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IN VITRO STUDIES OF METABOLISM OF FAT CELLS ISOLATED FROM BLACK AND WHITE OBESE SUBJECTS

E.P. BUTHELEZI

IN VITRO STUDIES OF METABOLISM OF FAT CELLS ISOLATED FROM BLACK AND WHITE OBESE SUBJECTS

ERNEST PHILANI BUTHELEZI

A dissertation submitted to the Faculty of Health Sciences of the University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

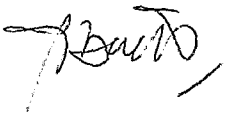
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ABSTRACT

The study aimed to quantify differences in lipid metabolism and insulin sensitivity in black and white subjects to explain ethnic clinicopathological differences in type 2 diabetes. The *in vitro* inhibitory effect of insulin on lipolytic activity and of its stimulating effect on glucose uptake by adipocytes isolated from obese black and white women were investigated. Fasting plasma levels of insulin and free fatty acid (FFA) in black and white women were 67±1pmol/l vs. 152±4pmol/l ($p<0.01$); 890±131μmol/l vs. 425±4 μmol/L ($p<0.01$) respectively. Isolated femoral adipocytes from white women were more responsive to insulin than those from black women with 0.72nmol/l causing a 55±4% inhibition of isoprotorenol-stimulated lipolysis compared to a 27±10% inhibition in black women ($p<0.01$). Furthermore, isolated femoral adipocytes from white women showed improved glucose uptake (247±36%) than those from black women (128±28%) in the presence of 7.0nmol/l insulin ($p<0.05$). The low responsiveness of adipocyte lipolytic activity to insulin in black women in the presence of relatively low insulin levels, may account for the high plasma FFA levels observed in these women which may, in turn account for their higher *in vivo* insulin resistance. It is proposed that the pathogenesis of glucose intolerance within the black obese population is strongly influenced by their adipocyte metabolism.

I, Ernest Philani Buthelezi, declare that this dissertation is my own work and has not been submitted or incorporated in another dissertation or thesis for any other degree.

E. P. BUTHELEZI



The work reported in this dissertation was carried out in the School of Pathology, Department of Chemical Pathology, of the University of the Witwatersrand, Johannesburg, South Africa.

In memory of my late parents, Flora and Albert Buthelezi

and

my aunt Annacletta and my late uncle Caleb Ndaba

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TABLE OF ABBREVIATIONS

°C	-	degree celsius
µL	-	microlitre(s)
µm	-	micrometre(s)
aP2	-	acid binding protein 2
ApoC	-	apolipoprotein C
ASP	-	asylation stimulating protein
ATP	-	adenosine triphosphate
BMI	-	body mass index
C	-	complement
C_3	-	complement 3
cAMP	-	cyclic adenosine monophosphate
cm^3	-	cubic centimetre(s)
CoA	-	coenzyme A
DMEM	-	Dulbecco's modified Eagle's medium
DNA	-	deoxyribunucleic acid
ED_{50}	-	50% effective dose
EDTA	-	ethylene diaminetetraacetate
ELISA	-	enzyme-linked immunosorbent assay
ER	-	endoplasmic reticulum
ERK	-	extracellular signal-regulated protein kinase
FFA	-	free fatty acids
g	-	unit of gravity
GAP	-	GTPase-activating protein

GLUT 2	-	glucose transporter 2
GLUT 4	-	glucose transporter 4
GRB 2	-	growth factor receptor-bound protein 2
Hck	-	haematopoietic cell kinase
HBSS	-	Hank's balanced salt solution
HDL	-	high density lipoprotein
Hepes	-	N-2-Hydroxyethypiperazine-N'-2-ethane sulphonic acid
HSL	-	hormone-sensitive lipase
IRS-1	-	insulin receptor substrate-1
IU	-	International unit
K_m	-	Michaelis constant
LDL	-	low density lipoprotein
LPL	-	lipoprotein lipase
M	-	Molar
meq	-	milliequivalent
ml	-	millilitre(s)
mol	-	mole(s)
mol wt	-	molecular weight
M_r	-	relative molecular mass
mRNA	-	messenger RNA
NAD	-	nicotinamide adenine nucleotide
NADH	-	nicotinamide adenine dehydrogenase
No.	-	number
NPY	-	neuropeptide Y

N.S.	-	not significant
P	-	probability
Pi	-	pyrophosphate
PFK-I	-	phosphofructokinase-1
PI3-Kinase	-	phosphatidylinositol-3-kinase
PK	-	pyruvate kinase
PKA	-	protein kinase A
PPAR-γ	-	peroxisome proliferation activator receptor-γ
PPP	-	pentose phosphate pathway
r	-	correla ıon coefficient
RIA	-	radioimmunoassay
RNA	-	ribunucleic acid
rpm	-	revolutions per minute
SD	-	standard deviation
SEM	-	standard error of the mean
Sx	-	standard error of the mean
SH2	-	Src homologous protein 2 domain
SH3	-	Src homologous protein 3 domain
SHC	-	Src homologous protein C
Sos	-	son of sevenless homologue
sp act	-	specific activity
$t_{1/2}$	-	half-life
TCA	-	trichloroacetic acid
TG	-	triglyceride
TNF-α	-	tumour necrosis factor alpha

TNFR1	-	tumour necrosis factor receptor 1
TZD	-	thiazolidinedione
VLDL	-	very low density lipoprotein
V_{max}	-	maximum velocity
vol	-	volume

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CHAPTER 1

Overview and Hypothesis

Obesity is a major cause of morbidity and mortality in many societies (Kissebach and Krakower, 1994), and is associated with an increased risk of chronic diseases such as type 2 diabetes and cardiovascular disease (Macor *et al* ,1997; Ferannini,1995). In South Africa the incidence of obesity (body mass index >30kg/m^2) is in the order of 31-54% for black and white adult women (Steyn *et al*, 1995; Roussouw *et al*, 1983).

Studies of obese black and white women in South Africa have shown that there are distinct differences between these two groups in terms of the incidences of diseases that are usually associated with obesity. Black women have higher incidences of hypertension and type 2 diabetes (Seedat, 1983) than white obese women who have a higher incidence of ischaemic heart disease (Walker *et al*, 1993).

Clinical studies performed in South Africa on obese black and white women have shown major metabolic differences between these two groups of women. Obese black women had higher fasting plasma free fatty acid (FFA) and lower insulin concentrations compared with their white counterparts (van der Merwe *et al*, 1996). Based on the degree of weight loss, it appeared that the *in vivo* lipolytic rate of the black women was higher than that for the white women.

These observations have subsequently been confirmed during microdialysis studies of the subcutaneous adipose tissue (van der Merwe *et al,* 1998).

It was not clear from these studies whether the higher lipolytic rate observed in black women was due to their lower insulin levels, or resistance to the antilipolytic effect of insulin. It has also not been established whether regional differences in the lipolytic rate between the gluteo-femoral and abdominal areas in the black women can be explained by differences in tissue sensitivity to insulin.

This study was designed to quantify differences in lipid metabolism and insulin sensitivity in black and white obese subjects to explain ethnic clinicopathological differences in the obese state and in type 2 diabetes. *In vitro* lipolytic activity of adipocytes isolated from black and white obese women were assessed. Adipocytes were also examined for their rate of glucose uptake using different concentrations of insulin.

CHAPTER 2

Literature Review

2.1 The Adipose Tissue

Adipose tissue is a complex, organised structure in which adipocytes interact with the vascular and nervous systems, resulting in a high degree of co-ordination of metabolic activities.

The main function of adipose tissue is to store energy in the form of triglycerides. During periods of starvation triglycerides are hydrolysed to release free fatty acids, which are used as an alternative energy source to conserve depleted glucose levels.

In the adipose tissue triglycerides are formed by the reaction of two molecules of fatty acyl coenzyme A (acyl-CoA) with glycerol-3-phosphate to form phosphatidate, which is dephosphorylated to diacylglycerol then acylated by a third molecule of acyl-CoA to yield a triglyceride molecule.

Insulin and acylation stimulation protein (ASP) are principal determinants of the rate of fatty acid uptake (Cianflone *et al*, 1989). Adipocytes synthesize triglycerides from the fatty acids delivered to them by the triglyceride-rich lipoproteins, chylomicrons, and very low density lipoproteins (VLDLs).

Adipocytes synthesize and secrete the three proteins of the alternate complement pathway v.i.z. the third component of complement (C3), factor B and factor D (or adipsin) (Sniderman *et al*, 1998). These proteins interact and produce a fragment of C3 which is processed to produce an ASP molecule. ASP (as a stimulator of triglyceride synthesis) achieves its effects by increasing the activity of diacylglycerol acyltransferase (Yasruel *et al*, 1991), the last enzyme involved in the synthesis of a triglyceride molecule. ASP also increases specific membrane transport of glucose by stimulating the translocation of glucose transporters from the cytosol to the cell membrane (Sniderman *et al*, 1998).

To trap dietary fatty acids in adipocytes, first, the fatty acids must be released from the chylomicron triglyceride by the action of lipoprotein lipase (LPL); next, these fatty acids must be taken up by the adipocyte and incorporated into triglyceride (Sniderman *et al*, 1998). The rate at which LPL can hydrolyse lipoprotein triglycerides is determined by the number of active LPL molecules in contact with the particle, and by the rate at which fatty acids that are liberated can be removed from the capillary environment. Fatty acids that do not enter the adipocytes enter the general circulation, from which they reach the liver.

Triglycerides that are stored in adipose tissue are mobilised by the action of adrenaline or glucagon through a hormone-sensitive lipase (HSL). The fatty acids released by this enzyme bind to serum albumin and are carried in the

blood to the tissues that use fatty acids for fuel. Increased cortisol and growth hormone (GH) levels caused by stress may result in increased lipolysis in adipose tissue (Newsholme and Leech, 1994), but the mechanism is not known.

Another function of adipose tissue is to provide insulation against cold temperature and to increase body temperature by dissipating stored energy as heat.

Adipocytes also secrete factors known to play a role in immunological responses, vascular disease, and appetite regulation. These include TNF-α, plasminogen activator (PAI) –1, macrophage migration inhibitory factor, leptin and adiponectin (Kihara *et al,* 1998).

2.2 Control of Lipolysis and Lipogenesis

A wide range of hormones and other substances have been recognised as regulators of lipolysis and lipogenesis, but insulin and the catecholamines appear to be the most important.

2.2.1 Insulin

Postprandially lipid metabolism is under the control of insulin which suppresses the release of FFA from adipose tissue, and inhibition of VLDL-triglyceride secretion from the liver.

Lipolysis of circulating lipoprotein-triglyceride via lipoprotein lipase (LPL) occurs in the capillary lumen prior to cellular uptake of fatty acids. The fatty acids transported into the cell as a result of this process may either be re-esterified into triglyceride or immediately oxidized. Re-esterification is dependent on a supply of glycerol-1-phosphate from glycolysis and therefore is dependent on glucose uptake (Björntop, 1994).

Insulin activates LPL, which is the rate-limiting enzyme for chylomicron-triglyceride hydrolysis. LPL is bound to the luminal side of the capillary endothelium, and is activated by circulating plasma apolipoprotein C-II (apoC-II). ApoC-II is synthesized in the liver and small intestine and is found predominantly in chylomicrons, VLDL and HDL (Staels *et al*, 1994).

Insulin inhibits intracellular HSL, the rate-limiting enzyme in the hydrolysis of stored triglycerides, by inhibiting cAMP production by adenyl cyclase thereby preventing phosphorylation of cAMP-dependent protein kinase A (PKA). Insulin also promotes re-esterification of fatty acids within adipose tissue by increasing fatty acyl synthase activity.

2.2.2 Catecholamines

Catecholamines regulate fat cell function by stimulating three subtypes of β-adrenergic receptors ($\beta_{1,2,3}$). The adrenergic system plays a key role in regulating energy balance through the stimulation of thermogenesis and lipid

mobilisation in brown and white adipose tissues in humans and various other mammals (Gagnon *et al*, 1996).

Using agonists for β_1 and β_2 it has been shown that β_1 and β_2-adrenergic receptors are involved in sympathetically-mediated thermogenesis (Blaak *et al*, 1993). β_2 agonists have been reported to increase thermogenesis in rats (Holloway *et al*, 1991) and humans (Henny *et al*, 1987). Three groups of investigators reported a missense mutation in codon 64 of the human gene for the β_3 -adrenergic receptor (Jequier, 1996); however, there is no evidence that the mutation is more frequent among obese subjects and the activity of β_3 -adrenegic receptor has not been studied in these patients (Arner, 1995). α_1- and α_2-adrenergic receptors are of minor significance in mediating the thermogenic response to catecholamines.

Lipolysis is stimulated by the β -adrenergic adenyl cyclase-coupled receptors. The receptor-controlled increment of intracellular cAMP concentrations promotes activation of cAMP-dependent PKA, which phosphorylates a serine residue on the HSL and promotes its activation and its translocation towards the lipid droplet (Lafontan and Berlan, 1993). A newly discovered family of phosphoproteins, perilipins, may also be involved in the process (Londos *et al*, 1996).

2.2.2 Glucocorticoids

Glucocorticoids are known to increase the sensitivity of adipose tissue to catecholamines, increasing lipolysis (Bjöntorp, 1994) and to blunt the antilipolytic effect of insulin. The expression of LPL is also increased in glucocorticoids (Appel and Fried *et al*, 1992). Visceral fat is more responsive to glucocorticoids due to high level of expression of glucocorticoid receptors in the fat depot (Rebuffé-Scrive *et al*, 1985) and this may explain the high level of visceral fat deposition observed in Cushing's Syndrome patients.

2.3 Receptor Binding and Insulin Signal Transduction

Insulin acts on cells to stimulate glucose, protein and lipid metabolism by modifying the activity of a variety of enzymes and transport processes. Binding of insulin to the receptor is followed by endocytic internalisation of the hormone-receptor complex. This requires the presence of specific amino acids in the cytoplasmic portion of the ß-subunit adjacent to the membrane and functionally normal receptor kinase (see Figure 1). Once internalised, some receptors are degraded, but most are recycled to the cell surface (Möller and Flier, 1991).

The insulin receptor is made up of two heterodimeric glycoprotein subunits consisting of one α-subunit and one ß-subunit. Insulin binds to the α-subunit of the receptor and this is thought to cause the autophosphorylation of tyrosines at positions 1146, 1150, and 1151 on the ß-subunit (Kahn and White,

1988). This stimulates the tyrosine kinase activity of the receptor by increasing the catalytic velocity (V_{max}). Cellular substrates for the insulin receptor tyrosine kinase (Roth *et al* 1992) include insulin receptor substrate 1 (IRS-1) (Sun *et al*, 1991), SHC (Pellici *et al*, 1992; Kovacina and Roth,1993), pp120/ecto ATPase (Margolis *et al*, 1993), pp62 (Sung *et al*, 1994) and the Ras GTPase-activating protein (GAP).

2.4 Src Homology 2 (Sh2) Domains In Insulin Signalling

SH2 domains are protein domains containing ~100 amino acids that share homology with a particular noncatalytic region of the *src* protooncogene product (Xu *et al*, 1997). The SH2 domains mediate the formation of specific protein complexes (Pawson, 1997), and are found in a range of cytoplasmic signalling proteins including those involved in growth factor signalling (Quon *et al*, 1994).

Src homology 3 (SH3) domains are protein domains containing ~ 50 amino acids that share homology with a particular noncatalytic region (distinct from SH2 domain) on the *src* gene product (Quon *et al*, 1994). The biochemistry of the SH3 domains is not as well understood as SH2 domains. However, SH3 domains are known to bind proline-rich sequences (see Figure 1)and may have a role in targeting proteins to specific subcellular locations (Bari-Sagi *et al*, 1993).

Insulin receptor signalling to SH2-containing proteins can occur via substrates of the insulin receptor such as IRS-1 and Src homology protein C (SHC). When these substrates are phosphorylated by the receptor kinase, they are able to interact with signalling molecules containing SH2 domains. Binding interactions with SH2 domains provide a means to regulate SH2-containing proteins. In the case of phosphatidylinositol 3-kinase (PI 3-kinase) (see Figure 1), binding of the SH2-containing regulatory subunit, p85α of PI 3-kinase to IRS-1 causes activation of the PI 3-kinase catalytic subunit, p110. Binding of growth factor receptor-bound protein 2 (GRB2) to IRS-1 facilitates the formation of signalling complexes with downstream elements such as son of sevenless homologue (Sos) via interactions with SH3 domains on GRB2 (see Figure 1). GRB2 and p85α bind IRS-1 only after it is phosphorylated.

2.5 Mitogenic-Activated Phoshatidylinositol (MAP) Kinase Phosphorylation Cascade in The Mitogenic and Metabolic Actions of Insulin

Insulin binding to its own receptor causes an increase in activity of cellular concentrations of GTP-activated Ras leading to the stimulation of a serine-threonine kinase cascade that involves the proteins Raf-1 kinase, mitogenic extracellular protein kinase (MEK), MAP kinase and RSK S6 kinase (see Figure 1). The Ras-MAP kinase triggers the phosphorylation of transcription factors that regulate gene expression, cell growth and differentiation. MAP kinase also phosphorylates RSK S6 kinase resulting in phosphorylation of

protein phosphatase 1 and subsequent dephosphorylation and activation of glycogen synthase.

2.6 Insulin-Stimulated Glucose Transport

The ability of insulin to stimulate glucose transport involves a series of subcellular events. Glucose transporter 4 (GLUT4) is predominantly responsible for insulin-mediated glucose transport in muscle and adipose tissue (Shepherd and Kahn, 1993, Abel *et al*, 1995). The activation of glucose transport is associated with the translocation of GLUT 4 containing vesicles from an intracellular pool to the plasma membrane (Holman *et al*, 1994).

PI 3-kinase, is reported to be the signalling candidate in the pathway to GLUT 4 translocation, given the sequence similarity of the p110 catalytic subunit with yeast Vps34 protein that is involved in vesicle exocytosis (Schu *et al*, 1993). Also, studies using compounds that inhibit PI 3-kinase confirmed the association of PI-3 kinase and GLUT 4 translocation (Cheatham *et al*, 1994; Clarke *et al*, 1994; Gould *et al*, 1994; Okada *et al*, 1994).

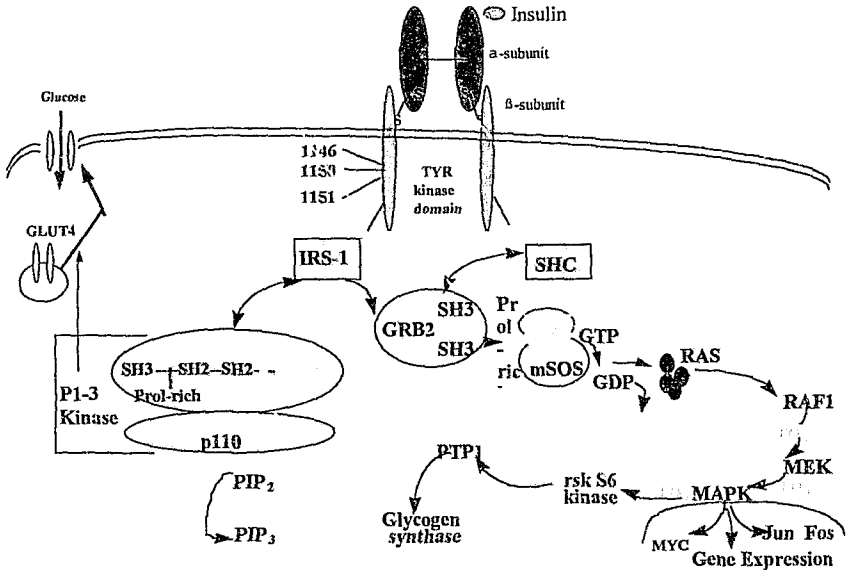


Figure 1. Molecular mechanisms of insulin signal transduction (Modified from Quon *et al* , 1994).

2.7 Control of Leptin Synthesis and Secretion

Leptin, an adipocyte-derived secretory factor that regulates food intake and thermogenesis, was identified by positional cloning of the murine obese (*ob*) gene and its human homologue (Zhang *et al*, 1994; Pelleymounter *et al*, 1995). Modulation of leptin synthesis and secretion is summarised in Figure 2.

Leptin controls appetite through the hypothalamus and may affect many other tissues because of the widespread distribution of its receptors. Expression of functional leptin receptor mRNA in mouse pancreatic islets (Emilsson *et al*,

1997), the human liver (Shimizu *et al,* 1998) and several other tissues in the mouse has been reported.

Insulin and glucocorticoids increase the production of leptin by fat cells (Bray, 1996). Leptin binds to receptors and enters the brain choroid plexus by a saturable process (Banks *et al,* 1996; Schwartz *et al,* 1996). Systemic leptin lowers the hypothalamic expression of neuropeptide Y (NPY) in ob/ob mice (Brown and Goldstein, 1997), which have abnormally raised levels of NPY mRNA, but has no effect on the obese db/db mice, lacking functional leptin receptors (Schwartz *et al,* 1996).

Overexpression of NPY in ob/ob mice leads to increased appetite and injection of leptin into the hypothalamus inhibits NPY; daily injections of recombinant leptin inhibit NPY (Banks *et al,*1996; Schwartz *et al,* 1996) and cause a decrease in food intake and a reduction in weight and fat mass in ob/ob mice and normalise blood glucose concentrations (Halaas *et al,* 1995; Pelleymounter *et al, 1995).*

It has been postulated that exogenous administration of leptin to treat obesity might be ineffective if endogenous leptin has already saturated its transporters (Caro *et al,* 1996). Thus it has been suggested that the reduced leptin transport into the CSF in obese individuals may provide a mechanism for leptin resistance (Caro *et al, 1996;* Schwartz *et al, 1996).*

Leptin seems to have additional roles, including inhibition of insulin secretion (Emilsson *et al*, 1997), modulation of insulin action in the liver (Cohen *et al*, 1996), production of steroids in the ovary (Zachow and Magoffin, 1997), and direct effects on adrenocortical steroidogenesis (Bornstein *et al*, 1997). Leptin also has a role in reproductive physiology (Chehab *et al*, 1997, Mounzih *et al*, 1997) and is involved in haemotopoietic and immune system development (Bennett *et al*, 1996).

Cold stimulation of β_3 –adrenergic receptors which commonly involves an increase in cAMP, lowers the synthesis of leptin in adipose tissue and this effect is partially reversed by a temperature increase (Remesar *et al*, 1997). The effects of leptin on other systems and pathways may give support to a major role for leptin as a counter-regulatory hormone that protects adipose tissue and perhaps other organs and tissues from excessive lipid synthesis induced by high insulin and ample energy availability.

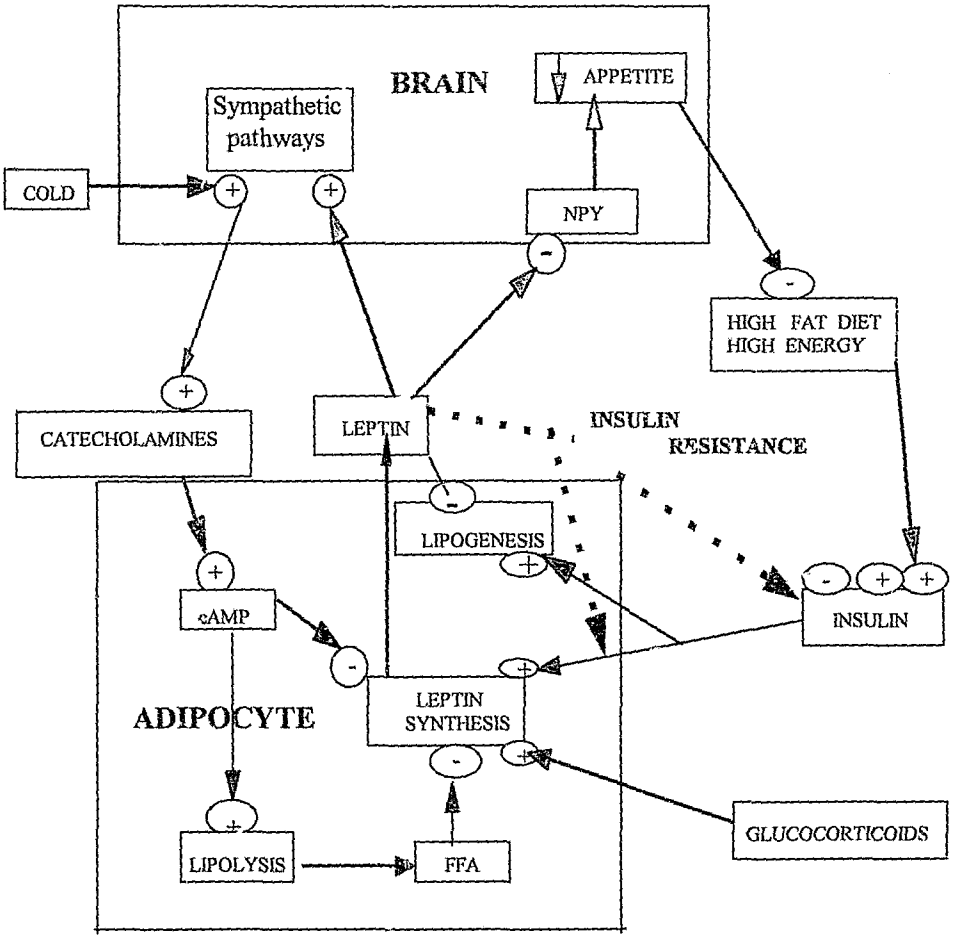


Figure 3. Modulation of leptin synthesis in adipose tissue by insulin, melanocyte-stimulating hormone, catecholamines and glucocorticoids (Adapted from Remesar *et al*, 1997).

2.8 Adipocyte Nuclear Transcription Factors.

PPAR-γ is a member of a nuclear hormone superfamily that influences adipocyte differentiation. PPAR-γ has also been identified as the major functional receptor for the thiazolidinedione (TZD) class of insulin-sensitizing

drugs (Spiegelman, 1998). It has been proposed that PPAR-γ activation controls one or more genes that regulate systemic insulin sensitivity and such genes include the leptin and TNF-α genes (Spiegelman, 1998). Evidence has been gathered that shows that treatment of obese mice with PPAR-γ ligands results in decreased adipose tissue TNF-α; TZDs have also been shown to block the ability of TNF-α to interfere with the most proximal events of insulin signalling (Spiegelman, 1998).

It has been shown that the application of TZDs *in vivo* or to cultured adipocytes can cause a reduction in the expression of leptin mRNA (Emilsson *et al,* 1997). Furthermore, in differentiated cells and tissues, leptin (Nolan *et al*, 1994) and TNF-α are reduced by increased PPAR-γ expression (Spiegelman, 1996). PPAR-γ activates , LPL and the fatty acid binding protein aP2. The expression of GLUT 4 gene is increased in cultured adipocytes and fat tissue through PPAR-γ activation by TZDs (Spiegelman, 1998) implying that GLUT 4 could be another potentially important target gene for PPAR-γ

Leptin exerts negative effects on other members of the nuclear hormone-receptor superfamily which influence adipocyte differentiation. These include glucocorticoid, 3,3',5-triodothyronine and retinoic acid receptors (Gregoire *et al, 1998*). Leptin indirectly suppresses PPAR-γ expression that is induced by glucocorticoids by increasing the expression of C/EBP-δ (another transcription

factor). The formation of C/EBP-δ/EBP-β heterodimers, lead to PPAR-γ expression (Wu *et al*, 1996).

2.9 Aetiology of obesity

Obesity can be defined as a syndrome characterised by an increase in body fat stores, its generation depending on an imbalance between energy intake and energy expenditure. Molecular mechanisms and genetic factors (Kahn, 1996), lifestyle (Kissebach and Krakower, 1994) and environmental factors (Ravusin, 1995) have been implicated in the aetiology of obesity. Several studies on familial inheritance of obesity have considered the hypothesis that a single major gene may be involved. Table 1 below shows the results from some of these studies.

Table 1. Overview of segregation analysis results for BMI and obesity phenotypes

Study	Multifactoral transmision	Major effect ?	Major gene ?	Gene frequency
Province *et al*	41%	Yes, 20%	Yes	0.25
Price *et al*	34%	Yes	Yes	0.21
Moll *et al*	42%	Yes, 35%	Yes	0.25
Tiret *et al*	39%	Yes	No	Non-Mendelian
Rice *et al*	42%	Yes, 20%	No	Non-Mendelian
Borecki *et al*	Yes	Yes	Age related	0.22

Adapted from Bouchard C, 1996.

2.10 Obesity and Disease

Obesity is associated with a cluster of metabolic aberrations involving impairment in insulin action (Kahn, 1996). This plurimetabolic disorder of insulin action has been named syndrome X and it includes insulin resistance,

dyslipidaemia, type 2 diabetes, hypertension and ischaemic heart disease (Reaven, 1995a, 1995b).

Insulin resistance is defined as a reduced biological response to insulin, (Kolaczynski and Caro, 1996) and, although more common in obese subjects, it is also found in the lean population (Ferrannini, 1995). It can result from reduced cellular sensitivity or responsiveness to insulin (Ferrannini, 1995; Kolaczynski and Caro, 1996).

Obesity provided the first recorded instance of insulin resistance, documented more than 30 years ago (Rabinowitz and Zierler, 1962). When insulin was infused through the brachial artery, the glucose uptake by forearm tissues in response to insulin was diminished in obese patients compared with lean individuals.

Studies showed that patients with high blood pressure are glucose intolerant and hyperinsulinaemic when compared with a matched group of individuals with normal blood pressure (Reaven, 1988, 1991). Also, it has been established that resistance to insulin-stimulated glucose uptake may be present in these individuals (Reaven, 1988, 1991). As with hyperinsulinaemia, the defect in insulin action is present in both obese and nonobese patients with hypertension and can still be detected when antihypertensive treatment has effectively controlled blood pressure (Reaven, 1988, 1991).

Insulin resistance is associated with dyslipidaemia, particularly elevated plasma triglycerides (triglyceride) and decreased high density lipoprotein-cholesterol concentrations (HDL-C) and the presence of small dense low density lipoprotein (LDL) particles. These changes stem from the disruption of the normal co-ordination of postprandial lipid metabolism by insulin and they are summarised in Table 2.

Table 2. Alterations of lipid metabolism associated with insulin resistance.

Elevated plasma triglyceride concentration (particularly elevation of VLDL-triglyceride and VLDL).
Decreased plasma HDL-C concentration (particularly HDL_2-C).
Elevation of plasma FFA concentration (particularly impaired postprandial suppression).
Small dense LDL particle distribution increase in numbers.

The association between insulin resistance and diabetes is not well understood. However, patients with type 2 diabetes have been found to have increased fasting plasma FFA levels due to increased rates of lipolysis and the reduced ability of insulin to suppress lipolysis (Jéquier, 1996). It has also been reported that there is a decrease use of FFAs by muscles in patients with type 2 diabetes which is accompanied by an increased hepatic lipid oxidation, which can in turn lead to increased gluconeogenesis (Jéquier, 1996). Increased FFA levels may

lead to increased insulin resistance which may also be mediated other factors including reduced insulin signalling, high leptin and TNF-α levels and hyperglycaemia (see section 2.12).

Insulin resistance is thought to promote atherosclerosis, in part through associated metabolic abnormalities such as hypertension, hyperglycaemia, hyperinsulinaemia and dyslipidaemia.

2.11 Molecular Mechanism of Insulin Resistance in Obesity

Insulin resistance and obesity have strong genetic components, indicating that they are the consequence of permanent, heritable defects (Bouchard *et al*, 1988). The mode of inheritance has been called complex because the magnitude of expression of the multiple molecular defects is thought to depend upon a complex interaction of environmental and heritable factors.

Cellular defects in insulin sensitivity were first suggested by the observation that insulin binding and action were reduced in cultured fibroblasts derived from some patients with *in vivo* insulin resistance (Reaven, 1995). Since the action of insulin involves many gene products (see Figure 1), target cell defects could be due to mutations affecting any protein between the receptor and the final insulin-regulated proteins (see Table 3).

Table 3. Causes of insulin resistance (Möller and Flier, 1991).

1. Defects to target cells

(a) Mutations of the insulin-receptor gene

(b) Defects in other genes important for insulin action

-Glucose transporters

-Substrates for insulin-receptor kinase or signalling intermediates

-Cellular inhibitors of insulin-receptor kinase e.g. PC1, Leptin

2. Secondary factors affecting target cells

(a) Abnormal physiologic states - stress (e.g. fever, sepsis), fasting or starvation, uraemia, cirrhosis, obesity, ketoacidosis, diabetes or hyperglycaemia.

(b) Normal physiologic states - Puberty, advanced age, pregnancy.

3. Specific hormonal or metabolic factors

(a) Glucocorticoids (e.g. Cushing's syndrome),

(b) growth hormone (e.g. acromegaly), catecholamine (e.g. pheochromocytoma),

(c) glucagon (e.g. glucagonoma),

(e) thyroid hormone (e.g. thyrotoxicosis),

(f) hyperinsulinaemia (e.g. obesity, type 2 diabetes, insulinoma),

(g) hyperglycaemia (e.g. obesity, diabetes),

(h) FFAs (e.g obesity, type 2 diabetes).

2.12 TNF-α, FFAs and Leptin in Insulin Resistance.

TNF-α is a peptide constitutively expressed and secreted by adipose tissue, and the level of which is increased in obese subjects. Increased expression of TNF-α in adipose tissue has been suggested as a key mediator of obesity-linked insulin resistance (Hotamisligil *et al*, 1993; Hotamisligil and Spiegelman, 1994; Hoffmann *et al*, 1994).

The molecular mechanism by which tumour necrosis factor-α causes insulin resistance is unclear but studies on rodents suggest that TNF-α reduces insulin receptor autophosphorylation and IRS-1 phosphorylation (Hotamisligil *et al*, 1994, Liu *et al*, 1998). Studies on human adipocytes showed that TNF-α induces a rapid inhibition of insulin signalling at the level of PI 3-kinase (Hauner *et al*, 1998), an enzyme which plays a pivotal role for downstream insulin signalling, including translocation of GLUT 4 and the regulation of DNA synthesis by activation of pp70 S6 kinase (Cheatham and Kahn, 1995).
It has also been shown that chronic exposure of newly differentiated human fat cells to TNF-α results in downregulation of GLUT 4 expression and a complete loss of insulin-stimulated glucose uptake (Hauner *et al*, 1995).

There is growing evidence that plasma FFA flux link insulin resistance, type 2 diabetes and obesity (Fraze *et al*, 1985; Chen, Y-DI *et al*, 1987; Skowronski *et al*, 1991). Increased plasma FFA concentrations produce peripheral and hepatic insulin resistance (Ferrannini *et al*, 1983), which under normal circumstances is

compensated by FFA-induced potentiation of glucose-stimulated insulin secretion (Boden, 1997).

In vitro studies showed that FFAs promote gluconeogenesis (Boden, 1997) by increasing production of ATP and NADH and the activation of pyruvate carboxylase by the acetyl-CoA that is generated via fatty acid oxidation (Jomain-Baum and Hanson, 1975; Williamson *et al*, 1969).

There is evidence that insulin inhibits hepatic glucose output (HGO), in part by suppressing plasma FFA levels. This suggests that impaired suppression of plasma FFA levels after glucose ingestion would impair HGO suppression and increase the systemic delivery of glucose (Kruszynska *et al*, 1997). It has been proposed that in the development of type 2 diabetes FFAs fail to stimulate insulin secretion which leaves hepatic and peripheral insulin resistance unchecked resulting in hepatic overproduction and peripheral underutilisation of glucose (Boden, 1997).

Randle *et al* (1963) first demonstrated the inhibitory effect of FFAs on carbohydrate oxidation. According to this hypothesis, under conditions when the glycogen store in the liver is depleted, fatty acids are mobilised from adipose tissue so that their rate of oxidation by muscle increases and this, in turn, decreases the rate of glucose utilisation (Newsholme, 1994). Conversely, after a period of fasting on refeeding, the rate of fatty acid release by adipose tissue is reduced, decreasing their rate of oxidation, so that the rate of glucose

utilisation by the muscle increases. These re... nses serve to stabilise the blood glucose concentration (see Figure 3).

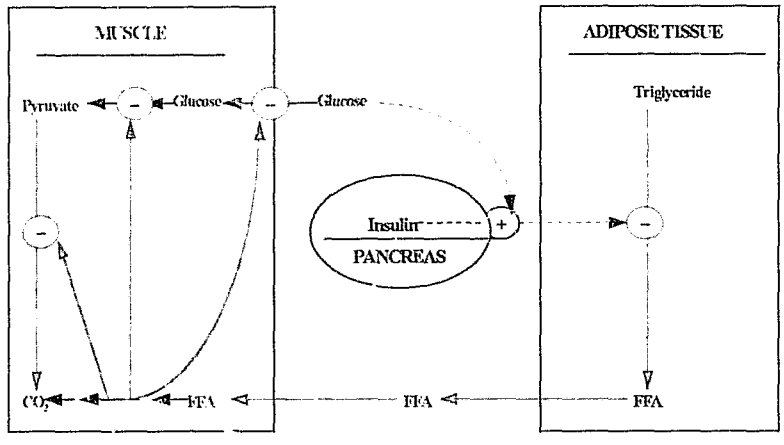


Figure 3. The glucose/fatty acid cycle (Adapted from Newsholme and Leech, 1994).

FFA release is more rapid from visceral than subcutaneous (sc) fat due to regional variations in lipolysis regulation and visceral fat has a higher density of glucocorticoids receptors (Fried *et al*, 1993). Visceral fat is directly connected to the liver through the portal vein system. Increased portal FFAs may stimulate gluconeogenesis and triglyceride synthesis by the liver and also inhibit insulin degradation in this organ (Arner, 1998). This may explain why

abdominal obesity is strongly associated with type 2 diabetes and cardiovascular disease (Kissebach and Krakower).

The role of leptin in the pathogenesis of obesity is impairment of several metabolic actions of insulin, i.e. stimulation of glucose transport, glycogen synthase, lipogenesis, protein synthesis, inhibition of isoproterenol-induced lipolysis and PKA activation (Möller *et al,* 1997).

Biochemical data characterising leptin's effect on insulin action suggests that leptin inhibits insulin receptor kinase activity and phosphorylation of IRS-1. These studies were carried out on insulin stimulated Rat-1 fibroblasts overexpressing the human insulin receptor (Kroder *et al,* 1995). Cohen *et al* (1996) also reported that leptin downregulates insulin-dependent phosphorylation of IRS-1. Leptin secretion is thought to be mediated by TNF-α signalling through TNF receptor 1(TNFR1) and this is thought to represent a novel mechanism contributing to obesity related hyperleptinaemia (Sethi *et al,* 1998).

2.13 The Effect of Race on Body Fat Distribution and Glucose Metabolism

Lovejoy *et al* reported differences in abdominal fat distribution between ethnic groups. Thus, African-American women have less visceral fat than their Europid counterparts (Lovejoy *et al,* 1996). However, African-American women have a higher prevalence of diagnosed diabetes than Europid women

and are more insulin resistant than Europid women matched for age, degree of obesity and waste-hip-ratio (Lovejoy *et al*, 1996; Dowling *et al*, 1995). These findings are unusual in that visceral fat is normally associated with higher incidences of type 2 diabetes and a greater level of insulin resistance (Björntop, 1991). Similar findings were reported in South Africa where obese white women tend to have more visceral fat than obese black women, which is more lipolytically active, in the white subjects. Also the black women have lower plasma insulin, C-peptide and lactate levels but higher FFA levels than the white women (van der Merwe *et al,* 1996). Other studies have also shown that in general the South African black population are more insulinopaenic than the white population (Rubenstein *et al*, 1969; Shires *et al*, 1978). Furthermore, the prevalence of obesity-associated diseases are different within these ethnic groups. Thus, hypertension is more common within the black community (Seedat, 1997) whereas ischaemic heart disease is rare in comparison with the white population (Isles and Milne, 1987; Walker *et al*, 1993). The low mortality associated with obesity in the black population of South Africa prompted Walker *et al* (Walker *et al,* 1989) to describe it as "benign' obesity. However, the rising incidence of type 2 diabetes in the black South African population (Bonnici *et al*, 1997) counters this argument.

Studies of the black and white population of Southern Africa have also indicated that there could be some differences in the pathology of type 2 diabetes in these two ethnic groups. In the former group the age of onset is

earlier, disease decompensation is more acute and plasma insulin levels are lower (van der Merwe *et al,* 1996; Osei and Schuster, 1995; Joffe *et al,* 1992).

The reasons for these differences in type 2 diabetes pathology are unknown, but differences in metabolism between the black and white obese subjects may be relevant, particularly the high FFA and leptin levels observed in obese black females (van der Merwe *et al*, 1996 and 1999). The present study was therefore designed to investigate these ethnic metabolic differences by looking at *in vitro* adipocyte metabolism.

CHAPTER 3

Methods

3.1 Anthropometric Measurements

3.1.1 Signed Informed Consent

The procedure was explained to the participants and they all gave informed consent, and the study was approved by the Committee for Research on Human Subjects of The University of the Witwatersrand.

3.1.2 The Population Sample

Ten black and ten white women were matched for age, waist-hip ratio, BMI, percentage body fat, duration of obesity (10-12 years) and number of offspring (no more than 3). None of the women were postmenopausal or on an oral contraceptive. Exclusion criteria included diseases of the major organs on the basis of clinical examination and history (e.g. regular menstrual cycles), smoking in excess of 5 cigarettes per day and alcohol consumption in excess of 4 gm alcohol per day or history of eating disorders. Patients fasted for 10 hours and did not exercise for 12 hours prior to the tests.

3.1.3 Body Composition Analyses

Bio-electrical impedance analyses were carried out with the Bodystat machine (Bodytrach Pty. Ltd., South Africa), to assess body composition of fat (percentage level). For accurate results the patients did not eat or drink for 10

hours prior to the test nor did they consume alcohol for 24 hours prior to the test or partake in vigorous exercise for 12 hours prior to the test.

3.1.4 Definition of Obesity

Obesity was assessed using the World Health Organisation (W.H.O.) criteria: BMI <18.5kg.m^{-2} $\Rightarrow$ underweight; BMI range 18.5 - 24.9 kg.m^{-2} $\Rightarrow$ normal; BMI range 25.0 - 29.9 kg.m^{-2} $\Rightarrow$ overweight; BMI range 30.0 - 39.9 kg.m^{-2} $\Rightarrow$ obese; BMI $\geq$ 40.0 kg.m^{-2} $\Rightarrow$ extremely obese. All subjects used in this study had BMI's above 25 kg.m^{-2}.

3.2 Fat Biopsy and Adipocyte Isolation

3.2.1 Fat Biopsy

The women were fasted from 23h00 the previous night. At 09h00 the next morning needle biopsies of the subcutaneous adipose tissue were taken from the abdominal area (5 cm lateral to the umbilicus) and the femoral area at the level of the midfemur. The biopsies were obtained under local anaesthesia (2% Lignocaine HCl; Adcock Ingram Ltd) by using 1.8mm x 14 mm needle (Promex, SA) and a 50ml syringe. Due to the finding that local anaesthesia may affect lipolysis (Arner *et al,* 1973), care was taken not to infiltrate the excised tissue. The interval between aspirations was approximately seven minutes (Biopsies were carried out by Dr. M-T. van der Merwe).

3.2.2 Reagents used in adipocyte isolation

Fat cell isolation and incubation medium consisted of medium 199 (M199) with Hank's salts (Gibco, cat. No. 10012-037) 16.4mg/ml; bovine serum albumin (BSA fraction V, Sigma, Poole, England cat. No. 4503) 4g/100ml; glucose 1mg/ml and sodium bicarbonate 35mg/100ml (Both SAARCHEM, South Africa). The above constituents were weighed and dissolved in 500 ml distilled water. The pH of the solution was adjusted to 7.15 using sodium hydroxide, filter-sterilised using 0.22μm pore size filters to give the final pH of 7.4. The medium can be stored at 4°C for up to 4 weeks.

3.2.3 Development of Adipocyte Isolation Method

All adipocyte isolation protocols use collagenase digestion of adipose tissue (*Marshal et al*, 1984; Eriksson *et al*, 1992). Incubation with collagenase has the disadvantage of causing cell damage, particularly the digestion of cell surface proteins. Therefore, each batch of collagenase must be used in test digestions to determine optimum collagenase concentrations and optimum incubation times. The end point of tissue digestion is reached when all adipose tissue clumps have been digested into single cells, and this can be determined by removing aliquots from the digestion mixture and studying under a light microscope. The digestion is stopped when only single cells are visible.

Fat tissue biopsies (ranging in size from 300 to 500mg) were incubated with different concentrations of collagenase ranging from 0.6-0.8mg/ml. The 0.8mg/ml concentration was found to give complete tissue digestion without

changing the morphology of adipocytes and this concentration was used in all experiments. An incubation period of 40 minutes with 0.8mg/ml collagenase was found to be the optimal time period, producing minimal tissue clumping and maximal tissue digestion.

3.2.4 Adipocytes Isolation Protocol

Aspirated adipose tissues from abdominal and femoral areas were washed once in M199 with Hank's salts. Each adipose tissue biopsy was then well-minced with scissors and transferred to 50 ml Falcon tubes to which 16mg collagenase (Boehringer Mannheim, cat. No. 10 88 793) was added. Then 20ml of clean M199 was added and the contents were incubated for 40 minutes at 37°C in a shaking waterbath. The same collagenase batch was used for all the experiments to avoid variability in activity.

Once digested the isolated adipocytes were filtered through a nylon mesh (500μm, Filter Pure SA) and washed four times with collagenase-free M199 kept at 37°C. The adipocytes were then suspended in 850μl of M199. All glassware used in this procedure was silanised with Sigmacote (Sigma, cat. No. SL-2)

3.3 Estimation of Fat Cell Numbers

Adipocytes are very large cells (~ 100μm in diameter) and therefore numbers cannot be estimated using a haemocytometer. Instead, the number of cells is calculated by measuring fat cell diameter, which is used to calculate fat cell volume and mass. Triglyceride mass of 50μl adipocyte cell suspension is then measured and each cell is considered to be 100% triglyceride.

3.3.1 Measurement of Fat Cell Diameter

Fat cell mass was assessed using the method of Smith *et al* (1972). Using a Pasteur pipette 50μl of an adipocyte suspension was removed and placed in a glass chamber. A coverslip was placed on top of the chamber without rupturing the adipocytes. The diameter of 100 adipocytes was determined using an Olympus microscope fitted with an ocular lens-mounted graticule. Mean cell diameter was determined.

3.3.2 Triglyceride Quantification

Triglyceride extraction solution consisted of 80ml isopropanol, 20ml heptane and 2ml of 0.5 M H_2SO_4. The triglyceride content of each adipocyte preparation was measured as described by Hirsch and Gallian (1968). A 50μl aliquot of each adipocyte preparation was transferred into glass tubes in duplicate. To these cell suspensions, 2.8ml of extraction solution was added and incubated at room temperature for 2 hours. This was followed by the

addition of 1.8ml heptane and 1ml distilled water. The contents were mixed and centrifuged at 123g for 3min. A scintillation vial (Packard PONY vial, cat. No. 6000292) was weighed and 1ml of the top phase (heptane) from each glass tube was added into each vial and evaporated under nitrogen gas. Each tube was re-weighed and the weight differences were assumed to be due to triglyceride.

3.3.3 Calculations

Assuming that each cell is a perfect sphere composed of material with the density of triolein (0.915), then a sphere of any given diameter, d (in microns), would weigh:

$$\text{(i) Mass of cell} = \left[\frac{0.915}{6}\right] \times \frac{\pi}{10^6} \times d^3, \quad \text{or} \quad \left[\frac{0.4791}{10^6}\right] \times d^3$$

$$\text{(ii) No of cells per tube} = \frac{\text{triglyceride content per tube}}{\text{mass of cell}}$$

3.4 Assessment of Adipocyte Function

Adipocyte function was assessed by measuring the lipolytic activity of the isolated cells in the presence of varying concentrations of the catecholamine

derivative, isoproterenol (see figure 5). Isoproterenol is a powerful activator of lipolysis (Honnor *et al*, 1985a, 1985b). The proteolytic actvity of collagenase preparations can lead to cell damage and therefore it was important to ensure that adipocytes isolated using collagenase were fully functional. Isolating cells using collagenase has the advantage of keeping adipocytes in their spherical shape without a tendency to form conglomerates and this makes the method suitable for use in metabolic as well as morphological studies of human adipocytes.

Triglycerides are composed of a glycerol backbone to which are attached 3 fatty acid units. Triglyceride [illegible] down results in the release of FFAs and glycerol. Thus, glycerol levels are a good measure of adipocyte lipolytic activity. Adipocyte lipolytic metabolism was assessed in this study by measuring glycerol release in the presence of a lipolytic activator i.e. isoproterenol.

3.4.1 Preparation of Isoproterenol Solutions

1.8mg of isoproterenol (Sigma, I 2760) was weighed and dissolved in M199 to give a 1mM solution. This 1mM solution was further diluted to 250μM. Serial dilutions (M199) of the 250μM isoproterenol solution were made to give 50, 10, 2 and 0.4μM solutions. Then 6μl of each solution was added in duplicate to Packard PONY vials containing adenosine deaminase (see below) and M199. Adipocytes (50μl per tube) were added to give a final volume of 300μl.

3.4.2 Adenosine Deaminase

The solution comes as a 2000U/ml stock solution (Boehringer Mannheim Cat. No. 102 121) from which 20µl was aliquoted into a microcentrifuge tube. This was centrifuged for 2 minutes in a microcentrifuge at 739g. The supernatant was removed and the pellet reconstituted with 800µl of M199 giving an activity of 50U/ml. A 6µl aliquot of the solution was added to all tubes. Adenosine deaminase is used to remove endogenous adenosine, a potent antilipolytic compound that accumulates in the medium during adipocyte incubations (Honnor *et al*, 1985a).

3.4.3 Adipocyte Incubations with Isoproterenol

Adipocytes were isolated as described in Chapter 3; Section 3.2.4 and 50µl aliquots were incubated at 37°C for 60 minutes in the presence of varying concentrations of isoproterenol and 50U/ml adenosine deaminase prepared as described above. After the incubation, 1ml silicon oil (Silinkov DC 200 100 CST, Kebo Lab. Stockholm, Sweden) was added to each tube and centrifuged at 739g for 3 minutes. The adipocytes rise to the top of the silicon oil layer which remains above the M199. The M199 was recovered by piercing the bottom of the microfuge tube and transferring the media to fresh Eppendorf tubes. The M199 was stored at -20°C until such time as the glycerol assay was performed.

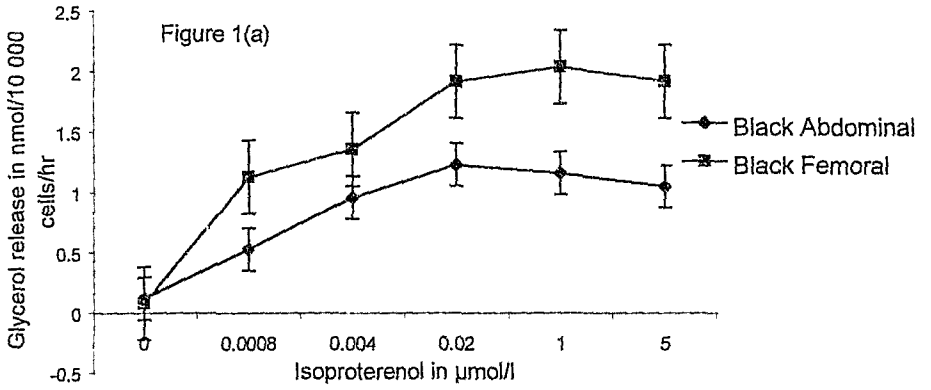
Figure 1a and b represent dose-response curves of glycerol release versus isoproterenol from abdominal and femoral adipocytes of a black obese woman (Figure 1a) and from abdominal adipocytes from white obese woman (Figure 1b). Isoproterenol concentration of 0.2-1.0μmol/l elicited the maximum lipolytic response. Furthermore, later experiments (see chapter 4) showed that abdominal and femoral adipocytes from both subject groups were very responsive to 1.0μmol/l isoproterenol thus demonstrating normal lipolytic activity. Previous investigators found that 1.0μmol/l of isoproterenol produced maximum stimulation of lipolysis (Honnor *et al*, 1985; Lönnroth *et al*, 1993).

3.5 Glycerol Assay Protocol

A sensitive radiometric glycerol assay was used based on the method of Bradley and Kaslow (Bradley and Kaslow, 1989).

3.5.1 Reagents and equipment

Packard Tri-Carb Scintillation counter; shaking waterbath; Albumin (Sigma - A7030); ATP (Boehringer Mannheim, disodium salt, 126 88); Radiolabelled ATP (DuPont, [γ-^{32}P]-ATP, 250μCi); Glycerol (BDH); Glycerol kinase (Boehringer Mannheim, 127 159); 20% Perchloric acid (Fluka); 85% Phosphoric acid (Merck); Sodium chloride (SAARCHEM, S.A.); Triethanolamine (Merck); 2ml Eppendorf tubes (with caps), Ammonium molybdate (Merck); Triethylamine (BDH).



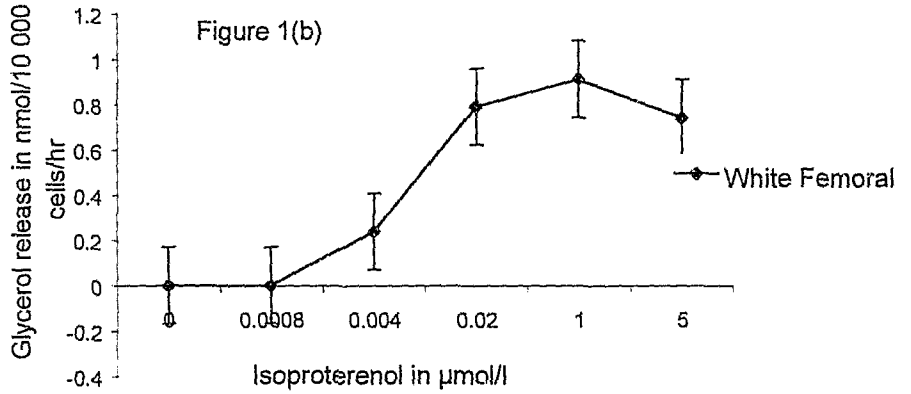


Figure 1(a) and (b). Assessment of adipocyte function using different concentrations of isoproterenol. (a) Lipolytic activity of adipocytes isolated from obese black abdominal and femoral regions (b); adipocytes isolated from obese white femoral region. 1.0µmol/l concentration of isoproterenol showed the maximal lipolysis rate.

3.5.2 Principle

Glycerol levels can be measured by the formation of [^{32}P]L-α-glycerol phosphate from [γ-^{32}P]ATP and glycerol, via the action of glycerol kinase. Unreacted ^{32}P-ATP is hydrolysed using a perchloric-phosphoric acid solution and resultant ^{32}P is precipitated using ammonium molybdate and triethylamine.

3.5.3 Procedure

A 1μCi/μl stock solution of [γ-^{32}P]ATP solution was prepared by diluting 50μl of 250μCi of [γ-^{32}P]ATP with 200μl of a 0.9% NaCl solution. A reaction solution was prepared by addition of 80μl of glycerol kinase to a 2mM magnesium chloride hexahydrate/0.1% BSA solution containing 750μl non-radiolabelled (73mg) ATP and 20μl of 1μCi/μl stock solution of [γ-^{32}P]-ATP. The reaction was initiated by addition of 50μl of the reaction solution to 50μl aliquots of undiluted glycerol samples and standards diluted in 0.9% NaCl solution in Eppendorf tubes. The contents were placed in a shaking waterbath at 37°C for 30min. The reaction was terminated by addition of pre-warmed (1ml) 1M perchloric acid containing 1mM phosphoric acid. The tubes were then placed into 95°C dry blocks for 1 hour to hydrolyse the remaining [γ-^{32}P]ATP while the [^{32}P]L-α-glycerol phosphate remained unhydrolysed (Thorner and Henry, 1971). The tubes were cooled on ice and 200μl of ice-cold 0.1M ammonium molybdate, and 200μl 0.2M triethylamine were added. The orthophosphate precipitated as a bright yellow complex while the [^{32}P]L-α-glycerol phosphate remained in solution. The precipitate was removed by

centrifugation at 4°C for 15 minutes at 739g and 1ml of the supernatant was removed into a PONY vial for scintillation counting with 4 ml distilled water in a Packard Tri-Carb scintillation counter. This method has been reported to be 10-100 times more sensitive than assays based on the detection of NADH by absorbance (Bradley and Kaslow, 1989). It is tedious to perform but cheaper than more common spectrophotometric assays.

3.6 Analytical Procedures

3.6.1 Insulin assay:

The Medgenix (Medgenix Lab Diagnostics, Belgium) ELISA method, a solid phase two-site enzyme immunoassay was used. It is based on the direct sandwich technique in which monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule. During incubation insulin in the samples and standards react with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microtitre plate wells. The bound conjugate is detected by reaction with 3,3',5,5'-tetramethylbenzidine. It is reported that this method does not crossreact with proinsulin and split proinsulins. The reaction is terminated by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

4.3.2 Free Fatty Acid Assay:

Free fatty acid was assayed enzymatically using the optimised colorimetric measurement kit (Boehringer Mannheim, Germany). The assay is based on the

principle that, in the presence of the enzyme acyl-coenzyme A (CoA) synthetase and the co-factors ATP and CoA, free fatty acid are converted into acyl coenzyme A, resulting in AMP and Pi production. Acyl coenzyme A reacts with O_2 in the presence of acyl coenzyme A oxidase to form 2,3-enoyl-CoA and H_2O_2. H_2O_2 converts 2,4,6-tribromo-3-hydroxybenzoic acid and 4-aminoantipyrine to a red colour in the presence of peroxidase. The colour formed is measured spectrophotometrically at 546nm.

3.6.3 Glucose Assay

Plasma glucose levels were measured by glucose oxidase method using a Hitachi® autoanalyser (Boehringer Mannheim, Germany).

CHAPTER 4

Antilipolytic Effect of Insulin in Adipocytes Isolated from Obese Black and White Women

4.1 Introduction

Obesity, is a major cause of morbidity and mortality (Kissebach and Krakower, 1994), is associated with increased risk of chronic diseases such as Type 2 diabetes (non-insulin-dependent) *diabetes mellitus* and cardiovascular disease including hypertension (Ferannini, 1995; Macor *et al*, 1997). In South Africa the prevalence of obesity, i.e. body mass index (BMI) > 30kg/m^2, is 47.7% for black and 15.6% for white women within the age group of 35-44 years (Steyn *et al*, 1991; Walker, 1995). The prevalence of type 2 diabetes amongst the black community is 6-7% (Bonnici, *et al*, 1997).

Obesity is strongly associated with insulin resistance (Reaven, 1995; Kahn, 1996) which may be a major cause of chronic diseases, particularly type 2 diabetes (Walker, 1995; Reaven, 1995). The mechanism by which obesity gives rise to insulin resistance is unknown, however, excessive circulating plasma free fatty acids may play a role (Randle *et al*, 1963; Boden, 1997).

A recent clinical study has shown that obese black South African women have higher plasma FFA levels, lower serum insulin concentrations and higher *in vivo* lipolytic rates compared with whites (van der Merwe *et al*, 1996). The

present study, using isolated adipocytes sought to clarify whether higher systemic lipolytic rate observed in black women is due to lower insulin concentrations or to resistance to the antilipolytic effect of insulin.

4.1.2 **Subjects and Methods**

4.2.1 Selection criteria and other measurements

Ten obese black and white women were used for the study (see details in Chapter 3 ; section 3.1.2 to 3.1.4 for selection criteria and anthropometric measurements).

4.2.2 Preparation of insulin dilutions

Serial dilutions of insulin (Actrapid Insulin, Novo-Nordisk, Denmark 100IU/ml) were made from stock solution. A 2.6µl aliquot of the stock solution was diluted to 1ml in M199 with Hank's salts to give a 1800nM solution. Serial dilutions of insulin were made to obtain: 360nM, 120nM, 36nM and 3.6nM concentrations of insulin, and 6µl of each aliquot of insulin from the above preparations were added to appropriately marked Packard PONY vials to give a final volume of 300µl, following the addition of 50µl of adipocyte suspension, 6µl of a 50µM solution of isoproterenol and 6µL of adenosine deaminase (see Chapter 3; sections 3.4.1/2).. The volume was made up to 300µl with M199. This gave final insulin concentrations of 7.2, 2.4, 0.72 and 0.07nM and 1µM isoproterenol. Tubes were also set up containing

adipocytes with no insulin and no isoproterenol and another duplicate set with adipocytes and 1μM isoproterenol but no insulin.

4.2.3 Estimation of insulin resistance

Insulin resistance was estimated using the homeostasis assessment model method as described by Mathews *et al* (1995). The formula is:

$$\frac{[\text{Fasting insulin level (pmol/l)} \div 7] \times \text{Fasting glucose level (mmol/l)}}{22.5}$$

4.2.4 Data presentation:

Glycerol output was expressed in two ways: glycerol released per mm^2 of adipocyte surface area per hour (Figure 2a and 2b) or as percentage inhibition of glycerol release by insulin (Figure 3a and 3b), which was calculated according to the following formula:

$$\left[1 - \frac{\text{glycerol release in presence of INS and ISO}}{\text{glycerol release in presence of ISO only}}\right] \times 100 ,$$

where INS = insulin; ISO = isoproterenol.

Expressing lipolytic activity in this way made comparisons between ethnic groups of the effect of insulin on the inhibition of isoproterenol-stimulated lipolysis easier and clearer.

4.2.6 Calculation of ED_{50} Values

ED_{50} is the concentration of insulin that causes inhibition of lipolysis that is 50% of the maximum level of inhibition. Insulin sensitivity and insulin

responsiveness were determined from the dose-response curves for lipolysis (see figure 2a and 2b) from which the ED_{50} value was obtained. Figure 1 shows how ED_{50} was calculated.

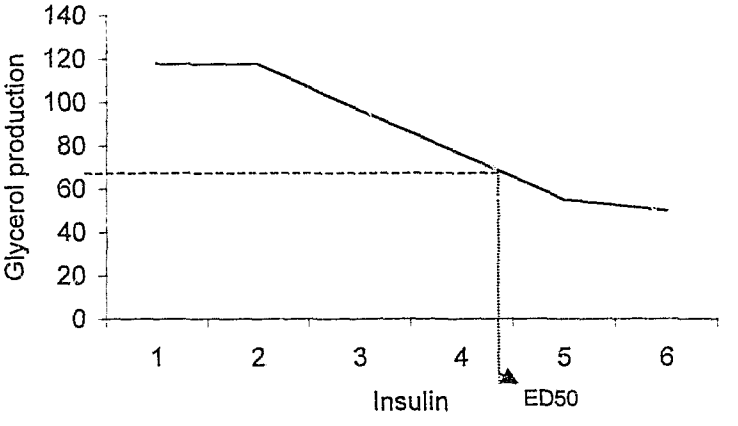


Figure 1. An illustration of how ED_{50} was calculated using a dose-response curve for lipolysis.

4.2.7 Statistical Analysis

Data are expressed as means ± SEM. Student's unpaired t-test was used to analyse the differences between the two groups whilst a paired t-test was used for the analysis of differences within the groups.

4.3 Results

4.3.1 Clinical Measurements

Figure 2. Insulin inhibition of lipolysis in (A) abdominal and (B) femoral adipocytes

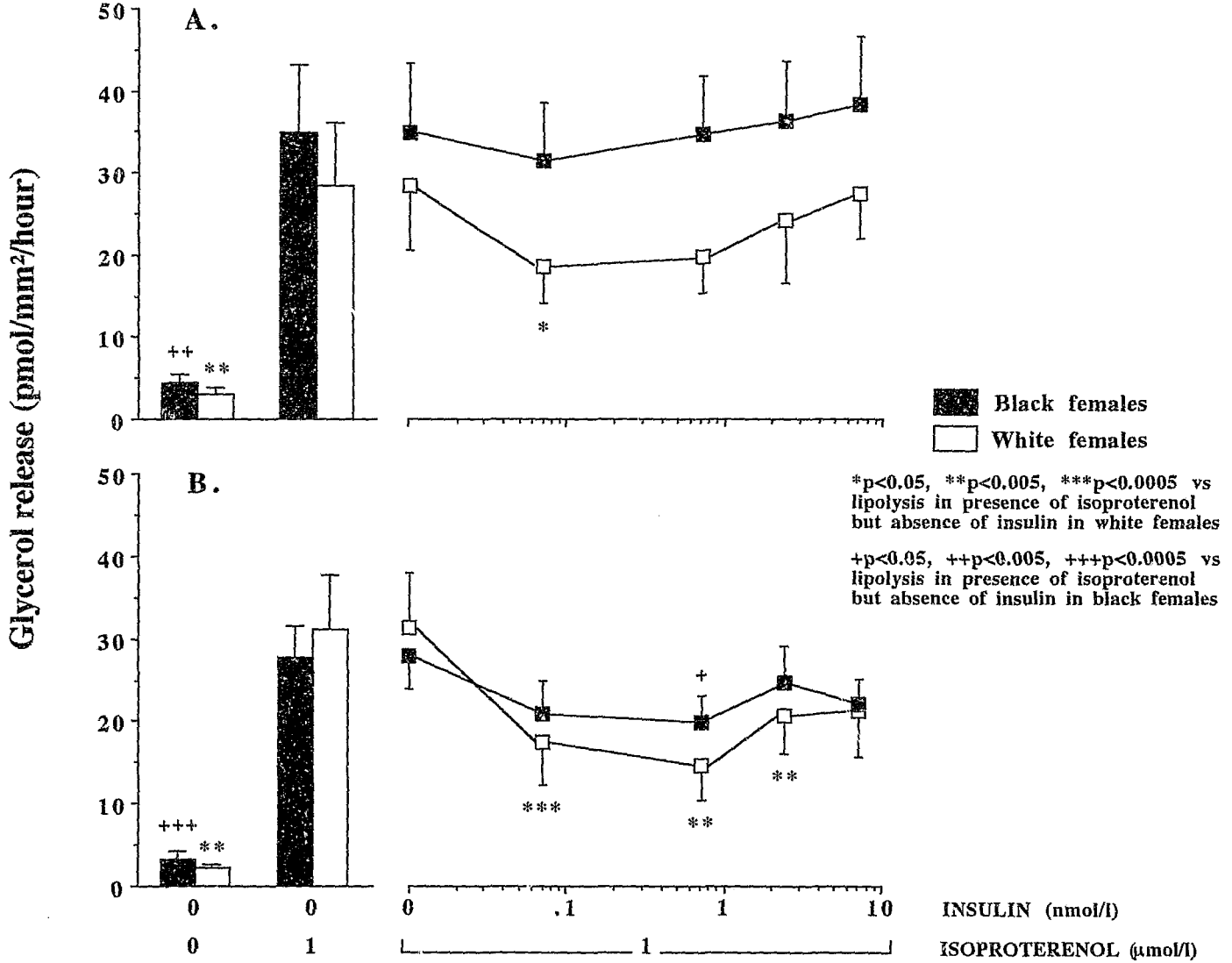


Figure 3(a)

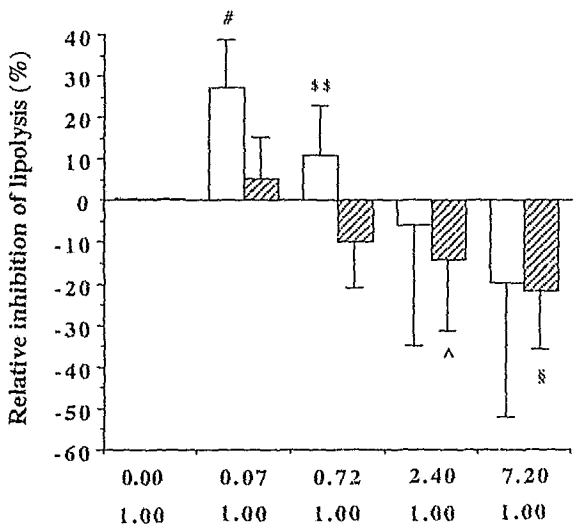


Figure 3(b)

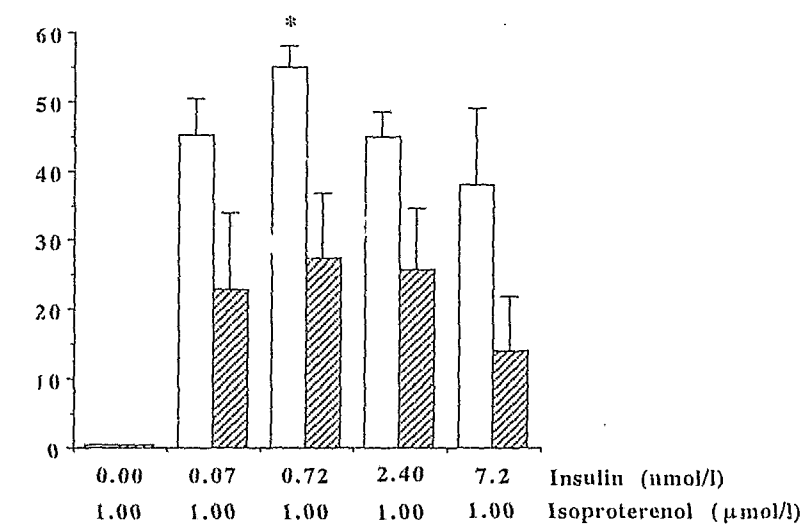


Figure 3. The percentage inhibition of lipolysis in: (a) abdominal and (b) femoral adipocytes. ☐ Adipocytes isolated from white, obese women; ▨ Adipocytes isolated from black, obese women; *$p < 0.05$ versus lipolysis rate in the presence of 0.72 nmol/l insulin of femoral adipocytes isolated from black subjects; $^{\#}p < 0.05$ and $^{\$\$}p < 0.005$ versus lipolysis rate in the presence of 0.07 nmol/l and 0.72 nmol/l insulin respectively of femoral adipocytes isolated from white subjects; $^{\wedge}p < 0.05$ and $^{\S}p < 0.05$ versus lipolysis rate in the presence of 2.40 nmol/l and 7.20 nmol/l insulin respectively of femoral adipocytes isolated from black subjects.

The subjects were well-matched for age, BMI, waist-hip ratio, and percentage body fat (Table 1).

4.3.2 Insulin, Glucose and Free Fatty Acid Levels

Table 2 shows that the obese black women had an almost two-fold higher fasting plasma FFA concentration ($p<0.01$) compared with the white women. In addition, the obese black women were relatively insulinopaenic in comparison to their white counterparts ($p<0.001$) ; in this study the term insulinopaenia is used to describe a relatively lower insulin level in obese black subjects when they are compared to the white obese subjects. Glucose levels were higher in the obese black women, but not statistically significantly so.

4.3.3 Fat Cell Size

The sizes of abdominal and femoral adipocytes in both groups were not statistically different (Table 3).

4.3.4 Antilipolytic effect of insulin

Absolute glycerol release by adipocytes from black women stimulated by 1μmol/l isoproterenol was statistically inhibited by insulin only in femoral adipocytes and by 0.72nmol/l insulin (Figure 2b). However in the white women there was statistically significant inhibition of lipolysis in both abdominal (by 0.07nmol/l insulin – Figure 2a) and femoral (by 0.07, 0.72 and 2.40nmol/l insulin – Figure 2b) adipocytes. The abdominal and femoral adipocytes isolated

from white subjects were more responsive to the antilipolytic effect of insulin compared to the adipocytes taken from black subjects and statistically significant difference in the inhibition of lipolytic activity by insulin between ethnic groups were seen in the femoral adipocytes at an insulin concentration of 0.72nmol/l ($p<.05$) Figure 3b). Furthermore, abdominal adipocytes from both ethnic groups were more resistant to the effects of insulin than were femoral adipocytes. Thus, femoral adipocytes demonstrated statistically significantly higher percentage inhibition of lipolysis compared to abdominal adipocytes in white women at insulin concentrations of 0.07 ($p<0.05$) and 0.72 ($p<0.005$) nmol/l and in black women insulin concentrations of 2.40 and 7.20nmol/l ($p<0.05$ for both (Figure 3a and Figure 3b).

4.3.5 ED_{50} values

There were not statistically significant differences between the ED_{50} values for the antilipolytic effect of insulin in any of the adipocyte populations that were studied. The ED_{50} values are shown in table 4 (units are pmol/l ±SEM).

Measurements	Black Women	White Women	p-value
Age (years)	37.0 ± 1.7	35.0 ± 0.8	N.S.
BMI (kgm^2)	34.2 ± 1.8	35.3 ± 1.8	N.S.
Waist-hip ratio	0.82 ± 0.03	0.79 ± 1.10	N.S.
Body Fat (%)	46.1 ± 1.6	44.1 ± 1.6	N.S.
n number	10	10	

Table 1. Clinical characteristics of two population groups. Values are means ±SEM; N.S. => not significant.

Measurements	Black Women	White Women	p-value
FFA (µmol/l)	897 ± 89.8 (560 – 1300)	515.0 ± 52.9 (290 – 740)	<0.01
Insulin (pmol/l))	64.3 ± 0.7 (33.6 – 106.4)	164.7 ± 12.5 (7[illegible]3 – 226.8)	<0.01
Glucose (mmol/l)	4.6 ± 0.2	4.5 ± 0.2	N.S.
Insulin resistance (HOMA)	1.9 ± 0.2 (1.7 - 2.1)	4.7 ± 0.6 (4.4 - 5.6)	<0.01

Table 2. Biochemical characteristics of the two population groups. Values are means ± SEM. N.S. = not significant. HOMA = HOMA = homeostasis model assessment. Numbers in parentheses are ranges.

Measurements	Black Women	White Women	p-value
Age (years)	37.0 ± 1.7	35.0 ± 0.8	N.S.
BMI (kgm^2)	34.2 ± 1.8	35.3 ± 1.8	N.S.
Waist-hip ratio	0.82 ± 0.03	0.79 ± 1.10	N.S.
Body Fat (%)	46.1 ± 1.6	44.1 ± 1.6	N.S.
n number	10	10	

Table 1. Clinical characteristics of two population groups. Values are means ±SEM; N.S. => not significant.

Measurements	Black Women	White Women	p-value
FFA (μmol/l)	863 ± 93.1 (560 - 1300)	412.0 ±34.2 (290 - 740)	<0.01
Insulin (pmol/l))	66.7 ± 4.7 (33.6 - 106.4)	152.0 ± 20.1 (79.8 - 226.8)	<0.01
Glucose (mmol/l)	4.5 ± 0.2	4.0 ± 0.2	N.S.
Insulin resistance (HOMA)	1.9 ± 0.2	3.8 ± 0.5	<0.01

Table 2. Biochemical characteristics of the two population groups. Values are means ± SEM; N.S. => not significant. Insulin, glucose and free fatty acids values are all fasting values. Numbers in parentheses are ranges.

Adipocyte Population	Black Women: adipocyte diameter (μm)	White Women: adipocyte diameter (μm)	p-value
Abdominal	132.0 ± 14.0 (97.7 – 220.2)	115.0 ± 1.8 (101.5 – 120.0)	N.S.
Femoral	130.7 ± 12.7 (102.1 – 210.5)	111.5 ± 1.3 (106.4 – 123.2)	N.S.

Table 3. Size of adipocytes isolated from obese black and white women. Values are mean ± SEM. Numbers in parentheses are ranges.

	Black Women:	White Women:	p-value
Abdominal	33 ± 6	42 ± 14	N.S.
Femoral	40.5 ± 5	56 ± 12	N.S.

Table 4. ED_{50} values for the antilipolytic effect of insulin in the populations studied. Values are mean ± SEM. N.S. => not significant.

4.4 Discussion

The present study shows that the fasting metabolism of white and black obese women is markedly different with the latter group having lower plasma insulin but higher plasma FFA levels than the former group. This is a confirmation of previous studies (van der Merwe *et al,* 1996; Rubenstein *et al,* 1969; Joffe *et al,* 1992) and a recent study has shown that these metabolic differences are not confined to the fasting state but also exist after oral administration of a glucose load (van der Merwe *et al,* 1998). Insulinopaenia may be due to a decreased β-cell mass as a result of fetal or neonatal protein-energy malnutrition and/or genetic factors (Joffe *et al,* 1992). Several studies, including one carried out in South Africa (Crowther *et al,* 1998), have shown an association between low birth weight, which is common amongst black South Africans, and poor glucose tolerance (Barker *et al,* 1993; Valdez *et al,* 1994, Lithel *et al,* 1996).

This *in vitro* study shows that abdominal and femoral adipocytes isolated from obese black women are relatively unresponsive to insulin. Adipocytes taken from white subjects did respond to insulin and therefore the experimental protocol that was used in this study cannot be the reason for the insulin insensitivity of the adipocytes taken from the black women. This study is in agreement with other investigators who obtained similar results for the insulin-responsiveness of adipocytes from white subjects (Lönnroth *et al,* 1993).

Estimation of whole body insulin resistance using the hyperinsulinaemic euglycaemic clamp technique on obese black and white women also showed that black women were more insulin resistance than obese white women (Buthelezi *et al*, 2000),

	Black Women	White Women	p-value
Glucose infusion rate	1.3±0.2	2.2±0.3	$p<0.05$

Insulin sensitivity was also measured using the HOMA method (Mathews *et al*, 1995). HOMA predicted higher insulin resistance in the white subjects, which contradicts the results obtained from the glucose clamp studies. This difference may reflect inaccuracy of fasting glucose and insulin levels as a measure of insulin resistance and also HOMA does not take into account FFAs levels. High circulating FFAs may cause insulin resistance via the Randle Cycle (Randle *et al*, 1963) and may also reduce β-cell insulin secretory function (Boden, 1997).

The ED_{50} values (Table 4) for insulin inhibition of lipolysis are similar for all adipocyte populations studied but the response of adipocytes from black subjects to the antilipolytic effect of insulin is blunted in comparison to those of white subjects. Thus the higher serum FFA concentrations observed in the black subjects in the post absorptive state are explained since insulin is a major determinant of post absorptive lipolytic rates (Karlsson *et al*, 1995). These

results also suggest that the ethnic difference in insulin inhibitory activity may be due to an effect distal to insulin binding to its receptor i.e. differences in insulin responsiveness of the adipocytes rather than insulin sensitivity may be involved. However, it should be noted that the ED_{50} values were calculated in this study using only four different insulin concentrations and this will introduce some inaccuracy. Furthermore, the dose response curves for insulin inhibition of lipolysis shown in figure 2a and 2b demonstrate that in femoral adipocytes from both ethnic groups maximal inhibition of lipolysis occurs in the presence of 0.72nmol/l insulin (this is true in 72% of subjects) whilst in the abdominal adipocytes it occurs at 0.07nmol/l (this is true in 61% of subjects). This subject-to-subject variability causes the ED_{50}'s to be not significantly different between the four adipocyte populations. However, the dose response curves for the femoral adipocytes mirror each other quite closely as do those for the abdominal adipocytes and therefore despite the variability it is believed that there are no inter ethnic differences in adipocyte insulin sensitivity. In order to confirm this statement the measurement of insulin receptor numbers and binding affinities by Scatchard analysis is required.

A study comparing African-Americans to Caucasians-Americans demonstrated ethnic differences in the ED_{50} values for insulin inhibition of lipolysis (Dowling *et al*, 1995). Thus, abdominal adipocytes taken from upper-body obese Caucasian-Americans were less insulin sensitive than those from upper body obese African-Americans. This relationship was reversed in lower-body obese subjects. These results again demonstrate ethnic differences in adipocyte

sensitivity to insulin inhibition of lipolysis. Unfortunately these results could not be compared to those obtained in the American study because the lower number of subjects in this study meant that it was not possible to separate the populations into upper-body and lower-body obese groups.

The abdominal and femoral adipocytes isolated from black women are larger, but not statistically significantly so, than those isolated from white women (Table 3) and it has been shown that larger adipocytes should be more responsive to the antilipolytic activity of insulin (Jacobsson *et al*, 1976). This is not the case in the present study where adipocytes from black subjects are less responsive to the antilipolytic effect of insulin than those isolated from white subjects. Therefore the ethnic differences in the responsiveness of adipocytes to insulin-inhibition of lipolysis cannot be explained by differences in adipocyte size, but must be due to other factors.

The lipolytic response to isoproterenol of all the adipocyte populations studied is similar with the basal lipolytic rate increasing 10-fold on addition of isoproterenol (see Figure2a and 2b). The basal and isoproterenol-stimulated lipolytic rates are higher in the black women but the differences between ethnic groups are not statistically different. A previous study has shown that the *in vivo* lipolytic rate is higher in obese black women compared to their white counterparts (van der Merwe *et al*, 1996) and our data suggests that this may be a result of the insulin insensitivity of adipocytes taken from black obese

women. β-adrenergic agents stimulate lipolysis by increasing intracellular levels of cAMP (Steinberg *et al,* 1976) whilst the antilipolytic action of insulin involves reduction of cAMP levels via an insulin receptor-mediated process (Kuroda *et al,* 1987). Therefore, the reduced response to insulin in the adipocytes taken from the black subjects may be due to high intracellular cAMP levels and/or a pertubation of insulin receptor signalling.

Abdominal adipocytes were less responsive to insulin compared to femoral adipocytes in both the black and white population groups. This metabolic feature of subcutaneous abdominal adipose tissue in combination with its high lipolytic rate may explain results from a study showing that this fat depot predicts insulin sensitivity more strongly than does visceral fat (Goodpaster *et al,* 1997). These data emphasise the important contribution of both visceral and subcutaneous fat to the pathology of type 2 diabetes (Kahn, 1996) and other obesity-associated disorders.

Insulin displayed a biphasic effect on isoproterenol-stimulated lipolysis. This effect has been reported before (Fain *et al,* 1966; Chlouverakis, 1967; Kono, 1972; Solomon *et al,* 1970), and it has been suggested that the antilipolytic effect of insulin is achieved by inhibition of adenyl cyclase activity and activation of low Km 3'5'-cAMP phosphodiesterase. The lipolytic effect of insulin probably reflects enhancement of adenyl cyclase activity to an extent

that overrides any activation of low km 3'5'-cAMP phosphodiesterase activity, resulting in an increase in peak adipocyte 3'5'- camp levels (Desai *et al*, 1973).

In summary, the higher FFA levels of the black obese women result from a combination of insulin insensitivity of adipose tissue and insulinopaenia. The raised FFA levels may in turn contribute to the higher *in vivo* insulin resistance of the black compared the white obese women. Thus, the pathogenesis of type 2 diabetes particularly within the black obese subjects may be strongly influenced by adipose tissue metabolism.

CHAPTER 5

Effect of Insulin on Glucose Uptake in Adipocytes Isolated from Obese Black and White Women

5. 1 Introduction

Experiments described in Chapter 4 demonstrated that adipocytes from black obese women are more resistant to the antilipolytic effect of insulin than adipocytes from white obese women and therefore the present study was carried out to determine if glucose uptake was similarly affected.

The main effects of insulin on adipocytes are to inhibit lipolysis, increase triglyceride synthesis and increase glucose uptake and metabolism. Glucose uptake is primarily via the glucose transporter 4 (GLUT 4) protein which is translocated to the plasma membrane under the influence of insulin. Adipocytes also contain GLUT 1which acts in the absence of insulin. Glucose uptake was therefore studied in the absence and presence of insulin, using ^{14}C glucose.

5.2 Subjects and methods

5.2.1 Selection of subjects

Selection criteria for this study was the same as that described in Chapter 3; (section 3.1.1 to 3.1.8). Ten black and white women took part in the study. Only two black women were used to determine insulin binding levels of adipocytes.

5.2.2 Preparation of solutions

A 100µl of D-[U-^{14}C] glucose solution (50µCi) with a concentration of 310mCi/mmol was added to 1.8ml of M199 and mixed. Insulin dilutions were prepared as described in Chapter 4; 4.2.2. A stock solution (690µM) of insulin (Actrapid) was diluted to give 0.36µM and 0.036µM solutions which were used to find concentrations of 7nmol/l and 0.7nmol/l. These concentrations were chosen because they were shown to cause inhibition of lipolysis in adipocytes isolated from black and white obese women (see Chapter 4).

5.2.3 Glucose uptake determination

Isolated fat cells were prepared as described in Chapter 3; section 3.2.4. After the adipocytes had been digested with collagenase all the washes were carried out with M199 containing no glucose. After the final wash all the M199 was removed and the pellet resuspended in 450µl of M199 without glucose.

The incubation of adipocytes with D-[U-^{14}C] glucose was carried out in Packard PONY vials. Non-specific binding (NSB) and zero, 0.7nmol/l and 7nmol/l insulin tubes were prepared in duplicate. A 50µl aliquot of adipocyte suspension was added to all the tubes and the total incubation volume adjusted to 290µl with M199. After 15 minutes pre-incubation at 37°C in a shaking waterbath, 10µl of D-[U-^{14}C] glucose solution was added, and further incubated for 1 hour. NSB tubes were not subjected to the 1 hour incubation;

immediately after addition of D-[U-^{14}C] glucose the contents of the NSB tubes were poured into Eppendorf tubes and 1ml silicone oil added and spun at 123g for 3min in a microcentrifuge. The adipocytes that float to the top of the silicone oil layer were removed from the tubes and transferred into Packard PONY vials. A 4ml aliquot of scintillant (Beckman) was added and the radioactivity determined. After the 1 hour incubation the zero and insulin tubes were treated the same way as NSB tubes. Total count tubes containing 10µL of D-[U-^{14}C] glucose plus 4ml scintillant and background tubes containing 50µl silicone oil were also prepared and counted.

5.2.4 Assessment of Insulin Binding to Adipocytes

The method used to determine insulin binding capacity of adipocytes was modified from the method described by Gammeltoft (1984). Preparation and isolation of adipocytes is as described in Chapter 4, Section 4.2.4. A 100µl aliquot of the adipocyte suspension was transferred to each of four Eppendorf tubes. Then, 20µl of 7000nM insulin solution plus 30µl M199 were added to two tubes and 50µl of M199 (NSB) was added to the remaining two tubes. To each tube 50µl of iodinated insulin (prepared as in Chapter 5; section 5.5) containing 8mM potassium cyanide solution was added. Potassium cyanide solution was added to help remove insulin and other agents from pre-incubation period which might directly interfere with binding. Incubation was carried out for 4 hours at 16°C. After incubation the tubes were centrifuged for 3 minutes at 123g in the presence of silicone oil. The cells were removed from

the oil layer for determination of the cell-bound radioactivity. Non-specific binding was measured as the amount of ^{125}I-insulin remaining in the cell layer in the presence of 7000nM unlabelled insulin. All data were corrected for non-specific binding.

5.2.5 Preparation of radiolabelled insulin

Iodogen [1,2,4,6-tetrachloro3α, 6α-diphenylglycoluril (Sigma)] mediates rapid iodination in the solid phase of free hydroxyl groups of peptides present in an aqueous solution. The reaction is stopped by addition of excess sodium iodide.

Preparation of iodogen tubes was carried out in a well-ventilated fume hood, at room temperature. Iodogen (5mg,) was dissolved in 10ml dichloromethane (Sigma) and 20µl aliquots were dispensed into 1.5ml Eppendorf tubes which were left in the fume hood, at room temperature, overnight. Tubes were prepared in advance and stored at room temperature in a sealed, covered container until date of use.

5.2.6 Iodination protocol

Apparatus and solutions: Microfuge tubes, Iodogen coated tube(s), PD10 column (Pharmacia Cat No. 17-0851-01), 500mM sodium phosphate buffer (pH 7.0), Single strength veronal buffer (SSV- which contains 1g/100ml sodium barbitone, 0.5gBSA/100ml, 0.9% sodium chloride and 10mg/100ml

sodium azide pH7.4), 10% Trichloroacetic acid solution. PD10 column was pre-equilibrated by running 25ml of SSV buffer through it.

Procedure: To iodogen tube (prepared above) was added 20µl of 50mM sodium phosphate buffer, 200 pmoles of insulin and 300µCi (11.1Mbq) of sodium ^{125}I, mixed and incubated for 15 minutes at room temperature in a fume cupboard. The reaction was stopped by addition of excess sodium iodide. A 10µl aliquot of iodination reaction was diluted in 990µl SSV and 100µl of this was diluted to 1ml with SSV buffer (total count tube), and 10µl was counted on a gamma counter and used for estimation of total ^{125}I incorporated into the peptide. The remainder of the iodination reaction was poured onto the PD10 column and the initial eluate was discarded. Then, the SSV buffer was added to the column and 20 × 0.5ml fractions were collected in twenty 1.5ml Eppendorf tubes.

5.2.7 Evaluation

A 10µl aliquot from each of the 20 fractions was removed and added to 990µl of the SSV buffer in appropriately labelled tubes. This was mixed and 10µl transferred to appropriately labelled Eppendorf tubes and tested for radioactivity using a gamma counter. Following this 100µl of the BSA-containing SSV buffer was added to each tube plus 1ml of 10% TCA solution to precipitate out all proteins. This was incubated at 4°C for 30 minutes. The contents were then centrifuged at 2000g for 10 minutes at 4°C. Supernatants were aspirated and the pellets counted on the gamma counter. Fractions

showing >90% trichloroacetic acid precipitatable counts were kept. The total count tube was treated exactly as described above. The following formula was used to calculate the specific activity of ^{125}I-insulin:

Specific activity (μCi/μg) =

$$\left[\frac{\text{Post-TCA precipitation total count value}}{\text{Pre-TCA precipitation total count value}}\right] \times 300 \times \frac{1}{1.16}$$

300μCi was the amount of label used in the experiments; 200pmoles of insulin was used in the reaction which equals 1.16μg of insulin. Specific activity of resultant ^{125}I-insulin was in the range of 120-130μCi/μg. The radioiodination is easy to perform and exposure of peptide to the oxidant in the two-phase system is reduced, a product of high specific activity is obtained, oxidative damage of the peptide is minimized, and the half life ($t_{1/2}$ = 60 days) of the ^{125}I-insulin is reasonably long.

5.2.5 Data presentation

Glucose uptake was expressed in two ways: Glucose uptake in pmol/10^6cells/hour (Figure 1) or as a percentage (Figure 2) of the glucose uptake in the absence of insulin. Glucose uptake in pmol/10^6cells/hour was calculated as follows:

$$\left[\frac{[(\text{sample counts} - (\text{background} + \text{NSB})]}{100\,000}\right] \times 322 \times 10^6 \quad \frac{}{\text{cells per tube}}$$

The ^{14}C glucose has a specific activity of 100 000cpm per 322 pmoles glucose. The percentage glucose uptake level was calculated as follows:

$$\left[\frac{\text{glucose uptake in presence of insulin}}{\text{glucose uptake in absence of insulin}}\right] \quad X\ 100$$

Insulin binding was expressed as cpm per 10 000 cells.

5.2.6 Statistical Analysis

Data are expressed as means ± SEM. Student's unpaired t-test was used to analyse the differences between the two groups whilst a paired t-test was used for the analysis of differences within the groups.

5.3 Results

5.3.1 Clinical measurements

The clinical measurements (i.e. BMI, waist-hip-ratio and age) results were similar to those shown in Chapter 4, section 4.3.1 (Table 1).

5.3.2 Free fatty acid measurements

Table 2 shows that the obese black women had a 56% increase in fasting plasma FFA concentration ($p<0.01$) compared with the obese white women. Insulin glucose levels were slightly increased in the obese black women, but not statistically significantly so. These results are comparable with those shown in Chapter 4, section 4.3.2.

5.3.3 Glucose uptake

In both ethnic groups, adipocytes from the abdominal region did not show any significant increase in the rate of glucose uptake at either insulin concentration. The femoral adipocytes from white obese subjects, however, showed a significant increase in glucose uptake when basal (no insulin added) and 0.7nmol/l insulin concentrations were compared ($p<0.05$). There was no significant increase in glucose uptake in the presence of insulin by femoral adipocytes from obese black subjects. Basal glucose uptake in adipocytes from abdominal area of obese black subjects was significantly higher than in abdominal adipocytes from obese white subjects ($p<0.01$).

The percentage glucose uptake by femoral adipocytes at 7.0nmol/l insulin concentration was significantly higher in the white subjects when compared with their black counterparts ($p<0.05$).

5.3.4 Insulin binding

Studies were carried out on only 2 black obese women and the results are shown in Table 4. Insulin binding levels were similar for abdominal and femoral adipocytes.

5.4 Discussion

In obese black women there was an insignificant increase in glucose uptake in the presence of insulin by abdominal and femoral adipocytes. In obese white

women there was a significant increase in glucose uptake in femoral adipocytes in the presence of 0.7nmol/l insulin, but no significant increase above basal levels were observed in abdominal adipocytes. When glucose uptake was expressed as a percentage increase above basal glucose uptake there was a significant difference between black and white women in femoral adipocytes at an insulin concentration of 7.0nmol/l, again emphasising the difference in insulin sensitivity between the two groups of women.

The reason for the lower insulin sensitivity of glucose uptake in adipocytes isolated from black obese women compared to adipocytes from white obese women is unknown. It is believed that this difference is unlikely to be due to insulin receptor numbers as our previous studies have shown that the ED_{50} values for insulin-inhibition of lipolysis are similar between the ethnic groups. An alternative hypothesis would be that ethnic differences exist in insulin receptor signalling. The control of both glucose uptake and inhibition of lipolytic activity by insulin is mediated by the insulin receptor PI 3-kinase pathway (Degerman *et al*, 1997).

Insulin binding to adipocytes was analysed in the present study, but only two obese, black women, comparing femoral to abdominal adipocytes. The insulin binding level was similar between these two populations, but because of the low n number it is difficult to draw conclusions. However, a recent study by Zierath *et al* (1998), has shown that differences in insulin sensitivity between subcutaneous and omental fat depots was due to differences in insulin

insulin receptor autophosphorylation levels and PI 3-kinase activity, and not due to differences in insulin receptor numbers. Whether such differences exist between these two ethnic groups is not known and obviously requires further study.

In the absence of insulin, glucose transport can be mediated by the constitutive glucose transporter , GLUT 1, which is expressed in all tissue types (Shepherd and Kahn, 1999). Whether levels of GLUT 1 are higher in black compared to white obese women is unknown and future studies must include an analysis of GLUT 1 and GLUT 4 (insulin-sensitive glucose transporter) levels in adipocytes from these ethnic groups.

Measurements	Black Women	White Women	p-value
Age (years)	36.0 ± 1.8	39.0 ± 0.8	N.S.
BMI (kgm^2)	36.5 ± 1.8	37.8 ± 1.8	N.S.
Waist-hip ratio	0.81 ± 0.03	0.81 ± 0.10	N.S.
Body Fat (%)	45.1 ± 1.7	46.7 ± 1.6	N.S.
n number	10	10	

Table 1. Clinical characteristics of two population groups. Values are means ±SEM; N.S. => not significant.

Measurements	Black Women	White Women	p-value
FFA (μmol/l)	897 ± 89.8 (560 – 1300)	516.0 ± 52.9 (290 – 740)	<0.01
Insulin (pmol/l))	64.4 ± 0.7 (33.6 – 106.4)	164.8 ± 12.5 (79.8 – 226.8)	<0.01
Glucose (mmol/l)	4.6 ± 0.2	4.5 ± 0.2	N.S.
Insulin resistance (HOMA)	1.9 ± 0.2 (1.7 - 2.1)	4.7 ± 0.6 (4.4 - 5.6)	<0.01

Table 2. Biochemical characteristics of the two population groups. Values are means ± SEM. N.S. = not significant. HOMA = homeostasis model assessment. Numbers in parentheses are ranges.

Adipocyte Population	Black Women: adipocyte diameter (μm)	White Women: adipocyte diameter (μm)	p-value
Abdominal	128 ± 14.6 (99.8 – 225.1)	111 ± 2.2 (103.8 – 122.0)	N.S.
Femoral	123.3 ± 12.2 (105.3 – 216.5)	110.0 ± 1.0 (108.4 – 123.0)	N.S.

Table 3. Size of adipocytes isolated from obese black and white women. Values are mean ± SEM. Numbers in parentheses are reference ranges.

Adipose Site	Binding (cpm/10 000 cells	p-value
Abdominal	3899±2243	N.S.
Femoral	3045±1123	N.S.

Table 4. Insulin binding to abdominal and femoral adipocytes isolated from two obese black women. Values are means ± SEM; N.S. => not significant.

Figure 1(a)

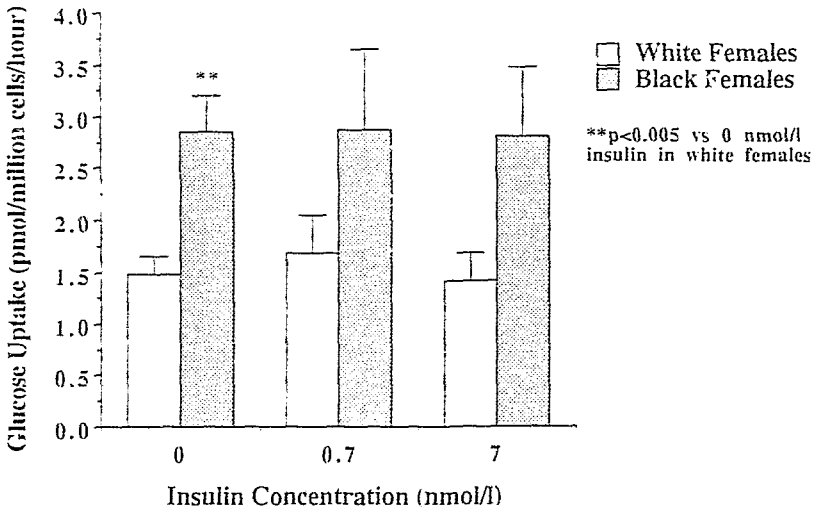


Figure 1(b)

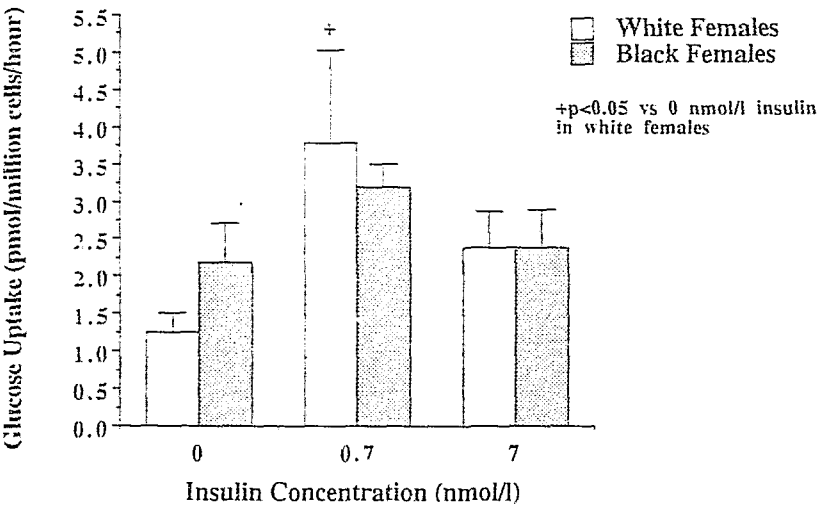


Figure 1. Glucose uptake in (a) abdominal and (b) femoral adipocytes isolated from obese black and white women.

Figure 2(a)

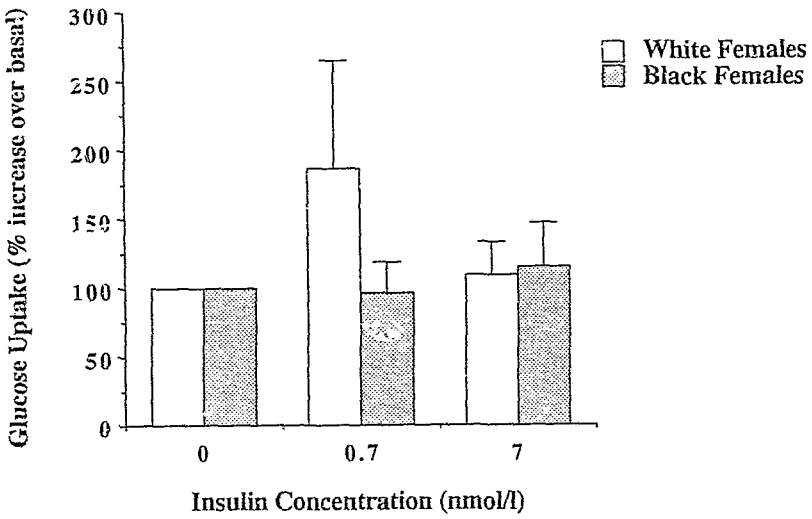


Figure 2(b)

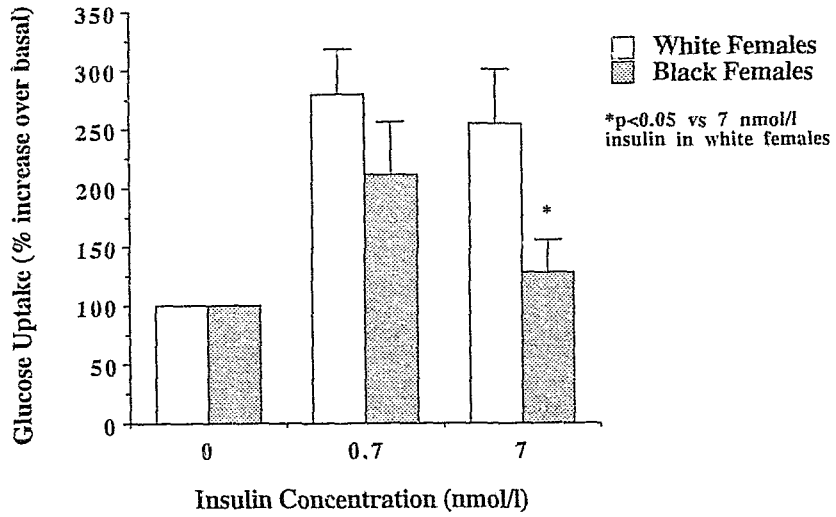


Figure 2. Percentage glucose uptake in (a) abdominal and (b) femoral adipocytes isolated from obese black and white women.

Following oral administration of glucose, the liver, muscle and adipose tissue are the main organs involved in glucose clearance. It has been calculated that adipose tissue accounts for 10 to 20% of whole body glucose disposal (Frost, 1996), and 60-70% of the glucose taken up by adipocytes is converted to lactate (Kashiwagi *et al*, 1983). Thus, adipose tissue plays a major role in the control of blood glucose levels. In this study, fasting blood glucose levels were similar in the obese white and black women, despite higher whole body insulin resistance in the black women, and lower *in vitro* stimulation of glucose uptake by insulin in adipocytes from these subjects. However, basal glucose uptake, measured in the absence of insulin was higher in the black women and this may explain why fasting blood glucose levels were similar to those of the white females. Again, the role of the GLUT 1 transporter in maintaining euglycaemia in the face of increased insulin resistance in the black females needs to be studied.

This study confirms the data obtained from investigations of the sensitivity of adipocytes to insulin-inhibition of lipolysis. In both studies, femoral adipocytes, particularly from white obese women, were more sensitive to insulin and in both studies adipocytes from black obese women were less sensitive to the action of insulin on both lipolysis and glucose uptake. The possible reasons for the interethnic difference in adipocyte insulin-sensitivity will be discussed in the next chapter.

CHAPTER 6

Discussion and Conclusion

In summary this study has shown that adipocytes particularly those isolated from femoral adipose depot of black obese women are less responsive to the inhibitory effect of insulin on lipolysis compared to adipocytes isolated from white obese subjects. The glucose uptake data demonstrated impairment in insulin-stimulated glucose uptake by adipocytes isolated from black obese women when compared with the adipocytes isolated from obese white women. However, basal glucose uptake in the absence of insulin is greater in abdominal adipocytes from black than white women. Furthermore, fasting insulin levels were lower but fasting FFA levels were higher in obese black than white women.

The pathogenesis of type 2 diabetes of the black population in South Africa appears to be different from that of the white population as evidenced by an earlier age of onset, more acute disease decompensation and lower plasma insulin concentrations (Shires *et al*, 1978; Joffe *et al*, 1992). Obesity is strongly associated with the development of type 2 diabetes and studies carried out in obese white and black South African women have shown that the latter group have lower fasting insulin levels and higher fasting free fatty acid and leptin levels (van der Merwe *et al*, 1998; van der Merwe *et al*, 1999).

The data presented in this study demonstrates further metabolic differences between these two population groups. Adipocytes from obese black women are more insulin resistant than those from white obese women both in terms of inhibition of lipolysis and stimulation of glucose uptake. The glucose clamp studies showed that the obese black women were more insulin resistant than the white women. This contradicts our results and shows that the pathophysiology of insulin resistance within the black African population is different to that observed in American blacks. This is further demonstrated by a study showing that insulin levels in native Nigerians are lower than those in African-Americans (Osei *et al*, 1993).

Our studies have demonstrated increased peripheral and whole body insulin resistance in black obese women. The cause of the greater insulin resistance in the black women is not known. However, these results suggest two possible explanations: the first is that high plasma free fatty acid levels in the black women may play a role. Increased plasma free fatty acid concentrations cause acquired insulin resistance via the Randle Cycle (Randle *et al*, 1963; Boden, 1997) and impairment of ß-cell function in humans (Zhou and Gill, 1995; Padisso *et al*, 1995). This may explain the higher insulin resistance of the black women despite lower plasma insulin levels when compared to the white women. The high free fatty acid levels observed in the black women is probably a result of insulin insensitivity of the adipocytes in combination with relative insulinopaenia. The second factor that may be involved in the higher insulin resistance of the black women is leptin. Two previous studies (van der Merwe

et al, 1999 and Punyadeera *et al*, 1999) have shown that leptin levels are higher in black than white obese women.

It has been suggested that excessive secretion of leptin by adipose tissue may be one mechanism whereby increased adiposity causes insulin resistance (Cohen *et al*, 1996). The mechanism by which leptin contributes to insulin resistance could be its antagonistic effect on insulin signalling by decreasing insulin-induced tyrosine phosphorylation of IRS-1 leading to downregulation of PI 3-kinase (Taylor *et al*, 1996). Cohen *et al* (1996), showed that leptin antagonises insulin's ability to decrease mRNA encoding phosphoenolpyruvate carboxykinase (PEPCK), the enzyme catalysing the rate-limiting step in gluconeogenesis. Whether leptin plays any role in the insulin insensitivity of adipocytes from black obese women is not known and obviously warrants further investigations, as does the level of PI 3-kinase activity in adipocytes from black and white obese women.

In adipocytes, insulin increases glucose uptake and inhibits lipolysis via the activation of the insulin receptor-PI 3-kinase signal transduction pathway. Therefore, the reduced insulin sensitivity of adipocytes from black obese women compared to those from white obese women in terms of glucose uptake and inhibition of lipolysis may be due either to fewer insulin receptors or reduced PI 3-kinase and PI 3-kinase-related kinase activity. The similar ED50 values for insulin inhibition of lipolysis in adipocytes from both population groups suggest that lower PI 3-kinase activity may be involved. This must be

proven via Scatchard analysis of insulin binding and measurement of insulin receptor kinase and PI 3-kinase activity in adipocytes from black and white obese women. A recent study has shown that differences in insulin sensitivity between different fat depots is due to differences in insulin receptor signalling activity and not insulin receptor numbers (Zierath *et al*, 1998).

Differences in adipocyte metabolism between obese black and white women have therefore been identified, but the reasons for these differences have not. However, studies carried out in rodents may provide some clues. It has been shown that the offspring of female rats fed a low protein diet during gestation and weaning have altered adipocyte metabolism compared to offspring of rats fed a normal protein level during this period (Ozanne *et al* 1997; Shepherd *et al*, 1997). Poor diet during pregnancy may be more common in the black South African population than the white population due to poorer social circumstances and this may affect adipocyte metabolism. Studies in the rats showed that adipocytes from 3-month old animals born of mothers fed a low protein diet during pregnancy had higher GLUT 4 levels and higher PI 3-kinase activity than adipocytes isolated from rats born of mothers given a normal protein level during pregnancy. The insulin sensitivity of these adipocytes was not studied but it was shown that the animals exposed to a low protein diet were more glucose tolerant compared to animals who were exposed to higher protein levels, in utero. Neither group of animals were obese at this age. When these two groups of animals were studied at 15 months of age when they were obese the situation was reversed, with low-protein group being less glucose

tolerant than the high-protein group. Also, adipocytes isolated from the low protein group were more insulin resistant and had lower PI 3-kinase activity levels than adipocytes taken from the high protein exposed group (Ozanne *et al*, 1999). Recent studies have shown that lean, 30- year old black women are more glucose tolerant than 30-year old white females (Punyadeera *et al*, personal communication), and this study has shown that the situation is reversed in older, obese women. The parallels between the human and rodent studies are clear and must be investigated more thoroughly to determine if the rodent model can be used to make accurate inferences about the aetiology and pathology of type 2 diabetes in human populations in developing countries.

Fasting insulin levels were lower in the black than in the white obese women. Other studies have shown similar results (Joffe *et al*, 1992; van der Merwe *et al* 1996). The reason for the relative insulinopaenia of the black women is not known, however it has been hypothesised by Joffe *et al* (1992) that this may be due to low ß-cell numbers in the black population resulting from poor fetal nutrition particularly in terms of protein intake. In support of this, a study has shown that small for gestational age births are common within the black population (Cooper *et al*, 1996) and that in 7-year old African children, birthweight correlates positively with glucose tolerance (Crowther *et al*, 1998). As mentioned earlier, the high FFA levels observed in the black women may also contribute to their relative insulinopaenia. Post-mortem on pancreatic

sections from subjects with insulinomas must be studied to determine whether there are ethnic differences in β-cell numbers.

Hypertension like type 2 diabetes has also been linked to poor fetal growth (Barker *et al*, 1993, Valdez *et al*, 1994, Lithell *et al*, 1996, Roseboom *et al*, 1999) and a study carried out in South Africa has confirmed this in an African population (Levitt *et al*, 1999). It is also known that hypertension is more common in the black than the white South African population (Seedat, 1983).

The prevalence of ischaemic heart disease (IHD) is higher in the white population of South Africa than is the black population (Isles and Milne, 1987; Walker *et al*, 1983). The reason for the low prevalence of IHD within the black community especially in the presence of high plasma FFA levels is not known although it has been reported that the clearance rate of triglycerides is higher in black obese subjects compared to lean subjects (Shires *et al*, 1978) and that lipid profiles in the black population are less atherogenic than is the white population (Steyn *et al*, 1995; van der Merwe *et al*, 1996). The lower plasma insulin levels observed in the black population may also influence the incidence of ischaemic heart disease (IHD). The higher incidence of IHD among obese white women may well be associated with their larger amount of visceral fat (Björntorp, 1991; van der Merwe *et al*, 1998). Furthermore, several investigators have shown that higher FFA, in the presence of hypoinsulinaemia will lead to lower cardiovascular disease morbidity when compared with the

combination of elevated FFA and hyperinsulinaemia (Arner *et al*, 1981; Bolinder *et al*, 1983).

It has been shown that obese black women are more insulin resistant and their adipocytes are less insulin-sensitive than those from white obese women. These findings demonstrate that the best treatment for type 2 diabetes in black obese subjects may be via drugs that improve insulin sensitivity , rather those that increase insulin secretion. This may be especially true if black subjects have fewer pancreatic islet ß -cells. The thiazolidinedione group of drugs may be particularly useful for treating black, obese, type 2 diabetics because they are insulin-sensitizers that act mainly by improving adipocyte insulin sensitivity. It has been suggested that they accomplish this by reducing leptin and TNF-α production by adipocytes (Spiegelman, 1998). Therefore, thiazolidinediones may reduce the high leptin and high free fatty acid levels observed in black obese subjects, by acting directly on the tissue responsible for their production.

This study has shown that adipocytes from obese black women are less sensitive to the action insulin with respect to inhibition of lipolysis and stimulation of glucose uptake than adipocytes from obese white women. One cosequence of this may be higher FFA levels in the black women, which may contribute to their higher whole-body insulin resistance (Buthelezi *et al*, 2000). Other studies have demonstrated higher leptin levels in obese black women (van der Mewe, *et al*, 1999; Punyadeera *et al*, 1999) and lower visceral fat

mass (van der Merwe *et al*, 1996) than in white women and these factors may play a role in the higher insulin resistance and the lower incidence of IHD respectively within the black South African population. Ethnic differences in adipocytes metabolism and body fat distribution may therefore play an important role in the different pathologies and incidence of obesity-related disorders observed in these two populations and it is important to determine what environmental factors may contribute to these observations in particular the role of fetal nutrition and socio-economic studies.

The main drawback of the present study is that adipocyte metabolism was investigated under non-physiological conditions, although many other studies have used *in vitro* techniques to examine adipocyte function. An *in vivo* study by van der Merwe et al (1999) of abdominal and femoral fat depots of obese black and white women by analysis of glycerol levels in the extra cellular fluid confirms the results from the present study; *in vivo* lipolysis rates were higher in the fat depots of black than white women. Also, using the euglycaemic hyperinsulinaemia clamp method (Buthelezi *et al*, 2000) black obese women were found to be more insulin resistant than white obese women confirming that the higher insulin resistance of adipose tissue from black women is mirrored by higher whole body insulin resistance. The reduced insulin sensitivity of adipocytes from black obese women can be further confirmed by analysing insulin receptor numbers by Scatchard analysis and function by *in vitro* analysis of insulin receptor autophosphorylation and phosphorylation of IRS-1 and IRS-1-associated PI3-kinase activity. The lower insulin-stimulated

glucose uptake of femoral adipocytes from black obese women could be further investigated by analysis of GLUT-4 levels in plasma membrane and intra cellular membrane fractions. The hypothesis that increased GLUT-1 levels may explain the higher non-insulin stimulated basal glucose uptake rates in adipocytes from black women could be confirmed by measuring GLUT-1 mRNA and protein levels in both populations.

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Ethnic Differences in the Responsiveness of Adipocyte Lipolytic Activity to Insulin

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Abstract

BUTHELEZI, ERNEST P., MARIA-TERÉSA VAN DER MERWE, PETER N. LÖNNROTH, I. PETER GRAY, AND NIGEL J. CROWTHER. Ethnic differences in the responsiveness of adipocyte lipolytic activity to insulin. *Obes Res.* 2000;8:171–178.

Objective: The goal of this study was to quantify differences in lipid metabolism and insulin sensitivity in black and white subjects to explain ethnic clinicopathological differences in type 2 diabetes.

Research Methods and Procedures: The in vitro lipolytic activity of adipocytes isolated from obese black and white women was measured in the presence of insulin and isoproterenol. Insulin resistance was assessed in vivo using the euglycemic hyperinsulinemic clamp technique.

Results: Fasting plasma levels of insulin and nonesterified fatty acid (NEFA) in black and white women were 67 ± 5 pM vs. 152 ± 20 pM ($p < 0.01$) and 863 ± 93 μM vs. 412 ± 34 μM ($p < 0.01$), respectively. Euglycemic hyperinsulinemic clamp studies showed that obese black subjects were more insulin-resistant than their white counterparts (glucose infusion rates: 1.3 ± 0.2 vs. 2.2 ± 0.3 mg/kg per min; $p < 0.05$). Isolated adipocytes from white women were more responsive to insulin than those from black women with 0.7 nM insulin causing a 55 ± 4% inhibition of isoproterenol-stimulated lipolysis compared with 27 ± 10% in black women ($p < 0.05$).

Discussion: The low responsiveness of adipocyte lipolytic activity to insulin in black women in the presence of a relative insulinopenia may account for the high plasma NEFA levels seen in these women, which may, in turn, account for their higher in vivo insulin resistance. High NEFA levels may also contribute to the low insulin secretory activity observed in the obese black females. These data suggest that the pathogenesis of insulin resistance and type 2 diabetes within the black obese community is strongly influenced by their adipocyte metabolism.

Key words: adipocytes, lipolysis, insulin sensitivity, ethnic differences

Introduction

Obesity, a major cause of morbidity and mortality (1), is associated with increased risk of chronic diseases such as type 2 diabetes and cardiovascular disease, including hypertension (2,3). In South Africa the prevalence of obesity, i.e., body mass index (BMI) > 30 kg/m^2, is 47.7% for black and 15.6% for white females within the age group 35 to 44 years (4,5). The prevalence of type 2 diabetes among the black community is 6% to 7% (6).

Obesity is strongly associated with insulin resistance (7,8), which may be a major cause of chronic diseases, particularly type 2 diabetes (9,10). The mechanism by which obesity gives rise to insulin resistance is unknown; however, excessive circulating plasma nonesterified fatty acids (NEFA) may play a role (11,12).

A recent clinical study has shown that obese black South African women have higher plasma NEFA levels, lower serum insulin concentrations and higher in vivo lipolytic rates compared with whites (13). The present study, using isolated adipocytes and glucose clamp studies to measure whole body insulin sensitivity, sought to clarify whether the higher systemic lipolytic rate observed in black women is due to lower insulin concentrations or to resistance to the antilipolytic effect of insulin and also to measure whole body insulin sensitivity in the two population groups.

Research Methods and Procedures

Subjects

Ten black and ten white women were matched for age, waist-hip ratio, BMI, percentage of body fat, duration of

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obesity (10 to 12 years), and number of offspring (no more than three). None of the women were postmenopausal or taking any oral contraceptives. Exclusion criteria included diseases of the major organs on the basis of clinical examination and history (e.g., regular menstrual cycles), smoking in excess of five cigarettes per day, and alcohol consumption in excess of 4 g of alcohol per day or a history of eating disorders. Patients fasted for 10 hours, abstained from alcohol for 24 hours, and did not exercise for 12 hours before the tests. All patients gave informed consent, and the study was approved by the Committee for Research on Human Subjects of the University of the Witwatersrand.

Analytical Methods

Blood glucose and plasma NEFA levels were measured using commercially available assay kits (Boehringer Mannheim, Germany). Insulin levels were measured with an enzyme amplified immunoassay that does not cross-react with proinsulin (Mercodia, Belgium).

Body Composition Analyses

Anthropometric measurements were as previously described (13): the waist measurement was taken at the midpoint between the lowest rib and the suprailiac crest. Bioelectrical impedance was measured in the fasted state using the Bodystat device (Bodytrach Pty. Ltd., Johannesburg, South Africa) to assess fat and muscle mass.

Euglycemic Hyperinsulinemic Clamp Studies

These were completed on a separate day, within 2 weeks of the fat biopsy studies, during the luteal phase of the menstrual cycle. The patients were asked to fast from 10:00 PM the previous night, and the investigations started at 8:00 AM the next morning. The patients were studied in the supine position. Two polyethylene catheters were placed intravenously, one in each forearm. One catheter was used for infusion of insulin (Actrapid; Novo-Nordisk, Copenhagen, Denmark) and the other for 20% glucose. The contralateral forearm was placed under a heating pad to ensure the sampling of arterialized venous blood (14,15). The insulin infusion rate was set at 80 mL/m^2 per minute. The glucose infusion rate was varied at 10-minute intervals as calculated from a computerized algorithm based on a minimal model of glucose kinetics (16). The fasting glucose was reduced or increased steadily over 1 hour to a targeted concentration of 5.5 mM, and the blood glucose was clamped at these levels. The glucose infusion rates during steady state were calculated over the last 60 minutes and reported in milligrams per kilogram per minute. A fasting blood sample was taken, and further samples were taken at 20-minute intervals throughout the steady-state period.

Fat Tissue Biopsy

Needle biopsies of the subcutaneous adipose tissue were taken from the abdominal area (5 cm lateral to the umbilicus) and at the level of the midfemur under local anesthetic (2% Lignocaine HCL (remicaine); Adcock Ingram Generics, Johannesburg, South Africa) using a 1.8- by 14-mm needle (Promex, South Africa) and a 50-mL syringe. Care was taken not to infiltrate the tissue to be excised. The interval between abdominal and femoral aspirations was approximately 7 minutes.

Isolation of Adipocytes

Medium 199 (M199) containing Hanks' salts (Life Technologies, Gaithersburg, MD) supplemented with glucose (1 mg/mL), sodium bicarbonate (0.35 mg/mL, Saarchem, South Africa), and 40 mg/mL bovine serum albumin (Sigma, Poole, England) was used throughout. Glass tubes used for washing adipocytes were silanized before use.

Aspirated adipose tissue was washed once and then digested using collagenase (0.8 mg/mL; Boehringer Mannheim, Germany) at 37 °C for 40 minutes, in a shaking water bath under normal atmospheric conditions. The adipocytes were filtered through a nylon mesh of pore size 500 μm and washed four times with M199 (37 °C) that was free of collagenase. After the final wash the adipocytes were resuspended in M199.

Estimation of Triglyceride Content

The triglyceride content of each adipocyte preparation was measured as previously described (17). Fat cell size was estimated using the method of Smith et al. (18) in which the diameter of 100 adipocytes was measured using a calibrated ocular lens-mounted graticule and a microscope (CK light; Olympus, Tokyo, Japan).

Assessment of Adipocyte Function

Adipocyte function was assessed by measurement of lipolytic activity in the presence of isoproterenol, a stimulant of lipolysis by virtue of its nonselective β-adrenergic agonist activity (19).

The antilipolytic effect of insulin was assessed by measuring adipocyte glycerol production in response to 1.0 μM isoproterenol in the presence of varying concentrations of insulin (0.07, 0.72, 2.40, and 7.20 nM Actrapid insulin; Novo-Nordisk). Incubations lasted for 1 hour at 37 °C in a shaking water bath in air (20). Glycerol was measured using an enzymatic method (21).

Data Presentation

Lipolytic activity was expressed as glycerol released per mm^2 of adipocyte surface area per hour (Figure 1). The antilipolytic response to insulin was expressed as the percentage inhibition of lipolysis (Figure 2), which was calculated according to the following formula:

$$1 - \left[\frac{\text{lipolytic activity in presence of insulin and isoprotorenol}}{\text{lipolytic activity in presence of isoprotorenol only}} \right] \times 100$$

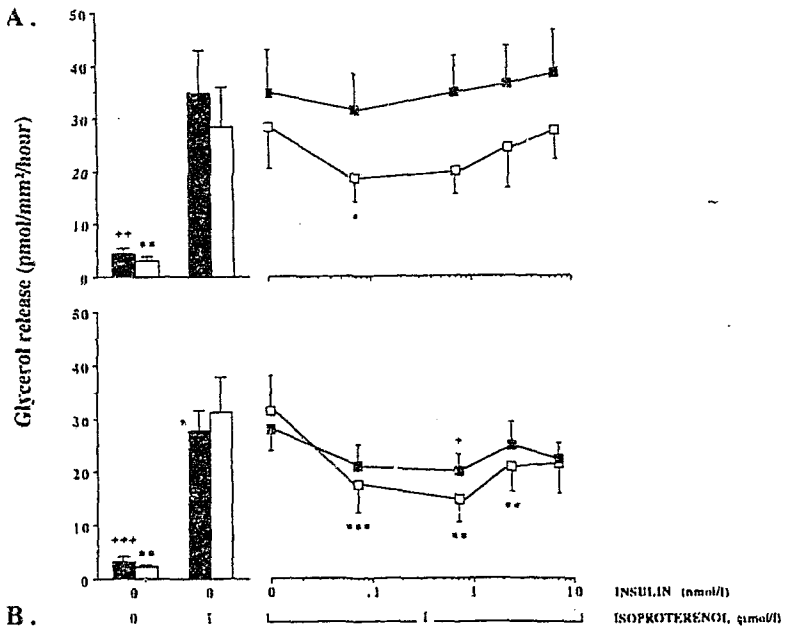


Figure 1. Glycerol release of adipocytes isolated from abdominal (A) and femoral (B) adipose tissue. ■, adipocytes from black obese females; □, adipocytes from white obese females; $*p < 0.05$, $**p < 0.005$, and $***p < 0.0005$ vs. lipolysis rate in the presence of isoproterenol but the absence of insulin of adipocytes taken from white females; $+p < 0.05$, $++p < 0.005$, and $+++p < 0.0005$ vs. lipolysis rate in the presence of isoproterenol but the absence of insulin of adipocytes taken from black females. Data are presented as means ± SEM ($n = 8–10$).

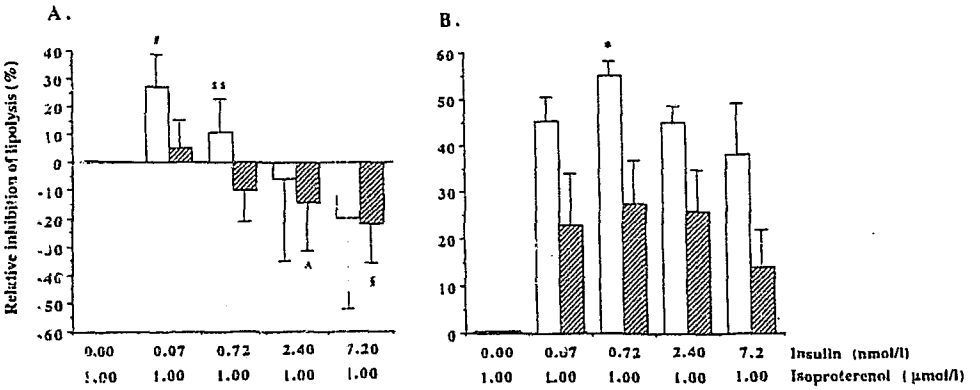


Figure 2. The percentage inhibition of lipolysis in abdominal (A) and femoral (B) adipocytes. ▨, adipocytes from black, obese females; □, adipocytes from white, obese females; $*p < 0.05$ vs. lipolysis rate in the presence of 0.72 nM insulin of femoral adipocytes isolated from black subjects; $^{\#}p < 0.05$ and $^{\$\$}p < 0.005$ vs. lipolysis rate in the presence of 0.07 and 0.72 nM insulin, respectively, of femoral adipocytes isolated from white subjects; $^{\wedge}p < 0.05$ and $^{\$}p < 0.05$ vs. lipolysis rate in the presence of 2.40 and 7.20 nM insulin, respectively, of femoral adipocytes isolated from black subjects. Data are presented as means ± SEM ($n = 8–10$).

Expressing lipolytic activity in this way made comparisons between ethnic groups of the effect of insulin on the inhibition of isoproterenol-stimulated lipolysis easier and clearer.

The ED_{50} values, which were used as a measure of insulin sensitivity of the adipocytes to the antilipolytic effect of insulin, were calculated for each subject using standard procedures (22).

Statistical Analyses

Data are expressed as means ± SEM unless otherwise stated. Student's unpaired *t* test was used to analyze differences between the two ethnic groups, whereas a paired *t* test was used for the analysis of differences within groups.

Results

Clinical Measurements

The clinical characteristics were similar in the two groups (Table 1).

Insulin, Glucose, and NEFA Concentrations

Obese black women had an almost 2-fold higher fasting plasma NEFA concentration ($p < 0.01$) than did the white women (Table 2). In addition, the obese black women were relatively insulinopenic in comparison with their white counterparts ($p < 0.001$).

Measurement and Estimation of Insulin Resistance

Estimations of in vivo insulin resistance in obese black and white women are shown in Table 2. The euglycemic clamp technique demonstrated that the obese black women were more insulin-resistant than the white women ($p < 0.05$). Plasma insulin levels were similar in both groups of subjects during steady state (624 and 639 pM, respectively).

Adipocyte Analyses

The size of abdominal and femoral adipocytes in both groups were not statistically significantly different (Table 3), although adipocytes isolated from black females did tend to be larger than those from white females. This was due to two outliers in the black subject group who had abdominal and femoral adipocytes with diameters above 200 μm.

A dose-response curve of glycerol release vs. isoproterenol demonstrated that 1.0 μM isoproterenol elicited the maximum lipolytic response (data not shown). Furthermore, Figure 1 shows that abdominal and femoral adipocytes from both subject groups were responsive to 1.0 μM isoproterenol thus demonstrating normal lipolytic activity. Previous investigators also found that 1.0 μM isoproterenol produced maximum stimulation of lipolysis (19,20).

Ethnic Differences in the Antilipolytic Effect of Insulin

Absolute glycerol release by adipocytes from black women stimulated by 1 μM isoproterenol was statistically significantly inhibited by insulin only in femoral adipocytes and by 0.72 nM insulin (Figure 1B). However, in the white females there was statistically significant inhibition of lipolysis in both abdominal (by 0.07 nM insulin; Figure 1A) and femoral (by 0.07, 0.72, and 2.40 nM insulin; Figure 1B) adipocytes. The abdominal and femoral adipocytes isolated from white subjects were more responsive to the antilipolytic effect of insulin than were those taken from black subjects and statistically significant differences in the inhibition of lipolytic activity by insulin between the ethnic groups were seen in the femoral adipocytes at an insulin concentration of 0.72 nM ($p < 0.05$) (Figure 2B). Furthermore, abdominal adipocytes from both ethnic groups were more resistant to the effects of insulin than were femoral adipocytes. Thus, femoral adipocytes demonstrated a higher statistically significant percentage of inhibition of lipolysis than did abdominal adipocytes in white females at insulin concentrations of 0.07 ($p < 0.05$) and 0.72 ($p < 0.005$) nM and in black females at insulin concentrations of 2.40 and 7.20 nM ($p < 0.05$ for both) (Figure 2, A and B).

Glycerol release was also expressed as a function of adipocyte cell number and triglyceride content, and the results obtained were similar to those shown in Figure 1. This demonstrates that ethnic differences in the responsiveness of adipocyte lipolysis to insulin are not due to differences in adipocyte morphology.

There were no statistically significant differences between the ED_{50} values for the antilipolytic effect of insulin in any of the adipocyte populations that were studied. The ED_{50} values were as follows (units are pM ± SEM): white abdominal adipocytes, 42 ± 14; black abdominal, 33 ± 6; white femoral, 56 ± 12; black femoral, 40 ± 5.

Table 1. Anthropometric indices of study populations

Measurements	Black females	White females	*p* value
Age (years)	37.0 ± 1.7*	35.0 ± 0.8	NS
BMI (kg/m^2)	34.2 ± 1.8	35.3 ± 1.8	NS
Waist circumference (cm)	96.7 ± 2.1	100.3 ± 4.4	NS
Hip circumference (cm)	118.7 ± 2.2	119.4 ± 3.8	NS
Waist-hip ratio	0.82 ± 0.03	0.83 ± 0.02	NS
Body fat (%)	46.1 ± 1.6	44.1 ± 1.6	NS

* Values are means ± SEM; NS, not significant.

Discussion

The pathogenesis of type 2 diabetes of the black population of South Africa appears to be different from that of the

Table 2. Biochemical indices of study populations

Measurements	Black females	White females	*p* value
NEFA (μM)	863.0 ± 93.1* (560–1300)†	412.0 ± 34.2 (290–740)	<0.01
Insulin (pM)	66.7 ± 4.7 (33.6–106.4)	152.0 ± 20.1 (79.8–226.8)	<0.01
Glucose (mM)	4.5 ± 0.2	4.0 ± 0.2	NS
Glucose infusion rate (mg/kg/min)	1.3 ± 0.2	2.2 ± 0.3	<0.02

* Values are means ± SEM; NS, not significant. Insulin, glucose, and NEFA values are all fasting levels.
† Numbers in parentheses are ranges.

white population as evidenced by an earlier age of onset, more acute disease decompensation, lower plasma insulin concentrations, and higher plasma free fatty acid concentrations (13,23,24). There are also ethnic differences in diseases associated with type 2 diabetes; hypertension is more common within the black community (25), whereas ischemic heart disease (IHD) is rare in comparison with the white population (26,27). Insulinopenia may be due to a decreased β-cell mass as a result of fetal or neonatal protein-energy malnutrition and/or genetic factors (24). Several studies, including one carried out in South Africa (28), have shown an association between low birth weight, which is common among black South Africans, and poor glucose tolerance (29–31). Hypertension has also been linked to poor fetal growth (29). The reason for the low incidence of IHD within the black community, especially in the presence of high plasma free fatty acids, is not known. It has been reported that the clearance rate of triglycerides is higher in black obese subjects than in lean subjects (32) and the lower plasma insulin levels observed in the black population may also influence the incidence of IHD.

Table 3. Size of adipocytes isolated from obese black and white females

	Adipocyte diameter (μm)		
Adipocyte population	Black females	White females	*p* value
Abdominal	132.0 ± 15.0* (99.8–225.1)†	115.0 ± 2.0 (103.8–122.0)	NS
Femoral	134.0 ± 13.7 (105.3–216.5)	115.0 ± 4.4 (108.4–123.0)	NS

* Values are means ± SEM; NS, not significant.
† Numbers in parentheses are ranges.

The present study shows that the fasting metabolism of white and black obese females is markedly different with the latter group having lower plasma insulin but higher plasma NEFA levels than the former group. This is a confirmation of previous studies (13,23,24), and a recent study has shown that these metabolic differences are not confined to the fasting state but also exist after oral administration of a glucose load (33).

The data presented in this study demonstrate a further ethnic difference in type 2 diabetes pathology, namely, that obese black women are more insulin-resistant than their white counterparts. African Americans have also been shown to be more insulin-resistant than white Americans but insulin levels observed in the former group were higher than those observed in the latter (34,35). This contradicts our results and shows that the pathophysiology of insulin resistance within the black African population is different to that observed in African Americans, perhaps due to genetic admixture (36) and different levels of acculturation (37). This is further demonstrated by a study showing that insulin levels in native Nigerians are lower than those in African Americans (38).

The greater degree of insulin resistance in the black group observed in our study may be caused by high NEFA concentrations. Increased plasma NEFA concentrations cause acquired insulin resistance (11,12) and impairment of β-cell function in humans (39,40). This may explain the higher insulin resistance of the black females despite lower plasma insulin levels when compared with the white females. The in vitro studies confirmed the insulin resistance showing that abdominal and femoral adipocytes isolated from obese black women are less responsive to insulin than those isolated from obese white females.

The ED_{50} values for insulin inhibition of lipolysis were similar for all the adipocyte populations studied, but the response of adipocytes from black subjects to the antilipolytic effect of insulin is blunted in comparison with those of the white subjects. Thus the higher serum NEFA

concentrations observed in the black subjects in the postabsorpative state are explained because insulin sensitivity is a major determinant of postabsorpative lipolytic rates (41). These results also suggest that the ethnic difference in insulin inhibitory activity may be due to an effect distal to insulin binding to its receptor, i.e., differences in insulin responsiveness of the adipocytes rather than insulin sensitivity may be involved. However, it should be noted that the ED_{50} values were calculated in this study using only four different insulin concentrations, and this will introduce some inaccuracy. Furthermore, the dose-response curves for insulin inhibition of lipolysis shown in Figure 1 demonstrate that in femoral adipocytes from both ethnic groups maximal inhibition of lipolysis occurs in the presence of 0.72 nM insulin (this is true in 72% of subjects), whereas in the abdominal adipocytes it occurs at 0.07 nM (this is true in 61% of subjects). This subject-to-subject variability causes the ED_{50} values to be not significantly different between the four adipocyte populations. However, the dose-response curves for the femoral adipocytes mirror each other quite closely as do those for the abdominal adipocytes; therefore, despite the variability, we believe that there are no interethnic differences in adipocyte insulin sensitivity. In order to confirm this statement the measurement of insulin receptor numbers and binding affinities by Scatchard analysis is required.

A study comparing African Americans to white Americans demonstrated ethnic differences in the ED_{50} values for insulin inhibition of lipolysis (42). In the same study abdominal adipocytes taken from upper-body obese white Americans were less insulin-sensitive than those from upper-body obese African Americans. This relationship was reversed in lower-body obese subjects. These results are at variance with our study and again suggest that the expressed phenotype of insulin resistance in black obese South Africans is different to that seen in obese African Americans.

The abdominal and femoral adipocytes isolated from black females are larger, but not statistically significantly so, than those isolated from white females, and it has been shown that larger adipocytes should be more responsive to the antilipolytic activity of insulin (43). This is not the case in the present study where the adipocytes from the black subjects are less responsive to the antilipolytic effect of insulin than those isolated from white subjects. Therefore, the ethnic differences in the responsiveness of adipocytes to insulin inhibition of lipolysis cannot be explained by differences in adipocyte size but must be due to other factors.

The lipolytic response to isoproterenol of all the adipocyte populations studied is similar with the basal lipolytic rate increasing 10-fold on addition of isoproterenol (see Figure 1). The basal lipolytic rate in both abdominal and femoral adipocytes and the isoproterenol-stimulated lipolytic rate in abdominal adipocytes are higher in the black females, but the differences between ethnic groups are not statistically significant. A previous study has shown that the in vivo lipolytic rate is higher in obese black females than in their white counterparts (13), and our data suggest that this may be a result of the insulin insensitivity of adipocytes taken from black obese females. β-Adrenergic agents stimulate lipolysis by increasing intracellular levels of adenosine 3′-cyclic monophosphate (cAMP) (44), whereas the antilipolytic action of insulin involves reduction of cAMP levels via an insulin receptor-mediated process (45). The reduced response to insulin in the adipocytes taken from the black subjects may be due to high intracellular cAMP levels and/or a perturbation in insulin receptor signalling.

Abdominal adipocytes were less responsive to insulin compared with femoral adipocytes in both the black and white population groups. This metabolic feature of subcutaneous abdominal adipose tissue in combination with its high lipolytic rate may explain results from a study showing that this fat depot predicts insulin sensitivity more strongly than does visceral fat (46). These data emphasize the important contribution of both visceral and subcutaneous abdominal fat to the pathology of type 2 diabetes (8) and other obesity-associated disorders.

In summary, the higher NEFA levels of the black obese females result from a combination of insulin insensitivity of adipose tissue and insulinopenia. The raised NEFA levels may in turn contribute to the higher in vivo insulin resistance of the black compared with the white obese females. Thus, the pathogenesis of type 2 diabetes, particularly within black obese subjects, may be strongly influenced by adipose tissue metabolism.

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 (Registry)

CLEARANCE CERTIFICATE PROTOCOL NUMBER M940104

PROJECT In vivo metabolism of fat tissue regions in black urban subjects.

INVESTIGATORS Dr M T van der Merwe

DATE CONSIDERED Internal Medicine

DATE CONSIDERED 940128

DECISION OF THE COMMITTEE

Approved with the attached conditions.

DATE 940215 CHAIRMAN .....
(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE 2/6/94 SIGNATURE .....

Ethnic Differences in the Responsiveness of Adipocyte Lipolytic Activity to Insulin

Ernest P. Buthelezi, Maria-Terésa van der Merwe,† Peter N. Lönnroth,‡ I. Peter Gray,* and Nigel J. Crowther**

Abstract

BUTHELEZI, ERNEST P., MARIA-TERÉSA VAN DER MERWE, PETER N. LÖNNROTH, I. PETER GRAY, AND NIGEL J. CROWTHER. Ethnic differences in the responsiveness of adipocyte lipolytic activity to insulin. *Obes Res.* 2000;8:171–178.

Objective: The goal of this study was to quantify differences in lipid metabolism and insulin sensitivity in black and white subjects to explain ethnic clinicopathological differences in type 2 diabetes.

Research Methods and Procedures: The in vitro lipolytic activity of adipocytes isolated from obese black and white women was measured in the presence of insulin and isoproterenol. Insulin resistance was assessed in vivo using the euglycemic hyperinsulinemic clamp technique.

Results: Fasting plasma levels of insulin and nonesterified fatty acid (NEFA) in black and white women were 67 ± 5 pM vs. 152 ± 20 pM ($p < 0.01$) and 863 ± 93 μM vs. 412 ± 34 μM ($p < 0.01$), respectively. Euglycemic hyperinsulinemic clamp studies showed that obese black subjects were more insulin-resistant than their white counterparts (glucose infusion rates: 1.3 ± 0.2 vs. 2.2 ± 0.3 mg/kg per min; $p < 0.05$). Isolated adipocytes from white women were more responsive to insulin than those from black women with 0.7 nM insulin causing a 55 ± 4% inhibition of isoproterenol-stimulated lipolysis compared with 27 ± 10% in black women ($p < 0.05$).

Discussion: The low responsiveness of adipocyte lipolytic activity to insulin in black women in the presence of a relative insulinopenia may account for the high plasma NEFA levels seen in these women, which may, in turn, account for their higher in vivo insulin resistance. High NEFA levels may also contribute to the low insulin secretory activity observed in the obese black females. These data suggest that the pathogenesis of insulin resistance and type 2 diabetes within the black obese community is strongly influenced by their adipocyte metabolism.

Key words: adipocytes, lipolysis, insulin sensitivity, ethnic differences

Introduction

Obesity, a major cause of morbidity and mortality (1), is associated with increased risk of chronic diseases such as type 2 diabetes and cardiovascular disease, including hypertension (2,3). In South Africa the prevalence of obesity, i.e., body mass index (BMI) > 30 kg/m^2, is 47.7% for black and 15.6% for white females within the age group 35 to 44 years (4,5). The prevalence of type 2 diabetes among the black community is 6% to 7% (6).

Obesity is strongly associated with insulin resistance (7,8), which may be a major cause of chronic diseases, particularly type 2 diabetes (9,10). The mechanism by which obesity gives rise to insulin resistance is unknown; however, excessive circulating plasma nonesterified fatty acids (NEFA) may play a role (11,12).

A recent clinical study has shown that obese black South African women have higher plasma NEFA levels, lower serum insulin concentrations and higher in vivo lipolytic rates compared with whites (13). The present study, using isolated adipocytes and glucose clamp studies to measure whole body insulin sensitivity, sought to clarify whether the higher systemic lipolytic rate observed in black women is due to lower insulin concentrations or to resistance to the antilipolytic effect of insulin and also to measure whole body insulin sensitivity in the two population groups.

Research Methods and Procedures

Subjects

Ten black and ten white women were matched for age, waist-hip ratio, BMI, percentage of body fat, duration of

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obesity (10 to 12 years), and number of offspring (no more than three). None of the women were postmenopausal or taking any oral contraceptives. Exclusion criteria included diseases of the major organs on the basis of clinical examination and history (e.g., regular menstrual cycles), smoking in excess of five cigarettes per day, and alcohol consumption in excess of 4 g of alcohol per day or a history of eating disorders. Patients fasted for 10 hours, abstained from alcohol for 24 hours, and did not exercise for 12 hours before the tests. All patients gave informed consent, and the study was approved by the Committee for Research on Human Subjects of the University of the Witwatersrand.

Analytical Methods

Blood glucose and plasma NEFA levels were measured using commercially available assay kits (Boehringer Mannheim, Germany). Insulin levels were measured with an enzyme amplified immunoassay that does not cross-react with proinsulin (Mercodia, Belgium).

Body Composition Analyses

Anthropometric measurements were as previously described (13): the waist measurement was taken at the midpoint between the lowest rib and the suprailiac crest. Bioelectrical impedance was measured in the fasted state using the Bodystat device (Bodytrach Pty. Ltd., Johannesburg, South Africa) to assess fat and muscle mass.

Euglycemic Hyperinsulinemic Clamp Studies

These were completed on a separate day, within 2 weeks of the fat biopsy studies, during the luteal phase of the menstrual cycle. The patients were asked to fast from 10:00 PM the previous night, and the investigations started at 8:00 AM the next morning. The patients were studied in the supine position. Two polyethylene catheters were placed intravenously, one in each forearm. One catheter was used for infusion of insulin (Actrapid; Novo-Nordisk, Copenhagen, Denmark) and the other for 20% glucose. The contralateral forearm was placed under a heating pad to ensure the sampling of arterialized venous blood (14,15). The insulin infusion rate was set at 80 mL/m^2 per minute. The glucose infusion rate was varied at 10-minute intervals as calculated from a computerized algorithm based on a minimal model of glucose kinetics (16). The fasting glucose was reduced or increased steadily over 1 hour to a targeted concentration of 5.5 mM, and the blood glucose was clamped at these levels. The glucose infusion rates during steady state were calculated over the last 60 minutes and reported in milligrams per kilogram per minute. A fasting blood sample was taken, and further samples were taken at 20-minute intervals throughout the steady-state period.

Fat Tissue Biopsy

Needle biopsies of the subcutaneous adipose tissue were taken from the abdominal area (5 cm lateral to the umbilicus) and at the level of the midfemur under local anesthetic (2% Lignocaine HCL (remicaine); Adcock Ingram Generic Johannesburg, South Africa) using a 1.8- by 14-mm needle (Promex, South Africa) and a 50-mL syringe. Care was taken not to infiltrate the tissue to be excised. The interval between abdominal and femoral aspirations was approximately 7 minutes.

Isolation of Adipocytes

Medium 199 (M199) containing Hanks' salts (Life Technologies, Gaithersburg, MD) supplemented with glucose (1 mg/mL), sodium bicarbonate (0.35 mg/mL, Saarchem, South Africa), and 40 mg/mL bovine serum albumin (Sigma, Poole, England) was used throughout. Glass tubes used for washing adipocytes were silanized before use.

Aspirated adipose tissue was washed once and then digested using collagenase (0.8 mg/mL; Boehringer Mannheim, Germany) at 37 °C for 40 minutes, in a shaking water bath under normal atmospheric conditions. The adipocytes were filtered through a nylon mesh of pore size 500 μm and washed four times with M199 (37 °C) that was free of collagenase. After the final wash the adipocytes were resuspended in M199.

Estimation of Triglyceride Content

The triglyceride content of each adipocyte preparation was measured as previously described (17). Fat cell size was estimated using the method of Smith et al. (18) in which the diameter of 100 adipocytes was measured using a calibrated ocular lens-mounted graticule and a microscope (CK light; Olympus, Tokyo, Japan).

Assessment of Adipocyte Function

Adipocyte function was assessed by measurement of lipolytic activity in the presence of isoproterenol, a stimulant of lipolysis by virtue of its nonselective β-adrenergic agonist activity (19).

The antilipolytic effect of insulin was assessed by measuring adipocyte glycerol production in response to 1.0 μM isoproterenol in the presence of varying concentrations of insulin (0.07, 0.72, 2.40, and 7.20 nM Actrapid insulin; Novo-Nordisk). Incubations lasted for 1 hour at 37 °C in a shaking water bath in air (20). Glycerol was measured using an enzymatic method (21).

Data Presentation

Lipolytic activity was expressed as glycerol released per mm^2 of adipocyte surface area per hour (Figure 1). The antilipolytic response to insulin was expressed as the percentage inhibition of lipolysis (Figure 2), which was calculated according to the following formula:

$$1 - \left[\frac{\text{lipolytic activity in presence of insulin and isoprotorenol}}{\text{lipolytic activity in presence of isoprotorenol only}} \right] \times 100$$

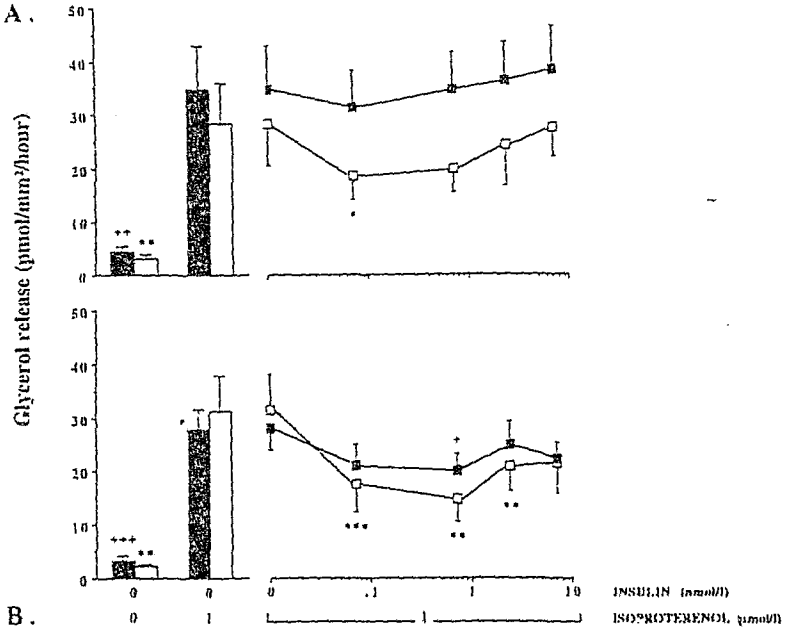


Figure 1. Glycerol release of adipocytes isolated from abdominal (A) and femoral (B) adipose tissue. ▩, adipocytes from black obese females; □, adipocytes from white obese females; $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$ vs. lipolysis rate in the presence of isoproterenol but the absence of insulin of adipocytes taken from white females; $+p < 0.05$, $++p < 0.005$, and $+++p < 0.0005$ vs. lipolysis rate in the presence of isoproterenol but the absence of insulin of adipocytes taken from black females. Data are presented as means ± SEM (n = 8–10).

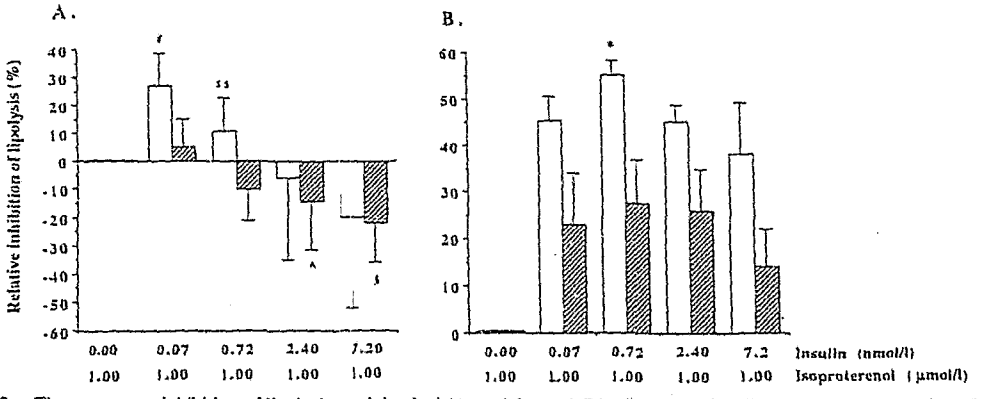


Figure 2. The percentage inhibition of lipolysis in abdominal (A) and femoral (B) adipocytes. ▩, adipocytes from black, obese females; □, adipocytes from white, obese females; $^{*}p < 0.05$ vs. lipolysis rate in the presence of 0.72 nM insulin of femoral adipocytes isolated from black subjects; $^{\#}p < 0.05$ and $^{\S\S}p < 0.005$ vs. lipolysis rate in the presence of 0.07 and 0.72 nM insulin, respectively, of femoral adipocytes isolated from white subjects; $^{\wedge}p < 0.05$ and $^{\S}p < 0.05$ vs. lipolysis rate in the presence of 2.40 and 7.20 nM insulin, respectively, of femoral adipocytes isolated from black subjects. Data are presented as means ± SEM (n = 8–10).

Expressing lipolytic activity in this way made comparisons between ethnic groups of the effect of insulin on the inhibition of isoproterenol-stimulated lipolysis easier and clearer.

The ED_{50} values, which were used as a measure of insulin sensitivity of the adipocytes to the antilipolytic effect of insulin, were calculated for each subject using standard procedures (22).

Statistical Analyses

Data are expressed as means ± SEM unless otherwise stated. Student's unpaired *t* test was used to analyze differences between the two ethnic groups, whereas a paired *t* test was used for the analysis of differences within groups.

Results

Clinical Measurements

The clinical characteristics were similar in the two groups (Table 1).

Insulin, Glucose, and NEFA Concentrations

Obese black women had an almost 2-fold higher fasting plasma NEFA concentration ($p < 0.01$) than did the white women (Table 2). In addition, the obese black women were relatively insulinopenic in comparison with their white counterparts ($p < 0.001$).

Measurement and Estimation of Insulin Resistance

Estimations of in vivo insulin resistance in obese black and white women are shown in Table 2. The euglycemic clamp technique demonstrated that the obese black women were more insulin-resistant than the white women ($p < 0.05$). Plasma insulin levels were similar in both groups of subjects during steady state (624 and 639 pM, respectively).

Adipocyte Analyses

The size of abdominal and femoral adipocytes in both groups were not statistically significantly different (Table 3), although adipocytes isolated from black females did tend to be larger than those from white females. This was due to two outliers in the black subject group who had abdominal and femoral adipocytes with diameters above 200 μm.

A dose-response curve of glycerol release vs. isoproterenol demonstrated that 1.0 μM isoproterenol elicited the maximum lipolytic response (data not shown). Furthermore, Figure 1 shows that abdominal and femoral adipocytes from both subject groups were responsive to 1.0 μM isoproterenol thus demonstrating normal lipolytic activity. Previous investigators also found that 1.0 μM isoproterenol produced maximum stimulation of lipolysis (19,20).

Ethnic Differences in the Antilipolytic Effect of Insulin

Absolute glycerol release by adipocytes from black women stimulated by 1 μM isoproterenol was statistically significantly inhibited by insulin only in femoral adipocytes and by 0.72 nM insulin (Figure 1B). However, in the white females there was statistically significant inhibition of lipolysis in both abdominal (by 0.07 nM insulin; Figure 1A) and femoral (by 0.07, 0.72, and 2.40 nM insulin; Figure 1B) adipocytes. The abdominal and femoral adipocytes isolated from white subjects were more responsive to the antilipolytic effect of insulin than were those taken from black subjects and statistically significant differences in the inhibition of lipolytic activity by insulin between the ethnic groups were seen in the femoral adipocytes at an insulin concentration of 0.72 nM ($p < 0.05$) (Figure 2B). Furthermore, abdominal adipocytes from both ethnic groups were more resistant to the effects of insulin than were femoral adipocytes. Thus, femoral adipocytes demonstrated a higher statistically significant percentage of inhibition of lipolysis than did abdominal adipocytes in white females at insulin concentrations of 0.07 ($p < 0.05$) and 0.72 ($p < 0.005$) nM and in black females at insulin concentrations of 2.40 and 7.20 nM ($p < 0.05$ for both) (Figure 2, A and B).

Glycerol release was also expressed as a function of adipocyte cell number and triglyceride content, and the results obtained were similar to those shown in Figure 1. This demonstrates that ethnic differences in the responsiveness of adipocyte lipolysis to insulin are not due to differences in adipocyte morphology.

There were no statistically significant differences between the ED_{50} values for the antilipolytic effect of insulin in any of the adipocyte populations that were studied. The ED_{50} values were as follows (units are pM ± SEM): white abdominal adipocytes, 42 ± 14; black abdominal, 33 ± 6; white femoral, 56 ± 12; black femoral, 40 ± 5.

Table 1. Anthropometric indices of study populations

Measurements	Black females	White females	*p* value
Age (years)	37.0 ± 1.7*	35.0 ± 0.8	NS
BMI (kg/m^2)	34.2 ± 1.8	35.3 ± 1.8	NS
Waist circumference (cm)	96.7 ± 2.1	100.3 ± 4.4	NS
Hip circumference (cm)	118.7 ± 2.2	119.4 ± 3.8	NS
Waist-hip ratio	0.82 ± 0.03	0.83 ± 0.02	NS
Body fat (%)	46.1 ± 1.6	44.1 ± 1.6	NS

* Values are means ± SEM; NS, not significant.

Discussion

The pathogenesis of type 2 diabetes of the black population of South Africa appears to be different from that of the

Table 2. Biochemical indices of study populations

Measurements	Black females	White females	*p* value
NEFA (μM)	863.0 ± 93.1* (560–1300)†	412.0 ± 34.2 (290–740)	<0.01
Insulin (pM)	66.7 ± 4.7 (33.6–106.4)	152.0 ± 20.1 (79.8–226.8)	<0.01
Glucose (mM)	4.5 ± 0.2	4.0 ± 0.2	NS
Glucose infusion rate (mg/kg/min)	1.3 ± 0.2	2.2 ± 0.3	<0.02

* Values are means ± SEM; NS, not significant. Insulin, glucose, and NEFA values are all fasting levels.
† Numbers in parentheses are ranges.

white population as evidenced by an earlier age of onset, more acute disease decompensation, lower plasma insulin concentrations, and higher plasma free fatty acid concentrations (13,23,24). There are also ethnic differences in diseases associated with type 2 diabetes; hypertension is more common within the black community (25), whereas ischemic heart disease (IHD) is rare in comparison with the white population (26,27). Insulinopenia may be due to a decreased β-cell mass as a result of fetal or neonatal protein-energy malnutrition and/or genetic factors (24). Several studies, including one carried out in South Africa (28), have shown an association between low birth weight, which is common among black South Africans, and poor glucose tolerance (29–31). Hypertension has also been linked to poor fetal growth (29). The reason for the low incidence of IHD within the black community, especially in the presence of high plasma free fatty acids, is not known. It has been reported that the clearance rate of triglycerides is higher in black obese subjects than in lean subjects (32) and the lower plasma insulin levels observed in the black population may also influence the incidence of IHD.

Table 3. Size of adipocytes isolated from obese black and white females

Adipocyte population	Adipocyte diameter (μm) Black females	White females	*p* value
Abdominal	132.0 ± 15.0* (99.8–225.1)†	115.0 ± 2.0 (103.8–122.0)	NS
Femoral	134.0 ± 13.7 (105.3–216.5)	115.0 ± .4 (108.4–123.0)	NS

* Values are means ± SEM; NS, not significant.
† Numbers in parentheses are ranges.

The present study shows that the fasting metabolism of white and black obese females is markedly different with the latter group having lower plasma insulin but higher plasma NEFA levels than the former group. This is a confirmation of previous studies (13,23,24), and a recent study has shown that these metabolic differences are not confined to the fasting state but also exist after oral administration of a glucose load (33).

The data presented in this study demonstrate a further ethnic difference in type 2 diabetes pathology, namely, that obese black women are more insulin-resistant than their white counterparts. African Americans have also been shown to be more insulin-resistant than white Americans but insulin levels observed in the former group were higher than those observed in the latter (34,35). This contradicts our results and shows that the pathophysiology of insulin resistance within the black African population is different to that observed in African Americans, perhaps due to genetic admixture (36) and different levels of acculturation (37). This is further demonstrated by a study showing that insulin levels in native Nigerians are lower than those in African Americans (38).

The greater degree of insulin resistance in the black group observed in our study may be caused by high NEFA concentrations. Increased plasma NEFA concentrations cause acquired insulin resistance (11,12) and impairment of β-cell function in humans (39,40). This may explain the higher insulin resistance of the black females despite lower plasma insulin levels when compared with the white females. The in vitro studies confirmed the insulin resistance showing that abdominal and femoral adipocytes isolated from obese black women are less responsive to insulin than those isolated from obese white females.

The ED_{50} values for insulin inhibition of lipolysis were similar for all the adipocyte populations studied, but the response of adipocytes from black subjects to the antilipolytic effect of insulin is blunted in comparison with those of the white subjects. Thus the higher serum NEFA

concentrations observed in the black subjects in the postabsorpative state are explained because insulin sensitivity is a major determinant of postabsorpative lipolytic rates (41). These results also suggest that the ethnic difference in insulin inhibitory activity may be due to an effect distal to insulin binding to its receptor, i.e., differences in insulin responsiveness of the adipocytes rather than insulin sensitivity may be involved. However, it should be noted that the ED_{50} values were calculated in this study using only four different insulin concentrations, and this will introduce some inaccuracy. Furthermore, the dose-response curves for insulin inhibition of lipolysis shown in Figure 1 demonstrate that in femoral adipocytes from both ethnic groups maximal inhibition of lipolysis occurs in the presence of 0.72 nM insulin (this is true in 72% of subjects), whereas in the abdominal adipocytes it occurs at 0.07 nM (this is true in 61% of subjects). This subject-to-subject variability causes the ED_{50} values to be not significantly different between the four adipocyte populations. However, the dose-response curves for the femoral adipocytes mirror each other quite closely as do those for the abdominal adipocytes; therefore, despite the variability, we believe that there are no interethnic differences in adipocyte insulin sensitivity. In order to confirm this statement the measurement of insulin receptor numbers and binding affinities by Scatchard analysis is required.

A study comparing African Americans to white Americans demonstrated ethnic differences in the ED_{50} values for insulin inhibition of lipolysis (42). In the same study abdominal adipocytes taken from upper-body obese white Americans were less insulin-sensitive than those from upper-body obese African Americans. This relationship was reversed in lower-body obese subjects. These results are at variance with our study and again suggest that the expressed phenotype of insulin resistance in black obese South Africans is different to that seen in obese African Americans.

The abdominal and femoral adipocytes isolated from black females are larger, but not statistically significantly so, than those isolated from white females, and it has been shown that larger adipocytes should be more responsive to the antilipolytic activity of insulin (43). This is not the case in the present study where the adipocytes from the black subjects are less responsive to the antilipolytic effect of insulin than those isolated from white subjects. Therefore, the ethnic differences in the responsiveness of adipocytes to insulin inhibition of lipolysis cannot be explained by differences in adipocyte size but must be due to other factors.

The lipolytic response to isoproterenol of all the adipocyte populations studied is similar with the basal lipolytic rate increasing 10-fold on addition of isoproterenol (see Figure 1). The basal lipolytic rate in both abdominal and femoral adipocytes and the isoproterenol-stimulated lipolytic rate in abdominal adipocytes are higher in the black females, but the differences between ethnic groups are not statistically significant. A previous study has shown that the in vivo lipolytic rate is higher in obese black females than in their white counterparts (13), and our data suggest that this may be a result of the insulin insensitivity of adipocytes taken from black obese females. β-Adrenergic agents stimulate lipolysis by increasing intracellular levels of adenosine 3′-cyclic monophosphate (cAMP) (44), whereas the antilipolytic action of insulin involves reduction of cAMP levels via an insulin receptor-mediated process (45) The reduced response to insulin in the adipocytes taken from the black subjects may be due to high intracellular cAMP levels and/or a perturbation in insulin receptor signalling.

Abdominal adipocytes were less responsive to insulin compared with femoral adipocytes in both the black and white population groups. This metabolic feature of subcutaneous abdominal adipose tissue in combination with its high lipolytic rate may explain results from a study showing that this fat depot predicts insulin sensitivity more strongly than does visceral fat (46). These data emphasize the important contribution of both visceral and subcutaneous abdominal fat to the pathology of type 2 diabetes (8) and other obesity-associated disorders.

In summary, the higher NEFA levels of the black obese females result from a combination of insulin insensitivity of adipose tissue and insulinopenia. The raised NEFA levels may in turn contribute to the higher in vivo insulin resistance of the black compared with the white obese females. Thus, the pathogenesis of type 2 diabetes, particularly within black obese subjects, may be strongly influenced by adipose tissue metabolism.

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COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 (Registry)

CLEARANCE CERTIFICATE PROTOCOL NUMBER M940104

PROJECT	In vivo metabolism of fat tissue regions in black urban subjects.
INVESTIGATORS	Dr M T van der Merwe
DEPARTMENT	Internal Medicine
DATE CONSIDERED	940128

DECISION OF THE COMMITTEE

Approved with the attached conditions.

DATE 940215 CHAIRMAN .....
(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

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DECLARATION OF INVESTIGATOR(S)

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I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

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