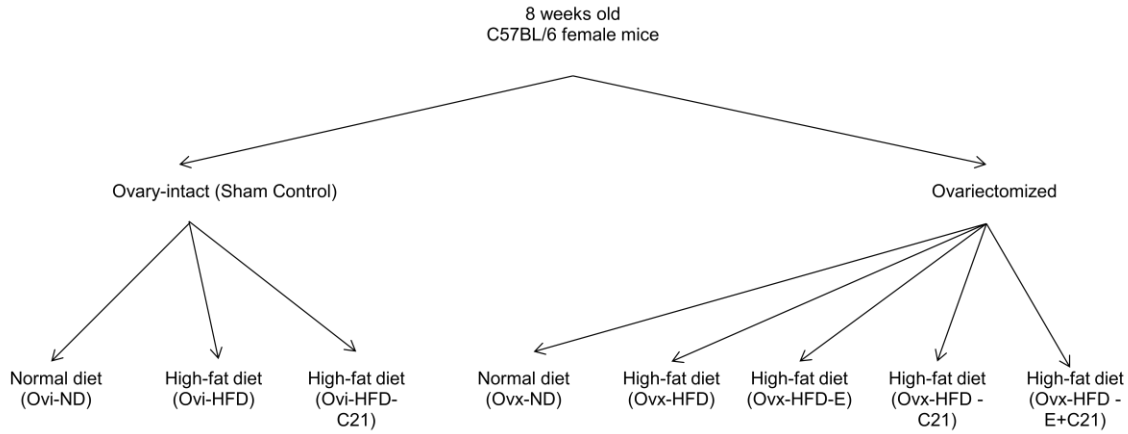


Fig. 3: (A) High-fat diet, C21 and E2 treatment protocol and (B) Animal groups using 8 week old ovary-intact (Ovi) and ovariectomized (Ovx) female C57BL/6 mice.

3.1.2 Food intake and body weight

The mice were housed in groups of 3-4 animals in each cage. Weekly 100 g of food in the form of pellet (Harlan, Indianapolis, IN) was placed in the food slot of the cages. At the end of each week the remaining food was weighed on an electronic balance (Denver instrument P-602) and the amount subtracted from the initial 100 g to account for the food consumed. The total intake of each cage was then divided by the number of mice in the cage. The food consumption, water intake and urine output were recorded after placing the individual mice in metabolic cage for 5 days before sacrificing the animals. The food intake of different groups was calculated in terms of calories consumed. The changes in body weight were measured to assess the effect of the diet and treatment on the body weight.

3.1.3 Free fatty acid (FFA) and triglyceride (TAG) estimation

Plasma FFA and TAG level was measured by a colorimetric method using the respective quantification kit as per the manufacturer's instructions. In the FFA assay, plasma fatty acids were first converted to their CoA derivatives. These were subsequently oxidized with the proportionate generation of the color.

In the TAG assay, plasma TAG was first converted to FFA and glycerol. Then the glycerol was oxidized to generate a product which reacted with the probe to proportionately generate the color. Both plasma FFA and TAG were expressed in nmol/µL.

3.1.4 ELISA for plasma leptin and insulin

Plasma leptin and insulin in different samples were determined using respective ELISA kit as per the manufacturer's instructions. For leptin estimation, plasma samples were first bound to the wells of the plate by the immobilized antibody. The wells were then washed and biotinylated anti-mouse leptin antibody was added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin was pipetted into the wells. After washing the wells again, a 3,3',5,5' - tetramethylbenzidine (TMB) substrate solution was added to the wells and blue color developed in proportion to the amount of leptin bound. Finally, the addition of the stop solution changed the blue color to yellow and the intensity of the color was measured.

For insulin, plasma samples with sample diluent were added to the appropriate wells. The plate was incubated at room temperature. The wells were washed and anti-insulin enzyme conjugate was added to each well and incubated for again at room temperature. The wells were washed, enzyme substrate was added and incubated in dark. Finally, the stop solution was added to stop the reaction and absorbance was measured. Both plasma leptin and insulin were expressed in ng/mL.

3.1.5 Urinary 17,β-estradiol (E2) extraction and enzyme immunoassay (EIA) for E2

Extraction from urine samples was performed by mixing individual samples with methylene chloride and allowing layers to separate. The extraction was repeated twice and the methylene chloride layer was separated followed by evaporation at 30°C. The residue was dissolved in 0.5 ml of EIA buffer. Estradiol level in different groups were determined by pipetting 50 µl of appropriate blank, standard or sample into assigned wells in duplicates. After adding estradiol tracer and EIA antiserum to appropriate wells, the plate was incubated at room temperature for 1 hour. The plate was developed by adding 200 µl of Ellman's reagent to each well and incubating in dark on orbital shaker for 90 min. The absorbance was read. The data was calculated as 17-β-estradiol excreted in 24 hr urine.

3.1.6 Tissue sample preparation

3.1.6.1 Adipose tissue sample preparation: The epididymal or parametrial white adipose tissue (50 mg) samples were homogenized in 2.5 mL buffer (10mM HEPES, 0.25M sucrose, 1mM EDTA, 1X protease inhibitor cocktail having aprotinin, calpain inhibitors, leupeptin, pepstatin and trypsin inhibitor, pH 7.4). The homogenates were centrifuged at 1000g at 4°C for 25 minutes. The liquid (containing proteins) between the fat-cake and pellet were collected gently. This process of collection was repeated 5-6 times until the liquid was assured to be lipid-free. The lipid free liquid exhibits a visibly clear solution with no further cake formation taking place. The protein concentrations extracted from

adipose tissue samples were determined by BCA method using a kit (Pierce, Rockford, IL).

3.1.6.2 Liver sample preparation: The liver (25 mg) samples were homogenized in 2.5 mL phosphate buffered saline (PBS) buffer (with 1X protease inhibitor cocktail, pH 7.4). The homogenates were centrifuged at 1000g for 10 minutes at 4°C. The pellets were discarded and the supernatant liquid (containing proteins) was collected gently. The protein concentrations extracted from liver tissue samples were determined by BCA method using the kit (Pierce, Rockford, IL).

3.1.7 Western Blotting

The levels of specific proteins in adipose or liver samples were determined by standard western blotting technique. Equal amounts of total protein (20µg) from various treatment groups were subjected to 4-12% SDS-PAGE and were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA). The blots were blocked with 5% fat-free milk in PBST and incubated with primary antibodies as listed in Table 1. For chemiluminescent detection, appropriate HRP anti-IgG secondary antibody and enhanced chemiluminescence (ECL) substrate were used. The signals were recorded and analyzed by Quantity One Analysis Software (Bio-Rad, CA) for the densitometry of the bands. For the loading controls, the blots were stripped and re-probed with anti-β-actin.

3.1.8 Histological analysis

The eWAT and pWAT samples, preserved in formalin buffer solution, were used to prepare the paraffin-embedded sections of 5 µm thickness. After staining the sections with hematoxylin and eosin (H&E), the size of adipocytes was measured under three microscopic fields separated by at least 100 µM distance in the tissue (in single blinded condition) using Nikon Eclipse TS 100 light microscope (10X) fitted with a Nikon (*Infinity 1*) camera with NIS-Elements D 3.2 software (Nikon Instruments Inc., Melville, NY). The adipocyte size was expressed in μm^2.

3.1.9 Physical activity assessment

C57BL/6 ovary-intact (Ovi) and ovariectomized (Ovx) female mice were placed in animal activity chambers (60x60 cm open field surrounded by 50 cm high walled Plexiglas chambers) for 2½ h. The first 30 min was considered the acclimation phase and data were calculated for the next 2 h. The data for total activity and the distance travelled were analyzed using a computer-operated Opto-Varimex Micro Activity Meter v2.00 system (Optomax, Columbus Instruments, OH).

3.2 Adipocytes and experimental protocols

3.2.1 Isolation and collection of primary adipocytes

Male C57BL/6 (4 weeks old) mice from Harlan (Indianapolis, IN) were used to obtain isolated primary white adipocytes. The mice were euthanized by cervical dislocation and epididymal fat pads were removed. The epididymal fat was minced

into smaller pieces and white adipocytes were isolated using collagenase - type II (Sigma Aldrich, St Louis, MO) digestion using Dulbecco's phosphate buffered saline modified medium with 0.2% bovine serum albumin buffer (pH 7.4) in a 15 mL Falcon tube at 37°C for 1 h. At the end of 1 h, the Falcon tube was centrifuged at 500Xg at 37°C for 5 minutes. The pellet of the stromal vascular fraction (SVF) was discarded and the supernatant buffer containing the floating adipocytes was filtered and collected using a nylon filter mesh (Spectrum Lab, Rancho Dominguez, CA) of 200 µm. Finally the adipocytes were re-suspended in Dulbecco's phosphate buffered saline modified medium with 0.2% bovine serum albumin buffer and 1M HEPES buffer (pH 7.4) and washed three times before use. The viability of the adipocytes was tested using the trypan blue solution and cell numbers were counted using cell counting chamber (Hausser Sci. Co., Horsham, PA).

3.2.2 Drug treatment protocol and fatty acid uptake assay in adipocytes

Isolated mouse primary epididymal adipocytes (40,000 cells/well/90 µL of serum free Dulbecco's phosphate buffered saline modified medium) were treated with 10 µL of test compounds or serum free Dulbecco's phosphate buffered saline modified medium at 37°C for 10 min. Finally 100 µL of the loading buffer (prepared from the fatty acid uptake assay kit) was added to each well of the 96 well plate and immediately transferred to a microplate reader (BMG LABTECH, Inc. Cary, NC) for fatty acid uptake kinetics readings for 1 h. Excitation and emission filters were set at 485 and 515 nm respectively. Insulin and fatty acid uptake inhibitor

(Triacsin C) were used as positive and negative controls respectively for the fatty acid uptake assay.

In principle, fatty acid uptake assay kit is supplied with a loading buffer that contains fluorescent fatty acid analogs with a quenching dye to reduce the fluorescence. As this loading buffer is added to the adipocytes, the transportation of fluorescent fatty analog inside the adipocytes causes an increase in fluorescence signal via removal of the quenching dye. The test compounds can increase or decrease this fluorescence signal as they activate or inhibit the fatty acid uptake process respectively.

3.3 Chemicals

Compound C21, 97% pure, was custom synthesized by SPS-Alfachem (Lexington, MA) using a synthesis scheme previously published (Wan *et al*., 2004). Dulbecco's phosphate buffered saline modified medium (catalog # D4031), bovine serum albumin (catalog # A8806), collagenase - type II (catalog # C6885), HEPES (catalog # H4034) and Triacsin C (catalog # I3536) were purchased from Sigma Aldrich, St Louis, MO. Fatty Acid Uptake Assay kit (catalog # R8132) for estimation of fatty acid uptake activity was purchased from Molecular Devices, Sunnyvale, CA. Losartan (AT1R blocker) and PD123310 (AT2R blocker) were generous gifts from Merck Sharp & Dohme and Pfizer respectively. Test compounds namely: L-NG-Nitroarginine Methyl Ester / L-NAME (catalog # 0665/100) was purchased from R&D Systems Inc., Minneapolis, MN; 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one / ODQ (catalog # O3636) and Rp-8-Br-PET-cGMPS / PKG inhibitor / PKG-I

(catalog # B6684) activity were purchased from Sigma Aldrich, St Louis, MO. The HFD feed was Teklad custom research diet (catalog # TD.06414) with adjusted calorie diet (60/Fat) containing 18.4% protein, 21.3% carbohydrate and 60.3% fat, and normal diet 7022 with isocaloric 29% protein, 56% carbohydrate and 15% fat were purchased from Harlan, Indianapolis, IN. Both the diet contained similar levels of mineral mix AIN-93G-MX (94046) and vitamin mix AIN93-VX (94047). Plasma free fatty acid quantification assay kit (catalog # ab65341, Abcam, MA) was used for estimation of free fatty acids. Triglyceride quantification assay kit (catalog # K622-100, Biovision) was used for estimation of triglyceride. The standards for Ang(1-7), acetonitrile, deionized water (HPLC grade) and formic acid were purchased from Sigma Aldrich, St Louis, MO). C18 solid phase extraction (SPE) cartridge was purchased from Waters Corporation (Milford, MA). The leptin mouse ELISA kit (catalog # ab100718, Abcam, MA) and ultra sensitive insulin mouse ELISA kit (catalog # 90080, Crystal Chem Inc., Downers Grove, IL) were used for estimation of plasma leptin and insulin, respectively. The Estradiol EIA kit (catalog # 582251, Cayman Chemical, Ann Arbor, MI) was used for estimation of estradiol. The primary antibodies used for western blotting and their sources are enlisted in Table 1.

3.4 Statistical Analysis

The data were analyzed using GraphPad Prism 4 (GraphPad software, San Diego, CA) and subjected to one-way ANOVA with Newman-Keuls post hoc test and Student' unpaired *t* test. A *p* value of less than 0.05 was considered statistically significant. Data are presented as means ± SEM.

Table 1: List of antibodies

Protein	Company	Catalog #	Conc. of protein loaded	Dilution (Primary antibody)
AT2R	EZ Biolab, Carmel, IN	Custom Raised	30 µg	1:500
FABP4	Abcam, Cambridge, MA	Ab92501	20 µg	1:1000
FASN	Abcam, Cambridge, MA	Ab22759	20 µg	1:750
ATGL	Cayman, Ann Arbor, MI	10006409	20 µg	1:500
HSL	Sigma Aldrich, St Louis, MO	SAB4501762	20 µg	1:1000
CPT1A	Abcam, Cambridge, MA	ab128568	20 µg	1:1000
UCP1	Abcam, Cambridge, MA	ab10983	40 µg	1:1000
β-Actin	BioVision, Milpitas, CA	3598-100	-	1:1000

4: CHAPTER 1 – AT2R ACTIVATION AND MALE ADIPOSITY

4.1: Does pharmacological activation of AT2R regulate lipogenic and lipolytic enzymes/proteins and reduce adiposity in 5 week old (young) male mice?

4.1.1: RESULTS from five week old mice:

4.1.1.1: Effect of C21 (AT2R agonist) on calorie intake

Food intake was calculated in terms of total kilo calorie (kcal) consumed in 2 weeks. As shown in Fig. 4, HFD-fed group had significantly higher Kcal intake in 2 weeks (ND 64±4.0 vs HFD 130±5.7 Kcal) compared with ND group. The C21 treatment did not affect the Kcal intake under ND or HFD conditions in these mice.

4.1.1.2: Effect of C21 on epididymal WAT (eWAT) weight-to-body weight ratio

The epididymal WAT (eWAT) weight-to-body weight ratio reflects the eWAT weight. HFD caused an increase (94%) in the eWAT weight-to-body weight ratio (Fig 5). C21-treatment reduced (34%) the HFD-induced increase in eWAT weight-to-body weight ratio, but had no effect in mice on ND.

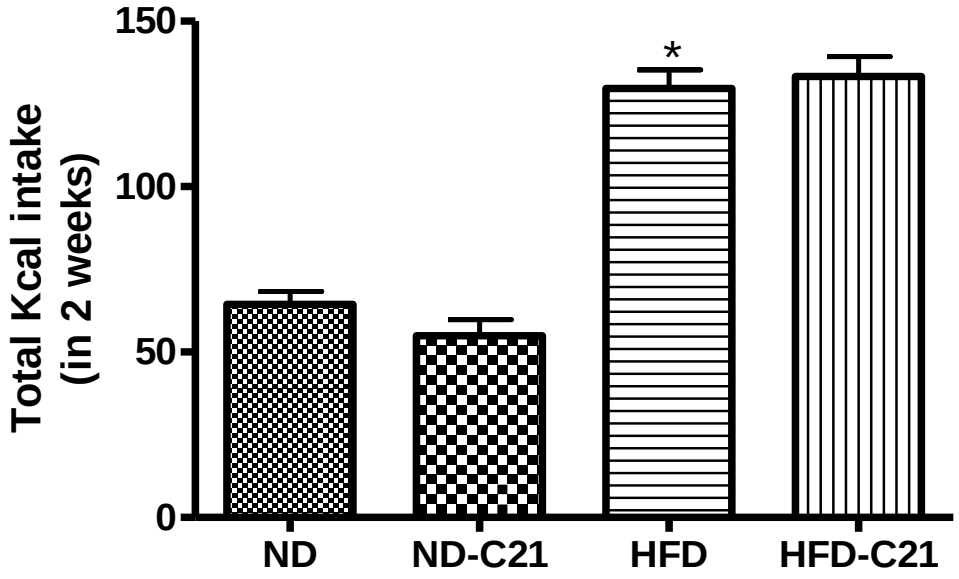


Fig. 4: Total Kcal intake in control and C21-treated 5 week old male C57BL/6 mice fed with ND or HFD. Results are means ± SEM; *significantly different compared with ND. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=6 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

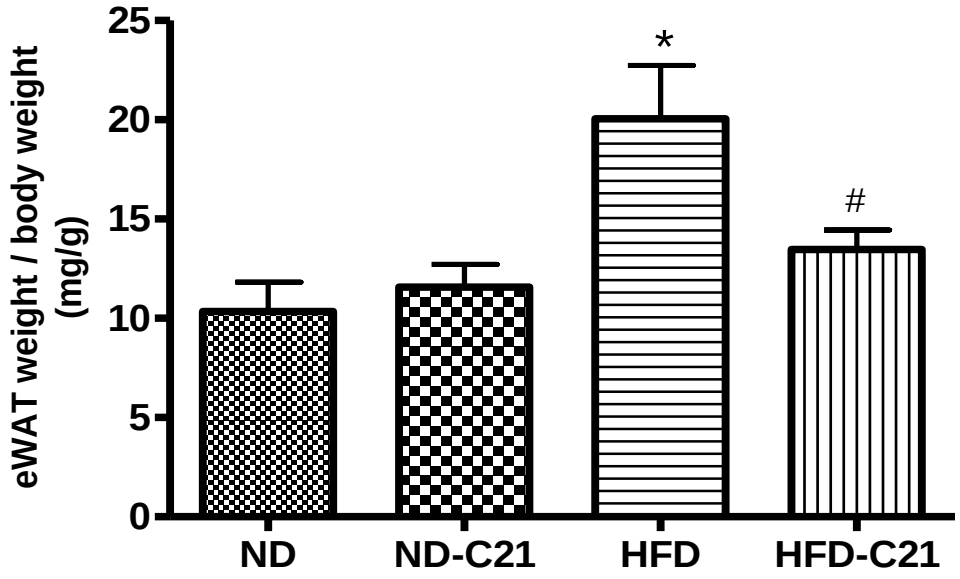


Fig. 5: Epididymal WAT weight-to-body weight ratio of control and C21-treated 5 week old male C57BL/6 mice fed with ND or HFD. Results are means ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=6-8 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

4.1.1.3: Effect of C21 on epididymal adipocyte size

The Fig. 6A shows the H&E staining of the epididymal adipose tissue sections from control and C21-treated 5 weeks old male C57BL/6 mice on ND and HFD. Compared with ND, HFD had increased epididymal adipocyte size (ND 1118±54 vs HFD 2636±79 μm^2) (Fig. 6B). The C21-treatment reduced HFD-induced increase in epididymal adipocyte size (HFD 2636±79 vs HFD-C21 1407±102 μm^2), but had no effect in ND fed mice.

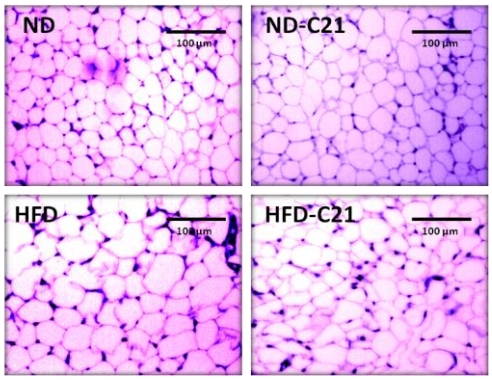
4.1.1.4: Effect of C21 on plasma free fatty acid (FFA)

Obesity is known to be associated with enhanced plasma FFA. We measured the effect of C21 on plasma FFA in young male C57BL/6 mice (Fig. 7) and observed that HFD significantly increased the plasma FFA (ND 2.30±0.20 vs HFD 5.24±0.36 nmol/μL) in these mice. The C21-treatment under HFD reduced the increase in plasma FFA (HFD 5.24±0.36 vs HFD-C21 3.27±0.19 nmol/μL) and had no effect in ND fed mice.

4.1.1.5: Effect of C21 on plasma triglyceride (TAG)

Similar to FFA, the plasma TAG was also significantly increased by HFD compared with ND (ND 1.96±0.23 vs HFD 4.03±0.25 nmol/μL) in these mice (Fig. 8). C21 treatment significantly reduced the plasma TAG (HFD 4.03±0.25 vs HFD-C21 3.3±0.18 nmol/μL) under HFD condition.

(A)



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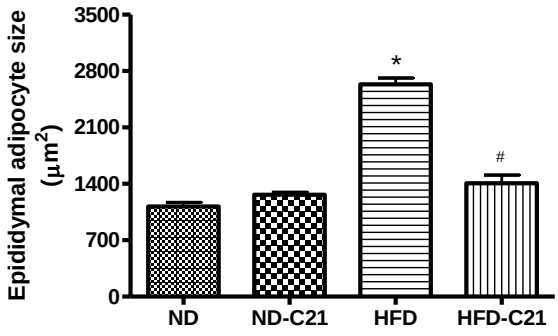


Fig. 6: Epididymal adipocyte size of control and C21-treated 5 week old male C57BL/6 mice fed with ND or HFD. (A) Representative micrograph of epididymal adipose tissue (magnification, 10X) (B) Epididymal adipocyte size was expressed as cell area (μm^2). Results are means ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=3 (3 sections per slide from 3 mice). (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

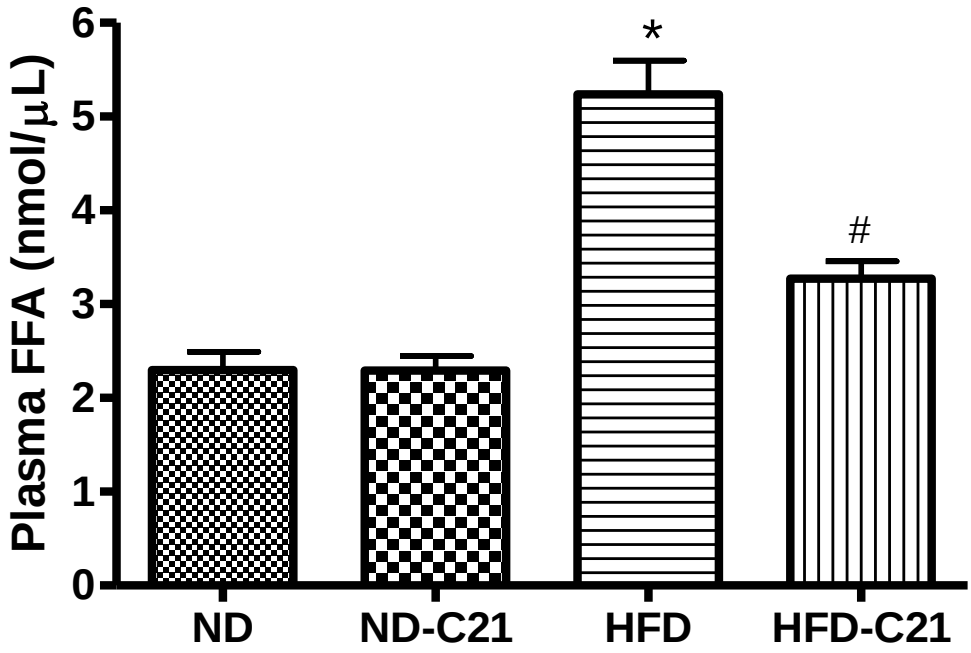


Fig. 7: Plasma FFA concentrations in control and C21-treated 5 week old male C57BL/6 mice fed with ND or HFD. Results are means ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=5-7 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

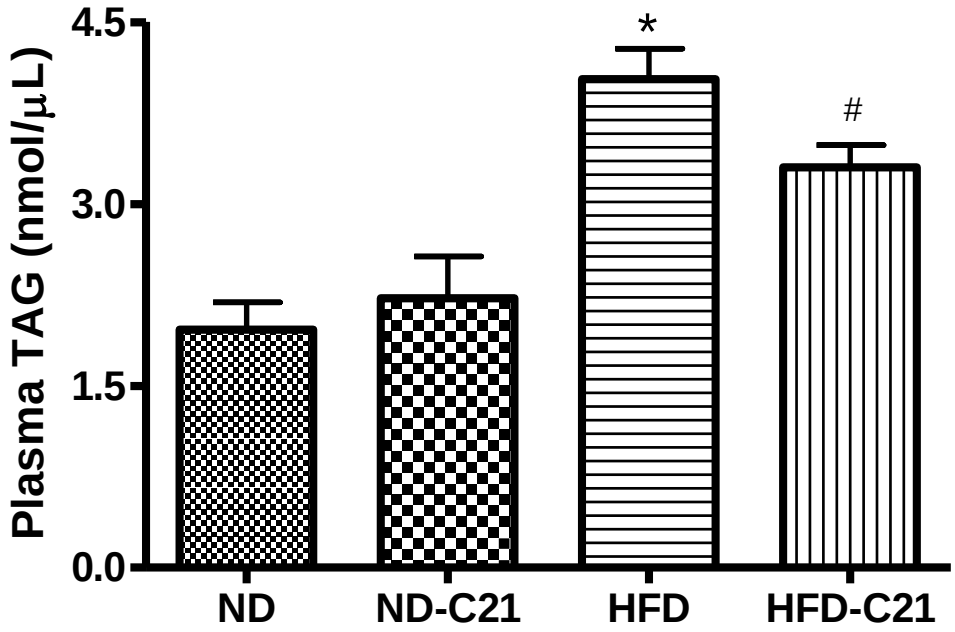


Fig. 8: Plasma TAG concentrations in control and C21-treated 5 week old male C57BL/6 mice fed with ND or HFD. Results are means ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=4-6 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

4.1.1.6: Effect of C21 on plasma insulin

Hyperinsulinemia is an index of insulin resistance which is known to correlate with obesity. Also, AT2R deficiency has been shown to prevent diet-induced insulin resistance. So we expected that activating AT2R will not prevent diet-induced insulin resistance: HFD will cause an increase serum insulin levels in the presence of C21. Therefore, we measured the effect of C21 on plasma insulin levels (Fig. 9). The plasma insulin level was nearly three-fold increased in HFD (ND 0.78±0.06 vs HFD 2.34±0.09 ng/mL) fed mice. Interestingly, the plasma insulin level was attenuated by C21 treatment (HFD 2.34±0.09 vs HFD-C21 1.33±0.19 ng/mL) in HFD condition and had no effect in ND fed mice, consistent with our expectation.

4.1.1.7: Effect of C21 on plasma TNFα

Obesity induces an inflammatory state in which pro-inflammatory cytokines levels increase in the circulation. As a marker of systemic inflammation, we measured plasma TNFα levels. In young male mice, HFD significantly increased plasma TNFα levels (ND 35±2.6 vs HFD 72±15.8 pg/mL) (Fig. 10), which were significantly reduced (HFD-C21 43±4.5 pg/mL) by C21 treatment under HFD condition.

4.1.1.8: Effect of C21 on plasma adiponectin

We also measured plasma adiponectin levels as an anti-inflammatory marker. Plasma adiponectin was significantly decreased by HFD compared with ND (ND 4.41±0.19 vs HFD 2.68±0.43 ng/mL) in young mice (Fig.11). C21 treatment significantly

increased the adiponectin (HFD 2.68±0.43 vs HFD-C21 5.24±0.68 ng/mL) under HFD condition.

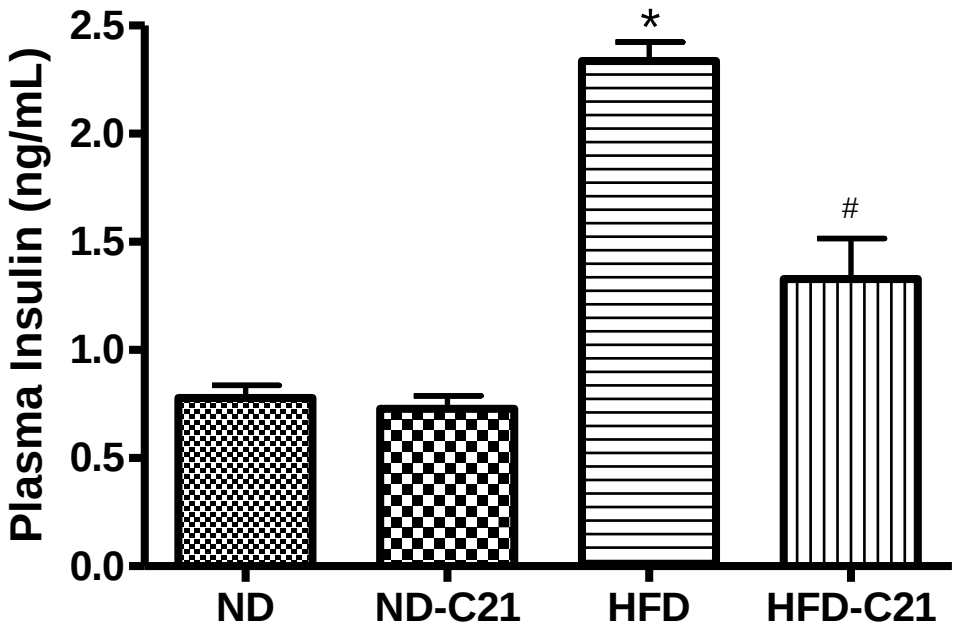


Fig. 9: Plasma insulin levels in control and C21-treated 5 week old male C57BL/6 mice fed with ND or HFD. Results are mean ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=4-5 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

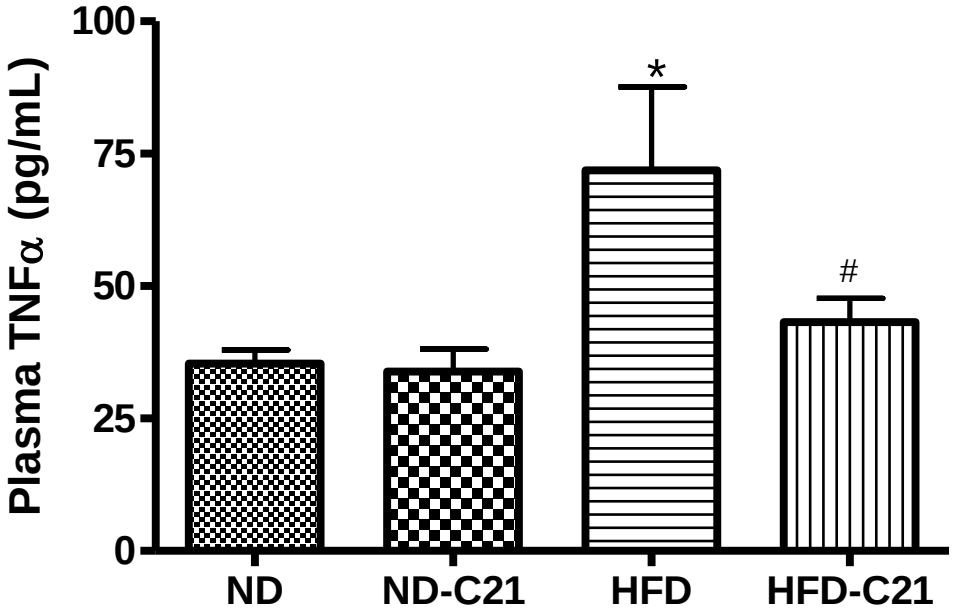


Fig. 10: Plasma TNFα levels in control and C21-treated 5 week old male C57BL/6 mice fed with ND or HFD. Results are means ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=4-5 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

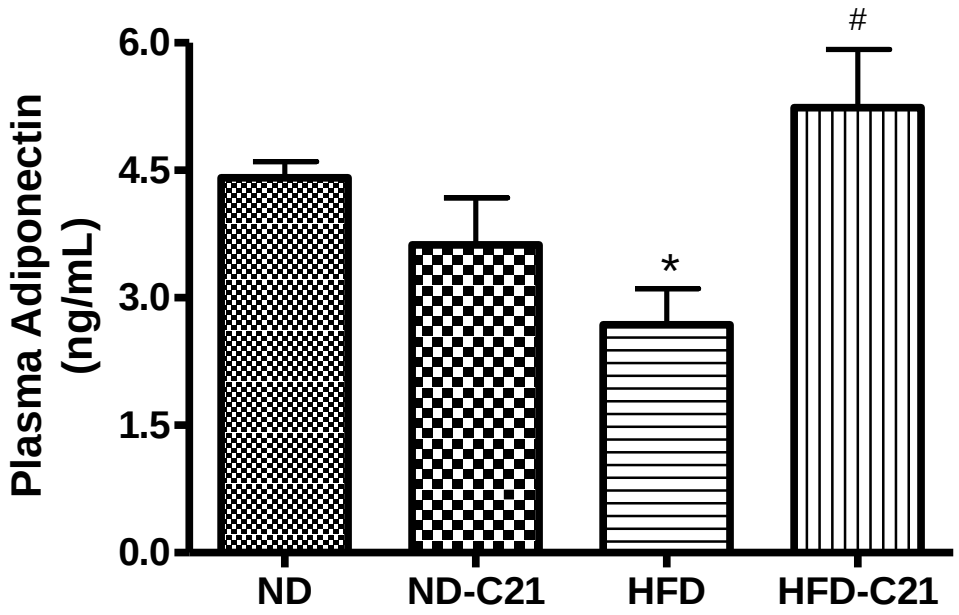


Fig. 11: Plasma adiponectin concentrations in control and C21-treated 5 week old male C57BL/6 mice fed with ND or HFD. Results are means ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=3-4 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

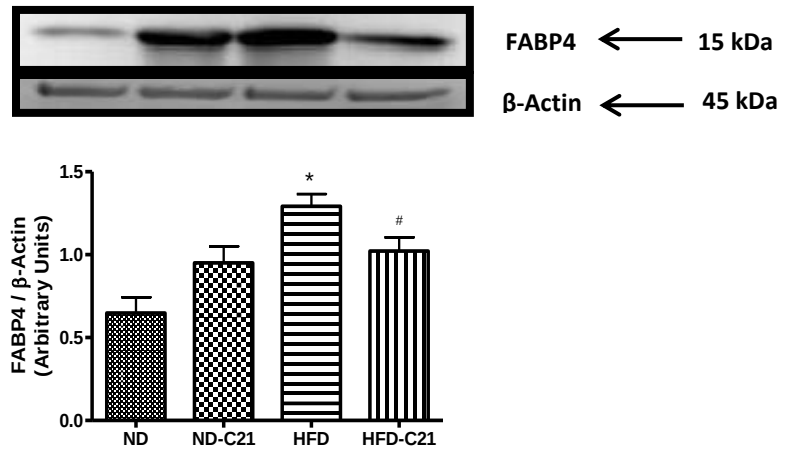
4.1.1.9: Effect of C21 on epididymal WAT (eWAT) lipogenic regulators' protein expression

Western blotting revealed the presence of the lipogenic regulators FABP4 (15 KDa) and FASN (273 KDa) protein expression in the eWAT of young male mice. Densitometric analysis of the bands revealed that HFD (compared with ND) increased FABP4 (199%) and FASN (225%) protein expression in eWAT (Fig. 12A and B respectively). C21-treatment under HFD reduced the HFD-induced increase in the protein expression of both the proteins - FABP4 (79%) and FASN (54%).

4.1.1.10: Effect of C21 on epididymal WAT (eWAT) lipolytic regulators' protein expression

Distinct bands for ATGL and HSL were detected approximately at 56 and 84 KDa, respectively in eWAT of 5 weeks old male C57BL/6 mice by western blotting. Densitometric analysis of the bands revealed that compared with ND, HFD decreased ATGL (62%) and increased HSL (191%) protein expression in eWAT (Fig. 13A and B respectively). The C21 under HFD increased protein expression of ATGL (167%) and decreased HSL (50%) in eWAT. Surprisingly, C21 treatment under ND condition significantly increased the protein expression of HSL (166%) in eWAT (Fig. 13B).

(A)



(B)

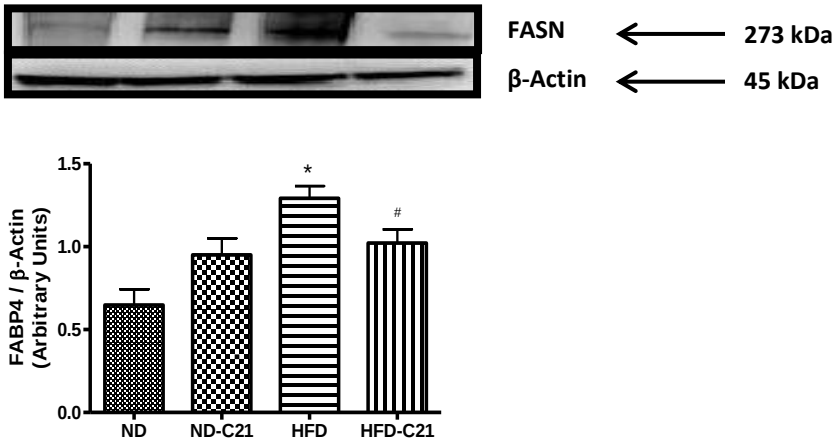
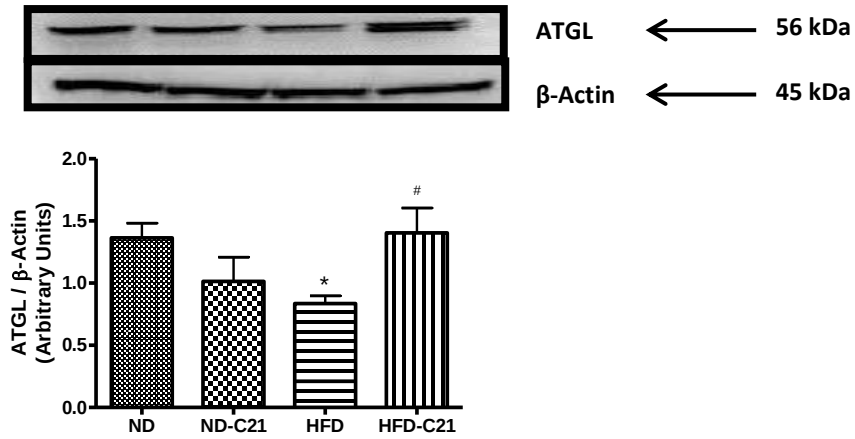


Fig 12: Protein expression of lipogenic regulators in the epididymal WAT of control and C21-treated 5 week old male C57BL/6 mice fed with ND or HFD. Representative western blots for (A) FABP4 with loading control β-actin and (B) FASN with loading control β-actin. The bar graphs represent the ratios of densities of FABP4 and FASN normalized with β-Actin protein bands. *significantly different compared with ND, #significantly different compared with HFD. Results are means ± SEM; Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=4-5 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

(A)



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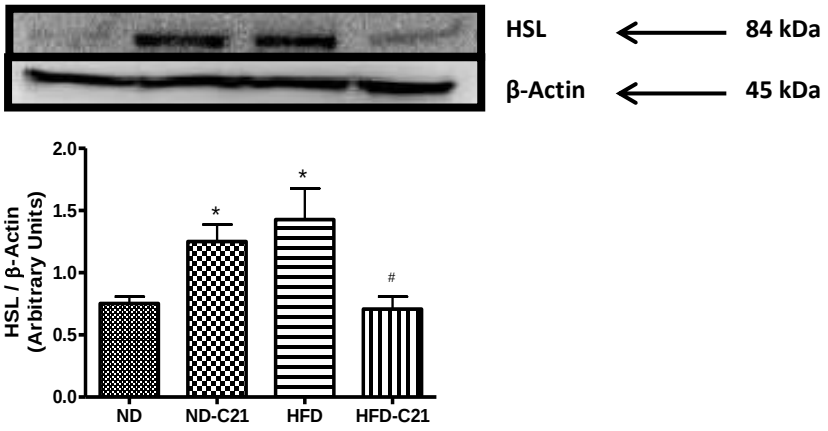


Fig 13: Protein expression of lipolytic regulators in the epididymal WAT of control and C21-treated 5 week old male C57BL/6 mice fed with ND or HFD. Representative western blots for (A) AGTL with loading control β-actin and (B) HSL with loading control β-actin. The bar graphs represent the ratios of densities of AGTL and HSL normalized with β-Actin protein bands. *significantly different compared with ND, #significantly different compared with HFD. Results are means ± SEM; Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=4-6 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

4.1.1.11: Effect of C21 on epididymal WAT (eWAT) AT2R protein expression

As shown in Fig. 14, distinct bands for AT2R were detected approximately at 44 KDa by western blotting in eWAT of young male C57BL/6 mice. Densitometric analysis of the bands revealed that HFD (compared with ND) increased AT2R (54%) protein expression in eWAT. C21 treatment did not affect the protein expression of AT2R in ND or HFD conditions.

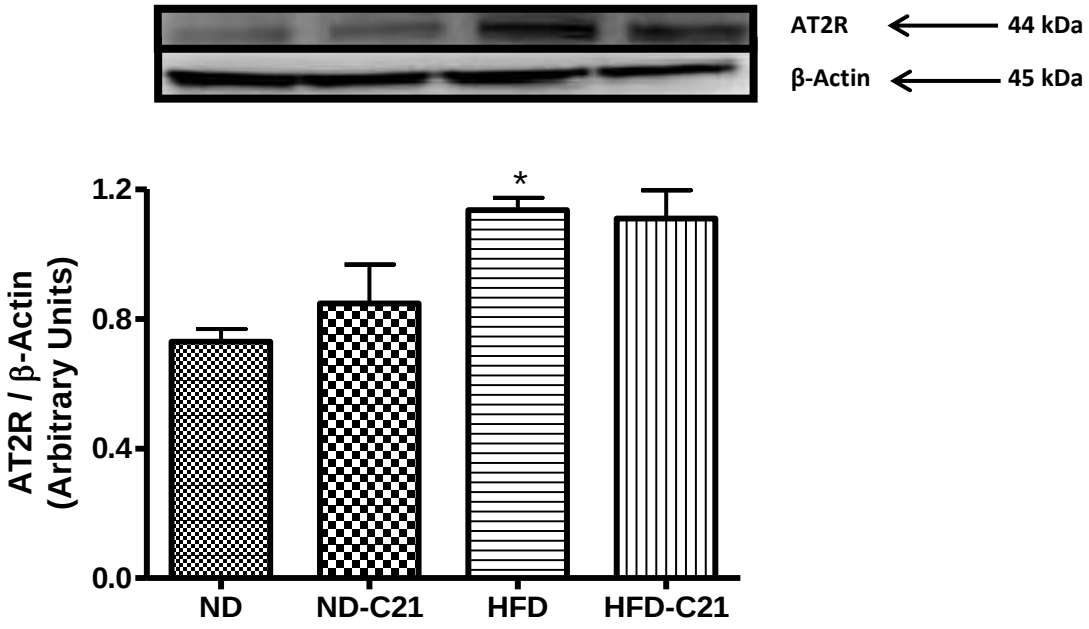


Fig. 14: Protein expression of angiotensin II type 2 receptor (AT2R) in the epididymal WAT of control and C21-treated 5 week old male C57BL/6 mice fed with ND or HFD. Representative western blots for AT2R with loading control β-actin. The bar graphs represent the ratios of densities of AT2R normalized with β-Actin protein bands. *significantly different compared with ND. Results are means ± SEM; Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=4-5 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

4.2: Does pharmacological activation of AT2R reduce fatty acid uptake via NO-dependent pathway in isolated mouse primary epididymal adipocytes?

4.2.1: RESULTS from five weeks old male mice primary epididymal adipocytes:

4.2.1.1: FA uptake by isolated primary epididymal adipocytes and validation of FA assay

The Fig. 15 depicts that there was an epididymal adipocyte number-dependent increase in FA uptake responses. We also observed that there was a decrease or plateau after 3600 sec possibly due to loss in cells viability. For rest of the studies, we chose 40,000 cells and terminated the assay at 4200 sec.

The FA uptake was inhibited by the FA transport protein (FATP) inhibitor, triacsin C (Fig. 16). Complete inhibition was achieved at 10μM concentrations of triacsin C indicating that FATP mediated the uptake of FA in the epididymal adipocytes. Further, FA uptake was increased dose-dependently by insulin in the epididymal adipocytes (Fig. 17). The FA uptake was significantly increased by 10 nM concentrations of insulin.

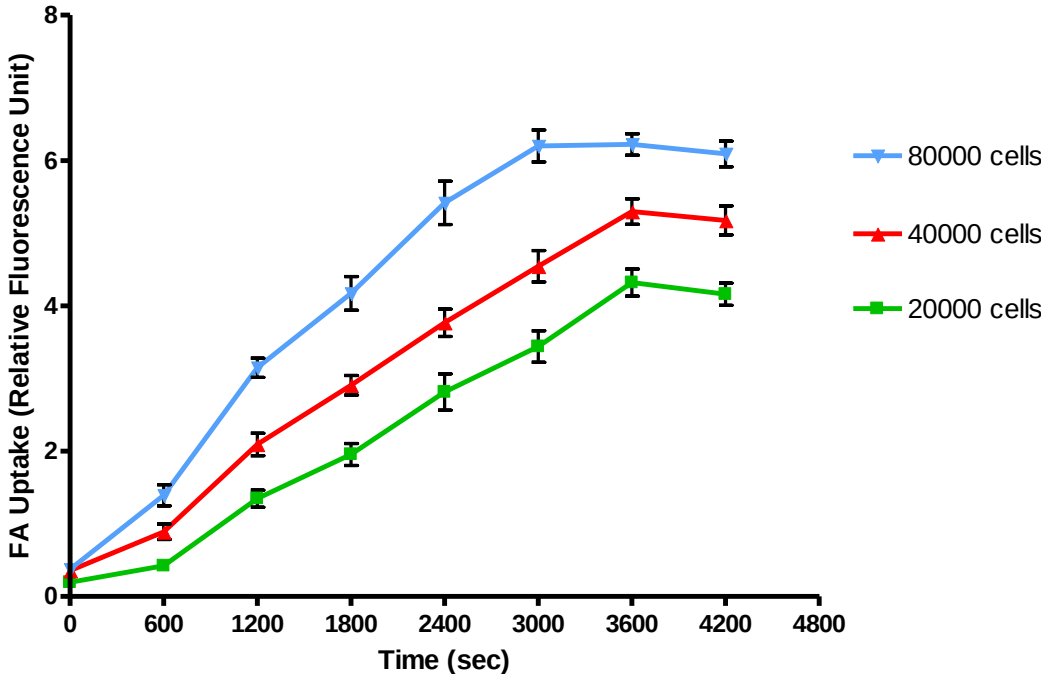


Fig. 15: Fatty acid (FA) uptake assessed in isolated primary epididymal adipocytes using fluorescence assay. Different numbers of isolated primary epididymal adipocytes (80,000, 40,000 and 20,000 cells) were used to obtain the FA uptake response curves. Results are means ± SEM; N=3 in each group.

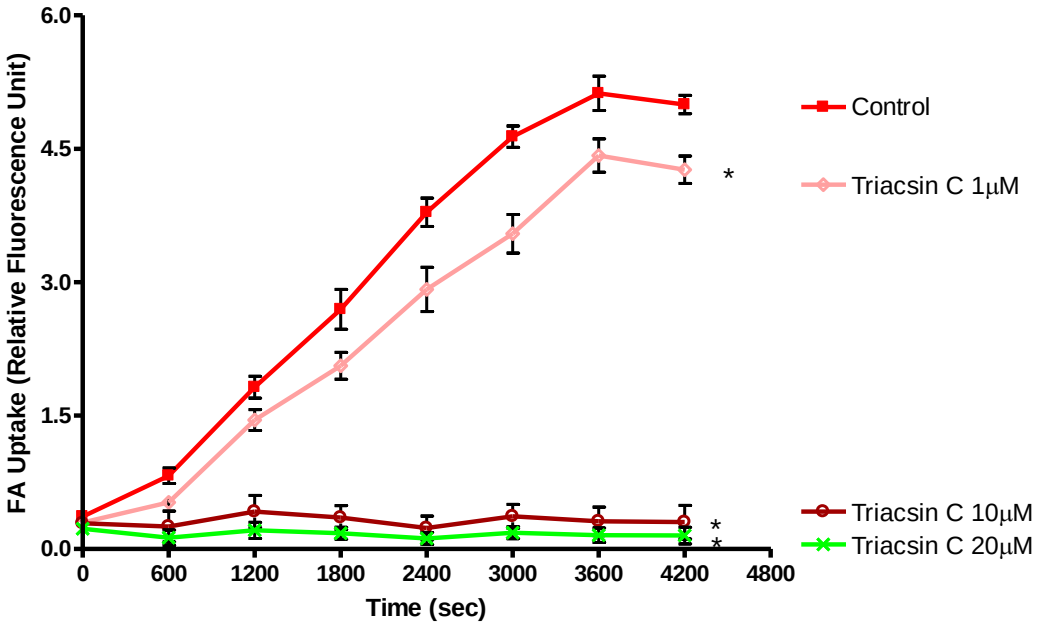


Fig. 16: Fatty acid uptake in isolated primary epididymal adipocytes assessed using fluorescence assay in absence and presence of different concentrations (1, 10 and 20 μM) of triacsin C, the FATP-inhibitor. *significantly different control adipocytes (without triacsin C treatment). Results are means ± SEM; Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=3 in each group.

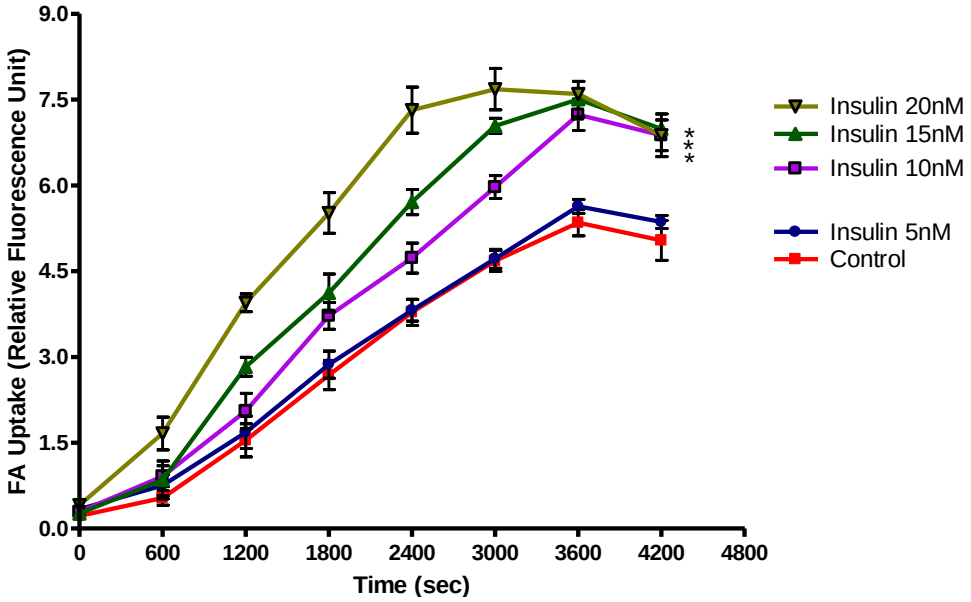


Fig. 17: Fatty acid uptake in isolated primary epididymal adipocytes assessed using fluorescence assay in absence and presence of different concentrations (5, 10, 15 and 20 nM) of insulin, the FATP-activator. *significantly different control adipocytes (without insulin treatment). Results are means ± SEM; Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=3 in each group.

4.2.1.2: Effect of C21 on FA uptake

The Fig. 18 depicts that FA uptake was significantly decreased by C21 at 1 μM concentration in epididymal adipocytes. A lower dose (0.1 μM) of C21 was not effective and a higher dose (5 μM) did not cause any further reduction in FA uptake. In further experiments, we used 1 μM of C21.

4.2.1.3: Effect of PD123319 and losartan on C21-induced reduction in FA uptake

The C21 treatment-induced decrease in FA uptake in the epididymal adipocytes was blocked by PD (PD123,319), the AT2R antagonist at 10 μM concentration (Fig. 19). This inhibition indicated that the C21 treatment-induced effect in FA uptake was AT2 receptor mediated. Furthermore, we observed that the AT1R antagonist losartan did not affect the C21-induced decrease in FA uptake in the epididymal adipocytes (Fig. 20) suggesting no involvement of AT1R in FA uptake.

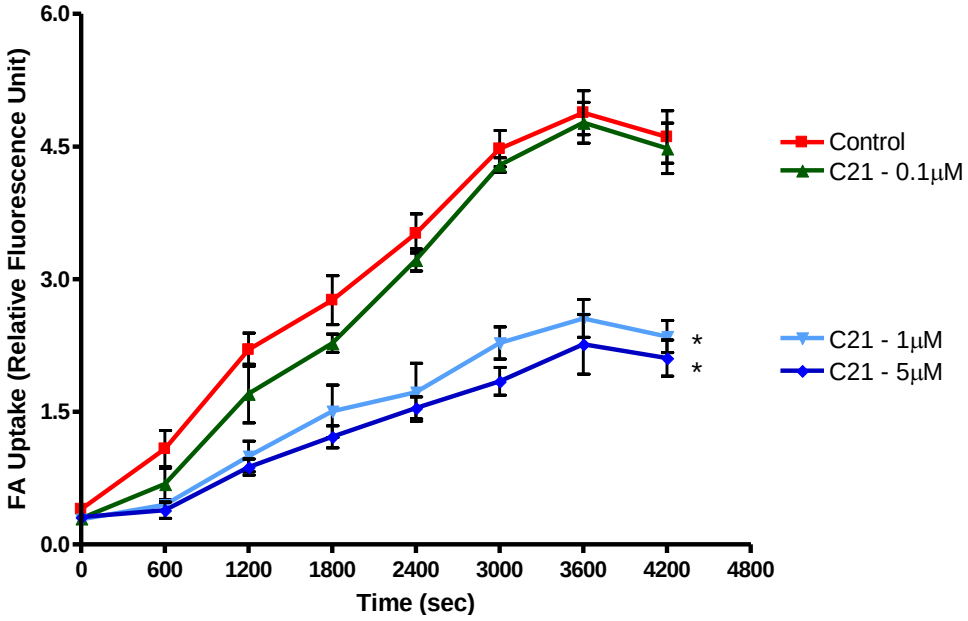


Fig. 18: Fatty acid uptake in isolated primary epididymal adipocytes assessed using fluorescence assay in absence and presence of different concentrations (0.1, 1.0 and 5.0 µM) of C21, the AT2R agonist. *significantly different control adipocytes (without C21 treatment). Results are means ± SEM; Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=3 in each group.

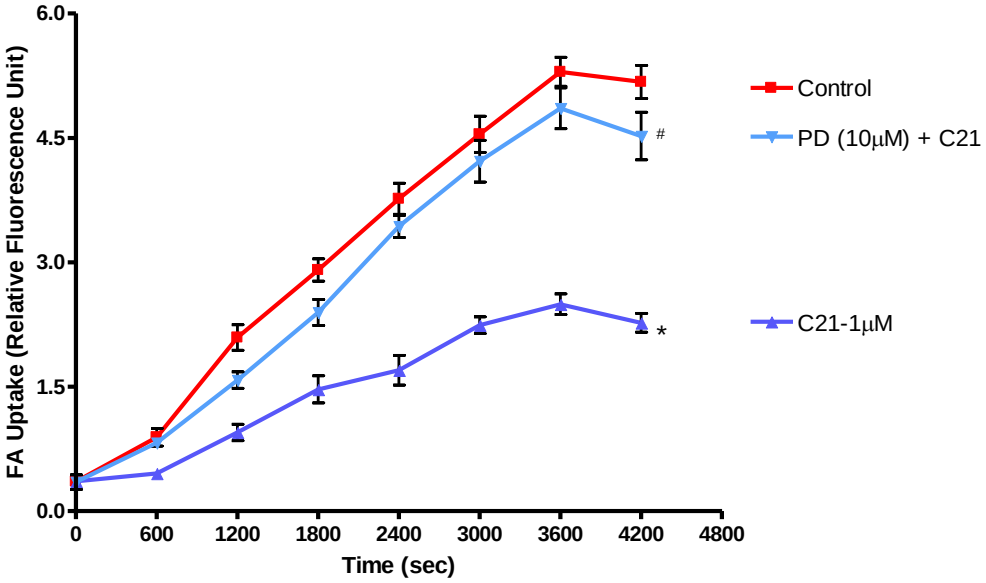


Fig. 19: Fatty acid (FA) uptake in isolated primary epididymal adipocytes assessed using fluorescence assay in absence, presence of C21 (AT2R agonist) and presence of PD (PD123,315 - AT2R antagonist) + C21. *significantly different control adipocytes (without any treatment), #significantly different from C21 treatment group. Results are means ± SEM; Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=3 in each group.

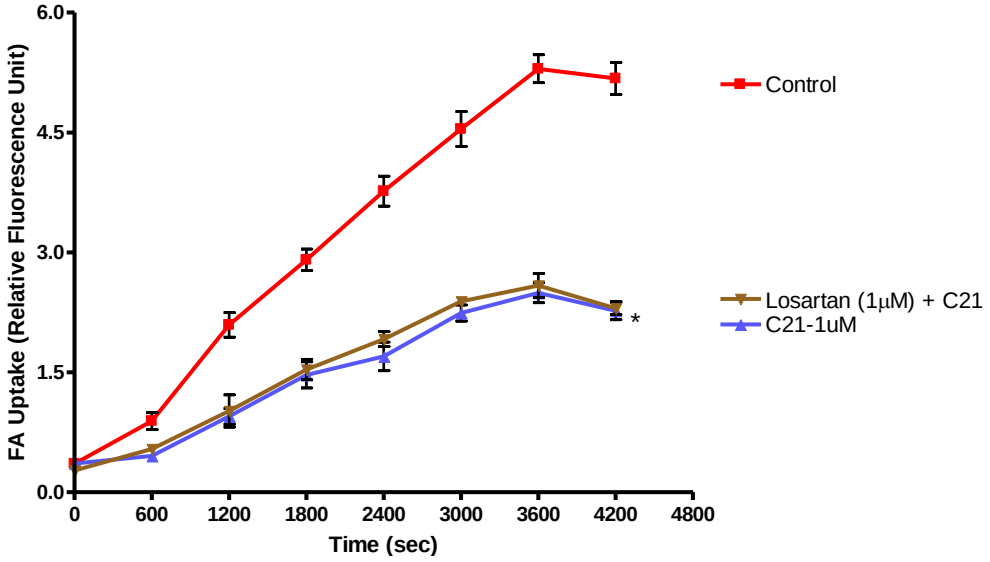


Fig. 20: Fatty acid (FA) uptake in isolated primary epididymal adipocytes assessed using fluorescence assay in absence, presence of C21 (AT2R agonist) and presence of losartan (AT1R agonist) + C21. *significantly different control adipocytes (without any treatment). Results are means ± SEM; Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=3 in each group.

4.2.1.4: Effect of L-NAME, ODQ and Rp-8-Br-PET-cGMPS on C21-induced decrease in FA uptake

The Fig. 21 depicts that the C21-induced decrease in FA uptake in the epididymal adipocytes was blocked by L-NAME, the NO synthase (NOS) inhibitor at 1 mM concentration. Also, ODQ, the guanylyl cyclase (GC) inhibitor at 10 mM concentration blocked the C21-induced decrease in FA uptake in the epididymal adipocytes (Fig. 22). Finally, we observed that Rp-8-Br-PET-cGMPS, the cGMP-dependent protein kinase (PKG)-inhibitor at 1 μM concentration also blocked the C21-induced decrease in FA uptake (Fig. 23). These results indicated the involvement of NOS, GC and PKG in C21-induced decrease in FA uptake in the epididymal adipocytes.

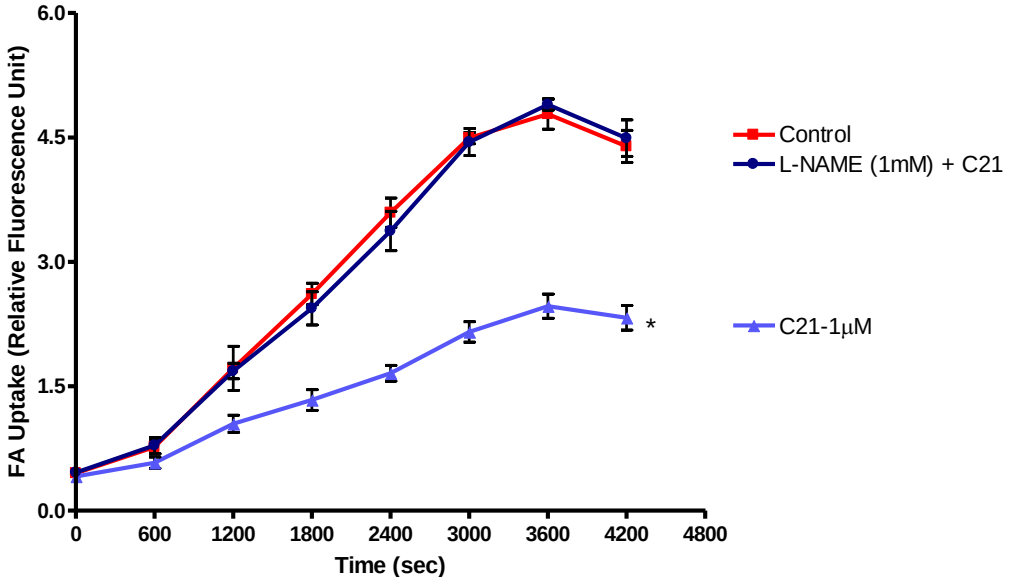


Fig. 21: Fatty acid uptake in isolated primary epididymal adipocytes assessed using fluorescence assay in absence, presence of C21 (AT2R agonist) and presence of L-NAME (NO synthase inhibitor) + C21. *significantly different control adipocytes (without any treatment). Results are means ± SEM; Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=3 in each group; L-NAME - L-NG-Nitroarginine Methyl Ester.

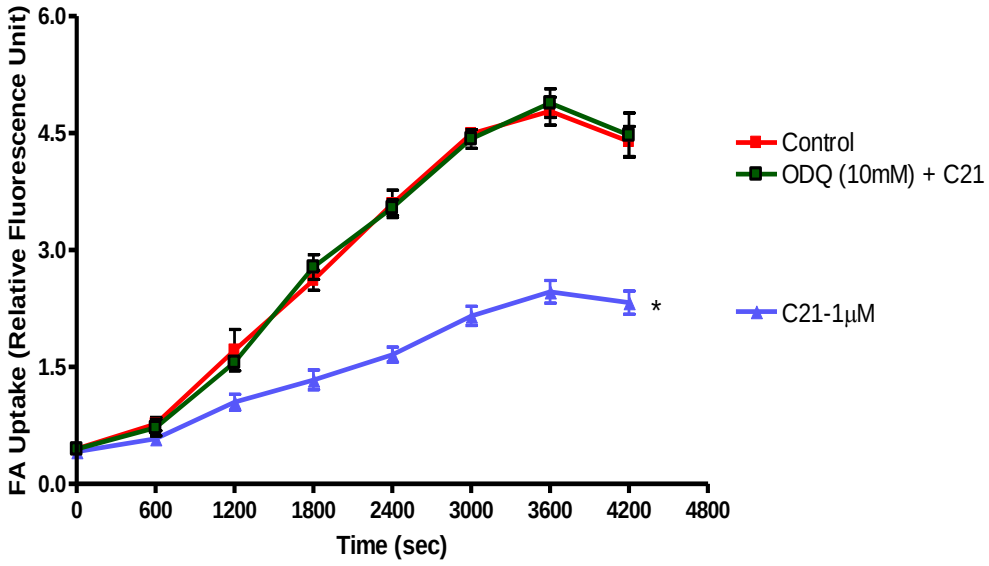


Fig. 22: Fatty acid (FA) uptake in isolated primary epididymal adipocytes assessed using fluorescence assay in absence, presence of C21 (AT2R agonist) and presence of ODQ (guanylyl cyclase inhibitor) + C21. *significantly different control adipocytes (without any treatment). Results are means ± SEM; Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=3 in each group; ODQ - 1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one.

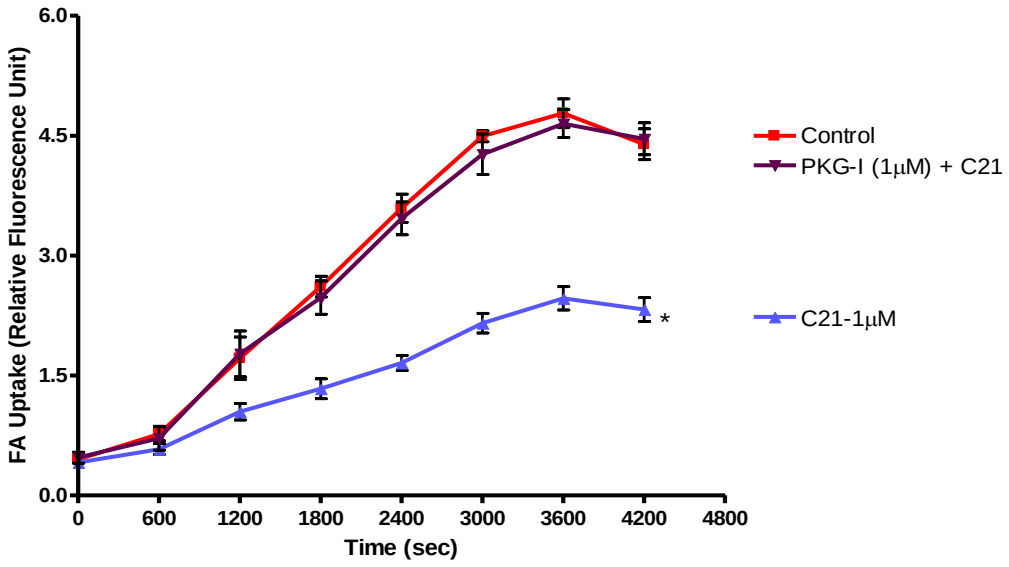


Fig. 23: Fatty acid (FA) uptake in isolated primary epididymal adipocytes assessed using fluorescence assay in absence, presence of C21 (AT2R agonist) and presence of Rp-8-Br-PET-Cgmps (PKG-inhibitor) + C21. *significantly different control adipocytes (without any treatment). Results are means ± SEM; Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=3 in each group; PKG-I - PKG-inhibitor / Rp-8-Br-PET-Cgmps.

4.3: Does pharmacological activation of AT2R prevent adiposity in 12 week old (adult) male mice?

4.3.1: RESULTS from twelve week old male mice:

4.3.1.1: Effect of C21 on calorie intake

The Kcal intake was significantly increased in HFD-fed mice in 2 weeks (ND 123±5.2 vs HFD 220±5.7 Kcal) than the HD-fed mice (Fig. 24). Similar to that in young groups, the C21 treatment did not affect the Kcal intake in adult mice on ND (ND-C21 119±6.7 Kcal) or HFD (HFD-C21 218±6.3 Kcal).

4.3.1.2: Effect of C21 on epididymal WAT (eWAT) weight-to-body weight ratio

As shown in Fig 25, HFD increased (97%) the eWAT weight-to-body weight ratio and C21-treatment under HFD condition prevented the HFD-induced increase in eWAT weight-to-body weight ratio (35%) in adult male mice. C21 treatment in mice on ND had no effect on the eWAT weight-to-body weight ratio.

4.3.1.3: Effect of C21 on epididymal adipocyte size

The Fig. 26A shows H&E staining of the epididymal adipose tissue sections from control and C21-treated 12 weeks old male mice on ND and HFD. The HFD caused an increase in epididymal adipocyte size (ND 2516±184 vs HFD 5948±151 μm^2) compared with ND. The C21-treatment prevented HFD-induced increase in epididymal adipocyte size (HFD 5948±151 vs HFD-C21 3386±66 μm^2) (Fig. 26B).

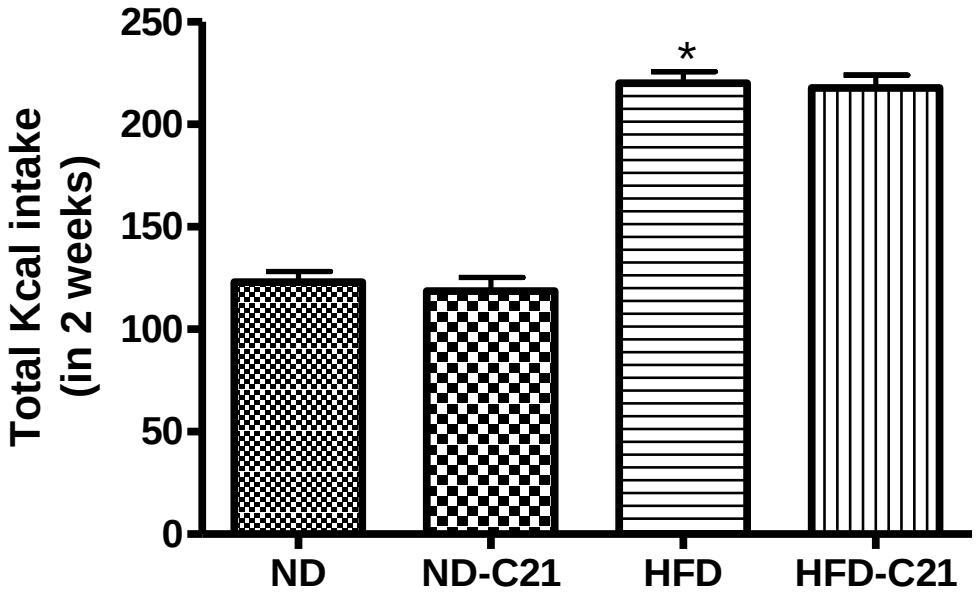


Fig. 24: Total Kcal intake in control and C21-treated 12 week old male mice fed with ND or HFD. Results are means ± SEM; *significantly different compared with ND. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=6 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

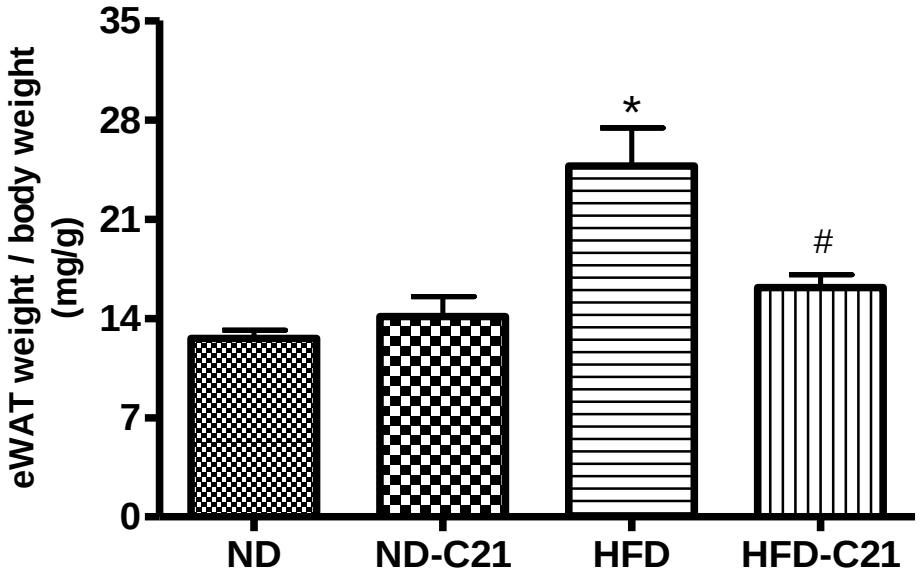
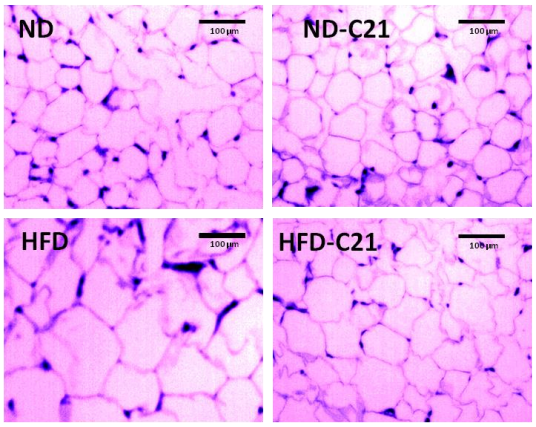


Fig. 25: Epididymal WAT to body weight ratio of control and C21-treated 12 week old male mice fed with ND or HFD. Results are means ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=6-7 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

(A)



(B)

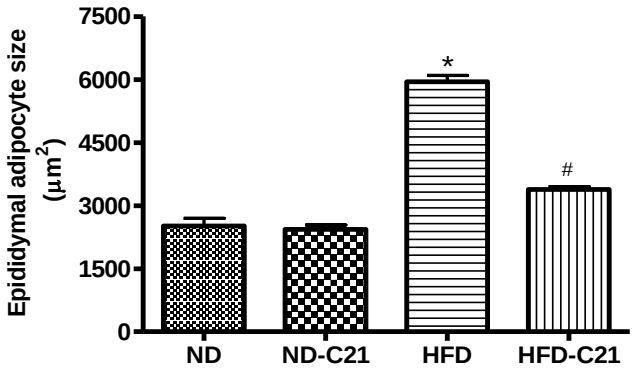


Fig. 26: Epididymal adipocyte size of control and C21-treated 12 week old male mice fed with ND or HFD. (A) Representative micrograph of epididymal adipose tissue (magnification, 10X) (B) Epididymal adipocyte size was expressed as cell area (μm^2). Results are means ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=3 (3 sections per slide from 3 mice). (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

4.3.1.4: Effect of C21 on plasma free fatty acid (FFA) and triglyceride (TAG)

The Fig. 27 depicts the effect of C21 on plasma FFA in 12 weeks old male mice. Compared with ND, mice on HFD had significantly increased plasma FFA (ND 3.31±0.25 vs HFD 6.47±0.61 nmol/μL), which was rescued by the C21-treatment under HFD (HFD-C21 4.37±0.78 nmol/μL). Similarly, the plasma TAG was also significantly increased by HFD compared with ND (ND 2.3±0.29 vs HFD 4.1±0.23 nmol/μL) (Fig. 28). The C21 significantly reduced the plasma TAG (HFD-C21 3.2±0.34 nmol/μL) under HFD condition.

4.3.1.5: Effect of C21 on plasma insulin

As shown in Fig. 29, the non-fasting plasma insulin level was significantly higher in HFD (ND 0.72±0.23 vs HFD 3.77±0.38 ng/mL) fed mice. Interestingly, the plasma insulin level increase under HFD was attenuated by C21 treatment (HFD 3.77±0.38 vs HFD-C21 2.45±0.27 ng/mL).

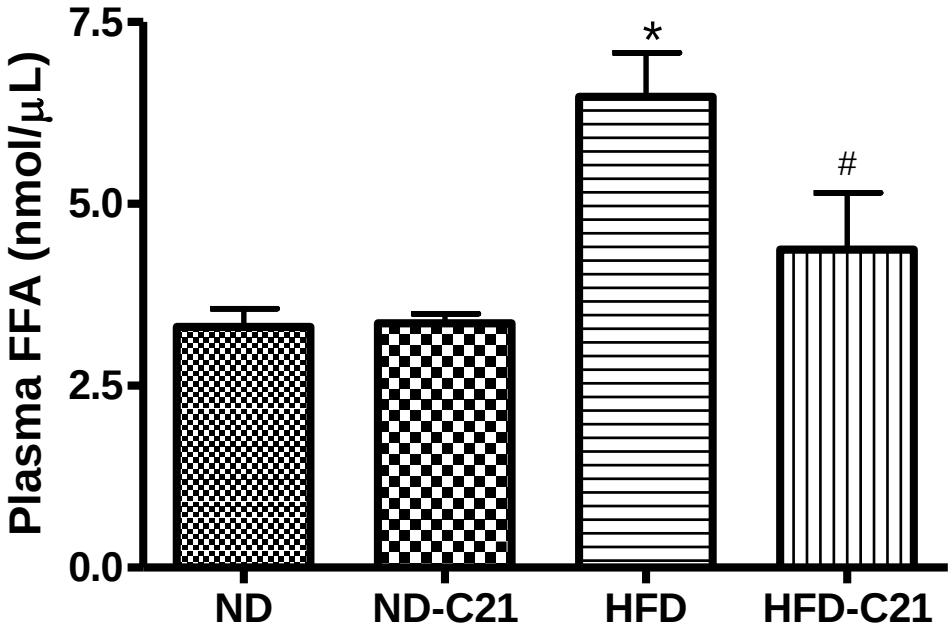


Fig. 27: Plasma FFA concentrations in control and C21-treated 12 week old male mice fed with ND or HFD. Results are means ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=5 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

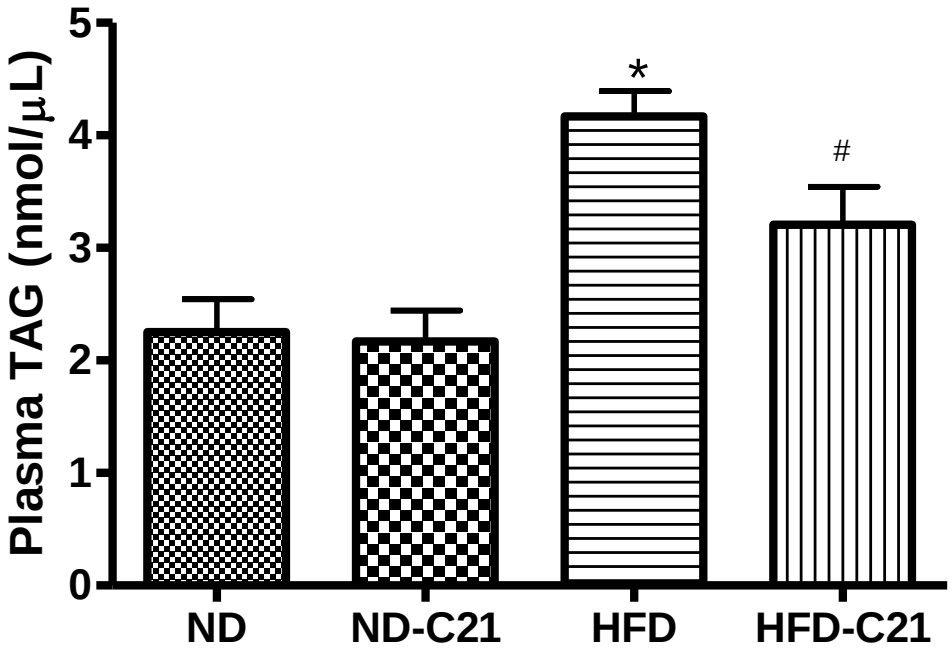


Fig. 28: Plasma TAG concentrations in control and C21-treated 12 week old male mice fed with ND or HFD. Results are means ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=5 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

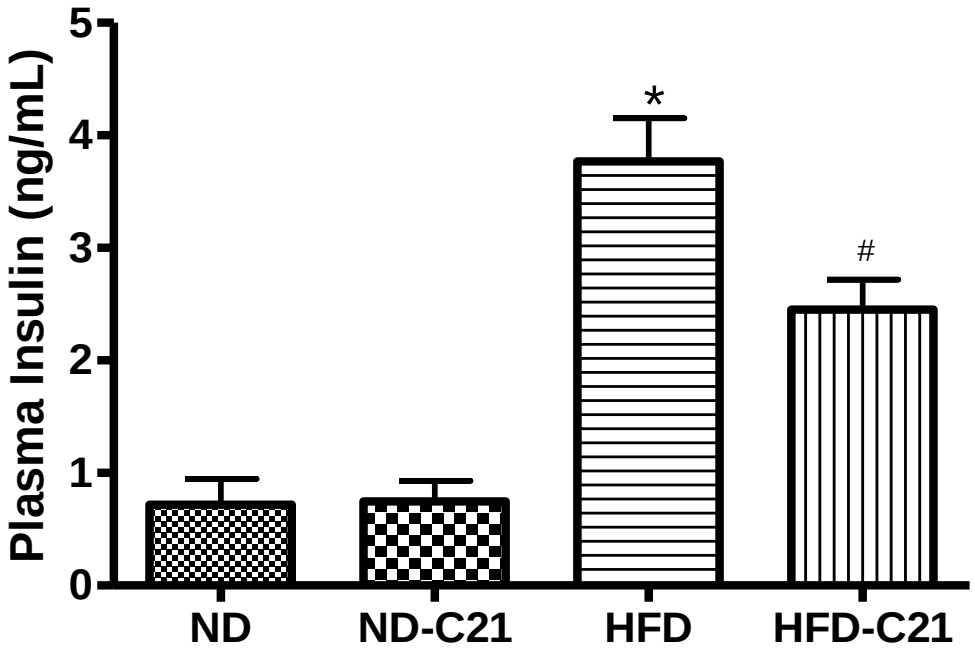


Fig. 29: Plasma insulin levels in control and C21-treated 12 week old male mice fed with ND or HFD. Results are means ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=4-5 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

4.3.1.6: Effect of C21 on plasma TNFα and adiponectin

The HFD significantly increased the plasma TNFα levels (ND 54±10.3 vs HFD 105±21.6 pg/mL) in adult male mice (Fig. 30). The C21-treatment rescued the plasma TNFα levels increase (HFD 105±21.6 vs HFD-C21 57±11.7 pg/mL) under HFD condition. On the other hand, the plasma adiponectin was significantly decreased by HFD compared with ND (ND 5.94±0.52 vs HFD 1.55±0.31 ng/mL) in these mice (Fig. 31). Interestingly, the C21-treatment significantly increased the plasma adiponectin levels (HFD 1.55±0.31 vs HFD-C21 4.47±0.48 ng/mL) under HFD condition.

4.3.1.7: Effect of C21 on epididymal WAT (eWAT) AT2R protein expression

As shown in Fig. 32, distinct bands for eWAT-AT2R were detected approximately at 44 KDa by western blotting. Densitometric analysis of the bands revealed that HFD (compared with ND) increased AT2R (41%) protein expression in eWAT. The C21 treatment did not affect the eWAT-AT2R protein expression in ND or HFD conditions.

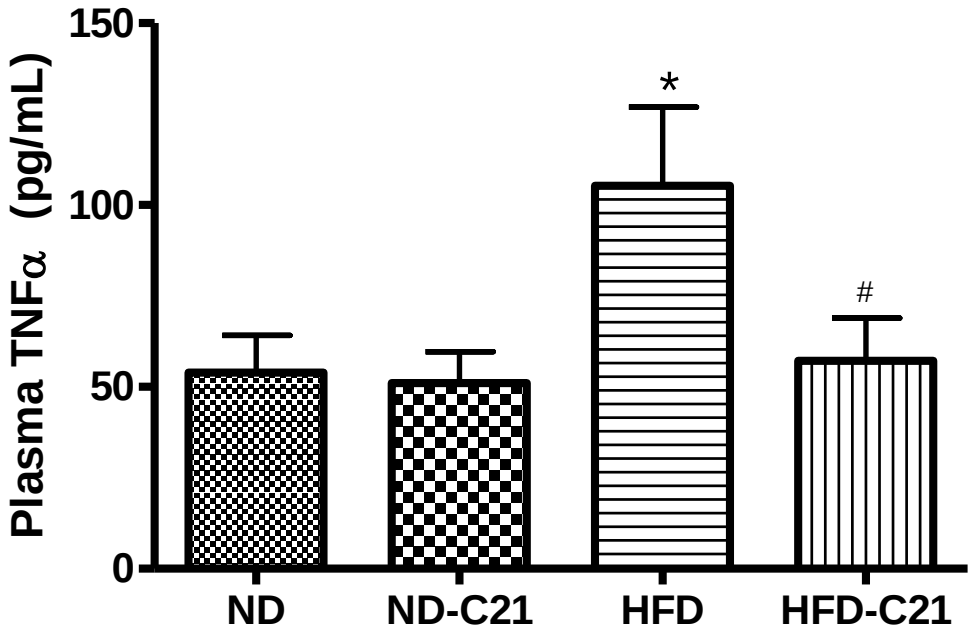


Fig. 30: Plasma TNFα levels in control and C21-treated 12 week old male mice fed with ND or HFD. Results are means ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=4-6 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

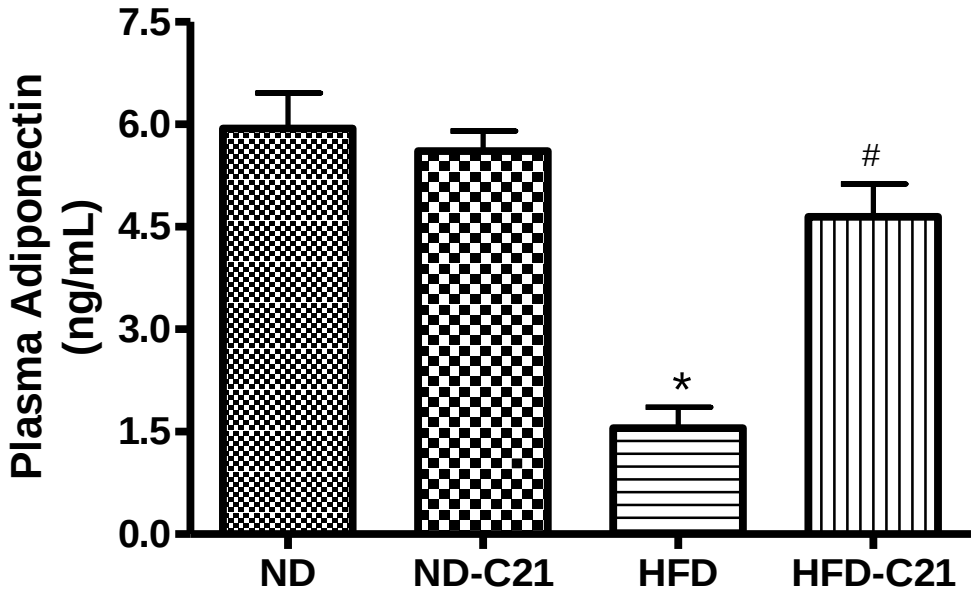


Fig. 31: Plasma adiponectin concentrations in control and C21-treated 12 week old male mice fed with ND or HFD. Results are means ± SEM; *significantly different compared with ND, #significantly different compared with HFD. Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=5 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

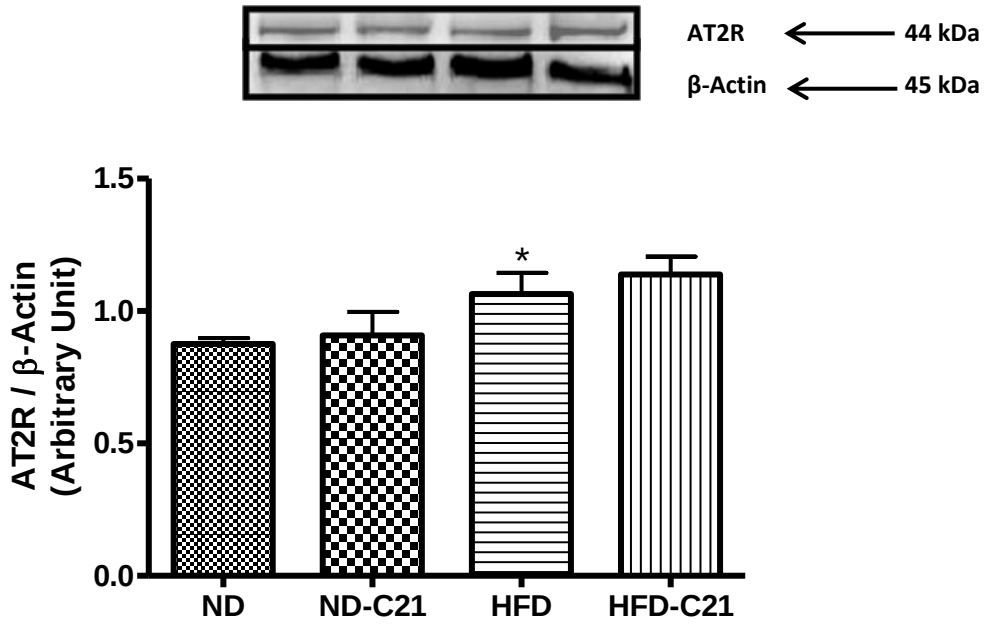


Fig. 32: Protein expression of angiotensin II type 2 receptor (AT2R) in the epididymal WAT of control and C21-treated 12 week old male mice fed with ND or HFD. Representative western blots for AT2R with loading control β-actin. The bar graphs represent the ratios of densities of AT2R normalized with β-Actin protein bands. *significantly different compared with ND. Results are means ± SEM; Data were analyzed using one-way ANOVA followed by Neuman-Keuls post hoc test and Student's t test, $p<0.05$; N=3-5 in each group. (ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

Table 2: Metabolic and hormonal parameters of control and C21-treated 5 week old male C57BL/6 mice fed with ND or HFD.

Parameters/Mice groups	ND	ND-C21	HFD	HFD-C21
Total Kcal intake (in 2 weeks)	B	↔	↑*	↔
eWAT weight / body weight (mg/g)	B	↔	↑*	↓#
Epididymal adipocyte size (μm^2)	B	↔	↑*	↓#
Plasma FFA (nmol/µL)	B	↔	↑*	↓#
Plasma TAG (nmol/µL)	B	↔	↑*	↓#
Plasma insulin (ng/mL)	B	↔	↑*	↓#
Plasma TNFα (pg/mL)	B	↔	↑*	↓#
Plasma adiponectin (ng/mL)	B	↔	↓*	↑#
FABP4 / β-actin (protein expression)	B	↔	↑*	↓#
FASN / β-actin (protein expression)	B	↔	↑*	↓#
ATGL / β-actin (protein expression)	B	↔	↓*	↑#
HSL / β-actin (protein expression)	B	↑*	↑*	↓#
AT2R / β-actin (protein expression)	B	↔	↑*	↔

ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21; B - basal under ND, ↑ - increased, ↓ - decreased, ↔ - not-significantly different from ND, ↔ - not-significantly different from HFD, * - significantly different from ND, # - significantly different from HFD; data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$).

Table 3: Metabolic and hormonal parameters of control and C21-treated 12 week old male C57BL/6 mice fed with ND or HFD.

Parameters/Mice groups	ND	ND-C21	HFD	HFD-C21
Total Kcal intake (in 2 weeks)	B	↔	↑*	↔
eWAT weight / body weight (mg/g)	B	↔	↑*	↓#
Epididymal adipocyte size (μm^2)	B	↔	↑*	↓#
Plasma FFA (nmol/µL)	B	↔	↑*	↓#
Plasma TAG (nmol/µL)	B	↔	↑*	↓#
Plasma insulin (ng/mL)	B	↔	↑*	↓#
Plasma TNFα (pg/mL)	B	↔	↑*	↓#
Plasma adiponectin (ng/mL)	B	↔	↓*	↑#
AT2R / β-actin (protein expression)	B	↔	↑*	↔

ND - Normal Diet, ND-C21 - Normal Diet on C21, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21; B - basal under ND, ↑ - increased, ↓ - decreased, ↔ - not-significantly different from ND, ↔ - not-significantly different from HFD, * - significantly different from ND, # - significantly different from HFD; data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$).

4.4: Discussion:

Obesity is the malfunctioning of the hormonal regulation of metabolism (Eyster, 2011). One such hormonal system is RAS. The adipose RAS has been implicated in obesity (Yvan-Charvet and Quignard-Boulange, 2011). Ang II, the active product of RAS, increases lipogenesis culminating in adipose size increase (Jones *et al.*, 1997). The lipogenic effect and adipocyte size increase by Ang II is rescued by the blockade of AT1R (Jones *et al.*, 1997; Tomono *et al.*, 2008). However the role of AT2R in adipocyte size regulation is controversial. We and others have reported that the AT2R knockout male mice on HFD have decrease epididymal fat mass indicating that AT2R contributes to adiposity (Yvan-Charvet *et al.*, 2005). Contrary to this, deficiency of AT2R in Apolipoprotein E knockout mice (a model of atherosclerosis) increased epididymal adipose tissue weight in male mice indicating that AT2R prevents adiposity (Iwai *et al.*, 2009). In current study, we planned to explore the role of AT2R on adiposity using the pharmacological stimulation of AT2R by C21. Furthermore, as the relative expression of AT2R decreases during adolescence and adulthood, we investigated the effect of pharmacological stimulation of AT2R using C21 on adiposity in two different age (5 and 12 weeks) groups of mice.

In obesity, the body weight gain is associated with deposition of fat in the abdominal region and the gonadal (epididymal in males) white adipose tissue (WAT) is the most vulnerable type (Foster *et al.*, 2013). In the current study, we observed that HFD significantly increased the weight of epididymal WAT in both

5 and 12 weeks old male mice. Interestingly the AT2R agonist, C21 treatment significantly reduced the HFD-induced increase in the epididymal WAT weight in both 5 and 12 weeks old male mice despite having the relative expression of AT2R decreased in 12 weeks compared to the 5 weeks old male mice. Also the HFD-induced increase in epididymal adipocyte size was significantly reduced by C21 treatment in both 5 and 12 weeks old male mice. These changes in adiposity caused by the C21 treatment were observed despite the similar calorie intake under HFD condition. Thus, C21 treatment reduces the HFD-induced increase in adiposity (epididymal adipocyte weight and size) in both 5 and 12 weeks old male mice. We decided to explore the effects of C21 on lipid metabolism using in 5 weeks old male mice only.

The size of the adipocyte is built-up by the process of lipogenesis. In the postprandial phase, the fatty acids are first transported inside the adipocytes by the FATPs (Schaffer and Lodish, 1994). Once inside the aqueous cytosol, FFAs are then bound to the fatty acid binding proteins namely FABP4 for cytosolic transportation (Thompson *et al.*, 2009). These FFAs along with the de-novo synthesized FFAs from the Kreb's cycle (by fatty acid synthase/FASN) are packed and stored as TAG in the lipid droplets of adipocytes (Wakil and Abu-Elheiga, 2009). The AT2R knockout mice with the smaller adipocytes were having decreased FABP4 and FASN gene expressions (Yvan-Charvet *et al.*, 2005). In the present study we observed, HFD significantly increased the protein expression of the epididymal WAT lipogenic regulators (FABP4 and FASN)

explaining the increase of adipocyte size under HFD condition. Thus, in contrast to the proposed lipogenic functions of AT2R (Yvan-Charvet and Quignard-Boulange, 2011), the stimulation of AT2R under HFD condition reduces the epididymal adipocyte size increase potentially by reducing the epididymal WAT lipogenesis.

The adipocyte size is also regulated by lipolysis. With the energy demand from the peripheral organs like the skeletal muscle, the breakdown of stored lipids in adipocytes is triggered. In adipocytes, TAG (stored form of lipid) is catabolized first to produce diacylglycerols (DAGs) and FFA / non-esterified fatty acid (NEFA) mainly by ATGL (Ahmadian *et al.*, 2007; Zechner *et al.*, 2009). ATGL is the rate-limiting enzyme in the TAG catabolism cascade and is crucial for energy homeostasis (Haemmerle *et al.*, 2006). The DAG are further catabolized to produce monoacylglycerols (MAGs) and another NEFA by HSL (Hajer *et al.*, 2008). Finally, MAGs are cleaved to release the glycerol and third NEFA by monoglyceride lipase (MGL). Recently, it has been observed that the ATGL KO mice have severely increased adipose mass and adipose size (Haemmerle *et al.*, 2006). In contrast to ATGL KO mice, the HSL KO mice do not have increased fat deposition, body weight and adipocyte size (Osuga *et al.*, 2000; Sekiya *et al.*, 2004). A non-coordinated regulation of ATGL and HSL mRNA as well as protein expressions have been reported in lipid mobilization phase (Phase 2) and protein breakdown phase (Phase 3) of fasting (Bertile and Raclot, 2011). In concurrence with these observations, along with the increased

epididymal WAT weight, in the present study we observed, HFD significantly decreased the protein expression of epididymal WAT lipolytic regulator (ATGL) while increasing the protein expression of epididymal WAT lipolytic regulator (HSL). Thus, the eWAT lipolytic regulators protein expressions were observed to be differential regulated by the HFD. In these animals, HFD seems to trigger the blocking of the basal lipolysis by decreasing the ATGL protein expression but increasing the protein expression of lipolytic enzyme HSL. HFD-induced increased expression of HSL plausibly accounts for the increased levels of plasma FFA and TAG. However, the AT2R agonist treatment significantly altered the protein expression of both the epididymal WAT lipolytic regulators. Although AT2R has been proposed to have lipogenic function (Yvan-Charvet and Quignard-Boulange, 2011), we observed the stimulation of AT2R under HFD condition reduce the epididymal adipocyte size increase by altering the protein expression of the epididymal WAT lipolytic regulators as well. The increased level of ATGL by C21 under HFD condition might be critical in regularizing the imbalanced energy homeostasis state in obesity. However, the significant increase in protein expression of HSL by C21 treatment under ND condition might be plausibly due to the body's physiological compensatory increase of the protein levels to compensate the reduction of FA uptake and restore the lipid metabolism under ND condition.

The lipogenic and lipolytic regulators are governed by the function of the fatty acid transport into the adipocytes (Glatz *et al.*, 2010). In adipocytes, plasma

membrane fatty acid transport protein (FATP) facilitates the long-chain fatty acids (LCFAs) uptake (Schaffer and Lodish, 1995). Modulation of FATP function results in alteration of energy homeostasis and insulin sensitivity (Gimeno, 2007). Treatment with Ang II alone and with PD123319 (AT2R blocker) has been shown to increase the uptake of palmitic acid (a long-chain fatty acid) in immortalized cardiomyocytes (HL-1 cells) (Alfarano *et al.*, 2008); this indicates that the AT1R is involved in facilitating the fatty acid uptake. To test whether the AT2R plays a direct role on fatty acid transport regulation, we performed the in-vitro study with the isolated mouse primary epididymal adipocytes. Our data showed that the fatty acid uptake was reduced by the AT2R agonist C21 in the primary epididymal adipocytes. Moreover, the C21-induced decrease in fatty acid uptake was blocked by PD123319 (AT2R blocker), indicating an AT2R mediated effect of C21. Furthermore at the molecular level, studies have shown AT2R increases nitric oxide synthase (NOS) activity (Taguchi *et al.*, 2012). Also it has been shown that the overexpression of NOS prevents adiposity (Sansbury *et al.*, 2012). So, we further explored whether the pharmacological activation of AT2R reduces fatty acid uptake via NOS-dependent pathway. We observed that the pre-treatment of primary epididymal adipocytes with L-NG-Nitroarginine Methyl Ester (L-NAME / NOS-inhibitor) or 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ / guanylyl cyclase (GC)-inhibitor) or Rp-8-Br-PET-cGMPS (protein kinase G-inhibitor / PKG-I) blocked the C21-induced decrease in fatty acid uptake indicating the involvement of NOS/GC/PKG pathway. Thus our in-vitro data suggest that C21 acting on adipocyte AT2R via NOS/GC/PKG-dependent

pathway reduces the fatty acid transportation in adipocytes. This reduction in fatty acid transport possibly as a consequence prevents the HFD-induced changes in lipogenic and lipolytic regulators and thus prevents epididymal adipocyte size, eWAT weight increase under HFD. However, the role of AT2R-mediated reduction in fatty acid transport and consequently prevention of HFD-induced changes in lipogenic and lipolytic regulators needs a further systematic investigation.

Increased adiposity is associated with hyperlipidemia, hyperinsulinemia and increased inflammation. The larger adipocytes under HFD not only have impaired lipid metabolism but also trigger the pro-inflammatory state of obesity leading to insulin resistance (Hajer *et al.*, 2008; Guilherme *et al.*, 2008; Gustafson *et al.*, 2009). In postprandial phase, the presence of diet stimulates the increase in plasma insulin to facilitate the uptake of glucose in cells. However, in obesity as adipocytes become resistant to insulin, the plasma FFA and TAG levels increase. The larger adipocytes also trigger the inflammation resulting in increased plasma TNF-α, the pro-inflammatory cytokine and decreased plasma adiponectin, the anti-inflammatory, insulin-sensitizing cytokine. The AT2R knockout mice developed insulin resistance with decreased adiponectin (Yvan-Charvet *et al.*, 2005) while the AT2R knockout in atherosclerotic mice have increased plasma FFA (Iwai *et al.*, 2009). Also pharmacological stimulation of AT2R with CGP42112A (a peptide AT2R agonist) has been reported to increase plasma FFA and TAG in rats (Ran *et al.*, 2005),

though experiments were conducted in ND condition. In the present study we observed that HFD significantly increased plasma FFA, TAG, insulin, TNF-α and decreased plasma adiponectin in both the 5 and 12 week old male mice. Interestingly, the C21 treatment in HFD condition prevented all the HFD-induced plasma parameters - FFA, TAG, insulin, TNF-α and adiponectin levels. Thus C21 treatment can prevent not only adiposity but also hyperlipidemia, hyperinsulinemia/insulin resistance and increased inflammation under HFD condition. Similar to the lipogenic/lipolytic regulation, all these effects may be a consequence of reduced adipocyte fatty acid uptake in response to the AT2R agonist C21. However, a direct role of AT2R in reducing inflammation and improving insulin resistance cannot be ruled out. There are studies which support that the AT2R exerts anti-inflammatory effects and also protects against insulin resistance (Ohshima *et al.*, 2012; Shum *et al.*, 2013; Dhande *et al.*, 2013; Shao *et al.*, 2013).

Chronic activation of the G-protein coupled receptor (GPCR) using agonists is known to cause receptor internalization and desensitization, which impede the chronic use of GPCR-agonists. Although AT2R belongs to the GPCR family of receptors, ligand binding to AT2R has been shown to cause no desensitization or downregulation (Hein *et al.*, 1997), possibly because the third intracellular loop of AT2R is short and does not offer enough binding site for G protein-coupled receptor kinase. Consistent with previous findings (Ali *et al.*,

2013), we observed that the AT2R protein expression was not reduced with the chronic AT2R agonist, C21 treatment.

In conclusion, the activation of AT2R by C21 prevents HFD-induced increase in epididymal WAT weight and adipocyte size in mice. These protective effects of AT2R agonist in mice on HFD are accompanied by alteration in the epididymal WAT lipogenic and lipolytic regulators (Fig. 33) as well as plasma FFA, TAG, insulin, TNFα, adiponectin (Table 2 and 3). We propose that the AT2R-mediated reduction in fatty acid uptake may be a site of action of C21 leading to the prevention of lipid accumulation and reduced inflammation and insulin resistance. Collectively, we propose that C21 or other AT2R agonists may serve as therapeutics for controlling obesity and obesity-associated disorders like type-2 diabetes and cardiovascular disorders. However, more studies need to be systematically performed to establish the anti-obesity effect of AT2R agonist and involved molecular mechanism(s).

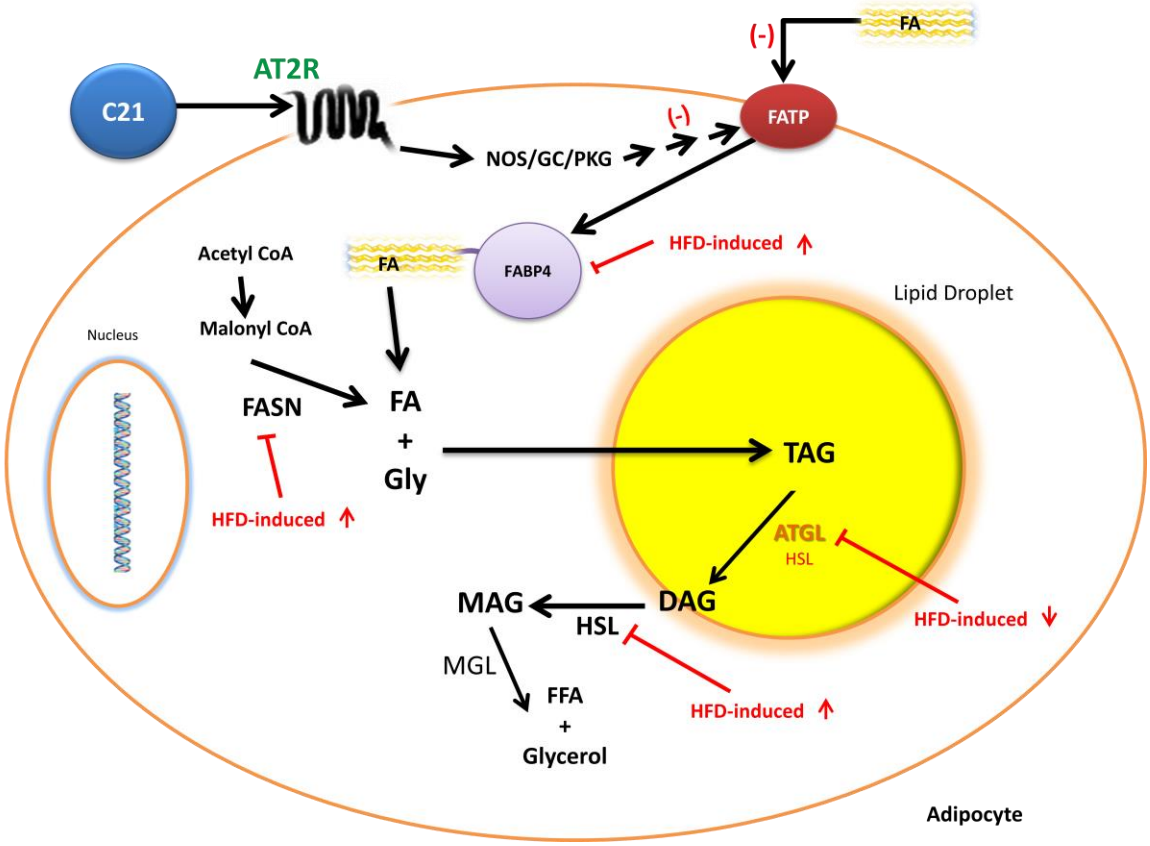


Fig. 33: Hypothetical proposed scheme for mechanism of action of AT2R agonist, C21 on lipid metabolism regulators in an epididymal adipocyte. C21 acting on AT2R via NOS/GC/PKG-dependent pathway reduces fatty acid uptake in the adipocyte. In consequence of the events, C21 prevents HFD-induced increase in lipogenic regulators namely FABP4, FASN while prevents HFD-induced changes in lipolytic regulators - decrease in AGTL and increase in HSL and prevents epididymal adipocyte size increase under HFD.

5: CHAPTER 2 – AT2R ACTIVATION AND FEMALE ADIPOSITY

5.1: Does estrogen play a role in AT2R agonist mediated effect on adiposity and lipid metabolism?

5.1.1: RESULTS from eight week old female mice:

5.1.1.1: Effect of C21 on calorie intake

The Fig. 34 depicts that the total Kcal intake was significantly higher in the HFD group as compared to ND group of Ovi mice (Ovi-ND 98±5.0 vs Ovi-HFD 132±5.8 Kcal). The Kcal intake was also significantly higher in Ovx mice on ND as compared to Ovi mice on ND (Ovi-ND 98±5.0 vs Ovx-ND 120±7.1 Kcal). Placing the ovariectomized mice on HFD further increased the total Kcal intake as compared to ovariectomized mice on ND (Ovx-ND 120±7.1 vs Ovx-HFD 153±5.3 Kcal). The C21 treatment did not impact the Kcal intake in Ovi and Ovx mice with HFD. The E2 supplementation alone (Ovx-HFD 153±5.3 vs Ovx-HFD-E 125±3.0 Kcal) as well as along with C21 (Ovx-HFD-E+C21 123±4.9 Kcal) significantly reduced the total Kcal intake in ovariectomized mice on HFD.

5.1.1.2: Effect of C21 on plasma leptin

The HFD group had significantly increased plasma leptin levels than the ND group of Ovi mice (Ovi-ND 1.7±0.5 vs Ovi-HFD 8.7±1.2 ng/mL) (Fig. 35). The Ovx mice on ND also had increased plasma leptin levels than the ND group of Ovi mice (Ovi-ND 1.7±0.5 vs Ovx-ND 3.4±0.4 ng/mL). The plasma leptin levels were further increased in

Ovx mice on HFD (Ovx-HFD 8.4±0.1 ng/mL). The C21 treatment did not affect the plasma leptin levels in Ovi and Ovx mice on HFD.

5.1.1.3: Effect of C21 on urinary 17β-estradiol (E2) levels

As shown in Fig. 36, the urinary E2 level was significantly lower (Ovi-ND 41.04±2.4 pg/24 h urine vs Ovi-HFD 12.22±1.6 pg/24 h urine) in HFD fed compared with ND fed Ovi mice. The C21 treatment did not affect the urinary E2 levels in Ovi mice under HFD condition. Except for the groups supplemented with E2 pellets (Ovx-HFD-E 71.41±9.7 and Ovx-HFD-E+C21 76.73±9.3 pg/24 h), the urinary E2 in Ovx-ND and Ovx-HFD were not detected.

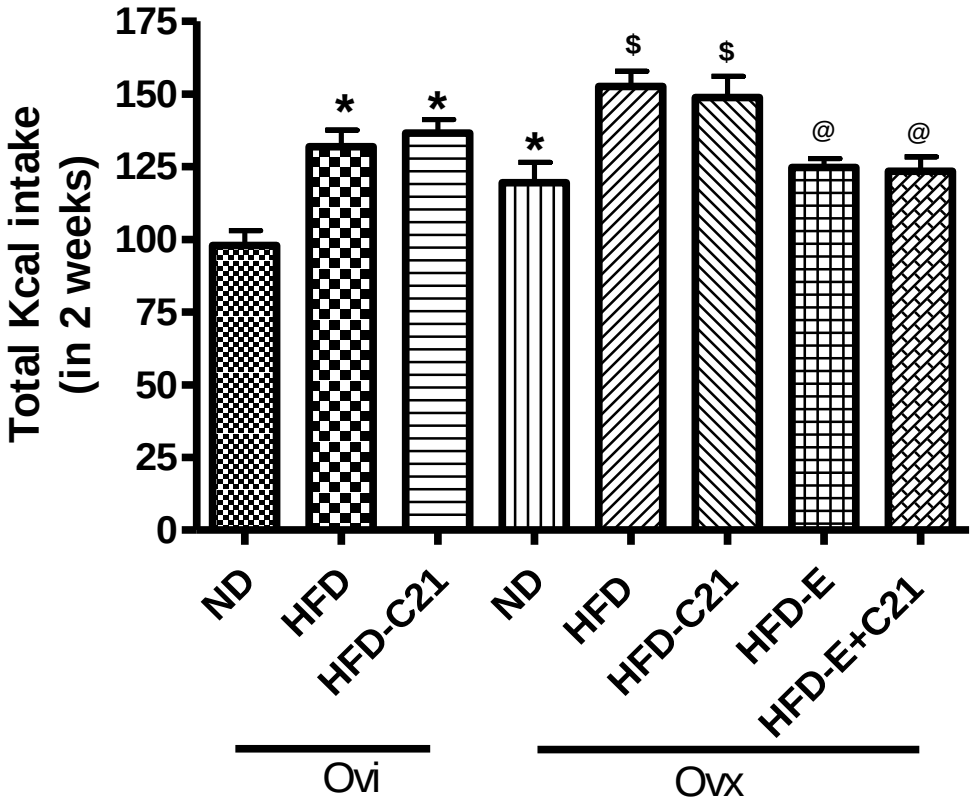


Fig. 34: Total Kcal intake in different treatment groups of ovary-intact (Ovi) and ovariectomized (Ovx) mice fed with ND or HFD. Results are means ± SEM; * - significantly different from Ovi on ND, $ - significantly different from Ovx on ND, @ - significantly different from Ovx on HFD. Data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); N=5-6 in each group. (ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21, HFD-E - High-Fat Diet on E2, HFD-E+C21 - High-Fat Diet on E2+C21).

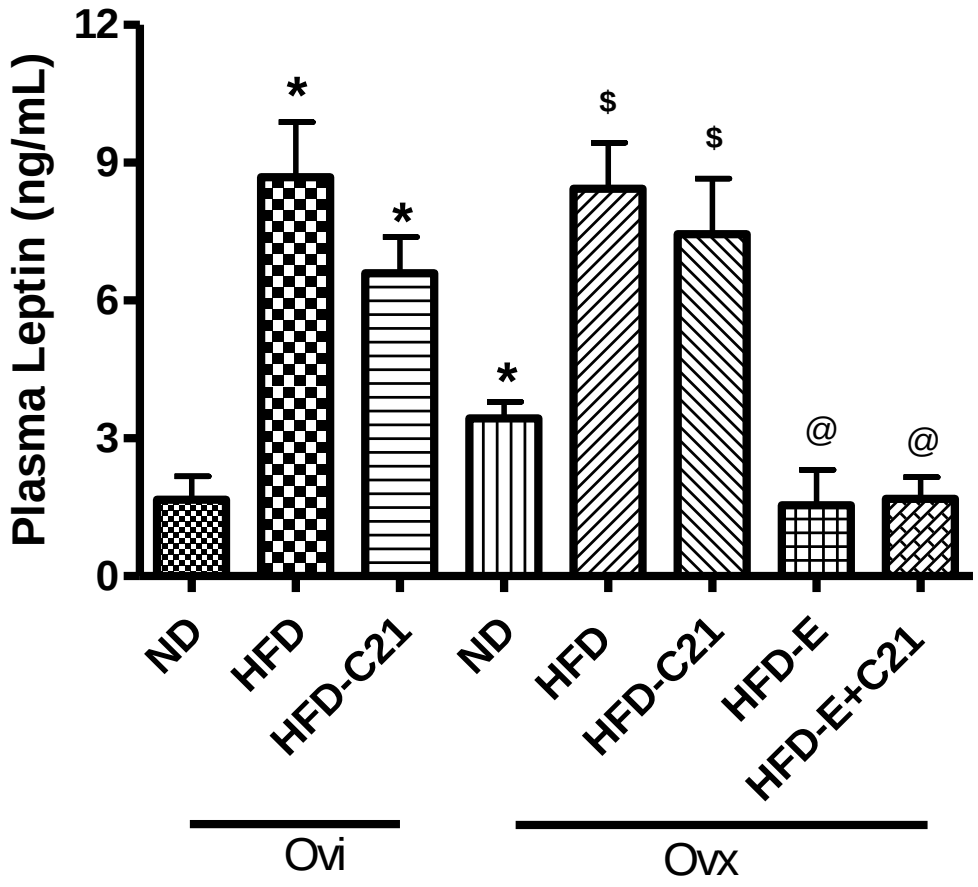


Fig. 35: Plasma leptin levels in different treatment groups of ovary-intact (Ovi) and ovariectomized (Ovx) mice fed with ND or HFD. Results are means ± SEM; * - significantly different from Ovi on ND, $ - significantly different from Ovx on ND, @ - significantly different from Ovx on HFD. Data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); N=6-10 in each group. (ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21, HFD-E - High-Fat Diet on E2, HFD-E+C21 - High-Fat Diet on E2+C21).

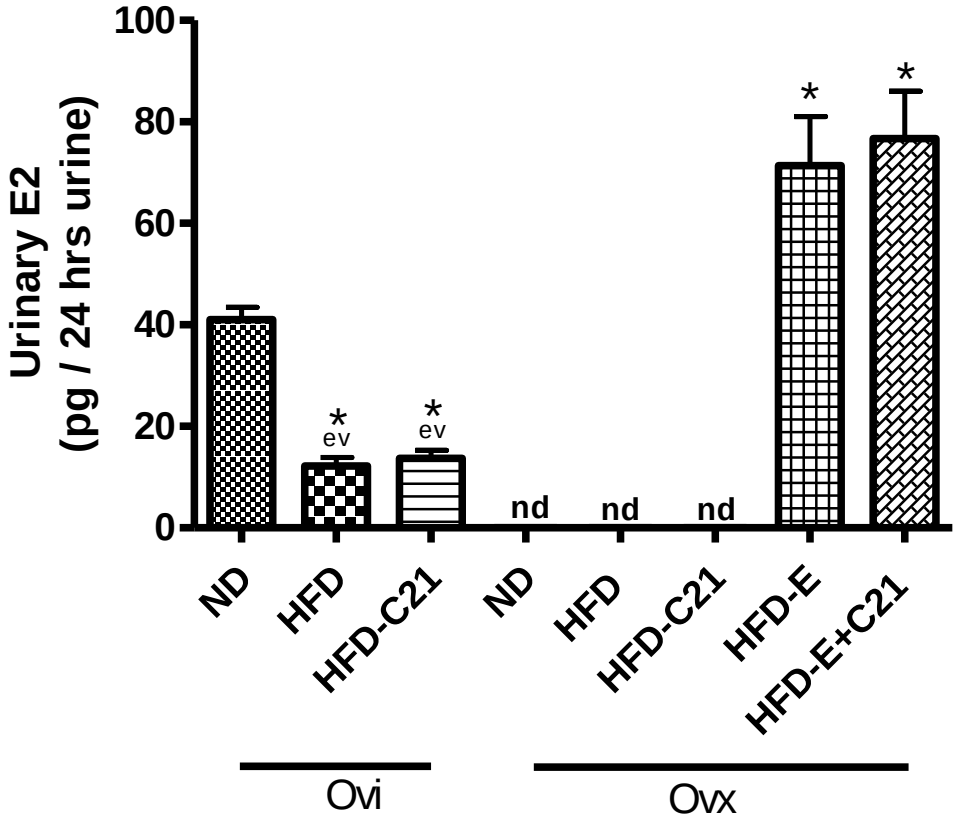


Fig. 36: Urinary 17β-estradiol (E2) levels in different treatment groups of ovary-intact (Ovi) and ovariectomized (Ovx) mice fed with ND or HFD. Results are means ± SEM; * - significantly different from Ovi on ND, nd – not detectable, ev – extrapolated value from standard curve. Data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); N=5. (ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21, HFD-E - High-Fat Diet on E2, HFD-E+C21 - High-Fat Diet on E2+C21).

5.1.1.4: Effect of C21 on parametrial WAT (pWAT) weight and adipocyte size

As evidenced by pWAT weight-to-body weight ratio in Fig. 37, the pWAT weight was significantly increased (168%) by HFD in Ovi mice. Compared to Ovi mice, Ovx itself caused an increase (159%) in the pWAT weight which was further increased (62%) by HFD. The C21 treatment attenuated the pWAT weight increase in both Ovi (28%) and Ovx (37%) mice on HFD.

Similarly as shown in Fig. 38A and B, HFD significantly increased the parametrial adipocyte size in Ovi mice (Ovi-ND 1558$\pm$92 vs Ovi-HFD 2788$\pm$139 μm^2). Ovariectomy caused an increase in the parametrial adipocyte size (Ovx-ND 2448$\pm$79 μm^2) which was further increased by HFD (Ovx-HFD 4694$\pm$151 μm^2). The HFD-induced increase in parametrial adipocyte size in both Ovi (Ovi-HFD 2788$\pm$139 vs Ovi-HFD-C21 2264$\pm$66 μm^2) and Ovx (Ovx-HFD 4694$\pm$151 vs Ovx-HFD-C21 3657$\pm$152 μm^2) mice were attenuated by C21 treatment. In Ovx mice, the E2 supplementation (Ovx-HFD 4694$\pm$151 vs Ovx-HFD-E 1775$\pm$76 μm^2) prevented the HFD-induced increase in the parametrial adipocyte size.

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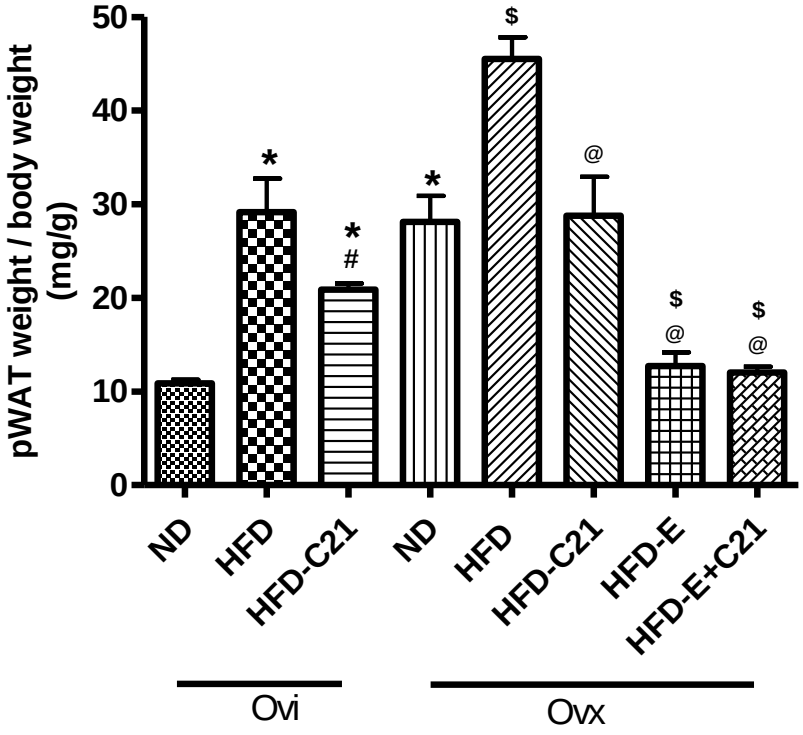
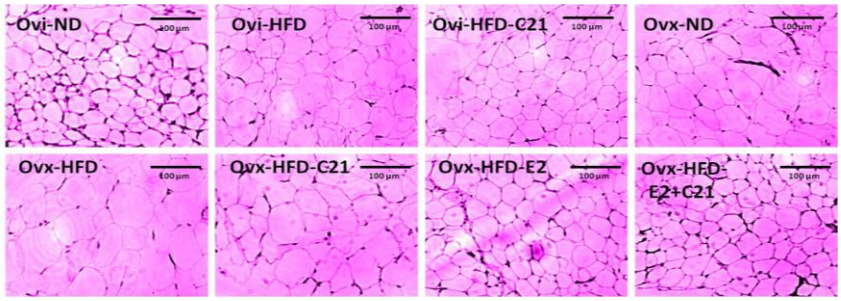


Fig. 37: The pWAT / body weight ratio in different treatment groups of ovary-intact (Ovi) and ovariectomized (Ovx) mice fed with ND or HFD. Results are means ± SEM; * - significantly different from Ovi on ND, # - significantly different from Ovi on HFD, $ - significantly different from Ovx on ND, @ - significantly different from Ovx on HFD. Data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); N=6-10 in each group. (ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21, HFD-E - High-Fat Diet on E2, HFD-E+C21 - High-Fat Diet on E2+C21).

(A)



(B)

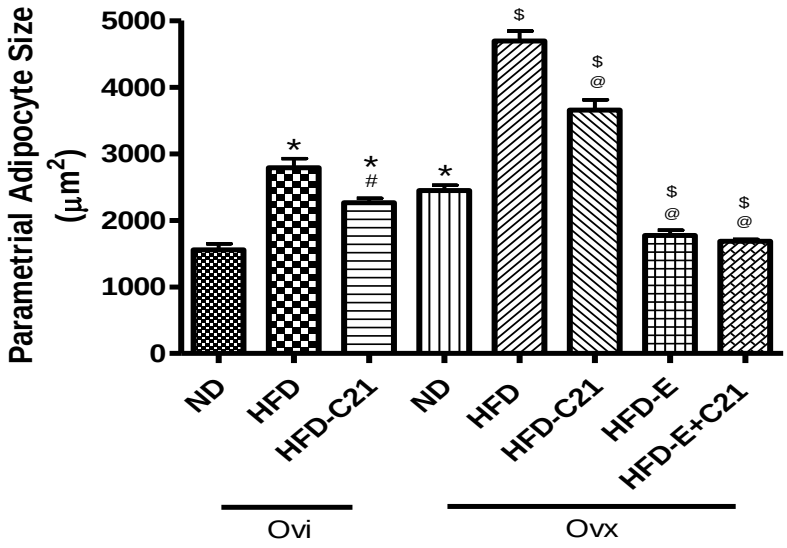


Fig. 38: The parametrial adipocyte size in different treatment groups of ovary-intact (Ovi) and ovariectomized (Ovx) mice fed with ND or HFD. Results are means ± SEM; * - significantly different from Ovi on ND, # - significantly different from Ovi on HFD, $ - significantly different from Ovx on ND, @ - significantly different from Ovx on HFD. Data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); N=3 (3 sections per slide from 3 mice). (ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21, HFD-E - High-Fat Diet on E2, HFD-E+C21 - High-Fat Diet on E2+C21).

5.1.1.5: Effect of C21 on plasma free fatty acid (FFA) and triglyceride (TAG)

The HFD significantly increased the plasma FFA in Ovi mice (Ovi-ND 3.99±0.30 vs Ovi-HFD 6.90±0.23 nmol/µL) as well as the Ovx mice (Ovx-ND 8.54±0.38 vs Ovx-HFD 10.52±0.75 nmol/µL) (Fig. 39). The HFD-induced increase in FFA in Ovi and Ovx mice were attenuated by C21 treatment. In Ovx mice, the E2 supplementation (Ovx-HFD 10.52±0.75 vs Ovx-HFD-E 6.91±0.54 nmol/µL) prevented the HFD-induced increase in the plasma FFA.

Similarly, the plasma triglyceride (TAG) was significantly increased by HFD in Ovi mice (Ovi-ND 1.63±0.12 vs Ovi-HFD 4.37±0.8) (Fig. 40). Compared to Ovi mice, Ovx itself caused an increase in the plasma TAG (Ovi-ND 1.63±0.12 vs Ovx-ND 4.98±0.16) which was further increased by HFD (Ovx-ND 4.98±0.16 vs Ovx-HFD 8.36±0.37). The C21 treatment attenuated the plasma TAG increase in both Ovi (Ovi-HFD 4.37±0.8 vs Ovi-HFD-C21 2.41±0.15) and Ovx (Ovx-HFD 8.36±0.37 vs Ovx-HFD-C21 5.51±0.41) mice on HFD.

5.1.1.6: Effect of C21 on plasma insulin

The HFD significantly increased the plasma insulin in Ovi mice (Ovi-ND 0.69±0.02 vs Ovi-HFD 2.34±0.16 ng/mL) as well as the Ovx mice (Ovx-ND 0.82±0.04 vs Ovx-HFD 2.37±0.16 ng/mL) (Fig. 41). Interestingly, the HFD-induced increase in plasma insulin were attenuated by C21 treatment in both Ovi and Ovx mice.

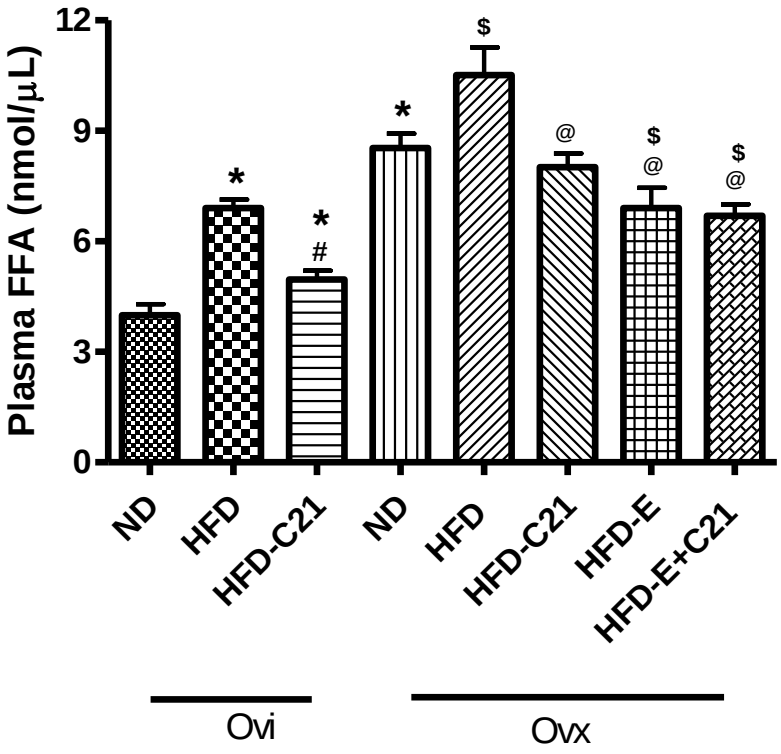


Fig. 39: Plasma free fatty acid (FFA) level in different treatment groups of ovary-intact (Ovi) and ovariectomized (Ovx) mice fed with ND or HFD. Results are means ± SEM; * - significantly different from Ovi on ND, # - significantly different from Ovi on HFD, $ - significantly different from Ovx on ND, @ - significantly different from Ovx on HFD. Data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); N=5-6 in each group. (ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21, HFD-E - High-Fat Diet on E2, HFD-E+C21 - High-Fat Diet on E2+C21).

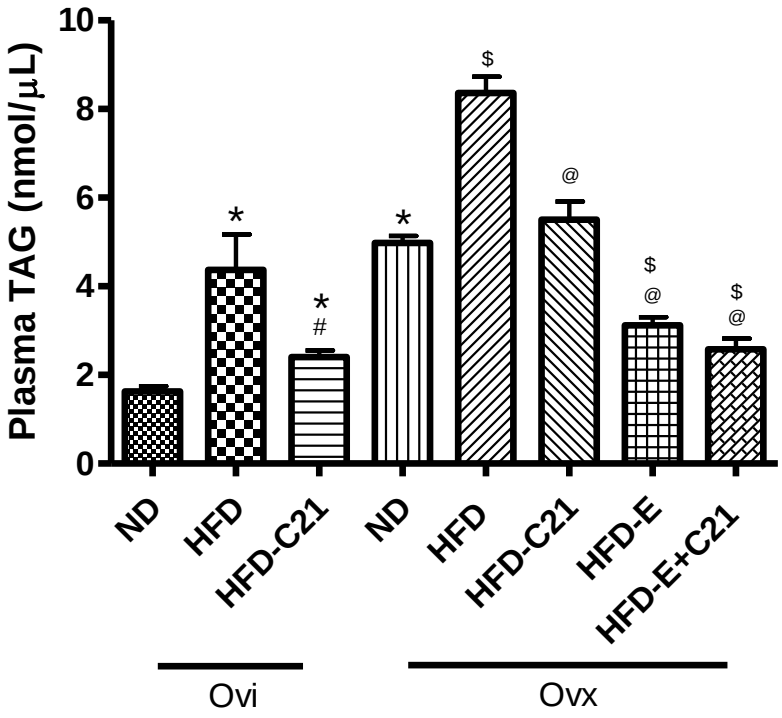


Fig. 40: Plasma triglyceride (TAG) level in different treatment groups of ovary-intact (Ovi) and ovariectomized (Ovx) mice fed with ND or HFD. Results are means ± SEM; * - significantly different from Ovi on ND, # - significantly different from Ovi on HFD, $ - significantly different from Ovx on ND, @ - significantly different from Ovx on HFD. Data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); N=4-6 in each group. (ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21, HFD-E - High-Fat Diet on E2, HFD-E+C21 - High-Fat Diet on E2+C21).

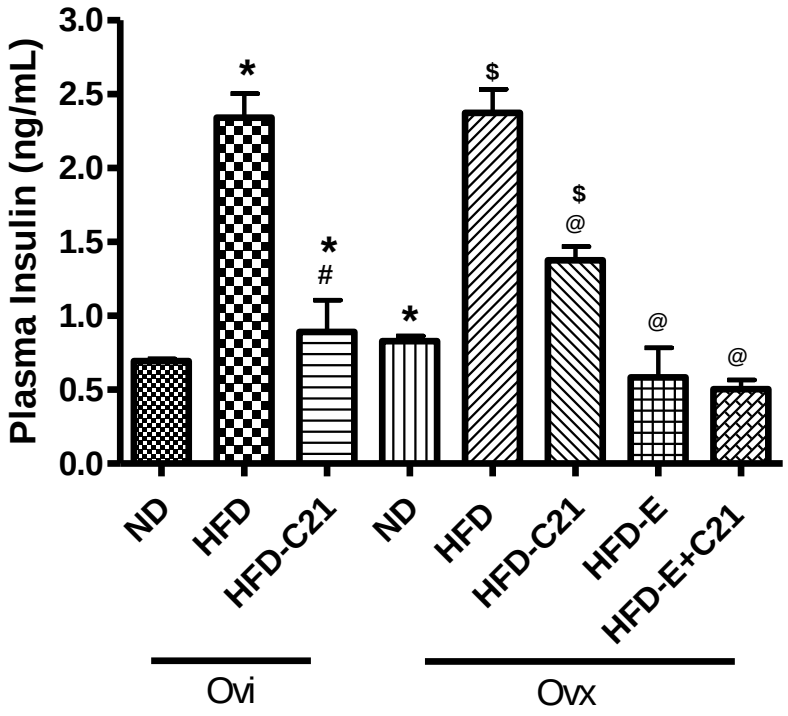


Fig. 41: Plasma insulin concentration in different treatment groups of ovary-intact (Ovi) and ovariectomized (Ovx) mice fed with ND or HFD. Results are means ± SEM; * - significantly different from Ovi on ND, # - significantly different from Ovi on HFD, $ - significantly different from Ovx on ND, @ - significantly different from Ovx on HFD. Data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); N=5-6 in each group. (ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21, HFD-E - High-Fat Diet on E2, HFD-E+C21 - High-Fat Diet on E2+C21).

5.1.1.7: Effect of C21 on uncoupling protein1 (UCP1) protein expression in pWAT

Western blotting revealed the presence of the uncoupling protein1 (UCP1) (32 KDa) protein expression in the pWAT. Densitometric analysis of the bands revealed that HFD (compared with ND) decreased UCP1 (26%) protein expression in pWAT of Ovi mice (Fig. 42). UCP1 protein expression in pWAT was also significantly decreased (34%) in Ovx mice on ND (as compared to Ovi mice on ND). C21-treatment under HFD did not impact the protein expressions of pWAT UCP1 in Ovi or Ovx mice. However, E2 treatment under HFD increased the protein expressions of pWAT UCP1 in Ovx mice.

5.1.1.8: Effect of C21 on parametrial WAT (pWAT) AT2R protein expression

As shown in Fig. 43, distinct bands for pWAT AT2R were detected approximately at 44 KDa by western blotting. Densitometric analysis of the bands revealed that HFD (compared with ND) increased AT2R (32%) protein expression in pWAT of Ovi mice. The C21 treatment did not affect the AT2R protein expression in pWAT of Ovi or Ovx mice. However, E2 treatment under HFD increased protein expression of pWAT AT2R in Ovx mice.

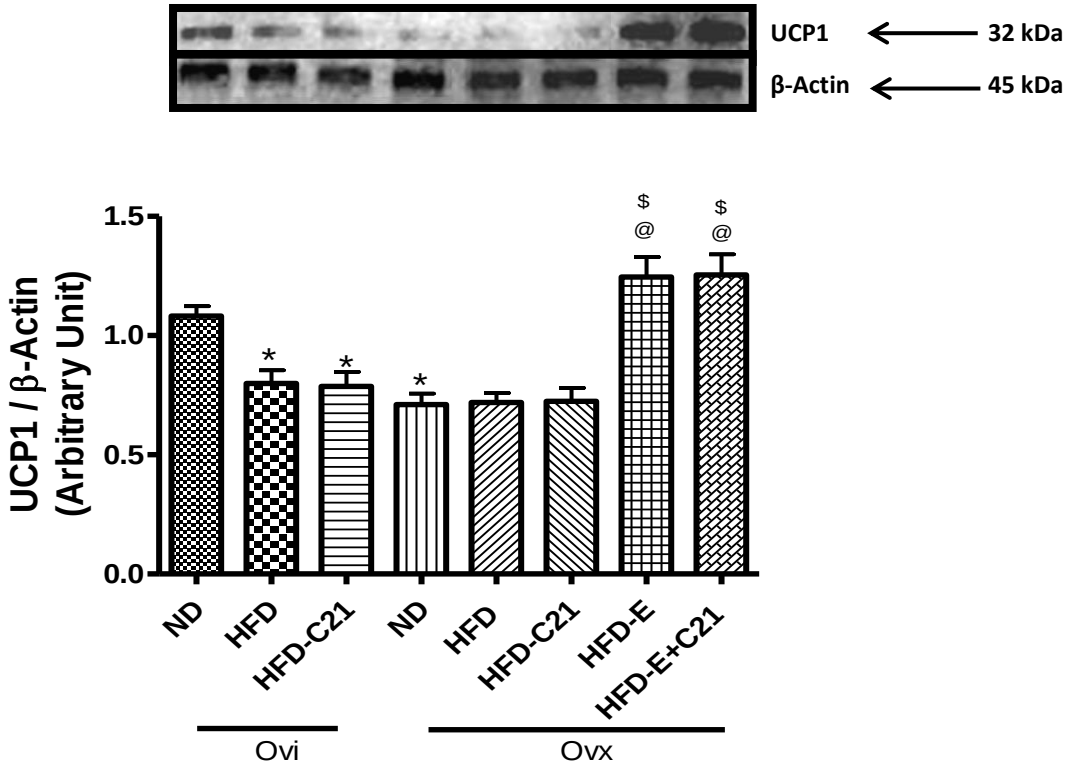


Fig. 42: Protein expression of uncoupling protein1 (UCP1) in the parametrial WAT of ovary-intact (Ovi) and ovariectomized (Ovx) mice fed with ND or HFD. Upper panel: Representative western blots for UCP1 with loading control β-actin. Bar graphs represent the ratios of densities of UCP1 and β-Actin protein bands i.e. UCP1/β-Actin. Results are means ± SEM; * - significantly different from Ovi on ND, $ - significantly different from Ovx on ND, @ - significantly different from Ovx on HFD. Data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); N=3 in each group. (ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21, HFD-E - High-Fat Diet on E2, HFD-E+C21 - High-Fat Diet on E2+C21).

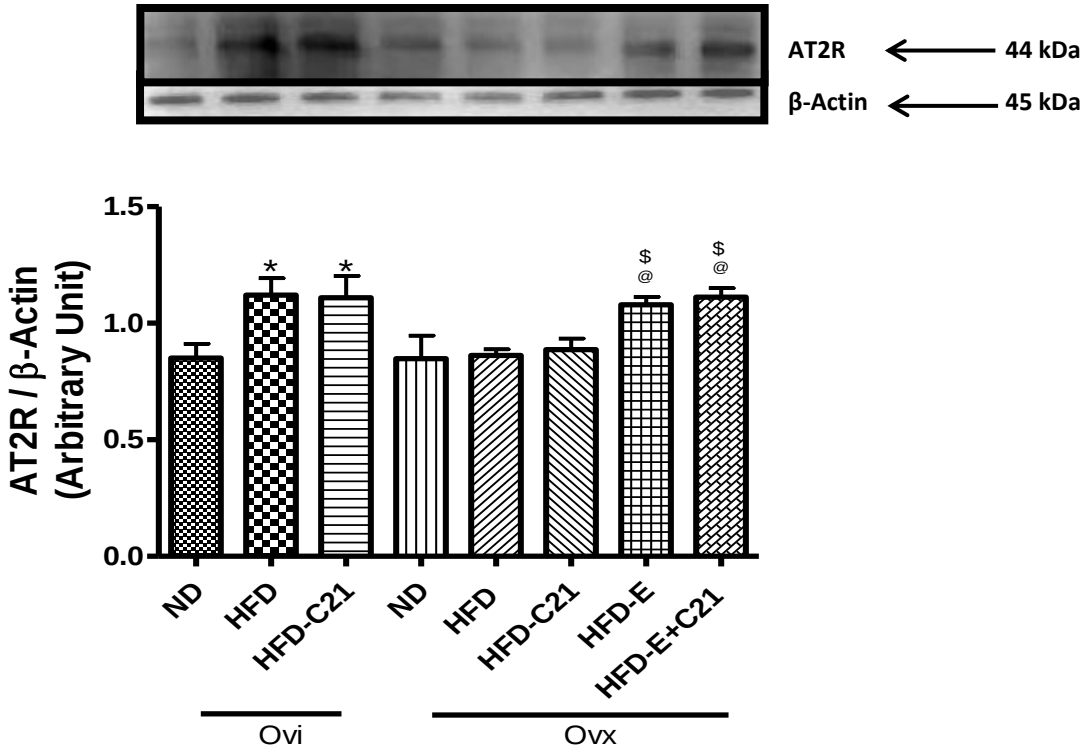


Fig. 43: Protein expression of angiotensin II type 2 receptor (AT2R) in the parametrial WAT of ovary-intact (Ovi) and ovariectomized (Ovx) mice fed with ND or HFD. Upper panel: Representative western blots for AT2R with loading control β-actin. Bar graphs represent the ratios of densities of AT2R and β-Actin protein bands i.e. AT2R/β-Actin. Results are means ± SEM; * - significantly different from Ovi on ND, $ - significantly different from Ovx on ND, @ - significantly different from Ovx on HFD. Data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); N=3-5 in each group. (ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21, HFD-E - High-Fat Diet on E2, HFD-E+C21 - High-Fat Diet on E2+C21).

5.1.1.9: Effect of C21 on liver weight and CPT1-A protein expression

As evidenced by liver weight-to-body weight ratio, liver weight was significantly increased (32%) by HFD in Ovi mice (Fig. 44). Compared to Ovi mice, Ovx itself caused an increase (19%) in the liver weight which was further increased (21%) by HFD. C21 treatment attenuated the liver weight increase in both Ovi (18%) and Ovx (21%) mice on HFD.

Furthermore, western blotting revealed the presence of CPT1-A (88 KDa) protein expression in the liver (Fig. 45). CPT1-A protein expression in pWAT was significantly decreased (21%) in Ovx mice on ND (as compared to Ovi mice on ND). Densitometric analysis of the bands also revealed that HFD (compared with ND) decreased CPT1-A protein expression in liver of Ovi mice by 23% and Ovx mice by 30%. The C21-treatment under HFD increased the protein expressions of liver CPT1-A in Ovi (20%) or Ovx (57%) mice. As expected, E2 treatment under HFD increased the protein expressions of liver CPT1-A in Ovx mice.

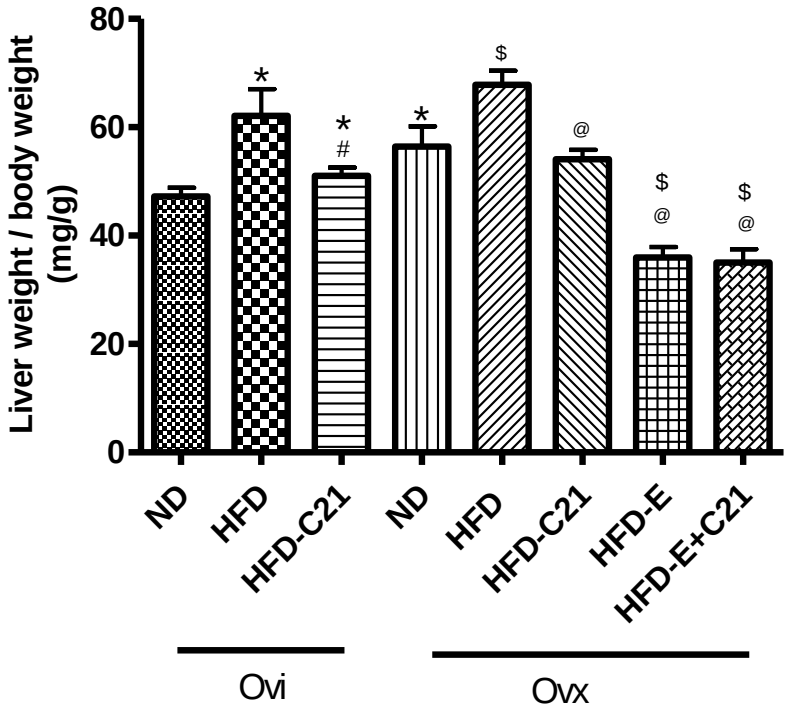


Fig. 44: The liver / body weight ratio in different treatment groups of ovary-intact (Ovi) and ovariectomized (Ovx) mice fed with ND or HFD. Results are means ± SEM; * - significantly different from Ovi on ND, # - significantly different from Ovi on HFD, $ - significantly different from Ovx on ND, @ - significantly different from Ovx on HFD. Data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); N=6-10 in each group. (ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21, HFD-E - High-Fat Diet on E2, HFD-E+C21 - High-Fat Diet on E2+C21).

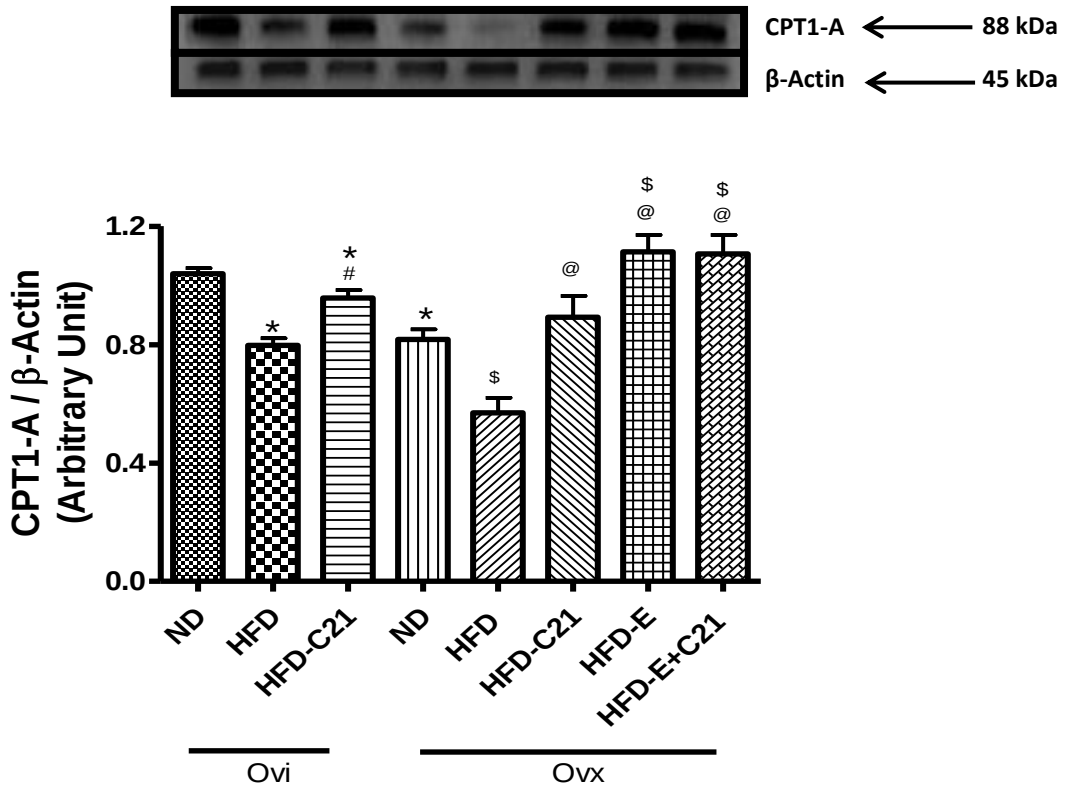


Fig. 45: Protein expressions of CPT1-A in liver of ovary-intact (Ovi) and ovariectomized (Ovx) mice fed with ND or HFD. Upper panel: Representative western blots for CPT1-A with loading control β-actin. Bar graphs represent the ratios of densities of CPT1-A and β-Actin protein bands i.e. CPT1-A /β-Actin. Results are means ± SEM; * - significantly different from Ovi on ND, # - significantly different from Ovi on HFD, $ - significantly different from Ovx on ND, @ - significantly different from Ovx on HFD. Data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); N=3-4 in each group. (ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21, HFD-E - High-Fat Diet on E2, HFD-E+C21 - High-Fat Diet on E2+C21).

5.1.1.10: Effect of C21 on physical activity

The total activity was significantly decreased by HFD in Ovi mice (Ovi-ND 19533±2723 vs Ovi-HFD 7011±1387) (Fig. 46). C21 treatment prevented HFD-induced decrease (Ovi-HFD 7011±1387 vs Ovi-HFD-C21 13267±1134 counts) of total activity in Ovi mice.

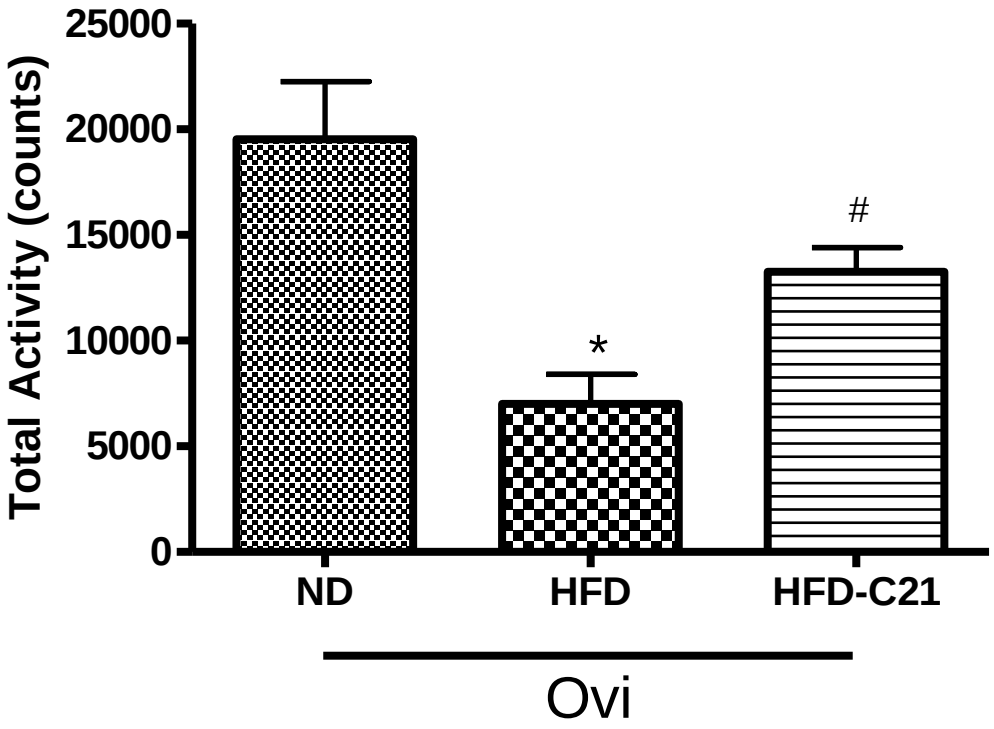


Fig. 46: The total activity in different groups of ovary-intact (Ovi) mice fed with ND or HFD. Results are means ± SEM; * - significantly different from Ovi on ND, # - significantly different from Ovi on HFD. Data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); N=6 in each group. (ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21).

Table 4: Metabolic and hormonal parameters of ovary-intact (Ovi) and overectomized (Ovx) C57BL/6 mice fed with ND or HFD.

	Ovi			Ovx				
Parameters/Mice groups	ND	HFD	HFD-C21	ND	HFD	HFD-C21	HFD-E	HFD-E+C21
Total Kcal intake (in 2 weeks)	B	↑*	↔	↑*	↑$	↔	↑@	↑@
Plasma leptin (ng/mL)	B	↑*	↔	↑*	↑$	↔	↓@	↓@
Urinary E2 (pg/24 h urine)	B	↓*	↔	nd	nd	nd	↑*	↑*
pWAT weight / body weight (mg/g)	B	↑*	↓#	↑*	↑$	↓@	↓@	↓@
Parametrial adipocyte size (μm^2)	B	↑*	↓#	↑*	↑$	↓@	↓@	↓@
Plasma FFA (nmol/µL)	B	↑*	↓#	↑*	↑$	↓@	↓@	↓@
Plasma TAG (nmol/µL)	B	↑*	↓#	↑*	↑$	↓@	↓@	↓@
Plasma insulin (ng/mL)	B	↑*	↓#	↑*	↑$	↓@	↓@	↓@
UCP / β-actin (protein expression)	B	↓*	↔	↓*	↔	↔	↑@	↑@
AT2R / β-actin (protein expression)	B	↑*	↔	↔	↔	↔	↑@	↑@
pWAT weight / body weight (mg/g)	B	↑*	↓#	↑*	↑$	↓@	↓@	↓@
CPT1-A / β-actin (protein expression)	B	↓*	↑#	↓*	↓$	↑@	↑@	↑@
Total Activity (counts)	B	↓*	↑#					

ND - Normal Diet, HFD - High-Fat Diet, HFD-C21 - High-Fat Diet on C21, HFD-E - High-Fat Diet on E2, HFD-E+C21 - High-Fat Diet on E2+C21; B - basal under ND in Ovi, ↑ - increased, ↓ - decreased, ↔ - not-significantly different from Ovi on ND, ↔ - not-significantly different from Ovi on HFD, ↔ - not-significantly different from Ovx on ND, ↔ - not-significantly different from Ovx on HFD, * - significantly different from Ovi on ND, # - significantly different from Ovi on HFD, $ - significantly different from Ovx on ND, @ - significantly different from Ovx on HFD; data were analyzed using one-way ANOVA with Newman-keuls post hoc test and Student's *t* test ($p<0.05$); nd – not detectable.

5.2: Discussion:

Undue increased adiposity causes obesity which is known as an underlying basis for the pathogenesis of diabetes and various CVDs (Dole, 1956; Randle *et al.*, 1963; Black *et al.*, 1991). According to World Health Organization (WHO), women are more likely to be obese than men (http://www.who.int/gho/ncd/risk_factors/obesity_text/en/) and post-menopausal women are associated with higher incidence of obesity (Lambrinoudaki *et al.*, 2010). Interestingly, it was observed that female mice express a substantially higher level of AT2R than male mice (Baiardi *et al.*, 2005). We observed that knocking out AT2R increased the gonadal or parametrial WAT (pWAT) and body weight in female mice compared with control animals on HFD (Samuel *et al.*, 2013), suggesting a protective role of AT2R on adiposity in female mice under HFD. In accordance with our knock out study, in the present study we observed a significantly reduced adiposity (as measured by pWAT weight and parametrial adipocyte size) in HFD fed ovary-intact female mice treated with the AT2R agonist C21. Furthermore, C21 treatment also prevented the pWAT weight and parametrial adipocyte size increase in ovariectomized mice fed HFD. This finding indicates that activation of AT2R can prevent HFD-induced adiposity in ovary-intact as well as ovariectomized conditions.

The increased adiposity and obesity can be an outcome of increase in food intake or hyperphagia. In rodents, ovariectomy induces hyperphagia and body weight gain while exogenous administration of estrogen (E2) prevents hyperphagia culminating in prevention of body weight gain (Wade and Zucker, 1970). High-fat

diet is reported to reduce E2 (Bryzgalova *et al.*, 2008) and E2 deficiency is known to cause leptin resistance (Ainslie *et al.*, 2001). Low levels of E2 could lead to accumulation of fat mass and development of obesity and hyperlipidemia (Ainslie *et al.*, 2001; Riant *et al.*, 2009). On the other hand, E2 supplementation in ovariectomized mice has been shown to reduce adipocyte size by decreasing the adipocyte lipogenic enzymes such as lipoprotein lipase, ACC, fatty acid synthase and increasing catecholamine-induced lipolysis (D'Eon *et al.*, 2005). In the present study we observed that, in ovary-intact as well as ovariectomized mice, HFD-induced adiposity was associated with increased Kcal intake. As documented previously (Brown and Clegg, 2010), we also observed that E2 supplementation prevented HFD-induced adiposity via reducing the Kcal intake through the leptin-hyperphagia axis. However unlike E2 supplementation, C21 treatment prevented HFD-induced adiposity without impacting the Kcal intake and plasma leptin levels in ovary-intact as well as ovariectomized mice suggesting that C21 does not impact adiposity via the leptin-hyperphagia axis. Furthermore, E2 has been shown to positively regulate AT2R expression (Yoshimura *et al.*, 1996; Baiardi *et al.*, 2005). In our previous study we reported that the deletion of AT2R in female mice caused an increase in adiposity with a parallel decrease in urinary E2 level (Samuel *et al.*, 2013). These studies indicated a potential inter-regulation between E2 and AT2R. However in the present study, mice fed on HFD had reduced urinary E2 which was not affected by C21 in Ovi mice suggesting that the role of AT2R on adiposity could be independent of estrogen. This finding is further supported by our observation in

Ovx mice. In these mice, urinary E2 were not detectable, yet C21 treatment prevented the adiposity to the same extent as in the Ovi mice.

Decreased oxidation of fatty acids to generate energy (β-oxidation) is associated with adiposity and obesity (Koves *et al.*, 2005). In accordance with this finding, increased β-oxidation has been shown to prevent adiposity and obesity (Fiamoncini *et al.*, 2013). CPT1 mediates transport of fatty acids across the mitochondrial membrane to channelize the β-oxidation (Gyamfi *et al.*, 2012). We observed an increase in CPT1 protein expression in the liver of HFD-fed ovary-intact as well as ovariectomized mice treated with C21, suggesting a potential increase in β-oxidation by C21 treatment in female mice. Furthermore, we observed a reduction in the liver weight of HFD fed ovary-intact as well as ovariectomized mice treated with C21, suggesting that C21 treatment potentially prevents fatty liver induced by HFD perhaps via increased β-oxidation in liver. However, recent report indicates that liver mitochondrion is devoid of AT2R (Astin *et al.*, 2013). Thus plausibly, C21-mediated increase of β-oxidation in liver might be secondary to C21-mediated changes in adiposity.

Obesity is also symptomatically characterized by enhanced plasma FFA and hyperinsulinemia which in turn contribute further towards insulin resistance and type 2 diabetes (Dole, 1956; Randle *et al.*, 1963; Black *et al.*, 1991). Recently, pharmacological activation of AT2R has been reported to improve insulin sensitivity in male animals (Ohshima *et al.*, 2012; Shum *et al.*, 2013). In our previous study, we reported that AT2R deficiency in female mice led to hyperinsulinemia, increase in

FFA and hepatic TAG (Samuel *et al.*, 2013). In concert, in the present study, C21 treatment improved hyperinsulinemia along with decreased plasma FFA as well as TAG in ovary-intact and in ovariectomized mice. These findings indicate that C21 treatment prevents not only adiposity but it also improves HFD-induced metabolic syndrome in female mice.

In the event of lower accumulation of fatty acids in adipocytes (smaller adipocyte size) and decreased plasma FFA and TAG under HFD by C21, the fate of fatty acids needed further investigation. Possibly, the fatty acids were being utilized in heat (via browning of WAT) and/or energy (β-oxidation in skeletal muscles or physical activity) generation. Thus to assess the fate of fatty acids in these animals, we performed western blotting for UCP1 in pWAT to study whether C21 treatment triggers the browning of WAT to utilize the fatty acids for heat generation. We observed that C21 treatment did not impact UCP1 protein expression in the pWAT of ovary-intact as well as ovariectomized mice. Also, a recent report has showed that stimulation of AT2R in adipose tissue suppress the UCP1 (Grobe *et al.*, 2013). Collectively, these findings indicate that C21 treatment does not promote browning of WAT and may not be utilizing the fatty acids for heat generation; we have not however assessed the body temperature in these mice.

Further to study the utilization of fatty acids by C21 in our animals, we assessed the physical activity of mice. To our surprise, we observed that HFD significantly reduced the physical activity which was significantly increased by C21 treatment in the HFD fed ovary-intact mice. This shows that the C21 treatment

utilizes fatty acids for energy generation and utilizes the energy in physical activity to prevent adiposity in female mice. As the AT2R are expressed by skeletal muscles (Chai *et al.*, 2010) which are energy utilizing organs, possibly C21 treatment increases β-oxidation in skeletal muscle and utilizes the energy in physical activity. However, it is also possible that C21-mediated increase in physical activity might be secondary to C21-mediated changes in adiposity, like C21-mediated effects on liver. A further systematic investigation is required to explore these possibilities.

In conclusion, the activation of AT2R by C21 prevents HFD-induced increase in parametrial WAT weight and adipocyte size in female mice independent of estrogen. These protective effects of AT2R agonist in female mice on HFD is accompanied by alterations in the plasma FFA, TAG and insulin similar to male mice (Table 4). Further, C21 treatment also prevented HFD-induced increase in liver weight and possibly β-oxidation in these animals. Most interestingly we observed that AT2R agonist treatment increased physical activity of HFD-fed mice. This study indicates that the activation of AT2R may serve as a potential therapeutic target to control obesity and improve associated metabolic disorders in females as well.

6: SUMMARY AND CONCLUSION:

Summary:

- In adipocytes, C21 acting on AT2R via NOS/GC/PKG pathway reduces fatty acid uptake.
- C21 treatment in male mice prevents HFD-induced changes in lipogenic and lipolytic regulators namely HFD-induced increase in FABP4 and FASN, HFD-induced decrease in ATGL and HFD-induced increase in HSL, thus preventing adiposity (adipocyte size and adipose tissue weight) under HFD (Table 2).
- C21 treatment prevents HFD-induced increase in plasma FFA, TAG and insulin in mice, both male and females (Table 2, 3 and 4).
- In female mice, C21 treatment prevents HFD-induced increase in adiposity (adipocyte size and adipose tissue weight) independent of estrogen.
- In female mice liver, C21 treatment increases CPT1A expression which is an index of β-oxidation and prevents liver weight increase under HFD.
- C21 treatment increases HFD-induced lowering of physical activity in female mice.

Conclusion:

From the present study we conclude that pharmacological activation of AT2R reduces fatty acid uptake in adipocytes and thus prevents HFD-induced changes in lipid metabolism, adiposity and plasma parameters in young and adult male as well as female mice. Pharmacological activation of AT2R increases physical activity and possibly β-oxidation in liver of mice placed on HFD. We propose that AT2R activation may serve as a potential therapeutic target to control obesity and as a consequence may improve obesity-associated metabolic disorders. However, these significant findings need further systematic investigation.

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