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Department of Medicine
Division of Diabetes
University of Helsinki
Helsinki, Finland

Minerva Foundation Institute
for Medical Research
Biomedicum Helsinki
Helsinki, Finland

MOLECULAR MECHANISMS OF INSULIN RESISTANCE IN HUMAN SKELETAL MUSCLE AND LIPODYSTROPHIC ADIPOSE TISSUE

Elena V. Korsheninnikova

ACADEMIC DISSERTATION

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Supervisors

Professor Hannele Yki-Järvinen, MD, PhD, FRCR
Department of Medicine
Division of Diabetes
University of Helsinki
Helsinki, Finland

and

Docent Antti Virkamäki, MD, PhD
Minerva Foundation Institute for Medical Research
Biomedicum
Helsinki, Finland

Reviewers

Docent Heikki Koistinen, MD, PhD
Department of Medicine, Division of Cardiology
Helsinki University Central Hospital and Biomedicum
Helsinki, Finland

Professor J. Antonie Maassen, PhD
Department of Molecular Cell Biology
Leiden University Medical Centre and Vrij University Medical Centre, Amsterdam
Leiden, The Netherlands

Official opponent

Assistant Professor Olavi Ukkola, MD, PhD
Department of Internal Medicine and Biocenter Oulu
University of Oulu
Oulu, Finland

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Abstract

Background and aims: 'Fat in the wrong place', i.e. accumulation of lipid in insulin sensitive tissues such as skeletal muscle and the liver has been shown to be associated with insulin resistance independent of subcutaneous fat. The mechanisms underlying these associations are poorly understood. Hypothetically, insulin resistance could be mediated via direct effects of intracellular lipid on insulin signalling or indirectly via release of adipocytokines such as adiponectin. Studies I and II were undertaken to determine, whether fasting insulin concentration and accumulation of intramyocellular lipid (IMCL) is associated with alterations in insulin signalling and whether such an association is independent of obesity and physical fitness. Study III was undertaken to characterize gene expression in adipose tissue of patients with lipodystrophy and insulin resistance due to treatment of HIV-infection by highly active antiretroviral therapy (HAART). In study IV, we wanted to determine whether adiponectin expression and circulating concentrations are abnormal in patients with HAART-associated lipodystrophy (HAL) and if so, whether such alterations are related to intrahepatocellular lipid.

Experimental material, subjects and methods: In study I, for method development, we compared insulin signalling assays in rat gastrocnemius muscle [insulin receptor (IR), insulin receptor substrate-1 (IRS-1), p85 subunit of phosphatidylinositol-3 kinase (PI 3-kinase), and kinase activity assay (PI 3-kinase)] using freeze-dried and purified, or frozen specimens. In study II, insulin signalling in human muscle was compared between 10 subjects with IMCL higher and 10 subjects lower than the median. Insulin sensitivity (euglycaemic hyperinsulinaemic clamp), body composition and maximal aerobic capacity were also measured. IMCL content was measured by proton nuclear magnetic resonance spectroscopy (^{1}H-MRS). In studies III and IV, liver fat was measured by ^{1}H-MRS and gene expression in subcutaneous adipose tissue biopsies by real-time polymerase-chain reaction in 30 patients with HAL and 13 HIV patients without lipodystrophy matched for age, gender and body mass. Total abdominal, subcutaneous and intra-abdominal were measured by magnetic resonance imaging.

Results: Insulin signalling in rat freeze-dried muscle was comparable but less variable than in conventionally proceeded biopsies. Human muscle biopsies had on the average 11 % non-muscle contaminants (I). Subjects with lower than median IMCL had higher rates of glucose uptake, lower serum fatty acid concentrations during hyperinsulinaemia and higher insulin-stimulated tyrosine phosphorylation of IR and IRS-1-associated PI 3-kinase activity than subjects with IMCL above median (II). The patients with HAL had several features of insulin resistance, including fasting hyperinsulinaemia and hypertriglyceridaemia compared to the HIV patients without lipodystrophy. In patients with HAL expression of the transcription factors (peroxisome proliferator activated receptor γ and δ, sterol regulatory binding protein 1c, peroxisome proliferator activated receptor γ co-activator (PGC-1), lipoprotein lipase, acyl CoA synthase and glucose transport protein 4 (GLUT4) were lower, whereas markers of inflammation (interleukin-6, cluster of differentiation 45) higher in patients with than without lipodystrophy (III). mRNA concentrations of fatty acid transport proteins 1 and 4 and of adipocyte and keratinocyte lipid binding proteins were similar in both groups (III). In study IV in the same patients, adiponectin gene expression in subcutaneous adipose tissue was lower in patients with than without lipodystrophy. Both serum adiponectin and mRNA concentrations correlated closely with features of insulin resistance, including liver fat content.

Conclusions: Freeze-drying and purification of human skeletal muscle biopsies allows more accurate measurement of insulin signalling than use of non-purified muscle. High IMCL in insulin-resistant non-obese non-diabetic men is associated with defects in early insulin signalling independent of body weight and physical fitness. Lipodystrophy accompanying HAART is characterized by insulin resistance and decrease of mRNA concentrations of several adipogenic transcription factors, PGC-1 and GLUT-4, an increase in inflammatory markers and a decrease in adiponectin. The relationship between adiponectin and liver fat raises the possibility that humoral factors released by adipose tissue regulate liver fat content.

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ORIGINAL PUBLICATIONS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. Korsheninnikova E, Seppälä-Lindroos A, Vehkavaara S, Goto T, Virkamäki A. Elevated fasting insulin concentrations associate with impaired insulin signaling in skeletal muscle of healthy subjects independent of obesity. *Diabetes/Metabolism Research and Reviews* 18: 209-216, 2002.

II. Virkamäki A, Korsheninnikova E, Seppälä-Lindroos A, Vehkavaara S, Goto T, Halavaara J, Häkkinen AM, Yki-Järvinen H. Intramyocellular lipid is associated with resistance to *in vivo* insulin actions on glucose uptake, antilipolysis and early insulin signaling pathways in human skeletal muscle. *Diabetes* 50: 2337-2343, 2001.

III. Kannisto K, Sutinen J, Korsheninnikova E, Fisher R, Ehrenborg E, Gertow K, Virkamäki A, Nyman T, Vidal H, Hamsten A, Yki-Järvinen H. Expression of adipogenic transcription factors, PGC-1, IL-6 and CD45 in subcutaneous adipose tissue in HAART-associated lipodystrophy. *AIDS,* in press 2003.

IV. Sutinen J, Korsheninnikova E, Funahashi T, Matsuzawa Y, Nyman T, Yki-Järvinen H. Circulating concentration of adiponectin and its expression in subcutaneous adipose tissue in patients with highly active antiretroviral therapy-associated lipodystrophy. *J Clin Endocrinol Metab* 88: 1907-1910, 2003.

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ABBREVIATIONS

ACS	acyl CoA synthase
ADD	adipocyte differentiation and determination factor
ALBP	adipocyte lipid binding protein
AMPK	adenosine monophosphate-activated protein kinase
AU	arbitrary units
BMI	body mass index
cAMP	cyclic adenosine monophosphate
CAP	Cbl-associated protein
c-Cbl	protooncogene Cbl
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT enhancer binding protein
CPT	carnitine palmitoyltransferase
DAG	diacylglycerol
ECL	enhanced chemiluminescence
EMCL	extramyocellular lipid
ERK	extracellular signal-regulated protein kinase
FATP	fatty acid transport protein
FFA	free fatty acid
Gab-1	Grb2-associated binder-1
GFA	glutamine fructose-6-phosphate amidotransferase
GLUT	glucose transport protein
G6P	glucose-6-phosphate
Grb-2	growth factor receptor bound 2
GS	glycogen synthase
GSK3	glycogen synthase kinase 3
HAART	highly active antiretroviral therapy
HAL	highly active antiretroviral therapy-associated lipodystrophy
HI	high fasting serum insulin
HiIMCL	high intramyocellular lipid
HIV	human immunodeficiency virus
^{1}H-MRS	proton nuclear magnetic resonance spectroscopy

HRP	horseradish peroxidase
11-β-HSD	11-beta-hydroxysteroid dehydrogenase
IB	immunoblotting
IGT	impaired glucose tolerance
IL	interleukin
IMCL	intramyocellular lipid
IP	immunoprecipitation
IR	insulin receptor
IRS	insulin receptor substrate
IRTK	insulin receptor tyrosine kinase
JNK	c-Jun N-terminal kinase
KLBP	keratinocyte lipid binding protein
LAR	leucocyte common antigen
LCACoA	long-chain fatty acid-CoA
LD	lipodystrophy
LO	low fasting serum insulin
LoIMCL	low intramyocellular lipid
LPL	lipoprotein lipase
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated extracellular-signal-regulated kinase kinase
MRI	magnetic resonance imaging
mtDNA	mitochondrial DNA
PAI-1	plasminogen activator inhibitor 1
PBS	phosphate buffered saline
PCR	polymerase-chain reaction
PDH	pyruvate dehydrogenase
PDK	pyruvate dehydrogenase kinase
PGC	peroxisome proliferator activated receptor γ coactivator
PH	pleckstrin homology
PI 3-kinase	phosphatidylinositol 3-kinase
PIP	phosphatidylinositol phosphate
PKB	protein kinase B (Akt)
PKC	protein kinase C
PP	protein phosphatase

PPAR	peroxisome proliferator activated receptor
PTP	protein tyrosine phosphatase
PKB	protein kinase B
PTB	phosphotyrosine binding protein
pY	phosphotyrosine
mRNA	messenger ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate/polyacrylamide gel electrophoresis
SH2	Src homology 2
SOCS	suppressor of cytokine signalling
SREBP	sterol regulatory element binding protein
TG	triglyceride
TNF	tumor necrosis factor
UDP-GlcNAc	uridine diphosphate-N-acetyl-glucosamine
VO_2max	maximal aerobic capacity

1. INTRODUCTION

Insulin resistance, a state where one or several of insulin's biological actions are impaired, characterizes both patients with excessive amounts of subcutaneous fat (obesity) and those who lack subcutaneous or visceral fat altogether (lipoatrophy). Both obese subjects and patients with lipoatrophy are at increased risk of developing type 2 diabetes and cardiovascular disease (104,254). Therefore, it is important to understand the molecular mechanisms underlying insulin resistance.

In recent years, studies of humans (275,283,284) and genetically engineered animals (48) have suggested that accumulation of triglycerides (TG) in insulin-sensitive tissues such as skeletal muscle and the liver may be the most proximal correlate of insulin resistance. However, as TGs themselves are unlikely to act as signalling molecules, it is likely that interactions with insulin action stem more from the metabolically active forms of lipid in muscle, such as long-chain fatty acid-CoA (LCACoA). They could accumulate either from a dietary source or could be derived from adipose tissue depots. The LCACoAs can, based on animal studies, regulate insulin signalling in muscle via multiple mechanisms including direct effects on enzyme activities (47,270), and effects mediated via diacylglycerol (DAG)-induced activation of protein kinase C (PKC), which in turn downregulate insulin signalling (32,52,53,99,126,126,137). There are, however, no data in humans which would link any defects in insulin signalling in skeletal muscle to intramyocellular lipid (IMCL).

The use of highly active antiretroviral theraphy (HAART) has dramatically improved survival of patients with HIV but is, in approximately 50 % of the patients, associated with serious metabolic side effects characterized by lipodystrophy (LD) and insulin resistance (42). The pathogenesis of this side effect is poorly understood. It is thought that HAART primarily affects metabolism in adipose tissue but how this results in whole-body insulin resistance in addition to LD is unclear. One possibility is that lipodystrophic adipose tissue either over- or undersecretes mediators, which affect insulin sensitivity of other tissues such as the liver.

The first aim of this thesis was to investigate the molecular mechanisms of insulin action in skeletal muscle in apparently healthy men with IMCL higher and lower than the median. The second aim was to characterize alterations in gene expression in patients with HAL and to determine whether the observed alterations in adipose tissue are related to fat accumulation in the liver.

2. REVIEW OF THE LITERATURE

2.1. Insulin signal transduction in skeletal muscle

2.1.1. Insulin signalling cascade

The first event in the insulin signalling cascade is binding of insulin to its receptor. The insulin receptor (IR) is a transmembrane glycoprotein that belongs to a receptor tyrosine kinase family of receptors. IR is composed of two extracellular α-subunits and two transmemrane β-subunits joined by disulfide cross-bridges. The IR is expressed in two isoforms (A and B) differing by 12 amino acids due to alternative splicing of exon 11. The type B receptor displays greater insulin-stimulated tyrosine kinase activity and ability to phosphorylate insulin receptor substrate (IRS) proteins (146) and predominates in classical insulin-sensitive tissues such as the liver, muscle and adipose tissues, whereas the type A isoform is found mostly in fetal tissues, the adult central nervous system and haematopoetic cells (93,195,198,256). After insulin binds to the α-subunit of the IR, insulin receptor tyrosine kinase activity (IRTK) is activated, and the IR undergoes autophosphorylation on several tyrosine residues located in the cytoplasmic portion of the β-subunit (306). In the absence of its ligand, the IR resides in the plasma membrane. Upon insulin binding, the ligand-receptor complex is internalized into endosomes within minutes (38). The acidic pH of endosomes induces dissociation of insulin from its receptor and allows the degradation of insulin by endosomal acidic insulinase (18). The IR is then recycled back to the cell surface.

Activated IR phosphorylates a number of intracellular substrates on tyrosine residues, including *Gab-1, Shc, Cbl* and members of the *IRS* family (**Fig. 1**). These substrates interact with IR by specific domains including PH, PTB and SH2 proteins. Pleckstrin homology (PH) domain, found in most of the proteins, interacts with the IR and is involved in targeting of the IRS proteins to the membrane and the IR (324). The phosphotyrosine binding protein (PTB) domain, also present in most of the IRS proteins, recognizes an *asparagine-proline-any amino acid-phosphotyrosine* (NPXpY) motif in the IR (75,318). Grb2-associated binder-1 (*Gab-1)* has no PTB domain and is phosphorylated by the epidermal growth factor receptor rather than the IR (118). *Shc* has no PH domain and is mainly bound by Grb2 which leads mainly to activation of a Ras/mitogen activated protein kinase (MAPK) pathway (245) (see below, MAPK pathway). The tyrosine phosphorylation of protooncogene *Cbl* seems to be required for insulin-stimulated glucose uptake in addition to the classic IRS-pathway (21).

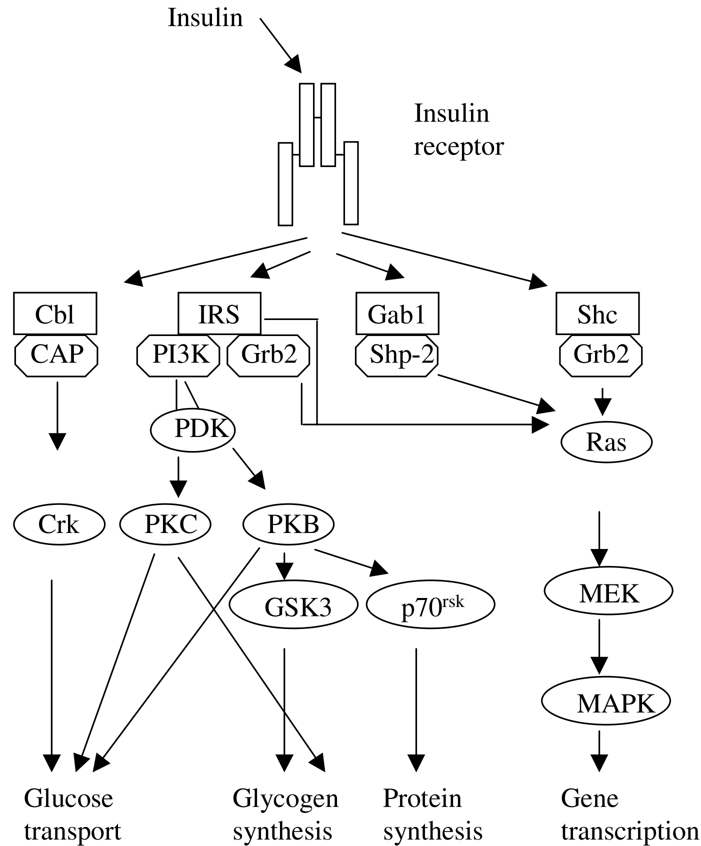

Fig 1. *An overview of insulin signalling pathways. Upon binding insulin induces autophosphorylation of the IR on tyrosine residues. Activation of the IRTK leads to phosphorylation of a number of cellular substrates, including Cbl, IRS, Gab1, and Shc. After phosphorylation, the signal is conducted downstream via interaction of the substrates with SH2-containing proteins. The IR phosphorylates Cbl via the adaptor protein CAP. After phosphorylation, Cbl translocates to the lipid raft subdomain of the plasma membrane, where it interacts with the adaptor protein Crk. This pathway is also necessary for the stimulation of glucose transport by insulin. The IRS family of substrates interacts with PI 3-kinase (PI3K), which produces PIP2 and PIP3 which activate downstream cascades, including PKB/p70^{rsk} which stimulates protein synthesis, and atypical PKCs or PKB/GSK3 which activate glucose uptake and glycogen synthesis. Gab1 interacts with phopshotyrosine phosphatase SHP2 which activates the MAPK pathway. Phosphorylation of Shc results in its binding to the adaptor protein Grb2, activation of the protooncogene Ras, and the MAPK pathway.*

There are at least four distinct *IRS* proteins in mammals, with IRS-1 and IRS-2 found in most tissues and apparently having major roles in co-ordinating actions of insulin in peripheral carbohydrate and lipid metabolism, and β-cell function (157). The IRS-3 isoform is not found in humans (26). **Table 1** lists mice knockout models that clarify the role of different IRSs in metabolism and mitogenesis. Mice lacking the IRS-1 protein exhibit generalized pre- and post-natal growth retardation, as well as insulin resistance but do not develop overt diabetes (12,278). On the contrary, IRS-2 deficient mice have defective growth in only some tissues (138,245,313) and develop type 2 diabetes (313). IRS-3- and IRS-4-knockout mice have normal or nearly normal growth and metabolism (78).

Table 1. *Murine phenotypes characterized by deficiency of specific isoforms of IRS. Adapted from reference 39.*

Genotype	Body weight	Fasting glucose	Insulin levels	Glucose tolerance	Insulin resistance	β-cell mass
$IRS1^{-/-}$	-50%	Normal	Increased	Normal	Muscle	Two-fold
$IRS2^{-/-}$	-10%	-	Decreased	Impaired	Liver, muscle	-50%
$IRS3^{-/-}$	Normal	Normal	Normal	Normal	No	-
$IRS4^{-/-}$	-10%	Decreased	Normal	Normal	No	-

Many proteins have SH2 domains and associate with IRS-1. These include the PI 3-kinase and Grb2. The docking sites of PI 3-kinase and Grb2 in IRS-1 are, however, different. Grb2 can also associate with Shc and activate the Sos/Ras complex, which is the first step of the MAPK pathway and mitogenesis (247).

PI 3-kinase pathway

The phosphatidyl inositol 3-kinase (PI 3-kinase) pathway is mainly involved in mediating the metabolic effects of insulin, such as glucose transport, glycogen and protein synthesis, ion and amino acid transport, and lipid metabolism (245). The PI 3-kinase is a heterodimer composed of a p110 catalytic subunit and a p85 regulatory subunit. After the SH2 domain of the p85 regulatory subunit of PI 3-kinase attaches to IRS-1, p110 catalytic subunit, which is responsible for the lipid kinase activity of PI 3-kinase, catalyzes the phosphorylation of membrane-bound PIP2 to PIP3. This step has been shown to be a critical step which ultimately leads to insulin-dependent glucose transporter 4 (GLUT4) translocation to plasma

membrane. Increased PIP3 activates a protein kinase cascade, stimulating the piruvate dehydrogenase kinase (PDK) (6), which phosphorylates and activates two classes of serine/threonine kinases: protein kinase B (PKB, also known as Akt) and the atypical protein kinase C (PKC) (isoforms ζ and λ) (162,244). Members of the PKC family of serine/threonine kinases have been implicated in several of insulin's actions (162). Different isoforms of PKC have been shown to undergo translocation from the cytosol to the membrane in response to insulin stimulation in different tissues. Atypical PKCs (ζ and λ) have been proposed to play a role in insulin-dependent glucose transport (148,266) and protein synthesis (187). It is also known that PKCs can activate the MAPK pathway and the transcription factor nuclear factor-kB, leading to increased gene expression and protein synthesis (139). PKB phosphorylates and regulates the function of many cellular proteins involved in processes that include metabolism, apoptosis and proliferation (206). Among the targets of PKB are glycogen synthase kinase 3 (GSK3) and glycogen-associated protein phosphatase 1 (PP1). GSK3 is phosphorylated and inactivated in response to PKB stimulation. Decreased activity of GSK3 leads to the dephosphorylation and activation of glycogen synthase (GS). Insulin activates GS by promoting its dephosphorylation, through the inhibition of kinases such as protein kinase A or GSK3, and activation of PP1 (34). Upon its activation downstream of PI 3-kinase, PKB is transmitting the insulin signal by phosphorylation of GSK3, the forkhead transcription factors and cAMP response element-binding protein (49).

MAPK pathway

The MAPKs are evolutionary conserved enzymes that play an important role in regulating cell proliferation, differentiation and apoptosis. Four groups of MAPKs have been identified in mammalian cells. These are extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), the p38 kinases and ERK5/Big MAPK 1. While ERK is mainly activated by mitogens and growth factors including insulin, p38 and JNK are activated by many environmental stress stimuli (e.g. ultraviolet and ionizing radiation), resulting in apoptosis (50,213). ERK pathway involves the tyrosine phosphorylation of IRS proteins and/or Shc, which interact with the adapter protein Grb2 and recruit son-of-sevenless exchange protein to the plasma membrane for activation of Ras. Activated Ras binds to and activates the MAPK kinase kinase (Raf-1). Activated Raf-1 phosphorylates and activates the MEK, which in turn phosphorylates and activates ERK (271) (**Fig.1**). Activated ERK can translocate into the nucleus, where it catalyzes the phosphorylation of transcription factors

and initiates a transcriptional programme that leads to cellular proliferation or differentiation (33).

Other pathways

In addition to PI 3-kinase activity, other signals seem to be required for insulin-stimulated glucose uptake. This alternative pathway appears to involve tyrosine phosphorylation of the protooncogene Cbl, the substrate of the IRTK. Cbl-associated protein (CAP), which is not phosphorylated in response to insulin, targets Cbl to the IR. After phosphorylation, Cbl translocates to caveolae, a specialized subdomain of the plasma membrane. Inhibition of the CAP-Cbl interaction by dominant negative CAP correlates with inhibition of insulin-stimulated glucose transport and GLUT4 translocation in a wortmannin-independent fashion, suggesting that Cbl participates in a PI 3-kinase-independent pathway where insulin stimulates GLUT4 translocation in adipocytes (21).

The insulin signalling cascade is regulated by protein tyrosine phosphotyrosine phosphatase (PTP) activity. In rat skeletal muscle, inmmunodepletion studies have demonstrated that leucocyte common antigen (LAR), phosphotyrosine phosphatase SHP2 and PTP-1B are the three major enzymes responsible for PTP activity (5). The expression levels and/or activity of these specific PTPs are increased in insulin resistant obese patients (4). LAR negatively regulates insulin signalling through dephosphorylation of IRS-2 (or other IRS proteins), although IR and IRS-1 may be affected in other tissues or physiological states. PTP-1B knockout mice have enhanced insulin sensitivity, increased phosphorylation of the IR in liver and muscle tissue after insulin injection in comparison to PTP-1B $^{+/+}$ mice (76).

The adenosine monophosphate-activated protein kinase (AMPK) has been implicated in the stimulation of glucose uptake into skeletal muscle and inhibition of liver gluconeogenesis. AMPK is a heterotrimeric protein, composed of one catalytic (α) and two noncatalytic (β and γ) subunits (311) which is activated by cellular stress associated with ATP depletion (114). AMPK mediates several critical exercise-induced mitogenic and metabolic events in skeletal muscle. An increase in AMPK activity in response to muscle contraction or exercise correlated with GLUT4 translocation and glucose transport in skeletal muscle (23,24,115,160). In rats, metformin activates AMPK in hepatocytes resulting in a decrease in the activity of acetyl-CoA carboxylase, an increase in fatty acid oxidation and suppression of lipogenic enzymes and sterol regulatory binding protein 1c (SREBP-1c) expression (335).

2.1.2. Acquired defects in insulin signalling

Obesity

Insulin sensitivity related to lowering of whole-body glucose levels decreases with increasing body weight (35). Molecular mechanisms for this association are poorly understood (245) but obese subjects show muscle insulin resistance and appear to have insulin signalling defects in skeletal muscle. Morbidly obese subjects have reduced IRS-1 content, insulin-stimulated IRS-1 phosphorylation and PI 3-kinase activation which is paralleled by a decrease in insulin-stimulated glucose uptake in skeletal muscle strips (95). Pregnant obese women with or without gestational diabetes have been reported to have impaired insulin-stimulated IRS-1 phosphorylation, which was primarily due to a decrease in IRS-1 protein, while IRS-2 expression appeared to be increased (84). In another study, obese subjects with impaired glucose tolerance (IGT) had lower IRS-1 tyrosine phosphorylation than normal subjects, significantly impaired IRS-2 tyrosine phosphorylation, defective IRS-2-associated PI 3-kinase activation, and impaired PKC activation in myotubes (295). PTPase could more rapidly dephosphorylate IRS-2 in myotubes from patients with IGT than in normal subjects. PTPase activity is increased in skeletal muscle in obese subjects (4,185). PTPase activity was also found to be 2-fold increased in omental fat compared to subcutaneous fat (319). In morbidly obese individuals, the reduced insulin-stimulated glucose transport may be explained partly by reduced GLUT4 expression in skeletal muscle (67).

An increase of certain intracellular lipid molecules can activate signalling pathways that downregulate insulin action. Increased diacylglycerol (DAG) levels in muscle are associated with activation of one or more isoforms of the PKC family, which is known to attenuate insulin signalling, especially at the level of IRS-1 (250). *De novo* synthesis of ceramide can inhibit more distal sites by activating PTP 2A and thereby promoting dephosphorylation and inactivation of PKB (250). The JNK activity which is abnormally elevated in obesity (116), can interfere with insulin action in cultured cells (116). These mechanisms may account at least in part for the reduced insulin sensitivity occurring in obesity and type 2 diabetes.

Physical inactivity

The ability of physical exercise to improve glucose metabolism has been known for more than 100 years, but the exact mechanisms of this effect have only recently started to become clear. *Contraction* causes no stimulation of autophosphorylation of isolated IRs (287) or IRTK activity in human muscle (316), and has no effect on IRS-1-associated PI 3-kinase activity in human skeletal muscle (316). *Short-term endurance training* did not alter IR or IRS-1 mRNA expression (297). *Chronic exercise* is associated with increased insulin sensitivity, enhanced insulin-stimulated phosphotyrosine-associated PI 3-kinase activity and decreased levels of IRS-2 in skeletal muscle (122,143).

MAPK signalling pathways are activated in human skeletal muscle in response to acute, short-term exercise (17,152,307,308) and endurance running (31,333). MAPK signalling pathways appear to play a critical role in the transcriptional regulation of muscle genes in response to exercise (308). For example, activation of ERK1/2 and JNK by exercise and contraction is associated with the rapid induction of immediate early genes such as c-fos (261) and c-jun (15,16).

Despite contradictory data on effects of physical training on insulin signalling in humans, it is well established in rats that exercise stimulates glucose uptake by enhancing the translocation of GLUT4 (70,117). Such glucose transporter recruitment can occur independent of PI 3-kinase (163,175,323). This documents that exercise and insulin trigger GLUT4 translocation in muscle by effects on different signalling mechanisms.

AMPK is an important mediator of muscle contraction-induced glucose transport (311). An increase in AMPK activity in response to muscle contraction or exercise correlates with GLUT4 translocation and glucose transport in skeletal muscle (336). The greater the force production generated by contraction (125), or the greater the intensity of treadmill running exercise (226), the greater the activation of AMPK. In humans, moderate-intensity cycle exercise (86,312,317), as well as high-intensity ’sprint’ exercise (51) increases skeletal muscle AMPK activity. Transgenic overexpression of a dominant inhibitory mutant of AMPK in skeletal muscle completely blocks the ability of hypoxia to activate glucose uptake, whereas only partially reducing contraction-stimulated glucose uptake (202). Thus, AMPK-dependent and AMPK-independent pathways contribute to the regulation of glucose uptake in skeletal muscle in response to exercise.

Hyperglycaemia

Type 1 diabetes. Patients with type 1 diabetes are insulin resistant compared to age, gender and weight-matched normal subjects, if glycaemic control is poor (330). Improvement of glycaemic control by continuous subcutaneous insulin infusion therapy enhances insulin action despite reduced insulin requirements (329). Insulin sensitivity is also normal in clinical remission of type 1 diabetes (330). These observations suggest that hyperglycaemia itself induces insulin resistance and this indeed has been shown to be the case (328).

Type 2 diabetes. Although the pathogenesis of insulin resistance in type 2 diabetes almost certainly is multifactorial, the degree of insulin resistance in type 2 diabetes is proportional to glycaemic control (326) as is the case in type 1 diabetes. Indeed, the insulin resistance which characterizes patients with type 2 diabetes, but not equally obese non-diabetic patients with a family history of diabetes, can largely be attributed to chronic hyperglycaemia (167).

Possible mediators of hyperglycaemia-induced insulin resistance.

Except the hyperglycaemia-induced activation of PKC, which leads to the inhibition of the IRTK activity (32) and thus, insulin resistance, the molecular mechanism of hyperglycaemia remained, however, a mystery until 1991 when the hexosamine pathway was discovered. The rate-limiting enzyme of the hexosamine pathway, glutamine fructose-6-phosphate amidotransferase (GFA) converts glutamine and fructose-6-phosphate to glucosamine-6-phosphate (285). The end-product of the hexosamine pathway, uridine diphosphate-N-acetyl-glucosamine (UDP-GlcNAc) is attached to the OH group of threonine and serine residues of proteins, which results in *O*-glycosylation of proteins (110). Glucosamine bypasses the rate-limiting reaction in the hexosamine pathway. In rats, infusion of glucosamine increases muscle UDP-GlcNAc concentration, and decreases insulin-stimulated glucose transport and glycogen storage. It has multiple effects on insulin signalling molecules: reduction in insulin-stimulated IRS-1 tyrosine phosphorylation, IRS-1 association with the p85 regulatory subunit of PI 3-kinase and IRS-1-associated PI 3-kinase activity, as well as decrease in insulin-stimulated GS activity (215). Glucosamine also increases *O*-linked UDP-GlcNAc modification of IRS-1 and IRS-2 (215). In humans, GFA activity is increased in proportion to the degree of hyperglycaemia in skeletal muscle of patients with type 2 diabetes (327). Transgenic mice overexpressing GFA in muscle and adipose tissue exhibit whole-body insulin resistance (59).

Insulin signalling defects in diabetic subjects.

Data are conflicting regarding insulin signalling in skeletal muscle of non-obese or moderately obese type 2 diabetic subjects. IR tyrosine phosphorylation has been found to be normal in the quadriceps femoris muscle of type 2 diabetic subjects incubated *in vitro* for 40 min in the absense or presence of insulin (2.4-60 nmol/l), with the peak phosphorylation at 8 min (151). Other studies have found impaired phosphorylation of IR or decreased IRTK activity (14,177,207). In other studies IRTK activity was similar in subjects with and without type 2 diabetes (144). Insulin-stimulated GLUT4 translocation and PI 3-kinase activity have been reported to be decreased (27,61,151) or normal (106,216).

Excess of counterregulatory hormones

Catecholamines, growth hormone and cortisol all decrease muscle glucose uptake and induce insulin resistance *in vivo* in skeletal muscle and in the liver (165,232,233).

Chronic *growth hormone* excess in rodent models decreases IR, IRS-1, and IRS-2 tyrosine phosphorylation in response to insulin in skeletal muscle (69). Growth hormone also acts directly in fat cells by increasing lipolysis, which may exacerbate insulin resistance in the liver and skeletal muscle. Growth hormone therefore induces insulin resistance and causes hyperglycaemia, and is recognized as the main cause of the clinical Dawn phenomenon in type 1 diabetic subjects (40).

β-adrenergic agents such as isoprenaline (isoproterenol), when incubated *in vitro* with adipocytes decreases apparent insulin-binding affinity, with no change in receptor number (219). This effect is mimicked by cAMP analogues and agents such as the phosphodiesterase inhibitor methylxanthine, which increases intracellular cAMP levels (219). Interestingly, epinephrin also causes translocation of GLUT-4 to the plasma membrane in muscle, but glucose transport in inhibited at high physiological concentrations because it also breaks down muscle glycogen, thus increasing intracellular glucose-6-phosphate (G6P) levels (164). α-Adrenergic agonists do not elicit these effects.

Hypercortisolaemia is associated with increased glucose production by the liver (9), decreased peripheral glucose uptake and utilization (63,209), decreased protein synthesis, and increased protein degradation in muscle (224). There are no data on effects of glucocorticoid treatment or endogenous hypercortosolaemia on insulin signalling in human skeletal muscle.

Cortisol is also produced outside the adrenal gland in tissues such as subcutaneous and visceral adipose tissue and the liver (255) in humans in a reaction catalyzed by 11β-HSD-1. Transgenic mice overexpressing 11β-HSD-1 in subcutaneous adipose tissue develop a full-blown metabolic syndrome characterized by visceral obesity and insulin resistance (183). 11β-HSD-1 activity is increased in adipose tissue of obese as compared to non-obese subjects (225). The role, of any of excess of 11β-HSD-1 in causing insulin resistance in humans is unclear but under intense investigation.

Elevated circulating FFAs

Plasma FFAs modulate insulin sensitivity and metabolic actions of insulin in skeletal muscle, heart and the liver (25,29,155,208). In the liver, FFAs stimulate glucose production and induce hepatic insulin resistance (80). FFAs also impair insulin-stimulated glucose uptake in human skeletal muscle and the heart (208).

Dresner et al. reported the reduction of glucose transport activity, IRS-1 tyrosine phopshorylation and IRS-1-associated PI 3-kinase activity after acute elevations in FFAs in healthy non-obese individuals (72). Similar finding were found by Griffin et al. in rats after a 5 h infusion of lipids to raise plasma FFA levels (99).

The mechanism underlying insulin resistance in human skeletal muscle induced by FFAs is controversial. First, Randle proposed that FFAs compete with glucose as the major energy substrate in cardiac muscle, leading to decreased glucose oxidation when FFAs are elevated (223). He showed that in the heart FFAs increased citrate and G6P concentration and the latter inhibited glucose phosphorylation by hexokinase (223). Since visceral fat is observed to be less sensitive to insulin than subcutaneous fat, the resulting direct flux of FFAs from visceral adipocytes through the portal vein to the liver can stimulate glucose production in the liver (244) - a single signal for insulin action and insulin resistance to the liver. These findings led Bergman and coworkers to propose the 'single gateway hypothesis' (25). However, G6P levels and intracellular glucose decrease rather than increase in response to increased lipid availability in human skeletal muscle (72,150). In type 2 diabetic subjects the intracellular glucose concentration was 2-fold reduced, a value that was 1/25 of it what be if hexokinase were the rate-controlling enzyme in glucose metabolism (56). The latter suggests a rate-limiting step at the level of glucose transport rather than phosphorylation.

Finally, infusion of intralipid to rats seems to activate the hexosamine pathway (239) and induce insulin resistance, which is why the hexosamine pathway has been called a nitrient-sensing' pathway. In humans, Gumbiner et al. examined IR tyrosine phosphorylation in muscle biopsies taken before and after lipid/heparin infusion and found no reduction in IR autophosphorylation (103).

Taken together, the molecular mechanisms underlying FFA-induced insulin resistance in human skeletal muscle are still unresolved, although glucose uptake seems rate-limited at the level of transport rather than phosphorylation.

IMCL

Recently, several studies have shown an association between lipid accumulation in skeletal muscle and insulin resistance (83,94,128,153,181,214,221). In four of these studies, this relation was shown to be caused by intramyocellular rather than extramyocellular lipids, as measured by ^{1}H-MRS (83,128,153,218). In humans, it is unclear whether IMCL is associated with insulin resistance independent of obesity. In 20 Europeans, Forouhi et al. (83) found the relationship between IMCL and insulin resistance to disappear after adjusting for obesity. In another study, no relation was found between IMCL and whole-body insulin sensitivity measured using the insulin tolerance test (221). In two other studies performed in offspring of type 2 diabetic patients, insulin resistance could not be attributed to obesity (128,218). Krssak et al. (153) found that IMCL did not correlate with insulin sensitivity, but in that study, both men and women were included and analyzed as one group. Women appear to have more IMCL than men for the same BMI (218) but similar insulin sensitivity; women also have greater adiposity than men with similar BMI (325). That's why, there is a lack of a correlation between IMCL and insulin sensitivity as it may have been confounded by the mixing of sexes.

At the cellular level, IMCL can interfere with insulin signalling via several mechanisms. They include inhibition via PKC activation and ceramide formation. In addition, LCACoAs can directly inhibit enzyme activity such as hexokinase (270) and GS (47). Increased glucose metabolism in muscle will exacerbate these effects, as this will enhance production of malonyl CoA, which inhibits oxidation of fatty acids. *DAG* inhibits insulin-stimulated IRS-1-associated PI 3-kinase activity (137,169,199). Treating various cell lines with phorbol esters, which mimic the effects of DAG, inhibits insulin signalling to IRS-1 (137) and PKB (199). Moreover, several PKC isoforms including PKC α (52,53), β1 and β2 (32,126), δ (126,137), γ

(52), and τ (99,137) are downstream effectors of DAG that antagonize insulin signalling by phosphorylating IRS-1 on inhibitory serine residues. Rodent models of insulin resistance showed elevated levels of DAG (250). DAG derived from polyunsaturated FFAs is a stronger activator of PKC, than that from saturated FFAs (298). *Ceramide* blocks insulin signalling by preventing the activation of PKB (251,274) by accelerating PKB dephosphorylation via PTP 2A (243,253,280).

In humans, IMCL can be rapidly modified since infusion of FFAs can increase IMCL in soleus and tibialis muscle in healthy men during a *5 h* hyperinsulinaemic euglycaemic clamp (36). The changes in plasma FFAs and IMCL-TG also induced insulin resistance in those subjects (36). Infusion of insulin for *67 h* has also been shown to increase IMCL content in soleus muscle and intrahepatic lipid content in diabetic patients (11). Fasting for *72 h* increases plasma FFA concentration and accumulation of IMCL in the vastus lateralis muscle (267). *Chronic interventions*, like weight loss following gastric bypass surgery, have been shown to decrease IMCL in type I and II muscle fibers and enhance insulin action (96). Biliopancreatic diversion which was accompanied by weight loss normalized insulin sensitivity, GLUT4 expression in skeletal muscle, leptin concentrations and markedly decreased IMCL (97).

Infections

Acute and chronic infections impair glucose tolerance (246) and may complicate maintenance of good glycaemic control in diabetic patients (228). This deterioration is due to insulin resistance in both skeletal muscle and the liver (293). The exact mediators of insulin resistance are unclear but could involve increased secretion of counterregulatory hormones, TNFα, and interleukins (189-191,258). TNFα-induced insulin resistance is due to reduced tyrosine phosphorylation of IRS-1 (120) because of increased serine phosphorylation of IRS-1 (134,276). The mechanisms via which counterregulatory hormones induce insulin resistance have been discussed above.

IL-6 inhibits insulin signalling in hepatocytes (257). Members of the suppressor of cytokine signalling (SOCS) family are associated with the IR, and their ectopic expression inhibits IR signalling (258). Several SOCS proteins are induced by IL-6. Ectopically expressed SOCS-3 leads to a decrease in insulin-dependent IR phosphorylation, IRS-1 tyrosine phosphorylation, association of IRS-1 with the p85 subunit of PI 3-kinase, and activation of PKB in

hepatocytes. These data suggest that induction of SOCS-3 in liver may be an important mechanism of IL-6 mediated insulin resistance (258).

2.1.3. Genetic defects in regulators of insulin action in humans

Genes encoding key elements of the insulin signal transduction pathway may be responsible for both the defects of insulin action and insulin secretion. Several of these genes are also expressed in pancreatic β-cells, and recent results from knockout animals have demonstrated that insulin signalling itself may play an important role in the mechanisms of insulin secretion (156,313).

More than 100 different mutations have been found in the coding regions of the IR gene on chromosome 19p (279,294). These patients have severe insulin resistance associated with leprechaunism, or with acanthosis nigricans, hirsutism and marked hyperinsulinaemia (82). Mutations of the α-subunit of the IR lead to complete loss of its function and development of leprechaunism (147) or partial loss of IR function and development of Rabson-Medenhall syndrome (91). Mutations in the tyrosine kinase domain of the IR lead to type A insulin resistance (hyperandrogenism, polycystic ovary syndrome, oligo- or amenorrhoea, anovulation, acne, baldness, short stature, muscle cramps) (133).

Arg972 polymorphism of IRS-1 in its heterozygous form is associated in obese non-diabetic subjects with a 50 % reduction in insulin sensitivity compared to obese subjects without polymorphism, indicating that the polymorphism potentiates obesity-associated insulin resistance (54). Expression of the Arg972 IRS-1 variant in L6 myocytes results in a significant decrease in GSK3 phosphorylation and inactivation by PKB (124). Overall, these results suggest that the Arg972 IRS-1 polymorphism might contribute to the development of insulin resistance by impairing the ability of insulin to activate IRS-1/PI 3-kinase/PKB/GSK3 signalling pathway, thus leading to defects in glucose transport, glucose transporter translocation and glycogen synthesis. Several additional polymorphisms and amino acid substitutions have been described, but their frequency is lower than that of the Arg972 variant (259).

In contrast to the insulin-resistant phenotype of IRS-2 $^{-/-}$ mice, polymorphisms of the human IRS-2 gene do not appear to contribute to the pathogenesis of either the common form of type 2 diabetes or early-onset autosomal dominant type 2 diabetes (259). IRS-3 is not expressed in human tissues (26). Polymorphisms of the IRS-4 gene are common in Caucasians but do not seem to be associated with type 2 diabetes or insulin resistance (8). Similarly, an association between polymorphisms of the muscle GS gene on chromosome 19q and type 2 diabetes has been observed in Finnish (101) and Japanese (159) but not in French subjects (337). Taken together, these results suggest that the IRS-1 and muscle GS genes may act in some populations as minor susceptibility genes for insulin resistance. Recently, a rare P387L variant in PTB-1B has been described and associated with a 3.7-fold genotype-relative risk of type 2 diabetes in a Danish Caucasian population. This variant impairs *in vitro* serine phosphorylation of the PTP-1B protein (74).

The regulatory G-subunit of the glycogen-associated form of PP1 plays a crucial role in muscle tissue glycogen turnover. Gene encoding for this protein is polymorphic at codon 905 and has been associated with insulin resistance and insulin hypersecretion in Danish patients with type 2 diabetes (108).

The β3-adrenergic receptor, located mostly in visceral fat, is involved in the regulation of lipolysis and thermogenesis (172). A point mutation in the gene encoding this receptor is associated with increased susceptibility to gain weight in a population of morbidly obese subjects (55), with a reduced metabolic rate and early onset of type 2 diabetes (300), and with the development of upper body obesity and insulin resistance (309) in patients with type 2 diabetes.

Peroxisome proliferator activated receptor (PPAR) γ2 gene has a Pro12Ala polymorphism that may cause a reduction in the transcriptional activity of PPARγ, and, hence, is associated with decreased insulin resistance and decreased risk of type 2 diabetes (272). In an obese group, subjects with Ala12 were more insulin sensitive than those without. The frequency of Ala12 was significantly lower in the diabetic group, suggesting that this polymorphism protects against type 2 diabetes (131).

2.2. Insulin signal transduction in adipose tissue

2.2.1. Overview of insulin action in adipose tissue

Adipocytes possess receptors for growth factors (IGF-I and –II, epidermal, platelet-derived, and fibroblast growth factors), hormones (insulin, glucagon, growth hormone, glucocorticoids, androgen, estrogen, progesterone, triiodothyronine, thyroid-stimulating hormone), cytokines (leptin, TNF-α, IL-6), vitamin D, very low density, low-density, and high-density lipoproteins, nuclear factors (PPARγ, RAR/RXR), catecholamines (β-1,2,3, α-1,2) and peptides (gastrin, gastric inhibitory peptide, glucagon-like peptide-1, neuropeptide Y-Y1, atrial natriuretic peptide, neuropeptide) (85).

Insulin is a key regulator of adipocyte metabolism. Insulin promotes lipogenesis by several mechanisms, including fostering of differentiation of preadipocytes to adipocytes and, in mature adipocytes, by stimulating glucose transport and TG synthesis and by inhibiting lipolysis (231). Insulin stimulates adipocyte lipogenesis by stimulating the activities of pyruvate dehydrogenase (PDH), acetyl CoA carboxylase and fatty-acid synthetase. Insulin increases glucose uptake and hence the availability of pyruvate for *de-novo* fatty-acid synthesis, glycerol-phosphate for FFA esterification by inhibiting adipocyte lipolysis via hormone-sensitive lipase and by promoting FFA re-esterification. *In vivo*, a small increment of 10-15 mU/l in plasma insulin above fasting is sufficient to suppress circulating FFA concentrations (30).

The antilipolytic effect of insulin requires much lower insulin concentrations than stimulation of glucose transport. Hence, even in insulin resistant states in which glucose transport is impaired, sensitivity to insulin's antilipolytic effect is relatively preserved, resulting in maintenance or expansion of adipose stores (132). The sensitivity of adipocytes to lipolytic triggers depends on their localizations. Lipolytic activity is the highest in visceral, lower in abdominal subcutaneous, and the lowest in peripheral subcutaneous adipocytes. Human subcutaneous adipocytes express β1- and β2-adrenoreceptors (154). Visceral adipocytes also express β3-adrenoreceptors, which contribute to lipolysis. Unlike in humans, peripheral white adipocytes of rats and mice contain high levels of the β3-adrenoreceptors, while the expression of β3-adrenoreceptors in visceral adipocytes of the obese Zucker rat and the obese ob/ob mouse is decreased (154). Several studies suggest that subjects with larger subcutaneous adipocytes are more prone to the development of insulin resistance and type 2

diabetes, independently of total body fat mass or distribution. This observation has recently been confirmed in a prospective study of Pima Indians (304,305).

Insulin action in adipocytes involves changes in gene transcription that involves regulation of a variety of transcription factors. These proteins are expressed in a cascade in which CCAAT enhancer binding protein (C/EBP) β and δ are among the earliest seen. These two proteins induce expression of PPARγ, which in turn activates C/EBPα. C/EBPα feeds back on PPARγ to maintain the differentiated state. C/EBP β and δ double knockout mice express normal levels of PPARγ and C/EBPα, suggesting the existence of a C/EBP-independent mechanism for inducing PPARγ. This may involve a transcription factor known as adipocyte differentiation and determination factor 1 (ADD1)/SREBP. It can activate PPARγ by inducing its expression as well as by promoting the production of an endogenous PPARγ ligand. These factors contribute to the expression of genes which characterize the terminally differentiated adipocyte phenotype (237). Transcription factors of the forkhead family may also play a major role in transducing insulin signals to the nucleus (145). Moreover, the differentiation of human preadipocytes to mature adipocytes was characterized by a marked (>10-fold) increase in IRS-1 protein expression whereas IRS-2 expression was only modestly increased (>2-fold) (217). These changes were associated with full development of the human adipocyte phenotype, including increased insulin-stimulated glucose transport capacity and increased expression of GLUT4 (217).

Insulin resistance in obesity and type 2 diabetes is manifested by decreased insulin-stimulated glucose transport and metabolism in adipocytes and skeletal muscle and by impaired ability of insulin to suppress hepatic glucose output (46). These functional defects may result from impaired insulin signalling in all three target tissues and, in adipocytes, also from downregulation of the major insulin-responsive glucose transporter GLUT4. In both muscle and adipocytes, insulin binding to its receptor, receptor phosphorylation and tyrosine kinase activity, and phosphorylation of IRSs are reduced (46). In adipocytes from obese humans with type 2 diabetes, IRS-1 expression is reduced, resulting in decreased IRS-1-associated PI 3-kinase activity, and IRS-2 becomes the the main docking protein for PI 3-kinase (235). In contrast, in skeletal muscle of obese, type 2 diabetic subjects, IRS-1 and IRS-2 protein levels are normal but PI 3-kinase activity associated with both IRSs is impaired (142).

Mice with fat-specific disruption of the insulin receptor gene (FIRKO mice) have low fat mass and are protected against age-related and hypothalamic lesion-induced obesity, and obesity-related glucose intolerance (28). Primary adipocytes from knockout mice lacking IRS-1 showed a decrease in glucose transport and GLUT4 translocation to the plasma membrane in response to insulin (278). In agreement with these results, it has been shown in human adipocytes that IRS-1 is the main docking protein for the binding and activation of PI 3-kinase in response to insulin (235). Recent studies show reduced IRS-1 expression in adipocytes of not only in morbidly obese and type 2 diabetic, but also of healthy first-degree relatives of type 2 diabetic subjects (44,45) as well as insulin-stimulated PI 3-kinase activity and insulin-stimulated glucose transport and GLUT4 expression (45).

2.2.2. Adipocytokines

Adipocytes secrete a wide range of products (85), which may impair insulin action through endocrine effects on distant target tissues and/or have paracrine actions on neighbouring cells. Cytokines are soluble proteins synthesized by immune or non-immune cells, which mediate intercellular communication by transmitting information to target cells through receptor-ligand interactions.

TNFα expression is increased in obesity, and interferes with insulin signalling by inducing phosphorylation of IRS-1 at serine residues and thus, inhibiting IR activity (119,135). TNFα rapidly inhibits insulin signalling at the level of PI 3-kinase and insulin-stimulated glucose transport in isolated human adipocytes (170). The effect of TNFα correlated with inhibition of tyrosine phosphorylation of IRS-1 while autophosphorylation of the IR β-subunit was unaffected (170).

IL-6 production is positively correlated with BMI (291). Human omental fat produces more IL-6 than subcutaneous adipose tissue. TNFα increases IL-6 production in differentiated 3T3-L1 adipocytes (102).

Adiponectin (also known as Acrp30 and AdipoQ) is a cytokine-like protein with some structural homology to TNFα. Adiponectin expression and/or secretion is increased by insulin like growth factor-1 and PPARγ agonists, and decreased by TNFα, glucocorticoids, β-adrenergic agonists and cAMP (268). In rodents, adiponectin expression and secretion fall with increasing fat mass, and its replenishment improves insulin resistance in various models of genetic and dietary-induced obesity (22). In lipodystrophic mice models, adiponectin and leptin ameliorated insulin resistance (263,321). In both lipoatrophic and obese mice, infusion of adiponectin increased β-oxidation of FFA in muscle and decreased TG content in skeletal muscle and the liver (321). Treatment with insulin-sensitizing PPARγ agonists such as rosiglitazone increases adiponectin expression (58).

In humans, circulating adiponectin levels are low in obesity (13) and type 2 diabetes (121), and increase with weight loss (22,322). However, an increase in subcutaneous fat mass in obesity may not explain such a reduction since serum adiponectin concentrations are also reduced in generalized forms of non-HIV lipodystrophy (109). It is possible that insulin resistance in both obese subjects and patients with LD is determined by the amount of fat in the liver and skeletal muscle (227).

Adiponectin inhibits the inflammatory process and possibly atherogenesis by suppressing the migration of monocytes/macrophages and their transformation into foam cells (212). Hypoadiponectinaemia was found as an independent risk factor for coronary artery disease in age-matched Japanese subjects (158).

Adiponectin enhances insulin sensitivity in both muscle and the liver by activating AMPK (320). This is accompanied by an inhibition of acetyl-CoA carboxylase and increased FFA oxidation in myocytes and a reduction of molecules involved in gluconeogenesis in the liver (320). The adiponectin gene is localized on human chromosome 3q27, a locus that has been associated with susceptibility to type 2 diabetes and the metabolic syndrome in genetic studies (273). Furthermore, adiponectin is a sticky molecule which accumulates in vessel walls and inhibits TNF-α-induced cell adhesion, and may therefore protect against endothelial dysfunction (211).

Leptin, a protein product of the *ob* gene, is secreted by fat cells in proportion to body fat stores (286). Leptin decreases food intake and increases energy expenditure. Leptin mRNA expression is higher in subcutaneous than omental adipocytes (196). Large fat cells contain more leptin than small adipocytes of the same individual (3,60). Insulin *in vivo* increases leptin secretion. This action of insulin does not seem to be affected by insulin resistance in type 2 diabetes (180,289). PPARγ agonists inhibit leptin expression (64).

PAI-1 is a member of the family of serine protease inhibitors and is the main regulator of the endogenous fibrinolytic system (310). Elevated plasma concentrations of PAI-1 have been found in obese (129) as well as lipodystrophic subjects (332). Increased PAI-1 concentrations may contribute to the prothrombotic state in obesity and type 2 diabetes (262).

Resistin is the product of an adipocyte-specific gene whose expression is regulated by PPARγ agonists and insulin (111,112,197,301) and was shown to cause insulin resistance in mice (269). The human homologue of resistin is expressed in circulating monocytes and at very low levels in adipose tissue (204). Thus, data regarding the role of resistin in the development of obesity and insulin resistance are contradictory (269,301).

2.2.3. Lipodystrophy and insulin resistance

Human lipodystrophies

The LDs are a heterogeneous group of disorders characterized by local LD and loss of body fat (lipoatrophy) (88). Atrophy can be localized ('partial' lipodystrophy) or complete ('generalized' lipodystrophy) (230). LDs can be genetic, autoimmune, inflammatory or acquired. The most common lipodystrophic condition in humans is that caused by HAART in patients with HIV (**Table 2**). The phenotype associated with LD is variable and characterized by hypertriglyceridaemia, insulin resistance, and increased risk of type 2 diabetes and cardiovascular disease. Insulin resistance is present in patients with genetic LDs, specifically autosomal dominant partial lipodystrophy of the Dunnigan type and autosomal recessive complete LD of the Berardinelli-Seip type.

Recently, a new form of genetic LD was identified when three lipodystrophic patients were found to have dominant negative mutations in the PPARγ gene (19). The affected subjects had severe insulin resistance, hypertension, dyslipidaemia, and hyperuricaemia. The LD is

similar to that seen in familial partial Dunnigan LD and HIV-associated, with a paucity of limb and gluteal fat, but normal facial and abdominal fat (91).

Table 2. *Characterization of human lipodystrophies. Adapted from reference 88.*

Type of lipodystrophy	Insulin resistance	Age of onset	Localization of fat loss	Localization of fat excess
1. Familial/genetic types				
1.1.Congenital generalized LD (Berardinelli-Seip syndrome)	+ + +	Birth	Face, neck, trunk, limbs, intra-thoracic and intra-abdominal, bone marrow	
1.2.Familial partial LD				
1.2.1.Dunnigan variety	+ +	Puberty	Trunk, limbs	None
1.2.2.Köberling variety	+	Not known	Limbs	Trunk
1.2.3.Mandibuloacral dysplasia	- (+)	Not known	Limbs	
2. Acquired types				
2.1.Acquired generalized LD (Lawrence syndrome)	+ + +	Childhood	Face, neck, trunk, limbs, palms, soles	None
2.2.Acquired generalized LD (Barraquer-Simons syndrome)	– (+)	Childhood	Face, neck, upper trunk, upper limbs	Lower abdomen, hips, lower limbs
2.3.LD in HIV infected patients	+ +	All ages	Face, trunk, limbs	Neck, upper body, intra-abdominal
2.4.Localized LD: induced by drugs	-	All ages	Depends on the cause	
2.5.Idiopathic	- (+)	All ages		

A striking syndrome of peripheral LD, hyperlipidaemia and insulin resistance characterizes patients receiving HAART. Up to 53 % of patients using HAART may experience some degree of LD within the first year of treatment, making this the most common form of human LD (42,43). Typical features include fat wasting over the face, limbs and buttocks, with fat accumulation in the abdomen and/or dorsocervical spine ('buffalo hump'). This is associated with insulin resistance, which in some cases progresses to diabetes and dyslipidaemia. Although the LD has been most commonly associated with the use of protease inhibitors, nucleoside analogues may also cause the syndrome (43,100). The mechanism of developing LD is not understood.

Lessons from genetically engineered animals

Studies in lipoatrophic mice have provided insight into the pathogenesis of insulin resistance characterizing not only obese but also lipoatrophic subjects. There are three mouse models of complete lipoatrophy. The first model was created by Ross et al. and used targeted expression of an attenuated diphtheria toxin to adipose tissue (238). All mice died within a day of birth with chylous ascites. The second model was created by Moitra et al. by expression of an artificial transcription factor in adipose tissue, which prevented DNA binding of β-ZIP transcription factors such as C/EBP and Jun which are critical for adipocyte growth and differentiation (194,292). The resulting A-ZIP/F-1 transgenic mice lacked visible white adipose tissue during development. The third transgenic mice model of LD was created by overexpression of SREBP1c in adipose tissue (264). It is unclear why overexpression of an active SREBP1c would result in white adipose tissue deficiency. The second and the third models are similar in that both feature absent white adipose tissue, insulin resistance, hyperglycaemia, hyperlipidaemia, organomegaly and a fatty liver. The models differ in that the A-ZIP/F1 mice lack both white and brown adipose tissue, while SREBP1c mice have increased amounts of immature brown adipose tissue and Pref-1, the biochemical marker of immature white adipose tissue.

Insulin signalling abnormalities in transgenic models. A-ZIP/F1 mice had a defect in insulin activation of IRS-1 in muscle and IRS-1-associated PI 3-kinase activity in liver (141). In SREBP1c overexpressing mice, baseline levels of IRS-1 and IRS-2 were low in adipocytes, as was IRS-2 in hepatocytes (264,265).

Leptin and adiponectin. In A-ZIP/F1 mouse model of lipoatrophic diabetes leptin overexpression partially improved insulin action (73). Leptin replacement has also recently been shown to reverse insulin resistance in human subjects with lipoatrophic diabetes and low circulating leptin levels (210). All murine models of LD showed both insulin resistance and decreased leptin levels. In mice with adipocyte specific SREBP1c overexpression, leptin administration improved insulin sensitivity, decreased plasma glucose levels, decreased excess hepatic fat, but did not correct the lipodystrophy (263). In contrast, leptin administration only minimally improved insulin resistance in A-ZIP/F1 mice (90), while offspring from crosses with leptin overexpressing mice showed decreased insulin and improved hepatic steatosis (73). Thus, leptin may not be the sole factor contributing to improved insulin resistance in LD. Treatment of lipoatrophic diabetes with adiponectin improved insulin resistance, and the effects were additive to those of leptin (321).

Fat transplantation. Implantation of white adipose tissue into A-ZIP/F-1 mice completely corrected hyperglycaemia and hyperinsulinaemia, and glucose uptake (89). Euglycaemic and hyperinsulinaemic clamp studies of white adipose tissue transplanted A-ZIP/F-1 mice showed normalization of insulin activation of IRS-1 and IRS-2-associated PI 3-kinase activity in muscle and liver respectively, along with decreased TG levels in liver and muscle (89). This demonstrates that subcutaneous fat contributes to maintenance of normal insulin sensitivity, and also that intrahepatocellular and intramyocellular lipid cause insulin resistance.

3. AIMS OF THE STUDY

The present study was undertaken to identify:

1. Early defects of insulin signalling in skeletal muscle of healthy lean subjects, specifically their association with potential signs of subclinical insulin resistance such as elevated fasting insulin concentrations (study I) and increased intramyocellular lipid content (study II).

2. Potential defects in markers of adipogenesis, inflammation and secretion of adipocytokines in adipose tissue of insulin resistant patients with HAL, specifically alterations in the expression of adipogenic transcription factors, PGC-1 and inflammatory markers (study III), and circulating concentration of adiponectin and its expression in the adipose tissue (study IV).

4. STUDY DESIGNS

4.1. Measurement of key insulin signalling molecules in freeze-dried and purified muscle in animals and healthy subjects (study I)

Animals. Male Wistar rats (95-115 g) were purchased from the Helsinki University Animal Laboratory. The animals were kept in a 12-h light-dark cycle for 1 week before the experiments. After an overnight fast the rats were anaesthetized by an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body weight) and then given an intraportal injection of either insulin (15 IU) or saline (control group) to obtain tissue samples representing insulin-stimulated and non-stimulated states.

Human subjects. Thirteen healthy men (42 ± 2 yrs) with a BMI ranging from 21 to 32 kg/m^2 (25.7 ± 0.7 kg/m^2, mean ± SEM) with no family history of type 2 diabetes were studied on two occasions. On the first occasion, insulin sensitivity was measured using the euglycaemic hyperinsulinaemic clamp technique. Before and after 30 min of the insulin infusion, muscle biopsies were taken from the left and right vastus lateralis muscles, for determination of early insulin signalling steps. On the second study occasion, maximal oxygen consumption (VO_2max) was measured directly using an incremental bicycle ergometer test. The subjects were divided by their median fasting insulin concentration in two groups (**Table 3**).

Table 3. *Characteristics of the groups in study I*

	Fasting insulin < median	**Fasting insulin > median**
Number of subjects	6	7
Fasting serum insulin (mU/l)	4 ± 1	8 ± 1**
Age (years)	45 ± 3	44 ± 3
Body mass index (kg/m^2)	24 ± 1	25 ± 1
VO_2max (ml/kg min)	37 ± 4	34 ± 2
M-value (mg/kg·min)	5.5 ± 0.4	3.4 ± 0.4**
Fasting plasma glucose (mmol/l)	5.2 ± 0.1	5.9±0.3*
Serum triglycerides (mmol/l)	0.9 ± 0.1	1.4 ± 0.2*
HDL cholesterol (mmol/l)	1.7 ± 0.1	1.3 ± 0.1*

Data are means ± SEM, *$p < 0.05$, **$p < 0.005$

4.2. IMCL and insulin signalling defects in human skeletal muscle (study II)

A total of 20 healthy men (42 ± 2 years), with a BMI ranging from 21 to 32 kg/m^2 (25.7 ± 0.7 kg/m^2, mean ± SEM) and no family history of type 2 diabetes, were studied on three occasions. On the first visit, IMCL content was measured using proton spectroscopy of the vastus lateralis muscle. On this visit, visceral and subcutaneous fat were also quantified using MRI. The subjects were divided into two groups: low IMCL (LoIMCL; 3.0 ± 0.5 resonance of intramyocellular CH_2 protons in lipids/resonance of CH_3 protons of total creatine (Cr_T) [IMCL/Cr_T]) and high IMCL (HiIMCL; 9.5 ± 0.9 IMCL/Cr_T) based on their median IMCL content (6.1 IMCL/Cr_T) (**Table 4**). The LoIMCL and HiIMCL groups were comparable with respect to several parameters thought to cause insulin resistance. On the second study occasion, maximal oxygen consumption (VO_2max) was measured directly using an incremental bicycle ergometer test. On the third occasion, insulin sensitivity was measured using the euglycaemic hyperinsulinaemic clamp technique. Before and after 30 min of insulin infusion, biopsies of the left and right vastus lateralis muscles were taken, respectively, for determination of early insulin signalling steps.

Table 4. *Characteristics of the groups in study II*

	IMCL > median	**IMCL < median**
Number of subjects	10	10
Fasting serum insulin (pmol/l)	55 ± 10	38 ± 4*
Age (years)	43 ± 3	40 ± 3
Body mass index (kg/m^2)	26 ± 1	26±1
VO_2max (ml/kg min)	33 ± 2	36 ± 3
Lean body mass (kg)	67 ± 3	64 ± 3
Fasting plasma glucose (mmol/l)	5.8 ± 0.2	5.4 ± 0.1
Serum triglycerides (mmol/l)	1.6 ± 0.3	1.1 ± 0.2
HDL cholesterol (mmol/l)	1.3 ± 0.1	1.5 ± 0.1

Data are means ± SEM, *$p < 0.05$

4.3. Expression of adipogenic transcription factors, PGC-1 and inflammatory markers in subcutaneous adipose tissue in patients with HAL (study III)

The patients were recruited from the HIV outpatient clinic of the Helsinki University Central Hospital. Patients' characteristics are given in **Table 5.** The subjects had had to be treated with HAART for at least 18 months prior to enrollment. Those belonging to the lipodystrophy group (HAART+LD+) had self-reported symptoms of loss of subcutaneous fat with or without increased girth, breast size or development of a buffalo hump. A single investigator (Jussi Sutinen) confirmed these findings before inclusion in the study. Patients without lipodystrophy (HAART+LD-) had received HAART without developing changes in body composition.

All patients were studied after an overnight fast at 8 a.m. A blood sample was taken for measurement of serum concentrations of glucose, insulin, cholesterol, triglycerides, and HDL cholesterol. Thereafter a needle aspiration biopsy of subcutaneous fat was taken under local anaesthesia as previously described (331). The fat sample was immediately frozen and stored in liquid nitrogen until analysis. In order to quantify any blood contamination of the samples, the concentration of haemoglobin was measured based on the absorbance of the homogenized sample at 560 nm, which was corrected for circulating haemoglobin concentration and homogenized sample size. The groups did not differ in this respect. In addition, in each patient the volumes of intra-abdominal and subcutaneous fat depots were determined using MRI, and waist-to-hip ratio was measured as detailed below.

4.4. Adiponectin expression in subcutaneous adipose tissue and adiponectin circulating concentration in patients with HAL (study IV)

The same subjects as in study III participated in study IV (**Table 5**). Hepatic fat was measured using ^{1}H-MRS. Adiponectin expression in adipose tissue and its circulating concentrations in serum were measured.

The purpose, nature, and potential risks of studies I-IV were explained to the patients before their written informed consent was obtained. The studies protocols were approved by the ethical committee of the Helsinki University Central Hospital.

Table 5. *Characteristics of the groups in studies III and IV*

Variable	HAART+LD+	HAART+LD-
Number of subjects (males/females)	25 / 5	9 / 4
Age (years)	43 ± 2	39 ± 2
Weight (kg)	73 ± 2	69 ± 4
Body mass index (kg/m^2)	23.6 ± 0.5	22.4 ± 1.1
Total abdominal fat/MRI (cm^3)	3100 ± 300	2700 ± 500
Subcutaneous fat / MRI (cm^3)	1100 ± 200	1800 ± 300*
Intra-abdominal fat/MRI (cm^3)	1900 ± 200	900 ± 300**
Waist-to-hip ratio	0.99 ± 0.01	0.89 ± 0.03***
Skinfold thickness (mm)	38 ± 3	54 ± 5***
Plasma glucose (mmol/l)	5.5 ± 0.3	5.0 ± 0.1
Serum insulin (mU/l)	11 ± 1	6 ± 1**
C-peptide (nmol/l)	0.81 ± 0.06	0.51 ± 0.09**
Serum triglycerides (mmol/l)	3.4 ± 0.4	1.2 ± 0.1***
Serum HDL cholesterol (mmol/l)	1.1 ± 0.1	1.6 ± 0.1***
Liver fat (%)	7.6 ± 1.7	2.1 ± 1.1***
Time from diagnosis of HIV (yrs)	8.4 ± 0.6	8.7 ± 1.3
Duration of HAART (yrs)	4.3 ± 0.2	3.8 ± 0.4
Currently receiving a protease Inhibitor	73 %	69 %
Most recent viral load (log_{10} copies/ml)	1.9 ± 0.1	1.6 ± 0.2
Most recent CD4 count (cells/mm^3)	572 ± 54	516 ± 70

HAART+LD+: HIV-positive patients with HAART-associated lipodystrophy; HAART+LD-: HIV-positive patients using HAART but without lipodystrophy; MRI: magnetic resonance imaging. Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

5. METHODS

5.1. Insulin signaling assays (studies I, II)

5.1.1. Processing of muscle specimens

Animal experiments (study I). Three minutes after the insulin injection (15 U Actrapid intraportally) and pentobarbital anaesthesia rat gastrocnemius muscle was dissected out and snap frozen in liquid nitrogen after which the rats were sacrificed with CO_2. Gastrocnemius muscle was divided into two pieces and snap-frozen in liquid nitrogen. The first piece ('wet') was thawed and homogenized as such (100 mg muscle/1 ml buffer A containing (in mM) 25 Tris-HCL (pH 7.4), 10 Na_3VO_4, 100 NaF, 10 $Na_4P_2O_7$, 10 EGTA, 10 EDTA, 1 phenylmethylsulfonyl fluoride (PMSF), and 5 µg/ml leupeptin, 5 µg/ml aprotinin, and 1 % NP40. The other piece ('dry') was lyophilized (200-400 Pa, Edward freeze-dryer, Crawley Sussex, England) for 14 h, macroscopically dissected free of fat, blood and connective tissue at room temperature. The tissue taken for the analysis was a macroscopically pure muscle fiber preparate. Both wet and dry samples were homogenized (HETO, Birkerod, Denmark) for 2 min (**Fig. 2**).

Human skeletal muscle (studies I, II). Biopsies of human vastus lateralis muscle were taken under local lidocaine anaesthesia with a Bergström needle before and after 30 min of the insulin infusion. The two biopsies were taken from opposite sites. The specimen was removed from muscle with suction applied through a syringe attached to the needle and then shot by rapidly emptying the syringe into liquid nitrogen (296). This procedure allowed the samples to be frozen within 3 seconds. The specimens were stored in liquid nitrogen until freeze-drying. To minimize the variability due to non-muscle contaminants, the muscle biopsy (~50 mg) was lyophilized, macroscopically dissected, and pure muscle fibers were homogenized in buffer A described earlier. After centrifugation (55 000 rpm, 1 h, +4 ^{o} C, 70.1 TI rotor, Beckman Instruments, Palo Alto, CA), the supernatant was transferred to another tube and its protein content was determined (Pierce, Rockford, IL). The supernatant was thereafter aliquoted for direct immunoblotting and immunoprecipitation experiments.

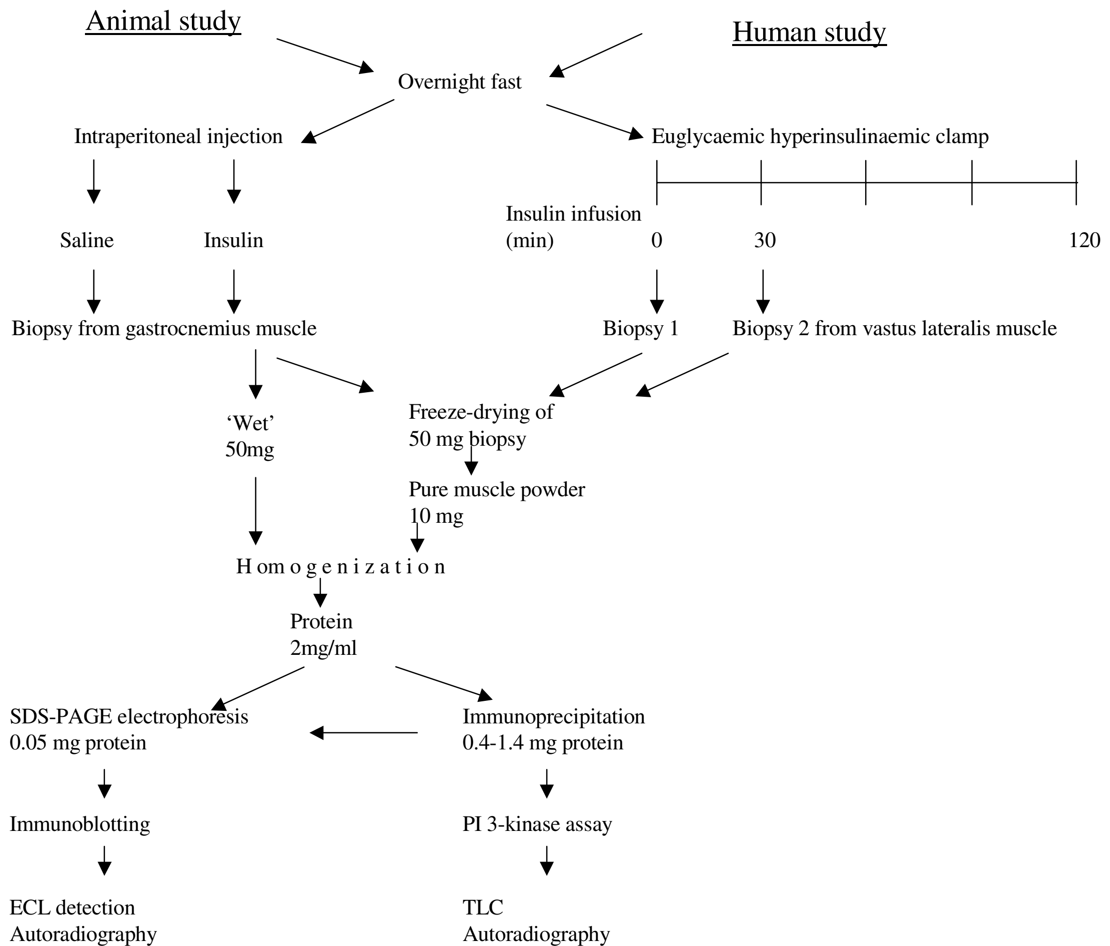

Fig. 2. *Processing rat and human muscle biopsies. Insulin was administered after overnight fast. In rats, a gastrocnemius muscle was dissected out and snap-frozen in liquid nitrogen. Each piece was divided in two parts: one was homogenized as such, and the other was freeze-dried and purified of non-muscle contaminants. After homogenization, ultracentrifugation and measurement of the protein concentration, the supernatant was aliquoted for immunoprecipitation and SDS-PAGE electrophoresis. Immunoblotting was done using specific antibodies and molecules of interest were detected by ECL or autoradiography. The PI 3-kinase assay was done using IRS-1 immunoprecipitates. In humans, normolycaemic hyperinsulinaemia was maintained for 120 min. Biopsies of vastus lateralis muscle were taken before and after 30 min of hyperinsulinaemia. Biopsies were snap-frozen in liquid nitrogen, freeze-dried and processed as in the animal experiments.*

5.1.2. Immunoprecipitation

To define posttranslational modification (tyrosine phosphorylation of IR) and IRS-1 associated PI 3-kinase enzymatic activity the muscle homogenates were immunoprecipitated with an appropriate antibody (anti-IR or anti-IRS-1) and 40 µl of 50 % protein A-Sepharose slurry (6 MB) for 2 h at 4 ^{o} C. The beads were washed 3 times with lysis buffer and dried before proceeding. For determination of tyrosine phosphorylation (pY) 25 µl of 2x Laemmli sample buffer was added on the dried immunoprecipitates, heated (+96 ^{o} C) for 3 min and stored in –80 ^{o} C. For IRS-1-associated PI 3-kinase *in vitro* assay, the washed and dried immunoprecipitates were stored at –80 ^{o} C without denaturing the sample (**Fig. 2**).

5.1.3. SDS-PAGE and immunoblotting

Supernatants and immunoprecipitates stored at –80 ^{o} C in Laemmli sample buffer were heated for 3 min before protein separation by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE gel acrylamide strength was chosen based on the size of the protein of interest. After electrophoresis the proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P) by transfer using buffer, which contained 10 % SDS, 99 % methanol, 1.9 M glycine and 0.25 M Trizma-base. After Immobilon-P membranes were blocked in PBS containing 0.1 % Tween-20 and 5 % non-fat milk for at least 30 min at room temperature and washed 3 times in PBS-Tween, they were subjected to immunoblotting: incubated with primary antibody (against phosphorylated tyrosine (pY20) or IR, 1:2 000 or 1:500 dilution) for 1 h, washed 3 times for 10 min, and then incubated with secondary HRP-linked rabbit anti-mouse antibodies (1:1 000 dilution), washed 3 times and detected by ECL. The films were scanned with tabletop scanner and analyzed by Image Gauge software (version 3.12, Fuji Photo Film, Tokyo, Japan).

5.1.4. PI 3-kinase assay

The dried protein-A Sepharose beads were washed twice with PI 3-kinase reaction buffer containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.5 mM EGTA, dried quickly and then resuspended in the same buffer supplemented with the PI 3-kinase substrate PI, which was freshly suspended in 50 µl of PI 3-kinase reaction buffer by sonication for 10 min at 4 ^{o} C. PI 3-kinase reaction was initiated by addition of 5 µl of $MgCl_2$-ATP mixture (200 mM $MgCl_2$, 200 µM ATP) containing 5 µCi of [γ-^{32}P]-ATP to the immunoprecipitates and incubated for 25 min at room temperature. The reaction was terminated by adding 150 µl of

chloroform-methanol-11.6 N HCl (100:200:2). After adding 120 µl of chloroform and 120 µl of methanol-1 N HCl (1:1) twice the organic phase was separated by centrifugation at 14 000 rpm for 1 min, the resulting organic phase was dried in SpeedVac (UVS400A, Savant Instruments, NY) dissolved in 15 µl of chloroform, spotted on thin layer chromatography plates and developed in chloroform-methanol-28 % ammonium hydroxide-water (43:38:5:7) for 1 h. The phosphorylated products were visualized by autoradiography after an overnight exposure and quantified by Bio-Imaging analyzer (BAS-5000; Fuji, Tokyo).

5.1.5. Materials for insulin signalling assays

Regular insulin was from Novo Nordisk (Actrapid, Bagsvaerd, Denmark). Polyclonal antibodies against the insulin receptor (IR, JD 15, JD 433) and insulin receptor substrate-1 (IRS-1, JD 288) were generous gifts from Professor C. Ronald Kahn (Joslin Diabetes Center). The antibody against the p85 subunit of PI 3-kinase was from Upstate Biotechnology (Lake Placid, NY). HRP-linked rabbit anti-mouse and donkey anti-rabbit antibodies, the ECL kit and [^{125}I] protein A were from Amersham Pharmacia Biotech (Amersham Place Little Chalfont Buckinghamshire, England). [γ-^{32}P] ATP was from NEN Life Science Products, Zaventem, Belgium; PI-from Avanti Polar Lipids (Alabaster, Alabama); silica gel thin layer chromatography plates-from Merck (Darmstadt, Germany); Immobilon-P transfer membranes -from Millipore Corporation (Bedford, MA); protein A Sepharose 6 MB - from Amersham Pharmacia Biotech AB (Uppsala, Sweden); reagents for SDS-PAGE-from Bio-Rad Laboratories (Richmond, CA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

5.2. Human studies (studies I, II)

5.2.1. Whole-body glucose uptake

Whole-body glucose uptake was measured by the euglycaemic hyperinsulinaemic clamp technique (66). After an overnight fast, two indwelling catheters were inserted, one in an antecubital vein for infusion of insulin and glucose, and another in a hand vein, which was kept in a heated chamber (65 ^{o}C) to arterialize venous blood (184). The rate of the continuous insulin infusion was 40 mU/m^2 · min, or 1 mU/kg·. min. The rate of the glucose infusion was adjusted to maintain euglycaemia based on plasma glucose measurements that were performed every 5 minutes from arterialized venous blood. Whole-body insulin sensitivity (M-value) was calculated from the glucose infusion rate needed to maintain euglycaemia after

correction for changes in the glucose pool size (66). Blood samples were taken at 30 min intervals for measurement of serum free insulin concentrations. The muscle biopsy from vastus lateralis muscle from one leg was taken at the beginning of the clamp and from the other leg after 30 min of insulin infusion. The biopsies were freeze-dried, purified and processed as described in section 5.1.1 (**Fig. 2**).

5.2.2. Maximal aerobic power

Maximum aerobic power (VO_2max) was measured directly, using an incremental work-conducted upright exercise test with an electrically braked cycle ergometer (Ergometer Ergoline 900ERG, Germany) combined with continuous analysis of expiratory gases and minute ventilation (Vmas 229 series, SensorMedics). Exercise was started at a workload of 50 watts. The workload was then increased by 50 watts every 3 min until perceived exhaustion or a respiratory quotient of 1.10 was reached. The highest maximal aerobic power observed during a 30 sec period was defined as VO_2max.

5.2.3. Body composition

IMCL (^{1}H-MRS)

Magnetic resonance (MR) images for localization and ^{1}H-MR spectroscopy were acquired using a 1.5 T MR system (Magnetom Vision; Siemens, Erlangen, Germany). A loop surface coil was used for detection. The subjects lay supine, with the left leg immobilized by firm padding. One-third of the distance from the trochanter major of the femur to the middle of the patella was measured, and the center of the coil was placed in contact with that spot. The ^{1}H-spectra were obtained from quadriceps femoris (vastus lateralis) muscle (**Fig. 3**). The volume of interest, voxel (13 x 13 x 20 mm^3), was placed inside the lateral part of the vastus lateralis muscle to ensure parallel fiber orientation. The position of the voxel was chosen on the T_1-weighted MR images so that it did not contain any visible fat or fasciae known to contain significant amounts of adipocytes which would affect the resonance-frequency shift data (**Fig. 3**). Spatial localization was achieved by using a stimulated echo acquisition mode applied with a repetition time of 3 000 ms, with an echo time of 20 ms, and a mixing time of 30 ms. We used 128 excitations with water presaturation. The resonance at 1.5 ppm originates from the extramyocellular CH_2-protons of lipids (TGs and FFAs) and the resonance at 1.3 ppm from intramyocellular CH_2-protons of lipids (249,277). An example of the proton spectra is shown in **Fig. 3.** Resonance intensities were expressed relative to the resonance at 3.0 ppm, which is derived from the CH_3-protons of total creatine (Cr_T; creatine and phosphocreatine).

The spectral data were fitted in time domain with the program MRUI, based on the solving of nonlinear least squares problems (62). The IMCL data were quantified by using Cr_T signal intensity as a reference. The total water-to-Cr_T ratios were constant between subjects (signal intensity ratio 829 ± 46, correlation coefficient for signal intensity of water vs. Cr_T 0.96) and the Cr_T-to-water ratio (x10^4) averaged 12.9 ± 0.9 and 12.3 ± 1.2 in the LoIMCL and HiIMCL groups, respectively (ns). We chose IMCL/Cr_T for grouping criterion because both signals are of intramyocellular origin and of comparable magnitude of intensity.

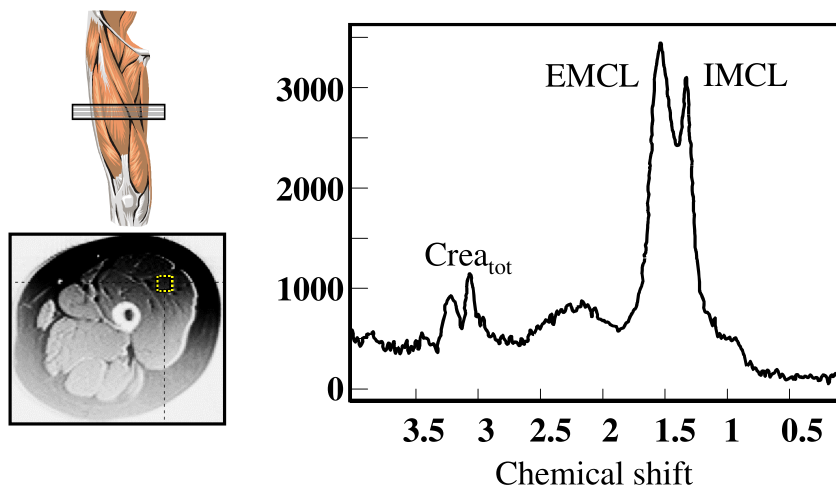

Fig. 3. *Proton magnetic resonance spectroscopy of human vastus lateralis muscle. The volume of interest, voxel, was placed inside the lateral part of the vastus lateralis muscle. The resonance at 1.5 ppm (EMCL) originates from the extramyocellular CH_2-protons of lipids (triglycerides and fatty acids) and the resonance at 1.3 ppm (IMCL) from intramyocellular CH_2-protons of lipids. Resonance intensities are expressed relative to the resonance at 3.0 ppm (Crea$_{tot}$), derived from the CH3-protons of total creatine (Cr_T; creatine and phosphocreatine).*

Intra-abdominal, subcutaneous and liver fat

A series of T1-weighted transaxial scans for the determination of *visceral and subcutaneous fat* were acquired from a region extending from 8 cm above to 8 cm below the 4th and 5th lumbar interspace (16 slices, field of view 375 x 500 mm^2, slice thickness 10 mm, and breath-hold repetition time divided by the echo time 138.9 ms/4.1 ms), as previously described (242). Visceral and subcutaneous fat areas were calculated using an image analysis program (Alice 3.0, Parexel, Walthham, MA). A histogram of pixel intensity in the intra-abdominal region was displayed, and the intensity corresponding to the nadir between the lean and fat peaks was used as a cut point. Visceral adipose tissue was defined as the area of pixels in the intra-abdominal region above this cut point. For calculation of subcutaneous adipose tissue area, a region of interest was first manually drawn at the demarcation of subcutaneous adipose tissue and visceral tissue (242). To measure *liver fat content by ^{1}H-MRS,* localized single voxel (2 x 2 x 2 cm^3) proton spectra were acquired using a 1.5 T whole body system (Siemens Magnetom Vision, Erlangen, Germany), which consisted of a combination of whole-body and loop surface coils for radio frequency transmitting and signal receiving (**Fig. 4**).

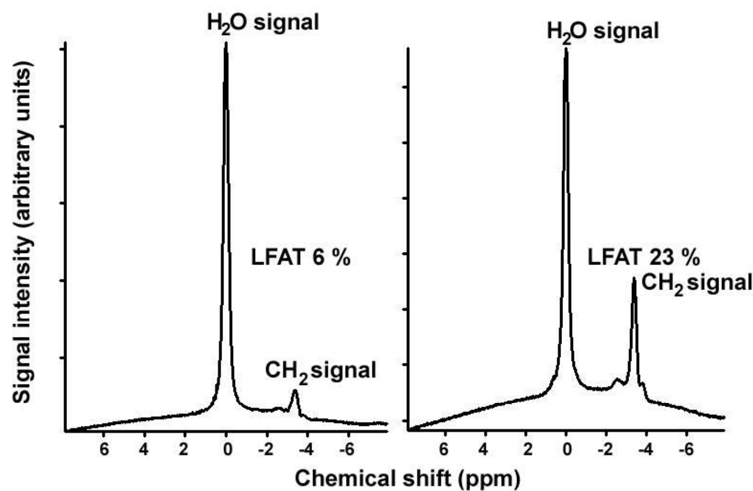

Fig. 4. *Proton magnetic resonance spectra from 2 patients with different percent of liver fat (LFAT). The CH_2 signal is derived from intrahepatocellular protons of lipids. The percent LFAT was calculated by dividing the area under the CH_2 signal by the sum of the areas under the water and CH_2 signals.*

T1-weighted spin echo MR images were used for localization of the voxel of interest within the right lobe of the liver. Vascular structures and subcutaneous fat tissue were avoided in localization of the voxel. Subjects were lying on their stomach on the surface coil, which was embedded in a mattress in order to minimize abdominal movement due to breathing. The single voxel spectra were recorded by using the stimulated echo-acquisition mode sequence with an echo-time of 20 ms, a repetition time of 3 000 ms and a mixing time of 30 ms. A total of 1024 data-points over 1000 kHZ spectral width with 32 acquisitions were collected. Water-suppressed spectra with 128 acquisitions were also recorded to detect weak lipid signals. The short echo-time and the long repetition time were chosen to ensure a fully relaxed water signal, which was used as an internal standard. Chemical shifts were measured relative to water at 4.80 ppm. The methylene signal, which represents intracellular triglyceride, was measured at 1.4 ppm. The spectra were fitted in the time domain using the variable projection method (VARPRO) (62). Spectroscopic intracellular triglyceride content was expressed as a ratio of the area under the methylene peak to that under the methylene and water peaks x 100 (= % liver fat, LFAT). This measurement of percent hepatic fat by proton spectroscopy has been validated against histologically determined lipid content of liver biopsies in humans (282), animals (277) and against estimates of fatty degeneration or infiltration by X-ray computed assisted tomography (171). Examples of two spectra of subjects with low and high liver fat are shown in **Fig. 4.**

Body fat content and other measurements

Body composition (fat mass, fat free mass and percentage of body fat) was determined by bioelectrical impedance plethysmography (BioElectrical Impedance Analyzer System model #BIA-101A; RJL Systems, Detroit, Michigan) (174). *Waist circumference* was measured midway between the lower rib margin and the iliac crest, and hip circumference over the greater trochanters (205). *Skinfold thicknesses* (sum of mean values of triplicate measurements) were determined at 6 sites (triceps, biceps, subscapular, iliac crest, thigh and cheek).

5.2.4. Analytical methods

Plasma glucose concentrations were measured in duplicate using the glucose oxidation method (Beckman Glucose Analyzer II, Beckman Instruments, Fullerton, CA) (130). *Serum free insulin concentrations* were determined by double antibody radioimmunoassay (Pharmacia Insulin RIA Kit, Pharmacia, Uppsala, Sweden) after precipitation with polyethylene glycol. *Serum FFA concentrations* were determined by the fluorometric method (192). *Serum high density lipoprotein cholesterol and TG concentrations* were determined with respective enzymatic kits from Roche Diagnostics using an autoanalyzer (Roche Diagnostics Hitachi 917, Hitachi Ltd, Tokyo, Japan). *HIV viral load* was measured using the HIV-1 Monitor Test (Roche Diagnostics, Branchburg, NJ) with a detection limit of 50 copies/ml. *Serum IL-6 concentration* was measured using an enzyme-linked immunoassay (Quantikine, R&D Systems, Minneapolis, MN). *Serum adiponectin concentration* was measured using a commercial enzyme-linked immunosorbent assay (Human Adiponectin ELISA kit, B-Bridge International, San Jose, CA).

5.3. RNA isolation and measurement of gene expression adipose tissue (studies III, IV)

5.3.1. Processing of fat biopsy and total RNA isolation

Approximately 100 mg (41-137 mg) of frozen fat tissue was homogenized in 2 ml of RNA STAT-60 (Tel-Test, Friendswood, TX) and total RNA was prepared according to manufacturer's instructions. After DNase treatment (RNase-free DNase set, Qiagen, Hilden, Germany) RNA was subjected to cleanup (RNeasy mini kit, Qiagen, Hilden, Germany). **Fig. 5** depicts how RNA was used.

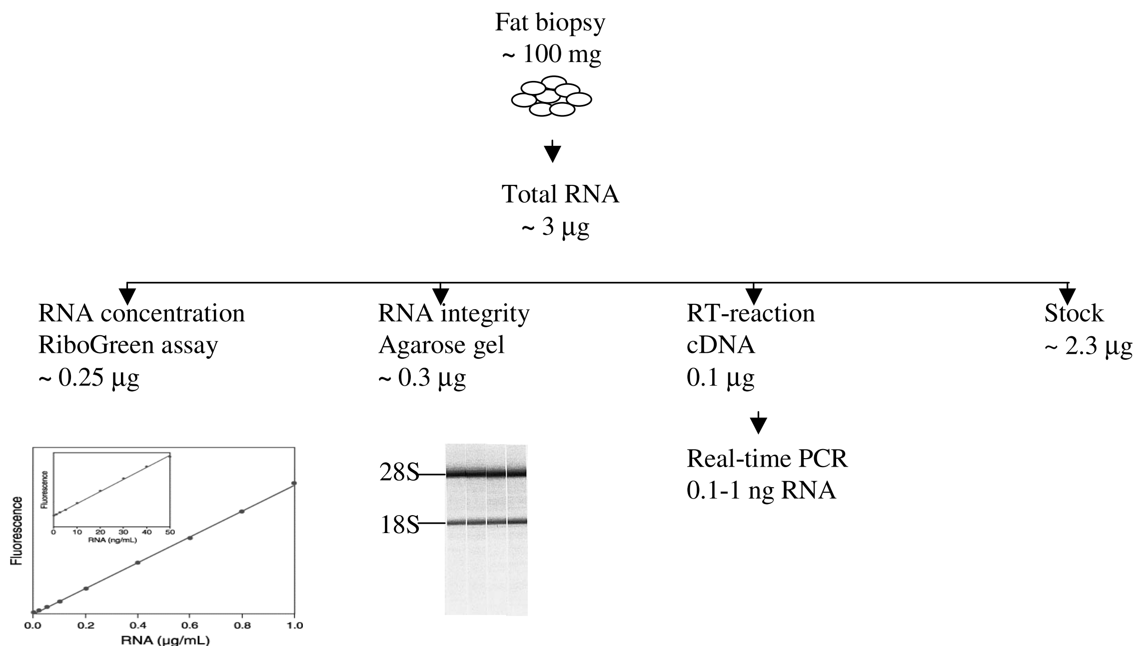

Fig. 5. *Design of the experiments using total RNA from human fat. Fat obtained by an aspiration biopsy (around 100 mg) was snap-frozen in liquid nitrogen and stored in liquid nitrogen until analysis. Total RNA was isolated, cleaned, and used for: RNA concentration measurements using RiboGreen assay, assessment of RNA quality using agarose gel or bioanalyzer and the reverse-transcriptase (RT) reaction for cDNA synthesis and PCR. The remaining total RNA was stored in liquid nitrogen.*

5.3.2. Quantification of RNA concentration and synthesis of cDNA

RNA concentrations were measured using the RiboGreen fluorescent nucleic acid stain (RNA quantification kit, Molecular Probes, Eugene, OR). Average yields of total RNA were 3.3 ± 0.4 µg per 100 mg of adipose tissue wet weight, and were not significantly different between HIV positive patients with or without lipodystrophy, and healthy controls. Total RNA was suspended in water and stored at –80 ^{o}C until the reverse-transcriptase reaction. 0.1 µg total RNA was transcribed into cDNA using M-MLV reverse transcriptase (Life Technologies, Paisley, UK) and oligo $(dT)_{12-18}$ (0.5 µg/µl) primers.

5.3.3. Real-time PCR

Relative quantification of the mRNAs was performed by real-time PCR using LightCycler technology (Roche Diagnostics GmbH, Mannheim, Germany) for quantification of β-actin, PPARγ, LPL, SREBP1c and adiponectin (at the Minerva Institute for Medical Research), and TaqMan technology using ABI PRISM 7000 Sequence Detection System (PE Applied Biosystem, Foster City, CA) for quantification all the other genes (at the King Gustaf V Research Institute, Atherosclerosis Research Unit, Karolinska Institute, Stockholm, Sweden). PCR allows amplification of DNA from a selected region of the genome a billionfold. 2 µl of diluted cDNA was brought to a volume of 20 µl with reaction mix (Roche Diagnostics), containing a final concentration of 3 mM $MgCl_2$, 2 µl of LightCycler-FastStart DNA Master SYBR Green I and 0.5 µM of primers. SYBR Green is a fluorofor, a double-stranded DNA binding dye, which fluoresces when only bound to DNA and thus makes the signal much more specific than in conventional PCR.

5.3.4. Quantification of mRNA using real-time PCR

β-actin, PPARγ, LPL, SREBP-1c and adiponectin

Quantification of the mRNAs was performed by real-time PCR using LightCycler technology (Roche Diagnostics GmbH, Mannheim, Germany). 2 µl of 1:10 diluted cDNA was brought to a final volume of 20 µl, which contained 3 mM $MgCl_2$, 2 µl of LightCycler-FastStart DNA SYBR Green I Mix (Roche Diagnostics), and 0.5 µM of primers. After initial activation of the DNA polymerase at 95 ^{o}C for 10 min, the amplification conditions were as follows: 40 cycles consisting of denaturation at 95 ^{o}C for 15 sec, annealing for 5 sec at 57 ^{o}C (β-actin), 56 ^{o}C (PPARγ), 58 ^{o}C (LPL), for 10 sec at 60 ^{o}C (SREBP-1c), or for 5 sec at 58 ^{o}C (adiponectin) and extension at 72 ^{o}C. The extension times (sec) were calculated from the amplicon size

(base pairs/25). Fluorescent data were acquired at the end of each extension phase. After amplification, a melting curve analysis from 65 ^{o}C to 95 ^{o}C with a heating rate of 0.1 ^{o}C/sec with a continuous fluorescence acquisition was made.

The primers for PPARγ, LPL, SREBP-1c and β-actin are listed in **Table 6**. A standard curve for PPARγ was created using purified cloned plasmid cDNA (QIAquick PCR purification kit, Qiagen, Hilden, Germany). For human β-actin, LPL and SREBP-1c expression, standard curves were created from a specific PCR product. To account for differences in RNA loading, PPARγ, SREBP-1c and LPL were expressed relative to β-actin. The mRNA concentration of human β-actin was not different between the groups (217 ± 44 in the HAART+LD+ and 180 ± 40 in the HAART+LD- group, ns).

Human β2-microglobulin, GLUT1, GLUT4, PGC-1, PPARδ, ALBP, KLBP, FATP-1 and –4, ACS, CD45 and IL-6 gene expression

TaqMan real-time semiquantitative PCR was performed according to the manufacturer's protocol using ABI PRISM® 7000 Sequence Detection System instrument and software (PE Applied Biosystem, Foster City, CA). Primer and probe sets were designed using the manufacturer's software and sequences available in GeneBank I (**Table 6**). IL-6 was measured using Pre-Developed TaqMan Assay Reagents (PE Applied Biosystem, Foster City, CA). The GLUT4 primer set has been published (229). Differences in loading of RNA were adjusted for by expressing results relative to β2-microglobulin. mRNA concentrations of β2-microglobulin were not different between the groups (606 ± 71 in the HAART+LD+ and 645 ± 73 in the HAART+LD- group, ns). Expression levels were quantified (AU) by generating a six-point serial standard curve (302). The mRNA concentrations of GLUT1, GLUT4, PGC-1, PPARδ, ALBP, KLBP, FATP-1 and FATP–4, ACS, CD45 and IL-6 are given relative to β2-microglobulin mRNA concentration.

Table 6. *Primers and probes used for mRNA analyses*

mRNA Gene Bank Number	Sense primers	Antisense primers	Probe
PPARγ U79012	5'-CTCATATCCGAGGGCCAA	5'-TGCCAAGTCGCTGTCATC	*
β-actin M10277	5'-CACACTGTGCCCATCTACGA	5'-CCATCTCTGCTCGAAGTCC	*
SREBP-1c NM004176	5'-GCGGAGCCATGGATTGCAC	5'- CTCTTCCTTGATACCAGGCCC	*
LPL NM000237	5'-GGTCGAAGCATTGGAATCCAG	5'-TAGGGCATCTGAGAACGAGTC	*
Adiponectin XM 003191	5'-CAGAGATGGCACCCCTGGTG	5'-TTCACCGATGTCTCCCTTAG	*
β2microglobulin AH002619	5'-GCCTGCCGTGTGAACCAT	5'- TTACATGTCTCGATCCCACTTAACTAT	5'-TGACTTTGTCACAGCCCA
GLUT1 NM006516	5'-CCTGTGGGAGCCTGCAAA	5'-TCTATACACAACAGGGCAGGAGTCT	5'-CACTGCTCAAGAAGAC
GLUT4 14758189	5'-GCTACCTCTACATCATCCAGAATCTC	5'-CCAGAAACATCGGCCCA	5'-CTGCCAGAAAGAGTCTGAAGCGCCT
PGC-1 AH008808	5'-AGAGACAAATGCACCTCCAAAAA	5'-AAAGTTGTTGGTTTGGCTTGTAAGT	5'-AAGTCCCACACACAGTCGCAGTCACAA
PPARδ NM006238	5'-CACACGGCGCCCTTTG	5'-CCTTCTCTGCCTGCCACAA	5'-ATCCACGACATCGAGAC
CD45 NT004612	5'-TCTTGGCATTTGGCTTTGC	5'-GGAGACTGACTGCGTGTGTGA	5'-CTGGACACAGAAGTATTT
ALBP NM001442	5'-TGATAAACTGGTGGTGGAATGC	5'-CCCTTGGCTTATGCTCTCTCA	5'-TCATGAAAGGCGTCACTTCCACGAGA
KLBP NM001444	5'-GGGAAGGAAAGCACAATAACAAGA	5'-CGAGTACAGGTGACATTGTTCATG	5'-CACACTCCACCACTAATTTCCCATCTTTCAATT
FATP-1 AX015323	5'-AGATGCCCGCTCACTTCG	5'-GCTAAGGCCCTGATCTTTGGA	5'-CCACCGCCGCCACCATTTCTC
FATP-4 AF055899	5'-CAAGACCATCAGGCGCG	5'-CGCACCTTTGCCTTCACCT	5'-TATCTTTGGCGGCCTGGTCCTCCT
ACS L09229	5'-CAAAGACGGCTGGTTACACACA	5'-TCCGGTCGATAATTTTCAAGGT	5'-CCATTTGGTAACCATTTTCCGATGTCCC

5.4. Statistical analyses

Student's *t-test* was used for comparison signals in wet versus dry muscle samples in study I and in high versus low intramyocellular lipid content groups in study II. For comparison of FFA concentrations between the groups in study II, analysis of variance for repeated measures was used. Correlation coefficients were calculated using Pearson's correlation coefficient in study II. In studies III and IV the unpaired *t-test* was used to compare differences between the groups after logarithmic transformation when necessary. Correlations were calculated using Spearman's rank correlation coefficient. Categorical variables were compared using Fisher's exact test. All calculations were performed using the Systat statistical package, version 10.0 (Systat, Evanston, IL) or GraphPad Prism, version 2.01 (GraphPad Inc, San Diego, CA). In all the studies data are shown as mean ± standard error of mean. A *p*-value less than 0.05 was considered statistically significant.

6. RESULTS

6.1. Insulin signalling in freeze-dried rat skeletal muscle (study I)

The non-muscle contaminants varied between 1 and 40 % (mean 11 %) of dry muscle weight. To test the feasibility of insulin signalling assays in freeze-dried purified muscle, we measured insulin signalling molecules in rat gastrocnemius muscle and compared the results with those obtained using conventionally prepared muscle samples.

Protein expression of signalling molecules

Protein expression of the IR, IRS-1 and p85 subunit of PI 3-kinase in freeze-dried rat gastrocnemius muscle were of the same order of magnitude as in wet, conventionally processed muscle samples (**Fig. 6**).

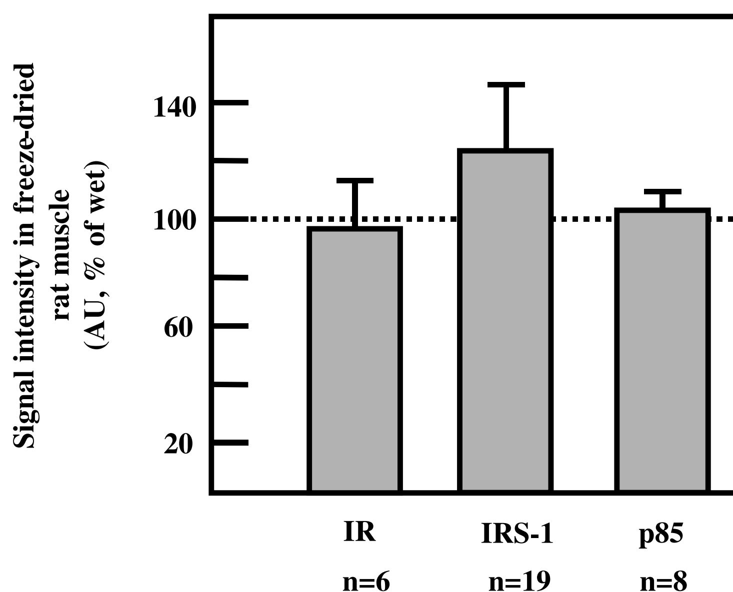

Fig. 6. *Protein expression of the IR, IRS-1 and the p85 subunit of the PI 3-kinase in freeze-dried rat gastrocnemius muscle relative to wet specimens (set as 100 %). Equal amounts of protein (0.05 mg) were separated by SDS-PAGE and immunoblotted with anti-IR, anti-IRS-1 and anti-p85 antibodies, respectively.*

Tyrosine phosphorylation of IR and IRS-1-associated PI 3-kinase activity in dry versus wet rat muscle preparates

To determine the extent of IR tyrosine phosphorylation, the tissue lysates were immunoprecipitated with IR antibodies and then immunoblotted with antiphosphotyrosine antibodies. Insulin increased tyrosine phosphorylation of the -subunit of the IR 9.4 ± 1.0-fold in freeze-dried and 6.4 ± 0.9–fold in wet muscle ($p < 0.05$, dry vs wet, **Fig. 7, panel A**). IR protein expression was slightly lower in freeze-dried (87 ± 8 AU) than in wet (113 ± 8 AU, $p < 0.05$, $n = 6$) muscle. As shown in **Fig. 7, panel B,** PI 3-kinase activity in IRS-1 immunoprecipitates in dry and wet rat muscle samples was preserved during freeze-drying. The large dose of insulin (15 IU) given to rats 3 minutes prior to removal of gastrocnemius muscle increased IRS-1-associated PI 3-kinase activity similarly in wet (4.0 ± 0.4-fold relative to basal) and dry (3.8 ± 0.8-fold relative to basal, ns) samples. IRS-1 protein expression was slightly lower in freeze-dried (86 ± 9 AU) than in wet (114 ± 9 AU, $p < 0.05$, $n = 6$) muscle.

Coefficient of variation of repeated measurements in freeze-dried samples

To examine whether freeze-drying reduces variation of repeated measurements, we compared variation of protein expression of the p85 regulatory subunit of PI 3-kinase after 4 different homogenizations using freeze-dried and wet samples of the same rat gastrocnemius muscle. Protein expression of freeze-dried and wet muscle p85 was identical (57600 ± 2358 AU and 57480 ± 6875 AU) but the coefficient of variation was significantly lower in freeze-dried and purified (8.2 %) as compared to wet (23.9 %) samples ($p < 0.05$).

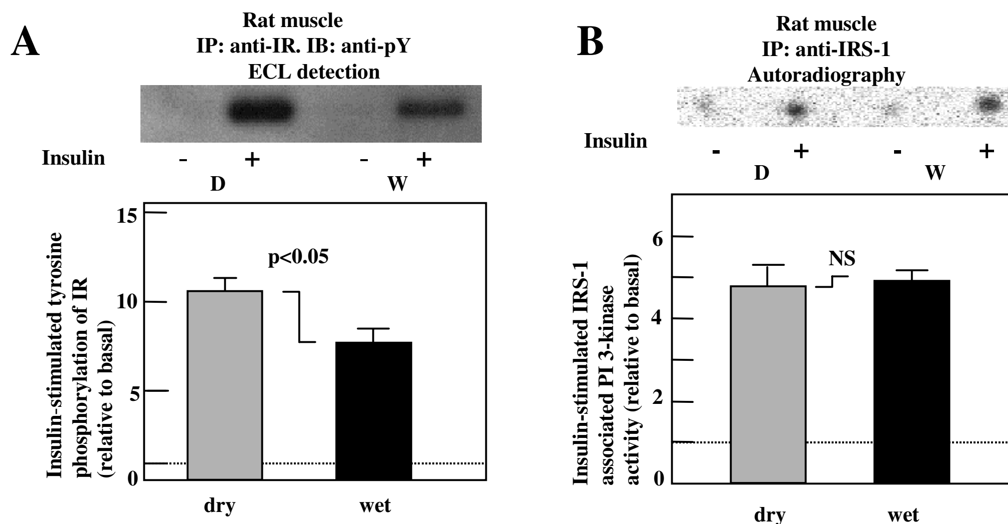

Fig. 7, panel A: *Insulin-stimulated tyrosine phosphorylation of IR in wet and freeze-dried (dry) rat skeletal muscle. Equal amounts of protein (0.4 mg) were immunoprecipitated with anti-IR antibodies, separated by SDS-PAGE, and immunoblotted with anti-pY antibodies. The band migrating at 95 kDa represents the tyrosine phosphorylated IR signal. Data (mean ± SEM, n = 8) are expressed relative to basal which is shown as the broken line.* ***Panel B:*** *IRS-1-associated PI 3-kinase activity in wet and freeze-dried (dry) rat gastrocnemius muscle. Equal amounts of protein (1.4 mg) were immunoprecipitated with anti-IRS-1 antibodies. The kinase activities were measured in the presence of PI and [^{32}P] ATP, and PIP3 was separated by TLC. Data (mean ± SEM, n = 6) are expressed relative to basal activity. The upper panel shows a representative TLC. The lower panel shows PI 3-kinase activity 3 min after a 15 IU intraportal injection of insulin.*

6.2. Insulin signalling in freeze-dried human skeletal muscle (study I)

Healthy men without family history of type 2 diabetes (n = 13) were divided into two groups by the median fasting serum insulin concentration, as it is the most physiological measurement of insulin sensitivity: low insulin group (LI) and high insulin insulin (HI). The men with HI were more insulin-resistant than were men with LI. The HI group was characterized by a significantly lower rate of whole-body glucose uptake (3.4 ± 0.4 vs 5.5 ± 0.4, HI vs LI, $p < 0.005$), higher serum TGs (1.4 ± 0.2 vs 0.9 ± 0.1, HI vs LI, $p < 0.05$), lower high density lipoprotein cholesterol (1.3 ± 0.1 vs 1.7 ± 0.1, HI vs LI, $p < 0.05$) and higher fasting plasma glucose (5.9 ± 0.3 vs 5.2 ± 0.1, HI vs LI, $p < 0.05$) concentrations than the LI group. The subjects were matched for age, gender and BMI and had similar VO_2max (see characteristics of the subjects in **Table 1** of original publication I and **Table 3**).

As methodological experiments on rats demonstrated the feasibility of the freeze-drying, all muscle biopsies from humans were freeze-dried prior to the analysis. In freeze-dried human skeletal muscle, insulin-stimulated tyrosine phosphorylation of the IR was higher (200 ± 40 %) in the below median insulin group compared to the above median insulin group (81 ± 22 %, $p < 0.05$, **Fig. 8, panel A**). IRS-1-associated PI 3-kinase activity was also higher in the below median insulin group (109 ± 23 %) than in the above median insulin group (59 ± 19 %, $p < 0.05$, **Fig. 8, panel B**). Individual insulin-stimulated IRS-1-associated PI 3-kinase activities varied from 46 to 176 AU (3.8-fold) and were significantly inversely correlated with fasting serum insulin concentrations (**Fig. 9**) which varied from 3 to 9 mU/l (3-fold).

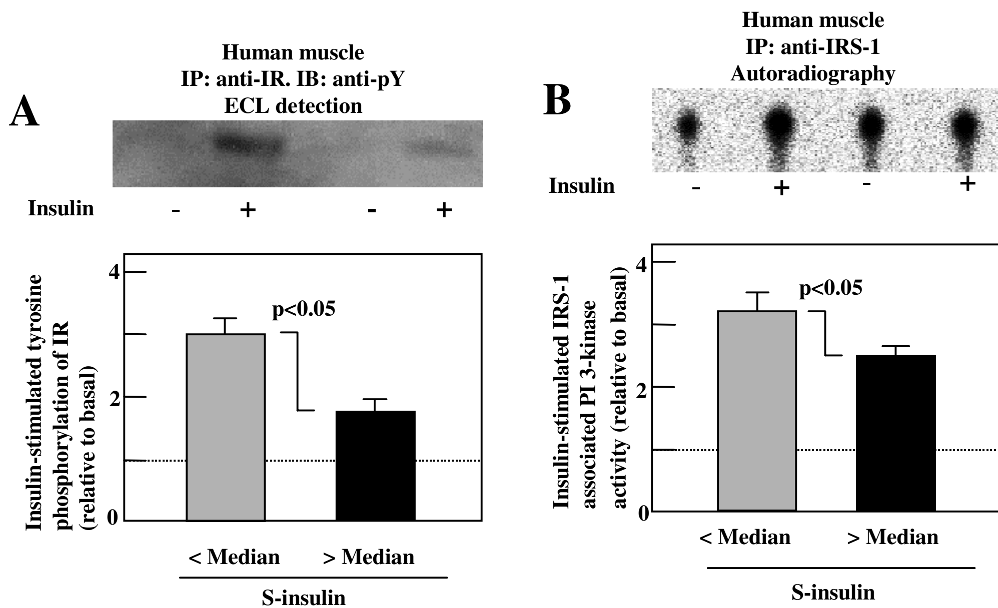

Fig. 8, panel A: *Insulin-stimulated tyrosine phosphorylation of IR in human vastus lateralis muscle from healthy subjects subdivided based on their median serum insulin concentration. Equal amounts of protein (1.4 mg) were immunoprecipitated with anti-IR antibodies, separated by SDS-PAGE, and immunoblotted with anti-pY antibodies. The band migrating at 95 kDa represents the tyrosine phosphorylated IR signal. Data (mean ± SEM, n = 8) are expressed relative to basal that is shown as broken line.* ***Panel B:*** *IRS-1-associated PI 3-kinase activity in human skeletal muscle. Equal amounts of protein were immunoprecipitated with anti-IRS-1 antibodies. The kinase activities were measured in the presence of PI and [^{32}P] ATP, and PIP3 was separated by TLC. Data (mean ± SEM, n = 6) are expressed relative to basal activity. The upper panel shows a representative TLC. The lower panel shows PI 3-kinase activity 3 min after 30 min insulin infusion.*

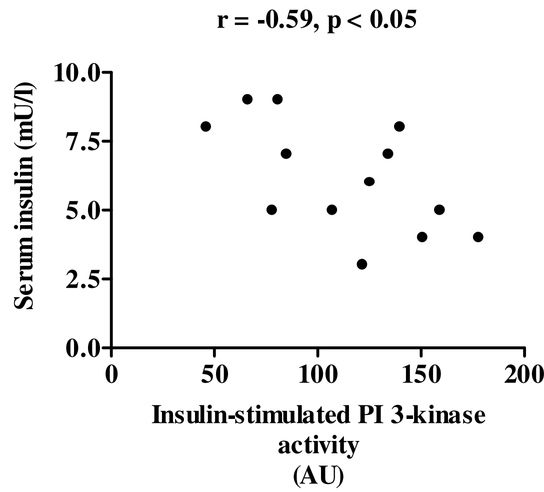

Fig. 9. *The relationship between insulin-stimulated PI 3-kinase activity in freeze-dried and purified muscle of human vastus lateralis biopsies taken after 30 min of physiological hyperinsulinaemia (S-insulin 0.4 nmol/l), and fasting serum insulin concentrations.*

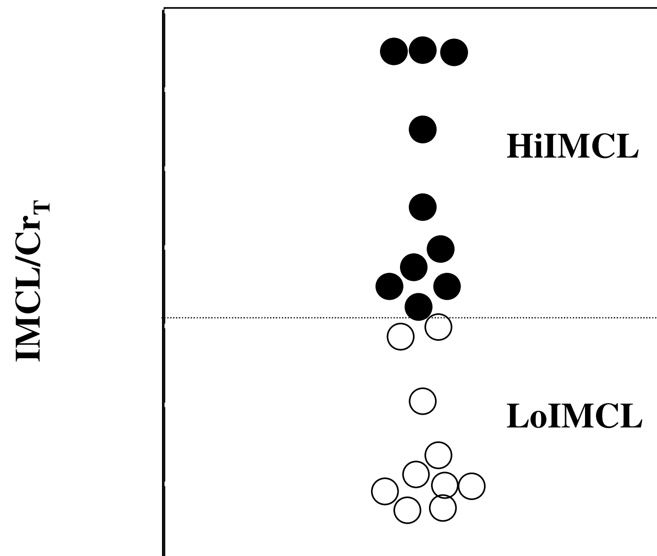

Fig. 10. Distribution of IMCL in human vastus lateralis muscle measured by proton nuclear magnetic resonance spectroscopy in 20 healthy men. The median value is shown by the horizontal line.

6.3. Insulin signalling pathways and IMCL (study II)

20 healthy subjects with no family history of type 2 diabetes were divided into groups with high IMCL (HiIMCL; 9.5 ± 0.9 IMCL/Cr_T, *n* = 10) and low IMCL (LoIMCL; 3.0 ± 0.5 IMCL/Cr_T, *n* = 10), the cut point being median IMCL (6.1 IMCL/Cr_T, **Fig. 10**). The LoIMCL and HiIMCL groups were comparable with respect to age (43 ± 3 vs 40 ± 3 years, ns, HiIMCL vs LoIMCL), BMI (26 ± 1 vs 26 ± 1 kg/m^2, ns, **Fig. 11**, **panel B**), and maximal oxygen consumption (33 ± 2 vs 36 ± 3 ml· kg^{-1}· min^{-1}, ns, **Fig. 11**, **panel D**) (Table I in original publication II and **Table 4**). The groups also had similar volumes of visceral (3.4 ± 0.5 vs 2.9 ± 0.3 l, LoIMCL vs HiIMCL, ns) and subcutaneous (2.9 ± 0.3 vs 2.5 ± 0.5 l, ns) fat. Plasma TG concentrations were slightly higher in the HiIMCL group than in the LoIMCL group, but other lipid concentrations were comparable between the groups.

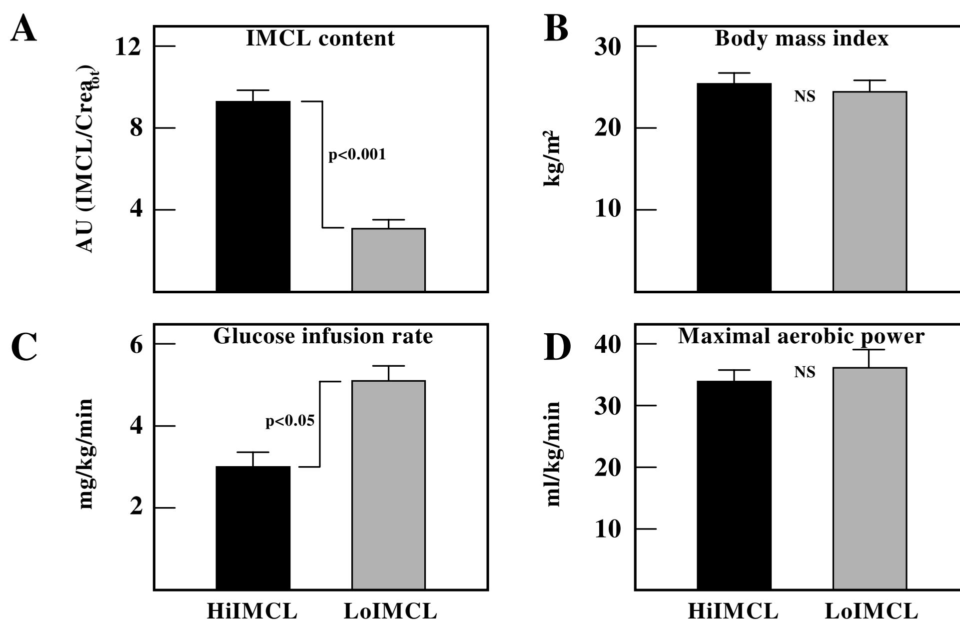

Fig. 11. *IMCL* ***(panel A)****, BMI* ***(panel B)****, insulin sensitivity* ***(panel C)****, and VO_2max* ***(panel D)*** *in subjects with low (LoIMCL) and high (HiIMCL) IMCL. NS, $p < 0.001$, and $p < 0.05$ denote differences between the groups.*

Insulin action on glucose and lipid metabolism

The fasting plasma glucose concentrations as well as the steady-state mean plasma glucose concentrations during the euglycaemic hyperinsulinaemic clamp study (5.2 ± 0.1 vs 5.2 ± 0.1 mmol/l, HiIMCL vs LoIMCL, ns) were comparable between the groups. The HiIMCL group had a 46 % higher fasting insulin concentration (Table 1 in original publication II and **Table 4**), and the insulin concentration remained slightly but significantly higher during the euglycaemic hyperinsulinaemic clamp in the HiIMCL group (450 ± 24 vs 382 ± 18 pmol/l, HiIMCL vs LoIMCL, $p < 0.05$), despite a similar insulin priming and infusion protocol. The serum insulin concentrations at the time of the muscle biopsy (30 min) were comparable between the groups (382 ± 26 vs 340 ± 20 pmol/l, HiIMCL vs LoIMCL, respectively, ns). Whole-body insulin-stimulated glucose uptake was lower in the HiIMCL group (3.0 ± 0.4 mg $\cdot$ kg$^{-1}\cdot$ min^{-1}) than the LoIMCL group (5.1 ± 0.5 mg$\cdot$ kg$^{-1}\cdot$ min^{-1}, $p < 0.05$, **Fig. 11**, **panel C**). There was significant inverse correlation between whole body glucose uptake and IMCL (**Fig. 12**).

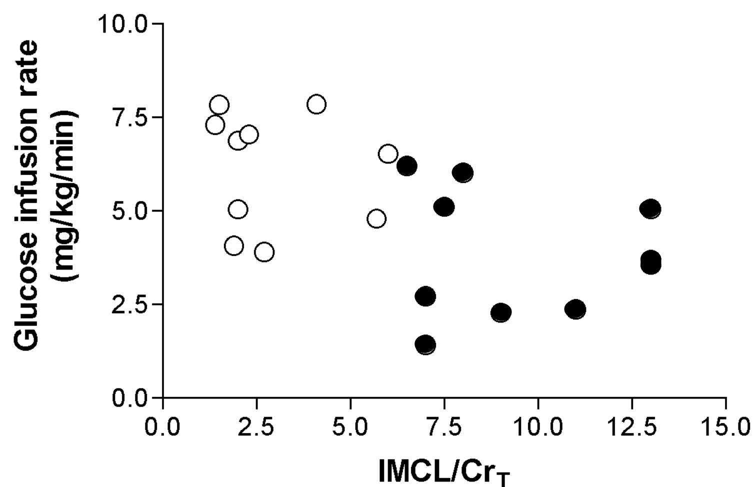

Fig. 12. *The relationship between IMCL and in vivo insulin sensitivity (Pearson's correlation coefficient).*

Basally, before the insulin infusion, serum FFA concentrations were comparable between the groups. During hyperinsulinaemia, the FFA concentrations decreased slower (0.47 ± 0.04 vs 0.31 ± 0.21 mmol/l at 20 min, HiIMCL vs LoIMCL, $p < 0.01$) and remained higher (0.23 ± 0.02 vs 0.17 ± 0.02 mmol/l at 120 min) in the HiIMCL compared with the LoIMCL group (**Fig. 13**). IMCL was significantly correlated with serum FFA at all time points during the insulin infusion and with the mean FFA concentration during hyperinsulinaemia ($r = 0.53$, $p < 0.02$) but not with the fasting FFA concentration ($r = 0.22$, ns).

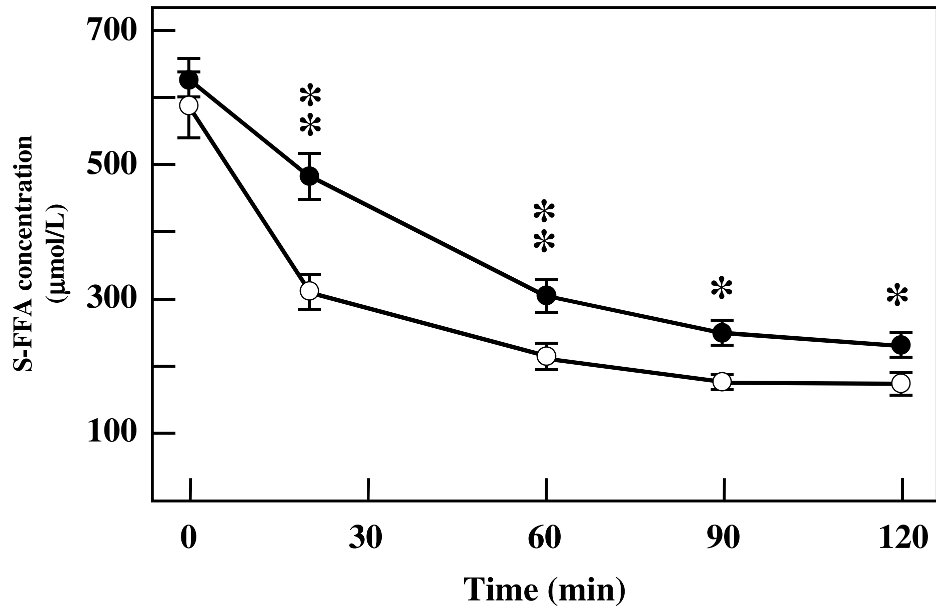

Fig. 13. *Serum FFA concentrations in LoIMCL (open circles) and HiIMCL (black circles) groups during the euglycaemic hyperinsulinaemic clamp study. *p < 0.05, **p < 0.01 for difference between the groups at a given time point.*

IR tyrosine phosphorylation and PI 3-kinase activity

Insulin-induced IR tyrosine phosphorylation was determined using immunoblotting from anti-IR immunoprecipitates after SDS-PAGE by determining the increase above basal phosphorylation in each subject. There were no detectable differences in the IR expression level by direct immunoblotting from the tissue homogenates. Normalized for respective protein expression level in each subject, the HiIMCL group had a blunted insulin–stimulated IR tyrosine phosphorylation (from 0.28 ± 0.07 to 0.44 ± 0.10, from basal to insulin-stimulated, respectively; ns) compared with the LoIMCL group (from 0.22 ± 0.08 to 0.55 ± 0.07, $p < 0.05$) or 57 ± 7 % increase in HiIMCL vs 142 ± 8 % in LoIMCL, $p < 0.001$ (**Fig. 14**, **panel A**). The insulin-stimulated increase in the IRS-1-associated PI 3-kinase activity above basal activity was markedly decreased in the HiIMCL group (42 ± 13 % above basal) compared with the LoIMCL group (88 ± 13 % above basal, $p < 0.05$, **Fig. 14**, **panel B**).

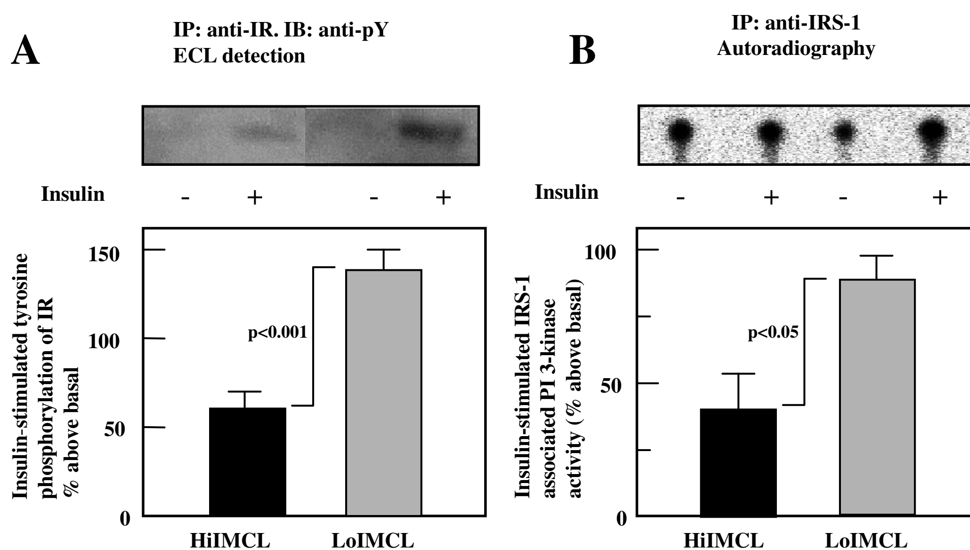

Fig. 14. *The stimulatory effect of insulin on IR tyrosine phosphorylation (**panel A**) normalized by protein expression and IRS-1-associated PI 3-kinase activity (**panel B**) in the HiIMCL and LoIMCL groups. Data are expressed as the percent increase from basal. P values refer to the statistical significance of the difference between the groups.*

6.4. Gene expression in adipose tissue in HAL (study III)

Characteristics of HIV subjects

Clinical and biochemical characteristics of the patients are given in Table 2 in original publication III and **Table 5**. BMI and the total amount of abdominal fat measured by MRI were similar between the groups. HIV lipodystrophic patients on HAART (HAART+LD+ group) had approximately 2-fold less subcutaneous and 2-fold more intra-abdominal fat than those without lipodystrophy (HAART+LD- group). The HAART+LD+ group also had features of insulin resistance. HIV- and HAART-related characteristics did not differ between the groups (**Table 5**).

Adipose tissue gene expression of PPARγ, SREBP-1c, PPARδ and PGC-1

The mRNA concentrations of PPARγ, SREBP-1c, PPARδ, and PGC-1 are shown in **Fig. 15**. Expression of all these genes was significantly lower in the HAART+LD+ than the HAART+LD- group. PPARγ and SREBP-1c expressions were significantly interrelated in the HAART+LD+ group ($r = 0.53$, $p < 0.01$).

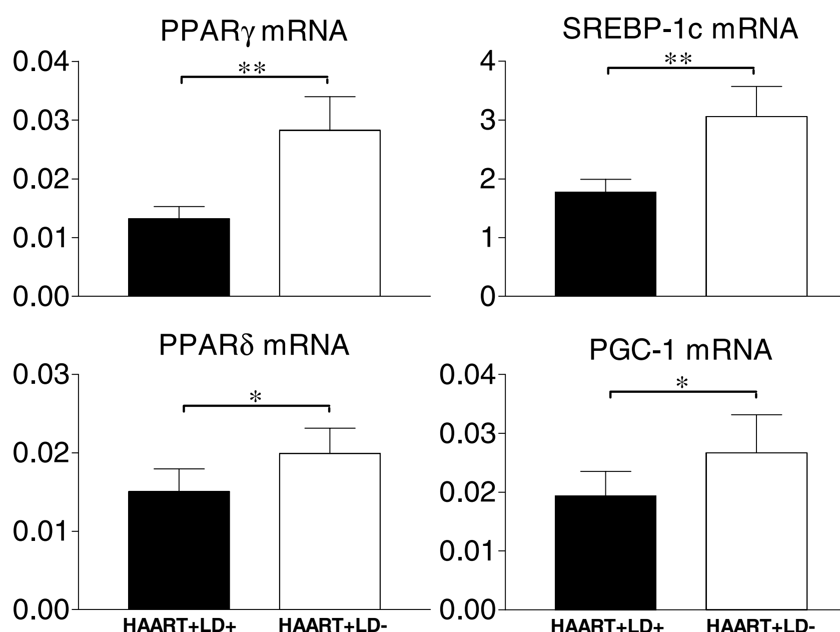

Fig. 15. *The mRNA concentrations of PPARγ, SREBP-1c, PPARδ and PGC-1 relative to β-actin and β2-microglobulin expression in subcutaneous adipose tissue in HAART+LD+ and HAART+LD- groups. *p < 0.05, **p < 0.01 for comparisons as indicated.*

Genes of lipogenesis and fatty acid metabolism: LPL, ACS, ALBP, FATP-1, FATP-4, and KLBP

Expression of LPL and ACS were significantly decreased in the HAART+LD+ group compared to the HAART+LD- group (**Fig. 16**). The mRNA concentrations of FATP-1 and ALBP did not differ between the groups (**Fig. 16**). The mRNA concentrations of FATP-4 (1.2 ± 0.2 vs 1.1 ± 0.1, HAART+LD+ vs HAART+LD-, ns) and KLBP (1.3 ± 0.1 vs 1.1 ± 0.2, respectively, ns) also did not differ between the groups.

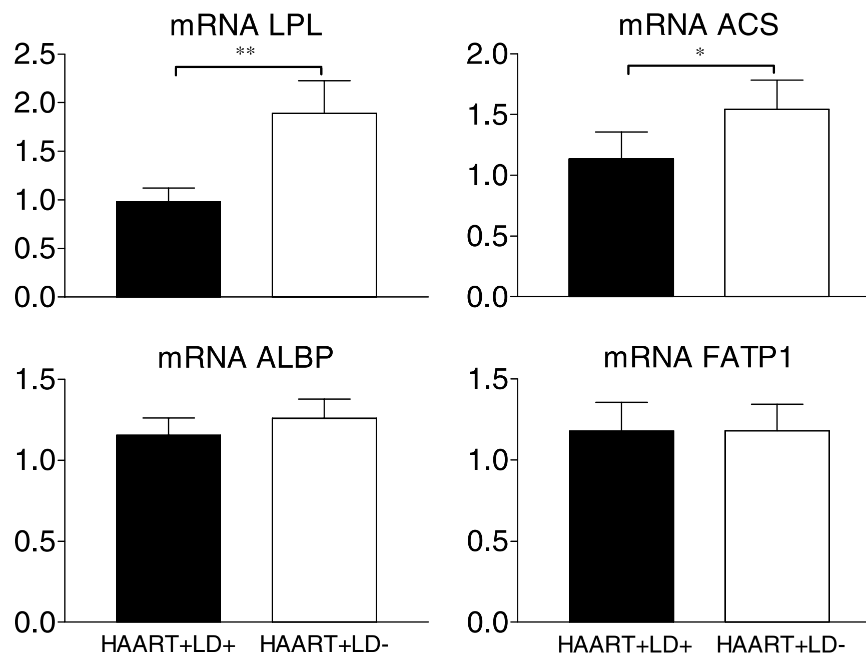

Fig. 16. *The mRNA concentration of LPL, ACS, ALBP and FATP-1 relative to β-actin and β2-microglobulin expression in subcutaneous adipose tissue in HAART+LD+ and HAART+LD- groups. *p < 0.05, ***p < 0.001 for comparisons as indicated.*

Glucose transport proteins: GLUT1 and GLUT2

The mRNA concentrations of GLUT1 were not different between the groups while GLUT4 mRNA concentrations were significantly decreased in patients with lipodystrophy (**Fig. 17**). Expression of PGC-1 correlated closely with that of GLUT4 within the HAART+LD+ group (**Fig. 17**).

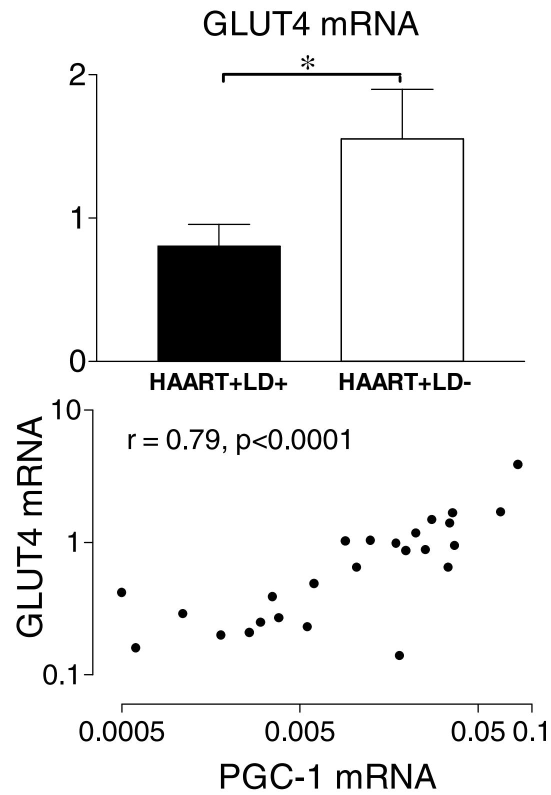

Fig. 17. *The mRNA concentration of GLUT4 relative to β2-microglobulin expression in subcutaneous adipose tissue in HAART+LD+ and HAART+LD- groups* ***(upper panel)*** *and the correlation between the mRNA concentrations of GLUT4 and PGC-1 in the HAART+LD+ group (****lower panel****). *p < 0.05 for HAART+LD+ vs. HAART+LD-.*

Markers of inflammation: IL-6 and CD45

The mRNA concentrations of CD45 and IL-6 were significantly higher in the HAART+LD+ than the HAART+LD- group (**Fig. 18**).

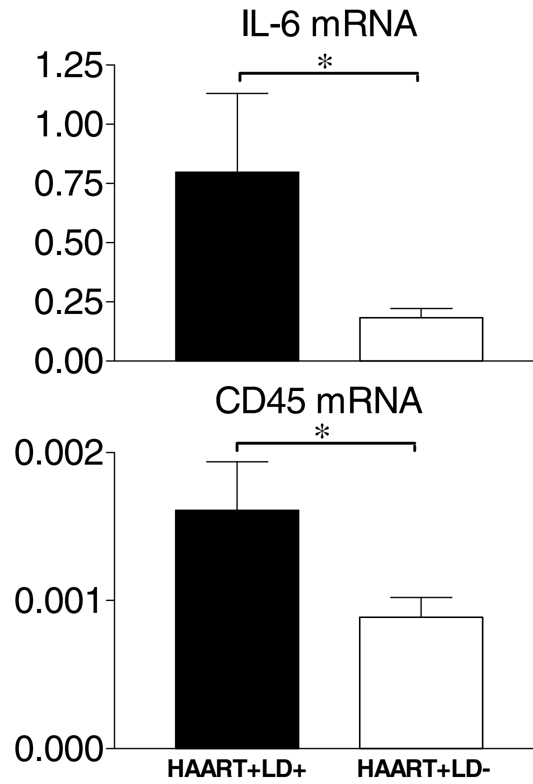

Fig. 18. *The mRNA concentrations of IL-6 and CD45 relative to β2-microglobulin expression in subcutaneous adipose tissue in HAART+LD+ and HAART+LD- groups.* $*p < 0.05$ *for comparisons as indicated.*

6.5. Adiponectin mRNA in adipose tissue and circulating adiponectin concentration in HAL (study IV)

The same patients participated in study IV as in study III. Liver fat content was significantly higher in HAART+LD+ than in HAART+LD- group (**Table 5**). Liver fat content correlated closely with serum fasting insulin concentration (r = 0.60, p < 0.001). The mRNA concentration of adiponectin in subcutaneous adipose tissue and adiponectin concentration in serum were significantly decreased in the HAART+LD+ group when compared with the HAART+LD- group and correlated inversely with liver fat content (**Fig. 19**).

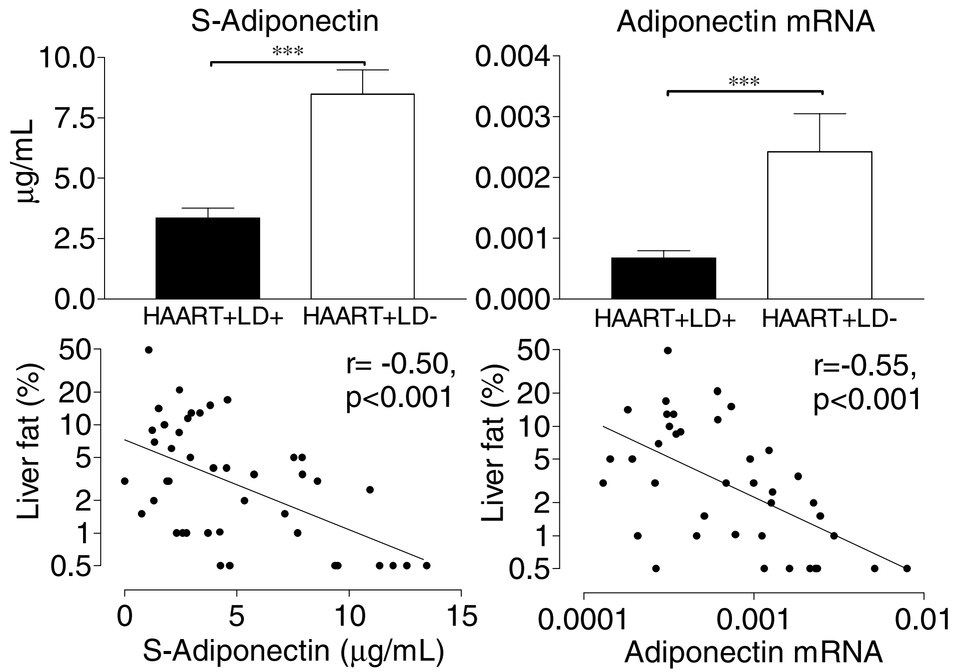

Fig. 19. *Serum adiponectin and adipose tissue adiponectin mRNA concentrations relative to β-actin expression in subcutaneous adipose tissue* ***(upper panels),*** *and liver fat content* ***(lower panels)****. Data are means ± SEM. ***p < 0.001 for HAARTLD+ vs. HAART+LD-.*

7. DISCUSSION

7.1. Methods

7.1.1. Insulin signalling assays

Currently, the *in vivo* effect of insulin on insulin signal transduction pathway in humans can only be studied in tissue biopsies. During intravenously maintained hyperinsulinaemia skeletal muscle accounts for 60-70 % whole-body glucose uptake (65), and is therefore an important target tissue for insulin action. In clinical studies, where the Bergström needle is used to obtain the muscle biopsy like in studies I and II, the sample contains non-muscle components, such as blood, fat, connective tissue and blood vessels. Based on earlier experience on metabolite assays, non-muscle contaminants decrease analytical precision by several mechanisms. First, the protein concentration used generally for normalization of the results may be over- or underestimated, depending on the nature of the contaminant. Second, non-muscle components may express the same intracellular proteins than muscle, but may be differentially regulated. This is especially important in signal transduction assays, where stimulation by insulin is known to trigger a rapid phosphorylation cascade of specific signalling proteins like IRS-1 in myocytes, but not in contaminating blood cells. In studies involving invasive procedures, like muscle biopsies, the ultimate reason to aim for better precision is to reduce the number of subjects needed to study. As stated above, conventionally the muscle biopsy is taken, snap-frozen in liquid nitrogen and then homogenized as such ('wet' sample). Study I describes an alternative and improved way to handle the muscle biopsy – freeze-drying and purification of the muscle biopsy specimen ('dry' sample). The purification after freeze-drying was done at room temperature and for 10 to 15 minutes per sample. The absolute signal intensities in all measured parameters, including IRS-1-associated PI 3-kinase activity and stimulation, were comparable between the 'wet' and 'dry' samples, which shows that the freeze-drying process does not significantly degrade signalling molecules, or influence the enzymatic activity of PI 3-kinase. To assess the coefficient of variation of measurement of a specific protein, we used immunoblotting with anti-p85 antibodies to determine expression of the regulatory subunit of PI 3-kinase in rat gastrocnemius muscle. The reason for choosing this particular protein to determine the effect of contamination was due to the superb band integrity, quantifiability, and minimal inter-assay variation in immunoblotting (data not shown) compared to other protein blots. The variation was found to be significantly higher in non-purified 'wet' samples. Regarding other insulin signalling molecules, we found protein expression of IR and IRS-1 to be slightly

lower, p85 similar, tyrosine phosphorylation of the IR significantly higher and PI 3-kinase activity similar in freeze-dried as compared to wet samples. We calculated by using the p85 signal variation data, how many subjects would be needed to detect a 30 % difference in expression between the groups with 95 % confidence. The 'dry' technique (n = 10) would reach this theoretical goal by approximately 50 % fewer samples than the 'wet' technique (n = 21). For this reason, muscle samples were freeze-dried and purified prior to insulin signalling assays done in study II.

7.1.2. Insulin signalling during *in vivo* insulin stimulation

Study I is one of the first to study effects of *in vivo* hyperinsulinaemia on insulin signalling in human skeletal muscle. *In vitro* studies used 150-250-fold higher insulin concentrations for stimulation insulin signalling molecules (95,151,260). As study I was begun in 1998, there were not enough data regarding the ideal time point of taking the biopsy for insulin signalling measurements. The 30-min time-point was chosen based on studies performed *in vitro* with surgically obtained human muscle strips, where 2-fold activation of PI 3-kinase and IR tyrosine phosphorylation has been observed between 20 and 60 min (260) and after 30 min (95), respectively. The designs of *in vitro* and *in vivo* studies included stimulation of different muscle groups by insulin, which may further complicate interpretation of data. Even though human muscle is a tissue, that is more mixed regarding muscle fibers, than animal tissue, some muscles have a predominant amount of either of the fiber types, e.g. soleus muscle contains more type I than type II fibers. In more recent studies examining the time-course for insulin signalling by performing a muscle biopsy, 6 times during a 120-min insulin infusion (315), a 2-fold increase in IRS-1-associated PI 3-kinase was observed at 30 min and a 5-fold increase at 120 min. Another study found 1.3-fold increase in PI 3-kinase activity after 40 min of hyperinsulinaemia (122), 1.8-fold after 120 min (142), and 2.0-fold increase - after 60 min (27). These data are consistent with our finding of a 1.7-fold increase in IRS-1-associated PI 3-kinase activity in 30 min in normal subjects. The fold stimulation of IR tyrosine phosphorylation in response to physiological insulin infusion in study I was 1.4-fold, while Cusi et al. found 1.5-fold (61), and Koval et al. 1.7-fold stimulation (149).

7.1.3. ^{1}H-MRS spectroscopy for IMCL

In study II intramyocellular lipid was measured by a novel non-invasive method - ^{1}H-MRS. Lipid quantitated intracellularly by electron microscopic morphometry and ^{1}H-MRS has been shown to be closely correlated in tibialis muscle (123). Tibialis anterior muscle fibers run parallel, while in vastus lateralis muscle, which was used in the present study, muscle fibers are more randomly orientated (123). This implies that many fibers are located at an angle to each other. Whenever fiber orientation is 45 to 90 degrees relative to the magnet axis, some fibers cross over the "magic angle", and the bulk magnetic susceptibility-shift between EMCL and IMCL is lost, with the EMCL signal being detected at the IMCL frequency. In our study, the volume of interest, the voxel was placed inside the lateral part of the vastus lateralis muscle to optimise parallel fiber orientation. The position of the voxel was chosen on the T1-weighted magnetic resonance images so that it did not contain any visible fat or fasciae that would contain adipocytes.

7.1.4. Total RNA isolation from adipose tissue and real-time PCR

Adipocytes contain a relatively low amount of total RNA. In studies III and IV we measured the mRNA concentration of several genes that might be important for development of insulin resistance in HAL. This was particularly challenging given the paucity of subcutaneous fat in lipodystrophic patients. We isolated 3.1 ± 0.3 µg of RNA from 99 ± 2 mg, or 3.3 ± 0.4 µg RNA per 100 mg tissue (n = 81). A reverse-transcriptase reaction was done as the SYBER Green assay for the real-time PCR LightCycler can be only done using cDNA. Other methods for measuring mRNA concentrations include competitive PCR, the ribonuclease protection assay, Northern blotting, and *in situ* hybridization, but they are all much less sensitive than real-time PCR, when one can see the amplification process in real time and define the specificity of the product by analyzing the melting curve. Like with other methods, one has to run a gel to verify the integrity of the product. In the LightCycler this analysis is performed automatically at the end of each run. Regarding sample volume, the method is very economical. For cDNA synthesis we used 0.1 µg of RNA (of a total of 3 µg), and then used only 0.1-1 ng of cDNA for each sample in the LightCycler. Since air has virtually no mass, the amplification process is significantly faster than cycling with the conventional thermal block. To measure the relative expression of the gene of interest, we first made a standard curve of the gene of interest and then included one standard point from this curve in each run. Expression of each gene of interest was calculated relative to a house-keeping gene.

7.2. Association of *in vivo* insulin resistance with impaired insulin signalling (study I)

Increased fasting insulin concentration is the most physiological surrogate marker of insulin resistance. Therefore, we investigated a cohort of healthy men matched for age, body weight and physical fitness, and divided them by their median fasting insulin into two groups with low or high fasting insulin concentrations, respectively. The majority of previous studies have been performed *in vitr*o with surgically obtained human muscle strips and pharmacological insulin concentrations (95,151,260). Thus, study I is among the first studies showing that physiological insulin concentrations stimulate key insulin signalling molecules *in vivo* in needle biopsies. After 30 minutes of physiological hyperinsulinaemia subjects with lower fasting insulin had 118 % higher insulin stimulated IR tyrosine phosphorylation and 50 % higher IRS-1-associated PI 3-kinase activity than those subjects with higher fasting insulin. These signalling defects were part of a cluster of abnormalities in insulin action, which included not only glucose but also lipid metabolism (**Table 3**). Moreover, there was a significant negative correlation between IRS-1-associated PI 3-kinase activity and fasting insulin concentrations. Thus, these data are among the first to document defects in insulin signalling in human skeletal muscle independent of body weight and physical fitness. It is possible that subjects with reduced insulin action had more intramyocellular fat than more insulin sensitive subjects. This possibility was investigated in study II.

7.3. Insulin signalling and IMCL (study II)

In the present study, both IR tyrosine phosphorylation and IRS-1-associated PI 3-kinase activation by insulin were significantly lower in the HiIMCL group than in the LoIMCL group. The defect in IRS-1-associated PI 3-kinase activity is similar to that recently found in fatless mice with a twofold increase in muscle TGs (141). In humans, an elevation of circulating FFA acutely, about 4-fold above basal, decreases whole-body glucose uptake by 50 % and abolishes insulin stimulation of IRS-1-associated PI 3-kinase activity (72). IR tyrosine phosphorylation was not measured in these two studies. The present data imply that regardless of whether FFAs originate from intramyocellular TGs or from the circulation, they may similarly influence early insulin signalling events. In our study, the concentration of serum FFAs during hyperinsulinaemia, but not under fasting conditions, significantly correlated with IMCL. This finding is consistent with acute FFA infusion studies in which IMCL increased during hyperinsulinaemia but not under basal conditions, perhaps because the combined effects of increased FFA availability and increased inhibition of intramyocellular lipolysis by insulin favour IMCL accumulation (36). It is thus possible that

insulin resistance of lipolysis in adipose tissue or other sites, which contribute to circulating FFA, may be responsible for IMCL accumulation. On the other hand, because FFA fluxes were not quantitated in the present study, it is also possible that impaired insulin stimulation of FFA uptake in skeletal muscle or adipose tissue (87) was responsible for the higher FFA levels during hyperinsulinaemia in the HiIMCL group compared with the LoIMCL group. Whether it is IMCL or circulating FFA that causes the signalling defects, or whether the signalling defects precede lipid accumulation, cannot be determined in a cross-sectional study.

Several possible mechanisms could link IMCL or circulating FFA to impaired insulin signalling. These include activation of signalling cascades known to downregulate either IR tyrosine phosphorylation or IRS-1 association with PI 3-kinase, such as activation of the hexosamine pathway (215), various PTPs (92), or PKC (136). The hexosamine pathway can be activated by infusion of either lipid or glucosamine (113). Both agents also decrease IRS-1-associated PI 3-kinase activity (72,215). Activation of the hexosamine pathway also increases *O*-glycosylation of IRS-1 (215), which may reduce IRS-1 tyrosine phosphorylation and association with PI 3-kinase, but whether the effect of FFAs on the hexosamine pathway is important in mediating FFA-induced alterations in early insulin signalling and glucose uptake is not known and requires more definite experiments. Finally, it is also possible that increases in metabolites, such as malonyl-CoA (241) and LCACoA may mediate effects of increased lipid availability on glucose metabolism and early insulin signalling. Infusion of FFA in rats increases muscle LCACoA(240). This increase has been suggested to increase PKC, a serine/threonine kinase, which is associated with decreased tyrosine and increased serine phosphorylation of IRS-1 (99). However, in humans, PKC is lower in obese subjects than in lean subjects (127). LCACoA may also interact directly with glucose metabolism through the inhibition of enzymes such as GS (314) and hexokinase (281).

7.4. Adipose tissue in HAL

7.4.1. Adipogenic transcription factors and PGC-1

Expression of all transcription factors (PPARγ, SREBP-1c and PPARδ) and the transcription factor co-activator PGC-1 were decreased in lipoatrophic adipose tissue as were the lipogenic enzymes ACS and LPL, and GLUT4. In contrast, IL-6 expression and CD45 (a common leukocyte surface marker) expression were significantly higher in the lipodystrophic than the non-lipodystrophic patients.

The HAART+LD+ group had almost 2-fold less subcutaneous and 2-fold more intra-abdominal fat than the HAART+LD- group. Features of insulin resistance were found only in the HAART+LD+ group. In the face of comparable body mass indexes, these significant differences in fat distribution confirmed the self-reported and investigator-confirmed diagnosis of LD in the HAART+LD+ group.

PPARγ is a transcription factor that is necessary for the development of mature adipocytes both *in vitro* and *in vivo* (236). PPARγ expression can be induced by C/EBPβ and δ (173). PPARγ and C/EBPα have positive cross-regulation, i.e. C/EBPα induces PPARγ expression and vice versa. PPARγ expression can also be induced by SREBP-1 (77). Expression of both PPARγ and SREBP-1c were decreased in subcutaneous adipose tissue in patients with HAL compared with non-lipodystrophic patients. Low PPARγ mRNA expression may be partly due to low SREBP-1c expression, as was suggested by the positive correlation between PPARγ and SREBP-1c mRNAs, but possibly also due to decreases in C/EBPβ and C/EBPα expression as was found in the study comparing lipodystrophic patients with normal subjects (20). In the present study, PPARδ mRNA concentrations were decreased in HAART+LD+ patients. A role of PPARδ in adipose tissue development is supported by diminished adipose tissue volume in PPARδ-null mice compared with wild-type mice (220). Since PPARδ activation promotes mitotic clonal expansion of 3T3-L1 preadipocytes, it has been suggested that PPARδ may affect the proliferation of adipocyte precursor cells and have less impact on terminal adipocyte differentiation (107). These data would suggest that low PPARδ expression may play a role in the loss of subcutaneous fat in HAART+LD+ patients.

PGC-1 expression has not been previously studied in patients with HAL. In mice, PGC-1 is exclusively expressed in brown adipose tissue (237), whereas PGC-1 expression has been found in white adipose tissue in humans (161). In the present study, PGC-1 expression in subcutaneous white adipose tissue was decreased in the HAART+LD+ group compared with the HAART+LD- group. PGC-1 was first identified as a co-activator of the nuclear receptor PPARγ (222). The decrease in PGC-1 expression may therefore have contributed to low transcriptional activity of PPARγ in these patients. In mice, ectopic expression of PGC-1 in white adipose cells increases expression of uncoupling protein-1, key enzymes of the respiratory chain, and the cellular content of mtDNA (222). The mtDNA content is decreased in subcutaneous adipose tissue of patients with HAL (299). Nucleoside analogue reverse transcriptase inhibitors appear to inhibit human mtDNA polymerase γ which could decrease mtDNA content (10,37). The present data suggest that low PGC-1 expression may contribute to decreased mitochondrial biogenesis in these patients. Since mitochondrial dysfunction may lead to apoptosis (98), mitochondrial damage may be responsible for the loss of subcutaneous adipose tissue in these patients (68,299). PGC-1 also induces GLUT4 expression in cultured muscle cells (188), and animals overexpressing PGC-1 in muscle are characterized by conversion of type II to type I muscle fibers, which are known to be insulin sensitive and express high amounts of GLUT4 (168). Our finding of a close correlation between adipose tissue PGC-1 and GLUT4 expression ($r = 0.79$, $p < 0.0001$) is in keeping with such data. Decreased expression of PGC-1 could thus also contribute to insulin resistance in these patients.

7.4.2. Genes of lipogenesis and fatty acid metabolism

The mRNA concentrations of proteins associated with fatty acid transport (FATP-1, FATP-4) and fatty acid intracellular binding (ALBP, KLBP) were similar in HAART+LD+ and HAART+LD- groups. Uptake of circulating FFAs into cells is likely to occur both by passive diffusion and by active transport (1). Three groups of membrane proteins have been implicated in the transport process: FATP, plasma membrane FA-binding protein (FABP) and FAT, also known as CD36 (1). FATP and FAT expressions are induced by PPARγ in mice (200). Despite lower expression of PPARγ in the HAART+LD+ than the HAART+LD- group, expression of FATP-1, FATP-4, ALBP or KLBP were unchanged. Whether this is because regulation of these genes differs in mice as compared to man is unclear. Both ALBP and KLBP are expressed in human macrophages (179). Thus it is possible that the similar

expression of FABPs in adipose tissue biopsies in the two patient groups could reflect a decrease in expression in adipocytes and an increase in macrophages in the HAL subjects. A recent mouse model, in which macrophages were deficient in aP2 (mouse analogue of human ALBP), revealed that the absence of aP2 was associated with decreased expression of proinflammatory cytokines (TNFα, IL-1β, IL-6) (179). Consequently, the unchanged expression of ALBP in the HAART+LD+ patients may have contributed to the increased expression of IL-6. An alternative explanation for the unchanged pattern of fatty acid binding protein expression in the HAART+LD+ and HAART+LD- groups is that a normally inactive pathway was activated in adipocytes undergoing apoptosis, which resulted in upregulation of genes, the expression of which is aimed at maintaining fatty acids in adipose tissue.

Expression of ACS and LPL genes was reduced in the HAART+LD+ group compared with HAART+LD- group. ACS catalyzes conversion of long-chain fatty acids to their acyl-CoA esters that can then be used either for synthesis of cellular lipids such as TGs, or for β-oxidation (248). Formation of acyl-CoA esters also prevents efflux of FFAs (248). PPARγ increases ACS expression both in preadipocytes *in vitro* and in adipose tissue in the rat *in vivo* (182). Proinflammatory cytokines (TNFα, IL-1) decrease the expression of ACS in the liver and adipose tissue in hamsters (186). The low ACS expression observed in patients with HAL may be a consequence of a low expression of PPARγ and an increased expression of TNFα as described in a previous study (20). Regulation of LPL expression resembles that of ACS in adipose tissue. Both SREBP-1 and PPARγ induce LPL expression *in vitro* and in rat adipose tissue (140,252), whereas proinflammatory cytokines (TNFα, IL-6) decrease the expression of LPL (85). Thus, both the decreases in PPARγ and SREBP-1 expression and the increase in IL-6 expression could have contributed to the low LPL expression.

7.4.3. IL-6 and CD 45 expression

Expression of IL-6 in subcutaneous adipose tissue was increased in the HAART+LD+ group. IL-6 is a multifunctional cytokine produced by many different cell types including immune cells, adipocytes, fibroblasts, stromal-vascular cells, endothelial cells, myocytes, and a variety of endocrine cells (85). As much as a third of the total circulating concentration of IL-6 has been estimated to originate from adipose tissue (85). However, of the IL-6 secreted from adipose tissue, only 10 % is secreted by adipocytes (85). The HAART+LD+ group had a higher number of white blood cells capable of IL-6 production in their adipose tissue as

judged from the significantly higher concentration of the mRNA encoding for CD45. CD45 is a tyrosine phosphatase, which is expressed exclusively in the white blood cell lineage (7). However, CD45 is expressed on these cells regardless of whether they are activated or resting. The increase in CD45 expression therefore may or may not reflect an increase in inflammation in lipodystrophic adipose tissue. The data do raise the possibility that the increased mRNA concentration of TNFα found in adipose tissue biopsies of these patients by Bastard et al may have originated from macrophages in addition to adipocytes (20). TNFα has pronounced catabolic effects in adipose tissue, including inhibition of C/EBPα, PPARγ, and LPL expression and induction of apoptosis (85). Interestingly, high levels of TNFα mRNA in adipose tissue and a high circulating concentration of TNFα were described in an adiponectin knockout mouse model (176). Adiponectin is a protein secreted by adipocytes, the plasma concentration of which is reduced in insulin resistant conditions and in type 2 diabetes (176), and according to very recent data also in non-HIV and HIV-LDs (2,203). It is therefore also possible that the increased TNFα expression in the study of Bastard et al. was due to low adiponectin in HAL (20). Since TNFα is known to induce expression of IL-6 (290), it is also possible that the low levels of adiponectin may contribute to increased expression of IL-6.

Study III does not clarify the chain of events leading to the described alterations in adipose tissue gene expression. We confirm and extend the findings by Bastard et al., who compared adipose tissue gene expression between patients with HAL and HIV-negative controls (20). In the current comparison of two HIV-positive, HAART-treated groups with and without LD, it is possible that gene expression was affected by the HIV-infection or HAART in both groups since an HIV-negative group or an HIV-positive, but treatment-naive group was not studied. However, since the groups did not differ with respect to HIV-related characteristics, the observed differences most likely were attributable to LD. This implies that individual susceptibility may contribute to the development of HAL. Consistent with this, a functionally active polymorphism in the promoter region of the TNFα gene and a single nucleotide polymorphism in SREBP-1c gene have been shown to be associated with development of LD and hyperlipidaemia in these patients (178,193).

In vitro, both protease and nucleoside reverse transcriptase inhibitors inhibit adipocyte differentiation (41,71,166,234,303,334). The suggested mechanisms include both SREBP-1/PPARγ dependent (41,71) and independent mechanisms (303,334). In addition, protease inhibitors have been shown to cause adipocyte death (71). Since antiretroviral combinations in clinical practice always include several agents, these *in vitro* data evaluating the effects of a single drug may not be applicable to *in vivo* situation. This is exemplified by a study which showed that the effects on adipocytes, produced by combinations of nucleoside reverse transcriptase and protease inhibitors are different from those elicited by each drug separately (234).

7.4.4. Adiponectin expression and circulating concentration

Adiponectin expression in adipose tissue and its circulating concentrations are significantly decreased in HIV-positive, HAART-treated patients with LD when compared with HAART-treated patients without LD. Both serum and mRNA concentrations of adiponectin correlated closely with features of insulin resistance including liver fat content, suggesting that adiponectin deficiency may contribute to hepatic insulin resistance in these patients.

Our findings of low serum concentration of adiponectin in HAL confirm those of Mynarcik et al. (203). Although we did not measure adiponectin protein concentrations in subcutaneous fat, the present study extends the data of Mynarcik et al. by demonstrating low expression of adiponectin in subcutaneous fat.

Regarding the possible causes of adiponectin deficiency in the HAART+LD+ group, both groups were HIV-positive, had similar viral loads, CD4 cell counts and used comparable HAART regimens. The decrease in adiponectin therefore was a consequence of LD rather than the HIV-infection or antiretroviral therapy *per se*. The decrease in serum adiponectin in the HAART+LD+ group was not due to overall obesity, as both groups had similar absolute and relative weights. Healthy lean subjects with low subcutaneous fat volume have higher serum adiponectin concentrations than obese subjects (22). Therefore, the observed low serum adiponectin concentration in the HAART+LD+ group is more likely to reflect dysfunction of the remaining subcutaneous adipose tissue rather than reduced subcutaneous fat volume.

Current data are limited regarding expression of adiponectin in subcutaneous vs. intra-

abdominal fat. Adiponectin mRNA and protein levels were reduced in omental fat compared with those of subcutaneous fat in the study performed by Fisher et al. (81). In another study, human omental adipocytes secreted more adiponectin than subcutaneous adipocytes after insulin or rosiglitazone stimulation (201). Visceral fat has also been hypothesized to release an as yet unidentified factor that destabilizes adiponectin mRNA (105). In the present study, the significantly decreased serum adiponectin concentration together with the significantly increased intra-abdominal fat mass in the HAART+LD+ group compared to the HAART+LD- group and the strong inverse correlation between serum adiponectin concentration and intra-abdominal fat mass imply an inhibitory rather than a stimulatory effect of intra-abdominal fat on adiponectin production.

We found a close correlation between serum adiponectin and liver fat content, and liver fat content correlated closely with fasting serum insulin concentrations. In mice, infusion of adiponectin enhances insulin sensitivity, β-oxidation in muscle, and decreases liver and muscle fat content (321). In another mouse model, adiponectin infusion has been shown to inhibit the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose production (57). In isolated primary rat hepatocytes, physiological doses of adiponectin have been shown to enhance insulin-induced suppression of glucose production (22). These data would support the idea that low adiponectin contributed to hepatic insulin resistance and fasting hyperinsulinaemia in the HAL patients.

Adiponectin is a structural homologue of the TNFα family of trimeric cytokines and has anti-inflammatory properties, which antagonize those of TNFα (288). TNFα decreases expression of adiponectin in 3T3-adipocytes (79). In an adiponectin knockout mouse model, lack of adiponectin was associated with high levels of TNFα mRNA in adipose tissue and high plasma TNFα concentrations (176). TNFα expression is increased in subcutaneous adipose tissue in patients with HAL (20). Increased TNFα could therefore contribute to low adiponectin expression in HAL, or vice versa.

7.5. Concluding remarks

Insulin resistance is the main feature of type 2 diabetes mellitus. Skeletal muscle is the primary site of glucose disposal accounting for up to 70-80 % postprandial glucose disposal during hyperinsulinaemia (65). Consequently, skeletal muscle is a relevant target tissue to study the mechanisms of insulin resistance. In studies I and II we analyzed insulin signalling in healthy men without family history of type 2 diabetes, matched for body weight and physical fitness. In study I, we found a relationship between fasting plasma insulin concentrations and defects in early insulin signalling events. Thus, defects in proximal insulin signalling events are coupled to impaired insulin action in apparently healthy men. In study II, early insulin signalling events were impaired in healthy men with high intramyocellular lipid content, which was independent of body weight, aerobic capacity or genetic background. Thus, these data provide evidence that environmental factors, such as fat accumulation in the muscle, contribute to the development of insulin resistance.

During recent years, it has become evident that not only obesity but also the opposite state – lipodystrophy - is associated with insulin resistance. Currently, the most common form of lipodystrophy is the one associated to highly-active antiretroviral therapy (HAART) for HIV infection. HAART therapy has dramatically improved the prognosis of HIV infection in industrial countries during the last decade. However, HAART-associated insulin resistance and potentially increased risk for cardiovascular mortality have become of great clinical concern. In the current cross-sectional study (studies III and IV), expression of adipogenic transcription factors, co-activator of PPARγ and adiponectin in subcutaneous adipose tissue was decreased in HAART-treated patients with lipodystrophy (HAL) compared to those without lipodystrophy. Thus, these data suggest that observed alterations in gene expression may contribute to lipodystrophy in these subjects. In study IV, adiponectin expression was inversely correlated with liver fat content. Liver fat content was correlated with fasting serum insulin concentrations. Thus, these associations suggest that decreased adiponectin concentration may contribute to the development of insulin resistance in HAL patients via increased fat content in the liver.

8. SUMMARY AND CONCLUSIONS

The results of studies I-IV can be summarized as follows:

I. In rat skeletal muscle, phosphorylation, expression and enzymatic activity of insulin signalling molecules were similar or higher in freeze-dried purified muscle compared to conventionally proceded biopsies. In human skeletal muscle, elevated fasting insulin concentrations associate with impaired insulin signalling independent of body weight. Thus, defects in early insulin signalling contribute to insulin resistance in healthy men.

II. Healthy subjects with increased IMCL are less sensitive to insulin *in vivo* than those with IMCL below median, and have impaired insulin signalling independent of body weight and physical fitness. Thus, enhanced accumulation of intramyocellular lipid may contribute to the development of insulin resistance via impaired insulin signalling.

III. Expression of adipogenic transcription factors, GLUT4, LPL, ACS and co-activator of PPARγ are decreased in HAART-patients with lipodystrophy compared to those without lipodystrophy while expression of IL-6 is increased. These multiple alterations in gene expression in subcutaneous adipose tissue of HAART-lipodystrophic patients may contribute to development of lipodystrophy and insulin resistance in these patients.

IV. The concentration of adiponectin in serum and its expression in adipose tissue are lower in subcutaneous adipose tissue of HAART-lipodystrophic subjects than in HIV patients without lipodystrophy. Both serum adiponectin and adipose tissue adiponectin mRNA concentrations correlated with features of insulin resistance, including liver fat content. Adiponectin deficiency may therefore contribute to hepatic insulin resistance.

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Elena V Korsheninnikova

10. REFERENCE LIST

1. Abumrad, N.C., and A. Ibrahimi. 1999. Membrane proteins implicated in long-chain fatty acid uptake by mammalian cells: CD36, FATP and FABPm. *Biochim Biophys Acta* 1441:4-13.

2. Addy, C.L., A. Gavrila, S. Tsiodras, K. Brodovicz, A.W. Karchmer, and C.S. Mantzoros. 2003. Hypoadiponectinemia is associated with insulin resistance, hypertriglyceridemia, and fat redistribution in human immunodeficiency virus-infected patients treated with highly active antiretroviral therapy. *J Clin Endocrinol Metab* 88:627-636.

3. Ahima, R.S. and J.S. Flier. 2000. Leptin. *Annu Rev Physiol* 62:413-437.

4. Ahmad, F., J.L. Azevedo, R. Cortright, G.L. Dohm, and B.J. Goldstein. 1997. Alterations in skeletal muscle protein-tyrosine phosphatase activity and expression in insulin-resistant human obesity and diabetes. *J Clin Invest* 100:449-458.

5. Ahmad, F. and B.J. Goldstein. 1995. Purification, identification and subcellular distribution of three predominant protein-tyrosine phosphatase enzymes in skeletal muscle tissue. *Biochim Biophys Acta* 1248:57-69.

6. Alessi, D.R., M. Deak, A. Casamayor, F.B. Caudwell, N. Morrice, D.G. Norman, P. Gaffney, C.B. Reese, C.N. MacDougall, D. Harbison, A. Ashworth, and M. Bownes. 1997. 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. *Curr Biol* 7:776-789.

7. Alexander, D.R. 2000. The CD45 tyrosine phosphatase: a positive and negative regulator of immune cell function. *Semin Immunol* 12:349-359.

8. Almind, K., S.K. Frederiksen, M.G. Ahlgren, S. Urhammer, T. Hansen, J.O. Clausen, and O. Pedersen. 1998. Common amino acid substitutions in insulin receptor substrate-4 are not associated with Type II diabetes mellitus or insulin resistance. *Diabetologia* 41:969-974.

9. Amatruda, J.M., J.N. Livingston, and D.H. Lockwood. 1985. Cellular mechanisms in selected states of insulin resistance: human obesity, glucocorticoid excess, and chronic renal failure. *Diabetes Metab Rev* 1:293-317.

10. Anderson, K.S. 2002. Perspectives on the molecular mechanism of inhibition and toxicity of nucleoside analogs that target HIV-1 reverse transcriptase. *Biochim Biophys Acta* 1587:296-299.

11. Anderwald, C., E. Bernroider, M. Krssak, H. Stingl, A. Brehm, M.G. Bischof, P. Nowotny, M. Roden, and W. Waldhausl. 2002. Effects of insulin treatment in type 2 diabetic patients on intracellular lipid content in liver and skeletal muscle. *Diabetes* 51:3025-3032.

12. Araki, E., M.A. Lipes, M.E. Patti, J.C. Bruning, B. Haag, R.S. Johnson, and C.R. Kahn. 1994. Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature* 372:186-190.

13. Arita, Y., S. Kihara, N. Ouchi, M. Takahashi, K. Maeda, J. Miyagawa, K. Hotta, I. Shimomura, T. Nakamura, K. Miyaoka, H. Kuriyama, M. Nishida, S. Yamashita, K. Okubo, K. Matsubara, M. Muraguchi, Y. Ohmoto, T. Funahashi, and Y. Matsuzawa. 1999. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 257:79-83.

14. Arner, P., T. Pollare, H. Lithell, and J.N. Livingston. 1987. Defective insulin receptor tyrosine kinase in human skeletal muscle in obesity and type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 30:437-440.

15. Aronson, D., M.D. Boppart, S.D. Dufresne, R.A. Fielding, and L.J. Goodyear. 1998. Exercise stimulates c-Jun NH2 kinase activity and c-Jun transcriptional activity in human skeletal muscle. *Biochem Biophys Res Commun* 251:106-110.

16. Aronson, D., S.D. Dufresne, and L.J. Goodyear. 1997. Contractile activity stimulates the c-Jun NH2-terminal kinase pathway in rat skeletal muscle. *J Biol Chem* 272:25636-25640.

17. Aronson, D., M.A. Violan, S.D. Dufresne, D. Zangen, R.A. Fielding, and L.J. Goodyear. 1997. Exercise stimulates the mitogen-activated protein kinase pathway in human skeletal muscle. *J Clin Invest* 99:1251-1257.

18. Authier, F., R.A. Rachubinski, B.I. Posner, and J.J. Bergeron. 1994. Endosomal proteolysis of insulin by an acidic thiolmetalloprotease unrelated to insulin degrading enzyme. *J Biol Chem* 269:3010-3016.

19. Barroso, I., M. Gurnell, V.E. Crowley, M. Agostini, J.W. Schwabe, M.A. Soos, G.L. Maslen, T.D. Williams, H. Lewis, A.J. Schafer, V.K. Chatterjee, and S. O'Rahilly. 1999. Dominant negative mutations in human PPARgamma associated with severe insulin resistance, diabetes mellitus and hypertension. *Nature* 402:880-883.

20. Bastard, J.P., M. Caron, H. Vidal, V. Jan, M. Auclair, C. Vigouroux, J. Luboinski, M. Laville, M. Maachi, P.M. Girard, W. Rozenbaum, P. Levan, and J. Capeau. 2002. Association between altered expression of adipogenic factor SREBP1 in lipoatrophic adipose tissue from HIV-1-infected patients and abnormal adipocyte differentiation and insulin resistance. *Lancet* 359:1026-1031.

21. Baumann, C.A., V. Ribon, M. Kanzaki, D.C. Thurmond, S. Mora, S. Shigematsu, P.E. Bickel, J.E. Pessin, and A.R. Saltiel. 2000. CAP defines a second signalling pathway required for insulin-stimulated glucose transport. *Nature* 407:202-207.

22. Berg, A.H., T.P. Combs, and P.E. Scherer. 2002. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends Endocrinol Metab* 13:84-89.

23. Bergeron, R., S.F. Previs, G.W. Cline, P. Perret, R.R. Russell, III, L.H. Young, and G.I. Shulman. 2001. Effect of 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside infusion on in vivo glucose and lipid metabolism in lean and obese Zucker rats. *Diabetes* 50:1076-1082.

24. Bergeron, R., R.R. Russell, III, L.H. Young, J.M. Ren, M. Marcucci, A. Lee, and G.I. Shulman. 1999. Effect of AMPK activation on muscle glucose metabolism in conscious rats. *Am J Physiol* 276:E938-E944.

25. Bergman, R.N. and M. Ader. 2000. Free fatty acids and pathogenesis of type 2 diabetes mellitus. *Trends Endocrinol Metab* 11:351-356.

26. Bjornholm, M., A.R. He, A. Attersand, S. Lake, S.C. Liu, G.E. Lienhard, S. Taylor, P. Arner, and J.R. Zierath. 2002. Absence of functional insulin receptor substrate-3 (IRS-3) gene in humans. *Diabetologia* 45:1697-1702.

27. Bjornholm, M., Y. Kawano, M. Lehtihet, and J.R. Zierath. 1997. Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle from NIDDM subjects after in vivo insulin stimulation. *Diabetes* 46:524-527.

28. Bluher, M., M.D. Michael, O.D. Peroni, K. Ueki, N. Carter, B.B. Kahn, and C.R. Kahn. 2002. Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance. *Dev Cell* 3:25-38.

29. Boden, G., B. Lebed, M. Schatz, C. Homko, and S. Lemieux. 2001. Effects of acute changes of plasma free fatty acids on intramyocellular fat content and insulin resistance in healthy subjects. *Diabetes* 50:1612-1617.

30. Bonadonna, R.C., L.C. Groop, K. Zych, M. Shank, and R.A. DeFronzo. 1990. Dose-dependent effect of insulin on plasma free fatty acid turnover and oxidation in humans. *Am J Physiol* 259:E736-E750.

31. Boppart, M.D., S. Asp, J.F. Wojtaszewski, R.A. Fielding, T. Mohr, and L.J. Goodyear. 2000. Marathon running transiently increases c-Jun NH2-terminal kinase and p38 activities in human skeletal muscle. *J Physiol* 526 Pt 3:663-669.

32. Bossenmaier, B., L. Mosthaf, H. Mischak, A. Ullrich, and H.U. Haring. 1997. Protein kinase C isoforms beta 1 and beta 2 inhibit the tyrosine kinase activity of the insulin receptor. *Diabetologia* 40:863-866.

33. Boulton, T.G., S.H. Nye, D.J. Robbins, N.Y. Ip, E. Radziejewska, S.D. Morgenbesser, R.A. DePinho, N. Panayotatos, M.H. Cobb, and G.D. Yancopoulos. 1991. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell* 65:663-675.

34. Brady, M.J., A.C. Nairn, and A.R. Saltiel. 1997. The regulation of glycogen synthase by protein phosphatase 1 in 3T3-L1 adipocytes. Evidence for a potential role for DARPP-32 in insulin action. *J Biol Chem* 272:29698-29703.

35. Bray, G.A. 1998. Obesity: a time bomb to be defused. *Lancet* 352:160-161.

36. Brechtel, K., D.B. Dahl, J. Machann, O.P. Bachmann, I. Wenzel, T. Maier, C.D. Claussen, H.U. Haring, S. Jacob, and F. Schick. 2001. Fast elevation of the intramyocellular lipid content in the presence of circulating free fatty acids and hyperinsulinemia: a dynamic 1H-MRS study. *Magn Reson Med* 45:179-183.

37. Brinkman, K., J.A. Smeitink, J.A. Romijn, and P. Reiss. 1999. Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy. *Lancet* 354:1112-1115.

38. Burgess, J.W., I. Wada, N. Ling, M.N. Khan, J.J. Bergeron, and B.I. Posner. 1992. Decrease in beta-subunit phosphotyrosine correlates with internalization and activation of the endosomal insulin receptor kinase. *J Biol Chem* 267:10077-10086.

39. Burks, D.J. and M.F. White. 2001. IRS proteins and beta-cell function. *Diabetes* 50 Suppl 1:S140-S145.

40. Campbell, P.J., G.B. Bolli, P.E. Cryer, and J.E. Gerich. 1985. Pathogenesis of the dawn phenomenon in patients with insulin-dependent diabetes mellitus. Accelerated glucose production and impaired glucose utilization due to nocturnal surges in growth hormone secretion. *N Engl J Med* 312:1473-1479.

41. Caron, M., M. Auclair, C. Vigouroux, M. Glorian, C. Forest, and J. Capeau. 2001. The HIV protease inhibitor indinavir impairs sterol regulatory element-binding protein-1 intranuclear localization, inhibits preadipocyte differentiation, and induces insulin resistance. *Diabetes* 50:1378-1388.

42. Carr, A. and D.A. Cooper. 2000. Adverse effects of antiretroviral therapy. *Lancet* 356:1423-1430.

43. Carr, A., K. Samaras, A. Thorisdottir, G.R. Kaufmann, D.J. Chisholm, and D.A. Cooper. 1999. Diagnosis, prediction, and natural course of HIV-1 protease-inhibitor-associated lipodystrophy, hyperlipidaemia, and diabetes mellitus: a cohort study. *Lancet* 353:2093-2099.

44. Carvalho, E., P.A. Jansson, M. Axelsen, J.W. Eriksson, X. Huang, L. Groop, C. Rondinone, L. Sjostrom, and U. Smith. 1999. Low cellular IRS 1 gene and protein expression predict insulin resistance and NIDDM. *FASEB J* 13:2173-2178.

45. Carvalho, E., P.A. Jansson, I. Nagaev, A.M. Wenthzel, and U. Smith. 2001. Insulin resistance with low cellular IRS-1 expression is also associated with low GLUT4 expression and impaired insulin-stimulated glucose transport. *FASEB J* 15:1101-1103.

46. Cederberg, A. and S. Enerback. 2003. Insulin resistance and type 2 diabetes-an adipocentric view. *Curr Mol Med* 3:107-125.

47. Chalkley, S.M., M. Hettiarachchi, D.J. Chisholm, and E.W. Kraegen. 1998. Five-hour fatty acid elevation increases muscle lipids and impairs glycogen synthesis in the rat. *Metabolism* 47:1121-1126.

48. Chen, H.C., S.J. Stone, P. Zhou, K.K. Buhman, and R.V.J. Farese. 2002. Dissociation of obesity and impaired glucose disposal in mice overexpressing acyl coenzyme A:diacylglycerol acyltransferase 1 in white adipose tissue. *Diabetes* 51:3189-3195.

49. Chen, R., O. Kim, J. Yang, K. Sato, K.M. Eisenmann, J. McCarthy, H. Chen, and Y. Qiu. 2001. Regulation of Akt/PKB activation by tyrosine phosphorylation. *J Biol Chem* 276:31858-31862.

50. Chen, Y.R., X. Wang, D. Templeton, R.J. Davis, and T.H. Tan. 1996. The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and gamma radiation. Duration of JNK activation may determine cell death and proliferation. *J Biol Chem* 271:31929-31936.

51. Chen, Z.P., G.K. McConell, B.J. Michell, R.J. Snow, B.J. Canny, and B.E. Kemp. 2000. AMPK si gnaling in contracting human skeletal muscle: acetyl-CoA carboxylase and NO synthase phosphorylation. *Am J Physiol Endocrinol Metab* 279:E1202-E1206.

52. Chin, J.E., M. Dickens, J.M. Tavare, and R.A. Roth. 1993. Overexpression of protein kinase C isoenzymes alpha, beta I, gamma, and epsilon in cells overexpressing the insulin receptor. Effects on receptor phosphorylation and signaling. *J Biol Chem* 268:6338-6347.

53. Chin, J.E., F. Liu, and R.A. Roth. 1994. Activation of protein kinase C alpha inhibits insulin-stimulated tyrosine phosphorylation of insulin receptor substrate-1. *Mol Endocrinol* 8:51-58.

54. Clausen, J.O., T. Hansen, C. Bjorbaek, S.M. Echwald, S.A. Urhammer, S. Rasmussen, C.B. Andersen, L. Hansen, K. Almind, and K. Winther. 1995. Insulin resistance: interactions between obesity and a common variant of insulin receptor substrate-1. *Lancet* 346:397-402.

55. Clement, K., C. Vaisse, B.S. Manning, A. Basdevant, B. Guy-Grand, J. Ruiz, K.D. Silver, A.R. Shuldiner, P. Froguel, and A.D. Strosberg. 1995. Genetic variation in the beta 3-adrenergic receptor and an increased capacity to gain weight in patients with morbid obesity. *N Engl J Med* 333:352-354.

56. Cline, G.W., K.F. Petersen, M. Krssak, J. Shen, R.S. Hundal, Z. Trajanoski, S. Inzucchi, A. Dresner, D.L. Rothman, and G.I. Shulman. 1999. Impaired glucose transport as a cause of decreased insulin-stimulated muscle glycogen synthesis in type 2 diabetes. *N Engl J Med* 341:240-246.

57. Combs, T.P., A.H. Berg, S. Obici, P.E. Scherer, and L. Rossetti. 2001. Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. *J Clin Invest* 108:1875-1881.

58. Combs, T.P., J.A. Wagner, J. Berger, T. Doebber, W.J. Wang, B.B. Zhang, M. Tanen, A.H. Berg, S. O'Rahilly, D.B. Savage, K. Chatterjee, S. Weiss, P.J. Larson, K.M. Gottesdiener, B.J. Gertz, M.J. Charron, P.E. Scherer, and D.E. Moller. 2002. Induction of adipocyte complement-related protein of 30 kilodaltons by PPARgamma agonists: a potential mechanism of insulin sensitization. *Endocrinology* 143:998-1007.

59. Cooksey, R.C. and D.A. McClain. 2002. Transgenic mice overexpressing the rate-limiting enzyme for hexosamine synthesis in skeletal muscle or adipose tissue exhibit total body insulin resistance. *Ann N Y Acad Sci* 967:102-111.

60. Couillard, C., P. Mauriege, P. Imbeault, D. Prud'homme, A. Nadeau, A. Tremblay, C. Bouchard, and J.P. Despres. 2000. Hyperleptinemia is more closely associated with adipose cell hypertrophy than with adipose tissue hyperplasia. *Int J Obes Relat Metab Disord* 24:782-788.

61. Cusi, K., K. Maezono, A. Osman, M. Pendergrass, M.E. Patti, T. Pratipanawatr, R.A. DeFronzo, C.R. Kahn, and L.J. Mandarino. 2000. Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. *J Clin Invest* 105:311-320.

62. De Beer, R., B.A. van den, D. van Ormondt, W.W. Pijnappel, J.A. den Hollander, A.J. Marien, and P.R. Luyten. 1992. Application of time-domain fitting in the quantification of in vivo 1H spectroscopic imaging data sets. *NMR Biomed* 5:171-178.

63. De Pirro, R., A. Green, M.Y. Kao, and J.M. Olefsky. 1981. Effects of prednisolone and dexamethasone in vivo and in vitro: studies of insulin binding, deoxyglucose uptake and glucose oxidation in rat adipocytes. *Diabetologia* 21:149-153.

64. De Vos, P., A.M. Lefebvre, S.G. Miller, M. Guerre-Millo, K. Wong, R. Saladin, L.G. Hamann, B. Staels, M.R. Briggs, and J. Auwerx. 1996. Thiazolidinediones repress ob gene expression in rodents via activation of peroxisome proliferator-activated receptor gamma. *J Clin Invest* 98:1004-1009.

65. DeFronzo, R.A., E. Jacot, E. Jequier, E. Maeder, J. Wahren, and J.P. Felber. 1981. The effect of insulin on the disposal of intravenous glucose. Results from indirect calorimetry and hepatic and femoral venous catheterization. *Diabetes* 30:1000-1007.

66. DeFronzo, R.A., J.D. Tobin, and R. Andres. 1979. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* 237:E214-E223.

67. Dohm, G.L., C.W. Elton, J.E. Friedman, P.F. Pilch, W.J. Pories, S.M.J. Atkinson, and J.F. Caro. 1991. Decreased expression of glucose transporter in muscle from insulin-resistant patients. *Am J Physiol* 260:E459-E463.

68. Domingo, P., X. Matias-Guiu, R.M. Pujol, E. Francia, E. Lagarda, M.A. Sambeat, and G. Vazquez. 1999. Subcutaneous adipocyte apoptosis in HIV-1 protease inhibitor-associated lipodystrophy. *AIDS* 13:2261-2267.

69. Dominici, F.P. and D. Turyn. 2002. Growth hormone-induced alterations in the insulin-signaling system. *Exp Biol Med* 227:149-157.

70. Douen, A.G., T. Ramlal, A. Klip, D.A. Young, G.D. Cartee, and J.O. Holloszy. 1989. Exercise-induced increase in glucose transporters in plasma membranes of rat skeletal muscle. *Endocrinology* 124:449-454.

71. Dowell, P., C. Flexner, P.O. Kwiterovich, and M.D. Lane. 2000. Suppression of preadipocyte differentiation and promotion of adipocyte death by HIV protease inhibitors. *J Biol Chem* 275:41325-41332.

72. Dresner, A., D. Laurent, M. Marcucci, M.E. Griffin, S. Dufour, G.W. Cline, L.A. Slezak, D.K. Andersen, R.S. Hundal, D.L. Rothman, K.F. Petersen, and G.I. Shulman. 1999. Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest* 103:253-259.

73. Ebihara, K., Y. Ogawa, H. Masuzaki, M. Shintani, F. Miyanaga, M. Aizawa-Abe, T. Hayashi, K. Hosoda, G. Inoue, Y. Yoshimasa, O. Gavrilova, M.L. Reitman, and K. Nakao. 2001. Transgenic overexpression of leptin rescues insulin resistance and diabetes in a mouse model of lipoatrophic diabetes. *Diabetes* 50:1440-1448.

74. Echwald, S.M., H. Bach, H. Vestergaard, B. Richelsen, K. Kristensen, T. Drivsholm, K. Borch-Johnsen, T. Hansen, and O. Pedersen. 2002. A P387L variant in protein tyrosine phosphatase-1B (PTP-1B) is associated with type 2 diabetes and impaired serine phosphorylation of PTP-1B in vitro. *Diabetes* 51:1-6.

75. Eck, M.J., S. Dhe-Paganon, T. Trub, R.T. Nolte, and S.E. Shoelson. 1996. Structure of the IRS-1 PTB domain bound to the juxtamembrane region of the insulin receptor. *Cell* 85:695-705.

76. Elchebly, M., P. Payette, E. Michaliszyn, W. Cromlish, S. Collins, A.L. Loy, D. Normandin, A. Cheng, J. Himms-Hagen, C.C. Chan, C. Ramachandran, M.J. Gresser, M.L. Tremblay, and B.P. Kennedy. 1999. Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. *Science* 283:1544-1548.

77. Fajas, L., K. Schoonjans, L. Gelman, J.B. Kim, J. Najib, G. Martin, J.C. Fruchart, M. Briggs, B.M. Spiegelman, and J. Auwerx. 1999. Regulation of peroxisome proliferator-activated receptor gamma expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: implications for adipocyte differentiation and metabolism. *Mol Cell Biol* 19:5495-5503.

78. Fantin, V.R., Q. Wang, G.E. Lienhard, and S.R. Keller. 2000. Mice lacking insulin receptor substrate 4 exhibit mild defects in growth, reproduction, and glucose homeostasis. *Am J Physiol Endocrinol Metab* 278:E127-E133.

79. Fasshauer, M., J. Klein, S. Neumann, M. Eszlinger, and R. Paschke. 2002. Hormonal regulation of adiponectin gene expression in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 290:1084-1089.

80. Ferrannini, E., E.J. Barrett, S. Bevilacqua, and R.A. DeFronzo. 1983. Effect of fatty acids on glucose production and utilization in man. *J Clin Invest* 72:1737-1747.

81. Fisher, F.M., P.G. McTernan, G. Valsamakis, R. Chetty, A.L. Harte, A.J. Anwar, J. Starcynski, J. Crocker, A.H. Barnett, C.L. McTernan, and S. Kumar. 2002. Differences in adiponectin protein expression: effect of fat depots and type 2 diabetic status. *Horm Metab Res* 34:650-654.

82. Flier, J.S. 1992. Lilly Lecture: syndromes of insulin resistance. From patient to gene and back again. *Diabetes* 41:1207-1219.

83. Forouhi, N.G., G. Jenkinson, E.L. Thomas, S. Mullick, S. Mierisova, U. Bhonsle, P.M. McKeigue, and J.D. Bell. 1999. Relation of triglyceride stores in skeletal muscle cells to central obesity and insulin sensitivity in European and South Asian men. *Diabetologia* 42:932-935.

84. Friedman, J.E., T. Ishizuka, J. Shao, L. Huston, T. Highman, and P. Catalano. 1999. Impaired glucose transport and insulin receptor tyrosine phosphorylation in skeletal muscle from obese women with gestational diabetes. *Diabetes* 48:1807-1814.

85. Fruhbeck, G., J. Gomez-Ambrosi, F.J. Muruzabal, and M.A. Burrell. 2001. The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am J Physiol Endocrinol Metab* 280:E827-E847.

86. Fujii, N., T. Hayashi, M.F. Hirshman, J.T. Smith, S.A. Habinowski, L. Kaijser, J. Mu, O. Ljungqvist, M.J. Birnbaum, L.A. Witters, A. Thorell, and L.J. Goodyear. 2000. Exercise induces isoform-specific increase in 5'AMP-activated protein kinase activity in human skeletal muscle. *Biochem Biophys Res Commun* 273:1150-1155.

87. Furler, S.M., G.J. Cooney, B.D. Hegarty, M.Y. Lim-Fraser, E.W. Kraegen, and N.D. Oakes. 2000. Local factors modulate tissue-specific NEFA utilization: assessment in rats using 3H-(R)-2-bromopalmitate. *Diabetes* 49:1427-1433.

88. Garg, A. 2002. The lipodystrophies and other primary disorders of adipose tissue. In Harrison's Principles of Internal Medicine. E. Brauwald, A.S. Fauci, D.L. Kasper, A.S. Houser, D.L. Longo, and J.L. Jameson, editors. New York. Pages 2316-2319.

89. Gavrilova, O., B. Marcus-Samuels, D. Graham, J.K. Kim, G.I. Shulman, A.L. Castle, C. Vinson, M. Eckhaus, and M.L. Reitman. 2000. Surgical implantation of adipose tissue reverses diabetes in lipoatrophic mice. *J Clin Invest* 105:271-278.

90. Gavrilova, O., B. Marcus-Samuels, L.R. Leon, C. Vinson, and M.L. Reitman. 2000. Leptin and diabetes in lipoatrophic mice. *Nature* 403:850-851.

91. George, S., D. Savage, and S. O'Rahilly. 2003. Hereditary and acquired syndromes of severe insulin resistance. In Textbook of Diabetes. G. Williams and J.C. Pickup. Blackwell Science, chapter 29.

92. Goldstein, B.J., F. Ahmad, W. Ding, P.M. Li, and W.R. Zhang. 1998. Regulation of the insulin signalling pathway by cellular protein-tyrosine phosphatases. *Mol Cell Biochem* 182:91-99.

93. Goldstein, B.J. and C.R. Kahn. 1989. Analysis of mRNA heterogeneity by ribonuclease H mapping: application to the insulin receptor. *Biochem Biophys Res Commun* 159:664-669.

94. Goodpaster, B.H., F.L. Thaete, and D.E. Kelley. 2000. Thigh adipose tissue distribution is associated with insulin resistance in obesity and in type 2 diabetes mellitus. *Am J Clin Nutr* 71:885-892.

95. Goodyear, L.J., F. Giorgino, L.A. Sherman, J. Carey, R.J. Smith, and G.L. Dohm. 1995. Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J Clin Invest* 95:2195-2204.

96. Gray, R.E., C.J. Tanner, W.J. Pories, K.G. MacDonald, and J.A. Houmard. 2003. Effect of weight loss on muscle lipid content in morbidly obese subjects. *Am J Physiol Endocrinol Metab* 284:E726-E732.

97. Greco, A.V., G. Mingrone, A. Giancaterini, M. Manco, M. Morroni, S. Cinti, M. Granzotto, R. Vettor, S. Camastra, and E. Ferrannini. 2002. Insulin resistance in morbid obesity: reversal with intramyocellular fat depletion. *Diabetes* 51:144-151.

98. Green, D.R. and J.C. Reed. 1998. Mitochondria and apoptosis. *Science* 281:1309-1312.

99. Griffin, M.E., M.J. Marcucci, G.W. Cline, K. Bell, N. Barucci, D. Lee, L.J. Goodyear, E.W. Kraegen, M.F. White, and G.I. Shulman. 1999. Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes* 48:1270-1274.

100. Grinspoon, S. Insulin resistance in the HIV-lipodystrophy syndrome. 2001. *Trends Endocrinol Metab* 12:413-419.

101. Groop, L.C., M. Kankuri, C. Schalin-Jantti, A. Ekstrand, P. Nikula-Ijas, E. Widen, E. Kuismanen, J. Eriksson, A. Franssila-Kallunki, C. Saloranta, et al. 1993. Association between polymorphism of the glycogen synthase gene and non-insulin-dependent diabetes mellitus. *N Engl J Med* 328:10-14.

102. Grunfeld, C. and K.R. Feingold. 1991. The metabolic effects of tumor necrosis factor and other cytokines. *Biotherapy* 3:143-158.

103. Gumbiner, B., J.F. Mucha, J.E. Lindstrom, I. Rekhi, and J.N. Livingston. 1996. Differential effects of acute hypertriglyceridemia on insulin action and insulin receptor autophosphorylation. *Am J Physiol* 270: E424-E429.

104. Hadigan, C., J.B. Meigs, C. Corcoran, P. Rietschel, S. Piecuch, N. Basgoz, B. Davis, P. Sax, T. Stanley, P.W. Wilson, R.B. D'Agostino, and S. Grinspoon. 2001. Metabolic abnormalities and cardiovascular disease risk factors in adults with human immunodeficiency virus infection and lipodystrophy. *Clin Infect Dis* 32:130-139.

105. Halleux, C.M., M. Takahashi, M.L. Delporte, R. Detry, T. Funahashi, Y. Matsuzawa, and S.M. Brichard. 2001. Secretion of adiponectin and regulation of apM1 gene expression in human visceral adipose tissue. *Biochem Biophys Res Commun* 288:1102-1107.

106. Handberg, A., A. Vaag, P. Damsbo, H. Beck-Nielsen, and J. Vinten. 1990. Expression of insulin regulatable glucose transporters in skeletal muscle from type 2 (non-insulin-dependent) diabetic patients. *Diabetologia* 33:625-627.

107. Hansen, J.B., H. Zhang, T.H. Rasmussen, R.K. Petersen, E.N. Flindt, and K. Kristiansen. 2001. Peroxisome proliferator-activated receptor delta (PPARdelta)-mediated regulation of preadipocyte proliferation and gene expression is dependent on cAMP signaling. *J Biol Chem* 276:3175-3182.

108. Hansen, L., T. Hansen, H. Vestergaard, C. Bjorbaek, S.M. Echwald, J.O. Clausen, Y.H. Chen, M.X. Chen, P.T. Cohen, and O. Pedersen. 1995. A widespread amino acid polymorphism at codon 905 of the glycogen-associated regulatory subunit of protein phosphatase-1 is associated with insulin resistance and hypersecretion of insulin. *Hum Mol Genet* 4:1313-1320.

109. Haque, W.A., I. Shimomura, Y. Matsuzawa, and A. Garg. 2002. Serum adiponectin and leptin levels in patients with lipodystrophies. *J Clin Endocrinol Metab* 87:2395.

110. Hart, G.W. 1997. Dynamic O-linked glycosylation of nuclear and cytoskeletal proteins. *Annu Rev Biochem* 66:315-335.

111. Hartman, H.B., X. Hu, K.X. Tyler, C.K. Dalal, and M.A. Lazar. 2002. Mechanisms regulating adipocyte expression of resistin. *J Biol Chem* 277:19754-19761.

112. Haugen, F., A. Jorgensen, C.A. Drevon, and P. Trayhurn. 2001. Inhibition by insulin of resistin gene expression in 3T3-L1 adipocytes. *FEBS Lett* 507:105-108.

113. Hawkins, M., N. Barzilai, R. Liu, M. Hu, W. Chen, and L. Rossetti. 1997. Role of the glucosamine pathway in fat-induced insulin resistance. *J Clin Invest* 99:2173-2182.

114. Hayashi, T., M.F. Hirshman, N. Fujii, S.A. Habinowski, L.A. Witters, and L.J. Goodyear. 2000. Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. *Diabetes* 49:527-531.

115. Hayashi, T., M.F. Hirshman, E.J. Kurth, W.W. Winder, and L.J. Goodyear. 1998. Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport. *Diabetes* 47:1369-1373.

116. Hirosumi, J., G. Tuncman, L. Chang, C.Z. Gorgun, K.T. Uysal, K. Maeda, M. Karin, and G.S. Hotamisligil. 2002. A central role for JNK in obesity and insulin resistance. *Nature* 420:333-336.

117. Hirshman, M.F., H. Wallberg-Henriksson, L.J. Wardzala, E.D. Horton, and E.S. Horton. 1988. Acute exercise increases the number of plasma membrane glucose transporters in rat skeletal muscle. *FEBS Lett* 238:235-239.

118. Holgado-Madruga, M., D.R. Emlet, D.K. Moscatello, A.K. Godwin, and A.J. Wong. 1996. A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature* 379:560-564.

119. Hotamisligil, G.S., P. Peraldi, A. Budavari, R. Ellis, M.F. White, and B.M. Spiegelman. 1996. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. *Science* 271:665-668.

120. Hotamisligil, G.S. and B.M. Spiegelman. 1994. Tumor necrosis factor alpha: a key component of the obesity-diabetes link. *Diabetes* 43:1271-1278.

121. Hotta, K., T. Funahashi, Y. Arita, M. Takahashi, M. Matsuda, Y. Okamoto, H. Iwahashi, H. Kuriyama, N. Ouchi, K. Maeda, M. Nishida, S. Kihara, N. Sakai, T. Nakajima, K. Hasegawa, M. Muraguchi, Y. Ohmoto, T. Nakamura, S. Yamashita, T. Hanafusa, and Y. Matsuzawa. 2000. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol* 20:1595-1599.

122. Houmard, J.A., C.D. Shaw, M.S. Hickey, and C.J. Tanner. 1999. Effect of short-term exercise training on insulin-stimulated PI 3-kinase activity in human skeletal muscle. *Am J Physiol* 277:E1055-E1060.

123. Howald, H., C. Boesch, R. Kreis, S. Matter, R. Billeter, B. Essen-Gustavsson, and H. Hoppeler. 2002. Content of intramyocellular lipids derived by electron microscopy, biochemical assays, and (1)H-MR spectroscopy. *J Appl Physiol* 92:2264-2272.

124. Hribal, M.L., M. Federici, O. Porzio, D. Lauro, P. Borboni, D. Accili, R. Lauro, and G. Sesti. 2000. The Gly-->Arg972 amino acid polymorphism in insulin receptor substrate-1 affects glucose metabolism in skeletal muscle cells. *J Clin Endocrinol Metab* 85:2004-2013.

125. Ihlemann, J., T. Ploug, Y. Hellsten, and H. Galbo. 1999. Effect of tension on contraction-induced glucose transport in rat skeletal muscle. *Am J Physiol* 277:E208-E214.

126. Itani, S.I., N.B. Ruderman, F. Schmieder, and G. Boden. 2002. Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha. *Diabetes* 51:2005-2011.

127. Itani, S.I., Q. Zhou, W.J. Pories, K.G. MacDonald, and G.L. Dohm. 2000. Involvement of protein kinase C in human skeletal muscle insulin resistance and obesity. *Diabetes* 49:1353-1358.

128. Jacob, S., J. Machann, K. Rett, K. Brechtel, A. Volk, W. Renn, E. Maerker, S. Matthaei, F. Schick, C.D. Claussen, and H.U. Haring. 1999. Association of increased intramyocellular lipid content with insulin resistance in lean nondiabetic offspring of type 2 diabetic subjects. *Diabetes* 48:1113-1119.

129. Juhan-Vague, I. and M.C. Alessi. 1997. PAI-1, obesity, insulin resistance and risk of cardiovascular events. *Thromb Haemost* 78:656-660.

130. Kadish, A.H., R.L. Litle, and J.C. Sternberg. 1968. A new and rapid method for the determination of glucose by measurement of rate of oxygen consumption. *Clin Chem* 14:116-131.

131. Kadowaki, T., K. Hara, N. Kubota, K. Tobe, Y. Terauchi, T. Yamauchi, K. Eto, H. Kadowaki, M. Noda, R. Hagura, and Y. Akanuma. 2002. The role of PPARgamma in high-fat diet-induced obesity and insulin resistance. *J Diabetes Complications* 16:41-45.

132. Kahn, B.B. and J.S. Flier. 2000. Obesity and insulin resistance. *J Clin Invest* 106:473-481.

133. Kahn, C.R., J.S. Flier, R.S. Bar, J.A. Archer, P. Gorden, M.M. Martin, and J. Roth. 1976. The syndromes of insulin resistance and acanthosis nigricans. Insulin-receptor disorders in man. *N Engl J Med* 294:739-745.

134. Kanety, H., R. Feinstein, M.Z. Papa, R. Hemi, and A. Karasik. 1995. Tumor necrosis factor alpha-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1. *J Biol Chem* 270:23780-23784.

135. Kanety, H., R. Hemi, M.Z. Papa, and A. Karasik. 1996. Sphingomyelinase and ceramide suppress insulin-induced tyrosine phosphorylation of the insulin receptor substrate-1. *J Biol Chem* 271:9895-9897.

136. Kellerer, M., R. Lammers, and H.U. Haring. 1999. Insulin signal transduction: possible mechanisms for insulin resistance. *Exp Clin Endocrinol Diabetes* 107:97-106.

137. Kellerer, M., J. Mushack, E. Seffer, H. Mischak, A. Ullrich, and H.U. Haring. 1998. Protein kinase C isoforms alpha, delta and theta require insulin receptor substrate-1 to inhibit the tyrosine kinase activity of the insulin receptor in human kidney embryonic cells (HEK 293 cells). *Diabetologia* 41:833-838.

138. Kido, Y., D.J. Burks, D. Withers, J.C. Bruning, C.R. Kahn, M.F. White, and D. Accili. 2000. Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. *J Clin Invest* 105:199-205.

139. Kido, Y., J. Nakae, and D. Accili. 2001. Clinical review 125: The insulin receptor and its cellular targets. *J Clin Endocrinol Metab* 86:972-979.

140. Kim, J.B. and B.M. Spiegelman. 1996. ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Genes Dev* 10:1096-1107.

141. Kim, J.K., O. Gavrilova, Y. Chen, M.L. Reitman, and G.I. Shulman. 2000. Mechanism of insulin resistance in A-ZIP/F-1 fatless mice. *J Biol Chem* 275:8456-8460.

142. Kim, Y.B., S.E. Nikoulina, T.P. Ciaraldi, R.R. Henry, and B.B. Kahn. 1999. Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. *J Clin Invest* 104:733-741.

143. Kirwan, J.P., L.F. del Aguila, J.M. Hernandez, D.L. Williamson, D.J. O'Gorman, R. Lewis, and R.K. Krishnan. 2000. Regular exercise enhances insulin activation of IRS-1-associated PI3-kinase in human skeletal muscle. *J Appl Physiol* 88:797-803.

144. Klein, H.H., H. Vestergaard, G. Kotzke, and O. Pedersen. 1995. Elevation of serum insulin concentration during euglycemic hyperinsulinemic clamp studies leads to similar activation of insulin receptor kinase in skeletal muscle of subjects with and without NIDDM. *Diabetes* 44:1310-1317.

145. Kops, G.J. and B.M. Burgering. 1999. Forkhead transcription factors: new insights into protein kinase B (c-Akt) signaling. *J Mol Med* 77:656-665.

146. Kosaki, A., T.S. Pillay, L. Xu, and N.J. Webster. 1995. The B isoform of the insulin receptor signals more efficiently than the A isoform in HepG2 cells. *J Biol Chem* 270:20816-20823.

147. Kosztolanyi, G. 1997. Leprechaunism/Donohue syndrome/insulin receptor gene mutations: a syndrome delineation story from clinicopathological description to molecular understanding. *Eur J Pediatr* 156:253-255.

148. Kotani, K., W. Ogawa, M. Matsumoto, T. Kitamura, H. Sakaue, Y. Hino, K. Miyake, W. Sano, K. Akimoto, S. Ohno, and M. Kasuga. 1998. Requirement of atypical protein kinase C lambda for insulin stimulation of glucose uptake but not for Akt activation in 3T3-L1 adipocytes. *Mol Cell Biol* 18:6971-6982.

149. Koval, J.A., K. Maezono, M.E. Patti, M. Pendergrass, R.A. DeFronzo, and L.J. Mandarino. 1999. Effects of exercise and insulin on insulin signaling proteins in human skeletal muscle. *Med Sci Sports Exerc* 31:998-1004.

150. Krebs, M., M. Krssak, P. Nowotny, D. Weghuber, S. Gruber, V. Mlynarik, M. Bischof, H. Stingl, C. Furnsinn, W. Waldhausl, and M. Roden. 2001. Free fatty acids inhibit the glucose-stimulated increase of intramuscular glucose-6-phosphate concentration in humans. *J Clin Endocrinol Metab* 86:2153-2160.

151. Krook, A., M. Bjornholm, D. Galuska, X.J. Jiang, R. Fahlman, M.G.J. Myers, H. Wallberg-Henriksson, and J.R. Zierath. 2000. Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. *Diabetes* 49:284-292.

152. Krook, A., U. Widegren, X.J. Jiang, J. Henriksson, H. Wallberg-Henriksson, D. Alessi, and J.R. Zierath. 2000. Effects of exercise on mitogen- and stress-activated kinase signal transduction in human skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 279:R1716-R1721.

153. Krssak, M., P.K. Falk, A. Dresner, L. DiPietro, S.M. Vogel, D.L. Rothman, M. Roden, and G.I. Shulman. 1999. Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. *Diabetologia* 42:113-116.

154. Kruszynska, Y.T. 2003. Normal metabolism: the physiology of fuel homeostasis. In Textbook of Diabetes. J.C. Pickup and G. Williams, editors. Blackwell Science. Chapter 9.

155. Kruszynska, Y.T., D.S. Worrall, J. Ofrecio, J.P. Frias, G. Macaraeg, and J.M. Olefsky. 2002. Fatty acid-induced insulin resistance: decreased muscle PI3K activation but unchanged Akt phosphorylation. *J Clin Endocrinol Metab* 87:226-234.

156. Kulkarni, R.N., J.C. Bruning, J.N. Winnay, C. Postic, M.A. Magnuson, and C.R. Kahn. 1999. Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell* 96:329-339.

157. Kulkarni, R.N., J.N. Winnay, M. Daniels, J.C. Bruning, S.N. Flier, D. Hanahan, and C.R. Kahn. 1999. Altered function of insulin receptor substrate-1-deficient mouse islets and cultured beta-cell lines. *J Clin Invest* 104:R69-R75.

158. Kumada, M., S. Kihara, S. Sumitsuji, T. Kawamoto, S. Matsumoto, N. Ouchi, Y. Arita, Y. Okamoto, I. Shimomura, H. Hiraoka, T. Nakamura, T. Funahashi, and Y. Matsuzawa. 2003. Association of hypoadiponectinemia with coronary artery disease in men. *Arterioscler Thromb Vasc Biol* 23:85-89.

159. Kuroyama, H., T. Sanke, S. Ohagi, M. Furuta, H. Furuta, and K. Nanjo. 1994. Simple tandem repeat DNA polymorphism in the human glycogen synthase gene is associated with NIDDM in Japanese subjects. *Diabetologia* 37:536-539.

160. Kurth-Kraczek, E.J., M.F. Hirshman, L.J. Goodyear, and W.W. Winder. 1999. 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. *Diabetes* 48:1667-1671.

161. Larrouy, D., H. Vidal, F. Andreelli, M. Laville, and D. Langin. 1999. Cloning and mRNA tissue distribution of human PPARgamma coactivator-1. *Int J Obes Relat Metab Disord* 23:1327-1332.

162. Le Good, J.A., W.H. Ziegler, D.B. Parekh, D.R. Alessi, P. Cohen, and P.J. Parker. 1998. Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* 281:2042-2045.

163. Lee, A.D., P.A. Hansen, and J.O. Holloszy. 1995. Wortmannin inhibits insulin-stimulated but not contraction-stimulated glucose transport activity in skeletal muscle. *FEBS Lett* 361:51-54.

164. Lee, A.D., P.A. Hansen, J. Schluter, E.A. Gulve, J. Gao, and J.O. Holloszy. 1997. Effects of epinephrine on insulin-stimulated glucose uptake and GLUT-4 phosphorylation in muscle. *Am J Physiol* 273:C1082-C1087.

165. Lembo, G., B. Capaldo, V. Rendina, G. Iaccarino, R. Napoli, R. Guida, B. Trimarco, and L. Sacca. 1994. Acute noradrenergic activation induces insulin resistance in human skeletal muscle. *Am J Physiol* 266:E242-E247.

166. Lenhard, J.M., D.K. Croom, J.E. Weiel, and D.A. Winegar. 2000. HIV protease inhibitors stimulate hepatic triglyceride synthesis. *Arterioscler Thromb Vasc Biol* 20:2625-2629.

167. Lillioja, S., D.M. Mott, B.V. Howard, P.H. Bennett, H. Yki-Jarvinen, D. Freymond, B.L. Nyomba, F. Zurlo, B. Swinburn, and C. Bogardus. 1988. Impaired glucose tolerance as a disorder of insulin action. Longitudinal and cross-sectional studies in Pima Indians. *N Engl J Med* 318:1217-1225.

168. Lin, J., H. Wu, P.T. Tarr, C.Y. Zhang, Z. Wu, O. Boss, L.F. Michael, P. Puigserver, E. Isotani, E.N. Olson, B.B. Lowell, R. Bassel-Duby, and B.M. Spiegelman. 2002. Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* 418:797-801.

169. Lin, Y., S.I. Itani, T.G. Kurowski, D.J. Dean, Z. Luo, G.C. Yaney, and N.B. Ruderman. 2001. Inhibition of insulin signaling and glycogen synthesis by phorbol dibutyrate in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 281:E8-E15.

170. Liu, L.S., M. Spelleken, K. Rohrig, H. Hauner, and J. Eckel. 1998. Tumor necrosis factor-alpha acutely inhibits insulin signaling in human adipocytes: implication of the p80 tumor necrosis factor receptor. *Diabetes* 47:515-522.

171. Longo, R., P. Pollesello, C. Ricci, F. Masutti, B.J. Kvam, L. Bercich, L.S. Croce, P. Grigolato, S. Paoletti, B. de Bernard, et al. 1995. Proton MR spectroscopy in quantitative in vivo determination of fat content in human liver steatosis. *J Magn Reson Imaging* 5:281-285.

172. Louis, S.N., G.P. Jackman, T.L. Nero, D. Iakovidis, and W.J. Louis. 2000. Role of beta-adrenergic receptor subtypes in lipolysis. *Cardiovasc Drugs Ther* 14:565-577.

173. Lowell, B.B. 1999. PPARgamma: an essential regulator of adipogenesis and modulator of fat cell function. *Cell* 99:239-242.

174. Lukaski, H.C., P.E. Johnson, W.W. Bolonchuk, and G.I. Lykken. 1985. Assessment of fat-free mass using bioelectrical impedance measurements of the human body. *Am J Clin Nutr* 41:810-817.

175. Lund, S., G.D. Holman, O. Schmitz, and O. Pedersen. 1995. Contraction stimulates translocation of glucose transporter GLUT4 in skeletal muscle through a mechanism distinct from that of insulin. *Proc Natl Acad Sci USA* 92:5817-5821.

176. Maeda, N., I. Shimomura, K. Kishida, H. Nishizawa, M. Matsuda, H. Nagaretani, N. Furuyama, H. Kondo, M. Takahashi, Y. Arita, R. Komuro, N. Ouchi, S. Kihara, Y. Tochino, K. Okutomi, M. Horie, S. Takeda, T. Aoyama, T. Funahashi, and Y. Matsuzawa. 2002. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nat Med* 8:731-737.

177. Maegawa, H., Y. Shigeta, K. Egawa, and M. Kobayashi. 1991. Impaired autophosphorylation of insulin receptors from abdominal skeletal muscles in nonobese subjects with NIDDM. *Diabetes* 40:815-819.

178. Maher, B., A. Alfirevic, F.J. Vilar, E.G. Wilkins, B.K. Park, and M. Pirmohamed. 2002. TNF-alpha promoter region gene polymorphisms in HIV-positive patients with lipodystrophy. *AIDS* 16:2013-2018.

179. Makowski, L., J.B. Boord, K. Maeda, V.R. Babaev, K.T. Uysal, M.A. Morgan, R.A. Parker, J. Suttles, S. Fazio, G.S. Hotamisligil, and M.F. Linton. 2001. Lack of macrophage fatty-acid-binding protein aP2 protects mice deficient in apolipoprotein E against atherosclerosis. *Nat Med* 7:699-705.

180. Malmstrom, R., M.R. Taskinen, S.L. Karonen, and H. Yki-Jarvinen. 1996. Insulin increases plasma leptin concentrations in normal subjects and patients with NIDDM. *Diabetologia* 39:993-996.

181. Manco, M., G. Mingrone, A.V. Greco, E. Capristo, D. Gniuli, A. De Gaetano, and G. Gasbarrini. 2000. Insulin resistance directly correlates with increased saturated fatty acids in skeletal muscle triglycerides. *Metabolism* 49:220-224.

182. Martin, G., K. Schoonjans, A.M. Lefebvre, B. Staels, and J. Auwerx. 1997. Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARalpha and PPARgamma activators. *J Biol Chem* 272:28210-28217.

183. Masuzaki, H., J. Paterson, H. Shinyama, N.M. Morton, J.J. Mullins, J.R. Seckl, and J.S. Flier. 2001. A transgenic model of visceral obesity and the metabolic syndrome. *Science* 294:2166-2170.

184. McGuire, E.A., J.H. Helderman, J.D. Tobin, R. Andres, and M. Berman. 1976. Effects of arterial versus venous sampling on analysis of glucose kinetics in man. *J Appl Physiol* 41:565-573.

185. McGuire, M.C., R.M. Fields, B.L. Nyomba, I. Raz, C. Bogardus, N.K. Tonks, and J. Sommercorn. 1991. Abnormal regulation of protein tyrosine phosphatase activities in skeletal muscle of insulin-resistant humans. *Diabetes* 40:939-942.

186. Memon, R.A., J. Fuller, A.H. Moser, P.J. Smith, K.R. Feingold, and C. Grunfeld. 1998. In vivo regulation of acyl-CoA synthetase mRNA and activity by endotoxin and cytokines. *Am J Physiol* 275:E64-E72.

187. Mendez, R., G. Kollmorgen, M.F. White, and R.E. Rhoads. 1997. Requirement of protein kinase C zeta for stimulation of protein synthesis by insulin. *Mol Cell Biol* 17:5184-5192.

188. Michael, L.F., Z. Wu, R.B. Cheatham, P. Puigserver, G. Adelmant, J.J. Lehman, D.P. Kelly, and B.M. Spiegelman. 2001. Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1. *Proc Natl Acad Sci USA* 98:3820-3825.

189. Michie, H.R., T.J. Eberlein, D.R. Spriggs, K.R. Manogue, A. Cerami, and D.W. Wilmore. 1988. Interleukin-2 initiates metabolic responses associated with critical illness in humans. *Ann Surg* 208:493-503.

190. Michie, H.R., P.J. Guillou, and D.W. Wilmore. 1989. Tumour necrosis factor and bacterial sepsis. *Br J Surg* 76:670-671.

191. Michie, H.R., D.R. Spriggs, K.R. Manogue, M.L. Sherman, A. Revhaug, S.T. O'Dwyer, K. Arthur, C.A. Dinarello, A. Cerami, and S.M. Wolff. 1988. Tumor necrosis factor and endotoxin induce similar metabolic responses in human beings. *Surgery* 104:280-286.

192. Miles, J., R. Glasscock, J. Aikens, J. Gerich, and M. Haymond. 1983. A microfluorometric method for the determination of free fatty acids in plasma. *J Lipid Res* 24:96-99.

193. Miserez, A.R., P.Y. Muller, L. Barella, M. Schwietert, P. Erb, P.L. Vernazza, and M. Battegay. 2001. A single-nucleotide polymorphism in the sterol-regulatory element-binding protein 1c gene is predictive of HIV-related hyperlipoproteinaemia. *AIDS* 15:2045-2049.

194. Moitra, J., M.M. Mason, M. Olive, D. Krylov, O. Gavrilova, B. Marcus-Samuels, L. Feigenbaum, E. Lee, T. Aoyama, M. Eckhaus, M.L. Reitman, and C. Vinson. 1998. Life without white fat: a transgenic mouse. *Genes Dev* 12:3168-3181.

195. Moller, D.E., A. Yokota, J.F. Caro, and J.S. Flier. 1989. Tissue-specific expression of two alternatively spliced insulin receptor mRNAs in man. *Mol Endocrinol* 3:1263-1269.

196. Montague, C.T., J.B. Prins, L. Sanders, J. Zhang, C.P. Sewter, J. Digby, C.D. Byrne, and S. O'Rahilly. 1998. Depot-related gene expression in human subcutaneous and omental adipocytes. *Diabetes* 47:1384-1391.

197. Moore, G.B., H. Chapman, J.C. Holder, C.A. Lister, V. Piercy, S.A. Smith, and J.C. Clapham. 2001. Differential regulation of adipocytokine mRNAs by rosiglitazone in db/db mice. *Biochem Biophys Res Commun* 286:735-741.

198. Mosthaf, L., K. Grako, T.J. Dull, L. Coussens, A. Ullrich, and D.A. McClain. 1990. Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *EMBO J* 9:2409-2413.

199. Motley, E.D., S.M. Kabir, K. Eguchi, A.L. Hicks, C.D. Gardner, C.M. Reynolds, G.D. Frank, and S. Eguchi. 2001. Protein kinase C inhibits insulin-induced Akt activation in vascular smooth muscle cells. *Cell Mol* 47:1059-1062.

200. Motojima, K., P. Passilly, J.M. Peters, F.J. Gonzalez, and N. Latruffe. 1998. Expression of putative fatty acid transporter genes are regulated by peroxisome proliferator-activated receptor alpha and gamma activators in a tissue- and inducer-specific manner. *J Biol Chem* 273:16710-16714.

201. Motoshima, H., X. Wu, M.K. Sinha, V.E. Hardy, E.L. Rosato, D.J. Barbot, F.E. Rosato, and B.J. Goldstein. 2002. Differential regulation of adiponectin secretion from cultured human omental and subcutaneous adipocytes: effects of insulin and rosiglitazone. *J Clin Endocrinol Metab* 87:5662-5667.

202. Mu, J., J.T. Brozinick, Jr., O. Valladares, M. Bucan, and M.J. Birnbaum. 2001. A role for AMP-activated protein kinase in contraction- and hypoxia-regulated glucose transport in skeletal muscle. *Mol Cell* 7:1085-1094.

203. Mynarcik, D.C., T. Combs, M.A. McNurlan, P.E. Scherer, E. Komaroff, and M.C. Gelato. 2002. Adiponectin and leptin levels in HIV-infected subjects with insulin resistance and body fat redistribution. *J Acquir Immune Defic Syndr* 31:514-520.

204. Nagaev, I. and U. Smith. 2001. Insulin resistance and type 2 diabetes are not related to resistin expression in human fat cells or skeletal muscle. *Biochem Biophys Res Commun* 285:561-564.

205. National Food and Nutritional Institute. 1987. Measuring Obesity - Classification and Description of Antropometric Data. Report on a WHO Consultation on the Epidemiology of Obesity. *WHO* 1-22.

206. Nicholson, K.M. and N.G. Anderson. 2002. The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal* 14:381-395.

207. Nolan, J.J., G. Freidenberg, R. Henry, D. Reichart, and J.M. Olefsky. 1994. Role of human skeletal muscle insulin receptor kinase in the in vivo insulin resistance of noninsulin-dependent diabetes mellitus and obesity. *J Clin Endocrinol Metab* 78:471-477.

208. Nuutila, P., V.A. Koivisto, J. Knuuti, U. Ruotsalainen, M. Teras, M. Haaparanta, J. Bergman, O. Solin, L.M. Voipio-Pulkki, and U. Wegelius. 1992. Glucose-free fatty acid cycle operates in human heart and skeletal muscle in vivo. *J Clin Invest* 89:1767-1774.

209. Olefsky, J.M., J. Johnson, F. Liu, P. Jen, and G.M. Reaven. 1975. The effects of acute and chronic dexamethasone administration on insulin binding to isolated rat hepatocytes and adipocytes. *Metabolism* 24:517-527.

210. Oral, E.A., V. Simha, E. Ruiz, A. Andewelt, A. Premkumar, P. Snell, A.J. Wagner, A.M. DePaoli, M.L. Reitman, S.I. Taylor, P. Gorden, and A. Garg. 2002. Leptin-replacement therapy for lipodystrophy. *N Engl J Med* 346:570-578.

211. Ouchi, N., S. Kihara, Y. Arita, K. Maeda, H. Kuriyama, Y. Okamoto, K. Hotta, M. Nishida, M. Takahashi, T. Nakamura, S. Yamashita, T. Funahashi, and Y. Matsuzawa. 1999. Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. *Circulation* 100:2473-2476.

212. Ouchi, N., S. Kihara, Y. Arita, M. Nishida, A. Matsuyama, Y. Okamoto, M. Ishigami, H. Kuriyama, K. Kishida, H. Nishizawa, K. Hotta, M. Muraguchi, Y. Ohmoto, S. Yamashita, T. Funahashi, and Y. Matsuzawa. 2001. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* 103:1057-1063.

213. Owuor, E.D. and A.N. Kong. 2002. Antioxidants and oxidants regulated signal transduction pathways. *Biochem Pharmacol* 64:765-770.

214. Pan, D.A., S. Lillioja, A.D. Kriketos, M.R. Milner, L.A. Baur, C. Bogardus, A.B. Jenkins, and L.H. Storlien. 1997. Skeletal muscle triglyceride levels are inversely related to insulin action. *Diabetes* 46:983-988.

215. Patti, M.E., A. Virkamaki, E.J. Landaker, C.R. Kahn, and H. Yki-Jarvinen. 1999. Activation of the hexosamine pathway by glucosamine in vivo induces insulin resistance of early postreceptor insulin signaling events in skeletal muscle. *Diabetes* 48:1562-1571.

216. Pedersen, O., J.F. Bak, P.H. Andersen, S. Lund, D.E. Moller, J.S. Flier, and B.B. Kahn. 1990. Evidence against altered expression of GLUT1 or GLUT4 in skeletal muscle of patients with obesity or NIDDM. *Diabetes* 39:865-870.

217. Pederson, T. and C.M. Rondinone. 2000. Regulation of proteins involved in insulin signaling pathways in differentiating human adipocytes. *Biochem Biophys Res Commun* 276:162-168.

218. Perseghin, G., P. Scifo, F. De Cobelli, E. Pagliato, A. Battezzati, C. Arcelloni, A. Vanzulli, G. Testolin, G. Pozza, A. Del Maschio, and L. Luzi. 1999. Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a 1H-13C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. *Diabetes* 48:1600-1606.

219. Pessin, J.E., W. Gitomer, Y. Oka, C.L. Oppenheimer, and M.P. Czech. 1983. Beta-adrenergic regulation of insulin and epidermal growth factor receptors in rat adipocytes. *J Biol Chem* 258:7386-7394.

220. Peters, J.M., S.S. Lee, W. Li, J.M. Ward, O. Gavrilova, C. Everett, M.L. Reitman, L.D. Hudson, and F.J. Gonzalez. 2000. Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor beta (delta). *Mol Cell Biol* 20:5119-5128.

221. Phillips, D.I., S. Caddy, V. Ilic, B.A. Fielding, K.N. Frayn, A.C. Borthwick, and R. Taylor. 1996. Intramuscular triglyceride and muscle insulin sensitivity: evidence for a relationship in nondiabetic subjects. *Metabolism* 45:947-950.

222. Puigserver, P., Z. Wu, C.W. Park, R. Graves, M. Wright, and B.M. Spiegelman. 1998. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 92:829-839.

223. Randle P.J., Garland P.B., Hales C.N., and Newsholme E.A. 1963. The glucose fatty-acid cycle; its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet i* 785-789.

224. Rannels, S.R. and L.S. Jefferson. 1980. Effects of glucocorticoids on muscle protein turnover in perfused rat hemicorpus. *Am J Physiol* 238:E564-E572.

225. Rask, E., B.R. Walker, S. Soderberg, D.E. Livingstone, M. Eliasson, O. Johnson, R. Andrew, and T. Olsson. 2002. Tissue-specific changes in peripheral cortisol metabolism in obese women: increased adipose 11 beta-hydroxysteroid dehydrogenase type 1 activity. *J Clin Endocrinol Metab* 87:3330-3336.

226. Rasmussen, B.B. and W.W. Winder. 1997. Effect of exercise intensity on skeletal muscle malonyl-CoA and acetyl-CoA carboxylase. *J Appl Physiol* 83:1104-1109.

227. Ravussin, E. and S.R. Smith. 2002. Increased fat intake, impaired fat oxidation, and failure of fat cell proliferation result in ectopic fat storage, insulin resistance, and type 2 diabetes mellitus. *Ann N Y Acad Sci* 967:363-378.

228. Rayfield, E.J., M.J. Ault, G.T. Keusch, M.J. Brothers, C. Nechemias, and H. Smith. 1982. Infection and diabetes: the case for glucose control. *Am J Med* 72:439-450.

229. Razeghi, P., M.E. Young, J.L. Alcorn, C.S. Moravec, O.H. Frazier, and H. Taegtmeyer. 2001. Metabolic gene expression in fetal and failing human heart. *Circulation* 104:2923-2931.

230. Reitman, M.L., M.M. Mason, J. Moitra, O. Gavrilova, B. Marcus-Samuels, M. Eckhaus, and C. Vinson. 1999. Transgenic mice lacking white fat: models for understanding human lipoatrophic diabetes. *Ann N Y Acad Sci* 892:289-96.

231. Rhodes, C.J. and M.F. White. 2002. Molecular insights into insulin action and secretion. *Eur J Clin Invest* 32:3-13.

232. Rizza, R.A., L.J. Mandarino, and J.E. Gerich. 1982. Cortisol-induced insulin resistance in man: impaired suppression of glucose production and stimulation of glucose utilization due to a postreceptor detect of insulin action. *J Clin Endocrinol Metab* 54:131-138.

233. Rizza, R.A., L.J. Mandarino, and J.E. Gerich. 1982. Effects of growth hormone on insulin action in man. Mechanisms of insulin resistance, impaired suppression of glucose production, and impaired stimulation of glucose utilization. *Diabetes* 31:663-669.

234. Roche, R., I. Poizot-Martin, C.M. Yazidi, E. Compe, J.A. Gastaut, J. Torresani, and R. Planells. 2002. Effects of antiretroviral drug combinations on the differentiation of adipocytes. *AIDS* 16:13-20.

235. Rondinone, C.M., L.M. Wang, P. Lonnroth, C. Wesslau, J.H. Pierce, and U. Smith. 1997. Insulin receptor substrate (IRS) 1 is reduced and IRS-2 is the main docking protein for phosphatidylinositol 3-kinase in adipocytes from subjects with non-insulin-dependent diabetes mellitus. *Proc Natl Acad Sci USA* 94:4171-4175.

236. Rosen, E.D., P. Sarraf, A.E. Troy, G. Bradwin, K. Moore, D.S. Milstone, B.M. Spiegelman, and R.M. Mortensen. 1999. PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol Cell* 4:611-617.

237. Rosen, E.D. and B.M. Spiegelman. 2000. Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol* 16:145-171.

238. Ross, S.R., R.A. Graves, and B.M. Spiegelman. 1993. Targeted expression of a toxin gene to adipose tissue: transgenic mice resistant to obesity. *Genes Dev* 7:1318-1324.

239. Rossetti, L. 2000. Perspective: Hexosamines and nutrient sensing. *Endocrinology* 141:1922-1925.

240. Ruderman, N.B., A.K. Saha, D. Vavvas, T. Kurowski, D.R. Laybutt, C. Schmitz-Peiffer, T. Biden, and E.W. Kraegen. 1998. Malonyl CoA as a metabolic switch and a regulator of insulin sensitivity. *Adv Exp Med Biol* 441:263-270.

241. Ruderman, N.B., A.K. Saha, D. Vavvas, and L.A. Witters. 1999. Malonyl-CoA, fuel sensing, and insulin resistance. *Am J Physiol* 276:E1-E18.

242. Ryysy, L., A.M. Hakkinen, T. Goto, S. Vehkavaara, J. Westerbacka, J. Halavaara, and H. Yki-Jarvinen. 2000. Hepatic fat content and insulin action on free fatty acids and glucose metabolism rather than insulin absorption are associated with insulin requirements during insulin therapy in type 2 diabetic patients. *Diabetes* 49:749-758.

243. Salinas, M., R. Lopez-Valdaliso, D. Martin, A. Alvarez, and A. Cuadrado. 2000. Inhibition of PKB/Akt1 by C2-ceramide involves activation of ceramide-activated protein phosphatase in PC12 cells. *Mol Cell Neurosci* 15:156-169.

244. Saltiel, A.R. 2001. New perspectives into the molecular pathogenesis and treatment of type 2 diabetes. *Cell* 104:517-529.

245. Saltiel, A.R. and C.R. Kahn. 2001. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414:799-806.

246. Sammalkorpi, K. 1989. Glucose intolerance in acute infections. *J Intern Med* 225:15-19.

247. Sasaoka, T. and M. Kobayashi. 2000. The functional significance of Shc in insulin signaling as a substrate of the insulin receptor. *Endocr J* 47:373-381.

248. Schaffer, J.E. and H.F. Lodish. 1994. Expression cloning and characterization of a novel adipocyte long chain fatty acid transport protein. *Cell* 79:427-436.

249. Schick, F., B. Eismann, W.I. Jung, H. Bongers, M. Bunse, and O. Lutz. 1993. Comparison of localized proton NMR signals of skeletal muscle and fat tissue in vivo: two lipid compartments in muscle tissue. *Magn Reson Med* 29:158-167.

250. Schmitz-Peiffer, C. 2002. Protein kinase C and lipid-induced insulin resistance in skeletal muscle. *Ann N Y Acad Sci* 967:146-157.

251. Schmitz-Peiffer, C., D.L. Craig, and T.J. Biden. 1999. Ceramide generation is sufficient to account for the inhibition of the insulin-stimulated PKB pathway in C2C12 skeletal muscle cells pretreated with palmitate. *J Biol Chem* 274:24202-24210.

252. Schoonjans, K., J. Peinado-Onsurbe, A.M. Lefebvre, R.A. Heyman, M. Briggs, S. Deeb, B. Staels, and J. Auwerx. 1996. PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J* 15:5336-5348.

253. Schubert, K.M., M.P. Scheid, and V. Duronio. 2000. Ceramide inhibits protein kinase B/Akt by promoting dephosphorylation of serine 473. *J Biol Chem* 275:13330-13335.

254. Schunkert, H. 2002. Obesity and target organ damage: the heart. *Int J Obes Relat Metab Disord* 26 Suppl 4:S15-S20.

255. Seckl, J.R. and B.R. Walker. 2001. Minireview: 11beta-hydroxysteroid dehydrogenase type 1- a tissue-specific amplifier of glucocorticoid action. *Endocrinology* 142:1371-1376.

256. Seino, S. and G.I. Bell. 1989. Alternative splicing of human insulin receptor messenger RNA. *Biochem Biophys Res Commun* 159:312-316.

257. Senn, J.J., P.J. Klover, I.A. Nowak, and R.A. Mooney. 2002. Interleukin-6 induces cellular insulin resistance in hepatocytes. *Diabetes* 51:3391-3399.

258. Senn, J.J., P.J. Klover, I.A. Nowak, T.A. Zimmer, L.G. Koniaris, R.W. Furlanetto, and R.A. Mooney. 2003. Suppressor of cytokine signaling-3 (SOCS-3): A potential mediator of interleukin-6 dependent insulin resistance in hepatocytes. *J Biol Chem 30.*

259. Sesti, G., M. Federici, M.L. Hribal, D. Lauro, P. Sbraccia, and R. Lauro. 2001. Defects of the insulin receptor substrate (IRS) system in human metabolic disorders. *FASEB J* 15:2099-2111.

260. Shepherd, P.R., B.T. Nave, J. Rincon, L.A. Nolte, A.P. Bevan, K. Siddle, J.R. Zierath, and H. Wallberg-Henriksson. 1997. Differential regulation of phosphoinositide 3-kinase adapter subunit variants by insulin in human skeletal muscle. *J Biol Chem* 272:19000-19007.

261. Sherwood, D.J., S.D. Dufresne, J.F. Markuns, B. Cheatham, D.E. Moller, D. Aronson, and L.J. Goodyear. 1999. Differential regulation of MAP kinase, p70(S6K), and Akt by contraction and insulin in rat skeletal muscle. *Am J Physiol* 276:E870-E878.

262. Shimomura, I., T. Funahashi, M. Takahashi, K. Maeda, K. Kotani, T. Nakamura, S. Yamashita, M. Miura, Y. Fukuda, K. Takemura, K. Tokunaga, and Y. Matsuzawa. 1996. Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. *Nat Med* 2:800-803.

263. Shimomura, I., R.E. Hammer, S. Ikemoto, M.S. Brown, and J.L. Goldstein. 1999. Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature* 401:73-76.

264. Shimomura, I., R.E. Hammer, J.A. Richardson, S. Ikemoto, Y. Bashmakov, J.L. Goldstein, and M.S. Brown. 1998. Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes Dev* 12:3182-3194.

265. Shimomura, I., M. Matsuda, R.E. Hammer, Y. Bashmakov, M.S. Brown, and J.L. Goldstein. 2000. Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and ob/ob mice. *Mol Cell* 6:77-86.

266. Standaert, M.L., L. Galloway, P. Karnam, G. Bandyopadhyay, J. Moscat, and R.V. Farese. 1997. Protein kinase C-zeta as a downstream effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes. Potential role in glucose transport. *J Biol Chem* 272:30075-30082.

267. Stannard, S.R., M.W. Thompson, K. Fairbairn, B. Huard, T. Sachinwalla, and C.H. Thompson. 2002. Fasting for 72 h increases intramyocellular lipid content in nondiabetic, physically fit men. *Am J Physiol Endocrinol Metab* 283:E1185-E1191.

268. Stefan, N. and M. Stumvoll. 2002. Adiponectin-its role in metabolism and beyond. *Horm Metab Res* 34:469-474.

269. Steppan, C.M., S.T. Bailey, S. Bhat, E.J. Brown, R.R. Banerjee, C.M. Wright, H.R. Patel, R.S. Ahima, and M.A. Lazar. 2001. The hormone resistin links obesity to diabetes. *Nature* 409:307-312.

270. Stewart, J.M. and J.A. Blakely. 2000. Long chain fatty acids inhibit and medium chain fatty acids activate mammalian cardiac hexokinase. *Biochim Biophys Acta* 1484:278-286.

271. Stork, P.J. and J.M. Schmitt. 2002. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends Cell Biol* 12:258-266.

272. Stumvoll, M. and H. Haring. 2002. The peroxisome proliferator-activated receptor-gamma2 Pro12Ala polymorphism. *Diabetes* 51:2341-2347.

273. Stumvoll, M., O. Tschritter, A. Fritsche, H. Staiger, W. Renn, M. Weisser, F. Machicao, and H. Haring. 2002. Association of the T-G polymorphism in adiponectin (exon 2) with obesity and insulin sensitivity: interaction with family history of type 2 diabetes. *Diabetes* 51:37-41.

274. Summers, S.A., L.A. Garza, H. Zhou, and M.J. Birnbaum. 1998. Regulation of insulin-stimulated glucose transporter GLUT4 translocation and Akt kinase activity by ceramide. *Mol Cell Biol* 18:5457-5464.

275. Sutinen, J., A.M. Hakkinen, J. Westerbacka, A. Seppala-Lindroos, S. Vehkavaara, J. Halavaara, A. Jarvinen, M. Ristola, and H. Yki-Jarvinen. 2002. Increased fat accumulation in the liver in HIV-infected patients with antiretroviral therapy-associated lipodystrophy. *AIDS* 16:2183-2193.

276. Sykiotis, G.P. and A.G. Papavassiliou. 2001. Serine phosphorylation of insulin receptor substrate-1: a novel target for the reversal of insulin resistance. *Mol Endocrinol* 15:1864-1869.

277. Szczepaniak, L.S., E.E. Babcock, F. Schick, R.L. Dobbins, A. Garg, D.K. Burns, J.D. McGarry, and D.T. Stein. 1999. Measurement of intracellular triglyceride stores by H spectroscopy: validation in vivo. *Am J Physiol* 276:E977-E989.

278. Tamemoto, H., T. Kadowaki, K. Tobe, T. Yagi, H. Sakura, T. Hayakawa, Y. Terauchi, K. Ueki, Y. Kaburagi, S. Satoh, et al. 1994. Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* 372:182-186.

279. Taylor, S.I. 1992. Lilly Lecture: molecular mechanisms of insulin resistance. Lessons from patients with mutations in the insulin-receptor gene. *Diabetes* 41:1473-1490.

280. Teruel, T., R. Hernandez, and M. Lorenzo. 2001. Ceramide mediates insulin resistance by tumor necrosis factor-alpha in brown adipocytes by maintaining Akt in an inactive dephosphorylated state. *Diabetes* 50:2563-2571.

281. Thompson, A.L. and G.J. Cooney. 2000. Acyl-CoA inhibition of hexokinase in rat and human skeletal muscle is a potential mechanism of lipid-induced insulin resistance. *Diabetes* 49:1761-1765.

282. Thomsen, C., U. Becker, K. Winkler, P. Christoffersen, M. Jensen, and O. Henriksen. 1994. Quantification of liver fat using magnetic resonance spectroscopy. *Magn Reson Imaging* 12:487-495.

283. Tiikkainen, M., R. Bergholm, S. Vehkavaara, A. Rissanen, A.M. Hakkinen, M. Tamminen, K. Teramo, and H. Yki-Jarvinen. 2003. Effects of identical weight loss on body composition and features of insulin resistance in obese women with high and low liver fat content. *Diabetes* 52:701-707.

284. Tiikkainen, M., M. Tamminen, A.M. Hakkinen, R. Bergholm, S. Vehkavaara, J. Halavaara, K. Teramo, A. Rissanen, and H. Yki-Jarvinen. 2002. Liver-fat accumulation and insulin resistance in obese women with previous gestational diabetes. *Obes Res* 10:859-867.

285. Traxinger, R.R. and S. Marshall. 1991. Coordinated regulation of glutamine:fructose-6-phosphate amidotransferase activity by insulin, glucose, and glutamine. Role of hexosamine biosynthesis in enzyme regulation. *J Biol Chem* 266:10148-10154.

286. Trayhurn, P. 1996. New insights into the development of obesity: obese genes and the leptin system. *Proc Nutr Soc* 55:783-791.

287. Treadway, J.L., D.E. James, E. Burcel, and N.B. Ruderman. 1989. Effect of exercise on insulin receptor binding and kinase activity in skeletal muscle. *Am J Physiol* 256:E138-E144.

288. Tsao, T.S., H.F. Lodish, and J. Fruebis. 2002. ACRP30, a new hormone controlling fat and glucose metabolism. *Eur J Pharmacol* 440:213-221.

289. Utriainen, T., R. Malmstrom, S. Makimattila, and H. Yki-Jarvinen. 1996. Supraphysiological hyperinsulinemia increases plasma leptin concentrations after 4 h in normal subjects. *Diabetes* 45:1364-1366.

290. Vanden Berghe, W., L. Vermeulen, G. De Wilde, K. De Bosscher, E. Boone, and G. Haegeman. 2000. Signal transduction by tumor necrosis factor and gene regulation of the inflammatory cytokine interleukin-6. *Biochem Pharmacol* 60:1185-1195.

291. Vgontzas, A.N., D.A. Papanicolaou, E.O. Bixler, A. Kales, K. Tyson, and G.P. Chrousos. 1997. Elevation of plasma cytokines in disorders of excessive daytime sleepiness: role of sleep disturbance and obesity. *J Clin Endocrinol Metab* 82:1313-1316.

292. Vinson, C.R., T. Hai, and S.M. Boyd. 1993. Dimerization specificity of the leucine zipper-containing bZIP motif on DNA binding: prediction and rational design. *Genes Dev* 7:1047-1058.

293. Virkamaki, A., I. Puhakainen, V.A. Koivisto, H. Vuorinen-Markkola, and H. Yki-Jarvinen. 1992. Mechanisms of hepatic and peripheral insulin resistance during acute infections in humans. *J Clin Endocrinol Metab* 74:673-679.

294. Virkamaki, A., K. Ueki, and C.R. Kahn. 1999. Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J Clin Invest* 103:931-943.

295. Vollenweider, P., B. Menard, and P. Nicod. 2002. Insulin resistance, defective insulin receptor substrate 2-associated phosphatidylinositol-3' kinase activation, and impaired atypical protein kinase C (zeta/lambda) activation in myotubes from obese patients with impaired glucose tolerance. *Diabetes* 51:1052-1059.

296. Vuorinen-Markkola, H., V.A. Koivisto, and H. Yki-Jarvinen. 1992. Mechanisms of hyperglycemia-induced insulin resistance in whole body and skeletal muscle of type I diabetic patients. *Diabetes* 41:571-580.

297. Wadley, G.D., R.J. Tunstall, A. Sanigorski, G.R. Collier, M. Hargreaves, and D. Cameron-Smith. 2001. Differential effects of exercise on insulin-signaling gene expression in human skeletal muscle. *J Appl Physiol* 90:436-440.

298. Wakelam, M.J. 1998. Diacylglycerol-when is it an intracellular messenger? *Biochim Biophys Acta* 1436:117-126.

299. Walker, U.A., M. Bickel, V.S. Lutke, U.P. Ketelsen, H. Schofer, B. Setzer, N. Venhoff, V. Rickerts, and S. Staszewski. 2002. Evidence of nucleoside analogue reverse transcriptase inhibitor--associated genetic and structural defects of mitochondria in adipose tissue of HIV-infected patients. *J Acquir Immune Defic Syndr* 29:117-121.

300. Walston, J., K. Silver, C. Bogardus, W.C. Knowler, F.S. Celi, S. Austin, B. Manning, A.D. Strosberg, M.P. Stern, N. Raben, et al. 1995. Time of onset of non-insulin-dependent diabetes mellitus and genetic variation in the beta 3-adrenergic-receptor gene. *N Engl J Med* 333:343-347.

301. Way, J.M., C.Z. Gorgun, Q. Tong, K.T. Uysal, K.K. Brown, W.W. Harrington, W.R.J. Oliver, T.M. Willson, S.A. Kliewer, and G.S. Hotamisligil. 2001. Adipose tissue resistin expression is severely suppressed in obesity and stimulated by peroxisome proliferator-activated receptor gamma agonists. *J Biol Chem* 276:25651-25653.

302. Way, J.M., W.W. Harrington, K.K. Brown, W.K. Gottschalk, S.S. Sundseth, T.A. Mansfield, R.K. Ramachandran, T.M. Willson, and S.A. Kliewer. 2001. Comprehensive messenger ribonucleic acid profiling reveals that peroxisome proliferator-activated receptor gamma activation has coordinate effects on gene expression in multiple insulin-sensitive tissues. *Endocrinology* 142:1269-1277.

303. Wentworth, J.M., T.P. Burris, and V.K. Chatterjee. 2000. HIV protease inhibitors block human preadipocyte differentiation, but not via the PPARgamma/RXR heterodimer. *J Endocrinol* 164:R7-R10.

304. Weyer, C., J.E. Foley, C. Bogardus, P.A. Tataranni, and R.E. Pratley. 2000. Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. *Diabetologia.* 43:1498-1506.

305. Weyer, C., J.K. Wolford, R.L. Hanson, J.E. Foley, P.A. Tataranni, C. Bogardus, and R.E. Pratley. 2001. Subcutaneous abdominal adipocyte size, a predictor of type 2 diabetes, is linked to chromosome 1q21-q23 and is associated with a common polymorphism in LMNA in Pima Indians. *Mol Genet Metab* 72:231-238.

306. White, M.F. and C.R. Kahn. 1994. The insulin signaling system. *J Biol Chem* 269:1-4.

307. Widegren, U., X.J. Jiang, A. Krook, A.V. Chibalin, M. Bjornholm, M. Tally, R.A. Roth, J. Henriksson, H. Wallberg-Henriksson, and J.R. Zierath. 1998. Divergent effects of exercise on metabolic and mitogenic signaling pathways in human skeletal muscle. *FASEB J* 12:1379-1389.

308. Widegren, U., J.W. Ryder, and J.R. Zierath. 2001. Mitogen-activated protein kinase signal transduction in skeletal muscle: effects of exercise and muscle contraction. *Acta Physiol Scand* 172:227-238.

309. Widen, E., M. Lehto, T. Kanninen, J. Walston, A.R. Shuldiner, and L.C. Groop. 1995. Association of a polymorphism in the beta 3-adrenergic-receptor gene with features of the insulin resistance syndrome in Finns. *N Engl J Med* 333:348-351.

310. Wiman, B., J. Chmielewska, and M. Ranby. 1984. Inactivation of tissue plasminogen activator in plasma. Demonstration of a complex with a new rapid inhibitor. *J Biol Chem* 259:3644-3647.

311. Winder, W.W. 2001. Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle. *J Appl Physiol* 91:1017-1028.

312. Winder, W.W., B.F. Holmes, D.S. Rubink, E.B. Jensen, M. Chen, and J.O. Holloszy. 2000. Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. *J Appl Physiol* 88:2219-2226.

313. Withers, D.J., J.S. Gutierrez, H. Towery, D.J. Burks, J.M. Ren, S. Previs, Y. Zhang, D. Bernal, S. Pons, G.I. Shulman, S. Bonner-Weir, and M.F. White. 1998. Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* 391:900-904.

314. Wititsuwannakul, D. and K.H. Kim. 1977. Mechanism of palmityl coenzyme A inhibition of liver glycogen synthase. *J Biol Chem* 252:7812-7817.

315. Wojtaszewski, J.F., B.F. Hansen, Gade, B. Kiens, J.F. Markuns, L.J. Goodyear, and E.A. Richter. 2000. Insulin signaling and insulin sensitivity after exercise in human skeletal muscle. *Diabetes* 49:325-331.

316. Wojtaszewski, J.F., B.F. Hansen, B. Kiens, and E.A. Richter. 1997. Insulin signaling in human skeletal muscle: time course and effect of exercise. *Diabetes* 46:1775-1781.

317. Wojtaszewski, J.F., P. Nielsen, B.F. Hansen, E.A. Richter, and B. Kiens. 2000. Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle. *J Physiol* 528 Pt 1:221-226.

318. Wolf, G., T. Trub, E. Ottinger, L. Groninga, A. Lynch, M.F. White, M. Miyazaki, J. Lee, and S.E. Shoelson. 1995. PTB domains of IRS-1 and Shc have distinct but overlapping binding specificities. *J Biol Chem* 270:27407-27410.

319. Wu, X., J. Hoffstedt, W. Deeb, R. Singh, N. Sedkova, A. Zilbering, L. Zhu, P.K. Park, P. Arner, and B.J. Goldstein. 2001. Depot-specific variation in protein-tyrosine phosphatase activities in human omental and subcutaneous adipose tissue: a potential contribution to differential insulin sensitivity. *J Clin Endocrinol Metab* 86:5973-5980.

320. Yamauchi, T., J. Kamon, Y. Minokoshi, Y. Ito, H. Waki, S. Uchida, S. Yamashita, M. Noda, S. Kita, K. Ueki, K. Eto, Y. Akanuma, P. Froguel, F. Foufelle, P. Ferre, D. Carling, S. Kimura, R. Nagai, B.B. Kahn, and T. Kadowaki. 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 8:1288-1295.

321. Yamauchi, T., J. Kamon, H. Waki, Y. Terauchi, N. Kubota, K. Hara, Y. Mori, T. Ide, K. Murakami, N. Tsuboyama-Kasaoka, O. Ezaki, Y. Akanuma, O. Gavrilova, C. Vinson, M.L. Reitman, H. Kagechika, K. Shudo, M. Yoda, Y. Nakano, K. Tobe, R. Nagai, S. Kimura, M. Tomita, P. Froguel, and T. Kadowaki. 2001. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 7:941-946.

322. Yang, W.S., W.J. Lee, T. Funahashi, S. Tanaka, Y. Matsuzawa, C.L. Chao, C.L. Chen, T.Y. Tai, and L.M. Chuang. 2001. Weight reduction increases plasma levels of an adipose-derived anti-inflammatory protein, adiponectin. *J Clin Endocrinol Metab* 86:3815-3819.

323. Yeh, J.I., E.A. Gulve, L. Rameh, and M.J. Birnbaum. 1995. The effects of wortmannin on rat skeletal muscle. Dissociation of signaling pathways for insulin- and contraction-activated hexose transport. *J Biol Chem* 270:2107-2111.

324. Yenush, L., K.J. Makati, J. Smith-Hall, O. Ishibashi, M.G.J. Myers, and M.F. White. 1996. The pleckstrin homology domain is the principal link between the insulin receptor and IRS-1. *J Biol Chem* 271:24300-24306.

325. Yki-Jarvinen, H. 1984. Sex and insulin sensitivity. *Metabolism* 33:1011-1015.

326. Yki-Jarvinen, H. 1992. Glucose toxicity. *Endocr Rev* 13:415-431.

327. Yki-Jarvinen, H., M.C. Daniels, A. Virkamaki, S. Makimattila, R.A. DeFronzo, and D. McClain. 1996. Increased glutamine:fructose-6-phosphate amidotransferase activity in skeletal muscle of patients with NIDDM. *Diabetes* 45:302-307.

328. Yki-Jarvinen, H., E. Helve, and V.A. Koivisto. 1987. Hyperglycemia decreases glucose uptake in type I diabetes. *Diabetes* 36:892-896.

329. Yki-Jarvinen, H. and V.A. Koivisto. 1984. Continuous subcutaneous insulin infusion therapy decreases insulin resistance in type 1 diabetes. *J Clin Endocrinol Metab* 58:659-666.

330. Yki-Jarvinen, H. and V.A. Koivisto. 1986. Natural course of insulin resistance in type I diabetes. *N Engl J Med* 315:224-230.

331. Yki-Jarvinen, H., E.A. Nikkila, K. Kubo, and J.E. Foley. 1986. Assay of glucose transport in human fat cells obtained by needle biopsy. *Diabetologia* 29:287-290.

332. Yki-Jarvinen, H., J. Sutinen, A. Silveira, E. Korsheninnikova, R.M. Fisher, K. Kannisto, E. Ehrenborg, P. Eriksson, and A. Hamsten. 2003. Regulation of plasma PAI-1 concentrations in HAART-Associated lipodystrophy during rosiglitazone therapy. *Arterioscler Thromb Vasc Biol* 23:688-694.

333. Yu, M., E. Blomstrand, A.V. Chibalin, A. Krook, and J.R. Zierath. 2001. Marathon running increases ERK1/2 and p38 MAP kinase signalling to downstream targets in human skeletal muscle. *J Physiol* 536:273-282.

334. Zhang, B., K. MacNaul, D. Szalkowski, Z. Li, J. Berger, and D.E. Moller. 1999. Inhibition of adipocyte differentiation by HIV protease inhibitors. *J Clin Endocrinol Metab* 84:4274-4277.

335. Zhou, G., R. Myers, Y. Li, Y. Chen, X. Shen, J. Fenyk -Melody, M. Wu, J. Ventre, T. Doebber, N. Fujii, N. Musi, M.F. Hirshman, L.J. Goodyear, and D.E. Moller. 2001. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108:1167-1174.

336. Zierath, J.R. 2002. Invited review: Exercise training-induced changes in insulin signaling in skeletal muscle. *J Appl Physiol* 93:773-781.

337. Zouali, H., G. Velho, and P. Froguel. 1993. Polymorphism of the glycogen synthase gene and non-insulin-dependent diabetes mellitus. *N Engl J Med* 328:1568.