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Insulin resistance in obese patients with type 2 diabetes mellitus : effects of a very low calorie diet

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Insulin Resistance in Obese Patients with Type 2 Diabetes Mellitus:

Effects of a Very Low Calorie Diet

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Insulin Resistance in Obese Patients with Type 2 Diabetes Mellitus:

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ABBREVIATIONS

Acrp	Complement-related protein 30
ADD1/SREBP	Adipocyte determination and differentiation factor/sterol regulatory element-binding protein
AgRP	Agouti-related protein
AMPK	Adenosine monophosphate kinase
aP2	Fatty-acid binding protein
apM1	Adipose most abundant gene transcript-1
ASBG	Adjustable silicone gastric banding
AS160	Akt substrate of 160 kD
ASP	Acylation-stimulating protein
ATP	Adenosine triphosphate
AUC	Area under the plasma concentration-time curve
BAT	Brown adipose tissue
bHLH	Basic helix-loop-helix
BIA	Bioelectrical impedance analysis
BMI	Body mass index
BPD	Biliopancreatic diversion
BSA	Body surface area
cAMP	Cyclic adenosine monophosphate
CART	Cocaine-amphetamine-related transcript
C/EBP	CCAAT (is piece of DNA)/enhancer-binding protein
CHD	Coronary heart disease
CNS	Central nervous system
COS cells	Monkey cells immortalised with simian V40 virus
CRH	Corticotropin-releasing hormone
CT-scan	Computer tomography scan
Cys	Cysteine
DAG	Diacetylglycerol
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
EGP	Endogenous glucose production
ER	Energy restriction
FAS	Fatty acid synthase
FAT/CD36	Fatty acid transporter/CD36
FFA	Free fatty acids
FFM	Fat free mass
FIZZ	Found in inflammatory zone

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FPG	Fasting plasma glucose
GAP	GTP-ase activating protein
Gdp 28	Gelatin-binding protein
GH	Growth hormone
GLUT-4	Glucose transporter-4
G6Pase	Glucose-6-phosphatase
GS	Glycogen synthase
GSK-3	Glycogen synthase kinase-3
GTP	Guanosine triphosphate
HbA_{1C}	Glycosylated haemoglobin
HDL	High density lipoprotein
HGO	Hepatic glucose output
HNF	Hepatic nuclear factor
IL-6	Interleukin-6
IMCL	Intramyocellular lipids
IRS	Insulin receptor substrate
JAK	Janus kinase
kD	Kilo Dalton
LBM	Lean body mass
LCFA	Long-chain fatty acids
LDL	Low density lipoprotein
M	Glucose metabolised
MCR_i	Metabolic clearance rate of insulin
MODY	Maturity-onset diabetes of the young
mRNA	messenger-RNA (ribonucleic acid)
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
α-MSH	Alpha-melanocyte-stimulating hormone
MW	Molecular weight
N	Nitrogen
NEFA(s)	Non-esterified fatty acid(s)
NMR	Nuclear magnetic resonance
NOGD	Non-oxidative glucose disposal
NPY	Neuropeptide Y
Ob-Rb	Long isoform of the leptin receptor
PDK-1	Phosphoinositide-dependent kinase-1
PEPCK	Phospho-enolpyruvate carboxykinase
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidyl-inositol-3,4-biphosphate

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PIP3	Phosphatidyl-inositol-3,4,5-triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
POMC	Pro-opiomelanocortin
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PRAS 40	Proline-rich Akt substrate of 40 kDA
PTEN	Phosphatase and tensin homologue
PTP1B	Protein tyrosine phosphatase 1B
R_a	Rate of appearance of a flux
R_d	Rate of disappearance of a flux
RELM	Resistin-like molecule
RIA	Radioimmuno assay
RR	Relative risk
RQ	Respiratory quotient
RXR	Retinoid X receptor
SD	Standard deviation
SEM	Standard error of the mean
SHIP	SH2-domain-containing inositol 5-phosphatase
STAT	Signal transducers and activators of transcription
TC	Total cholesterol
TG	Triglycerides
TNF-α	Tumour necrosis factor alpha
TZD	Thiazolidinedione derivative
$\dot{V}co_2$	CO_2 production
$\dot{V}o_2$	O_2 consumption
VBG	Vertical banding gastroplasty
VLCD	Very low calorie diet
VLDL	Very low density lipoprotein
WAT	White adipose tissue
WHO	World health organization
WHR	Waist to hip ratio

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"Wie schrijft die blijft"

Ter herinnering aan mijn moeder

" Zij die menen zonder trainen kans te hebben op succes
Oh die dommerds, oh die stommerds leren straks een harde les"

Voor mijn vader

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CHAPTER 1

Introduction and outline of the thesis

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1.1. OBESITY AND TYPE 2 DIABETES MELLITUS: DEFINITIONS, EPIDEMIOLOGY AND HEALTH PROBLEMS

The enormous increase in overweight and obesity, defined as a body mass index (BMI, calculated as weight in kilograms divided by the length in meters squared) > 25 and > 30 kg/m^2 respectively [Table 1]), has reached epidemic proportions. Worldwide 1 billion people are overweight and 300 million people are obese (http://www/who.int/nut/#obs, obesity and overweight: fact sheet). Of even greater concern is the increase of overweight and obesity in children: worldwide 22 million children under the age of 5 years and 155 million school-age children (http://www.worldheart.org/pdf/press.factsheets.children.obesity.pdf.).

The reason for this concern is that overweight and obesity are associated with increased morbidity and mortality (Tables 2 and 3)[1-4]. Relative risks for the development of type 2 diabetes mellitus[5,6], hypertension[7], coronary heart disease[8,9], stroke[10,11], gallstones[12], osteoarthritis and arthrosis[13,14], infertility[15] and certain types of cancer (breast, colon, endometrium)[16-18] are substantially increased in this patient group (Table 2). Even after correction for diabetes mellitus, high blood pressure and other cardiovascular risk factors, overweight and obesity are in themselves independent risk factors for increased mortality[19]. The association between BMI and mortality has been described as a J-shaped curve with the lowest mortality for BMI values between 18.5 and 24.9 kg/m^2; below 18.5 kg/m^2 the risk is increased and above 24.9 kg/m the risk increases, and rises steeply when the BMI gets over 40 kg/m^2 [20].

Insulin resistance is probably the common denominator, relating obesity with type 2 diabetes mellitus. Obesity somehow (visceral fat deposition?) evokes insulin resistance, a condition predisposing for type 2 diabetes mellitus[21], a chronic disease characterised by impaired insulin secretion and insulin resistance of target organs leading to chronic hyperglycaemia[22]. In fact, in obese women who develop type 2 diabetes mellitus, in 53% of the cases the condition (diabetes) can be ascribed to obesity (Table 2). Therefore, it is not surprising that, along with the increased prevalence of overweight and obesity, the prevalence of type 2 diabetes mellitus has also steadily increased. It is estimated that nowadays over 190 million people worldwide have diabetes mellitus[23], more than 90-95% of them having type 2 diabetes melli-

Table 1. Classification of overweight in adults according to WH01 criteria

Classification	BMI (kg/m2)	Risk of comorbidities
Normal weight	18.5-24.9	average
Overweight	25.0-29.9	increased
Obesity		
Level I	30.0-34.9	moderately increased
Level II	35.0-39.9	severely increased
Level III (morbid)	≥ 40	very severely increased

[1] World Health Organisazation. Obesity: preventing and managing the global epidemic. Technical Report Series,#894,2000.

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Table 2. Estimated health risk for obese (BMI ≥ 30 kg/m^2) adults

	Women		Men	
	Prevalence 9.6%*		Prevalence 8.5%*	
	RR	PAR (%)	RR	PAR (%)
Type 2 diabetes	12.7	52.9	5.2	26.3
Hypertension	4.2	23.5	2.6	12.0
Myocardial infarction	3.2	17.4	1.5	4.1
Coloncarcinoma	2.7	14.0	3.0	14.5
Ischemic heart disease	1.8	7.1	1.8	6.4
Gallstones	1.8	7.1	1.8	6.4
Ovariumcarcinoma	1.7	6.3	-	-
Arthrosis	1.4	3.7	1.9	7.1
Stroke	1.3	2.8	1.3	2.5

Prevalence rates concerning obesity are derived from the MORGEN-project RIVM, Int J Obes Rel Metab Dis 2002:1218. The relative risks (RR), are derived from "Tackling Obesity in England. Report by the comptroller and auditor general. London: National Audit Office 2001". This table was derived from the Executive Summary: obesity and overweight, Health Council of the Netherlands, 2003. PAR = population attributable risk, i.e part of the disease that can be attributed to obesity.

Table 3. Body mass index and relative risk of death.

BMI	Relative risk of death
25.0-26.9	1.3
27.0-28.9	1.6
29.0-31.0	2.1

tus. It has been predicted that in the year 2030 366 million subjects worldwide will suffer from diabetes mellitus[24]. These are crude estimates, however, that have not taken into account the increase in overweight and obesity; hence, actual numbers may even be much higher.

Genetic factors are without doubt of major significance in the development of obesity and type 2 diabetes mellitus. However, because the human genome does not change over just decades, genetic predisposition cannot explain the explosive increase in obesity and type 2 diabetes mellitus of recent years. Environmental and social factors, like a lack of physical exercise and high caloric intake, are more likely explanations for the epidemic. A chronic imbalance between energy intake and energy expenditure eventually leads to obesity.

In obese and obese type 2 diabetic patients, insulin resistance is of paramount pathogenetic significance[21,25]. Insulin resistance not only impairs glucose homeostasis, but is also associated with hypertension[26-28], dyslipidaemia[29-31] and abnormalities in coagulation and fibrinolysis[32,33], conditions that are independent cardiovascular risk factors[34-38], seen in both obesity and type 2 diabetes. In addition, insulin resistance in (severely) obese type 2 diabetic patients makes it often difficult to achieve adequate glycaemic regulation. Sooner or later, insulin therapy will be instituted because normalisation of plasma glucose levels cannot be achieved with oral blood glucose-lowering agents alone. Insulin, however, induces weight gain[39], which in turn aggravates insulin resistance, thus requiring higher doses of insulin: a

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vicious circle has arisen. Furthermore, insulin therapy can also induce or aggravate already existing hyperinsulinaemia, which could be an independent cardiovascular risk factor[37,38,40,41], although the relation may be week[42].

Weight reduction improves insulin resistance and its associated metabolic features (hypertension, dyslipidaemia, hyperglycaemia)[43,44]. In obese patients this will lead to a lower risk for associated co-morbid conditions (Table 2). It has also been demonstrated that lifestyle intervention programmes (often combinations of behaviour therapy, diet therapy and exercise) in overweight and obese patients reduces the number of patients that develop type 2 diabetes mellitus[45,46]. In severely obese type 2 diabetic patients weight loss is, in fact, the only reasonable therapeutic approach. By reducing insulin resistance, glycaemic regulation can be restored often with much less blood glucose-lowering medication.

Calorie restriction remains the hallmark for weight loss. However, only substantial caloric restriction or more moderate caloric restriction for a longer period of time, will lead to the considerable weight loss (probably > 15 kg[47]) needed to restore peripheral insulin sensitivity in morbidly obese patients and (severely) obese type 2 diabetic patients[47,48]. This can either be achieved through a very low calorie diet (VLCD) or bariatric surgery. The latter is very effective in improving insulin resistance and associated cardiovascular risk factors[43,49-53]. In addition, bariatric surgery can prevent the development of type 2 diabetes mellitus[43,54] (review bariatric surgery:[56,57]). However, the procedure is invasive, costly and (also for logistic reasons) available for a limited number of subjects only. VLCDs are safe[58], commercially available, relatively cheap, and easy accessible. Given the enormous increase in incidence of obesity and (obese!) type 2 diabetes mellitus, VLCDs are, therefore, an interesting therapeutic option. Thus, the main focus of the studies described in this thesis was to investigate the short-term and long-term effects of calorie restriction *per se versus* weight loss *per se* on glucose and lipid metabolism, both at the whole-body and at the molecular level in obese patients with type 2 diabetes mellitus.

In this introduction, firstly the main actions of the "master" hormone in glucoregulation, insulin, will be discussed. Secondly, the normal regulation of blood glucose levels will be considered, both at the whole-body level as well as at the molecular level. Thirdly, the pathophysiology of type 2 diabetes mellitus is discussed, with specific focus on insulin resistance, both at the whole-body and the molecular level, and potential mechanisms of insulin resistance will be stressed. Fourthly, the reason and goals of therapeutic interventions will be attended, along with possible therapies. Fifthly, our research aims will be formulated and the outline of this thesis will be presented.

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1.2. INSULIN

1.2.1 Hormone production

Insulin is a hormone produced by the β-cells of the Islets of Langerhans in the pancreas. At birth about $3x10^{-5}$ islets are present, increasing to $1x10^{-6}$ islets during the first years of life. The islets contain various cell types which each produce different hormones. The β-cell produces insulin. Other important hormones are somatostatin, produced in the δ-cell, and glucagon, produced in the α-cell. The latter counteracts the effect of insulin in many ways. The β-cell is situated central in the islet of Langerhans whereas the other cells are located peripherally.

The human insulin gene is located on the short arm of chromosome 11. *Via* DNA/RNA re-synthesis, a precursor molecule known as pre-pro-insulin (98 amino acids, molecular weight [MW] 11.500) is produced in the endoplasmatic reticulum of the pancreatic β-cells. It is cleaved to proinsulin (86 amino acids, MW approximately 9000) directly after the molecule has left the ribosome. The proinsulin is transported to the Golgi apparatus, where packaging into clathrin-coated secretory granules takes place. Maturation of the secretory granule is associated with the loss of the clathrin coating. In addition, the proinsulin is converted into insulin and C-peptide (MW 3000) by proteolytic cleavage at two sites. Normal granules shed insulin and C-peptide in equimolar amounts, along with some proinsulin and so-called split-products (only partially cleaved proinsulin). Insulin (MW 5808) itself consists of an A-chain of 21 amino acids and a B-chain of 30 amino acids, which are connected by two disulfide bonds. The secreted insulin first passes the liver where a proportion of insulin is cleared *via* a receptor-mediated process after exerting its action[59-61] The proportion of insulin cleared during first-pass through the liver has been estimated to be about 50% in dogs[60] and approximately 40 to 80% in humans[62-65]. The plasma half-life time ($t_{½}$) of insulin is only 5-10 minutes. C-peptide, the 31 amino acid residue, has no known biological function. Since C-peptide is produced in equimolar amounts with insulin it can be used as a marker for insulin secretory capacity, because it is not cleared by the liver but by the kidney and has a longer $t_{½}$ than insulin[66,67].

1.2.2. Hormone secretion

The main trigger for insulin release is an increase in the plasma glucose concentration in the portal circulation. Plasma glucose is sensed and taken up by the β-cell *via* facilitated diffusion by the specific glucose transporter (GLUT)-2. Subsequently, glucose is metabolised by the cell, which sets free energy in the form of adenosine tri-phosphate (ATP). The increase in intracellular ATP induces a closure of the ATP-dependent potassium channel at the cell membrane of the β-cell. This causes a depolarisation of the cell membrane, which leads to an opening of the voltage-dependent calcium channels and an inflow of calcium ions into the cell. The increase in intracellular calcium concentration eventually leads to the release of insulin from the granulae *via* exocytosis (Fig. 1)[66,67]

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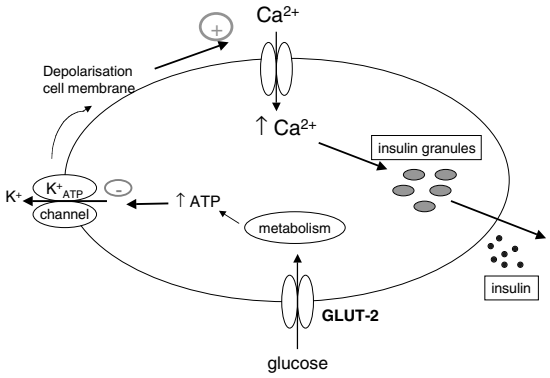

Figure 1.
See text for explanation (section 1.2.2 insulin secretion, page 18).

Several phases of insulin secretion can be identified: (i) basal insulin secretion is the way insulin is released in the post-absorptive state; (ii) the cephalic phase of insulin secretion is evoked by the sight, smell, and taste of food (before any nutrient is absorbed by the gut), and is mediated by pancreatic innervation; (iii) first-phase insulin secretion is defined as the initial burst of insulin, which is released in the first 5–10 min after the β-cells are exposed to a rapid increase in glucose (or other secretagogues); (iv) after the acute response, there is a second-phase insulin secretion, which rises more gradually and is directly related to the degree and duration of the stimulus; (v) finally, a third phase of insulin secretion has been described, albeit only *in vitro*. During all these stages, like many other hormones, insulin is secreted in a pulsatile fashion, resulting in oscillatory concentrations in peripheral blood. Oscillations include rapid pulses (recurring every 8-15 min) superimposed on slower, ultradian oscillations (recurring every 80-120 min) that are closely related to fluctuations in the glucose concentration[68-71]. This pulsatile pattern of insulin delivery to the liver is regulated mainly by modulation of insulin pulse mass in response to stimuli. The mass of insulin pulses through the liver is the predominant determinant of hepatic insulin clearance[65].

Table 4. Metabolic actions of insulin at the whole-body level.

	Stimulation of	**Inhibition of**
Liver	glycogen synthesis	gluconeogenesis
	protein synthesis	glycogenolysis
	lipogenesis	ketogenesis
Muscle	glucose transport	
	glycogen synthesis	
	protein synthesis	proteolysis
Adipose tissue	glucose transport	
	lipogenesis	lipolysis

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1.2.3 Hormone action

Insulin is an anabolic hormone, which means that insulin facilitates the storage of energy sources, such as fat and glycogen, and stimulates protein synthesis. Because, physiologically, insulin is secreted following energy intake, insulin not only directs these energy sources towards storage, but simultaneously prevents endogenous release of energy sources (free fatty acids through lipolysis, proteolysis, *de novo* glucose production by the liver and ketogenesis), because these substrates are redundant in times of plenty. The effects of insulin on the various tissues are depicted in Table 4[66,67].

1.3 NORMAL GLUCOSE REGULATION

1.3.1. Glucose homeostasis at the whole-body level

Blood glucose levels are usually tightly regulated between 4-8 mmol/L. Low blood glucose levels are dangerous because brain function depends on glucose, and lack of glucose in the brain can cause seizures, loss off consciousness and death. On the other hand, elevated blood glucose levels can lead to either ketoacidosis or hyperglycaemic hyperosmolar dehydration in the acute situation, which can both eventually result in a coma. Furthermore, prolonged elevation of blood glucose levels can result in micro- (retinopathy, nefropathy, neuropathy) and macrovascular long-term complications.

The tight regulation of plasma glucose levels is achieved by the finely tuned hormonal regulation of glucose uptake by the tissues (rate of disappearance, R_d) on the one hand and glucose production on the other hand (rate of appearance, R_a)[72].

Glucose uptake by peripheral tissues is either insulin-independent (in the brain) or insulin-dependent (in muscle and adipose tissue). The brain cannot store glucose and, as mentioned before, is critically dependent on glucose for its function. Therefore, in the non-fed (= post-absorptive) state a certain level of endogenous glucose production is necessary. Glucose appearing in the post-absorptive state is mainly derived from the liver[73], although the kidney is also capable of glucose production. The amount of glucose produced by the kidney has been reported to be less than 5% after an overnight fast to 20% after a 60-h fast[73]. However, higher estimates of the contribution of the kidney to total post-absorptive gluconeogenesis have been reported. These differences depend on the techniques used to quantify renal glucose production. A significant role for the kidney in carbohydrate metabolism in type 2 diabetes has recently been proposed[74,75]. In healthy individuals the amount of endogenous glucose production (EGP, both liver and kidney) in the post-absorptive state averages 1.8-2.3 $mg.kg^{-1}.min^{-1}$ [73,76-78], which is about 10.0-12.8 $\mu mol.kg^{-1}.min^{-1}$.

Endogenous glucose production comprises 2 pathways: glycogenolysis, which is the breakdown of glucose stored as glycogen, and gluconeogenesis, which is the synthesis of new glucose molecules from precursor molecules like amino acids (mainly alanine), glycerol and lactate.

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Endogenous glucose production is mainly regulated by fluctuations in the insulin/glucagon ratio in the portal vein[79,80]. Following a meal, insulin secretion is stimulated and the increase in portal vein insulin concentration inhibits endogenous glucose production *via* inhibition of glycogenolysis and gluconeogenesis. When the meal has been absorbed, plasma glucose levels decrease, even to a level a little below normal post-absorptive levels. This relative hypoglycaemia leads to increased secretion of glucagon. The subsequent elevation in portal vein glucagon concentration stimulates glycogenolysis and hepatic glucose production[81]. Endogenous glucose production is also influenced by other hormones (cortisol, growth hormone), free fatty acids (FFA), gluconeogenic precursors, paracrine substances (cytokines, prostaglandins) and the autonomic nervous system. All these factors keep endogenous glucose production relatively constant, a process called hepatic autoregulation[82-84].

Insulin-stimulated glucose uptake primarily takes place in skeletal muscle and amounts about 0.5 $mg.kg^{-1}.min^{-1}$ (the remainder of the average basal glucose uptake of 2.0-2.2 $mg.kg^{-1}.min^{-1}$ being utilised by the brain [1.0-1.2 $mg.kg^{-1}.min^{-1}$] and red blood cells)[85,86]. Glucose taken up in the muscle can either be oxidised to pyruvate (aerobic glycolysis) or lactate (anaerobic glycolysis) or stored as glycogen (non-oxidative glucose metabolism). Insulin-stimulated glucose oxidation seems to be bound to a maximum, making non-oxidative glucose disposal quantitatively the most important[87].

Of the three, for diabetes mellitus pathogenetically important, insulin-sensitive tissues, adipose tissue is the most sensitive for insulin. The EC_{50} value (i.e., the molar concentration of insulin that produces 50% of the maximum possible response that insulin is capable of) for suppression of lipolysis by insulin is between 7 and 16 μU/mL[76,88-92], whereas the EC_{50} values

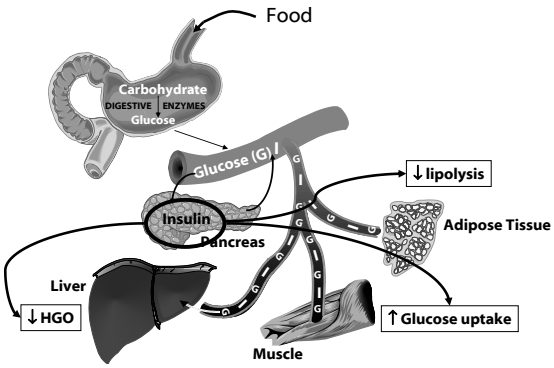

Figure 2.

The sight, smell and taste of food already stimulate insulin secretion. However, the rise of serum glucose levels following the consumption of a meal elicits a much more pronounced response (see text on page 19). Subsequently, insulin suppresses endogenous glucose production and lipolysis and stimulates whole-body glucose uptake. The duration of the increased insulin secretion following a meal is related to the degree and duration of hyperglycaemia.

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for suppression of EGP of the liver and stimulation of glucose uptake in skeletal muscle, in normal subjects, are 26 μU/mL and 58 μU/mL, respectively[93].

The differences in the insulin dose-response curve between the various tissues are necessary for normal glucose and lipid metabolism. During an overnight fast, serum insulin levels are sufficiently low as to not to inhibit lipolysis (which provides free fatty acids and hence ketone bodies for the brain and glycerol for gluconeogenesis) and endogenous glucose production (providing glucose for the brain), but, on the other hand, are not high enough for maximum stimulation of (skeletal muscle) glucose uptake. After a meal, serum insulin levels rise, which stimulates glucose uptake and inhibits lipolysis and glucose production. The latter is achieved directly, by inhibition of gluconeogenesis and glycogenolysis, as well as indirectly, *via* inhibition of lipolysis, which diminishes the supply of glycerol and free fatty acids to the liver[66,67]. Fig. 2 shows what happens when a meal has been consumed.

1.3.2. Insulin signalling, molecular mechanisms regulating glucose uptake

Glucose transport and metabolism, protein synthesis and gene expression are all regulated by activation of the insulin-signalling pathway. Insulin signalling aimed at increasing the rate of glucose transport will be discussed below.

Glucose cannot pass the lipid bilayers of the cell membrane and needs a transporter to enter the cell. GLUT-4 is the main insulin-responsive glucose transporter and is located primarily in skeletal muscle cells and adipocytes. In unstimulated fat or muscle cells, 3-10% of GLUT-4 is located at the cell surface and more than 90% is located inside the cell in distinct vesicles[94]. In response to insulin, exercise and contraction, GLUT-4- containing vesicles move to and fuse with the plasma membrane, thereby increasing the number of GLUT-4 molecules in the membrane and, hence, increasing the rate of glucose transport into the cell[94]. Insulin elevates the exocytic rate of GLUT-4 and reduces its endocytotic rate only minimally. A review[95] on the different intracellular compartments containing GLUT-4 and the proteins that form the cytoskeleton along which GLUT-4 travels is beyond the scope of this thesis; it has not been investigated here.

Insulin is an important mediator of insulin-stimulated glucose transport that begins with binding of insulin at its receptor leading to a signalling cascade that eventually leads to the translocation of GLUT-4 to the cell membrane.

The heterotetrameric insulin receptor consists of 2 extracellular, ligand binding α-subunits and 2 transmembrane β-subunits containing tyrosine kinase domains[96,97]. When insulin binds to specific regions of the α-subunit, a rapid conformational change results in phosphorylation of the intracellular tyrosine residues on one half of the receptor dimer by the kinase domain of the other half, a process called autophosphorylation[98-100]. The phosphotyrosines on the insulin receptor can now serve as docking sites for phosphotyrosine binding (PTB)-domains on other proteins, such as insulin receptor substrates (IRS-1 to 4), Shc and Gab-1[101].

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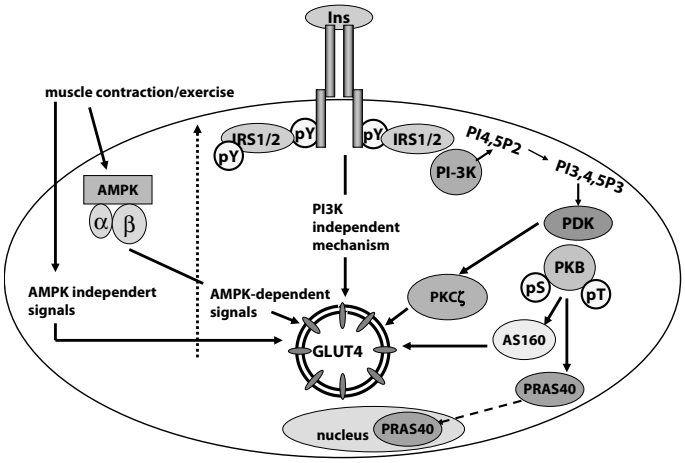

Figure 3.

Binding of insulin at the insulin receptor leads to phosphorylation of the receptor and insulin receptor substrates (IRS). Activated IRS-1 and -2 form a complex with phosphatidylinositol 3-kinase (PI3K) and this IRS/PI3K complex subsequently catalyses the formation of 3′-phosphoinositides (phosphatidyl-inositol-3,4-biphosphate [PIP2] and phosphatidyl-inositol-3,4,5-triphosphate [PI3P]). PIP3 attracts phosphoinositide-dependent kinase-1 (PDK-1) to the cell membrane and the complex subsequently activates protein kinase C (PKC) or protein kinase B (PKB/Akt), which are both involved in GLUT-4 trafficking to the cell membrane. The PKB/Akt substrate AS160 has recently been discovered as an intermediate in this process. Insulin-independent pathways involved in GLUT-4 translocation involve adenosine monophosphate-activated kinase (AMPK)-dependent (contraction, hypoxia) and -independent pathways.

IRS-1 and -2 appear to be the important mediators of insulin signalling in humans. IRS-1 is specifically involved in skeletal muscle and IRS-2 in adipose tissue insulin signalling[102]. Tyrosine phosphorylated IRS recruits and activates signalling molecules with src2-homology (SH2) domains, including phosphatidylinositol 3-kinase (PI3K)[103].

The IRS-PI3K complex catalyses the formation of 3′-phosphoinositides (phosphatidyl-inositol-3,4-biphosphate [PIP2] and phosphatidyl-inositol-3,4,5-triphosphate [PI3P]). PI3P serves as an allosteric regulator of phosphoinositide-dependent kinase (PDK), attracting PDK-1 to the cell membrane. There, PDK-1 activates (by phosphorylation) downstream mediators, such as protein kinase B (PKB/Akt) and atypical protein kinase C (aPKC, PKCζ/λ).

PKB/Akt is a serine/threonine kinase with 3 different isoforms, Akt 1, 2 and 3. Akt 2 is essential for normal glucose homeostasis[104,105]. After co-localisation with PDK-1[106], PKB/Akt is activated by phosphorylation of its two principal regulatory sites, Thr308 and Ser473[107]. Phosphorylation of both sites is essential for activation of PKB/Akt. Following activation, PKB/Akt dissociates from the cell membrane to affect metabolic processes[108,109]. Parts of the activated PKB/Akt also translocate to the nucleus to affect gene expression (see Fig. 3). The metabolic processes affected by PKB/Akt are glucose transport (*via* a stimulatory effect on GLUT-4 translocation) and glycogen synthesis. By inactivating glycogen synthase kinase-3 (GSK-3) the inhibitory action of GSK-3 on glycogen synthase[110] is abrogated and glycogen synthesis is stimulated[111].

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With respect to the stimulatory effect of activated PKB/Akt on the translocation of GLUT-4 to the cell membrane, numerous studies have linked PKB/Akt to the regulation of glucose metabolism but the endogenous substrates regulating these responses are only beginning to be identified. Recent evidence suggests that the protein Akt substrate of 160 kDa (AS160) is involved as an intermediary in this process. AS160 is a protein containing a GTPase-activating domain (GAP) for Rab proteins, which are small G-proteins required for membrane trafficking[112,113]. Phosphorylation of AS160 is required for the insulin-induced translocation of GLUT4 to the plasma membrane in 3T3-L1 adipocytes[114]. Another recently discovered PKB/Akt substrate, proline-rich Akt-substrate 40 (PRAS40, also known as Akt1 substrate 1(Akt1S1))[115,116], is ubiquitously expressed and appears to be localised in the nucleus[116,117]. In response to growth factors, PRAS40 is phosphorylated on Thr246 *via* a PI3K- and PKB/Akt-dependent mechanism[115,117]. Phosphorylation of PRAS40 facilitates the binding of 14-3-3-proteins *in vitro*, and this protein complex has been implicated in nerve growth factor (NGF) mediated neuroprotection from ischaemia[117]. Although, PRAS40 is phosphorylated in response to insulin-treatment of cultured cell lines[115,118], it is as yet unknown whether this protein is involved in physiological insulin action.

As mentioned earlier, GLUT-4 translocation and, hence, glucose uptake can also be mediated *via* insulin-independent pathways, involving AMP-activated protein kinase (AMPK)[119] and other intermediates[120]. Interestingly, AS160 contains motifs similar to sequences of proteins that are phosphorylated by protein kinase C (PKC)[121] and AMPK[122]. In fact, muscle contraction phosphorylated AMPK, Akt and AS160 in isolated rodent muscle and chemical activation of AS160 caused AS160 phosphorylation[123]. Possibly, AS160 may act as a common effector of insulin and exercise signalling to recruit GLUT-4 to the plasma membrane.

Another PDK-1 substrate (*via* PI3-kinase) is atypical protein kinase C. In the liver aPKC regulates the expression of sterol regulatory element binding protein-1c (SREBP-1c), a transcription factor that activates numerous genes, including fatty acid synthase (FAS) and acetyl-coenzyme A carboxylase, that control lipid synthesis in the liver[124].

The insulin signal also has to be terminated in order to maintain metabolic control; this is established *via* specific phosphatases. Protein tyrosine phosphatase-1B (PTP1B) is a physiologic negative inhibitor of insulin signalling. By dephoshorylating the activated insulin receptor it terminates the insulin signal transduction[125]. In addition, SH2-domain-containing inositol phosphatases SHIP1 and SHIP 2 terminate PI3K signalling *via* dephosphorylation of the 5-position of the inositol ring of PIP3, to produce $PI(3,4)P_2$. The phosphatase PTEN (phosphatase and tensin homologue) dephosphorylates the 3-position on PIP3, producing $PI(4,5)P_2$ [126]. All three phosphatases can be regarded as potential therapeutic targets for type 2 diabetes mellitus.

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1.4. TYPE 2 DIABETES MELLITUS

Type 2 diabetes mellitus is a chronic, multifactorial disease characterised by a combination of impaired insulin secretion by the pancreatic β-cells and insulin resistance of target organs, leading to hyperglycaemia. A diagnosis of diabetes mellitus is made when at least one of these three criteria is met: (i) symptoms of diabetes (polyuria, polydipsia, unexplained weight loss) with a casual blood glucose concentration > 11.1 mmol/L, (ii) fasting plasma glucose (FPG) level over 7.0 mmol/L, (iii) 2-h plasma glucose level > 11.1 mmol/L during an oral glucose tolerance test (OGTT)[127,128]. If no symptoms are present, one of these criteria must be present on a subsequent day.

Both conditions, i.e., deficient insulin secretion and insulin resistance, are necessary for diabetes mellitus to occur. Insulin resistance and a disturbed first-phase insulin response occur at an early stage in the development of type 2 diabetes mellitus. There seems to be a continuum from normal glucose tolerance to diabetes mellitus. Insulin resistance leads to increased insulin secretion by the pancreatic β-cell. This increase in insulin secretion is sufficient to offset hepatic insulin resistance (thereby maintaining a normal rate of basal hepatic glucose production) and to overcome the defect in muscle glucose uptake. At this moment, normal glucose levels are achieved at the expense of elevated serum insulin levels. In the second phase, the β-cells fail to compensate for the insulin resistance during glucose loads (as occurs during meals), leading to a condition known as impaired glucose tolerance (IGT). The cause is a disturbed first-phase insulin response, which normally suppresses endogenous glucose production. Over the years, the β-cell function deteriorates and when insulin secretion is no longer able to compensate for the insulin resistance hyperglycaemia ensues and a diagnosis of type 2 diabetes mellitus is made[22,129,130]. The relation between insulin secretion and insulin sensitivity is shown in Fig. 4 and the time-course of type 2 diabetes mellitus in Fig. 5.

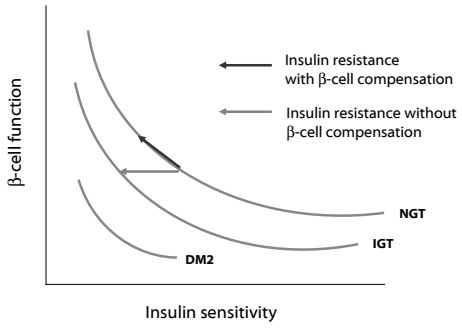

Figure 4.
In people with normal glucose tolerance (NGT), the relation between insulin sensitivity and β-cell function is curvilinear. See text for explanation (page 25).

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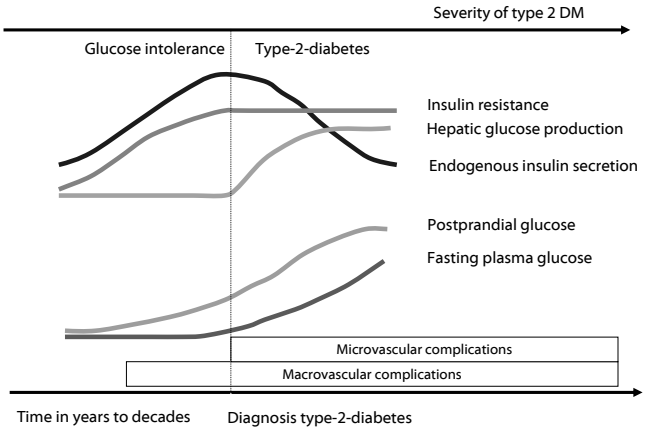

Figure 5.
Time course of type 2 diabetes mellitus. See text (page 25) for explanation.

1.4.1. Insulin resistance at the whole-body level

Insulin resistance at target organs leads to decreased glucose uptake, increased glucose production and increased whole-body lipolysis. Therefore, in patients with type 2 diabetes mellitus, basal glucose production is significantly elevated, leading to fasting hyperglycaemia. In addition, following a meal, insulin resistance leads to inadequate stimulation of (skeletal muscle) glucose uptake and insufficient suppression of endogenous glucose production and lipolysis. The result is postprandial hyperglycaemia.

The incapability to suppress whole-body lipolysis substantially contributes to the increased endogenous glucose production and diminished glucose uptake. Firstly, NEFAs increase endogenous glucose production by stimulating key enzymes involved in gluconeogenesis and by providing the energy needed for glucose production[22]. Secondly, the glycerol formed by triglyceride hydrolysis serves as a gluconeogenic substrate. Thirdly, free fatty acids impair insulin stimulated glucose uptake. Besides substrate competition (Randle effect)[131], impairment of insulin signalling appears to be responsible for this effect[132] (see next section).

1.4.2 Molecular mechanisms of insulin resistance

Skeletal muscle

Over 80% of insulin-stimulated glucose disposal takes place in skeletal muscle[86]. The main defect in patients with type 2 diabetes mellitus seems to reside in non-oxidative glucose disposal (NOGD), i.e., glycogen synthesis[133], the major pathway for overall glucose metabolism. With increasing obesity and insulin resistance, insulin-stimulated NOGD becomes more

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impaired[134,135]. In patients with overt diabetes mellitus, the rate of glycogen formation was 60% that of normal subjects[133].

Possible mechanisms involved in decreased glycogen synthesis could either be decreased hexokinase activity, diminished glycogen synthase activity or impaired GLUT-4 translocation. Shulman *et al*. using ^{31}P-and ^{13}-C-nuclear magnetic resonance (NMR) spectroscopy showed that the defects were not at the level of hexokinase[136] or glycogen synthase[137] activity, but that impaired glucose transport appears to be the prime defect in insulin-stimulated glycogen synthesis in type 2 diabetic patients. The defects in glucose transport can either be due to defects in the glucose transporter itself or in translocation of GLUT-4 to the cell membrane.

Polymorphisms of the gene encoding GLUT-4 are very rare[138-140] in patients with type 2 diabetes and have the same prevalence in non-diabetic subjects. In addition, GLUT-4 protein and mRNA expression are equal[141,142] or even higher[143] as compared with normal subjects. However, GLUT-4 does have an abnormal subcellular distribution in insulin-resistant subjects with or without diabetes[144]. This indicates that translocation of GLUT-4 from intracellular compartments to the plasma membrane is the prime defect. Hence, defects in the signalling cascade leading to GLUT-4 translocation have been extensively investigated. It appeared that exercise (i.e., non-insulin dependent)-induced GLUT-4 translocation is normal in type 2 diabetic patients[145], but that insulin-stimulated GLUT-4 translocation is impaired[146]. Several defects in the insulin-signalling pathway have already been found and will be discussed below.

Insulin binding at the insulin receptor and protein expression of the insulin receptor are normal in skeletal muscle of patients with type 2 diabetes[147-149]. Both impaired[147,150,151] and normal[149,152,153] receptor tyrosine kinase phosphorylation and/or activity have been reported in subjects with diabetes. However, it is widely believed that the disturbance in GLUT-4 translocation in type 2 diabetes mellitus is due to a post-receptor defect.

IRS-1 is the first molecule downstream in the insulin-signalling cascade and plays a key role in skeletal muscle insulin signalling. In humans, IRS-1 polymorphisms are significantly more common in type 2 diabetic patients than in controls[154,155], but their role in the development of insulin resistance and type 2 diabetes is unclear[103]. Furthermore, in obese insulin- resistant subjects[156,157] and moderately overweight type 2 diabetic patients[149,156,158-160], insulin-stimulated IRS-1 phosphorylation in skeletal muscle is decreased as compared to control subjects, whereas protein expression is not altered[149,156,159]. This defect can already be found in normoglycaemic relatives of type 2 diabetic patients[161]. The cause seems to be serine/threonine phosphorylation of IRS-1, which thereby loses its ability to act as a substrate for the tyrosine kinase activity of the insulin receptor and inhibits its capacity to bind to and activate downstream effector molecules such as PI3K[162,163]. Here, a link with adipocyte biology (and obesity) can be made, since circulating FFAs and TNF-α have been found to increase serine phosphorylation of IRS-1[132].

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PI3-kinase is central in the insulin-signalling cascade; however, its activation is necessary but not sufficient for the metabolic actions of insulin. A common polymorphism of the p85-α subunit of PI3K (Met326Ile) was found in two percent of a Caucasian study population in homozygous form, leading to a 32% reduction in insulin sensitivity during an intravenous glucose tolerance test as compared to wild type and heterozygous carriers. The frequency of the polymorphism is not increased in diabetes however[164], but insulin-stimulated PI3K activity is impaired in obese subjects [156], as well as in moderately overweight type 2 diabetic patients[156,158,159,165].

Little is known about the physiological regulation of PDK-1, but thus far insulin action on PDK-1 appears to be normal in insulin-resistant skeletal muscle[158]. With respect to PKB/Akt, unravelling its role in insulin resistance has been complicated by the existence of three isoforms. It appears that Akt 2 is essential in glucose homeostasis, Akt 2 knockout mice having insulin resistance and a diabetes mellitus-like syndrome[104]. In humans, recent studies have detected a missense mutation in the kinase domain of PKB-β (Akt2) in a family of severely insulin-resistant patients that was preserved over three generations[166]. Not only was the mutant Akt unable to phosphorylate downstream effectors in the insulin-signalling pathway, but it also inhibited phosphoenolpyruvate carboxykinase (PEPCK), a gluconeogenic enzyme. In humans with type 2 diabetes mellitus, basal PKB/Akt activity was similar to controls. Two *in vivo* studies showed normal insulin-stimulated activation of PKB/Akt[165,167] in patients with type 2 diabetes mellitus, although one study used supraphysiological concentrations of insulin[165]. In contrast, *in vitro* experiments showed decreased insulin-stimulated PKB/Akt activity at high levels and normal activity at low insulin levels[168] in human muscle strips of type 2 diabetic patients. The defect seems to be isoform specific[169] and a defect in one isoform might be masked by increased activity of the other.

With respect to the recently discovered Akt substrate AS160, Karlsson *et al*. showed that AS160 phosphorylation is impaired in skeletal muscle from type 2 diabetic patients[170].

Liver

Insulin signalling in the liver differs from that in skeletal muscle (and adipose tissue). In muscle, IRS-1 (*via* PI3K) controls both activation of aPKC and PKB/Akt, whereas in the liver aPKC is controlled (again *via* PI3K) by IRS-2 and PKB/Akt by IRS-1. In muscle and adipocytes, aPKC and PKB/Akt stimulate the transportation of GLUT-4 to the cell membrane. In the liver, aPKC regulates the expression of SREBP-1c, a transcription factor that activates numerous genes, including FAS and acetyl-coenzyme A carboxylase, that control lipid synthesis in the liver. PKB/Akt in the liver is involved in the control of glucose production[124].

When insulin activates PKB/Akt (*via* IRS-1), this results in the phosphorylation of Foxo-family transcription factors (Foxo-1a,-3a and -4). These Foxo-transcription factors can bind to so-called insulin response elements (IRE) on the promotor regions of (among others) two key gluconeogenic enzymes: PEPCK and the glucose-6-phosphatase catalytic subunit (G6Pase),

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thereby inhibiting their expression[171,172]. Defective IRS-1 signalling to PKB/Akt leads to lack of inhibition of these two enzymes and increased glucose production[124,173].

IRS-2-mediated signalling to aPKC in the liver of diabetic rodents is largely intact or elevated. This might explain the increased very-low-density lipoprotein (VLDL)-triglyceride synthesis in type 2 diabetes[124].

Hepatocyte nuclear factor (HNF) may also play a role in insulin-mediated glucose metabolism in the liver. HNF-1 enhances the effect of insulin on the promoter of the gene encoding G6Pase *via* interaction with an IRE[174]. Knockout mice that are homozygous for a null mutation in the HNF-3 gene have a complex impairment of glucose metabolism with persistent hypoglycaemia[175]. Finally, HNF-4 is involved in the PI3K/PKB/Akt-dependent stimulation of glucokinase gene expression by insulin, a mechanism involved in increasing glycolysis[176]. On the molecular level HNF-4 seems to interact with Foxo-1[177]. However, although genetic defects of some of the HNF transcription factors play a role in some forms of maturity-onset diabetes of the young (MODY), thus far no evidence exists that HNF-transcription factors are involved in type 2 diabetes mellitus.

GSK-3, an enzyme regulating glycogen synthesis, is a substrate of PKB/Akt. Normally, GSK-3 is constitutively active, phosphorylating glycogen synthase (GS), which becomes inactive and thus glycogen synthesis is inhibited. Insulin promotes glycogen synthesis *via* PKB-mediated inhibition of GSK-3. Defective glycogen synthesis is not only evident in skeletal muscle of patients with insulin resistance but also in the liver. Polymorphisms in the glycogen synthase gene have been described in insulin-resistant patients, the most frequent being the XbaI and Met416Val mutations within intron 14 and exon 10, respectively[178].

In conclusion, in the liver impaired insulin signalling from IRS-1 to PKB/Akt leads to increased glucose production *via* inhibition of gluconeogenic enzymes. In addition, glycogen synthesis is inhibited and, at least in rodents, impaired IRS-2 signalling to aPKC leads to increased VLDL synthesis. Unfortunately, ethical considerations do not permit liver biopsies in humans to study the pathogenetic abnormalities in patients with type 2 diabetes mellitus.

Adipose tissue

About 10% of whole-body glucose uptake occurs in adipose tissue. This might suggest that adipose tissue is of minor importance in insulin-stimulated glucose disposal and in insulin resistance. However, in mice, adipose-tissue-specific GLUT-4 knockout leads to a similar degree of insulin resistance in muscle and liver as muscle-specific GLUT-4 ablation[179,180]. In addition, muscle GLUT-4 depletion is associated with a markedly enhanced glucose uptake in adipose tissue[181]. Hence, there seems to be cross-talk between adipose tissue and skeletal muscle, and adipose tissue seems to be of major importance in the development of insulin resistance. This will be discussed in Chapter 2.

Insulin-stimulated glucose uptake in adipose tissue takes place *via* the same mechanism as in skeletal muscle: insulin signalling leading to GLUT-4 translocation. However, discrepan-

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cies have been found as to the defects in the insulin-signalling cascade in type 2 diabetic patients, between adipose tissue and skeletal muscle cells. In adipose tissue defects are related to decreased protein expression, whereas this is normal in skeletal muscle. Hence, IRS-1 phosphorylation in adipose tissue of patients with type 2 diabetes is decreased because of a decreased IRS-1 protein expression (by 70%) and PI3K activity is decreased to the same extent by decreased protein expression[182]. In addition, in adipose tissue IRS-2 is capable to compensate for changes in IRS-1[182], a phenomenon that does not seem to occur in skeletal muscle[149].

PKB/Akt activation is also impaired in adipose tissue of type 2 diabetic subjects, primarily *via* a reduction in insulin-stimulated serine phosphorylation[183]. GLUT-4 protein and mRNA expression are also substantially reduced in adipose tissue of type 2 diabetic patients[184], in contrast to the normal expression in skeletal muscle[141,142,185].

The main interest in the role of adipose tissue in whole-body insulin resistance has been on so called adipocytokines (or even better, adipokines, since not all proteins secreted by adipocytes are cytokines), proteins secreted by the adipocyte that might induce insulin resistance. This will be discussed shortly below and more extensively in Chapter 2.

1.4.3 How are changes in skeletal muscle insulin resistance induced?

Both FFAs and several adipokines derived from adipose tissue can influence insulin sensitivity.

It has been recognised for some time that insulin sensitivity is inversely related to fasting plasma FFA levels[186-188]. Furthermore, a strong inverse relationship has been demonstrated between intramyocellular lipid (IMCL) levels and skeletal muscle insulin sensitivity[189-192]. Endurance-trained athletes also have high levels of IMCLs, but they have a high insulin sensitivity[193]. It seems that the capacity to oxidise these IMCL is of prime importance in inducing insulin resistance. This has also been called metabolic flexibility[194,195]. It appears that metabolically-flexible persons (lean, aerobically fit, healthy individuals) have a preference for fat oxidation in muscle during fasting and that during insulin stimulation this fat oxidation is suppressed and glucose oxidation is stimulated[196]. In metabolically-inflexible people there is both a blunted preference for fat oxidation in the fasted state and a blunted suppression of fat oxidation upon insulin stimulation[197-199]. Hence, athletes appear to have a high IMCL content because they prefer to oxidise fat, with the intramyocellular triglycerides (present in high concentration) serving as an energy reservoir. Whereas in obese and/or type 2 diabetic patients, elevated IMCL seem to be secondary to a structural imbalance between plasma FFA availability, fatty acid re-esterification and oxidation. The defect in fat oxidation seems to reside in the mitochondria[200].

Apart from defects in intracellular fatty acid oxidation and or re-esterification, another mechanism leading to increased IMCL might be *via* increased fatty acid uptake. Long-chain fatty acids (LCFA) enter cells mainly by protein-mediated membrane transport, along with

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passive diffusional uptake[201]. One of these proteins is the fatty acid transporter (FAT)/CD36. FAT/CD 36, like GLUT-4, is usually located in the cytoplasm and can be acutely translocated to the sarcolemma by stimuli such as contraction and insulin[202-206]. Both in animal models[207] of insulin resistance, as well as in obese non-diabetic and non-obese diabetic humans[202], FAT/CD36 membrane expression was increased as compared to lean controls. Moreover, this increased sarcolemmal FAT/CD36 expression was associated with an increase in LCFA uptake[202,208]. In the human study, the increase in LCFA transport led to a 3-fold increase in fatty acid esterification, whereas fatty acid oxidation remained the same, again indicating that the core defect is in mitochondrial fatty acid oxidation[202].

Hence, any perturbation that leads to a defect in mitochondrial fatty acid oxidation (aging, potential type 2 diabetes genes) and/or increased delivery of fatty acids (increased caloric intake, obesity, increase in FAT/CD36) can lead to intramyocellular lipid accumulation.

ICML, in turn, can impair insulin signal transduction. It has been proposed that fatty acid metabolites induce a sustained activation of serine/threonine kinases, like protein kinase C isoforms[209-211], IκB kinase-β[212,213] and Jun N-terminal kinase[163,214], which phosphorylate IRS-1 and IRS-2 on serine and threonine sites. Serine-phosphorylated forms of IRS-1 and-2 cannot associate with and activate PI3K, resulting in a decreased activation of GLUT4-regulated glucose transport.

Another adipocyte product, TNF-α, also induces insulin resistance *via* serine/threonine phosphorylation of IRS-1, thereby inhibiting insulin signalling[215-217].

An extensive review of adipokines and their potential impact on insulin sensitivity is presented in Chapter 2.

1.4.4. Visceral obesity and insulin resistance

A chronic imbalance between energy intake and energy expenditure will eventually lead to obesity. Epidemiological studies have shown an association between severe obesity and increased mortality[20,218,219]. In more moderate obesity, regional distribution of fat seems to play an important role in the risk for (cardiovascular) morbidity and mortality[220-224]. As early as 1947 Vague put forward that "android or male-type obesity", is more often associated with increased mortality and risk for diabetes, hypertension, hyperlipidaemia and atherosclerosis than the "gynoid" (lower body or gluteofemoral) female-type of fat distribution[225]. Later, studies using imaging techniques (computer tomography [CT] and magnetic resonance imaging [MRI]) showed that the detrimental influence of abdominal obesity on metabolic processes is related to the intra-abdominal, i.e., visceral, fat depot and not to subcutaneous fat deposition[226-230]. However, other investigators have challenged a primary role for visceral adipose tissue in insulin resistance and showed that truncal subcutaneous adipose tissue is also strongly and inversely related to insulin-stimulated glucose disposal (reviewed by Garg *et al*.[231]). Moreover, given the fact that visceral adipose tissue contributes only 10-15% of the total systemic free fatty acid flux (the majority of FFAs being derived from non-splanchnic

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adipose tissue from the rest of the body)[232,233], they questioned the impact of excess visceral adipose tissue on peripheral insulin sensitivity. However, liposuction of subcutaneous abdominal adipose tissue does not improve insulin sensitivity[152]. Moreover, although only 10-15% of fatty acids are derived from visceral adipose tissue, their drainage *via* the portal vein directly to the liver could imply another, more deleterious mechanism of action than delivery of FFAs (and adipokines) to the liver *via* the hepatic artery. Hence, it is not clear yet whether visceral adipose tissue is the culprit or whether the combination of truncal subcutaneous adipose tissue with visceral adipose tissue are involved in insulin resistance. Finally, it is also unclear whether abdominal obesity causes insulin resistance or is merely the reflection of the pathologic state.

Notwithstanding these uncertainties, available evidence does support an important role for adipose tissue in, possibly, generating and, at least, maintaining whole-body insulin resistance. Several theories have been put forward to explain the link between obesity and insulin resistance. The portal/visceral hypothesis[234] states that visceral fat cells are metabolically more active (especially lipolytic activity) and are less responsive to the antilipolytic effects of insulin as compared to other adipose tissue depots. Subsequently, the high flux of FFAs and glycerol derived from these visceral fat cells, through their unique drainage directly into the liver *via* the vena portae, would induce hepatic insulin resistance, increase hepatic glucose production and increase VLDL-triglyceride production. However, as mentioned in the previous paragraph, the portal/visceral hypothesis cannot link visceral adiposity to peripheral insulin resistance given the fact that only 10-15% of the total FFA flux is derived from visceral adipose tissue, unless some other factor released by visceral adipose tissue induces peripheral insulin resistance and/or visceral fat cells have impaired functioning in insulin-resistant states leading to decreased triglyceride storage and partitioning of fat storage into other organs. This is where 2 new theories emerge: (i) the adipocyte as an endocrine organ and (ii) the ectopic fat storage theory[235].

To begin with the first theory, adipose tissue not merely stores triglycerides but actively secretes lipid moieties such as FFAs and proteins that are called adipokines[236,237]. Quantitatively, FFAs are the most important. Moreover, elevated FFAs play a major role in inducing whole-body insulin resistance. Chronically elevated FFA levels stimulate hepatic glucose production and VLDL-triglyceride synthesis, leading to hyperglycaemia and dyslipidaemia[22]. Furthermore, chronically elevated FFA concentrations impair insulin signalling *via* serine/threonine phosphorylation of IRS-1, thereby decreasing insulin-stimulated glucose transport[132]. In addition, chronic exposure to high FFA levels to the pancreas can impair insulin secretion[238-240]. Several of the adipokines produced by adipose tissue (adiponectin, leptin, TNF-α) can also induce insulin resistance, this will be discussed in Chapter 2.

The theory of ectopic fat storage states that a diminished capacity of fat cells to store fat as triglycerides leads to lipid storage in other organs, such as the liver, pancreas and muscle (overflow hypothesis[241]/ectopic fat storage[235]). This causes steatosis hepatis with hepatic in-

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sulin resistance, impaired insulin secretion and skeletal muscle insulin resistance (*via* IMCL and impaired insulin signalling, see previous section)[242]. The cause of ectopic fat storage is unclear but an association with enlarged adipocytes has been found[243]. This might be the result of impaired proliferation or differentiation of adipocytes. On the other hand, impaired whole-body fat oxidation might account for the ectopic accumulation of fat[244].

Hence, adipose tissue plays an important role in generating and maintaining insulin resistance *via* the excessive production of FFAs and insulin-resistance-provoking adipokines (TNF-α, IL-6, resistin, leptin and many others), possibly related to specific fat depots (visceral fat mass) and/or malfunctioning of adipocytes (in these specific depots?). Moreover, a diminished capacity to store fat leads to ectopic fat storage with lipotoxicity-induced impairments in function of insulin-responsive tissues such as the liver, muscle and pancreas.

1.5. OBESITY AND TYPE 2 DIABETES; TREATMENT REASONS, GOALS AND OPTIONS

Both obesity associated with insulin resistance (Table 1) and type 2 diabetes mellitus impose a major health risk. Patients with type 2 diabetes mellitus have an increased morbidity and mortality due to long-term micro- (retinopathy, neuropathy, nefropathy) and macrovascular complications. Patients with type 2 diabetes have a 2-4 fold increased relative risk (RR) for the development of myocardial infarction (MI), peripheral arterial disease and stroke[220] and approximately 65% of patients with type 2 diabetes die as a result of a cardiovascular event[245]. This increased risk is associated with chronic hyperglycaemia and an increase in cardiovascular risk factors such as hyperglycaemia, dyslipidaemia and hypertension. Hypertension occurs in up to 60% of patients with diabetes[246], and if diabetes and hypertension co-exist they exert a multiplicative effect on the absolute risk of a cardiovascular event[247]. Small dense LDL-cholesterol, high serum triglycerides and low HDL-cholesterol characterise diabetic dyslipidaemia. Hence, treatment of patients with type 2 diabetes should not only focus on glucoregulation but also on hypertension and dyslipidaemia.

Mainly based on two large prospective randomised studies investigating the effect of intensive blood glucose-lowering therapy on glycaemic control and occurrence of micro-and macrovascular complications in type 1 and type 2 diabetic patients[248,249], the treatment goals for glucoregulation in patients with type 2 diabetes as set by the ADA are: fasting plasma glucose level < 7.0 mmol/L, postprandial glucose level < 10 mmol/L and HbA_{1c} < 7%. In addition, systolic blood pressure should be lower than 130 mmHg and diastolic blood pressure under 80 mmHg. LDL-cholesterol should be < 2.6 mmol/L, triglycerides < 1.7 mmol/L and HDL-cholesterol > 1.1 mmol/L[250].

Theoretically, treatment of hyperglycaemia in patients with type 2 diabetes can consist of decreasing the need for insulin and/or increasing available insulin. The need for insulin

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can be diminished either by decreasing postprandial glucose levels (diet, acarbose[251]) or improving insulin sensitivity. The latter can be achieved *via* restriction of caloric intake[252], weight loss[252], exercise[253], or with drugs: metformin[254,255] or thiazolidinediones[256,257] (perhaps also rimonabant[258] and sibutramine[259], because of their weight-loss-inducing properties, their anorexic effects and possibly *via* a direct beneficial effect on insulin sensitivity). Increasing available insulin can be achieved with insulin secretagogues (sulfonylureaderivatives[254], meglitinides[260]) or by giving exogenous insulin.

Weight loss improves multiple aspects of insulin resistance: glucoregulation, dyslipidaemia, hypertension and others. In addition, it decreases the risk for arthrosis, low back pain, gallstones, cancer, etc. So ideally, weight loss should always be a component of the treatment regimen in obese patients.

Weight loss also improves insulin resistance in obese non-diabetic patients. A beneficial effect of even 5-10% loss of overweight has been shown on dyslipidaemia, hypertension, hyperinsulinaemia and glucose values[261-263]. To date, no effect on incidence rates of myocardial infarction, stroke, cancer and mortality has been demonstrated, however.

Weight loss regimens have been proven difficult to adhere to. In addition, weight loss achieved through diet is often followed by weight regain. Regimens combining a hypocaloric diet (500 to 600 kCal less than needed per day) with behaviour therapy and exercise have been proven the most beneficial with respect to outcomes after 1 year[264]. However, hypocaloric diets often lead to only modest weight loss, whereas morbidly obese patients and obese type 2 diabetic patients need larger weight losses to restore peripheral insulin sensitivity[47,48]. VLCDs and bariatric surgery have been advocated for this purpose.

1.5.1 Bariatric surgery

Surgical procedures to treat obesity have been performed since the 1950s[265] and include truncal vagotomy[266], jaw wiring[267], intragastric balloons and liposuction. Bariatric (weight loss) surgery can be divided into purely restrictive procedures (vertical banded gastroplasty [VBG], laparoscopic adjustable silicone gastric banding [LASBG]) and combined restrictive and malabsorptive procedures (Roux-en-Y gastric bypass [GBP], biliopancreatic diversion [BPD])[57,268]. The latter induce larger weight losses and, hence, greater improvements in hypertension, dyslipidaemia, glucose metabolism and hyperinsulinaemia as compared to the purely restrictive techniques[50,56]. However, they are irreversible, sometimes leading to greater weight losses than necessary and also to nutritional deficiencies. Patients have to take vitamin supplements for the rest of their lives. LASBG is the most popular form of bariatric surgery in the Netherlands (and the rest of Europe), because it can be performed laparoscopic and therefore has fewer perioperative complications and it is reversible. In addition, some influence as to the amount of food intake can be exerted *via* inflation/deflation of the saline-filled gastric ring[57,268]. This procedure also has disadvantages however, an estimated 7-17% of the

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patients has to be re-operated because of band erosion, dislocation or leakage or because of esophageal dilatation[269,270].

Bariatric surgery can induce large weight losses (20-50% of body weight) with a higher likelihood of maintaining weight loss (especially the combined restrictive and malabsorptive procedures) as compared to other weight loss interventions[55,271].

The Swedish Obese Subjects (SOS) study showed that surgically-treated obese subjects had about 25% percent greater weight loss at 10 years follow-up, along with a greater number of persons who no longer had diabetes (if present), hypertriglyceridaemia, low HDL-cholesterol concentrations, hypertension and hyperurikaemia as compared with conventionally treated obese subjects. The surgery group also had lower 2- and 10-year incidence rates of diabetes and hypertriglyceridaemia, but not hypercholesterolaemia[43]. Others have reported similar beneficial metabolic effects of bariatric surgery.

Bariatric surgery has also been performed in patients with type 2 diabetes. Although in some studies the number of patients with diabetes were small[52,55], the impressive results found were confirmed in larger studies[51,53]. A recent meta-analysis by Buchwald *et al*. showed that 1417 out of 1846 patients (76.8%) completely recovered from their diabetes following bariatric surgery (in the studies that mentioned complete resolution). The mean reduction in BMI was 14 kg/m^2 and a graded response with respect to diabetes resolution was noted with the greatest effect with BPD, whereas gastric banding was the least effective[56]. A recently published, retrospective chart review of 312 obese patients with type 2 diabetes that underwent biliopancreatic surgery (gastric bypass with biliopancreatic diversion), showed that the beneficial effects on glucose metabolism, dyslipidaemia and hypertension were maintained in most patients even after 10 years follow-up[53].

With respect to the underlying metabolic processes leading to the improvement in glucose metabolism following bariatric surgery, studies in morbidly obese patients have shown an improvement in insulin-stimulated glucose disposal, as assessed with the hyperinsulinaemic euglycaemic clamp technique[47,50,272,273]. Data on endogenous glucose production and whole-body lipolysis are not available. Moreover, in obese type 2 diabetic patients no studies using either of these sophisticated techniques have been performed to date.

1.5.2 Very low calorie diets

VLCDs typically provide less than 800 kCal/day. This can be achieved *via* adjustments of "normal" food intake or *via* commercially available packages. The advantage of the latter is that these products contain all the necessary vitamins, minerals and trace elements, so patients need not to figure out what to eat and what not.

VLCDs are safe[58] and can be used for several weeks to months or even up to one year([274] and own observations). VLCDs can also induce large weight losses[275]. Maintenance of weight loss is usually a problem, necessitating the need for regular dietary counselling and preferably also behaviour therapy.

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Both in obese patients and in obese patients with type 2 diabetes mellitus, VLCDs lead to substantial weight loss and improvements in hyperglycaemia, hyperinsulinaemia, dyslipidaemia and hypertension[58,275-280].

In obese type 2 diabetic patients hyperglycaemia improves already within 4-10 days after the beginning of an energy restricted diet[277,278,281,282]. This appears to be due primarily *via* a decrease in endogenous glucose production. These studies have been performed when some (4-5 kg) weight loss had already occurred, with varying degrees of calorie restriction or in mild type 2 diabetic patients. Surprisingly, there are no studies documenting to what extent carbohydrate and lipid metabolism improve in obese, insulin-treated type 2 diabetic patients after substantial weight loss using a sophisticated method such as the hyperinsulinaemic euglycaemic clamp technique with [6,6-$^{2}H_{2}$]-glucose and [$^{2}H_{5}$]-glycerol.

1.6. AIMS OF THE STUDIES AND OUTLINE OF THE THESIS

Most patients with type 2 diabetes mellitus are obese and both obesity and type 2 diabetes mellitus are associated with insulin resistance. Therefore our **first aim** was to evaluate the role of adipose tissue (which indeed is present in excess in obese and obese diabetic patients) in insulin resistance. For this purpose we reviewed the literature and present a hypothesis which links adipose tissue to insulin resistance (**Chapter 2**).

In **Chapter 3**, we present an example of a hormone produced by adipose tissue (leptin) that is associated with insulin resistance. The relation between serum insulin and leptin is well established in obese patients and patients with diabetes, but not in very obese, largely insulin-treated patients with diabetes. Our **second aim** was to evaluate the relation between fasting serum leptin and fasting serum insulin levels, as well as between fasting serum leptin levels and insulin secretion in a group severely obese type 2 diabetic patients at various moments of energy restriction and weight loss.

Insulin resistance in very obese type 2 diabetic patients makes it often difficult to achieve adequate glycaemic regulation. Energy restriction and weight loss improve insulin resistance and its associated metabolic abnormalities. VLCDs can induce large weight losses but most type 2 diabetic patients are afraid to use these diets along with their blood glucose-lowering medication for fear of hyperglycaemia. Therefore, we wanted to stop all blood glucose-lowering agents at the start of the VLCD. This would also facilitate weight loss and enable us to study glucose metabolism without interfering medication. However, we did not want to induce severe hyperglycaemia or other metabolic derangements. Therefore, our **third aim** was to evaluate whether it is safe to treat very obese, insulin-treated type 2 diabetic patients with a VLCD (Modifast®, 450 kCal/day) and simultaneously discontinue all blood glucose-lowering medication, including insulin (**Chapter 4**).

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Other studies mentioned a decline in blood glucose levels before weight loss occurred, even as early as 7 days after the initiation of a VLCD. Our own clinical observations suggested that blood glucose levels decrease already within 2 days after starting a VLCD. Because we wanted to differentiate later on between the effects of energy restriction *per se* and weight loss *per se* on glucose metabolism, our **fourth aim** was to establish whether blood glucose levels indeed decline as early as 2 days after the initiation of a VLCD and the discontinuation of all blood glucose-lowering agents, including insulin (**Chapter 4**).

Because we wanted to study the effect of calorie restriction and weight loss on lowering blood glucose levels, the patients entering our later studies should preferentially react to the VLCD with a decline in blood glucose levels. Therefore, our **fifth aim** was to find out whether there are discriminating factors that will tell in advance which patients will show a decline in blood glucose levels during weight loss with a VLCD and which patients will not (**Chapter 4**).

Subsequently, our **sixth aim** was to investigate, using the hyperinsulinaemic euglycaemic clamp technique with stable isotopes, at the whole-body level, the mechanisms by which calorie restriction *per se* (2-day VLCD) decreases blood glucose levels in obese insulin-treated type 2 diabetic patients in whom all blood glucose-lowering medication was discontinued at the start of the VLCD (**Chapter 5**). In this same study, our **seventh aim** was to unravel the blood glucose-lowering effect of a 2-day VLCD at the molecular level. To this end, we studied components of the insulin-signalling cascade, GLUT-4 and FAT-CD36 translocation and intramyocellular triglycerides in skeletal muscle biopsies taken on day 0 and day 2 of the diet, both in the basal as well as in the insulin-stimulated situation (**Chapter 6**).

In addition, our **eighth aim** was to differentiate between the effects of calorie restriction *per se* (day 2 of a VLCD) and those of weight loss *per se* (until 50% of overweight was lost), on whole-body glucose and lipid metabolism in obese insulin-treated type 2 diabetic patients in whom again all blood glucose-lowering medication was discontinued at the start of the VLCD (day 0) (**Chapter 7**). Our **ninth aim**, carried out in the same study, was to investigate whether calorie restriction *per se* and weight loss have differential effects on insulin signalling, GLUT-4 and FAT/CD36 translocation and the amount of intramyocellular triglycerides in skeletal muscle biopsies obtained on day 2 of a VLCD and again when 50% of overweight was lost, in the basal situation and during hyperinsulinaemia (**Chapter 8**).

Our **tenth aim** was to investigate whether the weight loss and beneficial metabolic effects of a once-only 30-day VLCD in obese type 2 diabetic patients, who were taken off all blood glucose-lowering therapy during that diet and who received standard outpatient care thereafter (blood glucose-lowering therapy was restarted if deemed necessary by their own doctor), were sustained at 18 months regular outpatient follow-up (**Chapter 9**).

In **chapter 10** the results of our studies are discussed and integrated.

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CHAPTER 2

Adipose tissue as an endocrine organ: impact on insulin resistance

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ABSTRACT

It is well known that obesity is associated with insulin resistance and an increased risk for type 2 diabetes mellitus. Formerly it was postulated that increased lipolysis and consequently free fatty acid (FFA) production, from with triglycerides overloaded fat cells would disrupt glucose homeostasis *via* Randle's hypothesis. Lipodystrophy, however, also leads to insulin resistance. Recently it has become clear that adipose tissue functions as an endocrine organ and secretes numerous proteins in response to a variety of stimuli. These secreted proteins exert a pleiotropic effect. The proteins that are involved in glucose and fat metabolism and, hence, can influence insulin resistance are discussed in this paper. They include leptin, resistin, adiponectin, acylation-stimulating protein, tumour necrosis factor-α and interleukin-6. The stimuli for production and the site and mechanism of action in relation to insulin resistance will be discussed. None of these proteins are, however, without controversy with regard to their mechanism of action. Furthermore, some of these proteins may influence each other *via* common signalling pathways. A theory is presented to link the interrelationship between these adipocyte secretory products and their effect on insulin resistance.

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INTRODUCTION

Type 2 diabetes mellitus is a chronic disease characterised by insulin resistance of the muscle, liver and adipose tissue and an impaired function of the β-cell of the pancreas[1].

The incidence of type 2 diabetes mellitus (type 2 DM) has increased dramatically over the last decades. Nowadays it is the most frequently occurring metabolic disease, affecting over 140 million people worldwide with an expected rise to about 300 million patients in 2025[2]. Epidemiological studies assessing the explanation for this explosion point to an excess caloric intake over metabolic demand and decreased physiological activity as plausible causes. A chronic imbalance between energy intake and energy expenditure eventually leads to obesity, a condition predisposing to insulin resistance and type 2 DM. Of type 2 diabetic patients, 80% are overweight or obese, as defined by a body mass index > 25 and 30 kg/m^2, respectively[3].

In the past, adipose tissue was merely viewed as a passive organ for storing excess energy in the form of triglycerides. Recently, however, it has become clear that the adipocyte actively regulates the pathways responsible for energy balance and that this function is controlled by a complex network of hormonal and neuronal signals.

To discuss all the adipocyte secretory products (Table 1) and all their effects is beyond the scope of this paper. In this review we will focus on the function of the adipocyte in relation to

Table 1. Proteins secreted by adipocytes.

Molecule	Effect
Leptin*	Feedback effect on hypothalamic energy regulation; maturation of reproductive function
Resistin*	Appears to impair insulin sensitivity
Adiponectin*	Improves insulin sensitivity if administered to rodent models of insulin resistance; improves fatty acid transport and utilization
Adipsin*	Required for the synthesis of ASP, possible link between activation of the complement pathway and adipose tissue metabolism.
ASP*	Activates diacylglycerol acyltransferase, inhibits hormone sensitive lipase, stimulates GLUT-4 translocation to the cell surface.
TNF-α*	Mediator of the acute phase response. Inhibits lipogenesis, stimulates lipolysis and impairs insulin-induced glucose uptake, thus leading to insulin resistance and weight loss.
IL-6*	Increases hepatic glucose production and triglyceride synthesis, role in insulin resistance unclear
PAI-1	Potent inhibitor of the fibrinolytic system
Tissue factor	Initiator of the coagulation cascade
Angiotensinogen	Regulator of blood pressure and electrolyte homeostasis.
PGI_2 and $PGF_2\alpha$	Implicated in inflammation and blood clotting, ovulation and menstruation, acid secretion
TGF-β	Regulates growth and differentiation of numerous cell types
IGF-1	Stimulates cell proliferation and mediates many of the effects of growth hormone
MIF	Involved in proinflammatory processes and immunoregulation
aP_2	Involved in intracellular trafficking and targeting of fatty acids
agouti	Might be involved in inducing insulin resistance through increasing intracellular free calcium concentrations

Proteins discussed in this chapter.

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insulin resistance and obesity. Firstly, the differentiation process of the adipocyte will be discussed. Then, some of the adipocyte secretory products that are involved in energy balance regulation and their function will be considered. Finally, some interactions between adipocyte-derived factors that could be involved in inducing insulin resistance will be described.

ADIPOCYTE DIFFERENTIATION

There are two forms of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). BAT serves primarily to dissipate energy, which is done *via* uncoupling protein 1 (UCP-1) in the mitochondria of BAT. Adult humans have only a small amount of BAT. WAT stores energy in the form of triglycerides. It has recently become evident that WAT also secretes a vast amount of so-called adipocytokines, which are involved in maintaining energy homeostasis. This will be discussed in this article.

In humans, the formation of WAT begins during late embryonic development, with a rapid expansion shortly after birth as a result of increased fat cell size as well as fat cell number. Even in adults the potential to generate new fat cells persists. The origin of the adipose cell and adipose tissue are still poorly understood. Our current understanding indicates that a pluripotent stem cell precursor gives rise to a mesenchymal precursor cell, which has the potential to differentiate along mesodermal lineages of myoblast, chondroblast, osteoblast and adipocyte (Fig. 1)[4]. Given appropriate stimuli the preadipocyte undergoes clonal expansion and subsequent terminal differentiation into a mature adipocyte.

In vitro, adipogenesis follows an orderly and well-characterised temporal sequence[4,5]. Initially there is growth arrest of proliferating preadipocytes induced by the addition of a prodifferentiative hormonal mixture (including insulin, a glucocorticoid, an agent that elevates cAMP levels and fetal bovine serum). Growth arrest is followed by one or two rounds of cell division, known as clonal expansion. At about the second day after differentiation induction there is a second, permanent period of growth arrest. Growth-arrested cells are committed to becoming adipocytes and begin to express late markers of adipocyte differentiation at day 3. Cells eventually become spherical, accumulate fat droplets and become terminally differentiated adipocytes by day 5 to 7.

Most of the changes that occur during adipocyte differentiation take place at the gene expression level. Several reports[4,5] have attempted to schematise the stages of adipocyte differentiation as we have here in Fig. 1.

Three major classes of transcription factors that directly influence fat cell development have been identified: the peroxisome proliferator-activated receptor-γ (PPAR-γ), CCAAT/enhancer binding proteins (C/EBPs) and the basic helix-loop-helix family (ADD1/SREBP1c).

The C/EBPs belong to the basic-leucine zipper class of transcription factors which function through homodimeric and heterodimeric complexes with C/EBP family members. Six

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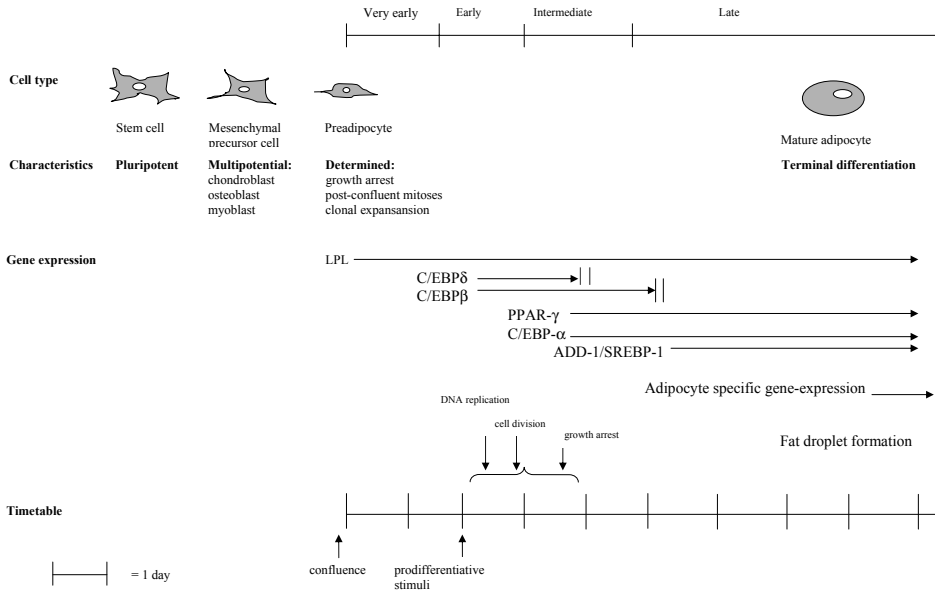

Figure 1.

Addition of mitogens and hormonal stimuli to 3T3-L1 cells leads to a cascade of transcriptional events that account for the expression of most proteins mediating adipocyte function. See text on page 58 to 60 for further explanation.

isoforms have been identified with varying tissue distribution. C/EBP α, β and δ are expressed in both white and brown adipose tissue and are involved in the regulation of adipogenesis[5].

The peroxisome proliferator-activated receptor (PPAR) belongs to the nuclear hormone receptor family. Three isotypes have been identified thus far, PPPAR α, β and γ, each with a different tissue distribution, ligand and metabolic action. All PPARs form a heterodimer with the retinoid X receptor (RXR) and bind to a PPAR-RXR response element on the DNA. Their actions upon ligand binding, however, are completely different. PPAR-γ exists as three isoforms, γ1, γ2 and γ3. PPAR-γ2 is highly expressed in adipose tissue. The thiazolidinediones (TZDs, a new class of oral blood glucose-lowering drugs), which are high affinity synthetic ligands for PPAR-γ, strongly induce adipogenesis and activate the expression of multiple genes encoding for proteins involved in lipid and glucose metabolism[6,7].

Adipocyte determination and differentiation factor 1(ADD1) and sterol regulatory element binding protein 1c (SREBP-1c), which are rodent and human homologues respectively, belong to the basic helix-loop-helix (bHLH) family of transcription factors. ADD1/SREBP1c is expressed in brown adipose tissue, the liver, WAT and the kidney[5]. The expression of ADD1-SREBP-1c is increased early during adipocyte differentiation[4,5]. The protein seems to exert its adipogenic effect through upregulation of PPAR-γ. Furthermore the protein might be involved in the production of an endogenous ligand for PPAR-γ[8]. In addition to its effect on adipogenesis, ADD1/SREBP-1c clearly stimulates many genes involved in fatty acid and cholesterol metabolism[9].

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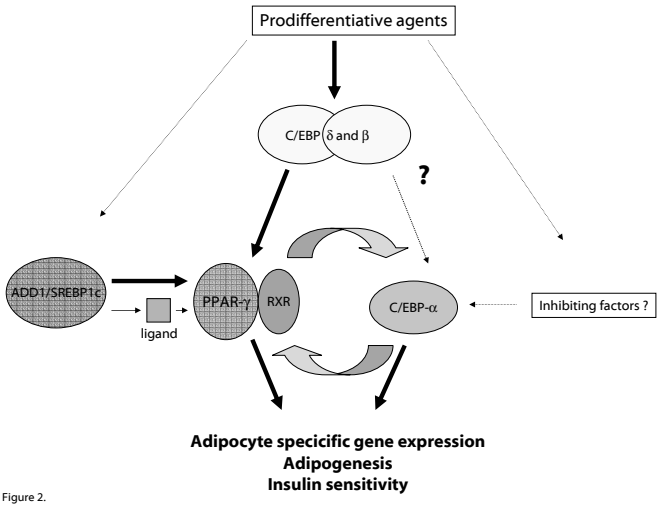

Figure 2.

Figure 2[4,5].

Solid lines indicate direct or indirect transcriptional events. Broken lines indicate less clear interactions. The addition of prodifferentiative agents to 3T3-L1 cells leads to a significant and transient increase of the transcription factors C/EBP β and δ, which in turn mediate the expression of another transcription factor: PPAR-γ. PPAR-γ is also activated by ADD1/SREBP$_{1c}$[8] although the events leading to the activation of ADD1/SREBP$_{1c}$ are not fully understood. PPAR-γ on turn activates C/EBP-α, these two proteins seem to cross regulate each other, thus maintaining their gene expression despite a decline in C/EBP β and δ. Activation of PPAR-γ and C/EBP α leads to the expression of many adipocyte specific proteins involved in glucose and lipid metabolism (LPL, aP2, fatty acid synthase, etc.), adipocyte differentiation and an increase in insulin sensitivity, either via a decrease in triglycerides and fatty acids or via a direct effect on proteins involved in glucose metabolism (PEPCK, GLUT-4).

A summary of the molecular events of adipocyte differentiation, based on our current knowledge, is depicted in Fig. 1 and 2.

ADIPOCYTE SECRETORY PRODUCTS

Leptin

Discovery, structure, genetic locus and sites of expression of leptin

The discovery of leptin (from the Greek *leptos* which means thin) in 1994[10] has led to a renewed and intensified interest in the adipocyte and its role in energy homeostasis. Leptin acts on hypothalamic neuropeptide-containing regions and increased leptin signalling leads to decreased food intake, increased energy expenditure and increased thermogenesis, all promoting weight loss. Apart from these effects, leptin is also involved in glucose metabolism, normal sexual maturation and reproduction, and has interactions with the hypothalamic-pituitary-adrenal, thyroid and growth hormone axes.

Leptin is a protein consisting of 167 amino acids and has a helical structure similar to cytokines. Leptin is the product of the *ob* gene, which is located on chromosome 7q31. Leptin

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is expressed mainly in white adipose tissue. The protein circulates as both free and bound hormone and is cleared among others by the kidneys[11-13].

Modulators of leptin production[12,13]

Leptin levels are positively correlated with the amount of energy stored as fat, so leptin levels are higher in obese people[14,15]. Leptin levels rapidly decrease during fasting[16] and remain low until four to six hours after eating when they begin to rise again[17]. Plasma leptin levels show a diurnal pattern with a nocturnal peak shortly after midnight and a midmorning through between 10 AM and 12 noon[18]. Insulin also plays a role in the regulation of leptin secretion: prolonged insulin infusions markedly increase leptin levels[19,20]. Finally, even after adjustment for body fat mass, women have higher leptin levels than men[15]. At the gene promotor level, it is known that stimulation of PPAR-γ downregulates leptin production[21] whereas C/EBP-α stimulates leptin production[22].

Site of action of leptin and its role as part of an adipostat

Leptin acts through binding at and activation of specific leptin receptor isoforms, which belong to the class I cytokine receptor family[23]. Only the long isoform (*ob-rb*) is able to activate the JAK-(Janus kinase)-STAT (signal transducers and activators of transcription) signal transduction pathway upon leptin binding (Fig. 3). The long form of the leptin receptor is found in several peripheral tissues and in many areas of the brain, including the arcuate, ventromedial and dorsomedial hypothalamic nuclei[24]. These hypothalamic regions are known to be involved in the regulation of appetite, food intake, temperature regulation and body weight. Intracerebral administration of leptin alters the expression of many hypothalamic neuropeptides[25]. By modulating these neurotransmitter systems, leptin has a major role in maintaining energy balance and thus serves as part of an adipostat. During fasting, serum insulin levels fall and the uptake of glucose and lipids by the adipocyte diminishes. This leads to a decreased expression of the *ob*-gene, which is responsible for leptin formation and, hence, the plasma leptin concentration falls. Reduced leptin signalling leads to an increased expression of neuropeptide Y (NPY) and agouti-related protein (AgRP) in the arcuate nucleus of the hypothalamus. NPY and AgRP promote body weight gain by stimulating food intake and decreasing energy expenditure. Another neuronal cell type co-produces cocaine-amphetamine related transcript (CART) and pro-opiomelanocortin (POMC), from which α-melanocyte stimulating hormone (α-MSH) is cleaved. CART and α-MSH are both anorexigens and reduced leptin signalling inhibits the synthesis of CART and POMC (Fig. 4)[26,27]. Finally, corticotropin-releasing hormone (CRH), which is also produced in the hypothalamus, might be important in mediating the effects of leptin, presumably *via* activation of sympathetic outflow to BAT, WAT, liver and muscle. Intracerebral injection of CRH stimulates thermogenesis and oxygen consumption and reduces food intake and body weight. CRH mRNA levels are increased by the intraventricular administration of leptin[28].

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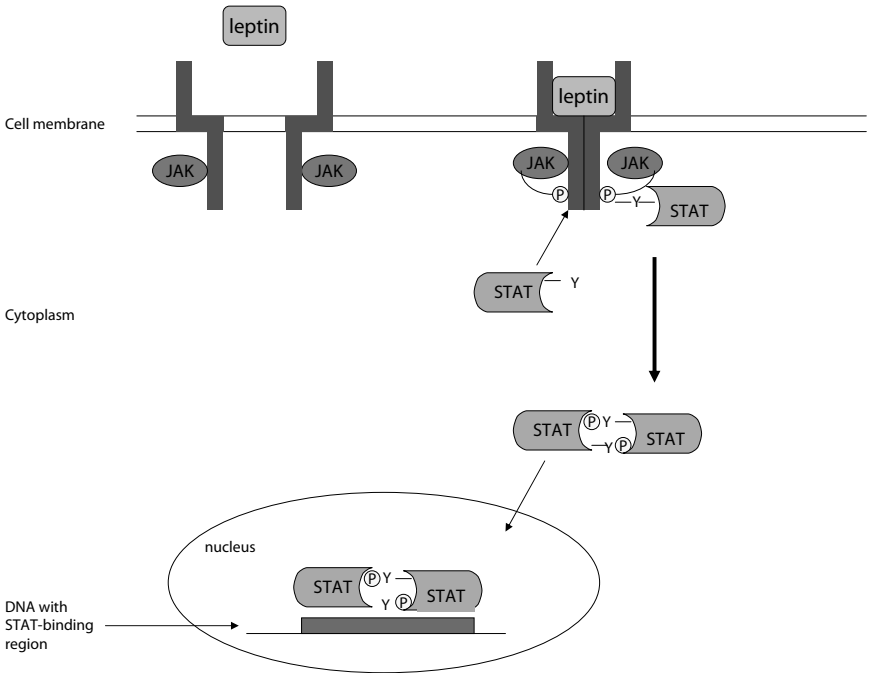

Figure 3.

The leptin receptor is a transmembrane receptor belonging to the class I cytokine receptors. The receptor consists of two parts. The intracellular domain is associated with the Janus kinase, a tyrosine kinase. Binding of leptin to the receptor results in the fusion of the two receptor parts, which results in trans-phosphorylation of the JAK-molecules, which subsequently phosphorylate the terminus of the leptin receptor. The phosphorylated receptor then forms a docking site for a variety of Src homology 2 (SH2) domain containing proteins, including a novel family of cytoplasmatic transcription factors termed STATs (signal transducers and activators of transcription). STATs are then phosphorylated on a single tyrosine residue by JAKs, after which STATs dimerise, migrate to the nucleus and regulate gene transcription.

Role of leptin in obesity

The initial conception of leptin as an anti-obesity hormone, whose primary role was to increase the metabolic rate and decrease food intake and appetite through action in the brain, was based on the following observations: (i) leptin deficient *ob/ob* mice and leptin receptor deficient *db/db* mice exert marked hyperphagia, decreased energy expenditure, morbid obesity and insulin resistance[29,30]; (ii) administration of intravenous or intracerebroventricular leptin decreases body weight and fat mass through inhibition of food intake and increased energy expenditure in *ob/ob* but not in *db/db* mice[31]; (iii) there is a threshold level of serum leptin (25-30 ng/mL) above which increases in serum levels are not translated into proportional increases in cerebrospinal or brain leptin levels, i.e., the transport system must be saturable[32]; (iv) the discovery of leptin receptors in the hypothalamus, the region involved in regulation of food intake and energy balance[27].

However, in most obese humans the gene encoding leptin is normal: up till now only two families with a mutation in the leptin gene have been identified[33,34]. In contrast, most obese humans have increased serum leptin levels[14,15], indicating that obesity is a leptin-resistant

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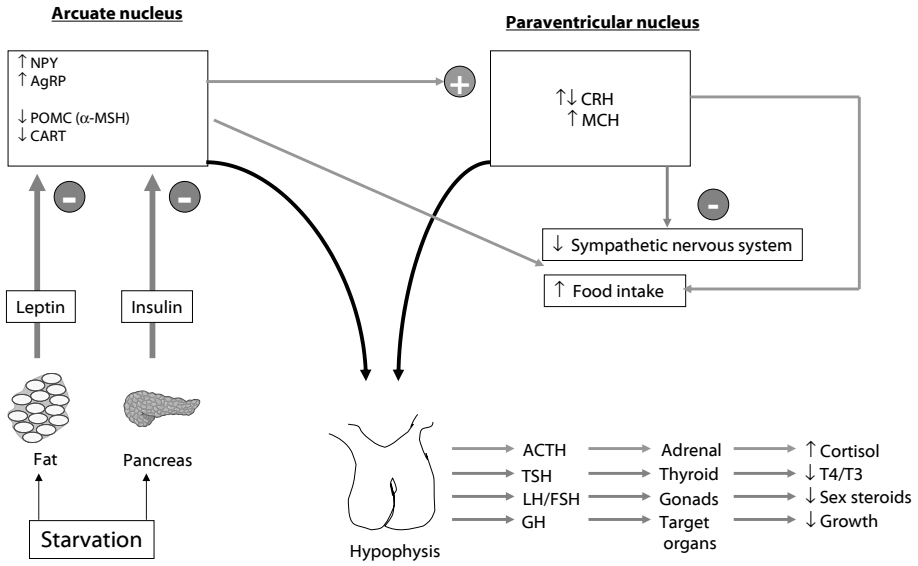

Figure 4.

Starvation leads to a decrease in serum insulin levels and a decreased expression of the *ob*-gene leading to a decrease in serum leptin levels. This subsequently leads to an increased expression of neuropeptide-Y (NPY) and agouti-related protein (AgRP) in the hypothalamus and a decrease in pro-opiomelanocortin (POMC) and cocaine-amphetamine related transcript (CART) in the hypothalamus. These hormones are involved in food intake and energy expenditure, leading to an increase in food intake and a decrease in energy expenditure. Furthermore, the hypothalamic hormones have either a direct or an indirect (via corticotropin-releasing hormone [CRH] and α-melanocyte-stimulating hormone [α-MSH]) effect on various hormones secreted by the pituitary. Thus, leptin has multiple effects, not only on food intake and energy metabolism but also on the hypothalamic-pituitary-adrenal axis, thyroid function and sex steroids.

state. Such a resistance could theoretically occur at several levels of the leptin signal transduction pathway, but this has not been resolved yet.

Leptin and insulin resistance

Since obesity is associated with insulin resistance, it is interesting to look at the role of leptin in the development of insulin resistance and diabetes. A strong correlation between serum leptin and insulin levels, independent of body fatness, has been demonstrated in human studies[35,36]. Hyperinsulinaemia induced by clamp techniques increases serum leptin levels, though not acutely[19]. Serum leptin levels are increased by insulin therapy as well, both in type 1 and type 2 diabetic patients[36,37]. Vice versa, a fair amount of evidence points to the fact that leptin has insulin- and glucose-lowering properties, although some studies find just the opposite. An extensive review on the association between leptin and insulin resistance has recently been published[38].

In both normal rodents[39] and rodents with obesity and insulin resistance[40-42], leptin therapy improves hyperinsulinaemia and hyperglycaemia. These effects are already apparent before weight loss occurs and are not due to energy restriction as was shown in pair-fed control studies[41,43].

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Most obese humans have increased serum leptin levels[14,15] and thus far the overall effect of leptin therapy on weight loss and metabolic parameters has been modest[44]. It is likely that very high plasma levels of the hormone are needed to overcome the leptin-resistant state. A final point directing to an antidiabetogenic effect of leptin is that both in lipodystrophic rodents[45] and humans (who have an extreme deficit of subcutaneous adipose tissue)[46], a condition associated with severe insulin resistance with hyperglycaemia, hyperinsulinaemia and hypertriglyceridaemia, leptin therapy corrects all these metabolic abnormalities, independent of the accompanying reduction in food intake.

Hypotheses with regard to the glucose and insulin lowering effect of leptin

As mentioned before, leptin seems to have an insulin-sensitising effect on the whole-body level but conflicting results were reported when individual tissues were examined. Most *in vitro* experiments suggest a diabetogenic effect of leptin[38]. Beside the differences between animals and humans, sources of leptin and time of exposure to this hormone might also play a causative role in the differences found. Furthermore, the fact that leptin exerts a glucose- and insulin-lowering effect and improves insulin sensitivity *in vivo*, suggests involvement of centrally acting mechanisms. This concept is further supported by the observation that leptin fails to reverse insulin resistance and lipid accumulation in mice with ventromedial hypothalamic lesions[47]. The peripheral mechanism by which leptin exerts its glucose- and insulin-lowering effect might be *via* promoting fatty acid oxidation and triglyceride synthesis. Indeed, leptin administration activates 5'-AMP-activated protein kinase (AMPK) in skeletal muscle, leading to the inhibition of acetyl coenzyme A carboxylase and subsequently stimulation of fatty acid oxidation. The resulting intramyocellular lipid depletion will enhance insulin sensitivity[48].

Apart from insulin-sensitising effects, leptin diminishes hyperinsulinaemia probably *via* inhibition of insulin secretion. Functional leptin receptors have been demonstrated on insulin secreting β-cells of the pancreas[49]. Leptin inhibits glucose-stimulated insulin secretion both *in vitro*[50] and *in vivo*[51]. The mechanism involved is activation of the ATP-sensitive potassium channels in the β-cell. Finally, leptin shares intracellular pathways with insulin, both in peripheral tissues and in the CNS[52]. Many effects of both insulin and leptin are mediated *via* activation of PI-3 (phosphatidylinositol-3-phosphate) kinase, so degree of cross talk between insulin and leptin may exist at the level of PI-3 kinase. Effects of leptin on insulin signalling have been studied and support an inhibitory effect of leptin on insulin signalling at the level of tyrosine phosphorylation of IRS-1 and PI3-kinase binding to IRS-1[38]. The effect of hyperinsulinaemia on intracellular leptin signalling has rarely been addressed but in one study supraphysiogical concentrations of insulin completely cancelled out the leptin-induced insulin response[53].

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Conclusion

Thus, leptin is an adipocyte secretory product that is not only involved in food intake and energy metabolism but clearly also has a role in glucose metabolism. Since plasma leptin levels are positively correlated with BMI, obesity seems to reflect a leptin-resistant state. Resistance for the action of leptin could promote obesity *via* decreased energy expenditure and a failure to diminish food intake. Furthermore, since leptin has a glucose- and insulin-lowering effect on the whole-body level *in vivo*, resistance for this effect could induce insulin resistance. One explanation for the insulin resistance seen in obesity might be that the high leptin levels interfere with insulin signalling. Another possibility is that there is a diminished activation of AMPK due to impaired leptin signalling. The resultant decrease in fatty acid oxidation will lead to an increase in intramyocellular lipids and thus to insulin resistance. Finally, both peripheral and central leptin resistance must be involved in insulin-resistant states since leptin treatment fails to correct insulin resistance in mice with ventromedial hypothalamic lesions.

Resistin

Discovery, structure, genetic locus, sites and modulators of expression of resistin

Resistin is a unique protein with cysteine-rich residues[54], which belongs to a class of tissue-specific secreted proteins termed the RELM (resistin-like molecule)/FIZZ (found in inflammatory zone) family. Resistin/FIZZ 3 is specifically expressed and secreted by adipocytes. The gene encoding resistin in mice has been named *Retn*. The regulation of resistin gene expression is controversial, see Table 2.

Resistin in obesity and insulin resistance

The initial report by Steppan *et al.*[54] suggested that resistin might constitute the link between obesity and insulin resistance. Resistin serum levels were increased in obese mice and resistin gene expression was induced during adipocyte differentiation. In addition, administration of resistin impaired glucose tolerance and insulin action in wild-type mice and *in vitro* in 3T3-L1 adipocytes whereas resistin antibody improved insulin sensitivity. The fact that thiazolidine-

Table 2. Regulators of resistin expression.

Factor	Decreasing resistin	Increasing resistin	No effect
Thiazolidinediones	[54-56,58]	[59]	[60]
Insulin	[56,58]	[59,61]	
Glucose		[58]	
Dexamethasone		[56,58]	
β-adrenergic agonists	[62]		[56]
TNF-α	[58,63]		
Epinephrine	[58]		

Factors that have been reported to increase or decrease resistin expression with their references.

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diones suppressed resistin secretion led to the hypothesis that these insulin-sensitisers exert their effect *via* downregulation of resistin gene expression. An increase in adipocyte gene expression during 3T3-L1 adipocyte differentiation[61] and after the induction of high-fat-diet induced obesity[57] was found in two other studies. Several other investigators, however, found a decreased resistin gene expression in WAT in different models of rodent obesity and insulin resistance[59,64,65], and resistin did not seem to be involved in the aetiology of insulin resistance in Fischer 344 rats, a good model for the metabolic syndrome in humans[66].

Studies in humans are even more controversial. One study could not detect any resistin mRNA in human fat cells at all in subjects with varying degrees of insulin resistance and obesity[67]. Another investigator found increased resistin mRNA in adipose tissue of obese humans, compared with lean controls, but decreased mRNA in freshly isolated human adipocytes[60]. In addition, resistin mRNA was undetectable in a severely insulin resistant subject. Janke *et al*. found an increased resistin gene expression in cultured human preadipocytes compared with mature adipocytes but again no relationship between resistin gene expression and either insulin resistance or body weight could be detected[68]. Although the higher resistin mRNA levels found in abdominal fat tissue compared with thigh, could explain the increased metabolic abnormalities in abdominal obesity, the fact that resistin mRNA expression is very similar in subcutaneous and omental adipose tissue suggests that it is unlikely that resistin is the link between (visceral) adiposity and insulin resistance[69].

Conclusion

The conclusion must be that many questions have to be resolved. Conflicting results have been reported with regard to the factors regulating resistin gene expression (Table 2). This is probably due to the difference between 3T3-L1 cell lines and *in vivo* models. Furthermore, the observed relation between resistin mRNA, serum resistin levels and insulin resistance in rodents cannot readily be extrapolated to humans. Murine resistin is only about 56% identical to human resistin at the amino acid level. Even in mouse models it is still unclear whether resistin plays a causal role in insulin resistance. Experiments in resistin knockout mice and in transgenic mice (which overexpress resistin) will be needed to solve this problem, but even then the relevance of resistin to human diabetes remains unclear, especially because some groups have found only minimal expression of the hormone in human fat[69]. Furthermore it would be interesting to know how resistin exerts its presumed insulin-antagonising effects and what its target organs are. For that purpose the resistin receptor would have to be found and downstream signalling pathways have to be unravelled.

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Adiponectin

Discovery, sites of expression and stimuli leading to adiponectin production

Adiponectin is a recently identified[70,71] adipocyte-specific secretory protein of about 30 kDa that appears to be involved in the regulation of energy balance and insulin action and also seems to have anti-inflammatory and anti-atherogenic properties.

Adiponectin is the product of the adipose tissue most abundant gene transcript-1 (apM1), which is exclusively expressed in WAT and is located on chromosome 3q27. Adiponectin is specifically expressed during adipocyte differentiation and is not detectable in fibroblasts. The expression of adiponectin is stimulated by insulin[70,72], IGF-1[72] and the TZDs[73]. Corticosteroids[72], TNF-α[74] and β-adrenerg stimulation[75] inhibit adiponectin gene expression in 3T3-L1 adipocytes.

Serum and mRNA levels of adiponectin in obesity and insulin resistance

Serum adiponectin levels are decreased in humans with obesity[76,77] and type 2 diabetes[76,78] as well as in obese and insulin-resistant rodents[79]. In addition, adiponectin gene transcription is decreased in adipocytes from obese[71] and diabetic[80] humans and rodents[71,79]. Plasma adiponectin concentrations increase after weight reduction in obese diabetic and non-diabetic patients[78]. The degree of plasma hypoadiponectinemia was more closely related to the degree of hyperinsulinaemia and insulin resistance than to the degree of adiposity[76]. Low plasma adiponectin concentrations predicted a decrease in insulin sensitivity[81] and an increase of type 2 diabetes[82] in Pima Indians as well as in a German population[83]. In non-diabetics, plasma adiponectin levels are also positively correlated with insulin sensitivity[84]. A recent study confirmed that the relation between low adiponectin levels and insulin resistance is not determined by obesity since low plasma adiponectin levels at baseline did not predict future obesity[85]. Finally, the fact that the insulin-sensitising TZDs strongly increase plasma adiponectin[73,86] further supports a role of adiponectin in insulin sensitivity.

Theory with regard to the possible mechanism of action of adiponectin

Administration of recombinant adiponectin to normal, obese and diabetic rodents led to acute normalisation of serum glucose levels[79,87,88]. Both decreased gluconeogenesis of the liver[87] and an increased fatty acid oxidation in muscle[79,88] have been proposed as underlying mechanisms. Recently, Yamauchi underscored his previous hypothesis[89]. Administration of adiponectin led to an increase of glucose utilisation and fatty acid oxidation in cultured myocytes and in soleus muscle of mice *in vivo*. In hepatocytes AMPK was activated as well, leading to a reduction in gluconeogenesis.

In addition, it has been shown that administering only the globular domain of adiponectin instead of full-length adiponectin is much more effective in improving insulin sensitivity because this fragment augments insulin-induced phosphorylation of insulin receptor substrate

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1 (IRS-1) and protein kinase B in skeletal muscle[79]. Thus, adiponectin might exert its insulin-sensitising effect *via* the following mechanisms: (i) increased fatty acid oxidation, leading to a lower muscle triglyceride content and lower plasma concentrations of free fatty acids which will both improve insulin signalling; (ii) direct improvement of insulin signalling; (iii) inhibition of gluconeogenesis, partly *via* reduced substrate delivery and partly *via* reduction of molecules involved in gluconeogenesis by activation of AMPK.

Disappointingly, no positive correlation between plasma adiponectin levels and 24-hour respiratory quotient (RQ) measurement (pointing to an increase in carbohydrate metabolism) could be demonstrated in healthy nondiabetic Pima Indians[90]. This does not rule out, however, that administration of adiponectin to subjects with low levels of this hormone will increase RQ and energy expenditure.

The acylation-stimulating protein (ASP)- pathway

ASP production and site of action

Acylation-stimulating protein (ASP) is a 76 amino acid protein identical to C3adesArg, a cleavage product of complement factor 3 (C3) formed *via* interaction of C3 with factor B and adipsin. C3, factor B and adipsin are all components of the alternative complement pathway and are produced by the adipocyte in a differentiation-dependent manner[91].

The major site of action of ASP appears to be on the adipocytes themselves, which have a specific saturable receptor for ASP[92]. In human adipocytes there are differentiation and site-specific differences in ASP binding which are proportional to the ASP response: differentiated adipocytes bind more ASP and have a greater response to ASP than undifferentiated adipocytes[93]. Furthermore, subcutaneous adipose tissue has greater affinity and greater specific binding to ASP than undifferentiated adipocytes[94].

ASP promotes triglyceride storage

ASP promotes triglyceride storage in adipocytes *via* three mechanisms. Firstly, ASP increases fatty acid esterification in adipocytes by increasing the activity of diacylglycerol acyltransferase, which is the final enzyme involved in triglyceride synthesis[91]. Secondly, ASP stimulates glucose transport in human and murine adipocytes and preadipocytes[93]. This effect on glucose transport is accomplished *via* translocation of cell-specific glucose transporters to the cell membrane. Thirdly, ASP decreases lipolysis *via* inhibition of hormone-sensitive lipase[95]. The effects of ASP are independently of and additional to the action of insulin[95].

Stimuli leading to ASP production

In vitro studies in cultured adipocytes indicate that insulin[96] and even more so chylomicrons[96,97] increase ASP production. *In vivo*, plasma ASP concentrations seem to show little change after an oral fat load[98]. There is, however, postprandially an increased venoarterial

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gradient of ASP across a subcutaneous abdominal tissue bed with a maximum after 3 to 5 hours, indicating increased adipose tissue ASP production[98]. This increase in ASP postprandially is substantially later than the increase in insulin but shows a close temporal relationship with maximal plasma triacylglycerol clearance[98].

Plasma ASP levels in obesity

An excellent review on the physiology of ASP in humans and rodents has recently been published[99]. Plasma levels of ASP are 225-fold lower (weighted average 28.3 nM) than its precursor C3. Studies measuring plasma ASP levels should therefore be interpreted with caution while it might very well be that ASP acts as a paracrine hormone[99]. Plasma ASP levels are increased in obese humans[100-103] and are reduced after fasting or weight loss[101;103]. ASP has also been shown to be significantly increased in type 2 diabetes[102,104] but since type 2 diabetes is often associated with obesity this might be a confounding factor. On the other hand, plasma ASP levels were inversely correlated to glucose disposal during a euglycaemic clamp in humans[102]. Adipocytes from obese humans are as responsive to ASP as adipocytes from lean people[105]. Thus the increased levels of ASP in human obesity in the face of a similar responsiveness to ASP compared with lean subjects, may promote energy storage, leading to adiposity.

Relation between ASP-enhanced triglyceride clearance and insulin resistance

ASP production is increased in obese mice. Intraperitoneal (i.p.) administration of ASP to normal mice resulted in accelerated postprandial triglyceride (TG) and non-esterified fatty acid (NEFA) clearance after an oral fat load[106]. In addition, plasma glucose levels returned faster to basal levels. C3 knockout mice (KO), which are unable to produce ASP, showed delayed plasma triglyceride clearance after an oral fat load in the absence of any change in fasting plasma TG levels. Administration of exogenous ASP enhanced plasma TG clearance[107]. Remarkably, these C3 KO mice were more insulin sensitive, had a reduced fat mass and yet an increased food intake. It was later shown that the hyperphagia/leanness was balanced by an increase in energy expenditure[108].

Conclusion

In summary, ASP promotes storage of energy as fat. Decreased ASP production decreases lipid storage and induces an obesity-resistant state and improved insulin sensitivity. Plasma ASP levels are increased in obese humans; whether this is the effect or cause of the increased adipose tissue mass remains to be elucidated. *Post or propter*, increased ASP levels together with a continuing responsiveness of the ASP receptor will lead to further triglyceride storage. Although enhanced fatty acid trapping will decrease free fatty acid levels and hence diminish hepatic gluconeogenesis, increased ASP functioning in skeletal muscle will lead to an increase in skeletal muscle triglyceride storage leading to insulin resistance.

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Tumour necrosis factor-α (TNF-α)

Structure of TNF-α, sites of production and receptor interaction

TNF-α is a cytokine produced mainly by activated macrophages in response to invasive stimuli, but also by non-immune cells such as muscle and adipose tissue. Furthermore, TNF-α has a variety of biological effects in various tissues and cell-types, and can thus be considered a multifunctional cytokine[109].

TNF-α is produced as a 26-kDa membrane-bound precursor that is proteolytically cleaved to a 17-kDa soluble form[109]. The cytokine interacts with two membrane-bound receptors, a 60-kD and an 80-kD subtype also called type I and type II receptor (TNFR1 and TNFR2). These receptors have different cellular and tissue distribution patterns and can bind other cytokines as well. TNF-α has a higher affinity for TNFR-1 than for TNFR-2[109]. Due to the high affinity for its receptor TNF-α can act either as an autocrine or paracrine cytokine at low concentrations or as an endocrine cytokine at high concentrations.

In addition to the membrane-bound receptors, soluble forms of the two receptors exist for which TNF-α has an even higher affinity. When TNF-α is bound to these soluble receptors no interaction can take place with the membrane-bound forms and thus TNF-α action is inhibited. Therefore, the physiological role of the soluble receptors may be to regulate TNF-α action.

Modulators of TNF-α production

In macrophages and monocytes, the expression and production of TNF-α is stimulated by endotoxins such as lipopolysacharide (LPS). LPS resulted in a fivefold stimulation of TNF-α in human adipose tissue and isolated adipocytes *in vitro,* the latter indicating that it is unlikely that the response is entirely due to macrophages and monocytes in the stromal vascular fraction of adipose tissue. Insulin and glucocorticoids did not have a significant effect on TNF-α release from human adipose tissue or isolated adipocytes *in vitro*[110]. Thiazolidinediones reduced adipocyte TNF-α release in obese rodents[111] but no effect was seen in human adipose tissue *in vitro*[110]. Since high-fat diets resulted in a significant increase in TNF-α mRNA and protein in epididymal and retroperitoneal fat pads in rats, free fatty acids and/or triglycerides may play an important role as inducers of TNF-α expression[112].

Effect of TNF-α on glucose and lipid metabolism

Firstly, TNF-α inhibits preadipocyte differentiation by downregulating the expression of two important adipocyte transcription factors: PPAR-γ and CEBP/α[113]. Secondly, TNF-α reduces the expression of GLUT-4, glycogen synthase and fatty acid synthase, which are essential for insulin-mediated glucose uptake and the subsequent conversion of glucose to glycogen or fatty acids. Furthermore, genes involved in the uptake of free fatty acids and the subsequent conversion to triglycerides, such as lipoprotein lipase, long-chain fatty acyl-CoA synthethase

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and diacylglycerol acyltransferase, were also downregulated by TNF-α[113]. The above-mentioned changes in gene expression lead to a diminished insulin-stimulated glucose uptake and an altered lipid metabolism which can, *via* accumulation of triglycerides in various organ systems, eventually lead to insulin resistance of the muscle and liver.

In addition, insulin resistance can be induced *via* a direct toxic effect of TNF-α on intracellular insulin signalling[114]. TNF-α reduces the insulin-stimulated autophosphorylation of the insulin receptor in a variety of cell types. It does so by phosphorylation of serine residues at the insulin receptor substrate-1 (IRS-1); this modified IRS-1 subsequently interferes with the insulin signalling capacity of the insulin receptor[114].

Relation between TNF-α, obesity and insulin resistance

A positive relationship between obesity, insulin resistance and adipose tissue mRNA levels of TNF-α has clearly been established in rodent models[115]. Furthermore, mice with no functional copy of the TNF-α gene (TNF-α $^{-/-}$) although developing marked obesity on a high-fat, high-energy diet, remained highly insulin sensitive as compared to their control litter mates (TNF-α $^{+/+}$)[116].

In contrast to rodents, the role of TNF-α in the induction of insulin resistance in humans is less clear. Although there seems to be a positive relationship between obesity and TNF-α mRNA and protein levels in adipose tissue in humans *in vitro*[117-119], TNF-α is expressed at much lower levels in humans as compared to rodents[117-119]. In addition, no difference in TNF-α concentration was found in a vein draining subcutaneous adipose tissue as compared to a peripheral vein, suggesting no or very low TNF-α production *in vivo*[120]. Furthermore, circulating TNF-α concentrations in obese diabetic and non-diabetic patients are not substantially elevated[118,120]. With regard to a direct relationship between TNF-α and insulin sensitivity *in vivo*, two studies found a strong and positive correlation between adipose tissue mRNA levels and hyperinsulinaemia[117,118]. When the relation between adipose tissue TNF-α secretion and insulin-stimulated glucose transport was examined, a strong inverse relationship was found that was independent of fat cell volume, age and BMI[122].

However, other studies[121,123] showed no significant relationship between adipose tissue mRNA for TNF-α and insulin sensitivity. Furthermore, treatment of insulin-resistant subjects with anti-TNF-α antibodies did not improve insulin sensitivity[124]. All these results implicate that TNF-α might have an effect on insulin resistance but that it must be a local factor. Interestingly, TNF-α is also produced by muscle, and muscle TNF-α production is increased in obesity[125]. Since adipose tissue dispersed within muscle is correlated with insulin resistance, the effect of fat cell secretory products on insulin signalling in skeletal muscle cells was recently studied in a model in which muscle cells were co-cultured with adipocytes. A disturbance of insulin signalling was found, but TNF-α did not seem to be involved[126].

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Conclusion

In conclusion, TNF-α is a multifunctional cytokine produced by adipocytes in proportion to the percentage body fat. TNF-α has a variety of metabolic effects, including increased lipolysis, decreased lipogenesis and decreased insulin-stimulated glucose transport, contributing to insulin resistance. These effects are induced by modulation of genes involved in glucose and lipid metabolism. Furthermore, TNF-α directly interferes with early steps of insulin signalling. However, the role of TNF-α in obesity-induced insulin resistance in humans is not quite clear yet, as might be obvious from the contradicting results mentioned in the previous paragraph. The low plasma levels of TNF-α in humans indicate that the hormone most likely acts in a paracrine and/or autocrine manner. This might be the reason why treatment with anti-TNF-α did not improve insulin sensitivity in humans *in vivo*.

Interleukin-6 (IL-6)

Structure, genetic locus and site of production of IL-6

IL-6 is a circulating, multifunctional cytokine that is produced by a variety of cell types including fibroblasts, endothelial cells, monocytes/macrophages, T-cell lines, various tumour cell lines and adipocytes. The protein has a molecular mass of 21 to 28 kDa, depending on the cellular source and preparation. The gene encoding IL-6 is localised on chromosome 7p21 in humans[127].

Although human adipocytes do produce IL-6, adipocytes accounted for only 10% of total adipose tissue when IL-6 production by isolated adipocytes prepared from omental and subcutaneous fat depots was examined[128]. This means that cells in the stromal vascular fraction of adipose tissue have a major contribution in adipose tissue IL-6 release. The concentrations of IL-6 in adipose tissue are up to 75 ng/mL, which is well within the range to elicit biological effects[129]. Furthermore, plasma levels of IL-6 are markedly elevated in obesity and up to 30% of plasma levels could be derived from adipocytes[130].

Modulators of IL-6 production

The stimuli leading to IL-6 production differ with the cell type; here only IL-6 production by adipocytes will be discussed. Both in rodent and human adipocytes, IL-6 production is stimulated by catecholamines and inhibited by glucocorticoids, whereas insulin has no effect whatsoever[128,131,132]. Finally, another stimulator of IL-6 release is TNF-α, which has been reported to produce a 30-fold [113] increase in IL-6 production in 3T3-L1 adipocytes. Interestingly, IL-6 in turn inhibits the release of TNF-α!

IL-6 acts via receptor interaction

IL-6 acts through binding at and activation of a specific receptor, belonging to the class I cytokine receptors, which act through JAK-STAT signalling (see Fig. 3 where leptin signalling

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is explained)[133]. The IL-6 receptor consists of two membrane glycoproteins, a 80-kDa ligand binding component and a 130-kDa signal-transducing component (gp130). The 80-kDa component binds IL-6 with low affinity; this complex subsequently binds with high affinity to gp130 after which signal transduction can take place[127].

Soluble forms of the IL-6 receptor have been found but neither their functional significance nor the regulation of their production is understood.

Metabolic effects of IL-6

IL-6 has pleiotropic effects on various cell types. Here, we will only focus on its role in glucose and lipid metabolism. Infusion of rhIL-6 to humans increased whole-body glucose disposal and glucose oxidation but increased hepatic glucose production[134] and the fasting blood glucose concentration in a dose-dependent manner[135]. With regard to lipid metabolism, IL-6 decreases adipose tissue lipoprotein lipase (LPL) activity[129] and has been implicated in the fat depletion taking place during wasting disorders, such as cancer, perhaps *via* an increase in plasma norepinephrine, cortisol, resting energy expenditure and fatty acid oxidation as was assessed in eight renal cancer patients[134]. In rats, IL-6 increased hepatic triglyceride secretion partly because the increase of adipose tissue lipolysis resulted in an increased delivery of free fatty acids to the liver[136]. This increased release of free fatty acids following rhIL-6 infusion was observed in humans as well[134].

IL-6 in obesity and insulin resistance

In both mice [132]and humans, IL-6 mRNA in adipose tissue[137,138] but even more so plasma levels of IL-6 are positively correlated with BMI[132,137,138]. Weight loss is associated with a reduction in serum and IL-6 mRNA levels. After one year of a multidisciplinary programme of weight reduction, obese women lost at least 10% of their original weight and this was associated with a reduction of basal serum IL-6 levels from 3.18 to 1.7 pg/mL ($p<0.01$)[138]. In another study, both IL-6 mRNA in adipose tissue and IL-6 serum levels were reduced with weight loss after three weeks of a very low calorie diet in obese women[138]. In this study, insulin sensitivity as assessed by the fasting insulin resistance index (FIRI= fasting glucose x fasting insulin/25) improved as well. The reduction in IL-6 levels could play a role in this improvement, since several studies found a significant correlation between circulating IL-6 levels and insulin sensitivity measured by either an intravenous glucose tolerance test[137] or the fasting insulin resistance index[138]. Recently this correlation between circulating IL-6 and insulin sensitivity was confirmed using the "gold standard for insulin sensitivity": the hyperinsulinaemic euglycaemic clamp[140]. In addition, a high correlation between adipose tissue IL-6 content and insulin sensitivity was found, both *in vivo* and *in vitro*. Furthermore, for the first time IL-6 receptors were demonstrated in 60% of the subcutaneous adipocytes suggesting that IL-6 can alter adipocyte metabolism *via* autocrine or paracrine mechanisms and have a local influence on insulin sensitivity[140]. Further support for a relationship between IL-6 and insulin sensitivity

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comes from a genetic study. It appeared that subjects with an IL-6 gene polymorphism had lower IL-6 levels, a lower area under the glucose curve after an oral glucose tolerance test, lower glycosylated haemoglobin (HbA_{1c}) and fasting serum insulin levels and an increased insulin sensitivity index as compared with carriers of the normal IL-6 allele, despite similar age and BMI[141]. Finally, basal serum IL-6 levels are higher in type-2-diabetic patients[142].

In contradiction with the abovementioned positive correlation of IL-6 with BMI and inverse relation with insulin sensitivity is the observation that the lack of IL-6 also leads to obesity and a disturbed glucose tolerance, at least in mice.

Conclusion

Various studies show a clear relationship between increased IL-6 levels and obesity[132,137,138], and between IL-6 levels and insulin resistance[137,138,140] even when corrected for BMI[137]. Furthermore, basal plasma IL-6 levels are higher in patients with type 2 diabetes[142] and subjects with an IL-6 gene polymorphism clearly have lower serum IL-6 levels and this is correlated with improved insulin sensitivity and postload glucose levels[141]. IL-6 does have different effects on the various end-organ tissues, however, with on the one hand improved glucose uptake in adipocytes and whole-body glucose disposal, and on the other hand an increased hepatic glucose output, decreased LPL activity (leading to decreased triglyceride clearance) and increased hepatic triglyceride synthesis. How then does IL-6 fit in the insulin resistance syndrome? Is there a causal effect or are the increased IL-6 levels found in obesity and insulin resistance merely a reflection of the pathogenetic state or the increased adipose tissue mass? Is IL-6 detrimental to health or does it have a positive role in health. If we start from the principle that IL-6 production is increased in obesity and that it is involved in inducing insulin resistance, what would be the mechanisms by which IL-6 causes insulin resistance? Firstly, it has to be noted that omental fat produces threefold more IL-6 than subcutaneous adipose tissue[128]. Because venous drainage of omental tissue flows directly to the liver and IL-6 is known to increase hepatic triglyceride secretion[134,136] this might explain the hypertriglyceridaemia associated with visceral obesity. As mentioned before, increased triglyceride content of muscle and liver leads to insulin resistance. Secondly, IL-6 signal transduction is mediated *via* JAK-STAT signalling; it is possible that feedback mechanisms interfering with insulin signalling exist. Thirdly, IL-6 has opposing effects to those of insulin on hepatic glycogen metabolism[143] and increases hepatic glucose production[135]. On the contrary, despite an increase of IL-6 in obesity, insulin resistance and type 2 diabetes, there is evidence that IL-6 improves insulin sensitivity; (i) IL-6 increases glucose uptake in 3T3-L1 adipocytes[144]; (ii) infusion of rhIL-6 to humans increased whole-body glucose disposal and glucose oxidation[134]; (iii) IL-6 inhibits TNF-α production, a cytokine with deleterious effects on insulin sensitivity; and (iv) physical exercise, which is related to an improvement in insulin sensitivity, is coupled with an increased IL-6 secretion[145]. It might be that muscle-derived IL-6 downregulates TNF-α[145].

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So, in conclusion, it is still not clear whether IL-6 has a positive or a negative metabolic role in health. One of the reasons of the contradicting results might be that there is a difference in the acute and chronic exposure to IL-6 with regard to health implications. Furthermore, local and CNS-acting effects of IL-6 might be different. More transgenic mice studies can help shed light on the role of IL-6 in insulin resistance. Up until now, it is quite possible that the increased IL-6 levels observed in adiposity and type 2 diabetes are the cause of an increased production by the enlarged adipose tissue mass and/or an attempt to overcome either insulin resistance or another metabolic effect, for example IL-6 resistance.

DISCUSSION

Obesity, defined as a BMI > 30 kg/m^2, is the consequence of a chronic imbalance between energy intake and energy expenditure. This is partly due to modern society with excess ('fast') food intake and a sedentary life style. The role that should be ascribed to primary defects in energy storage caused by adipocyte secretory products or impaired hypothalamic functioning remains to be elucidated. At the moment a combination of the two seems the most likely. It is well known that obesity is associated with insulin resistance and type 2 diabetes mellitus. An overwhelming amount of evidence indicates that visceral fat is associated with glucose intolerance and insulin resistance[146-151], along with other facets of the metabolic syndrome such as dyslipidaemia. Therefore, in the past, the predominant theory used to explain the link between obesity and insulin resistance was the portal/visceral hypothesis[152], which states that increased visceral adiposity leads to an increased free fatty acid flux into the portal system and inhibition of insulin action *via* Randle's effect[153]. However, several investigators have challenged the singular importance of visceral adiposity in inducing insulin resistance. They found an independent association between total fat mass and subcutaneous truncal fat mass and insulin resistance[154-156]. Furthermore, the observations that (i) triglyceride content within skeletal muscle cells is increased in obesity[157] and type 2 diabetes mellitus[157,158] and is a strong predictor of insulin resistance[159]; and (ii) lipodystrophy is associated with insulin resistance as well[160,161], necessitated the need to develop new theories to explain the link between adipose tissue and insulin resistance[162]. A well-accepted theory is that of ectopic fat storage[162,163]. A limitation in the capacity of adipose tissue to store triglycerides would divert triglycerides to be deposited in liver cells and skeletal muscle cells[162,163]. The cause of the ectopic fat storage is unclear. It might be due to impaired fat oxidation[162], since inhibition of fat oxidation in rodents increased intracellular lipid content and decreased insulin action[164]. Furthermore, a mutation in the AGPAT2 gene encoding 1-acylglycerol-3-phosphate O-acyltransferase inhibits triacylglycerol synthesis and storage in adipocytes but not in hepatocytes, thus leading to hepatosteatosis, because the latter can accumulate triacylglycerol *via* AGPAT-1[165]. Another possibility is the central and/or peripheral action of leptin, since leptin therapy has been as-

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sociated with the reversal of insulin resistance and hepatic steatosis in patients with lipodystrophy[46] and also with improvement of intramyocellular lipid content[163]. Finally, a defect in the proliferation and/or differentiation of adipocytes, whether or not due to alterations in the expression of transcription factors[166] can lead either to impaired adipocyte triglyceride storage and/or adipocyte hypertrophy. This is where the third hypothesis emerges: the adipocyte as an endocrine organ[162]. Adipocytes secrete a large number of cytokines and hormones that act in a paracrine, autocrine and endocrine manner on adipocyte- and whole-body metabolism. It is plausible that these enlarged adipocytes are deregulated in their transcriptional setting and secrete a different pattern of hormones or different amounts of them compared with small adipocytes. On the other hand, enlarged adipocytes might merely be a manifestation of other, yet to be defined, pathogenetic factors[162].

In obese humans and rodents there is, besides numerous other proteins and cytokines that have not been discussed here, overproduction of leptin[14,15], IL-6[132,137,138], TNF-α[115,117-119], ASP[100,101] and resistin[54,60], and a decreased production of adiponectin[71,77,78,80] (see Fig. 5). Of leptin[23], TNF[74] and IL-6[127] it is known that they act *via* receptors on the cell surface and subsequent intracellular signalling cascades. As can be seen in Fig. 5, all three adipocytokines decrease food intake and increase energy expenditure and lipolysis together with a decrease in lipogenesis. These are well-adaptive mechanisms to prevent further weight gain. Since all these adipocytokines are increased in adiposity it is unlikely that they are the cause of adiposity unless there is an impairment in (adipo)cytokine signalling. Interestingly, leptin and TNF-α have opposing effects with regard to insulin sensitivity. TNF-α interferes with insulin signalling and downregulates many genes encoding for proteins involved in glucose and free fatty

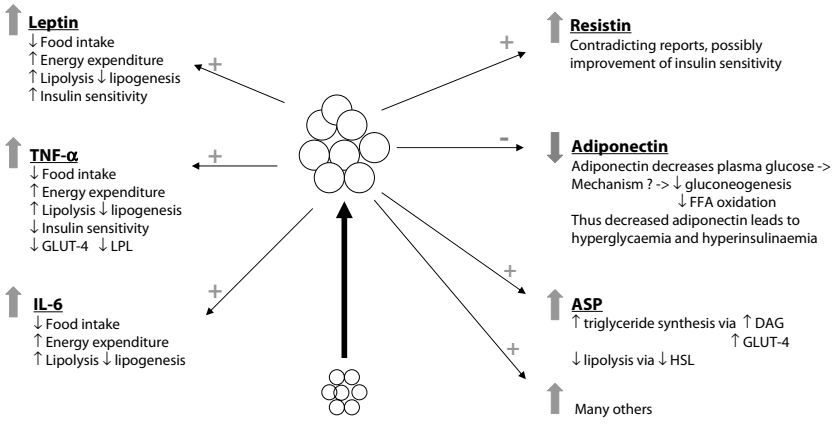

Figure 5.

Hyperplasia and hypertrophy of adipocytes, as seen in adiposity, leads to an increased production of leptin, TNF-α, IL-6, resistin, ASP and many other proteins, and a decreased production of adiponectin. The results of these increases, respectively decrease, are mentioned below each protein.

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acid uptake[113]. Leptin can act through some components of the insulin-signalling cascade as well[52]. The relation between TNF-α and leptin in humans is not clear. Infusion of TNF-α to patients has been reported to acutely raise serum leptin levels[167], whereas chronic exposure of cultured human adipocytes to TNF-α resulted in a decrease in leptin production[168]. If TNF-α increases leptin production this might be an adaptive mechanism to compensate for the TNF-α induced impaired insulin signalling.

When we take a further look at the mutual coherence of the adipocyte secretory factors it is striking that both insulin and TNF-α are, somehow, involved in the regulation of all of the adipocyte secretory products. Insulin increases the production of leptin[19,20,36,37], adiponectin[70,72] and ASP[96], whereas no effect has been recorded with regard to TNF-α[110] and a potentially positive effect on resistin levels[61]. TNF-α downregulates resistin[58] and stimulates the production of leptin[169], adiponectin[74] and IL-6[113]. The problem is that some of these factors lead to an improvement of insulin sensitivity, whereas others have just the opposite effect. This makes it extremely difficult to elucidate which factors are most important in regulating insulin sensitivity. Furthermore, the time of exposure to a stimulus seems to be important. Thus it seems that leptin and insulin are long-term regulators with regard to food intake and energy expenditure, whereas insulin has a direct effect on glucose uptake and lipolysis.

How do these adipocyte-derived factors mediate their effects? What they all seem to have in common is a change in the expression of genes encoding for proteins involved in glucose and lipid metabolism. Transcription of genes can only take place if they are activated, which always occurs *via* some kind of ligand-receptor interaction followed by an intracellular signal transduction. Cytokine signalling proceeds in part *via* the JAK-STAT pathway[170]. The actions of leptin, TNF-α and IL-6 may influence each other *via* common signalling steps. Furthermore, it is known that leptin can signal through some components of the insulin-signalling cascade such as IRS-1 and -2, PI3K and MAPK and can modify insulin-induced changes in gene expression *in vitro* and *in vivo*[171]. TNF-α can interfere with the early steps of insulin signalling as well[114]. So, more and more evidence exists that the adipocyte secretory products leptin, IL-6 and TNF-α not only interact with each other but also with insulin on the level of intracellular signal transduction.

In the case of obesity and hyperinsulinaemia there is an increase in hormones and cytokines produced by the adipose tissue. These hormones subsequently mediate a change in the expression of genes encoding for proteins involved in glucose and lipid metabolism. In case of ASP these changes promote triglyceride uptake. However, in case of leptin, IL-6, TNF-α and adiponectin there is a deleterious effect on glucose uptake and fatty acid oxidation leading to insulin resistance. The effect of increased serum resistin levels remains to be elucidated. Everything seems to come down to interference with intracellular signal transduction, not only of insulin but also of the various adipocyte secretory products, with a subsequent change in the expression of genes involved in glucose and lipid metabolism leading to a diminished glucose uptake and fatty acid oxidation. The latter will, *via* accumulation of tri-

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glycerides in liver cells and muscle cells, enhance insulin resistance, thus further impairing glucose uptake.

CONCLUDING REMARKS

It is now well established that adipose tissue not only has an important function in the storage and release of triglycerides but also has an important effect on whole-body metabolism and energy homeostasis *via* the production of various hormones and cytokines.

Adipose tissue not only responds to insulin, glucagon, cortisol and catecholamines but also to cytokines and products that it produces itself, thereby regulating its own metabolism and cell size. Some of the products produced by the adipocytes, such as TNF-α and leptin, are clearly involved in the induction of insulin resistance. The role of others (resistin, IL-6) has yet to be defined. Their increase in obesity is at least a manifestation of the increased adipose tissue mass itself. Further research is needed to come to a better understanding of the molecular pathways regulating the production of these hormones, their individual actions and target organs, and finally their mutual interaction and role in insulin resistance. These new insights provide the basis for the development of improved therapies for obesity and insulin resistance-related diseases as type 2 diabetes and cardiovascular complications.

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CHAPTER 3

The relation between leptin and insulin remains when insulin secretion is disturbed

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ABSTRACT

Serum insulin and leptin levels correlate positively. It is unknown whether this relation remains the same in cases of severely disturbed insulin secretion and after rapid weight loss. We therefore studied the relation between insulin and leptin in obese type 2 diabetic patients before and after considerable weight loss.

In 17 obese (BMI 37.6 ± 1.4 kg/m^2, mean ± SEM) type 2 diabetic patients (duration 8.0 ± 1.4 years, fasting plasma glucose [FPG 12.9 ± 0.8 mmol/L, HbA_{1c} 8.6 ± 0.4%), blood glucose-lowering medication was discontinued (day -1) and a 30-day very low calorie diet (VLCD, 450 kCal/day) was started. On days 0, 2 and 30, body weight, body fat mass (with bioelectrical impedance analysis [BIA]), fasting serum glucose, insulin and leptin were determined. Homeostatic model assessment was used to estimate insulin resistance (HOMA-IR) and β-cell function (HOMA-β). On days 2 and 30, an intravenous glucose tolerance test (IVGTT) was performed.

Fasting serum leptin levels correlated positively with fasting serum insulin levels ($r = 0.72$, $p = 0.001$ on day 2; $r = 0.78$, $p = 0.001$ on day 30) and area under the curve (AUC) of insulin ($r = 0.74$, $p = 0.001$ on day 2; $r = 0.84$, $p = 0.0001$ on day 30), as well as HOMA-β, as a measure of insulin secretion, even after correction for body mass index (BMI) and body fat mass, with which serum leptin levels were also positively correlated.

In conclusion, in a group of obese type 2 diabetic patients with a wide range of residual endogenous insulin secretion, we found a positive relation between fasting serum leptin and insulin levels (fasting as well as AUC), even after correction for BMI and body fat mass. This was true both before weight loss and during energy restriction with weight loss.

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INTRODUCTION

Leptin, the product of the *ob*-gene[1], is a 16 kDa protein that is mainly synthesised by white adipose tissue[1,2]. Leptin acts on hypothalamic neuropeptide-containing regions[3] and regulates body weight by controlling energy expenditure and food intake[1,4,5]. Serum leptin levels are positively correlated with body mass index (BMI) and body fat mass in both rodents and humans[6-8]. For any given body weight, serum leptin levels are higher in women than in men. However, after correction for fat mass, these differences seem to disappear[6,7], although not all authors agree[9]. Serum leptin levels show a diurnal pattern with a nocturnal peak shortly after midnight, and a midmorning low between 10:00 AM and 12:00 noon[10,11]. Serum leptin levels fluctuate with changes in body weight. Remarkably, with weight reduction, serum leptin levels fall before significant weight loss has occurred[12,13], suggesting that factors other than body fat mass regulate serum leptin levels in the short term. Possible regulators of the early decrease in serum leptin levels are energy restriction itself and/or serum insulin levels. The latter are also positively correlated with BMI and body fat mass.

A positive relation between serum leptin and serum insulin levels has been described in normal weight and obese subjects with or without impaired glucose tolerance[9,14-18] and in type 2 diabetic patients[16,19-21]. This positive relation has also been found before and after weight loss in obese men and women[14,22-24]. However, data on the effect of weight loss in type 2 diabetic patients, especially obese type 2 diabetic patients, are scarce[15,25]. It has been postulated[26] that during progressive β-cell failure, the relation between serum insulin and serum leptin levels is lost, either because of lower serum insulin levels or because of the developing hyperglycaemia, which might have a deleterious effect on both insulin production by β-cells and leptin production by adipose tissue.

In this study, we investigated both the effect of energy restriction (2 days of 450 kCal/day, minimal weight loss) and the effect of energy restriction plus weight loss (30 days of 450 kCal/day) on the relationship between serum leptin levels and serum insulin levels. Our study group was unique in the sense that we studied a group severely obese type 2 diabetic patients with varying degrees of endogenous insulin secretion, as assessed by an intravenous glucose tolerance test (IVGTT). We were, therefore, also able to address the relation between serum leptin levels and residual endogenous insulin secretory capacity.

PATIENTS AND METHODS

In 17 obese (BMI 37.6 ± 1.4 kg/m^2, mean ± SEM) type 2 diabetic patients (duration 8.0 ± 1.4 years) who had persistent high blood glucose levels (12.9 ± 0.8 mmol/L) and HbA_{1c} percentages (8.6 ± 0.4%) despite maximal doses of oral blood glucose lowering medication and/or

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insulin (66 to 400 units/day), all blood glucose-lowering medication was stopped (day -1) and a very low calorie diet (VLCD, Modifast®, 450 kCal/day) was started for 30 days.

On days 0, 2, 10 and 30, weight and length were measured, and fasting serum glucose, insulin, C-peptide and leptin levels were determined. Body fat mass was measured using bio-electrical impedance analysis (BIA, Bodystat, Bodystat®, Bodystat Ltd. Douglas, Isle of Man). An IVGTT (25 g of i.v. in 4 min with blood sampling at 0, 2, 4, 6, 8, 10, 12, 20, 30, 40, 50 and 60 min) was performed after an overnight fast on days 2 and 30 of the VLCD[27,28].

We chose day 2 instead of day 0 for the first IVGTT because most patients had used NPH insulin on the evening before the start of the study. For the same reason, we used laboratory measures taken on day 2 for baseline values of fasting plasma insulin and C-peptide. In addition, we used the data for body fat mass achieved *via* a BIA on day 0 also on day 2. The reason we did so was that the BIA was not reliable on day 2 due to fluid shifts (the natriuresis of "fasting" induces a new fluid and salt balance in the first few days of a diet). Furthermore, body fat mass would not have changed yet during 2 days of a VLCD; thus, data obtained on day 0 would be applicable on day 2 as well.

Blood Chemistry

All blood chemistry was measured at the Laboratory for Clinical Chemistry of Leiden University Medical Centre. Serum glucose was measured using a fully automated Hitachi 747 (Hitachi, Tokyo, Japan) system. Serum insulin was measured by immunoradiometric assay (Medgenix, Fleurus, Belgium) with a detection limit of 3.0 mU/L. The interassay coefficient of variation (CV) was below 6%. Serum leptin concentrations were determined by a standardised radio immunoassay (Linco Research, St. Charles, MO, USA), with a detection limit of 0.5 µg/L and a coefficient of variation of 3-5% at different levels.

Statistical analysis and mathematical calculations

Values are presented as mean ± standard error of the mean (SEM).

The glucose disappearance rate (k-value) was determined by (natural) log-linear regression of the glucose concentrations against time over the period from 10 to 60 minutes post-glucose loading[27]. The areas under the curve (AUC) of glucose and of insulin were determined over the periods from 0 to 60 and 10 to 60 min, respectively, post-glucose loading from zero level using the linear trapezoidal rule.

Estimates of insulin resistance and β-cell function by HOMA score were calculated with the formulas as described by Matthews *et al.*[29].

For comparisons between study days a Student's *t*-test for paired samples was used. The relation between serum leptin and serum insulin levels, as well as with the AUC of insulin were evaluated with a two-tailed Pearson's correlation. In addition, two-tailed partial correlations were carried out for adjustment of BMI, fat mass, age and gender. All analyses were

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performed using SPSS for Windows version 11.0 (SPSS, Chicago, IL, USA). A p value of < 0.05 was considered statistically significant.

RESULTS

Patient characteristics are presented in Table 1. Fourteen of the 17 patients completed the 30-day VLCD; the other 3 patients were not able to adhere to the diet and stopped within just a few days. We did not have any follow-up data from these three patients; so they were left out of the analysis comparing differences in various parameters between day 2 and day 30. Therefore, data on day 2 (fasting insulin, AUC insulin) in Table 2 (n=14) may differ from data on day 2 in Table 1 (n=17). For the correlation analysis between fasting serum leptin and insulin (fasting and AUC) all available data were used, resulting in 17 patients being analysed for this relation on day 2 and 14 patients on day 30 (Table 3).

The 14 patients who completed the study showed a gradual weight loss, amounting -2.5 ± 0.2 kg on day 2 (reflecting mainly salt and fluid loss) and -12.2 ± 0.8 kg on day 30 of the diet. This is equal to a reduction in BMI from 38.3 ± 1.5 kg/m^2 on day 0 to 37.5 ± 1.5 kg/m^2 on day 2 and 34.1 ± 1.5 kg/m^2 on day 30 of the diet ($p = 0.0001$ from day 0 to day 2, and day 0 to day 30, as well as from day 2 to day 30, see also Table 2). The decline in fasting serum leptin levels from day 0 to day 2 was highly significant, with a mean of 6.8 ± 1.6 ng/mL ($p = 0.001$, n=14). On day 30, the drop in fasting serum leptin levels in the 14 patients who completed the diet was also significant (Table 2).

Table 1. Patient characteristics (n=17).

Sex (male/female)	9 : 8
Age (years)	59.0 ± 1.9
Weight (kg)	110.7 ± 4.2
BMI (kg/m^2)	37.6 ± 1.4
Fat mass (kg)	42.6 ± 3.2
Fasting plasma glucose day 0 (mmol/L)	12.9 ± 0.8
HbA_{1c} (%)	8.6 ± 0.4
Duration type 2 diabetes (years)	8.0 ± 1.4
Fasting C-peptide day 0 (ng/mL)	1.3 ± 0.16
Fasting insulin day 2 (mU/L)	21.2 ± 3.5
Fasting leptin day 0 (ng/mL)	27.3 ± 5.3
AUC of insulin day 2 (mU*50 min)	1357 ± 224
Blood glucose lowering therapy	
only insulin	n = 4 (mean 167 units/day)
oral glucose-lowering therapy	n = 6
combination therapy	n = 7 (mean 168 units of insulin/day)

Data are presented as mean ± SEM.

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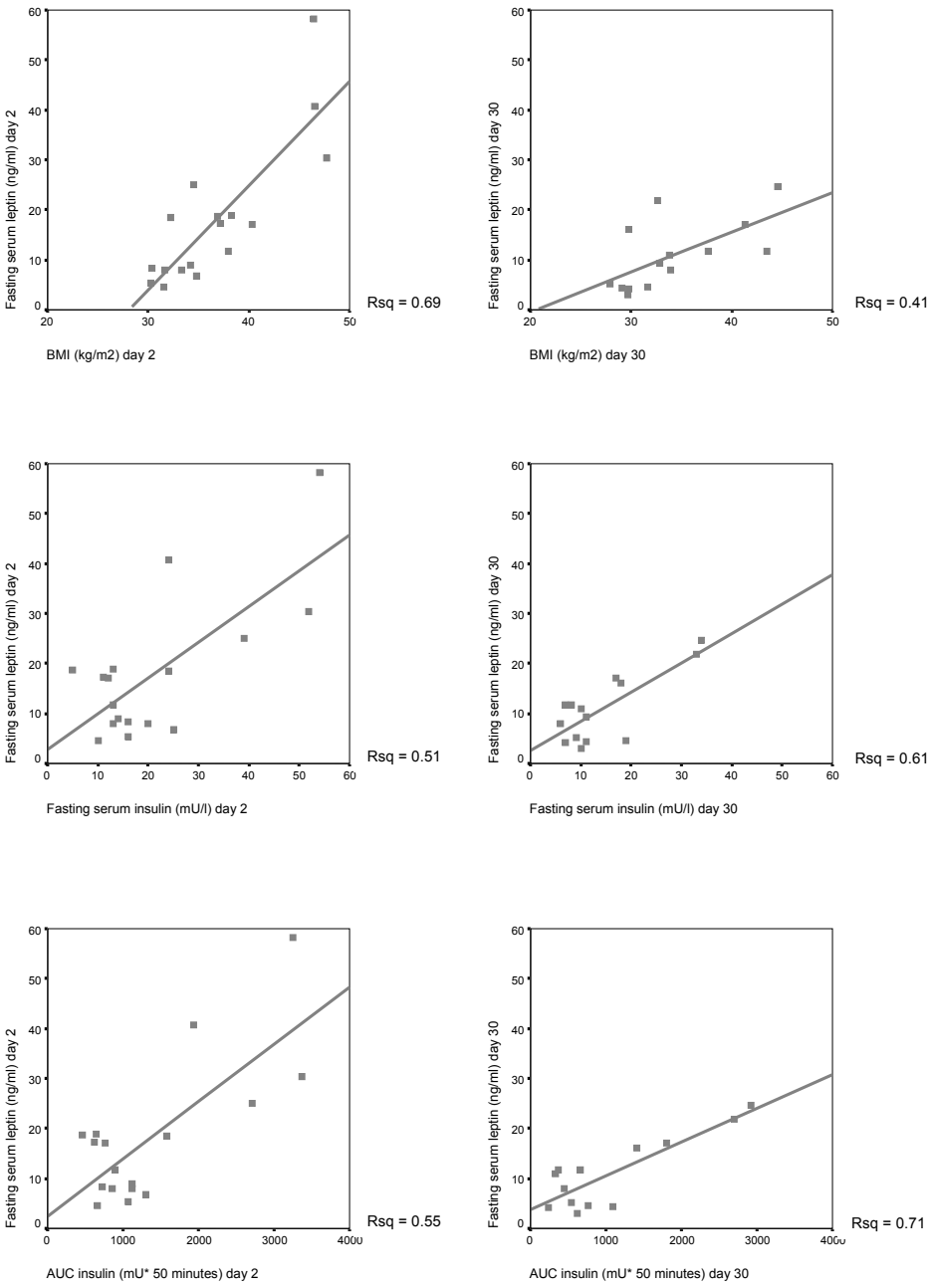

Figure 1.

Scatterplots of the correlation analysis between fasting serum leptin and BMI (top row), fasting serum insulin (middle row) and AUC of insulin (bottom row) on day 2 (left side, n=17) and day 30 (right side,n=14) of the VLCD.

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Table 2. Changes in anthropometric values, fasting serum insulin and leptin concentrations and estimates of insulin secretion and insulin sensitivity.

	Day 2 (n=14)	**Day 30** (n=14)	**P**
Weight (kg)	109.3 ± 5.0	99.7 ± 4.8	0.0001
BMI (kg/m^2)	37.5 ± 1.5	34.1 ± 1.5	0.0001
Fat mass (kg)	44.5 ± 3.8	38.6 ± 3.9	0.0001
Fasting serum glucose (mmol/L)	14.9 ± 1.1	12.0 ± 1.5	0.007
Fasting serum insulin (mU/L)	23.0 ± 4.1	14.3 ± 2.4	0.010
Fasting serum leptin (ng/mL)	20.3 ± 3.9	10.9 ± 1.8	0.008
AUC of insulin (mU*50 min)	1537.5 ± 242.4	1068.5 ± 247.6	0.005
AUC of glucose (mmol*60 min)	1194.4 ± 63.6	1721.0 ± 690.8	NS
k-Value (%/min)	0.46 ± 0.03	0.48 ± 0.04	NS
HOMA-IR	13.9 ± 2.2	6.6 ± 0.9	0.002
HOMA-β	49.4 ± 10.9	55.2 ± 13.4	NS

This table shows the changes in various parameters from day 2 to day 30 of the diet. A paired Student's *t*-test was used since all patients served as their own controls. Because only 14 patients have completed the study these data represent only those 14 patients. Hence, values might differ from Table 1, because on day 0 data from 17 patients were available.
Values are presented as mean ± SEM. NS = not significant.

The decline in fasting serum leptin levels was paralleled by a decline in fasting serum insulin levels. On both day 2 and day 30 fasting serum leptin levels correlated positively with fasting serum insulin levels and AUC of insulin (Table 3 and Fig. 1). The change in fasting serum leptin levels from day 2 to day 30 (delta leptin 2-30) also correlated positively with the change in fasting serum insulin levels from day 2 to day 30 (delta insulin 2-30) ($r = 0.71$, $p = 0.005$) and the change in AUC of insulin from day 2 to day 30 (delta AUC insulin 2-30) ($r = 0.81$, $p = 0.001$).

Fasting serum leptin levels were positively correlated with body weight ($r = 0.52$, $p = 0.033$ on day 2; $r = 0.60$, $p = 0.024$ on day 30) and BMI ($r = 0.84$, $p = 0.0001$ on day 2; $r = 0.64$, $p = 0.014$ on day 30). Fasting serum insulin levels correlated positively with body weight and BMI on day 2, whereas the correlation with BMI was lost on day 30. After adjustment for BMI, gender and age, the positive correlation between fasting serum leptin and fasting serum insulin levels and AUC of insulin remained (Table 3). The decrease in fasting serum leptin levels from day 2 to day 30 was also positively correlated with the decrease in fasting serum insulin levels and the decrease in AUC of insulin from day 2 to day 30 after adjusting for BMI, gender and age. After correction for fat mass, the positive relation between fasting serum leptin and serum insulin (fasting and AUC) remained (Table 3).

No correlation was found between fasting serum leptin levels and either fasting plasma glucose (FPG) or k-values (as a measure of the glucose disposal rate). Fasting serum leptin levels also showed no correlation with the AUC of glucose during an IVGTT, HbA_{1c} levels, duration of type 2 diabetes, or fasting C-peptide levels. Fasting serum leptin levels were positively correlated with HOMA-IR ($r = 0.57$, $p = 0.017$ on day 2; $r = 0.64$, $p = 0.013$ on day 30) and HOMA-β ($r = 0.83$, $p = 0.0001$ and $r = 0.76$, $p = 0.001$ on day 2 and day 30, respectively),

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however. After correcting for BMI in a partial correlation analysis, these relations remained significant with the exception of HOMA-IR on day 2.

Table 3. Partial correlation analysis of fasting serum leptin with the fasting serum insulin and AUC of insulin.

		Day 2 (n=17)	Day 30 (n=17)
Fasting serum insulin (mU/L)	Unadjusted	r = 0.72, p = 0.001	r = 0.78, p = 0.001
	Adjusted for BMI	r = 0.51, p = 0.042	r = 0.81, p = 0.001
	Adjusted for BMI and gender	r = 0.58, p = 0.024	r = 0.80, p = 0.002
	Adjusted for BMI, gender and age	r = 0.60, p = 0.023	r = 0.90, p = 0.0001
	Adjusted for fat mass, gender and age	r = 0.58, p = 0.030	r = 0.86, p = 0.003
AUC of insulin (mU*50min)	Unadjusted	r = 0.74, p = 0.001	r = 0.84, p = 0.0001
	Adjusted for BMI	r = 0.54, p = 0.030	r = 0.83, p = 0.001
	Adjusted for BMI and gender	r = 0.61, p = 0.016	r = 0.84, p = 0.001
	Adjusted for BMI, gender and age	r = 0.64, p = 0.015	r = 0.93, p = 0.0001
	Adjusted for fat mass, gender and age	r = 0.60, p = 0.023	r = 0.94, p = 0.001

DISCUSSION

This study shows that, even in patients with a severely disturbed endogenous insulin secretion, a positive relation between fasting serum insulin and fasting serum leptin levels exists, even after correcting for BMI and body fat mass. This was true both during energy restriction (day 2) and during weight loss plus energy restriction (day 30). Furthermore, fasting serum leptin levels also correlated with HOMA-β and the AUC of insulin as measures of insulin secretory capacity.

We found a sharp decline in both fasting serum leptin and fasting serum insulin levels after only 2 days of the VLCD. Other investigators have also seen a rapid decrease in serum leptin levels with energy restriction[12,13,30-32]. Since fat mass can hardly have decreased significantly in such a short period of time, this decline in fasting serum leptin and insulin levels more likely reflects a signal to the brain that the body is in negative energy balance. Support for this concept can be found in the study of Chin-chance *et al.*[33]. Six healthy normal weight subjects were included in a 12-day study with four consecutive dietary treatment periods of 3 days each. A baseline period (feeding at 100% of total energy expenditure [TEE]) was followed by random crossover periods of overfeeding (130% TEE) or underfeeding (70% TEE), separated by a eucaloric period (100% TEE). Serum leptin levels responded acutely to modest changes in energy intake (declining during 70% TEE and increasing during 130% TEE) and, remarkably, returned to baseline values only after completion of the complementary feeding periods, indicating that leptin levels were a marker of short-term cumulative energy balance. In contrast, in the long-term, when weight loss occurs, serum leptin levels once again reflect body fat stores. Wadden *et al*.[34] showed that, in the first 6 weeks of a diet, serum leptin levels were primarily determined by the degree of caloric restriction, whereas at 40 weeks weight

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loss accounted for 47% of the variance in serum leptin levels. In addition, Cella *et al*.[13] found a gradual rise in serum leptin levels with further weight loss.

We also studied the relation between fasting serum leptin and fasting serum insulin levels after a period of energy restriction plus weight loss. To our knowledge, with regard to the relation between serum leptin and serum insulin levels after weight loss, few studies have been performed in obese type 2 diabetic patients. Moreover, these studies had either included very few patients[15,25] or patients with only mild diabetes and obesity[15,25].

Our study is, therefore, unique with regard to the extreme patient population (severely obese type 2 diabetic patients, inadequately regulated on maximal oral blood glucose-lowering medication and/or insulin therapy) and the fact that we performed a dynamic test in the form of an IVGTT. We were, therefore, able to demonstrate that the relation between fasting serum leptin and insulin levels, even after correction for BMI and fat mass, holds true over a wide range of residual endogenous insulin secretory capacity (as defined by the AUC of insulin).

What we were not able to demonstrate was whether this positive relationship between serum insulin and serum leptin levels is due to leptin regulating insulin levels or *vice versa*. Several facts point to the latter. Firstly, when octreotide is given to patients with an insulinoma, serum leptin levels fall within half an hour of the decline in serum insulin levels[35]. Secondly, during a prolonged hyperinsulinaemic euglycaemic clamp, serum leptin levels show a dose-dependent[36] increase[37]. Thirdly, serum leptin levels are increased by insulin therapy, both in patients with type 1 and type 2 diabetes[19,38]. Fourthly, when patients were stratified to high and low serum insulin groups, serum leptin levels were higher in the high insulin group than in the low insulin group, while BMI was the same[14]. Fifthly, conversely argued, leptin therapy does not increase serum insulin levels[39]; in fact, leptin probably diminishes insulin levels by directly inhibiting insulin secretion. To that end, functional leptin receptors are present on the cell membranes of pancreatic β-cells[40].

In conclusion, even in patients with a highly disturbed endogenous insulin secretion, a positive relation between fasting serum leptin and serum insulin levels (fasting and AUC) can be found. This relation was found during both energy restriction and weight loss. Whether insulin regulates leptin levels or *vice versa,* or, whether both are regulated in concert to reflect changes in energy balance cannot be deduced from this study. However, the evidence at hand makes it seem most likely that insulin regulates leptin.

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CHAPTER 4

Factors predicting the blood glucose-lowering effect of a 30-day very low calorie diet in obese type 2 diabetic patients.

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ABSTRACT

Calorie restriction and weight loss improve hyperglycaemia in some but not all obese patients with type 2 diabetes mellitus. To identify specific endocrine and metabolic markers that predict a favourable response to a very low calorie diet (VLCD), 17 obese (BMI 37.6 ± 5.6 kg/m^2 [mean ± SD]) type 2 diabetic (FPG 12.9 ± 3.1 mmol/L, HbA_{1c} 8.6 ± 1.6%) patients were studied on day 0, 2, 10 and 30, of a VLCD (Modifast®, 450 kCal/day). A responder was a priori defined as a patient with a fasting plasma glucose concentration (FPG) < 10 mmol/L on day 30. All blood glucose-lowering medication (including insulin) was discontinued on day -1. On day 2 and 30 of the VLCD an intravenous glucose tolerance test (IVGTT) was performed.

Of the 14 patients who completed the 30-day VLCD, eight qualified as responder. Responders and non-responders could be distinguished by day 2. Responders had a shorter duration of type 2 diabetes and higher fasting serum insulin, C-peptide and HOMA-β-values. In addition, responders displayed a more prominent second-phase insulin response following i.v. glucose loading and higher k-values. In a stepwise discriminant analysis, the change in FPG from day 0 to day 2 (responders + 0.64 ± 2.3, non-responders + 4.15 ± 3.3 mmol/L, p = 0.035) in combination with the area under the curve of insulin (AUC) above baseline during an IVGTT on day 2 (responders 571 ± 236, non-responders 88 ± 65 mU*50min, p < 0.001), distinguished responders completely from non-responders.

In conclusion, preservation of the capacity of β-cells to secrete insulin predicts a favourable metabolic response to a VLCD in obese type 2 diabetic patients. Already on day 2 a decline in FPG levels can be found in those patients that react favourably to the diet. Nevertheless, even in patients who qualified as non-responders, no gross hyperglycaemia (> 20 mmol/L) or any other side effects were observed.

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INTRODUCTION

Over 80% of type 2 diabetic patients are obese[1]. Numerous studies have shown that calorie restriction and weight loss can reverse their metabolic abnormalities[2-10]. After initiation of a very low calorie diet (VLCD), hyperglycaemia decreases within 4-10 days, even before significant weight loss has occurred[4,5,8]. In one study, a decrease in fasting plasma glucose (FPG) was detected within 2 days[11]. Another[4] study reported patients who failed to respond but an explanation was not given.

Neither the mechanism nor the factors that predict the blood glucose-lowering effect of energy restriction and weight loss have been established. The current study was undertaken to determine (i) if a decrease in FPG would occur within 2 days after the initiation of a VLCD and (ii) which factors predict a favourable metabolic response (defined as a FPG< 10 mmol/L on day 30) during a prolonged VLCD in obese type 2 diabetic patients when all blood glucose-lowering medication is discontinued.

PATIENTS AND METHODS

In 17 obese (BMI 37.6 ± 5.6 kg/m^2, mean ± SD) type 2 diabetic patients (duration 8.0 ± 5.8 years) who had persistent high blood glucose levels (12.9 ± 3.1 mmol/L) and HbA_{1c} percentages (8.6 ± 1.6%) despite maximal doses of oral blood glucose-lowering medication and/or insulin (66-340 units/day), all blood glucose-lowering medication was stopped (day –1) and a very low calorie diet (Modifast®, Novartis Consumer Health, Breda, the Netherlands, 450 kCal/day) was started for 30 days.

On days 0, 2, 10 and 30, body weight was measured, and fasting glucose, insulin, C-peptide and leptin were determined. In addition, an intravenous glucose tolerance test (IVGTT, 25 g of glucose i.v. in 4 min with blood sampling at 0, 2, 4, 6, 8, 10, 12, 20, 30, 40, 50 and 60 min) was performed after an overnight fast at days 2 and 30 of the VLCD[12,13]. We chose day 2 instead of day 0 for the first IVGTT because most patients had used NPH insulin the evening before the start of the study. For the same reason we used laboratory measures taken on day 2 for baseline values of fasting plasma insulin and C-peptide.

Statistical analysis and mathematical calculations

Values are presented as mean ± standard deviation (SD).

The glucose disappearance rate (k-value) was determined by (natural) log-linear regression of the glucose concentrations against time over the period from 10 to 60 min post-glucose loading[12]. The area under the curve (AUC) of glucose and of insulin were determined over the period from 0 to 60, respectively 10 to 60 min post-glucose loading from zero level using the linear trapezoidal rule. The AUC of glucose and insulin above baseline were also calculated. Baseline was defined as plasma glucose and insulin levels at time 0 min.

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Table 1. Metabolic response to a VLCD in responders and non-responders.

	Responders (n=8)			Non-responders (n=6)		
	Day 0	**Day 30**	**P**	**Day 0**	**Day 30**	**P**
FPG (mmol/L)	12.3 ± 2.3	7.9 ± 1.2*	0.001	13.4 ± 3.8	17.3 ± 4.4*	NS
Leptin (mg/mL)	31.7 ± 24.7	12.4 ± 8.9	NS	22.2 ± 8.3	8.2 ± 3.1	0.003
Body weight (kg)	119.4 ± 21.2	107.2 ± 20.3	0.0001	101.9 ± 6.9	89.7 ± 6.8	0.0001
BMI (kg/m^2)	39.3 ± 7.1	35.3 ± 6.7	0.0001	37.1 ± 3.6	32.7 ± 3.4	0.0001
	Day 2	**Day 30**	**P**	**Day 2**	**Day 30**	**P**
FI (mU/L)	30.6 ± 16.0†	18.8 ± 9.9†	0.034	12.8 ± 5.0†	8.3 ± 2.0†	NS
FCP (nmol/L)	1.8 ± 0.7†	1.1 ± 0.4	0.003	0.8 ± 0.1†	0.6 ± 0.2	0.042
AUC of insulin (mU*50 min)	2014 ± 978†	1494 ± 906†	0.042	775 ± 201†	388 ± 115†	0.040
AUC of insulin above baseline (mU*50 min)	571 ± 236‡	552 ± 425†	NS	88 ± 65‡	66 ± 73†	NS
AUC of glucose (mmol*60 min)	1094 ± 132†	860 ± 81‡	0.0001	1355 ± 274†	1305 ± 240‡	NS
AUC of glucose above baseline (mmol*60 min)	344 ± 107	372 ± 40†	NS	372 ± 32	280 ± 78†	NS
k-Value (%/min)	0.51 ± 0.08†	0.55 ± 0.08†	NS	0.37 ± 0.13†	0.36 ± 0.12†	NS
HOMA-IR	17.4 ± 9.2	6.7 ± 3.9	0.004	9.3 ± 2.7	6.5 ± 2.6	NS
HOMA-β	69.9 ± 42.4†	86.8 ± 44.8†	NS	22.2 ± 15.6†	13.1 ± 5.4†	NS

Data are presented as mean ± standard deviation. FI: fasting serum insulin; FCP: fasting serum C-peptide.

* P < 0.0001; † P < 0.05; ‡ P < 0.001, all responders *versus* non-responders

Estimates of insulin resistance and β-cell function by HOMA score were calculated with the formulas as described by Matthews *et al*.[14].

Comparisons between groups (i.e., responders *versus* non-responders) were made with the Student's *t*-test for independent samples. Within groups comparisons were made with the Student's *t*-test for paired samples. Stepwise discriminant analysis was performed to determine prognostic factors for distinction between responders and non-responders. *A priori*, a responder was defined as a patient with a FPG < 10 mmol/L on day 30.

A p-value of < 0.05 was considered statistically significant.

RESULTS

Fourteen out of the 17 patients completed the 30-day VLCD.

By 2 days of a VLCD, when weight loss was still minimal (responders -2.8 ± 0.7 kg, non-responders -2.4 ± 0.7 kg, NS), a distinction between responders and non-responders could be made. Responders showed only a minimal rise or even a decrease in FPG at day 2 (+0.64 ± 2.3 mmol/L), whereas non-responders had an increase in FPG (+4.15 ± 3.3 mmol/L), p = 0.035. On day 10, FPG had improved in responders (-2.7 ± 2.9 mmol/L) and remained more or less

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the same in non-responders (+4.2 ± 5.5), p = 0.011. After 30 days, FPG improved further in responders (-4.3 ± 2.4 mmol/L) whereas FPG remained elevated in non-responders (+3.9 ± 5.2 mmol/L), p = 0.002. All values given are compared with day 0 (Table 1).

Responders had a significantly higher fasting serum insulin and C-peptide concentration and HOMA-β on day 2 compared with non-responders (Table 1). During an IVGTT, responders had a significantly higher AUC and AUC above baseline of insulin (second-phase insulin response) on day 2 than non-responders. A first-phase insulin response was lacking in both groups on day 2 and day 30 (Fig. 1).

Neither the initial weight and fat mass nor the extent of weight loss (-12.2 ± 3.6 kg in responders, -12.2 ± 2.5 kg in non-responders, NS), or the decline in serum leptin were different between responders and non-responders. Previous blood glucose-lowering therapy and initial FPG were also similar in responders and non-responders.

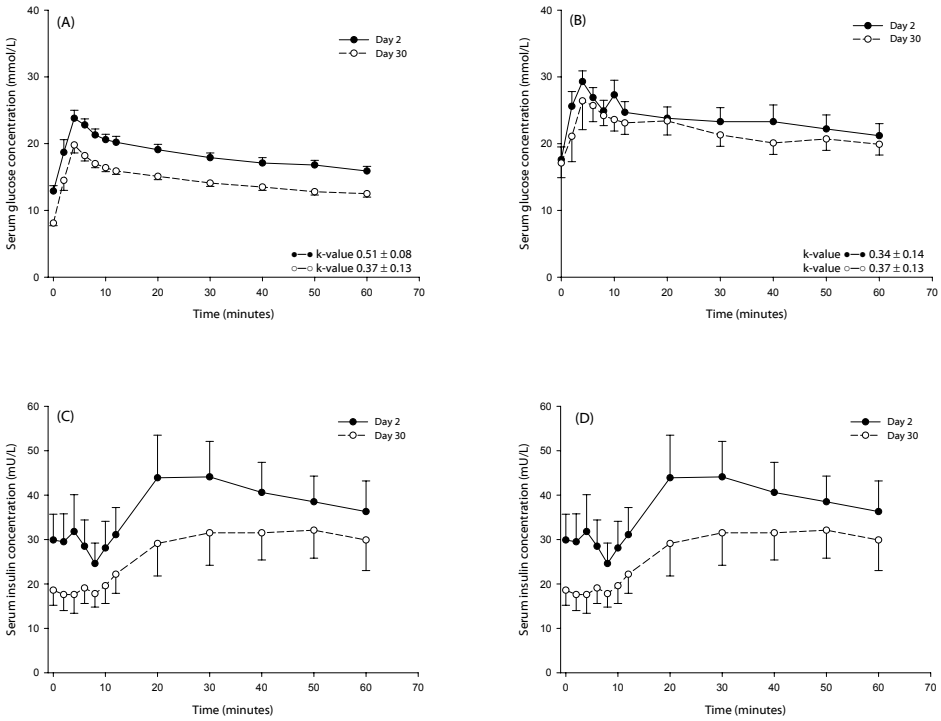

Figure 1.

Glucose excursions (top, A, B) and insulin secretion (bottom, C,D) of responders (left, A, C) and non-responders (right, B, D) after an intravenous glucose load on day 2 (closed circles) and day 30 (open circles) of a VLCD. Responders have a lower area under the curve (AUC) of glucose, a higher AUC of insulin and a higher k-value. After a 30-day VLCD, fasting plasma glucose (FPG) and fasting serum insulin decrease but incremental AUC of glucose and insulin do not change and neither do the k-values. Note that both responders and non-responders lack a first-phase insulin response. Data are presented as mean ± SEM.

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Stepwise discriminant analysis was performed to determine prognostic factors for distinction between responders and non-responders. The change in FPG from day 0 to day 2 combined with the AUC of insulin above baseline during an IVGTT on day 2 completely separated responders from non-responders. When IVGTT data were left out of the analysis, fasting C-peptide on day 2 and duration of diabetes were identified as discriminating factors although in this analysis two responders were misclassified as non-responders.

DISCUSSION

We examined the effect of a 30-day VLCD on FPG levels and glucose handling after an intravenous glucose load in obese type 2 diabetic patients in whom all blood glucose-lowering medication was discontinued. *A priori*, responders were defined as those patients who would have a FPG level less than 10 mmol/L on day 30.

It was found that within 2 days of a VLCD, when weight loss was still minimal (reflecting salt and fluid loss), a distinction between responders and non-responders could be made. Responders exhibited only a minimal increase or even a decrease in FPG at day 2 whereas non-responders showed a considerable increase in FPG.

Preservation of β-cell function appeared to predict a favourable response to a VLCD. Thus, responders had higher fasting serum insulin and C-peptide levels and a higher HOMA-β than non-responders on day 2. In addition, responders had a higher second-phase insulin response during an IVGTT. Other factors associated with a favourable response were a shorter duration of type 2 diabetes mellitus and higher k-values. Weight loss and the fall of serum leptin concentrations were not discriminating. A stepwise discriminant analysis showed that change in FPG from day 0 to day 2 combined with the AUC of insulin above baseline during an IVGTT on day 2 could fully discriminate responders from non-responders.

The fact that FPG improved by 2 days of a VLCD confirms earlier observations[2,4-6,8] that reduced caloric intake and not weight loss is of prime importance to the early blood glucose reduction. The mechanism of this early beneficial effect on glucose metabolism is unclear although several studies have reported a close association of FPG with hepatic glucose output (HGO)[3-5,7,8,15].

After 30 days of a VLCD, both responders and non-responders had lost about 12 kg of body weight. Both groups had a decrease in fasting serum insulin but it remained significantly higher in responders than in non-responders. HOMA-β was also higher in responders compared with non-responders and did not change significantly in either group after a 30-day VLCD. HOMA-IR was similar in both groups after 30 days of a VLCD.

In a dynamic test (IVGTT), AUC of glucose above baseline, k-values and the amount of insulin secreted remained the same after 30 days of a VLCD in both responders and non-responders. Thus, the only factors that changed favourably after 30 days of a VLCD were a lower FPG in

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responders and lower fasting serum insulin concentrations in both groups. This lower FPG in responders, in the presence of a lower serum insulin concentration, might have been caused by an increased sensitivity of the liver for insulin suppression of HGO. Because the k-values did not improve, we have no arguments for an increased peripheral glucose disposal.

This study again stresses the potential of diet therapy in obese type 2 diabetic patients. Eight out of the 14 (57%) patients had a decrease in FPG levels and none of those eight had to be restarted on insulin during a weight-maintaining diet (data not shown). We are aware, however, that our study included small numbers and follow-up was limited.

In conclusion, this study shows that by 2 days of a VLCD a distinction can be made between those who will react favourably to the diet and those who will not. Responders can be identified on the basis of a preserved capacity of the β-cell to secrete insulin. In this study, the change of the fasting plasma glucose concentration during the first 2 days of the VLCD in combination with the AUC of insulin above baseline during an IVGTT on day 2 could separate responders completely from non-responders.

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CHAPTER 5

Two days of a very low calorie diet reduces endogenous glucose production in obese type 2 diabetic patients despite the withdrawal of blood glucose-lowering therapies, including insulin.

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ABSTRACT

The mechanism of the blood glucose-lowering effect of a 2-day very low calorie diet (VLCD; 1890 kJ/d [450 kCal/day]) in combination with the cessation of all blood glucose-lowering agents was studied in 12 (7 women, 5 men) obese (body mass index 36.3 ± 1.0 kg/m^2 [mean ± SEM]) type 2 diabetic patients (age 55 ± 4 years; HbA_{1c}, 7.3 ± 0.4%) undergoing insulin therapy. Endogenous glucose production (EGP) and whole-body glucose disposal ([6,6 $^2H_2$]-glucose), lipolysis ([$^2H_5$]-glycerol), and substrate oxidation (indirect calorimetry) rates were measured before and after the intervention in basal and hyperinsulinaemic euglycaemic conditions.

After 2 days of a VLCD and discontinuation of all blood glucose-lowering therapies, fasting plasma glucose levels did not increase (11.3 ± 1.3 versus 10.3 ± 1.0 mmol/L). Basal EGP significantly declined (14.2 ± 1.0 to 11.9 ± 0.7 μmol.kg^{-1}.min^{-1}, p = 0.009). Basal metabolic clearance rate of glucose and rate of basal lipolysis did not change. During hyperinsulinaemia, EGP (5.5 ± 0.8 to 5.2 ± 0.5 μmol.kg^{-1}.min^{-1}), whole-body glucose disposal (12.1 ± 0.7 to 11.3 ± 1.0 μmol.kg^{-1}.min^{-1}), the metabolic clearance rate of glucose, and the rate of lipolysis did not change after the 2-day intervention.

In conclusion, cessation of blood glucose-lowering therapy in combination with a 2-day VLCD does not lead to hyperglycaemia and is associated with a reduction in basal EGP. Insulin-stimulated whole-body glucose disposal did not improve, nor did insulin suppressibility of EGP and lipolysis.

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INTRODUCTION

There is a strong relationship between type 2 diabetes and obesity[1], more than 70% of type 2 diabetic patients are overweight and obese[2]. In obese patients, insulin resistance is the most important underlying defect leading to glucose intolerance and, subsequently, when hyperinsulinaemia is insufficient to overcome insulin resistance, type 2 diabetes develops[3]. Numerous studies have shown that weight loss diminishes the metabolic abnormalities of obese type 2 diabetic patients[4-10]. Because patients usually find it difficult to adhere to a diet, very low calorie diets (VLCDs) have been advocated. The rapid weight loss achieved with these diets is an important stimulus for patients to continue. The simultaneous discontinuation of a blood glucose-lowering therapy facilitates weight loss and minimises the risk of hypoglycaemia but raises concern about possible hyperglycaemia. We recently showed in a group of obese type 2 diabetic patients, in whom we discontinued all blood glucose-lowering therapies including insulin, that a VLCD (Modifast®; 450 kCal/day) does not lead to a deterioration of fasting plasma glucose (FPG) levels[11]. In fact, in most patients, a decrease in FPG occurred already after 2 days of the VLCD, when weight loss was minimal.

A decline in FPG levels before significant weight loss occurred has been described before[5,6,9,12]. Several studies have shown that FPG declined in parallel with hepatic glucose output[5,6,8,12]. However, to our knowledge, no one has studied this effect in detail after only 2 days of a VLCD. In addition, few studies address the patient group we are interested in: severely obese type 2 diabetic patients inadequately regulated on insulin therapy. We therefore studied obese type 2 diabetic patients undergoing insulin therapy with or without oral blood glucose-lowering agents before and after 2 days of a VLCD in combination with the cessation of these medications.

We used the isotope dilution technique to measure endogenous glucose production (EGP) in combination with the hyperinsulinaemic euglycaemic clamp technique to study insulin-mediated peripheral glucose disposal and insulin suppressibility of EGP. In addition, we measured total-body lipolysis *via* the infusion of deuterium-labelled glycerol and substrate oxidation rates *via* indirect calorimetry.

RESEARCH DESIGN AND METHODS

Subjects

A total of 12 obese type 2 diabetic patients, 5 men and 7 women with a mean age of 55 ± 4 years (mean ± SEM) and a body mass index (BMI) of 36.3 ± 1.0 kg/m^2 (range 31.3 – 43.9 kg/m^2), participated in this study, which was approved by the Medical Ethical Committee of the Leiden University Medical Centre. Written informed consent was obtained from all patients. Patients underwent a medical screening including a physical examination and resting

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electrocardiogram. Patients used at least 30 units of exogenous insulin with or without oral blood glucose-lowering medication and had a BMI > 30 kg/m^2. In addition, they had to have remaining endogenous insulin secretion defined as a fasting plasma C-peptide level greater than 0.8 ng/mL or a 2-times increase of the basal C-peptide level after 1 mg glucagon i.v.[13].

Patients had to have a stable weight for at least 3 months and were instructed not to alter life style habits (eating, drinking, exercise) from screening until the start of the study. None of the patients were smokers and the use of any other medication (than that used specifically for its glucose-lowering effect) known to alter glucose or lipid metabolism was prohibited.

Protocol

Three weeks before the start of the study, all oral blood glucose-lowering medication was discontinued. On day -1, only short-acting insulin was given, evening doses of intermediate and long-acting insulin were omitted. On day 0, patients were admitted to the research centre for baseline investigations (day 0) as outlined below. Insulin therapy was restarted after this study day until the start of the VLCD (again, only short-acting insulin was given on the day before the start of the diet) and remained stopped during the 2-day VLCD. To ensure complete washout of the stable isotopes, the second study had to be undertaken 1 week later. This meant that patients started the 2-day VLCD (1890 kJ/d) on day 5 and had the second study on day 7 (day 2). (See Fig. 1)

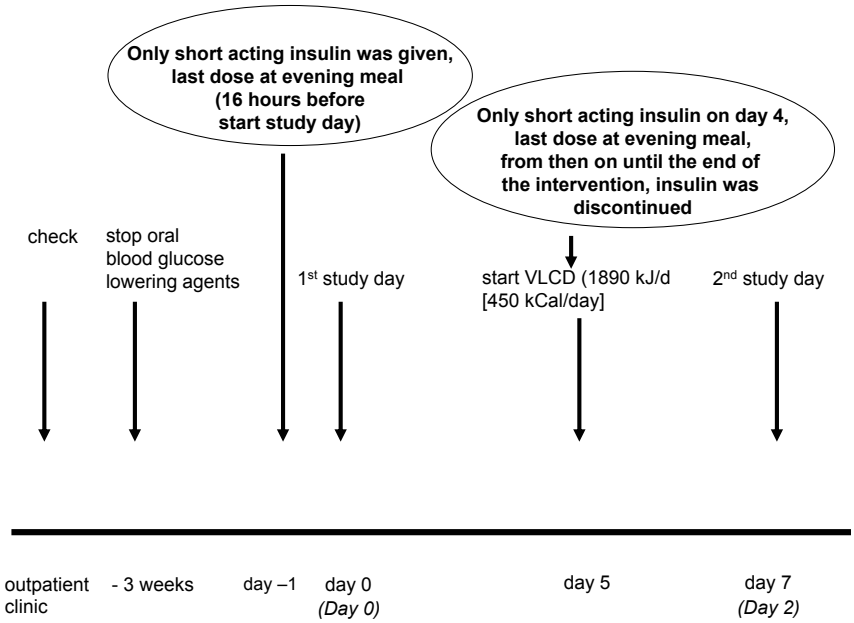

Figure 1.
Protocol outline. See text (methods) for explanation.

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STUDY DAYS

All studies started at 7:00 AM after an overnight fast. Length (meters [m]), weight (kilograms [kg]), BMI (weight [kg] / length^2 [m]) and waist-hip circumference were measured according to WHO recommendations[14].

Patients were subsequently requested to lie down on a bed in a semirecumbent position. A polyethylene catheter was inserted into an antecubital vein for infusion of test substances. Another catheter was inserted into a contralateral dorsal hand vein for blood sampling. This hand was kept in a heated box (60°C) throughout the test to obtain arterialised venous blood samples[15]. Basal blood samples for glucose, insulin, C-peptide, non-esterified fatty acids (NEFAs), glycerol, and background enrichment of [6,6-$^2H_2$]-glucose and [$^2H_5$]-glycerol were taken. At 7:30 AM (t = 0 minutes), an adjusted primed (17.6 µmol/kg x actual plasma glucose concentration (mmol/L)/5(normal plasma glucose)[16] continuous (0.33 µmol/kg per minute) infusion of [6,6-$^2H_2$]-glucose (enrichment 99.9%; Cambridge Isotopes, Cambridge, Mass, USA) was started and continued throughout the study. At 9:00 AM (t= 90 minutes) a primed (1.6 µmol/kg) continuous (0.11 µmol/kg per minute) infusion of [$^2H_5$]-glycerol (Cambridge Isotopes) was started and continued throughout the study. During this period, indirect calorimetry with a ventilated hood (Oxycon Beta, Mijnhardt Jaegher, Breda, The Netherlands) was performed for 30 minutes for basal glucose and lipid oxidation rates[17]. At the end of the basal period, 3 blood samples were taken at 7-minute intervals for the determination of plasma glucose, glycerol, insulin, and [6,6-$^2H_2$]-glucose- and [$^2H_5$]-glycerol-specific activitities. In addition, blood samples for the determination of NEFAs, triglycerides, lactate, the counterregulatory hormones (growth hormone [GH], cortisol, and glucagon), as well as some of the adipokines involved in glucose metabolism (leptin, resistin and adiponectin) were taken. Subsequently, a primed continuous infusion of insulin (Actrapid®, Novo Nordisk Pharma, The Netherlands, 40 mU/m^2 per minute)[18] was started (t = 180 minutes). Exogenous glucose 20% enriched with 3% [6,6-$^2H_2$]-glucose was infused at a variable rate to maintain the plasma glucose level at 5.0 mmol/L. A second indirect calorimetry was performed at the end of the hyperinsulinaemic clamp (t = 390 minutes). From t = 420 to 450 minutes, blood was drawn every 10 minutes for the determination of [6,6-$^2H_2$]-glucose- and [$^2H_5$]-glycerol-specific activity, glucose, insulin, glycerol, C-peptide, NEFAs, triglycerides, lactate, GH, cortisol, glucagon, leptin, resistin and adiponectin.

All blood samples, except serum samples, were immediately put on ice and centrifuged promptly (2000×g at 4°C for 20 minutes). Serum samples first had to coagulate before undergoing the same procedure. Samples were subsequently put in plastic tubes and frozen (–20°C) until assay.

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BLOOD CHEMISTRY

Serum insulin, C-peptide, glucagon, GH, cortisol, leptin, resistin, adiponectin, triglycerides, and lactate were measured in one batch. Serum insulin was measured with an ultrasensitive Human Insulin assay (Linco Research, St Charles, MO) with a detection limit of 0.1 mU/L. The interassay coefficient of variation (CV) was below 6%.

C-peptide, glucagon, leptin, resistin and adiponectin were measured with radioimmunoassays from Linco Research. For C-peptide the interassay coefficient of variation (CV) varied between 4.2% and 6.0% at different levels with a sensitivity of 0.03 nmol/L. The CV for glucagon ranged between 4.0% and 6.8% with a sensitivity of 20 ng/L. For leptin, the CV was 3.0% to 5.1% and the sensitivity was 0.5 µg/L. For resistin, the interassay CV was 3.2% to 5.4% at different levels, with the lowest detection level of 0.15 µg/L. Adiponectin had an interassay CV of 6.3% to 8.1% with the lowest detection level of 1 µg/L.

Growth hormone was measured with a time-resolved immunofluorescent assay (Wallace Inc, Turku, Finland) specific for the 22-kDa GH. The CV varied from 5.3% to 8.4%, sensitivity was 0.03 mU/L. Cortisol was also measured with a radioimmunoassay (Sorin Biomedica, Milan, Italy) with a CV between 2.3% and 4.2% and a detection limit of 25 nmol/L. Serum triglycerides and lactate were determined with a fully automated Hitachi 747 system (Hitachi, Tokyo, Japan).

Serum glucose and [6,6-$^{2}H_{2}$]-glucose as well as serum glycerol and [$^{2}H_{5}$]-glycerol were determined in a single analytical run, using gas chromatography coupled to mass spectrometry as described previously[19,20].

Serum non-esterified fatty acids were measured using the enzymatic colorimetric acyl-CoA synthase/ acyl-CoA oxidase assay (Wako Chemicals, Neuss, Germany) with a detection limit of 0.03 mmol/L. The interassay coefficient of variation was below 3%.

Very low calorie diet

The diet consisted of 3 sachets of Modifast® (Novartis Consumer Health, Breda, The Netherlands) per day. Modifast® is a commercially available VLCD packaged in powder form. One sachet is mingled with 250 mL of water and is used to replace each of the 3 conventional meals. We provided patients with shakes, muesli, pudding and potage in various tastes. One hundred grams of Modifast® contains 1402.8 kJ [334] kcal and about 35 g protein, 6 g fat and 38 g carbohydrates. Since sachets vary from 42 to 50 gram, energy intake could range from 1764 to 2062.2 kJ/d depending on the products used. Patients were allowed to drink calorie-free substances *ad libitum* and were encouraged to drink at least 2 L of these liquids per day.

Calculations

In all subjects, both plasma glucose concentrations and tracer/tracee ratios of [6,6-$^{2}H_{2}$]-glucose and [$^{2}H_{5}$]-glycerol were stable during the last half hour before the clamp (t = 150-180

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minutes) and during the last hour of the clamp (*t* = 390-450 minutes). In addition, the plasma glucose concentration did not decline during the last hour before the clamp and the last hour of the euglycaemic clamp. Therefore, the rate of appearance (R_a) for glucose and glycerol were calculated using Steele's steady-state equation as adapted for stable isotopes using a single-compartment kinetic model[21].

Endogenous glucose production during the basal steady state is equal to the Ra of [6,6-$^2H_2$]-glucose, whereas endogenous glucose production during the clamp was calculated as the difference between R_a and the glucose infusion rate.

The metabolic clearance rate (MCR) of glucose was calculated as the rate of disappearance of glucose (R_d; identical to R_a under steady-state conditions) divided by the serum glucose concentration (average of steady-state measurements at *t* = 150-180 and *t* =420-450 minutes, respectively).

Total lipid and carbohydrate oxidation rates were calculated as described by Simonson and DeFronzo[17]. For the conversion of fat oxidation from milligram per kilogram per minute to micromole per kilogram per minute, an average molecular weight of 270 was assumed for serum NEFAs[12]. Non-oxidative glucose metabolism was calculated by subtracting the glucose oxidation rate (determined by indirect calorimetry) from R_d.

Statistical analysis

Data are presented as mean ± SEM unless stated otherwise. Differences before (day 0) and after (day 2) the VLCD were analysed by the Student *t*- test for paired samples. Correlation analysis was carried out using Pearson's correlation. All analyses were performed using SPSS for Windows version 11.0 (SPSS Inc, Chicago, IL, USA). Significance was accepted at $p < 0.05$.

RESULTS

Of the 12 patients participating in this study, clamp data from one female patient had to be excluded from the analysis because of errors in the infusion rate in the afternoon of the second study day. Basal data from this patient and substrate oxidation rates could be and were used, however. Patient characteristics can be found in Table 1.

Weight

After 2 days of a VLCD, patients had lost -2.9 ± 0.4 kg. Presumably, this weight loss reflects mostly salt and fluid loss.

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Table 1. Patient characteristics.

Sex (male/female)	5 : 7
Age (years)	55 ± 4
BMI (kg/m^2)	36.3 ± 1.0
Waist circumference (cm)	120 ± 3
Waist-hip ratio	1.02 ± 0.03
Fasting plasma glucose (mmol/L)	11.3 ± 1.3
HbA_{1c} (%)	7.3 ± 0.4
Fasting serum insulin (mU/L)	20.7 ± 2.1
Fasting serum C-peptide (ng/mL)	1.0 ± 0.1
Duration of type 2 diabetes (years)	7.9 ± 1.3
Units of insulin injected per day	78 ± 9
Additional use of oral glucose-lowering medication	6 metformin 1 rosiglitazone

Data are presented as mean ± SEM.

Fasting plasma glucose and insulin concentration

After 2 days of a VLCD, despite minimal weight loss (see above) and the cessation of all blood glucose-lowering agents, FPG did not increase. Basal serum insulin levels declined from 20.7 ± 2.3 to 15.9 ± 1.8 mU/L (p = 0.033) (Table 2).

Endogenous glucose production, whole-body glucose disposal, and MCR of glucose

Basal EGP declined from 14.2 ± 1.0 to 11.9 ± 0.7 mmol/L (p = 0.008). On both study days, serum glucose was clamped at identical levels (5.0 ± 0.4 mmol/L on day 0 and 4.9 ± 0.4 mmol/L on day 2, p = NS) and the same degree of hyperinsulinaemia was obtained (88.1 ± 5.9 mU/L on day 0 and 83.7 ± 4.8 mU/L on day 2, p = NS) (see also Table 2). Insulin decreased EGP (from 14.2 ± 1.0 to 5.5 ± 0.8 μmol.kg^{-1}.min^{-1} on day 0) but could not completely suppress it. A 2-day

Table 2. Metabolic parameters at baseline (day 0) and after 2 days of a VLCD (day 2) in obese type 2 diabetic patients.

	Day 0	Day 2	P
Fasting serum glucose (mmol/L)	11.3 ± 1.3	10.3 ± 1.0	NS
Fasting serum insulin (mU/L)	20.7 ± 2.3	15.9 ± 1.8	0.033
Fasting serum cortisol (nmol/L)	570 ± 69	612 ± 58	NS
Fasting serum GH (mU/L)	1.9 ± 0.9	1.2 ± 0.4	NS
Fasting serum glucagon (ng/L)	57.3 ± 7.7	64.2 ± 8.6	NS
Fasting serum glycerol (μmol/L)	137 ± 19	186 ± 32	NS
Fasting serum NEFA (mmol/L)	1.1 ± 0.1	1.5 ± 0.1	NS
Fasting serum triglycerides (mmol/L)	1.8 ± 0.2	2.0 ± 0.2	NS
Fasting serum lactate (mmol/L)	0.9 ± 0.1	0.8 ± 0.04	NS
Clamp serum glucose (mmol/L)	5.0 ± 0.4	4.9 ± 0.4	NS
Clamp serum insulin (mU/L)	88.1 ± 5.9	83.7 ± 4.8	NS
Clamp serum glycerol (μmol/L)	60.0 ± 6.2	56.3 ± 7.0	NS
Clamp serum NEFA (mmol/L)	0.39 ± 0.07	0.35 ± 0.04	NS

Values are presented as mean ± SEM.

NS indicates not significant.

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Table 3. Metabolic parameters at baseline (day 0) and after 2 days of a VLCD (day 2) in obese type 2 diabetic patients.

	Day 0	Day 2	P
Basal EGP[a]	14.2 ± 1.0	11.9 ± 0.7	0.008
Clamp glucose $R_a = R_d$	12.1 ± 0.7	11.3 ± 1.0	NS
Clamp EGP	5.5 ± 0.8*	5.2 ± 0.5*	NS
Basal MCR	1.5 ± 0.1	1.4 ± 0.1	NS
Clamp MCR	2.6 ± 0.2*	2.4 ± 0.3*	NS
Basal whole-body glucose oxidation	6.1 ± 0.8	3.0 ± 0.4	0.0001
Clamp whole-body glucose oxidation	8.8 ± 1.0†	6.4 ± 0.6*	0.015
Basal non-oxidative glucose metabolism	8.6 ± 1.0	8.9 ± 0.7	NS
Clamp non-oxidative glucose metabolism	3.0 ± 1.3‡	5.2 ± 1.0‡	NS
Basal glycerol R_a	5.2 ± 1.0	4.0 ± 0.6	NS
Clamp glycerol R_a	1.9 ± 0.2‡	1.8 ± 0.2‡	NS
Basal whole-body lipid oxidation	3.8 ± 0.2	4.5 ± 0.1	0.002
Clamp whole-body lipid oxidation	2.9 ± 0.2*	3.4 ± 0.2*	0.022

All values are presented as mean ± SEM. [a] Units are in $umol.kg^{-1}.min^{-1}$.

Clamp compared to basal values: * $p = 0.0001$; † $p = 0.001$; ‡ $p < 0.008$

R_a = glucose rate of appearance, R_d = glucose rate of disappearance, MCR = metabolic clearance rate of glucose

VLCD showed no improvement of insulin suppressibility of EGP (see also Table 3). Glucose Rd did not increase during hyperinsulinaemia on both day 0 and day 2, indicating that patients remained severely insulin resistant. Serum glucose MCR, both basal as well as during hyperinsulinaemia, also did not reveal any significant change between study days (Table 3, Fig. 2).

Non-esterified fatty acids, lactate, glycerol, triglycerides, and hormones

Basal plasma NEFA levels increased from 1.1 ± 0.1 to 1.5 ± 0.1 mmol/L after 2 days of a VLCD (p = NS). Plasma NEFAs were suppressed during the hyperinsulinaemic euglycaemic clamp to 0.4 ± 0.06 and 0.4 ± 0.04 on day 0 and day 2, respectively (change between study days, NS). Basal and hyperinsulinaemic glycerol, triglyceride, and lactate levels did not significantly change after a 2-day VLCD as well (Table 2).

We also measured the serum concentrations of the counterregulatory hormones: glucagon, cortisol and GH. None of these hormones showed significant changes between day 0 and day 2 in either the basal or insulin-stimulated state.

Basal serum leptin levels showed a significant decline after a 2-day VLCD. Only serum leptin levels showed a significant correlation with BMI ($r = 0.73$, $p = 0.007$ on day 0; $r = 0.81$, $p = 0.001$ on day 2). None of the 3 adipokines (leptin, resistin, and adiponectin) showed (before and after the intervention) a correlation with measures of insulin resistance such as fasting serum insulin, MCR and R_d of glucose (data not shown).

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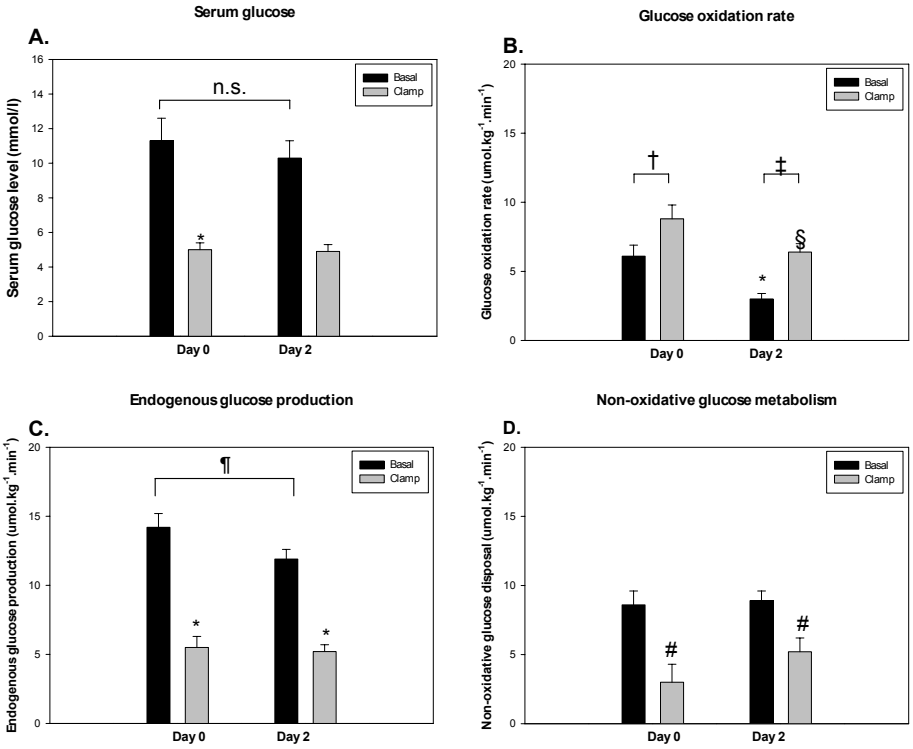

Figure 2.

Plasma glucose levels (A), endogenous glucose production (C) and oxidative (B) and non-oxidative (D) glucose disposal in 12 obese type 2 diabetic patients before and after a 2-day VLCD. Black bars represent basal values, grey bars represent values during the hyperinsulinaemic clamp. Values are presented as mean ± SEM. Note the decrease in FPG (A, black bars) due to a decrease in basal EGP (C, black bars), and the switch from glucose (B) to lipid oxidation (D).

Clamp compared with basal: * p= 0.0001; $^{\#}$ p < 0.008; § p = 0.015

Day 0 compared wiht day 2: † p = 0.001; ‡ p = 0.0001; ¶ p = 0.008

n.s. indicates not significant.

Glycerol R_a

Basal glycerol R_a did not change significantly after a 2-day VLCD. Insulin significantly suppressed glycerol R_a (5.2 ± 1.0 to 1.9 ± 0.2 μmol.kg^{-1}.min^{-1} on day 0 [p= 0.004] and from 4.0 ± 0.6 to 1.8 ± 0.2 μmol.kg^{-1}.min^{-1} on day 2 [p=0.002]). Glycerol R_a during hyperinsulinaemia was not different between study days (Table 3).

Glucose and lipid oxidation rates

Both basal and insulin-stimulated glucose oxidation rates significantly decreased after a 2-day VLCD, whereas lipid oxidation rates (both basal and insulin stimulated) increased. Basal as well as clamp non-oxidative glucose disposal remained the same before and after the 2-day VLCD (Table 3).

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DISCUSSION

In this study, we assessed the determinants of the blood-glucose lowering effect of 2 days of energy restriction (VLCD; 1890 kJ/d [450 kCal/day]) in severely obese type 2 diabetic patients in whom all blood glucose-lowering agents including insulin were discontinued.

In the absence of a deterioration of blood glucose levels, we demonstrated a decrease of basal EGP. Insulin-stimulated whole-body glucose disposal did not improve, nor did insulin suppressibility of EGP and lipolysis.

Several studies have proven that energy restriction leads to a reduction in FPG levels[4-10] and even that FPG is closely and positively correlated to basal EGP[5,6,8]. However, these studies were either incapable of distinguishing between the effects of energy restriction and those of weight loss on glucose metabolism or were performed in a patient group with mild type 2 diabetes. Only one study[12] closely matches our study with regard to patient population (i.e., severely obese type 2 diabetic patients undergoing insulin therapy) and timing of the first study day (although still on day 5, in comparison with day 2 in our study). However, their patients were probably provided with more calories compared with our patients, who received on average 1890 kJ/d [450 kCal/d]. In addition, it is not clear how much insulin the patients in the Christiansen *et al*. study used. Given that oral glucose-lowering medication and/or insulin were discontinued 2 weeks before the start of the study with no major dysregulation of their blood sugar levels despite the fact that they still ate their usual amount of calories suggests that these patients used little medication and had milder diabetes than did our patients. Nonetheless, in the study of Christiansen *et al*., the short period of energy restriction also led to a decrease in FPG levels caused by a reduction in basal EGP. Remarkably, the reduction in EGP was entirely caused by a decrease in glycogenolysis.

We only measured total EGP and could not discriminate between gluconeogenesis and glycogenolysis. The finding of Christiansen *et al.*[12] that a decreased glycogenolysis accounts for the decline in EGP after energy restriction is further supported by Clore *et al.*[22] and Clore and Blackard[23]. They repeatedly show that liver glycogen stores are preserved in type 2 diabetic patients after a 3-day fast, suggesting that glycogenolysis is suppressed. However, another study investigated type 2 diabetic patients and control subjects between 14 and 22 hours of fasting[24]. In that study, both gluconeogenesis and glycogenolysis declined during the fast, with a greater reduction of gluconeogenesis in diabetic subjects compared with control subjects. We believe that a decrease in glycogenolysis would be more obvious because higher doses of insulin are needed to suppress gluconeogenesis as compared to glycogenolysis[25,26]. So, we postulate that, in our study, the decreased basal EGP can be ascribed to a decrease in glycogenolysis, particularly because the decrease in basal EGP occurred despite lower basal serum insulin levels on day 2. This would suggest that the liver, in the postabsorptive state, has become more sensitive to insulin, at least with respect to glycogenolysis. However, 2 days of energy restriction had no effect whatsoever on insulin's capacity to suppress EGP dur-

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ing the hyperinsulinaemic clamp. This inability to demonstrate an effect of 2 days of energy restriction on insulin action in the liver (and in adipose tissue) may have been caused by the relatively high insulin levels (88 mU/L [528 pmol/L] and 84 mU/L [504 pmol/L] on day 0 and day 2, respectively) achieved during the clamp. These concentrations might have been high enough for a near-maximal suppression of the glucose and glycerol R_a. Perhaps a differentiating effect between the 2 study days would be found if glucose and glycerol R_a were studied at lower insulin concentrations.

Basal EGP showed a significant decrease of 16% after 2 days of a VLCD whereas basal FPG levels decreased only by 8%. Normally, a close correlation is found between FPG and basal EGP[5,27]. Our patient group, however, had higher FPG levels than that in the study of Fery[27] and the number of patients we studied was much smaller than that of Henry *et al.*[5], who also pooled the data of 4 time point measurements from each patient (giving 58 measurements). Hence, one possible explanation for the discrepancy between the results from our study and those from other studies[5,27] regarding the relation between EGP and FPG could be the small sample size in our study. On the other hand, although the change was not significant, FPG levels did decrease and, hence, the substrate-driven glucose uptake could have decreased after 2 days of a VLCD (clamp glucose disposal tended to decrease on day 2; see Table 3), which might have partly counteracted the decrease in EGP levels.

Another finding of this study was a lack of improvement in whole-body glucose disposal and glucose MCR. This is also in accordance with the study of Christiansen *et al.*[12]. They found an increase in MCR not before day 20 of energy restriction. In patients with mild diabetes (undergoing a diet or oral blood glucose-lowering medication only) a 4-day energy-restricted diet (but still providing 4620 +/- 1050 kJ/d [1100 ± 250 kCal/day]) even resulted in a deterioration of basal MCR of glucose and of insulin-stimulated glucose disposal[9]. The latter is in accordance with fasting[28,29] and low caloric feeding[30] studies in lean normal glucose-tolerant subjects who show a decreased peripheral glucose disposal as well. From an evolutionary perspective, this is understandable since more glucose will now be available for the brain. The fact that this response is not apparent in obese type 2 diabetic patients is probably the result of the already severely insulin-resistant state.

The fact that NOGD decreased during the hyperinsulinaemic euglycaemic clamp was unexpected. In healthy subjects, NOGD increases, along with total glucose disposal during hyperinsulinaemia, whereas the rate of increase in glucose oxidation seems to be bound to a limit[31], indicating that NOGD is quantitatively the most important. In obese and type 2 diabetic patients, NOGD is disturbed. With increasing obesity and insulin resistance, total glucose disposal and NOGD during hyperinsulinaemia are much lower compared with control subjects[32,33]. Our patients had severe insulin resistance. Despite clamp insulin levels of 88 and 83 mU/L on day 0 and day 2 respectively, glucose disposal did not change significantly and NOGD decreased. There was apparently some room for a slight increase in glucose oxidation

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during hyperinsulinaemia. These findings reflect the severely insulin-resistant state of our subjects with a core defect in glucose storage as glycogen (NOGD).

We showed, in accordance with Markovic *et al*.[9] and Christiansen *et al*.[12], a switch from carbohydrate to lipid oxidation. What we had not expected beforehand was that the rate of basal lipolysis did not increase. This is in contrast to data found in lean nondiabetic subjects who show an increase in whole-body glycerol turnover and whole-body lipid oxidation after 5 days of energy restriction[34]. However, 2 other studies in obese[35] and obese diabetic[12] patients (albeit performed after a longer period of energy restriction [5-20 days]), also found no increase in basal lipolysis. This might be indicative of a disturbed lipid metabolism in obese and obese diabetic subjects. On the other hand, the R_a of glycerol might have been already maximally elevated in these insulin resistant subjects, leaving no room for further increment of lipolysis during fasting. The increased lipid oxidation might therefore be counterbalanced by a decrease in lipogenesis.

We found no arguments for a role of the counterregulatory hormones we measured in the blood glucose-lowering effect of the VLCD because the concentrations of these hormones remained unchanged. This is also true for the adipokines adiponectin and resistin. Whereas the role of resistin in insulin resistance in human beings is controversial[36], it is well established that adiponectin concentrations are negatively correlated with insulin resistance, even independently of BMI[37,38]. Adiponectin levels increase with weight loss in parallel with insulin sensitivity[39]. We found no change in serum adiponectin levels after 2 days of a VLCD, which is consistent with the fact that we also found no change in insulin sensitivity and only a small amount of weight loss, mainly reflecting salt and fluid loss. Leptin, another adipocyte-derived hormone has a major role in maintaining energy homeostasis but is also thought to have glucose- and insulin-lowering properties[40,41]. The decrease in serum leptin levels we found most likely reflects the negative energy balance and is consistent with findings in other studies.

We were particularly interested in obese type 2 diabetic patients undergoing insulin therapy because adequately regulated blood glucose levels are usually not achieved in these patients, instead, insulin usually aggravates the insulin-resistant state by inducing weight gain. The fact that plasma glucose levels do not deteriorate despite the cessation of all blood glucose-lowering agents offers therapeutic options. The current study was designed to study the mechanism underlying the early reduction in blood glucose levels after energy restriction and not its long-term effect. We observed, however, that 2 patients had increasing blood glucose levels during the first few days of the VLCD but ended up normoglycaemic (without any form of medication) after continuation of this diet and substantial weight loss. We are currently investigating the effect on glucose metabolism of short-term energy restriction *versus* longer-term energy restriction with substantial weight loss, again in obese type 2 diabetic patients undergoing insulin therapy. Further studies are warranted to determine if any factor can predict *a priori* which patients will benefit from the diet on the long term. This might withhold doctors to treat potentially nonresponsive patients with a demanding VLCD.

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In conclusion, despite the cessation of large doses of insulin and oral blood glucose-lowering medication in obese type 2 diabetic patients, FPG levels do not increase and even tend to decline already after 2 days of a VLCD, when weight loss is minimal. The mechanism underlying this early effect of a VLCD is a reduction in basal EGP and not an improvement in whole-body glucose disposal.

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CHAPTER 6

Effect of a 2-day very low energy diet on skeletal muscle insulin sensitivity in obese type 2 diabetic patients on insulin therapy.

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ABSTRACT

This study investigates the molecular mechanisms underlying the blood glucose-lowering effect of a 2-day very low-energy diet (VLED, 1890 kJ/d = very low calorie diet [VLCD, 450 kCal/day]) in 12 obese (body mass index 36.3 ± 1.0 kg/m^2 [mean ± SEM]) type 2 diabetic (HbA_{1c} 7.3 ± 0.4%) patients simultaneously taken off all glucose-lowering therapy, including insulin.

Endogenous glucose production (EGP) and glucose disposal ([6,6 $^2H_2$]-glucose) were measured before and after the VLED in basal and hyperinsulinaemic (insulin infusion rate 40 mU/m^2/min) euglycaemic conditions. Insulin signalling and expression of GLUT4, FAT/CD36 and triglycerides were assessed in muscle biopsies, obtained before the clamp and after 30 minutes of hyperinsulinaemia.

Fasting plasma glucose decreased from 11.3 ± 1.3 to 10.3 ± 1.0 mmol/L because of a decreased basal EGP (14.2 ± 1.0 to 11.9 ± 0.7 μmol.kg^{-1}.min^{-1}, p = 0.009). Insulin-stimulated glucose disposal did not change. No diet effect was found on the expression of the insulin receptor and insulin receptor substrate-1 or on phosphatidylinositol 3'-kinase activity, or on FAT/CD36 expression pattern, GLUT4-translocation or triglyceride distribution in either the basal or insulin-stimulated situation. Unexpectedly, basal PKB/Akt-phosphorylation on T308 and S473 increased after the diet, at equal protein expression.

In conclusion, a 2-day VLED lowers fasting plasma glucose *via* a decreased basal EGP without an effect on glucose disposal. Accordingly, no changes in activation of phosphatidylinositol 3'-kinase, triglyceride distribution, FAT/CD36 expression and GLUT-4 translocation were found in skeletal muscle biopsies.

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INTRODUCTION

Energy restriction (ER) and weight loss[1,2] improve the insulin resistance (IR) seen in obese type 2 diabetic patients[3]. Because skeletal muscle is the primary site of insulin-stimulated glucose disposal[4] with glucose transport over the membrane as rate limiting step[5], skeletal muscle IR might play an important role in obese type 2 diabetic patients.

Intramyocellular lipid (IMCL) accumulation is strongly associated with IR[6]. The cause for IMCL accumulation might include an increased sarcolemmal expression of the fatty acid transporter FAT/CD36 in obese and non-obese type 2 diabetic patients[7], leading to an increased rate of fatty acid transport[7,8].

Intramyocellular lipids, in turn, can impair insulin signal transduction[5]. It has been proposed that fatty acid metabolites induce a sustained activation of serine/threonine kinases, such as protein kinase C isoforms, IκB kinase-β and Jun N-terminal kinase, which phosphorylate the insulin receptor substrates (IRS) IRS-1 and IRS-2 on serine and threonine sites[5]. Serine-phosphorylated forms of IRS1/2 cannot associate with and activate phosphatidylinositol 3'-kinase (PI3K), resulting in a decreased activation of GLUT-4-regulated glucose transport.

Energy restriction improves blood glucose values and insulin-stimulated glucose disposal in humans with type 2 diabetes as early as 7 days after the initiation of a 3347 kJ/d [800 kCal/day] diet[1]. The molecular mechanism underlying this improvement in insulin sensitivity is largely unknown. In rat skeletal muscle, 20 days of ER enhanced insulin-stimulated GLUT-4 translocation[9]. However, this effect occurred independent of activation of PI3K, indicating that ER ameliorates insulin-stimulated GLUT-4 translocation *via* other mechanisms, possibly down-stream of PI3K. In this regard, PKB/Akt is an attractive candidate given its putative role in insulin-stimulated glucose transport[10,11] and the observation that 20 days of ER led to an increased activation of this protein in rat skeletal muscle[12].

We found that a very low energy (calorie) diet (VLED = very low calorie diet [VLCD], Modifast®, Novartis Consumer Health, Breda, The Netherlands, 1883 kJ/d [450 kCal/day]) improves fasting plasma glucose (FPG) levels as early as 2 days after the initiation of the diet in obese type 2 diabetic patients simultaneously taken off all blood glucose-lowering medication, including insulin[13]. The present study was conducted to elucidate the mechanism underlying this effect. At the whole-body level, the blood glucose-lowering effect of a 2-day VLED appeared to be due to a decrease in basal endogenous glucose production (EGP) with no effect on whole-body insulin-stimulated glucose disposal, as assessed with the hyperinsulinaemic euglycaemic clamp technique with stable isotopes[14]. However, because no effect on whole-body insulin-stimulated glucose disposal does not preclude any effect (or a beginning effect) on skeletal muscle at the molecular level, we also took muscle biopsies. In fact, beforehand we assumed a beginning effect of ER on insulin signal transduction that might become apparent at the whole-body level after 7 to 10 days.

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We therefore examined IRS-1-associated PI3K-activity and PKB/Akt phosphorylation in skeletal muscle biopsies taken before and after 2 days of a VLED, both in the basal and in the insulin-stimulated situation. In addition, we determined the expression and translocation of the fuel transporters GLUT-4 and FAT/CD36. Finally, we examined intramyocellular triglyceride content with an oil red O staining.

RESEARCH DESIGNS AND METHODS

Subjects

Twelve obese type 2 diabetic patients, 5 male and 7 female (age 55 ± 4 years [mean ± SEM], body mass index [BMI] 36.3 ± 1.0 kg/m^2) participated in this study, which was approved by the Medical Ethical Committee of Leiden University Medical Centre. Written informed consent was obtained from all patients after the study was explained.

Patients used at least 30 units of exogenous insulin with or without oral blood glucose-lowering medication. Only subjects with remaining insulin secretion, defined as a fasting plasma C-peptide level of more than 0.8 ng/mL or a 2 times increase of the basal C-peptide level after 1 mg glucagon iv[15], were included.

Patients had to have stable body weight for at least 3 months and were instructed not to alter life style habits (eating, drinking, exercise) from screening until the start of the study. None of the patients were smokers, and the use of medication known to alter glucose or lipid metabolism was prohibited.

Diet and protocol outline

Three weeks before the start of the study, all oral blood glucose-lowering medication was discontinued. At day -1 and day 4, only short-acting insulin was given. On day 0, baseline investigations (day 0) were performed as outlined below. Insulin therapy was restarted after this study day until the start of the 2-day VLED on day 5 (to ensure complete washout of stable isotopes) and remained stopped during the 2-day VLED. On day 7 the second study day (day 2) took place. The VLED consisted of 3 sachets of Modifast® per day, amounting approximately 1883 kJ/d [450 kcal/day]. Patients were provided with muesli, shakes, and potage, from which they could chose freely. The exact amount of carbohydrates, protein, and fat in the Modifast® sachets varies a little between the different substances; but with 3 sachets of Modifast® per day, patients receive about 50 g protein, 50 to 60 g carbohydrates, 7 to 9 g lipids, and 10 g of dietary fibres. Patients followed the VLED at home and were only admitted to the research ward for study days.

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Study days

All studies started at 7:00 AM after an overnight fast. Length (m), weight (kg) and BMI (BMI = length [kg] / length^2 [m]) were measured according to WHO recommendations[16].

Metabolic studies were performed as described previously[14]. In short, basal rates of glucose and glycerol turnover were assessed after 3 hours of an adjusted primed (17.6 µmol/kg × actual plasma glucose concentration [mmol/L]/5 (normal plasma glucose)[17] continuous (0.33 µmol/kg per min) infusion of [6,6-$^2H_2$]-glucose (Enrichment 99.9%, Cambridge Isotopes, MA, USA) and 1.5 hours of a primed (1.6 µmol/kg) continuous (0.11µmol/kg per min) infusion of [$^2H_5$]-glycerol (Cambridge Isotopes). Insulin-stimulated rates of glucose and glycerol turnover were assessed after 4.5 hours of a hyperinsulinaemic-euglycaemic clamp (Actrapid®, Novo Nordisk Pharma, Alphen aan de Rijn, The Netherlands, rate 40 mU/m^2/min[18]). Glucose values were clamped at 5 mmol/L by the infusion of a variable rate of 20% glucose enriched with 3% [6,6-$^2H_2$]-glucose.

Blood chemistry

Serum insulin was measured by an ultrasensitive Human Insulin assay (Linco Research, St Charles, MO, USA) with a detection limit of 0.1 mU/L. The interassay coefficient of variation was below 6%. Serum C-peptide was measured with a radioimmunoassay from Linco Research. Serum triglycerides were determined with a fully automated Hitachi 747 system (Hitachi, Tokyo, Japan).

Serum glucose and [6,6-$^2H_2$]-glucose were determined in a single analytical run, using gas chromatography coupled to mass spectrometry as described previously[19,20].

Serum non-esterified fatty acids (NEFA) were measured using the enzymatic colorimetric acyl-CoA synthase, acyl-CoA oxidase assay (Wako Chemicals, Neuss, Germany) with a detection limit of 0.03 mmol/L. The interassay coefficient of variation was below 3%.

Muscle biopsies

Muscle biopsies were taken from the vastus lateralis muscle, after localised anaesthesia with 1% lidocaine, with a modified Bergström needle (Maastricht Instruments, Maastricht, The Netherlands) using applied suction[21]. The muscle biopsies were taken in the basal situation (8:00 AM, i.e., 1 hour after patients came in and were in a semirecumbent position) and 30 minutes after the start of the insulin infusion (10 minute prime followed by a constant rate of 40 mU/m^2/min[18]), while blood glucose levels were kept at initial values during these first 30 minutes *via* the infusion of 20% glucose at a variable rate. Muscle samples were snap-frozen in isopentane chilled on dry ice and stored at -80°C until further analysis.

Insulin Signalling

Muscle biopsies were homogenised in PI3K lysis buffer using an ultraturrax mixer and centrifuged (15 minutes, 14.000 rpm, 4°C), then protein content was determined using a BCA-kit

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(Pierce, Rockford, IL)[22]. Insulin receptor substrate-1 (IRS-1) was immunoprecipitated overnight (4°C) from 1.5 mg protein using IRS-1 antibody K6, and PI3K-activity was determined as described previously[22].

To determine expression and phosphorylation of other components of the insulin signalling system, proteins (25 µg/lane) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and blotted on polyvinylidene difluoride membranes (Millipore, Bedford, MA). Filters were incubated overnight (4°C) with phospho-specific PKB/Akt-Thr308, PKB/Akt-Ser473 (Cell Signalling Technology, Beverly, MA), IRS1 K6 and Akt-1 antibody (Upstate, Lake Placid, USA). Bound antibodies were detected using appropriate horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI) in a 1:10.000 dilution, followed by visualization by enhanced chemiluminescence. Blots were quantitated by densitometric analysis of the films using Scion Image beta 4.02 software.

Immunofluorescence assay for FAT/CD36 and GLUT-4 and Oil Red O staining

Routine indirect (double) immunofluorescence assays were performed as described previously[23]. Serial cryosections were fixed and incubated overnight at 4°C with the following primary antibodies: MO25, a monoclonal antibody directed against human FAT/CD36[23]; sc-7309 (Santa Cruz, TeBu-Bio, Heerhugowaard, the Netherlands), a mouse IgM monoclonal antibody reactive to FAT/CD36 of human origin; GLUT-4-BW, a polyclonal rabbit antibody directed against the final 12 amino acids of the C-terminus of the human GLUT-4 protein[24]; a polyclonal laminin antibody (L-9393, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands); a monoclonal caveolin-3 antibody (clone 26; BD Biosciences Pharmingen, Alphen aan de Rijn, The Netherlands); and a mouse monoclonal antibody directed against adult human slow myosin heavy chain (A4.840; developed by Dr. Blau[25]).

After washing the slides with phosphate-buffered saline (PBS), sections were incubated with the appropriate secondary fluorescent-labelled antibodies and thereafter mounted with Mowiol.

According to Koopman *et al*.[26], tissue sections were stained with oil red O combined with an immunofluorescence assay. Oil red O epifluorescence signal was quantified for each muscle cell of each cross section as described before[27]. Lipid droplet density was calculated by dividing the total numbers of droplets by the total (IMCL) area measured. Statistical significance of differences between trials was assessed by paired *t*-tests.

Images were examined in a Nikon E800 microscope (Uvikon, Bunnik, the Netherlands) and were digitally captured using a 1.3 Megapixel Basler A101C progressive scan colour CCD colour camera, driven by LUCIA laboratory image processing and analysis software (Laboratory Imaging, Prague, Czech Republic).

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Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting for FAT/CD36 and GLUT-4

Western blotting analyses were performed as described before for GLUT-4[24] and FAT/CD36[23]. Briefly, forty 20-µm-thick cryosections of muscle biopsies were sampled and homogenised. After centrifugation, the membrane fraction (pellet) and cytosol fraction (supernatant) were separated and both suspended in PBS.

For SDS-polyacrylamide gel electrophoresis and Western blotting, 1 part of the samples was boiled for 4 min in 2 parts of SDS-sample buffer (Bio-Rad Laboratories, Veenendaal, The Netherlands). Equal amounts of proteins were loaded on 10% polyacrylamide SDS-gels (Bio-Rad Laboratories). After electrophoretic separation, the proteins were transferred to nitrocellulose in Western blotting, then the blots were preincubated for 20 min with 5% non-fat dry milk in 0.05% Tween 20 (Sigma-Aldrich Chemicals) in PBS and incubated overnight at room temperature with the polyclonal GLUT-4-BW antibody[24] or the MO25 monoclonal antibody specific for FAT/CD36[23]. Chemiluminescence detection was performed after incubation with the appropriate horseradish-conjugated secondary antibodies. Proteins bands were analysed by densitometry using Image Master (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Calculations

The rate of appearance (R_a) and rate of disappearance (R_d) for glucose were calculated using the steady state equation by Steele as adapted for stable isotopes using a single-compartment kinetic model[28].

Endogenous glucose production during the basal steady state is equal to the R_a of [6,6-$^2H_2$]-glucose, whereas EGP during the clamp was calculated as the difference between R_a and the glucose infusion rate.

Statistical analysis

Data are presented as mean ± SEM. Differences before (day 0) and after (day 2) the VLED were analysed by the Student's *t*-test for paired samples. Correlation analysis was carried out using Pearson's correlation. All analyses were performed using SPSS for Windows version 11.0 (SPSS Inc., Chicago, IL, USA). Significance was accepted at $p < 0.05$.

RESULTS

Clinical and metabolic characteristics

Patient characteristics can be found in Table 1.

After 2 days of a VLED, FPG levels decreased (11.3 ± 1.3 to 10.3 ± 1.0 mmol/L), despite the cessation of all blood glucose-lowering medication. At that moment, weight loss amounted 2.9 ± 0.4 kg ($p = 0.001$). The decrease in FPG was accompanied by a significant decrease in basal EGP (Table 2) although basal insulin levels had also significantly decreased.

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Table 1. Patient characteristics.

Sex (male/female)	5 : 7
Age (years)	55 ± 4
BMI (kg/m^2)	36.3 ± 1.0
Waist circumference (cm)	120 ± 3
Waist-hip ratio	1.02 ± 0.03
Fasting plasma glucose (mmol/L)	11.3 ± 1.3
HbA_{1c} (%)	7.3 ± 0.4
Fasting serum insulin (mU/L)	20.7 ± 2.1
Fasting serum C-peptide (ng/mL)	1.0 ± 0.1
Duration of type 2 diabetes (years)	7.9 ± 1.3
Units of insulin injected per day	78 ± 9
Additional use of oral glucose-	6 metformin
lowering medication	1 rosiglitazone

Data are presented as mean ± SEM.

Table 2. Metabolic parameters on day 0 and after 2 days of a VLED in obese type 2 diabetic patients.

	Day 0			Day 2		
	Basal	Clamp	P	Basal	Clamp	P
Glucose (mmol/L)	11.3 ± 1.3	5.0 ± 0.4	0.0001	10.3 ± 1.0	4.9 ± 0.4	0.0001
Insulin (mU/L)	20.7 ± 2.3*	88.1 ± 5.9	0.0001	15.9 ± 1.8*	83.7 ± 4.8	0.0001
NEFA (mmol/L)	1.1 ± 0.1	0.39 ± 0.07	0.001	1.5 ± 0.1	0.35 ± 0.04	0.0001
Triglycerides (mmol/L)	1.8 ± 0.2	2.1 ± 0.2	0.028	2.0 ± 0.2	2.0 ± 0.2	NS
Glucose R_d $^\Delta$	14.2 ± 1.0†	12.1 ± 0.7	NS	11.9 ± 0.7†	11.3 ± 1.0	NS
EGP$^\Delta$	14.2 ± 1.0†	5.5 ± 0.8	0.0001	11.9 ± 0.7†	5.2 ± 0.5	0.0001
Glycerol R_a $^\Delta$	5.2 ± 1.0	1.9 ± 0.2	0.008	4.0 ± 0.6	1.8 ± 0.2	0.008

Basal $R_d = R_a = EGP$. During insulin stimulation, the amount of 20% glucose has to be subtracted from the R_d to get EGP.
The data in this table have already been published [14].
$^\Delta$ data in μmol.kg^{-1}.min^{-1}
Basal day 0 versus day 2: * p = 0.033, † p = 0.008

On both study days, we achieved comparable clamp serum glucose and insulin values (Table 2, these data have already been published[14]). Neither insulin suppressibility of EGP nor insulin stimulation of whole-body glucose disposal differed significantly after 2 days of a VLED (Table 2). Serum NEFA levels were more, but not significantly (p = 0.057), suppressed during hyperinsulinaemia on day 2. In line with this finding, the capacity of insulin to suppress whole-body lipolysis as measured by R_a of glycerol, also did not change after 2 days of a VLED (data not shown).

Effect of a 2-day VLCD on insulin signalling in skeletal muscle

To study the effect of 2 days of a VLED on insulin signalling, we examined IRS-1 associated PI3K activity in skeletal muscle biopsies obtained before and 30 minutes after the initiation

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of a hyperinsulinaemic euglycaemic clamp. Of the 12 patients, 4 showed a higher basal PI3K activity after 2 days of a VLED, which was not associated with an increase in insulin-stimulated PI3K activity nor with an increase in insulin-stimulated glucose disposal both before and after the VLED. Only in 5 out of 12 subjects, insulin increased IRS-1-associated PI3K activity, and a 2-day VLED did not improve the magnitude of this insulin response. Collectively, IRS-1-associated PI3K activity did not change after 2 days of a VLED, neither in the basal nor in the insulin-stimulated situation (Fig. 1). In addition, there was no effect of the VLED on the protein expression of the insulin receptor and IRS-1 (data not shown).

Basal PKB/Akt phosphorylation (both on T308 and S473) was significantly higher after 2 days of a VLED (Fig. 2), whereas the capacity of insulin to stimulate PKB/Akt activation was not significantly different between study days. When we looked at the individual data, none of the patients showed an increase in PKB/Akt phosphorylation during hyperinsulinaemia before the diet, whereas after the 2-day VLED, 3 of the 12 patients showed a 2-fold increase with hyperinsulinaemia. Protein expression of PKB/Akt (Fig. 2E) did not differ between study days, neither in the basal nor in the insulin-stimulated situation.

In line with the finding that insulin-stimulated whole-body glucose disposal did not change, we also found no change in the total amount of GLUT-4 expression (Fig. 3A) nor in translocation of GLUT-4 from the cytoplasm to the sarcolemma (Fig. 3B-E) as assessed by immunofluorescence staining (Fig. 3B-E) and Western blotting (Fig. 3A) in the skeletal muscle biopsies. Insulin-stimulated GLUT-4 translocation was monitored by a previously published

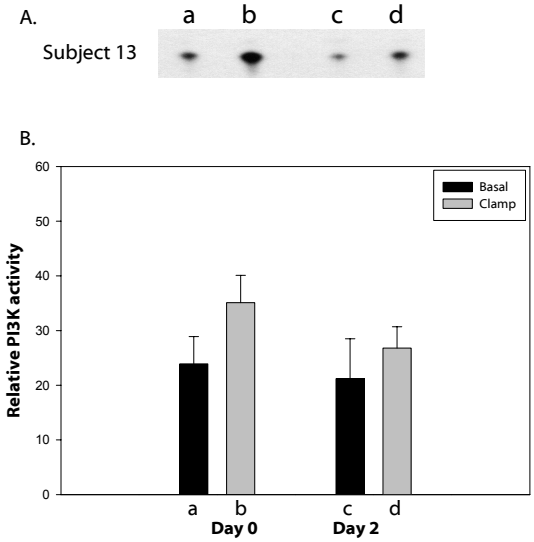

Figure 1.

Autoradiograph (A) and quantification (B) of IRS-1-associated PI3K activity in vastus lateralis muscle biopsies obtained before (a and b) and after a 2-day VLED (c and d) in basal (a and c) and hyperinsulinaemic euglycaemic conditions (b and d), changes are not significant. Data are expressed as mean±SEM .

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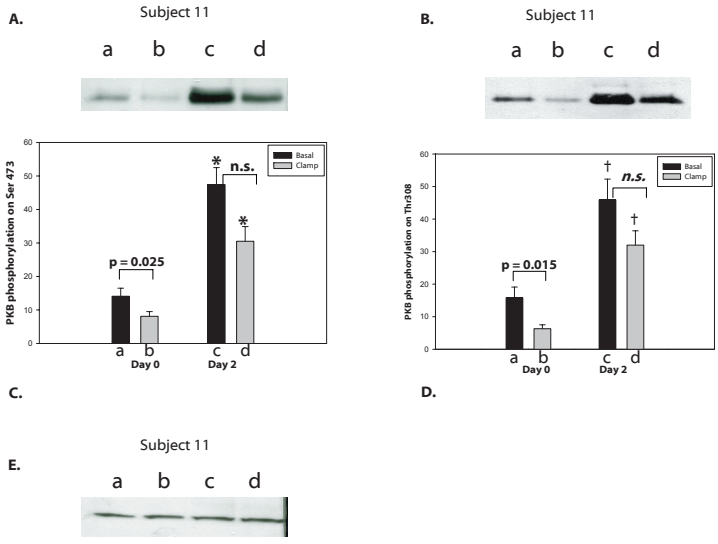

Figure 2.

Immunoblot and quantification of Akt/PKB phosphorylation at Ser 473 (A and C) and Thr 308 (B and D) in vastus lateralis muscle biopsies obtained before (a and b) and after a 2-day VLED (c and d) in basal (a and c) and hyperinsulinaemic (b and d) conditions. An immunoblot of PKB protein expression is given in E. Data are expressed as mean ± SEM . Note the increase in basal PKB/Akt phosphorylation, at SER 473 as well as Thr 308, after 2 days of a VLED. * $p < 0.001$, † $P < 0.005$, day 2 compared to day 0.

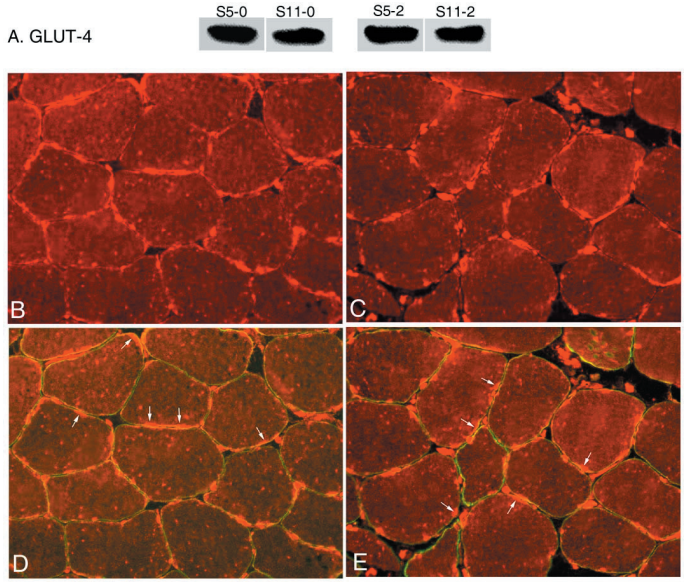

Figure 3.

Immunoblotting (A) of total muscle fractions of two subjects (S5 and S11) before (0) and after a 2-day (2) VLED. Double-immunofluorescence staining (B-E) of GLUT-4 (red) and caveolin-3 (green) in insulin-stimulated cryosections of human vastus lateralis muscle before (B, D) and after a 2-day VLED (C, E).

B and C, GLUT4. D and E, GLUT4 and caveolin-3. Note the GLUT-4 accumulations near the plasmalemma both before and after the 2-day VLED (arrows).

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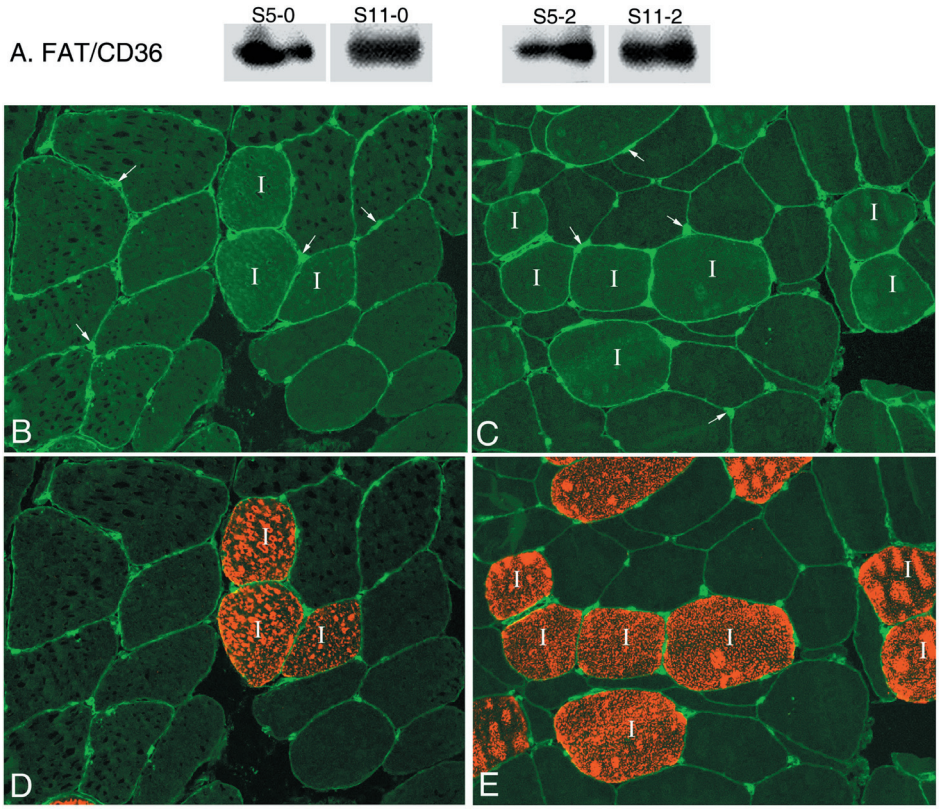

Figure 4.

Immunoblotting (A) of the muscle cell membrane fraction. Shown are two subjects (S5 and S11) before (0) and after a 2-day (2) VLED. Double-immunofluorescence staining of FAT/CD36 (green) and myosin heavy chain type 1 (MHC-1) (red) in insulin-stimulated cryosections of vastus lateralis muscle before (A, C) and after 2-days of diet intervention (B, D). A and B, FAT/CD36. C and D, FAT/CD36 and MHC-1.
I , indicates type-1 muscle fibers. No changes were observed between study days.

immunofluorescence method, albeit in a different model (increased GLUT-4 translocation upon 36 hours of pharmacologic blocking of fat oxidation using CPT1[29]). Using this methodology, we also were able to detect, in a semi-quantitative manner, insulin-induced GLUT-4 translocation after 2 hours of a hyperinsulinaemic euglycaemic clamp in healthy human subjects. Given these data (reflecting a positive control) we are also confident that the method used is of sufficient sensitivity to detect insulin-mediated changes in GLUT-4 localization.

Immunofluorescence staining showed that FAT/CD 36 was expressed at the sarcolemma as well in the cytoplasm of muscle cells (Fig. 4B-E) and that FAT/CD 36 staining was more intense in type 1 muscle fibres. Neither the VLED nor hyperinsulinaemia affected the FAT/CD 36 staining pattern. A Western blot analysis confirmed the findings of the immunofluorescence staining (Fig. 4A).

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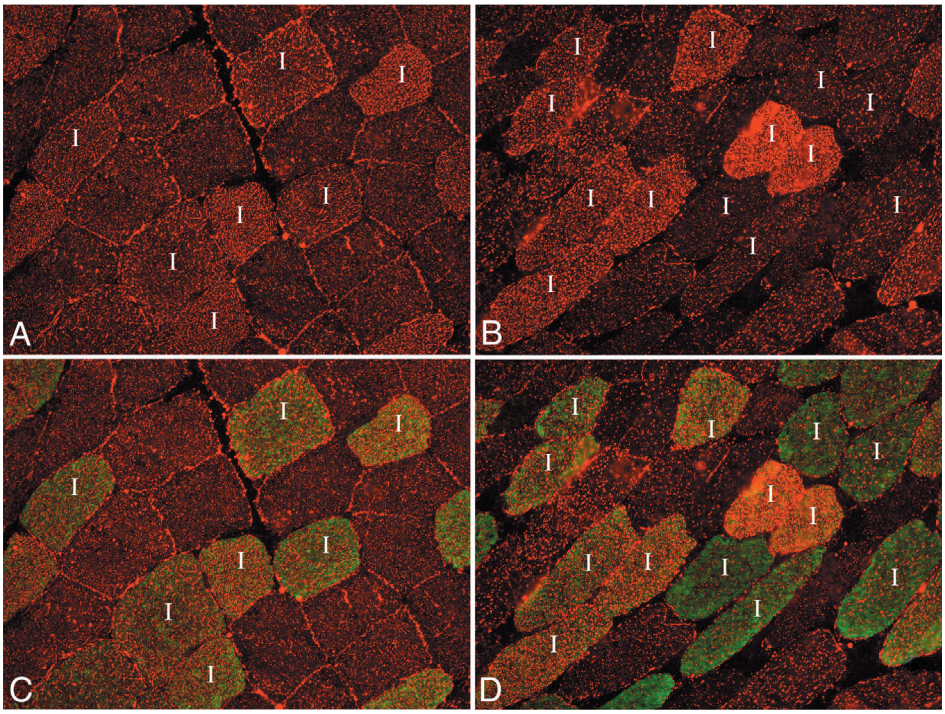

Figure 5.
Oil red O staining (A, B) in combination with myosin heavy chain type 1 (MHC-1) immunofluorescence assay (C, D) in cryosections of vastus lateralis muscle before (A, C) and after 2-days of diet intervention (B, D). No changes were observed between study days.

Triglyceride content in skeletal muscle cells, as assessed with oil red O staining, did not change between study days, neither in the basal nor in the insulin-stimulated situation (Fig. 5).

DISCUSSION

This study was performed to elucidate the molecular mechanism underlying the blood glucose-lowering effect of a 2-day VLED in insulin-treated obese type 2 diabetic patients. In line with our previous observations[13], this study again shows that 2 days of a VLED, in combination with the cessation of all blood glucose-lowering medication in obese type 2 diabetic patients lowers FPG levels. At the whole-body level this decrease in FPG could be explained by a decrease in basal EGP without an improvement in insulin-stimulated glucose disposal. These results are described elsewhere[14].

Although we did not find any improvement in insulin-stimulated glucose disposal at the whole-body level, we did analyse the muscle biopsies we took during this study because we still expected a beginning effect of the VLED at the molecular level in skeletal muscle

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biopsies. However, we did not find a significant diet effect either in GLUT-4 content or in GLUT-4 translocation from the cytoplasm to the plasma membrane (Fig. 3) in skeletal muscle biopsies. In addition, no diet effect was found on the protein expression of IRS-1 and on IRS-1-associated PI3K activation. Of the 12 patients, 4 showed a higher basal PI3K activity after 2 days of a VLED, which was not associated with an increase in insulin-stimulated PI3K activity nor with an increase in insulin-stimulated glucose disposal both before and after the VLED. Remarkably, 7 of 12 patients lacked an increase in insulin-stimulated PI3K activity. This is in accordance with several other studies in which a decreased insulin-stimulated tyrosine phosphorylation of IRS-1 and PI3K activity was found in skeletal muscle of type 2 diabetic patients compared to control subjects[30-32]. The fact that we did not find any stimulation of PI3K activation during hyperinsulinaemia in most of our patients probably reflects their severely insulin-resistant state with a grossly disturbed insulin signal transduction. A 2-day VLED does not (yet) improve this.

With regard to PKB/Akt we, unexpectedly, found a markedly enhanced phosphorylation on T308 and S473 after 2 days of a VLED in the basal situation, whereas we failed to observe insulin-stimulated PKB/Akt phosphorylation under our experimental conditions. Other studies found both decreased[33] and normal[34] insulin-stimulated PKB/Akt activity in patients with type 2 diabetes as compared with controls. In the latter study, supraphysiological doses of insulin have been used however (infusion rate of 120-300 mU/m^2/min). Another problem with the comparison of our results with those of others is that some studies, like we did, used biopsies taken during *in vivo* physiological hyperinsulinaemia, whereas others take muscle biopsies and incubate the muscle strips *in vitro*[33] with varying insulin concentrations. With regard to the increase in basal PKB/Akt phosphorylation, another study[35] showed that obese patients presenting with atypical diabetes had impaired Akt-2 expression and activation that increased after normalisation of glycaemia with intensive insulin therapy. There are 3 Akt isoforms (insulin action in muscle predominantly involves Akt-1 and Akt-2 stimulation) with Akt-2 knockout mice having impaired glucose homeostasis[11]. We did not measure Akt isoforms, and the interventions (VLED *versus* insulin therapy) are different but both are aimed at lowering blood glucose levels, and it might have been interesting to see whether 2 days of caloric deprivation would have the same results on PKB/Akt phosphorylation in these newly diagnosed type 2 diabetic patients.

Despite the fact that we found no changes in IRS-1 tyrosine phosphorylation and PI3K activity, basal PKB/Akt phosphorylation was increased after 2 days of a VLED, at equal PKB/Akt protein expression. This observation suggests that factors other than the IR-IRS-PI3K pathway also modulate the activity of PKB/Akt. In the liver, PKB/Akt has been shown to be involved in gluconeogenesis[36]. If the increased basal PKB/Akt activation we found in skeletal muscle also holds for the liver, this might explain the lower basal glucose production after 2 days of ER.

Studies regarding the expression pattern of FAT/CD 36 in humans are scarce[37,38]. Recently, 2 morphologic studies[23,39] using immunofluorescence microscopy showed that FAT/CD 36 is

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indeed expressed at both the sarcolemma and in the cytoplasm in human skeletal muscle. In both studies it became apparent that FAT/CD 36 is more abundant in type 1 muscle fibres. In line with the study of Keizer *et al*.[23] we show, for the first time in obese, very insulin-resistant patients, a similar dual expression pattern of FAT/CD 36, which was also more prominent in type 1 muscle fibres. Unlike other studies, we did not find an effect of hyperinsulinaemia. This might be because many studies used the so-called giant vesicles method[40,41] or it might reflect the severely insulin-resistant state of our subjects. Recently, Bonen *et al*.[7], found a 4-fold increase in long-chain fatty acid (LCFA) transport along with an increased intramuscular triacylglycerol content in giant sarcolemmal vesicles prepared from skeletal muscle of relatively lean (BMI 25 ± 1.1 kg/m^2) type 2 diabetic subjects (on diet or oral blood glucose-lowering agents only) compared with control subjects. This increased LCFA transport was associated with an increased expression of FAT/CD 36 at the sarcolemma at equal total FAT/CD 36 expression. This study supports the concept that augmented LCFA transport along with an imbalance between fatty acid reesterification and oxidation leads to an excess accumulation of triacylglycerols in the skeletal muscle cell, a marker for insulin resistance. It also shows that impaired trafficking of FAT/CD 36 between the sarcolemma and the cytosol (with an increased expression at the sarcolemma) might be the underlying pathogenetic mechanism. Because FAT/CD 36 can, at least partly, be stimulated *via* the insulin signal transduction pathway[42], a possible link with the altered GLUT-4 trafficking (which in contrast has a decreased expression at the sarcolemma as a pathogenic state) might be the cause of the impairment seen in both FAT/CD 36 and GLUT-4 trafficking in type 2 diabetic patients. We did not include control subjects and hence cannot confirm that our patients also had relatively more FAT/CD36 at the sarcolemma compared with control subjects.

One might argue that we studied patients while they were not normoglycaemic. Indeed, hyperglycaemia may have deleterious effects on insulin signalling[43,44], but each patient was his/her own control, and we were only looking for changes in signal transduction after 2 days of a VLED. Moreover, although we discontinued all blood glucose-lowering agents, FPG tended to decline and certainly did not increase after 2 days of a VLED. Another criticism may be that the timing of the muscle biopsies might have been to soon after initiating hyperinsulinaemia. Serum samples showed that maximal insulin concentrations had already been achieved at the time of the biopsy (data not shown) although this does not mean that steady state insulin concentrations in the interstitium had been achieved. In addition, several studies have shown that the effect of hyperinsulinaemia on activation of insulin-signal transduction molecules such as IRS-1, PI3K and PKB/Akt occur as early as 15 minutes[45-47] and that over 50% of the maximal effect already occurred at this time although maximal activity was reached at 60 minutes[47].

Kelley *et al*.[1] showed that peripheral glucose uptake increases and contributes to the blood glucose-lowering effect of a VLED already after 7 days. Because we had seen a decrease in FPG levels after only 2 days of a VLED[13], we presumed a change in muscle glucose uptake or

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at least already some changes at the molecular level in skeletal muscle biopsies. Our study shows that the very early (2 days) glucose-lowering, insulin-sparing effect of a VLED is predominantly due to a decreased EGP. Studies with a longer duration of the VLED have to be performed to detect the moment that an increased muscular glucose uptake contributes to the blood glucose-lowering effect, what the underlying molecular mechanisms are, and when these underlying molecular mechanisms become apparent.

In conclusion, this is one of the very few human studies investigating the short-term effect of ER on insulin-stimulated glucose disposal both at the whole-body and at the molecular level in obese type 2 diabetic patients in whom all blood glucose-lowering medication was discontinued. The participants in our study exhibit marked clinical insulin resistance. The clamp data indicate that two days of reduced food intake does not significantly affect basal and insulin-stimulated peripheral glucose disposal. This observation is in line with the inability of hyperinsulinaemia to activate PKB/Akt and the lack of an effect of the diet on other components of the insulin-signalling pathway such as PI3K activation and GLUT-4 expression and degree of GLUT-4 translocation. Remarkably, basal PKB/Akt phosphorylation is significantly increased after 2 days of reduced food intake indicating a link between the energy status and basal PKB/Akt activity. In the liver, PKB/Akt has been shown to be involved in regulating gluconeogenesis[36]. If this elevated basal PKB/Akt activation also holds for the liver, a situation difficult to test in the human situation, this could explain the observed significant decrease in EGP in the basal state after two days of reduced food intake.

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CHAPTER 7

Loss of 50% overweight substantially improves insulin sensitivity in obese insulin-treated type 2 diabetic patients using a very low calorie diet.

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ABSTRACT

Calorie restriction *per se* improves hyperglycaemia primarily via a reduction in basal endogenous glucose production (EGP) in obese patients with type 2 diabetes mellitus. To evaluate the effect of weight reduction as opposed to calorie restriction, on insulin sensitivity, 10 obese (body mass index [BMI] 40.2 ± 1.6, mean ± SEM) insulin-treated type 2 diabetic patients (HbA_{1c} 7.7 ± 0.4%, FPG 11.1 ± 0.8 mmol/L) were studied during a very low calorie diet (VLCD, 450 kCal/day) on day 2 and again after losing 50% of their overweight (50% OWR). Oral blood glucose-lowering agents and insulin were discontinued 3 weeks prior to the VLCD and at the start of the VLCD, respectively. EGP and whole-body glucose disposal ([6,6-$^2H_2$]-glucose), lipolysis ([$^2H_5$]-glycerol) and substrate oxidation rates were measured on both study days in basal and hyperinsulinaemic (insulin infusion rate 40mU/m2/min) euglycaemic conditions.

From day 2 to day 50% OWR, weight loss amounted 20.3 ± 2.2 kg. FPG decreased from 12.5 ± 0.5 to 7.8 ± 0.5 mmol/L (p = 0.0001), while basal EGP was restored to normal levels (20.0 ± 0.9 to 16.4 ± 1.2 µmol.kg fat free mass [FFM]$^{-1}$.min^{-1}, p = 0.001). Insulin-stimulated glucose disposal increased from 18.8 ± 2.0 to 39.1 ± 2.8 µmol.kgFFM^{-1}.min^{-1} (p = 0.001), due to an improvement in both oxidative and non-oxidative glucose metabolism. The ability of insulin to suppress EGP also improved: EGP during hyperinsulinaemia decreased from 8.5 ± 0.9 µmol.kgFFM^{-1}.min^{-1} on day 2 to 4.6 ± 1.2 µmol.kgFFM^{-1}.min^{-1} on day 50% OWR. Finally, insulin suppressibility of whole lipolysis also improved as indicated by a lower R_a of glycerol and lower serum glycerol and non-esterified fatty acid concentrations during hyperinsulinaemia on day 50% OWR.

In conclusion, as opposed to caloric restriction *per se*, which only decreases basal EGP, weight loss also considerably improves insulin sensitivity, especially insulin-stimulated glucose uptake, in severely obese insulin-treated type 2 diabetic patients. This occurred despite the fact that all blood glucose-lowering agents were discontinued and patients were still obese (BMI 32.3 kg/m^2). This observation stresses the fundamental importance of dietary intervention in this patient group.

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INTRODUCTION

Most type 2 diabetic patients are obese[1]. Insulin resistance plays a pivotal pathogenetic role in inducing and maintaining hyperglycaemia in this patient group and often leads to difficulties in achieving adequate glycaemic regulation.

It is well known that weight reduction improves hyperglycaemia[2-5] in obese patients with type 2 diabetes mellitus. In fact, blood glucose levels decline in response to caloric restriction even before significant weight loss has occurred[2,3,6,7], and improve further with ongoing weight loss[2,8]. In a previous study, we showed that blood glucose levels decline already after 2 days of a very low calorie diet in obese insulin-treated type 2 diabetic patients. The mechanism underlying this blood glucose-lowering effect of a VLCD was a decrease in basal endogenous glucose production (EGP), while hepatic and peripheral insulin sensitivity were unaffected[9].

The present study was conducted to evaluate, again in obese insulin-treated type 2 diabetic patients, whether a prolonged VLCD (Modifast®, 450 kCal/day) leading to substantial weight loss (50% of overweight [50% OWR]) has a different blood glucose-lowering mechanism as compared to caloric restriction only (2-day VLCD). By establishing baseline metabolic status at day 2 of a VLCD, we aimed to largely negate the effects of caloric restriction *per se* so as to specifically determine the impact of body weight reduction. During the study all blood glucose-lowering agents, including insulin, were discontinued. We used [6,6-$^2H_2$]-glucose to measure EGP, and the hyperinsulinaemic euglycaemic clamp technique to assess insulin-mediated peripheral glucose disposal and the capacity of insulin to suppress EGP. In addition, we measured whole-body lipolysis via infusion of [$^2H_5$]-glycerol, and substrate oxidation rates via indirect calorimetry.

RESEARCH DESIGN AND METHODS

Subjects

We studied 10 obese (BMI 40.2 ± 1.6 kg/m^2, mean ± SEM) patients with type 2 diabetes mellitus (FPG 11.1 ± 0.8 mmol/L, HbA_{1c} 7.7 ± 0.4%, duration of type 2 diabetes mellitus 8 ± 3 years), 8 women and 2 men, with a mean age of 54 ± 3 years. Subjects were recruited via local advertisements. All patients underwent a medical screening including a physical examination, resting electrocardiogram and blood chemistry tests to make sure that they were otherwise healthy and did not have liver-or renal function abnormalities. Patients had to use at least 30 units of insulin per day (mean 94 ± 14 units/day; 8 patients also used metformin and 2 patients used rosiglitazone with the insulin therapy) and had to have a BMI > 30 kg/m^2. In addition, patients had to have remaining endogenous insulin secretion defined as a fasting plasma C-peptide level of more than 0.8 ng/mL and/or a 2-fold increase of the basal C-peptide level after administration of 1 mg glucagon i.v.

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Patients had to have a stable body weight for at least 3 months and were instructed not to alter life style habits (eating, drinking, exercise) from screening until the start of the study. None of the patients were smokers and the use of other medication (than that used specifically for the treatment of hyperglycaemia) known to alter glucose or lipid metabolism was prohibited.

Written informed consent was obtained from each subject after oral and written explanation of the study had been given. The study was approved of by the Medical Ethical Committee of Leiden University Medical Centre.

Diet and protocol outline

3 weeks prior to the start of the study all oral blood glucose-lowering medication was discontinued. At day -1 only short acting insulin was given, evening doses of intermediate and long acting insulin were omitted. On day 0, patients started a VLCD (450 kCal/day) consisting of 3 sachets of Modifast® (Nutrition & Santé, Antwerpen, Belgium) per day, providing about 50 g protein, 50 to 60 g carbohydrates, 7 to 9 g lipids and 10 g dietary fibres daily.

Insulin therapy remained stopped from the start of the VLCD on. After 48 h of the VLCD, patients were admitted to the research centre for the metabolic studies (day 2) as outlined below. After this study day patients continued the VLCD until they had lost 50% of their overweight (see Calculations*)*. Then the second study day took place (day 50% overweight-reduced [OWR]) (See Fig. 1)

During the VLCD patients visited the research centre on a weekly basis for measurement of body weight, waist-hip ratio, blood pressure and blood glucose regulation.

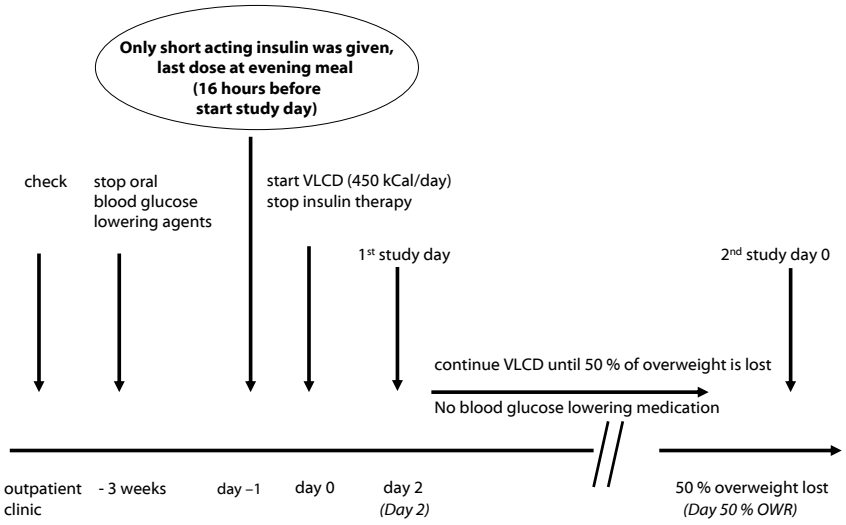

Figure 1

Protocol outline. See text for explanation.

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Assessments of body composition

On both study days (day 2 and day 50% OWR), body fat mass (FM) and fat free mass mass (FFM) were measured by Bioelectrical Impedance Analysis (BIA, Bodystat® 1500, Bodystat Ltd., Douglas, Isle of Man,UK). The impedance measurements were performed first thing in the morning after subjects had voided; while they were fasting and resting in bed. On a separate day, close to (1 or 2 days before) day 2 and day 50% OWR, total body fat mass and FFM were also assessed using dual-energy X-ray absorptiometry (Hologic QDR 4500, Hologic, Waltham, MA, USA). The scanner had a coefficient of variation for FM of 2.1% and of 1.0% for LBM. Data obtained for FM and FFM with either technique correlated greatly on both study days ($r = 98$, $p = 0.0001$). Because we did not obtain the correct data in 2 patients on day 50% OWR for the DEXA-scan (only bone mineral density was measured accidentally), we used the data obtained from the BIA for further calculations.

Length (meters [m]) and weight (kilograms [kg]), body mass index (BMI= weight (kg) / length^2 (m)) and waist circumference were measured according to WHO recommendations[10].

Hyperinsulinaemic euglycaemic clamp studies

Metabolic studies were performed as described previously[9]. In short, basal rates of glucose and glycerol turnover were assessed after 3 hours of an adjusted primed (17.6 µmol/kg x actual plasma glucose concentration (mmol/L)/5 (normal plasma glucose)[11] continuous (0.33 µmol/kg per min) infusion of [6,6-$^2H_2$]-glucose (Cambridge Isotopes, enrichment 99.9% Cambridge, USA) and 1.5 hours of a primed (1.6 µmol/kg) continuous (0.11 µmol/kg per min) infusion of [$^2H_5$]-glycerol (Cambridge Isotopes, Cambridge, USA). Subsequently, insulin-stimulated rates of glucose and glycerol turnover were measured after 4.5 hours of a hyperinsulinaemic euglycaemic clamp ((Actrapid®, Novo Nordisk Pharma, Alphen aan de Rijn, The Netherlands; rate 40 mU/m^2/min)[12]. Glucose values were clamped at 5 mmol/L via the infusion of a variable rate of 20% glucose enriched with 3% [6,6-$^2H_2$]-glucose.

Arterialised venous blood samples[13] were collected before the beginning of the tracer infusion, during the last 30 minutes of the basal period (3 times, with 7-minute intervals, *t* = 150-180 minutes after the start of the [6,6-$^2H_2$]-glucose infusion) and during the last 30 minutes of the euglycaemic hyperinsulinaemic clamp (4 times, with 10 minute intervals, *t* = 420-450 minutes). At these time points, blood samples were taken for the determination of [6,6-$^2H_2$]-glucose- and [$^2H_5$]-glycerol-specific activity, glucose, insulin, glycerol, C-peptide, non-esterified fatty acids (NEFAs), triglycerides, lactate, growth hormone (GH), cortisol, glucagon, leptin, resistin and adiponectin.

All blood samples, except serum samples, were immediately put on ice and centrifuged promptly (2000×*g* at 4°C for 20 minutes). Serum samples first had to coagulate before undergoing the same procedure. Samples were subsequently put in plastic tubes and frozen (-20°C) until assay.

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At the end of both the basal and the clamp period indirect calorimetry with a ventilated hood (Oxycon Beta, Mijnhardt Jaegher, Breda, The Netherlands) was performed for 30 minutes for the determination of glucose and lipid oxidation rates[14].

Blood chemistry

Serum insulin was measured with an immunoradiometric assay (IRMA, Biosource, Nivelles, Belgium). The detection limit was 3 mU/L and the interassay coefficient of variation was below 6%.

C-peptide, glucagon, leptin, resistin and adiponectin were measured with radioimmuno assays from Linco Research (St. Charles MO, USA). For C-peptide the interassay coefficient of variation (CV) varied between 4.2 and 6.0% at different levels with a sensitivity of 0.03 nmol/L. The CV for glucagon ranged between 4.0 and 6.8% with a sensitivity of 20 ng/L. For leptin the CV was 3.0-5.1% and the sensitivity 0.5 µg/L. For resistin the interassay CV was 3.2- 5.4% at different levels, the lowest detection level was 0.15 µg/L. Adiponectin had an interassay CV of 6.3-8.1% with a lowest detection level of 1 µg/L.

GH was measured with a time-resolved immunofluorescent assay (Wallac, Turku, Finland) specific for the 22 kDa GH. The CV varied from 5.3 to 8.4%, sensitivity 0.03 mU/L. Cortisol was measured with a radioimmunoassay (Sorin Biomedica, Milan, Italy) with CV between 2.3 and 4.2% and a detection limit of 25 nmol/L. Serum triglycerides were measured with a fully automated Modular P 800, serum lactate and fructosamine with a Modular I 800 system, both from Hitachi (Hitachi, Tokyo, Japan) with interassay CVs below 3%.

Serum glucose and [6,6-$^{2}H_{2}$]-glucose as well as serum glycerol and [$^{2}H_{5}$]-glycerol were determined in a single analytical run, using gas chromatography coupled to mass spectrometry as described previously[15,16].

Serum non-esterified fatty acids (NEFA) were measured using the enzymatic colorimetric acyl-CoA synthase/acyl-CoA oxidase assay (Wako Chemicals, Neuss, Germany) with a detection limit of 0.03 mmol/L. The interassay coefficient of variation was below 3%.

Calculations

In all subjects, a physiologic and isotopic steady state was achieved during the last half hour before the clamp (t = 150-180 minutes) and during the last hour of the clamp (t = 390-450 minutes). Therefore, the rate of appearance (R_a) for glucose and glycerol were calculated using Steele's steady-state equation as adapted for stable isotopes using a single-compartment kinetic model[17].

Endogenous glucose production (EGP) during the basal steady state is equal to the R_a of glucose, whereas EGP during the clamp was calculated as the difference between R_a of glucose and the glucose infusion rate.

The hepatic insulin resistance index was calculated as the product of the EGP (µmol.kgFFM^{-1}.min^{-1}) and the plasma insulin concentration (mU/L)[18].

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The metabolic clearance rate (MCR) of insulin was calculated as the constant infusion rate of insulin divided by the steady-state serum insulin concentration (SSI). The steady-state insulin concentration was corrected for endogenous insulin secretion using the following formula: SSI = steady-state insulin concentration (basal insulin concentration x [steady state C-peptide/basal C-peptide concentration])[19,20].

Total lipid and carbohydrate oxidation rates were calculated as described by Simonson and DeFronzo[14]. For the conversion of fat oxidation from milligram per kilogram per minute to micromole per kilogram per minute, an average molecular weight of 270 was assumed for serum NEFAs[7]. Non-oxidative glucose metabolism was calculated by subtracting the glucose oxidation rate (determined by indirect calorimetry) from R_d.

Percentage overweight was calculated as 100x(weight/ideal body weight) – 100. Ideal body weight for height was determined according to the Metropolitan Life Insurance tables (1983).

Homeostatic Model Assessment (HOMA) of insulin resistance (IR, normal values approach 1) and β-cell function (% β, 100% is normal) were calculated with the updated computer version (HOMA2) of the formulae of Matthews *et al*[21].

Statistical analysis

Data are presented as mean ± SEM. Differences between day 2 and day 50% OWR were analysed by the Student's *t*-test for paired samples. Non-parametric (Wilcoxon signed-rank test) tests for paired samples were performed when appropriate. All analyses were performed using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA). Significance was accepted at $p < 0.05$.

RESULTS

Weight and body composition

Weight loss during the first 2 days (day 0 to day 2) amounted –2.1 ± 0.3 kg, reflecting mainly salt and fluid loss. From day 2 until the second study day, patients lost an additional 20.3 ± 2.2 kg ($p = 0.0001$). BMI decreased from 39.7 ± 1.7 on day 2 to 32.3 ± 1.2 kg/m^2 on day 50% OWR ($p = 0.0001$). Mean time to weight loss of 50% of overweight was 17 weeks (range 4-35 weeks).

Body fat mass decreased from 51.0 ± 3.9 kg on day 2 to 32.7 ± 3.0 kg on day 50% OWR ($p = 0.0001$). This indicates that 85% of weight loss was loss of FM, and that LBM was relatively spared. Waist circumference was also reduced significantly (Table 1).

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Table 1. Effect of a VLCD on body composition and glycaemic regulation in obese type 2 diabetic patients.

	Before VLCD	Day 2 VLCD	50% reduction of overweight
BMI (kg/m^2)	40.2 ± 1.6	39.7 ± 1.7[*]	32.3 ± 1.2[†]
Weight (kg)	113.0 ± 7.1	110.9 ± 6.9[‡]	90.6 ± 5.0[†]
Fat mass (kg)	51.0 ± 3.9	50.1 ± 3.7	32.7 ± 3.0[†]
Waist circumference (cm)	126.8 ± 3.3	126.2 ± 3.5	107.7 ± 3.3[†]
FPG (mmol/L)	11.1 ± 0.8	12.5 ± 0.5	7.8 ± 0.5[†]
Fructosamine (nmol/L)	(HbA_{1c} 7.7 ± 0.4%)	329 ± 11	283 ± 12[§]
Fasting serum insulin (mU/L)	[a]	24.2 ± 2.2	15.2 ± 1.3[‖]
HOMA-IR	[a]	4.1 ± 0.3	2.1 ± 0.2[¶]
HOMA-β	[a]	42.9 ± 4.0	70.9 ± 9.4[#]

Data are presented as mean ± SEM.

[a] values likely to be unreliable because patients had used short-acting insulin therapy until the evening before the start of the VLCD (day 0)

[†] p = 0.0001 compared to both before VLCD and Day 2 VLCD

[§] p = 0.035; [‖] p = 0.001; [¶] p = 0.0001, [#] p = 0.009 day 50% OWR compared to day 2 VLCD.

[‡] p = 0.0001, [*] p = 0.049 day 2 compared to day 0

Fasting plasma glucose and insulin concentration

FPG levels declined significantly from day 2 of the VLCD until 50% of the overweight was reduced (12.5 ± 0.5 to 7.8 ± 0.5 mmol/L, p = 0.0001). In addition, serum insulin concentrations declined significantly between the two study days from 24.2 ± 2.2 to 15.2 ± 1.3U/L (p = 0.001).

Serum fructosamine levels, a measure for prolonged (2-4 weeks) glucose regulation, declined from 329 ± 11 to 283 ± 12 nmol/L (p = 0.035). HOMA-IR declined significantly whereas HOMA-β increased significantly (Table 1).

Endogenous glucose production and whole-body glucose disposal.

On both study days, serum glucose was clamped at identical levels (5.1 ± 0.3 and 5.4 ± 0.3 mmol/L on day 2 and day 50% OWR respectively, NS). The degree of hyperinsulinaemia was lower on day 50% OWR (80.8 ± 4.0 mU/L) as compared to day 2 (90.2 ± 3.3 mU/L, p = 0.023). This is probably the result of the increased metabolic clearance rate of insulin (see Table 2). The lower clamp serum insulin concentration on day 50% OWR does not negatively affect the results of our study. In fact, at equal and, thus, higher serum insulin levels on day 50% OWR the differences between study days on measures of insulin sensitivity would become even greater.

Basal EGP decreased significantly from day 2 to day 50% OWR (20.0 ± 0.9 and 16.4 ± 1.2 μmol.kgFFM^{-1}.min^{-1} on day 2 and day 50% OWR, respectively, p = 0.001, Fig. 2). During the hyperinsulinaemic euglycaemic clamp EGP was significantly lower on day 50% OWR, although the amount of suppression (from basal to clamp) was not significantly different between study days. However, basal and clamp hepatic insulin resistance indexes were significantly lower on day 50% OWR (Table 2).

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Table 2. Metabolic parameters during a VLCD on day 2 and after 50% of overweight (50% OWR) was lost, in obese type 2 diabetic patients.

	Day 2	Day 50% OWR	P
Basal EGP$^{\Delta}$	20.0 ± 0.9	16.4 ± 1.2	0.001
Clamp EGP$^{\Delta}$	8.5 ± 0.9*	4.6 ± 1.2*	0.005
Basal HIR$^{\square}$	485 ± 39	249 ± 28	0.0001
Clamp HIR$^{\square}$	756 ± 72	362 ± 91	0.001
Glucose R_d^{Δ}	18.8 ± 2.0	39.1 ± 2.8	0.001
MCR insulin (ml/m^2/min)	0.47 ± 0.02	0.53 ± 0.03	0.028
Basal whole-body glucose oxidation$^{\Delta}$	6.7 ± 1.4	4.2 ± 0.4	NS
Clamp whole-body glucose oxidation$^{\Delta}$	6.1 ± 0.9	12.7 ± 1.5†	0.002
Basal non-oxidative glucose metabolism$^{\Delta}$	14.8 ± 1.1	12.4 ± 1.1	0.036
Clamp non-oxidative glucose metabolism$^{\Delta}$	12.2 ± 1.6	27.7 ± 2.8‡	0.002
Basal glycerol R_a°	16.4 ± 2.3	14.6 ± 1.4	NS
Clamp glycerol R_a°	11.5 ± 2.3	7.5 ± 1.6§	NS
Basal whole-body lipid oxidation$^{\Delta}$	8.0 ± 0.5	7.1 ± 0.5	NS
Clamp whole-body lipid oxidation$^{\Delta}$	8.3 ± 0.3	5.5 ± 0.8$^{\|}$	0.008

Data are presented as mean ± SEM. NS indicates not significant.

$^{\Delta}$values are in µmol.kgFFM^{-1}.min^{-1} ; $^{\circ}$ values are in µmol.kgFM^{-1}.min^{-1}

$^{\square}$ EGP in µmol.kgFFM^{-}1.min^{-1} was multiplied with plasma insulin in mU/L

$^{*}p = 0.0001$, $^{\dagger}p = 0.001$, $^{\ddagger}p = 0.005$, $^{\S}p = 0.012$, $^{\|}p = 0.011$ clamp versus basal values

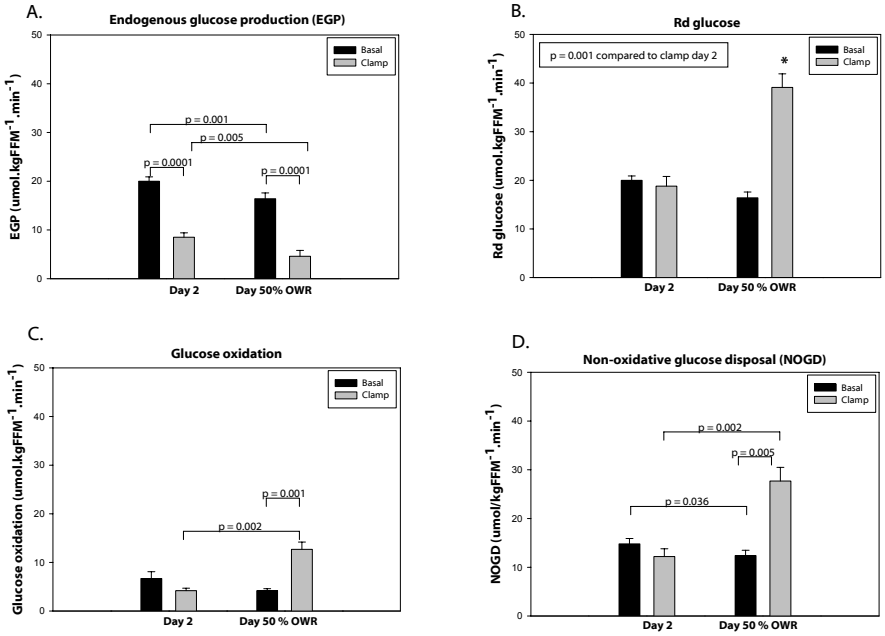

Figure 2.

Endogenous glucose production (EGP) [A], glucose disposal rates (Rd glucose) [B], oxidative [C] and non-oxidative [D] glucose disposal rates in 10 obese type 2 diabetic patients on day 2 of a VLCD and after a weight loss of 50% of the overweight (day 50% OWR). Black bars represent basal values; grey bars represent values during the hyperinsulinaemic euglycaemic clamp. Values are presented as mean ± SEM. Note the decrease in FPG levels and a decrease in basal EGP as well as a better suppression of EGP during insulin stimulation.

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Table 3. Effects of weight loss on hormones, substrate levels and adipokines in obese type 2 diabetic patients.

	Day 2	Day 50% OWR	P
Fasting serum cortisol (nmol/L)	451 ± 30	419 ± 34	NS
Fasting serum GH (mU/L)	1.2 ± 0.4	3.7 ± 1.5	0.012
Fasting serum glucagon (ng/L)	63.2 ± 8.3	70.7 ± 5.1	NS
Fasting serum glycerol (µmol/L)	150 ± 15	108 ± 12	0.008
Fasting serum NEFA (mmol/L)	1.6 ± 0.2	1.2 ± 0.1	0.018
Fasting serum triglycerides (mmol/L)	2.7 ± 0.5	1.2 ± 0.1	0.005
Fasting serum leptin (µg/L)	26.9 ± 4.4	11.4 ± 2.8	0.005
Fasting serum resistin (µg/L)	13.3 ± 1.1	11.5 ± 1.0	NS
Fasting serum adiponectin (µg/L)	5.2 ± 0.4	6.6 ± 0.6	0.012
Clamp serum glucose (mmol/L)	5.1 ± 0.3	5.4 ± 0.3	NS
Clamp serum insulin (mU/L)	90.2 ± 3.3	80.8 ± 4.0	0.023
Clamp serum glycerol (µmol/L)	114 ± 18	65 ± 12	0.011
Clamp serum NEFA (mmol/L)	1.1 ± 0.3	0.3 ± 0.1	0.017

Data are presented as mean ± SEM. NS indicates not significant.

Of the hormones involved in the regulation of hepatic glucose production, fasting serum cortisol and glucagon concentrations did not change with weight loss, whereas fasting growth hormone levels (as expected) increased (Table 3).

Insulin stimulated glucose disposal increased from 18.8 ± 2.0 µmol.kgFFM^{-1} on day 2 to 39.1 ± 2.8 µmol.kgFFM^{-1}.min^{-1}on day 50% OWR, p = 0.001 (Fig. 2). This is an increase of 107%.

The MCR of insulin was significantly greater on day 50% OWR, which could explain the lower steady state serum insulin values at the end of the clamp procedure on day 50% OWR (while the insulin infusion rate of 40 mU/m2/min was the same on both study days).

Glycerol R_a and non-esterified fatty acids, glycerol and triglycerides

Basal glycerol R_a decreased from 16.4 ± 2.3 to 14.6 ± 1.4 µmol.kgFFM^{-1}.min^{-1} (p = NS) between study occasions. The R_a of glycerol during the clamp was lower on day 50% OWR as compared to day 2, but this difference also did not reach significance. The Glycerol R_a was suppressed to a lower level by insulin on day 50% but if the change in glycerol R_a from basal to hyperinsulinaemia was calculated, statistical significance was not reached (–4.8 ± 2.7 on day 2 *versus* -7.1 ± 2.2 µmol.kgFFM^{-1}.min^{-1} after 50% of overweight was lost) (Table 2 and Fig. 3).

However, fasting levels of NEFAs, triglycerides and glycerol declined significantly, and clamp values of serum NEFA and glycerol were also significantly lower at day 50% OWR, reflecting a better suppressibility of lipolysis by insulin (Table 3).

Glucose and lipid oxidation rates

On day 50% OWR, insulin infusion increased the rate of glucose oxidation significantly as compared to day 2. Basal, as well as insulin-stimulated non-oxidative glucose disposal (NOGD) also increased significantly after the weight loss. The capacity of insulin to suppress lipid oxidation was improved with weight loss (Table 2 and Fig. 3).

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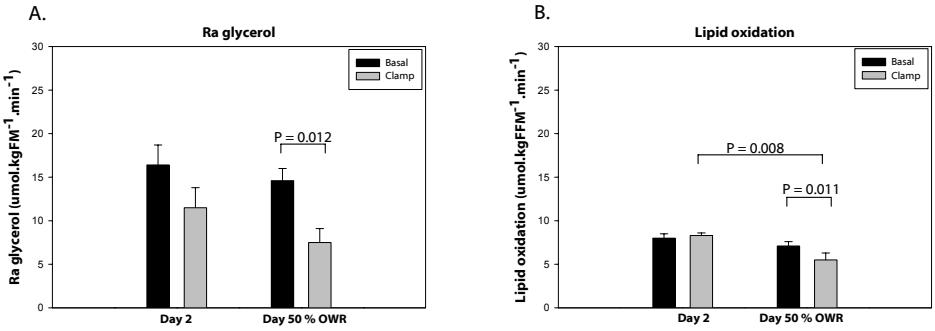

Figure 3.

Glycerol Ra [A] and lipid oxidation [B] rates in 10 obese type 2 diabetic patients on day 2 of a VLCD and after a weight loss of 50% of the overweight (day 50% OWR). Black bars represent basal values, grey bars represent values during the hyperinsulinaemic euglycaemic clamp. Values are presented as mean ± SEM. Note that values for R_a of glycerol are presented in µmol.kgFM^{-1}.min^{-1}, while those for lipid oxidation are in µmol.kgFFM^{-1}.min^{-1}. Weight loss resulted in a decrease in basal whole-body lipolysis and lipid oxidation, with a better suppression during hyperinsulinaemia of both parameters.

Adipokines

As expected with weight loss, serum leptin levels were significantly lower at day 50% OWR. Serum resistin levels were not significantly different between study days but serum adiponectin was significantly higher on day 50% OWR.

DISCUSSION

The aim of the present study was to evaluate the underlying mechanisms by which weight reduction *per se* improves hyperglycaemia in obese insulin-treated type 2 diabetic patients. As compared to caloric restriction *per se* (2-day VLCD[9]), a prolonged VLCD leading to a loss of 50% of overweight led to a substantial improvement in insulin-stimulated glucose disposal, despite the cessation of all blood glucose-lowering medication (including insulin) and the fact that patients were still obese. This improvement in insulin-stimulated glucose uptake was due an improvement in both oxidative and non-oxidative glucose disposal. In addition, insulin sensitivity of the liver and adipose tissue, reflected in the rate of insulin-suppressibility of EGP and lipolysis (R_a glycerol, and hyperinsulinaemic serum FFA and glycerol concentrations), respectively, also improved. This study indicates that prolonged use of a VLCD, resulting in major weight loss, induces additional adaptations in fundamental aspects of glucose metabolism in obese patients with type 2 diabetes mellitus compared to those induced by short-term use of a VLCD.

The increase in insulin-stimulated glucose uptake was due to an increase in both insulin-stimulated glucose oxidation as well as non-oxidative glucose disposal (NOGD). A 2-day VLCD not only had no effect on insulin-stimulated glucose uptake but even decreased NOGD[9]. In

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obese and type 2 diabetic patients, total glucose disposal and NOGD during hyperinsulinaemia are much lower compared to controls[22-24]. Since the increase in insulin-stimulated glucose oxidation seems to be bound to a maximum[25], NOGD is quantitatively the most important. Hence the improvement in NOGD is an important finding, indicating that patients were better able to store glucose as glycogen after weight loss. Others found either an increase[5,6,26,27] or no effect[28,29] on NOGD with weight loss following low calorie diets in obese type 2 diabetic[6,27,28,30] patients. The mechanisms underlying an improvement in NOGD are unclear, since several studies failed to demonstrate an effect of weight loss on glycogen synthase activity in skeletal muscle biopsies[26,28,30].

As compared to a 2-day VLCD, basal EGP was reduced further to normal levels. Because we did not measure between day 2 and day 50% OWR we do not know at what time-point normal values for basal EGP were obtained. Given the fact that basal EGP decreased substantially within 2 days of a VLCD[9], and the fact that others found that the greatest reduction in EGP takes place in the first 7-10 days of caloric restriction[2,3] the normalisation of basal EGP probably took place early during the course of the VLCD. The improvement in insulin suppressibility of EGP has been found before[3,6] and occurs already with modest (approximately 8 kg) weight loss[31]. However, Laakso *et al.*[27] did not find an effect of weight loss on insulin sensitivity of the liver. With respect to the causes of the improvement in basal EGP and insulin-suppressibility of EGP, of the hormones we measured, the concentration of glucagon and cortisol did not change while the GH concentration (a hormone known to stimulate EGP) was decreased with weight loss. In addition, the decrease in serum NEFAs and glycerol, and probably also a decrease in intrahepatic fat, might contribute. Furthermore, in rodents and in *in vitro* studies, adiponectin (levels of which were increased with weight loss in our study) can inhibit gluconeogenesis[32,33]. In humans, serum adiponectin levels are negatively correlated with EGP[34].

We found a lower basal and hyperinsulinaemic R_a of glycerol, as well as lower basal and hyperinsulinaemic serum NEFA and glycerol concentrations after weight loss, altogether indicative of a lower basal rate of lipolysis and an improved capacity of insulin to suppress whole-body lipolysis. In healthy and obese humans, short-term fasting increases the basal rate of lipolysis, whereas it remains the same or even decreases following short-term severe caloric restriction in obese type 2 diabetic patients[7,9]. Caloric restriction for a longer period of time in obese patients (VLCD 615 kcal/day during 28 days)[35] and obese patients with type 2 diabetes (10 days 25% of energy requirements and 10 days 75%)[7] has no effect on the basal rate of glycerol R_a. The fact that we found a decline in the basal rate of lipolysis cannot be explained by the lower total body fat mass because we expressed the R_a of glycerol per kilogram fat mass. We presume that the rate of lipolysis has been higher at the beginning of the VLCD, but that with ongoing caloric restriction, because of a lower metabolic rate, utility (lipid oxidation) has decreased, and, because there is a balance between lipolysis (production) and lipid oxidation (utility), lipolysis has also decreased. The cause of the decrease in basal metabolic rate during calorie restriction is unknown but intracellular enzymatic pro-

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cesses must be involved. These processes are in themselves regulated by several hormones and the autonomic nervous system. The novelty of our study is that we also documented the effect of a prolonged VLCD leading to substantial weight loss on insulin suppressibility of whole-body lipolysis, measured with [$^2H_5$]-glycerol, in obese insulin-treated obese type 2 diabetic patients, and showed that insulin suppressibility of lipolysis improves with weight loss. We could not compare these results with those of others because data are lacking for this intervention and patient group.

We also documented, with a hyperinsulinaemic euglycaemic clamp technique combined with [6,6-$^2H_2$]-glucose, the magnitude of the improvement in insulin-stimulated glucose disposal (107%) following substantial weight loss in very obese insulin-treated patients with type 2 diabetes. Several studies using the hyperinsulinaemic euglycaemic clamp technique (but without stable isotopes) have been performed in morbidly obese non-diabetic patients before and after substantial weight loss following bariatric surgery. M-values in the lean control groups in these studies were around 50 µmol.kg $LBM^{-1}.min^{-1}$ (LBM = lean body mass)[36-38]. After significant weight loss (50-60 kg) M-values in obese patients increased from 7-19 µmol.kg $LBM^{-1}.min^{-1}$ to around 35 µmol.kg $LBM^{-1}.min^{-1}$ in 2 studies[36,39] and even above 50 µmol.kg $LBM^{-1}.min^{-1}$ in 2 other studies[37,38], while their BMI remained in the obese range after weight loss (30-39.9 kg/m^2), like in our patients. When we calculated M-values in our study, patients increased from 9.9 ± 2.3 to 37.2 ± 4.6 µmol.kg $LBM^{-1}.min^{-1}$. Although the effectiveness of bariatric surgery in improving type 2 diabetes has been established in several studies[40-42] (review in[43]), unfortunately again no data on glucose disposal rates are available in obese type 2 diabetic patients.

Hence, short-term energy, or, more likely, carbohydrate restriction, improves hyperglycaemia primarily via a reduction in basal EGP[9,44]. Modest weight loss (approximately 8 kg) also improves hepatic insulin sensitivity[31], and substantial weight loss improves all aspects of glucose metabolism (this study). Given the fact that weight loss induced by subcutaneous liposuction does not lead to an improvement in insulin sensitivity (and adipokines such as leptin and adiponectin)[45], whereas weight loss with a decrease in waist circumference (like we found) does, indicates a role for energy restriction and/or upper body obesity (i.e., visceral adipose tissue and/or the deep layers of abdominal subcutaneous tissue). Unfortunately, we did not measure visceral fat mass and hence could not investigate whether the improvement in glucose and lipid metabolism we found, is correlated with a decrease in visceral fat mass. The decline in fasting as well as clamp levels of NEFA and triglycerides suggests a decrease in lipotoxicity.

In conclusion, prolonged caloric restriction leading to 50% reduction of overweight in obese type 2 diabetic patients simultaneously taken off all blood glucose-lowering medication (including insulin), considerably improves insulin sensitivity of endogenous glucose production, peripheral glucose uptake and lipolysis, even though patients were still obese (BMI 32.3 ± 1.2 kg/m^2). These observations stress that weight-reducing strategies, especially diets, should be a cornerstone of therapy in obese type 2 diabetic patients.

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CHAPTER 8

Effect of loss of 50% overweight on insulin-stimulated glucose disposal, insulin signalling and intramyocellular triglycerides in obese, insulin-treated type 2 diabetic patients using a very low calorie diet.

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ABSTRACT

To investigate the effect of considerable weight loss on skeletal muscle glucose disposal, both at the whole body and at the molecular level, 10 obese (BMI 40.2 ± 1.6 kg/m^2 [mean ± SEM]) insulin-treated type 2 diabetic patients (HbA_{1c} 7.7 ± 0.4%, FPG 11.1 ± 0.8 mmol/L) were studied during a very low calorie diet (VLCD, 450 kCal/day) on day 2 and again after losing 50% of their overweight (50% OWR). Oral blood glucose-lowering agents and insulin were discontinued 3 weeks prior to the VLCD and at the start of the VLCD, respectively. Endogenous glucose production (EGP) and whole-body glucose disposal (6,6-$^2H_2$-glucose), lipolysis ($^2H_5$-glycerol) and substrate oxidation rates were measured on both study days in basal and hyperinsulinaemic (insulin infusion rate 40mU/m2 per minute) euglycaemic conditions. In addition, skeletal muscle biopsies were obtained from the vastus lateralis muscle, in the basal situation and 30 min after the start of the insulin infusion for determination of insulin signalling, insulin-mediated expression of GLUT-4 and FAT/CD36 at the cell membrane and intramyocellular triglyceride content.

Weight reduction (20.3 ± 2.2 kg from day 2 to day 50% OWR) not only normalised basal EGP, but also improved insulin sensitivity, especially insulin-stimulated glucose disposal (18.8 ± 2.0 to 39.1 ± 2.8 μmol.kgFFM^{-1}.min^{-1}, p = 0.001). At the myocellular level, insulin-stimulated phosphatidylinositol 3'-kinase (PI3K)-activity over basal was significantly higher after weight loss. In addition, 2 down-stream effectors, AS160 and PRAS40, showed an absolute increase after weight loss. The improvement in insulin signalling was accompanied by a tendency for increased GLUT-4 content at the sarcolemma during hyperinsulinaemia. Intramyocellular triglyceride content decreased, with no significant change in insulin-stimulated sarcolemmal FAT/CD36 content. Time to weight loss of 50% overweight was negatively correlated with the number of type I muscle fibres at baseline.

In conclusion, the increase in insulin-stimulated glucose disposal after considerable weight loss in obese type 2 diabetic patients is associated with a tendency to improved insulin signalling at the level of PI3K, and a significant improvement in signalling towards the more downstream components, AS160 and PRAS40. The observed decrease in intramyocellular triglyceride content might have contributed to this effect. The fact that GLUT-4 content at the sarcolemma did not change significantly indicates that it is not GLUT-4 content that is important in insulin-stimulated glucose disposal, but rather its insulin-stimulated translocation or the intrinsic activity of GLUT-4. Alternatively, another glucose transporter or increased glucose uptake in adipose tissue might be responsible for the observed increase in insulin-stimulated glucose uptake.

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INTRODUCTION

About 80% of insulin-stimulated glucose disposal takes place in skeletal muscle[1], with glucose transport over the membrane as the rate limiting step[2]. In type 2 diabetic patients, insulin-stimulated glucose disposal is disturbed due to defects in the insulin-signalling pathway regulating the translocation of the glucose transporter GLUT-4 to the cell membrane. Notably, defects in insulin-induced phosphorylation of insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI3K)[3-6] and in translocation of GLUT-4 to the cell membrane[7,8] have been found in skeletal muscle of patients with type 2 diabetes, whereas total GLUT-4 protein and mRNA levels in type 2 diabetic patients have repeatedly shown to be normal[9,10]. The involvement of the PI3K substrate protein kinase B (PKB/Akt) in skeletal muscle insulin resistance is less clear, as is illustrated by studies reporting either normal[4,11] or impaired activation[12,13] by insulin. However, the recently characterised Akt substrate 160 (AS160)[14,15] has been implicated in linking PKB/Akt activation to GLUT-4[16] trafficking and insulin-mediated AS160 phosphorylation is impaired in skeletal muscle of type 2 diabetic patients[17]. Collectively these studies highlight the importance of the PI3K-PKB/AKT-AS160-signalling pathway regulating GLUT-4 trafficking.

Caloric restriction and weight loss both improve hyperglycaemia in type 2 diabetic patients. We previously reported that a 2-day very low calorie diet (VLCD, Modifast®, 450 kCal/day) decreased basal endogenous glucose production (EGP) in obese insulin-treated type 2 diabetic patients in whom all blood glucose-lowering medication was discontinued[17]. These changes were neither accompanied by an improvement in whole-body peripheral insulin sensitivity, nor by changes in insulin signalling, fuel transporter (GLUT-4, FAT/CD 36) localisation and triglyceride content in skeletal muscle biopsies[19].

In the present study, we assessed whether a prolonged VLCD in obese insulin-treated type 2 diabetic patients leading to substantial weight loss (50% of overweight) has a different blood glucose-lowering mechanism as compared to caloric restriction only (2-day VLCD). During the study all blood glucose-lowering agents, including insulin, were discontinued. Insulin sensitivity was determined by hyperinsulinaemic euglycaemic clamp (insulin infusion: 10 minute prime followed by a constant rate of 40 mU/m^2/min) on day 2 of the VLCD and after loss of 50% of the overweight. Insulin signalling, insulin-mediated expression of GLUT-4 and FAT/CD36 at the cell membrane and intramyocellular triglyceride content were determined in skeletal muscle biopsies obtained on both study days in the basal situation and 30 minutes after the start of the insulin infusion.

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RESEARCH DESIGN AND METHODS

Subjects

10 obese (BMI 40.2 ± 1.6 kg/m^2, [mean ± SEM]) patients with type 2 diabetes mellitus (FPG 11.1 ± 0.8 mmol/L, HbA_{1c} 7.7 ± 0.4%, duration of type 2 diabetes mellitus 8 ± 3 years), 8 women and 2 men (age 54 ± 3 years) participated in this study, which was approved by the Medical Ethical Committee of Leiden University Medical Centre. Written informed consent was obtained from all patients after the study was explained.

Patients had to use at least 30 units of insulin per day (mean 94 ± 14 units/day, 8 patients also used metformin and 2 patients used rosiglitazone with the insulin therapy) and had to have a BMI > 30 kg/m^2. In addition, patients had to have remaining endogenous insulin secretion defined as a fasting plasma C-peptide level of more than 0.8 ng/mL or a 2-fold increase of the basal C-peptide level after administration of 1 mg glucagon i.v.

Patients had to have a stable body weight for at least 3 months and were instructed not to alter life style habits (eating, drinking, exercise) from screening until the start of the study. None of the patients were smokers and the use of other medication (than that used specific for the treatment of hyperglycaemia) known to alter glucose or lipid metabolism was prohibited.

Diet and protocol outline

Three weeks before the start of the study, all oral blood glucose-lowering medication was discontinued. At day -1 only short acting insulin was given, evening doses of intermediate- and long-acting insulin were omitted. On day 0, patients started a VLCD (450 kCal/day) consisting of 3 sachets of Modifast® (Nutrition & Santé, Antwerpen, Belgium) per day, providing about 50 gram protein, 50 to 60 g carbohydrates, 7 to 9 g lipids, and 10 g of dietary fibres. Insulin therapy remained stopped from the start of the VLCD on. After 48 hours of the VLCD patients were admitted to the research centre for the metabolic studies (day 2) as outlined below. After this study day patients continued the VLCD until they had lost 50% of their overweight (see *Calculations)*. Then the second study day took place (day 50% overweight-reduced [OWR]).

During the VLCD patients visited the research centre on a weekly basis for measurement of body weight, waist-hip ratio, blood pressure and blood glucose regulation.

Study days

All studies started at 7:00 AM after an overnight fast. Length (meters [m]), weight (kilograms [kg]) and body mass index (BMI= weight (kg) / length^2 (m)) were measured according to WHO recommendations[20]. Body fat mass (FM) and fat free mass (FFM) were measured by Bioelectrical Impedance Analysis (BIA, Bodystat® 1500, Bodystat Ltd., Douglas, Isle of Man, UK). The impedance measurements were performed first thing in the morning after subjects had voided and while they were fasting and resting in bed.

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Metabolic studies were performed as described previously[18]. In short, basal rates of glucose and glycerol turnover were assessed after 3 hours of an adjusted primed (17.6 µmol/kg x actual plasma glucose concentration (mmol/L)/5(normal plasma glucose)[21] continuous (0.33 µmol/kg per min) infusion of [6,6-$^2H_2$]-glucose (Cambridge Isotopes, enrichment 99.9% Cambridge, USA) and 1.5 hours of a primed (1.6 µmol/kg) continuous (0.11µmol/kg per min) infusion of [$^2H_5$]-glycerol (Cambridge Isotopes, Cambridge, USA). Insulin-stimulated rates of glucose and glycerol turnover were assessed after 4.5 hours of a hyperinsulinaemic euglycaemic clamp (Actrapid®, Novo Nordisk Pharma, Alphen aan de Rijn, The Netherlands, rate 40 mU/m^2/min [22]). Glucose values were clamped at 5 mmol/L via the infusion of a variable rate of 20% glucose enriched with 3% [6,6-$^2H_2$]-glucose.

Blood chemistry

Serum insulin was measured with an immunoradiometric assay (IRMA, Biosource, Nivelles, Belgium). The detection limit was 3 mU/L en the interassay coefficient of variation was below 6%.

Serum C-peptide was measured with a radioimmuno assay from Linco Research, St. Charles MO, USA. The interassay coefficient of variation (CV) varied between 4.2 and 6.0% at different levels with a sensitivity of 0.03 nmol/L. Serum triglycerides were determined with a fully automated Hitachi 747 system (Hitachi, Tokyo, Japan).

Serum glucose and [6,6-$^2H_2$]-glucose as well as serum glycerol and [$^2H_5$]-glycerol were determined in a single analytical run, using gas chromatography coupled to mass spectrometry as described previously[23,24].

Serum non-esterified fatty acids (NEFAs) were measured using the enzymatic colorimetric acyl CoA synthase/acyl-CoA oxidase assay (Wako Chemicals, Neuss, Germany) with a detection limit of 0.03 mmol/L. The interassay coefficient of variation was below 3%.

Muscle biopsies

Muscle biopsies were taken from the vastus lateralis muscle after localised anaesthesia with 1% lidocaine, with a modified Bergström needle (Maastricht Instruments, Maastricht, The Netherlands) using applied suction[25]. The muscle biopsies were taken in the basal situation (8:00 AM, i.e., 1 hour after patients came in and were in a semirecumbent position) and 30 minutes[26] after the start of the insulin infusion (10 minute prime followed by a constant rate of 40 mU/m^2/min), while blood glucose levels were kept at initial values during these first 30 minutes *via* the infusion of 20% glucose at a variable rate. Muscle samples were snap-frozen in isopentane chilled on dry ice and stored at -80°C until further analysis.

Insulin Signalling

Muscle biopsies were homogenised in PI3K lysis buffer using an ultraturrax mixer and centrifuged (15 minutes; 14.000 rpm; 4°C), then protein content was determined using a BCA-kit

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(Pierce, Rockford, IL)[27]. Insulin receptor substrate-1 (IRS-1) was immunoprecipitated overnight (4°C) from 1.5 mg protein using IRS-1 antibody K6, and PI3K-activity was determined as described previously[27].

To determine expression and phosphorylation of other components of the insulin signalling system, proteins (25 µg/lane) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and blotted on polyvinylidene difluoride-membranes (Millipore, Bedford, MA). Filters were incubated overnight (4°C) with IRS-1 K6 and Akt-1 antibody (Upstate, Lake Placid, USA), anti-phospho-Proline rich Akt substrate 40 (PRAS40)-Thr246 (#44-100G), anti-phospho-AS160 (#44-1071G) (Biosource International, Camarillo, CA, USA) and anti-AS160/TBC1D4-antibody (Abcam, Ltd, Cambridge, UK). Bound antibodies were detected using appropriate horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI, USA) in a 1:10.000 dilution, followed by visualization by enhanced chemiluminescence. Blots were quantitated by densitometric analysis of the films using Scion Image beta 4.02 software.

Oil Red O staining

According to Koopman *et al*.[28] tissue sections of basal biopsies were stained with Oil Red O (ORO) combined with a double-immunofluorescence assay. Briefly, after fixation with 4% formaldehyde in mQ-water, sections were incubated for 45 minutes at room temperature with a mixture of the polyclonal rabbit antiserum directed to laminin (L-9393, Sigma, Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) and a mouse monoclonal antibody directed against adult human slow myosin heavy chain (Developmental Studies Hybridoma Bank, Iowa City, IO, USA). After three washing steps with phosphate-buffered saline (PBS), sections were incubated for 30 minutes at room temperature with the appropriate secondary antibodies, i.e., Goat anti-Rabbit AlexaFluor350 and Goat anti-Mouse IgM AlexaFluor488 (Molecular Probes, Invitrogen, Breda, The Netherlands). After three final washing steps with PBS, sections were stained with Oil Red Oil according to Koopman *et al*. [28]. Finally, the sections were mounted in Mowiol.

Images were examined in a Nikon E800 microscope (Uvikon, Bunnik, the Netherlands) and were digitally captured using a 1.3 Megapixel Basler A101C progressive scan colour CCD colour camera, driven by LUCIA laboratory image processing and analysis software (Laboratory Imaging, Prague, Czech Republic).

Oil Red O epifluorescence signal was quantified for each muscle cell of each cross section as described before[29]. Lipid droplet density was calculated by dividing the total number of droplets by the total (IMCL) area measured.

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Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blotting for FAT/CD36 and GLUT-4

For Western blotting analyses, muscle membrane fractions and total muscle protein fractions were prepared as described before for GLUT-4[30] and FAT/CD36 [19,31] in biopsies taken during the insulin-stimulated situation.

Equal amounts of proteins were loaded on 10% polyacrylamide SDS-gels and after electrophoretic separation, the proteins were transferred to nitrocellulose in Western blotting. Then the blots were preincubated for 60 min with Odyssey Blocking Buffer (Licor, Westburg, Leusden, The Netherlands) 1:1 diluted in PBS and incubated overnight at room temperature with the polyclonal GLUT-4-BW antibody[30] or the MO25 monoclonal antibody specific for FAT/CD36[31]. Then, after incubation with the appropriate secondary antibodies Donkey anti-Rabbit IRDye800 and Donkey anti-Mouse IRDye800 (Rockland, TeBu-bio, Heerhugowaard, The Netherlands), protein bands were detected and quantified with an Odyssey Infrared Imager (Licor). Primary and secondary antibodies were diluted in Odyssey Blocking Buffer. Finally, protein bands were detected and quantified with an Odyssey Infrared.

Calculations

The rate of appearance (R_a) and rate of disappearance (R_d) for glucose and glycerol were calculated using the steady state equation by Steele[32] as adapted for stable isotopes using a single compartment kinetic model.

Endogenous glucose production (EGP) during the basal steady state is equal to the R_a of glucose, whereas EGP during the clamp was calculated as the difference between R_a and the glucose infusion rate.

Total lipid and carbohydrate oxidation rates were calculated as described by Simonson and DeFronzo[33]. For the conversion of fat oxidation from milligram per kilogram per minute to micromole per kilogram per minute an average molecular weight of 270 was assumed for serum NEFAs. Non-oxidative glucose metabolism was calculated by subtracting the glucose oxidation rate (determined by indirect calorimetry) from R_d.

Percentage overweight was calculated as: 100 x (weight/ideal body weight) – 100. Ideal body weight for height was determined according to the Metropolitan Life Insurance tables (1983).

Statistical analysis

Data are presented as mean ± SEM. Differences between day 2 and day 50% OWR, as well as differences between basal and insulin-stimulated biopsies were analysed by the Student's *t*-test for paired samples. Non-parametric (Wilcoxon signed-rank test) tests for paired samples were performed when appropriate. Correlation analysis was carried out using Pearson's correlation. All analyses were performed using SPSS for Windows version 12.0 (SPSS Inc., Chicago, IL, USA). Significance was accepted at $p < 0.05$.

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RESULTS

Clinical and metabolic characteristics

Patient characteristics can be found in Table 1. Mean weight loss from day 2 to day 50% OWR amounted 20.3 ± 2.2 kg, average time to weight loss of 50% of overweight was 17 weeks (range 4 to 35 weeks). FPG levels declined significantly from day 2 to day 50% OWR (12.5 ± 0.5 to 7.8 ± 0.5 mmol/L, p = 0.0001). Basal EGP decreased from 20.0 ± 0.9 to 16.4 ± 1.2 µmol. $kgFFM^{-1}.min^{-1}$, p = 0.001. Weight loss to 50% OWR also improved insulin sensitivity (Table 2),

Table 1. Patient characteristics.

Sex (male/female)	2 : 8
Age (years)	54 ± 3
Weight (kg)	113.0 ± 7.1
BMI (kg/m^2)	40.2 ± 1.6
Waist circumference (cm)	126.8 ± 3.3
Fat mass (kg)	51.0 ± 3.9
Fasting plasma glucose (mmol/L)	11.1 ± 0.8
HbA_{1c} (%)	7.7 ± 0.4
Duration type 2 diabetes (years)	8 ± 3
Units of insulin injected per day	94 ± 14
Additional use of oral glucose-lowering medication	8 metformin 2 rosiglitazone

Data are presented as mean ± SEM.

Table 2. Metabolic parameters during a VLCD on day 2 and after 50% reduction of overweight in obese type 2 diabetic patients.

	Day 2			Day 50% OWR		
	Basal	Clamp	P	Basal	Clamp	P
Glucose (mmol/L)	12.5 ± 0.5	5.1 ± 0.3	0.0001	7.8 ± 0.5*	5.4 ± 0.3	0.003
Insulin (mU/L)	24.2 ± 2.2	90.2 ± 3.3	0.0001	15.2 ± 1.3†	80.8 ± 4.0‡	0.0001
NEFA (mmol/L)	1.6 ± 0.2	1.1 ± 0.3	NS	1.2 ± 0.1§	0.3 ± 0.1‖	0.012
Triglycerides (mmol/L)	2.7 ± 0.5	2.5 ± 0.5	NS	1.2 ± 0.1¶	0.9 ± 0.1#	0.0001
Glycerol (µmol/L)	150 ± 15	114 ± 18	NS	108 ± 12**	65 ± 12††	0.011
Glucose R_d Δ	20.0 ± 0.9	18.8 ± 2.0	NS	16.4 ± 1.2†	39.1 ± 2.8†	0.001
EGPΔ	20.0 ± 0.9	8.5 ± 0.9	0.0001	16.4 ± 1.2†	4.6 ± 1.2¶	0.0001
Glycerol R_a □	16.4 ± 2.3	11.5 ± 2.3	NS	14.6 ± 1.4	7.5 ± 1.6	0.012
Glucose oxidationΔ□	6.7 ± 1.4	6.1 ± 0.9	NS	4.2 ± 0.4	12.7 ± 1.5‡‡	0.001
NOGDΔ	14.8 ± 1.1	12.2 ± 1.6	NS	12.4 ± 1.1§§	27.7 ± 2.8‡‡	0.005
Lipid oxidationΔ	8.0 ± 0.5	8.3 ± 0.3	NS	7.1 ± 0.5	5.5 ± 0.8**	0.011

Δ values in value in $\mu mol.kgFFM^{-1}.min^{-1}$; □value in $\mu mol.kgFM^{-1}.min^{-1}$

NEFA = non-esterified fatty acids, R_d = rate of disappearance (= peripheral glucose disposal); EGP = endogenous glucose production, NOGD = non-oxidative glucose disposal rate, FFM = fat free mass, FM = fat mass

Day 2 versus day 50% OWR:

* p = 0.0001; † p = 0.001; ‡ p = 0.023; § p = 0.018; ‖ p = 0.017; ¶ p = 0.005; # p = 0.019; ** p = 0.008, †† p = 0.011; ‡‡ p = 0.002; §§ p = 0.036

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especially insulin-stimulated glucose disposal increased by 107% (18.8 ± 2.0 to 39.1 ± 2.8 μmol.kgFFM^{-1}.min^{-1} (p=0.001)).

Effect of weight loss on insulin signalling in skeletal muscle

Absolute levels of insulin-stimulated IRS-1-associated PI3K were equal on both study days but the magnitude of the insulin-effect compared to basal was greater and only significantly enhanced after weight loss (p = 0.01, Fig. 1). To corroborate this finding, we also assessed the phosphorylation of two more distal components of the insulin signalling system, i.e., the recently identified PKB/Akt substrates AS160 and proline-rich Akt substrate 40 (PRAS40). Basal as well as insulin-stimulated AS160 phosphorylation, corrected for AS160 protein expression, was significantly higher after weight loss as compared to day 2 of the VLCD (Fig. 2). In addition, basal and hyperinsulinaemic levels of PRAS40 phosphorylation, were also significantly increased on day 50% OWR as compared to day 2 (Fig. 3).

Effect of weight loss on the fuel transporters GLUT-4 and FAT-36

Weight reduction had no significant effect on the abundance of the fuel transporters GLUT-4 (Fig. 4) and FAT/CD36 (Fig. 5) at the plasma membrane following hyperinsulinaemia. However, it should be noted that 7 out of the 10 patients showed a higher GLUT-4 density at the

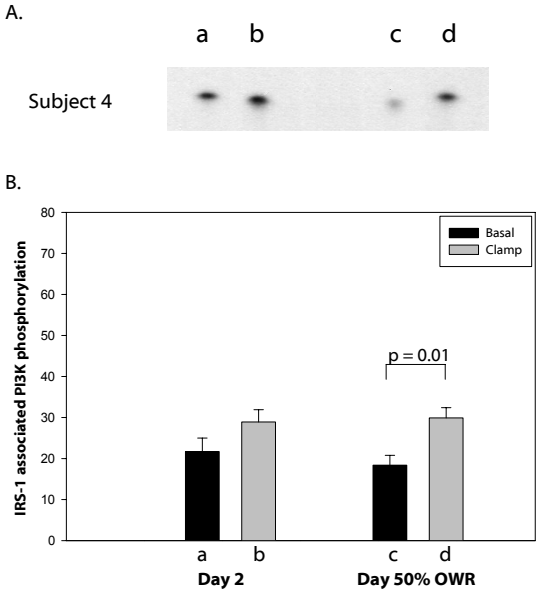

Figure 1

Immunoblot (A) and quantification (B) of IRS-1-associated PI3K activity in vastus lateralis muscle biopsies obtained on day 2 of a VLCD (a and b) and after 50% of overweight was lost (c and d) in basal (a and c) and hyperinsulinaemic euglycaemic conditions (b and d). Data are expressed as mean±SEM. Note that only insulin-stimulated increase over basal is significant on day 50% OWR.

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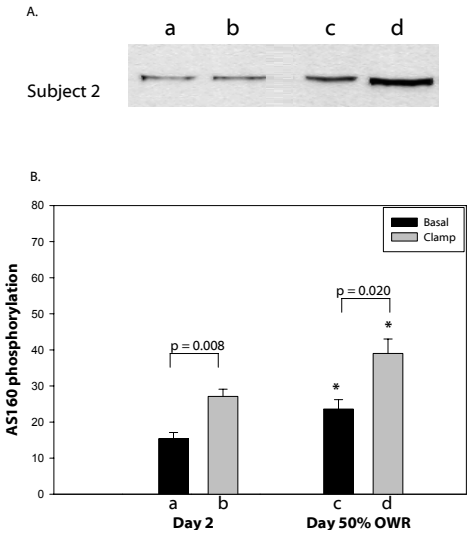

Figure 2

Immunoblot (A) and quantification (B) of AS160 phosphorylation in vastus lateralis muscle biopsies obtained on day 2 of a VLCD (a and b) and after 50% of overweight was lost with the VLCD (c and d) in basal (a and c) and hyperinsulinaemic (b and d) conditions. Data are expressed as mean ± SEM .

Note the absolute increase in AS160 phosphorylation following weight loss, both in the basal as well as in the insulin-stimulated situation. $^{*}P = 0.026$, day 50% OWR compared to day 2 , basal as well as insulin-stimulated.

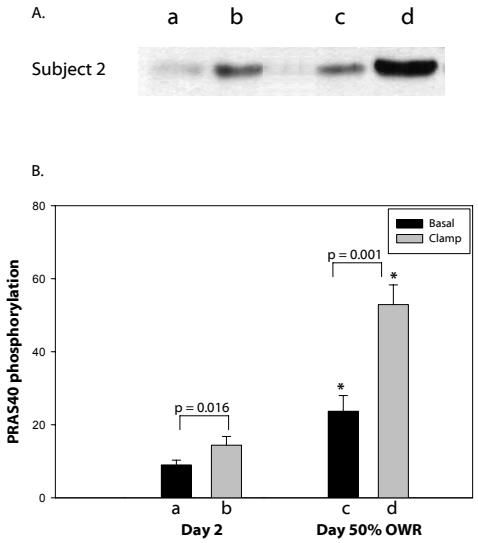

Figure 3

Immunoblot (A) and quantification (B) of PRAS40 phosphorylation in vastus lateralis muscle biopsies obtained on day 2 of a VLCD (a and b) and after 50% of overweight was lost with the VLCD (c and d) in basal (a and c) and hyperinsulinaemic (b and d) conditions. Data are expressed as mean ± SEM.

Note that PRAS 40 phosphorylation is increased in the basal and insulin-stimulated situation after weight loss. $^{*}P = 0.046$, $^{\dagger}p = 0.018$, day 50% OWR compared to day 2.

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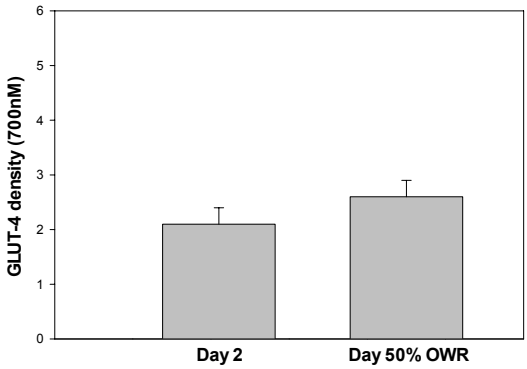

Figure 4

Quantification of GLUT-4 at the cell membrane during insulin-stimulated conditions on day 2 of a VLCD and after 50% of the overweight was lost with the VLCD (50% OWR). Data are expressed as mean ± SEM. Changes between study days were not significant.

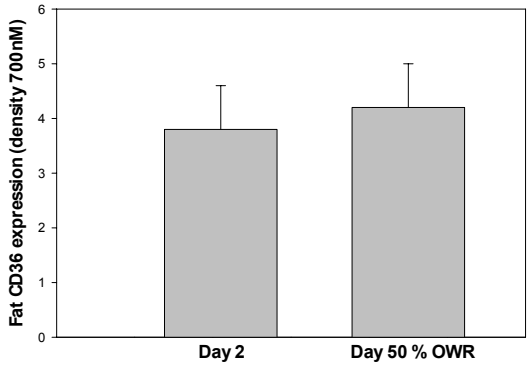

Figure 5

Quantification of FAT/CD36 at the cell membrane during insulin-stimulated conditions on day 2 of a VLCD and after 50% of the overweight was lost with the VLCD (50% OWR). Data are expressed as mean ± SEM. Changes between study days were not significant.

cell membrane after weight loss. As to FAT/CD 36 the results were much less coherent: 4 patients showed an increase, 4 a decrease and 2 had equal FAT/CD36 expression at the cell membrane after weight loss.

In a correlation analysis, insulin-stimulated GLUT-4 content at the cell membrane did not correlate with the rate of glucose disposal on either study day. Neither did the change in insulin-stimulated sarcolemmal GLUT-4 content between study days correlate with the change in insulin-stimulated glucose disposal. Also no correlation between insulin-stimulated plasmalemmal GLUT-4 content and body weight, age or duration of type 2 diabetes was found.

FAT/CD36 at the cell membrane during insulin infusion did not correlate with whole-body lipolysis, lipid oxidation or insulin-stimulated glucose disposal. However, a negative correlation was found between insulin stimulated sarcolemmal FAT/CD36 and the serum concentration of NEFAs during insulin stimulation ($r = -0.88$, $p = 0.004$ on day 2 and $r = -0.72$, $p = 0.045$).

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Intramyocellular triglyceride content as assessed with an Oil red O Staining

Oil-red-O staining, as a measure of intramyocellular lipids, showed a reduction in intramyocellular lipids after weight loss (Fig. 6, Fig. 7). Type I and type II muscle fibres were also examined separately. Type I muscle fibres contained significantly more intramyocellular triglycerides on either study day as compared with type II muscle fibres. In both fibre types however, the amount of intramyocellular triglycerides decreased with weight loss. The percentage type I fibres did not change with weight loss although a slight, non-significant increase was observed (46.8 ± 4.9% to 51.5 ± 4.1%, p = NS, Fig. 7B), and accordingly, a decrease in type II muscle fibres. Interestingly, time to weight loss of 50% overweight correlated negatively with the number of type I fibres at the start of the diet (day 2), r = -0.64, p = 0.046. The amount of intramyocellular triglycerides correlated significantly with lipid oxidation (r = 0.74, p = 0.024) and whole-body insulin-stimulated glucose disposal (negative correlation, r = -0.63, p = 0.049) on day 50% OWR. In addition, the change in intramyocellular triglyceride concentration did not correlate with change in body weight, glucose and lipid metabolism (variables as shown in Table 2), insulin signalling or FAT/CD36 content.

DISCUSSION

This study shows that, as opposed to a 2-day VLCD, which only decreased basal EGP, prolonged caloric restriction leading to a loss of 50% of overweight also improves insulin sensitivity, especially insulin-stimulated glucose disposal (see Chapter 8 for the discussion of the clamp studies). Over 80% of insulin-stimulated glucose disposal takes place in skeletal muscle[1], with glucose transport over the membrane being the rate-limiting step[2]. We found improved insulin signalling, reflected by a small insulin-stimulated increase over basal with respect to IRS-1-associated PI3K activity and a clear absolute increase in two of its downstream components, AS160 and PRAS40. The amount of GLUT-4 at the cell membrane during insulin stimulation showed a tendency to increase after weight loss, however, this small increase in sarcolemmal GLUT-4 seems not in accordance with the clear improvement (107% increase as compared to day 2) in insulin-stimulated glucose disposal.

The reason why the increase in insulin-stimulated glucose disposal at the whole-body level was not reflected by a significant improvement in GLUT-4 translocation to the cell membrane is unclear and may reflect changes in intrinsic activity of GLUT-4. Others have also reported a dissociation between insulin-stimulated glucose disposal and either insulin signalling and/or GLUT-4 content at the cell membrane. Ryder *et al*.[7] found that although insulin-stimulated glucose disposal was 50% lower in patients with type 2 diabetes compared with lean controls, insulin-stimulated cell surface GLUT-4 content over basal amounted only 10% that of healthy controls in type 2 diabetic patients. In another study, Karlsson *et al*. found a significant, 36% improvement in insulin-stimulated whole-body glucose uptake after 26 weeks of

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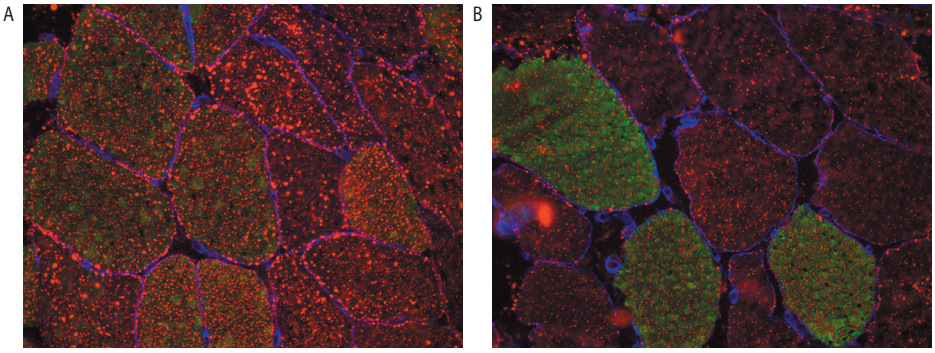

Figure 6

Oil red O staining (red) in combination with myosin heavy chain type 1 (MHC-1) immunofluorescence- (green) and lamin staining (blue) in cryosections of vastus lateralis muscle on day 2 of a VLCD (A) and after 50% of overweight was lost with the VLCD (B). Note the decrease in intramyocellular triglyceride content on day 50% OWR (Fig. 6B).

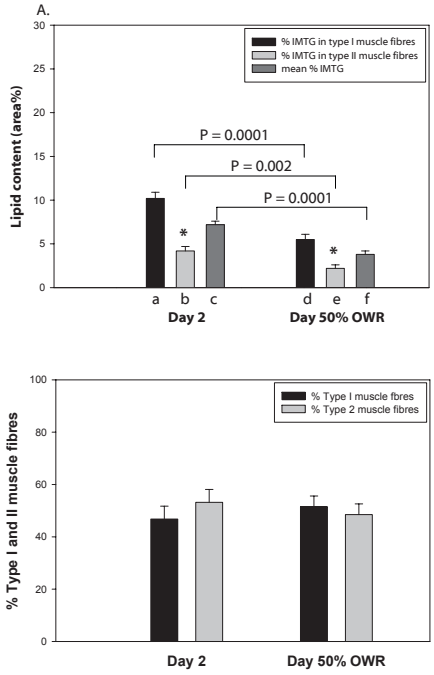

Figure 7

Quantification of the percentage intramyocellular triglycerides (IMTG, Fig. 7A) in type I muscle fibres (black bars), type II muscle fibres (light grey bars) and mean % IMTG (i.e., type I and II fibres combined, dark grey bars) on day 2 (a,b,c) of a VLCD and after 50% of overweight was lost (d,e,f). Note the significant decrease in IMTG after weight loss in both fibre types. Figure 7B shows the number of type I (black bars) and type II (grey bars) fibres on either study day. Note the significant increase in type I muscle fibres after weight loss.

treatment with 8 mg rosiglitazone daily in newly diagnosed type 2 diabetic patients, that was not accompanied by improved signalling of IRS-1 associated PI3K, PKB/AKT or AS160[34]. Finally, Friedman *et al.*[35] showed that weight loss of 36% of initial body weight by gastric

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bypass surgery improved whole-body glucose disposal by 3-fold and maximal glucose transport activity *in vitro* by 50% in 3 non-diabetic and 4 type 2 diabetic morbidly obese individuals, without an effect on total GLUT-4 protein content in skeletal muscle biopsies. Collectively, this indicates that not the amount of GLUT-4 at the cell membrane but its function and, consequently, the velocity of glucose transport over the membrane are the main determinants of insulin-stimulated glucose disposal. Alternatively, another glucose transporter, either GLUT-1[7], or a yet unidentified one, may have contributed to the increase in glucose uptake seen after weight loss. Another possible explanation is that insulin-stimulated glucose disposal in adipose tissue is greatly enhanced with weight loss. In 4 out of 8 patients from whom we obtained abdominal subcutaneous adipose tissue biopsies, an increase in insulin-stimulated PI3K-activity was observed after weight loss (data not shown).

Insulin-stimulated phosphorylation of AS160, a recently discovered substrate of PKB/Akt, has previously been reported to be disturbed in skeletal muscle of moderately obese (BMI 27 kg/m^2) type 2 diabetic patients with relatively mild diabetes (9 out of 10 used oral agents, only 1 patient on insulin therapy, HbA_{1c} 6.0 ± 0.5%)[17]. We did not use control subjects and can therefore not compare insulin-stimulated AS160 phosphorylation in our patients with that of healthy lean subjects. However, our patients were much more obese (BMI 40.2 ± 1.6 kg/m^2) and severely insulin-resistant (glucose disposal rate 18.8 ± 2.0 μmol.kgFFM^{-1}.min^{-1}; M-value 9.9 ± 2.3 μmol.kgFFM^{-1}.min^{-1}) as the patients in the study mentioned above[17] and, notwithstanding, we found a significant effect of insulin on AS160 phosphorylation on both study days. This study also shows that significant weight reduction (50% of overweight) enhances insulin-stimulated AS160 phosphorylation.

Notably, we observed that weight reduction significantly increases insulin-stimulated PRAS 40 phosphorylation, another substrate for PKB/Akt. PRAS40 is a nuclear protein, [36,37] and phosphorylation of PRAS40 facilitates the binding of 14-3-3-proteins *in vitro*. Studies in animal models and cultured cell lines suggest that PRAS40 regulates cell survival and protection from ischaemia. Although the physiological function of PRAS40 in insulin action is still unclear, we recently observed that phosphorylation of this protein is induced by physiological hyperinsulinaemia in insulin target tissues, and blunted under conditions of high-fat-diet-induced insulin resistance (E.B.M. Nascimento *et al*., submitted). Together, these findings suggest an important role for PRAS40 in physiological insulin action.

It has been hypothesised, that accumulation of intramyocellular lipids (IMCLs) are involved in the cause of impaired insulin signalling via phosphorylation of IRS-1 and IRS-2 on serine residues by fatty acid metabolites, thereby rendering these serine-phosphorylated IRSs unable to associate with and activate PI3K[41]. In our study substantial weight loss was associated with a significant decrease in IMCL. Only intramyocellular lipid content on day 50% OWR correlated negatively with the glucose disposal rate, but not with any other metabolic parameter we measured, nor with insulin signalling. Indeed, several other studies have also reported a decrease in IMCL following substantial weight reduction after bariatric surgery in

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morbidly obese, non-diabetic subjects[39-41], which was also associated with improved insulin-stimulated glucose disposal. On the other hand, more moderate weight loss (approximately 8 to 10 kg) in obese patients did not affect total IMCL content in 2 other studies[42,43]. In obese type 2 diabetic patients, Goodpaster *et al*. found a 41% reduction in IMCL following weight loss of approximately 14 kg[44].

Several studies have shown that patients with type 2 diabetes have a decreased percentage type I (oxidative) muscle fibres and an increased percentage type IIb (glycolytic) muscle fibres[45,46], like in our patients. A low capacity to oxidise fat due to a low percentage of type I muscle fibres might lead to obesity. However, whether the altered fibre type composition is the cause of obesity and type 2 diabetes or an effect of these pathologic states is unclear. Weight loss resulted in a slight, albeit non-significant, increase in the percentage of type I (and hence decrease in type II) muscle fibres. Only one other study[47] reported a tendency to increased type I fibres following weight loss, whereas the remainder of studies showed no changes in type I muscle fibres with weight loss[48-50]. None of these studies were performed in type 2 diabetic patients however. Interestingly, like one other study[51], we also found a positive relation between the amount of type I (oxidative) muscle fibres on day 2 and time to loss of 50% of overweight. The fact that type I muscle fibres contain more IMCL than type II muscle fibres and that IMCL in both muscle type fibres decrease with weight loss has been observed before[47].

IMCL might accumulate *via* increased fatty acid uptake and/or decreased fatty acid oxidation and/or –re-esterification. Weight loss did not change the amount of the fatty acid transporter FAT/CD36 at the cell membrane in our study (if any it was a tendency to increase). This is in contradiction with the hypothesis of Bonen *et al.*, who observed an increased sarcolemmal expression of FAT/CD36 in skeletal muscle of obese and type 2 diabetic patients along with an increased long-chain fatty acid uptake and proposed that this could contribute to increased IMCL and hence impaired insulin signalling[52]. With this hypothesis in mind, beforehand we expected to find a decreased sarcolemmal expression of FAT/CD36 after substantial weight loss. However, like GLUT-4, FAT/CD36 traffics between the sarcolemma and the cytoplasm and has even been demonstrated in mitochondria[53]. The trafficking can be regulated by insulin and exercise, involving PI3K/Akt[54] and adenosine monophosphate activated protein kinase (AMPK)[54] signalling pathways, respectively. Therefore, a weight-loss-induced improvement in insulin signalling could also enhance insulin-induced FAT/CD36 translocation to the sarcolemma. However, in our study, insulin-stimulated sarcolemmal FAT/CD36 did not change. Plasma NEFA levels and IMCL decreased, in combination with a decrease in whole-body lipolysis and lipid oxidation. Although highly speculative, one could assume that as a consequence of the considerable loss of adipose tissue whereby a new steady state has developed in our patients, the total release of NEFAs by fat cells is diminished and, therefore, the uptake by myocytes, leading to decreased IMCL and lipid oxidation.

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In conclusion, substantial weight loss in obese, insulin-treated type 2 diabetic patients, improves insulin sensitivity of skeletal muscle, adipose tissue and the liver. Especially insulin-stimulated glucose disposal improved considerably. At the cellular level, this was accompanied by improved insulin signalling. The observed decrease in IMCL might have contributed to the improved insulin signalling.

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CHAPTER 9

Sustained beneficial metabolic effects 18 months after a 30-day very low calorie diet in severely obese patients with type 2 diabetes.

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ABSTRACT

Very low calorie diets (VLCDs) induce rapid weight loss and improve glycaemia, dyslipidaemia and blood pressure in obese patients with type 2 diabetes mellitus. It is unclear how long the beneficial effects of a once-only VLCD will last in these patients.

We therefore looked at the long-term effect (18 months) of a once-only 30-day VLCD (450 kCal/day) on body weight, glycaemic regulation, hypertension and dyslipidaemia in 22 obese (BMI 37.7 ± 1.1 kg/m^2, mean ± SEM) type 2 diabetic patients (mean duration of diabetes 7.4 ± 1.0 years, fasting plasma glucose [FPG] 12.4 ± 0.8 mmol/L, HbA_{1c} 8.3 ± 0.3%), who participated in 2 other studies in which a 30-day VLCD was the intervention. During the 30-day VLCD, all blood glucose-lowering medication (including insulin) was stopped. After the 30-day VLCD, caloric intake was slowly increased to eucaloric and patients were encouraged to maintain weight loss. Medication for their diabetes, blood pressure or dyslipidaemia was reinstituted if deemed necessary by their own physician. On day 0 and 30 of the VLCD and after 18 months follow-up, anthropometric measures, blood pressure, glucose, HbA_{1c}, insulin, C-peptide and lipid levels were measured.

The 30-day VLCD significantly reduced body weight (-11.4 ± 0.6 kg) with an improvement in dyslipidaemia, hypertension and glycaemia (although all blood glucose-lowering medication was discontinued). As a group, patients had sustained loss of body weight and improvement in blood pressure and lipids, at 18 months follow-up. HbA_{1c} levels were also significantly lower (-0.7% compared to day 0), although patients used less blood glucose-lowering medication, especially insulin (18 patients on day 0 [112 ± 21 units/day]; 6 patients at 18 months [23 ± 9 units/day]). The 6 patients on insulin therapy at 18 months all had regained weight to prediet levels, but still had a better cardiovascular risk profile as compared to before the dietary intervention.

In conclusion, a once-only 30-day VLCD leads to a sustained improvement in glycaemia, dyslipidaemia and blood pressure up to 18 months follow-up in obese type 2 diabetic patients, even, although to a lesser extent, in patients who regained body weight.

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INTRODUCTION

Type 2 diabetes mellitus is a major health problem, both qualitatively and quantitatively. The number of patients with type 2 diabetes is increasing steadily worldwide with an estimated 366 million patients in 2030[1]. Especially worrisome is the increasing number of children and adolescents with type 2 diabetes mellitus[2,3]. It is estimated that over 80% of adult patients with type 2 diabetes are overweight (defined as a body mass index [BMI] between 25 and 30 kg/m^2) or obese (BMI > 30 kg/m^2)[4] and almost all children and adolescents who develop type 2 diabetes are overweight or obese[2,5]. Genetic factors are without doubt of major significance in the development of type 2 diabetes but environmental and social factors, like a lack of physical exercise and high caloric intake, are equally important and are pivotal targets for therapy.

Both impaired insulin secretion and insulin resistance of target organs are involved in the cause of type 2 diabetes mellitus. Insulin resistance is thought to be of major pathogenetic importance in obese type 2 diabetic patients[6], making it often difficult to achieve adequate glycaemic regulation. Insulin therapy in this patient group induces further weight gain, hence aggravating insulin resistance. Weight loss reduces insulin resistance and its associated metabolic abnormalities (hyperglycaemia, hyperinsulinaemia, dyslipidaemia and hypertension)[7-9] and, therefore, the only reasonable approach in (very) obese patients with type 2 mellitus is weight reduction.

Caloric restriction remains the hallmark for weight loss. However, only substantial caloric restriction or less severe caloric restriction of longer duration, will lead to the considerable weight loss (≥ 5-10 kg) needed to improve peripheral insulin sensitivity in morbidly obese[10] and obese type 2 diabetic patients[11]. Substantial caloric restriction has the advantage of rapid weight loss, which stimulates patients to adhere to their diet. Very low calorie diets (VLCD, < 800 kCal/day) can be used for this purpose. Nowadays, these diets are commercially available and safe[12]. Several strategies can be followed: continuously for several weeks to months or intermittently[13,14]. Both these regimens will lead to weight loss and improvement of blood glucose levels. However, the question is: how long will these effects of a VLCD on body weight, glycaemic control and other metabolic derangements last in obese type 2 diabetic patients?

The purpose of this study was to evaluate the long-term (18 months) effect of a once-only 30-day VLCD (Modifast®, 450 kCal/day) on body weight and glycaemic control in obese type 2 diabetic patients with inadequate glycaemic regulation, despite the fact that most patients used large doses of insulin before the dietary intervention, in addition to oral blood glucose-lowering agents. The effects on dyslipidaemia and blood pressure were also evaluated.

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PATIENTS AND METHODS

Patients

Twenty-two obese (BMI 37.7 ± 1.1 kg/m^2, mean ± SEM) patients (12 female and 10 male) with type 2 diabetes mellitus (mean duration 7.4 ± 1.0 years, fasting plasma glucose (FPG) level 12.4 ± 0.8 mmol/L, HbA_{1c} 8.3 ± 0.3%), age 56 ± 2 years, who participated in 2 different studies[15,16], in which a 30-day VLCD was either used as the intervention or offered as a therapy after finishing the initial study, were followed (as an observational study) for 18 months after they completed these 2 studies. The 2 studies were approved by the Medical Ethical Committee of Leiden University Medical Centre. Inclusion criteria for these 2 studies were a diagnosis of type 2 diabetes mellitus and obesity (BMI > 30 kg/m^2). In addition, glycaemic regulation had to be poorly controlled, defined as an HbA_{1c} level > 7% and FPG levels > 10 mmol/L. Eighteen of the twenty-two patients used insulin (mean dosage 112 ± 21 units/day) with or without oral blood glucose-lowering agents.

All patients underwent a medical screening, including a physical examination, blood chemistry testing and an electrocardiogram. None of the patients had a history of cardiovascular disease, nor did they have liver or kidney function abnormalities. The use of antihypertensive or lipid-lowering medication was allowed. None of the patients used other drugs, were smokers or suffered from any other disease that might interfere with the study.

Protocol

Study measurements, as outlined in the methods, were performed on day 0 and 30 of a 30-day VLCD and 18 months after the completion of the VLCD. All measurements were performed in the morning after an overnight fast while patients were still in the fasting state. Three weeks before the start of the study, all oral blood glucose-lowering agents were discontinued. If patients also used insulin, the insulin dosage was adjusted if necessary after the discontinuation of the oral blood glucose-lowering agents. On day 0, a VLCD (Modifast®, 450 kCal/day) was started and from that moment on insulin therapy was discontinued as well, at least for

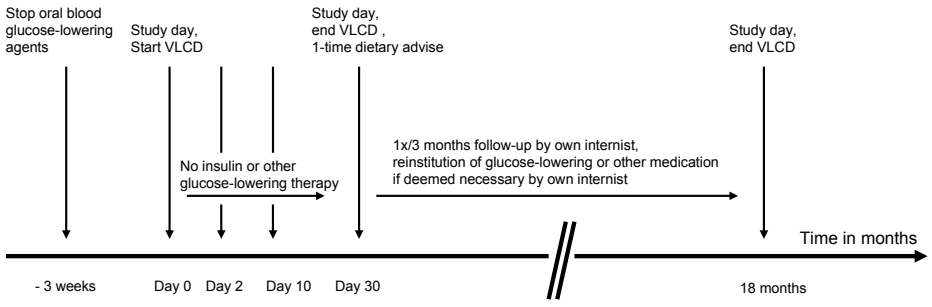

Figure 1

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the duration of the 30-day VLCD. Patients followed the VLCD for 30 days. During the 30-day VLCD, patients visited the outpatient clinic every week for support to keep up with their diet and control of body weight, blood pressure and blood glucose levels. After the 30-day VLCD, a normal diet was slowly reintroduced (reinstitution of 1 normal meal per 2-4 weeks, with an increase of 200 kCal/ 2-4 weeks until a caloric intake aimed at weight maintenance (energy requirements measured by bioelectrical impedance measurement) was achieved (around 1500 kCal/day) and patients were advised to maintain their weight loss. Thereafter patients were seen every 3 months at the outpatient clinic (Fig. 1).

The VLCD consisted of three sachets of Modifast® (Nutrition & Santé, Antwerpen, Belgium) per day (450 kCal/day), providing about 50 g protein, 50-60 g carbohydrates and 7 g lipids daily. During the diet patients were allowed to drink calorie-free substances *ad libitum*.

METHODS

Length (meters [m]), weight (kilograms [kg]), body mass index (BMI= weight (kg) / length^2 (m)) and waist/hip circumference were measured according to WHO recommendations[17].

Blood pressure was measured with an Omron 705IT blood pressure device (Omron Matsusaka, Mie, Japan) and recorded with 1 mmHg accuracy.

Serum insulin was measured with a radioimmunoassay (RIA) (Medgenix, Fleurus, Belgium), with an interassay coefficient of variation (CV) below 5%.

Serum glucose, total cholesterol, HDL-cholesterol, and triglyceride concentrations were measured with a fully automated Modulari system consisting of a P800, an I800 and an E170 (Roche, Almere, The Netherlands). HbA_{1c} levels were measured with an HPLC system (Variant, Biomed, Hercules, CA, USA). C-peptide levels were measured with a radioimmunoassay (Linco Research, St. Charles, MO, USA). The interassay CV was 4.2 to 6.0% with a sensitivity of 0.03 ng/mL.

Calculations

Data are presented as mean ± SEM.

Homeostatic Model Assessment (HOMA) of insulin resistance (IR, normal values approach 1) and β-cell function (% β, 100% is normal) were calculated with the updated computer version (HOMA2) of the formulae of Matthews *et al*.[18].

10 year coronary heart disease (CHD) risk was calculated according to both the Framingham risk score[19] and the United Kingdom Prospective Diabetes Study (UKPDS) risk engine[20].

Differences between study days were calculated with the Student's *t*-test for paired samples. Differences between groups were calculated with the Student's *t*-test for independent samples. A non-parametric test (Wilcoxon signed-rank test) was performed when appropriate. All analyses were performed using SPSS for Windows version 12.0 (SPSS Inc.,Chicago, IL, USA).

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RESULTS

Intervention period (day 0 to day 30 of the VLCD)

Baseline characteristics of the patients, as well as changes after 30 days VLCD and 18 months follow-up are given in Table 1. All 22 patients completed the 30-day VLCD without problems, no deterioration of glycaemic control occurred and no side effects were noted. Neither oral blood glucose-lowering agents nor insulin therapy had to be restarted during the VLCD.

Patients lost 11.4 ± 0.6 kg ($p = 0.0001$) and waist circumference decreased 8.6 ± 1.0 cm ($p = 0.0001$). Despite the fact that all blood glucose-lowering medication was discontinued, both FPG levels as well as HbA_{1c} levels decreased, although not significant. Fasting serum insulin concentrations declined from 19.7 ± 3.0 mU/L on day 2 to 15.0 ± 2.0 mU/L on day 30 ($p = 0.013$). HOMA-IR decreased from 3.4 ± 0.4 to 2.4 ± 0.3 ($p = 0.001$), whereas HOMA-β increased from 39.4 ± 6.0 to 53.4 ± 10.0 ($p = 0.031$). We used day 2 for the measurement of the serum insulin concentration to avoid interference with long-acting insulin, which had been used until one day before the start of the VLCD.

Systolic and diastolic blood pressure decreased significantly. Total cholesterol and triglyceride concentrations also decreased significantly. HDL-cholesterol, as is often seen at the initiation of weight loss, decreased a little.

Post-intervention period (day 30 to 18 months following the VLCD)

As the patients were free to choose their diet, we were not informed about their caloric intake during this period. No patient was lost to follow-up. As a group, patients did not gain weight

Table 1. Anthropometric measures, glycaemic regulation, lipid levels and blood pressure before, at the end and 18 months after a 30-day VLCD in obese type 2 diabetic patients.

	Day 0	Day 30	18 months
Weight (kg)	111.4 ± 3.5	99.3 ± 3.3*	99.1 ± 3.4†
BMI (kg/m^2)	37.7 ± 1.1	33.8 ± 1.0*	33.4 ± 1.1†
Waist circumference (cm)	122 ± 2	113 ± 2*	114 ± 2†
FPG (mmol/L)	12.4 ± 0.8	10.7 ± 0.9	10.9 ± 1.0
HbA_{1c} (%)	8.3 ± 0.4	7.9 ± 0.4	7.6 ± 0.4‡
Systolic blood pressure (mmHg)	168 ± 7	143 ± 7§	145 ± 4$^{\|}$
Diastolic blood pressure (mmHg)	95 ± 4	83 ± 3§	81 ± 2$^{\|}$
Total cholesterol (mmol/L)	5.9 ± 0.4	4.7 ± 0.2$^{\#}$	5.4 ± 0.2**
HDL-cholesterol (mmol/L)	1.1 ± 0.05	1.0 ± 0.05$^{\#}$	1.3 ± 0.07$^{\|,\dagger\dagger}$
Cholesterol/HDL-cholesterol ratio	5.6 ± 0.4	5.1 ± 0.4	4.6 ± 0.3‡‡
Triglycerides (mmol/L)	4.9 ± 1.5	1.9 ± 0.3§§	2.5 ± 0.4$^{\|\|,\#\#}$
Units of insulin (no. of patients treated with insulin)	112 ± 21 (n=18)	0	23 ± 9 (n=6)

* $p = 0.0001$, § $p = 0.004$, $^{\#}$ $p = 0.007$, §§ $p = 0.001$: day 30 as compared to day 0.

† $p = 0.0001$, ‡ $p = 0.027$, $^{\|}$ $p = 0.0001$, $^{\|\|}$ $p = 0.009$: 18 months versus day 0.

** $p = 0.007$, †† $p = 0.0001$, ‡‡ $p = 0.004$, $^{\#\#}$ $p = 0.014$: 18 months versus day 30

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from the end of the VLCD up to 18 months follow-up. In addition, waist circumference, as a measure of visceral fat mass, also remained the same. During the follow-up period, 1 patient experienced an acute coronary syndrome, and 1 patient developed prostate cancer. Some patients intermittently used a hypocaloric (1 sachet of Modifast for breakfast in combination with 2 normal meals a day) but not a very low calorie diet. Furthermore, no weight-control drugs were used.

FPG levels and HbA_{1c} levels did not increase during the follow-up period and although most patients were restarted on oral blood glucose-lowering therapy, the dose was less than before the diet. Since 6 patients were on insulin therapy again at 18 months follow-up and, hence, their fasting serum insulin level would no be accurate, we did not use their data for comparison with the fasting serum insulin concentration on day 30 (and day 2, next section). In addition, serum insulin levels at 18 months were lacking in 2 patients. We can therefore only compare data on endogenous insulin levels of 14 patients and, hence, HOMA-IR and HOMA-β could also only be calculated for 14 patients. Nevertheless, in these 14 patients serum insulin (15.3 ± 2.3 mU/L on day 30 to 14.4 ± 2.1 mU/L at 18 months), HOMA-IR (2.4 ± 0.3 on day 30 to 2.2 ± 0.3 at 18 months) and HOMA-β (53.4 ± 10.0 on day 30 to 55.7 ± 9.0 at 18 months) did not change significantly between day 30 and 18 months follow-up.

Systolic and diastolic blood pressure did not differ between day 30 and 18 months follow-up. Total cholesterol and triglyceride levels increased to some extent whereas HDL-cholesterol levels were significantly higher at 18 months as compared to day 30 ($p = 0.007$).(Table 1)

When looking more closely at the data, 8 patients had stable body weight (plus or minus 5 kilogram [kg]), 6 patients lost more than 5 kg of body weight and 8 patients regained more

Table 2. Cardiovascular risk factors at 18 months, according to post-intervention (day 30 to 18 months follow-up) weight changes.

	From day 30-18months :		
	Stable weight (n=8)	**Weight loss > 5 kg** (n=6)	**Weight gain > 5 kg** (n=8)
FPG (mmol/L)	10.1 ± 1.2	10.2 ± 2.6	12.5 ± 1.6
HbA_{1c} (%)	7.8 ± 0.4	7.1 ± 0.9	8.1 ± 0.6
Systolic blood pressure (mmHg)	148 ± 4	132 ± 6[*]	153 ± 7[†]
Diastolic blood pressure (mmHg)	84 ± 2	75 ± 3[‡]	82 ± 4
Total cholesterol (mmol/L)	5.3 ± 0.5	5.2 ± 0.4	5.6 ± 0.3
HDL-cholesterol (mmol/L)	1.3 ± 0.1	1.4 ± 0.2	1.2 ± 0.1
Triglycerides (mmol/L)	2.5 ± 0.8	1.7 ± 0.1	3.1 ± 0.4[§]
C-peptide (ng/mL)	1.2 ± 0.2	0.9 ± 0.1	0.8 ± 0.2
Insulin (mU/L)	16.0 ± 4.1	12.5 ± 1.9	13.0 ± 0.6
HOMA-IR	2.4 ± 0.6	1.7 ± 0.2	2.4 ± 0.4
HOMA-β	53.5 ± 9.6[‖]	85.4 ± 17.9	21.4 ± 4.1[¶]

Data are presented as mean ± SEM

[*] $p = 0.033$, [‡] $p = 0.017$ weight stable versus weight loss

[†] $p = 0.022$, [§] $p = 0.011$, [¶] $p = 0.031$ weight gain versus weight loss

[‖] $p = 0.016$ weight stable versus weight gain

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Table 3. Use of blood glucose-lowering, lipid-lowering and antihypertensive agents before and 18 months after a 30-day VLCD in obese type 2 diabetic patients.

	Day 0	**18 months**
	number of patients	number of patients
Insulin only	6	1
Insulin + oral blood glucose lowering agent	12	5
Metformin only	1	7
Metformin + SU-derivative	3	5
No blood glucose lowering therapy	0	4
Statin	6	4
Fibrate	3 (1 also statin)	1
Beta-blocker	9	7
ACE-inhibitor of ATII-antagonist	11	11
Diuretic	10	3
Number of antihypertensive agents	**Day 0**	**18 months**
	number of patients	number of patients
0 antihypertensive agents	6	9
1 antihypertensive agent	7	7
2 antihypertensive agents	5	5
3 antihypertensive agents	4	2
4 antihypertensive agents	1	0

than 5 kg of body weight from day 30 to 18 months follow-up (see Table 2). When these 3 groups were compared, the patients that had gained body weight had worse glycaemic control and dyslipidaemia and a higher (systolic) blood pressure as compared to the other 2 groups. Because the groups were small, significance was not always reached.

Day 0 versus 18 months follow-up

As a group, body weight and waist circumference were significantly lower at 18 months follow-up as compared to day 0.

FPG and HbA_{1c} levels for the whole group were also significantly lower at 18 months, despite the fact that patients used less blood glucose-lowering medication (see Table 3). Four patients used no blood glucose-lowering therapy at all at 18 months. Most of the patients on oral blood glucose-lowering therapy were on metformin only. In addition, only 6 patients were on insulin therapy at 18 months (5 patients had already been on insulin therapy before the VLCD, 1 patients used insulin for the first time) with a mean dose of 23 ± 9 units per day, whereas before the VLCD 18 patients were on insulin therapy with a mean dose of 112 ± 21 units/day.

Fasting serum insulin concentrations and HOMA-IR and HOMA-β could be compared in 14 patients (see section above). Fasting serum insulin levels were significantly lower at 18 months (14.4 ± 2.1 mU/L) as compared to day 2 (20.2 ± 3.5 mU/L), p = 0.045. HOMA-IR was sig-

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nificantly lower at 18 months (2.2 ± 0.3) as compared to day 2 (3.4 ± 0.4), p = 0.019, whereas HOMA-β did not significantly change between day 2 and 18 months. Serum C-peptide levels, another indirect measure for β-cell function, also did not change significantly between day 0 and 18 months (1.1 ± 0.1 ng/mL on day 0 to 1.0 ± 0.1 ng/mL at 18 months).

At 18 months follow-up both systolic and diastolic blood pressure were significantly lower than before the start of the diet. Although total cholesterol and triglyceride concentrations had increased between day 30 and 18 months, they were still significantly lower at 18 months as compared to day 0. HDL-cholesterol and the total cholesterol/HDL ratio were also significantly improved at 18 months follow-up (Table 1).

Surprisingly, the 8 patients who had gained more than 5 kg body weight still had a significantly lower systolic (183 ± 10 mmHg on day 0 to 152 ± 8 mmHg at 18 months, p = 0.004) and diastolic blood pressure (99 ± 5 mmHg on day 0 to 80 ± 4 mmHg at 18 months, p = 0.013), lower triglycerides (4.4 ± 0.8 mmol/L on day 0 and 3.1 ± 0.3 mmol/L at 18 months, p = 0.025) and a higher HDL-cholesterol (0.9 ± 0.08 mmol/L on day 0 to 1.2 ± 0.1 mmol/L at 18 months, p = 0.005) as compared to the start of the study. In addition, although not significant, FPG levels (14.1 ± 1.6 mmol/L on day 0 to 12.5 ± 1.6 mmol/L at 18 months) and HbA_{1c} levels (9.1 ± 0.6% on day 0 to 8.1 ± 0.6%) were also lower at 18 months follow up, as compared to before the dietary intervention.

Factors discriminating the patients on insulin therapy from those not on insulin therapy, at 18 months follow-up.

The 6 patients on insulin therapy at 18 months all had regained body-weight to prediet levels. They also had a longer duration of type 2 diabetes (10.7 ± 1.9 *versus* 6.2 ± 1.0 years, p = 0.04) with lower serum insulin (12.2 ± 3.7 *versus* 22.0 ± 2.7 mU/L, NS) and C-peptide levels (0.8 ± 0.1 versus 1.19 ± 0.16 ng/mL, NS) at the start of the study as compared to patients who were not restarted on insulin therapy.

The long-term influence of a once-only 30-day VLCD on the Framingham and UKPDS risk score for coronary heart disease

10 year coronary heart disease risk (CHD) risk estimates according to the Framingham Risk Tables declined from 11.3 ± 2.2 to 8.0 ± 1.5%, p = 0.007. CHD risk estimates according to the UKPDS risk engine were higher than Framingham risk estimates but were also lower at 18 months follow-up (23.8 ± 4.0 to 17.8 ± 3.0%, p = 0.002).

DISCUSSION

This study demonstrates that a 30-day VLCD in severely obese, mostly insulin-treated, type 2 diabetic patients is well tolerated and that the simultaneous discontinuation of all blood

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glucose-lowering agents is safe. The diet resulted in a considerable loss of weight and waist circumference. Glycaemic control improved, as did cardiovascular risk factors such as blood pressure and plasma lipid levels.

During the 18 months regular follow-up in an outpatient setting, weight loss and the decrease in waist circumference were maintained for the whole group. Glycaemic control deteriorated to some extent but remained considerably better than before the VLCD whereas patients used less blood glucose-lowering medication, especially insulin (see Table 3 and below). Blood pressure and serum lipid levels also remained lower than before the dietary intervention while patients used fewer antihypertensive and lipid-lowering agents at 18 months follow-up.

Six of the 22 patients were started on insulin therapy (5 already had insulin therapy before the VLCD was instituted, 1 patient used insulin for the first time) during the 18 months follow-up. All these patients had regained body weight to pre-intervention levels. In addition, they had a longer duration of type 2 diabetes and lower serum insulin and C-peptide levels at the start of the study, underscoring our previous observation that remaining endogenous insulin secretion is important as well[15]. Nevertheless, even the patients who gained more than 5 kg body weight (n=8) still had better glycaemic control and improved lipid levels and blood pressure as compared to before the dietary intervention. We do not have a good explanation for this, other than that at least for some period of time between day 0 and 18 months follow-up, their body-weight has been lower.

Few studies have addressed the long-term effect of a VLCD in obese type 2 diabetic patients and most used the VLCD for a much longer period of time than we did (8-20 weeks)[21-23] or also included a behaviour therapy programme[24]. The results of these other studies are also less favourable, resulting from an increase in weight to no increase in weight but deterioration of glycaemic control 1 year after the VLCD. One study[22] also extended follow-up to 1.5 years, but found a deterioration in glycaemic control in most of the patients. One of the reasons that our results are so impressive might be that most patients did not want to be restarted on insulin therapy and, hence, were very motivated to maintain their weight loss. In addition, a normal diet was reintroduced very slowly once the 30-day VLCD had been completed. Finally, regular counselling (every week during the diet, once every 3 months thereafter) seems to be an important factor.

Whether the lower HbA_{1c} levels, blood pressure, triglyceride and total cholesterol levels, along with the increased HDL-cholesterol level, at 18 months follow-up will lead to a lower risk for cardiovascular disease in our patients, remains to be elucidated. Most studies investigating the effects of lowering cardiovascular risk factors on morbidity and mortality from cardiovascular disease have follow-up periods of at least 3 years, whereas our group showed sustained improvement in cardiovascular risk factors for 18 months. Nevertheless, if our patients are able to sustain their weight loss and/or their improved cardiovascular risk profile, a reduction in risk for cardiovascular disease might be expected given the evidence of several

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large trials, also in patients with diabetes, that lower(ing) blood pressure[25,26], total[27] and LDL-cholesterol[28-30] and decreasing triglycide levels while increasing HDL-cholesterol[31-33] significantly reduces the risk for cardiovascular disease. Although not designed for this purpose, one might also estimate cardiovascular risk according to the Framingham risk score[19] or the UKPDS risk engine[20]. 10-year CHD risk estimates in our patients were lower at 18 months follow-up as compared to day 0, both according to the Framingham and the UKPDS risk score. Risk percentages calculated with the Framingham risk score were relatively low, probably because the original Framingham cohort contained only 237 patients with diabetes. Values obtained with the UKPDS risk engine are more likely to reflect the true cardiovascular risk in our group of middle-aged patients with type 2 diabetes, hypertension and dyslipidaemia (at least at the start of the study) and a duration of type 2 diabetes of 7.4 years.

Treatment goals for glycaemic regulation, blood pressure and serum lipids as set by the American Diabetes Association (ADA)[34] were not reached for all parameters but came very close (HbA_{1c} 7.6 ± 0.4%, total cholesterol 5.4 ± 0.2 mmol/L, triglycerides 2.5 ± 0.4 mmol/L, HDL cholesterol 1.3 ± 0.07 mmol/L, blood pressure 145 ± 4 mmHg / 81 ± 2 mmHg) and were substantially improved as compared to before the intervention (Table 1).

We are aware that the group of patients is relatively small, follow-up of limited duration and that a control group is lacking. Nevertheless, the observation in these 22 patients, that a once-only 30-day VLCD (with at the end a weight-maintaining advise, followed by regular outpatient clinic visits) has sustained beneficial metabolic effects that might extend over and beyond the weight loss/weight maintenance observed, is interesting and needs further investigation in a (randomised) controlled prospective study. We used a VLCD, to be able to discontinue all blood glucose-lowering medication at the start of the diet to avoid hypoglycaemia. Perhaps the same results can be obtained with a formula diet of greater energy content. In addition, varying the degree of calories with time (i.e. start with 450 kCal/day for 4 weeks, continue with 600 kCal/day and so on) or an intermittent VLCD might be as successful.

In conclusion, we show that a once-only 30-day VLCD in very obese, largely insulin-treated type 2 diabetic patients, leads to a sustained improvement in HbA_{1c}, total cholesterol, HDL-cholesterol, triglyceride levels and blood pressure at 18 months follow-up, even in patients who regained more than 5 kg body-weight. Although bariatric surgery is more effective in establishing sustained weight loss[35-37], this is an invasive and costly procedure available for only a limited number of patients. VLCDs are cheap, safe, easy to use and can also lead to large weight losses[38]. Given the enormous increase in obesity and type 2 diabetes, VLCDs can be an important therapeutic strategy.

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CHAPTER 10

Summary and conclusions

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INTRODUCTION

The increased worldwide incidence and prevalence of type 2 diabetes mellitus has reached epidemic proportions. Nowadays over 190 million people worldwide have diabetes mellitus[1], the majority having type 2 diabetes mellitus. Of type 2 diabetic patients, more than 80% are obese[2].

In obese type 2 diabetic patients, insulin resistance contributes substantially to the pathogenesis of hyperglycaemia[3]. Moreover, in very obese type 2 diabetic patients, insulin resistance makes it often extremely difficult to achieve adequate glycaemic regulation. Most oral blood glucose-lowering agents and exogenous insulin therapy induce weight gain, hence aggravating insulin resistance.

Caloric restriction and weight loss improve insulin resistance and its associated metabolic abnormalities[4-8] and are in fact the only reasonable therapeutic options in very obese type 2 diabetic patients.

Given the enormous increase in obese type 2 diabetic patients it is of utmost importance to find the optimal therapeutic strategy for this patient group. The aim of this thesis was to gain more insight in the pathofysiology of insulin resistance induced by adipose tissue, the safety and feasibility of very low calorie diets (VLCDs), and in the short-term and long-term effects of a VLCD on insulin resistance of the liver, adipose tissue and skeletal muscle. The findings of our studies will be discussed in view of the aims we put forward in Chapter 1.

FIRST AIM

Because of the association of obesity with insulin resistance and the fact that most type 2 diabetic patients are obese, our **first aim was to evaluate the role of adipose tissue in insulin resistance**.

When adipose tissue is discussed here, we refer to white adipose tissue (WAT), since adult humans hardly have any brown adipose tissue. WAT contains mature adipocytes, pre-adipocytes and fibroblasts, connective tissue, nerve tissue, stromal vascular cells and immune cells. The functions of these components are highly integrated, making adipose tissue a true endocrine organ. Adipose tissue responds to afferent signals of several well-known hormones (insulin, glucagon, cortisol) and the autonomous nervous system (catecholamines), but also to several of the proteins that it secretes itself, thereby regulating its own metabolism and cell size.

It is unknown whether obesity causes insulin resistance or is merely a reflection of a primary pathogenetic (insulin-resistant) state. However, given the fact that lipodystrophy also causes whole-body insulin resistance[9] and that transplantation of adipose tissue back into lipodystrophic animals reverses glucose intolerance and diabetes[10] suggests an important role for adipose tissue.

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Adipose tissue can modulate glucose homeostasis *via* the production of free fatty acids (FFA) and so-called adipocytokines (or rather adipokines, since many of the secreted products are not cytokines). Quantatively, FFA secretion is the most important. Elevated serum FFA concentrations can induce skeletal muscle insulin resistance *via* an impairment in insulin signalling[11]. In addition, chronically elevated FFAs lead to a decrease in insulin secretion by the pancreatic β-cells[12-14]. Finally, increased delivery of FFAs to the liver increases gluconeogenesis and might induce hepatic insulin resistance[15]. These FFA-induced metabolic disturbances are also referred to as lipotoxicity.

In Chapter 2, several of the so-called adipokines have been discussed. In obesity, increased production of leptin, restin, IL-6, TNF-α and ASP are found that correlate positively with insulin resistance, whereas adiponectin levels are decreased and correlate negatively with insulin resistance. New adipokines are being identified continuously, among them apelin[16], visfatin[17] and zinc-α2-glycoprotein (ZAG)[18,19], the first two of these being increased in obesity. The mechanism by which these adipokines induce insulin resistance is unclear but might involve impaired insulin signalling since several of the adipokines (leptin, TNF-α, possibly IL-6) can interfere with the insulin-signalling pathway. The elucidation of the exact role of adipokines in insulin resistance is further complicated by the heterogeneity between the various adipose tissue depots. Although a primary role for visceral adipose tissue as opposed to subcutaneous abdominal adipose tissue has recently been challenged[20], it is a fact that adipocytes in these various fat depots have a different secretion pattern[21,22] (see Table 1). Moreover, these secretion patterns might be different in obesity and diabetes mellitus. For example, adiponectin production in healthy humans is higher in subcutaneous adipose tissue in comparison to visceral adipose tissue. However, in both insulin-resistant rodents[42], as well as in humans it seems that omental adiponectin secretion is impaired, whereas it is preserved in subcutaneous adipose tissue[31,45,46].

Table 1. Characteristics of adipocytes derived from visceral adipose tissue (VAT) in comparison to those of subcutaneous adipose tissue (SAT).

Biochemical factors	**Regional differences**	**Physiological effect**
Lipolytic response to catecholamines	VAT > SAT[23,24]	
Antilipolytic effect of insulin	SAT > VAT[22,25]	↑ NEFA and TG turnover
Leptin secretion	SAT > VAT[26-28]	less CNS regulation of VAT, ↓ insulin sensitivity
Adiponectin secretion	SAT > VAT[29-31]	↑ insulin sensitivity
Acylation stimulating protein (ASP)	VAT > SAT[26]	
IL-6	VAT > SAT[32,33]	inflammation, cardiovascular risk
TNF-α	VAT = SAT[26,34-36]	
Resistin	Abdominal > tigh[37,38]	
PAI-1	VAT > SAT[32,39,40]	cardiovascular risk
Innate characteristics of preadipocytes		
Preadipocyte differentiation and fat cell-function gene expression	SAT > VAT[41,42]	
Apoptosis	VAT > SAT[43]	

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It has been noted that the size of adipocytes correlates better with insulin resistance than any other measure of adiposity. Weyer *et al.* reported that enlarged abdominal adipocytes predicted the development of type 2 diabetes mellitus, independent of emerging insulin resistance and (impaired) insulin secretion, in 108 previously normal glucose tolerant Pima Indians followed for 9.3 ± 4.1 years, of whom 33 developed type 2 diabetes[47]. It has been proposed that a diminished capacity for proliferation and differentiation of mesenchymal precursor cells leads to hypertrophy of mature adipocytes under conditions of energy excess[48]. These enlarged adipocytes are thought to secrete a different, insulin-resistance and atherogenesis provoking, pattern of adipokines and lead to ectopic fat storage because of a diminished capacity to store triglycerides. This ectopic storage of fat in liver, muscle and pancreas then leads to decreased insulin-mediated suppression of hepatic glucose production, decreased insulin-stimulated glucose uptake and decreased insulin secretion in these organs, respectively[49].

In conclusion, given the fact that both obesity[50] and lipodystrophy[9] are associated with insulin resistance and that transplantation of fat in lipodystrophic mice restores the metabolic abnormalities[10], supports an important role for adipose tissue in insulin resistance. As to the mechanism by which obesity induces insulin resistance, several theories have been proposed. The portal/visceral hypothesis[51], which proposes a primary role for visceral adipose tissue that would be deleterious because produced FFAs drain directly to the liver *via* the vena portae, has recently been challenged but, given this unique drainage of visceral FFAs and adipokines (that show a fat depot specific secretion pattern) directly to the liver, cannot be completely rejected. Notwithstanding, whether derived from visceral or truncal adipose tissue, elevated serum FFA levels, are involved in the pathogenesis of insulin resistance *via* the concept of lipotoxicity[49]. Two new paradigms involve the "theory of ectopic fat storage"[48,49,52] and that of "the adipocyte as an endocrine organ"[53]. These paradigms can also be explained using the concept of dysfunctioning adipose tissue. In this model, a defect in proliferation and differentiation of preadipocytes leads to enlarged mature adipocytes that secrete a different, insulin-resistance inducing, pattern of adipokines and have a diminished capacity to store triglycerides, leading to an ectopic storage of fat. If fat oxidation does not increase in these organs, then intracellular accumulation of lipids, with insulin resistance will occur. Further research is needed to investigate the interactions between the environment and adipose tissue leading to this impaired functioning of adipose tissue.

SECOND AIM

Leptin is secreted by adipocytes in direct proportion to adipose tissue mass[54-56] and nutritional status[57,58]. The primary role of leptin is to serve as a metabolic signal of energy deficiency rather than excess[59]. Serum leptin levels rapidly decrease during caloric restriction

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and weight loss[57,60], which leads to increased appetite and decreased energy expenditure. In obesity, serum leptin levels are increased[54,56], indicating a state of leptin resistance. Unfortunately, the leptin response to caloric restriction is preserved in obesity.

Serum insulin levels are also positively related to BMI and fat mass[61]. Moreover, several studies have shown a close correlation between serum leptin and serum insulin[62-65]. It is unknown, however, whether this relation also holds in patients with a severely disturbed insulin secretion. Moreover, most studies have evaluated the relation between fasting serum levels of leptin and insulin and did not study the relation between leptin and insulin secretion. Finally, data about the effect of weight loss on the relation between serum leptin and insulin in obese type 2 diabetic patients are scarce[64,66].

Therefore, we have studied the relation between fasting serum leptin and fasting serum insulin, as well as the area under the curve of insulin following an intravenous (i.v.) glucose load in obese (BMI 37.6 ± 1.4 kg/m^2, mean ± SEM) type 2 diabetic patients (duration 8.0 ± 1.4 years, fasting plasma glucose [FPG 12.9 ± 0.8 mmol/L, HbA_{1c} 8.6 ± 0.4%) on day 2 and day 30 of a very low calorie diet (VLCD, Modifast®, 450 kCal/day). During the VLCD, all blood glucose-lowering medication, including insulin, was discontinued. It was found that, even when insulin secretion was severely disturbed, the relation between serum leptin and serum insulin and insulin secretion remained. This was also true during energy restriction with weight loss. Whether insulin regulates leptin levels or *vice versa*, or alternatively, whether both are regulated in concert to reflect changes in energy balance, cannot be deduced from this study. From circumstantial evidence, however, it seems most likely that insulin regulates leptin.

AIMS 3 TO 5

These aims were investigated in a single study, presented in Chapter 3. In short, seventeen obese (BMI 37.6 ± 5.6 kg/m^2, mean ± SEM) patients with type 2 diabetes (duration 8 ± 5.8 years) with persistent high blood glucose levels (FPG 12.9 ± 3.1 mmol/L, HbA_{1c} 8.6 ± 1.6%) despite maximal doses of oral blood glucose-lowering medication and/or insulin (66 to 340 units per day) started a VLCD (Modifast®, 450 kCal/day) for 30 days during which all blood glucose-lowering medication was discontinued. On days 0, 2, 10 and 30, of the diet, body weight was measured and fasting serum samples of glucose, insulin, C-peptide and leptin were taken. An intravenous glucose tolerance test was performed on day 2 and day 30. *A priori*, a responder was defined as a patient with a FPG level < 10 mmol/L on day 30.

The third aim of this thesis was to test whether it is safe to start a VLCD in obese type 2 diabetic patients undergoing insulin therapy and simultaneously discontinue all blood glucose-lowering medication, including insulin. The latter was an important issue, since discontinuation of all blood glucose-lowering agents would minimise the risk for hypoglycaemia and facilitate weight loss[67].

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During the study presented in Chapter 3 and outlined in short above, no side effects were noted during the VLCD. Especially, no overt hyperglycaemia (glucose levels > 20 mmol/L) was noted, despite the fact that all blood glucose-lowering medication was discontinued. In addition, no hypoglycaemia, hypotension, vasovagal collaps, gall-bladder disease or cardiac events were observed. The 3 patients that did not complete the VLCD, all quit the study in the first few days because they did not like the Modifast®.

Meanwhile, over the years, more than 40 very obese insulin-treated type 2 diabetic patients have been treated with a VLCD and the simultaneous discontinuation of all blood glucose-lowering agents in a study setting, and also several patients in a non-study setting. In none of these patients adverse events were noted. Patients with known coronary artery disease were excluded, but diet therapy might be safe in these patients as well. Patients tolerated the diet very well, even for up to 8 months. Notably, women found it easier to adhere to the VLCD than men, probably because most of the women were not in the working process and because they found it more important to lose weight for esthetical reasons.

Our fourth aim was to establish whether blood glucose levels do indeed decline already after 2 days of a VLCD and the fifth aim to find factors that would discriminate responders from non-responders.

The study described in Chapter 3 showed a dichotomy in the blood glucose-lowering response to the VLCD: of the 14 patients that completed the 30-day VLCD, 8 patients could be defined as responders and 6 patients were classified as non-responders. The difference in blood glucose-lowering response to a VLCD was already apparent on day 2 of the VLCD: responders had only a small increase or a decline in fasting plasma glucose (FPG, +0.64 ± mmol/L [mean ± SEM]) whereas non-responders had an increase in FPG levels (4.15 ± 3.3 mmol/L), $p = 0.035$. It appeared that non-responders had a longer duration of type 2 diabetes mellitus (12.3 ± 2.6 *versus* 5.0 ± 1.4 years), lower fasting serum insulin, C-peptide and HOMA-β values and a lower second-phase insulin response following an i.v. glucose load on both day 0 and day 30. In a step-wise discriminant analysis, the change in FPG from day 0 to day 2 in combination with the area under the curve (AUC) of insulin above baseline during an intravenous glucose tolerance test (IVGTT) on day 2, completely distinguished responders from non-responders. We also found that the disappearance rate of glucose (k-value), as a measure of peripheral insulin sensitivity, neither differed between responders and non-responders, nor did it change with weight loss.

Therefore, the following conclusions can be drawn from this study with respect to the aims we put forward. With respect to the **fourth aim**, one can conclude that blood glucose levels can indeed decline already within the first few days of a VLCD. However, it seems that remaining endogenous insulin secretory capacity (rather than insulin sensitivity, since no difference in k-values was observed) determines the magnitude of this improvement. Later studies (Chapter 5 and 7) have confirmed that blood glucose levels decrease within 2 days of a VLCD in patients with remaining endogenous insulin secretion. With respect to the **fifth aim**, we

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found that non-responders had a lower capacity to secrete insulin. Given the fact that they also had a longer duration of type 2 diabetes mellitus, this is probably due to ongoing failure of the pancreatic β-cell. Furthermore, non-responders can already be discriminated from responders on day 2 of a VLCD on the basis of an increase in FPG levels from day 0 to day 2 and a low area under the curve of insulin following an i.v. glucose load on day 2 of the VLCD. For practical purposes, however, the fasting C-peptide level is an easier indicator of whether or not a patient will show a glucose-lowering response to weight loss: patients with a fasting C-peptide level < 0.8 ng/mL are less likely to have a decrease in FPG levels during the VLCD as compared to patients with a fasting C-peptide level > 0.8 ng/mL. In patients with a C-peptide level < 0.8 ng/mL, one can choose to either continue (or start, if not yet part of the therapy) metformin during the VLCD or stop all blood glucose-lowering agents at the start of the VLCD and if blood glucose levels do not decline within a few days, start metformin therapy (or another oral blood glucose-lowering agent).

Given the observations of this study, we decided to include only patients with remaining endogenous insulin secretion (defined as a fasting C-peptide level greater than 0.8 ng/mL and/or a 2 times increase of the basal C-peptide level [cut-off value 0.5 ng/mL] after 1 mg glucagon i.v.) in our subsequent studies. The reason was that we did not want to expose the patients to high blood glucose levels for a longer period of time, and obviously, if remaining endogenous insulin secretion is low, blood glucose levels rise even at low caloric intake (patients have become insulin-dependent). However, as already described above, a low C-peptide level does not exclude the use of a VLCD, but, if C-peptide levels are low, oral blood glucose-lowering agents should either be continued during the VLCD or stopped, but restarted when blood glucose levels do not decline within 7-10 days of the VLCD. We did not want to risk the chance of having to start oral blood glucose-lowering agents because they could disturb the results of our metabolic studies. Therefore, only subjects with remaining insulin secretory capacity, as defined above, were included in later studies.

AIM 6 AND 7

To study the short-term blood glucose-lowering effect of a VLCD, both on the whole-body level and at the molecular level, 12 obese (BMI 36.3 ± 1.0 kg/m^2, [mean ± SEM]) type 2 diabetic (age 55 ± 4 years; HbA_{1c} 7.3 ± 0.4%) patients undergoing insulin therapy were studied on day 0 and day 2 of a VLCD (Modifast®, 450 kCal/day). Three weeks before the study all oral blood glucose-lowering medication was discontinued and from day -1 on, insulin therapy was stopped as well. Endogenous glucose production (EGP) and whole-body glucose disposal (6,6 $^2H_2$-glucose), lipolysis ($^2H_5$-glycerol), and substrate oxidation (indirect calorimetry) rates were measured before and after the VLCD in basal and hyperinsulinaemic (insulin infusion: 10 min prime followed by a constant rate of 40 mU/ m^2 per minute[68]) eug-

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lycaemic conditions. Insulin signalling and expression of GLUT4, FAT/CD36 and triglycerides were assessed in skeletal muscle biopsies, obtained before the clamp and 30 min after the start of the insulin infusion.

With respect to the **sixth aim**, we found that short-term energy restriction without weight loss, lowers blood glucose levels due to a decrease in EGP with no effect on peripheral insulin sensitivity. As to the mechanism by which basal EGP was reduced; fasting serum glucagon, cortisol and growth hormone levels, as well as fasting serum non-esterified fatty acids, glycerol, triglycerides and lactate, were similar between study days. Although the lower fasting serum insulin levels we found suggested a better insulin sensitivity of the liver, this was not supported by the clamp studies. The reason that we did not find a better suppressibility of EGP by insulin during the hyperinsulinaemic euglycaemic clamp might have been due to the relatively high serum insulin levels achieved during the clamp (88 mU/L and 84 mU/L on day 0 and day 2, respectively). These concentrations might have been high enough for a near-maximal suppression of the glucose (and glycerol) R_a, making it difficult to observe changes between study days. Table 2 summarises some other studies of short-term, and longer-term energy restriction in obese type 2 diabetic patients. Only studies using a hyper-insulinaemic euglycaemic clamp, in combination with the isotope dilution technique as a measure of peripheral glucose disposal and endogenous glucose production, were included.

As described in Chapter 1 (section 1.4.2), insulin-stimulated glucose uptake is disturbed in patients with type 2 diabetes mellitus. This seems to be due to disturbances in the insulin-signalling cascade leading to GLUT-4 translocation. Table 3 summarises defects, known to date, in insulin signalling in obese, non-obese diabetic and obese diabetic patients. Few, if any, studies have been performed in humans evaluating the effect of short-term (Chapter 6) and long-term (Chapter 8) effects of energy restriction on the insulin-signalling pathway and GLUT-4 translocation. Although we did not observe an effect of calorie restriction *per se* on whole-body glucose disposal, we still analysed the muscle biopsies because we expected to find changes at the molecular level that were not yet translated to an effect on the whole-body level. However, no diet effect was found on the expression of the insulin receptor and insulin receptor-1 (IRS-1) or on IRS-1 associated phosphatidylinositol 3'-kinase (PI3K) activity; on FAT/CD36 expression pattern, GLUT4-translocation or triglyceride distribution, in either the basal or insulin-stimulated situation in skeletal muscle biopsies. Unexpectedly, basal PKB/Akt-phosphorylation on T308 and S473 increased after the diet. The meaning of this finding is unclear. However, as outlined in Chapter 1, PKB/Akt is also involved in the regulation of hepatic gluconeogenesis[88]. Hence, if our findings also apply to the liver, higher basal PKB/Akt concentrations in the liver might explain the observed decrease in basal EGP. Unfortunately, ethical considerations prohibit us to take liver biopsies in humans for study purposes.

In conclusion, with respect to the **seventh aim** we show that a 2-day VLCD has no effect on insulin stimulation of key signalling molecules or on translocation of the fuel transporters

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Table 2. Effect of energy restriction on glucose and lipid metabolism in obese patients with type 2 diabetes.

	Henry [4],a	**Laakso** [5], b	**Kelley**[7],,b	**Markovic**[6],b
Year	1985	1988	1993	1998
Number of patients	30	8	7	10
Age (yrs)	53 ± 11	52.6 ± 2.0	58.7 ± 3.3	48.3 ± 4.4
Duration DM2 (yrs)	9 ± 5	10.8 ± 1.7	< 5	?
Diabetes medication Diet Oral Insulin	3 11 16	1 7 -	2 5 -	7 2 -
FPG (mmol/L)	16.5 ± 3.9	11.4 ± 0.5	12.3 ± 1.4	7.3 ± 0.7
HbA1c (%)	12.3 ± 2.2	10.8 ± 0.5	8.8 ± 0.5	-
Weight (kg)	99.1 ± 14.2	92.8 ± 3.1	92.7 ± 4.7	-
BMI (kg/m^2)	37.1 ± 4.9	33.7 ± 0.8	32.8 ± 1.9	32.3 ± 0.8
Intervention (I)	40-day VLCD (330 kCal/d, liquid formula)	12 days 500 kCal/d (formula diet) followed by 3 days 800 kCal/d	7 days eucaloric, 7days 800kCal/day, 8 weeks VLCD (400kCal/d) + 3 weeks increasing intake, 7 days balance†	28 days (- 1000 kCal/d§ 1100 ± 250/day)
Diabetic medication during the intervention	Oral blood glucose-lowering medication and insulin were stopped 3 weeks and 1-3 days before the start of the study, resp.	Unclear, only mention is made that patients were in secondary drug failure	Oral blood glucose lowering agents were discontinued 3 weeks before the start of the study	Oral blood glucose lowering medication stopped 2 weeks before the start of the study
FPG (mmol/L) after I	7.6 ± 0.5 on day 10 (weight loss 4.6±0.2 kg)	9.6 ± 0.5 (weight loss ~ 5.1 kg)	9.5 ± 0.9, 7 days 800kCal/d (weight -2.2kg) 7.0 ± 0.7 at 13 weeks (weight -14.8 kg)	6.2 ± 0.5 on day 4 (weight loss 1.7 ± 2.2kg) 5.3 ± 0.4 on day 28 (weight loss 6.3 ± 0.4kg)
Basal EGP before I	149 ± 13 $mg.m^{-2}.min^{-1}$	2.49 ± 0.15 $mg.kg^{-1}.min^{-1}$*	158 ± 13 $mg.m^{-2}.min^{-1}$	14.0 ± 1.1 $\mu mol.kgFFM^{-1}.m^{-1}$
Basal EGP after I	81 ± 5 $mg.m^{-2}.min^{-1}$ on day 10	2.04 ± 0.1 $mg.kg^{-1}.min^{-1}$	125 ± 9 $mg.m^{-2}.min^{-1}$ after 7 days 800kCal/d 100 ± 6 $mg.m^{-2}.min^{-1}$ at 13 weeks	11.3 ± 1.3 $\mu mol.kgFFM^{-1}.m^{-1}$ on d 4 12.7 ± 1.3 $\mu mol.kgFFM^{-1}.m^{-1}$ on d 28
Clamp EGP before I	-	-	-	3.8 ± 2.1 $\mu mol.kgFFM^{-1}.m^{-1}$‖
Clamp EGP after I	-	-	-	0.6 ± 1.8 $\mu mol.kgFFM^{-1}.m^{-1}$ on d 4‖ 0.7 ± 1.6 $\mu mol.kgFFM^{-1}.m^{-1}$ on d 28‖
Glucose Rd before I	-	2.34 ± 0 0.15 $mg.kg^{-1}.min^{-1}$*	142 $mg.m^{-2}.min^{-1}$‡	18.9 ± 2.0 $\mu mol.kgFFM^{-1}.m^{-1}$‖
Glucose Rd after I	-	4.01 ± 0 0.4 $mg.kg^{-1}.min^{-1}$*	188 ± 17 $mg.m^{-2}.min^{-1}$, 7 days 800kCal/d‡ 244 ± 21 $mg.m^{-2}.min^{-1}$, at 13 weeks‡	15.8 ± 1.8 $\mu mol.kgFFM^{-1}.m^{-1}$, d 4‖ 19.6 ± 1.9 $\mu mol.kgFFM^{-1}.m^{-1}$, d 28‖
Basal glycerol Ra before I	-	-	-	-
Basal glycerol Ra after I		-	-	-
Remarks	No data on EGP are given on day 40. Greatest reduction in FPG within 10 days. A meal tolerance test suggested improved peripheral insulin sensitivity and insulin secretion	After 2 weeks of a 500 kCal diet, peripheral insulin sensitivity improved, relatively week improvement in basal EGP as compared to other studies	7 days of CR led to half of the improvement in FPG, HPG, insulin sensitivity and insulin secretion	4 days CR improved HGO, prolonged CR also improved insulin sensitivity

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Table 2, continued.

	Christiansen[69],b	**Jazet**[70],b	**Jazet**b
Year	2000	2005	In preparation for submission
Number of patients	8	12	10
Age (yrs)	51 ± 4	55 ± 4	54 ± 3
Duration DM2 (yrs)	5 ± 3	7.9 ± 1.3	8 ± 3
Diabetes medication Diet Oral Insulin	Oral and/or insulin	All patients used insulin (mean 78 ± 9 U/day), 6 also used metformin and 1 also used rosiglitazone	All patients used insulin (mean 94 ± 14 U/day), 8 also used metformin and 2 also used rosiglitazone
FPG (mmol/L)	11.9 ± 1.4	11.3 ± 1.3	11.1 ± 0.8
HbA1c (%)	8.1 ± 0.5	7.3 ± 0.4	7.7 ± 0.4
Weight (kg)	107 ± 14	107.9 ± 2.9	113.0 ± 7.1
BMI (kg/m^2)	36 ± 3	36.3 ± 1.0	40.2 ± 1.6
Intervention (I)	5 days eucaloric, 10 days 25% of ECN, 10 days 75% of ECN	2 days of a VLCD (formula, 450 kCal/day)	VLCD (formula, 450 kCal/day) until 50% of overweight was lost (50% OWR). Study days on day 2 and day 50% OWR
Diabetic medication during the intervention	All blood glucose-lowering medication (including insulin) was discontinued 2 weeks before the start of the study	Oral blood glucose-lowering agents were stopped 3 weeks before the start of the study, only short-acting insulin on day –1, insulin stopped at the start of the study	Oral blood glucose-lowering agents were stopped 3 weeks before the start of the study, only short-acting insulin on day –1, insulin stopped at the start of the study
FPG (mmol/L) after I	8.9 ± 1.6 , day 5 (of 25% ECN) (weight loss 2 kg) 7.4 ± 1.4, day 10 (of 25% ECN) (weight loss 3 kg) 8.8 ± 1.3, day 20 (d 10 of 75% ECN) (weight loss 3 kg)	10.3 ± 1.0 on day 2 (weight loss 2.9 ± 0.4 kg)	7.8 ± 0.5 on day 50% OWR (weight loss 20.3 ± 2.2 kg day 2 compared to day 50% OWR)
Basal EGP before I	22 ± 2 µmol.kgFFM^{-1}.m^{-1}	14.2 ± 1.0 µmol.kg^{-1}.min^{-1}	20.0 ± 0.9 µmol.kgLBM^{-1}.min^{-1}
Basal EGP after I	18 ± 2 µmol.kgFFM^{-1}.m^{-1} on d 5 17 ± 2 µmol.kgFFM^{-1}.m^{-1} on d 10 22 ± 2 µmol.kgFFM^{-1}.m^{-1} on d 20	11.9 ± 0.7 µmol.kg^{-1}.min^{-1}¶	16.4 ± 1.2 µmol.kgLBM^{-1}.min^{-1}
Clamp EGP before I	-	5.5 ± 0.8 µmol.kg^{-1}.min^{-1}¶	8.5 ± 0.9 µmol.kgLBM^{-1}.min^{-1}#
Clamp EGP after I	-	5.2 ± 0.5 µmol.kg^{-1}.min^{-1}¶	4.6 ± 1.2 µmol.kgLBM^{-1}.min^{-1}#
Glucose Rd before I	c	12.1 ± 0.7 µmol.kg^{-1}.min^{-1}¶	18.8 ± 2.0 µmol.kgLBM^{-1}.min^{-1}#
Glucose Rd after I	c	11.3 ± 1.0 µmol.kg^{-1}.min^{-1}¶	39.1 ± 2.8 µmol.kgLBM^{-1}.min^{-1}#
Basal glycerol Ra before I	9 ± 1 µmol.kgFFM^{-1}.m^{-1}	5.2 ± 1.0 µmol.kg^{-1}.min^{-1}	16.4 ± 2.3 µmol.kg fat mass^{-1}.min^{-1}
Basal glycerol Ra after I	9 ± 2 µmol.kgFFM^{-1}.m^{-1}on d 5 7 ± 1 µmol.kgFFM^{-1}.m^{-1}on d 10 7 ± 1 µmol.kgFFM^{-1}.m^{-1} on d 20	4.0 ± 0.6 µmol.kg^{-1}.min^{-1}	14.6 ± 1.4 µmol.kg fat mass^{-1}.min^{-1}
Remarks	Short-term CR reduces EGP. Longer term CR also improves glucose disposal. EGP rapidly rises with increase in caloric intake	2-day VLCD improved FPG due to a decrease in basal EGP with no effect on insulin sensitivity.	Considerable weight loss not only restores basal EGP to normal levels but also greatly enhances peripheral insulin sensitivity, especially insulin-stimulated glucose disposal, despite the fact that patients were still obese and used no blood glucose-lowering medication

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Legend to Table 2

Weight losses given are compared to day 0.

[a]Values are presented as mean ± SD; [b]Values are presented as mean ± SEM

* insulin infusion rate 40 mU/m^2/minute (clamp serum insulin concentration 89 ± 5 mU/L before and after the intervention)

† basal (before) data are after 7 days eucaloric, then data after 7 days 800 kcal/day and data following a 12 week weight reducing programme (8 weeks 400kCal/day liquid formula diet, 3 weeks increase with 200 kCal/day, followed by 1 week eucaloric :third study day) are presented

‡ insulin infusion rate 100 mU/m^2/minute (clamp serum insulin concentrations varied from 200-210 mU/L during the various clamps)

§ 1000 kCal/d less than patients used to consume as assessed by a 4-day dietary record

‖ relatively low insulin levels were obtained during the clamp (250 pmol/L ≅ 35 mU/L)

[c] Rd glucose measured by Christiansen *et al* . were non-insulin stimulated values, also presented divided by plasma glucose levels (metabolic clearance rate of glucose), values were 2.0 ± 0.2, 2.1 ± 0.2, 2.1 ± 0.3 and 2.7 ± 0.3 ml.kgLBM^{-1}.min^{-1} at baseline, day 5, 10 and 20, respectively.

ECN= eucaloric needs, LBM = lean body mass, CR = calorie restriction, EGP = endogenous glucose production, FPG = fasting plasma glucose

¶ glucose infusion rate 40 mU/m^2/min (clamp serum insulin values 88.1 ± 5.9 and 83.7 ± 4.8 mU/L on day 0 and day 2, respectively, p= NS)

♯ glucose infusion rate 40 mU/m^2/min (clamp serum insulin values 90.2 ± 3.3 and 80.8 ± 4.0 mU/L on day 2 and day 50% OWR, respectively, p = 0.023. Difference probably due to increased clearance of insulin)

FAT/CD36 and GLUT-4. We did observe a decrease in basal PKB/Akt phosphorylation, however, that might be linked to the decrease in basal EGP.

AIM 8 AND 9

To investigate the effect of weight reduction induced by caloric restriction as opposed to caloric restriction only, on insulin sensitivity, 10 obese (BMI 40.2 ± 1.6 kg/m^2 [mean ± SEM]) insulin-treated type 2 diabetic patients (HbA_{1c} 7.7 ± 0.4%, FPG 11.1 ± 0.8 mmol/L) were studied on day 2 of a very low calorie diet (VLCD, Modifast®, 450 kCal/day) and again after losing 50% of their overweight (50% OWR). Oral blood glucose-lowering agents and insulin were discontinued 3 weeks prior to the VLCD and at the start of the VLCD, respectively. Endogenous glucose production (EGP) and whole-body glucose disposal (6,6-$^2H_2$-glucose), lipolysis ($^2H_5$-glycerol) and substrate oxidation rates were measured on both study days in basal and hyperinsulinaemic (insulin infusion: 10 min prime followed by a constant infusion rate of 40mU/m2 per minute[68]) euglycaemic conditions. In addition, skeletal muscle biopsies were obtained from the vastus lateralis muscle, in the basal situation and 30 min after the initiation of the insulin infusion.

With respect to the **eighth aim** we showed that considerable weight reduction (20.3 ± 2.2 kg from day 2 to day 50% OWR), as opposed to caloric restriction *per se*, not only normalised basal EGP, but also improved insulin sensitivity, especially insulin-stimulated glucose disposal (increase 107% as compared to day 2, p = 0.001). The magnitude of the improvement in insulin-stimulated glucose disposal was comparable to that observed in some studies in morbidly obese patients undergoing bariatric surgery[89,90].

Although it is common knowledge that weight loss improves insulin sensitivity, the magnitude of this response has not been investigated before with state-of-the-art techniques (hyperinsulinaemic euglycaemic clamp technique with [6,6-$^2H_2$]-glucose and [$^2H_5$]-glycerol)

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Table 3. Insulin signal transduction in skeletal muscle of obese, non-obese diabetic and obese diabetic subjects as compared to lean insulin sensitive subjects.

		Non-obese diabetic	Obese diabetic	Obese
IR	Binding or protein level	= [71], [72], [73]	= [71]	= [71]
	Phosphorylation	= [73], [74]	= [75], [76], [77]	= [78]
		↓ [71], [79]	↓ [71], [78]	↓ [71], [76]
IRS-1	Binding or protein level	= [73], [80], [81]	= [82]	= [82]
	Tyrosine phosphorylation	↓ [73], [80]	↓ [75], [81], [83]	= [81]
	Serine phosphorylation		↑ [83]	
PI3K	p85 protein level	= [84]	= [81], [82], [85]	= [81], [82], [85]
	Activity	↓ [73],[80], [83], [84]	↓ [77], [81], [82], [85]	= [77], [85]
				↓ [81]
Akt	Protein level	= [86]	= [83], [87]	
	Phosphorylation	↓ [86]	= [83], [85]	
			↓ [87]	
AS160	Protein level	= [87]		
	Phosphorylation	↓ [87]		

in this patient group: severely obese, insulin treated type 2 diabetic patients. In Table 2 an overview is presented of studies investigating the effect of varying degrees of energy restriction, during a variable period of time (4 days up till 8 weeks) on glucose and lipid metabolism in obese patients with type 2 diabetes. As can be deduced from Table 2, our patients were more severely obese, used more medication and were more severely insulin resistant as compared to the type 2 diabetic patients in most other studies, with the exception of the studies of Henry *et al*.[4] and Christiansen *et al*. [69]. Moreover, studies investigating the effect of considerable weight loss on peripheral insulin sensitivity, using state-of-the-art techniques are lacking.

The fact that the impressive improvement in insulin sensitivity in our patients occurred despite the fact that patients did not use any blood glucose- (or lipid-) lowering medication and were still obese (BMI 32.3 kg/m^2), underscores the importance of a dietary intervention in this patient group.

Our **ninth aim** was to investigate, in skeletal muscle biopsies, the effect of considerable weight loss on insulin signalling, the expression of the fuel transporters GLUT-4 and FAT/CD36 at the cell membrane, as well as the concentration of intramyocellular triglycerides.

In this study, we found equal insulin-stimulated PI3K activation on both study days, but the magnitude of the insulin-induced increase over basal was greater after weight loss ($p = 0.010$). Two down-stream effectors of PI3K, the PKB/AKT substrates AS160 and PRAS 40, also

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showed an improved insulin-stimulated response with weight loss. Weight reduction had no significant effect on the abundance of the fuel-transporters GLUT-4 and FAT/CD 36 at the plasma membrane following hyperinsulinaemia. However, 7 out of the 10 patients showed a higher GLUT-4 density at the cell membrane after weight loss. An oil red O staining showed a significant decrease in intramyocellular triglycerides after weight loss in both type I and type II muscle fibres. Interestingly, time to weight loss of 50% overweight correlated negatively with the number of type I fibres at the start of the diet. We also find a trend towards an increase in the percentage of type I (and hence decrease in type II) muscle fibres with weight loss, a finding that has not been described before in patients with diabetes.

The reason why the increase in insulin-stimulated glucose disposal at the whole-body level was not reflected by a significant improvement in GLUT-4 translocation to the cell membrane is unclear and may reflect changes in intrinsic activity of GLUT-4. Others have also reported a dissociation between insulin-stimulated glucose disposal and either insulin signalling and/or GLUT-4 content at the cell membrane[90-93]. Several hypotheses can be put forward with respect to the relatively low concentration of GLUT-4 at the cell membrane. Firstly, it is possible that not the amount of GLUT-4 at the cell membrane but rather its function and, subsequently, the velocity of glucose transport over the membrane are the main determinants of insulin-stimulated glucose disposal. Secondly, another glucose transporter, either GLUT-1[93] or a yet unidentified one, may have contributed to the increase in glucose uptake seen after weight loss. Thirdly, it is possible that the increase in insulin-stimulated glucose disposal does not only take place in skeletal muscle but also in adipose tissue. The weight loss in our patients mainly reflected a decrease in body fat mass. This is most likely due to a depletion of intracellular triglyceride stores and not to a decrease in adipocyte number. The smaller adipocytes following weight loss might be better able to take up glucose as compared with the greater, lipid-laden adipocytes before weight loss. In our study, 4 out of the 8 patients from whom we obtained adipose tissue biopsies showed increased insulin-stimulated PI3K phosphorylation after weight loss.

AIM 10

Our tenth aim was to investigate the long-term effect of a once-only 30-day VLCD on body weight, hyperglycaemia, dyslipidaemia and blood pressure in obese type 2 diabetic patients.

To that end, we looked at the long-term effect of a once-only 30-day VLCD in 22 obese (BMI 37.7 ± 1.1 kg/m^2, mean ± SEM) type 2 diabetic patients (mean duration of diabetes 7.4 ± 1.0 years, fasting plasma glucose [FPG] 12.4 ± 0.8 mmol/L, HbA_{1c} 8.3 ± 0.3%) who participated in 2 other studies in which a 30-day VLCD was either used as the intervention or offered as a therapy after finishing the initial study. During the VLCD all oral blood glucose-lower-

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ing medication and insulin therapy were discontinued. After the 30-day VLCD, caloric intake was slowly increased to eucaloric and patients were encouraged to maintain weight loss, but no specific diet was prescribed. Patients were followed at the outpatient clinic at 3-monthly intervals and medication for their diabetes (and blood pressure and/or dyslipidaemia) was reinstituted as deemed necessary by their own physician. Anthropometric parameters, blood pressure, glucose, HbA_{1c}, insulin, C-peptide and lipid levels were measured on day 0 and day 30 of the VLCD and after 18 months follow-up.

Surprisingly, after 18 months regular follow-up, as a group, patients had managed to maintain the loss of body weight achieved during the 30-day VLCD (-11.4 ± 0.6 kg). In addition, the improvement in systolic and diastolic blood pressure and serum lipids obtained during the 30-day VLCD was also largely sustained at 18 months follow-up. With respect to glycaemic regulation, HbA_{1c} levels were 0.7% lower as compared to the situation before the start of the diet, despite the fact that patients used less blood glucose-lowering medication, especially insulin (18 patients on day 0 [112 ± 21 units/day]; 6 patients at 18 months [23 ± 9 units/day]). The 6 patients using insulin therapy at 18 months follow-up had all regained weight to prediet levels.

In a subanalysis, it appeared that 8 patients had stable body weight (plus or minus 5 kilogram [kg]), 8 patients regained more than 5 kg of body weight and 6 patients lost more than 5 kg of body weight from day 30 to 18 months follow-up. The patients who had regained body weight to prediet levels had worse glycaemic control and dyslipidaemia and a higher (systolic) blood pressure as compared to the other two groups, but these parameters were still better than the values these patients had at the start of the study.

Treatment goals for glycaemic regulation (HbA_{1c} < 7%), blood pressure (< 130/80 mmHg) and serum lipids (LDL-cholesterol < 2.6 mmol/L, triglycerides < 1.7 mmol/L, HDL-cholesterol > 1.1 mmol/L) as set by the American Diabetes Association (ADA)[94] were not reached for all parameters but came very close (HbA_{1c} 7.6 ± 0.4%, total cholesterol 5.4 ± 0.2 mmol/L, triglycerides 2.5 ± 0.4 mmol/L, HDL cholesterol 1.3 ± 0.07 mmol/L, blood pressure 145 ± 4 mmHg / 81 ± 2 mmHg) and were very much improved as compared to before the intervention.

Thus, with regard to the **tenth aim** we conclude that a once-only 30-day VLCD in combination with the cessation of all blood glucose-lowering agents leads to a sustained improvement in glycaemic control, blood pressure and serum lipids at least up to 18 months follow-up even, albeit to a lesser extent, in patients who regained body weight.

OVERALL CONCLUSIONS WITH RESPECT TO THE USE OF VLCDs

The following conclusions with respect to the use of VLCDs, as a means to induce weight loss and improve glycaemic control, can be drawn from our findings. Firstly, VLCD therapy in obese, insulin-treated type 2 diabetic patients is safe, even when continued for up to 8

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months. Secondly, the simultaneous discontinuation of all blood glucose-lowering agents does not lead to a deterioration of blood glucose levels, provided that patients do have residual endogenous insulin secretion. For practical purposes, this was defined as a fasting C-peptide level > 0.8 ng/mL and/or a two times increase of the fasting C-peptide level after 1 mg glucagon i.v.. Thirdly, in patients with remaining endogenous insulin secretion, FPG levels declined already within 2 days of a VLCD, when weight loss was minimal and despite the fact that all blood glucose-lowering agents were discontinued. Fourthly, this early (day 2) decline in FPG levels appeared to be due to a decrease in basal EGP without an effect on peripheral insulin sensitivity. Hence, in skeletal muscle biopsies no improvement in insulin signalling at the level of IRS-1-associated PI3K and PKB/Akt was seen and no increase in insulin-stimulated GLUT-4 translocation was observed. Fifthly, as opposed to short-term energy restriction, prolonged energy restriction leading to a loss of 50% of overweight, also improved peripheral insulin sensitivity, especially insulin-stimulated glucose disposal. Sixthly, at the molecular level this was accompanied by increased PI3K phosphorylation over basal after weight loss as compared to day 2 and a significant total AS 160 and PRAS40 phosphorylation after weight reduction. The amount of GLUT-4 at the cell membrane was higher in 7 out of 10 patients, although the group effect was not significant. An oil red O staining showed a significant reduction in intramyocellular triglycerides. Interestingly, the amount of type I muscle fibres before weight loss correlated negatively with time to weight loss of 50% overweight. In addition, a slight, non-significant increase in type I muscle fibres was observed after weight loss. Seventhly, in an observational analysis we found that the effect of a once-only 30-day VLCD on body weight, glycaemic control, blood pressure and dyslipidaemia was sustained after 18 months regular follow-up, even in patients who regained body weight to prediet levels.

Our findings stress the importance of diet therapy in obese (insulin-treated) type 2 diabetic patients. The fact that insulin-stimulated glucose disposal improved by 107%, despite the fact that patients were still obese, raises the question whether it can be fully restored with weight loss up to ideal body weight. On the other hand, thiazolidinediones (TZDs) and exercise can also improve insulin sensitivity, albeit *via* a different mechanism[84,95-97]. Perhaps the combination of a VLCD, exercise and a TZD can fully restore insulin sensitivity. In a new study we will investigate this, again in obese type 2 diabetic patients, during a 16-week intervention in which all patients will follow a VLCD and subgroups will receive either exercise and/or rosiglitazone. Again, hyperinsulinaemic euglycaemic clamp studies with stable isotopes and skeletal muscle biopsies will be performed before and after the intervention to accurately measure changes at the whole-body and molecular level.

Type 2 diabetes mellitus is associated with micro-and macrovascular long-term complications that are related to the increased morbidity and mortality seen in these patients[98]. Approximately 65% of patients with type 2 diabetes die as a result of a cardiovascular event[99]. Patients with type 2 diabetes have a 2-4 fold increased relative risk (RR) for the development of myocardial infarction, peripheral arterial disease and stroke[100]. This increased risk is as-

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sociated with an increase in various metabolic and other cardiovascular risk factors such as hyperglycaemia, dyslipidaemia and hypertension. We observed a sustained beneficial effect of a once-only 30-day VLCD on these risk factors up to 18 months follow-up, even in patients that regained body weight. It remains to be evaluated how long these beneficial effects will persist and if the intermittent use of a VLCD (on demand, i.e., when body weight increases over a predefined weight [studied in obese non-diabetic patients[101]], or 5 days every 5 weeks[102]), or other stategies (addition of exercise and or an insulin-sensitising drug) will even be more beneficial or leads to a longer duration of the beneficial effects. Given the results of several large trials, also in patients with diabetes, that lower(ing) blood pressure[103,104], total[105] and LDL-cholesterol[106-108] and decreasing triglycide levels while increasing HDL-cholesterol[109-111] significantly reduces the risk for cardiovascular disease, a sustained improvement in these parameters could also reduce the risk for cardiovascular disease and, hence, reduce health care costs and usage in obese type 2 diabetic patients following a VLCD.

When the beneficial effects of a VLCD and the abovementioned considerations with respect to cardiovascular risk are taken into account, a VLCD can be an attractive, cost-effective therapy. VLCDs are in themselves relatively cheap (30 days of Modifast® costs approximately 160 Euro) and all blood glucose-lowering medication can be discontinued. Moreover, the improvement in cardiovascular risk factors are likely to lead to a decreased incidence of cardiovascular disease with less hospital admissions and interventions (and, hence, less days staying away from the economic process) which will lead to a much greater saving in health care costs. On the cost-side are the expenses of regular counselling. These can be minimised however, when a diabetic nurse performs most of the controls. It would be interesting to do a study at the outpatient clinic, which also takes into account the cost-effectiveness of a VLCD. It will be our job to convince insurance companies of the benefits of the VLCD and to persuade them to compensate for the costs of a VLCD.

Finally, although we only studied the VLCD in obese, mostly insulin-treated (in the study of Chapter 4 also patients on oral blood glucose-lowering agents only participated) type 2 diabetic patients, it is likely that the same treatment will be successful in obese patients with type 2 diabetes treated with diet and/or oral blood glucose-lowering agents. Because these patients are in an earlier phase of the disease process, results with respect to the improvement in insulin sensitivity will probably be even more impressive. We hypothesise that considerable weight loss in obese, non-diabetic but insulin-resistant patients will normalise insulin sensitivity.

In conclusion, a VLCD in combination with the simultaneous discontinuation of all blood glucose-lowering agents in obese, insulin-treated patients with remaining endogenous insulin secretion is safe, can increase insulin sensitivity to a great extent and the improvement in metabolic parameters is sustained up to 18 months follow-up. Our observations stress the importance of weight-reducing therapies, especially diet, because of its safety, low costs and availability, in this patient group.

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CHAPTER 11

Samenvatting

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GLUCOSE(SUIKER)STOFWISSELING EN DIABETES MELLITUS (SUIKERZIEKTE)

Normaal gesproken worden de bloedsuikerwaarden binnen nauwe grenzen (4-8 mmol/L) gehandhaafd. De reden hiervoor is dat de hersenen afhankelijk zijn van glucose (suiker) voor hun functioneren. Bij een te lage bloedsuikerspiegel (hypoglycaemia) zullen dan ook trekkingen, bewustzijnsverlies en uiteindelijk een coma optreden. Aan de andere kant zijn te hoge bloedsuikerspiegels (hyperglycaemie) ook schadelijk voor het lichaam. Een acute forse verhoging van de bloedsuikerspiegel (vaak concentraties van meer dan 20-30 mmol/L) kan leiden tot uitdroging, bewustzijns-veranderingen en ook coma. Langdurige blootstelling aan te hoge bloedsuikerwaarden kan, in genetisch gepredisposeerde patiënten, leiden tot micro- en macrovasculaire complicaties. Met microvasculaire complicaties bedoelen we schade in de kleine bloedvaatjes van de ogen, nieren en benen. Dit leidt tot schade aan het netvlies van de ogen, verminderde nierfunctie, verminderd gevoel in de voeten en handen en,mede door het verminderde gevoel, wondjes met vaak slechte wondgenezing. Met macrovasculaire schade bedoelen we de grote vaten. Dit leidt tot een verhoogde kans op een hartinfarct, herseninfarct en verminderde doorbloeding van de benen (leidend tot wat in de volksmond "etalagebenen" wordt genoemd).

De bloedsuikerspiegel is een balans tussen glucose dat het bloed inkomt en glucose dat het bloed uitgaat. Glucose kan in het bloed komen via een maaltijd of door aanmaak door het lichaam. De lever, en in mindere mate ook de nier, zijn in staat glucose aan te maken (endogene glucose productie [EGP]). Deze eigen glucose-productie speelt een rol gedurende de nacht en tijdens vasten, het is een adequate reactie van het lichaam om een te lage bloedsuikerspiegel (hypoglycaemie) te voorkomen. Glucose verdwijnt weer uit het bloed doordat het wordt opgenomen door de verschillende lichaamsweefsels. De grootste opname vind plaats in skeletspierweefsel.

Bij het in balans houden van glucose dat in het bloed komt en dat er weer uit gaat, en derhalve het binnen de normale grenzen houden van de bloedsuikerspiegel, is het hormoon insuline van groot belang. Dit hormoon wordt gemaakt en uitgescheiden door de alvleesklier (pancreas). Na de maaltijd stijgt de bloedsuikerspiegel en geeft de alvleesklier insuline af. Dit insuline zorgt er aan de ene kant voor dat glucose in de weefsels kan worden opgenomen. Aan de andere kant zorgt het ervoor dat de lever minder glucose gaat aanmaken (dat is immers niet nodig als je net hebt gegeten). Tenslotte remt insuline ook de afbraak van vet (lipolyse), vet als brandstof is immers ook niet nodig als je net gegeten hebt. Andere hormonen, onder anderen glucagon en cortisol, zijn als tegenregulerende hormonen ook bij de bloedsuikerregulatie betrokken.

Bij mensen met suikerziekte (diabetes mellitus) is er een tekort aan insuline. Er zijn 2 soorten suikerziekte. Bij type 1 diabetes mellitus is er een absoluut tekort aan insuline, deze patiënten moeten insuline gaan spuiten. Bij mensen met type 2 diabetes mellitus (DM2), voorheen ook wel ouderdomssuikerziekte genoemd, is er een relatief tekort aan insuline. Het blijkt dat die

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mensen minder gevoelig zijn voor de werking van insuline, dit noemen we insulineresistentie. Deze insulineresistentie hangt samen met overgewicht. Overgewicht wordt uitgedrukt in de zogenaamde body mass index (BMI) die wordt berekend uit het gewicht (in kilogram) gedeeld door de lengte (in meters) in het kwadraat. We spreken van overgewicht bij een BMI > 25 kg/m^2 en van obesitas bij een BMI > 30 kg/m^2. Meer dan 80% van de mensen met DM2 hebben overgewicht of obesitas.

De laatste decennia is er wereldwijd een enorme toename van het aantal mensen met overgewicht en obesitas. Wereldwijd zijn er nu 1 miljard mensen met overgewicht en 300 miljoen met obesitas. Ook het aantal kinderen met overgewicht en obesitas is sterk gestegen: wereldwijd 22 miljoen kinderen onder de leeftijd van 5 jaar en 155 miljoen kinderen op schoolgaande leeftijd. Een dieet met een hoge energiedichtheid en een zittend bestaan zijn de belangrijkste oorzaken van deze enorme stijging.

Doordat overgewicht, waarschijnlijk via insulineresistentie, kan leiden tot DM2 is het aantal mensen met DM2 ook sterk gestegen. Op dit moment zijn er ongeveer 191 miljoen mensen met diabetes wereldwijd, 90-95% hiervan heeft DM2. Geschat is dat dit aantal zal stijgen tot 366 miljoen in 2030, bij deze schatting is de toename van het aantal mensen met overgewicht en obesitas niet doorberekend dus waarschijnlijk zal het werkelijke aantal mensen met diabetes in 2030 veel hoger zijn. Door de sterke toename van overgewicht en obesitas op kinderleeftijd en het feit dat overgewicht, via insulineresistentie, predisponeert voor het krijgen van DM2, komt ook op steeds jongere leeftijd DM2 voor. Vandaar dat we liever niet meer spreken van ouderdomssuikersziekte.

Zowel overgewicht/obesitas alswel diabetes mellitus (door de eerder genoemde korte- en lange termijn complicaties) zijn geassocieerd met een verhoogde morbiditeit (ziekte) en mortaliteit (sterfte). Het is daarom van het grootste belang deze ziekten adequaat te behandelen. Bij obese patiënten met DM2 speelt, zoals reeds gezegd, resistentie voor de werking van insuline een belangrijke rol in het ontstaan en onderhouden van hyperglycaemie. Deze insulineresistentie maakt het moeilijk om een goede regulatie van de bloedsuikerwaarden te krijgen (uitgedrukt in het geglycosileerd hemoglobinegehalte: HbA_{1c} dat onder de 7% , liefst onder de 6.5% moet zijn om de kans op micro-en macrovasculaire lange-termijn complicaties te voorkomen). Indien wordt geprobeerd met insulinetherapie een goede instelling te krijgen, leidt dit vaak tot verdere gewichtstoename en dus verergering van de insulineresistentie: er ontstaat een vicieuze cirkel.

Caloriebeperking en gewichtsvermindering verbeteren de insulineresistentie. In obese DM2 is gewichtsreductie dan ook de aangewezen therapie. Omdat het volhouden van een dieet vaak moeilijk is zijn er zeer laagcalorische diëeten (very low calorie diet, VLCD) op de markt gekomen. De snelle daling in lichaamsgewicht die hiermee bereikt kan worden is een goede stimulans voor patiënten om door te gaan. In dit proefschrift worden enkele studies beschreven die gaan over de veiligheid en de korte- en lange- termijn effecten en effectiviteit van een VLCD bij obese patiënten met DM2. De eerste 2 hoofdstukken gaan over de

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relatie tussen vetweefsel en het ontstaan van insulineresistentie. Korte samenvattingen van de hoofdstukken beschreven in dit proefschrift worden hieronder gegeven.

HOOFDSTUK 2

Overgewicht en obesitas zijn geassocieerd met insulineresistentie en een verhoogd risico op het ontwikkelen van DM2. Omdat teveel vetweefsel een kenmerk is van overgewicht en obesitas is er de laatste jaren veel onderzoek gedaan naar de rol van vetweefsel in het ontstaan van insulineresistentie. Het is namelijk gebleken dat vetweefsel niet zomaar een opslagdepot voor energie in de vorm van triglyceriden(vetten) is maar dat het een groot aantal eiwitten produceert. Deze eiwitten hebben verschillende functies. In hoofdstuk 2 worden enkele eiwitten besproken die betrokken zijn bij het glucose- en vetmetabolisme en op die manier insulineresistentie kunnen induceren. Deze eiwitten zijn leptine, adiponectine, resistine, acylation-stimulating protein (ASP), tumour necrosis factor-α (TNF-α) en interleukine-6 (IL-6). Bij mensen met overgewicht is de productie van leptine, resistine, TNF-α, IL-6 en ASP verhoogd en die van adiponectine verlaagd. Deze verhoogde, respectievelijk verlaagde plasmaconcentraties zijn gecorreleerd met insulineresistentie. Het bestuderen en interpreteren van de rol van deze hormonen in insulineresistentie wordt bemoeilijkt door het feit dat de productie van deze eiwitten verschilt per vetdepot. Hierbij onderscheiden we 2 grote groepen van vetopslagplaatsen: in de buik (visceraal vet, ook wel mannelijk vetopslagpatroon genoemd) en onder de huid (subcutaan, met name op de heupen: vrouwelijk vetopslagpatroon). Daarnaast is het zo dat de productie van sommige van deze eiwitten zich niet vertaalt in een hoge concentratie in het bloed: met andere woorden deze eiwitten lijken met name een lokaal effect uit te oefenen.

Concluderend kan op dit moment gezegd worden dat vet in het bovenste lichaamsgedeelte gerelateerd is aan insulineresistentie. Het lijkt erop dat vetcellen in de buik en in de diepe lagen van het vet onder de buikhuid zich anders gedragen. Of dit de oorzaak is van insulineresistentie en DM2 of juist een uiting van deze ziekten is momenteel onduidelijk.

HOOFDSTUK 3

Leptine is een van de eiwitten die door vetweefsel worden geproduceerd. De productie van leptine is positief gecorreleerd met het lichaamsgewicht. De belangrijkst rol van leptine lijkt het aangeven van energietekort te zijn. De concentratie leptine in het bloed daalt snel in reactie op energiebeperking en gewichtsverlies. Dit leidt tot daling van het energieverbruik en toename van het hongergevoel. Bij mensen met overgewicht is de concentratie leptine in het bloed verhoogd, waarschijnlijk omdat er resistentie is voor de werking van leptine.

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Helaas daalt ook in deze groep mensen de leptineconcentratie gedurende gewichtsverlies. Mogelijk is dit een oorzaak waarom afvallen in deze groep vaak moeilijk is.

De insulineconcentratie is ook positief gecorreleerd met overgewicht en vetmassa. Verschillende studies hebben een positieve relatie aangetoond tussen leptine en insuline. Echter over de relatie tussen deze 2 hormonen in zeer dikke DM2 met een sterk gestoorde insulinesecretie is niet veel bekend. Evenmin is in deze groep patiënten gekeken naar het effect van gewichtsreductie middels een VLCD op de relatie tussen insuline(secretie) en serum leptine.

In deze studie tonen wij in 14 obese (BMI 37.6 ± 1.4 kg/m^2 , gemiddelde ± SEM) type 2 DM (duur diabetes 8.0 ± 1.4 years, nuchtere bloedsuiker [fasting plasma glucose, FPG, 12.9 ± 0.8 mmol/L, HbA_{1c} 8.6 ± 0.4%) die 30 dagen een VLCD (Modifast®, 450 kCal/dag) volgden aan, dat zelfs in patiënten met een gestoorde insulinesecretie de relatie tussen nuchter serum leptine en insuline blijft bestaan, ook na gewichtsverlies. Daarnaast hebben wij ook de insulinesecretie gemeten middels een intraveneuze glucose belastingstest en ook de hierbij gemeten insulinesecretie was positief gecorreleerd aan het nuchtere serum leptine. De opzet van deze studie maakte het niet mogelijk om uit te maken of leptine insuline aanstuurt of andersom, hoewel het meest waarschijnlijk is dat insuline leptine beinvloedt.

HOOFDSTUK 4

In hoofdstuk 4 hebben we gekeken naar factoren waarmee we, liefst vantevoren, konden zien welke patiënten gunstig reageren op een VLCD (Modifast®, 450 kCal/dag) en welke niet. Een gunstige reactie werd *a priori* gedefinieerd als een nuchtere bloedsuiker (fasting plasma glucose [FPG]) kleiner dan 10 mmol/L op dag 30 van het dieet. Daarnaast was deze studie opgezet om de veiligheid van een VLCD in combinatie met het stoppen van alle bloedsuikerverlagende medicatie te bestuderen en om te kijken hoe snel na het starten van het dieet een daling optrad in de nuchtere bloedsuikerspiegels.

Hiertoe kregen 17 obese (BMI 37.6 ± 5.6 kg/m^2) patienten met DM2 (duur diabetes 8 ± 5.8 jaar, FPG 12.9 ± 3.1 mmol/L, HbA_{1c} 8.6 ± 1.6%) gedurende 30 dagen een VLCD. Gedurende het VLCD werden alle bloedsuikerverlagende medicijnen gestopt. Op dag 2 en dag 30 vond een intraveneuze glucose tolerantietest plaats.

Deze studie toonde aan dat al op dag 2 van het VLCD een onderscheid kon worden gemaakt tussen patiënten die gunstig reageren (responders) en die niet gunstig reageren (non-responders). Non-responders bleken zelf niet voldoende insuline meer aan te maken (nuchtere C-peptide < 0.8 ng/mL; lagere insuline secretie na een glucosebelasting) en ook een langere diabetesduur te hebben, in vergelijking tot responders (12.3 ± 2.6 vs. 5.0 ± 1.4 jaar). De verandering in FPG van dag 0 naar dag 2 (+4.15 ± 3.3 mmol/L in non-responders versus +0.64 ± 2.3 mmol/L responders, p = 0.035) in combinatie met de insulinesecretie na een glucosebelasting (non-responders 88 ± 65 mU*50 minutes, responders 571 ± 236 mU*50

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minutes, $p < 0.001$) onderscheidde volledig de responders van de non-responders. Op basis van deze studie zijn in de daaropvolgende studies alleen patiënten geïncludeerd met een nuchter C-peptide > 0.8 ng/mL en/of een tweevoudige stijging van de nuchtere C-peptide (ondergrens basale waarde 0.5 ng/mL) na een glucagonstimulatietest.

HOOFDSTUK 5

Het doel van de studie beschreven in dit hoofdstuk was om het mechanisme dat ten grondslag ligt aan de snelle daling van de bloedsuikers na het starten van een VLCD te onderzoeken. Twaalf obese (BMI 36.3 ± 1.0 kg/m^2) DM2 (leeftijd 55 ± 4 jaar; HbA_{1c} 7.3 ± 0.4%) die insuline gebruikten (gemiddelde dosering 78 ± 9 eenheden/dag) werden onderzocht op dag 0 en dag 2 van een VLCD (Modifast®, 450 kCal/dag). Drie weken vantevoren werden alle orale bloedsuikerverlagende middelen gestopt, op dag –1 werd alleen kortwerkende insuline gegeven en vanaf dag 0 werd de insuline gestopt. De endogene glucoseproductie (EGP) en glucose-opname ([6,6-$^2H_2$]-glucose), lipolyse ([$^2H_5$]-glycerol) (dit zijn stabiele isotopen) en glucose-en vetverbranding (indirecte calorimetrie) werden gemeten voor en na het 2-daagse VLCD zowel in basale als hyperinsulinaemische euglycaemische omstandigheden (insuline infusiesnelheid 40 mU/m^2/min, serum clamp concentratie van insuline 88.1 ± 5.9 en 83.7 ± 4.8 mU/L op respectievelijk dag 0 en dag 2, p = ns, clamp concentratie glucose 5.0 ± 0.4 en 4.9 ± 0.4 mmol/L op respectievelijk dag 0 en dag 2, p = ns).

Ook in deze studie bleek de nuchtere bloedsuiker al na 2 dagen te dalen (van 11.3 ± 1.3 mmol/L op dag 0 naar 10.3 ± 1.0 mmol/L op dag 2), ondanks het feit dat er op dag 2 nog nauwelijks gewichtsverlies was (-2.9 ± 0.4 kg) en alle bloedsuikerverlagende medicatie was gestopt. De oorzaak was een daling van de EGP van 14.2 ± 1.0 naar 11.9 ± 0.7 $\mu mol.kg^{-1}.min^{-1}$, p = 0.009. Na 2 dagen VLCD bleek de insulinegevoeligheid van de perifere weefsels nog niet verbeterd te zijn: de insuline-gestimuleerde glucose opname en de mate van onderdrukking van de EGP en de vetafbraak (lipolysis) door insuline waren onveranderd.

HOOFDSTUK 6

Het grootste deel van de insuline-gestimuleerde glucose-opname vindt plaats in skeletspierweefsel. Glucose wordt hierbij in de cel opgenomen via de speciale glucose transporteur, GLUT-4. Dit GLUT-4 bevindt zich voor 90% in de cellen. Binding van insuline aan zijn receptor op de celmembraan leidt, via een reeks eiwitphosphoryleringen (proces van signaaltransductie), tot translocatie van het GLUT-4 naar de celmembraan en vervolgens tot opname van glucose in de cel. Bij patiënten met DM2 is het signaaltransductie proces en de translocatie van GLUT-4 naar de celmembraan gestoord.

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Omdat we het bloedsuikerverlagende mechanisme van een 2-daags VLCD wilden bestuderen in obese DM2 werden bij de patiënten uit Hoofdstuk 5, ook spierbiopten genomen op dag 0 en dag 2 van het VLCD, zowel in de rustsituatie (basaal) als na 30 minuten van een insuline infuus (10 minuten bolus gevolgd door een constante infusiesnelheid van 40 mU/m^2/minuut).

Hoewel op totaal lichaamsniveau geen effect van het 2-daagse VLCD op de insuline-gestimuleerde glucoseopname werd geobserveerd zou het kunnen dat er al een beginnend effect zichtbaar zou zijn op cellulair niveau. Echter de spierbiopten lieten geen dieet-effect zien op de expressie van de insuline-receptor, IRS-1, IRS-1-geassocieerde PI3K activiteit en GLUT-4 translocatie. Ook werd geen effect gezien op de expressie van het vettransporterend eiwit FAT/CD36 noch op de hoeveelheid vet in de spiercel (ook gecorreleerd met insulineresistentie). Een onverwachte bevinding was de stijging van de basale activiteit van het signaaltransductie-eiwit PKB/Akt. Aangezien dit eiwit ook betrokken is bij de EGP zou het zo kunnen zijn dat als deze activiteit ook hoger is in de lever na 2 dagen VLCD, dit de daling van de EGP die werd gevonden kan verklaren. Om ethische redenen is het echter niet mogelijk om leverbiopten bij mensen te verrichten in studieverband.

HOOFDSTUK 7

Om het bloedsuikerverlagende effect van aanzienlijke gewichtsreductie te bestuderen werden 10 obese (BMI 40.2 ± 1.6 kg/m^2) DM2 (HbA_{1c} 7.7 ± 0.4%, FPG 11.1 ± 0.8 mmol/L) die met insuline behandeld werden (gemiddelde dosering 90 ± 14 eenheden per dag) onderzocht gedurende een VLCD (Modifast®, 450 kCal/dag) op dag 2 en opnieuw nadat ze 50% van hun overgewicht kwijt waren (50% OWR = overgewicht reductie). Alle orale bloedsuikerverlagende middelen en insuline werden 3 weken, respectievelijk, 1 dag, voor de start van de studie gestopt. De endogene glucoseproductie (EGP) en glucose-opname ([6,6-$^2H_2$]-glucose), lipolyse [$^2H_5$]-glycerol en glucose-en vetverbranding (indirecte calorimetrie) werden gemeten op beide studiedagen (dag 2 en dag 50% OWR) zowel in basale als hyperinsulinaemische euglycaemische omstandigheden (insuline infusiesnelheid 40 mU/m^2/min, serum clamp concentratie van insuline 90.2 ± 3.3 en 80.8 ± 4.0 mU/L op respectievelijk dag 0 en dag 2, p = 0.023, clamp glucosewaarden 5.1 ± 0.3 en 5.4 ± 0.3 op resp. dag 2 en dag 50% OWR, p = ns).

Het bleek dat aanzienlijk gewichtsverlies (20.3 ± 2.2 kg van dag 2 tot dag 50% OWR), in tegenstelling tot alleen caloriebeperking (2-daags VLCD, Hoofdstuk 5 en 6) dat uitsluitend de basale EGP verlaagde, ook leidt tot een sterke verbetering van de perifere insulinegevoeligheid. Met name de insuline-gestimuleerde glucoseopname nam sterk toe (toename van 107% ten opzichte van dag 2; 18.8 ± 2.0 naar 39.1 ± 2.8 µmol.kg vetvrijemassa (fat free mass=FFM)$^{-1}$.min^{-1},p = 0.001). Hoewel de insulineconcentratie gedurende de hyperinsulinaemische euglycaemische clamp significant lager was op dag 50% OWR (waarschijnlijk door

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een betere klaring van insuline door de lever) beïnvloedt dit onze resultaten niet in negatieve zin. Immers, een gelijke, en dus hogere, insulineconcentratie tijdens de clamp op dag 50% OWR zou alleen maar een nog hogere insuline-gestimuleerde glucoseopname geven. Deze studie onderstreept opnieuw het belang van gewichtsreductie en dieet in de behandeling van obese patiënten met DM2.

HOOFDSTUK 8

In de studie beschreven in Hoofdstuk 7, werden ook spierbiopten genomen op dag 2 van het VLCD en na reductie van 50% van het overgewicht, zowel in basale omstandigheden als tijdens hyperinsulinaemie (30 minuten na de start van het insuline infuus, bolus van 10 minuten, gevolgd door een constante infusiesnelheid van 40 mU/m^2/min).

De sterke toename van de insulinegestimuleerde glucoseopname op totaal lichaamsniveau ging gepaard met een tendens tot stijging van de hoeveelheid GLUT-4 aan de celmembraan: 7 van de 10 patiënten lieten een toename zien van de hoeveelheid GLUT-4 aan de celmembraan gedurende insuline-stimulatie. Wat betreft de insuline signaaltransductie vonden we een grotere insuline-gestimuleerde stijging van IRS-1-geassocieerde PI3K activatie (phosphorylering) ten op zichte van de basaalwaarde na gewichtsreductie. De insuline-gestimuleerde AS160 en PRAS40 activatie was zowel in absolute zin als qua stijging ten opzichte van de de basaalwaarde, significant hoger na gewichtsreductie. Na gewichtsreductie was de concentratie FAT/CD36 aan de celmembraan gelijk doch de hoeveelheid triglyceriden in de spiercel was significant lager na gewichtsreductie, zowel in type I (langzame, insuline-gevoelige, oxydatieve) als in type II (snelle, glycolytische) spiervezels. Een interessante bevinding was dat het aantal type I vezels bij het begin van het dieet negatief correleerde met de tijd die het duurde voor een gewichtsverlies van 50% van het overgewicht werd bereikt. Ook was er een lichte, niet significante stijging van het percentage type I spiervezels na gewichtsreductie.

Concluderend is er op cellulair niveau een verbetering in insuline signaaltransductie en een trend tot stijging van de hoeveelheid GLUT-4 aan de celmembraan. De lagere triglyceridenconcentratie in de cel speelt mogelijk een rol bij de verbeterde insuline signaaltransductie. Het lijkt erop dat niet de hoeveelheid GLUT-4 aan de celmembraan, maar de functie, en derhalve de snelheid van transport over de celmembraan, de belangrijkste factor is voor de insulinegestimuleerde glucoseopname. Anderzijds, is er misschien een andere, tot nu toe nog onbekende glucosetransporteur, die verantwoordelijk is voor de toegenomen glucoseopname. Een derde verklaring zou kunnen zijn dat er meer glucoseopname in vetweefsel plaatsvindt. In vetcellen van onze patiënten (data niet getoond) vond in 4 van de 8 patiënten waarin een vetbiopt werd genomen, een verbetering van de insulinegestimuleerde PI3K-activatie op na gewichtsverlies.

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HOOFDSTUK 9

Het lange-termijn effect van een eenmalig, 30-dagen durend VLCD (Modifast®, 450 kCal/dag) op gewichtsreductie, hyperglycaemia, dyslipidaemie en bloeddruk werd geobserveerd in 22 obese (BMI 37.7 ± 1.1 kg/m^2) DM2 (duur diabetes 7.4 ± 1.0 jaar, FPG 12.4 ± 0.8 mmol/L, HbA_{1C} 8.3 ± 0.3%) die in 2 andere studies hadden geparticipeerd waarin een 30-daags VLCD de interventie was (studie uit Hoofdstuk 3 en 4), respectievelijk vrijwillig kon worden gevolgd na een 2-daags interventie (studie uit Hoofdstuk 5 en 6). Gedurende het 30-dagen-durende VLCD werd alle bloedsuikerverlagende medicatie gestopt. Na het 30-dagen VLCD werd langzaam een normaal dieet geintroduceerd (1 Modifast-maaltijd vervangen door 1 normale maaltijd per 2-4 weken, met toename van het aantal kCal met 200 per 2-4 weken tot eucalorisch). Patiënten waren verder vrij in hun keuze van het dieet, hoewel advies werd gegeven tenminste het gewichtsverlies vast te houden en liefst nog verder af te vallen. De patiënten werden vervolgens iedere 3 maanden gecontroleerd door hun eigen internist. Indien nodig werd, naar inzicht van de eigen internist, de bloedsuikerverlagende medicatie hervat. Op dag 0 (voor start dieet), dag 30 (dag 30 van het VLCD) en 18 maanden (18 maanden na de start van het 30-dagen VLCD) werden gewicht, middelomtrek, bloeddruk en nuchtere serum-waarden van glucose, insuline, C-peptide en lipiden gemeten.

Het 30-dagen VLCD gaf een gewichtsreductie van -11.4 ± 0.6 kg wat gepaard ging met een verbetering van de bloedsuikers, lipiden en bloeddruk. Na 18 maanden bleek de groep als geheel dit gewichtsverlies vastgehouden te hebben. Ook de bloeddruk en het lipidengehalte waren, hoewel ze iets verslechterd waren ten opzichte van dag 30 van het VLCD, nog steeds beter dan voor de start van het dieet. Daarnaast bleek het HbA_{1c}-gehalte 0.7% lager te liggen dan voor de start van het VLCD, ondanks het feit dat de patiënten veel minder bloedsuikerverlagende medicatie gebruikten (18 patienten op insulinetherapie op dag 0 [112 ± 21 eenheden/dag]; 6 patienten na 18 maanden [23 ± 9 eenheden/dag].

Binnen de groep waren er patiënten die vanaf dag 30 van het VLCD tot aan de 18 maanden, weer aankwamen in gewicht terwijl anderen gelijk bleven of juist nog verder afvielen. Echter, zelfs de patiënten die weer in gewicht aankwamen tot hun uitgangsgewicht hadden nog steeds een betere HbA_{1c}-waarde, bloeddruk en lipidengehalte ten opzichte van de waarden die ze voor de start van het 30-dagen VLCD hadden.

Concluderend is dus zelfs het eenmalig volgen van een 30-dagen VLCD door obese DM2 gunstig voor de bloedsuikerinstelling, bloeddruk en de dyslipidaemie op lange termijn (18 maanden), zelfs als patiënten na 18 maanden weer terug zijn op hun uitgangsgewicht. Het mechanisme hiervan is onduidelijk. In ieder geval hebben deze patiënten onderricht gehad in het volgen van een dieet en zijn zij op het belang van gewichtsreductie gewezen en hebben zij daarvan ook de positieve effecten zelf ondervonden. In hoeverre op cellulair nivo opgetreden veranderingen als gevolg van het éénmaal gebruikte VLCD een rol spelen bij dit gunstige effect na 18 maanden is onbekend.

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CONCLUSIES

De volgende conclusies met betrekking tot het laagcalorisch dieet in obese DM2 kunnen worden getrokken: 1) het is veilig om een VLCD aan deze groep patiënten te geven; 2) het bloedsuikerverlagende effect is beperkt tot die patiënten die zelf nog voldoende insuline aanmaken; 3) bij patiënten met resterende endogene insulineproductie daalt de nuchtere bloedsuiker binnen 2 dagen na het starten van het dieet, op een moment dat het gewichtsverlies nog minimaal is en ondanks het feit dat alle bloedsuikerverlagende medicatie werd gestaakt; 4) het onderliggende mechanisme van de snelle daling van de bloedsuikers is een daling van de endogene glucoseproductie zonder verbetering van de insulinegevoeligheid; 5) een gewichtsreductie van 50% van het overgewicht verbetert ook de insulinegevoeligheid, met name de insuline-gestimuleerde glucoseopname; 6) op cellulair niveau is er een verbetering te zien in de insuline signaaltransductie en GLUT-4 translocatie na gewichtsverlies. De verbetering in insuline signaaltransductie hangt mogelijk samen met de geobserveerde afname in de intramyocellulaire triglyceridenconcentratie.

Al met al is het VLCD een waardevolle, veilige therapie in obese DM2. Indien nog resterende insulinesecretie aanwezig is (nuchter C-peptide > 0.8 ng/mL en/of 2-voudige stijging vanaf een basaalwaarde ≥ 0.5 ng/mL na 1 mg glucagon iv.[hebben wij in onze studies aangehouden]) kan de bloedsuikerverlagende medicatie tegelijkertijd worden gestopt. Dit vergemakkelijkt het gewichtsverlies en vermijdt het risico op hypoglycaemieën. Indien er nauwelijks resterende endogene insulinesecretie is, is het verstandig een oraal middel te continueren. Liefst metformine omdat dit niet leidt tot gewichtstoename en een laag risico op hypoglycaemieën geeft.

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CURRICULUM VITAE

Ingrid M. Jazet werd geboren op 6 juli 1968 te Schiedam. Na het behalen van haar eindexamen Atheneum β aan het Petrus Canisius College te Alkmaar in 1987, studeerde zij eerst 2 jaar Engels en Europese studies aan de Universiteit van Amsterdam wegens uitloting voor de studie Geneeskunde. In 1989 kon alsnog worden aangevangen met de studie Geneeskunde aan dezelfde universiteit. In 1996 behaalde zij Cum Laude het Artsexamen waarna zij startte met de opleiding tot internist in het Rijnland Ziekenhuis te Leiderdorp (opleiders dr. W.J. Molendijk en dr. F.H.M Cluitmans). De opleiding werd in 1998 voortgezet in het Leids Universitair Medisch Centrum (Opleider Prof. dr. A.E. Meinders). Tijdens de opleiding werd gestart met het in dit proefschrift beschreven onderzoek onder leiding van Prof.dr. A.E. Meinders. De registratie als internist vond plaats in 2002. Sinds 2004 heeft zij een vaste aanstelling als staflid bij de afdeling Algemene Interne Geneeskunde van het Leids Universitair Medisch Centrum.

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PUBLICATIONS

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