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UNIVERZITA KARLOVA v Praze
Přirodovědecká fakulta
Studijní oddělení
Albertov 6, 128 43 Praha 2
IČO: 00216208, DIČ: CZ 00216208
UK 89

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Eva KLIMČÁKOVÁ

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Regulation of human adipose tissue gene expression in relation to obesity and insulin resistance

Supervisors: **Docent Vladimír ŠTICH**

: **Professor Dominique LANGIN**

Jury

Professor Petr GOETZ

Doctor Hubert VIDAL, Director of Research

Docent Marie KUNEŠOVÁ

Docent Vladimír ŠTICH

Professor Dominique LANGIN

Chair

Reviewer

Reviewer

Supervisor

Supervisor

Declaration

I hereby declare that this thesis is of my own composition, and that it contains no material previously submitted for the award of any other degree.

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Nothing is clear...

&

Everything is possible...

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ABBREVIATIONS

- AMPK:** 5'-AMP-activated protein kinase
- AP-1:** activator protein 1
- apM1:** adiponectin
- AR:** adrenergic receptor
- ASK1:** apoptosis signal regulating kinase 1
- AT:** adipose tissue
- ATGL:** adipose triglyceride lipase
- BMI:** body mass index
- BPD:** biliopancreatic diversion
- cAMP:** cyclic adenosine monophosphate
- CB-1:** cannabinoid receptor 1
- CBT:** cognitive-behavior therapy
- cGMP:** cyclic guanosine monophosphate
- CIDEA:** cell death-inducing DNA fragmentation factor- α -like effector A
- CNS:** central nervous system
- CRP:** C-reactive protein
- CSF:** colony stimulating factor
- CT:** computer tomography
- CVD:** cardiovascular disease
- EC:** endothelial cells
- ER:** endoplasmic reticulum
- ERK:** extracellular regulated mitogen-activated protein kinase
- FA:** fatty acid
- FAT:** fatty acid translocase
- FATP:** fatty acid transport protein
- FFA:** free fatty acid

FFM: fat-free mass
FIAF/PGAR: fasting-induced adipose factor
FOXO: phosphorylation of forkhead box protein O
GH: growth hormone
GI: glycemic index
GLUT4: glucose-transporter 4
GBS: gastric bypass surgery
Grb2: growth receptor-binding protein 2
GSK: glycogen synthase kinase
GyK: glycerol kinase
HDL: high-density lipoprotein
HGF: hepatocyte growth factor
HMW: high-molecular weight
HOMA: homeostasis model assessment
HSL: hormone-sensitive lipase
ICAM-1: intracellular adhesion molecule 1
IGF: insulin-like growth factor
IKK: inhibitor of NF- κ B
IL: interleukin
IL-1Ra: interleukin 1 receptor antagonist
IMTG: intramuscular triacylglycerol
IP-10: interferon-gamma (IFN- γ) inducible protein
IR: insulin resistance
IRS: insulin receptor substrate
IS: insulin sensitivity
JAK-STAT: janus kinase-signal transducers and activators of transcription
JNK: c-jun-NH₂-terminal kinase
LAR: leukocyte antigen-related phosphatase
LCD: low calorie diet
LDL: low-density lipoprotein
LIF: leukemia inhibitory factor
LMW: low-molecular weight

MAPKs: Ras-mitogen-activated protein kinases

MC4R: melanocortin-4 receptor

MCP-1: monocyte chemoattractant protein 1

MGL: monoglyceride lipase

MCH: melanin-concentrating hormone

MIF: macrophage migration inhibitory factor

MIP-1: macrophage inflammatory protein 1

MRI: magnetic resonance imaging

NEFA: non-esterified fatty acid

NF- κ B: nuclear factor-kappa B

NP: natriuretic peptide

oGTT: oral glucose tolerance test

p38: p38 mitogen-activated protein kinase

PAI-1: plasminogen activator inhibitor 1

PCSK1: pro-hormone convertase-1

PDK: 3-phosphoinositide-dependent protein kinase

PECAM-1: platelet-endothelial cell adhesion molecule 1

PEPCK: phosphoenolpyruvate carboxykinase

PH: pleckstrin homology

PI3K: phosphatidylinositol 3-kinase

PIP₃/PtdIns(3,4,5)P₃: phosphatidylinositol 3,4,5 triphosphate

PKB: protein kinase B

PKC: conventional protein kinase C

POMC: pro-opiomelanocortin

PPAR: peroxisome proliferator-activated receptor

PPRE: peroxisome proliferator-activated receptor response elements

PTB: phosphotyrosine binding

PTP: protein tyrosine phosphatase

RANTES: regulated upon activation, normal T-cell expressed, and presumably secreted

RAR: retinoic acid receptors

RBP4: retinol-binding protein 4

ROS: reactive oxygen species

rQUICKI: revised quantitative insulin sensitivity check index
RT-qPCR: reverse transcription- real-time quantitative polymerase chain reaction
RXR: retinoic acid-X receptors
SCAT: subcutaneous adipose tissue
SHP: src-homology-phosphatases
SIM1: single-minded homolog 1
Sir2: silent mating type information regulator 2
SIRT1: sirtuin 1
SNPs: single nucleotide polymorphisms
SOCS: suppressor of cytokine signaling
STAT: signal transducers and activators of transcription
SVF: stromal vascular fraction
TG: triglyceride
TNF- α : tumor necrosis factor-alpha
TTR: transthyretin
TZD: thiazolidinedione
UPR: unfolded protein response
VAT: visceral adipose tissue
VCAM-1: vascular cell adhesion molecule 1
VLCD: very low calorie diet
VLDL: very-low density lipoproteins
VO₂max: maximal aerobic capacity/maximal oxygen uptake
WHO: world health organization
WHR: waist to hip ratio
WM: weight maintenance

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PREFACE

For the first time ever, the number of overweight people living on earth exceeded the number of underweight people. More than 1.1 billion adults worldwide are overweight, and 312 million of them are clinically obese. If adjusted for ethnic differences, the prevalence is higher and 1.7 billion people would be classified as overweight. Obesity and overweight affects an alarming 50-65% of countries in the worldwide scale. Obesity is not anymore a threat only in developed countries, but its prevalence spreads rapidly in economically advanced regions of developing countries as well and affects increasingly children. The estimates for underweight are between 700 and 800 million people around the world. In the world health organization (WHO) European Region, 145 million people are obese, while 23 million are undernourished. Both the overweight and the underweight suffer from malnutrition, an excess or a deficiency of a food intake. The WHO estimates for 2005, standardized for body mass index (BMI) $\geq 30 \text{ kg/m}^2$ and ages 15 -100 years, show a prevalence of 20.7% females and 18.5% males in the Czech Republic and 6.6% females and 7.8% males in France suffering from obesity.

The rising levels of overweight and obesity, this “modern disease”, drive the prevalence of chronic non-communicable diseases, including cardiovascular disease and type 2 diabetes mellitus. In most developed countries, heart disease and stroke is the first and diabetes mellitus is the fourth leading cause of death, what is more deaths each year than AIDS. The recent large-scale international IDEA study including 170 000 patients from 63 countries confirmed the pandemic of abdominal obesity and showed the independent association of both the waist circumference and BMI with the presence of cardiovascular disease. Obesity is almost invariably linked with hyperinsulinemia and as a consequence, the resistance to insulin-mediated glucose disposal develops. Insulin resistance is considered as a core early abnormality in the pathogenesis of type 2 diabetes. Obesity, insulin resistance (IR) or diabetes together with other factors form a cluster of conditions referred to as insulin resistance syndrome. Originally termed as Syndrome X by Dr. Reaven, the syndrome has been assigned internationally as the dysmetabolic syndrome

and the prevalence data clearly show that it is a large problem everywhere in the world; only in the U.S. it concerns 70-80 million people.

The epidemics of obesity and diabetes have emerged as the global public health issue of the 21st century. Excess weight gain and associated disorders may however be reversible. Through the regular physical exercise and the control of food intake, the patients can benefit from weight reduction. Even a 5% weight loss results in improvement of several metabolic parameters, mostly due to increased insulin sensitivity (IS). Changing peoples' lifestyles is simple in theory, but brings practically poor outcomes. People live in an "obesogenic environment", i.e. face a wealth of foods rich in fat and sugar, an affluent sedentary lifestyle and long working hours. The search for effective pharmacological therapies for the treatment of obesity and its complications is a major goal in pharmaceutical companies. Obesity is not an easy problem to tackle and it requires the action at the government level. Nevertheless without fundamental changes in national strategies in non-government sectors as food industry, the media and communities, the enormous costs of obesity (direct, indirect and intangible) will be hard to reduce. Therefore, in 2004, WHO Member States adopted the Global Strategy on Diet, Physical Activity and Health that targets the lifestyle modifications to curb the increase in non-communicable diseases.

Recent findings suggest that the pathogenesis of obesity and related diseases are due to the alterations of metabolic and endocrine functions of adipose tissue (AT) in obese subjects. Besides fatty acid (FA) release, AT secretes a variety of substances termed adipokines, which might affect whole-body metabolism and play a role in the pathogenesis of obesity and IR. In our laboratory, we aimed at investigating the impact of non-pharmacological interventions on the regulation of AT metabolism in populations with features of the insulin resistance syndrome.

In the first part of my work, we studied several groups of obese patients in different long-term nutritional and physical fitness conditions and their phenotypic data were related to the expression of genes (at mRNA and protein level) potentially involved in the regulation of the whole-body IS and/or low-grade inflammation. In the second part of the work, we applied pharmacological approach on human AT secretome in an in vitro study and particularly investigated the regulatory role of the three known peroxisome proliferator activated receptor (PPAR) isotypes on the production of adipokines by subcutaneous adipose tissue (SCAT).

CHAPTER 1

INTRODUCTION / REVIEW OF THE LITERATURE

1.1 OBESITY AND ADIPOSE TISSUE PHYSIOLOGY

1.1.1 Etiopathogenesis of obesity

Obesity is a complex multi-factorial disease, characterized as a state with excess amount of body fat associated with health risks. Obesity is most commonly assessed by a single index of weight-for-height, the BMI (weight (kg)/height (m²)), that applies to both adult men and women, independently of age. The WHO classifies adults into several categories according to BMI (www.who.int) (Table 1). The BMI value is a useful guideline, but has several limitations, as well as other tools used, e.g. weight circumference or waist to hip ratio (WHR) to identify parameters of abdominal obesity, or skin fold thickness to measure percent body fat. The formal WHO universal definition of the “normal” range of BMI, based on North American mortality data has been later revised and adjusted for ethnic differences, and a BMI>23 is recognized as critical cut-off for high risk of type 2 diabetes mellitus and cardiovascular disease within Asian populations (www.who.int). The same is needed to establish the relationship between anthropometric data and visceral and subcutaneous adiposity in various populations.

The etiology of obesity is very broad and varies from purely genetic (e.g. monogenic obesity due to leptin deficiency or obesity syndromes such as Prader-Willi) to purely behavioural (e.g. sumo wrestlers) basis [1]. It represents a complex interaction of genetics and environment. The genetic contribution can have a rare monogenic character (monogenic obesities), nonetheless more common forms of obesity are polygenic (polygenic/common obesities). Monogenic obesities are caused by mutations in single genes, they are rare (less than 1% of all obesity cases), very severe and generally start in childhood [2]. Currently, genetic defects in six different genes were identified to be responsible for different monogenic forms of human obesity: the leptin (LEP), leptin receptor (LEPR), pro-opiomelanocortin (POMC), pro-hormone convertase-1 (PCSK1), melanocortin-4 receptor (MC4R) and single-minded homolog 1 (SIM1) [3]. Mutations of the MC4R gene are the leading cause of oligogenic obesity with

frequency of mutations assessed to 0.5 to 4% [4]. As genetics has not changed in the last 100 years, the genes alone cannot explain the rapid increase in the global prevalence of obesity. In most cases, obesity is the outcome of the environment (that has become “obesigenic”) that affects subjects with susceptible genotype. Polygenic/common obesities are caused by interaction of several genetic variants and likely involve both gene-gene and gene-environment interactions; however most studies have often analysed genotype-phenotype associations without regarding the influence of environmental factors [2]. The degree to which hereditary factors contribute to the common forms of obesity is estimated to 30-70% [5,6]. Over 430 genes or chromosomal regions have been implicated in the etiology of obesity so far, and only 15 of them have been replicated by multiple studies (<http://depts.washington.edu/cgph/>). The very last discovery is FTO gene, a commonly occurring gene variant, which has been reported to predispose to obesity [7]. The environmental factors that affect obesity are food access, lifestyle, individual behavioral habits, and also economic situation of people. In addition, other factors have been incriminated, such as inadequate breast feeding with deficiency of long-chain polyunsaturated FA during a critical stage in the development of the brain, what might lead to obesity via increased production of pro-inflammatory cytokines [8]. Therefore, the inflammatory condition may play an important role as a potentiator in the pathophysiology of obesity. A genetic predisposition for people to gain weight is supposed to have the roots in our evolutionary history. The widely recognized theory, the “thrifty gene hypothesis” explaining the origin of obesity and being the basis for other hypotheses [9] has been recently challenged by Speakman, who presents the “predation release hypothesis” as a nonadaptive scenario explaining the genetic predisposition to obesity [10].

Table 1 The international classification of adult underweight, overweight and obesity according to BMI (adapted from WHO 1995, WHO 2000 and WHO 2004 (http://www.who.int/bmi/index.jsp?introPage=intro_3.html))

Classification	BMI(kg/m ²)	
	Principal cut-off points	Additional cut-off points
Underweight	<18.50	<18.50
Severe thinness	<16.00	<16.00
Moderate thinness	16.00 - 16.99	16.00 - 16.99
Mild thinness	17.00 - 18.49	17.00 - 18.49
Normal range	18.50 - 24.99	18.50 - 22.99
		23.00 - 24.99
Overweight	≥25.00	≥25.00
Pre-obese	25.00 - 29.99	25.00 - 27.49
		27.50 - 29.99
Obese	≥30.00	≥30.00
Obese class I	30.00 - 34.99	30.00 - 32.49
		32.50 - 34.99
Obese class II	35.00 - 39.99	35.00 - 37.49
		37.50 - 39.99
Obese class III	≥40.00	≥40.00

1.1.2 Regulation of energy stores

White AT is the main organ in human body for long-term energy storage, heat regulation and body cushioning. This characteristic as being rather a relative inert tissue has been considered the major AT feature for a long time. Many previous studies have examined AT from a metabolic point of view and concentrated mostly on the static basal condition changes in obese states. However, there is a whole array of events governing regulation of fat stores, e.g. physiological situations such as feeding, fasting or exercise [11]. In the post-absorptive state, FA are the main source of energy supply in the body whereas postprandially

the major fuel becomes exogenous glucose which gets to the plasma from diet containing fat and carbohydrate [12].

The biological function of adipocyte is to store the incoming energy in the form of triglycerides (TG) mostly in the postprandial period. Adipocyte contains 90-99% TG stored in a lipid droplet. The data on energy storage are, however limited and it is suggested that there are differences between lean and obese in terms of glucose and FA uptake [11]. Other major metabolic activities of white AT are lipogenesis (TG synthesis) for energy intake and lipolysis (TG breakdown) for energy release. Chronic imbalance between energy intake and energy expenditure favoring positive energy balance leads to obesity (Figure 1). The pathways of fat deposition and fat mobilization can be regulated in accordance with the integrated balance between whole-body energy intake and energy expenditure [13].

The process of lipogenesis is activated after feeding, when TG are formed inside adipocytes from circulating TG-rich lipoproteins, i.e. chylomicrons and very-low density lipoproteins (VLDL). Chylomicrons, formed from FAs produced postprandially in the intestine are entering the blood circulation and, in part, are drained through the portal vein to the liver. In the liver, TG released from chylomicrons together with those partly derived from endogenous de novo lipogenesis, are incorporated into VLDL particles, which are released to the plasma. During eucaloric conditions, chylomicrons and VLDL delivered to the tissues are hydrolyzed by lipoprotein lipase (LPL), thus enabling release and uptake of FA by AT or skeletal muscle for the storage or oxidation, respectively. In adipocytes, FA are bound on glycerol 3-phosphate coming mainly from the glycolysis, and form TG. In an opposite mechanisms, TG can be hydrolyzed during lipolysis and exported to other tissues when lipid energy is needed (e.g during fasting or exercise). AT has also the capacity for de novo lipogenesis, i.e. synthesis of FA from carbohydrates, however, this process appears to be low in humans [13].

Mobilization of FA from TG stores in AT by the catabolic process of lipolysis is of complex nature requiring lipolytic enzymes (lipases), plasma membrane transporters, fatty acid binding proteins and proteins associated with the lipid droplet [14]. Three lipases for complete TG breakdown are active in human AT, the adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL). ATGL and HSL both possess the capacity to catalyze the initial step in TG hydrolysis, i.e. the hydrolysis of TG into diglycerides (DG) *in vitro*. However, only HSL looks able to catalyze the hydrolysis of DG to

monoglycerides (MG). HSL has also the capacity of MG breakdown to FA and glycerol *in vitro*, however *in vivo* MGL is required for complete hydrolysis of MG. TG are hydrolyzed at a lower rate than DG, hence the first step of lipolysis is rate limiting. HSL and ATGL participate in catecholamine and natriuretic peptide-stimulated lipolysis. Dysfunctional lipolysis affects energy homeostasis and may contribute to the pathogenesis of obesity and IR.

Both lipogenesis and lipolysis are under the tight control of hormonal and nervous signals. In humans, the main regulators are insulin, catecholamines and natriuretic peptides. Insulin induces lipogenesis in the short-term and in the long-term via different mechanisms [15,16]. Catecholamines (the neurotransmitter, noradrenaline, and the hormone, adrenaline) regulate human lipolysis through lipolytic beta-adrenoceptor (beta1- and beta2- adrenergic receptors; β 1-2-AR) and anti-lipolytic alpha2-adrenoceptor (α 2-AR) via modulation of intracellular cAMP levels. Natriuretic peptides (atrial and brain natriuretic peptides) stimulate lipolysis through a cGMP-dependent pathway and this pathway is specific of primate fat cells [17]. Nevertheless, other lipolytic pathways are active in human fat cells [14].

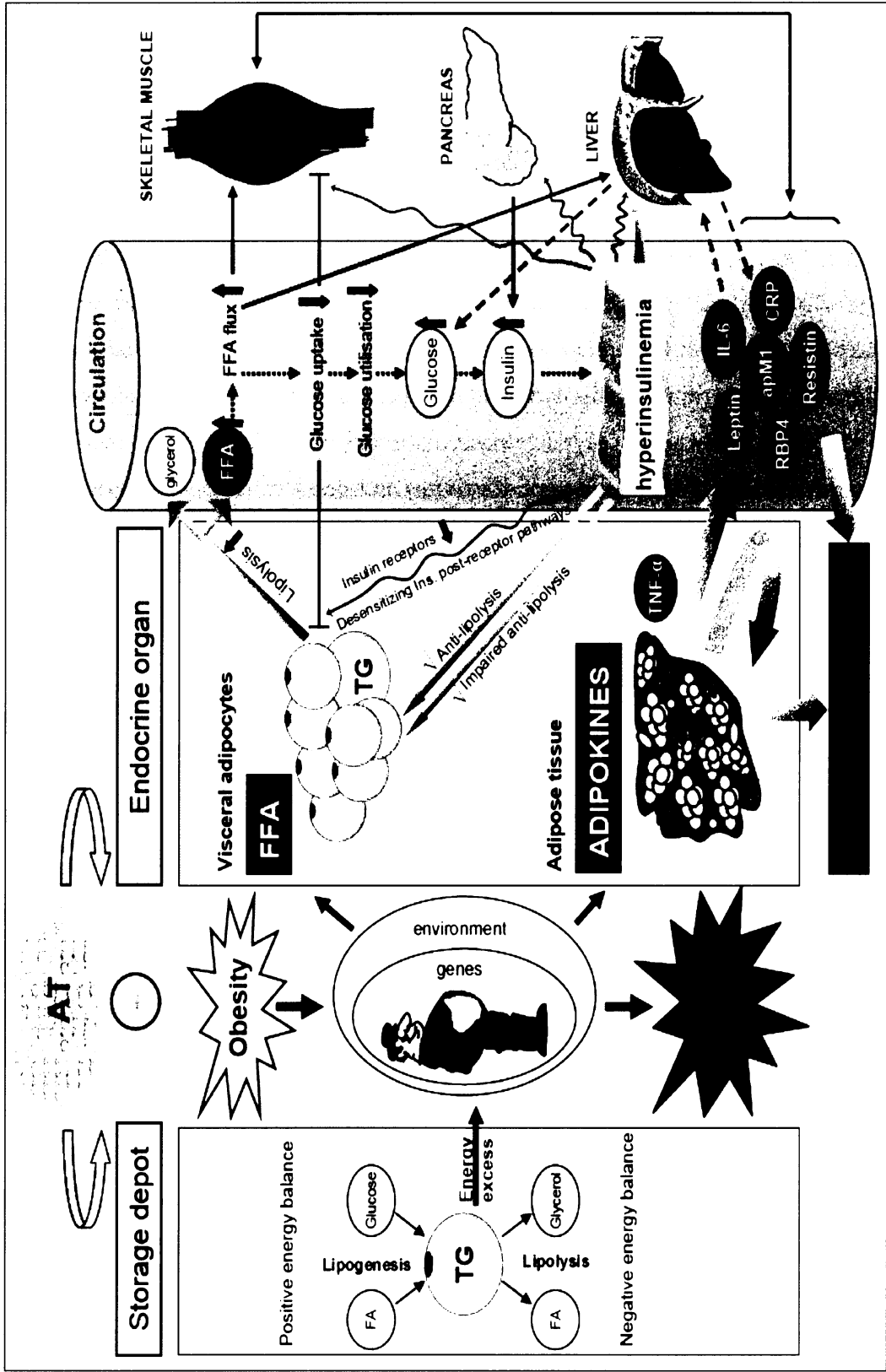


Figure 1 Role of adipose tissue in the development of insulin resistance.

Previously, the biological function of AT was considered to be the long-term repository for energy excess, having metabolic activities such as lipogenesis (storage of FFA in the form of TG) and lipolysis (release of TG in the form of FFA and glycerol). Recently, it has become clear that AT is an endocrine organ producing hormones, adipokines and other peptides. Sustained imbalance between energy intake and energy expenditure favoring positive energy balance leads to obesity. Obesity has genetic and environmental background. Increasing adiposity activates local, portal and systemic effects on inflammation in IR-states. Increased FFA amounts derived from enhanced AT-lipolysis (together with adipokines, drained from visceral adipocytes) delivered to the portal system contribute to hepatic inflammation and IR. Increased production of several adipokines (cytokines and chemokines) from AT causes local inflammation and IR. Sustained production of pro-inflammatory and decreased release of anti-inflammatory cytokines by AT, liver or associated immune cells creates a systemic low-grade inflammatory state that promotes IR at the periphery (skeletal muscle and other tissues).

AT, adipose tissue; IR, insulin resistance; FFA, free fatty acid; FA, fatty acid; TG, triglyceride; TNF- α , tumor necrosis factor- α ; IL-6, interleukin 6; apM1, adiponectin; RBP4, retinol-binding protein 4; CRP, C-reactive protein.

1.2 THE ROLE OF ADIPOSE TISSUE IN INSULIN RESISTANCE

Obesity and IR are strongly related and this association is often critical in the development of type 2 diabetes mellitus. The mechanisms of IR, as well as the cause of β -cell failure and insulin deficiency are complex. IR can develop in all insulin-responsive tissues, classically central (liver) and peripheral (AT and skeletal muscle), but also in the pancreas, kidney and brain among others, and therefore is highly heterogeneous in terms of the primary causes, development or biochemical pathways. In normal conditions, skeletal muscle mass represents the largest tissue mass in humans and contributes the most to total body glucose disposal. The identification of the link between adiposity and impaired IS in skeletal muscle has proved difficult. Interestingly, recent data from genetic rodent models tend us to speculate about AT as a primary site of IR, nevertheless the clear evidence that adipocyte IR alone is able to generate systemic IR is lacking [18,19].

In normal weight subjects the total body AT constitutes between 10-20% and 15-25% of total body mass in males and females, respectively [20]. AT is distributed in several specific regional depots throughout the human body and according to the updated classification of total body and regional AT, two main AT compartments can be distinguished, SCAT and internal AT [21]. Further, SCAT can be divided into superficial and deep SCAT, and internal AT comprises visceral AT (VAT) and nonvisceral AT. VAT is composed of intrathoracic AT (intrapericardial and extrapericardial) and intraabdominopelvic AT (intraperitoneal and extraperitoneal) [21]. However, very often in the literature, VAT is used for intraperitoneal AT. Other classifications are also used in the literature, as upper-body fat (SCAT and VAT in the upper abdominal region) and lower-body fat (lower SCAT deposits) [22]. The SCAT is major fat depot that comprises about 80% of total body fat [23], compared to VAT, which represents only about 20% in men and 5-8% in women [22]. Despite this fact, most investigators support the hypothesis of upper-body obesity and accumulation of fat in VAT (primarily intraperitoneal) as a main determinant of metabolic complications [24-32], but some notable exceptions exist [33]. Recently, three theories apply to explain the contribution of AT into the development of obesity-linked diseases, the theory of “portal paradigm”/“portal/visceral fat theory” [32,34,35], “endocrine paradigm” [36,37], and “ectopic fat storage syndrome”/“ectopic fat deposition” theory [38,39]. These theories are discussed in following sections with particular focus on humans when possible and one theory does not exclude another one.

Although an enormous progress has been done regarding better understanding of the mechanisms underlying obesity-linked IR during the past decade, none of these theories is universally accepted. New data are still expected to answer such questions as: “Is IR the consequence of obesity with respect to regional adiposity, or is it IR that contributes to obesity and visceral fat development?”

1.2.1 Proximal insulin signaling and insulin resistance

Insulin resistance, i.e. resistance to one or several insulin's biological functions, involves resistance to the effect of insulin to stimulate glucose uptake by skeletal muscle and adipocytes and to suppress hepatic endogenous glucose production [40]. The precise molecular mechanisms of the functional defects are tenuous, but recent body of literature links defective insulin signaling and metabolism in obesity with the alterations in insulin receptor substrate (IRS) proteins [41]. Hitherto, six IRS isoforms have been identified, IRS1-6 [42]. The major IRSs in the regulation of glucose homeostasis are the ubiquitously expressed IRS1 and IRS2 [41]. IRS3 is probably not expressed in humans [43] and the information about the role of IRS4 in insulin's metabolic effect in humans is rather limited. IRS5/DOK4 and IRS6/DOK5 were demonstrated to be expressed in human tissues, with high levels in kidney and liver (IRS5/DOK4), and muscle (IRS6/DOK5). Both seem to function in insulin signaling [44]. IRSs exert no intrinsic catalytic activity. They contain both domains, pleckstrin homology (PH) and phosphotyrosine binding (PTB) domain that interact with insulin receptor and mediate IRS main function, i.e. the interaction between insulin and IRS effectors, what finally leads to glucose uptake [41]. The principal events of insulin signaling cascade with the stimulatory effect on glucose uptake and cell proliferation [41,45], illustrated in Figure 2, are as follows: insulin binds to the α -subunit of the insulin receptor, activates insulin receptor tyrosine kinase in the β -subunit and induces its autophosphorylation. The activated insulin receptor phosphorylates major IRS isoforms, IRS1 and IRS2 on their tyrosine residues. IRSs possess over 20 potential tyrosine residues that can act as docking sites for downstream signaling proteins [46]. After IRSs phosphorylation, the signal is conducted downstream via binding of src homology (SH2) domain-containing downstream signaling proteins (PI3K, SHC, SHP2, Fyn, Grb2 among others) to tyrosine phosphorylated residues. Among the best studied SH2 proteins are adaptor molecules, such as the regulatory subunit p85 of phosphatidylinositol 3-

kinase (PI3K) and growth receptor-binding protein 2 (Grb2). These signaling molecules can associate with IRS-1 and activate two main signaling pathways, the PI3K and the ras-mitogen activated protein kinase (MAPK) pathway through several events. These events involve recruitment of PI3K heterodimer complex that consists of a regulatory subunit of ~ 85 kDa and a catalytic subunit of 110 kDa (p85-p110) to the plasma membrane, thus generating lipid second messenger phosphatidylinositol 3,4,5 triphosphate (PIP3/PtdIns(3,4,5)P3). Downstream molecules with PH domains such as 3-phosphoinositide-dependent protein kinases (PDK1 and PDK2) can bind to PIP3, and further phosphorylate and activate protein kinase B (PKB)/AKT or atypical protein kinase C (aPKC) [42]. This process promotes most of the metabolic actions of insulin, e.g. translocation of glucose transporter 4 (GLUT4) to the plasma membrane ensuing glucose uptake in adipocytes and skeletal muscle. A potential target responsible for the GLUT4 translocation is Rab-GTPase-activating protein AKT substrate of 160 kDa (AS160). However, the downstream signals of PI3K are not well documented. In addition to PI3K pathway, other alternative pathways seem to be required for insulin-stimulated glucose uptake [47,48]. The signaling molecule Grb2, which exerts large interaction with IRS1, can activate as well MAPK pathway that promotes mitogenic effects of insulin (cell growth and differentiation) by some of the four MAPKs (ERK, JNK, p38, ERK/Big MAPK 1), most probably through extracellular regulated mitogen-activated protein kinase (ERK) pathway [49].

IRS1 and IRS2 have distinctive contributions to glucose homeostasis and there are also tissue-specific differences. The current data support the IRS1 isoform as an appealing candidate regulating insulin-stimulated metabolic outcomes in muscle and AT, while IRS1 and IRS2 might play complementary roles in insulin signaling and metabolism in liver [41]. As mentioned above, insulin signal transduction can be impaired and contribute to IR in obesity. Insulin receptor and IRS proteins can be negatively regulated by several mechanisms, such as protein tyrosine phosphatases (PTPs), ligand-activated downregulation, and serine phosphorylation [42]. PTPs interact with insulin receptor and dephosphorylate tyrosine residues, thus reduce its activity and terminate stimulatory signaling. The most studied PTPs are PTP1B, leukocyte antigen-related phosphatase (LAR) and src-homology-phosphatases 1 and 2 (SHP1, SHP2), and their expression and/or activity has been found to be increased in muscle and AT of obese humans [50,51]. A ligand-induced internalization and degradation, leading to the downregulation of insulin receptor at protein level is present also in obese states [52].

Besides tyrosine residues, IRSs contain over 70 potential serine residues, phosphorylation of which, in general, accounts for the defects in insulin signaling. The stimuli for this inhibitory effect are various, including insulin, FAs, cytokines, or excess of nutrients [53]. The molecular mechanisms of IR in skeletal muscle, liver and fat are similar. Several serine/threonine IRS kinases (c-JUN NH2-terminal kinase (JNK), inhibitor factors of nuclear factor κ B kinase (IKK), S6 kinase 1, conventional protein kinase C (PKC θ in rodents, PKC- β and - δ in humans), and p38 MAPK), activated by the factors mentioned above, increase serine phosphorylation of IRS-1/2, thus making them poor substrates for insulin receptor-activating tyrosine kinases. This further inhibits insulin-induced PI3K activity, with ensuing reduction of insulin-stimulated AKT activity. The process leads to decreased activation of GLUT4 translocation and other downstream AKT-dependent events, and finally to diminished insulin-induced glucose uptake. In liver, it results in reduced insulin stimulation of glycogen synthase kinase (GSK3) and phosphorylation of forkhead box protein O (FOXO), which normally leads to decreased hepatic glucose uptake and production, and increased gluconeogenesis, respectively. This mechanism exacerbates IR in liver and results in fasting hyperglycemia [18,42,45,54,55]. The IRS tissue-specific alterations are regarding especially the expression of IRS1 isoform in muscle and adipocyte. Whereas insulin binding, tyrosine kinase activity, insulin receptor phosphorylation, and phosphorylation of IRS1 are reduced in both, skeletal muscle and AT, the expression of IRS1 in obese patients appears to be normal in skeletal muscle, but conversely diminished in adipocytes in insulin resistant subjects [45]. Furthermore, the downregulation of expression of the main glucose transporter in AT and skeletal muscle, GLUT4, is another factor that can trigger IR in humans. However, its decreased expression was demonstrated only in adipocytes, but not in skeletal muscle, where other mechanisms like impaired GLUT4 translocation are likely to stand behind [45]. Some studies reported also increased polymorphism of IRS1 in obese humans and associated it with IR and diabetes [56].

Among other molecular mediators that may impair insulin signaling and lead to IR, are oxidative stress, endoplasmic reticulum (ER) stress, and mitochondrial dysfunction [54,57,58]. All of these factors are associated with obesity; however, the triggering mechanisms and their downstream pathways of action in this condition are not clear. Reduced mitochondrial function (decrease in oxidative phosphorylation activity and mitochondrial ATP production), most likely attributed to reductions in mitochondrial density, results in intracellular lipid accumulation in

muscle and liver [54]. Elevated FA levels, especially intracellular FA metabolites fatty acyl-CoA and diacylglycerol can disrupt insulin signaling directly or via induction of both pathogenic stresses (oxidative and ER stress) and promote fat-induced IR in skeletal muscle and liver by the mechanisms discussed earlier [58]. Systemic oxidative stress is defined as „a persistent imbalance between the production of highly reactive molecular species (chiefly oxygen and nitrogen) and antioxidant defences“ [58]. High FA levels increase mitochondrial uncoupling and β -oxidation, thus generating high levels of reactive oxygen species (ROS), possibly at an early stage of IR-development, preceding mitochondrial dysfunction. Excess of ROS may aggravate mitochondrial function at a latter stage and activate inhibitory insulin signaling cascade (see above) [58]. ER stress response also increases ROS production and induce oxidative stress [59], otherwise it supresses insulin signaling through activation of JNK and IKK pathways [60-62].

Together, increased adipose mass in AT or ectopic sites (see section 1.2.4) induce many factors that are most likely interdependent and by their interplay form the basis of the pathogenesis of IR. The biggest advances have been done and the main focus is still being on the role of FAs and metabolically active proteins produced by AT (adipokines) as candidate culprits of IR. The obesity associated mediators of IR and their mechanisms of action are schematically shown in Figure 3.

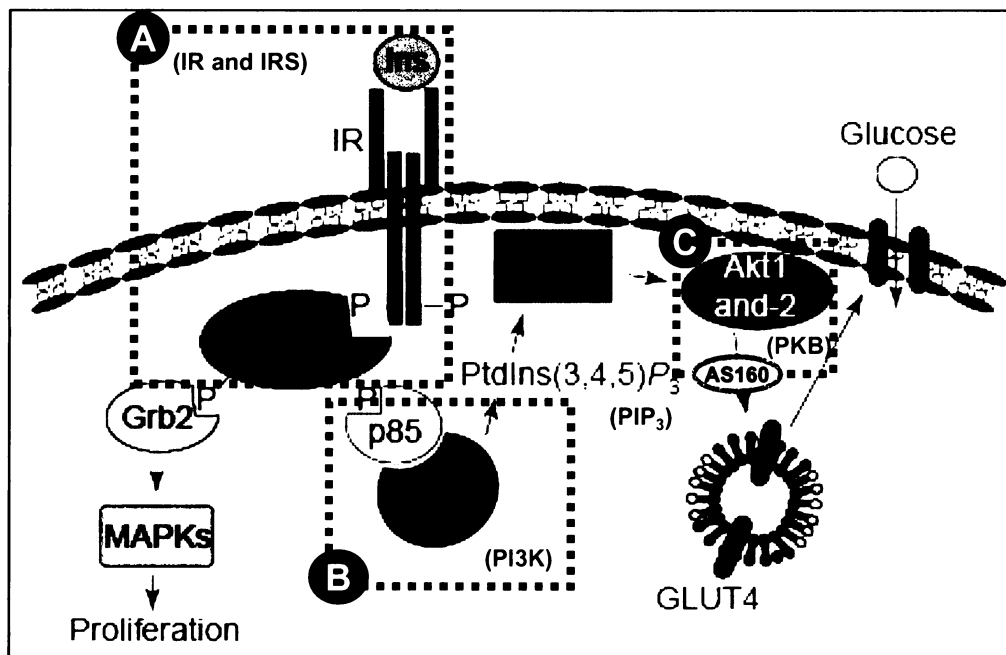


Figure 2 Insulin signaling in adipocyte and skeletal muscle (adapted from Thirone AC et al., *Trends Endocrinol Metab* 17 (2006) 72-78 and Taniguchi CM et al., *Nature Rev Mol Cell Biol* 7 (2006)).

Three best defined critical nodes can be distinguished in the insulin signaling pathway: **A.** IR and IRS [blue box], **B.** PI3K (p85-p110 heterodimer complex), [pink box], **C.** AKT/PKB isoforms [red box].

IR: insulin receptor; Ins: insulin; IRS: insulin receptor substrate; p85: regulatory subunit of phosphatidylinositol 3-kinase (PI3K); p110: catalytic subunit of PI3K; PtdIns(3,4,5)P₃: phosphatidylinositol 3,4,5-triphosphate (PIP₃); PDK1 and PDK2: 3-phosphoinositide-dependent protein kinase-1 and -2; Akt1 and Akt2: protein kinase B (PKB); AS160, GTPase activating protein AKT substrate of 160kDa; GLUT4: glucose transporter 4; Grb2: growth receptor-binding protein 2; MAPKs: Ras-mitogen-activated protein kinases.

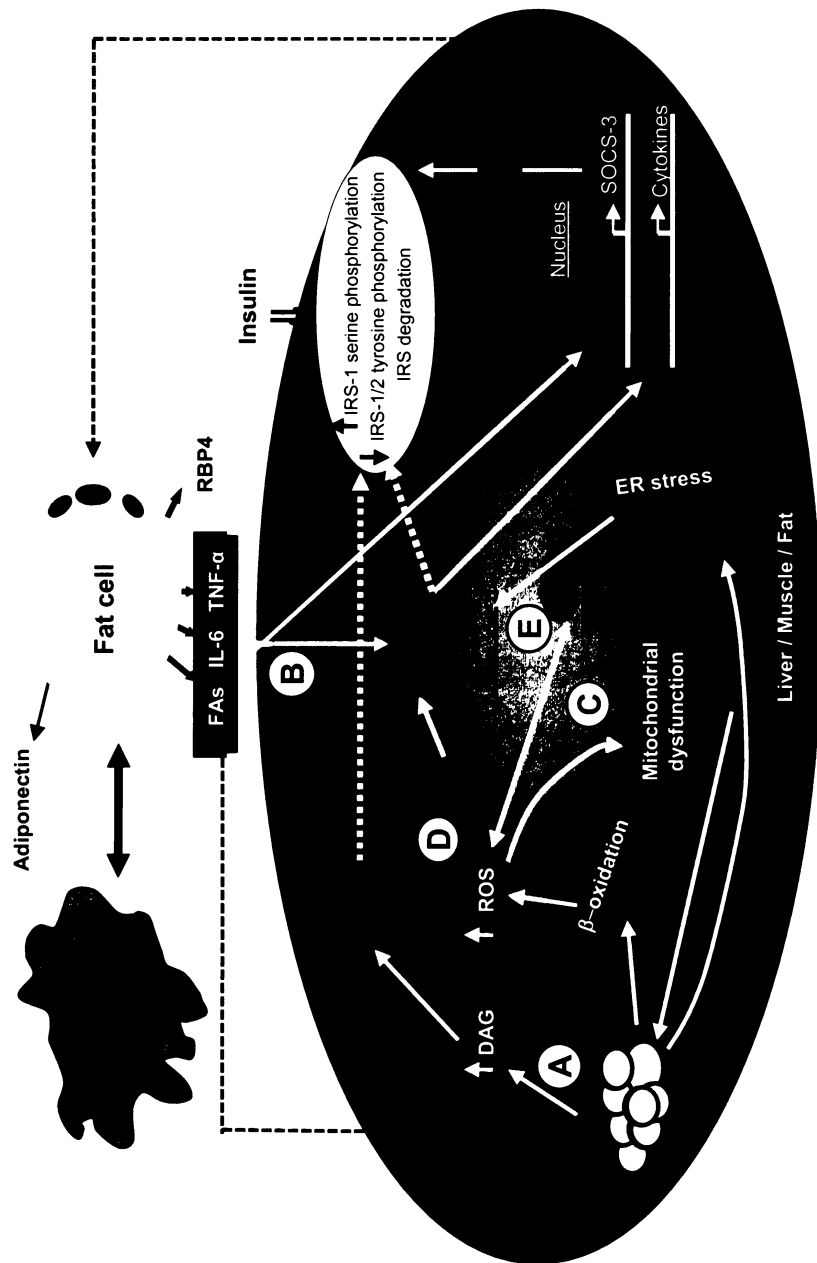


Figure 3 Candidate mediators of obesity-associated insulin resistance and mechanisms of impaired insulin signaling pathways in skeletal muscle, liver and adipose tissue (adapted from Qatanani M et al., *Genes & Dev* 21 (2007) 1443-1455).

A. Elevated FA metabolites (e.g. DAG) can inhibit insulin signaling via activation of PKC. **B.** Altered secretion of adipokines (e.g. increase in IL-6 or TNF- α) impairs insulin signaling via activation of Ser/Thr kinases or by induction of inflammatory SOCS proteins, which compose a negative feedback loop in cytokine signaling. **C.** Mitochondrial dysfunction present in obesity predisposes to intramyocellular and intrahepatic lipid accumulation and exacerbates IR. **D.** Increased levels of ROS may contribute to decreased mitochondrial function and lead to activation of Ser/Thr kinases (potentially via ASK1) that inhibit insulin signaling either directly via IRS-1/2 serine phosphorylation or indirectly via NF- κ B. **E.** Increased intracellular ER stress because of several stimuli, e.g. FA, may deteriorate insulin signaling through the activation of JNK, IKK or through induction of ROS.

ATM, adipose tissue macrophage; FA, fatty acid; DAG, diacylglycerol; PKC, conventional protein kinase C; Ser/Thr, serine/threonine; SOCS, suppressor of cytokine signaling; IR, insulin resistance; ROS, reactive oxygen species; ASK1, apoptosis signal regulating kinase 1 (evolutionarily conserved mitogen-activated protein 3-kinase); IRS, insulin receptor substrate; NF- κ B, nuclear factor- κ B; ER, endoplasmic reticulum; JNK, c-jun-NH₂-terminal kinase; IKK, inhibitor of NF- κ B; p38, p38 mitogen-activated protein kinase (p38MAPK); STAT, signal transducers and activators of transcription.

1.2.2 Fatty acids as candidate mediators of insulin resistance

Elevated fasting and postprandial levels of free fatty acids (FFAs) are typical hallmark of obesity and other IR states [55,63]. The first and earliest hypothesis of “portal paradigm”/“portal/visceral fat theory” highlights the central role of VAT, adipocyte and FFAs in the development of IR. It is based on two lines of investigations, an increased lipolytic activity of visceral adipocytes and a venous drainage of intraperitoneal VAT [33,34]. According to several studies, visceral adipocytes are more resistant to the anti-lipolytic effect of insulin and together with enhanced sensitivity to catecholamines’ lipolytic action, they feature a high release of FFAs [64-69]. Intraperitoneal VAT drains into portal vein and hence there is a direct FFA flux into the liver [33], which in turn may modify hepatic lipid metabolism (increase TG synthesis and VLDL), impair insulin-inhibition of hepatic glucose production and result in hepatic IR [70,71]. In 1963, Randle and colleagues postulated the concept of substrate competition (FFAs and glucose) to explain the adverse effect of FFAs at periphery, the so called glucose fatty-acid or Randle cycle [72]. They suggested that in skeletal muscle, which is the major site of insulin-mediated dietary glucose uptake (~75%) [73], chronically available FFAs become preferred substrate over glucose. They proposed increased FA oxidation (increased intramitochondrial acetyl CoA/CoA and NADH/NAD⁺ ratios) as a mechanism responsible for increased intracellular glucose concentration, decreased muscle glucose uptake, inhibition of insulin action and following muscle/peripheral IR [74,75]. *In vivo* studies performed in humans by Shulman et al. demonstrated, contrary to the Randle mechanism, that the rate-limiting step for FA-induced IR is glucose transport as a consequence of increase in intracellular FA metabolites, such as diacylglycerol, fatty acyl-CoA, and ceramides [55]. Sustained circulating glucose levels give a stimulus to pancreas to overproduce insulin and subsequently compensatory hyperinsulinemia ensues. Hyperinsulinemia *per se* can cause IR partly by downregulation of insulin receptors and desensitizing post-receptor pathways in all insulin-sensitive tissues, and/or by downregulating GLUT4 glucose transporter in adipocytes [45]. Hyperinsulinemia could lead to a constant lipolytic inhibition in adipocytes, but the ability of insulin to suppress FFA-release from expanded AT is conventionally believed to be impaired in insulin-resistant obese subjects [76]. Nevertheless, an anti-lipolytic effect of insulin has been reported even at low insulin concentrations in insulin-resistant states with defective glucose transport, what is not surprising, because it is well documented that human adipocytes are one

of the most highly insulin-sensitive cell types [45,63]. Insulin secreted by the pancreas is normally removed by the liver (about 50% on first pass) [77], but in insulin-resistant states increased FFA flux in the portal circulation reduces this insulin clearance, which contributes to peripheral hyperinsulinemia [78]. This effect might be seen also as an adaptive mechanism, to partially overcome the peripheral IR by providing a higher proportion of insulin to the periphery and to relieve the β -cells to compensate for the need of insulin imposed by IR [79]. The role of FFAs as potential culprits of IR in obesity is schematically depicted in Figure 1.

However, several issues that do not support the role of VAT and FFAs in the control of the whole-body IS were raised. Surprisingly, the generally accepted notion of increased FFA levels in insulin-resistant obese subjects was recently argued [76] referring to the study that demonstrated fasting FFA concentrations not to be increased in this population [80]. AT beds are metabolically heterogeneous and differ in lipolytic and anti-lipolytic metabolic activity [81] (Table 2). Catecholamine-induced lipolytic activity of visceral adipocytes is generally considered higher when compared with SCAT, but both increased as well as no difference in lipolytic response were reported [65-67,82,83]. The higher production of FFAs from VAT could be ascribed to increased expression or function of HSL or related proteins on one hand and to increased β 1-2- or decreased α 2-AR responsiveness on the other hand [84]. The association of increased fat cell size with IR [37] is another fact that might support the role of VAT in IS. *In vitro* studies showed that large hypertrophied adipocytes (typical in obesity) in general, hence hypertrophied visceral adipocytes, are characterized by a higher basal lipolytic rate [69,85]. It was also shown that increasing amount of visceral fat depots correlates with an increase of FFA delivery into the liver [85], where they may deteriorate hepatic metabolism. Further, hypertrophied subcutaneous adipose cells have low number of β 1-2-ARs and the highest number of α 2-ARs and exhibit the least responsiveness to the lipolytic effect of catecholamines [84]. Anti-lipolytic effect of insulin appears to be reduced in omental AT compared to SCAT depot [64,68]. However, these findings come from *in vitro* studies and does not necessarily reflect the effect *in vivo*, as it has been shown in a recent study of Samra et al. [86]. Another line of evidence that cast doubt on the portal fat hypothesis is the question of FFAs origin in the systemic circulation. It has been shown that SCAT remains the predominant contributor of systemic FFAs and accounts for ~70% of total FFAs in the circulation in lean subjects (lower amounts are seen in obese when compared to lean) [85,87]. If the role for VAT

is presumed, one would expect a substantially higher contribution of VAT into systemic FFA concentrations. Nevertheless, in lean men and women, ~ 5-10% of hepatic delivery comes from VAT lipolysis (20-25% in obese) and leg AT contributes by ~15-20% of basal, systemic release (by 28% in obese) [85]. Some investigators evaluated the relationships between regional fat beds and IR. By using reliable methods for the assessment of glucose disposal rates (euglycemic hyperinsulinemic clamp) and direct measurement of visceral fat by computer tomography (CT) or magnetic resonance imaging (MRI), the results suggest only a minor role of intraperitoneal VAT in the pathogenesis of IR in humans [33]. Moreover, the few human studies with omentectomy performed in obese subjects, did not bring the convicting evidence for the portal fat hypothesis neither [88].

In conclusion, the causal nature of relationship between VAT and IR remains a matter of debate and the prevailing opinion is that the portal paradigm theory, with elevated portal FFAs having detrimental effect on insulin action alone does not sufficiently explain the mechanisms of IR in obesity. Increased release of adipokines from VAT into the portal circulation is an alternative scenario linking VAT and IR via induction of systemic inflammation. The role of adipokines is being extensively studied and the recent findings of Fontana et al., based on the measurement of adipokine arteriovenous concentration differences across visceral fat, suggest that VAT could promote systemic inflammation and ensuing metabolic abnormalities through increased production of interleukin 6 (IL-6) in humans [89]. This issue has so far been addressed only in part and future studies are required to bring more insights.

Table 2 Metabolic heterogeneity of adipose tissue (adapted from Garg A [81])

STUDIES	Visceral (omental)	Subcutaneous (abdominal)
In vitro studies		
Adipocyte size	↓	↑
Basal lipolysis	= or ↓	= or ↑
Catecholamine stimulated lipolysis	= or ↑	= or ↓
Anti-lipolytic effect of insulin	↓	↑
In vivo studies		
Interstitial glycerol concentration	↓	↑
Glycerol release	=	=

↑ increase, ↓ decrease, = no change

1.2.3 Adipokines as candidate mediators of insulin resistance

The second theory “endocrine paradigm” was developed together with the hypothesis of “ectopic fat storage syndrome” (see sub-chapter 1.2.4). Based on the endocrine function of AT, the “adipocentric view” of the pathogenesis of IR has emerged, and implies bioactive molecules secreted by AT as the main culprits in IR. These factors were collectively named adipocytokines [90], or adipokines. It is important to define this term more specifically as it has been previously a matter of debate in the literature. The term “adipokine” is more appropriate since many, but not all AT-secretory products are cytokines, or cytokine-like. Further, it was supposed that various protein signals are secreted from the adipocyte itself. Ensuing data clearly showed that there are only few substances produced exclusively by adipocytes, and that the majority of proteins come from other cells present in AT. Accordingly, it seems more appropriate to adopt adipokines as molecules synthesized and secreted from the whole AT. However, the designation “adipokines” is inaccurate regarding the organ-specificity and does not restrict adipokines to be the products only of AT as many “adipokines” are expressed and released also from other organs and non-AT specific cells. To define a protein as an adipokine, the detection of its gene expression at mRNA level in AT is the initial required step which needs to be subsequently confirmed by the detection of protein secretion from adipocytes or other cells that constitute the tissue *in vitro*, or in the venous drainage from AT *in vivo* [91].

The endocrine feature of adipose organ underscores the fact that AT subserves multiple functions with a considerable role in the communication with other organs and tissues. A corollary to, a wide range of secreted adipokines with diverse structures and roles is highly possible to be involved into the development of obesity and/or IR and may impair or enhance insulin action on distant target tissues, such as skeletal muscle and liver (systemic effect) and/or have autocrine or paracrine actions (local effect). An intense effort has been done in identifying such adipokines, their physiological and metabolic function, and mechanisms of association with obesity-related disturbances. To date, over 100 substances have been reported as adipokines [92], but the nature and function of many of them remain poorly understood and largely speculative. However, what has been clearly established, is that the expression and secretion of number of AT-proteins is altered in different obese states. Not long ago, the concept of obesity as a chronic low-grade inflammatory state has emerged [93,94]. It is characterized by increased circulating levels of pro-inflammatory cytokines and acute-phase

proteins that may be causal in the genesis of obesity-linked IR. While this scenario is generally assumed, other line of research hypothesizes that origin of inflammation in obesity may be a result of resistance to insulin [8].

1.2.3.1 Adipose tissue structure and changes during obesity

AT is a highly heterogeneous organ with unique organization and dynamics. The knowledge of the basic organization of AT is essential for the understanding of its integrative function in the human body, including the role of adipokines. AT is composed of mature adipocytes and non-AT fraction, which consists of stromal-vascular cells, blood vessels, lymph nodes and nerves [95]. The cellular composition of AT and their relative amounts [92] are shown in Figure 4. The major cellular components of AT are mature white adipocytes (50-70%). The adipocytes are in close proximity to the vasculature that provides the oxygen and supply or removal of metabolic substances. White AT is relatively well vascularized with the number of capillaries per cytoplasm unit typically greater than in skeletal muscle [12]. The number of lymph nodes depends on the topography of AT and is generally limited in SCAT [95]. AT is innervated by the nerve endings of the autonomic nervous system that copy the course of blood vessels [84]. Thus, AT-components function together as an integrated unit.

The fat cells have a great size-variability, ranging from 15 to 150 μm in diameter [96]. A large variation in the cell diameter is possible due to the adipocyte's principal function to store TG. The ability to expand is unique of adipocytes and makes AT the only organ in the human body able to modify its mass after adult size is reached. This feature is remarkable during the weight gain in obesity, when AT undergoes multiple changes [11]. Typically, positive energy balance during the development of obesity is associated with expansion of AT stores and both increase in size (hypertrophy) and number (hyperplasia) of adipocytes [95]. It is likely that hypertrophied adipocyte reaches its maximum expansion capacity (up to 140-180 μm) during the time, after which gives way to adipocyte hyperplasia, which is unlimited and plays a substantial role in obesity [97,98]. The originally proposed "critical fat cell size hypothesis" [97,99] suggesting enlarged fat cells giving the signals for new adipocyte proliferation has been extended for other factors, such as paracrine internal factors (growth factors IGF-1, IGF binding proteins, TNF- α , angiotensin II, or macrophage colony-stimulating factor (MCSF), afferent neural inputs and/or circulating factors [95]. Fat mass can range from 2-3% (high-level athletes)

to 60-70% (severely obese patients) of total body weight, with average fat mass values being 9-18% (males) and 14-28% (females). Fat mass may exceed 22% or 32% of body weight in males and females, respectively [95]. Except of further histological (relative rarefaction of blood vessels or neural structures, angiogenesis, neovascularization, remodeling of extracellular matrix, macrophage infiltration) and macroscopic (visible or relative expansion of different AT depots) changes, there are many others characteristic for the development of obesity, such as different functional changes, changes in regulation of AT energy stores, or changes in AT secretory function [11]. Indeed, obesity is seen now as a state of impaired secretory function. Schematic representation of some AT histological changes associated with fat expansion in obesity is depicted in Figure 4.

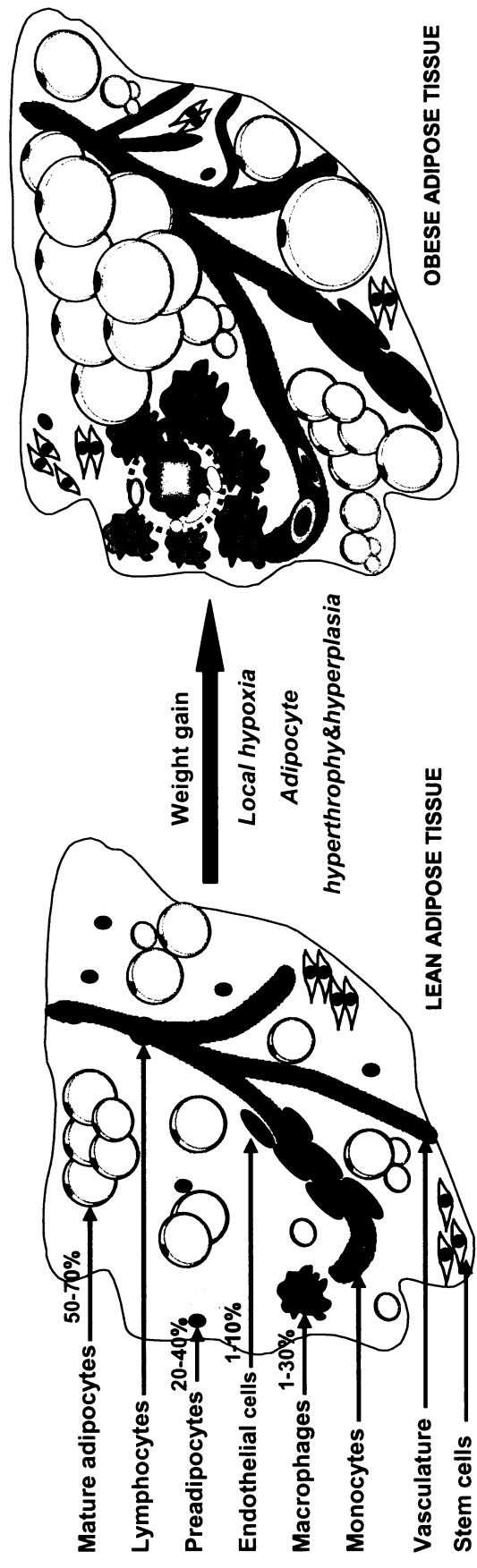


Figure 4 Human white adipose tissue cell composition and changes in obesity.

AT is composed of adipocytes and a non-adipocyte cellular component called the stromal vascular fraction. As obesity develops, white AT mass increases and undergoes multiple changes. The cellular development includes adipocyte hypertrophy and hyperplasia. Hypertrophy reflects the high amount of stored triglycerides in existing adipocytes and usually precedes hyperplasia. Hyperplasia (adipogenesis) is characterized by proliferation of stem cells in AT and their differentiation into new fat cells or vascular cells. Preadipocytes are adipocyte precursor cells able to proliferate and are believed to be present in AT throughout life. They can differentiate into mature adipocytes, rather incapable of further replication. AT expansion is accompanied by neovascularization and angiogenesis in parts of AT that suffer a local hypoxia due to insufficient amount of vasculature. AT is extensively reorganized, involving the process of extracellular matrix remodeling by the matrix metalloproteinases. Obese AT changes also the non-adipocyte cell content and increases number of macrophages, which infiltrate AT from the blood stream. Macrophages localize to individual „necrotic-like adipocytes“ and form crown-like structures, i.e. macrophage syncytia laden by residual lipid droplet and ingested debris from „dead adipocyte“. Adipocyte death shows necrotic degenerative features, such as disrupted plasma membrane, dilated ER and mitochondria, and degeneration of unilocular lipid droplet into numerous small lipid droplets. Of note, adipocyte nucleus exhibits no signs of apoptosis. AT, adipose tissue; ER, endoplasmic reticulum.

1.2.3.2 *Inflammatory background of insulin resistance in obesity*

Abnormal fat accumulation is supposed to be associated with broad inflammatory response. Both increased expression of inflammatory cytokines and increased metabolic stress (oxidative and ER stress) are markers proposed to be potential mediators leading to IR [100]. Yet currently, none of these candidate-presumptions has large body of evidence in the literature and the precise triggers need to be clarified by further studies. The breakthrough that directed researchers to focus on inflammation in relation to obesity were the findings of Hotamisligil et al. in their study with obese animals [101]. They identified the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF- α) to be an adipokine and demonstrated that its increased levels in obesity can be neutralized by TNF- α soluble antibody what resulted in amelioration of IS. Another important discovery was done by Weisberg et al., who described the influx of macrophages into obese AT and that macrophage number is positively correlated with adipocyte size and BMI [102].

The general picture is that the secretory dysfunction of obese AT is the result of macrophage infiltration, which could be the paramount event leading to inflammation and manifestation of obesity-complications [103]. Thus the precedent AT-muscle axis paradigm shifted to the appealing concept referred to as the adipocyte-macrophage-muscle axis [104]. Macrophages as monocytic phagocytes are present probably in all body tissues in normal physiological conditions. These tissue-resident macrophages have an important role in the tissue homeostasis and act occasionally to remove apoptotic cells, debris, or react against foreign pathogens [105]. In pathological conditions such as obesity, macrophages can adapt to the local “pathological” environment and acquire different functions or phenotypes. Resident macrophages become activated and a new supply of macrophages is drawn into AT from circulating monocytes [106]. The proposition that macrophages could differentiate *in situ* from the adipocyte progenitor cells in AT [107,108], was challenged and it was estimated that about 85% of macrophages is derived from the bone marrow [102]. Blood monocytes originate from the bone marrow precursors [109] and form either a subset of monocytes with short-life that can be actively recruited into the inflamed tissue, or a subset of monocytes that will be constitutive in the tissues [110]. Which subset is the the source of AT-infiltrating macrophages and what are the reasons of their activation or invasion into AT and not to other localizations is not yet well documented, but contributing factors are likely to be multifactorial. It is also not clear if the

enrichment of AT by macrophages is unique to the obesity state, or if it could be a feature of other inflammatory process in the human body. A recent study suggests that the recruitment of macrophages in omental AT, where macrophages are found in higher amounts compared to SCAT [111,112], is associated with hepatic damage in obese patients [112].

Among factors that may trigger macrophage recruitment, accumulation, and persistence in obesity are mechanical changes of AT, e.g. during adipocyte hypertrophy. Cinti et al. showed in a very interesting study that the increase in adipocyte size and obesity *per se* increases the frequency of death of adipocytes in human AT and suggested that macrophages are implicated in the process of sequestering and clearing of these adipocytes with necrotic-like phenotype. Thus, macrophage can have a local “beneficial” effect, such as to create a room for new adipocytes [113]. They report that the predominance (90%) of all macrophages within white AT in humans surround selectively dead adipocytes and form “lipid-laden macrophage syncytia”, resembling multinucleate giant cells which are a hallmark of serious chronic inflammatory states, thus promoting AT inflammation. The presence of macrophages localized to adipocytes in a similar manner as “crown-like structures” was observed concurrently by other researchers in a group of obese women [114]. In the same study, the authors suggest local adipose hypoxia present in some parts of obese AT as another potential inducer of AT macrophage infiltration. The molecular basis for inducing macrophage attraction and retention in AT are probably autocrine, paracrine, and endocrine signals from expanding fat mass. AT is a source of several chemoattractants, like monocyte chemoattractant protein 1 (MCP-1) (specific chemoattractant for monocytes and macrophages) or colony stimulating factor (CSF), that promote activation of endothelial cells (EC) resulting in adhesion of peripheral blood monocytes to the EC of AT capillaries, with subsequent transmigration inside the tissue, where they accumulate and further differentiate into macrophages [115]. Leptin overproduced by hypertrophied adipocyte appears to behave also as powerful chemoattractant because it was shown to increase monocyte diapedesis to AT-derived EC in a concentration dependent manner in an *in vitro* study [116]. Furthermore, leptin is able to enhance the expression and production of MCP-1 from EC [117,118]. The chemoattracting effect of leptin can be counteracted by adiponectin, which inhibits the activation of EC by inhibiting the adhesion of monocytes and downregulating the expression of adhesion molecules [119,120]. However, other molecules are expected to participate in the process of macrophage infiltration, e.g. the recently discovered













adipokine, macrophage migration inhibitory factor (MIF) [121]. Currently, it has been reported that activated adipocytes *per se* can promote increased monocyte adhesion and macrophage accumulation in AT [115].

AT macrophages during activation underlie biochemical, morphological and functional changes that are suspected to stand behind the origin of IR through the local, as well as systemic effects of adipokines produced by them [105]. Xu et al., as first separated AT into adipocyte- and stromal vascular fraction (SVF) and reported preponderance of inflammatory genes to be expressed in non-adipose SVF fraction [103]. Nevertheless, both macrophages and adipocytes may act synergistically to generate local AT inflammation with ensuing generalized chronic low-grade pro-inflammatory state. It is suggested that the accumulation of macrophages in AT triggers the concert of cell cross-talk and adipokine interplay, in other words a cross-talk between AT and the immune system [122,123]. The feedback loops are schematically summarized in Figure 5. Hypertrophied adipocytes and activated macrophages oversecrete numerous cytokines and chemokines that can impair local adipocyte IS, what can further lead to vascular and systemic IR and eventually to other serious metabolic diseases. The precise mechanisms whereby adipokines trigger IR and obesity complications are yet unclear. Chronic low-grade systemic inflammation is characterized by moderate, about 2- to 3- fold increase of TNF- α , IL-1, IL-6, IL-1Ra, soluble TNF- α receptors (sTNF-Rs), and C-reactive protein (CRP) plasma concentrations [124]. However, the main source of these inflammatory cytokines in circulation does not need to be necessarily AT itself and is difficult to determine in obesity. The liver and lymphoid system might be the principal site of cytokine production, or adipokines released by AT could act only as signal molecules for secretion of inflammatory markers from other organs, as liver, or it might be the result of all-above combination [91]. A recent *in vitro* study of Permana et al., provides evidence that AT macrophage-secreted factors, thus macrophages themselves have a direct effect on adipocyte inflammation and IR and that there is a vicious inflammatory cycle of cross-talk between adipocytes and macrophages [115]. They showed that macrophage-secreted factors increased transcription levels of inflammation-related chemokines, adhesion molecules and cytokines in adipocytes such as MCP-1, MIP-1 α , MIP-1 β , ICAM-1 and IL-6 among others, and that these molecules consequently increased monocyte adhesion to adipocytes. Macrophage-secreted factors also increased FFA levels and decreased glucose uptake in adipocytes, thus induced local IR in fat cells. A corollary to, it is conceivable

that inflammation-induced enhanced lipolysis with high circulating FFAs may have adverse effect on IS in periphery (liver and skeletal muscle).

Obesity leads to increased metabolic stresses, which similar to macrophages, are able to activate inflammatory signaling pathways, inhibit insulin receptor signaling pathways and hence contribute to IR development in humans [57,100]. It is not yet known why these stresses are present in obesity, but it is conceivable, that it might be the reaction of human body to fat accumulation, which might appear as a state reminiscent of infection and lead to local AT inflammation. As inflammation stimulates lipolysis, it has been also speculated, that moderate inflammation could keep AT homeostasis in terms of to avoid fat excess [100]. Two potential pathogenic metabolic stresses were reported to induce inflammation in obesity, i.e. ER stress and oxidative stress. The perturbations such as energy and nutrient fluctuations, pathogens or others increase demands on the ER and excess its functional capacity. ER becomes stressed, activates the unfolded protein response (UPR) that further via activation of JNK and IKK signaling pathways disrupt insulin action and deteriorate IS. [57,60-62]. A second mechanism, increased oxidative stress levels, may be the result of several conditions such as hyperglycemia, chronic inflammation, high tissue lipid levels, inadequate antioxidant defence, hyperleptinemia, and ER stress among others [59,125]. Elevated glucose levels in obesity give rise to increased glucose uptake by EC in AT and accordingly to increased production of ROS from mitochondria and ER. ROS bring along oxidative damage with ensuing activation of inflammatory pathways in EC, adipocytes or muscle cells. In adipocytes, ROS via macrophage attraction may deregulate adipokine production (increase expression of pro-inflammatory cytokines) and generate local inflammation [100]. However, as obesity is considered as chronic inflammatory state with increased pro-inflammatory adipokine levels, oxidative stress, ER stress and ROS formation may also be induced by adipokines (e.g. leptin, TNF- α), which in turn exacerbate local AT inflammation. Hence, it is plausible, that there is a vicious cycle provoking increased stresses in obese states [57,126]. The potential inflammatory signaling pathways that could contribute to local and systemic IR are schematically summarized in Figure 5.

Cell cross-talk
Adipokine interplay

STROMAL VASCULAR FRACTION CELLS	
	ICAM-1, VCAM-1, PECAM-1
	MCP-1, MIP-1 α
	TNF- α
	IL- (1 β , 6, 8, 10, 18)....
	IL-1Ra
	Resistin
	FIAF/PGAR
ADIPOCYTES	
	Leptin
	Adiponectin
	RBP4
	Serum amyloids
	FIAF/PGAR

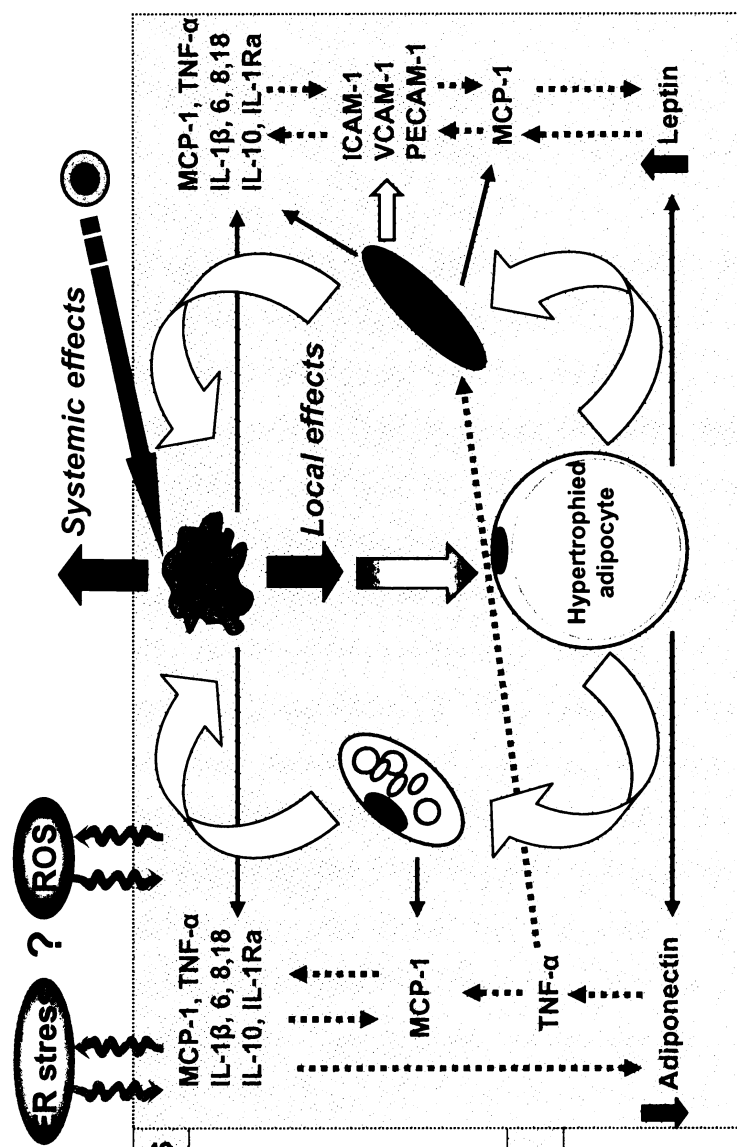


Figure 5 Inflammatory signaling pathways in adipose tissue during obesity.

Obesity corresponds to a chronic low-grade inflammation, which triggers the concert of AT cell cross-talk and adipokine interplay leading to local and systemic insulin resistance. In obesity, hypertrophied adipocytes produce high levels of leptin and low levels of adiponectin. Preadipocytes, EC and activated macrophages per se, or as a result of altered levels of adiponectin and leptin secrete MCP-1 and possibly other chemoattractants that upregulate expression of several adhesion molecules (ICAM-1, VCAM-1, PECAM-1) on EC, resulting in transmigration of bone marrow-derived circulating monocytes into AT where they differentiate to macrophages. Macrophages accumulate inside obese AT and oversecrete several pro-inflammatory cytokines and chemokines as TNF- α , IL-1 β , IL-6, IL-8, IL-18 and MCP1, which contribute to further increase of transcription levels of adhesion molecules, ensuing further monocyte/macrophage influx and vicious inflammatory cycle. Increased TNF- α can stimulate the production of MCP-1 by preadipocytes or EC. In contrast, the levels of the anti-inflammatory adiponectin fall with increased adiposity, thus its ability to downregulate the expression of adhesion molecules and inhibit the activation of EC is suppressed. Moreover, low adiponectin cannot counteract high TNF- α or IL-6 levels, on the contrary, both cytokines inhibit adiponectin expression.

AT, adipose tissue; EC, endothelial cells; ER, endoplasmatic reticulum; ROS, reactive oxygene species; ICAM-1, intracellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; PECAM-1, platelet-endothelial cell adhesion molecule 1; MCP-1, monocyte chemoattractant protein 1; MIP-1 α , macrophage inflammatory protein 1 alpha; TNF- α , tumor necrosis factor alpha; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; FIAF/PGAR, fasting-induced adipose factor; RBP4, retinol-binding protein 4 .

1.2.3.3 *Adipokines associated with insulin resistance*

The first mention of AT as a tissue producing protein signals dates to 1955, when LPL was identified by Korn et al. in several tissues including heart and AT [127-129]. This was followed in 1987 by adiponectin and later on, several other proteins were described in the literature to be released by AT, such as retinol-binding protein 4 (RBP4), plasminogen activator inhibitor 1 (PAI-1), or TNF- α . After the discovery of leptin in 1994, the classical view on AT as an inert reservoir devoted to energy storage has been abandoned, and the concept of white AT as a real endocrine organ was firmly established (Table 3). Thus, AT is now recognized as an active organ coupling (neuro)-endocrine and metabolic signaling, regulating many physiologic and pathophysiologic processes including immunity and inflammation-associated IR development. In 1997, Maeda et al. reported that 40% of genes expressed in human AT are novel and 20-30% of them might be secreted proteins [130]. The number of known AT-derived factors is still mounting but the list is far from being complete. Adipokines constitute a diverse group of bioactive peptides that can be divided into cytokines, chemokines and hormone-like proteins. These compounds can be further subdivided into different categories, according to many criteria, such as their putative function (metabolic, inflammatory, etc), principal effect (local and systemic), or others.

Cytokines are defined as soluble polypeptides of relatively low molecular weight and biologically active at very low levels [131,132]. They are produced by immune or non-immune cells after certain stimuli and exert autocrine, paracrine and endocrine effects through receptor-ligand interactions. Over 200 cytokine ligands have been described, but only few with regard to AT [133]. Chemokines constitute a large superfamily of small secreted proteins, chemotactic cytokines, that direct migration of inflammatory cells, activate inflammatory responses and have many other pleiotropic functions [134]. They are induced also by various stimuli, including cytokines and act essentially through paracrine manners [132]. In humans, there are about 50 known chemokines [134]. Cytokines appear to be major regulators of AT metabolism. The effects of cytokines within AT include action that may be defined as metabolic [135]. Among extensively studied adipokines potentially implicated into inflammation and/or pathogenesis of IR in obesity, besides leptin by adiponectin [136-139], are “classical cytokines” TNF- α [101,102,139-153], interleukins IL-6 [141,143-145,149,154-161], IL-1 β [135,143,149,153,159,162-164], IL-10 [156,165], IL-18 [166-168], then chemokines MCP-1

[92,169-172], IL-8 [157,169,171,173-175], also acute-phase proteins CRP [141,156,176,177], PAI-1 [92,178-184], and other peptides such as adiponectin [37,185-196], leptin [139,149,150,152,197-201], resistin [179,202-208], visfatin [208-214], and RBP4 [139,215-220] (Table 3). White AT is a heterogeneous organ in many regards, therefore to obtain ideas about the role of adipokines in general, several methodological aspects must be considered. Most remarkable, majority of data on adipokines were derived from studies in rodents and these often failed to be further confirmed in humans, e.g. the roles of adiponectin, resistin, or TNF- α in association with IR [221]. Of note, recent works highlight the role of several stromal vascular cells within AT, especially in inflammation, thus it is important to clarify the cellular origin of specific adipokines. Furthermore, anatomic heterogeneity of AT may contribute to functional heterogeneity of several adipokines. A considerably different gene expression profiles were found between the best characterized AT-sites in man, SCAT and VAT. Owing to the prevalent notion that VAT may be associated with unfavourable metabolic consequences of obesity, identification of adipokine depot origin is important. Only cytokines released into the circulation in detectable amounts can contribute to the increased adipokine plasma levels, therefore the identification of either local, or systemic action of adipokine is necessary. Adipokines that were related to inflammation and/or IS in obesity with respect to the facts mentioned above are shown in Table 3. A special attention was paid first to summarize our current knowledge in humans, second to depict both adipokine mRNA and plasma levels in obesity, and finally, to report the recent view on their effect and impact on IS. It is evident, that the hints are not unanimous throughout the literature, even for the basic notion of elevated adipokine levels in obesity. Many adipokines are reported to be elevated in obese states, but careful search reveals that the results are tenuous regarding both mRNA and adipokine protein levels. From numerous factors released by AT that may be relevant for the pathophysiology of obesity-associated complications, more detailed description will be limited in the following section to leptin, adiponectin, TNF- α , IL-6, IL-1 β and RBP4 with regard to their potential roles in humans whenever possible, because these factors were the subject of the present work.

Table 3 Selected adipokines related to insulin sensitivity and inflammation in humans

Adipokines	Discovery ^a	Physiologic concentration	Plasma/Serum (Obesity)	mRNA ^b (Obesity)	AT depof	AT cell type ^c	Effect ^d	Insulin sensitivity ^e
Adipsin	1985/86 [136,137]	1.2-1.6µg/mL [138]	↑ [138]	?	= (overw.-ob) [139]	○ [136]	S. Pro-IF	?
RBP4	1989 [215]	9.3-30.5µg/mL [216]	↑ [217] = [218,219]	↓ [218]	= [139] ↑ VAT(lean-ob) [220]	○ [139,218,219]	S. ↔	↔
PAI-1	1991 [178]	~5pg/mL [179]	↑ [179,180]	↑ [180,181]	↑ VAT (lean-sev.ob); [181,182] ↑ SC (ob); [183]	○ SVF [92,182,184]	S. Pro-IF	↓
TNF-α	1993 [101]	~1pg/mL [140]	↑ [140-143] = [144,145]	↑ [146,147] = [144,148]	= (overw.-sev.ob) [139,149,150] ↑ SC (ob); [151]	● [102,152,153]	L. Pro-IF	↔
Leptin	1994 [197]	1-10ng/mL [198]	↑ [199]	↑ [199]	↑ SC (lean-sev.ob) [139,149,150,200]	○ [152,201]	S. ↔	↑
Adiponectin	1995/96 [195,196,198,199]	0.5-30µg/mL [37]	↓ [186-188]	↓ [187,189,190] = [191,192]	= (ob) [190,193] ↑ SC (lean) [188]	○ [194]	S. Anti-IF	↑
IL-6	1996 [154]	~2pg/mL [155]	↑ [141,143,144,156] = [143,145]	↑ [161]	↑ VAT (ob-sev.ob) [149,157-159]	● [149,153,160]	S. ↔	↔
IL-8	2000 [173]	3.24±1.07pg/mL [174]	↑ [171,174]	?	↑ VAT (lean.ob) [157]	○ [169,175]	L. Pro-IF	↓
Resistin	2001 [202]	1.5-9.9ng/mL [203]	↑ [203] = [179]	↑ [204] = [204,205]	= (lean-sev.ob) [204,206,207]	○ [207,208]	L. Pro-IF	Norminor effect
MCP-1	2001 [169]	~75pg/mL [170]	↑ [171]	↑ [172]	?	○ [92,169,172]	L. Pro-IF	↓
IL-1β	2001 [153]	0.6±0.3pg/mL [143]	↑ [135] = [162]	↑ [163]	↑ VAT (sev.ob) [149,163]	○ SVF [153,159,164]	L. Pro-IF	↓
IL-18	2002 [166]	232±120pg/mL [167]	↑ [166,167]	?	= (ob) [168]	○ [168]	S. Pro-IF	↓
IL-10	2003 [156]	3.5-4.8pg/mL [165]	↑ [156] ↓ [165]	?	?	?	S. Anti-IF	↑
CRP	2003 [176]	<3mg/L [177]	↑ [141,156]	?	?	○ [176]	S. Pro-IF	↓
Visfatin	2005 [209]	15.9±3.4ng/mL [210]	↑ [209,211] ↓ [212]	↑ [211] ↑ [212] = [211]	= (lean-sev.ob) [211,213] ↑ VAT [209]	○ [208,214]	S. ?	Norminor effect

Table 3

^a Regarding mRNA or protein production from AT regardless the species and in some cases regarding only the association with obesity

^b AT depot and study group are defined in reference articles

^c Adipokine mRNA, protein, or secreted levels

^d ○ adipocyte; ● preadipocyte; 🍄 macrophage; ◎ monocyte; 🍄 EC, regardless AT depot and species

^e L, local effect; S, systemic effect; Pro-IF, pro-inflammatory; Anti-IF, anti-inflammatory, based on the data from the literature shown in this table

☺ Most plausible effect, based on the data from the literature shown in this table

↑ Increase; ↓ decrease; ↔ equivocal effect; ? no data; = no difference

AT, adipose tissue; SC, subcutaneous adipose tissue; VAT, visceral adipose tissue; EC, endothelial cells; SVF, stromal vascular fraction cells; overw., overweight; ob, obese; sev.ob, severely obese

Sex-related differences and age have not been considered

References are given in brackets [].

LEPTIN (OB protein)

Leptin (leptos (Greek): thin), the product of the ob (obese) gene [197] is a hormone expressed and secreted primarily, but not exclusively by white AT (adipocytes). At lower levels it is found produced by gastric epithelium, placenta, brain, skeletal muscle, bone, arterial endothelium and heart [126,222-224] and is cleared mainly by the kidney [225]. Leptin is a pleiotropic molecule, acting both centrally and peripherally, with pivotal role in regulating food intake, energy expenditure and neuroendocrine function. Subsequent studies showed that this “antiobesity hormone” [225], as proposed initially, has a more complex function, playing role also in immunity, inflammation, hematopoiesis, angiogenesis, thermogenesis or reproduction [226,227]. The leptin receptor (OB-R) was identified a year after the discovery of leptin itself [228]. The OB-R has at least six alternatively spliced isoforms in rodents (OB-Ra – Ob-Rf), which share a common extracellular leptin-binding domain and transmembrane domain, but differ in intracellular domain [225,229,230]. Leptin receptors are expressed in the brain and in peripheral tissues [228]. The OB-Ra is the predominant short isoform in most tissues and cells and is supposed to mediate otherwise inefficient leptin transport through the blood-brain barrier. However, it has also been shown in rats that leptin receptors are not essential for leptin transport into the cerebrospinal fluid [231]. After crossing the blood-brain barrier, leptin enters the central nervous system (CNS) and stimulates OB-Rb. OB-Rb (OB-RL; L=long) is a long isoform, expressed at high levels in hypothalamic neurons, where it mediates the anorectic effect of leptin, but is present also in several peripheral tissues [226]. OB-Re, a soluble receptor, lacks transmembrane as well as intracellular domain [225]. Leptin circulates either in a free form as a 16-kDa protein, or in a bound form [232]. In the bound form, leptin forms a complex with OB-Re, which function is not fully understood, but may act as leptin’s carrier protein to the membrane signaling receptors, or inhibit leptin’s activity. The bound form of circulating leptin is the major form present in lean individuals, whilst free leptin in obese people [232].

Plasma leptin exhibits a diurnal rhythm and a strong sexual dimorphism, being higher in females [224]. Normally, increased leptin concentrations result in negative energy homeostasis (inhibit appetite and feeding, increase energy expenditure), whereas decreased leptin levels in positive energy balance. Overfeeding leads to increased basal plasma leptin levels after several days and they decrease within hours after initiation of fasting [225]. Plasma leptin levels

correlate positively with body fat mass and fall during fasting or after weight loss [233,234]. Obesity is characterized by hyperleptinaemia (Table 3), but high leptin levels are not able to exert anorexigenic effect. This suggests that obesity could result from resistance to leptin at its sites of action, similar to IR with increased insulin levels in IR subjects. Nevertheless, 5-10% of obese people have relatively low plasma leptin concentrations, indicating probably inappropriate production of leptin [199,233]. Finally, obesity could be a result of missing leptin secretion. However, human *ob* gene mutations are rare and almost all obese subjects have at least some leptin production [234]. Mutations in leptin and leptin receptors cause hyperphagia, severe obesity, and hypothalamic hypogonadism, but unlike *ob/ob* [235-237] or *db/db* mice [238], hyperglycemia, hypercorticism, and hypothermia (decrease in energy expenditure) are not seen in leptin and leptin receptor-deficient humans [239-243]. Leptin therapy has been proved to be beneficial in leptin-deficient children and adults [239,242,244]. However, contrary to rodents [235-237], administration of high endogenous leptin levels failed to decrease body weight and body fat in obese humans and other obese mammals [199,233,245]. The reasons for these substantial differences in physiological actions of leptin between humans and rodents have not been fully addressed. Leptin exerts its central actions through actions in the brain, by involvement of many hypothalamic neuropeptides regulating the feeding behaviour, such as orexigenic (stimulates feeding) melanin-concentrating hormone (MCH), or neuropeptide Y (NPY) and anorexigenic (inhibit feeding) α -melanocyte stimulating hormone (α -MSH) [225,234].

Leptin has been proposed to play a major role in obesity and IR, and that is to increase IS. The effect of leptin at the periphery was demonstrated in some studies. In insulin-sensitive tissues such as skeletal muscle, it rapidly activates signaling pathways (activation of various kinases: JAK-STAT, PI3K, PKB/Akt, PKC, MAPK, Jun, ERK) directly by binding to the full-length OB-Rb, the only receptor believed to be involved in leptin signaling [246]. In rodents, leptin has an insulin sensitizing effect, through the suppression of hypothalamic-pituitary-adrenocortical axis and/or by direct effect in insulin sensitive tissues. Direct effects include the reduction of FA uptake and esterification, inhibition of lipid synthesis and promoting lipolysis, lipid oxidation, thus decreasing muscle lipid content and preventing excess lipid accumulation [45]. It has been shown that leptin directly stimulates FA oxidation in skeletal muscle in mice by activating the 5'-AMP-activated protein kinase (AMPK) (Figure 6). AMPK is an enzyme

that phosphorylates and represses the activity of acetyl-CoA carboxylase, what reduces malonyl-CoA formation and leads to increase in carnitine palmitoyl transferase-1 activity and FA oxidation. This early AMPK activation by leptin directly in muscle can be followed later by the leptin action on hypothalamic-sympathetic nervous system axis [247]. Nevertheless, factors other than AMPK might exist in the transmission of leptin effects. In humans, it has also been reported that leptin may have a stimulatory effect on fat oxidation [248]. However, Steinberg et al. demonstrated *in vitro* that leptin increases FA oxidation only in lean but not in obese human skeletal muscle, which could reflect some leptin resistance in the periphery [249,250]. The latter findings were recently supported in the *in vivo* human study of Blaak et al. [251]. They found a negative association between fasting serum leptin levels and fasting lipid oxidation in obese IR subjects what could indicate leptin resistance resulting in impaired capacity to regulate muscle fat oxidation. The effect of leptin on glucose metabolism appears to be synergistic with insulin action, i.e. increases glucose uptake in muscle, possibly via a CNS-mediated mechanism [198].

Leptin is constitutively produced by AT and its levels are regulated by variety of signals, chiefly by food intake and endocrine factors [234,252], but also by inflammatory stimuli [226]. As a cytokine (type I cytokine superfamily), leptin levels can be increased in response to infections, fever or systemic inflammation as well as stimulated by some cytokines [227] (Figure 6). In humans, TNF- α and IL-1 were found to induce leptin levels, what may contribute to the anorexia and weight loss accompanying these inflammatory conditions [198]. On the other hand, leptin can also directly regulate cytokine production, e.g. to increase MCP-1 in human umbilical vein EC [117], to up-regulate TNF- α , IL-1 β , IL-6, IL-12 and MIP-1 α , or to down-regulate IL-10 production in human dendritic cells, thus indicating a possible immunoregulatory role of leptin [253] (Figure 5). The general assumption is that leptin has a pro-inflammatory effect but at the same time might protect against infections [254]. Further studies are needed to establish precise mechanisms of leptin's actions in humans.

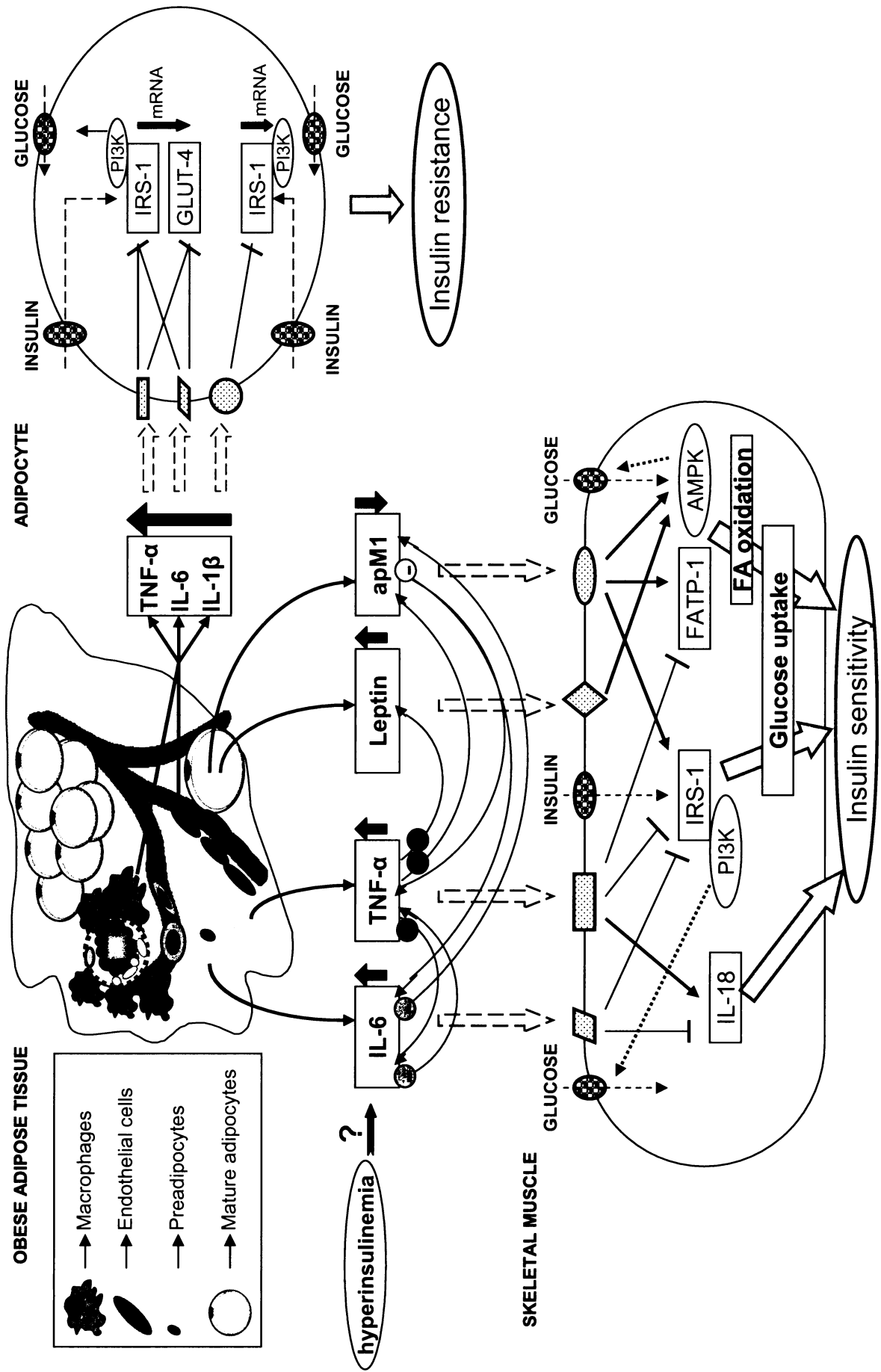


Figure 6 Role and mechanism of action of selected adipokines in the development of insulin resistance in adipose tissue and skeletal muscle.

AT produces adipokines, e.g. leptin, apM1, TNF- α , IL-6 or IL-1 β that are noted for the ability of their inter-regulation, especially in obese and IR-states. Insulin might be a regulator of adipokine production in AT, however the precise evidence needs to be established. Skeletal muscle is the major sink for glucose disposal, AT contributes only little to whole-body glucose disposal. In obesity, in skeletal muscle, IRS-1 tyrosine phosphorylation is impaired but not linked to a change in its expression. On the contrary, in AT, a down-regulation of IRS-1 mRNA levels is likely to be the major mechanism implicated in alteration in IRS-1 tyrosine phosphorylation. In insulin resistant states, the expression of GLUT4 is downregulated selectively in adipocytes but not in skeletal muscle; this leads to impaired insulin-induced glucose transport in adipocytes. The possible mechanisms of adipokine action shown in the figure are based on the data obtained mainly from animal studies and most findings require further confirmation in humans.

AT, adipose tissue; IR, insulin resistance; apM1, adiponectin; TNF- α , tumor necrosis factor alpha; IL-6, interleukin 6; IL-1 β , interleukin 1beta; IL-18, interleukin 18; FATP-1, fatty acid transport protein; IRS-1, insulin receptor substrate 1; PI3K, phosphatidylinositol 3-kinase; AMPK, 5'-AMP-activated protein kinase; GLUT4, glucose-transporter 4.

ADIPONECTIN (apM1, GBP28, Acrp30, AdipoQ)

Adiponectin has several synonyms, as it has been identified by four independent teams, using different approaches [255]. Two human adiponectin proteins were isolated, either from human AT library, named adipose most abundant gene transcript 1 (apM1), or from human plasma, called gelatin-binding protein 28 (GBP28) [185,195]. The two mouse homologues have been cloned, termed adipocyte complement-related protein of 30 kDa (Acrp30), or AdipoQ [189,196]. Adiponectin is a hormone expressed and secreted exclusively by AT (adipocytes), with mRNA levels being among the most abundant in the tissue [185,256] and with very high circulating amounts (0.01% of total plasma proteins) in human bloodstream [186]. Information on adiponectin functions come mostly from observations in rodents and culture models. Adiponectin is considered to have anti-inflammatory, anti-atherogenic and insulin-sensitizing effects, suggesting that it is involved in the pathogenesis of IR, atherosclerosis and inflammation [190]. Some studies reported that adiponectin might also be involved in the regulation of energy balance and body weight in mice, reducing weight gain, somehow paradoxically stimulating food intake, but simultaneously increasing body temperature, suggesting stimulation of energy expenditure [257,258]. As mentioned above, adiponectin is an approximately 30-kDa polypeptide, composed of four main domains: an NH₄-terminal signal sequence, a collagenous domain, a COOH-terminal globular domain, and a variable domain without homology to known proteins [259]. The molecule shares high structural homology to complement factor C1q, and is similar also to collagen VIII and X, and TNF- α [185,256]. Once synthesized, posttranslational modifications produce multiple isoforms: trimers, which oligomerize to form polymers. In human plasma, adiponectin circulates in different multimeric complexes, i.e. trimeric low-molecular weight (LMW), hexameric medium-molecular weight (MMW) and larger oligomeric structures of high-molecular weight (HMW). Some researchers have distinguished only two adiponectin isoforms, LMW and HMW [260-265], that are considered as the most clinically relevant oligomers. All isoforms, but monomers are usually present in plasma [254]. A proteolytic cleavage product with globular domain also circulates in trimeric form at physiologically significant levels (about 1% of total plasma adiponectin) [266]. Both the globular and the full-length adiponectin have biological activities. Mutations in the adiponectin gene can cause impaired multimer formation, changes in the HMW/LMW ratio in plasma or reduced abundance of all adiponectin isoforms. Two adiponectin mutations

associated with impaired formation of HMW complexes have been phenotypically linked with hypoadiponectinaemia and type 2 diabetes mellitus in humans, suggesting a possible role of HMW adiponectin in the pathogenesis of diabetes [267]. Besides potentially different properties of adiponectin isoforms and their relative levels in plasma, the biological effects of adiponectin might depend on the presence of different adiponectin receptors as well. Two cell-surface receptors were identified, AdipoR1 and AdipoR2 [268]. AdipoR1 is the major receptor in muscle with high-affinity for globular head of adiponectin. AdipoR2 is expressed primarily in liver with intermediate-affinity for both globular and full-length adiponectin [269]. Both receptors are expressed in AT [268,270].

Plasma adiponectin does not fluctuate during the day [271] and a sexual dimorphism exists with higher plasma concentrations in women [272]. HMW multimer levels appear also to be higher in women than in men [267]. Interestingly, unlike most adipokines, adiponectin serum levels are generally reduced in obese, IR and type 2 diabetic patients, and also in non-obese IR humans [186,188,189,260,271,273,274]. Considering distinct adiponectin multimers, our laboratory have recently shown no difference in HMW and decrease in MMW and LMW forms, as well as total plasma adiponectin in obese when compared with non-obese women, as analyzed by Western blot (unpublished data). Other studies using Western blot detection of multimeric complexes have reported a selective reduction in HMW forms in type 2 diabetic individuals [267,275,276]. However, HMW adiponectin, as measured by the novel commercially available ELISA assay system for the selective measurement of human adiponectin multimers, in a study of Bluher et al. did not differ between subjects with normal glucose tolerance, impaired glucose tolerance and type 2 diabetes mellitus [274]. These studies indicate that besides incompletely understood process regulating the production of adiponectin oligomers in various groups of people, there are significant differences when using different techniques to assess individual adiponectin isoforms in human plasma. Low adiponectin levels in IR states are due to either obesity or lipodystrophy, and adiponectin administration partly ameliorates metabolic parameters in these conditions [269]. The mechanisms that reduce adiponectin levels in humans are not well documented. Of note, not all studies report decreased mRNA levels in obese, and/or diabetic subjects (Table 3). Concentrations of plasma adiponectin correlate negatively with BMI, glucose, insulin, and TG levels and positively with high-density lipoprotein (HDL) cholesterol level and insulin-stimulated glucose disposal [277].

Recent findings showed that HMW adiponectin is predominantly responsible for the positive correlation between total adiponectin and HDL-cholesterol [261,263,278], perhaps through its impact on hepatic metabolism [261]. In our study, we observed a correlation between the MMW form and HDL-cholesterol [279]. Because of both latter forms are able to stimulate AMPK in primary culture hepatocytes [267], they may therefore have similar effects on hepatocytes. Several polymorphisms in the adiponectin gene were related to obesity and/or IR in murine models [269].

A strong inverse association between adiponectin and both IR and inflammatory states has been reported [269]. Adiponectin enhances IS in both muscle and liver (mouse model), at least in part, by increasing phosphorylation of insulin receptor, activating IRS-1 associated PI3K and stimulating AMPK. This is accompanied by a stimulation of glucose uptake (enhanced GLUT4 translocation), an inhibition of acetyl-CoA carboxylase and increased FA oxidation in myocytes (Figure 6), and a reduction of hepatic glucose production [280], thus resulting in plasma FFA and intracellular TG clearance in both organs. Furthermore, it was demonstrated that adiponectin increased muscle fatty acid transport protein 1 (FATP-1) mRNA levels, which may partially account for increased FFA uptake, enhanced FFA oxidation and clearance of plasma FFAs [266] (Figure 6). Adiponectin can improve FA catabolism also indirectly, by the stimulation of PPAR α , which regulates the enzymes involved in lipid metabolism [258]. Little is known about the peripheral effects of adiponectin in humans due to the absence of intervention studies with adiponectin administration. One study showed, that physiological concentrations of fasting plasma adiponectin are not related to fat oxidation or energy expenditure in resting conditions in obese humans [281]. It has been reported, that total adiponectin in plasma may not be the best indicator of IS [267] and that different adiponectin isoforms might have different biological activities. Isolated globular domain of adiponectin stimulates FA oxidation in skeletal muscle and improves insulin-stimulated glucose disposal via AdipoR1, whereas full-length adiponectin synergizes with insulin to inhibit glucose production in liver via AdipoR2 [257,258,282]. The HMW form has been suggested to be physiologically the most potent form of adiponectin, and might be the form responsible for its beneficial insulin-sensitizing and anti-atherosclerotic effects [264,283]. An association between the HMW form and whole-body IS has been reported [261,263,264,284-286]. Moreover, it has been hypothesized that HMW adiponectin may better predict metabolic parameters than total

adiponectin [286]. Nevertheless, a majority of recent studies (including our current unpublished study) support neither the predominant role of the HMW oligomers in influencing IS as measured by Western blot [261,279], nor the superiority of HMW form over total adiponectin in assessing IS and metabolic variables as quantified by the novel ELISA [274] either at baseline, or after different lifestyle interventions. It has been shown that the HMW/total adiponectin and HMW/LMW ratios are plausible indicators of thiazolidinedione (TZD)-induced changes in IS [264]. It can be hypothesized that associations of changes in HMW as well as MMW and LMW forms with changes in indices of IS might be more specific to TZD treatment and play only a minor role in LCD- induced changes in IS [264,283].

AT-derived cytokines, especially TNF- α and IL-6, were suggested to downregulate synthesis and release of adiponectin. The negative regulation of TNF- α on adiponectin was shown in 3T3-L1 cells [287] and in human preadipocytes [288]. On the contrary, TNF- α does not seem to inhibit release of adiponectin from human adipocytes [190]. IL-6 has also been shown to reduce adiponectin gene expression and secretion in 3T3-L1 adipocytes [160]. Cytokine cross-talk involves also a feed-back mechanism because adiponectin is also able to decrease TNF- α and IL-6 production (Figure 6) [254], partly by the reduction of nuclear factor- κ B (NF- κ B) signaling and ERK1/2 activity [123], and induce the anti-inflammatory cytokines IL-10 and IL1Ra in human leukocytes and macrophages [289,290]. Moreover, adiponectin is a sticky molecule which accumulates in vessel walls and inhibits TNF- α - induced monocyte adhesion to EC and EC activation by downregulating adhesion molecule expression (ICAM-1, VCAM-1), and may therefore protect against endothelial dysfunction [119] (Figure 5). Thus, adiponectin appears to exert mostly anti-inflammatory effects. However, based on the opposite action of adiponectin isomers on the transcription factor NF- κ B (LMW inhibits NF- κ B, MMW and HMW activate NF- κ B), this notion was lately enriched to the paradigm that not adiponectin as such is an anti-inflammatory protein, but that adiponectin is able to modulate the innate immunity in an isoform-specific way [123].

TUMOR NECROSIS FACTOR alpha (TNF- α)

TNF- α was initially characterized as a factor inducing tumor necrosis in septic animals and associated with cachexia-inducing states, such as cancer and infection, and subsequently shown to be identical to cachexin (hence also called cachectin), a factor secreted by

macrophages *in vitro* [133,269]. Therefore, its role in energy homeostasis was suspected. Now, it is known that the biological actions of TNF- α are broad. TNF- α is implicated in anorexia, inflammatory diseases, autoimmune diseases, tumorigenesis, sepsis, among others, and especially, TNF- α is linked to the development of obesity and IR [224,269]. TNF- α is a cytokine produced by a variety of cell types like immune cells, tumor cells, fibroblasts, muscle cells, and also by adipocytes [132]. Within human AT, TNF- α is expressed by adipocytes and preadipocytes. However, the majority of TNF- α secretion comes from stromavascular cells, especially from infiltrated macrophages [102,159,291]. TNF- α is expressed as a 26-kDa cell surface membrane-bound precursor that is posttranslationally cleaved off by matrix metalloproteinase (TNF- α - converting enzyme) into a 17-kDa soluble, biologically active form [133,292]. Both, transmembrane and soluble TNF- α , can interact with more than 20 receptors in humans, but it is unclear how many of them are active in AT [131]. It was shown that human AT expresses mRNA of two cell-surface receptors, TNF-R1/TNFR60/gp60 (p55 in mice) and TNF-R2/TNFR80/gp80 (p75 in mice) [133], which can be proteolytically cleaved to form soluble TNFR. Both soluble TNFR bind to TNF- α *in vitro* and inhibit its biological activity by competing with cell-surface TNFR for TNF- α [246]. The physiological relevance of these receptors is not clearly established.

TNF- α levels are generally believed to be slightly increased in obesity, although not without conflicting results in humans (Table 3). TNF- α mRNA and protein expression in AT are low, but were shown to correlate positively with BMI, percentage of body fat, and hyperinsulinemia [144,147]. Plasma TNF- α levels are also low relative to local tissue concentrations, but have been positively associated with obesity and IR. However, the correlation between plasma TNF- α and adiposity is relatively weak and some studies do not find these results at all [133]. In humans, TNF- α has been found not to be secreted into the systemic circulation and acts in local autocrine and paracrine manners [293]. One of the mechanisms that could be responsible for the elevated membrane-associated TNF- α in AT in obesity is its decreased processing rate in mature adipocytes combined with its increased production [294]. Otherwise, TG and FA are physiological inducers of TNF- α expression in rats [101,295].

TNF- α appears to have a critical role in the pathogenesis of IR, at least in rodents. In humans, there are reports of both, no association between TNF- α and whole-body IS [148,296],

or an association of TNF with glucose uptake and IR [297]. Its actual involvement in disrupted glucose and lipid metabolism in humans remains controversial. TNF- α is supposed to be a major player to trigger IR in AT [298] (Figure 6). Raised TNF- α concentrations may directly interfere with insulin signaling pathways by decreasing insulin receptor tyrosine kinase activity and IRS-1 tyrosine phosphorylation, while inducing phosphorylation of IRS-1 at serine residues, and/or by decreasing the GLUT4 synthesis and translocation [23]. TNF- α rapidly inhibits insulin signaling at the level of PI3K and insulin-stimulated glucose transport in isolated human adipocytes [299]. TNF- α has been associated also with inhibition of glucose uptake in adipocytes from lean individuals [300]. Indirectly, TNF- α could contribute to IR by stimulating lipolysis in human fat cells through several mechanisms (inhibition of anti-lipolytic insulin's action via inactivation of IRS-1, stimulation of basal lipolysis via phosphorylation and decrease of expression of perilipin, or downregulation of CIDEA expression), leading to the increase of FFAs in the circulation, what might further induce IR in other organs [23,301]. In skeletal muscle in animal and culture models, TNF- α may cause IR via similar mechanisms as in AT, involving activation of protein kinase p38 or JNK and IKK, which can phosphorylate IRS-1 on serine residues, probably through diacylglycerol activation of some PKC isoforms. These actions at the end impair insulin action and reduce glucose uptake [224,302,303]. Nevertheless, it has been demonstrated that TNF- α has no direct effect on FA uptake, oxidation, or esterification into TG [304]. Another mechanism by which TNF- α may affect its multiple metabolic effects is the capacity to influence gene expression in metabolically active tissues such as AT and liver [305]. TNF- α could aggravate IR also by counteracting of adiponectin's function in muscle [266]. The potential mechanisms of TNF- α - mediated IR in skeletal muscle and liver are not well documented in humans. One recent study investigating TNF- α and IL-6, demonstrated that TNF- α (not IL-6) is able to increase the expression of the pro-inflammatory cytokine IL-18 in skeletal muscle (not in AT) in humans and presents IL-18 as a factor interacting with TNF- α , that together may mediate IR in skeletal muscle [306] (Figure 6). Targeting TNF- α and its pathways represents a potential therapeutic strategy to decrease FFA circulating levels. However, at the opposite to the promising results in obese rats (decrease in circulating FFA levels) [101], neutralizing TNF- α (infusion of anti-TNF- α antibodies or recombinant TNF receptors) in obese type 2 diabetic patients did not ameliorate high glucose

levels or IR [307,308]. Taken together, clinical investigations are still required to define the role of TNF- α in humans.

TNF- α is a powerful local regulator of adipokine production in AT, standing on the top of the hierarchy of cytokines [91] (Figure 6). TNF- α upregulates leptin production. TNF- α and adiponectin induce local, reciprocal suppression in AT and suppress also each other's function remotely in muscle, thus contributing to the process of IR [266,298]. TNF- α stimulates cellular IKK, which activates the transcription factor NF- κ B, which further increases the production of pro-inflammatory cytokines like IL-1 β , IL-6, interferon and also its own synthesis [309]. As mentioned above, TNF- α has been shown to induce mRNA levels of IL-18, a recently found adipokine associated with IR in humans, *in vivo* in human skeletal muscle [168,306]. On the other hand, IL-18 is also able to stimulate the production of TNF- α , by mononuclear and mesenchymal cells [310]. All of these effects indicate that TNF- α is clearly a pro-inflammatory cytokine and plays a major role in mediating immune responses (Figure 5).

INTERLEUKIN 6 (IL-6)

The original characterization of interleukins as leukocyte-derived proteins with activity on other leukocytes has changed during the time and the term is now used for a broad range of cytokines produced by both immune and non-immune cells with diverse biological activities [246]. IL-6 is a cytokine secreted by numerous cell types like immune cells, fibroblasts, EC, skeletal muscle cells, and AT cells [311]. The majority of AT-derived IL-6 originates from stromal-vascular cells (macrophages and EC) [160] and only about 10% of total IL-6 comes from adipocytes [158]. It is a pleiotropic molecule with widespread multiple effect, e.g. is implicated in inflammation, host defence, tissue injury, or carbohydrate metabolism [312]. IL-6 circulates in multiple glycosylated 22- to 27-kDa forms. IL-6 receptor system is expressed in AT and is homologous to the leptin receptor. Receptor exists either in membrane-bound form as approximately 80-kDa protein (IL-6R), or in about 50-kDa soluble forms (IL-6Rs), arising from proteolytic cleavage of membrane-bound receptor. The biological activities of IL-6 are initiated by its binding to a high-affinity receptor complex consisting of two 80-kDa membrane proteins, which further induces homodimerization of other transmembrane component gp130, which finally triggers intracellular signal transduction cascade of IL-6. IL-6 downstream effects may be potentially mediated by leukemia inhibitory factor (LIF), which is a member of the IL-6

family of cytokines and activates JAK/STAT pathway by inducing tyrosine phosphorylation of JAK1, JAK2, Tyk2, STAT1 and STAT3 [246,269].

Although chronic low-grade systemic inflammation in obesity is characterized by moderate increase of IL-6 plasma concentrations, the same is true for IL-6 as for TNF- α levels, IL-6 amounts in serum are not found to be augmented in all human studies (Table 3). However, contrary to TNF- α , IL-6 is present in the circulation at high levels, and about 30% has been estimated to be derived from white AT [293]. Accordingly, besides local effects, IL-6 exerts systemic functions as well. IL-6 production and systemic concentrations were positively correlated with BMI and percent body fat [143,144,313]. Important modulators of IL-6 expression in different fat depots are glucocorticoids and catecholamines, among others [158,314]. It has been proposed that IL-6 has direct central effects, as its receptors were found in the hypothalamus in mice, and appears to exert anorectic action on the brain resulting in appetite suppression, increased energy expenditure and decreased body fat in rodents [315].

It was suggested that elevated IL-6 might have a causal role in IR, but there is some conflicting evidence and raised IL-6 may be only associative of IR [224]. Plasma IL-6 has been either associated with IR independently of BMI [144], or some works have failed to demonstrate this relationship [316]. Peripheral IL-6 infusion was shown to induce hyperlipidemia, hyperglycemia, and IR in rodents and humans [269,317]. Contradictory to the latter, IL-6 administration did not impair muscle glucose uptake, whole body glucose disposal, and endogenous glucose production in healthy subjects [316]. Additionally, IL-6 infusion in humans *in vivo* stimulated lipolysis and fat oxidation [318]. Also, IL-6- deficient mice were able to develop glucose intolerance and mature-onset obesity, which was reversed by IL-6 replacement [315]. IL-6 can influence IS by distinct mechanisms. Because IL-6 from visceral AT is drained into the liver, the metabolic effects of IL-6 are present also in this organ. One direct mechanism by which IL-6 antagonizes insulin action in 3T3-L1 adipocytes and primary mouse hepatocytes is through the inhibition of insulin-stimulated glucose uptake via decreased activation of IRS-1 and PI3K, downregulation of IRS-1 and GLUT4 mRNA expression, and impaired insulin-induced glycogenesis in liver cells [319,320]. In skeletal muscle in mice, IL-6 blunted glucose disposal and IRS-1- associated PI3K activity, and increased intramuscular fatty acyl-CoA [321]. Increased lipid availability, resulting from increased FA-uptake and decreased FA oxidation, has been proposed as a possible mechanism for IL-6 to mediate IR in obesity.

Indirectly, IL-6 can exert its adverse effects on IS in AT by downregulating LPL activity, increasing lipolysis with ensuing elevation of circulating FFAs [133,259] and, at least in part, by altering the levels of some adipokines (suppression of adiponectin). The possible impact of elevated IL-6 on AT and skeletal muscle is schematically depicted in Figure 6. IL-6 gene polymorphisms were also demonstrated to correlate with IR [322].

There is a feedback loop between IL-6 and TNF- α , TNF- α potently stimulates IL-6 mRNA and secretion from differentiated 3T3-L1 adipocytes [323,324], and IL-6 decreases TNF- α [325]. However, contradictory results have been observed in a study using isolated human adipocytes, where TNF- α did not stimulate IL-6 release [326]. IL-6 downregulates also adiponectin mRNA expression and protein secretion in 3T3-L1 adipocytes, hence might contribute to the genesis of IR [327]. Furthermore, IL-6 was shown to induce its own sustained expression in 3T3-L1 adipocytes [323]. The high IL-6 levels together with TNF- α and IL-1 are likely responsible for the increase in production of hepatic acute-phase proteins, such as CRP, hence provoking the acute-phase response [132]. In addition, IL-6 induces the expression of adhesion molecules by EC [92] (Figure 5). In humans, it has been demonstrated *in vivo*, that the infusion of a low physiological concentration of recombinant human IL-6 did not augment the pro-inflammatory cytokine TNF- α , but elevated the plasma levels of anti-inflammatory cytokines IL-10 and IL-1Ra [328]. Taken together, IL-6 appears to act as both pro- and anti-inflammatory cytokine. Some of the possible adipokine inter-regulations are shown in Figure 6.

INTERLEUKIN 1 beta (IL-1 β)

IL-1 β belongs to the IL-1 family, composed of three related cytokines (IL-1 α , IL-1 β , and IL-1Ra), which are products of different genes. IL-1 α and IL-1 β exert identical pro-inflammatory effects on most cell types, they play a central role in regulating the immune response [132]. IL-1Ra is a specific natural antagonist of IL-1, suppressing its pro-inflammatory activities by binding competitively to IL-1 receptor without inducing a signal [329]. IL-1 α and IL-1 β stimulate IL-1Ra, which is expressed and secreted from human AT, serum levels being markedly elevated in obese individuals and correlated with BMI and IR [149]. IL-1 receptor type I and IL-1 receptor accessory protein, required for IL-1 actions, are expressed in human AT [149]. After binding to its receptor, IL-1 β exerts its biological functions via the IKK/NF- κ B pathway and the three MAPKs (ERK, JNK, p38) [330]. IL-1 β looks to be

produced mainly by monocytes and activated macrophages during the inflammatory process [162].

Generally, the data on IL-1 β (patho)-physiological functions in human AT have only recently become more apparent. The current knowledge on IL-1 β and its role in inflammation and IR in obesity is summarized in Table 3. IL-1 β exerts central effects, inducing anorexia, even more potently than TNF- α , when administered into the brain [135].

Recent findings support the hypothesis that IL-1 β signaling in AT might be implicated into obesity-linked IR. Two current studies report that IL-1 β induced IR in murine and human adipocytes [162,164]. IL-1 β has been shown to decrease insulin-stimulated glucose transport and lipogenesis in human adipocytes via inhibition of tyrosine phosphorylation of insulin receptor and IRS-1 [162], and by decreasing IRS-1 mRNA expression through IL-1 β - activation of the ERK pathway [164]. Moreover, it was demonstrated in the latter study, that IL-1 β has only a modest effect on Glut4 expression, whereas TNF- α and IL-6 are more potent to downregulate its mRNA levels in adipocytes. However, these actions were present only after prolonged IL-1 β treatment and no inhibitory effect on glucose disposal was observed after an acute intervention [164]. The role of IL-1 β in human skeletal muscle is not yet known. IL-1 β signaling in AT is schematically shown in Figure 6. IL-1 β was also shown to increase lipolysis and diminish lipogenesis by downregulation of FATP and fatty acid translocase (FAT) mRNA in AT of Syrian hamsters [331]. In 3T3-L1 adipocytes, IL-1 β decreases LPL expression and activity, and impairs adipocyte differentiation through the inhibition of PPAR γ [332,333].

Importantly, Flower et al. has reported, that IL-1 β is not released as an endocrine signal from AT *in vivo*, but is a local autocrine and paracrine inducer of IL-6 from human adipocytes as well as from peripheral blood cells [326]. Regarding to other cytokine regulations, IL-1 β has been shown to suppress leptin from bone marrow adipocytes, and adiponectin expression and production from human AT explants or adipocytes [162,192,334]. TNF- α , a cytokine also with local effect, was demonstrated to upregulate IL-1 β mRNA levels and protein release from human preadipocytes and adipocytes [153]. Thus, the conceivable concept of cytokine cascade that regulates energy metabolism and immune response in AT during obesity could involve a TNF- α - IL-1 β - IL-6 axis. IL-1 β could also play a role in the increased production of MCP1 [335,336] (Figure 5).

RETINOL BINDING PROTEIN 4 (RBP4)

RBP4 is the specific and sole plasma transport protein of 21-kDa for retinol (vitamin A), which is taken up by hepatocytes from dietary retinoid and stored in the hepatic stellate cells as retinyl esters. According to the body needs, retinol is mobilized from the liver bound to RBP4 and is delivered to the target tissues [337]. Hence, serum RBP4 has been used clinically as a rapid turnover protein for assessing the short-term fluctuation of nutritional states [338]. In addition to the liver, which is the principal source of retinol and circulating RBP4, RBP4 mRNA was found to be present at low levels also in non-hepatic tissues, including kidney, lung, spleen, brain, stomach, small intestine, pancreas, testis and eye [337]. AT became one of the potential important source of RBP4 mobilization and transport after the discovery that RBP4 is strongly expressed in rat AT [215]. Under physiological conditions, RBP4 forms a complex with transthyretin (TTR, formerly called prealbumin), which stabilizes RBP4 in the circulation and prevents its otherwise rapid renal excretion [338]. Within AT, RBP4 is expressed almost exclusively in adipocytes [218,219]. The information on the RBP4 receptors is scarce, the only receptor identified to date in peripheral tissues is megalin/gp320, a low-affinity receptor. A high-affinity RBP4 receptor has not been identified [19].

Levels of RBP4 were reported to be increased in obesity. However, several studies do not support this findings, moreover the trend is opposite for mRNA, its levels were found to be decreased (Table 3). In one study, elevated serum concentrations of RBP4 correlated positively with BMI, serum TG, systolic blood pressure, WHR ratio, waist circumference and percent trunk fat [220].

RBP4 joined a growing list of adipokines implicated in the development of IR only very recently. Despite the evident results in rodents, studies in humans reveal rather profound differences between rodents and humans, and remain equivocal. Several studies reported elevated plasma RBP4 levels in subjects with impaired glucose tolerance and type 2 diabetes mellitus [216,217]. One research team has observed correlations between serum RBP4 levels and the magnitude of IR in different groups of subjects [217,220], whilst this relationship was not found in other cross-sectional studies [218,219,339,340]. The method for measuring circulating RBP4 was discussed in the literature as possible reason of methodological variances and different findings [341]. However, von Eynatten et al. demonstrated a strong correlation between RBP4 concentrations measured by commercial ELISA and the “gold standard”

quantitative western blotting. They support the notion that immunoblot gives higher values of RBP4 levels than ELISA (what may be critical for judging the importance of RBP4 as a valuable clinical marker) and that linearity between the two methods is less pronounced in non-IR control subjects [340]. Studies in mice suggest RBP4 as a factor produced by AT that induces IR in the liver and skeletal muscle [19]. The proposed model was based on the genetic manipulation of GLUT4 expression in AT and tight reciprocal regulation of RBP4 by glucose uptake. Decreased glucose uptake by adipocytes, as a result of genetic knockout of the GLUT4 transporter, results in increased RBP4 expression in AT and serum levels, what further leads to impaired whole-body IS. The latter study showed also that the overexpression of RBP4 or injection of recombinant RBP4 induced IR in mice whereas pharmacologically decreased serum levels of RBP4 improved IS in high fat diet-fed mice. The mechanisms of impaired insulin action are only partially elucidated. In muscle, increased RBP4 impairs insulin signaling by decreasing the phosphorylation of IRS-1 and activity of PI3K. In liver, RBP4 does not alter PI3K, but increases the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose output [342]. The regulation of PEPCK is an example of retinol-dependent mechanism of RBP4-induced IR, characterised by increased production or altered tissue metabolism of the active retinol forms that through the interactions with retinoic acid receptors (RAR) and retinoic acid-X receptors (RXR) regulate gene transcription. Retinol-independent mechanisms of RBP4-induced IR suggest that RBP4 binds to cell surface receptors [19]. A genetic association between single nucleotide polymorphisms (SNPs) in RBP4 gene and IR, impaired insulin secretion, and/or type 2 diabetes mellitus has recently been reported [220]. Graham et al. suggests that serum level of RBP4 is correlated more specifically with IR and changes in IS than with circulating levels of other adipokines such as leptin, adiponectin, IL-6, or CRP [217].

RBP4 does not appear as an adipokine involved in the process of obesity-linked inflammation, as infiltrating macrophages were found unlikely to contribute to RBP4 mRNA in SCAT and VAT, or to circulating levels in IR-humans [220]. The relation of RBP4 to other adipokines is very limited to date and awaits further research.

1.2.4 Ectopic fat storage syndrome

The third theory “ectopic fat storage syndrome”/“ectopic fat deposition”/“acquired lipodystrophy” represents an appealing concept, introduced also as “weapons of lean body mass destruction” [343] and gets recently more and more attention. Similar to the “portal paradigm theory”, it is based on the “lipocentric view” of the pathogenesis of IR. Lipids can easily accumulate in and/or around other tissues and organs than AT, called “ectopic sites” and the theory hypothesizes that this undesirable effect can evoke IR [55]. The latter situation may occur during the chronic energy surplus, typical in obesity, as well as paradoxically in lipodystrophy (lipoatrophy) when lipids accumulate in skeletal muscle, liver, heart and possibly also in kidney and pancreatic β -cells [55,343,344]. Several works studying lipodystrophic patients reported that SCAT is not a prerequisite for IR-development [38]. Whilst a small intracellular lipid stockpile resides in most tissues (as a source of energy in case of need and for the maintenance of cells’ basal functions), the redundant extra-lipids induce dysfunction in lean tissues or programmed cell death what is referred to as lipotoxicity or lipoapoptosis, respectively [345,346]. The first description about ectopic lipid storage appeared in 1964, when it was demonstrated in rat heart and muscle [347]. Simple explanation of the “ectopic fat storage syndrome” is following: AT by means of its unique ability to hypertrophy, is a pre-designed buffer to store the energy excess in the form of TG. The concept is to protect the non-adipose tissues against lipotoxicity. However, when AT becomes insulin resistant probably due to genetic factor, increased lipolysis and FFAs, and/or increased adipocyte size itself, it loses its fat storing monopoly, i.e. it is relatively unable to serve anymore as an “energy sink”. Thus AT re-directs the spillover of incoming FFAs to non-adipose organs, even if it they cannot be easily oxidized [37,39,77].

Intra- as well as extra-myocellular TG content and hepatic steatosis were associated with IR [76,348,349], and myocardial TG levels were shown to increase with increasing BMI [350], but the precise causes and mechanisms that drive ectopic fat accumulation and link with IR are not known. An abnormal redistribution of FA-binding and transport proteins (FAT/CD36, FABP) to the plasma membrane could be one of the possible culprits leading to excessive FA uptake and insufficient oxidation rates with ensuing lipid accumulation in skeletal muscle [351]. Elevated intramuscular triacylglycerol (IMTG) stores in skeletal muscle may be however only a marker of dysfunctional muscle FA metabolism and more reactive lipids (long-

chain fatty acyl-CoA, diacylglycerol, ceramides) accumulating in muscle could play a causative role in IR, via activation of PKC and serine/threonine kinases that inhibit insulin signaling [58,351]. It was also suggested, that IMTG may not deteriorate metabolism in skeletal muscle which exerts high capacity for efficient lipid utilization [352], such as that of highly physically trained athletes, where IMTG was paradoxically found to accumulate in the muscle [353]. The latter findings suggest that the primary defect causing IMTG storage and IR in different IR states could be impaired muscle FA oxidation due to excessive chronic exposure to FFAs [352]. Savage et al. speculates that increased lipid uptake and/or synthesis within the muscle and liver may result in the ectopic lipid deposition in obese and lipodystrophic populations whilst reduced mitochondrial FA oxidation would be the main course for fat accumulation in lean elderly subjects and lean insulin-resistant offsprings of type 2 diabetics [76]. The study on transgenic skinny mice overexpressing leptin showed that not the lack of AT alone, but the deficit of a wide variety molecules secreted from adipocytes may cause IR in lipoatrophy [354]. Hence, one could imagine that local fat depots could take effect on the surrounding tissues through the production of adipokines also in humans and together with possible mechanical impairment of non-adipose organs' functions (by organ compression) could exert influence on IR. Interestingly, Mazurek et al. showed that secretion levels of some adipokines from epicardial AT of defined group of patients were considerably higher when compared with SCAT [355]. Recent advances in identifying the local factors secreted from several anatomic locations of ectopic fat storage with subsequent putative consequences are depicted in Table 4.

Taken together, many unsolved questions remain in order to support this theory, e.g.: “Why some individuals do and others do not deposit lipids in extra-AT locations?”, or “Can VAT represent only a marker of defective fat partitioning?”

Table 4 Specific organ-associated white adipose tissue depots, “ectopic sites” (adapted from Thalmann S [132])

WHITE ADIPOSE TISSUE (locations)	LOCAL FACTORS (secretion)	PUTATIVE CONSEQUENCES
Muscle	TNF- α IL-6 FFAs	IR
Epicardial	IL-6 IL-1 β MCP-1 TNF- α	Local inflammation and chemotaxis
Perivascular	MCP-1 IL-1/IL-1Ra IL-6 IL-8 IP-10 RANTES TNF- α	Atherosclerosis systolic hypertension
Visceral	IL-8 MCP-1 IP-10 RANTES	Local and systemic inflammation

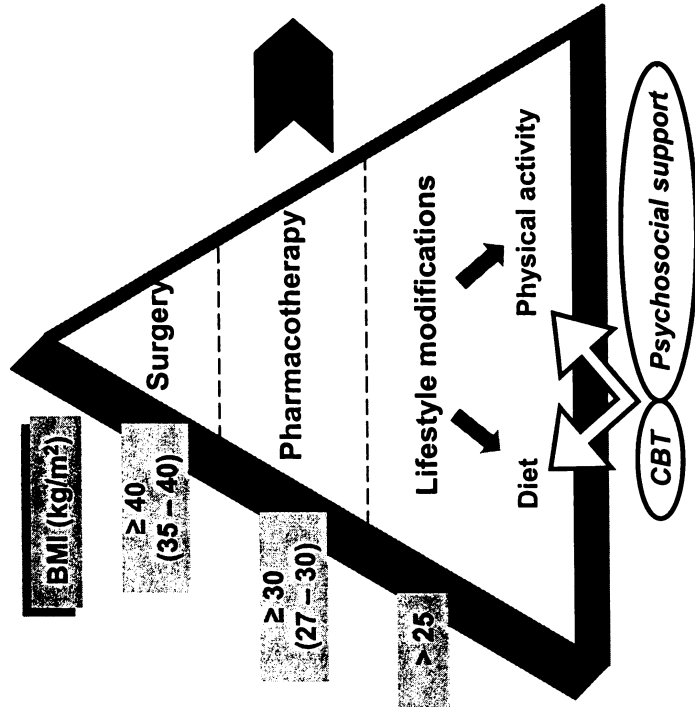
1.3 STRATEGIES TO TACKLE OBESITY AND INSULIN RESISTANCE

Obesity is a complex disease, clearly associated with many chronic non-communicable diseases (type 2 diabetes mellitus, atherosclerosis, hypertension, heart failure, stroke, kidney failure, gallbladder disease, cancer). It represents one of the biggest challenges to the health and quality of life of people in an increasing number of countries. Background of experience shows that most of the abnormalities of AT in obesity are, even if maybe not completely, reversible.

The conventional treatment of patients begins with primary intervention strategies, i.e. non-pharmacological means (diet and regular physical activity), accompanied commonly with cognitive behavioral therapy and psychosocial support. Though both diet and physical exercise were shown to have an important conducive impact in the prevalence of obesity, they are often not very effective and, even after successful loss of weight, most patients regain weight [356,357]. One of the reason is that AT of obese subjects is metabolically less responsive to physiological regulators, such as exercise or fasting/feeding [11]. It has been reported, that only less than 10% of those who lose weight are able to maintain the weight loss [95]. If lifestyle changes alone are insufficient, or fail (usually over 1-2 years period), a secondary interventions, i.e. drug therapy and surgery follows. Drugs can be classified into several categories, basically, into agents to reduce IR or to promote weight loss. In the case of severe/morbid obesity (BMI obese class III), not well controlled by any of previous means, bariatric surgery is the only strategy that can modify the weight and metabolic complications. Therapeutic options in the treatment of obesity and related disorders are depicted in Figure 7.

As emerging from the previous chapters, FA and adipokines are considered to be the main culprits in obesity and IR. Therefore, they could represent targets of new potential therapeutic strategies. Obesity is suggested to be a low-grade systemic inflammatory disease. Owing to this notion, the therapeutic implications focusing on obesity and linked complications in itself may aim also to reduce overall systemic inflammation accompanying obesity and IR. Besides classical pharmacotherapy, non-pharmacological interventions such as dietary and physical activity interventions are suggested to play a significant role in reducing systemic inflammation and/or improving IR and thus be of benefit in obesity. There are promising data that behavioural interventions may be as effective as medications for reducing overall inflammation [358]. In addition, targeting AT lipolysis and decreasing FA levels constitutes also a plausible therapeutic strategy to reverse IR in obesity and linked conditions. Inhibition of HSL, ATGL or TNF- α signaling pathway has likely therapeutic potential. One of the first hypolipidemic drug niacin (nicotinic acid) which has recently obtained a renewed interest because of the cloning of its cognate Gi-coupled receptor [359,360] is based on a similar concept, its mechanism of action is chiefly mediated via inhibition of lipolysis. However, while niacin lowers LDL cholesterol, TG, lipoprotein(a) and raises HDL-cholesterol, its benefits on IR are not clear. Whether inhibition of adipocyte lipases will reduce circulating FFA levels

without increasing fat mass is an important issue that has to be addressed in the future [301]. On the other hand, specific β 3-adrenergic stimulators also belong to a list of potentially perspective pharmaceutical agents. Besides their thermogenic effect they could favourably influence IR in some obese patients. However, clinical tests showed either low efficacy or insufficient specificity of these drugs in humans [361].



Drug class	Mechanisms of action
INSULIN SENSITIZERS TZDs (Rosiglitazone, Pioglitazone) Biguanides (Metformin)	↑ Insulin sensitivity, preservation of β -cell function ↓ Hepatic glucose output
Improvement of insulin secretion Insulin secretagogues Sulfonylureas (Glimperide, Glipizide, Glyburide) Nonsulfonylureas (Nateglinide, Repaglinide)	↑ Insulin secretion
WEIGHT REDUCTION Sibutramine Rimonabant	↑ Feeling of fullness after meal via CNS ↓ Food intake via CB-1 receptor
Reduction of nutrient absorption Orlistat Alpha-glucosidase inhibitors (Acarbose, Miglitol)	↓ Fat absorption in the gut ↓ Glucose absorption in gastrointestinal tract

Figure 7 Therapeutic options in the treatment of obesity and related disorders.

BMI, body mass index; CBT, cognitive-behavior therapy, TZDs, thiazolidinediones, CNS, central nervous system, CB-1, cannabinoid receptor 1

1.3.1 Lifestyle modifications as non-pharmacological means to combat obesity and insulin resistance

1.3.1.1 Dietary interventions in the treatment of obesity and insulin resistance

Diet has two faces, it is an important factor contributing to the development of obesity and IR, and can be also an important factor in controlling or reversing these diseases. Nowadays, the modern diet rich in fats and sugars increases the energy density in foods (i.e. the number of calories in a given volume of food) and promotes an excess of calorie intake with ensuing weight gain. On the other hand, several types of diets were developed for weight management (Table 5), all set for one aim, to lose weight. Diet restriction represents the most common method for obesity reduction [362]. Many works have studied the macronutrient composition in different diets, and it seems that the fat and carbohydrates of hypoenergetic diets are of minor importance for the outcome of dietary treatment [363-366]. It appears that it is still rather the reduction of total amount of calories than dietary composition to be the best way to achieve the loss of weight. Some body of literature highlights the importance of considering the glycemic index (GI) in dietary program. GI, the concept invented by Jenkins et al., is a ranking system for carbohydrates based on their effect on postprandial blood glucose levels [367]. It is an indicator of the ability of different types of foods that contain carbohydrates to raise the blood glucose levels within 2 hours. Carbohydrates that break down rapidly during digestion have the highest glycemic index. Carbohydrates that break down slowly, releasing glucose gradually into the blood stream, have a low glycemic index. Currently, there is no agreement on whether low GI foods are beneficial in weight reduction. The weight loss is a very important initial step, but much more of a challenge is weight maintenance. The result of dieting for many people is ratchet effect/ yoyo effect, i.e. each round of dieting is followed by a rebound of weight to a higher level than before, with higher body fat content and fall of caloric needs, thus making the next round of weight loss harder [368]. However, data regarding the weight maintenance in general, including the type of diet suitable for this period, are limited in the literature.

Apart from the ideal cosmetic impact, sustained weight loss brings along the improvement of many metabolic parameters as well as the decreased risk of complications such as heart disease (Figure 8). It has been proven that even as moderate as a 5-10% weight reduction is enough to produce a clinically positive health outcome in overweight or obese

people [369]. Potentially, the improvement could be the result of reduced visceral fat, as it has been shown that a weight loss of about 10% leads to visceral fat reduction of 30% [370]. The weight loss *per se* is able to improve IS by 30-60%, what is more than by the use of insulin-sensitizing drugs, and this amelioration persists as long as there is no weight relapse [371,372]. However, the mechanisms of diet actions with regard on improvement of the metabolic status are not clearly established. The data inconsistency in the literature makes it difficult to drive simple conclusions. Many clinical trials were performed with different set-ups, as cross-sectional or longitudinal studies with different duration and sample size, investigating single or mixed genders with varying age and BMI levels. A corollary to, notwithstanding the enormous number of existing diets, there is no main dietary method that can be applied universally in all cases to treat obesity or IR. Dietary treatment improves IS and some studies report that it can be influenced by diet composition [371]. The total fat intake was shown to be correlated positively with plasma insulin and negatively with IS [371]. One study has demonstrated that, when total fat intake does not exceed 38% of total energy, then high-monounsaturated-fat diet significantly improves IS compared to a high-saturated-fat diet [371]. Whilst dietary carbohydrate with a high GI increases blood glucose levels, particularly in the postprandial period, the low-GI foods and/or high-fibre have opposite effect; nevertheless the impact on IS is controversial. Other works point out, that moderate low-fat/high-carbohydrate diets rich in fibers could be a good choice regarding to the prevention of diabetes mellitus and CVD risk [373]. Moreover, in addition to diet composition, there are some reports that weight loss is associated with a reduction of several adipokine levels, what can be effective for reducing systemic inflammation in humans and enhance insulin action [224,374].

Table 5 The most common types of diet used for weight management (adapted from International Diabetes Federation (IDF) and International Association for the Study of Obesity (IASO) recommendations, 2004 [375])

DIET TYPE	COMMENTS
Starvation diet	<200 kcal/day; serious medical complications
VLCD (Very Low Calorie Diet)	~800 kcal/day; replace normal foods but supply all essential nutrients; requires medical supervision; ineffective for long-term weight maintenance
LCD (Low Calorie Diet)	~800-1500 kcal/day; use low-fat and high carbohydrate foods; more effective for long-term weight maintenance
Low-Fat Diet	Reduce diet's energy density; high in carbohydrates and proteins; modest weight loss
"Ad libitum" Low-Fat Diet	No restriction of energy intake; high in carbohydrates and proteins; food intake is reduced through satiating effect of fat
"Ad libitum" Low-Carbohydrate Diet	No restriction of energy intake; only 25-30 g of carbohydrates/day and high in fat; rapid initial weight loss (loss of body water) mainly by reducing appetite; in long-term usage is associated with increased risk of CVD

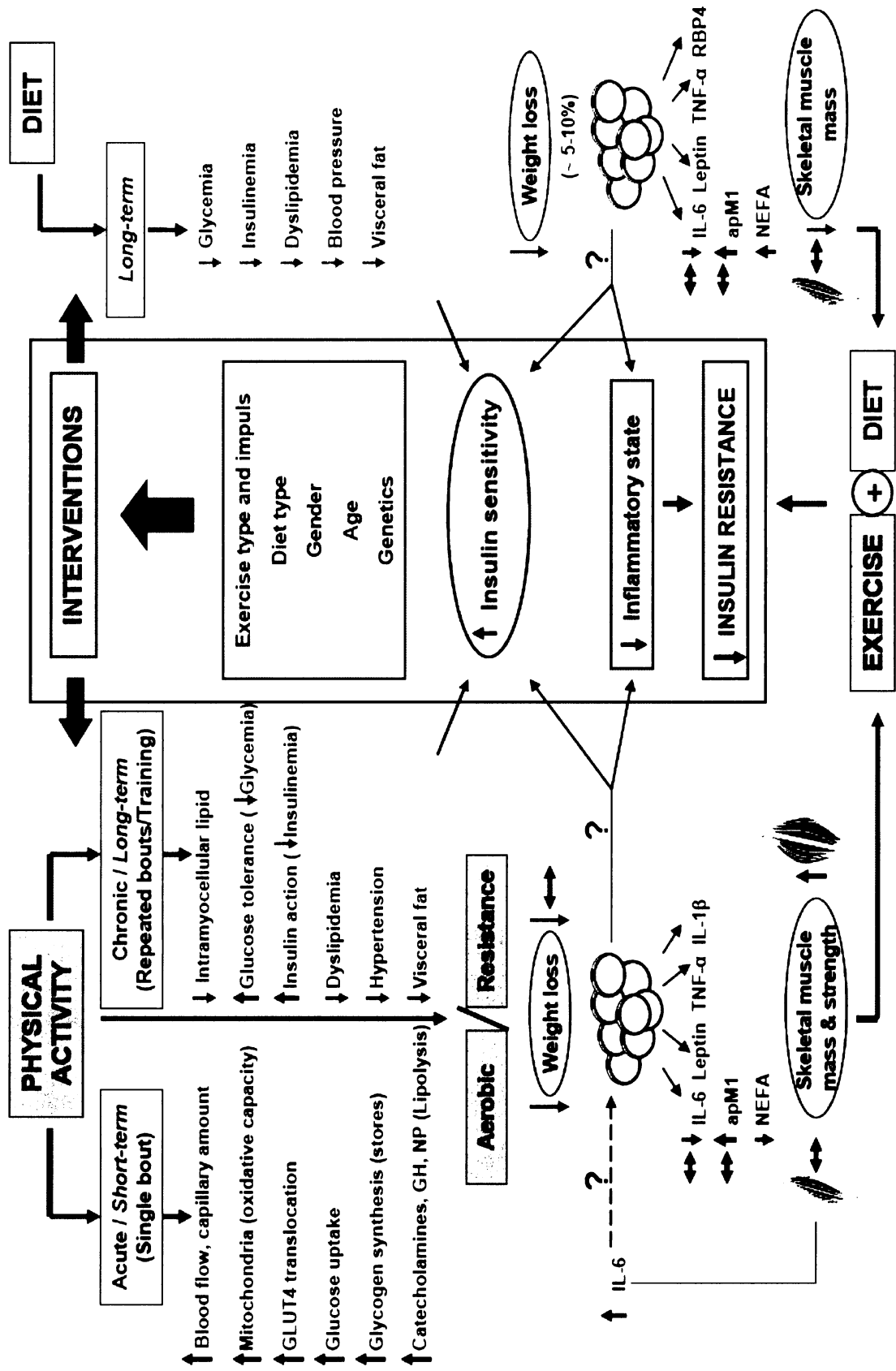


Figure 8 Non-pharmacological means to combat obesity and insulin resistance. Role of physical activity and diet.

Diet and exercise are considered as a cornerstone in the treatment regimen for obesity and associated diseases, such as type 2 diabetes. Diet appears to be more effective than exercise in lowering body weight. The reduction of body weight is more pronounced when diet is combined with aerobic exercise and this combination is the best long-term strategy for controlling body weight. Resistance training usually does not decrease the body weight, but significantly increases skeletal muscle mass and strength. Aerobic training does not influence muscle mass and strength, and diet-induced weight loss seems rather to decrease skeletal muscle mass. Exercise and diet may improve glucose metabolism and IS and may have a major positive impact on the „inflammatory state“ of patients with impaired glucose tolerance or IR via direct effects on AT through modification in adipokine production (downregulation of pro-inflammatory and upregulation of anti-inflammatory cytokines following exercise). A new insight was recently proposed for the role of IL-6 and that is, that IL-6 is a myokine exerting anti-inflammatory effect. It has been suggested that during exercise, muscle produces IL-6, that further plays a role in glucose and fat metabolism and is responsible for the anti-inflammatory effect of regularly performed exercise (discontinuous arrow). The hypothesis is that acute bout of exercise could inhibit the production of pro-inflammatory cytokine TNF- α and IL-1, which are known to impair insulin signaling.

↑ Increase, ↓ decrease, ↔ no change, ? equivocal effect

Sex-related differences and age have not been considered.

AT, adipose tissue; GLUT4, glucose-transporter 4; GH, growth hormone; NP, natriuretic peptides; IS, insulin sensitivity; IR, insulin resistance; IL-6, interleukin 6; IL-1 β , interleukin 1beta; apM1, adiponectin; TNF- α , tumor necrosis factor alpha, NEFA, non-esterified fatty acid.

1.3.1.2 Physical activity in the treatment of obesity and insulin resistance

Physical activity is a key factor in the energy balance equation. Increased physical inactivity is a phenomenon that dates far in the human evolution and is a strong typical hallmark of modern society, especially of obese people. Decreasing physical inactivity is a key factor in the successful treatment of obesity. Regularly performed exercise is also beneficial in the prevention and treatment of type 2 diabetes mellitus, atherosclerosis, hypertension, hyperlipidemia, ischemic heart disease, and some cancers [8,376]. Increasing daily physical activity does not significantly affect the rate of weight loss in the early stages, but it plays an important role in weight maintenance [377]. Generally, under the term exercise one can understand a single bout of exercise (acute exercise), and repeated bouts of exercise (chronic exercise) are referred to as training (exercise training) [378]. Exercise can be classified as either anaerobic (resistance, strength) or aerobic (endurance, dynamic, cardio) [379]. Otherwise, there are other types of exercise such as yoga, stretching, or other sports (mostly intermittent, anaerobic). Aerobic exercise (e.g. distance running) requires oxygen, places demands on the heart and evokes cardiovascular conditioning (improves condition of heart, lungs, all body muscles), and involves mostly the slow twitch oxidative fibres. Aerobic training programs involve large muscle groups in dynamic repetitive activities, designed specifically to increase aerobic endurance performance (usually a minimum of 20 minutes at a minimum of 60% maximum heart rate). Anaerobic exercise (e.g. sprint, weight lifting) does not require oxygen, does not bring about cardiovascular conditioning, and encompasses fast twitch glycolytic fibres. Strength training programs involve strength, weight, static and/or isometric repetitive movements performed against resistance, designed specifically to increase skeletal muscle strength, power, endurance and mass. In response to resistance training, affected muscle enlarges because it generates net protein accretion as their protein synthesis rate exceeds that of protein degradation. The type of exercise is critical to losing weight; aerobic exercise is considered to be more effective in body weight reduction than resistance training and is generally the prescription of choice for the treatment of obesity [377]. In addition to the type of exercise, the beneficial effects of physical activity depend on exercise-training impulse, i.e. volume (usually expressed as energy expenditure per week) x intensity (percentage of peak O₂ consumption [VO_{2peak}] x frequency [380], and exercise duration. Whereas intensity of training is important for the maximal aerobic capacity/maximal oxygen uptake (VO_{2max}) and

improvement of fitness, it looks that it has no impact on weight regulation. According to the American association of sports medicine, minimal training intensity necessary for increasing cardiorespiratory fitness is at the level of 50% VO_2max , and optimal intensity is set at the level of 60-75% VO_2max , depending on the initial body fitness [377]. Exercise of longer duration appears to improve IS more substantially than that of shorter-term, regardless of exercise intensity and volume [381].

Physical inactivity is directly and negatively associated with IR, impaired glucose tolerance and type 2 diabetes mellitus [380]. It is well established that both acute and chronic exercise improve insulin action in skeletal muscle in healthy, obese, IR-subjects, and also in individuals with type 2 diabetes mellitus [380,382] with some minor exceptions. Moreover, the latest studies point out also the fact that both types of exercise training endurance and strength, and not only aerobic training, have the same potential to ameliorate IR. However, several other factors might contribute, such as gender, genetic factors or obesity type for a certain type of exercise [377].

Skeletal muscle is the primary tissue for the peripheral glucose disposal in the human body. Glucose transport into the muscle and GLUT4 translocation to plasma membrane are regulated by insulin and insulin-like factors as well as by an insulin independent mechanism, activated by contractions/exercise, hypoxia, nitric oxide, or bradykinin [383]. There are some indications that the amount of GLUT4 translocation but not its protein level might be the limiting factor for the capacity of skeletal muscle to enhance glucose transport in IR-subjects [384-387]. Acute exercise and exercise training may work through different mechanisms that can help to control and alleviate IR (Figure 8). It has been reported that a single bout of exercise is associated with increased blood flow to skeletal muscle, mitochondrial biogenesis, muscle GLUT4 translocation, IRS-1 tyrosine phosphorylation, glucose uptake into muscle, reduced plasma glucose and insulin levels, while there is no difference in whole body glucose tolerance (oGTT) and glucose disposal, or GLUT4 and IRS1 protein expression [380,383,388]. Interestingly, one of the proposed mechanisms how physical activity can mediate beneficial metabolic and physiological effects is that contracting muscle during exercise can have an impact on other organs (e.g. AT and liver) via some humoral/exercise factor(s)/myokines produced and released into the circulation [389]. Contrary to acute exercise, exercise training leads to an enhancement of oGTT and glucose disposal. Enhanced IS can last for up to 20

hours, decreases within 3 days and is lost within a week, even after a period of long-term training [388]. Other general benefits of regular exercise trainings are reduced visceral fat [372], lowered blood pressure [390] and improved blood lipid profile [391] (Figure 8). The precise mechanisms are not fully known, but besides upregulation of GLUT4 expression and potential increase in GLUT translocation in IR subjects [383] are likely to involve, at least in aerobic exercise, also improvements in muscle oxidative capacity, decreases in muscle lipid content, and increases in whole-body rates of fat oxidation and/or turnover [382].

While the effect of acute and chronic exercise on glucose metabolism in skeletal muscle has been examined to a great extent, our knowledge of their impact on AT glucose metabolism is far more limited and is derived mostly from *in vitro* studies that investigated the effect of endurance training on lipolysis in adipocytes [392]. The principal difference between skeletal muscle and AT is during single bout of exercise; skeletal muscle uses energy for exercise from lipid and glucose influx, whereas AT provides energy by lipolysis. The “hot topic” that currently attracts researchers’ attention is the hypothesis that different types of trainings could decrease inflammation in obesity. The supposed mechanisms are via direct effect on innate immune system (reducing number of mononuclear cells in the blood) and via direct actions in AT (modulation of adipokine production and/or function, reduction of the number of macrophages and EC), thus improve IS [224,358,374]. Of note, acute bouts of exercise appear to have a transient pro-inflammatory effect while regular exercise training might elicit an anti-inflammatory effect [124,393].

1.3.2 Pharmacotherapy of obesity and related disorders

Drug therapy is the secondary intervention strategy to assist weight loss, weight loss maintenance, and/or to treat metabolic disturbances. It is recommended to be considered for patients with a BMI ≥ 30 kg/m² or a BMI 27-30 kg/m² with more than one obesity-related disorder [394]. Pharmacotherapy is an option for overweight and obese individuals in whom lifestyle changes alone failed or were insufficient. Drugs can be classified into several categories, generally into agents to promote weight loss or to reduce IR (Figure 7).

Currently, there are two drugs approved by the United States food and drug administration (U.S. FDA) and in many European countries for long-term use in the treatment of obesity: sibutramine and orlistat. **Sibutramine** (FDA approved in 1997) is a highly selective

inhibitor for the reuptake of serotonin and norepinephrine, and to a lesser degree, dopamine, at nerve endings in the CNS. It increases the feeling of fullness after meals and by its thermogenic effect blocks energy expenditure during very low calorie diet (VLCD). This leads to modest weight loss and helps in weight maintenance. **Orlistat** (FDA approved in 1999) inhibits lipases by binding to the catalytic site serine and reduces lipid absorption by ~30% in gastrointestinal tract. Orlistat is not absorbed in the gut to any significant degree and has little effect in subjects eating a low-fat diet. It produces a negative energy balance, which results in weight loss. Sibutramine and orlistat can be recommended for up to two years use. **Rimonabant** is seeking approval of the FDA. It is a specific antagonist of the cannabinoid 1 (CB-1) receptor, that is widely distributed throughout the body, including brain, fat cells, and the gastrointestinal tract. It inhibits food intake [361,395]. **Acarbose** (alpha-glucosidase inhibitor) delay carbohydrate absorption in the small intestine and reduces energy intake. However, its effect on blood glucose and weight loss in obese patients is modest and currently it is not licensed for use in people who do not have type 2 diabetes mellitus [375]. Of note, short-term clinical trials with antiobesity drugs report only modest weight loss and long-term trials are lacking [396].

Several classes of drugs are used in the treatment of IR, e.g. TZDs, biguanides, or insulin secretagogues. **TZDs** decrease IR by improving IS in skeletal muscle, liver and AT (Figure 9). They also have rejuvenating effect on the pancreatic β -cell, which normally deteriorates in function over time. The net effect of TZDs is to decrease circulating hyperinsulinemia while improving glycemic control. From the broad spectra of **biguanides**, there is practically only metformin in use. Its primary action is to reduce glucose production from the liver. Metformin also has an anorectic effect, helping to curb the weight gain associated with improved glycemic control. It is typically used as a first-line agent in the management of type 2 diabetes mellitus. **Insulin secretagogues** are antidiabetic drugs that work by augmenting insulin secretion and rapid lowering of blood glucose levels. There are two groups, sulfonylureas and nonsulfonylureas. The *sulfonylureas* are one of the oldest classes of antidiabetic agents, and there are many available. The *nonsulfonylureas* are a newer class and compared to sulfonylureas, they have a shorter half-life (shorter duration of action) and reduced risk of hypoglycemia [397,398]. Modern drugs for the treatment of obesity should fulfil several criteria. They should evoke specific reduction of AT (especially VAT), should lead to a weight reduction of at least 5% of initial body weight within a three month-period, should

favourably influence cardiovascular and metabolic risk factors, should be efficacious, safe and non-addictive in the long-term [361]. However, the obesity pipeline, i.e. from discovery to the market, is predominantly (~80%) in an early/discovery-stage.

Generally, one of the major targets for drug development is nuclear hormone receptors. Drugs for the treatment of obesity associated diseases such as type 2 diabetes mellitus, dyslipidemia, or hypercholesterolemia are targeted at PPARs [399]. This aspect will be developed further as it constituted part of my experimental work.

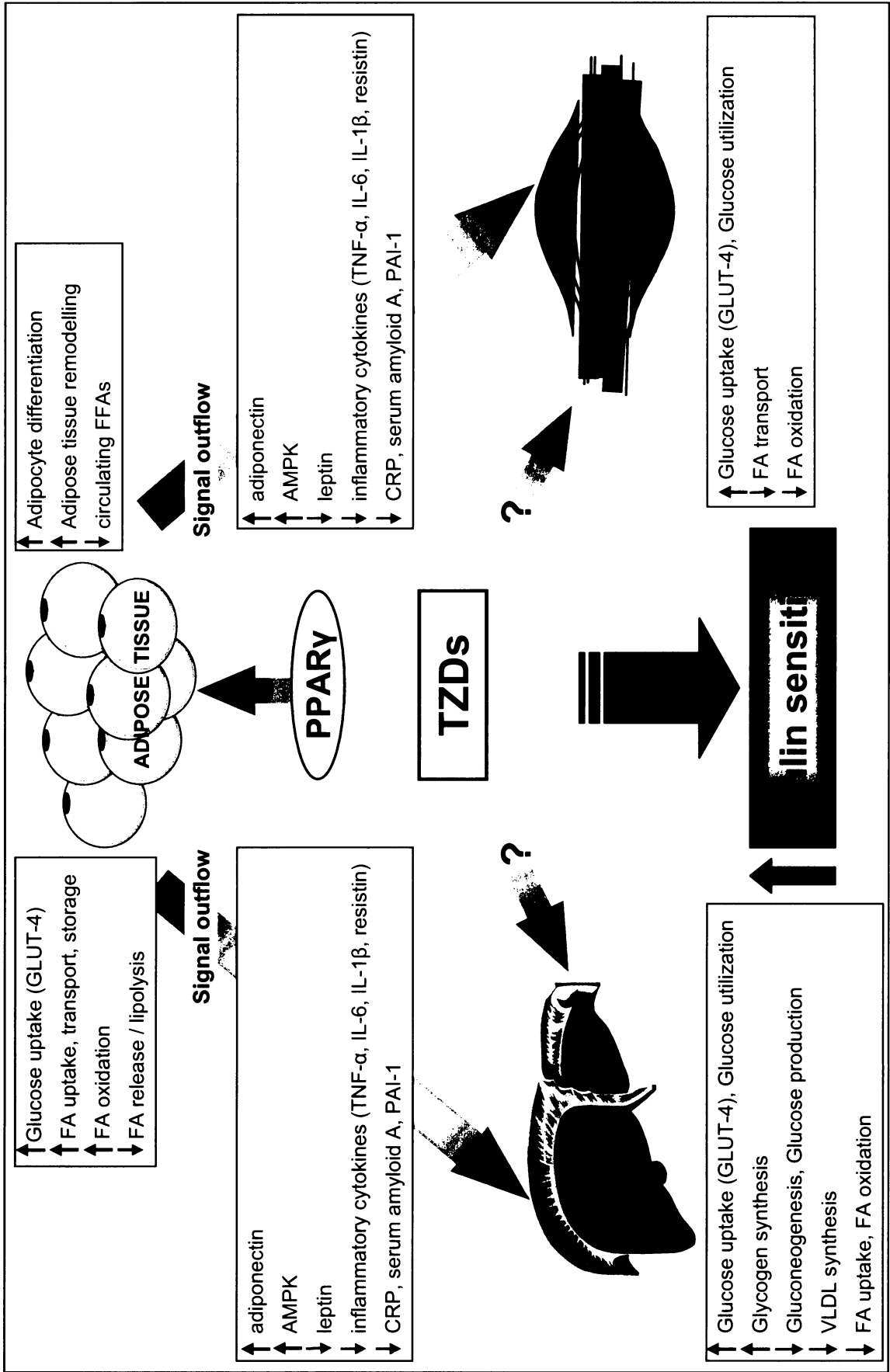


Figure 9 Role of PPAR γ in reducing insulin resistance

(adapted from Sinal Ch (<http://pharmacology.medicine.dal.ca/undergraduate/courses.cfm>), Furnsinn C, Waldhausl W, *Diabetologia* (2002) 45:1211-1223), and Blaschke F et al., *Arterioscler Thromb Vasc Biol* (2006) 26:28-40).

PPAR γ is one of the therapeutic targets for metabolic diseases and molecular target of a class of insulin-sensitizing drugs, TZDs. TZDs are potent PPAR γ -selective ligands, effective for treatment of type 2 diabetes. They require the presence of insulin and amplify its action, thus improve IS in several tissues, such as skeletal muscle, liver, or AT. In addition, they improve plasma lipid parameters (decrease triglycerides and FFA levels, increase HDL) and suppress hepatic glucose output. It appears that TZDs may exert direct and indirect actions to modulate glucose metabolism in insulin-sensitive tissues, predominantly in skeletal muscle and liver (as the two primary tissues responsible for insulin-mediated glucose disposal). TZD can act on muscle and liver directly via PPAR γ and/or other mechanisms independent of PPAR γ , or indirectly via signal output from AT, i.e. PPAR γ -induced mediators from AT. Evidence from animal models indicates that AT is a primary site for the systemic insulin-sensitizing actions of TZDs. Activation of PPAR γ promotes adipocyte differentiation, glucose uptake and FA storage, primarily in SCAT as opposed to VAT. This provides the mechanism for TZD- induced remodelling of AT, and the concurrent redistribution of lipids from VAT into SCAT. PPAR γ agonists alter also endocrine activity of AT by modulating the expression of adipokines that affect insulin signaling in other tissues. They inhibit the release of several inflammatory cytokines, thus decreasing inflammation. TZDs increase both plasma levels of adiponectin (which increases AMPK activity in AT, skeletal muscle and liver) and AMPK itself, what results in increased FFA oxidation and IS.

PPAR, peroxisome proliferator-activated receptor; AT, adipose tissue; IR, insulin resistance; IS, insulin sensitivity; HDL, high-density lipoprotein; FFA, free fatty acid; FA, fatty acid; SCAT, subcutaneous adipose tissue; VAT, visceral adipose tissue, TZD, thiazolidinedione.

1.3.2.1 *Peroxisome proliferator activated receptors*

PPARs are members of the large superfamily of nuclear hormone receptors, which comprises six subfamilies; PPARs belong to the group C in the largest subfamily 1 (NR1C) [400]. The first member of the PPAR family, PPAR α , was discovered in 1990 and from this time PPARs became the most intensively studied members of the nuclear hormone receptor family. As transcription factors, PPARs regulate DNA transcription by binding to the PPAR response elements (PPREs) in the promoters of target genes [401,402]. PPARs activate process of transcription only after binding of ligand on already formed PPAR-RXR heterodimer complex through the recruitment of coactivators. In the absence of ligands, PPARs can repress transcription through the recruitment of corepressors, or by other mechanisms such as by antagonizing other transcription factors, e.g. NF- κ B or activator protein-1 (AP-1) [396]. The efficacy of PPAR to alter gene expression depends on aforementioned factors and on the interplay between them [402]. A trio of PPAR isotypes encoded by separate genes, PPAR α (NR1C1), PPAR β/δ (NR1C2, NUC-1, FAAR) and PPAR γ (NR1C3) have been identified to date [403,404]. Paradoxically, only PPAR α is able to activate gene transcription in response to peroxisome proliferators (e.g. pesticides, phthalates, some hypolipidemic drugs), while the two other members are not [399].

PPARs great potency, different ligand-activation profiles and diverse cell, tissue and organ distribution gives them a broad range of physiological functions (reviewed in Ref. [396,401,405-407] (Figure 10). After identification of FA and FA-derived metabolites as endogenous ligands for all three of the PPARs, the prevalent view on their function is that they are implicated in energy homeostasis and act as lipid sensors [406,408]. Despite an intensive research, our understanding of PPAR roles in AT is far from being complete. PPAR γ expressed at high levels in AT controls adipogenesis and IS [409,410]. PPAR α regulates lipid catabolism in highly oxidative tissues but its role in AT has not been documented [411]. PPAR β/δ is the most ubiquitously distributed isotype. It is suggested to have a broad function, but remains the least well understood and further studies are required.

Besides natural ligands, which have very low affinity [412], many potent synthetic ligands have been identified to bind and activate PPAR isotypes (Figure 11). They represent an invaluable tool in investigating the molecular mechanisms of PPAR action in regard to discovery of new efficient drugs to target adiposity. However, the employment of a variety of

physiological and pharmacological agonists, that substantially differ also between synthetic PPAR agonists itself (e.g. different selectivity and affinity), makes difficult to establish an unequivocal conclusions determining PPAR functions. Only two classes of synthetic PPAR activators are in clinical use with some limitations, TZDs targeting PPAR γ (Figure 9) and fibrates as ligands for PPAR α . They are used for the treatment of type 2 diabetes mellitus and hyperlipidemia to improve IS and plasma lipid profile [413]. The first TZD was troglitazone but was withdrawn from the market because of its rare but detrimental hepatic side effects (hepatotoxicity). At present, two other TZDs are clinically available, rosiglitazone and pioglitazone that are not toxic to the liver, but have some other side effects such as weight gain and edema. Ligands for PPAR β/δ are currently under clinical development, and there is a first recent study reporting that the pharmacological PPAR β/δ agonist GW501516 was administered to healthy man and enhanced fat clearance in the circulation [414]. Recently, synthetic dual PPARs (a single ligand activating both γ and α) and panPPARs (activating all, α , γ , β/δ) are also emerging [396]. Taken together, the identification of specific, more efficacious and safer PPAR agonists as potential candidates for treatment of obesity and associated diseases is still being a big challenge.

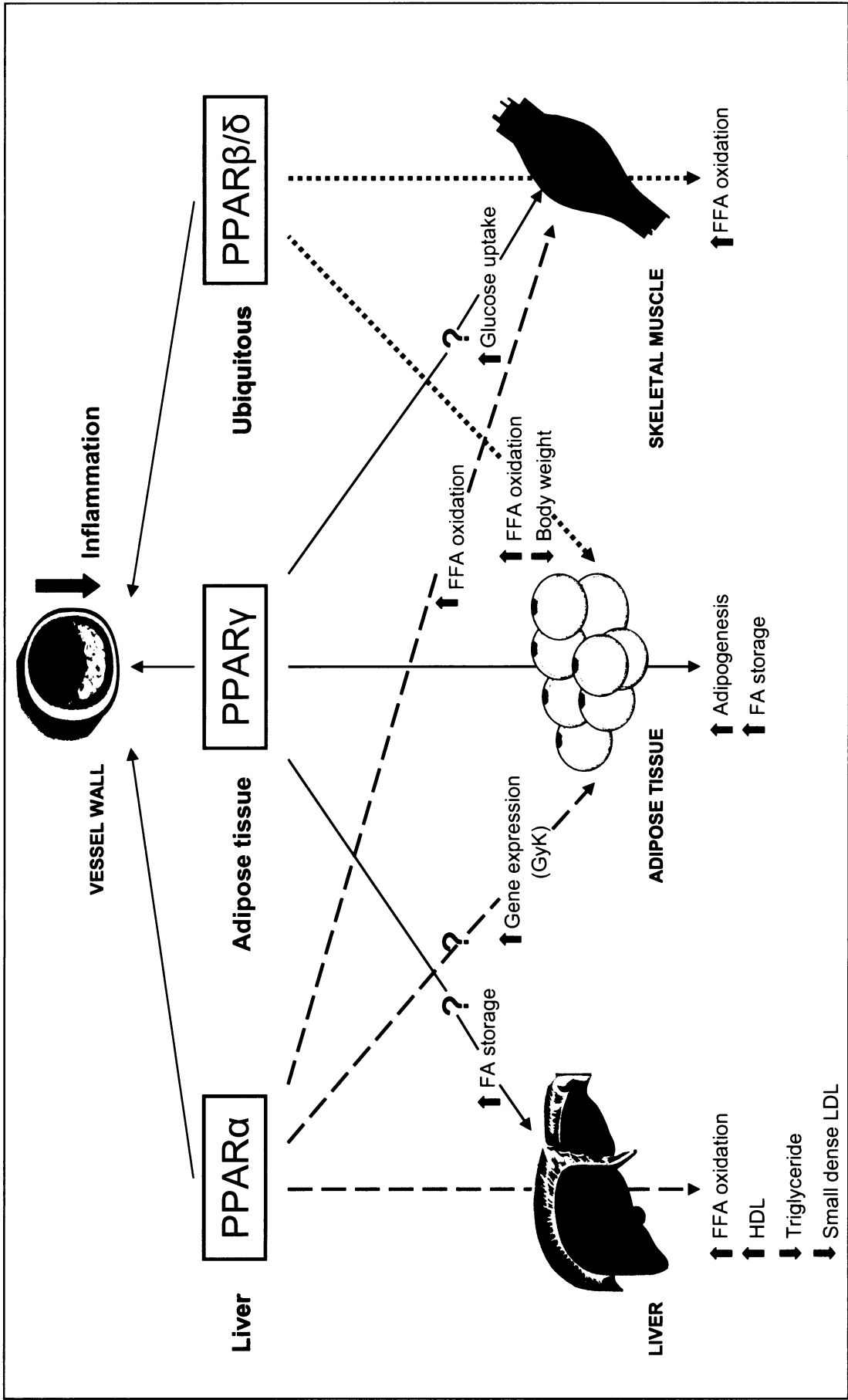


Figure 10 Pharmacological means to combat obesity and insulin resistance. Role of peroxisome proliferator activated receptors (adapted from Blaschke F et al., *Arterioscler Thromb Vasc Biol* (2006) 26:28-40).

All three of the PPAR isotypes (PPAR α , PPAR β/δ and PPAR γ) can participate in the regulation of inflammatory responses. They appear to exert anti-inflammatory properties, which might lead to the improvement of IR. The mechanism of action is possibly via suppression of activation of inflammatory genes by negatively interfering with the NF κ B, STAT-1, and AP-1 signaling pathways, and/or via inducing expression of genes involved in catabolism of pro-inflammatory lipid mediators. PPAR α functions as an important regulator of lipid metabolism and energy homeostasis; stimulates the uptake and oxidation of FA in liver and skeletal muscle for energy production, thereby diminishing circulating TG and LDL, and increasing HDL levels. Recent evidence from our laboratory uncovers that PPAR α may be directly involved in regulating white fat metabolism. PPAR γ has pivotal role in adipogenesis and lipogenesis in AT and PPAR γ -dependent lipid accumulation may occur also in other tissues, such as liver. PPAR γ activation may also interfere with glucose signaling and improve IS in skeletal muscle, AT and liver, and reduce hyperglycemia. Precise role for PPAR β/δ still remains to be elucidated, but it appears that it is implicated in fat-burning and thermogenesis; it increases FA oxidation and uncouples energy metabolism in skeletal muscle and potentially also in AT, thus suggesting PPAR β/δ agonists as candidates for treatment of obesity and linked disturbances.

AT, adipose tissue; PPAR, peroxisome proliferator-activated receptor; FFA, free fatty acid; FA, fatty acid; GyK, glycerol kinase; HDL, high-density lipoprotein; LDL, low-density lipoprotein; IR, insulin resistance; IS, insulin sensitivity; NF κ B, nuclear factor-kappa B; STAT-1, signal transducers and activators of transcription; AP-1, activator protein-1; TG, triglycerides.

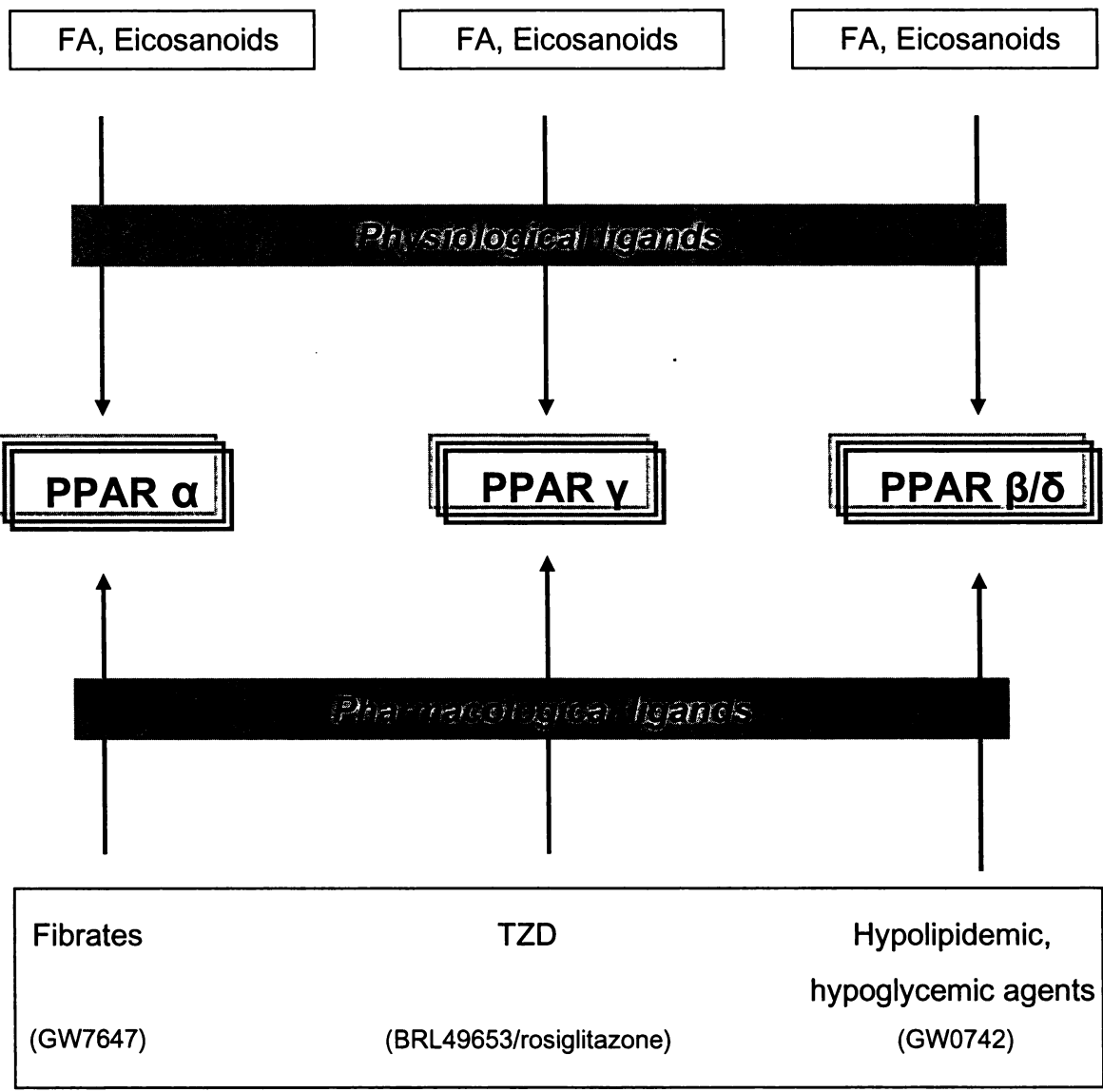


Figure 11 Ligands of peroxisome proliferator activated receptors.

PPAR, peroxisome proliferator-activated receptor; FA, fatty acid; TZD, thiazolidinedione

1.3.3 Other strategies to combat obesity and insulin resistance

Among secondary interventions for the treatment of obesity and metabolic complications beside drug therapy are strategies such as cognitive-behavior therapy (CBT) and surgery.

CBT has become a standard component of most treatment programs, including obesity treatment. The literature on CBT for obesity is extensive but elusive. CBT, rather than a specific intervention, it is a systemic application of principles of social cognitive theory to modify behaviors that are thought to contribute to or maintain obesity. There are several strategies in CBT (e.g. self-monitoring, goal setting, stimulus control) that were found to be helpful in improving short-term weight loss, but they are not very effective in the long-term. Nevertheless, these therapeutic techniques appear as a good predictor of long-term sustainable weight loss [415].

Surgery as a mean for the weight loss is indicated usually for people with severe obesity (BMI \geq 40). Bariatric surgery offers an effective, standardized and compared to other surgical operations in non-obese patients also a relatively safe way to produce weight loss and maintain the reduced weight in the long-term. One of the most commonly performed bariatric procedures is the restriction operation on the stomach, i.e. vertical gastroplasty and gastric banding. Other means of procedures are gastric bypass surgery (GBS) or biliopancreatic diversion (BPD), but these are more invasive and limited to a certain category of patients. The aim of bariatric surgery is to increase the feeling of fullness after meals by gastric banding or to decrease the absorption of nutrients by by-passing various sections of the gut [416].

Liposuction (lipoplasty, suction-assisted lipectomy) is another surgical technique of fat removal for aesthetical reasons but has been proposed also as a potential treatment for the metabolic complications of obesity. Klein et al. reported an absence of effects of abdominal liposuction on cardiovascular factors, inflammatory markers, and IR in obese women [417]. However, most studies report that liposuction is associated with significant amelioration of IS and inflammatory status in obese patients, reducing cardiovascular risk [418]. In general, metabolic effects of abdominal liposuction have been investigated in a handful of studies and usually on a small number of subjects, therefore it is difficult to make a clear conclusion.

CHAPTER 2

AIMS

Obesity promotes states of both chronic low-grade systemic inflammation and IR. Currently, the most appealing concept is that expanding AT *per se* initiates the inflammatory response that further contributes to local and peripheral IR and other metabolic complications such as impaired glucose tolerance or type 2 diabetes mellitus. It is clear that one of the mechanisms of action in inflammation-mediated IR is the inhibition of insulin receptor signaling pathways, nevertheless other pathways are likely to exist. Why and how it happens are questions still to be unravelled. The “hot” candidates that may trigger inflammation and IR are adipokines secreted from different AT cell types, including lipid-laden adipocytes, recruited activated macrophages or EC.

The first indications of association between inflammation and obesity or type 2 diabetes mellitus date to the late 1950s and the 1960s, when the levels of fibrinogen and other acute-phase reactants were reported to be increased [419-421]. However, the breakthrough came in 1993 with the discovery that TNF- α produced by AT is able to induce IR in obese animals. This finding started a new era in the obesity research and linked inflammation to the pathogenesis of IR. The early findings of increased levels of acute-phase reactants and markers of inflammation in obese states have been later confirmed, extended for other molecules and still nowadays, the list of bioactive adipokines implicated into the development of IR is not complete and the role in IR not fully understood.

On the one hand, it is important to understand which and how adipokines produced by AT can initiate the inflammatory process, which cell type is their main site of production, or how increasing fat mass leads to the recruitment of immune cells into AT, among others. On the other hand, it is of great clinical importance to elucidate if and how the latter could be modulated by interventional strategies (primary or secondary) that are known to improve whole-body IS in humans. Therefore, the overall aim of this thesis was to investigate the

mechanisms of changes in AT (metabolic and secretory characteristics) during different non-pharmacological interventions in different groups of obese subjects and in an *in vitro* study using pharmacological approach.

2.1 FIRST PART

The first part of my work encompasses three different clinical studies, which were designed as non-pharmacological longitudinal research studies. Both types of lifestyle interventions were applied, exercise training and dietary weight loss program. Regular physical activity and energy-restricted diets are recognized as effective therapies for improving the whole-body IS and there are suggestions that they could be effective in reducing overall inflammation. One of the possible mechanisms by which increased physical activity and weight loss could ameliorate inflammatory state and IS is via a decrease of pro-inflammatory and an increase of anti-inflammatory cytokine levels in plasma through modulation of their production in AT. It is conceivable, that chronic physical activity and dietary restriction might elicit local anti-inflammatory effect that may or may not be evident in the systemic circulation.

Each of the behavioural interventions is characterized by different designs with different impact and mechanisms of action but targeting especially one goal, to improve IS. According to the latest data, this can be achieved with or without weight reduction. However, the amount of weight loss may play a role for clinically meaningful reductions in inflammation. Hence, from the clinical point of view, there are several important aspects to consider, e.g. which type of training would be the best choice for a certain population (different gender, age, genetics), what would be the right duration and intensity of training, or which type of diet would be the most suitable to reduce and sustain the body weight. These questions are awaiting to be answered as is the major question, whether any of the lifestyle interventions should be used as a treatment for chronic inflammation.

The general objective of these studies was to investigate the potential role of SCAT (specifically the role of adipokines produced by SCAT) in the etiopathogenesis of IR. The main aim was to investigate the link between aerobic training, dynamic strength training or dietary intervention of relatively long durations (3 months), and IS in different groups of obese subjects. More precisely, our objective was to investigate whether the insulin sensitizing effect of lifestyle modifications is associated with changes in mRNA and plasma levels of adipokines

potentially involved in the regulation of IS and/or the proinflammatory status of the body. The attention was particularly paid to adiponectin, leptin, IL-6, IL-1 β , and RBP4. By the application of both types of exercise, we could investigate the above mentioned hypothesis in a model when the body weight is reduced (aerobic exercise program) or maintained (dynamic strength training). Compared to physical training, nutritional intervention is supposed to be more effective in the weight reduction. Thus, we designed a dietary intervention trial, where besides the weight-loss period, we could evaluate for the first time the adaptations also during a weight maintenance period (3 to 4 months). The widespread view by the general public is that aerobic exercise is effective in weight loss, hence is attractive and especially appreciated by women. Indeed, aerobic training is the predominant mode of regular physical activity advised for the treatment of obesity. Therefore, it appeared logical to motivate and recruit obese women in an aerobic exercise program. Strength training is a type of physical activity preferred by men, therefore they were recruited in a dynamic strength training program. Moreover, this type of exercise attracts more and more attention thanks to findings of its beneficial health-effect, and also becomes increasingly popular in obese subjects and type 2 diabetic patients as it may be easily practised in a rising number of fitness centers.

2.2 SECOND PART

The second part of my work was designed as *in vitro* study using a pharmacological approach. Well-characterized nuclear receptors PPAR γ and PPAR α are therapeutic targets for IR and hypertriglyceridemia, respectively, and drugs that modulate these receptors are currently in clinical practice. PPAR β/δ is a less described isotype but recent preclinical data suggest that PPAR β/δ agonists as well might be important candidates for the treatment of obesity, IR and dyslipidemia, and are presently clinically tested. In order to shed light upon the potential connection between PPARs and obesity, it is necessary to elucidate the roles of PPARs in AT. One of the fields that has not rigorously been examined and is of high importance, is the complex information on proteome of human AT affected by PPAR agonists. Therefore, the overall objective was to have a closer insight into the role of the three known PPAR isotypes in the regulation of human AT secretome. Our aim was to carry out a protein expression profiling in human subcutaneous fat interstitial fluid and to identify the effect of three potent PPAR agonists on expression of adipokines secreted by AT explants. From the large amount of

proteins produced by AT, we wished to discover the profile of new adipokines regulated by PPARs. The model of AT explants offers the unique possibility to study whole tissue secretion, i.e. from the different cell types present in the tissue. All these cell types generate factors regulating tissue remodelling, immune function or energy homeostasis in paracrine and endocrine manner. Given that the majority of AT-secreted products is produced by nonfat cells, the sub-objective of the study was to determine the cellular origin of adipokines that were found to be regulated by PPAR agonists.

CHAPTER 3

RESULTS

3.1 FIRST PART

IMPACT OF LIFESTYLE INTERVENTIONS ON THE REGULATION OF EXPRESSION OF ADIPOKINES AS PUTATIVE MEDIATORS OF CHANGES IN OVERALL INFLAMMATION AND INSULIN SENSITIVITY IN OBESE SUBJECTS



Effect of aerobic training on plasma levels and subcutaneous abdominal adipose tissue gene expression of adiponectin, leptin, interleukin 6, and tumor necrosis factor alpha in obese women.

Polak J, Klimcakova E, Moro C, Viguerie N, Berlan M, Hejnova J, Richterova B, Kraus I, Langin D, Stich V. *Metabolism*. 2006 Oct;55(10):1375-81.



Dynamic strength training improves insulin sensitivity without altering plasma levels and gene expression of adipokines in subcutaneous adipose tissue in obese men.

Klimcakova E, Polak J, Moro C, Hejnova J, Majercik M, Viguerie N, Berlan M, Langin D, Stich V. *J Clin Endocrinol Metab*. 2006 Dec;91(12):5107-12.



Plasma levels and adipose tissue messenger ribonucleic acid expression of retinol-binding protein 4 are reduced during calorie restriction in obese subjects but are not related to diet-induced changes in insulin sensitivity.

Vitkova M*, Klimcakova E*, Kovacikova M, Valle C, Moro C, Polak J, Hanacek J, Capel F, Viguerie N, Richterova B, Bajzova M, Hejnova J, Stich V, Langin D. *J Clin Endocrinol Metab*. 2007 Jun;92(6):2330-5.

* These authors contributed equally to the work.

3.1.1 Comment on the first part

The search for the pathophysiological mechanisms involved in the development of obesity and associated co-morbidities has undergone a dramatical shift during the last several years stimulated by the finding that obese states are likely to be low-grade inflammatory states. While lifestyle interventions have been considered as the primary strategies in the prevention and treatment of obesity and fortunately also as good strategies to control hyperglycemia and reduce the risk of type 2 diabetes mellitus, they have not been considered to be able to reduce systemic inflammation and/or ameliorate IR until recently. Thanks to the technological progress, the scientific community has moved from descriptive studies to studies investigating mechanisms whereby exercise or diet can impact on inflammation or IR. The research of today puts in the forefront of such potential mechanisms cytokines and other factors produced by AT. The first part of this thesis contains three non-pharmacological clinical studies, the results of which and discussions are presented in detail in the respective publications. Here, I present the synopsis and additional comments on our findings.

♣ **In the first study, we investigated the effect of 3 months of intensive aerobic training (5 times/week) on plasma and mRNA levels of adiponectin, leptin, TNF- α and IL-6 in SCAT in a group of 25 obese women.** The conventional recommendations for physical activity duration, frequency and intensity in the treatment of obesity are 45 minutes, 3-4 times/week, at the level of 50-70% of VO_2max [377]. The data on the amount of weight reduction induced by regular exercise training of aerobic type vary in the literature as they may depend on the above mentioned factors. Generally, very intensive programs of physical training (500-800 min/week) were shown to decrease the body weight notably, 5-7 kg within 8-16 weeks. By the application of less-intensive programs (90-200 min/week with intensity of 50-70% of VO_2max), the weight reductions reached at the most 3 kg in the period of 8-24 weeks. In a rare longitudinal physical exercise intervention lasting for 18 months, the participants lost as much as about 7 kg, but it has been markedly associated with the intensity and duration of physical activity [377]. The participants in our study decreased their body weight by 6% (~ 5 kg), what appears reasonable for this close to conventional type of training with higher frequency. The frequency has been set at intensity corresponding to 50% of individual VO_2max at the start and progressively increased to 55%, 60%, and 65% VO_2max every 3 weeks. As expected, endurance training resulted in an increase in aerobic fitness by 13% in our group of

premenopausal obese females. Body fat mass and waist circumference were significantly reduced by 6% and 4%, respectively, as well as plasma FFAs by 54%. Other indices of lipid metabolism were not changed during the physical exercise program. Fasting insulin and glucose levels remained unchanged by aerobic training, nevertheless IS measured by revised quantitative insulin sensitivity check index (rQUICKI) increased by 12%. rQUICKI takes into account plasma FFAs and shows a stronger relation with parameters measured by the euglycemic hyperinsulinemic clamp than QUICKI or HOMA indices [422,423]. In our study, we focused our attention to determine the impact of aerobic exercise training on adiponectin, leptin, TNF- α , and IL-6, i.e. adipokines supposedly involved in the regulation of lipid and carbohydrate metabolism and in the development of IR. Except for decreased plasma leptin concentrations (by 26%), our results showed that investigated adipokines were not influenced by this type of training, neither plasma, nor their mRNA levels. To further investigate the relationship between studied adipokines and IR, correlations between their mRNA or plasma levels and indices of IR were performed, but no associations were observed.



Effect of aerobic training on plasma levels and subcutaneous abdominal adipose tissue gene expression of adiponectin, leptin, interleukin 6, and tumor necrosis factor α in obese women

Jan Polak^{a,c,*}, Eva Klimcakova^{a,b,c}, Cedric Moro^{a,b}, Nathalie Viguerie^{a,b}, Michel Berlan^{a,e}, Jindriska Hejnova^{a,c}, Blanka Richterova^{a,c}, Ivan Kraus^d, Dominique Langin^{a,b}, Vladimir Stich^{a,c}

^aFranco-Czech Laboratory for Clinical Research on Obesity, Third Medical Faculty and INSERM Unite 586, Charles University, Prague, Czech Republic

^bObesity Research Unit (Institut National de la Sante et de la Recherche Medicale, Unite 586), Institut Louis Bugnard, Centre Hospitalier, University of Toulouse, Universite' Paul Sabatier, 31403 Toulouse, France

^cDepartment of Sport Medicine, Third Faculty of Medicine, Charles University, 10000 Prague, Czech Republic

^dGynaecology Department, Third Faculty of Medicine, Charles University, 10000 Prague, Czech Republic

^eLaboratory of Medical and Clinical Pharmacology, Faculty of Medicine, Purpan Hospital, 31073 Toulouse, France

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Abstract

Adipocytokines secreted by adipose tissue are suggested to play a role in the development of obesity-related complications. Regular aerobic exercise has been shown to reduce the risk of metabolic complications in obese subjects. The aim of this study was to investigate the effect of aerobic training on gene expression in subcutaneous abdominal adipose tissue (SCAAT) and on plasma levels of several adipocytokines in obese women. Twenty-five obese sedentary premenopausal women (body mass index, $32.18 \pm 3.17 \text{ kg/m}^2$) underwent a 12-week aerobic exercise program, with a frequency of 5 d/wk and intensity corresponding to 50% of individual maximal oxygen consumption ($\dot{V}O_2\text{max}$) consisting of 2 sessions per week of supervised aerobic exercise and 3 sessions per week of home-based exercise on a bicycle ergometer. Before and after the aerobic training, $\dot{V}O_2\text{max}$ and body composition were measured and plasma and SCAAT biopsy samples (in a subgroup of 8 subjects) were obtained for determination of plasma and messenger RNA levels of adipocytokines (leptin, adiponectin, interleukin 6, tumor necrosis factor α). The aerobic training resulted in an increase of subjects' $\dot{V}O_2\text{max}$ by 12.8% (24.6 ± 3.9 vs $27.7 \pm 4.8 \text{ mL} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, $P < .05$). Body weight and fat mass were reduced by 5.9% (88.5 ± 8.2 vs $83.3 \pm 7.7 \text{ kg}$, $P < .001$) and 6.4% ($38.8 \pm 4.2\%$ vs $36.3 \pm 4.6\%$, $P < .001$), respectively, and the revised QUantitative Insulin sensitivity Check Index (QUICKI) increased (0.43 ± 0.06 vs 0.48 ± 0.06 , $P < .05$) during the aerobic training. No aerobic training-induced changes in messenger RNA levels of the investigated genes in SCAAT were observed. A decrease of plasma leptin (24.3 ± 8.7 vs $18.1 \pm 8.3 \text{ ng/mL}$, $P < .05$) was detected, whereas plasma levels of other cytokines remained unchanged. In moderately obese females, 3 months' aerobic training did not promote changes in the adipose tissue gene expression or plasma levels of the adipocytokines (except for leptin) involved in a regulation of lipid and carbohydrate metabolism.

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1. Introduction

Obesity is known to be associated with a whole body pro-inflammatory state and a number of metabolic disturbances included in the metabolic syndrome. In the search for the mechanism of association between obesity and metabolic syndrome, a role for the adipocytokines secreted by

adipose tissue has been suggested. Plasma levels and the expression of adipocytokines in adipose tissue are modified in obese subjects [1-3]. The adipocytokines bring about a number of metabolic actions that might induce or prevent obesity-related metabolic disturbances. Adiponectin increases glucose uptake and fatty acid oxidation by muscle [4] and reduces hepatic gluconeogenesis [5]. A large part of these effects are mediated by the activation of adenosine monophosphate-activated protein kinase(s). Infusion of adiponectin together with leptin reversed insulin resistance in a lipotrophic mouse model [6]. Tumor necrosis factor α

* Corresponding author. Oddeleni Telovychovneho Lekarstvi, Ruska 87, 100 34, Praha 10, Czech Republic. Tel.: +42 731181599, +42 267102210; fax: +42 267102263.

E-mail address: jan.polak@totes.hyperlink.cz (J. Polak).

(TNF- α) induced insulin resistance in animals and in an *in vitro* model [7] by interacting with insulin receptor signaling and through activation of lipolysis and inhibition of lipoprotein lipase, the 2 effects concurring to cause increased plasma free fatty acid (FFA) levels [8,9]. Subcutaneous abdominal adipose tissue may not contribute to plasma TNF- α level as no net release from this area was detected [9]. Recently, TNF- α was proposed to have preferentially paracrine effects and to be a regulator of insulin resistance at the tissue level [10]. Association of interleukin 6 (IL-6) with fasting glucose, fasting insulin, and insulin sensitivity was observed in cross-sectional studies [2,11]. Its role in the development of insulin resistance is not completely clear as infusion of IL-6 at doses not higher than those achieved during exercise with stable concentrations of counterregulatory hormones results in unchanged glycemia and induces lipolysis in adipose tissue [12,13].

Aerobic training is considered to be a key part of the therapy for obesity, and numerous studies have shown improvement in the metabolic and cardiovascular status of obese subjects after an aerobic training period. It might be hypothesized that the effect of aerobic training on adipocytokine production and secretion mediates some of the beneficial effects of aerobic training in obesity. Recently, several studies have investigated the effects of the aerobic training on plasma levels of several adipocytokines in different groups of patients. Variable effects of physical training on plasma levels of adiponectin [12,14,15], tumor necrosis factor [16–18], and IL-6 [16,17,19] were found. Moreover, some of the adipocytokines such as TNF- α or IL-6 are also secreted by other tissues in addition to adipose tissue. Consequently, the plasma levels of adipocytokines are not a straightforward reflection of training-induced effects on their production in adipose tissue.

As recently pointed out [20,21], prospective studies of various types of treatment of obesity are necessary to clarify the effect of aerobic training on chronic, systemic inflammation. The aerobic training program is the predominant mode of regular exercise activity used in obese women. Therefore, the aim of this study was to investigate the effect of an intensive (5 days/wk) aerobic training program on plasma and messenger RNA (mRNA) levels of adipocytokines in subcutaneous adipose tissue (SCAAT) in obese female subjects.

2. Subjects and methods

2.1. Subjects

Twenty-five obese premenopausal women (age, 40.4 ± 6.7 years; weight, 88.5 ± 8.2 kg; body mass index [BMI], 32.2 ± 2.2 kg/m²) were included in the study. None of women had any chronic disease, and all were free of any medication. Pregnancy was excluded at the beginning of the study. All the subjects had been sedentary before this aerobic training program for at least 1 year. The body weight

of the subjects had not changed by more than 2 kg during the 3 months preceding the study. They all gave written informed consent before the experiments began. The study was performed according to the Declaration of Helsinki and approved by the ethical committee of the Third Faculty of Medicine, Charles University (Prague, Czech Republic).

2.2. Training program and maximal oxygen consumption determination

Five days before each investigation, maximum exercise test was performed on a bicycle ergometer (Ergoline 800, Ergoline GmbH, Bitz, Germany) in each subject to determine the maximal oxygen consumption ($\dot{V}O_2\text{max}$) (V_{max} , Sensor Medics, Yorba Linda, CA). An initial work rate of 60 W was followed by a sequential increase in work rate by 25 W every minute until exhaustion. Verbal encouragement was given to attain maximal performance. Heart rate was monitored continuously. Two criteria assessed that the subjects achieved their true $\dot{V}O_2\text{max}$ [22]. The maximal heart rate corresponded to that predicted for the given age (179.6 ± 6.34 beats per minute) before (180.6 ± 9.20 beats per minute; range, 165–205 beats per minute) as well as after (180.8 ± 10.36 beats per minute; range, 167–207 beats per minute) the training, and the achieved respiratory quotient was equal to 1.089 ± 0.025 (range, 1.03–1.15) before and to 1.090 ± 0.0271 (range, 1.01–1.16) after the training. Calibration check of the spiroergometry system has been performed every week using the commercially available ethanol combustion testing method.

The aerobic training program lasted for 12 weeks and consisted of sessions of aerobic exercise 5 d/wk: (1) twice a week aerobic exercise performed in gymnasium and supervised by an exercise instructor and (2) 3 times a week home-based exercise on an electrically braked bicycle ergometer. Subjects were instructed to exercise at each session for 45 minutes at the intensity corresponding to the individually recommended target heart rates; the individual target heart rate was determined as that corresponding to 50% of individual $\dot{V}O_2\text{max}$ as measured during a maximum exercise test.

Each participant was provided with a cardiometer (Polar Accurex Plus Cardiometer, Polar Electric Oy, Kempele, Finland) to check the actual heart rate during exercise sessions. Throughout the program, the intensity of exercise was progressively increased to target heart rates corresponding to 55%, 60%, and 65% $\dot{V}O_2\text{max}$, respectively, every 3 weeks. The compliance to the home-based part of the training was found to be good as checked by exercise instructors using training diaries of each subject.

2.3. Experimental protocol

Subjects were investigated at 08:00 AM after an overnight fast in a semirecumbent position before and 72 hours after the last day (to eliminate possible effects of the last bout of exercise) of a 12-week aerobic training program. After a 30-minute rest in a semirecumbent

position, blood samples were drawn from an indwelling venous catheter. In a subgroup of 8 women, a needle biopsy of abdominal SCAAT was performed 15 to 20 cm laterally from the umbilicus, as described before [23].

2.4. Dietary regimen

Before the initial examination and throughout the aerobic training program, patients were instructed to maintain their habitual diet. The dietary intake was estimated by a 7-day food record before the beginning of the study. The maintenance of the dietary regimen was controlled every 4 weeks of the study using a 3-day food record. Adherence to the habitual diet was confirmed by the analysis of these 3-day food records.

2.5. Body composition assessment

Body composition was assessed in fasting condition using dual-energy x-ray absorptiometry performed with a whole-body scanner (Hologic, Siemens, Waltham, MA, the scanner being calibrated daily with Phantom Model DPA/QDR-1). All the measurements were performed during the morning hours (08:00 to 11:00 am), with a difference of maximum 2 hours between the entry and final examination, by the same technician ascribed specifically to this study.

2.6. Messenger RNA analysis

The biopsies of abdominal SCAAT (about 1 g) were used for mRNA quantification. The samples were washed, homogenized in RLT lysis buffer (Qiagen, Courtaboeuf, France) and stored at -80°C until analysis. Total RNA was extracted using the RNeasy total RNA Mini kit (Qiagen). Integrity of RNA was checked on agarose gel, and RNA concentration was determined using a fluorometric assay (Ribogreen, Fluoroskan Ascent, Thermo Electron Corp, Waltham, MA). Reverse transcription was performed with 250 ng of total RNA using random hexamers as primers and Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France). Real-time quantitative polymerase chain reaction (qPCR) was performed on a GeneAmp 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). A set of primers was designed for adiponectin and leptin using the software Primer Express 1.5 (Applied Biosystems). For each primer pair, a standard curve was obtained using serial dilutions of human adipose tissue complementary DNA before mRNA quantification. For verification that genomic DNA was not amplified, qPCR was performed on reverse transcription reactions with no addition of reverse transcriptase. Primers and probes for IL-6 and TNF- α were obtained from Applied Biosystems. Each sample was taken in duplicate and 10 ng of complementary DNA was used as a template for real-time polymerase chain reaction. When the difference between the duplicates was more than 0.5 Ct, qPCR was performed again. We used 18S ribosomal RNA (Ribosomal RNA Control TaqMan Assay kit, Applied Biosystems) as control to normalize gene expression.

2.7. Blood analysis

Plasma glucose was determined with a glucose oxidase technique (Biotrol kit, Merck-Clevenot, Nogent-sur-Marne, France) (coefficient of variation [CV], 1.1%–2.0%) and FFA by an enzymatic procedure (Wako kit, Unipath, Dardilly, France) (CV, 2.7%). Plasma insulin concentrations were measured using radioimmunoassay kits from Sanofi Diagnostics Pasteur (Marnes la Coquette, France) (CV, 2.8%–4%).

Plasma triglycerides were determined by spectrophotometry. Adiponectin and leptin plasma levels were determined using radioimmunoassay kits from Linco Research (St Charles, MO) (CV for adiponectin kit, 9.3%; leptin, 3.4%–8.3%) according to the manufacturer's recommendations. Interleukin 6 and TNF- α plasma levels were determined using Quantikine IL-6 (CV 2%–4.2%) and Quantikine High Sensitivity TNF- α kits (CV, 4.6%–5.2%) (R&D Systems, Minneapolis, MN), respectively. The above-mentioned measurements were done in duplicates. Coefficients of variability in our laboratory were in the range of those indicated by kit producers (2%–9%) according to the analyzed substance.

2.8. Statistical analysis

All values are mean \pm SD. All analyses were performed using SPSS 12.0 for Windows (SPSS, Chicago, IL). Response of gene expression data was analyzed by a Wilcoxon signed rank test. Correlations were examined by the Pearson correlation coefficient. $P < .05$ was considered statistically significant. The homeostasis model assessment (HOMA) index was computed following this equation: [fasting glycemia (mmol/L) \cdot fasting insulin (mIU/L)]/22.5. The revised QUantitative Insulin sensitivity ChecK Index (QUICKI) was computed following this equation: $1/\log[\text{insulin } (\mu\text{IU/mL})] + \log[\text{glucose (mg/dL)}] + \log[\text{NEFA (mmol/L)}]$ [24].

Fold change in the gene expression analysis was calculated for the increasing changes by dividing relative mRNA level values after the intervention by values before the intervention and, for the decreasing changes, by dividing relative mRNA level values before the intervention by values after the intervention.

3. Results

3.1. Anthropometric and biochemical characteristics

Anthropometric, physical fitness, and related biochemical characteristics before and after the aerobic training program are shown in Table 1. An increase in aerobic fitness by 12.8% (24.59 vs 27.74 mL \cdot min $^{-1}$ \cdot kg $^{-1}$, $P < .05$) as evaluated by $\dot{V}\text{O}_2\text{max}$ was achieved during the aerobic training program. Body weight decreased by 5.9%, fat mass by 6.52%, and waist circumference by 3.9%. Indices of lipid metabolism remained unchanged except for plasma FFA that decreased after physical intervention.

Table 1
Anthropometric and biochemical characteristics of subjects before and after a 12-week aerobic training program

	Before training	After training	P
Body weight (kg)	88.5 ± 8.2	83.3 ± 7.7	<.001
BMI (kg/m ²)	32.2 ± 2.2	30.4 ± 2.4	<.001
Fat mass (%)	38.8 ± 4.2	36.3 ± 4.6	<.001
Waist circumference (cm)	92.9 ± 7.0	89.3 ± 6.4	<.05
Hip circumference (cm)	114.5 ± 5.8	111.0 ± 5.9	<.001
Waist-hip ratio	0.81 ± 0.04	0.79 ± 0.04	.09
VO ₂ max (mL · kg ⁻¹ · min ⁻¹)	24.6 ± 3.9	27.7 ± 4.8	<.05
Fasting glucose (mmol/L)	5.0 ± 0.5	5.1 ± 0.3	NS
Fasting insulin (mIU/L)	6.0 ± 3.9	5.5 ± 2.1	NS
Total cholesterol (mmol/L)	5.14 ± 0.9	5.09 ± 0.9	NS
HDL cholesterol (mmol/L)	1.4 ± 0.3	1.4 ± 0.4	NS
Triglycerides (mmol/L)	1.33 ± 0.7	1.30 ± 0.52	NS
FFAs (μmol/L)	670 ± 413	308 ± 128	<.05
HOMA	1.4 ± 0.9	1.2 ± 0.5	NS
rQUICKI	0.43 ± 0.06	0.48 ± 0.06	<.05

Data are presented as mean ± SD (N = 25). NS indicates not significant; HDL, high-density lipoprotein; rQUICKI, revised QUICKI.

3.2. Insulin sensitivity indices

The aerobic training did not influence fasting insulin (6.0 ± 3.9 vs 5.5 ± 2.1 mIU/L, *P* > .05) or fasting glucose level (5.0 ± 0.5 vs 5.1 ± 0.3 mmol/L, *P* > .05). Congruently with this finding, the HOMA index has not changed. Nonetheless, the revised QUICKI index, which takes into account plasma FFA levels, increased by 11.6% during the physical exercise program (0.43 ± 0.06 vs 0.48 ± 0.06, *P* < .05). Data are summarized in Table 1.

3.3. Adipocytokine plasma levels

Plasma leptin declined after the aerobic training program by 25.7% (*P* < .001). No change in plasma adiponectin, TNF-α, and IL-6 was observed. Data are summarized in Table 2.

When leptin plasma levels before and after the training program were adjusted to BMI or fat mass, the aerobic training-induced changes in leptin remained significant, suggesting independent effects of aerobic training on plasma leptin besides reduction of body fat. Plasma leptin, after adjustment to BMI, was reduced by 22.1% and after adjustment to fat mass by 14.6%.

3.4. Expression of adipocytokines in SCAAT

Gene expression was assessed by determining relative mRNA level for adiponectin, leptin, IL-6, and TNF-α in

Table 2
Plasma levels of adipocytokines before and after 12-week aerobic training program

	Before training	After training	P
Adiponectin (μg/mL)	10.9 ± 6.1	10.0 ± 4.4	NS
Leptin (ng/mL)	24.3 ± 8.7	18.1 ± 8.3	<.001
TNF-α (pg/mL)	6.1 ± 7.6	4.8 ± 4.5	.08
IL-6 (pg/mL)	3.1 ± 3.7	1.4 ± 1.5	NS

Data are presented as mean ± SD (N = 25).

SCAAT. No changes in mRNA in SCAAT for any of the studied cytokines were detected. Data are summarized in Table 3. It is to be noted that, with the number of biopsies analyzed in this study (n = 8), the power analysis shows the minimum detectable fold decrease is 1.9 for leptin, 2.03 for TNF-α, 1.9 for IL-6, and fold increase 1.58 for adiponectin.

3.5. Association of gene expression in SCAAT with plasma levels of adipocytokines and anthropometric variables

A marked association between TNF-α expression and IL-6 expression (*r* = 0.821, *P* = .023) as well as between TNF-α expression and plasma IL-6 level (*r* = 0.811, *P* = .027) was apparent at the beginning of the study. Adiponectin gene expression was positively associated with adiponectin plasma levels (*r* = 0.811, *P* < .05) and negatively associated with plasma TNF-α (*r* = -0.9, *P* < .05) at the end of the study. Plasma levels of IL-6, leptin, and TNF-α were not associated with their gene expression in subcutaneous adipose tissue either at the beginning or at the end of the study. No correlation between mRNA level and anthropometric variables (weight, BMI, fat mass, waist circumference) or indices of insulin resistance was observed for any of the adipocytokines.

3.6. Association of plasma levels of adipocytokines with anthropometric variables and indices of insulin resistance or metabolic syndrome

Plasma adiponectin concentration showed a close negative association with adiposity (*r* = -0.629, *P* < .05), FFA (*r* = -0.562, *P* < .05), and glycerol (*r* = -0.715, *P* < .05) at the beginning of the study. These correlations were not present at the end of the study. Plasma TNF-α was closely related to adiposity of subjects before and after aerobic training (*r* = 0.527 and *r* = 0.438, respectively, *P* < .05). No significant associations between plasma levels of adipocytokines (adiponectin, leptin, IL-6, or TNF-α) and any of the indices of metabolic syndrome (HOMA, revised QUICKI, fasting glucose, plasma triglycerides, waist circumference, high-density lipoprotein cholesterol, or blood pressure) were observed before or after aerobic training. However, changes in plasma adiponectin were

Table 3
Relative mRNA levels before and after 12-week aerobic training program (data are expressed as arbitrary units obtained after normalization by the 18S ribosomal RNA subunit)

	Percent change	Fold change	P	Minimum detectable fold change
TNF-α	-53.6	Fold decrease 1.49	NS	2.03
Adiponectin	+18.9	Fold increase 1.18	NS	1.58
IL-6	-3.1	Fold decrease 1.03	NS	1.9
Leptin	-32.1	Fold decrease 1.4	NS	1.9

Fold change in the gene expression analysis was calculated for the increasing changes by dividing relative mRNA level values after the intervention by values before the intervention and, for the decreasing changes, by dividing relative mRNA level values before the intervention by values after the intervention (N = 8).

significantly associated with changes in BMI, body weight, glycerol, and FFA ($r = -0.509$, $r = -0.494$, $r = -0.562$, $r = -0.775$, $P < .05$, for FFA $P = .07$). On the other hand, no association between aerobic training-induced changes in plasma adiponectin, leptin, IL-6, or TNF- α , and indices of insulin resistance (plasma insulin, glucose, HOMA, revised QUICKI), body weight, waist circumference, or adiposity were detectable.

4. Discussion

The main aim of this study was to investigate the effect of aerobic training on adipose tissue gene expression and plasma levels of a number of adipocytokines that might play a role in the pathogenesis of metabolic disturbances and the pro-inflammatory state in obese subjects. We focused our attention on adiponectin, leptin, TNF- α , and IL-6 as modifications of plasma, and SCAAT mRNA levels of these adipocytokines have been described in obese subjects [1,23].

In the present study, the aerobic training program induced a decrease of body weight, BMI, body adiposity, and waist circumference. Fasting insulin and fasting glucose levels were not changed by aerobic training, whereas it did promote a marked decrease of fasting plasma FFAs. Aerobic training induced a change in insulin sensitivity when evaluated by the revised QUICKI index; nevertheless, no changes in other indices of insulin resistance such as HOMA or QUICKI were observed. It has been shown previously that the revised QUICKI index, which takes into account the level of plasma FFAs, has a stronger relationship with parameters measured by euglycemic hyperinsulinemic clamp than QUICKI or HOMA indices alone [24,25].

Our results show that aerobic training with mild weight loss did not change plasma adiponectin levels. Studies on the effect of aerobic training on plasma adiponectin have, to date, provided conflicting data. Six months aerobic training under stable body weight had no effect on plasma adiponectin [26] despite decreased plasma insulin levels and increased insulin sensitivity [12,14]. Opposite effects have been published as well—increased plasma adiponectin and insulin sensitivity without changes in body weight [15] or with moderate weight reduction [27]. Such a controversy might partly be explained by the influence of other exercise-induced substances that might negatively influence the production or release of adiponectin from adipocytes. Among these, catecholamines and TNF- α have been shown to decrease adiponectin gene expression in visceral adipose tissue [28,29]. The plasma noradrenaline concentration was higher at the end of our study (215.3 ± 112.9 vs 296.6 ± 92.2 pg/mL, $P = .016$), which suggests that adiponectin gene expression was inhibited (or remained unchanged) due to catecholamine stimulation, thus preventing significant changes in plasma adiponectin.

A strong negative association between pretraining plasma adiponectin and plasma FFA (and glycerol) levels as well as between changes in plasma adiponectin and changes in FFA

during aerobic training was observed. A negative association between adiponectin and FFA was previously reported [30], and similarly to our study, this relationship disappeared after the intervention period with the very low-energy diet. Adiponectin intracellular signaling is connected with activation of adenosine monophosphate kinase [4], which has been previously shown to attenuate β -adrenergic stimulation of lipolysis in fat tissue and muscle [31].

Here, we observed neither a change in plasma IL-6 nor a change in IL-6 gene expression in SCAAT. It has been shown that short-term bouts of exercise increase both plasma IL-6 levels as well as IL-6 gene expression in muscle and adipose tissue of healthy subjects [32]. However, longer-term endurance training might have the opposite effect as shown by diminished exercise-induced elevation of muscle IL-6 gene expression and plasma IL-6 after endurance training [33]. Similar data were observed in a group of patients with chronic heart failure or coronary heart disease where plasma IL-6 decreased after 12 weeks or remained unchanged after 6 months of aerobic exercise [16,17]. No significant associations between plasma IL-6 and anthropometric parameters or indices of insulin sensitivity observed in other studies [2,11,34] were observed here.

Expression of IL-6 in SCAAT as well as plasma IL-6 level were closely associated with expression of TNF- α in SCAAT suggesting that, in the adipose tissue, paracrine interactions play an important role. Similar relationships between IL-6 and TNF- α have already been published by other groups [34,35] as well as an association between TNF- α plasma level and adiponectin gene expression [36]. The ability of TNF- α to activate the transcription of IL-6 gene through activation of nuclear factor κ B has been proposed [37].

Contradictory findings exist in literature describing reduction [16] as well as no change [2,17,18] of plasma TNF- α induced by diet or physical activity. Tumor necrosis factor α is predominantly secreted by adipose tissue macrophages [38] and is rather considered to be involved in paracrine regulations without being significantly secreted into the circulation [39].

In conclusion, modifications of anthropometric parameters (waist circumference, BMI, fat mass) and of a revised index of insulin resistance induced by aerobic training were not associated with significant changes in gene expression of adiponectin, leptin, TNF- α , or IL-6 in SCAAT. In plasma, only the leptin concentration decreased. The lack of any training-induced change in other circulating adipocytokines in the present study might be associated with the specific type and duration of the training in this study as well as with the sex and degree of obesity of the subjects. Further studies with different populations and longer training periods are warranted. Moreover, the response of adipocytokines to aerobic training might be influenced by interactions between adipocytokines as demonstrated by associations between TNF- α and IL-6 gene expression and plasma IL-6 and adiponectin concentration.

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♠♠ In the second study, we investigated the impact of 3 months of dynamic strength training (3 times/week) on plasma and mRNA levels of adiponectin, leptin, TNF- α , IL-6 and IL-1 β in SCAT in a group of 12 obese men. It is presumed that this type of training keeps the body weight and cardiorespiratory fitness unchanged, while it increases skeletal muscle mass and strength [377]. Congruently, we confirmed in our study that indices of muscle strength represented by bench and leg press markedly increased by 33% and 32%, respectively, but we have not observed a significant increase in lean body mass. Resistance training decreased blood pressure in our group of subjects, systolic by 9% and diastolic by 12%. Most of other anthropometric and clinical data were not altered. As expected, strength training had positive impact on insulin action in obese men. Evaluated by euglycemic-hyperinsulinemic clamp, the whole-body glucose disposal and IS calculated by SI_{Clamp} significantly increased by 24% and 31%, respectively. SI_{Clamp} is one of the indices of IS measured by the clamp technique [424]. It has been suggested that strength training could be more effective in amelioration of IR than aerobic physical activity. One of the hypothesis why it could be so, operates with the idea that the defect for IR is primarily located in skeletal muscle and therefore, resistance training targeting directly the muscles could improve skeletal muscle metabolism rather than cardiovascular conditioning [373]. Our study has not been aimed to compare aerobic versus resistance training in order to define their impact on IS. We hypothesized that AT is the main culprit in IR genesis, and similar to the first study, we investigated whether the improvement in IR by dynamic strength training may be associated with training-induced decrease in pro-inflammatory state via modulation of adiponectin, leptin, TNF- α , IL-6 and IL-1 β expression. Plasma and mRNA levels were not found to be affected by strength training for all adipokines but plasma leptin, which concentration was diminished by 21%. A good biomarker that reflects low-grade chronic inflammation is CRP [358], therefore was included and measured in plasma samples in our subjects. The baseline CRP levels indicated the low-grade systemic inflammation and high cardiovascular risk in our group of obese men, but no significant change in CRP concentration was detected at the end of training program. Similarly, no changes in CRP after 12 weeks exercise training without weight loss has been recently demonstrated [425]. When searching for the associations between adipokines and improved IS, we found no relation of their expression (mRNA or protein levels) to IS, except for the negative correlation between TNF- α mRNA and glucose disposal rate before the training program.

Dynamic Strength Training Improves Insulin Sensitivity without Altering Plasma Levels and Gene Expression of Adipokines in Subcutaneous Adipose Tissue in Obese Men

E. Klimcakova, J. Polak, C. Moro, J. Hejnova, M. Majercik, N. Viguerie, M. Berlan, D. Langin, and V. Stich

Franco-Czech Laboratory for Clinical Research on Obesity (E.K., J.P., C.M., J.H., N.V., M.B., D.L., V.S.), Institut National de la Santé et de la Recherche Médicale, 3rd Faculty of Medicine, and Department of Sports Medicine (E.K., J.P., J.H., M.M., V.S.), 3rd Faculty of Medicine, Charles University, CZ-100 00 Prague, Czech Republic; Institut National de la Santé et de la Recherche Médicale (E.K., C.M., N.V., M.B., D.L.), U586, Obesity Research Unit, and Louis Bugnard Institute IFR31 (C.M., N.V., M.B., D.L.), Paul Sabatier University, F-31432 Toulouse, France; Faculty of Medicine Purpan (C.M., M.B.), Laboratory of Medical and Clinical Pharmacology, F-31000 Toulouse, France; and Centre Hospitalier Universitaire Toulouse (D.L.), Laboratory of Biochemistry, Purpan Institute of Biology, F-31059 Toulouse, France

Context: Obesity is characterized by a low-grade inflammatory state, which could play a role in insulin resistance. Dynamic strength training improves insulin sensitivity.

Objective: The objective of this study was to investigate, in obese subjects, whether the insulin sensitizing effect of dynamic strength training is associated with changes in plasma levels and gene expression of adipokines potentially involved in the development of insulin resistance.

Design: Twelve obese male subjects were investigated before and at the end of 3 months of dynamic strength training. Insulin sensitivity was evaluated using euglycemic-hyperinsulinemic clamp. Blood samples and needle biopsy samples of sc abdominal adipose tissue were obtained. The plasma levels and adipose tissue mRNA levels of adiponectin, leptin, IL-1 β , IL-6, and TNF- α were determined.

Results: The training induced an increase in the whole-body glucose disposal rate by 24% ($P = 0.04$). The body weight was not altered during the training. Plasma levels of leptin decreased during the training (16.6 ± 6.3 vs. 13.1 ± 5.7 ng/ml) by 21% ($P < 0.02$), whereas no change in plasma levels of other adipokines and C-reactive protein was observed. Gene expression of the investigated adipokines was not changed in sc adipose tissue during the training.

Conclusions: In obese subjects, the dynamic strength training resulted in an improvement of whole-body insulin sensitivity. The increase in insulin sensitivity was not associated with training-induced modifications of plasma levels or adipose tissue gene expression of adipokines supposedly involved in the development of insulin resistance. (*J Clin Endocrinol Metab* 91: 5107–5112, 2006)

REGULAR PHYSICAL ACTIVITY is recognized as an effective nonpharmacological intervention with beneficial effect on insulin sensitivity and glucose tolerance in healthy, obese (1), insulin resistant (2), and type 2 diabetic individuals (3). Strength training (also called resistance training) is known to improve insulin sensitivity as is aerobic exercise training, although potentially via different mechanisms (4).

Obesity, as generally accepted now, is characterized by a low-grade inflammatory state that leads to insulin resistance and development of metabolic diseases such as diabetes and cardiovascular diseases (5). Chronic low-grade systemic inflammation is characterized by a 2- to 3-fold increase of

TNF- α , IL-1, IL-6, IL-1 receptor antagonist, soluble TNF- α receptors (sTNF-Rs), and C-reactive protein plasma concentrations (6). Higher plasma IL-6 and TNF- α mRNA and protein secretion from adipose tissue are associated with obesity and/or insulin resistance (7). They are assumed to play a role in the mechanism of insulin resistance. Adiponectin and leptin are secreted by adipocytes and may act like physiological insulin sensitizers. The suggested mechanisms of insulin sensitizing action of adiponectin are the suppression of hepatic glucose production (8) and the increase of fatty acid oxidation by skeletal muscle, thereby reducing plasma free fatty acid levels (9). Adiponectin stimulates fatty-acid oxidation and glucose uptake in adipocytes and muscle by activating 5'-AMP-activated protein kinase (10). Leptin also operates through activation of 5'-AMP-activated protein kinase and improves fatty acid oxidation in muscle (11). Little is known about the peripheral physiological effects of adiponectin and leptin in humans. Stefan *et al.* (12) suggested that physiological concentrations of fasting plasma adiponectin are not related to fat oxidation or energy expenditure in resting conditions in humans. A recent study of Blaak *et al.* (13) showed that serum leptin was negatively related to

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Abbreviations: BMI, Body mass index; hsCRP, high-sensitivity C-reactive protein; qPCR, quantitative PCR; 1-RM, one-repetition maximum strength; SCAAT, sc abdominal adipose tissue; SI_{Clamp}, clamp-derived index of insulin sensitivity; sTNF-R, soluble TNF- α receptor; VO_{2max}, maximal oxygen consumption.

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fasting fat oxidation. As shown recently, there is an increased amount of macrophages in the stroma vascular fraction of adipose tissue in obese subjects (14, 15). A substantial number of inflammatory genes in the stromavascular fraction of adipose tissue are expressed in macrophages, and it is hypothesized that the presence of resident macrophages is a reason for the inflammatory profile observed in sc (14) or visceral (15) adipose tissue of morbidly obese subjects.

The effects of strength training on insulin sensitivity in different populations have been examined already in a number of studies (16–18). There are a few studies indicating that exercise training might reduce the overall inflammatory state (19). However, as recently emphasized, the type and extent of physical activity necessary for antiinflammatory and insulin sensitizing effects are not clear. Strength training is an increasingly popular type of exercise in obese subjects and type 2 diabetic patients as it may be easily practiced in growing numbers of fitness centers. Moreover, strength training is a good model to investigate the effects of exercise while maintaining stable body weight.

To our knowledge, no study has investigated the effects of dynamic strength training simultaneously on gene expression and plasma levels of the adipokines with a suggested role in insulin resistance. Therefore, the aim of the present study was to investigate whether long-term dynamic strength training induces improvement in insulin sensitivity in obese middle-aged men and exerts antiinflammatory and insulin-sensitizing effects by altering plasma and mRNA levels of adiponectin, leptin, IL-1 β , IL-6, and TNF- α in sc abdominal adipose tissue (SCAAT).

Subjects and Methods

Subjects

Twelve obese, middle-aged, sedentary males [age 50.4 ± 2.3 yr, mean body mass index (BMI) 33.6 ± 1.2 kg/m²] were recruited for the study. Five subjects were previously diagnosed as type 2 diabetic, based on the World Health Organization criteria (20). Three of them were drug free; the two others were on hypotensive (amlodipin) therapy. The latter medication was not changed during the study. Among the remaining seven nondiabetic subjects, four of them had impaired oral glucose tolerance test, whereas the three remaining subjects had normal oral glucose tolerance test (20). None of the subjects had any disease other than those mentioned above. All patients had a stable weight for at least 3 months before inclusion. The sedentarity of patients was assessed during an interview. They were instructed to maintain their habitual diet throughout the whole study period. Dietary regimen was assessed using a 3-d food record in the beginning, at the sixth week, and at the end of the study. According to this evaluation, no changes in calorie intake and macronutrient composition of the diet were detected. The study was approved by the Ethical Committee of the 3rd Faculty of Medicine, Charles University, and all subjects gave their informed consent after detailed explanation.

Experimental protocol

The participants followed a program of dynamic strength training for 12 wk. The subjects were investigated twice: before and at the end of the intervention. On each occasion, they were investigated at 0800 h after an overnight fast. The second investigation was performed 48–72 h after the last exercise session to eliminate the effect of the last acute bout of exercise on insulin sensitivity. Anthropometric and body composition measurements and blood sampling for subsequent analysis were performed. Thereafter, hyperinsulinemic euglycemic clamp was carried out. Two days after this investigation at 0900 h, after an overnight fast, a needle microbiopsy of adipose tissue was performed under local

anesthesia (1% Xylocaine) from the abdominal region (14–20 cm lateral to the umbilicus) for subsequent gene expression analysis. In addition, 4–5 d before the clamp, the maximum exercise test for determination of maximum aerobic capacity was carried out. At the beginning of the dynamic strength training program, the test of maximum muscular strength was performed [see *maximal oxygen consumption (VO₂max)* and *maximum muscle strength determination* paragraph].

Training program

The dynamic strength training program started after completion of entry examinations. Based on the test of muscular strength, the individual prescription of physical activity (intensity, number of repetitions) was recommended according to conventional guidelines (21). Participants exercised in fitness centers three times a week, for 1 h, including warm-up and stretching. The training was performed using equipment (Technogym, Gambettola, Italy), with the minimum duration of 30–45 min of strength training itself. It consisted of 17 different single-joint and multijoint exercises (one set) involving large muscle groups. The intensity was set at 60–70% of their maximum muscle strength [one repetition maximum (1-RM)] for each exercise (*i.e.* for each muscle group), and 12–15 repetitions were performed. The subjects completed one set during the initial week. Afterward the number of sets was gradually adapted to the progression of fitness. To assess progress in muscle strength, maximum strength on upper (bench press) and lower body (leg press) was determined. Participants were supervised by professional fitness instructors twice a week during the initial period and once a week later on. All participants kept an exercise diary. The participants were instructed to limit other regular physical activity besides the dynamic strength training during the study.

Anthropometric measurements

Body weight and waist and hip circumference were measured regularly throughout the study. Body composition (fat mass, fat free mass) was assessed in the fasting condition by bioimpedance (QuadScan 4000; Bodystat, Douglas, UK). Coefficients of variation of fat mass, fat-free mass, and impedance were, respectively, 1.7, 0.8, and 1.5%.

VO₂max and maximum muscle strength determination

The maximum exercise test was performed on a bicycle ergometer (Ergoline 800) to determine VO₂max in each subject. An initial workload of 50 W was followed by a sequential increase in workload of 25 W every minute until exhaustion. Oxygen uptake was measured using Vmax Sensor Medics (Yorba Linda, CA), and attention was paid to reach one of the criteria for VO₂max as defined (22). The highest VO₂ achieved was taken as the VO₂max. Verbal encouragement was given to reach maximal performance. Heart rate was continuously monitored.

To determine maximal muscle strength (1-RM) on upper body (bench press) and lower body (leg press), the subjects performed, after warm-up and stretching, three to five initial exercises on 60–80% of their estimate 1-RM. Afterward, weight-balance was increased, and they performed one more lift. If they were successful in lifting, they continued the same way with 3- to 5-min breaks in between exercises until their maximum. The weight of the weight-balance of the last successful lift was taken as the maximal muscle strength (1-RM). Three to five attempts were allowed to reach the maximum.

Euglycemic-hyperinsulinemic clamp

The euglycemic-hyperinsulinemic clamp was performed according to the method of de Fronzo *et al.* (23). A catheter for insulin and glucose infusion was inserted into an antecubital vein, and a second catheter for blood sampling was placed in a dorsal vein of the ipsilateral hand. The hand was kept in a warm box (60 C) to provide arterialization of venous blood. Priming plus continuous infusion of crystalline human insulin (Actrapid Human; Novo A/S, Bagsvaerd, Denmark), 40 mU/m² body area/min, was given for 210 min. Euglycemia (the fasting blood glucose concentration) was maintained by a variable 20% glucose infusion. The infusion rate was determined by measuring arterialized plasma glucose every 5 min (glucose analyzer; Beckman Instruments, Fullerton, CA). Glucose consumption was calculated from the exogenous glucose in-

fusion rates during the last 30 min of the clamp and corrected for kilogram of fat-free mass ($\text{mg}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$ fat-free mass). Glucose clamp-derived index of insulin sensitivity (SI_{Clamp}) was defined as $M/(G \times \Delta I)$ corrected for body weight (24). M is the steady-state glucose infusion rate (milligram per minute), G is the steady-state blood glucose concentration (milligram per deciliter), and ΔI is the difference between basal and steady-state plasma insulin concentrations (microunits per milliliter).

mRNA quantification

The biopsies of SCAAT (about 1 g) were used for mRNA quantification. The samples were washed, homogenized in RLT lysis buffer (QIAGEN, Courtaboeuf, France) and stored at -80°C until analysis. Total RNA was extracted using the RNeasy minikit (QIAGEN). Integrity of RNA was checked on agarose gel, and RNA concentration was determined using a fluorometric assay (Ribogreen, RNA Quantification Kit, Invitrogen, Cergy Pontoise, France; Fluoroskan Ascent, Labsystem, Cergy Pontoise, France; Thermo Electron Corp., Waltham, MA). Reverse transcription was performed with $1\ \mu\text{g}$ of total RNA using random hexamers as primers and SuperScript II reverse transcriptase (Invitrogen). Real-time quantitative PCR (qPCR) was performed on ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). A set of primers was designed for adiponectin and leptin using the software Primer Express 1.5 (Applied Biosystems) and used at a final concentration of 300 nmol/liter with SYBR-Green-based chemistry. For each primer pair, a standard curve was obtained using serial dilutions of human adipose tissue cDNA prior mRNA quantification. To verify that genomic DNA was not amplified, qPCR was performed on reverse transcription reactions with no addition of reverse transcriptase. Primers and probes for IL-1 β , IL-6, and TNF- α were obtained from Applied Biosystems using TaqMan probe-based assays. The probes were labeled with a reporter dye (FAM) on the 5' end. We used 18S rRNA (Ribosomal RNA Control TaqMan assay kit; Applied Biosystems) as control to normalize gene expression. Each sample was performed in duplicate, and 10 ng cDNA was used as a template for real-time PCR. When the difference between the duplicates was above 0.5 Ct, qPCR was performed again.

Determination of plasma levels

Plasma glucose was determined with a glucose oxidase technique (Biotrol kit, Paris, France). Plasma insulin was measured using an insulin Irma kit (Immunotech, Prague, Czech Republic). Plasma triglycerides were determined by spectrophotometry. Plasma high-sensitivity C-reactive protein concentrations were assessed by immunoturbidimetry using an ultrasensitive kit (Orion-Diagnostica, Espoo, Finland) and Cobas Mira Plus analyzer (Roche, Stockholm, Switzerland). Adiponectin and leptin plasma levels were determined using RIA kits from Linco Research (St. Charles, MO) according to the manufacturer's recommendations. IL-6, IL-1 β , and TNF- α plasma levels were determined using quantikine IL-6, quantikine high sensitivity IL-1 β , and TNF- α kits (R&D Systems, Minneapolis, MN), respectively.

Statistical analysis

Data are presented as means \pm sd. Statistical analysis was performed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL). The differences in the responses to the training were analyzed using a Wilcoxon's signed rank test. Correlations were assessed by Spearman's correlation. The level of significance was set at $P < 0.05$.

Results

Clinical data

The training did not produce a significant change in body weight or other anthropometric variables (fat mass, fat free mass, waist and hip circumference) or VO_2max (Table 1). However, it did lead to a marked increase of indices of muscle strength represented by bench press and leg press (by 33.4 and 31.7%, respectively).

Effect of strength training on metabolic variables and insulin sensitivity

Metabolic and hormonal variables of the subjects before and after the training are shown in Table 2. Plasma insulin, blood glucose concentrations, and indices of lipid metabolism remained unchanged. No change in high-sensitivity C-reactive protein (hsCRP) concentration in blood was detected. Glucose disposal rate corrected for kg of fat-free mass increased after training by 24.4%. As shown in Fig. 1 and Table 2, the insulin sensitivity calculated by SI_{Clamp} also significantly increased by 30.8%.

Effect of strength training on plasma adipokine levels

Training did not induce changes in plasma levels of adiponectin, IL-1 β , IL-6, or TNF- α (Table 3). The plasma leptin level decreased by 21%. This decrease in leptin concentration was present in all but one subject, whose leptin levels increased after intervention (Fig. 2). The training-induced plasma leptin reduction remained significant after adjustment of plasma values to BMI (by 20%, $P = 0.02$).

Effect of strength training on gene expression of adipokines in SCAAT

The training did not induce changes in relative mRNA levels for any of the investigated adipokines in SCAAT (Table 4).

TABLE 1. Anthropometric and clinical characteristics of subjects before and after strength-training period

	Before training	After training	95% CI	P value
Body weight (kg)	109.2 \pm 12.6	109.5 \pm 12.9	-1.76, 2.08	0.878
BMI (kg/m^2)	33.6 \pm 3.9	33.7 \pm 4.0	-0.54, 0.66	0.799
Fat mass (%)	31.6 \pm 4.9	30.1 \pm 4.2	-0.23, 5.31	0.139
Fat-free mass (%)	68.5 \pm 4.8	69.9 \pm 4.2	-5.28, 0.50	0.139
Waist circumference (cm)	115.7 \pm 7.9	115.7 \pm 8.0	-2.75, 2.95	0.905
Hip circumference (cm)	112.2 \pm 7.4	112.4 \pm 4.7	-2.85, 3.05	1.000
Waist to hip ratio	1.03 \pm 0.1	1.03 \pm 0.04	-0.04, 0.04	0.905
Systolic blood pressure (mm Hg)	140.0 \pm 10.8	127.7 \pm 19.4	0.54, 24.12	0.002
Diastolic blood pressure (mm Hg)	92.5 \pm 9.2	81.3 \pm 9.8	3.57, 18.93	0.002
Heart rate (bpm)	66.6 \pm 7.4	65.7 \pm 7.6	-2.04, 3.81	0.552
VO_2max ($\text{ml}/\text{kg}\cdot\text{min}$)	21.4 \pm 2.9	22.9 \pm 4.1	-3.86, 0.96	0.358
Bench press 1-RM (kg)	51.8 \pm 8.9	69.1 \pm 8.7	-26.28, -11.50	0.005
Leg press 1-RM (kg)	174.9 \pm 46.8	230.4 \pm 56.9	-103.57, -21.98	0.005

Values are means \pm sd. CI, Confidence interval.

TABLE 2. Metabolic and hormonal characteristics of obese men before and after 12 wk of dynamic strength training

	Before training	After training	95% CI	P value
Fasting glucose (mmol/liter)	7.1 ± 3.1	6.6 ± 1.8	-7.44, 2.48	0.260
Fasting insulin (mU/liter)	10.9 ± 10.6	4.8 ± 4.78	-4.57, 15.77	0.237
Total cholesterol (mmol/liter)	5.2 ± 0.7	5.2 ± 1.0	-0.41, 0.44	0.646
HDL cholesterol (mmol/liter)	1.1 ± 0.2	1.07 ± 0.2	-0.16, 0.18	0.878
Triglycerides (mmol/liter)	1.8 ± 0.9	1.8 ± 0.8	-0.50, 0.43	0.878
Norepinephrine (pg/ml)	307.6 ± 66.1	320.9 ± 73.2	-47.72, 21.12	0.507
Epinephrine (pg/ml)	39.9 ± 14.3	40.6 ± 11.3	-6.37, 4.97	0.540
hsCRP (mg/liter)	3.3 ± 2.2	2.9 ± 1.7	-0.92, 1.68	0.929
Glucose disposal (mg·min ⁻¹ ·kg ⁻¹ fat-free mass)	4.5 ± 2.1	5.6 ± 1.8	-2.11, -0.09	0.04
SI _{Clamp}	5.2 ± 2.3	6.8 ± 3.1	-2.75, -0.43	0.01

Values are means ± SD. CI, Confidence interval; HDL, high-density lipoprotein.

Relationship between insulin sensitivity and adipokine levels

Marked association between the glucose disposal rate and TNF- α mRNA ($r = -0.697$, $P = 0.03$) was apparent before the training program. No relations between the indices of insulin sensitivity and plasma level and gene expression in SCAAT for other cytokines were observed.

Relationship between adipokine plasma levels and/or gene expression in SCAAT and anthropometric and metabolic variables

Plasma leptin was positively related to BMI at the beginning ($r = 0.683$, $P = 0.04$) but not at the end of the intervention. hsCRP was positively correlated with waist circumference as at baseline as after dynamic strength training ($r = 0.572$, $r = 0.628$, respectively, $P < 0.05$). No correlations between mRNA levels of adipokines before or after the training and anthropometric or metabolic variables were observed.

Discussion

The aim of the present study was to investigate the associations between the effects of dynamic strength training on insulin resistance/sensitivity and modifications of the cytokines produced in adipose tissue and supposedly related to the pathogenesis of insulin resistance in obese individuals. In the present study, the 3 months of dynamic strength training induced an improvement in insulin sensitivity as assessed by the euglycemic-hyperinsulinemic clamp. We investigated a sustained effect of exercise on plasma levels of relevant adipokines as well as the effect on gene expression of the cytokines in

SCAAT. The study did not reveal any significant changes, except the decrease of plasma leptin, in the measured variables induced by the training.

The positive muscular effects of dynamic strength training in the present study were demonstrated by a marked improvement in muscular strength (as evaluated by bench press and leg press tests). The improvement of insulin sensitivity in our subjects is in accordance with results of other studies investigating effects of strength training (18, 25). The training did not induce any significant change in total body weight or body adiposity evaluated by the bioimpedance method. We cannot exclude training-induced changes in the amount of visceral fat because no specific measures of visceral fat were performed in this study.

Obesity and/or type 2 diabetes mellitus are associated with low-grade inflammatory state characterized by altered levels of C-reactive protein and several cytokines produced and released from adipose tissue (adipokines). Compared with lean individuals, mRNA and plasma levels are found to be lower for adiponectin (26) and higher for leptin (27) in obese subjects. This corresponds to the findings of pretraining values in this study. The circulating levels of IL-6 have been reported to be higher in patients with obesity (27). The insulin stimulation of IL-6 gene expression in adipose tissue might play a role in this process (28). In obese subjects, TNF- α levels are found to be elevated (29). Circulating levels of IL-1 β in type 2 diabetic patients (30) were not found to be different from lean subjects. No reports concern obese individuals. Adipokines act as autocrine, paracrine, or endocrine substances and are thought to contribute to the pathogenesis of insulin resistance as well as the development of the low-grade inflammatory state observed in obese or diabetic subjects. In this regard, we also measured hsCRP as a classical inflammatory marker. In our study, base-

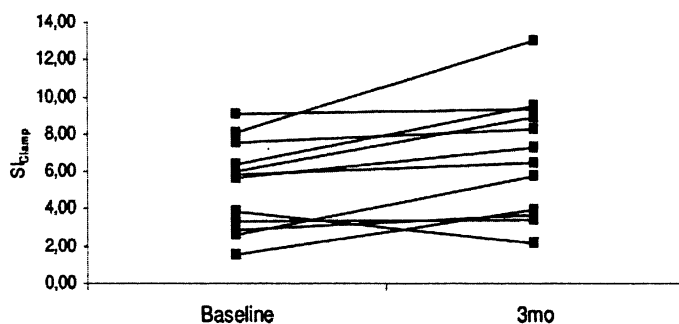


FIG. 1. Individual changes in insulin sensitivity in response to dynamic strength training. The mean of SI_{Clamp} is 5.2 ± 0.7 at baseline and 6.8 ± 0.9 after 3 months (3mo) of dynamic strength training in 12 subjects.

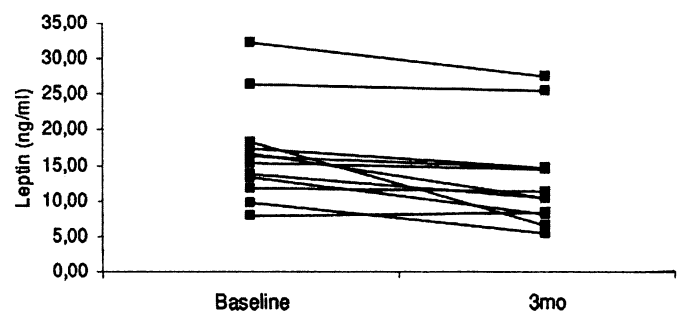


FIG. 2. Individual plasma leptin variations of 12 subjects in response to dynamic strength training.

TABLE 3. Plasma levels of adipokines before and after 12 wk of dynamic strength training

	Before training	After training	95% CI	P value
Adiponectin ($\mu\text{g/ml}$)	5.6 \pm 3.9	5.05 \pm 3.1	-0.67, 1.35	0.683
Leptin (ng/ml)	16.6 \pm 6.3	13.1 \pm 5.7	0.59, 6.36	0.02
IL-1 β (pg/ml)	1.6 \pm 1.2	1.0 \pm 0.5	-0.46, 2.58	0.063
IL-6 (pg/ml)	1.4 \pm 0.7	1.5 \pm 0.6	-0.97, 0.68	0.859
TNF- α (pg/ml)	2.0 \pm 1.5	2.3 \pm 2.2	-0.13, 0.37	0.261

Values are means \pm SD. CI, Confidence interval.

line hsCRP level corresponded to the reference range for indication of chronic low-grade inflammation state and high cardiovascular risk (31).

It may therefore be hypothesized that the training-induced changes in insulin sensitivity could be associated with modifications of adipokine production or release. However, in the present study, no training-induced modifications of either plasma levels (except of plasma leptin diminution) of adipokines or mRNA levels in SCAAT were found. To date, few studies have investigated the effect of strength training on plasma levels of adipokines, and no data on the effect of training on gene expression of adipokines in SCAAT are available. The absence of training-induced effects on plasma adipocytokines found in this study is in agreement with the results of previous studies (25, 32–34). A 4-month resistance training program was reported to increase glucose disposal and lean body mass independently of plasma levels of TNF- α , sTNF R1, and sTNF R2 in old hypertensive subjects (25). No change in plasma IL-6 or TNF- α levels was observed in a 12-wk resistance training program in the oldest of the aged study population, despite the improvement in muscle strength (32). A 12-wk high-intensity progressive resistance training program did not induce changes in plasma IL-1 β , TNF- α , or IL-6 in healthy young or elderly individuals or subjects with rheumatoid arthritis (33). In healthy obese males, 12 wk of resistance training did not produce a change in serum levels of TNF- α (34). The lack of effect on IL-1 β , TNF- α , and IL-6 plasma levels observed in this study was supported by the lack of change in adipose tissue mRNA. It is to be noted that the training-induced response of cytokine mRNA levels could be different in visceral adipose tissue. This might be relevant in respect to the insulin sensitivity changes (given the higher expression of some of the investigated adipokines in visceral adipose tissue as compared with the sc one, e.g. IL-6) (35).

We found a significant inverse association of TNF- α mRNA and insulin sensitivity in the beginning of the study. Although this could support the hypothesis of the role of TNF- α in the pathogenesis of whole-body insulin resistance, the relevance of TNF- α produced in adipose tissue is still not clear. No net release of TNF- α from the SCAAT bed was observed (36). TNF- α possibly acts in an autocrine and paracrine manner, thus

playing a local role in the regulation of adipose tissue metabolism, namely in the control of lipolysis (37).

Reduction of plasma leptin without any modification of mRNA SCAAT levels was found in this study. Decrease of plasma leptin was also demonstrated in the only study using resistance training (38). Importantly, we found that the reduction was independent of BMI changes, suggesting a direct effect of the training on leptin release, independent of changes in body weight. An alternative possibility for leptin diminution could be its enhanced removal from the blood. The lack of effect on fat mass may explain the discrepancy between leptin plasma levels and SCAAT mRNA expression and suggest a regulation at the level of leptin secretion or protein turnover. Such a discrepancy has been reported before (39).

Adiponectin mRNA and plasma levels have not been investigated in the context of dynamic strength training. Studies on the effect of aerobic exercise training on plasma adiponectin have provided conflicting results (40–42). The reported training-induced changes in adiponectin levels may be related to training-induced weight loss. However, in the present study, the adiposity of the participants remained unchanged and may explain the absence of changes in adiponectin expression. It is noteworthy that during very low-calorie diet-inducing weight loss and an improvement in insulin sensitivity, no change in plasma or mRNA levels of adiponectin was found (43).

In conclusion, the present study demonstrates that 3 months of dynamic strength training improves insulin sensitivity in obese middle-aged males. This confirms the beneficial effect of this type of training in obese individuals. In a search for mechanisms underlying the training-induced change in insulin sensitivity, the indices of proinflammatory state of the body, namely plasma cytokine levels and their expression in adipose tissue, were investigated. Because no training-induced changes of these variables were observed, it is suggested that the adipokines investigated are not mediators of the change in insulin sensitivity induced by dynamic strength training.

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TABLE 4. Relative mRNA levels before and after strength training period in SCAAT

	Before training	After training	Fold change	95% CI	P value
Adiponectin	7.1 \times 10 ⁻⁴ \pm 2.2 \times 10 ⁻⁴	7.7 \times 10 ⁻⁴ \pm 2.8 \times 10 ⁻⁴	1.08	-2.2 \times 10 ⁻⁴ , 2.0 \times 10 ⁻⁴	0.859
Leptin	2.6 \times 10 ⁻³ \pm 7.0 \times 10 ⁻⁴	2.6 \times 10 ⁻³ \pm 9.1 \times 10 ⁻⁴	1.01	-8.8 \times 10 ⁻⁴ , 6.7 \times 10 ⁻⁴	0.859
IL-1 β	2.1 \times 10 ⁻⁶ \pm 2.0 \times 10 ⁻⁶	4.4 \times 10 ⁻⁶ \pm 6.6 \times 10 ⁻⁶	2.10	-9.1 \times 10 ⁻⁶ , 3.3 \times 10 ⁻⁶	0.953
IL-6	3.1 \times 10 ⁻⁷ \pm 1.9 \times 10 ⁻⁷	4.5 \times 10 ⁻⁷ \pm 5.0 \times 10 ⁻⁷	1.47	-6.2 \times 10 ⁻⁷ , 2.5 \times 10 ⁻⁷	0.594
TNF- α	6.2 \times 10 ⁻⁷ \pm 2.5 \times 10 ⁻⁷	8.3 \times 10 ⁻⁷ \pm 4.8 \times 10 ⁻⁷	1.34	-7.2 \times 10 ⁻⁷ , 1.9 \times 10 ⁻⁷	0.208

Values are means \pm SD. Relative amounts of mRNA are expressed as arbitrary units obtained after normalization by the 18S rRNA subunit. Fold change was calculated by dividing gene expression values after the treatment by values before the intervention. CI, Confidence interval.

Address all correspondence and requests for reprints to: Dominique Langin, Unité de Recherches sur les Obésités, Institut National de la Santé et de la Recherche Médicale, UPS U586, IFR31, Institut Louis Bugnard, Bâtiment L3, 2ème étage, Centre Hospitalier Universitaire Rangueil, 1 Avenue Jean Poulhès, 31400 Toulouse, France. E-mail: langin@toulouse.inserm.fr.

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♣♣♣ In the third study, we investigated the effect of multiple phase dietary intervention program with a total duration of 6 to 7 months on plasma and mRNA levels of RBP4 in a group of 24 obese women. Three successive dietary phases were applied in this clinical study: 1 month of VLCD (800 kcal/day), 2 months of low calorie diet (LCD) (600 kcal less than the calculated daily energy requirements), and 3-4 months of a weight maintenance (WM) period (weight maintaining diet). VLCD induced a moderate weight loss of 7% (~ 7 kg) and after LCD phase we observed a reduction of 10% (~ 10 kg) of the initial weight, which remained stable at the end of WM phase. The amount of weight loss obtained in our study corresponds to values usually obtained by application of this kind of interventions [358]. For calorie reduction programs, it is typical that they lead to a partial reduction of skeletal muscle mass [377], what has also been seen in our studied cohort, it resulted in a decrease of about 5% of the initial fat-free mass (FFM). However, the loss of body weight in our obese women appears mostly due to a significant diminution in fat mass (by 13%, 23%, and 20% after VLCD, LCD, and WM, respectively). The weight loss period (VLCD and LCD) induced positive changes in the majority of clinical parameters, i.e. waist circumference, plasma insulin, glycerol, total cholesterol, TG, or cortisol were decreased after each dietary phase and stabilized during WM. Glycemia was significantly decreased only after VLCD by 5% and during other phases did not differ from the baseline values. The FFA concentration was not influenced by VLCD, but the levels were diminished after LCD (by 15%) and at the end of the protocol (by 19%). Circulating β -hydroxybutyrate as a marker of ketosis has also been measured and we observed a marked elevation during VLCD and LCD by 306% and 69%, respectively, and return to basal levels at the end of WM. It is known, that weight loss, even if modest, is generally associated with an improvement in IS [369] and our results confirmed this fact. Glucose consumption was measured at each of the dietary phases by euglycemic hyperinsulinemic clamp. Glucose disposal rate corrected per kg of body weight increased after VLCD (by 21%) and further increased throughout subsequent periods of LCD (by 36% of the pre-diet value) and WM (by 41% of the pre-diet value). Glucose disposal rate corrected for kg of FFM followed the same pattern. RBP4 is an adipokine, which has lately been characterized as a potential candidate implicated in the development of IR in rodents [19]. The longitudinal design of our study that leads to the improvement of IR offers a unique way to shed light on the association between RBP4 and IR in humans. We measured plasma and mRNA levels of RBP4

in SCAT before and after each of the dietary phase, and found that both parameters were decreased only after the VLCD period. During the following phases, the RBP4 profile changed, plasma concentrations remained significantly diminished during LCD and WM, while RBP4 mRNA levels returned to basal values. In this study, we also measured circulating adiponectin and leptin, and SCAT Glut4 mRNA levels. Plasma adiponectin level was not changed in the course of whole dietary intervention, and leptin concentration was, as expected, decreased by 51%, 42%, and 34% during VLCD, LCD, and WM, respectively. Given that Glut4 mRNA and protein levels are reduced in fat cells in several IR states and in type 2 diabetes mellitus [426], and that in mice with genetic ablation of Glut4 in adipocytes plasma RBP4 are increased [19], one could hypothesize an inverse relationship between Glut4 and RBP4. However, this negative association was not found in our study, the profile of Glut4 mRNA was identical with that of RBP4 mRNA. To gain further insight into the possible relation of RBP4 to IR, associations between RBP4 expression and indices of IR have been tested. No correlations were detected between RBP4 plasma levels and glucose disposal rate (by any of the glucose disposal rate calculations) at baseline as well as after VLCD or the whole dietary intervention in obese women. Even if the subjects were stratified into two groups according to the baseline IS or the amount of changes in glucose disposal rate during VLCD or the entire program, no differences in plasma RBP4 have been found between these groups. Moreover, a control group of lean women was recruited and compared with the obese cohort. We confirmed, as expected, a higher glucose consumption in the lean (~ 2 fold) than in the obese group when corrected for kg of body weight or kg of FFM, while there was no difference in plasma RBP4 between these two groups. As mentioned in the introduction, liver is the principal source of RBP4 in the circulation. Further studies are warranted to determine to which extent AT contributes to the total plasma RBP4 levels.

Plasma Levels and Adipose Tissue Messenger Ribonucleic Acid Expression of Retinol-Binding Protein 4 Are Reduced during Calorie Restriction in Obese Subjects but Are Not Related to Diet-Induced Changes in Insulin Sensitivity

Michaela Vitkova,* Eva Klimcakova,* Michaela Kovacicova, Carine Valle, Cédric Moro, Jan Polak, Jiri Hanacek, Frédéric Capel, Nathalie Viguerie, Blanka Richterova, Magda Bajzova, Jindra Hejnova, Vladimir Stich, and Dominique Langin

Institut National de la Santé et de la Recherche Médicale (M.V., E.K., M.K., C.V., C.M., J.P., F.C., N.V., B.R., M.B., J.He., V.S., D.L.), Franco-Czech Laboratory for Clinical Research on Obesity, Prague, CZ-10100 Czech Republic; Department of Sports Medicine (M.V., E.K., M.K., J.P., B.R., M.B., J.He., V.S.), and Division of Cell and Molecular Biology (M.K.), 3rd Faculty of Medicine, Charles University, Prague, CZ-100 00 Czech Republic; Institut National de la Santé et de la Recherche Médicale (M.V., E.K., C.V., C.M., F.C., N.V., D.L.), U858, Obesity Research Laboratory, Toulouse, F-31432 France; Institute for Mother and Child Care (J.Ha.), Prague, Czech Republic; Paul Sabatier University (E.K., C.V., C.M., F.C., N.V., D.L.), Louis Bugnard Institute, IFR31, Toulouse, F-31432 France; and Centre Hospitalier Universitaire de Toulouse (D.L.), Biochemistry Laboratory, Biology Institute of Purpan, Toulouse, F-31059 France

Context: Retinol-binding protein 4 (RBP4) may play a role in the development of insulin resistance.

Objective: We investigated whether RBP4 adipose tissue mRNA expression and plasma level are related to insulin sensitivity during a diet-induced weight loss.

Design, Setting, Patients, and Intervention: Obese women followed a dietary intervention composed of a 4-wk very low-calorie diet (VLCD), a 2-month low-calorie diet, and 3–4 months of a weight maintenance (WM) phase.

Main Outcome Measures: Clinical investigation was performed before and at the end of each phase. Insulin sensitivity was assessed with the euglycemic hyperinsulinemic clamp. Adipose tissue mRNA and plasma levels of RBP4 were determined using reverse transcription-quantitative PCR and ELISA, respectively.

Results: Weight and fat mass decreased during VLCD and were stabilized during WM. Glucose disposal rate increased during VLCD and remained elevated thereafter. Plasma levels of RBP4 decreased after VLCD and, although increasing at subsequent phases, remained lower than prediet values. Adipose tissue mRNA levels were diminished after VLCD, and increased during low-calorie diet and WM to reach basal values. Basal RBP4 levels or diet-induced variations of RBP4 were not different in lean women and two groups of obese women with high- and low-insulin sensitivity.

Conclusions: Severe calorie restriction promotes a reduction in adipose tissue and plasma levels of RBP4. The study does not bring evidence for a role for RBP4 in the regulation of diet-induced changes in insulin sensitivity. (*J Clin Endocrinol Metab* 92: 2330–2335, 2007)

TYPE 2 DIABETES is characterized by insulin resistance and relative insulin deficiency. The resistance to insulin action occurs in multiple tissues, including the liver with an increase in glucose production and skeletal muscles with a decrease in glucose use. Insulin resistance independent of overt diabetes is an important causative factor of the metabolic syndrome and constitutes an important risk factor for cardiovascular disease. Obesity is one of the principal causes

for insulin resistance and risk factors for type 2 diabetes. The excess of fat mass is associated with release of multiple molecules with paracrine or endocrine action by adipose tissue that may contribute to the development of insulin resistance (1). A recently characterized potential candidate is retinol-binding protein 4 (RBP4) (2). Adipose tissue RBP4 expression and circulating levels are increased in several mouse models of insulin resistance. Genetic knockout of the insulin-stimulated glucose transporter 4 (Glut4) selectively in adipocytes results in impaired whole-body insulin sensitivity (3). Because adipocytes contribute little to whole-body glucose disposal, the existence of a factor released by the adipocytes and acting on the liver and skeletal muscle was predicted. RBP4 has been identified as such a factor (2). Overexpression of RBP4 or injection of recombinant RBP4 induced insulin resistance in mice, whereas pharmacologically decreased serum levels of RBP4 improved insulin sensitivity in high-fat

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* M.V. and E.K. contributed equally to the work.

Abbreviations: BMI, Body mass index; Glut4, glucose transporter 4; LCD, low-calorie diet; PPAR, peroxisome proliferator-activated receptor; RBP4, retinol-binding protein 4; VLCD, very low-calorie diet; WM, weight maintenance.

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diet-fed mice. Indeed, RBP4 impairs insulin signaling in skeletal muscle and affects glucose output in the liver.

RBP4 has been used clinically as a rapid turnover protein for assessing the short-term fluctuation of nutritional states. RBP4 is the only specific transport protein for retinol (vitamin A) in the circulation (4). It is produced by hepatocytes, which are believed to contribute to a large part of circulating RBP4, although adipocytes have the second-highest expression level (5). Elevated RBP4 levels have been reported in subjects with insulin resistance and type 2 diabetes (6–9). Correlations have been observed between serum RBP4 levels and the magnitude of insulin resistance in different groups of subjects (8). However, the cross-sectional design of most of the clinical studies performed so far does not allow for a determination of a putative causal role of RBP4 in the pathogenesis of insulin resistance and type 2 diabetes in humans (10). Here, we studied RBP4 in obese subjects enrolled in a multiple-phase weight reduction program based on a 4-wk very low-calorie diet (VLCD), followed by a 2-month low-calorie diet (LCD), and 3–4 months of a weight maintenance (WM) phase. RBP4 mRNA expression in sc adipose tissue and plasma RBP4 levels were determined before and at the end of each phase, and related to anthropometric and biological parameters, including glucose disposal rate assessed by the euglycemic hyperinsulinemic clamp.

Subjects and Methods

In vitro adipose tissue studies

Subcutaneous abdominal adipose tissue obtained from overweight women undergoing plastic surgery was digested with collagenase. Mature adipocytes were separated from the stromavascular fraction by mild centrifugation. Isolation of different cell types (endothelial cells, preadipocytes, and macrophages) in the stromavascular fraction was performed using surface antigen-coupled magnetic microbeads (11). Human preadipocytes in primary culture were differentiated as described (12). At d 13, 60–80% of cells were differentiated into lipid droplet-containing adipocytes. For culture of human adipose tissue explants, sc abdominal adipose tissue was cut into small pieces weighing approximately 10 mg or less. After washing steps, explants were cultured in DMEM F12 medium (Cambrex Corp., East Rutherford, NJ) containing 33 μ mo/liter biotin, 17 μ mol/liter pantothenate, and 50 μ g/ml gentamycin supplemented with 10% of fetal calf serum. Explants were preincubated overnight to allow for removal of soluble factors and cellular debris released by cells broken during the preparation of the small pieces of adipose tissue. On d 2, explants were washed three times with PBS. Explants (200 mg/ml) were then incubated for 24 h in fresh medium. Aliquots of the medium were stored at -80°C for protein measurements. These studies were in agreement with the French National Institute of Health and Medical Research (Inserm) and the Toulouse University Hospital ethics regulation.

Subjects

Participants in the study were recruited at the Third Faculty of Medicine of Charles University and at the Institute for Mother and Child Care in Prague, Czech Republic. The clinical investigation was performed at the Department of Sports Medicine of the Third Faculty of Medicine. A group of 24 obese premenopausal women was included in the study. Exclusion criteria were weight changes of more than 3 kg within the 3 months before the start of the study, hypertension, diabetes, or hyperlipidemia treated by drugs, drug-treated obesity, pregnancy, participation in other trials, and alcohol or drug abuse. A control group of 12 lean women [age 38 ± 10 yr; body mass index (BMI) 21 ± 2 kg/m²] was also investigated. The studies were approved by the Ethical Committee of the Third Faculty of Medicine. Volunteers were informed on the study, and written consent was obtained before study participation.

Dietary intervention

During the first 4 wk of the dietary intervention program, the obese subjects received a 800 kcal/d VLCD (liquid formula diet; Redita, Promil, Czech Republic). During the next 2 months, a LCD was designed to provide 600 kcal/d less than the individually estimated energy requirement based on an initial resting metabolic rate multiplied by 1.3, the coefficient of correction for physical activity level. The final period was a WM phase of 3–4 months, during which the patients were instructed to keep on a weight-maintaining diet. Patients consulted a dietitian once a week during the first 3 months of the program and once a month during the WM phase. They provided a written 3-d dietary record at each dietary consultation.

Clinical investigation

A complete clinical investigation was realized before and at the end of each phase in the morning. Anthropometric and resting metabolic rate measurements were performed as previously reported (13). Body composition was determined with multifrequency bioimpedance (Bodystat QuadScan 4000; Bodystat Ltd., Isle of Man, British Isles). Blood samples were drawn from an indwelling catheter in the antecubital vein. Needle microbiopsy of sc adipose tissue was performed under local anesthesia (1% Xylocaine; AstraZeneca PLC, London, UK) from the abdominal region (14–20-cm lateral to the umbilicus) (14). The euglycemic hyperinsulinemic clamp was performed according to the DeFronzo method (15). Priming plus continuous infusion of crystalline human insulin (Actrapid Human; Novo, A/S, Bagsvaerd, Denmark), 40 mU/m² body area·min, was given for 210 min. Euglycemia (the fasting blood glucose concentration) was maintained by a variable 20% glucose infusion. The infusion rate was determined by measuring arterialized plasma glucose every 5 min (Beckman Glucose Analyzer; Beckman Coulter, Inc., Fullerton, CA). Glucose consumption was calculated from the exogenous glucose infusion rates during the last 30 min of the clamp and corrected for kilogram of body weight (mg·min⁻¹·kg⁻¹) or kilogram of fat-free mass (mg·min⁻¹·kg⁻¹ fat-free mass).

RNA analysis

Total RNA was extracted from adipose tissue biopsy samples, explants, and cells using the RNeasy Mini kit (QIAGEN, Inc., Valencia, CA). RT was performed with 500-ng total RNA using random hexamers (Promega Corp., Madison, WI) and Superscript II Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA). Real-time quantitative PCR was performed with TaqMan probe-based gene expression assays for RBP4, Glut4, peroxisome proliferator-activated receptor (PPAR) γ , and CD68, and a SYBR Green-based assay for adiponectin using ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). An 18S ribosomal RNA was used as control to normalize gene expression (Ribosomal RNA Control TaqMan Assay kit; Applied Biosystems). Each sample was performed in duplicate, and 10-ng cDNA was used as a template for real-time PCR. When the difference between the duplicates was above 0.5 Ct (threshold cycle), real-time PCR was performed again. Results are expressed as $2^{-\Delta\text{Ct}}$ values.

Determination of culture medium and plasma levels

RBP4 levels were measured using an ELISA kit (Immundiagnostik AG, Bensheim, Germany). Plasma samples were diluted so that the absorbance was in the middle of the range of linearity for the assay. Within-run coefficient of variation for RBP4 was 2.7%. Plasma glucose was determined by the glucose hexokinase technique (Konelab 60; Labsystems CLD, Konelab, Finland). Plasma insulin was measured using chemiluminescent immunometric assay (Immulite 2000 Insulin; DPC Czech sro, Brno, Czech Republic). Nonesterified fatty acid levels were determined using an enzymatic procedure (Wako; Unipath Ltd, Bedford, UK). Leptin and adiponectin levels were determined using ELISA kits (BioVendor Laboratory Medicine, Brno, Czech Republic). Plasma β -hydroxybutyrate was measured by an enzymatic "Liqui-Color" kit (Stanbio Laboratory, Boerne, TX). Plasma levels of other parameters were determined using standard clinical biochemistry methods. To determine cell damage in adipose tissue explant culture, mea-

surement of adenylate kinase activity was performed in the culture medium using a bioluminescent assay (Cambrex, Corp.).

Statistical analysis

Data were analyzed using the SPSS software (SPSS, Inc., Chicago, IL). Nonparametric Wilcoxon signed rank or Mann-Whitney *U* tests were used for comparison of paired and unpaired values, respectively. Correlations were analyzed by Spearman's nonparametric test. The level of significance was set at $P < 0.05$.

Results

Expression of RBP4 in human adipose tissue

Comparison of mRNA expression in mature adipocytes and stromavascular cells from human sc adipose tissue revealed that RBP4 is expressed almost exclusively in mature adipocytes (Fig. 1A). As a control of cell isolation, Glut4 and

adiponectin mRNA were detected mostly in adipocytes and CD68 mRNA, mostly in the stromavascular fraction. Direct detection on isolated endothelial cells, macrophages, and preadipocyte-like cells confirmed that the level of RBP4 expression was very low compared with that in mature adipocytes (data not shown). RBP4 gene expression was strongly induced during the conversion of human preadipocytes into adipocytes along with markers of adipocyte differentiation, such as PPAR γ and adiponectin (Fig. 1B). We also wished to determine whether RBP4 was produced by human sc adipose tissue. Primary culture of adipose tissue explants showed that the production rate of RBP4 *in vitro* was comparable to that of adiponectin, a factor secreted at high levels by adipocytes (Fig. 1C). The release of adenylate kinase from the explants was very low, indicating that cell damage was limited in our culture conditions (data not shown).

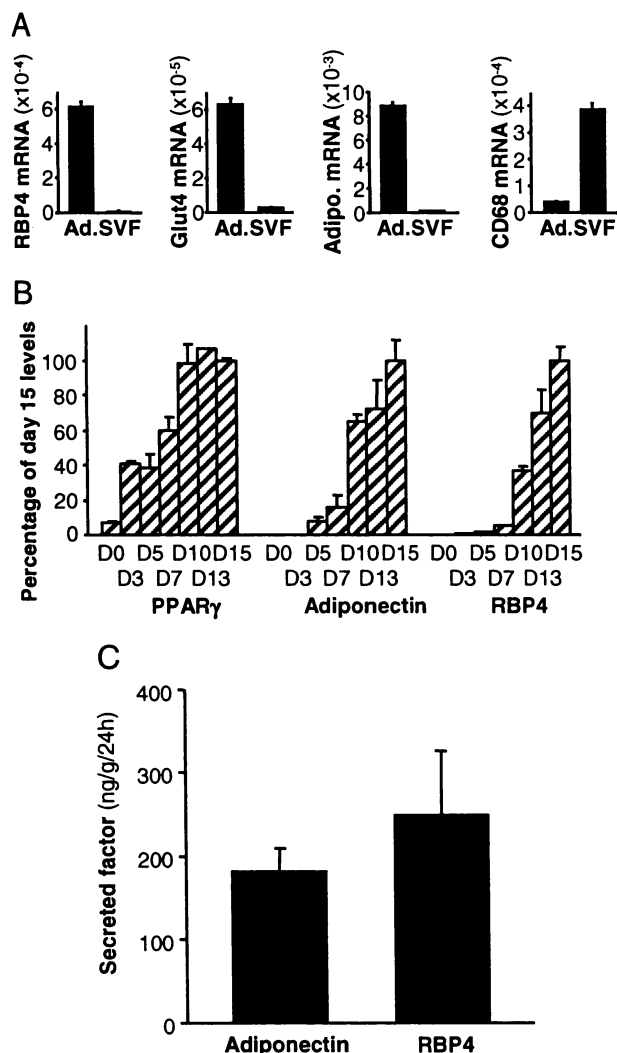


FIG. 1. RBP4 expression and production in human sc adipose tissue. A, RBP4, Glut4, adiponectin (Adipo), and CD68 mRNA expression was determined in mature adipocytes (Ad) and stromavascular cells (SVF) ($n = 6$). B, Time course of RBP4, adiponectin, and PPAR γ mRNA expression during differentiation of human preadipocytes into adipocytes from d 0 (D0) to d 15 (D15) ($n = 4$). C, Production of RBP4 and adiponectin by human adipose tissue explants ($n = 6$). The levels of secreted products were normalized to the mass of adipose tissue. Values are means \pm SEM.

Clinical parameters of obese subjects during a weight reduction program

Obese women followed a 6-month weight reduction program composed of three successive phases: a 4-wk 800 kcal/d VLCD, a 2-month LCD with 600 kcal less than the calculated daily energy requirements, and a 3–4 month WM diet. Anthropometric and plasma parameters were determined before and at the end of each dietary phase (Table 1). The subjects' body weight and BMI decreased during the VLCD and LCD phases. The body weight was stabilized during WM. The loss of weight was chiefly due to a decrease in fat mass. Evolution of waist circumference followed the same pattern as body weight. Regarding plasma parameters, there was a decrease in insulin, leptin, triglyceride, glycerol, and cortisol levels at all the time points. Glycemia was decreased only after VLCD, and the nonesterified fatty acid level was decreased at the end of the protocol. Circulating β -hydroxybutyrate was elevated during VLCD and then decreased during subsequent phases to reach basal levels at the end of the program. Plasma adiponectin level was not changed during the dietary intervention. To evaluate insulin sensitivity, euglycemic hyperinsulinemic clamps were performed at each phase (Table 1). The glucose disposal rate increased during VLCD, and remained elevated throughout the phases of LCD and WM.

Adipose tissue mRNA expression of RBP4 and Glut4 during a weight reduction program

Subcutaneous abdominal adipose tissue biopsies were performed before and at the end of each dietary phase. RBP4 mRNA expression decreased during VLCD (Fig. 2A). During LCD, there was an increase in RBP4 mRNA levels ($P < 0.01$) so that at the end of the LCD phase and at the end of WM, RBP4 mRNA values were not different from basal values. The profile of Glut4 mRNA expression was similar to that of RBP4 (Fig. 2B).

Plasma levels of RBP4 during a weight reduction program

Plasma RBP4 levels were decreased during VLCD (Fig. 2C). The LCD and WM phases were characterized by a gradual increase in RBP4 levels. At the end of the dietary intervention, plasma RBP4 levels were higher than VLCD values ($P < 0.01$).

TABLE 1. Clinical parameters of 24 obese women before and at the end of different phases of a weight reduction program

Parameter	Basal	VLCD 4 wk	LCD 8 wk	WM 12–16 wk
Weight (kg)	97 ± 16	90 ± 15 ^c	87 ± 15 ^c	87 ± 15 ^c
BMI (kg/m ²)	35 ± 5	33 ± 5 ^c	32 ± 4 ^c	32 ± 5 ^c
Fat mass (kg)	40 ± 12	35 ± 11 ^c	31 ± 9 ^c	32 ± 11 ^c
Fat-free mass (kg)	58 ± 6	55 ± 7 ^b	56 ± 8 ^b	55 ± 7 ^c
Waist (cm)	104 ± 13	99 ± 13 ^c	96 ± 13 ^c	96 ± 13 ^c
Glucose (mmol/liter)	5.6 ± 0.4	5.3 ± 0.6 ^a	5.4 ± 0.7	5.3 ± 0.8
Insulin (mU/liter)	13.6 ± 8.3	6.8 ± 3.7 ^c	5.8 ± 2.6 ^c	6.8 ± 2.8 ^c
Glycerol (μmol/liter)	217 ± 77	160 ± 39 ^b	145 ± 59 ^c	150 ± 43 ^c
Nonesterified fatty acid (μmol/liter)	693 ± 144	741 ± 126	589 ± 170 ^a	564 ± 171 ^a
β-hydroxybutyrate (mmol/liter)	0.16 ± 0.09	0.65 ± 0.36 ^c	0.27 ± 0.16 ^c	0.19 ± 0.17
Total cholesterol (mmol/liter)	4.82 ± 0.66	3.90 ± 0.76 ^c	4.29 ± 0.69 ^c	4.56 ± 0.66 ^a
Triglycerides (mmol/liter)	1.43 ± 0.69	1.06 ± 0.35 ^c	1.06 ± 0.37 ^b	1.03 ± 0.29 ^b
Leptin (ng/ml)	41 ± 15	20 ± 13 ^c	24 ± 14 ^c	27 ± 15 ^c
Adiponectin (μg/ml)	8.4 ± 3.9	8.4 ± 3.3	8.3 ± 3.3	9.2 ± 3.6
Cortisol (nmol/liter)	205 ± 96	157 ± 67 ^b	146 ± 61 ^b	177 ± 102 ^a
Glucose disposal rate (mg/kg·min)	2.98 ± 1.56	3.61 ± 1.65 ^b	4.05 ± 1.66 ^c	4.19 ± 1.74 ^c
Glucose disposal rate (mg/kg fat-free mass·min)	4.88 ± 2.29	5.75 ± 2.43 ^a	6.15 ± 2.31 ^c	6.48 ± 2.51 ^c

Values are means ± SD.

^a $P < 0.05$.

^b $P < 0.01$.

^c $P < 0.001$ compared to basal values.

Nevertheless, throughout the dietary protocol, plasma RBP4 levels remained lower than the levels at the beginning of the program. The evolution of RBP4 levels was different from that of the glucose disposal rate that shows steadily higher values at the different time points of the dietary intervention than at the beginning of the program (Fig. 2D).

Relationship between plasma RBP4 levels and insulin resistance

No correlations were found between RBP4 level and glucose disposal rate before the diet ($r = -0.31$; $P > 0.1$). Similarly, no correlations were found between the diet-induced changes of RBP4 and glucose disposal rate when considering either the VLCD phase or the whole dietary program ($r = -0.22$, $P > 0.3$; $r = -0.14$, $P > 0.5$, respectively). A similar conclusion was reached when the glucose disposal rate was corrected for fat-free mass. To investigate whether the lack of relationship between RBP4 and glucose disposal rate is dependent on the level of baseline insulin sensitivity, the 24 obese subjects were stratified into two groups according to prediet glucose disposal rate (Table 2). No differences in either basal plasma RBP4 levels or in the diet-induced decreases of plasma levels were observed between the two groups. Similarly, if the subjects were stratified into two groups according to the magnitude of the changes in glucose disposal rate during VLCD and the entire program, there was no difference in the diet-induced variation of plasma RBP4 levels between the groups (data not shown). No correlations were found between plasma RBP4 and plasma triglyceride, high-density lipoprotein cholesterol, or waist circumference when considering the diet-induced responses of the variables (data not shown). To investigate further the relationship between plasma RBP4 levels and insulin resistance, a group of control lean women (BMI 21 ± 2 kg/m²) was investigated and compared with the obese group. As expected, the glucose disposal rate was higher in the lean group than in the obese group (6.43 ± 1.61 vs. 2.98 ± 1.56 mg/kg/min, $P < 0.001$; and 8.27 ± 1.73 vs. 4.88 ± 2.29 mg/kg fat free mass/min, $P <$

0.001). However, plasma RBP4 levels did not differ between the two groups (26.8 ± 8.4 vs. 27.4 ± 7.4 mg/ml; $P > 0.8$).

Discussion

This study shows that RBP4 is strongly expressed in human adipocytes, as shown earlier in rat adipocytes (5). Negligible expression was detected in the stromavascular fraction of adipose tissue, as recently reported (16). During adipogenesis of human preadipocytes, there was a very strong induction of RBP4, which is typical of a marker of adipocyte differentiation (17). RBP4 is steadily secreted by human adipose tissue, as shown in experiments on adipose tissue explants. Therefore, RBP4 expression profile in human adipose tissue is similar to the profile described in rodents.

Studies in mice suggest that RBP4 is a factor produced by adipose tissue that induces insulin resistance in the liver and skeletal muscle (2). Plasma RBP4 was reported to be elevated in subjects with insulin resistance and type 2 diabetes, although the relationship between RBP4 and insulin resistance was not found in other cross-sectional studies on subjects with normal glucose tolerance or mildly insulin-resistant obese patients (6–8, 16, 18). Similarly, we did not find differences in plasma RBP4 levels between lean and obese women despite a lower glucose disposal rate in the latter group. To gain further insight into the relationship between insulin sensitivity and RBP4, we investigated, in a prospective study, nondiabetic obese women during different time points of a multiple-phase weight reduction program. Insulin sensitivity was assessed by the gold standard technique, the euglycemic hyperinsulinemic clamp. The subjects had a marked decrease in body weight and fat mass during VLCD, a further moderate diminution during LCD and a stabilization during the WM phase. The glucose disposal rate was increased during VLCD and remained elevated during the subsequent phases. The plasma levels of RBP4 were markedly diminished during VLCD and subsequently increased during the later phases while remaining lower than basal

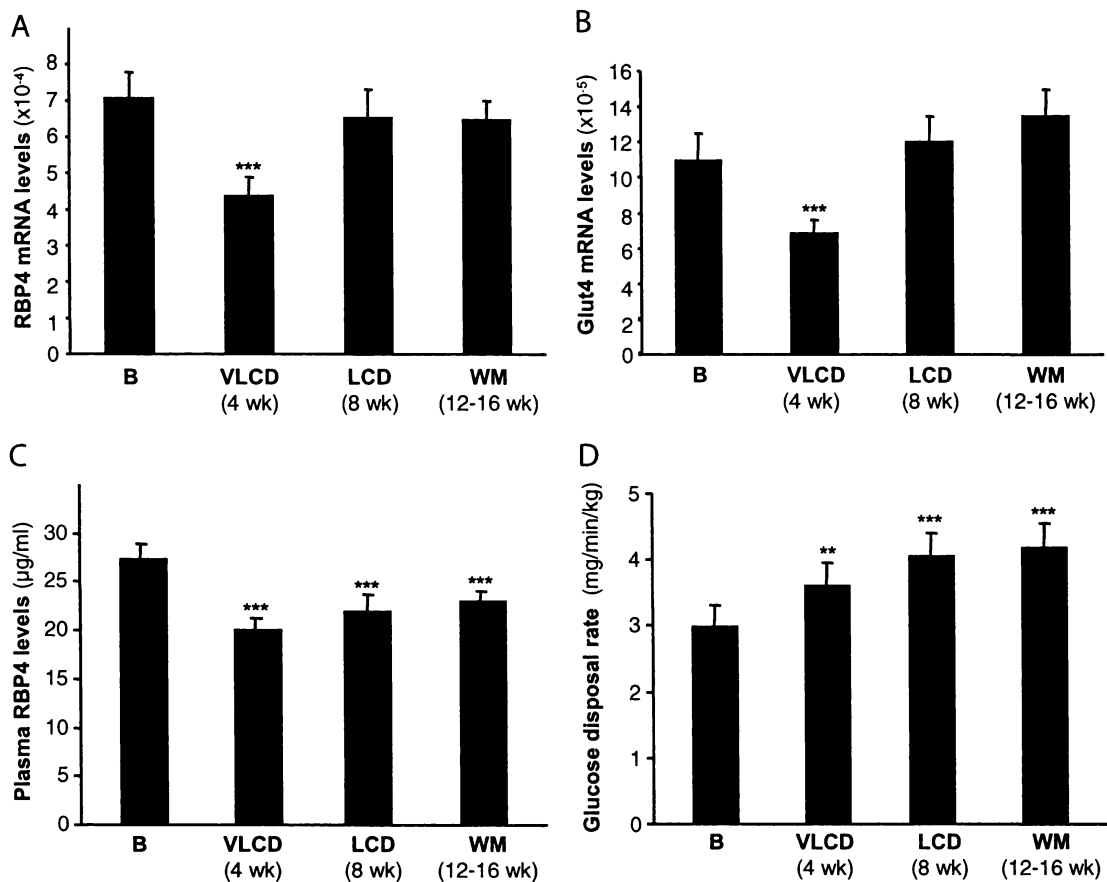


FIG. 2. Subcutaneous adipose tissue mRNA expression of RBP4 and Glut4, plasma level of RBP4, and glucose disposal rate during a weight reduction program in obese women ($n = 24$). A, Adipose tissue RBP4 mRNA levels. B, Glut4 mRNA levels. C, RBP4 plasma levels. D, Glucose disposal rate. Values are means \pm SEM. **, $P < 0.01$. ***, $P < 0.001$ compared with basal values. B, Basal conditions; LCD, end of the LCD; VLCD, end of the VLCD; WM, end of the WM phase.

values. Therefore, the profile of the plasma RBP4 time course was not superimposable to the variations in insulin sensitivity indices. Moreover, no correlations were found between RBP4 and glucose disposal rate. It was pointed out that the variability of the RBP4 response to an intervention might be influenced by the baseline insulin sensitivity of subjects (19). However, stratification of the population of obese women into a high and low-insulin sensitivity group was associated

TABLE 2. Diet-induced changes in plasma levels of RBP4 in two subgroups with high and low initial insulin sensitivity

	Obese subjects with low-insulin sensitivity ($n = 12$)	Obese subjects with high-insulin sensitivity ($n = 12$)
Glucose disposal rate (mg/min/kg)	1.77 \pm 0.12	4.18 \pm 0.35 ^a
Basal plasma RBP4 levels (μ g/ml)	29 \pm 3	26 \pm 1
Decrease of plasma RBP4 levels during VLCD (%)	23 \pm 8	26 \pm 4
Decrease of plasma RBP4 levels during WM (%)	17 \pm 5	13 \pm 4

Values are means \pm SEM.
^a $P < 0.001$.

neither with a significant difference in plasma RBP4 levels nor with different diet-induced responses of plasma levels of RBP4. When considering stratification according to the variations in insulin sensitivity induced by the dietary program, a similar conclusion was reached. Therefore, this kinetic study reveals that RBP4 is regulated by calorie restriction and weight loss but is not associated with insulin sensitivity in this population.

Plasma RBP4 levels are increased in mice with genetic ablation of Glut4 in adipocytes (2). Glut4 mRNA and protein levels are reduced in fat cells in several insulin-resistant states and in type 2 diabetes (20). In nonobese subjects with at least one first-degree relative with type 2 diabetes, a negative correlation was found between adipocyte Glut4 and plasma RBP4 levels (8). During the dietary intervention, the profile of variation of Glut4 mRNA levels was quite similar to that of RBP4 mRNA. Accordingly, a positive relationship between adipose tissue RBP4 and Glut4 gene expression was reported in adipose tissue from obese subjects (16). Therefore, the expected inverse relationship between adipose tissue Glut4 and RBP4 mRNA is not found in obese subjects.

Concentrations of serum proteins are used in assessing the

clinical status of patients with moderate-to-severe malnutrition. RBP4 is a clinically useful marker because it has a short half-life. In that respect, the decrease of plasma RBP4 levels during VLCD is in full agreement with earlier studies (21, 22). Moreover, this study shows that RBP4 gene expression in adipose tissue is subject to regulation by VLCD. Comparison of the evolution of adipose tissue mRNA and plasma levels suggests that the decrease of RBP4 levels during severe calorie restriction may be at least partly due to decreased adipocyte production. The down-regulation during VLCD may be related to ketosis because plasma β -hydroxybutyrate levels were increased specifically during this period. During LCD and WM, adipose tissue RBP4 mRNA levels returned to basal values, whereas the increase in plasma levels was more moderate and did not reach the prediet levels. Therefore, it may be hypothesized that, during these phases, the lower RBP4 levels reflect the reduction in fat mass. The magnitude of the decrease in fat mass during LCD and WM (22–23%) is indeed coherent with the decrease in RBP4 levels (15–20%). Another possibility is that the reduced plasma levels observed during long-term weight loss result from altered production by the liver, the probable major source of RBP4 in humans, but to date evidence is lacking for such a regulation (23). Finally, it may be noted that the pattern of changes in RBP4 and leptin levels were similar, raising the possibility of coregulatory mechanisms between the two adipokines.

To conclude, although RBP4 adipose tissue gene expression and plasma levels are reduced during severe calorie restriction, no relationship was observed between RBP4 and the improvement of insulin sensitivity induced during a weight reduction program in obese women. RBP4 is a marker of nutritional deficit but does not appear as a marker of insulin resistance during dietary intervention.

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Address all correspondence and requests for reprints to: Dominique Langin, Institut National de la Santé et de la Recherche Médicale Université Paul Sabatier U858, IFR31, BP 84225, 31432 Toulouse Cedex 4, France. E-mail: langin@toulouse.inserm.fr.

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In fine of the first part, we can conclude that any type of lifestyle modifications, nutritional intervention or physical activity with or without weight loss provided health benefits in terms of improvement of IS in obese subjects. If we try to sum up our findings and focus on the impact of lifestyle interventions during a 3 month period on IS in obese individuals regardless the gender and other factors, we could say that calorie restriction program and dynamic strength training are the most powerful means to ameliorate glucose metabolism. Aerobic training appears as the least efficient (increase in IS by 12%), but it needs to be noted that in this study contrary to the two others, IS was not assessed with the gold standard technique, the euglycemic hyperinsulinemic clamp. The improvement in insulin action was found similar in resistance training and dietary intervention study (increase by 24-31% and 26-36%, respectively). We must be careful to drive definitive conclusions about the possible mechanisms implied in the alleviation of IR, as the contribution and interplay of many factors is likely to be involved (weight loss, gender and age of subjects, level of obesity and disturbances, different training impulse, type of training and diet, etc.). One of these factors could be the loss of weight. As we have observed the moderate body weight reduction (6-10%) by aerobic training and dietary program, and stable weight during resistance training, we suppose that weight loss in itself is not a major determinant for the decrease in IR, however we do not exclude that it can have promoting effect.

Our primary aim was to test the hypothesis that the putative unfavourable profile of adipokines produced by obese AT and low-grade inflammation present in obese people could be influenced by lifestyle interventions with insulin sensitizing effects, i.e. that adipokines could play a role in the regulation of IS. The findings of our studies indicate that adiponectin, TNF- α , IL-6, IL-1 β and RBP4 are not the paramount mediators of changes in IS, induced either by physical activity or calorie restriction. **Adiponectin** has been proposed to play a central role in the regulation of glucose and lipid metabolism in humans, and to be a link between obesity and the development of IR. Later on, the adiponectin molecule has been found to circulate in human plasma in the form of different multimeric complexes and their distinct biological activities are recently a subject of intense research. Because of the lack of appropriate methods during the course of our studies, we have not investigated in our clinical work the effect of lifestyle interventions on adiponectin isoforms. This could be seen as a limitation. Several currently published studies were designed to identify the changes in plasma distribution of

adiponectin multimeric complexes following dietary intervention and have yielded contradictory results. They showed no changes in adiponectin oligomers distribution (including one recent study from our laboratory, unpublished data) [284], an increase in the HMW and MMW form [261,283], or an increase in all of the multimeric isoforms as observed in another recent study from our laboratory [279]. This discrepancy could be ascribed to many factors, especially to the use of different analytical methods, small study groups and sex differences. A few studies evaluated the effect of marked weight loss induced by bariatric surgery (BPD or GBS) in adiponectin multimerization but similarly to the effect of moderate weight loss induced by diet, they do bring equivocal data. Engl et al. reported an increase of total and MMW adiponectin, whereas HMW and LMW remained unchanged 1 year after surgical treatment [427]. Salani et al. showed increase in total adiponectin and HMW form 1 month after BPD [428]. Swarbrick et al. observed no change in total adiponectin but elevated amount of HMW form 1 month after GBS and at 1 year, both total and HMW concentrations were significantly increased [429]. To the best of my knowledge, only two very recent reports on physical activity-induced changes in plasma adiponectin oligomers have been published, assessing the levels of total adiponectin and HMW forms [274,278]. Bobbert et al. concluded that acute and chronic exercise directly affects neither circulating total adiponectin nor the HMW form distribution in lean and healthy subjects, as evaluated by ELISA and Western blot, respectively [278]. Bluher et al. used various commercially available ELISA assays to determine both the total and HMW adiponectin, and found substantially different results for total adiponectin concentrations, depending on the ELISA applied [274]. Exercise training did not change plasma levels of total adiponectin in subjects with normal glucose tolerance when measured by ELISAs from LINCO Research and Mediagnost, which is in agreement with the results of Bobbert et al. [278]. Using the same ELISA kits, there was a significant increase in total adiponectin in subjects with impaired glucose tolerance or type 2 diabetes mellitus after 1 month of exercise training. ELISA assay from ALPCO Diagnostics brought the opposite results, i.e. increase of total adiponectin in subjects with normal glucose tolerance and its unchanged levels in two other investigated groups. HMW form was assessed only by ELISA from ALPCO Diagnostics and was found to be increased in individuals with normal glucose tolerance and unaltered in two other groups of subjects. Based on the recent findings presented above, it is difficult to make any conclusion on the effect of insulin-sensitizing lifestyle interventions in adiponectin

multimerization and further studies are warranted to elucidate the physiological relevance and function of multimeric complexes of adiponectin with respect to obesity and IR. Contrary to unchanged levels of the majority of investigated adipokines, **RBP4** plasma levels were reduced after each phase of 3 months nutritional intervention program, and RBP4 mRNA levels were downregulated only during severe calorie restriction (VLCD). Despite its decrease, our study does not bring evidence for the role of RBP4 in the regulation of changes of IS. This notion was deduced from several findings in our study, such as no association between RBP4 and improvement of IS, opposite kinetics of plasma RBP4 and glucose disposal rate, lack of difference in basal or diet-induced decrease of plasma RBP4 levels between the groups stratified according to baseline IS, and no difference in plasma RBP4 levels between lean and obese women. A different situation is seen for **leptin**, which circulating levels have been decreased by each of the three lifestyle modalities. The aerobic or strength training-induced plasma leptin reduction remained significant after adjustment to BMI (reduction by about 20%), suggesting a direct effect of physical activity or factors other than weight loss on leptin release and/or clearance. Interestingly, leptin has been observed diminished while the body fat mass was maintained in our resistance training study, as well as during intensive training in studies of Steinacker et al. [430,431]. It is tempting to speculate that the amelioration of IS status seen in our subjects could be ascribed to the effect of this adipokine, even if no correlations between leptin and indices of IS were apparent in our clinical studies. Anyway, associations alone do not prove a causal relationship of investigated parameters and other reinforcing evidence is of need. As mentioned in the introduction, leptin improves IS, at least in part, by its action in the periphery. It has been demonstrated, that leptin increases FA oxidation in skeletal muscle in rodents and cultured skeletal muscle of lean humans, via activation of AMPK. Moreover, it is known that chronic hyperleptinemia, typical in obesity, blunts leptin's effect in skeletal muscle, i.e. leptin is not able to stimulate FA oxidation, thus reflecting skeletal muscle leptin resistance [432]. High baseline leptin levels may reflect also central leptin resistance [433]. Molecular mechanisms for leptin resistance may include dysregulation of leptin synthesis and/or secretion, abnormalities of brain leptin transport, and abnormalities of leptin receptors and/or post-receptor signaling, but are still remained to be clearly determined [225]. It has been suggested that SOCS3 may be one of the factors implicated in the inhibition of leptin signaling pathway in skeletal muscle, AT or brain [225]. This notion was recently

supported by data in skeletal muscle and AT in humans [250,434,435]. Another recent finding is that human skeletal muscle expresses at protein level the long isoform of the leptin receptor. Moreover, OB-Rb and OB-Ra mRNA are present in human AT. These data suggest that muscle and AT can respond to circulating leptin and support the possibility of a downregulation, desensitization and/or imbalance of OB-Rs associated with a defective leptin signaling cascade [434,435]. In normal conditions, when leptin levels are low, or normal, leptin exerts its insulin sensitizing effect [433]. Thus, it seems logical, that the reversion of leptin resistance can represent an important step to improve IS. Based on the results of our studies it is conceivable, that non-pharmacological interventions could restore leptin sensitivity, pulsatility and circulating levels, leading to normal functioning of leptin at the periphery (or centrally), i.e. improving muscle oxidative capacity and increasing IS. Given that leptin exerts inflammatory properties, its diminution might be beneficial in the term of breaking the inflammatory signaling pathways in AT, including decreased macrophage infiltration and contributing to the increase in whole-body IS. To determine whether these speculations are true, further investigations in humans must be conducted. A major task is to understand, which are the precise molecular mechanisms of leptin decrease and how, if at all, leptin can mediate these effects. On the other hand, it can also be imagined that the normalization of IS itself by unknown mechanisms restore leptin sensitivity. Nevertheless, it is expected that the process of improvement of IR is highly complex, involving more than a single molecule and multiple adaptation in glucose and lipid metabolism in several tissues. The general view is that both aerobic or resistance training and nutritional modifications lead to a decrease in the magnitude of VAT [370,372]. Design of our intervention trials did not allow us to assess the changes in regional AT depots, especially the reductions in total and VAT. Hence, we could investigate neither the longitudinal changes in the expression of the above mentioned adipokines in VAT nor the possible link between visceral adiposity and IR. The impact of lifestyle modifications, applied in the three different studies, on clinical and plasma parameters in obese subjects is summarized in Table 6.

Table 6 Summary of the effects of lifestyle interventions represented by aerobic exercise training, dynamic strength training and calorie restriction program on clinical and plasma parameters in obese individuals from three clinical trials performed in our laboratory.

	AEROBIC TRAINING (3 months, ♀)	RESISTANCE TRAINING (3 months, ♂)	DIETARY INTERVENTION (3 months, ♀)
Anthropometric and clinical data			
Body weight	↓	↔	↓*
BMI	↓	↔	↓*
Fat mass	↓	↔	↓*
Waist	↓	↔	↓*
VO2max	↑	↔	–
Skeletal muscle strength	–	↑	–
Biochemical and metabolic data			
Glucose	↔	↔	↔*
Insulin	↔	↔	↓*
Total cholesterol	↔	↔	↓*
Triglycerides	↔	↔	↓*
FFA	↓	↑	↓*
hsCRP	–	↔	–
Insulin sensitivity	↑	↑	↑*
Adipokines (plasma / SCAT mRNA)			
Leptin	↓ / ↔	↓ / ↔	↓ / –*
Adiponectin	↔ / ↔	↔ / ↔	↔ / –*
TNF-α	↔ / ↔	↔ / ↔	–
IL-6	↔ / ↔	↔ / ↔	–
IL-1β	–	↔ / ↔	–
RBP4	–	–	↓ / ↔*

↑ significant increase, ↓ significant decrease, ↔ no change, – analysis not performed

* the effect after the 3-4 months of WM phase (6-7 months of total intervention) was similar to that after 3 months, i.e. at the end of the LCD period

3.2 SECOND PART

IMPACT OF PPAR AGONISTS AS PHARMACOLOGICAL AGENTS ON THE REGULATION OF HUMAN ADIPOKINE PROTEIN EXPRESION



Profiling of adipokines secreted from human subcutaneous adipose tissue in response to PPAR agonists.

Klimcakova E, Moro C, Mazzucotelli A, Lolmede K, Viguerie N, Galitzky J, Stich V, Langin D.
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3.2.1 Comment on the second part

As a consequence of the difficulties to maintain long-term weight loss acquired by dieting or increased physical activity, it is expected that a major effort is made for the identification of regulatory systems and effective targets for drug development. A corollary to, AT represents the target organ to search for such markers, i.e genes and proteins expressed and/or secreted by different AT cell types. From the methodological point of view, expression profiling of AT appears as a necessary approach to identify the potential drug targets for obesity and other associated diseases. On one hand, rapid technological progress opened the possibility to analyze gene expression by DNA array methods, and on the other hand the big advance has been made in proteomic research. "Proteomics could be described as the global analysis of gene expression at the protein level (i.e. investigating expression level, post-translational modification, interactions, etc.) from a whole organism, cell culture or a tissue" [436,437]. Proteomic approach receives reasonably more and more attention because it enables to complete data from mRNA analyses and dissect the role of proteins globally in various physiological processes and metabolic pathways. In adipose biology, it opens a new possibility for the study of obesity and linked metabolic disturbances. The second part of this thesis is an *in vitro* study based on a pharmacological approach, which results and discussion are presented in detail in the publication. The synopsis and additional commentary regarding our finding is presented here.

◆◆◆◆ In the presented *in vitro* study, we were interested in a comprehensive survey of attractive targets for drug development, i.e. three PPAR isotypes in SCAT, as only circumstantial evidence currently exists with regard to their respective actions in human AT. One of the reasons for PPARs to be an interesting pharmaceutical target is their particular feature of large ligand-binding properties, making these receptors capable to accommodate and interact with a range of different ligands [438]. We investigated adipokine secretion in response to synthetic, highly selective PPAR α , PPAR β/δ and PPAR γ ligands (GW7647, GW0742 and BRL49653/rosiglitazone, respectively), by using cytokine antibody arrays. This technique allowed us to explore 120 cytokines spotted on the array membrane, and discover among secreted proteins from AT explants unknown factors that could be potentially involved in the regulation of energy metabolism and that could be putative new therapeutical targets. The main hypothesis in our study, based on the fact that PPAR isotypes are expressed in various cell types that are present in AT (e.g. adipocytes, EC and macrophages), was that each of the PPAR agonists can have an impact on AT to stimulate adipokine expression. All three of the PPAR isotypes are supposed to participate in the regulation of inflammatory responses, both *in vivo* and *in vitro*. Interestingly and not surprisingly for obese white AT, a majority (50%) of peptides identified in the culture media from human SCAT in our study is involved in immune response (according to the Human protein resource database) (<http://www.hprd.org>). We identified 16 proteins highly secreted in the medium, and the production of 5 of them was regulated by activated PPARs. Two of these factors were leptin and IL-6. After the treatment of AT explants with each of the PPAR agonists, the relative secretion levels of both leptin and IL-6, were found to be decreased by 12-16 % and 11-16%, respectively. PPAR α and PPAR β/δ ligands substantially enhanced hepatocyte growth factor (HGF) secretion by 37% and 22%, respectively. PPAR β/δ agonist markedly diminished angiogenin by 21% and increased TIMP-1 release by 30%. The best studied isotype PPAR γ , expressed at high levels in AT, is the main target for TZDs and PPAR γ -activated modulation of adipokine expression is one of the plausible mechanisms for indirect insulin sensitizing effect of TZD action on muscle and liver [439]. According to the best of my knowledge, there is only one study investigating the effect of TZDs on human AT explants [440]. Most studies use animal models for *in vitro* and *in vivo* experiments, or 3T3-L1 cell lines. Human studies compare plasma levels of subjects after oral

administration of different TZDs or placebo. Indeed, studies using rosiglitazone are not numerous.

Leptin is one of the candidate effectors for TZD-stimulated fat-to-muscle signaling, but findings derived from rodents cannot be straightforwardly applied to humans and consistent clinical data are missing. Toruner et al. showed that PPAR γ and PPAR α agonists decrease serum levels of leptin in diet-induced obese rats [441]. Our results show that leptin expression can be altered by PPAR α as well as by PPAR β/δ ligands, what has not previously been reported regarding human SCAT. Given that leptin can exhibit pro-inflammatory effects among others, its downregulation by PPAR agonists may be of high importance. From other adipokines, **adiponectin** might be the crucial candidate between PPAR γ -activation and the metabolic response of peripheral tissues, but its essential role in this process has not been confirmed so far. Yamauchi et al. demonstrated increased adiponectin mRNA and plasma levels *in vivo* in mice on high-fat diet and wild controls, and also upregulation of adiponectin mRNA *in vitro* in differentiated 3T3-L1 adipocytes after rosiglitazone treatment [258]. Also Combs et al. showed adiponectin elevation in plasma in different mice models and increased mRNA levels in 3T3-L1 cells in response to PPAR γ -specific TZD [442]. Bodles et al. in the only study using human adipose explants showed an increase in total adiponectin secretion in a dose-dependent manner after pioglitazone treatment and found that this increase was entirely due to the increase in HMW form [440]. In humans, it seems that oral rosiglitazone treatment consistently increases plasma adiponectin levels [442-444]. In our study, we did not observe any significant changes in adiponectin levels regardless of the PPAR agonist treatment. The disparity of results in the above mentioned studies could be ascribed to different experimental settings, differences at pre- and post-transcriptional level, and/or adiponectin post-translational formation of multiple isoforms. It can be seen from the human *in vivo* studies as discussed in the first part of the Results Chapter, that adiponectin oligomers might have distinct physiological functions and that associations of changes in HMW, MMW and LMW forms with changes in IS may be more specific to TZD treatment. A recent study showing that TZD treatment (pioglitazone) selectively stimulates secretion of the HMW adiponectin in human AT, human and mice adipocytes, and cell lines further supports this hypothesis [440]. The same limitation for our *in vitro* study can be raised as for our *in vivo* clinical studies, i.e. that we did not investigate possible changes in adiponectin multimeric complexes in response to various interventions.

However, while the application of cytokine antibody array method in our *in vitro* study allowed us to screen a large number of secreted factors, it did not allow us to evaluate the effect of PPAR agonists on adiponectin oligomers in the culture media from human AT explants. We suppose that antibodies used in RayBio Human Antibody Array detected the total adiponectin and other specific antibodies or techniques would be required to identify different adiponectin isoforms. Rosiglitazone treatment of patients with metabolic syndrome lowered IL-6 plasma levels [444], but there is no information on the effect of the two other agonists PPAR α and PPAR β/δ on IL-6 production, neither *in vivo* in humans nor *in vitro* from human SCAT. Likewise, the role of HGF, TIMP-1 and angiogenin produced by AT in relation to all PPAR isotypes has not been investigated and further studies are needed to clarify their function, with special reference to the complications of obesity in humans.

The sub-objective of the study was to define the origin of factors released by AT that were affected by PPAR agonists, and because of the lack of hints on PPAR isotype cellular distribution in human AT itself (except for PPAR γ), we measured also mRNA levels of all three of the PPARs. By real-time quantitative PCR gene expression analysis we confirmed the paramount expression of leptin and adiponectin in adipocytes, while HGF, IL-6 and TIMP-1 were predominantly expressed in AT stromal vascular fraction. Angiogenin was detected in both adipocytes and stromal cells. It is of note, that PPAR α mRNA level in human SCAT was higher (~ 4 fold) in adipocytes when compared with SVF. Hence, even if the expression of PPAR α in fat cells was about 6-fold less than PPAR γ mRNA levels, its potential contribution to the regulation of adipokine expression in AT needs to be taken into account. Accordingly, our recent study shows that PPAR α specifically regulates the expression of the Gyk gene in human adipocytes [445] (see Annex). It was out of the scope of our study to investigate the mechanisms of PPAR actions, therefore they remain largely speculative and other research is to be performed to make any statement.



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Profiling of adipokines secreted from human subcutaneous adipose tissue in response to PPAR agonists

Eva Klimcakova^{a,b,c,*}, Cedric Moro^{a,b,c}, Anne Mazzucotelli^{a,b,c}, Karine Lolmède^{b,c},
Nathalie Viguerie^{a,b,c}, Jean Galitzky^{b,c}, Vladimir Stich^a, Dominique Langin^{a,b,c,d}^a Franco-Czech Laboratory for Clinical Research on Obesity, Inserm and 3rd Faculty of Medicine, Charles University, Prague CZ-100 00, Czech Republic^b INSERM, U858, Obesity Research Laboratory, I2MR, Toulouse F-31432, France^c Paul Sabatier University, Louis Bugnard Institute IFR31, Toulouse F-31432, France^d CHU de Toulouse, Biochemistry Laboratory, Biology Institute of Purpan, Toulouse F-31059, France

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Abstract

The role of PPARs in the regulation of human adipose tissue secretome has received little attention despite its potential importance in the therapeutic actions of PPAR agonists. Here, we have investigated the effect of selective PPAR γ , PPAR α , and PPAR β/δ agonists on the production of adipokines by human subcutaneous adipose tissue. Antibody arrays were used to measure secreted factors in media from cultured adipose tissue explants. Sixteen proteins were produced in significant amounts. Activation of PPARs regulated the production of five proteins. Treatments with the three PPAR agonists decreased the secretion of leptin and interleukin-6. PPAR α and β/δ agonists markedly enhanced hepatocyte growth factor secretion whereas PPAR β/δ down-regulated angiogenin and up-regulated TIMP-1 release. Hepatocyte growth factor, interleukin-6, and TIMP-1 are chiefly expressed in cells from the stromal vascular fraction whereas angiogenin is expressed in both adipocytes and cells from the stromal vascular fraction. Our data show that PPAR agonists modulate secretion of bioactive molecules from the different cell types composing human adipose tissue.

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Keywords: PPAR agonist; Cytokines; Antibody array; Secretome; Subcutaneous adipose tissue; Obesity

Study of adipose tissue (AT) has attracted an enormous attention because of its role in the development of obesity and related complications. Although obesity can be prevented or treated by controlling the food intake and physical activity, these theoretically simple interventions fail to bring long-term results. The poor outcomes reflect the urgent need for more effective therapies. One of the major targets for drug development is nuclear hormone receptors. Peroxisome proliferator activated receptors (PPARs) are targets for several classes of drugs used in the treatment of obesity associated diseases such as type 2 diabetes, dyslipidemia, or hypercholesterolemia [1]. A trio of PPAR iso-

types, PPAR α (NR1C1), PPAR β/δ (NR1C2), and PPAR γ (NR1C3) have been identified to date [2,3]. The different ligand-activation profiles of PPARs and their diverse cell, tissue, and organ distribution gives them a broad range of physiological functions (reviewed in Refs. [4,5]). Despite an intensive research, our understanding of PPAR roles in AT is far from being complete. PPAR γ expressed at high levels in AT controls adipogenesis and insulin sensitivity [6,7]. PPAR α regulates lipid catabolism in highly oxidative tissues but its role in AT has not been documented [8]. PPAR β/δ is the most ubiquitously distributed isotype. It is suggested to have a broad function, but remains the least well understood. Of note, overexpression of PPAR β/δ in mouse AT leads to resistance to obesity [9].

The paracrine and endocrine function of AT has attracted a considerable interest. AT is an important source of adipokines, i.e. factors produced by adipocytes and

* Corresponding author. Address: Franco-Czech Laboratory for Clinical Research on Obesity, Inserm and 3rd Faculty of Medicine, Charles University, Prague CZ-100 00, Czech Republic. Fax: +420 267102263.

E-mail address: e.klimcakova@post.lf3.cuni.cz (E. Klimcakova).

other cells present in AT. The adipokines are thought to contribute to the development of an array of metabolic disturbances such as insulin resistance and type 2 diabetes [10,11]. To investigate adipokine secretion by AT, protein expression profiling is emerging as an important tool. To date, there is only a limited number of studies applying a proteome approach on human AT secretome [12–16]. They were largely aimed at the characterization of the peptide spectra secreted from various AT depots, but not at the regulation of protein secretion. In one recent study, our group has investigated the regulatory role of atrial natriuretic peptide on human AT cytokine secretion [16]. However, such information on PPARs is missing. PPAR γ is the most highly expressed subtype in adipocytes. The different PPAR isotypes are also expressed in macrophages and endothelial cells [17,18], i.e. cells that are present in AT. In endothelial cells, PPAR α and PPAR γ have anti-angiogenic, anti-inflammatory, and anti-proliferative properties [17] whereas PPAR β/δ induces angiogenesis and proliferation through vascular endothelial growth factor [18]. In macrophages, PPAR α and PPAR γ inhibit inducible nitric oxide synthase, MMP-9 and TNF α and, PPAR β/δ may regulate the inflammatory response [17]. Therefore, an impact of PPAR agonists in human AT on adipokine secretion is expected.

Most of the data on PPAR physiological functions were derived from animal experiments. Focusing on PPAR agonists in human AT is critical as important species differences have been reported. Distinct transduction pathways exist in human and rodent fat cells, notably for the control of lipolysis [19]. Major differences have also been described regarding adipokine production. For example, resistin which is produced by adipocytes in mouse AT is secreted from macrophages in humans [20].

In the present study, we wished to elucidate how the activation of the three PPAR isotypes by highly selective ligands is modulating the production of adipokines by human subcutaneous AT. We used cytokine antibody arrays that offer the possibility to screen more than hundred secreted factors [12,14,16]. Given that the majority of AT secreted products is not produced by adipocytes but by nonfat cells present in the stromavascular fraction (SVF) [21], we performed the experiments on whole AT explants. The large-scale proteomic profiling was completed by analysis of adipokine gene expression in the different cellular fraction of human AT.

Materials and methods

Culture of human adipose tissue explants. Human subcutaneous AT was obtained from 13 overweight and obese women undergoing plastic surgery in agreement with the declaration of Helsinki, the French National Institute of Health and Medical Research (INSERM), and the Toulouse University Hospital Ethics Regulation. Fresh surgical AT samples were cleared of the skin and vessels and rinsed in warm PBS under aseptic conditions. Culture of human AT explants was performed as previously reported [16]. Explants (~333 mg/ml) were either treated with the PPAR γ agonist BRL49653/rosiglitazone (Sigma), the PPAR β/δ agonist GW0742

(a kind gift from Glaxo-Smithkline-Beecham), the PPAR α agonist GW7647 (Sigma) or the vehicle (DMSO) as control. A treatment of 24 h was selected as our pilot experiments showed a significant induction in gene expression of known targets of PPAR γ (adiponectin, lipoprotein lipase, fatty acid translocase/CD36, fatty acid binding protein 4, and cytosolic phosphoenolpyruvate carboxykinase) when compared to 10 h incubation. Moreover, culture for 24 h does not induce a response associated to hypoxia and inflammation [21,22]. After incubation, aliquots of the media were taken for measurement of proteins released to the medium. Explants were washed in PBS and homogenized in lysis buffer for mRNA measurements. The samples were stored at -80°C until analysis.

Cytokine antibody arrays. The RayBiotech kit (RayBio[®] Human Cytokine Antibody Array C Series 1000, RayBiotech Inc.) composed of two array membranes with 2×60 cytokines (human cytokine antibody arrays VI and VII) was used for protein screening in adipose tissue explant medium. The complete list of cytokines and their full names is available at <http://www.raybiotech.com>. The experiment was performed according to the manufacturer's instructions. Briefly, 1 ml of medium was added to an antibody-coated membrane with cytokines spotted in duplicates and incubated overnight on a plate shaker at 4°C . After incubation with a cocktail of biotinylated antibodies and labeled-streptavidin, the signal was detected by chemiluminescence. The analysis of spot signal intensities was performed using the ScanAlyze software. Intra-assay variability range was 1–10% in our experiments. Positive controls were used to normalize the results from multiple membranes. The relative changes in cytokine levels are expressed in arbitrary units (AU)/g of AT.

Isolation of mature adipocytes and the stromavascular fraction of human adipose tissue. Mature adipocytes were separated from the SVF by collagenase digestion of AT pieces. The isolation of the different cell types from the SVF was performed using surface antigen-coupled magnetic microbeads [23].

mRNA quantification. Total RNA extraction and reverse transcription were performed as previously described [24] except that total RNA of different cell populations was treated with DNase I (DNase I amplification grade, Invitrogen). Real-time quantitative PCR (qPCR) was performed on ABI PRISM 7500 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA). A set of primers was designed for adiponectin, leptin, and tissue inhibitor of metalloproteinase 1 (TIMP-1) and used with SYBR-green based chemistry. For these primer pairs, a standard curve was obtained using serial dilutions of human adipose tissue cDNA prior mRNA quantification. Primers and probes for interleukin-6 (IL-6), angiogenin, hepatocyte growth factor (HGF), PPAR α , PPAR β/δ , and PPAR γ were obtained from Applied Biosystems using TaqMan probe-based assays. 18S ribosomal RNA was used as control to normalize gene expression using the TaqMan assay kit (Applied Biosystems). Each sample was performed in duplicate and 10 ng of cDNA was used as template for qPCR. When the difference between the duplicates was above 0.5 C_t , qPCR was performed again. Results are expressed as $2^{\Delta C_t}$ values.

Statistical analyses. Data are presented as means \pm SEM from 5 to 8 experiments for each data point. Statistical analysis was performed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Cytokine secretion profile from adipose tissue

The culture media from human subcutaneous AT explants were incubated with cytokine antibody arrays, designed to detect 120 cytokines, chemokines, and other secreted factors (Supplementary Fig. 1). We identified 16 factors abundantly present in the medium of human AT explants. Adiponectin, leptin, IL-6, IL-8, monocyte chemoattractant protein 1 (MCP-1), growth-related oncogene (GRO), HGF, angiogenin, TIMP-1, and TIMP-2 showed a high secretion level (Fig. 1). According to the human

protein resource database (<http://www.hprd.org>), a majority of identified peptides is involved in immune response (50%) and in the process of signal transduction and cell communication (25%).

Effect of PPAR agonists on adipose tissue protein secretion

Conditioned media of AT explants treated with 1 μ M of rosiglitazone, GW0742 and GW7647 were used to identify potential targets of PPAR γ , PPAR β/δ , and PPAR α agonist, respectively (Fig. 2). Treatment of AT explants with each of the PPAR agonist decreased the relative secretion levels of leptin (by 16%, 16%, and 12%, respectively) and IL-6 (by 14%, 11% and 16%, respectively). Both, PPAR α and β/δ agonists markedly enhanced HGF secretion (by 37% and 22%). PPAR β/δ significantly down-regulated angiogenin (by 21%) and up-regulated TIMP-1 (by 30%) release. We did not observe any significant changes in adiponectin levels regardless of the PPAR agonist treatment.

Cellular origin of secreted factors in human adipose tissue

Next, we sought to determine which fraction of AT expresses the cytokines regulated by PPAR agonists. The relative gene expression was measured in mature adipocytes, SVF, endothelial cells, and macrophages of human subcutaneous AT (Table 1). Adiponectin and leptin were expressed almost exclusively in adipocytes. The mRNA levels of PPAR γ but also PPAR α were significantly higher in adipocytes than in SVF. IL-6, HGF, TIMP-1, and PPAR β/δ were predominantly expressed in stromal cells. In SVF, PPAR γ , and IL-6 were expressed preferentially in endothelial cells. TIMP-1 was expressed at higher levels in macrophages. HGF, PPAR α , and PPAR β/δ did not show any SVF cell-specific gene expression pattern. Angiogenin was

expressed in adipocytes to the same extent than in stromal cells with a high expression in endothelial cells.

Discussion

The present study provides an overview of the profile of proteins that are secreted by human subcutaneous adipose tissue (AT) and for the first time of those that respond to the action of the three known PPAR isotypes activated by highly selective synthetic ligands. The screening was performed among 120 cytokines by using a cytokine antibody array approach. We have identified 16 proteins abundantly secreted by AT. Five of them were regulated by PPARs and we report here HGF, angiogenin, and TIMP-1 as new factors regulated by PPARs.

It is not surprising that most of the secreted factors found in the present study are chemokines and cytokines implicated in the immune response and inflammation. Obese white AT is characterized by the expansion of blood vessels and connective tissue and by macrophage infiltration, which locally contributes to the production and secretion of inflammatory markers [25,26]. The pro-inflammatory cytokine IL-6 was released from cultured explants in the highest amount of all secreted proteins. The same finding was observed by Fain et al. after 24 h incubation of human AT [21]. Moreover, other interleukins such as IL-8, chemokines such as GRO and MCP-1 and proteins participating in the remodelling of the extracellular matrix such as TIMPs were produced in significant quantities. These factors are normally present in the circulation at low levels. It therefore suggests that these molecules exert paracrine properties in the sub-clinical inflammatory state of AT.

Based on the real-time quantitative PCR gene expression analysis, we determined the cellular origin of several adipokines and confirmed predominant expression of leptin and adiponectin in adipocytes [26]. Other cytokines

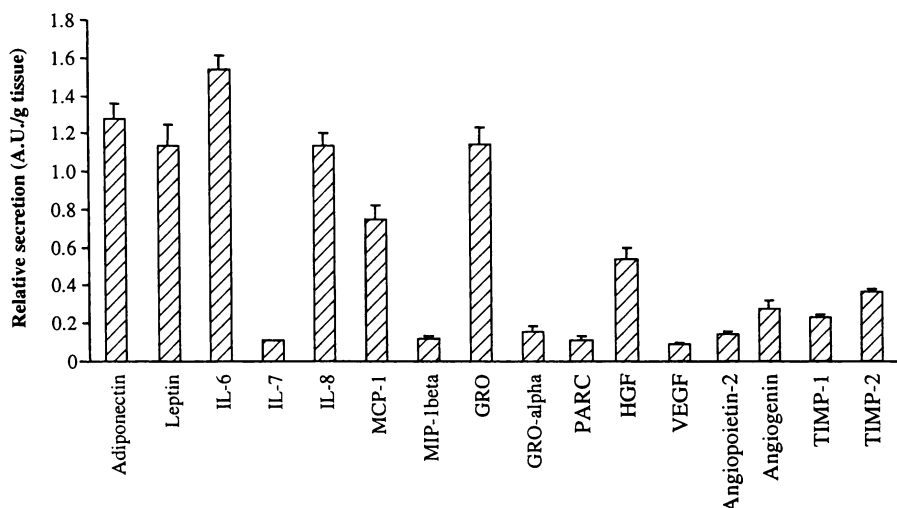


Fig. 1. Adipokines produced by human subcutaneous adipose tissue. Relative baseline secretion levels of various cytokines and other proteins in adipose tissue-conditioned media after 24 h of culture were normalized by grams of tissue. Data are means \pm SEM ($n = 8$).

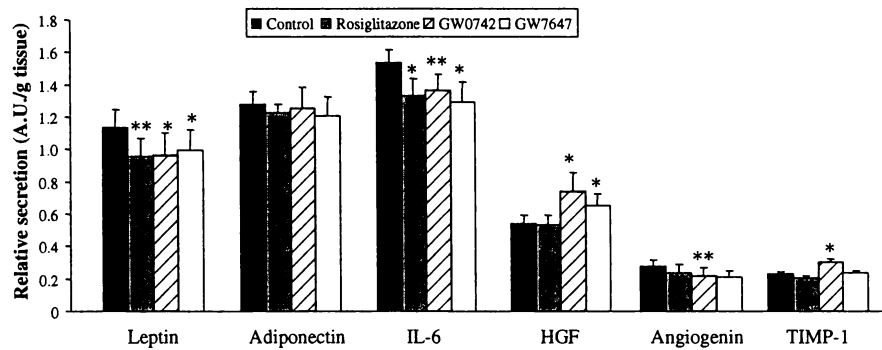


Fig. 2. Protein secretion by human adipose tissue explants in response to PPAR agonist treatments. Protein arbitrary units were expressed relative to adipose tissue explant weight. Data are means \pm SEM ($n = 8$). * $p < 0.05$ and ** $p < 0.01$ compared to control values.

Table 1

Comparison of mRNA levels in adipocytes, stromavascular fraction (SVF), macrophages, and endothelial cells from human subcutaneous adipose tissue

Gene	Adipocytes	SVF	Adipocytes-to-SVF ratio	Macrophages	Endothelial cells
<i>Adipokines</i>					
Adiponectin	1000 \pm 300**	0.71 \pm 0.24	1800 \pm 380	0.20 \pm 0.05	0.14 \pm 0.08
Leptin	1100 \pm 240**	1.6 \pm 0.6	1000 \pm 360	0.55 \pm 0.12	0.34 \pm 0.09
<i>Other proteins</i>					
IL-6	0.76 \pm 0.48	50 \pm 12**	0.016 \pm 0.006	40 \pm 17	160 \pm 45 ^s
Angiogenin	1.2 \pm 0.1	1.5 \pm 0.3	1.2 \pm 0.3	0.70 \pm 0.09	0.97 \pm 0.06 ^s
HGF	0.01 \pm 0.004	0.26 \pm 0.04**	0.071 \pm 0.023	0.39 \pm 0.08	0.25 \pm 0.03
TIMP-1	0.45 \pm 0.14	47 \pm 12**	0.011 \pm 0.002	56 \pm 5 ^s	25 \pm 2
<i>PPARs</i>					
PPAR α	0.55 \pm 0.05**	0.15 \pm 0.01	3.9 \pm 0.3	0.20 \pm 0.03	0.18 \pm 0.02
PPAR β/δ	0.10 \pm 0.01	0.42 \pm 0.10**	0.39 \pm 0.09	0.29 \pm 0.02	0.23 \pm 0.03
PPAR γ	3.1 \pm 0.3**	1.2 \pm 0.3	5.0 \pm 1.4	0.73 \pm 0.03	1.3 \pm 0.2 ^s

Data are means \pm SEM ($n = 5$). Relative mRNA levels are expressed as arbitrary units obtained after normalization by the 18S ribosomal RNA subunit ($2^{\Delta\Delta C_t} \times 100,000$). Ratio is the mean of individual mRNA ratios calculated by dividing adipocyte-mRNA values by SVF-mRNA values.

** $p < 0.01$ for adipocytes vs SVF.

^s $p < 0.05$ for macrophages vs endothelial cells.

secreted by AT were expressed mainly in SVF. While the tissue distribution of the PPAR genes is well elucidated, there is no information on PPAR cell-specific amounts in AT, except for PPAR γ which is expressed in the greatest amounts in adipocytes [27]. The levels of PPAR α were higher in adipocytes compared with SVF suggesting an unappreciated role of PPAR α in human fat cells. PPAR β/δ was expressed predominantly in stromal cells. The cellular distribution of PPARs clearly show that each subtype may play a role in the regulation of adipokine production by adipocytes, macrophages, and endothelial cells composing human AT.

Treatment of human AT with PPAR agonists modified the release of several adipokines. The secretions of IL-6 and leptin by human AT were diminished by the three PPAR agonists. In agreement, plasma IL-6 levels are decreased after in vivo treatments with PPAR γ agonists [28]. Moreover, treatment of obese rats with a PPAR α agonist decreased serum leptin levels to a similar extent as does a PPAR γ agonist [29]. Our work demonstrates the effect of PPAR α and β/δ on HGF secretion and PPAR β/δ on angiogenin and TIMP-1 levels. HGF secretion from human AT was stimulated by PPAR α and PPAR β/δ ago-

nists. Human AT has been found as a new source of HGF, which levels in the circulation are associated with obesity [16,21,30,31]. Both, beneficial properties on the cardiovascular system and adverse actions in atherosclerosis, hepatic disease and malignancy have been proposed for HGF [31,32]. In mesangial cells, PPAR γ agonists induced HGF gene and protein expression, thus supporting its anti-fibrotic action [33]. Angiogenin secretion was suppressed by PPAR β/δ agonist. Angiogenin is a potent angiogenic factor which circulating levels have been shown to be elevated in obesity [34] and positively correlated with pro-inflammatory cytokines such as IL-6 or TNF α [35]. A pathological angiogenesis is present in obesity and associated with chronic inflammatory processes [36]. In this context, it could be hypothesized that PPAR β/δ exerts positive effects in AT through the control of angiogenin production by endothelial cells. In contrast, TIMP-1 was predominantly expressed in macrophages and its release in the culture media was increased after PPAR β/δ agonist treatment. TIMP-1 has been proposed as a growth factor that promotes AT development and fat accumulation. Its levels are increased in obesity and IL-6 or TNF α are able to stimulate its expression [37]. Thus, our results suggest that

PPAR β/δ may influence AT growth through induction of TIMP-1 and its action on the remodelling of the extracellular matrix.

To conclude, this study used pharmacological and proteomic approaches to examine the role of three known PPARs in the regulation of adipokine secretion. The antibody array approach extends the spectrum of factors secreted by human AT and identifies new adipokines regulated by PPAR agonists. This work paves the way for future studies investigating the role of PPAR α and PPAR β/δ in the regulation of peptide secretion from the different cell types composing AT.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.05.012.

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In fine of the second part, we can conclude that the global analysis of PPAR target genes in the culture media from the whole AT by combining pharmacological and proteomic approach showed the potency of synthetic PPAR agonists to modulate production of bioactive molecules from different cells composing human AT. We have revealed HGF, angiogenin and TIMP-1 as new factors regulated by PPARs. Beyond these findings, however, we were not able to address the precise mechanisms whereby activated PPARs trigger their action, but we spreaded the insight on respective roles of PPAR isotypes, especially on less understood PPAR α and PPAR β/δ in human SCAT and extended the spectrum of molecules that could act as novel agents for therapy of obesity and related complications. Finally, it needs to be emphasized, that a heterogeneity of effects of PPARs on adipokine expression may be expected related to several parameters such as distinct effects in *in vitro* and *in vivo* conditions, differences between natural and synthetic PPAR ligands, or between synthetic agonists itself, different concentrations used, period of exposure, and generally by different settings of experimental conditions.

CHAPTER 4

CONCLUSIONS AND PERSPECTIVE

The initiation of the studies included in this thesis has been fostered by the gaps in our current knowledge of the etiopathogenesis of obesity and IR in humans and especially by the importance of adipokines in inflammatory conditions as obesity and associated disturbances are now assumed to be. Low-grade inflammation may have an important impact on insulin signaling in insulin target tissues and be a driver of IR. It seems appropriate to pause here and notice that this hypothesis is still being only a hypothesis and there is a great deal of research ahead to elucidate several key questions, which drive researchers to straits so far. A better understanding of this very complex issue at different levels (genetic, molecular, physiological) and from different points of view (clinical, social, environmental) is a beating board for improving or opening new therapeutic strategies (pharmacologic or non-pharmacologic) to prevent and/or treat obesity-related conditions. Our work brings small but important fragment into this intricate field by applying an integrative research approach and translating the information coming mostly from animal experiments to humans. Our findings disclose the minor role of several adipokines in the improvement of IS, observed during long-term non-pharmacological interventions (aerobic and dynamic strength training, multiple phase dietary program). Based on existing literature, these adipokines were supposed to characterize a chronic low-grade systemic inflammation (TNF- α , IL-6, IL-1 β) and to be causal mediators of changes in IS in obese subjects (TNF- α , IL-6, IL-1 β , RBP4, adiponectin). However, obese AT is a source of numerous other proteins, known and unknown awaiting to be explored, that may carry messages to the rest of the body and influence IS. This is indirectly evident from the results of our *in vitro* study, which emphasizes that the expression of fat-produced proteins has a big potential to be pharmacologically modulated and highlights the fact, that many unknown factors can appear as regulated by different drugs, including insulin sensitizers. Given that there are many more adipokines secreted by AT than presently characterized, and assuming that cytokines and chemokines work in network, the search for new molecules that can be

manipulated for medical use represents exciting, substantial and one of the most challenging but likely promising avenue to tackle obesity and its co-morbidities pharmacologically.



The basic hypothesis is that obesity is a low-grade systemic inflammatory disease. Together with obesity, also IR and type 2 diabetes mellitus are considered closely associated with chronic low-grade inflammation. Concerning the term “inflammation”, it needs to be distinguished between the “classic” inflammation, which can be classified as acute and chronic, and the “systemic low-grade” inflammation, a concept developed in relation to obesity. In acute inflammation, removal of the stimulus halts the recruitment of monocytes into the inflamed tissue, and existing macrophages exit the tissue via lymphatics. In chronically inflamed tissue, the stimulus is persistent, and therefore recruitment of monocytes is maintained, existing macrophages are tethered in place, and proliferation of macrophages is stimulated. However, systemic inflammation is not confined to a particular tissue but involves the endothelium and other organ systems. Systemic low-grade inflammation, also referred to as “chronic low-grade” or “subclinical” inflammation, could be seen as a subclass of classical chronic inflammation, because it likely involves similar factors and signaling pathways as those in classic inflammation. Because low-grade inflammatory state is possibly metabolically triggered (by nutrients and metabolic excess), a new term “metainflammation” has been recently proposed to designate this state [57]. Whilst the definition of traditional features of classic inflammation is clearly stated, the definition of markers of low-grade inflammatory state is somehow vague. CRP has been known for many years as a marker for acute inflammation, which concentration may rise above the normal value of less than 3 mg/L to 20-500 mg/L within 4 to 8 hours after an acute event (1000 mg/L after severe trauma) [446]. Thanks to the development of high sensitive immunoassay, it is now possible to detect CRP levels below 10 mg/L, what is indicative of low-grade inflammation (~3-10 mg/L) [447]. Nevertheless, CRP is the only known validated biomarker that can be used as a clinical measure of systemic inflammation at present. The confirmation of this state in overweight and obese people emerged from the study of Visser et al. who demonstrated that higher BMI is associated with higher circulating CRP in young adults [448]. However, CRP is not specific marker of obesity [126]. In obesity, CRP is secreted in high amounts from the liver and remains elevated in circulation in response to IL-6 stimulation, which production is increased from obese VAT and directly delivered to the portal

vein [89,449]. Besides CRP, chronic low-grade inflammation is characterized by abnormal expression (mRNA or protein) or circulation of a large array of other acute-phase reactants and cytokines produced by obese AT, but neither the validated clinical reference values for their plasma levels nor experimental reference values for mRNA or protein levels exist. Moreover, the most startling is the observation that, despite numerous published papers, the evidence remains equivocal for an increased levels of these markers especially regarding “main” regulators of inflammation such as TNF- α , IL-6, IL-1 β (Table 3). Therefore, the majority of longitudinal clinical trials (our studies including) are based on the “general agreement”, that obesity is a chronic inflammatory illness. To facilitate further research directions for developing better therapeutic strategies and to help the comparison of results obtained by different research teams, it would be of importance to bring unequivocal epidemiological, experimental and clinical data characterizing inflammatory biomarkers in obesity and associated complications, as well as biomarkers that are specific to obesity itself.

On the top of the measurement of adipokine mRNA or protein levels in different tissues and in the systemic circulation, assessment of the relative contribution of individual tissues into adipokine production is a necessary approach to relate specific organ in the pathogenesis of systemic inflammation in obesity. For example, adipokines such as RBP4 or serum amyloid A have been shown to be produced by AT but the main site of expression has been known for a long time to be the liver [219,450]. A simple *in vitro* method of this kind is the measurement of concentrations of adipokines secreted into media from tissue explants. However, the challenge is to use more sophisticated techniques, such as microdialysis or the measurement of arterio-venous differences of adipokine concentrations *in situ in vivo* across human SCAT, VAT or skeletal muscle. This field has not intensively been studied because of methodological hurdles. The first study investigating the interrelationship between VAT and systemic inflammation in humans appeared only very recently [89]. In that work, Fontana et al. investigates the secretion of several potential inflammatory messengers from VAT (TNF- α , MCP-1, resistin, leptin and adiponectin) and suggests that IL-6 brought to the liver via portal vein may be the trigger of low-grade inflammatory states. Basically, three main features contribute to form the concept of obesity as an inflammatory condition: alterations in adipokine circulating levels, changes in adipokine mRNA levels and the accumulation of macrophages in obese AT. Regarding macrophages, while monocyte infiltration into AT has been proven, our understanding of

macrophage phenotype in this tissue and functional involvement in systemic inflammation is still incomplete. Further studies are awaiting to confirm that the predominant role of macrophages is in the generation of pro-inflammatory cytokines and e.g. not only in clearing the debris from dead adipocytes in human obesity. The brief characteristics on known facts of inflammatory states are summarized in Table 7.

Table 7 Comparison of acute, chronic and systemic low-grade inflammation.

	ACUTE	CHRONIC	SYSTEMIC LOW- GRADE*
<i>Causative agent</i>	Pathogens, injured tissues	Persistent acute inflammation due to non-degradable pathogens, persistent foreign bodies, or autoimmune reactions	Nutrients, metabolic surplus ?
<i>Major cells involved</i>	Neutrophils	Mononuclear cells (monocytes, macrophages, lymphocytes, plasma cells), fibroblasts	Macrophages and probably other mononuclear cells
<i>Primary mediators</i>	Vasoactive amines, eicosanoids	IFN- γ and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes	Adipokines, fatty acids ?
<i>Onset</i>	Immediate	Delayed	Delayed
<i>Duration</i>	Few days	Up to many months, or years	Months, years
<i>Signs and symptoms</i>	Redness, swelling, pain, fever, loss of function	Infiltration of mononuclear immune cells (monocytes, macrophages, lymphocytes, plasma cells), tissue destruction, attempts at healing, which include angiogenesis and fibrosis.	Infiltration of monocytes and accumulation of macrophages in AT, organelle dysfunction, local and systemic effect of AT-derived molecules ?
<i>Outcomes</i>	Healing, abscess formation, chronic inflammation	Tissue destruction, fibrosis, systemic inflammatory response syndrome if inflammation overwhelms the host, that may lead to septic shock and death.	IR, type 2 diabetes mellitus, atherosclerosis, cardiovascular complications ?
<i>Markers (Effect)</i>	High systemic levels of acute-phase proteins, e.g. CRP, serum amyloids (beneficial)	High systemic levels of acute-phase proteins, e.g. CRP, serum amyloids (not beneficial)	CRP plasma levels 3-10mg/l Elevated plasma levels of IL-6, TNF- α , IL-8, IL-1, etc (not beneficial) ?

*Systemic low-grade inflammation as it is seen in the context of obesity and AT inflammation.

As already mentioned, systemic inflammatory status of the body may negatively influence the action of insulin in various tissues. It becomes increasingly evident that the ability of lifestyle interventions to reduce overall inflammation in obese subjects deserves a serious consideration. The “general assumption” that physical activity and calorie restriction improve IS does not need further confirmation. However, what still remains to be clarified are the

molecular mechanisms behind the beneficial effects of these non-pharmacological interventions. Application of two strategies in *in vivo* research could be envisaged, either a “lean-to-obese” or “obese-to-lean” approach. While both approaches can be used in animal experiments, preponderance of clinical trials is devoted to the second, “obese-to-lean” option. This application is logical and ethical, but on the other hand, it would be very interesting and conducive to look at the mechanisms of molecular adaptations of the “retrogressive” approach of overeating and underexercising in humans.

Regular training and weight loss induce adaptations at multiple levels in human body including e.g. regulations of gene expression in AT or skeletal muscle. Many longitudinal studies have been conducted in order to identify genes that are modified by different type of interventions. The traditional tools for gene profiling, such as reverse transcription- real-time quantitative polymerase chain reaction (RT-qPCR) were enriched with DNA microarrays techniques that have a capacity to analyze a plethora of genes in a single experiment using appropriate bioinformatic tools. They are widely used now and besides characterizing cluster of genes, they also provide a powerful tool to study specific metabolic pathways of complex diseases or cellular responses across samples derived from various biological sources and treatment regimens (e.g. training, nutritional, or drug treatment). Recent data from transcriptome analyses reveal rather different pattern of changes in potential AT inflammatory profile when comparing the effect of different types of non-pharmacological interventions. Based on the microarray analysis, it appears that VLCD programs improve inflammatory profile in SCAT [451], whereas studies investigating the effect of physical activity based on RT-qPCR method show no variations in mRNA levels of a majority of candidate adipokines involved in inflammation and/or genesis of IR (our studies). A number of works have examined global gene expression after some forms of exercise training in skeletal muscle, showing distinct expression profiles between different types of exercise [452-455]. However, to the best of my knowledge, no studies have investigated mRNA response in SCAT in obese subjects to neither aerobic nor strength training using DNA microarrays, and the two studies presented in this thesis are the only ones related to physical activity and mRNA adipokine expression in SCAT in humans. Hence, the future goal will be to apply pangenomic gene profiling in SCAT during different training programs, with a special emphasis on two aspects, which will be presented later here. Training has a substantial impact on AT, e.g. aerobic training has the

potential to reduce fat mass, while it does not affect the muscle mass and strength. Furthermore, if we assume the logical major effect of resistance training in skeletal muscle, we can also assume that muscle may communicate with AT via various pathways and induce the changes in AT gene transcription. Even if very interesting, for ethical reasons, VAT cannot be surveyed in long-term clinical trials, thus SCAT represents suitable source for microarray gene profiling as this tissue can be relatively easily obtained by needle-biopsy. Regarding the effect of calorie restriction on the inflammatory status of AT potentially underlying the metabolic disturbances in obesity, such as IR, a very attractive application of microarrays is to determine the dynamics of gene expression profile in SCAT, i.e. gene expression pattern during different time-points of different weight loss management programs. This project is being developed presently in our laboratory as a follow-up of nutritional intervention study presented in the thesis, targeting two aspects. Firstly, the improvement in IS is thought to induce changes in adipokine expression. As IS status of our subjects was improved after weight-reducing phases, we wish to identify subset of genes that are supposed to be involved in AT inflammation in each of these dietary phases and relate it to changes in IS. Secondly, we will perform systematic search of changes of all genes in each time-point that are suspected to be secreted. For this, programs predicting from the mRNA sequence whether a protein can be secreted will be a necessary part of an object. Using microarrays in non-pharmacological intervention studies is expected to provide new insight into the complex molecular mechanisms of diet or exercise-induced improvement of the whole-body IS and potential anti-inflammatory responses. Moreover, future research will probably aim at using gene expression profiling to characterize and predict variability of responses between individuals and the efficiency of training or dieting. This could result in individualized weight management programs.

Inasmuch as the adaptations in gene expression at the transcriptional level may be reflected in systemic circulation, intense effort has been done in characterizing changes in adipokine plasma profile induced by various non-pharmacological intervention trials. Candidate molecules with a suggested role in inflammation and IR are usually selected according to the previous experimental *in vitro* or *in vivo* findings. This strategy was employed also in our clinical studies. Data on the impact of physical activity or nutritional interventions on adipokine plasma levels in humans are not unanimous in the literature. Striking disparity can be ascribed to many factors, especially to different experimental settings, methods of analysis and

insufficient number of subjects in study groups. While experimental conditions or sample size can be controlled, research was limited until recently by insensitive methods for plasma measurements of several proteins, such as those implicated in AT inflammation and/or IR. Recently, besides high-sensitive ELISA kits for several factors, such as CRP, TNF- α , or IL-1 β , the advances have been made in other methodological approaches, “omics” technologies, e.g. proteomics, or metabolomics. Contrary to ELISA method, which allows measurement of single peptide in one experiment, the advantage of using e.g. cytokine antibody arrays or Luminex is similar to DNA microarrays, i.e. they allow measurement of hundreds of proteins in one assay. However, these methods are not commonly applied for determination of adipokine circulating levels yet. We used cytokine antibody arrays in our *in vitro* study to identify proteins released from human AT explants, in response to pharmacological treatment, but it is evident that this approach could be very useful in each of our clinical studies for measurements of plasma adipokines in large scale. Circulating levels of adipokines provide information on the total amount of protein that comes from different body tissues. A complementary approach that would bring hints on the contribution of AT itself into systemic levels is the measurement of adipokine concentrations secreted into media from AT explants. The methods mentioned above (ELISA, cytokine antibody arrays, Luminex) offer a large scale of possibilities and could be applied to study the pattern of changes in AT secretion in response to various types of lifestyle interventions. This represents an unexplored and even less understood area. For the simplicity of this “tissue secretion” method, when compared to the alternative microdialysis technique, we have started to investigate in our laboratory the profile of AT secretion (obtained by needle biopsy) during different dietary time-points of a weight loss program. Combining this approach with the specific analysis of DNA microarray gene expression data of putative secreted factors will provide an exhaustive view at the regulation of AT production during dietary intervention.



The basis for this thesis was the hypothesis that:

- obesity is characterized by **dysregulation of many adipokines** that serve as signals for other tissues,
- obesity, via dysregulated adipokines, **triggers IR** in insulin-sensitive tissues,
- obesity represents a **low-grade inflammatory state**,
- obesity is a condition that is **possible to tackle**, at least, to alleviate the above mentioned conditions by non-pharmacological and pharmacological interventions.

Based on the results from this thesis, I suggest here a hypothesis that could account for the beneficial effect of non-pharmacological and pharmacological therapies on the improvement of IS in obese subjects. In our clinical studies, we have assumed the first three points of the hypothesis and tried to disclose possible mechanisms by using the principal of the last point, i.e. to tackle obesity in different groups of adult patients by physical training or nutritional intervention programs as non-pharmacological treatments. The major outcome from all three clinical investigations is the improvement of IS status. A very interesting finding is that alleviation of IR always coincided with decrease in plasma leptin levels, while the concentrations of other investigated adipokines remained unchanged. These two facts urges us to ask, whether leptin could mediate the amelioration of IS and inflammation in humans. What is then the possible molecular explanation for the improvements in clinical outcomes?

The clue could be PPAR transcription factors. All three PPAR isotypes are expressed in human AT as well as in skeletal muscle. Recent works revealed that also leptin receptors are expressed in both tissues in humans, suggesting that AT and skeletal muscle are able to respond to circulating leptin [434,435]. However, only low or normal plasma leptin levels are able to promote insulin-sensitizing effect. Therefore, the primary goal would be to decrease elevated leptin levels typical in obese subjects and restore their leptin sensitivity and leptin pulsatility. Leptin (as many other hormones or NEFA and glycerol) is secreted into the blood stream in a pulsatile pattern that might be of importance for its biological effects, e.g. in the hypothalamic regulation of body weight. This field is not well documented, however one study of Mingrone et al. reports decreased leptin concentration and restoration of leptin ultradian variability (pulsatility) and sensitivity in formerly obese women after massive weight loss obtained by BPD operation. Importantly, increased pulsatility index and clearance of plasma leptin 14

months after BPD was accompanied by the improvement in IS [456]. While the authors suggest that the reversion of IR might be a causative factor for amelioration of leptin parameters, it is also conceivable that it is the restoration of plasma leptin characteristics, which lead to the enhancement of IS. The proposed mechanism for reduction of plasma leptin amounts and its proper functioning is activation of PPARs in AT and skeletal muscle. Our *in vitro* study provides evidence that all three of the PPAR isoforms significantly decrease leptin concentrations from human SCAT explants. To complete the mosaic of construed plausible mechanism, we suppose that physical training and possibly calorie restriction could stimulate PPAR-actions, either by their induction *per se* (increasing gene expression at mRNA or protein level), or by their activation (higher transcriptional activity). This hypothesis is supported, at least in part, for the activation of PPARs in AT and/or skeletal muscle with exercise by few recent works in rats [457] and humans [453,458,459]. The subject of human studies investigating PPAR gene expression changes (among other genes) after exercise was only skeletal muscle so far. Nevertheless, Petridou et al. showed increased PPAR γ -activity in SCAT and epididymal fat of the trained rats [457]. The role of PPAR α and PPAR β/δ in human AT is not as well understood as that of PPAR γ . However, several studies indicate that they might be involved in the regulation of human adipocyte gene expression. Recent evidence from our laboratory uncovers that PPAR α is indeed directly involved in regulating white fat metabolism [445] (see Annex). On one hand, it has been suggested that activation of PPAR α in white AT could prevent adipocyte hypertrophy and that PPAR α is essential for the lipopenic effect (disappearance of adipocyte fat) of hyperleptinemia in non-obese mice, and on the other hand that it might be a proximal mediator of leptin action [460,461]. However, in one of these studies, Lee et al. failed to explain PPAR α -dependent lipopenia in white AT by increased AMPK activity as a consequence of hyperleptinemia. It goes in line with our proposed concept based on the indication, that hyperleptinemia should be brought down first to the normal levels to be able to activate AMPK with ensuing improvement of IS. Taken together, it is likely that improvement in clinical profiles achieved by the exercise programs are paralleled by changes in expression of genes involved in lipid metabolism and mitochondrial biogenesis, such as PPARs, in the skeletal muscle and AT. This underscores the importance of future investigations to unravel whether exercise-mediated changes in PPAR expression may constitute a molecular mechanism by which different types of trainings enhance mitochondrial function and/or

biogenesis not only in skeletal muscle but also in AT. There are several future perspectives as a follow-up of our clinical studies. With the application of various methods mentioned previously, we could measure the gene expression of all PPARs at mRNA or protein levels in SCAT in obese subjects before and after lifestyle interventions represented by physical activity or during different dietary phases in long-term. Furthermore, it could be tested whether these interventions change the activity of PPAR isotypes in AT. DNA microarrays could be used to search for other transcriptional pathways, independent or additive to PPAR, that are activated by physical activity or dieting.

An exciting research direction raising from very recent studies [51,462] could be to test, whether insulin sensitizing lifestyle modifications could have an impact on sirtuin 1 (SIRT1). SIRT1 is a mammalian homologue of yeast silent mating type information regulator 2 (Sir2), the gene responsible for cellular regulation in yeast. This enzyme appears to be promising inducer of IS through repressing protein tyrosine phosphatase 1B (PTP1B). PTP1B negatively regulates insulin action directly as an insulin receptor phosphatase and indirectly by attenuation of leptin action [463]. SIRT1 is expressed in metabolically active tissues, including AT and skeletal muscle. It has been recently demonstrated that resveratrol, a plant-derived polyphenolic compound, is a potent activator of SIRT1 and improves IS *per se* [462]. As resveratrol can mimic caloric restriction in a Sir2-dependent manner [464], it is conceivable, that lifestyle interventions represented by nutritional intervention could elicit similar effect. However, the importance of SIRT1 as a regulator of insulin action *in vivo* in humans, as well as its ability to regulate PTP1B expression or insulin signaling in different metabolic tissues is so far unknown. In the frame of the European programs Diogenes and Nugenob, our laboratory together with the laboratory of Hubert Vidal in Lyon has investigated the effect of energy restriction and macronutrient composition on SCAT gene expression profile. The more pronounced effect of calorie restriction compared to fat or carbohydrate content suggests a role for the sirtuin pathway. While we did not observe significant changes in SIRT1 mRNA levels after two different types of diet, we have not performed the measurement of its enzymatic activity that may be increased (unpublished data).

This working hypothesis on the molecular benefits of non-pharmacological and pharmacological interventions to combat impairments in leptin sensitivity and insulin signaling in metabolic tissues is presented in Figure 12. As developed in Chapter 1, there are multiple

pathways potentially involved in the improvement of IS. The relative importance of the pathways described in this paragraph needs to be established as well as their interaction with existing mechanisms.

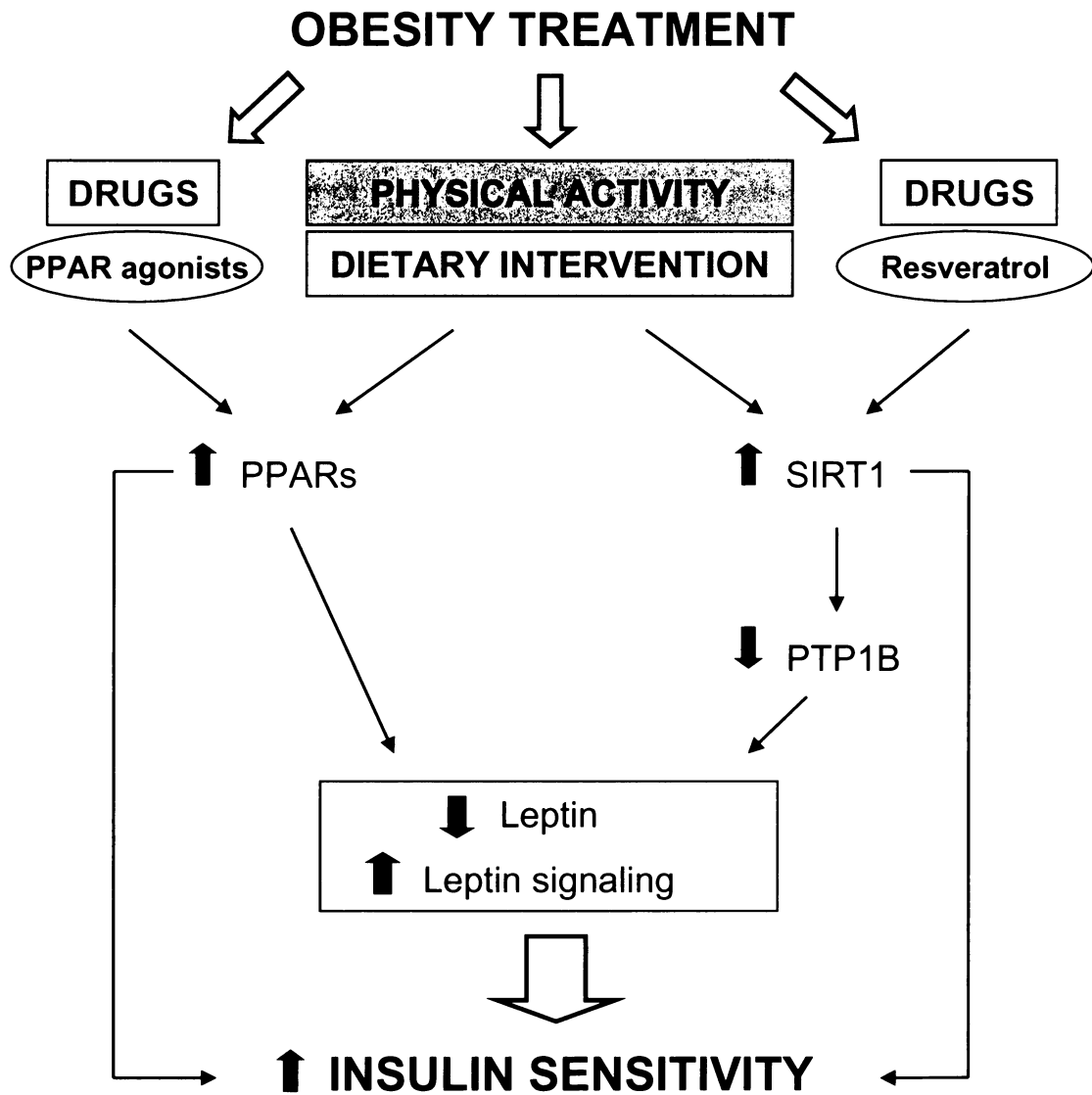


Figure 12 Working hypothesis on the molecular benefits of non-pharmacological and pharmacological interventions to combat impairments in leptin sensitivity and insulin signaling in metabolic tissues.

PPAR, peroxisome proliferator-activated receptor; SIRT1, sirtuin 1; PTP1B, protein tyrosine phosphatase 1B



This chapter opened with the message that the etiopathogenesis of obesity and linked disturbances is not fully understood. Within the past years, research generally has rapidly moved from an organ to a molecular level. Where does the future science go from here? In spite of enormous investments and effort, the progress in obesity research appears to be somehow slow, and the complexity of the problem could be depicted as the main decelerating factor. To advance our knowledge in the pathogenesis, prevention and treatment of obesity and related disorders, bridging the researchers' experience by increasing collaborations, integrating new technologies and disciplines, and the support at government level and other sectors is a key force of progress in this area. Treatment of obesity-related conditions usually focuses on losing the fat by the prescription of diet, exercise, or their combinations. By this approaches, numerous risk factors of obesity are improved. However, it appears that to treat these metabolic disturbances might be possible without shedding the fat itself. Direct evidence offers e.g. the strength training, a non-pharmacological modality that improves IS while the body fat keeps stable. The idea that obesity states could be tackled by drugs targeting adipokines is exciting future challenge, that would be very helpful for those people that have difficulties to lose weight. However, given that adipokines exert combinatorial and additive actions, there is a little probability that targeting a single molecule can generate beneficial systemic effect. Modulation of integrated molecular networks as well as possible "organelle therapy" (e.g. mitochondria and ER) are poorly explored and require further attention [57,465,466]. The notion of network is also found at a more integrated level. According to the recent study using the records of 12 067 participants of the Framingham Heart Study, network phenomena appears to touch obesity in a more global scale, suggesting that obesity is "socially contagious" and can spread in social networks, from person to person [467]. This opens a new avenue into the treatment of obesity, that the same force might be used to slow down the worldwide epidemic of obesity. In other words, it might be wholesome to treat obese people in groups instead of just the individual. An interesting and important current research questions remain whether lifestyle interventions may extend the maximum longevity or life expectancy, or whether conversely they could increase the mortality in obese and also in lean humans.

ANNEXE

Phosphodiesterase-5A and neutral endopeptidase activities in human adipocytes do not control atrial natriuretic peptide-mediated lipolysis.

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SUMMARY

Author: Eva KLIMČÁKOVÁ

Title: Regulation of human adipose tissue gene expression in relation to obesity and insulin resistance

Specialization: Molecular and cell biology, genetics and virology

: Innovations pharmacologiques / Ecole Doctorale Biologie-Santé-Biotechnologies

Supervisors: Doc. Vladimír Štich

: Prof. Dominique Langin

Address of laboratory:

: Department of Sports Medicine, 3rd Faculty of Medicine, Charles University, Ruska 87,
Prague CZ-100 00, Czech Republic

: Laboratoire de recherches sur les obésités, INSERM U858 - I2MR, IFR31, Institut Louis
Bugnard, Batiment L3, CHU Rangueil, 1 avenue Jean Poulhès, Toulouse F-31400, France

Date, time and place of defence: December 4th, 2007, at 10:00 a.m., 3rd Faculty of Medicine,
Charles University, Prague

Obesity is associated with insulin resistance (IR) and type 2 diabetes mellitus. Among possible mechanisms leading to IR are increased plasma levels of free fatty acids and altered levels of adipokines secreted from adipose tissue (AT). In the first part of the work, we studied obese patients during different nutritional and physical activity interventions. Phenotypic data were related to the expression of AT genes potentially involved in the regulation of insulin sensitivity (IS) and/or low-grade inflammation. We confirmed that aerobic and dynamic strength training improved IS and demonstrated that these interventions do not promote changes in subcutaneous AT gene expression or in plasma levels of adiponectin, interleukin-6, interleukin-1 beta and tumor necrosis factor-alpha, but decrease circulating leptin level. Very low calorie diet followed by low calorie diet and weight maintenance period enhanced IS in obese women and diminished retinol-binding protein 4 (RBP4) in plasma, but RBP4 mRNA

levels were reduced only after very low calorie diet. Our findings indicate that the investigated adipokines, except potentially leptin, might not be mediators of changes in IS induced by lifestyle interventions. In the second part of the work, we investigated the role of peroxisome proliferator-activated receptors (PPARs) on the protein secretion by human subcutaneous AT. We showed that PPARs regulated production of several proteins and identified new adipokines responding to activated PPARs. We demonstrated that PPARs modulate secretion of bioactive molecules from different AT cell types. These studies contribute to our understanding of the relationship between adipokines and IS.

Key words

obesity • adipose tissue • adipokines • gene expression • physical exercise • diet intervention • protein secretion • PPAR

RESUME EN FRANCAIS

Auteur: Eva KLIMČÁKOVÁ

Titre: Régulation de l'expression génique dans le tissu adipeux humain en relation avec l'obésité et l'insulino résistance

Discipline: Innovations pharmacologiques / Ecole Doctorale Biologie-Santé-Biotechnologies
: Biologie cellulaire et moléculaire, génétique et virologie

Directeurs de thèse: Prof. Dominique Langin
: Doc. Vladimír Štich

Intitulé et adresse du laboratoire:

: Laboratoire de recherches sur les obésités, INSERM U858 - I2MR, IFR31, Institut Louis Bugnard, Batiment L3, CHU Rangueil, 1 avenue Jean Poulhès, Toulouse F-31400, France

: Department of Sports Medicine, 3rd Faculty of Medicine, Charles University, Ruska 87, Prague CZ-100 00, Czech Republic

Date, heure et lieu de soutenance: 4 décembre 2007 à 10h à la 3^{ème} Faculté de Médecine, Université Charles de Prague, République Tchèque

Parmi les mécanismes possibles de l'insulinorésistance associée à l'obésité figure une altération de la production d'adipokines par le tissu adipeux (TA). Dans une première partie, nous avons étudié des patients obèses soumis à des programmes nutritionnels ou d'activité physique. Les données phénotypiques ont été reliées à l'expression de gènes du TA potentiellement impliqués dans la sensibilité à l'insuline. Nous avons confirmé qu'un entraînement en condition aérobie ou en force améliorait la sensibilité à l'insuline et démontré que ces interventions ne modifiaient pas l'expression génique dans le TA sous-cutané ou les niveaux plasmatiques d'adiponectine, d'interleukine 6, d'interleukine 1 beta et de *tumor necrosis factor alpha* mais diminuaient les concentrations circulantes de leptine. Différentes phases d'un programme de perte de poids améliorent la sensibilité à l'insuline et diminuent

transitoirement les concentrations plasmatiques de la protéine de liaison du rétinol RBP4. Les niveaux d'ARNm ne sont diminués qu'après la première phase à très basses calories. Nos résultats montrent que les adipokines, excepté peut-être la leptine, ne semblent pas des médiateurs des changements d'insulinosensibilité induits par une intervention diététique ou l'exercice physique. Dans une seconde partie, nous avons exploré le rôle des PPARs (*peroxysome proliferator-activated receptors*) sur la sécrétion de protéines par le TA sous-cutané humain. Il apparaît que les PPARs régulent la production de facteurs sécrétés provenant de différents types cellulaires du TA. Cet ensemble d'études contribuent à notre compréhension des relations entre adipokines et sensibilité à l'insuline.

Mots-clés

obésité • tissu adipeux • adipokines • expression génique • exercice physique • intervention diététique • sécrétion protéique • PPAR
