ABSTRACT

THE REGION-SPECIFIC INLUENCE OF ESTRADIOL ON IN-VIVO LIPOLYSIS IN SUBCUTANEOUS ADIPOSE TISSUE IN OVERWEIGHT-TO-MODERATELY-OBESE PREMENOPAUSAL WOMEN

By: Kathleen M. Gavin

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Director of Dissertation: Robert C. Hickner, PhD

Major Department: Department of Kinesiology

Premenopausal women demonstrate preferential accumulation of adiposity in the gynoid region, a distribution which shifts towards the abdominal region after the menopausal transition. Although estrogen is implicated as a major player in determining body fat distribution the mechanisms behind estrogenic action(s) in adipose tissue of women are still unclear. The global aim of this project was to determine if local estrogen influences regional adiposity in premenopausal women. Specifically, we investigated the influence of local estradiol on adipose tissue lipolysis as well as estrogen receptor content and adipocyte size in abdominal and gluteal subcutaneous adipose tissue of overweight-to-moderately-obese premenopausal women.

Eighteen overweight-to-moderately obese Caucasian (CA, n=9) and African American (AA, n=9) women were recruited. Between 15 and 17 of these women took part in each of the

three studies. We found the influence of estradiol on lipolysis to be adipose tissue depot specific and treatment dependent, with estradiol perfusion blunting the response to lipolytic stimulation under some conditions while potentiating this response in others. Furthermore, we found differences in abdominal and gluteal estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) protein content, potentially revealing region specific actions of estrogen through these estrogen receptors in adipose tissue. Finally, we found racial differences in adipose tissue morphology, as indicated by variations in adipocyte diameter populations in CA and AA women (higher proportion of medium size adipocytes in abdominal and gluteal subcutaneous adipose tissue of CA and higher proportion of small adipocytes in gluteal region of AA) which could underlie the increased chronic disease risk in AA compared to CA women for a given body mass index (BMI).

Our findings indicate depot specific influence of estradiol on lipid mobilization may play a role in the predominant gluteal-femoral body fat distribution of premenopausal women. It is possible that depot specific effects of estradiol may be a result of regional differences in estrogen receptor content. Racial divergence in adipocyte morphology is an adipose tissue characteristic potentially underlying differing disease risk between CA and AA women of similar BMI. The integration of these results provides insight towards a more complete understanding of regional adiposity in overweight-to-obese premenopausal women, but future studies must be conducted to uncover the interaction between estrogen receptor content and local estrogen action as well as the direct physiological consequences of these findings.

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Kathleen M. Gavin

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By: Kathleen M. Gavin

APPROVED BY:

DIRECTOR OF DISSERTATION:_____
Robert C. Hickner, Ph.D.

COMMITTEE MEMBER:_____
Robert M. Lust, Ph.D.

COMMITTEE MEMBER:_____
Ronald N. Cortright, Ph.D.

COMMITTEE MEMBER:_____
Abdel A. Abdel-Rahman Ph.D.

COMMITTEE MEMBER:_____
Jacques Robidoux, Ph.D.

CHAIR OF THE DEPARTMENT OF KINESIOLOGY:

_____ Stacey R. Altman, J.D.

DEAN OF THE COLLEGE OF HEALTH AND HUMAN PERFORMANCE:

_____ Glen G. Gilbert, Ph.D.

DEAN OF THE GRADUATE SCHOOL:

_____ Paul J. Gemperline, Ph.D.

DEDICATION

I dedicate this dissertation to my parents, Shawn and Donna.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
α2-AR	Alpha-adrenergic receptor subtype 2
α-AR	Alpha-adrenergic receptor
αERKO	Estrogen receptor-alpha knockout mouse model
β	Beta
β-actin	Beta-actin
β-AR	Beta-adrenergic receptor
βERKO	Estrogen receptor-beta knockout mouse model
°	Degree
μl	Microliter, $1 \cdot 10^{-6}$ liters
μm	Micrometer, $1 \cdot 10^{-6}$ meters
σ	Standard Deviation
17β-HSD	17-beta hydroxysteroid dehydrogenase
AA	African American
AB	Abdominal
ABHD5	Alpha/Beta-hydrolase domain-containing protein 5
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
AR	Adrenergic receptor
ARKO	Aromatase knockout mouse model
ATBF	Adipose tissue blood flow

ATGL	Adipose tissue triglyceride lipase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BMI	Body mass index, kg/m^2
BP	Blocking peptide
BSA	Bovine serum albumin
C	Celsius
C_{19}	19 carbon steroid, examples: androstenedione, testosterone
CA	Caucasian American
cAMP	3'-5'-cyclic adenosine monophosphate
CGI-58	Comparative gene identification-58
cGMP	3'-5'-cyclic guanosine monophosphate
CI	Confidence interval
cm	Centimeter
CT	Computed tomography
CTRC	Clinical and Translational Research Center
CV	Coefficient of variation
CVD	Cardiovascular disease
d	Diameter
DAG	Diacylglycerol, also called diglyceride
DERKO	Double estrogen receptor-alpha and -beta knockout mouse model
Dialysate	The fluid which has passed by the dialysis membrane and which is sampled at the outlet of the microdialysis probe

DPN	Diarylpropionitrile, selective estrogen receptor-beta agonist
DXA	Dual-energy x-ray absorptiometry
E	Estrogen
E+P	Estrogen plus progesterone
E_1	Estrone
E_2	Estradiol, 17β-Estradiol
$E_{2dialysate}$	Estradiol as measured in dialysate
E_{2IS}	Interstitial estradiol
ECDOI	East Carolina Diabetes and Obesity Institute
EDTA	Ethylenediaminetetraacetic acid
EE	Ethinyl estradiol
ELISA	Enzyme-linked immunosorbent assay
Epi	Epinephrine
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
Ex, EX	Exercise
FA	Fatty acid
FABP4	Fatty acid binding protein 4
FFA	Free fatty acid
FITT	Fitness, Instruction, Testing, and Training Building
FSH	Follicle stimulating hormone
g	Gram

G_i	Inhibitory G protein complex
GL	Gluteal
$Glyc_{dialysate}$	Glycerol as measured in dialysate
$Glyc_{IS}$	Interstitial glycerol
GPER	G protein-coupled estrogen receptor
G_s	Stimulatory G protein complex
HDL	High-density-lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, an organic chemical buffering agent
HERS	Heart Estrogen/Progestin Replacement Study
HOMA-IR	Homeostasis model of assessment of insulin resistance
HPLC	High-performance liquid chromatography
HSL	Hormone sensitive lipase
HT	Hormone therapy
IGF-1	Insulin-like growth factor-1
In situ	Latin phrase that translates literally into "in position" or "on site"
In vitro	Latin phrase that translates literally into "in glass." Studies that are conducted using components of an organism that have been isolated from their usual biological surroundings
In vivo	Latin phrase that translates literally into "within the living." Studies that are conducted using a whole, living organism.
ISO	Isoproterenol, beta-adrenergic receptor agonist
IV	Indwelling, intravenous polyethylene catheter
kDa	Kilodaltons, units of atomic mass. $1 \cdot 10^3$ daltons

kg	Kilograms, $1 \cdot 10^{3}$ grams
KRB	Krebs ringer bicarbonate buffer
LDL	Low-density-lipoprotein
LH	Luteinizing hormone
LPL	Lipoprotein Lipase
m	Meter
MAG	Monoacylglycerol
MCF7	Breast cancer cell line derived from a human breast adenocarcinoma, estrogen receptor positive.
MGL	Monoacylglycerol lipase
ml	Milliliter, $1 \cdot 10^{-3}$ liters
mmol/l	Millimolar concentration per liter, $1 \cdot 10^{-3}$ molar concentration
mRNA	Messenger ribonucleic acid
N, n	Sample size
NE	Norepinephrine
NEFA	Non-esterified fatty acid
NHANES	National Health and Nutritional Examination Survey
NIH	National Institutes of Health
nmol/l	Nanomolar concentration per liter, $1 \cdot 10^{-9}$ molar concentration
NO	Nitric oxide
NPR-C	Clearance receptor for atrial natriuretic peptide
O:I	Ethanol outflow-to-inflow ratio, outflow/inflow ratio
OVX	Ovariectomy, ovariectomized

P	Progesterone
$P450_{arom}$	Aromatase
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline containing 1% tween-20
PCR	Polymerase chain reaction
PDE3B	Cyclic nucleotide phosphodiesterase 3B
Perfusate	The fluid which is infused into the microdialysis probe, passing through the microdialysis probe from the inlet to the probe membrane
PHEN	Phentolamine, alpha-adrenergic receptor antagonist
PKA	Protein kinase A
PKG	Protein kinase G
pmol/l	Picomolar concentration per liter, $1 \cdot 10^{-12}$ molar concentration
PPT	Propylpyrazoletriol, selective estrogen receptor-alpha agonist
R_a	Rate of appearance
REE	Resting energy expenditure
RER	Respiratory exchange ratio
RPM	Revolution per minute
SAT	Subcutaneous adipose tissue
SDS	Sodium dodecyl sulfate
SE	Standard error
SEM	Standard error mean
SKBR3	Breast cancer cell line derived from a human breast adenocarcinoma. ER-alpha and -beta negative, G protein-coupled estrogen receptor positive

T2DM	Type 2 Diabetes Mellitus
TAG	Triacylglycerol
TC	Total cholesterol
TG	Triglycerides
TNFα	Tumor necrosis factor alpha
VAT	Visceral adipose tissue
VO_{2max}	Velocity of maximal oxygen uptake/consumption
WHI	Women's Health Initiative
WHR	Waist-to-hip ratio
WT	Wild type

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CHAPTER 1: Review of Literature

Obesity, Body Fat Distribution, and Disease Risk

Excess weight increases the risk of multiple disease states, including heart disease, hypertension, Type 2 diabetes, certain cancers (e.g. colon and breast) and stroke (1-4). The associations between adiposity and the Metabolic Syndrome and cardiovascular disease (CVD) are believed to follow a region-specific scale such that abdominal visceral adiposity predicts the highest risk, abdominal subcutaneous adiposity predicts an intermediate risk that is independent of the visceral depot, and femoral adiposity may actually predict metabolic protection (5, 6).

The idea that localization of body fat, and not merely total fat mass, holds high importance in the elevated health risks associated with obesity is not a new idea. Awareness of regional fat patterning has been demonstrated from the earliest recorded works of humankind. The development of systematic methodology for categorizing human body shape was pioneered in the 1930s (7). In 1947 J. Vague first suggested that the relative amount of upper- versus lower-body obesity was important (8) and could be quantified by an average of two ratios, contrasting the fat at the nape of the neck with that at the sacrum, and the ratio of fat-to-muscle area in the arm with that in the thigh (9). He further hypothesized that, although the gynoid (or lower-body) obesity typically seen in women is benign, android (or upper-body) obesity is

common among individuals with such health problems as diabetes, gout, and atherosclerosis. Evidence from more recent body composition studies suggests adipose tissue distributed in the intra-abdominal or visceral region (also known as "android", central, or male-pattern body fat distribution) carries a much greater risk for metabolic and cardiovascular disorders than does adipose tissue distributed subcutaneously (7, 10-13). There is also evidence that lower-body adiposity is actually protective against disease risk, rather than simply less harmful (5, 14-19).

Although central visceral and abdominal subcutaneous adipose tissue distribution is more closely associated with increased metabolic and CVD risk, research conducted on adipose tissue should not be limited to understanding fat deposition in these depots alone. Uncovering the physiology behind how and why premenopausal females preferentially carry adiposity peripherally, possibly protecting them from obesity associated diseases may help in development of abdominal obesity prevention strategies for women, particularly after menopause.

Racial Differences in Obesity and Disease Risk

According to data from the National Health and Nutrition Examination Study (NHANES) results from 2009-2010 the prevalence of age-adjusted obesity (Body Mass Index [BMI] $\geq$ 30 kg/m^2) and obesity and overweight (BMI $\geq$ 25 kg/m^2) in women at least 20 years of age or older in the United States is 35.8% and 63.7% respectively, with the mean age adjusted BMI for women being 28.7 kg/m^2 (20). African American women tend to be more obese than Caucasian women (20, 21), with obesity prevalence in non-Hispanic white women being 32.2% while in non-Hispanic black women it is 58.5% (20).

Caucasian (CA) and African American (AA) women are reported to have different body fat distributions for a given amount of total body adiposity. AA, in comparison to CA, women

have less visceral adipose tissue (VAT) for a similar age and BMI (22-24), and/or have greater amounts of subcutaneous adipose tissue (SAT) even after adjustment for total body fat (23, 25, 26). Visceral adipose is most commonly the depot implicated in elevated disease risk, yet AA women have more cardiovascular disease risk factors (e.g. increased BMI) (27) and a higher incidence of Type 2 diabetes mellitus (T2DM) and hypertension than CA women (28) despite preferential accumulation of adiposity in SAT. In fact, the relationship between central fat deposition and metabolic risk factors is weaker in African Americans in comparison to Caucasians (23). This evidence supports an important pathophysiological role for accumulation of adiposity even within the "less dangerous" subcutaneous depot.

The importance of the abdominal SAT depot contributing to disease risk is a point of particular significance in overweight and obese African American women, particularly in regards to risk of insulin resistance (29, 30). Decreased insulin sensitivity in black women is most closely associated with the increased abdominal SAT while in white women this association was equal between VAT and SAT (31, 32). The preferential accumulation of subcutaneous adiposity in AA women as well as the divergent association between subcutaneous adiposity and disease risk between CA and AA women potentially indicates an inherent difference in SAT characteristics between these groups, a topic requiring future investigation.

Regional Adiposity Association with Circulating Sex Hormones

The distribution of adipose tissue is divergent by sex. Men have a more central (abdominal) accumulation of fat, whereas women have more peripheral (gynoid, gluteal/femoral) accumulation (33-35). Men also have a higher incidence of CVD than women, until menopause when the incidence of CVD and central adiposity increases in women (36). This

epidemiological and clinical evidence suggests a large role for sex hormones in the regulation of adipose tissue distribution and disease risk (37).

Strong evidence from multiple large randomized controlled trials has accumulated to demonstrate that postmenopausal women on hormone therapy (HT) gain less weight than women on placebo (38-41). In the Heart Estrogen/progestin Replacement Study (HERS) (39) and the Women's Health Initiative Estrogen + Progesterone (WHI E+P) trial (41), women on HT lost significantly more weight than placebo-treated controls. In two other trials (38, 40) women on HT gained roughly 40% less weight than women on placebo. In the single trial (38) that included an E-only treatment arm, the attenuation of weight gain tended to be more effective with E-only (although not significant) than with E+P therapy, strongly suggesting that the mechanism for the attenuation of weight gain is estrogen mediated. Several smaller physiologic studies also found an attenuation of weight gain by HT, although not all differences were statistically significant (42-47). Importantly, although the large trials did not measure body composition, the smaller studies provide evidence that attenuation of weight gain by HT reflects a significant attenuation of fat gain, primarily in the trunk region (40, 42, 47) since leg fat mass either did not change (45) or increased (47) in response to hormone treatment.

If sufficient circulating estradiol (E_2) attenuates weight gain in postmenopausal women, it would be expected that suppressing circulating E_2 in young women would promote weight gain. In fact, evidence that this occurs comes from studies of premenopausal women on gonadotropin releasing hormone agonist ($GnRH_{AG}$) therapy to suppress sex hormone levels (48-52). Fat mass increases significantly in response to 4 to 6 months of $GnRH_{AG}$ therapy (48, 49, 52) and non-significantly in response to 3 months of therapy (50, 51). Specifically, 4 months of $GnRH_{AG}$ therapy has been shown to significantly increase trunk (7.5% to 10%) but not leg (<2%) fat mass

(49, 52). Collectively this evidence supports the hypothesis that the loss of circulating sex hormones, particularly estrogen, leads to a depot specific (abdominal versus gynoid) change in adiposity supporting the preferential accumulation of abdominal adipose tissue, however the mechanism behind this change remains unknown.

The Lipolytic Cascade

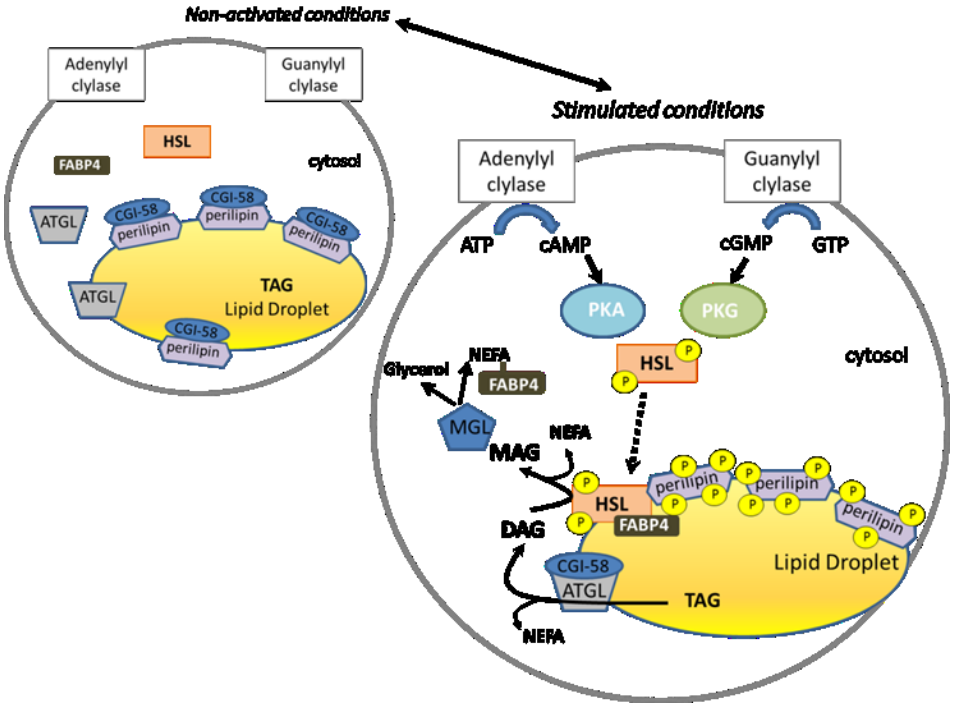

Figure 1.1. The lipolytic cascade. In the non-activated (i.e. un-stimulated) state, HSL is dispersed in the cytoplasm and perilipin coats the lipid droplets while binding CGI-58. ATGL is cytosolic and partly bound to the lipid droplet. Stimulation of lipolysis by catecholamines through adenylyl cyclase, or natriuretic peptides through guanylyl cyclase, increases intracellular cAMP and cGMP levels, in turn activating PKA and PKG, both leading to the phosphorylation of perilipin and HSL. HSL phosphorylation leads to its translocation from the cytosol to the surface of the lipid droplet. CGI-58 disassociates from phosphorylated perilipin into the cytosol where it can then bind to and activate ATGL to hydrolyze TAG leading to DAG production. HSL and MGL contribute to the final hydrolysis of DAG and MAG. Concomitant enhancement of ATGL, HSL and MGL activities is necessary for full hydrolysis of TAG and release of NEFAs and glycerol. Docking of FABP4 to HSL favors the outflow of NEFAs from the cell. Adapted from (53). ATGL, adipose triglyceride lipase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CGI-58, Comparative gene identification-58; FABP4, Fatty acid binding protein 4; HSL, hormone-sensitive lipase; MGL, monoacylglycerol lipase; NEFA, non-esterified fatty acid; PKA, protein kinase A; PKG, protein kinase G; (P), phosphorylation site in phosphorylated perilipin and phosphorylated HSL; TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol.

Adipose tissue is the human body's largest energy storage compartment, with most energy reserves being stored in fat cells as triacylglycerols (TAG). Regulation of storage and mobilization of TAG in adipocytes is important in regional fat accumulation (54), as an imbalance between the hydrolysis and synthesis of TAG can play a substantial role in the development and maintenance of obesity. The capacity of adipose tissue to buffer plasma TAG concentrations by increasing plasma TAG clearance through the activity of lipoprotein lipase (LPL) as well as suppressing the release of non-esterified fatty acids (NEFA) by decreased lipolysis, is of primary importance in maintaining cardiovascular and metabolic health (55). For example, it is typical for individuals with upper body obesity to experience excessive NEFA release along with impairments in NEFA utilization by liver and skeletal muscle, leading to metabolic abnormalities and increased risk of Type 2 diabetes (53).

Lipolysis is the catabolic process by which stored TAGs are broken down into NEFAs and glycerol (Figure 1.1). It has not been fully established what sets the inherent rate of adipose tissue lipolysis in the human body. Catecholamines, insulin, and adenosine are recognized as the major lipolytic regulators in humans, but it is likely that many other factors are also involved. There are three main lipases involved in hydrolysis of intracellular TAGs: 1) adipose triglyceride lipase (ATGL), 2) hormone sensitive lipase (HSL), and 3) monoacylglycerol lipase (MGL). Each TAG is hydrolyzed into three NEFAs and one glycerol. The NEFAs are either re-esterified into new TAG within the adipose tissue or efflux from the adipocyte and are transported in the blood to other organs for metabolism. Glycerol is primarily transported to the liver; the lack of the enzyme glycerol kinase does not allow for the adipocyte to utilize the glycerol produced through lipolysis (53).

The lipolytic cascade is activated or inhibited by regulators of lipolysis through their binding to plasma membrane receptors on the adipocyte. Multiple adipocyte cell surface receptors have a regulatory role on lipolysis (i.e. adrenergic receptors, adenosine receptors, etc.), most of which are coupled to adenylyl cyclase or guanylyl cyclase, and in turn stimulate lipolysis, by the stimulatory G-protein complex (G_s). However, some receptors, such as the alpha-2 adrenergic receptors (α2-AR) are coupled to the lipolytic enzymes by the inhibitory G-protein complex (G_i) and thereby inhibit lipolysis. The stimulation of adenylyl cyclase leads to an increase in 3'-5'-cyclic adenosine monophosphate (cAMP) (or cyclic guanosine monophosphate [cGMP] in the case of guanylyl cyclase) which in turn promotes activation of cAMP dependent protein kinase A (PKA) (or cGMP dependent protein kinase G [PKG]) promoting the progression of the lipolytic cascade. Just the opposite is true when the G_i complex is activated, which results in a decrease in cAMP/cGMP and inactivation of PKA/PKG, turning off progression of the lipolytic cascade (Figure 1.1) (56).

Modulation of Lipolysis

Regulation of lipolysis is a multifaceted process through which a large portion of the maintenance of fuel homeostasis in the human body takes place. Several hormonal and non-hormonal factors participate in the regulation of lipolysis in a complex and balanced system. Throughout a given day this system is under constant demand from the ever changing needs of lipid utilizing tissues, with times of rest, exercise, feeding, and fasting creating unending physiological situations requiring the response of the lipolytic cascade to meet new demands.

Stimulation of Lipolysis

Catecholamines are the primary hormones involved in stimulating lipolysis in humans. Catecholamine exposure to the adipose tissue occurs through general circulation and local blood flow (epinephrine, norepinephrine) (57) or through sympathetic innervation of the adipose tissue (norepinephrine) (58). Catecholamines stimulate lipolysis through beta-adrenergic receptors (β-AR), of which there are three subtypes (β1-AR, β2-AR, and β3-AR) present and active in human adipose tissue (59); however, in healthy subjects catecholamine induced lipolysis in adipose tissue is believed to be predominantly mediated by β2-ARs (60). Visceral adipose tissue expresses β3-ARs and it is in this depot where this β-AR subtype most likely has its greatest effects, playing only a minor role if any in subcutaneous adipose tissue (57).

Other known stimulators of lipolysis are atrial natriuretic peptide (ANP), glucocorticoids, thyroid hormones (61, 62), growth hormone (63, 64), sex steroid hormones (15, 37, 65), and tumor necrosis factor-alpha (TNF-α) (66, 67), but these factors will not be discussed further in this review.

Inhibition of Lipolysis

The balance between expression of pro-lipolytic β-ARs and the anti-lipolytic α-ARs within adipose tissue determines the net effect of catecholamines on lipolysis. Most studies of human adipose tissue conclude that the stimulatory effects of β-ARs predominate over the anti-lipolytic role of α2-ARs in times when fat mobilization is necessary (68). However, *in situ* microdialysis studies have revealed an anti-lipolytic role for α2-ARs during resting conditions, potentially helping to maintain low tonic levels of lipolysis (69).

Adenosine and insulin are the other two predominant regulatory factors demonstrating an anti-lipolytic effect (53, 56, 70-72), but insulin-like growth factor-1(IGF-1) (73), prostaglandins, neuropeptide Y (74-76), and nitric oxide (NO) also most likely have a role in decreasing lipolysis (77).

Pharmacological Modulators of Lipolysis

Investigations of lipolysis are commonly conducted *in vitro* either in primary adipocyte cell culture or isolated adipocytes from human adipose tissue biopsy samples. *In vivo* investigations of lipolysis can be conducted by measurement of systemic lipolytic rates or study of individual tissue depots (78). Local pharmacological or physiologic effects of drugs or hormones can be investigated via the microdialysis technique, through which measured changes in interstitial glycerol concentrations are an indicator of alterations in local lipolytic rate. *In vitro* incubations or microdialysis perfusion with pharmacological agents allows for controlled alterations in the local environment serving to stimulate or antagonize lipolysis. Adrenergic receptor targeted pharmacological agents such as isoproterenol and phentolamine are two of the most commonly used agents to specifically alter lipolytic rate through distinct adrenergic mediated mechanisms. The use of these targeted agents allows for determination of the relative contribution of the specific adrenergic receptor subtypes within the fat cells (69, 79) Isoproterenol is a non-specific β-AR agonist which leads to G_s stimulation of adenylyl cyclase and in turn increases signaling through the lipolytic cascade. On the other hand, phentolamine is a non-specific α-AR antagonist which binds to the α-AR receptors, in turn inhibiting the binding of catecholamines to these receptors and ultimately relieving inhibition of lipolysis that would

take place through activation of the α-AR receptor. Perfusion of each of these pharmacological agents is known to increase lipolysis as measured by microdialysis (69, 80).

Racial Differences in Lipolysis

Racial differences in lipolytic rate are evident but conflicting evidence exists. In a study by Albu et al. systemic lipolysis as measured by whole body glycerol turnover (rate of appearance) was lower in premenopausal obese black women than obese white women both at rest and in response to insulin suppression (pancreatic euglycemic clamp), with VAT accumulation being highly associated with the systemic resistance to the antilipolytic effect of insulin (81). A lower HSL mass and significantly lower basal lipolytic rate in both the SAT and VAT of AA compared with CA women was also reported by Barakat et al. (82), in further support of the belief that AA women have a lower basal lipolytic rate compared to CA women.

Conversely, adipocytes from postmenopausal AA exhibited a higher basal lipolytic rate and decreased insulin responsiveness in *in vitro* experiments conducted by Fried et al. (83). This decreased insulin responsiveness was highly related to the participants hyperinsulinemia. Two additional studies also found higher rates of lipolysis in black compared to white women from South Africa. The first found higher interstitial glycerol via *in situ* microdialysis (abdominal and femoral SAT) in the postabsorptive state and after an oral glucose tolerance test in black women (84) and the second found higher circulating fatty acids and decreased responsiveness to the antilipolytic effect of insulin in adipocytes isolated from the abdominal and femoral depots of the same women (85). Differences between *in vivo* and *in vitro* techniques in measurements of lipolysis as well as variations in participant populations may have led to the conflicting results in regards to black women demonstrating a lower basal lipolytic rate compared to white women.

Increased total numbers of β-ARs have been found in both the SAT and VAT from middle aged obese AA versus CA women signifying a higher potential for lipolysis (86). Barakat et al. found AA women demonstrated an increased lipolytic response to isoproterenol compared to CA women, despite having a lower basal lipolytic rate and HSL mass (82). An increased efficiency of the HSL signaling pathway was hypothesized to be the explanation behind the increased lipolytic response to isoproterenol even with a decreased HSL mass (82). Accordingly, it could be hypothesized that AA women would demonstrate an increased lipolytic response to submaximal exercise stimulation. However, there is a paucity of research investigating racial differences in exercise stimulated lipolysis in women and therefore evidence is lacking to support this hypothesis.

Although a full understanding is lacking, it seems clear that there is a divergence in both basal lipolytic rate and response to lipolytic stimulation in SAT of CA and AA women. This racial difference in lipolysis may play a role in preferential adipose accumulation in SAT compared to VAT in AA compared to CA women.

Regional Differences in Lipolysis

Evidence suggests that gluteal-femoral adipose tissue may actually be a fat sequestering storage depot in females. Femoral adipocytes, compared with abdominal adipocytes, have increased expression of α2-ARs as well as increased insulin sensitivity (87-89), promoting storage of TAG and inhibiting lipolysis in that region. Further, *in vivo* measures of basal free fatty acid release (lipolysis) indicate a lower lipolytic rate in the lower body compared with the upper body adipose tissue (90). However, investigations of regional differences in basal lipolysis (represented by interstitial glycerol) measured by the *in situ* microdialysis technique

have detected greater dialysate glycerol concentrations in the femoral versus abdominal SAT (91, 92). These unexpected findings of higher dialysate glycerol concentration from femoral adipose tissue may be explained by an increased adipocyte cell size in this region (93, 94) as rates of lipolysis are known to be directly related to fat cell size (95-98). It is possible that the lower response to lipolytic stimulation in the gluteal-femoral compared to abdominal SAT, and not basal differences, are primarily responsible for the characteristic fat sequestration of the lower body depots.

Regional differences in lipolytic activity may be a mechanism by which premenopausal women maintain their gynoid fat distribution. It is possible that the preference for adipose storage in the gynoid region in premenopausal women serves a specialized function, being an important source of energy supply during pregnancy and lactation (94, 99, 100).

Adipose Tissue Blood Flow

The importance of the microcirculation of adipose tissue is paramount in the regulation and function of adipose tissue metabolism and the maintenance of human health. The local blood flow is responsible for transporting hormones and other factors from the circulation to the adipose tissue, as well as allowing clearance of metabolites from the adipose tissue into the circulation. Adipose tissue blood flow (ATBF) is regulated by a multifaceted system which is highly responsive to metabolic conditions such as fasting, feeding, and exercise. In the fasted state blood flow through the adipose tissue is greater than that through resting skeletal muscle (101). During prolonged fasting or exercise ATBF is increased in order to supply albumin needed for the transport of released NEFA into circulation, and after feeding ATBF increases to enhance substrate delivery for TG clearance (101). Exercise can result in increased ATBF even

in adipose tissue that is distant from the working skeletal muscle (102). Blood flow is directly affected by local concentrations of substrates and hormones involved in lipid metabolism (103), as well as both β-AR (vasodilation) (104-106) and α2-ARs (vasoconstriction) (105, 107), sympathetic innervation of the vasculature as well as adipocytes (101, 108), endothelial nitric oxide (105), ANP (109, 110), and potentially many other factors.

It is clear that the rate of adipose tissue blood flow is not the same in all depots. Specifically, blood flow in the gluteal-femoral region is much lower than in the abdominal adipose tissue (111). Initially the lower blood flow in this region was attributed to the inert qualities of the lower body SAT; however, there are likely other mechanisms responsible, as this adipose tissue depot is still quite metabolically active and involved in metabolic homeostasis.

Sources of Estrogen in Women

Adipose tissue acts as a metabolic sink with the ability to take up estrogen from the circulation, but the circulating concentration is not the sole determinant of adipose tissue exposure to estrogen. Although the ovaries are the principal source of systemic estrogen in premenopausal women, other sites of estrogen biosynthesis are present throughout the body. These extragonadal sites such as the mesenchymal cells of the adipose tissue and skin, osteoblasts in bone, vascular endothelial and aortic smooth muscle cells, and a number of sites in the brain are sources of estrogen in both men and women (112). In fact, adipose tissue becomes the major source of estrogen production in postmenopausal women (113).

The estrogen synthesized within these extragonadal compartments is primarily active at the local tissue level in a paracrine or intracrine fashion (113), only entering the circulation if it escapes local metabolism (114). Accordingly, the total amount of estrogen synthesized by

extragonadal sites may be small, but the local tissue concentrations achieved are probably high and likely exert significant local biological influence (113). Thus, these sources of estrogen may play an important, but largely unrecognized, physiological and/or pathophysiological role within the adipose tissue (114).

The conversion of inactive precursor C_{19} steroids to produce active androgens and estrogens in human peripheral tissues is a process that depends on the expression of steroidogenic enzymes in the tissue, particularly aromatase (P450arom) (114) and 17β-hydroxysteroid dehydrogenase (17β-HSD) (115). The enzyme aromatase expressed in human adipose tissue is responsible for the conversion of testosterone to E_2 as well as androstenedione to estrone (E_1). Human adipose tissue also possesses the 17β-HSD enzyme which is responsible for the conversion of the weak estrogen E_1 to the more biologically active estrogen E_2 and androstenedione to the potent androgen testosterone (115).

Depot specific steroidogenic enzyme expression and activity allows for local regulation of the amounts of active steroids in the adipose tissue on a cellular basis (116) and may contribute to regulation of adipocyte metabolism at the local level (115). Importantly, aromatase enzyme activity differs between adipose tissue depots, with higher activity in the thigh, buttock and flank versus abdominal fat (113, 117) and higher in subcutaneous versus visceral preadipocytes (118) of premenopausal women. Therefore, fat depot-specific differences in intracrine conversion of testosterone to estradiol (i.e. aromatase activity) may play a role in mechanisms underlying region specific patterns of fat distribution (116, 118).

Estrogen Receptor Expression in Human Adipose Tissue

The estrogen receptor (ER) was first detected in human adipose tissue in 1993 (119). In 1998 multiple ER subtypes (ERα and ERβ) in human adipose tissue were first identified (120). Since then many studies have confirmed that ERα and ERβ mRNA and protein are expressed in human adipose tissue (121-125). Most recently mRNA for the G protein-coupled Estrogen Receptor (GPER) has also been detected in omental and subcutaneous abdominal adipose tissue of humans (126).

Expression of ERα (mRNA and protein) in adipocytes from normal weight men and women does not appear to be different between subcutaneous and visceral depots in either sex (123), but evidence for lower ERβ expression in VAT does exist (121). ERβ mRNA expression appears to be lower compared to ERα mRNA (123) in measurements from mature human adipocytes in both men and women, potentially indicating that ERα is the primary receptor responsible for estrogen action within adipose tissue. However, the importance of ERβ within adipose tissue cannot be dismissed due to lower expression. Support for a role of ERβ within adipose tissue is evident in studies conducted in ER knockout models (127). Potentially, it is the ratio of ERα-to-ERβ expression that may be the determining factor in the specific role of estrogen on adipose tissue metabolism within a given adipose tissue depot.

Interestingly, exposure to E_2 can alter ER expression itself. Anwar et al. demonstrated that chronic E_2 treatment lead to an up-regulation of both ERα and ERβ in stromal cells from SAT and VAT of healthy older women, but decreased ERα expression and increased ERβ in adipocytes from SAT (122). In VAT adipocytes E_2 exposure led to an increase in ERα expression (122). Because adipose tissue is a sink for estrogen storage as well as a producer of estrogen itself, it is possible that local concentrations of estradiol play a role in ER expression,

potentially in a region specific manner. It is not known if there is a relationship between regional adipose tissue estradiol concentration and ER expression in humans *in vivo*.

Regional Differences in ERα and ERβ Expression

Only one study has investigated regional differences in ER expression in peripheral adipose tissue depots, reporting only mRNA expression. Pedersen et al. found no difference in ERα mRNA expression between abdominal and gluteal SAT, but did find ERβ isoforms ERβ-4 and ERβ-5 to be significantly higher in the gluteal versus abdominal SAT in the same women (121). There are a paucity of studies investigating the expression of GPER in abdominal and gluteal SAT, but Hugo et al. did find 1.4 fold higher GPER mRNA expression from abdominal SAT compared to VAT of obese individuals (126).

Obesity and ER Expression

In premenopausal women obesity is associated with lower ERα mRNA levels compared to those of normal weight, and weight loss via caloric restriction results in increased ERα expression (128, 129). These alterations in ERα expression with obesity and weight loss indicate an association between ERα and lipid metabolism. Whether reduced ERα mRNA is a cause or consequence of obesity in women cannot be established from these previous investigations; therefore, more research is needed to understand the role of ER expression in obesity in women.

Further investigation into the role of estrogen receptors in adiposity comes from a study done in ovariectomized (OVX) female mice treated with vehicle, E_2, propylpyrazoletriol (PPT; an ERα agonist), and diarylpropionitrile (DPN; an ERβ agonist). Vehicle or DPN treatment in

OVX mice lead to an increase in body weight, whereas treatment with E_2 or PPT decreased body weight (130), supporting the role for ERα in the maintenance of adipose accumulation.

Evidence from ER Knockout Models for Direct ER Modulation of Adiposity

The development of transgenic mice in which ERα (131) and/or ERβ (132) have been knocked out allow investigation of the specific role of each receptor in various tissues throughout the body. Female ERα knockout (αERKO) mice demonstrate a marked increase in white adipose tissue, insulin resistance, and impaired glucose tolerance compared to wild type (133), implicating the E_2/ERα signaling pathway in regulation of female white adipose tissue deposition. In particular it seems that ERα, at least in the female mouse model, plays a critical inhibitory role in the development and total amount of white adipose (133). However, these changes in αERKO mice might not be a product solely of the lack of ERα, but also due to the resultant shift in ERα/ERβ ratio or other the actions of estrogen receptors such as GPER. αERKO mice also demonstrate increased circulating E_2 levels, potentially exaggerating the signaling and resultant actions of estrogen through other ERs in adipose tissue. Upon ovariectomy, αERKO mice (effectively void of E_2/ERβ signaling) demonstrate a reduction in body weight, fat pad weight and adipocyte size, and a normalization of circulating glucose and insulin, suggesting ERβ is a mediator of these negative adipose and glucoregulatory effects and confirming that both forms of ER have a role in estrogen effects in adipose tissue (127).

Female ERβ knockout (βERKO) mice do not demonstrate the obesity characteristics of ERα knockout models while double ERα/ERβ knockout (DERKO) models express similar increases in body fat content to αERKO (134). After ovariectomy, gonadal fat mass is reduced by estrogen treatment in wild type and βERKO but not αERKO or DERKO, again demonstrating

that ERα is responsible for this regulation of fat mass (133, 135, 136). An increased estrogenic response is also demonstrated in βERKO mice, indicating that ERβ may act as a repressor of the ERα mediated effects (135).

Opposing Effects of ERα and ERβ

ERβ has been reported to inhibit the transcriptional activity of ERα possibly through ERα/ERβ heterodimer formation (137), indicating the importance of the ratio of ERα-to-ERβ in the biological response to estrogens (123). Furthermore, differing tissue specific expression as well as varying phenotypes between ERα and ERβ knockout models confirm that each ERα and ERβ are responsible for different biological functions (133, 136). The relative expression of ERα and ERβ determine cellular sensitivity to estrogens (137) and although ERα is thought to be the more dominant of the two receptors, co-expression of ERβ results in decrements in estrogen stimulated responses (137). This evidence emphasizes the importance of determining the co-localization of these two receptors in defining the actions of estrogens in specific target tissues (137).

G protein-coupled estrogen receptor (GPER)

Very little is known about GPER, particularly within adipose tissue. GPER was first identified in the late 1990s by multiple groups (138, 139) and GPER deficient models have confirmed its role as an ER *in vivo* (140, 141). GPER mRNA has been detected in human abdominal SAT, VAT, and breast tissue from obese women (126), but to our knowledge the former is the only study to date to investigate GPER expression in human adipose tissue. Although controversial (140), GEPR deficiency has been associated with abdominal obesity in

both male and female animals (141). Together, GPER expression in human adipose tissue and its association with an obese phenotype in GPER deficient mice makes it a promising candidate for mediating at least some of the direct effects of estrogen within human adipose tissue.

Mechanisms of Estrogen Action in Adipose Tissue

The exact mechanism for sex steroid action within adipose tissue and subsequent regulation of regional adiposity is unknown. As discussed previously, estrogen can be taken up from the circulation into adipose tissue or produced within adipose tissue. Regardless of the origin of the local estrogen, there are two main regulatory actions it can have within the adipose tissue: 1) regulation of key proteins at the genomic level by transcriptional means and 2) acting on secondary messengers at the cell membrane by non-genomic effects. The presence of sex steroid receptors in the nucleus and cytosol of the adipocyte as well as on the plasma membrane prove the cellular machinery for both genomic and non-genomic mechanisms of action are present within adipose tissue. It is most likely a combination of these two pathways ultimately resulting in the many regulatory actions of sex steroids within adipose tissue.

The classical mechanism of action of sex steroid hormones involves the following cascade of events: 1) the steroid enters the target cell and 2) binds with high affinity to a specific receptor, 3) the steroid-receptor complex undergoes a conformation change and 4) is then able to bind to a specific DNA response element, 5) resulting in up- or down-regulation of the transcription of a given gene (37).

Although transcriptional regulation by sex steroid hormones has been the most highly studied mechanism of action, non-genomic mechanisms of sex steroid action within target tissues are also evident (142). Non-genomic actions are typically rapid, occurring in minutes,

whereas transcriptional regulation takes place on a slower timeline; over hours to days (143, 144). The mechanism of action in the non-genomic sequence of events involves a steroid receptor on the plasma membrane with a second messenger inside the cell to carry out the action of the steroid. Anwar et al. identified both ERα and ERβ in cellular membrane of abdominal SAT as well as omental human adipose tissue (122). Although the membrane associated ERα and ERβ are reportedly associated with caveolar endocytic vesicles, much remains to be determined about the exact mechanism of action of steroid hormones by membrane receptors (145-148). It is likely that many second messenger systems such as the cAMP cascade and the phosphoionositide cascade, play a role in actions of membrane associated ERs (149-151). It is important to remember that there is overlap between genomic and rapid signaling events in hormone mediated actions. Therefore estrogen receptor expression and cellular localization are of primary importance in determining the specific effects of estrogen in any given cell (152).

Effects of Estrogen on the Lipolytic Cascade

To date, most studies investigating estradiol's effects on lipolysis have been conducted in animal models, primarily in rats (153, 154). These studies have generated the overlying hypothesis that estradiol serves to stimulate lipolysis. Ovariectomy in rats leads to weight gain; E_2 replacement reverses that trend as well as increases adenylyl cyclase activity (155). In isolated rat adipocytes, E_2 treatment increases cAMP and lipolysis, as measured by glycerol release, within 5 minutes (156). In support of an alternative role of estrogen on lipolysis in animals, Pecquery et al. conducted a study in which estradiol decreased lipolysis in adipocytes from hamsters through a reduction in adenylyl cyclase activity (157).

Few *in vivo* human studies have been conducted that modulate local estrogen status while measuring real time changes in lipolytic rate. We know of only one study acutely modulating hormonal status *in vivo* to investigate changes in whole body and regional lipolysis. Van Pelt et al. found that acutely increasing circulating E_2 concentrations with an intravenous bolus of exogenous estrogen in postmenopausal women decreased basal lipolysis in femoral SAT, and to a lesser extent in abdominal SAT, using the microdialysis technique. These findings support estrogens in regional adipose metabolism and confirm an acute non-genomic effect of estrogen within the adipose tissue; at least on basal lipolysis (91).

A few notable studies have been conducted in humans or using isolated human adipocytes to investigate the chronic or genomic effects of estradiol on lipolysis, with most finding estrogen associated with lower stimulated lipolysis. Lindberg et al. isolated subcutaneous abdominal adipocytes from women before and after three weeks of oral ethinyl estradiol treatment, after which the adipocytes had decreased *in vitro* response to noradrenaline stimulated lipolysis in comparison to pretreatment (158). More recently, when adipocytes isolated from subcutaneous adipose tissue fragments of healthy premenopausal women were incubated in E_2 *in vitro* for 24 hours, glycerol release in response to epinephrine exposure was lower versus control conditions (159). The blunted response to stimulated lipolysis after E_2 treatment is attributed to an increase in anti-lipolytic α2-AR expression. Increased α2-AR expression is evident in subcutaneous adipocytes treated with E_2 for 24 hours, as well as in postmenopausal women treated with estrogen replacement for 3 years (159). On the other hand, estrogen treatment did not appear to have any effect on β-AR expression in SAT or α2-AR expression in VAT (159). The differential response of α2-AR up-regulation between SAT and VAT served as some of the earliest evidence to potentially explain how E_2 may have a

modulatory role in the maintenance the subcutaneous/gynoid fat patterning in women. Furthermore, α2-AR up-regulation with E_2 treatment in human SAT could help explain the divergent results between rat and human studies, as rats do not express α2-ARs in adipose tissue.

The primary adrenergic independent regulators of lipolysis in humans are insulin, adenosine, and ANP, but very few studies have investigated the potential role of estrogen in modulating these regulators of lipolysis. The one previous study which has investigated the role of estrogens in insulin mediated suppression of lipolysis in postmenopausal women did not see an alteration in whole body, abdominal or femoral SAT insulin mediated suppression of basal lipolysis after an acute intravenous bolus of estrogen (91). Both animal (160, 161) and human studies (162-164) have established an association between declines in circulating estrogen status and decreased circulating ANP levels as well as changes ANP-A receptor expression (165), but no mechanistic or depot specific effects of estrogen on ANP have been determined.

In SKBR3 cells (an ERα/ERβ negative but estrogen sensitive breast cancer cell line) *in vitro* GPER is recognized in mediating E_2-stimulated increases in cAMP and intracellular calcium, as well as E_2-promoted proliferative signaling (166-168), but to our knowledge, its involvement in modulation of the lipolytic cascade is not known.

Exercise-Stimulated Lipolysis

Fat serves a main energy source during prolonged physical activity (169). Low-to-moderate intensity exercise (up to about 60% maximal oxygen uptake [VO_2max]) increases fat oxidation on the whole body level, as well as increases mobilization of non-esterified fatty acid (NEFA) from the adipose tissue dramatically from what is seen at rest (170, 171). It is believed that SAT (170) along with intramuscular triglycerides (172, 173) are the primary sources of fatty

acids mobilized by the adipose tissue during moderate intensity exercise for oxidation, with a lesser contribution from the visceral depot (170). Importantly, only a very low intensity of exercise is required for significant increases in NEFA mobilization from abdominal SAT with further increases in exercise intensity leading to only small additional increases in NEFA mobilization (174, 175), if any at all (176).

The exercise induced release of NEFAs from adipose tissue is potentially influenced via multiple pathways such as adipose tissue lipolysis, fatty acid re-esterification, and adipose tissue blood flow (102). Decreased rates of fatty acid re-esterification play at least a small role in increased NEFA mobilization (172, 177). Lipolysis is the major pathway through which increased NEFA mobilization is modified during exercise. The traditional belief is that elevated catecholamine concentrations, as well as a small decrease in insulin concentration, are responsible for the exercise associated increase in lipolysis (69, 178). Even low intensity exercise increases circulating epinephrine levels enough to stimulate SAT lipolysis (179). Although some research has indicated that circulating mediators are the most important activators of lipolysis (180), others have found that sympathetic innervation of the adipose tissue and neural activation also play important roles in activation of lipolysis (181). The importance of catecholamine action on activation of β-AR receptors in SAT is demonstrated in the dramatic decrease (but not abolition) in exercise-induced lipolysis in young lean men and women undergoing selective β-AR blockade (69). Furthermore, some level of lipid mobilization is maintained during exercise even when epinephrine action is blocked (182), further supporting the role for AR-activation to play a large, but not the only, role in exercise mediated increases in fat mobilization.

One possible alternative modulator in exercise induced lipolysis is ANP, which is secreted primarily from the atrial cardiomyocytes in response to mechanical stretch and during exercise is secreted in an intensity-dependent manner (174, 183, 184). It is possible that the influence of ANP on exercise stimulated lipolysis is somehow related to regulation of adiposity (184). Furthermore, as the duration of low-to-moderate intensity exercise increases the actions of hormones such as cortisol and growth hormone (185-187) may also be involved in the increase in whole body lipolytic rate (170) and regional specific adipose tissue lipolysis (180, 188, 189) but more research is needed to determine the significance of these factors.

Estrogen Regulation of Exercise-Stimulated Lipolysis

Women utilize proportionally less carbohydrate and more lipid compared to men during exercise of mild to moderately high intensity (40-70% VO_2max) (190). Whole-body lipolysis appears to be higher, as measured by glycerol rate of appearance (R_a), for women compared with men during endurance exercise (191), and arterial glycerol levels in women are higher than men during the first 30 minutes of exercise (192), still, some studies have demonstrated no sex differences in circulating glycerol during exercise (190). Interestingly, during exercise levels of epinephrine and norepinephrine do not rise as much in women as they do in men (190). To explain this paradoxical relationship it is hypothesized that in women there is a greater sensitivity to the lipolytic action to one or both of the catecholamines leading to the equal or increased glycerol measured during exercise in women compared to men (190). Although these sex differences in whole-body lipolysis are primarily attributed to differences in circulating sex hormones, the direct mechanism of action for estrogen on these differences has not been full elucidated.

Regional Differences in Exercise-Stimulated Lipolysis

Given what is known about regional differences in basal lipolysis, adrenergic receptor density/sensitivity and adipose tissue blood flow, it is not surprising that there is a clear regional difference in exercise stimulation of lipolysis. Microdialysis studies have revealed exercise induced lipolysis is greater in abdominal versus lower body (gluteal or femoral) SAT regardless of the mode of exercise (69, 180, 193), a depot dependent effect that is much more apparent in women than in men (69). Interestingly, this sex divergence is manifested in differences in abdominal lipolysis, demonstrated by a larger increase in dialysate glycerol in the abdominal region in women as compared to men in response to 30 minutes of submaximal exercise, while in the gluteal region the increase in dialysate glycerol induced by exercise was almost identical between women and men (69). Variation in catecholamine sensitivity between adipose tissue depots are believed to be the primary modulators of this regional difference (89, 194, 195), but other mechanisms such as regional sympathetic outflow (196), local blood flow, or other unknown factors cannot be dismissed from having a potential role.

Conclusion of Literature Review

Premenopausal women have a distinctive gynoid body fat distribution which is associated with lower disease risk than accumulation of central adiposity (6, 197, 198). Circulating estrogen is recognized as playing a key role in determining regional adiposity, and the presence of estrogen producing enzymes (114) as well as estrogen receptors (119, 120) in adipose tissue supports a role for direct estrogen action within this tissue. Importantly, there is strong evidence in animal models that estrogen may influence lipolytic rate (153, 199), but the *in vivo* evidence

from human trials is limited and much of it conflicts with previous results from animal studies (91, 158, 159, 200).

African American premenopausal women accumulate less VAT and more SAT compared to Caucasian women of the same age and waist-to-hip ratio (22, 24). Paradoxically, AA women also have more CVD risk factors and an increased incidence of diabetes and hypertension than CA women despite displaying what would be considered a more metabolically favorable body fat distribution (27, 28). Racial differences in lipolysis, estrogen action within adipose tissue, or adipocyte size could be mechanisms involved in the divergent body composition and associated disease risk between CA and AA.

Central Hypotheses

The **global aim** of this project is to determine if local estrogens influence regional lipolysis in subcutaneous adipose tissue of premenopausal women. This will be the first step in developing a more complete understanding of the mechanisms behind the influence of sex hormones on regional adiposity. It is the **main hypothesis** of this project that exposure to high concentrations of estradiol will reduce lipolytic rate in SAT of premenopausal women, particularly in the gluteal region. This would support a role for estradiol in mediating the preferential subcutaneous, and chiefly gynoid, body fat distribution of premenopausal women. Determining characteristics of the adipose tissue such as estrogen receptor expression and adipocyte size will also help in understanding differences in regional lipid metabolism. Racial dissimilarities in regional adipose tissue accumulation and lipid metabolism lead to a complementary **secondary aim** for this project which is to conduct preliminary investigations into racial differences in estrogen action and adipose tissue characteristics such as estrogen

receptor content and adipocyte size between well-matched Caucasian and African American women.

Specific Aim 1

To determine if increasing the local subcutaneous adipose tissue E_2 concentration will reduce lipolytic rate in abdominal and gluteal SAT of overweight-to-obese premenopausal women. A secondary aim is to investigate if this effect of E_2 is different between Caucasian and African American women.

Lipolytic responsiveness in the gluteal-femoral region is typically lower than that of the abdominal region (201), but a full understanding of the mechanisms responsible for this regional difference remain unknown. Discovery of estrogen receptors within the adipose tissue confirms that estrogen may directly modulate fat metabolism (119, 123-125). Presently, measurement of SAT estradiol concentrations in humans is uncommon and the influence that this interstitial estrogen has locally on lipolytic rate *in vivo* is unknown. Completion of aim 1 will result in a more clear understanding of the largely unknown and unrecognized physiological (or pathophysiological) role of estrogen within the adipose tissue *in vivo* in premenopausal women, and if this role differs between CA and AA premenopausal women. Our hypothesis is that estradiol perfusion will decrease lipolysis, particularly in the gluteal region.

Specific Aim 2

To investigate regional differences in protein content of the estrogen receptors ERα, ERβ, and GPER within abdominal and gluteal subcutaneous adipose tissue of overweight-to-obese premenopausal women. A secondary aim was to determine if protein content of

these three estrogen receptors differs in Caucasian and African American premenopausal women.

Confirmation of ERα, ERβ and GPER expression in adipose has provided evidence that estrogen has direct actions within this tissue (119-121, 124, 126). This is the first study to report on protein content of all three major estrogen receptors from abdominal and gluteal subcutaneous adipose tissue of premenopausal women, creating a more complete picture of the potential pathways of estrogen action in adipose tissue. Racial differences in ER content may give insight into mechanisms behind differences in regional adiposity between CA and AA women. We hypothesize that ER content will be different between the abdominal and gluteal SAT, specifically that ERα content will be greater in the abdominal compared to gluteal region.

Specific Aim 3

To characterize mean adipocyte size and adipocyte diameter distribution in adipose samples from abdominal and gluteal SAT of overweight-to-obese premenopausal women. A secondary aim was to investigate if there are racial differences in adipocyte size and diameter distribution between Caucasian and African American women.

Recent advances in adipocyte sizing techniques have revealed the importance of determining not only the 'mean' adipocyte size for a given depot, but also the relative frequency distribution of adipocytes from the sample (202, 203). The size of an adipocyte is associated with lipolytic rate (204) and insulin sensitivity (205, 206) among other characteristics. Adipocyte diameter distribution comparisons are lacking between adipose tissue samples from the abdominal and gluteal SAT of overweight-to-obese premenopausal women, although an increased mean adipocyte size in the gluteal region is commonly reported in lower body obese

premenopausal women (93, 94). Differences in adipocyte size distribution between upper and lower body adipose depots and Caucasian and African American women will allow for a more complete understanding of regional and racial differences in lipolysis and adipose metabolism. We hypothesize that adipocytes from the gluteal region will be larger than those from the abdominal region.

Significance

The maintenance of normal adipose tissue metabolism is vital to human health. Understanding the mechanisms responsible for regional adiposity can help in development of strategies to prevent increases in central adiposity in aging women. The majority of research conducted on the association between obesity and disease risk is conducted in Caucasian women, but the elevated cardiometabolic disease risk and prevalence of diabetes in minority populations should not be ignored. Uncovering the direct role estrogen holds within adipose tissue and in maintenance of the premenopausal gynoid body fat distribution may eventually contribute to development of techniques in prevention of the menopause associated increase in central adiposity and thereby also the increase in disease risk associated with that shift.

CHAPTER 2: Estradiol Effect on Subcutaneous Adipose Tissue Lipolysis is Adipose Tissue Depot Specific and Treatment Dependent

Abstract

Regional differences in lipolytic rate have been implicated in preferential gynoid body fat distribution of premenopausal women. Estrogen has direct effects within adipose tissue and has been implicated as a modulator of regional adiposity; however its influence on *in vivo* lipolytic rate in premenopausal women is unclear. Therefore the purpose of this study was to investigate the effect of locally infused estradiol (E_2) on subcutaneous adipose tissue (SAT) lipolytic rate in premenopausal women. Our secondary aim was to investigate if the influence of E_2 on lipolysis was different between well matched subgroups of Caucasian (CA) and African American (AA) women. We measured *in vivo* lipolysis (indicated by dialysate glycerol) via microdialysis of subcutaneous AB and GL adipose tissue in 17 overweight/obese women (age: 27.4±2.0 yrs, BMI: 29.7±0.5 kg/m^2) at basal and during perfusion of the β-adrenergic (AR) agonist isoproterenol (ISO; lipolytic stimulator through β-AR activation), co-perfusion of ISO with the α-AR antagonist phentolamine (PHEN; relieves inhibition of lipolysis by α-AR), and physiological stimulation by submaximal aerobic exercise. Sixty-minutes of ISO perfusion resulted in a significant increase in dialysate glycerol from basal in all probes, with a significantly lower response in the GL region. Co-perfusion of E_2 with ISO blunted the stimulatory response to ISO in the AB region (195.9±30.6% versus 257.7±26.0% p=0.003), but did not change the response in the GL region (112.7±13.9% vs 110.6±12.4%, p=0.43). Thirty-minutes of submaximal exercise during ISO + PHEN perfusion significantly increased dialysate

glycerol from ISO + PHEN at rest in all probes. E_2 perfusion enhanced the percent increase in dialysate glycerol in response to ISO + PHEN + exercise in the AB region (89.7±9.3% vs 55.5±9.3%, p=0.007), but blunted the same response in the GL region (35.1±6.8% vs 62.0±12.0%, p=0.05). The effect of E_2 perfusion was similar in CA and AA women. These results indicate that E_2 effects on lipolysis are region-specific and may work through both β-adrenergic and potentially adrenergic-independent mechanisms to potentiate and/or blunt subcutaneous adipose tissue lipolysis in premenopausal women.

Introduction

Women have the propensity to accumulate more adipose tissue than men, particularly in the subcutaneous depot and gynoid region (34). With age an increase in abdominal obesity is commonly demonstrated in women, diminishing the sex divergence in body fat distribution common in earlier years of life (207). Reduced cardiometabolic disease risk in premenopausal women has been attributed to this differential body fat distribution (208) and the benign (8) or even protective effect of lower body adiposity (14, 209-211). Alterations in the circulating hormonal milieu with menopause, such as the decline in circulating estrogens, have been implicated in age related shifts towards a central body fat distribution and cardiometabolic risk (44, 47).

Although associative data supporting the role of sufficient circulating estrogen in maintaining gynoid body fat distribution is available, mechanisms of sex steroid action within adipose tissue and how this may impact body fat distribution and lipid metabolism remain unclear. Adipose tissue serves not only as a sink for hormones from the circulation, but also as an endocrine organ producing sex hormones which act in paracrine and autocrine fashion; more

recently termed intracrinology (116, 212). The ability of adipose tissue to regulate concentrations of sex steroids locally reveals what may be an important evolutionary mechanism to help determine or regulate regional adiposity. Of particular interest is the capacity of adipose tissue to produce estradiol (E_2) through two routes: 1) aromatization of testosterone or 2) reduction of estrone by 17β-hydroxysteroid dehydrogenase (17β-HSD). The discovery of estrogen receptor-α and -β (ERα and ERβ) (121, 123, 124), as well as the more recent detection of the G protein-coupled estrogen receptor (GPER) within human adipose tissue (126) further support the role of direct genomic and non-genomic actions of estrogens in maintaining homeostasis within adipose depots. Furthermore, increased adiposity in estrogen receptor alpha and aromatase knockout models (αERKO and ARKO) (133, 213) confirm the significance of E_2 in maintenance of lipid homeostasis.

The balance or imbalance of triacylglycerol accumulation and free fatty acid release (FFA) within a specific adipose depot is responsible for changes in local adiposity. Decreased lipolytic rate in the gluteal depot of premenopausal women has been implicated in the increased adiposity demonstrated in this region (90, 214). Moreover, acute intravenous administration of conjugated estrogens has been shown to decrease basal lipolysis in the abdominal and femoral subcutaneous adipose tissue of estrogen deficient postmenopausal women as measured by the *in situ* technique of microdialysis (91). However, similar studies in premenopausal women examining acute modulation of lipolysis by 17β-estradiol (E_2) are lacking. Therefore, the purpose of this study was to investigate the effect of locally infused E_2 on subcutaneous adipose tissue lipolytic rate in the abdominal and gluteal regions of overweight-to-obese premenopausal women. A secondary aim of the study was to determine if the effect of estradiol was race-dependent.

Methods and Procedures

Participants

Seventeen overweight/obese premenopausal women, (9 Caucasian/8 African American 27.4±2.0 years, 81.7±2.3 kg, BMI 29.7±0.5 kg/m^2) between 18 and 44 years old were studied. Participants enrolled in this study were eumenorrheic (average cycle length 30±1 days), not taking hormonal contraceptives (no use of hormonal contraceptives for at least 6 months at study entry), weight stable (< 3 kg weight change in the last 6 months) and sedentary, in that they did not engage in purposeful exercise training more than 30 minutes per day more than two days per week. Women were excluded from the study if they were trying to get pregnant, were pregnant or lactating, a smoker, had history of diagnosis of any metabolic or cardiovascular disease, or were taking any medications known to alter lipid metabolism or blood flow. Qualification for the study was determined by a pre-participation health history questionnaire completed by the participant and reviewed for accuracy with study personnel. All participants were informed both verbally and in writing of the purpose, risks, and benefits of the research and provided informed consent prior to enrollment in the study. This study was approved by the Medical Center Institutional Review Board at East Carolina University. Participant characteristics are presented in Table 2.1.

Study Design

Initial visit. Participants reported to the Fitness, Instruction, Testing, and Training (FITT) Facility of the Human Performance Lab at East Carolina University for their first study visit to obtain written informed consent and collect baseline information such as medical history, height,

weight, body composition measurements, waist and hip circumferences, and complete a maximal oxygen uptake (VO_2peak) test of aerobic fitness. Reported dates of menses for the previous 6-12 months were recorded by study personnel to confirm eumenorrhea. The microdialysis visit was subsequently scheduled according to the predicted start date of the next menstrual cycle; scheduling alterations were made if necessary to coincide with the early follicular phase of the menstrual cycle. If previous records of menstrual cycles were not available, participants tracked the succeeding 2-4 menstrual cycles, reporting back to the study coordinator on the first day of menses.

Body composition. Participants were weighed on an electronic scale with weight recorded to the nearest 0.1 kg and height was measured with a standard stadiometer to the nearest centimeter (cm). Minimal waist and hip circumferences were measured according to previously published guidelines and waist-to-hip ratio (WHR) was calculated (215). Fat-free mass (lean mass + bone mineral content), whole body and regional fat mass (android, gynoid), and whole body percent body fat were determined using dual-energy x-ray absorptiometry (DXA; GE Lunar Prodigy Advance, Madison, WI). The android region was bound on the lower border by the top of the iliac crest and on the upper border at a distance 20% of the distance between the top of the iliac crest and immediately below the chin; lateral borders were the arm lines. The gynoid region was bound on the upper border at a distance 1.5 times the height of the android region and on the lower border at a distance 2 times the height of the android region; lateral boundaries were the outer leg lines.

Maximal oxygen uptake exercise test (VO_2peak). The maximal exercise test was conducted on a Corival LODE cycle ergometer using a ParvoMedics TrueOne 2400 metabolic cart (Sandy, UT) for indirect calorimetry measurements. Expiratory gasses were monitored

throughout the test and heart rate (polar hear rate monitor, Polar Electro Inc, Lake Success, NY) and blood pressure (manual measurements) were monitored during exercise and recovery. Resistance started at 25 watts and increased 15-25 watts every 2 minutes until volitional fatigue or the participant asked to stop for another reason. The test was deemed successful by following the guidelines set forth by the American College of Sports Medicine (216). One test did not meet the criteria for a maximal test and was repeated at a later date.

Microdialysis visit. Participants reported to the East Carolina Diabetes and Obesity Institute (ECDOI) at the East Carolina Heart Institute at 8am after an overnight (≥10 hour) fast during the follicular phase of the menstrual cycle (day 2-8 after day 1 of menses, one participant was completed on day 9). On average, the microdialysis visit took place on day 5±1 of the menstrual cycle and cycle phase was confirmed by a blood draw the morning of the visit (Table 2.2). Food records were completed by all participants for the three days immediately preceding the microdialysis visit. All participants rested in a semi-recumbent position for the duration of the study with the exception of the 30 minute stationary cycling session at the end of the visit and specific timepoints assigned for restroom breaks. An indwelling polyethylene catheter (IV) was inserted into the antecubital vein for blood sampling.

Blood was collected in lithium heparin or EDTA (Ethylenediaminetetraacetic acid) tubes for plasma or in untreated vacutainers for serum. Plasma and serum were obtained by centrifugation, and aliquots were immediately stored at -80°C until later batch analysis to limit day to day assay variability. Dialysate samples were collected every 15-30 minutes and blood samples were collected at baseline and immediately before and during/after the exercise bout. The first blood draw of the day (baseline) was used for determination of all baseline participant characteristics and confirmation of menstrual cycle phase. Resting energy expenditure (REE)

measurements were made via indirect calorimetry (ParvoMedics TrueOne 2400 metabolic cart) for 25 minutes during the microdialysis visit while the participant was lying quietly undisturbed. The participants had been resting quietly for ~120 minutes after probe insertion before the REE measurement was made. Data points from the first five minutes of the REE measurement were excluded from analysis to allow for subject equilibration; the remaining 20 minutes of data points were averaged for final REE values.

The skin in a small area over the subcutaneous adipose tissue (SAT) region of interest was desensitized to pain using ethyl chloride spray and four microdialysis probes (CMA 20 MD Elite Probe 10 mm, 20 kDa cutoff, CMA Microdialysis/Harvard Apparatus, Holliston, MA) were first inserted unilaterally into the upper gluteal (GL) SAT ~3-10 cm right or left of the medial line of the buttock followed by insertion of four additional probes unilaterally into the abdominal (AB) SAT ~3-6 cm lateral to the umbilicus. Probes in both regions were placed at least 2 cm from one another to avoid interference from adjacent probes and at a depth of approximately 1cm into the SAT. No sample was collected during the first hour post-probe insertion to allow for equilibration of the microdialysis system (217, 218).

In vivo glycerol release (an indicator of lipolysis) was determined under basal conditions by continuously perfusing the microdialysis probes using CMA 107 microinfusion pumps (M Dialysis, N. Chelmsford, MA) at 2.0 μl/min with a 0.9% saline solution containing 10 mmol/l ethanol (for determination of local blood flow) (218, 219) which served as the base control solution for all stages of the visit. After exchange with the SAT the pumped perfusate was collected at the exit end of the probe (dialysate) and stored at 4°C for analysis of ethanol (index of local blood flow) within 48 hours and subsequently stored at -20°C for later batch analysis of dialysate glycerol (index of lipolysis) and dialysate E_2. The experimental timeline for the

microdialysis visit is shown in Figure 2.1, but involved perfusion of isoproterenol (ISO, β-adrenergic agonist; 1.0 μmol/l), phentolamine (PHEN, α-adrenergic antagonist; 0.1 mmol/l), and/or estradiol (E_2; 500 nmol/l) (all from Sigma Aldrich, St. Louis, MO). Previous microdialysis studies have demonstrated perfusion of these concentrations of ISO and PHEN induce maximal alterations in lipolysis (69, 80). Furthermore, use of these pharmacological agents allow for specific adrenergic receptor targeted perturbations of lipolysis to gain a more in depth understanding through which adrenergic pathway estradiol may act to influence lipolysis. A 15 minute equilibration period was observed between stages to allow for full infiltration of the probe and surrounding SAT with the new perfusate solution.

In the final portion of the visit (stage 5) participants were asked to cycle on a Monark Ergomedic 828E cycle ergometer for 30 minutes at 60% of their own previously determined VO_2peak at a constant speed of 65 RPMs (revolutions per minute). Submaximal exercise produces what is believed to be the one of, if not, the largest physiologic stimulus to increase adipose tissue lipolysis in humans and therefore was used within the current study to understand estradiol's influence on physiological stimulation of lipolysis. Heart rate was monitored throughout the exercise bout (polar hear rate monitor) and indirect calorimetry measurements (ParvoMedics TrueOne 2400 metabolic cart) were taken from the start of exercise until minute seven and repeated from minute 20 to minute 25 to confirm proper exercise intensity and steady state. Exercise at 60% VO_2max has previously been reported to elicit maximal lipolytic stimulation (220). Two 15 minute dialysate samples were collected during the exercise bout. After completion of exercise all eight microdialysis probes were removed.

Sample Analysis

Microdialysis samples and associated calculations. Dialysate glycerol ($Glyc_{dialysate}$) (index of *in vivo* lipolysis) concentration was analyzed using a CMA/600 automated microdialysis analyzer (M Dialysis). A concentration of 10 mmol/l ethanol has been shown to effectively detect blood flow changes around the microdialysis probe in SAT (221). The ethanol outflow-to-inflow ratio (O:I) is inversely related to blood flow and was calculated:

$$\text{Ethanol Outflow:Inflow} = [Ethanol_{dialysate}] / [Ethanol_{perfusate}]$$

Dialysate and perfusate ethanol (indicator of local blood flow) was measured in our laboratory using a previously described enzymatic, fluorometric assay (218).

In vitro studies previously conducted by our lab estimate the relative *in vitro* glycerol and ethanol recoveries over the microdialysis membrane to be 59.5±2.9% and 89.4±3.0% respectively at the flow rate of 2 µl/min through the CMA/Elite 10mm membrane probes used in both the *in vivo* and *in vitro* studies. The following formula was used for the calculation of interstitial glycerol ($Glyc_{IS}$):

$$Glyc_{IS} = [Glyc_{dialysate}] / ((1 - \textit{in vivo}\ \text{O:I})/(\textit{in vitro}\ \text{ethanol}_{\text{relative recovery}} / \textit{in vitro}\ \text{glycerol}_{\text{relative recovery}})).$$

Effects of E_2 perfusion on stimulated/disinhibited lipolysis are presented as percent change from the $Glyc_{dialysate}$ at the end of the previous stage to the $Glyc_{dialysate}$ measured at the end of the stage of interest (i.e. % change in $Glyc_{dialysate}$ = (($Glyc_{dialysate}$ 150min – $Glyc_{dialysate}$ 90min)/$Glyc_{dialysate}$ 90min)*100). Percent change in ethanol O:I between stages was calculated in the same manner.

Dialysate estradiol was measured using a Salivary Estradiol ELISA (SLV-4188, DRG Instruments GmbH, Marburg, Germany). The intra-assay coefficient of variation (CV) was 2.2%. Interstitial estradiol (E_{2IS}) was calculated identically to $Glyc_{IS}$, with *in vitro* recovery of E_2 over the microdialysis membrane estimated by previous *in vitro* experiments in our laboratory to be

21.5±4.3%. The *in vitro* relative recovery rate of E_2 and ethanol and *in vivo* relative recovery of ethanol were used to estimate the concentration of perfused E_2 passing from the perfusate over the microdialysis membrane into the adipose tissue *in vivo*. Our calculations determined that most likely 5-20% of the perfused E_2 passed over the microdialysis membrane. With the starting perfusate concentration at 500 nmol/l E_2 we estimate the concentration of E_2 passing into the local SAT surrounding the microdialysis probe to be ~25-100 nmol/l. A volume of 2 μl/min of perfusate passed into the adipose tissue dispersing around a volume of interstitial fluid surrounding the 10mm x 0.5mm (length x diameter) probe. Actual exchange of E_2 over the membrane and local concentration is also dependent on local blood flow immediately surrounding the microdialysis probe.

Blood samples. Blood was drawn at three timepoints throughout the microdialysis visit: baseline, pre-exercise (~3.5 hours after baseline) and exercise (~1 hour after pre-exercise). All blood measurements are listed in Tables 2.1, 2.2 and 2.7. Missed blood draws or IV failures for some participants resulted in decreased sample size for repeated measures analysis of humoral factors. Resting epinephrine was below the detection limit of the assay at baseline, but was measureable pre-exercise for most participants, consequently pre-exercise was used as the only resting epinephrine measurement. Exercise blood sampling was completed during the exercise bout, within 5 minutes of termination for seven participants; however, due to difficulty with the blood collection during the cycling bout, samples for five participants were drawn immediately post exercise. Unfortunately neither pre- nor post-exercise blood samples were obtained from five participants.

Fasting serum samples of glucose, total cholesterol (TC), triglycerides (TG), and high-density-lipoprotein (HDL) cholesterol were determined using enzymatic/colorimetric methods,

and low-density-lipoprotein (LDL) cholesterol was calculated using the Friedewald equation (222). Fasting serum E_2, follicle stimulating hormone (FSH), luteinizing hormone (LH), progesterone, testosterone, cortisol, and insulin were determined by Electrochemiluminescence Immunoassay (UniCel® DxC 600i Synchron® Access® Clinical System, Beckman Coulter, Inc., Brea, CA). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following equation [fasting glucose (mmol/l) x fasting insulin (μU/ml)]/22.5 (223).

Plasma glycerol (collected in lithium heparin) was determined in our laboratory using the Sigma quantitative enzymatic free glycerol determination kit (F6428 – Sigma-Aldrich Corp, St. Louis, MO). Serum free fatty acids (FFA), estrone, and plasma catecholamine measurements were performed by the University of Colorado Denver Clinical and Translational Research Center (CTRC) Core laboratory. FFA were determined by enzymatic colorimetric methods (Wako Diagnostics USA, Richmond, VA; Intra-assay CV 1.1%, Inter-assay CV 5.6%), estrone was measured by conventional radioimmunoassay (Diagnostic Systems Laboratories, Webster, TX; Intra-assay CV 8.7%, Inter-assay CV 11.7%) and epinephrine (Epi) and norepinephrine (NE) (collected in EDTA) were determined by high-performance liquid chromatography (HPLC) (BioRad, Hercules, CA; Intra-assay CV: Epi 5.2%, NE 4.1%; Inter-assay CV: Epi 5.4%, NE 5.4%).

Statistics

The skewed distribution of TG, circulating estradiol, estrone, and progesterone necessitated logarithmic transformation of these data; the re-exponentiated geometric mean and 95% confidence interval are presented. Paired t-tests were conducted to determine regional

differences in basal blood flow, dialysate and interstitial glycerol, dialysate and interstitial estradiol, and adipocyte diameter. Repeated measures ANOVA were conducted to determine changes in circulating factors throughout the study day as well as differences in dialysate glycerol values between stages. Two-way repeated-measure ANOVA tests (region x time, region x probe) were used to determine regional differences in dialysate glycerol response to pharmacological and physiological stimulated lipolysis with and without estradiol co-perfusion. When indicated by a significant F statistic, post hoc analyses to determine significant mean differences between the groups were conducted by t-tests with bonferroni correction for absolute differences in dialysate glycerol between stages or Newman-Keuls analyses for differences in percent change between stages. Three way ANOVA (race x probe x region) was used for preliminary analyses to determine racial and regional differences in dialysate glycerol response to pharmacological and physiological stimulated lipolysis with and without estradiol co-perfusion. Three way ANOVA (probe x time x region) was also used to determine probe and regional differences in dialysate glycerol percent change from baseline during basal (resting), exercise, and pharmacological perfusion. Analyses were completed using IBM SPSS statistics version 19. All data are presented as mean ± standard error (SE) unless otherwise noted, and alpha was set at 0.05.

Results

Participant Characteristics

Participant characteristics are presented in Table 2.1 and gynecologic and baseline sex hormones in Table 2.2. Baseline sex hormone concentrations confirmed all women were in the follicular phase of the menstrual cycle on the day of the microdialysis visit. Two African

American participants exhibited elevated circulating estradiol, estrone, and LH values, indicative of late follicular phase timing immediately preceding ovulation rather than the early-mid follicular phase timing of the other participants. However, neither FSH nor progesterone was significantly elevated in these two women; indicating ovulation had not yet occurred. Preliminary analyses of the data from these two participants revealed results consistent with the remainder of the study sample; therefore data from these participants has been included in all analyses. Inclusion of these two women resulted in higher baseline LH in the AA versus CA women (Table 2.2; p=0.03). Calculation of the mean LH value excluding these two women resulted in similar LH values between AA and CA women (data not shown).

Abdominal and gluteal interstitial E_2 concentration was determined from pooled dialysate samples consisting of dialysate from all basal timepoints in all four probes in each region in 13 women. Interstitial E_2 concentration was similar in the abdominal and gluteal SAT and CA and AA women (Table 2.2) but is higher than the normal circulating range for E_2 during the follicular phase of the menstrual cycle. This measurement also provided confidence that the concentration of E_2 perfused into the adipose tissue as part of the microdialysis protocol was of sufficient concentration to increase local SAT E_2 levels from basal concentrations.

Three day dietary analysis revealed that on average participants in the study consume 2003±155 kilocalories (kcal) per day, consisting of 49.1±2.2% carbohydrate, 13.7±0.6% protein, and 37.1±2.2% fat. There were no racial differences in total kcal/day (CA: 2143±244 kcal, AA: 1846±184 kcal; p=0.36), or percentage carbohydrate (CA: 49.8±2.9%, AA: 48.3±3.7%; p=0.74), protein (CA: 14.5±1.0%, AA: 12.7±0.7%; p=0.18), or fat (CA: 35.7±2.5%, AA: 38.6±3.9%; p=0.54).

Resting and Exercise Metabolic Characteristics

Metabolic characteristics are presented in Table 2.3. Women successfully exercised at ~60% of previously determined VO_2peak for 30 minutes to physiologically stimulate lipolysis (stage 5 of microdialysis). Performing submaximal exercise elicited a significant increase in absolute fat oxidation, respiratory exchange ratio (RER), and heart rate in all women (Table 2.3, $p<0.001$ vs resting). VO_2 (ml/kg/min) during submaximal exercise was lower in AA compared to CA women (Table 2.3). This was likely due to the slightly lower (although not statistically different) VO_2peak between the groups. Importantly, percentage of VO_2peak reached during the submaximal exercise bout was similar between races (Table 2.3) and therefore so was relative exercise intensity.

Regional Differences in Basal Interstitial Glycerol

Pooled group of participants. In the basal state average $Glyc_{dialysate}$ (pooled from all basal timepoints from all four probes) was not different in the abdominal and gluteal regions (AB 34.0±1.9 μmol/l; GL 35.0±1.5 μmol/l; p=0.58), however, mean outflow-to-inflow ratio from the same timepoints was significantly higher in the gluteal region (AB 0.79±0.01; GL 0.85±0.01; p=0.001). Calculation of $Glyc_{IS}$ did reveal a significantly higher interstitial glycerol concentration in the gluteal region versus the abdominal region (AB 200.1±29.7 vs GL 425.8±38.1 μmol/l; p=0.007). Raw data for $Glyc_{Idialysate}$ and O:I ratios for each stage and probe for the pooled population can be found in Tables 2.4 and 2.5 respectively. $Glyc_{dialysate}$ decreased throughout the microdialysis study day in all control probes similarly between the abdominal and gluteal regions (Figure 2.2A).

Racial comparison. Caucasian and African American women had similar dialysate and interstitial glycerol values in both the abdominal and gluteal regions (CA: AB 34.2±2.7 μmol/l, GL 35.1±1.9 μmol/l; AA: AB 33.7±2.8 μmol/l, GL 34.9±2.5 μmol/l; race x depot p=0.93). There was a tendency for the gluteal interstitial glycerol to be higher than the abdominal in both the CA and AA women (CA: AB 315.4±46.5 μmol/l, GL 452.1±54.9 μmol/l p=0.07; AA: AB 289.9±37.6 μmol/l, GL 396.2±54.1 μmol/l p=0.06). The lack of a significant difference between the regions was most likely due to a lack of statistical power with the reduced sample size for the racial comparison; therefore all results of racial analyses must be considered preliminary investigations. Both races demonstrated a significantly higher O:I in the gluteal versus abdominal region (CA: AB 0.79±0.01 vs GL 0.86±0.01 p=0.004; AA: AB 0.79±0.02 vs GL 0.83±0.02 p=0.04) .

Glycerol Response to Physiological and Pharmacological Modulation

Fifteen minutes of submaximal cycle ergometry exercise was sufficient to increase $Glyc_{dialysate}$ versus resting in both the abdominal and gluteal regions. At 30 minutes of exercise $Glyc_{dialysate}$ remained elevated versus resting in all probes (Figure 2.2A). The increase in $Glyc_{dialysate}$ with exercise was lower in the gluteal compared to abdominal region (Figure 2.2A).

Thirty minutes of ISO perfusion also significantly increased $Glyc_{dialysate}$ versus baseline in the abdominal and gluteal regions. $Glyc_{dialysate}$ remained elevated from basal in all probes at 60 minutes of ISO perfusion. Lipolytic stimulation due to ISO perfusion was lower in the gluteal compared to the abdominal region (Figure 2.2B). $Glyc_{dialysate}$ did not change from ISO perfusion alone after the addition of PHEN to the perfusate in either the abdominal or gluteal region (Figure 2.2B). Thirty minutes of submaximal exercise during ISO + PHEN perfusion was able

to further increase $Glyc_{dialysate}$ from its concentration during ISO + PHEN perfusion alone (Figure 2.2B).

There were no significant racial differences in the responses to pharmacological and/or exercise stimulation of lipolysis. Exercise (probe x time x race p=0.13), pharmacological modulation (probe x time x race p=0.5).

Effect of Local Estradiol Perfusion on Adipose Tissue Glycerol

Basal. In the pooled group basal resting $Glyc_{dialysate}$ measured via microdialysis was not affected by 180 minutes of perfusion of E_2 compared to measurements from control probe over the same time period in the abdominal or gluteal region (Figure 2.3A). There was a net effect for $Glyc_{dialysate}$ to decrease throughout the study day in all four probes. Preliminary analyses revealed a tendency for a racial difference in response to prolonged estrogen perfusion at rest (probe x time x race, p=0.06); estradiol tended to blunt the decrease in dialysate glycerol over time in the Caucasian group, but not the African American group (Figure 2.3B and C). Limited power in these racial analyses restricts our ability to draw definite conclusions about potential racial differences.

Exercise. Perfusion of E_2 did not alter the increase in $Glyc_{dialysate}$ in response to 30 minutes of cycle ergometry exercise compared to a no-E_2 (control) perfused probe in either the abdominal or gluteal region. The $Glyc_{dialysate}$ response to exercise was lesser in the gluteal region, but this diminished response was not different between control and E_2 probes (Figure 2.4A). There was no racial difference in the response to exercise, with or without estradiol perfusion (Table 2.6; depot x probe x race p=0.68).

Isoproterenol. SAT dialysate glycerol increased with perfusion of ISO (β-AR agonist) in the abdominal and gluteal regions. The effect of E_2 co-perfusion along with ISO on glycerol release was dependent on adipose tissue depot (probe x region p=0.003). In the abdominal region the addition of E_2 to the ISO perfusate resulted in a significantly blunted increase in $Glyc_{dialysate}$ compared to ISO perfusion without E_2 (Figure 2.4B; p=0.003). The increase in $Glyc_{dialysate}$ in response to ISO perfusion was lower in the gluteal compared to abdominal region, and this response was not different between the control and E_2 probes (Figure 2.4B, p=0.428). There were no racial differences in the response to ISO perfusion alone or with co-perfusion of E_2 (Table 2.6; depot x probe x race 0.09).

Isoproterenol and phentolamine. The addition of PHEN (α-AR antagonist) on top of ISO in the perfusate for 60 minutes did not significantly change local glycerol release in any of the probes (with or without co-perfusion of E_2 in AB or GL regions) as indicated by no change in $Glyc_{dialysate}$ concentration from ISO perfusion alone (Figure 2.4C). The same was true when analyses were broken down individually for Caucasian and African American women (Table 2.6; depot x probe x race p=0.38).

Isoproterenol, phentolamine, and exercise. Modulation of lipolysis by the addition of physiological stimulation (30 minutes of submaximal exercise) to ISO + PHEN perfusion (i.e. 'maximal' stimulation) resulted in a significant increase in $Glyc_{dialysate}$ from ISO + PHEN perfusion alone. $Glyc_{dialysate}$ measured from probes perfused with E_2 during ISO + PHEN + exercise responded divergently dependent on the region of interest (Figure 2.4D; probe x region p=0.001). In the abdominal region the percent increase in $Glyc_{dialysate}$ after 30 minutes of exercise along with ISO + PHEN perfusion the E_2 co-perfused probe was significantly greater than the increase in the control probe (p=0.007), whereas in the gluteal region, the E_2 perfused

probe had a smaller percent increase in $Glyc_{dialysate}$ versus the control probe (p=0.054) (Figure 2.4D). The $Glyc_{dialysate}$ response was similar between control probes in the abdominal and gluteal regions. Once again, there was no difference in the response to modulation with or without E_2 perfusion between the racial groups (Table 2.7; depot x probe x race p=0.18).

Adipose Tissue Blood Flow

Blood flow was monitored indirectly by outflow-to-inflow ratio throughout the microdialysis visit; raw data for all probes and timepoints for the pooled group can be found in Table 2.5. Percent change in O:I was calculated between stages to coincide with changes in dialysate glycerol over the same time periods (Figure 2.5 A-D). The percent decrease in O:I ratio did not differ between probes or regions during the ISO perfusion in stage 3 (Figure 2.5B), or during ISO + PHEN + exercise in stage 5 (Figure 2.5D). Exercise alone (stage 5, probes 1 and 2) increased blood flow surrounding the probe perfused with E_2 in the gluteal region, but did not change blood flow in the control probe in the same region or in either probe in the abdominal region (Figure 2.5A; region x probe, p=0.048). Co-perfusion of E_2 during the ISO + PHEN stage (stage 4) resulted in a significant decrease in local blood flow in the abdominal region, as indicated by an increase in O:I ratio versus the control probe (Figure 2.5C; main effect of E_2, p=0.009). This change in blood flow was not evident in the gluteal region.

There was no main effect of race on change in O:I ratio during exercise (race x probe p=0.88), ISO alone (race x probe p=0.78), ISO + PHEN (race x probe p=0.38), ISO + PHEN + exercise (race x probe p=0.56).

Circulating Humoral Factors

All blood values were similar between the two resting blood draws (baseline and pre-exercise) with the exception of an increase in circulating testosterone (Table 2.7). The values of most circulating factors from participants sampled post-exercise instead of during-exercise were similar and were combined into a single exercise timepoint (Table 2.7; n=7 during-ex + n=5 post-ex = n=12 exercise). The exceptions to this were insulin, which was lower, and norepinephrine, which was higher, when sampled during exercise compared to post-exercise (Table 2.7, both $p<0.05$). Therefore insulin and norepinephrine were kept separate during- and post-exercise timepoints for analysis. Exercise significantly increased all blood values in the pooled samples, except for estradiol, which did not change. Blood values were similar between races at all timepoints except for glucose at the exercise draw which was higher in the AA (CA: 4.6±0.15 mmol/l vs AA: 5.2±0.16 mmol/l, $p<0.05$).

Discussion

This is the first study to locally modulate the subcutaneous adipose tissue (SAT) sex steroid profile to determine how changes in E_2 may directly affect lipolysis. The novel finding of this study is that increasing the local SAT E_2 concentration does indeed change local lipolytic rate as measured via the *in situ* technique of microdialysis, reinforcing a modulatory role for estradiol in female adipose tissue metabolism. Importantly, our results indicate that the influence of E_2 is dependent upon the adipose tissue depot of interest as well as the specific regulatory mechanism targeted. We found that the β-adrenergic receptor mediated increase in lipolysis is blunted by estradiol (as indicated by smaller percent change in dialysate glycerol) in abdominal SAT, but is not affected in gluteal SAT. In addition, we demonstrated that estradiol blunts the

lipolytic response to 'maximal' stimulation in gluteal SAT while simultaneously increasing the lipolytic response to 'maximal' stimulation in abdominal SAT. Maximal stimulatory conditions consisted of pharmacological modulation (with local α-AR blockade and β-AR stimulation) coinciding with physiological stimulation (via submaximal exercise). Finally, we showed preliminary evidence that race does not appear to influence the effect of estradiol on stimulated lipolytic rate, but may alter estrogen's effect on basal lipolysis. Our variable findings within a single controlled study demonstrate the importance of specificity when investigating and reporting effects of estradiol on adipose tissue metabolism in women and may help clarify the heterogeneous results reported in previously published literature in this area.

Investigations utilizing isolated adipocytes *in vitro* and FFA release tracers *in vivo* in premenopausal women have established metabolic differences between adipocytes from gluteal-femoral versus abdominal SAT with the lower body regions being less lipolytically responsive (87, 88, 90, 93, 94, 224). Increased insulin sensitivity and α2-AR receptor expression are believed to promote triacylglycerol storage and inhibit lipolysis in the gluteal-femoral region (88), potentially playing a central role in the accumulation of lower body adiposity in premenopausal women. Specifically, increased α2-AR receptor expression leads to a reduced lipolytic effect of catecholamines in gluteal-femoral adipose tissue (87, 214). In agreement with this previous research, the current study also clearly demonstrates a blunted response to stimulated lipolysis in gluteal SAT under two conditions: 1.) β-AR agonist perfusion and 2.) submaximal exercise alone (believed to be primarily catecholamine mediated). It is possible that the preference for adipose storage in the gynoid region serves a specialized function in premenopausal women; accumulating energy stores for future utilization during pregnancy and lactation (94). Lower lipolytic response in the gluteal region may also be involved in the lack of,

or possibly negative, relationship between lower body obesity and cardiometabolic disease risk (5, 6, 16-19). Sequestering circulating lipids and releasing fewer free fatty acids into circulation may help to protect women with a propensity for storing adipose in the gluteal-femoral region from excess exposure to circulating lipids and the negative health consequences associated with them (i.e atherosclerosis, insulin resistance, diabetes, etc.). It is possible that local E_2 exposure may also play a role in regional differences in lipolytic rate and the preferential storage of adipose in the SAT depot in women. The importance of understanding estrogen action in adipose tissue is underscored by the fact that adipose tissue is an estrogen producing organ, particularly in postmenopausal women where adipose tissue is the major site of estrogen production (225).

Effects of E_2 on Basal Lipolysis

Studies involving acute effects of estradiol on lipolysis are lacking. We know of only one other study acutely modulating hormonal status *in vivo* to investigate changes in whole body and regional lipolysis and it was conducted in postmenopausal, rather than premenopausal, women. Van Pelt et al. found that acutely increasing circulating estradiol concentrations with an intravenous bolus of exogenous conjugated estrogens decreased basal lipolysis as determined using the microdialysis technique in SAT in the femoral region, and to a lesser extent in the abdominal region, supporting a role for estrogens in regional adiposity (91) and suggesting a potentially non-genomic effect of estradiol on basal lipolysis within the adipose tissue in humans. We were unable to detect an effect of estradiol perfusion on basal lipolysis in the present study, which was surprising considering the previous findings revealing a role for estradiol to decrease basal lipolytic rate within 90 minutes of the IV bolus (91). However, the

previous study was conducted in estrogen deficient postmenopausal women whereas the current study was conducted in normal, healthy, estrogen replete premenopausal women in which perfusion of supplementary estradiol may not lead to further alterations in an already estrogen saturated basal lipolytic pathway. It is also possible the mode of estrogen administration (SAT perfusion compared to IV bolus) could explain the differential results. The tendency for a racial difference in the response to E_2 perfusion during basal lipolysis measurements may have diminished our ability to clearly demonstrate an effect of estradiol on basal lipolysis. Further investigations are necessary to uncover if estradiol does in-fact depress basal lipolysis and if this differs in Caucasian and African American women, as our preliminary investigations suggest.

Effects of E_2 on Stimulated/Disinhibited Lipolysis

The first study to find exogenous steroids influenced stimulated lipolysis in humans involved a three week oral ethinyl estradiol treatment in overweight estrogen deficient postmenopausal women (158). Estrogen replacement in these women led to a decrease in norepinephrine stimulated lipolysis in adipocytes from the abdominal, but not gluteal, SAT compared to pretreatment values (158), providing evidence that estradiol may decrease lipolysis in subcutaneous adipose tissue of women in a region specific manner. Additional investigations have found that postmenopausal women on chronic estrogen replacement demonstrate a trend for lower hormone sensitive lipase (HSL) activity and increased α2-AR mRNA expression compared to a placebo treated group (159). In the same study, abdominal SAT samples from estrogen deplete postmenopausal women incubated *in vitro* for 24 hours with estradiol resulted in increased α2A-AR expression as well as a blunted lipolytic response to epinephrine, with no change in β-AR expression, versus control-treated adipose samples; potentially implicating the

increase in α2-AR expression in the decreased lipolytic response to epinephrine (159). In the present study acute SAT perfusion of estradiol led to blunting of lipolysis through a β-AR mediated mechanism, as demonstrated by reduced lipolytic responsiveness to ISO perfusion when estradiol was on board in the abdominal, but not gluteal, SAT. The addition of the α-AR blocker PHEN did not change the lipolytic response to ISO perfusion with or without estradiol co-perfusion in either depot; therefore, it does not appear that α-AR inhibition of lipolysis is involved in the E_2 modulation of ISO-stimulated lipolysis. The influence of E_2 specifically on α-AR inhibition of lipolysis cannot be determined from the current study design and future studies are warranted. As a whole, the results from the current and previous studies establish that estradiol can work through multiple adrenergic receptor mediated mechanisms to dampen lipolytic rate in subcutaneous abdominal adipose tissue of both pre- and post-menopausal women, potentially helping to maintain a subcutaneous adipose distribution in an estrogen replete population, and increase abdominal adipose accumulation in a postmenopausal population.

Depot Dependent Effects of E_2.

Of particular interest is our observation of a divergent response to estradiol perfusion in the abdominal and gluteal SAT. As discussed earlier, differences in lipolysis between the abdominal and gluteal-femoral regions in premenopausal women are well known and are believed to play a role in maintaining the gynoid body fat distribution in this population (93). Estradiol has also clearly been demonstrated to modulate lipolysis both in acute and chronic exposure conditions, with some limited evidence for regional differences in these effects (91, 158). Our study clearly provides evidence that estradiol's influence on lipolysis occurs in a

region specific manner. First, we found E_2 blunts ISO stimulated lipolysis in the abdominal region but not the gluteal region. Secondly, we found that E_2 blunts lipolysis in the gluteal region, but potentiates lipolysis in the abdominal region during 'maximally' stimulated/disinhibited conditions. Because previous investigations have demonstrated the perfusion of ISO and PHEN at the concentrations used in this investigation lead to maximal changes in lipolysis (69, 80) we believe that further increases in lipolysis with the addition of submaximal exercise along with perfusion of these pharmacological agents was most likely due to non-adrenergically mediated mechanism(s). We do however recognize the possibility that some, or all, of the additional stimulation of lipolysis could be a result of further catecholamine stimulation of the β-ARs. We hypothesize that the divergent regional effects of estradiol on lipolysis during 'maximal' stimulation occur through a yet to be determined adrenergic independent mediator of lipolysis. Through this yet to be determined pathway estradiol may be acting in a region specific manner to help maintain the gluteal-femoral body fat distribution in premenopausal women during times of increased lipid mobilizing signals. Furthermore, it appears estrogen acts through multiple mechanisms to influence lipolysis in a region specific manner.

Atrial natriuretic peptide, insulin, adenosine, cortisol, and nitric oxide are all well-known non-adrenergic regulators of lipolysis through which estrogen may potentially influence local lipolytic rate. It is also possible that alterations in second messenger systems such as the cyclic adenosine monophosphate (cAMP) cascade and the phosphoionositide cascade, play a role in actions of membrane associated ERs (149-151). More studies are necessary to fully elucidate the direct actions of estradiol on lipolysis and the mechanisms behind this influence, particularly

estrogen's actions through the three known adipose tissue estrogen receptors and its influence on both adrenergic and non-adrenergic modulators of lipolysis.

Adipose Tissue Blood Flow

Changes in adipose tissue blood flow must be considered when using microdialysis to study local lipolysis. The use of the ethanol technique provides a qualitative assessment of changes in microvascular exchange (nutritive blood flow), a key determinant in the concentration of substances recovered in the dialysate over the microdialysis membrane. In the current study, the gluteal SAT exhibited lower blood flow/microvascular exchange when compared to the abdominal region, indicating reduced tissue exposure to circulating hormones and humoral factors and decreased metabolic clearance in the gluteal adipose depot. These mechanisms could play a role in the decreased responsiveness to metabolic perturbations such as catecholamine stimulation during exercise in the gluteal-femoral region. Estradiol perfusion *per se* did not change SAT blood flow considerably in our study, even though estrogen is known to have vasodilatory effects (i.e. increase blood flow) in large vessels and even the coronary microvasculature in postmenopausal women (226-228). However, acute changes in circulating estrogen levels (i.e. the menstrual cycle) do not affect microvascular responses in normally cycling premenopausal women (229), consistent with our current findings with little change in blood flow around the probe with estradiol perfusion in SAT. Only under two conditions did the estradiol probe have a significantly different blood flow response than the no-estradiol probe, manifested in a lower blood flow than around the control probe in the abdominal region during ISO+PHEN perfusion and a higher blood flow than around the control probe in the gluteal region during exercise. Changes in blood flow with estradiol perfusion were inconsistent by both

region and stage and were not apparent during any stage in which estradiol had a significant effect on lipolysis, making it unlikely that observed changes in lipolysis were simply a result of changes in local blood flow.

Conclusions

In conclusion, this study suggests that exposure to high concentrations of estradiol leads to region and pathway dependent modulation of subcutaneous adipose tissue lipolysis in premenopausal women. Estradiol blunts β-adrenergic stimulation of lipolysis in the abdominal, but not gluteal, SAT of premenopausal women, possibly implicating local adipose estrogen concentrations in modulating abdominal SAT accumulation. In addition, estradiol may also have a potentiating effect on lipolysis in the abdominal SAT, but a suppressive effect on lipolysis in the gluteal SAT, when lipolysis is 'maximally' stimulated via potentially non-adrenergically mediated mechanisms. These divergent regional responses apparent with increased estrogen exposure may be involved in the maintenance of the gynoid body fat distribution in premenopausal women even during times of increased lipid mobilizing signals. Limited data infer that race does not appear to be a determining factor in the effect of estradiol on stimulated lipolysis in premenopausal women; however preliminary evidence suggests it may influence estrogen's effect on basal lipolysis. Our results shed light on why previous studies have demonstrated conflicting results in regards to pro- or anti-lipolytic effects of estradiol in adipose tissue of women. When investigating estrogen mediated modulation of lipolysis particular attention must be paid with regards to the specific lipolytic regulatory pathways as well as the adipose tissue depot(s) under investigation.

Table 2.1 Participant Characteristics

Variable	Pooled	Caucasian	African American
n	17	9	8
Age, yrs	27.4 ± 2.0	27.2 ± 2.9	27.6 ± 2.8
Weight, kg	81.7 ± 2.3	81.1 ± 2.0	83.4 ± 4.6
BMI, kg/m^2	29.7 ± 0.5	29.3 ± 0.6	30.3 ± 0.9
Waist Circumference, cm	89.3 ± 1.1	88.7 ± 1.5	90.0 ± 1.9
WHR	0.79 ± 0.01	0.79 ± 0.02	0.79 ± 0.01
Body fat, %	47.3 ± 1.0	46.0 ± 0.8	48.8 ± 2.0
Android Fat Mass, kg	3.1 ± 0.3	3.1 ± 0.2	3.1 ± 0.3
Gynoid Fat Mass, kg	7.0 ± 0.3	7.0 ± 0.3	7.0 ± 0.5
Total Cholesterol, mmol/l	3.9 ± 0.1	3.8 ± 0.2	4.0 ± 0.2
HDL Cholesterol, mmol/l	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
LDL Cholesterol, mmol/l	2.2 ± 0.1	2.1 ± 0.2	2.4 ± 0.1
Triglycerides, mmol/l #	0.73 (0.57-0.93)	0.78 (0.52-1.17)	0.67 (0.47-0.96)
Fasting Glucose, mmol/l	4.8 ± 0.1	4.9 ± 0.1	4.7 ± 0.1
Fasting Insulin, pmol/l	55.0 ± 5.5	58.8 ± 6.7	50.8 ± 9.3
HOMA-IR	1.7 ± 0.2	1.8 ± 0.2	1.5 ± 0.3

Data are presented as mean±SE unless otherwise indicated. # Skewed variable, re-exponentiated to geometric mean and 95% confidence interval. BMI, body mass index; WHR, waist-to-hip ratio; HDL, high density lipoprotein; LDL, low density lipoprotein; HOMA-IR, Homeostasis model assessment of insulin resistance.

Table 2.2 Gynecologic history and baseline sex hormones

Variable	Pooled	Caucasian	African American
n	17	9	8
Cycle length, days	30.4 ± 0.7	31.2 ± 0.7	29.5 ± 1.3
Age at Menarche, yrs	11.7 ± 0.3	11.4 ± 0.4	12.0 ± 0.5
Parity, yes/no	4/13	3/6	1/7
Past HC use, n yes/no	7/10	5/4	2/6
Time since HC use, yrs	5.6 ± 2.5	6.4 ± 3.5	3.5 ± 0.5
Estradiol, pmol/l #	226 (147-348)	181 (112-292)	290 (124-678)
Estrone, pmol/l #	223 (172-288)	205 (156-269)	242 (145-405)
Progesterone, nmol/l #	0.36 (0.24-0.54)	0.34 (0.20-0.59)	0.39 (0.19-0.82)
Testosterone, nmol/l	1.08 ± 0.13	1.20 ± 0.2	0.95 ± 0.2
FSH, Iμ/l	7.3 ± 0.9	5.9 ± 0.5	8.9 ± 1.7
LH, Iμ/l	5.6 ± 0.9	3.5 ± 0.2	7.8 ± 1.6*
Interstitial Estradiol Abdominal, pmol/l # [a]	415 (220-783)	397 (159-281)	437 (163-1169)
Interstitial Estradiol Gluteal, pmol/l # [a]	380 (268-540)	452 (281-728)	310 (186-519)

Data presented as mean ± SE. # Skewed variable, re-exponentiated to geometric mean and 95% confidence interval; *$p<0.05$ vs Caucasian; [a] n = 13 pooled, n = 7 Caucasian, n = 6 African American. HC, hormonal contraceptives; FSH, follicle-stimulating hormone; LH, luteinizing hormone.

Table 2.3 Metabolic characteristics at rest and during 30 minutes of submaximal cycle ergometry exercise

Variable	Rest			Submaximal Exercise		
Group	**Pooled**	**Caucasian**	**African American**	**Pooled**	**Caucasian**	**African American**
n	17	9	8	17	9	8
Resting Energy Expenditure, kcal/day	1457 ± 45	1509 ± 44	1397 ± 80	N/A	N/A	N/A
Respiratory Exchange Ratio	0.77 ± 0.01	0.77 ± 0.01	0.78 ± 0.02	0.88 ± 0.01*	0.88 ± 0.01*	0.88 ± 0.02*
Heart Rate, beats per minute	76 ± 3	70 ± 3	81 ± 4	154 ± 4*	156 ± 5*	151 ± 7*
VO_2peak, ml/kg/min	N/A	N/A	N/A	23.6 ± 1.4	26.0 ± 1.5	20.9 ± 2.2
Submaximal VO_2, ml/kg/min	N/A	N/A	N/A	13.8 ± 0.6	15.6 ± 0.7	11.8 ± 0.5†
Exercise Intensity, % of VO_2peak	N/A	N/A	N/A	60.0 ± 2.1	60.2 ± 0.9	59.8 ± 4.5

All data presented as mean ± SE. VO_2, oxygen uptake. * $p<0.001$ vs rest in same group, † $p<0.01$ vs Cacuasian during exercise.

Table 2.4 Dialysate glycerol concentrations measured by microdialysis in overweight-to-obese premenopausal women.

		Abdominal		Gluteal	
Stage		Mean (µmol/l)	SEM (µmol/l)	Mean (µmol/l)	SEM (µmol/l)
	Probe 1	n = 14		n = 16	
1	Basal	35.5	3.5	39.0	3.6
2	Basal	30.4	2.4	35.9	2.4
3	Basal	30.5	2.5	30.4	2.2
4	Basal	30.2	1.8	28.4	1.7
5	Basal + EX	85.1 [a,b]	7.8	57.4 [a]	6.7
	Probe 2	n = 14		n = 16	
1	Basal	32.5	2.0	37.1	3.0
2	E_2	31.3 [b]	1.8	37.3	2.3
3	E_2	27.3	2.4	31.9 [a]	2.3
4	E_2	28.2	2.2	30.3	2.1
5	E_2 + EX	67.6 [a]	6.0	56.8 [a]	3.7
	Probe 3	n = 15		n = 15	
1	Basal	32.8	2.2	36.9	2.3
2	Basal	30.6 [b]	1.4	37.0	2.6
3	ISO	106.6 [a,b]	6.6	71.2 [a]	3.6
4	ISO + PHEN	103.5 [b]	6.8	71.7	3.1
5	ISO + PHEN + EX	158.0 [a,b]	12.7	113.6 [a]	6.8
	Probe 4	n = 15		n = 15	
1	Basal	40.2	4.5	37.8	2.3
2	E_2	36.6	3.2	38.3	2.2
3	E_2 + ISO	98.5 [a,b]	7.8	78.2 [a]	2.8
4	E_2 + ISO + PHEN	93.2 [b]	6.2	76.7	3.0
5	E_2 + ISO + PHEN + EX	169.1 [a,b]	10.0	103.9 [a]	7.2

Data are from second timepoint in each stage. Basal, control perfusion at rest; ISO, isoproterenol; PHEN, phentolamine; EX, submaximal exercise.

[a] Different from previous stage within same probe ($p < 0.05$)

[b] Different from gluteal region within corresponding probe and stage ($p \leq 0.05$)

Table 2.5 Ethanol outflow-to-inflow ratio (inversely related to blood flow) as measured by microdialysis in overweight-to-obese premenopausal women.

		Abdominal		Gluteal	
Stage		**Mean**	**SEM**	**Mean**	**SEM**
	Probe 1	n = 14		n = 16	
1	Basal	0.81	0.02	0.85	0.01
2	Basal	0.82	0.02	0.85	0.02
3	Basal	0.74 [a,b]	0.02	0.83	0.02
4	Basal	0.79 [a]	0.02	0.84	0.02
5	Basal + EX	0.77 [b]	0.02	0.82	0.02
	Probe 2	n = 14		n = 16	
1	Basal	0.81	0.02	0.85	0.01
2	E_2	0.76	0.03	0.83	0.02
3	E_2	0.74 [b]	0.02	0.82	0.02
4	E_2	0.76 [b]	0.03	0.83	0.01
5	E_2 + EX	0.76	0.03	0.78	0.02
	Probe 3	n = 15		n = 15	
1	Basal	0.79 [b]	0.02	0.85	0.02
2	Basal	0.80 [b]	0.02	0.87	0.02
3	ISO	0.77	0.03	0.78 [a]	0.02
4	ISO + PHEN	0.75	0.02	0.79	0.02
5	ISO + PHEN + EX	0.72	0.02	0.77	0.02
	Probe 4	n = 15		n = 15	
1	Basal	0.78	0.02	0.82	0.02
2	E_2	0.80	0.02	0.78	0.02
3	E_2 + ISO	0.75 [a]	0.02	0.76	0.02
4	E_2 + ISO + PHEN	0.81 [a]	0.02	0.80 [a]	0.01
5	E_2 + ISO + PHEN + EX	0.75 [a]	0.02	0.78	0.01

Data are from second timepoint in each stage. Basal, control perfusion at rest; ISO, isopreterenol; PHEN, phentolamine; EX, submaximal exercise.

[a] Different from previous stage within same probe ($p < 0.05$)

[b] Different from gluteal region within corresponding probe and stage ($p < 0.05$).

Table 2.6 Percent change in dialysate glycerol in response to exercise and pharamacological modulation in Caucasian and African American premenopausal women

Variable	Abdominal				Gluteal			
Race	Caucasian		African American		Caucasian		African American	
Condition	Control	Estradiol	Control	Estradiol	Control	Estradiol	Control	Estradiol
Rest → EX (%)	173 ± 41	116 ± 38	209 ± 40*	192 ± 28	117 ± 26	80 ± 14	80 ± 21	117 ± 30
Basal → ISO (%)	266 ± 47*	224 ± 45*	251 ± 29*	171 ± 43	122 ± 22	113 ± 22	82 ± 10	113 ± 19
ISO → ISO + PHEN (%)	1.7 ± 5.5	-6.4 ± 2.5	-4.3 ± 10.0	-0.8 ± 6.1	7.3 ± 9.9	-4.2 ± 3.2	7.3 ± 9.9	0.6 ± 3.0
ISO + PHEN → ISO + PHEN + EX (%)	56 ± 8	77 ± 14*	56 ± 17	100 ± 27*	46 ± 16	31 ± 10	76 ± 17	39 ± 10

All data are mean percent change ± SE. Caucasian n=7, African American n=8. * $p \leq 0.05$ vs Gluteal in same race and probe.
ISO, isoproterenol; PHEN, phentolamine; EX, submaximal exercise.

Table 2.7 Circulating humoral factors at rest and with submaximal aerobic exercise

	Baseline	Pre-Exercise	Exercise (Combined During and Post Ex)	
Glucose, mmol/l (n=12)	4.5 ± 0.06	4.4 ± 0.07	4.9 ± 0.13*†	
Free Fatty Acids, uEq/l (n=10)	635 ± 44	765 ± 39	940 ± 123*	
Glycerol, µmol/l (n=10)	116 ± 11	119 ± 9	319 ± 43*†	
Estradiol, pmol/l # (n=12)	221 (133-367)	234 (147-373)	269 (163-443)	
Testosterone, nmol/l (n=12)	1.0 ± 0.16	1.20 ± 0.17*	1.53 ± 0.19*†	
Progesterone, nmol/l # (n=11)	0.37 (0.22-0.62)	0.46 (0.32-0.68)	1.27 (0.75-2.14)*†	
Cortisol, nmol/l (n=12)	183 ± 11	216 ± 18	455 ± 59*†	
Epinephrine, pmol/l (n=7)	< DL	172 ± 17	487 ± 75†	
			During-Ex[a]	Post-Ex[b]
Insulin, pmol/l (n=12)	43.1 ± 4.9	29.6 ± 4.0	29.3 ± 2.3	56.8 ± 9.0†‡
Norepinephrine, nmol/l (n=10)	1.9 ± 0.21	1.9 ± 0.18	8.3 ± 1.2*†	4.7 ± 0.4*†‡

Values are mean ± SE unless otherwise stated. *p<0.05 vs baseline, † p<0.05 vs pre-exercise, ‡p<0.05 vs during-exercise. # Skewed variable, presented as re-exponentiated to geometric mean and 95% confidence interval. [a] n=7 for insulin, n=5 for norepinephrine. [b] n=5 for insulin and norepinephrine. DL, detection limit; Ex, exercise.

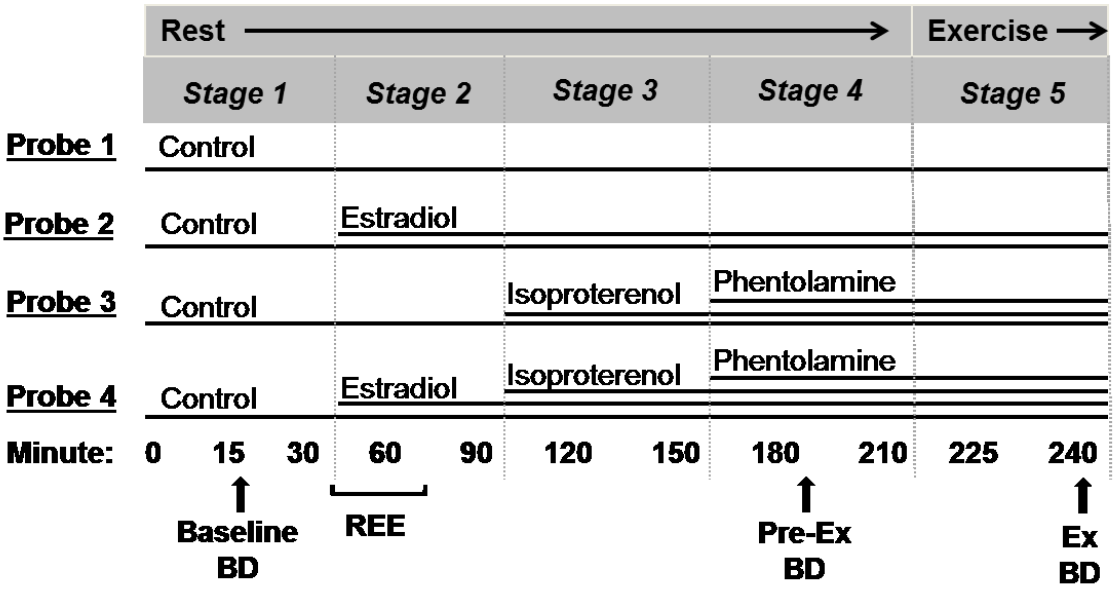

Figure 2.1. Microdialysis protocol. Four microdialysis probes were inserted in each the abdominal and gluteal subcutaneous adipose tissue undergoing identical treatments. Probes were perfused with the following pharmacological agents: Control (0.9% sodium chloride with 10mmol/l ethanol), isoproterenol (β-adrenergic agonist; 1.0 μmol/l), phentolamine (α-adrenergic antagonist; 0.1 mmol/l), and/or estradiol (500 nmol/l). Ten microdialysis samples were collected at the minutes listed above and blood was drawn three times throughout visit (black arrows). REE was measured for 25 minutes at the start of Stage 2. Stage 5 was 30 minutes of stationary cycle ergometry exercise at a previously determined 60% VO_2peak. BD, blood draw; REE, resting energy expenditure; Ex, exercise.

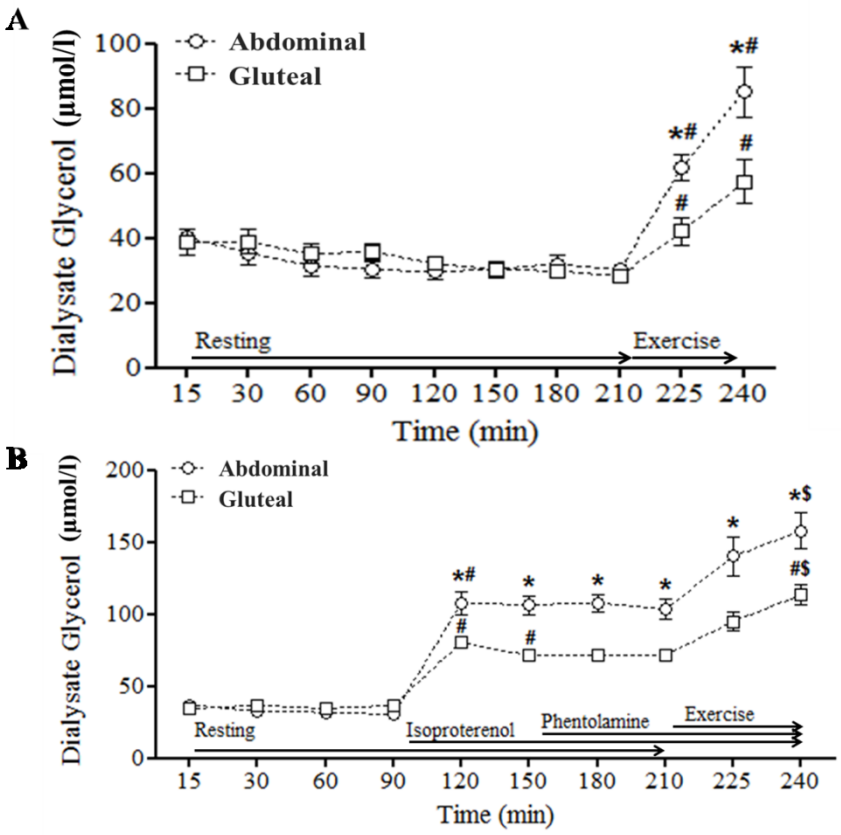

Figure 2.2. Dialysate glycerol as measured by microdialysis in response to (A) exercise and (B) pharmacological modulation of lipolysis in abdominal and gluteal subcutaneous adipose tissue in overweight-to-obese premenopausal women. Lipolysis was measured under control resting conditions or during physiological modulation by (A) 30 minutes of cycle ergometry exercise at 60% of predetermined VO_{2peak} or pharmacologically by (B) 60 minutes of perfusion of isoproterenol (ISO; 1.0 µmol/l), 60 minutes of perfusion of ISO + phentolamine (PHEN; 0.1mmol/l), and 30 minutes of ISO + PHEN + cycle ergometry exercise at 60% of predetermined VO_{2peak}. Open circles with dotted lines (○), represent abdominal and open squares with dotted lines (□) represent gluteal adipose tissue. All probes were perfused with a base control solution of 0.9% saline + 10 mmol/l ethanol. Data are mean ± SE. (A) AB n = 14, GL n = 16; (B) AB n = 15, GL n = 15. * $p<0.05$ abdominal vs gluteal, # $p<0.05$ vs previous timepoint in same region.

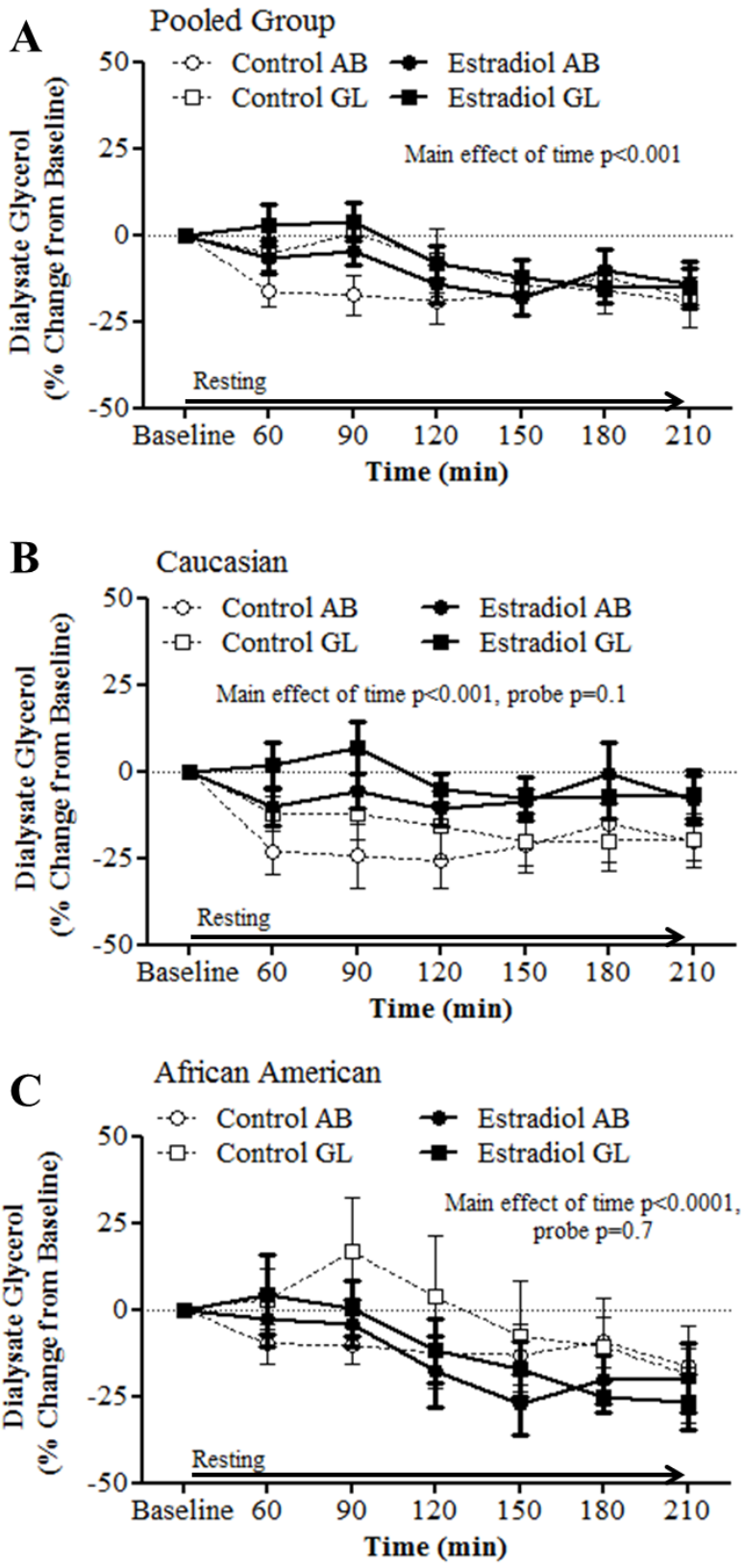
A
Pooled Group
Control AB
Estradiol AB
Control GL
Estradiol GL
Main effect of time p<0.001
Dialysate Glycerol (% Change from Baseline)
Resting
Baseline 60 90 120 150 180 210
Time (min)
B
Caucasian
Control AB
Estradiol AB
Control GL
Estradiol GL
Main effect of time p<0.001, probe p=0.1
Dialysate Glycerol (% Change from Baseline)
Resting
Baseline 60 90 120 150 180 210
Time (min)
C
African American
Control AB
Estradiol AB
Control GL
Estradiol GL
Main effect of time p<0.0001, probe p=0.7
Dialysate Glycerol (% Change from Baseline)
Resting
Baseline 60 90 120 150 180 210
Time (min)

Figure 2.3. Percent change in dialysate glycerol as measured by microdialysis at rest in abdominal (AB) and gluteal (GL) subcutaneous adipose tissue with-or-without perfusion of estradiol in overweight-to-obese premenopausal women. (A) Pooled population (AB, n=14; GL, n=16), (B) Caucasian women only (AB, n=7; GL, n=9), and (C) African American women only (AB, n=7; GL, n=7). Baseline is mean basal dialysate glycerol from min 15 and 30. Open symbols and dotted lines (○, □) represent the control perfused probes (0.9% saline + 10 mmol/l ethanol) and closed symbols and solid line (●, ■) represent the estradiol perfused probes (500 nmol/l in 0.9% saline + 10 mmol/l ethanol). Circles represent the abdominal region and squares represent the gluteal region.

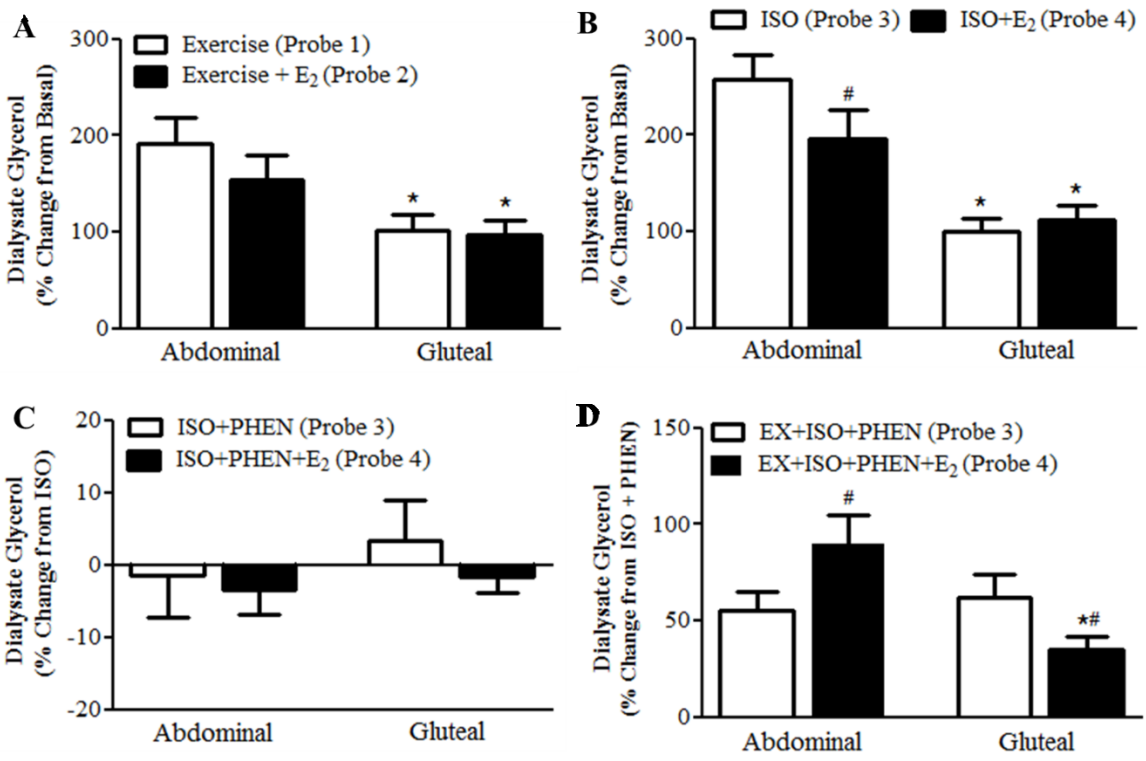

Figure 2.4. Effect of estradiol perfusion on the percent change in dialysate glycerol during exercise and pharmacological modulation as measured by microdialysis in the abdominal and gluteal subcutaneous adipose tissue of healthy overweight-to-obese premenopausal women. Percent change in dialysate glycerol was calculated: (A) from rest (basal) to after 30 minutes of submaximal cycle ergometry exercise (Ex, 60% VO_2peak), (B) from basal to after 60 minutes of perfusion of isoproterenol (ISO, 1.0 µmol/l), (C) from the end of ISO alone to after 60 minutes of perfusion of ISO + phentolamine (PHEN, 0.1 mmol/l), and (D) from the end of ISO + PHEN to after 30 minutes of ISO + PHEN + Ex. Dialysate glycerol was measured in all conditions (A-D) with or without estradiol co-perfusion (500 nmol/l E_2). Data are mean ± SE. Probe 1 & 2: abdominal, n=14; gluteal, n=16. Probe 3 & 4: abdominal, n=15; gluteal, n=15. See Figure 2.1 for details of study design containing key for probe numbers. * $p \leq 0.05$ vs abdominal region of same probe; # $p \leq 0.05$ vs no-E_2 probe in same region.

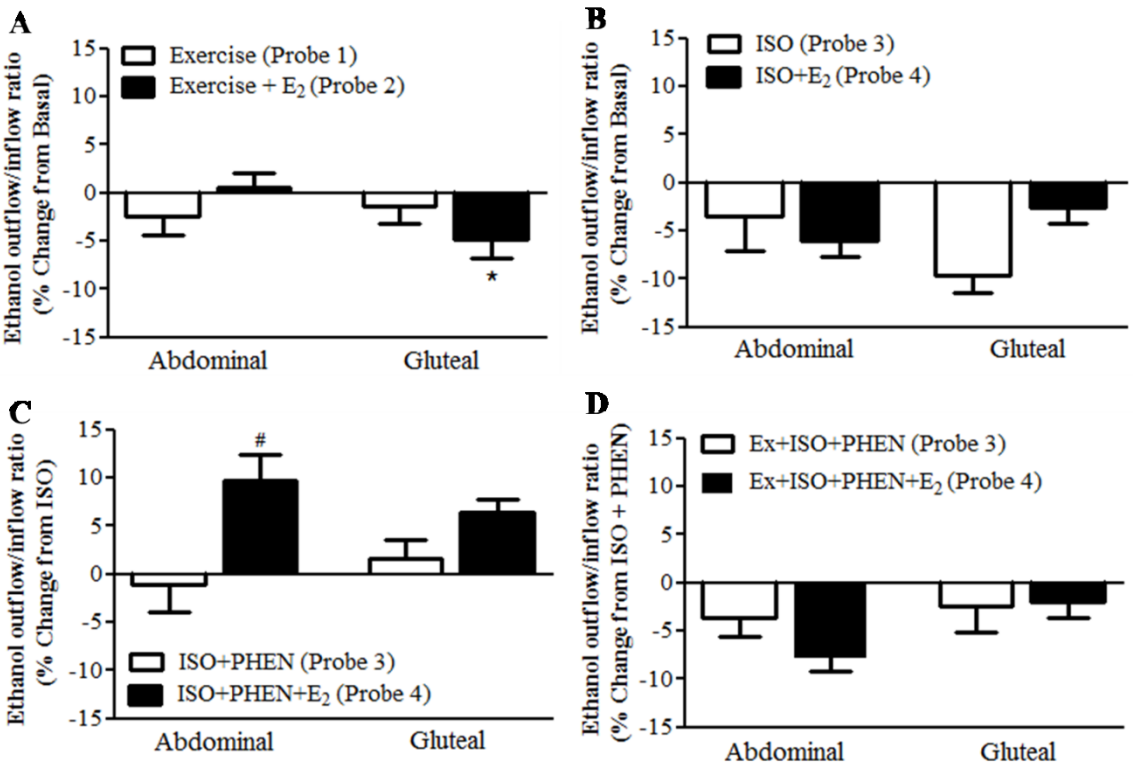

Figure 2.5. Effect of estradiol perfusion on the percent change in ethanol outflow-to-inflow ratio during exercise and pharmacological modulation as measured by microdialysis in the abdominal and gluteal subcutaneous adipose tissue of healthy overweight-to-obese premenopausal women. Percent change in ethanol outflow-to-inflow ratio was calculated: (A) from rest (basal) to after 30 minutes of submaximal cycle ergometry exercise (Ex, 60% VO_2peak), (B) from basal to after 60 minutes of perfusion of Isoproterenol (ISO, 1.0 µmol/l), (C) from the end of ISO alone to after 60 minutes of perfusion of ISO + Phentolamine (PHEN, 0.1 mmol/l), and (D) from the end of ISO + PHEN to after 30 minutes of ISO + PHEN + Ex. All conditions (A-D) were conducted with or without estradiol co-perfusion (500 nmol/l E_2). Data are mean ± SE. Probe 1 & 2: abdominal, n=14; gluteal, n=16. Probe 3 & 4: abdominal, n=15; gluteal, n=15. See Figure 2.1 for details of study design with key for probe numbers. * $p \leq 0.05$ vs abdominal region of same probe; # $p \leq 0.05$ vs no-E_2 probe in same region.

CHAPTER 3: Estrogen Receptor Protein Content is Different in Abdominal than Gluteal Subcutaneous Adipose Tissue of Overweight-to-Obese Premenopausal Women

Abstract

Premenopausal women demonstrate a distinctive gynoid body fat distribution and circulating estrogen status is associated with the maintenance of this adiposity patterning. Estrogen's role in modulation of regional adiposity may occur through estrogen receptors (ERs), which are present in human adipose tissue. The purpose of this study was to determine if there are regional differences in the protein content of ERα, ERβ, and the G protein-coupled estrogen receptor (GPER) between the abdominal (AB) and gluteal (GL) SAT of overweight-to-obese premenopausal women. A secondary aim was to determine if there are racial differences (CA vs AA) in regional ER protein content. We found that ERα protein was higher in AB vs GL ($p=0.02$), ERβ protein was higher in GL vs AB ($p=0.002$), ERα/ERβ ratio was higher in AB vs GL ($p=0.007$), and GPER protein content was similar in AB and GL ($p=0.80$). The regional content of ERβ and GPER was similar between CA and AA women, while ERα content was higher in AB vs GL SAT from CA ($p=0.04$) but not from AA women ($p=0.17$). These results indicate that the effects of estrogen on abdominal and gluteal adiposity in premenopausal women may be regulated by the depot specific expression of estrogen receptors.

Introduction

Premenopausal women demonstrate a distinctive gluteal-femoral body fat distribution (6, 197, 198). Although evolutionary reasoning behind this pattern of adiposity is not definitive one

hypothesis is that women accumulate energy reserves in the lower body in preparation for increased energy utilization and adipose mobilization from this depot during pregnancy and lactation (94, 99, 100). Sequestration of fat in the lower body region is associated with decreased risk for cardiovascular and metabolic disease versus accumulation of central adiposity (5, 6, 16-19). Therefore, a full understanding of the physiology behind why, and by what mechanisms, adipose accumulates in specific depots is important in efforts of obesity and chronic disease prevention.

Estrogen status is related to the maintenance of a gynoid body fat distribution (36). The loss of circulating estrogen over the menopause transition is associated with increases in central adiposity, a pattern of adiposity linked to increased cardio-metabolic disease risk (38-41). The expression of estrogen receptor alpha (ERα) (119, 124), estrogen receptor beta (ERβ) (120-123), and the G protein-coupled estrogen receptor (GPER) (126) within human adipose tissue indicates estrogen has direct effects within adipose tissue (37). Taken together, a shift toward increased central adiposity with decreases in circulating estrogen status and the presence of ERs within the adipose tissue present a strong case for modulation of adipose accumulation via estrogen, potentially in a region specific manner.

There have been few investigations into regional differences in estrogen receptor expression in human adipose tissue, with most of those focusing on differences in estrogen receptor gene expression between subcutaneous and visceral abdominal adipose tissue. To our knowledge studies are lacking which characterize the protein content of all three estrogen receptors in upper and lower body adipose tissue in overweight or moderately obese women. There is limited evidence that ERβ mRNA expression is higher in gluteal than abdominal subcutaneous adipose tissue (SAT) from overweight premenopausal women (121), supporting

the hypothesis that regional differences in ER expression may be a mechanism behind regional differences in adipose accumulation and/or mobilization. Importantly, ERα and ERβ are reported to have distinct actions and ERβ may even oppose the actions of ERα (121, 123, 137, 230), highlighting the need for a clear representation of the relative ERα to ERβ ratio in each adipose depot.

Therefore, the primary purpose of this study was to determine if there are regional differences in the protein content of ERα, ERβ, and GPER between the abdominal (AB) and gluteal (GL) SAT of overweight-to-obese premenopausal women demonstrating a gluteal-femoral body fat distribution (defined as a waist-to-hip ratio < 0.85). As a secondary aim, subgroup analyses investigated racial (Caucasian and African American) differences in regional ER protein content. Establishing regional SAT ER protein content is an important step towards understanding how estrogen may affect adipose depots in the upper and lower body differently, potentially playing a modulatory role in regional adipose accumulation.

Methods and Procedures

Participants

Fifteen overweight/obese premenopausal women, (7 Caucasian/8 African American, 25.1±1.8 years, 81.3±2.5 kg, BMI 29.5±0.5 kg/m^2) between 18 and 39 years old were studied. All but one participant also participated in the study from Chapter 2. Participants enrolled in this study were eumenorrheic (average cycle length 30.5±1 days), not taking hormonal contraceptives (no use of hormonal contraceptives for at least 6 months at study entry), weight stable (< 3 kg weight change in the last 6 months) and sedentary, in that they did not engage in purposeful exercise training more than 30 minutes per day more than 2 days per week. Women

were excluded from the study if they were trying to get pregnant, pregnant or lactating, a smoker, had history of diagnosis of any metabolic or cardiovascular disease, or were taking any medications known to alter lipid metabolism. Qualification for the study was determined by a pre-participation health history questionnaire completed by the participant and reviewed for accuracy with study personnel. All participants were informed both orally and in writing of the purpose, risks, and benefits of the research and provided informed consent prior to enrollment in the study. This study was approved by the Medical Center Institutional Review Board at East Carolina University. Participant characteristics are presented in Table 3.1.

Body Composition

Participants were weighed on an electronic scale with weight recorded to the nearest 0.1 kg and height was measured with a standard stadiometer to the nearest centimeter (cm). Minimal waist and hip circumferences were measured according to previously published guidelines and waist-to-hip ratio (WHR) was calculated (215). Total percent body fat, fat-free mass (lean mass + bone mineral content), and total and regional (android and gynoid) fat mass were determined using dual-energy x-ray absorptiometry (DXA; GE Lunar Prodigy Advance, Madison, WI). According to the manufacturer recommendations the android region was bound on the lower border by the top of the iliac crest and on the upper border at a distance 20% of the distance between the top of the iliac crest and immediately below the chin; lateral borders were the arm lines. The gynoid region was bound on the upper border at a distance 1.5 times the height of the android region and on the lower border at a distance 2 times the height of the android region; lateral boundaries were the outer leg lines.

Adipose Tissue Biopsy

Participants arrived at the East Carolina Diabetes and Obesity Institute (ECDOI) at the East Carolina Heart Institute between 7-8am after an overnight (≥10hr) fast during the follicular phase of the menstrual cycle (day 2-8 after the start of menses). On average the biopsy visit occurred on day 5±2 of the cycle. Approximately 1 gram of subcutaneous adipose tissue was removed using the needle aspiration technique (14 gauge needle) after local anesthesia by injection of 3-5cc of 1% lidocaine and under sterile conditions. Biopsies were taken from the abdominal (AB), approximately 5cm lateral to the umbilicus, and the upper gluteal (GL) regions, approximately ~5-10 cm from the medial line in the upper quadrant of the buttock. Fat tissue was immediately rinsed with Krebs Ringer Bicarbonate (KRB) buffer containing 1% bovine serum albumin (BSA). Clots and blood vessels were then removed; tissue was immediately frozen in liquid nitrogen and stored at -80°C for later analysis.

Western Blotting

Adipose tissue samples were homogenized glass on glass in a 2:1 (volume-to-weight) ratio of cold homogenization buffer (50 mmol/l HEPES [pH 7.4], 1% SDS [sodium dodecyl sulfate], 2% Triton X-100, 2 mmol/l Ethylenediaminetetraacetic acid, 1 mmol/l sodium orthovanadate, 50 mmol/l sodium fluoride, 1 mmol/l benzamidine, 2 mmol/l dithiothreitol and protease inhibitor cocktail [Sigma P8340]). Homogenized samples were rotated for 60 minutes and centrifuged for 30 minutes at 10,000 x g, both at 4°C. The supernatant was then removed and protein concentration determined by the Pierce BCA protein assay (Thermo Scientific, Rockford, IL). Samples were prepared in laemmli sample buffer (Bio-Rad, Hercules, CA) with 5% β-mercaptoethanol and heated in a water bath at 90°C for 5 minutes.

Seventy micrograms of protein was loaded and separated on a 10% SDS-polyacrylamide gel (Criterion precast gels; Bio-Rad) and electrotransferred to reduced-fluorescence polyvinylidene difluoride membranes (Immobilon-FL; Millipore, Bedford, MA). Membranes were blocked for 1 hour at room temperature with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) and then probed overnight at 4°C for ERα (1:400; sc-542; Santa Cruz Biotechnology, Santa Cruz, CA), ERβ (1:400; sc-8974; Santa Cruz Biotechnology) or GPER (1:300; sc-48524-R; Santa Cruz Biotechnology) and β-actin (1:2000; 926-42212; LI-COR). Primary antibodies were diluted in Odyssey blocking buffer containing 0.2% tween-20. Blots were then washed with phosphate buffered saline containing 1% tween-20 (PBS-T) and incubated for 1 hour at room temperature in fluorescent secondary detection antibodies (IRDye 800 CW-Donkey anti-Rabbit and IRDye 680LT Donkey anti-Mouse; LI-COR) diluted in Odyssey Blocking Buffer containing 0.2% tween-20 and 0.01% SDS. Fluorescence intensity data were quantified using the Odyssey infrared imaging system (LI-COR). All samples were normalized to β-actin protein as well an internal control sample run on each gel and presented in arbitrary units.

ERα and ERβ overexpression lysates (NBL1-10344 and NBL1-10345 respectively; Novus Biologicals, Littleton, CO), MCF 7 nuclear extract, and SKBR3 lysates (sc-2149, sc-2218; Santa Cruz Biotechnology) were used as positive controls to confirm detection of proper molecular weight bands for ERα, ERβ and GPER respectively. In addition, blocking peptides for ERα and GPER were utilized to further confirm proper band detection and quantification.

Blood Samples

A fasting blood sample was drawn the morning of the microdialysis visit. Fasting serum samples of glucose, total cholesterol (TC), triglycerides (TG), and high-density-lipoprotein (HDL) cholesterol were determined using enzymatic/colorimetric methods, and low-density-lipoprotein (LDL) cholesterol was calculated using the Friedewald equation (222). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following equation [fasting glucose (mmol/l) x fasting insulin (μU/ml)]/22.5 (223).

Statistics

Independent sample t-tests were used to determine racial differences in subject characteristics. Triglyceride was log transformed before analysis due to its skewed distribution. Paired t-tests were used to determine regional differences in estrogen receptor protein expression. Two-way repeated measure ANOVA (race x region) was used to determine racial differences (Caucasian versus African American) in estrogen receptor expression in the abdominal and gluteal SAT. Pearson correlation coefficient was used to determine significant bivariate relationships between estrogen receptor expression and measurements of body composition..

Results

Baseline characteristics of the participants in the study are included in Table 3.1. African American women had elevated android fat mass compared to Caucasian women; all other participant characteristics were similar between the racial subgroups.

Figure 3.1A-C presents evidence of correct band detection for ERα, ERβ, and GPER. Positive control lysate samples and blocking peptides were utilized to confirm detection of the appropriate representative band for each protein of interest with the exception of ERβ (no blocking peptide available). ERα was detected at a molecular weight just slightly below the expected 66 kDa, ERβ at the expected molecular weight of 56 kDa, and GPER at a molecular weight of approximately 40 kDa.

ER Protein Content

ERα protein content was higher in the abdominal compared to the gluteal SAT in the group as a whole (Figure 3.2a; p=0.02). Sub-analyses for racial and regional differences in regional ERα protein revealed ERα protein content to be higher in the abdominal compared to gluteal in the Caucasian, but not the African American, women (Figure 3.2b; region x race p= 0.04, main effect of race p=0.98, region p=0.008). Low statistical power due to small sample sizes in the racial sub-analyses may limit our ability to detect significant differences within the racial subgroups.

Conversely, ERβ protein content was significantly lower in abdominal versus gluteal SAT (Figure 3.3a; p=0.002) in this sample of overweight-to-obese premenopausal women. Abdominal and gluteal SAT from Caucasian and African American women expressed ERβ in similar patterns (Figure 3.3b; region x race p=0.405). ERβ protein was significantly higher in the gluteal SAT compared to abdominal in both Caucasian (p=0.04) and African American women (p=0.02).

GPER protein content was not different between abdominal and gluteal SAT in the group as a whole (Figure 3.4a; p=0.53) or within racial sub-analyses (Figure 3.4b; Main effect of race p=0.31, region p=0.54).

The ratio of ERα-to-ERβ protein was significantly higher in the abdominal compared to gluteal region (Figure 3.5a; p=0.007). Racial analyses revealed a tendency for higher ERα/ERβ ratio in abdominal compared to gluteal SAT in both Caucasian (Figure 3.5b; p=0.064) and African American (p=0.064) subgroups (Main effect of region p=0.007, race p=0.71).

Correlations

Correlation analyses were conducted to assess the relationship between ER protein content and anthropometric indices/body composition measurements and SAT estradiol concentrations. WHR was inversely related to gluteal ERβ protein (r^2=0.315, p=0.03) and positively related to gluteal ERα/ERβ ratio (r^2= 0.406, p=0.01). It is important to note that these correlations must be interpreted with caution due to lower statistical power resulting from a small sample size.

Discussion

This is the first study to characterize protein content of the three known estrogen receptors, ERα, ERβ, and GPER, in abdominal and gluteal subcutaneous adipose tissue samples from Caucasian and African American premenopausal women. Importantly, we found estrogen receptors had depot specific protein content pattern, in agreement with previously reported mRNA results (121). Abdominal SAT contained more ERα protein compared to gluteal, and gluteal SAT contained more ERβ protein when compared to abdominal. The resultant ERα-to-

ERβ ratio was also higher in the abdominal versus gluteal depot. We are the first to report GPER protein in human adipose tissue, to our knowledge only one previous study has measured GPER (mRNA) in human adipose tissue (126). We found protein content of the membrane protein GPER to be similar between the two SAT depots. Preliminary racial analyses revealed a similar ER distribution pattern for ERβ and GPER in the Caucasian and African American subgroups, but that Caucasian women have higher ERα in the abdominal versus gluteal while content of ERα was similar between the regions in the African American women. Our results indicate that estrogen may have depot specific effects manifested in abdominal and gluteal differences in ERα and ERβ protein and the resultant ERα-to-ERβ ratio.

Only one previous study has investigated regional differences in ER expression in peripheral adipose depots, reporting only mRNA data. Pedersen et al. found no difference in ERα mRNA expression between abdominal and gluteal SAT from overweight healthy premenopausal women, but did find ERβ isoforms ERβ-4 and ERβ-5 to be significantly higher in the gluteal versus abdominal SAT (121). In the current study we also found ERβ protein content to be higher in gluteal that abdominal SAT. It is important to note that our protein measurement did not distinguish between the multiple ERβ isoforms, but recognized a homologous sequence found in each of the ER isoforms. In opposition to the findings of Pedersen et al. we did find ERα content to be higher in the abdominal compared to gluteal region, this could indicate a post-translational modification occurring in ERα resulting in a regional difference in protein content, but not mRNA.

Investigations utilizing ER knockout models have allowed the determination of specific roles for ERα and ERβ in the maintenance of adipose tissue homeostasis. Female ERα knockout (αERKO) mice demonstrate a marked increase in white adipose tissue compared to wild type

(133), implicating the E_2/ERα signaling pathway in regulation of female white adipose tissue deposition. In particular it seems that ERα, at least in the female mouse model, plays an inhibitory role in the development and total amount of white adipose tissue (133). Conversely, female ERβ knockout (βERKO) mice do not demonstrate the obesity characteristics of ERα knockout models, while double ERα/ERβ knockout (DERKO) models express similar increases in body fat content to αERKO (134), reinforcing the importance of ERα. A specific modulatory role for estrogen acting through the ERs has also been investigated utilizing reduction and repletion of circulating estrogen as induced by ovariectomy and estrogen replacement therapy in the three knockout models. After ovariectomy, estrogen treatment reduces gonadal fat mass in wild type (WT) and βERKO but not αERKO or DERKO female mice, endorsing ERα's role in reduction of fat mass (135) and confirming similar results found in previous investigations (133, 136). In addition, βERKO mice demonstrated an increased response to estrogen treatment (greater loss in fat mass) compared to wild type, indicating that ERβ may act as a repressor of, or in opposition to, the ERα mediated effects (127, 135). From these knockout models ERα has been implicated as the primary ER in reduction of fat mass and ERβ may act to counteract or repress some of ERα's effects in adipose tissue.

Our results indicate the ERα/β ratio is higher in the abdominal versus gluteal region of overweight-to-obese premenopausal women. These findings seem to be in agreement with the aforementioned results in which ERα confers the primary role of adiposity regulation (limiting adipose accumulation) and ERβ may repress the actions of ER. The lower ERα/ERβ ratio (increased ERβ effects/decreased ERα effects) we report in the gluteal SAT may provide a favorable environment for adipose accumulation and storage compared to the abdominal SAT. In support of this we found that WHR is inversely related to gluteal ERβ protein and directly

related to gluteal ERα/ERβ ratio. Our results indicate that depot specific effects of estrogen may be a result of divergent ER expression, which could in turn be a mechanism behind preferential gynoid adipose accumulation in premenopausal women.

GPER is the most recently discovered estrogen receptor and its role in adipose tissue remains uncertain. This is only the second study conducted in human adipose tissue to confirm the presence of GPER, and the first to detect GPER protein. The similarity between GPER protein in the abdominal and gluteal SAT leads us to believe that this membrane associated ER is not a primary pathway through which estrogen has its effects on region specific adiposity in premenopausal women. However, this does not exclude GPER from potentially playing an important role in estrogen mediated effects on lipid metabolism within the adipose tissue. We did find a direct relationship between dialysate and interstitial E_2 and GPER protein content in the gluteal depot, providing evidence that local estrogen concentration may be involved in determining depot GPER content. Consistent with similar regional protein content of GPER, we did not find a regional difference in SAT E_2 concentration, but as adipose tissue becomes the primary source of estrogen after menopause, depot specific changes in the local E_2 concentration may result in altered regional expression in GPER (and ERα and ERβ) and reveal a region specific role for GPER in shifts in body fat distribution with menopause. However, future studies are needed to clarify the specific role of GPER in adipose tissue metabolism and the influence of local E_2 concentration on its expression.

Our small sample size and homogenous population may have limited our ability to detect racial differences in ER expression as part of the current study. Our findings of similar ERβ, and GPER protein content between our subgroups of CA and AA women does not rule out a potential role for estrogen to mediate some of the reported differences in subcutaneous and

visceral adipose tissue accumulation (22-24) and disease risk demonstrated between these groups (27, 231, 232). In fact, our preliminary finding that ERα content was higher in abdominal compared to gluteal SAT in Caucasian women but not African American women supports that possibility. Future studies investigating racial differences in ER protein, particularly differences between subcutaneous and visceral adipose tissue, are warranted

The associative nature of this study does not allow for direct cause and effect conclusions to be drawn about estrogen action through estrogen receptors in adipose tissue. It was not our intention to investigate regional differences in the signaling cascades resulting from estrogen activation of each of the three ERs. Nevertheless, we do provide strong preliminary evidence for region specific effects of estrogen to be mediated at least in part by differences in ER content. Comparisons of region specific protein content of ERα, ERβ, and GPER in premenopausal and postmenopausal women demonstrating clear upper and lower body fat distributions are needed for further confirmation of the association between regional ER expression regional adiposity. Furthermore, mechanistic studies clarifying intracellular signaling cascades resulting from activation of each of the estrogen receptors and their influence on adipose tissue metabolism are of great interest in further understanding region specific effects of estrogens on regional adiposity through ER signaling.

Conclusions

The novel results from the current study demonstrate clear differences in ERα and ERβ protein content in the abdominal and gluteal SAT of overweight-to-obese premenopausal women. The presence of all three ER subtypes in SAT as well as increased ERα expression in the abdominal, and increased ERβ expression in the gluteal, SAT suggests that ERα and ERβ

may both play a role in modulating regional adiposity. Furthermore, these results support the hypothesis that ERβ may counteract ERα mediated actions to decrease adipose accumulation, resulting in a favorable environment for adipose accumulation in the gluteal region of premenopausal women. Finally, we demonstrated well matched premenopausal Caucasian and African American women have similar regional expression ERβ and GPER, but that there may be a racial difference in regional expression of ERα. These results indicate that the effects of estrogen on adipose tissue metabolism in premenopausal women may depend on the depot specific protein content of the ERs, and more specifically the local ratio between ERα and ERβ.

Table 3.1. Participant characteristics

Variable	Pooled	Caucasian	African American
n	15	7	8
Age, yrs	25.1 ± 1.8	25.0 ± 2.7	25.1 ± 2.6
Weight, kg	81.3 ± 2.5	78.6 ± 1.5	83.8 ± 4.5
BMI, kg/m^2	29.5 ± 0.6	28.4 ± 0.3	30.5 ± 0.9
Waist Circumference, cm	88.4 ± 1.3	87.6 ± 1.5	89.2 ± 2.0
WHR	0.79 ± 0.01	0.79 ± 0.02	0.79 ± 0.02
Body fat, %	44.3 ± 1.2	43.7 ± 0.8	44.8 ± 2.2
Android Fat Mass, kg	3.1 ± 0.2	2.7 ± 0.2	3.4 ± 0.2*
Gynoid Fat Mass, kg	7.0 ± 0.2	6.9 ± 0.3	7.1 ± 0.3
Total Cholesterol, mmol/l	3.9 ± 0.2	3.8 ± 0.2	3.9 ± 0.3
HDL Cholesterol, mmol/l	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
LDL Cholesterol, mmol/l	2.3 ± 0.1	2.2 ± 0.2	2.4 ± 0.2
Triglycerides, mmol/l #	0.72 (0.53-0.98)	0.78 (0.49-1.27)	0.66 (0.43-1.02)
Fasting Glucose, mmol/l	4.8 ± 0.1	4.8 ± 0.1	4.8 ± 0.1
Fasting Insulin, pmol/l	58.4 ± 6.2	62.3 ± 8.2	54.5±9.8
HOMA-IR	1.7 ± 0.2	1.9 ± 0.2	1.6 ± 0.3

Data are mean ± SE unless otherwise indicated. * $p<0.05$ vs Caucasian; # Skewed variable, re-exponentiated to geometric mean and 95% confidence interval. BMI, body mass index; WHR, waist-to-hip ratio; HDL, high density lipoprotein; LDL, low density lipoprotein, HOMA-IR, homeostasis model assessment of insulin resistance.

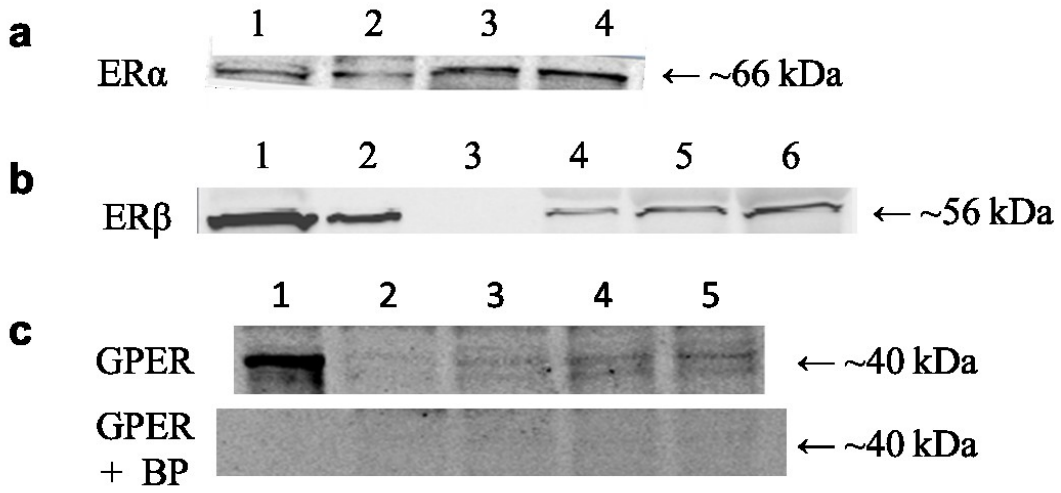

Figure 3.1. Western blotting of ERα, ERβ, and GPER from human subcutaneous adipose tissue (SAT). (a) ERα. Lane 1: 10µg ERα lysate, 2: 25µg MCF7 Nuclear Extract, 3: 40µg Human SAT, 4: 60µg Human SAT. (b) ERβ. Lane 1: 20µg ERβ lysate, 2: 25µg MCF7 Nuclear Extract, 3: Empty well – loading buffer only, 4: 20µg Human SAT, 5: 40µg Human SAT, 6: 60µg Human SAT. (c) GPER. Top panel, Lane 1: 20µg SKBR3 lysate, Lanes 2-5: 70µg Human SAT. Bottom panel, identical to top panel with blocking peptide (BP) added to immunize GPER primary antibody.

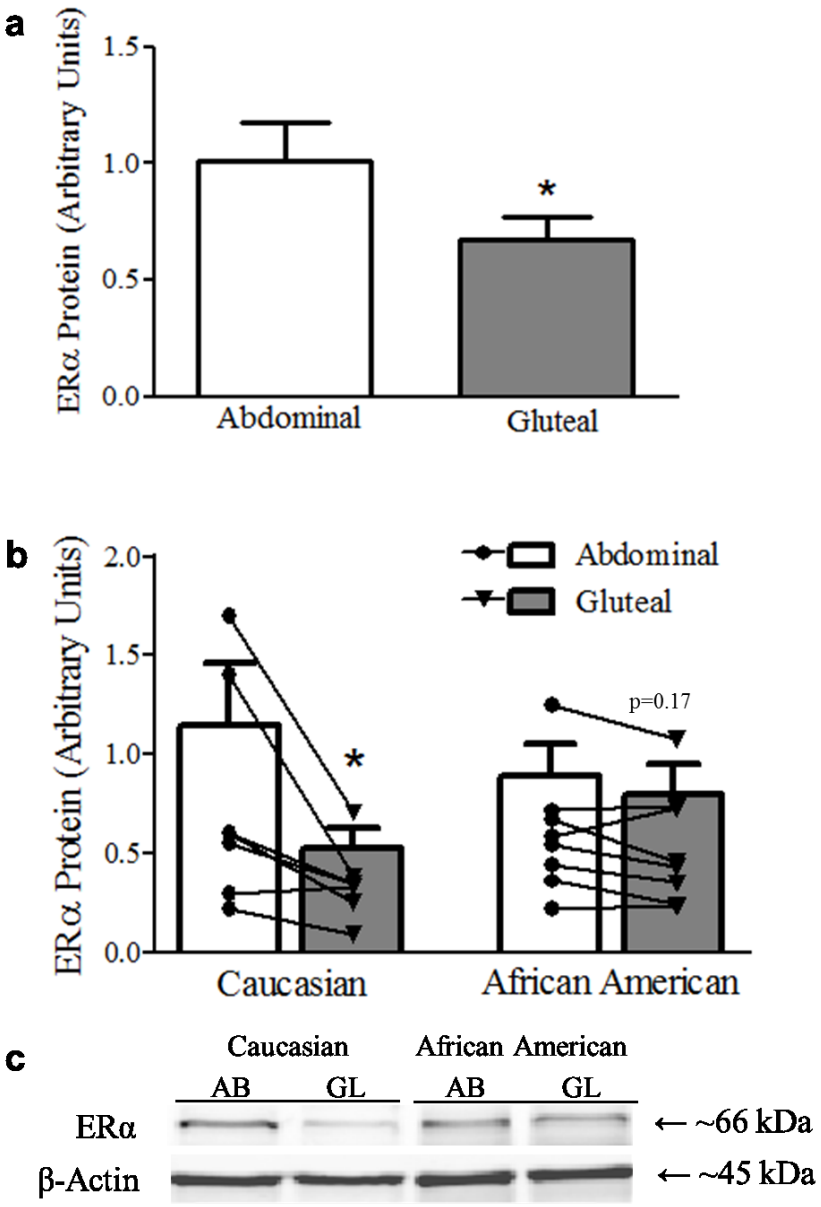

Figure 3.2. Estrogen receptor (ER) α protein content in abdominal and gluteal subcutaneous adipose tissue of overweight-to-obese premenopausal women. (a) Pooled group, n=15. (b) Caucasian (n=7) and African American (n=8) subgroups. Main effect of region p=0.08. (c) Representative western blot for ERα and β-actin. Data are normalized for β-actin and internal control sample and presented as mean ± SE. AB, abdominal; GL, gluteal.

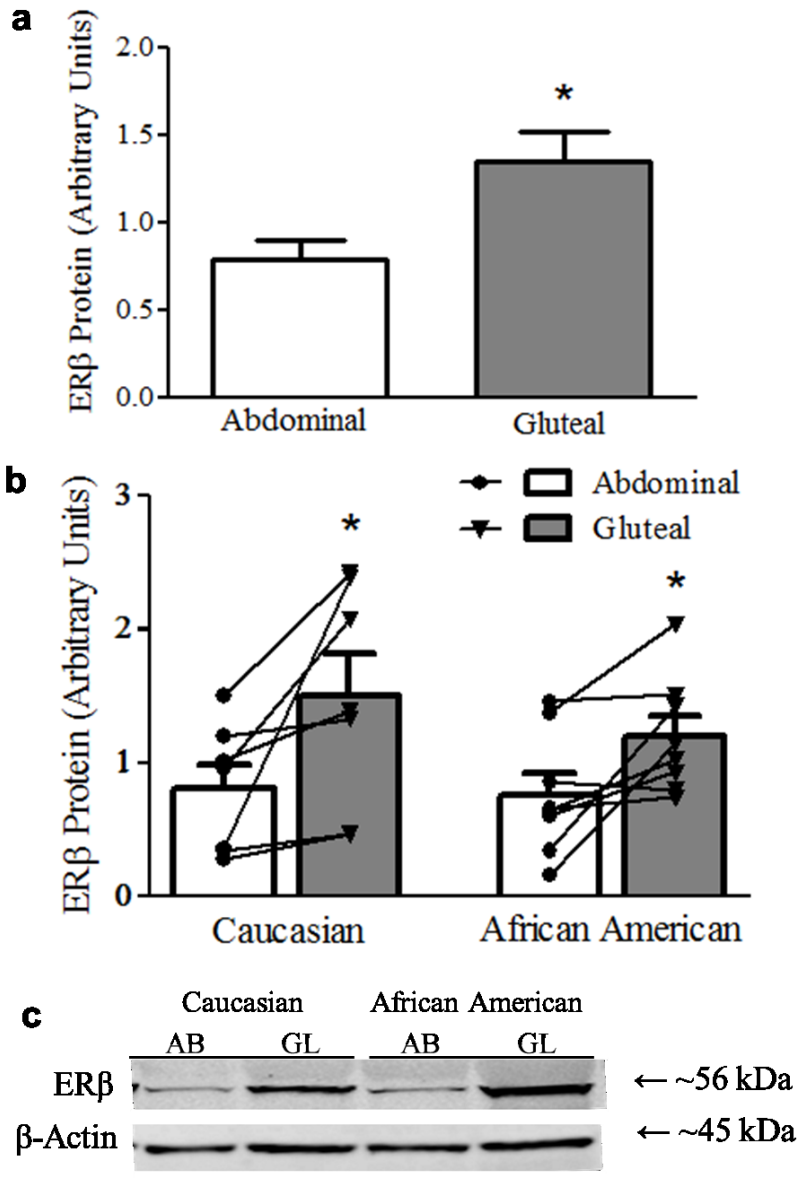

Figure 3.3. Estrogen receptor (ER) β protein content in abdominal and gluteal subcutaneous adipose tissue of overweight-to-obese premenopausal women. (a) Pooled group, * $p<0.05$ versus abdominal region. n=15. (b) Caucasian (n=7) and African American (n=8) subgroups. Main effect of region p=0.002. * $p<0.05$ vs abdominal region of same race. (c) Representative western blot for ERβ and β-actin. Data are normalized for β-actin and internal control sample and presented as mean ± SE. AB, abdominal; GL, gluteal.

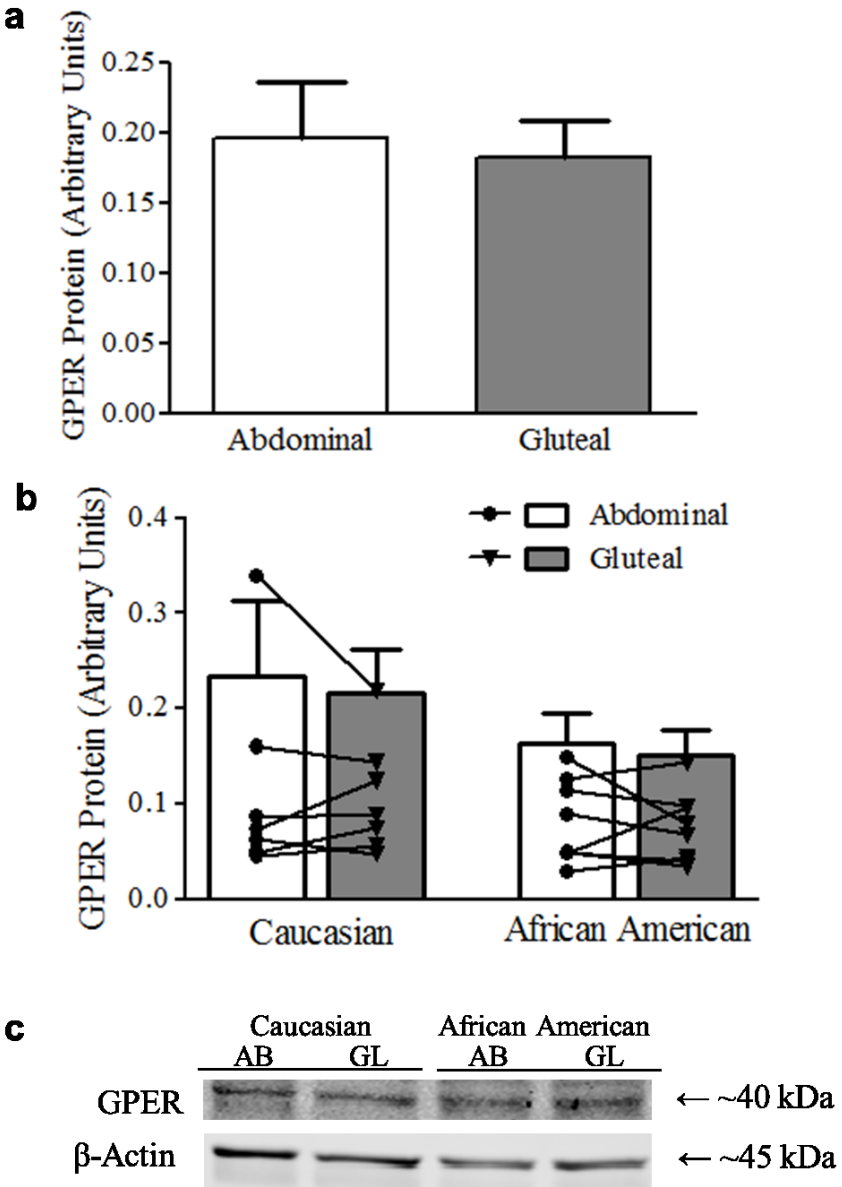

Figure 3.4. G protein-coupled estrogen receptor (GPER) protein content in abdominal and gluteal subcutaneous adipose tissue of overweight-to-obese premenopausal women. (a) Pooled group, n=15. (b) Caucasian (n=7) and African American (n=8) subgroups. (c) Representative western blot for GPER and β-actin. Data are normalized for β-actin and internal control sample and presented as mean ± SE. AB, abdominal; GL, gluteal.

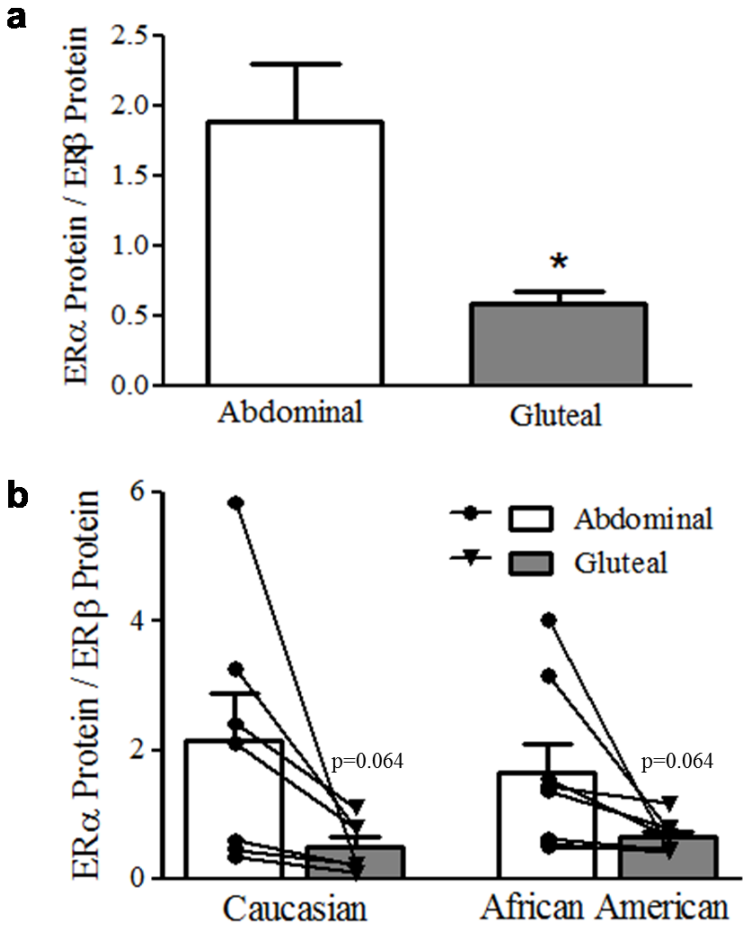

Figure 3.5. Ratio of ERα-to-ERβ protein in abdominal and gluteal subcutaneous adipose tissue in overweight-to-obese premenopausal women. (a) Pooled group, * $p<0.05$ vs abdominal region, n=15. (b) Caucasian (n=8) and African American (n=8) subgroups. Main effect of region p=0.007. Data are mean ± SE.

CHAPTER 4: Abdominal and Gluteal Adipocyte Diameter Distributions are Different in Overweight-to-Obese Caucasian and African American Premenopausal Women

Abstract

Regional adiposity is associated with disease risk, but this relationship is divergent by race. African American women (AA) have more cardiovascular disease risk factors and a higher incidence of Type 2 diabetes and hypertension than Caucasian women (CA) despite accumulating less visceral fat, for a given BMI. The primary purpose of this study was to determine if there are differences in adipocyte size populations (tertiles: small 25-83μm, medium 84-142μm, and large 143-200μm) in subcutaneous adipose tissue (SAT) from the abdominal (AB) and gluteal (GL) regions of overweight-to-obese premenopausal women, and if these regional adipocyte size distributions differ between CA and AA women. There was a trend for an interaction between region and race for mean adipocyte size (CA: AB 0.49±0.04 μg lipid/cell, GL 0.53±0.03 μg lipid/cell; AA: AB 0.59±0.06 μg lipid/cell, GL 0.47±0.04 μg lipid/cell; region x race, p=0.08). CA women had a higher percentage of medium adipocytes in both the AB (51±3% vs 43±3%; p=0.046) and GL regions (50±4% vs 37±4%; p=0.02) compared to AA women. AA women had a higher percentage of small adipocytes in the GL region compared to CA (55±4% vs 44±3%; p=0.044) and a trend for a higher percentage of large adipocytes in the AB region (13±2% vs 7±2%; p=0.065). In light of previous evidence indicating that both small and large adipocytes have been associated with unfavorable metabolic indices, we provide preliminary evidence from a small sample of women in support of the hypothesis that racial

divergence in adiposity related disease risk may be associated with the morphological differences in AB and GL SAT adipocyte size between CA and AA women.

Introduction

Subcutaneous adipose tissue (SAT) serves as the body's major energy storage depot. The size of the SAT depot is in constant flux, with weight gain leading to increases in adipocyte size (hypertrophy) and in some cases adipocyte number (hyperplasia) (233-235). Although not a new concept, the significance of upper versus lower body obesity has recently come to the forefront when trying to understand the health risks associated with increasing adiposity (7-9). People carrying excess adipose in their abdominal region, either subcutaneously or viscerally, tend to have increased risk of cardiovascular disease, diabetes and the Metabolic Syndrome compared to someone of a similar BMI or weight who has increased lower body adiposity (7, 10-13). Some studies have even found gynoid adiposity has a protective effect on disease risk (5, 6, 16-18).

Accumulating adiposity in the subcutaneous depot may not be as benign or favorable as previously believed, at least in regards to people of certain ethnicities. For a given BMI, African American (AA) women tend to accumulate more adipose subcutaneously than viscerally, but have a higher incidence of Type 2 diabetes and hypertension than Caucasian (CA) women (22-24, 28). Further, the relationship between central fat deposition and metabolic risk factors is weaker in African Americans in comparison to Caucasians (23), resulting in a racially divergent association between regional adiposity and disease risk. Inherent phenotypic and morphologic differences in the SAT depots between CA and AA women are likely to be involved in the apparent conflicting role of SAT adiposity in these groups. An elevated inflammatory profile (236) and increased adipocyte insulin resistance in SAT from black compared to white women

(85, 237) are two potential mechanisms behind the negative health outcomes associated with increased SAT in African American women.

Measurements of adipocyte size are commonly used to investigate depot specific differences in adipose tissue characteristics. These studies have established that abdominal subcutaneous adipocyte size is positively associated with unfavorable metabolic indices in both men and women independent of obesity and visceral adiposity (206, 238), while femoral adipocyte size displays a weaker association or no association at all (239).

Recent advances in adipocyte sizing techniques have resulted in the more common use of size distributions as opposed to mean cell size, revealing the complexities of adipocyte size (202, 203, 240, 241). Use of these new techniques have a revealed a role for small adipocytes in increased disease risk factors associated with adiposity, such as insulin resistance and inflammation (202, 203). As such, the use of adipocyte size distributions to determine the proportion of large and small adipocytes in adipose tissue depots is an important tool in establishing morphological differences between adipose tissue depots and between populations of varying disease risk. Therefore the primary purpose of this study was to determine if there are differences in adipocyte size populations in SAT from the abdominal and gluteal regions of overweight-to-obese premenopausal women. A secondary aim was to determine if there are differences in regional adipocyte size distributions between well matched subgroups of Caucasian and African American premenopausal women.

Methods and Procedures

Participants

Seventeen overweight-to-obese premenopausal women, (8 Caucasian/9 African American, 25.9±1.7 years, 81.4±2.3 kg, BMI 29.5±0.5 kg/m^2) between 18 and 39 years old were studied. All but one participant also participated in the studies from Chapters 2 and 3. Enrolled participants were eumenorrheic (average cycle length 30.5±1 days), not taking hormonal contraceptives (no use of hormonal contraceptives for at least 6 months at study entry), weight stable (< 3 kg weight change in the last 6 months) and sedentary, in that they did not engage in purposeful exercise training more than 30 minutes per day more than 2 days per week. Women were excluded from the study if they were pregnant or lactating, a smoker, had history of diagnosis of any metabolic or cardiovascular disease, or were taking any medications known to alter lipid metabolism. Qualification for the study was determined by a pre-participation health history questionnaire completed by the participant and reviewed for accuracy with study personnel. All participants were informed both orally and in writing of the purpose, risks, and benefits of the research and provided informed consent prior to enrollment in the study. This study was approved by the Medical Center Institutional Review Board at East Carolina University. Participant characteristics are presented in Table 4.1.

Body Composition

Participants were weighed on an electronic scale with weight recorded to the nearest 0.1 kg and height was measured with a standard stadiometer to the nearest centimeter (cm). Minimal waist and hip circumferences were measured according to previously published guidelines and waist-to-hip ratio (WHR) was calculated (215). Total and regional fat mass (android, gynoid),

fat-free mass (lean mass + bone mineral content), and total percent body fat were determined using dual-energy x-ray absorptiometry (DXA; GE Lunar Prodigy Advance, Madison, WI). According to the manufacturer recommendations the android region was bound on the lower border by the top of the iliac crest and on the upper border at a distance 20% of the distance between the top of the iliac crest and immediately below the chin; lateral borders were the arm lines. The gynoid region was bound on the upper border at a distance 1.5 times the height of the android region and on the lower border at a distance 2 times the height of the android region; lateral boundaries were the outer leg lines.

Blood samples

Blood was drawn in the morning after an overnight fast (≥ 10 hours) the morning of the day immediately before or after the biopsy visit (baseline blood draw from microdialysis visit in Study 1/Chapter 2). Fasting serum samples of glucose, total cholesterol (TC), triglycerides (TG), and high-density-lipoprotein (HDL) cholesterol were determined using enzymatic/colorimetric methods, and low-density-lipoprotein (LDL) cholesterol was calculated using the Friedewald equation (222). Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the following equation [fasting glucose (mmol/l) x fasting insulin (μU/ml)]/22.5 (223). Serum free fatty acid (FFA) measurements were determined by enzymatic colorimetric methods (Wako Diagnostics USA, Richmond, VA; Intra-assay CV 1.1%, Inter-assay CV 5.6%) performed by the University of Colorado Denver Clinical and Translational Research Center (CTRC) Core laboratory.

Adipose Tissue Biopsy

Participants arrived at the East Carolina Diabetes and Obesity Institute (ECDOI) at the East Carolina Heart Institute between 7-8am after an overnight (≥10hr) fast during the follicular phase of the menstrual cycle (day 2-8 after the start of menses). On average the biopsy visit occurred on day 5±2 of the cycle. Approximately 1 gram of subcutaneous adipose tissue was removed using the needle aspiration technique (14 gauge needle) after local anesthesia by injection of 3-5cc of 1% lidocaine and under sterile conditions. Biopsies were taken from the abdominal (AB), approximately 5cm lateral to the umbilicus, and the upper gluteal (GL) regions, approximately ~5-10 cm from the medial line in the upper quadrant of the buttock. Processing and cell sizing were conducted according to previously published methods (242) with modifications. Fat tissue was immediately rinsed with Krebs Ringer Bicarbonate (KRB) buffer containing 1% bovine serum albumin (BSA) on top of a 70µm cell strainer, clots and blood vessels were then removed. At least 300 milligrams of adipose tissue were transferred into fresh KRB buffer on ice and minced for 10 minutes. Collagenase Type 1 (CLS1 - Worthington Biochemical), 2 mg/mL, was added to the minced tissue and the sample was incubated for 30-60 minutes in a 37°C water bath (100 shakes/min). After collagenase digestion the floating adipocyte layer was washed two times with fresh KRB buffer and stained with 0.1% methylene blue. After five minutes, 200µl of the floating adipocyte layer was pipetted onto a glass slide and photographs of the cell suspension at 100x magnification were captured optically using a microscope at phase contrast with digital photography capability (Nikon Eclipse TS100; Nikon Inc, Melville, NY). Digital photographs containing ≥ 250 adipocytes were saved for subsequent analyses.

The diameter of 250 adipocytes was determined using the US National Institutes of Health (NIH) ImageJ software program (243). The scale was set using a digital photograph of a 1mm stage micrometer taken immediately preceding the adipocyte images for each participant. Twenty-five μm was set as the lower limit for adipocyte size (244). Frequency distributions with 10μm diameter bins were generated for each participant generating a relative frequency distribution for each biopsy sample. Tertiles for adipocyte size were also calculated resulting in sub-groups of small (25-83 μm), medium (84-142 μm), and large (143-200 μm) diameters. Adipocyte size (mean adipocyte weight; μg lipid/cell) was calculated according to Hirsch and Gallian (245) using the formula:

$$\text{Adipocyte size (μg lipid/cell)} = \frac{0.4791}{10^6}\left[3\sigma^2 \cdot d + (d)^3\right]$$

where d = mean diameter and σ = standard deviation and assuming that each cell is a sphere composed of material with the density of triolein (0.915 g/ml). For each adipose sample, adipocyte size (adipocyte weight) was calculated using the mean diameter and standard deviation for that individual sample, a mean value for each racial subgroup and region was calculated and is presented in Table 4.2.

Statistics

Group differences in subject characteristics were determined using two sample independent sample t-tests. Two-way analysis of variance (ANOVA) (2 x 2; race x region) was used to determine race and regional differences in mean adipocyte diameter and size. Two-way ANOVAs (2 x 3) were also used to determine regional (region x adipocyte size subgroup) and racial (race x adipocyte size subgroup) differences in adipocyte size distribution in each the

abdominal and gluteal regions. When indicated by a significant *F* statistic post hoc analyses were conducted with the bonferroni correction. Pearson correlation coefficient was used to determine significant bivariate relationships between mean adipocyte size and baseline circulating characteristics and body composition measurements. The small sample size of the current study limits the statistical power; therefore all analyses should be interpreted with caution and considered preliminary evidence in support of conducting larger investigations in this area in the future.

Results

Participant Characteristics

Seventeen overweight-to-obese premenopausal women took part in the study; there were no mean differences in any baseline participant characteristics between the Caucasian and African American women including BMI, WHR, and total percent body fat. Participant characteristics can be found in Table 4.1.

Mean Adipocyte Diameter and Adipocyte Size

Representative adipocyte photographs used for sizing analyses can be found in Figure 4.1. There were no mean differences in adipocyte diameter by region or race in abdominal and gluteal adipocytes of Caucasian and African American women. Similar results were found when analyses were conducted after calculation of adipocyte size (µg lipid/cell) although there was a tendency for a significant interaction between region and race ($p=0.08$) when data were expressed in this manner. Mean diameter and adipocyte size results can be found in Table 4.2.

Adipocyte Diameter Distribution

Pooled group of all participants. Frequency distribution of adipocyte diameters in the abdominal and gluteal regions in the pooled group can be found in Figure 4.2A. Tertile sub-population analyses revealed a significantly smaller proportion of large diameter adipocytes compared to small and medium diameter adipocytes (Figure 4.2B; main effect of size $p<0.001$). Percentage of adipocytes falling into the small, medium, and large diameter subgroups was similar between the abdominal and gluteal regions (main effect of region $p=0.33$, interaction size x region $p=0.15$).

Racial analyses. Racial comparison of adipocyte diameter frequency distributions from abdominal and gluteal SAT can be found in Figure 4.3. Diameter subgroup comparisons between Caucasian and African American women revealed differences in adipocyte diameter distributions between the races in both the abdominal and gluteal regions. In the abdominal region there was a trend for an interaction between adipocyte size and race (Figure 4.4A, size x race $p=0.064$, main effect of size $p<0.0001$, race $p=0.01$). AA women had a lower proportion of adipocytes in the medium diameter category ($p=0.046$), and tended to have a greater percentage of adipocytes in the large diameter group ($p=0.065$) compared to CA women. In the gluteal region, there was a significant interaction between size and race (Figure 4B, size x race $p=0.008$) with AA women having a higher percentage of adipocytes in the small category ($p=0.037$), and a lower percentage of adipocytes in the medium diameter group ($p=0.02$) compared to the CA women.

Correlations

Correlation analyses were conducted to assess the relationship between regional adipocyte size and anthropometric indices/body composition measurements and baseline circulating factors. Abdominal cell size was directly related to DXA weight (r^2 = 0.45), BMI (r^2 = 0.45), total body fat mass (r^2 = 0.27), and waist circumference (r^2 = 0.32) (all p<0.05). Gluteal cell size was negatively related to plasma FFA (r^2 = 0.40, power=0.82), a relationship primarily driven by the AA women. When separated by race, gluteal adipocyte size in AA women maintained a significant inverse correlation with FFA (r^2 = 0.73, p=0.007) while CA women did not (r^2 = 0.15, p=0.35). Correlation analyses must be interpreted with caution due to the limited sample size; however statistical power was greater than 0.80 for all correlations with exception of the relationships between abdominal cell size and total body fat mass as well as abdominal cell size and waist circumference.

Discussion

This is the first investigation of adipocyte diameter distribution in abdominal and gluteal subcutaneous adipose tissue from overweight/obese premenopausal Caucasian and African American women. The novel finding of this study was that, even when matched for BMI and WHR, the African American women had a higher proportion of small adipocytes (25-83 μm) in the gluteal SAT, and a trend for a higher proportion of large fat cells (143-200 μm) in the abdominal SAT, than the Caucasian women. Conversely, the Caucasian women had an increased proportion of medium sized adipocytes (84-142 μm) in both the abdominal and gluteal SAT compared to the African American women. Increased numbers of both large and small adipocytes can be indicative of an elevated metabolic risk profile (202, 203, 246-250). These

results emphasize the importance of analyzing not only mean adipocyte diameter when characterizing an adipose sample, but investigating the entire adipocyte diameter distribution. Future investigations on larger samples of Caucasian and African American women are warranted to confirm these preliminary findings and gain a better understanding of their physiological importance.

Analyses conducted on mean adipocyte size, the calculation of which takes into account the standard deviation of the mean adipocyte diameter (and therefore the large variation of the data), did show a trend for divergent mean abdominal and gluteal adipocyte size between the CA and AA groups; gluteal adipocytes tended to be larger in Caucasian women and abdominal adipocytes tended to be larger in African American women. Although this finding supports that mean adipocyte size can detect the racial regional differences in studies with sufficient sample size, the mean measurement lacks the detail of a size distribution for reporting distinct morphological differences between the groups, particularly with respect to the large and small adipocyte subgroups.

African American, in comparison to Caucasian, women have less visceral fat for a similar BMI, waist circumference, or waist-to-hip ratio (22-24). Visceral adipose tissue (VAT) volume is generally the depot implicated in increasing disease risk. However, AA have more cardiovascular disease risk factors (27) and a higher incidence of Type 2 diabetes and hypertension than CA women (28) even at the same BMI, despite the fact that more of their adipose accumulation occurs in the subcutaneous depots. This relationship between SAT/VAT and disease risk in AA women represents an inconsistency between traditional beliefs about regional adiposity and disease. The importance of excess abdominal SAT should not be overlooked, particularly in regards to increased insulin resistance risk (29, 30), a point that seems

to be of particular significance in overweight and obese African American women. Insulin sensitivity in black women is most closely associated with the subcutaneous abdominal adipose tissue while in white women the association with insulin sensitivity is equal between visceral and abdominal subcutaneous depots (31, 32). This divergent association between subcutaneous adipose tissue and disease risk between Caucasian and African American women at a given BMI may be related to our finding that women from these racial groups demonstrate different SAT adipocyte diameter distributions.

Maintenance of a healthy or "normal" adipocyte size, as opposed to excessive adipocyte hypertrophy, in SAT may help preserve a less negative association of SAT with insulin resistance and cardiometabolic disease risk factors. An increased proportion of medium sized adipocytes, representing the most benign subgroup of adipocyte, in Caucasian women may be linked to the reduced disease risk associated with SAT, compared to VAT, accumulation in Caucasian women for a given total adiposity. These normal medium size adipocytes are likely insulin sensitive and able to rapidly respond to metabolic demands for storage or mobilization of lipids throughout the day. In contrast, large, or hypertrophied, adipocytes, which tend to be in higher proportion in the AA women, are associated with insulin resistance (205, 246, 248), increased lipolytic rate (97, 204, 251), increased secretion of inflammatory adipokines (249, 250), as well as local adipose tissue macrophage infiltration (252, 253), and even increased cell death (252), all characteristics that may be associated with negative metabolic outcomes. We found abdominal adipocyte size was directly associated with DXA weight, BMI, fat mass, and waist circumference in our women, in support of previous evidence relating increased abdominal adipocyte size to increased cardiometabolic disease risk factors (205, 249, 250). Therefore, an abdominal adipose tissue depot displaying a high proportion of large diameter adipocytes, such

as abdominal SAT of AA women in the current study, would be more likely to be insulin resistant and/or metabolically dysfunctional compared to the abdominal SAT of CA women, which displays a higher proportion of medium diameter adipocytes.

In the gluteal region we found AA women had an increased relative percentage of small adipocytes compared to CA women. Contrary to more classical evidence discussed above, a recent study investigating overweight-to-obese insulin sensitive and insulin resistant, primarily Caucasian, adults found a predominance of small adipose cells in the insulin resistant group while unexpectedly finding no difference in the size of the large adipocytes between the groups (202). In a subsequent study McLaughlin et al. further established that an increased proportion of small (versus large) adipose cells independently predicted expression of inflammatory genes in moderately obese, again primarily Caucasian, adults (203), reemphasizing the importance of cell size on health risk factors. It is possible that the accumulation of small adipocytes in the gluteal region of AA results from an increased need to store triacylglycerol (TAG) despite the inability of the adipocyte to further progress in TAG storage (202). Impaired fat storage capacity or differentiation of small adipocytes may be related to the increased inflammatory profile of small cells (203) and/or the decreased exposure to local adipokines which are secreted from full size (medium size) adipocytes (250), of which there is a reduced proportion in our subgroup of AA women. Interestingly, we found gluteal adipocyte size to be negatively associated with plasma FFA levels, a relationship that was primarily driven by the AA women. Elevated circulating FFAs are related to impaired vascular function, insulin resistance, and elevated risk of Type 2 diabetes (254, 255). Therefore, the increased proportion of small adipocytes in the gluteal region of AA women may be associated with the negative health outcomes apparent in AA compared to CA women of similar obesity status.

There are limitations to the current study, the first of which is that we only characterized adipocyte diameter and size. No mechanistic or cause-and-effect conclusions can be drawn at this time. Although supporting evidence exists, more research needs to be conducted on the phenotypic and biochemical differences between small, medium, and large adipocytes in Caucasian and African American women in determining their relationship with regional adiposity associated disease risk. This study had a small sample size and narrow BMI range, limiting the applicability of our results solely to overweight-to-obese premenopausal women. However, the average BMI of our groups (Caucasian = 28.8 kg/m^2, African American = 30.2 kg/m^2) were similar to that of the current mean age adjusted BMI for women over the age of 20 in the United States, which is 28.7 kg/m^2 (20), indicating our study sample was a good representation of typical American women.

Conclusions

African American women demonstrated an increased proportion of small adipocytes in the gluteal region and a trend for an increased proportion of large adipocytes in the abdominal SAT compared to Caucasian women, who had increased proportions of medium sized adipocytes in both regions. Both large and small adipocytes have been associated with increased insulin resistance and inflammatory markers in previous investigations. This racial divergence in SAT adipocyte morphology may be an underlying contributing factor in the increased CVD risk factors and diabetes incidence in AA compared to CA of the same BMI. Although for investigations with a large sample size mean adipocyte size measurements may be indicative of general racial differences in regional adipocyte morphology, we conclude that characterizations of adipocyte diameter distributions are necessary to fully define the local adipose tissue depot

with respect to small, medium, and large adipocytes. Larger scale studies are needed to confirm these racial differences in adipocyte size distribution and further characterize differences in adipocyte size and its association with cardiometabolic disease risk.

Table 4.1 Participant characteristics

Variable	Pooled	Caucasian	African American
n	17	8	9
Age, yrs	25.9±1.7	25.1±2.3	26.6±2.7
Weight, kg	81.4±2.3	79.9±1.9	82.8±4.1
BMI, kg/m^2	29.5±0.5	28.8±0.4	30.2±0.8
Waist Circumference, cm	88.5±1.1	87.7±1.3	89.3±1.8
WHR	0.79±0.01	0.78±0.02	0.79±0.01
Body fat, %	44.9±1.1	44.2±0.9	45.6±2.0
Total Body Fat Mass, kg	36.8±1.6	35.4±1.5	38.0±2.8
Android Fat Mass, kg	3.0±0.2	3.1±0.2	2.9±0.3
Gynoid Fat Mass, kg	7.0±0.3	7.0±0.3	7.0±0.4
Total Cholesterol, mmol/l	3.9±0.2	3.8±0.2	4.0±0.2
HDL Cholesterol, mmol/l	1.3±0.1	1.3±0.1	1.2±0.1
LDL Cholesterol, mmol/l	2.2±0.2	2.1±0.2	2.4±0.1
Triglycerides, mmol/l #	0.73 (0.56-0.95)	0.79 (0.49-1.27)	0.67 (0.47-0.96)
Free Fatty Acids, uEq/l (n=10)	576 ± 35	541 ± 51	611 ± 47
Fasting Glucose, mmol/l	4.8±0.1	4.8±0.1	4.7±0.1
Fasting Insulin, pmol/l	55.3±5.9	59.8±7.5	50.8±9.3
HOMA-IR	1.7±0.2	1.8±0.2	1.5±0.3

Data are presented as mean±SE unless otherwise indicated. # Skewed variable, re-exponentiated to geometric mean and 95% confidence interval. BMI, body mass index; WHR, waist-to-hip ratio; HDL, high-density-lipoprotein; LDL, low-density-lipoprotein; HOMA-IR, Homeostasis model assessment of insulin resistance.

Table 4.2 Subcutaneous abdominal and gluteal adipocyte diameter size by race

	Adipocyte Diameter (μm)		Adipocyte Size (μg lipid/cell)	
	Abdominal	Gluteal	Abdominal	Gluteal
Pooled (n=17)	90.1±1.8	85.7±2.1	0.55±0.04	0.50±0.03
Caucasian (n=8)	88.6±2.5	89.1±2.1	0.49±0.04	0.53±0.03
African American (n=9)	91.4±2.6	82.7±3.3	0.59±0.06	0.47±0.04

Data are presented as mean±SE unless otherwise indicated.

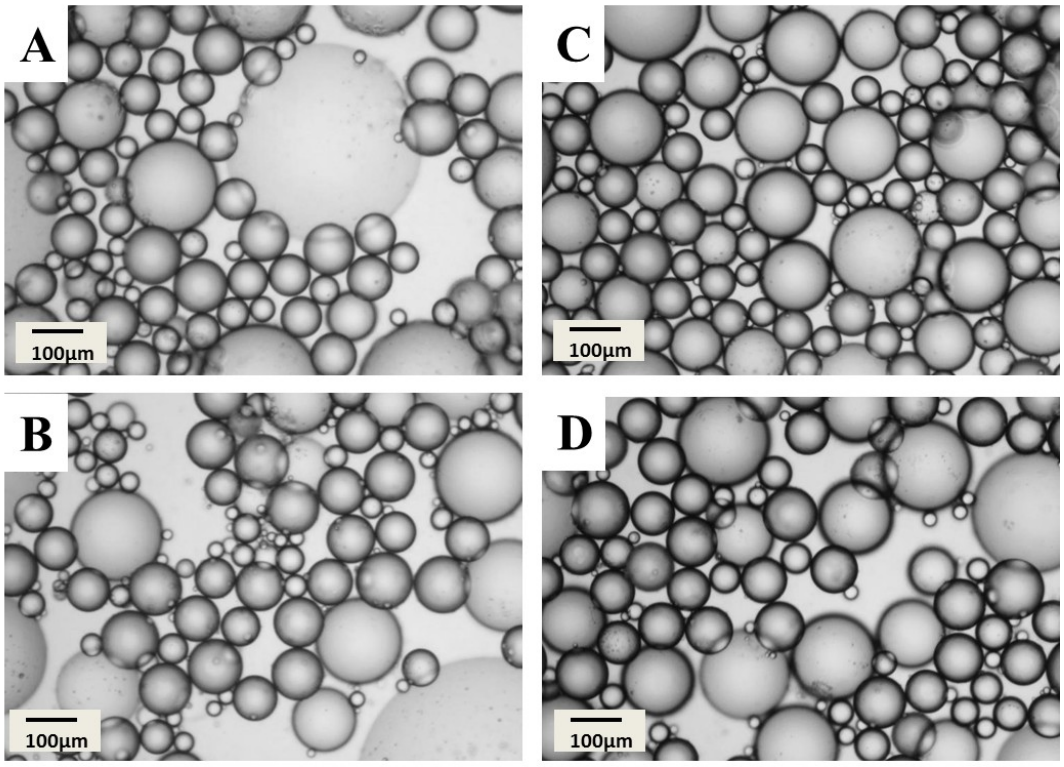

Figure 4.1. Representative images of adipocytes isolated from subcutaneous adipose tissue samples of overweight-to-obese premenopausal women. (A) Caucasian abdominal, (B) Caucasian gluteal, (C) African American abdominal, and (D) African American gluteal. 100x magnification.

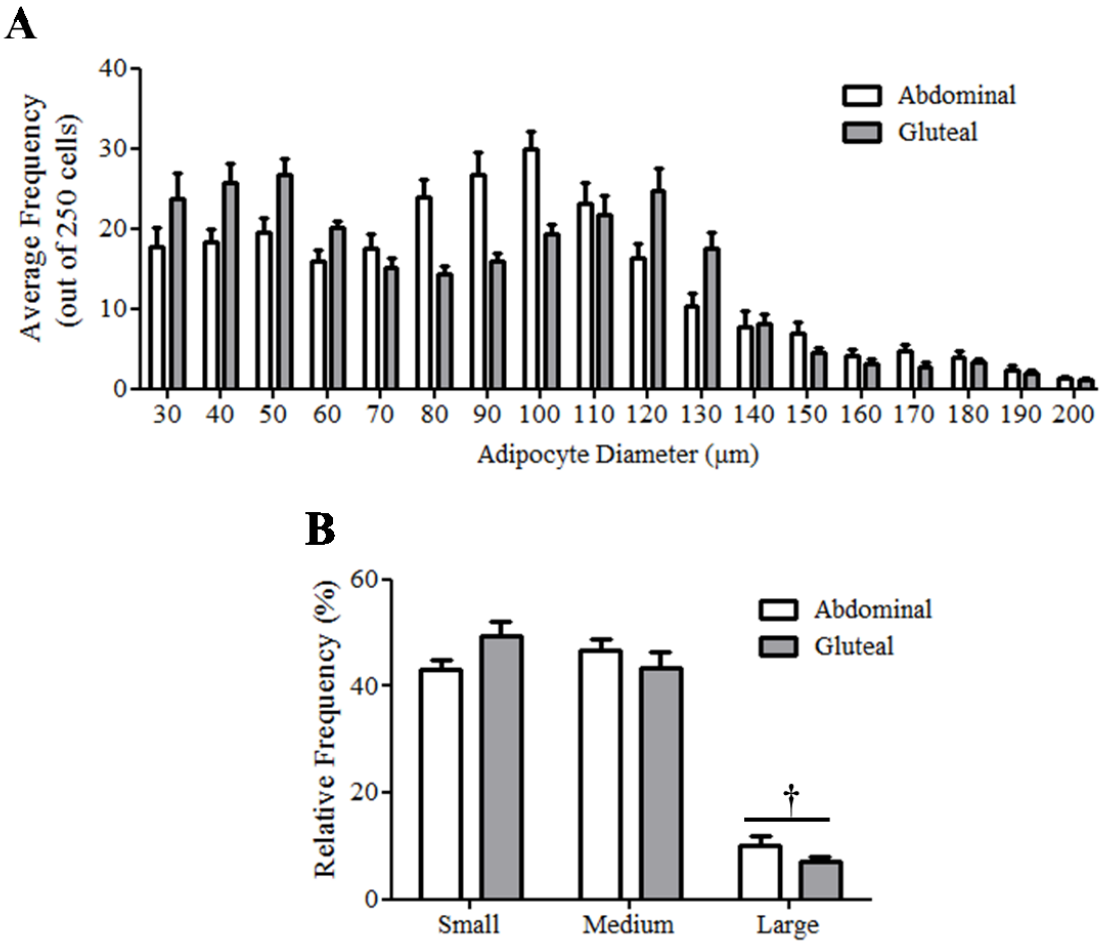

Figure 4.2. Frequency distributions of adipocyte diameters from abdominal and gluteal subcutaneous adipose tissue (SAT) samples from premenopausal women. (A) Average frequency histogram of adipocyte diameters in 10µm bins. (B) Relative frequency (in percent) of adipocytes in small (25-83µm), medium (84-142µm), and large (143-200µm) diameter subgroups in the abdominal and gluteal SAT. Open bars represent abdominal region, shaded (grey) bars represent gluteal region. Main effect of size $p<0.0001$. † $p<0.001$ vs small and medium subgroups; n=17. Data are mean ± SE.

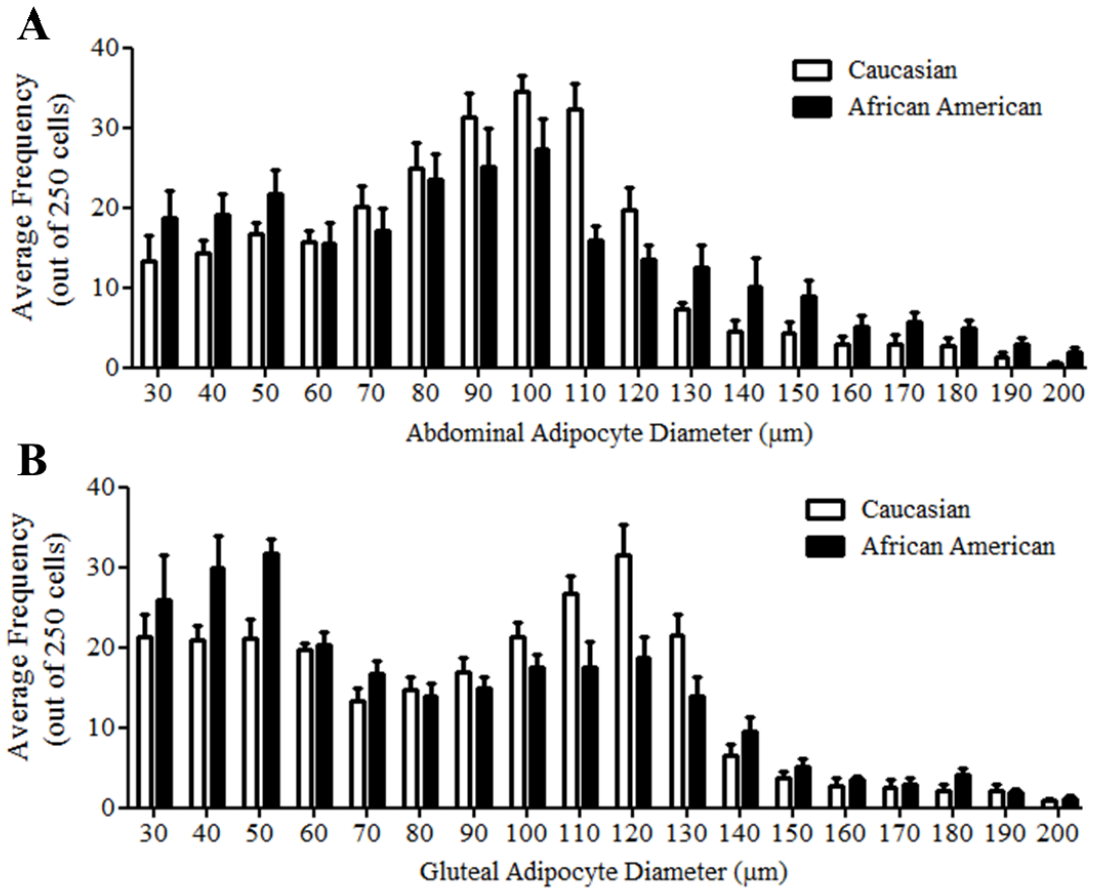

Figure 4.3. Racial differences in the frequency distribution of adipocyte diameters from (A) abdominal and (B) gluteal SAT samples of Caucasian and African American premenopausal women. Open bars represent Caucasian group, shaded (black) bars represent African American group. Diameter range 25µm-200µm; bins are 10µm. n = 8 Caucasian; n = 9 African American. Data are frequency out of 250 cells mean ± SE.

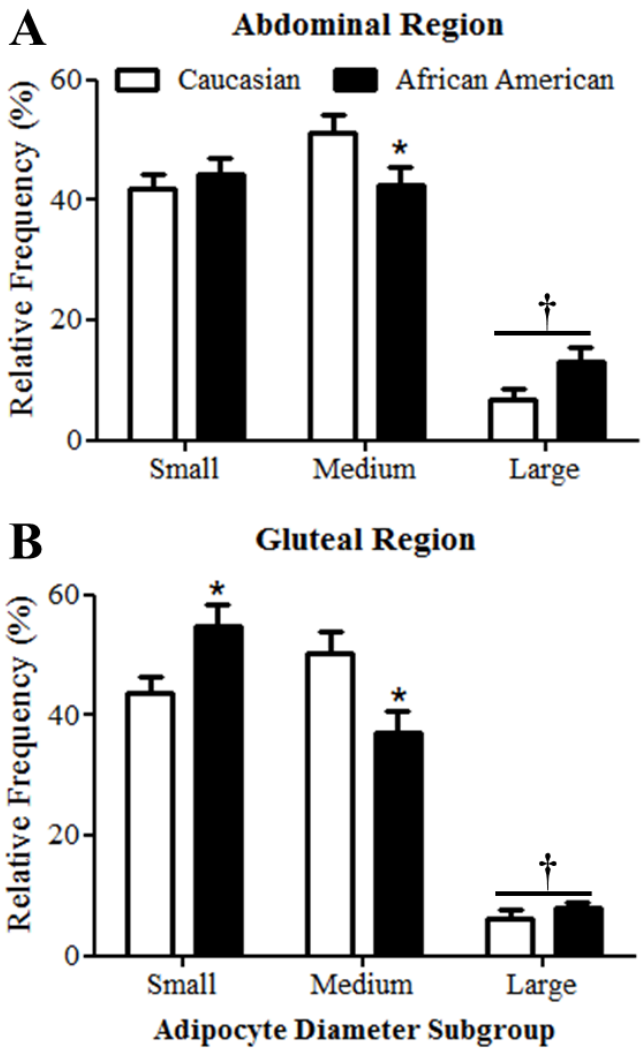

Figure 4.4. Relative frequency (in percent) of adipocytes in small (25-83µm), medium (84-142µm), and large (143-200µm) diameter subgroups in the (A) abdominal and (B) gluteal SAT from Caucasian and African American premenopausal women. (A) Size x race interaction $p=0.064$, main effect of size $p<0.0001$, race $p=0.01$. (B) Size x race interaction $p=0.03$, main effect of size $p<0.0001$, race $p=0.3622$. Open bars represent Caucasian group, shaded (black) bars represent African American group. * $p<0.05$ vs Caucasian in same subgroup, † $p<0.001$ vs small and medium subgroups; n = 8 Caucasian, n = 9 African American. Data are mean ± SE.

CHAPTER 5: Integrated Discussion

Effective regulation of adipose metabolism in the human body is a process that is essential for the maintenance of health. The study of obesity has garnered great attention for the multitude of implications for chronic diseases risk as well as the status of obesity as a modifiable disease risk factor. The mechanisms underlying regional adiposity at a most basic level are not completely understood. One of the classic examples of region specific adiposity is the sex divergent body fat distribution of men and premenopausal women. What makes this sex specific difference even more interesting is the shift from a preferential gynoid body fat distribution in premenopausal women to a more central body fat distribution after the menopausal transition (36). This shift in regional adiposity is of particular importance because increasing central adiposity is highly associated with increased risk of cardiovascular disease, the Metabolic Syndrome, Type 2 diabetes, and even some types of cancer (1, 3, 4). Although estrogen is implicated as a major player in determining body fat distribution, the mechanisms behind the estrogenic action(s) in adipose tissue of women are still unclear. Discovery of estrogen receptors as well as estrogen producing enzymes such as aromatase and 17β-HSD in human adipose tissue supports the hypothesis that estrogen has direct actions within adipose tissue and that concentrations of circulating estrogens are not necessarily representative of estrogen action at the local level (114).

The literature review in Chapter 1 suggests that one mechanism by which estrogen may influence adiposity in women is through effects on local lipolytic rate; the rate at which free fatty acids are mobilized from triacylglycerol storage in adipose tissue for fuel utilization in skeletal

muscle, liver and the heart. However, results from previous investigations in animal models and humans conflict in regards to conclusions about the pro-lipolytic or anti-lipolytic nature of this effect, nor do they clearly demonstrate if estrogen's influence occurs in a region specific manner to help maintain the preferential premenopausal gluteal-femoral body fat distribution. The global aim of studies 1, 2, and 3 (discussed in Chapters 2, 3, and 4 respectively) was centered on determining how local estrogens influence regional lipolysis in overweight-to-obese premenopausal women and how characteristics, such as estrogen receptor content and adipocyte size, of abdominal and gluteal adipose tissue may influence regional lipid metabolism. Furthermore, we addressed whether the effects of estrogen and regional adipose tissue characteristics were different in well-matched subgroups of Caucasian and African American women.

In study 1 (Chapter 2) we utilized the *in situ* microdialysis technique to determine the effect of increasing local subcutaneous adipose tissue estradiol concentrations on basal and stimulated/disinhibited lipolytic conditions in both the abdominal and gluteal regions of premenopausal women. We found that estrogen perfusion influenced stimulated lipolytic rate (as indicated by glycerol release) in both pro- and anti-lipolytic manners depending upon the stimulatory/disinhibitory conditions and adipose tissue depot. Estrogen served to blunt lipolytic stimulation by isoproterenol perfusion (β-adrenergic agonist) in the abdominal but not gluteal region. Paradoxically estrogen enhanced lipolytic response to 30 minutes of submaximal cycle ergometry exercise during isoproterenol and phentolamine (α-AR antagonist) perfusion in the abdominal region, but blunted lipolytic response in the gluteal region under the same conditions. These results demonstrate a clear region and treatment dependent effect of estradiol on *in vivo* lipolysis, potentially influencing upper and lower adiposity in premenopausal women. The

responses to estradiol perfusion were consistent between Caucasian and African American women, except for a tendency for racial differences in the effect of E_2 on basal lipolysis. These preliminary racial analyses indicate differential effects of estradiol on lipolysis may not be an underlying mechanism behind differences in body fat distribution in Caucasian and African American women, however further research needs to be conducted to confirm these results in a larger group of women.

Study 2 (Chapter 3) focused on region specific expression of the three known estrogen receptors in adipose tissue, ERα, ERβ, and GPER. Differences in abdominal and gluteal ER protein content were previously unknown. We found that ERα and ERβ protein both differed between the abdominal and gluteal SAT. GPER protein content, measured for the first time in human adipose tissue, was similar between the regions and between CA and AA women. ERα protein was higher in abdominal compared to gluteal, and ERβ protein was higher in gluteal compared to abdominal in both CA and AA women. ERα and ERβ have independent functions within adipose tissue, although knockout models have suggested that ERα confers the primary role of adiposity regulation (133, 135, 136), ERβ may repress or counteract the actions of ERα. Therefore, not only are the regional differences in the content of these receptors of significance, but also are the regional differences in the resultant ERα-to-ERβ ratio (127, 135). We also found that waist-to-hip ratio is inversely related to gluteal ERβ protein content and positively related to the ERα-to-ERβ ratio in the gluteal region. These data suggest it is possible that estrogen acting through ERβ (or decreased action through ERα) may be related to increased gluteal adiposity. This is the first evidence that there are regional differences in estrogen receptor protein content in SAT of women. It is possible that this regional difference in ER content may be involved in estrogen's region specific effects and potential modulation of regional adiposity, however, future

studies investigating ER signaling within abdominal and gluteal adipose tissue depots must be conducted before definitive conclusions can be drawn.

In study 3 (Chapter 4) we utilized measurements of mean adipocyte size and adipocyte diameter distribution to preliminarily characterize regional and racial (CA and AA) differences in adipocyte morphology. Although traditionally, according to association with disease risk factors, accumulating adiposity subcutaneously (in the abdominal, but more so, gluteal region) is considered preferable over increasing visceral adipose, this relationship is not apparent in obese AA women. AA women accumulate less VAT/more SAT but have increased incidence of Type 2 diabetes and hypertension compared to CA women for the same fat mass or BMI (23, 24). Both large and, more recently, small adipocytes have been implicated in contributing negatively to adipose tissue function and overall health (202, 203, 206, 238). Our analysis of mean adipocyte size did not reveal a difference between the abdominal and gluteal region in the pooled population but did expose a tendency for divergence by race in adipocyte size by region; with AA adipocytes larger in the abdominal than gluteal region and CA adipocytes larger in the gluteal than abdominal region. Adipocyte diameter distribution analyses broken down into tertiles allowed for a more detailed analysis of these racial differences. We found CA women to have an increased proportion of medium sized adipocytes (84-142μm) in both the abdominal and gluteal regions in comparison to AA women. On the other hand, AA women had an increased proportion of small adipocytes (25-83μm) in the gluteal region, and a trend for an increased proportion of large adipocytes in the abdominal region (143-200μm) compared to CA. These results indicate a clear difference in adipocyte morphology in CA and AA women that may underlie the divergent race associated disease risk with SAT accumulation between these two

races, however investigations on larger groups of CA and AA women must be conducted to confirm these preliminary findings.

In summary, the results of this project support the hypothesis that estrogen affects lipolysis through alterations in β-adrenergic stimulation as well as a potential non-adrenergically mediated mechanism, in a region specific manner. Furthermore, abdominal and gluteal differences in ERα and ERβ protein could underlie region specific actions of estrogen, possibly playing a regulatory role in the preferential accumulation of adipose tissue in the gynoid SAT in premenopausal women. Finally, morphologic differences in adipocyte size may be related to racial differences in disease risk associated with SAT adiposity in CA and AA women. All three of these findings are integral to gaining a more complete understanding of the regulation and characteristics of upper and lower body SAT in premenopausal women.

Although these results give further insight into the effects of estrogen within adipose tissue a significant amount of research is still necessary to clearly understand the multifaceted actions of estrogen within adipose tissue. It will be important for future investigations to clearly delineate the mechanisms through which estrogen influences lipolysis in each specific adipose depot. Although effects of estradiol on β-AR stimulation of lipolysis were confirmed here, actions through the α-AR receptors as well as potentially non-adrenergically mediated mechanisms such as ANP, insulin, adenosine, and cortisol (among others) must be considered and investigated thoroughly. Examination of direct estrogen action on the post-receptor lipolytic signaling cascade is also needed to fully uncover the mechanisms through which estradiol may influence lipolysis. In addition, determining the adipocyte sub-cellular distribution of ERα, ERβ, and GPER and the receptor specific signaling cascades that are initiated upon activation of these receptors by estrogen will help to connect regional differences in these receptors with tissue

specific effects of estrogen. Finally, preliminary racial analyses presented here provide support for the importance of future studies investigating racial differences in adipocyte phenotype and biochemical processes which may underlie racial differences in prevalence of obesity and disease risk.

The presence of estrogen converting enzymes as well as estrogen receptors within the adipose tissue of pre- and post-menopausal women leads to the hypothesis that there is a specific physiological purpose of the presence of estrogen within adipose tissue. It is possible that the distinctive pre- and post-menopausal regional adiposity patterns occur for a specific evolutionary reason and that direct influence of estrogen within adipose tissue on lipid metabolism are necessary to maintain health throughout the lifespan. Continuing investigations which will uncover the mechanisms underlying depot and pathway specific estrogen action within adipose tissue of both pre- and post-menopausal women is vital for our complete understanding of female health and regional adiposity related disease risk.

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APPENDIX: Medical Center Institutional Review Board at East Carolina University Human Subject Approval Letter

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board Office
1L-09 Brody Medical Sciences Building• 600 Moye Boulevard • Greenville, NC 27834
Office **252-744-2914** • Fax **252-744-2284** • **www.ecu.edu/irb**

TO: Kathleen Gavin, MS, Department of EXSS, ECU, 363 Ward Sports Medicine Building, Mailstop #158

FROM: UMCIRB JTC

DATE: May 2, 2011

RE: Full Committee Approval of a Study

TITLE: "Role of Subcutaneous Adipose Tissue Estradiol in Regional Lipoliysis in Premenopausal Women"

UMCIRB #11-077

The above referenced research study was initially reviewed by the convened University and Medical Center Institutional Review Board (UMCIRB) on **2/9/11.** The research study underwent a review and approval of requested modifications on **2/14/11** by expedited review. The UMCIRB deemed this **unfunded** study **more than minimal risk** requiring a continuing review in **12 months**. Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

The above referenced research study has been given approval for the period of **2/9/11** to **2/8/12**. The approval includes the following items:

- Internal Processing Form (revised, dated 2/14/11)
- Protocol (dated 1/19/11)
- Protocol summary
- Informed consent (revised, dated 2/14/11)
- COI disclosure form (dated 1/25/11)
- Recruitment flyer
- 3 day food record/instructions
- Medical history questionnaire

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study:
R. Hickner

NOTE: The following UMCIRB members with a potential Conflict of Interest did not attend this IRB meeting:
None

The UMCIRB applies 45 CFR 46, Subparts A-D, to all research reviewed by the UMCIRB regardless of the funding source. 21 CFR 50 and 21 CFR 56 are applied to all research studies under the Food and Drug Administration regulation. The UMCIRB follows applicable International Conference on Harmonisation Good Clinical Practice guidelines.