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MARJUKKA KOLEHMAINEN

Metabolic Studies on Adipose Tissue in Severe Obesity

Doctoral dissertation

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Department of Clinical Nutrition
University of Kuopio and Kuopio University Hospital



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Department of Public Health and General Practice
- Author's address:** Department of Clinical Nutrition
University of Kuopio
P.O.Box 1627
FIN-70211 KUOPIO
FINLAND
Tel. +358 17 162 781
Fax +358 17 162 792
- Supervisors:** Professor Matti Uusitupa, M.D., Ph.D.
Department of Clinical Nutrition
University of Kuopio
- Docent Jorma Ohisalo, M.D., Ph.D.
Department of Biomedicine
University of Helsinki
- Reviewers:** Professor Markku Koulu, M.D., Ph.D.
Department of Pharmacology and Clinical Pharmacology
University of Turku
- Professor Markku Savolainen, M.D., Ph.D.
Department of Internal Medicine
University of Oulu
- Opponent:** Professor Risto Huupponen, M.D., Ph.D.
Department of Pharmacology and Clinical Pharmacology
University of Turku

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ABSTRACT

Obesity is associated with metabolic alterations that can lead to insulin resistance, elevated blood pressure and disturbed lipid metabolism. There have been major advances made recently in our understanding of the metabolic and physiologic mechanisms behind obesity, i.e. the mechanisms that favour fat deposition and storage. Adipose tissue has been shown to be one of the major players in the regulation of body weight. The present studies were carried out to investigate the changes in adipose tissue metabolism that may contribute to development of obesity. Thirty-three (12 men, 21 women) severely obese (158.5 ± 37.0 kg for men, 139.2 ± 23.4 kg for women, mean \pm SD) subjects undergoing a gastric banding operation participated in the studies before the procedure and after one year of weight reduction.

Adipose tissue was studied *in vivo* using the microdialysis method and *in vitro* by lipolysis assay in isolated adipocytes. These methods gave concordant results with regard to abdominal subcutaneous adipose tissue metabolism. Gene expression was studied by RT-cPCR. Marked gender differences were found in adipose tissue lipolysis, and hormone sensitive lipase (HSL) and lipoprotein lipase (LPL) expression along with long term weight loss. In women, HSL expression decreased, this probably being attributable to simultaneous decrease in cell size. On the contrary, LPL expression increased slightly. HSL and LPL expression did not change in men. Basal and maximal lipolysis decreased and sensitivity to inhibition of lipolysis improved in men in conjunction with the weight loss, whereas women showed no changes in these measures.

The expression of sterol regulatory element binding protein (SREBP) 1c was lower in obese subjects than in lean subjects. The expression increased in parallel with the weight loss in obese, reaching almost the level of lean subjects. Furthermore, obesity was associated with reduced peroxisome proliferator activated receptor (PPAR) γ 1 expression, whereas no difference was found in PPAR γ 2 expression. In women, PPAR γ expression was higher in subcutaneous than in the omental adipose tissue depot. Weight loss did not change the level of PPAR γ expression. We did not detect any major differences in PPAR γ 2 or its target gene expressions between the groups with the Pro12Ala polymorphism of the PPAR γ 2 gene either before or after one year of weight loss.

To conclude, obesity and weight loss are associated with alterations in adipose tissue metabolism and gene expression. Moreover, the genders respond to the excess amount of body fat and to weight loss differently, this being reflected in the measures of lipolytic activity and in the expression of HSL and LPL. However, the changes in lipolytic activity cannot be explained by the changes in HSL and LPL expression. The changes seen in SREBP1c expression might reflect insulin resistance in obesity and improvement of insulin sensitivity along with weight loss. Furthermore, PPAR γ 1 and PPAR γ 2 expression revealed a gender difference with women having higher expression than men. The Pro12Ala polymorphism in the PPAR γ 2 gene did not have major effect on the expression of this gene or on its target genes, suggesting that the polymorphism has little influence on adipocyte function in morbidly obese subjects at least in its heterozygote form.

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Medical Subjects Headings: obesity/metabolism; obesity, morbid/metabolism; adipose tissue/metabolism; adipocytes/metabolism; gene expression regulation; RNA, messenger/genetics; lipolysis/genetics; microdialysis; weight loss; sex; human

*Maasta se pienikin ponnistaa, ja
pieniä saattaapi onnistaa.*

Elina Karjalainen: Uppo-Nalle

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Kuopio, April 2003



Marjukka Kolehmainen

ABBREVIATIONS

ADD1	adipose determination and differentiation factor 1
ALBP/aP2	adipocyte lipid-binding protein
ANP	atrial natriuretic peptide
AR	adrenergic receptor
A receptor	adenosine receptor
ASP	acylation stimulating protein
BMI	body mass index
bp	basepair
C3	third component of complement
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer binding protein
cGMP	cyclic guanosine monophosphate
DNA	deoxyribonucleic acid
FFA	free fatty acid
GH	growth hormone
HSL	hormone sensitive lipase
IGF-1	insulin like growth factor 1
LPL	lipoprotein lipase
mRNA	messenger ribonucleic acid
NEFA	non-esterified fatty acid
OGTT	oral glucose tolerance test
PCR	polymerase chain reaction
PDE3	phosphodiesterase 3B
PI3K	phosphatidylinositol 3 –kinase
PKA	protein kinase A
PPAR	peroxisome proliferator activated receptor
p85 α PI3	
-kinase	p85 α -subunit of phosphatidylinositol 3 –kinase
RA	retinoic acid
RNA	ribonucleic acid
RT	reverse transcriptase reaction
RT-cPCR	reverse transcriptase competitive polymerase chain reaction
RXR	retinoid X receptor
SREBP	sterol regulatory element binding protein
T ₃	triiodothyronine
TG	triglyceride
TNF – α	tumor necrosis factor α
UCP2	uncoupling protein 2
VLDL	very low density lipoprotein
Xe	xenon

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which will be referred to by their Roman numerals I-IV:

- I Kolehmainen M, Ohisalo JJ, Kaartinen JM, Tuononen V, Pääkkönen M, Poikolainen E, Alhava E, Uusitupa M. Concordance between *in vivo* and *in vitro* techniques in studying adipose tissue metabolism in severely obese subjects. *Int J Obes Relat Metab Disord*, 2000; 24: 1426-1432.
- II Kolehmainen M, Vidal H, Ohisalo J, Pirinen E, Alhava E, Uusitupa M: Hormone sensitive lipase expression and adipose tissue metabolism show gender difference in obese subjects after weight loss. *Int J Obes Relat Metab Disord*, 2002; 26: 6-16.
- III Kolehmainen M, Vidal H, Alhava E, Uusitupa M: Sterol regulatory element binding protein 1c (SREBP-1c) expression in human obesity before and after weight reduction. *Obes Res*, 2001; 9: 706-712.
- IV Kolehmainen M, Uusitupa M, Alhava E, Laakso M, Vidal H. Effect of the Pro12Ala polymorphism in the peroxisome proliferator-activated receptor γ 2 gene in the regulation of PPAR γ target genes expression in adipose tissues of massively obese subjects. *J Clin Endocrinol Metab*, 2003; 88: 1717-1722.

In addition, some unpublished results are presented.

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1. INTRODUCTION

Obesity is a growing problem on a worldwide scale (WHO 2000). It is associated with a variety of metabolic alterations that could lead to insulin resistance, elevated blood pressure, and altered lipid metabolism, and ultimately to increased morbidity and mortality (Kopelman 2000). Obesity is treated with energy restriction diets, behavioural modifications, physical activity, pharmacological agents, and with various combinations of these treatments. However, even after successful weight loss, weight maintenance has proven to be difficult, or almost impossible on the basis of studies, where different short- and long-term treatments have been investigated. This has increased research into the molecular and cellular mechanisms of obesity, e.g. to find factors that favour fat deposition and storage. Thus, investigation of the metabolic and physiologic basis of obesity has expanded rapidly during the past few years. New and developing methodologies have deepened our knowledge of the metabolic and genetic factors contributing to obesity. This progress has also revealed that adipose tissue itself is one of the major contributors in weight regulation. However, our understanding of the pathophysiology of obesity is still incomplete.

There are many factors that could predispose an individual to obesity and weight gain in humans. These factors include low resting metabolic rate, low fat-free mass, factors related to macronutrient utilization, energy expenditure, and hormonal status, including insulin sensitivity (Martinez 2000). These factors are modified by various environmental factors, for example by unhealthy food choices and low physical activity that predispose an individual to weight gain.

Adipocytes are highly specified cells that play a critical role in whole body energy regulation and homeostasis (Kim and Moustaid-Moussa 2000). The primary role of adipose tissue is to store energy when intake exceeds expenditure and to release energy in the form of fatty acids when it is needed by the other organs. Recent studies have shown that adipose tissue has an important role in energy expenditure via endocrine, paracrine and autocrine signals (Figure 1) (Mohamed-Ali et al. 1998). These functions enable adipose tissue to influence the activity of other tissues, but also by autocrine or paracrine manners to regulate adipocyte metabolism itself. Thus, it is evident that adipose tissue is a very important target for obesity research.

It has been shown (Foley 1988, Mauriège et al. 1991, Kaartinen et al. 1991, Coppack et al. 1992, Reynisdottir et al. 1994, Hellström et al. 1996, Lönnqvist et al. 1997, Large and Arner 1998, Arner 1999) that gaining excess fat and being in positive energy balance are associated with certain changes in adipose tissue metabolism. However, it is still unclear whether these changes are secondary to the gaining of fat or

primary with respect to the susceptibility to gain weight / fat. The discovery of leptin in 1994 (Zhang et al. 1994) opened a new avenue in obesity research. Leptin regulates long-term energy balance by inhibiting food intake via signals to the central nervous system (Campfield et al. 1995, Pelleymounter et al. 1995, Halaas et al. 1995). Adipose tissue secretes leptin in proportion to the amount of fat mass (Maffei et al. 1995, Considine et al. 1996). Thus, in obese subjects, the leptin concentration in plasma is increased, and it has been suggested that obese subjects are resistant to leptin (Havel 2000). In addition to leptin, several other agents are released from or expressed in adipose tissue that may affect many metabolic pathways of lipid and glucose metabolism (Mohamed-Ali et al. 1998, Frühbeck et al. 2001).

In the present work, the effect of obesity and weight reduction on adipose tissue metabolism was investigated to determine the changes that may affect the development of obesity. Adipose tissue metabolism was studied using *in vitro* analyses of isolated adipocytes from massively obese subjects, microdialysis for studying subcutaneous abdominal adipose tissue metabolism *in vivo* and, finally, mRNA expression of genes of interest in adipose tissue that might be related to the altered metabolism in obesity.

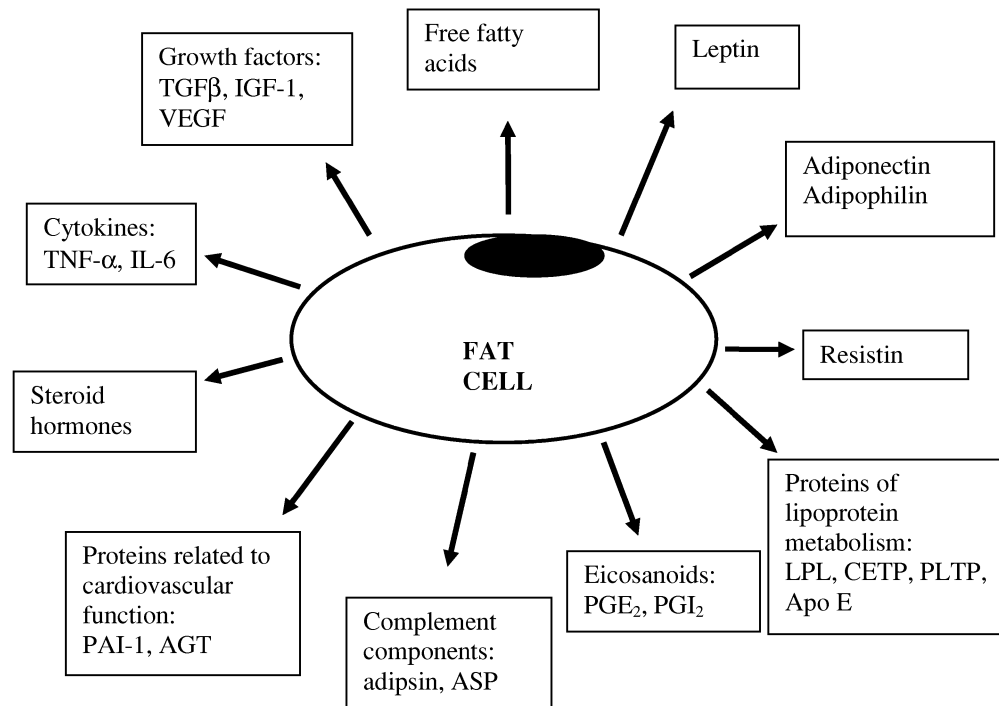


Figure 1. Some physiological regulators produced and secreted by adipocyte. Modified from Kim and Moustaid-Moussa, 2000 and Frühbeck et al. 2001. TNF- α : tumor necrosis factor α , IL-6: interleukin 6, PAI-1: plasminogen activator inhibitor 1, AGT: angiotensinogen, LPL: lipoprotein lipase, CETP: cholesteryl ester transfer protein, PLTP: phospholipid transfer protein, Apo E: apolipoprotein E, TGF β : transforming growth factor β , ASP: acylation stimulating protein, IGF-1 insulin like growth factor 1, VEGF: vascular endothelial growth factor, PGE₂: prostaglandin E2, PGI₂: prostaglandin I2.

2. REVIEW OF THE LITERATURE

2.1. Etiology of obesity

Obesity is caused by a long-term positive energy balance. If energy intake exceeds expenditure for a long period of time, excess energy is mostly stored as triglycerides (TG) in adipose tissue. Thus, the adipose depots become enlarged, and a state of obesity results.

There are many metabolic and hormonal factors that could predispose an individual to obesity. Insulin sensitivity has been found to promote weight gain in different populations (Swinburn et al. 1991, Valdez et al. 1994, Hoag et al. 1995). The peripheral sympathetic nervous system participates in the regulation of energy balance in humans (van Baak 2001). As a part of energy metabolism, it also controls adipose tissue lipolysis (Saris 1995). Low sympathetic nervous system activity and reactivity, and/or reduced sensitivity to sympathetic stimulation may play a role in the development and maintenance of obesity (van Baak 2001). Since these impairments have been shown to occur even after weight reduction, it has been claimed that this blunted reactivity and sensitivity may have a role in the etiology of obesity (van Baak 2001).

A low metabolic rate is also regarded as predisposing to obesity, since some studies (Ravussin et al 1988, Roberts et al. 1988) but not all (Goran et al. 1998), have shown low energy expenditure to be associated with subsequent weight gain. Other factors that may have a role are a relatively low leptin concentration, a low rate of fat oxidation and low level of spontaneous physical activity (Ravussin and Gautier 1999).

The above mentioned factors are mostly determined by the genetic factors, but do not necessarily lead to the development of obesity. These factors are, however, modified by environmental factors. Thus, the development of obesity is a complex multifactorial disease influenced by many susceptible genes, and gene-gene and gene-environmental factors. In Finnish people, a healthy lifestyle, i.e. physical activity, healthy food choices with no smoking and moderate alcohol consumption are associated with the maintenance of normal body weight (Lahti-Koski et al. 2002). Thus, if one has a sedentary lifestyle, physical inactivity and unhealthy food choices one will experience a high risk of gaining weight.

Obesity can be classified by the amount of the excess body weight in relation to height, body mass index (BMI) (kg/m^2) (Table 1). BMI is strongly connected to the amount of the fat mass (Garrow et al. 1985, Revicki et al. 1986). The index $>25 \text{ kg/m}^2$ is used as a cut-off point for overweight and obesity, since indices above the value of 25 associate with the higher rates of morbidity and mortality (WHO 2000).

Table 1. The classification of overweight and obesity according to the body mass index.

Body mass index (kg/m ²)	Classification	Risk of co-morbidities ¹
< 18.5	Underweight	Low
18.5 – 25	Normal range	Average
> 25	Overweight, obesity	
25-30	Pre-obese	Increased
30-35	Obesity class I	Moderate
35-40	Obesity class II	Severe
> 40	Obesity class III, morbid obesity	Very severe

¹ According to WHO Technical Report Series 2000.

Although obesity as such increases the risk of co-morbidities, excess truncal and visceral fat is especially associated with higher rates of insulin resistance, dyslipidemia and increased blood pressure etc. (Tchernof et al. 1996, Sniderman et al. 1998, Couillard et al. 1998, Frayn 2000, Després 2001). This may be caused by the differences in the adipose tissue metabolism in different regions. Visceral fat is considered as lipolytically more active than abdominal or femoral subcutaneous fat (Arner 1995). Therefore, this may directly expose the liver to an excess amount of free fatty acids (FFA) from visceral depots, causing increased production of very low density lipoproteins (VLDL) and glucose in the liver and reduced hepatic uptake of insulin and consequently hyperinsulinemia (Frayn and Coppack 1992, Sniderman et al. 1998, Kahn and Flier 2000).

2.2. Genetics of obesity

According to ‘The Human Obesity Gene Map’ (Chagnon et al. 2003), the number of genes, markers and chromosomal regions that have been associated or linked with human obesity is now above 300. This evidence comes from human single gene mutations, and through association, from case-control and linkage studies with identification of candidate genes or from genome-wide searches (Martinez 2000). These studies have dealt with various obesity linked phenotypes, such as body mass index, body fat mass, percentage of body fat, fat free mass, skinfolds, resting metabolic rate, plasma leptin levels, and other components of energy balance (Chagnon et al. 2003). Putative loci affecting obesity-related phenotypes have been found on all but

chromosome Y of the human chromosomes (Chagnon et al. 2003). Some genes are involved in food intake or the regulation of energy expenditure and thermogenesis, while some participate in signalling pathways such as adipocyte differentiation (Martinez 2000). Although these associations are significant, there is no single gene that would explain the increasing frequency of obesity which has emerged in many nations during the past few decades. According to the latest overview, only eighty-six human cases have been reported to have obesity that can be considered to be caused by single gene mutations. These include 36 mutations in six different genes (Chagnon et al. 2003).

In a classical study by Bouchard et al. (1990), twin pairs participated in a long-term overfeeding study. The response to overfeeding was significant with respect to body weight, percentage of fat, fat mass and estimated subcutaneous fat with about three times more variance among pairs than within pairs (Bouchard et al 1990). Thus, there is a clear genetic impact on the susceptibility to gain weight and fat mass. About half (40 – 70 %) of the variance in obesity-related phenotypes has been estimated to have genetic background (Comuzzie et al. 1998). However, the estimates of genetic impact on total body fat have been somewhat lower, only about 25 %, and, furthermore, when adjusted for age, gender, and height, the level was about 15 % (Bouchard et al. 1993). When genetic background was studied for truncal abdominal fat, the estimates were again higher, showing 35 – 50 % heritability, which was identical for maternal and parental transmission (Bouchard et al. 1993).

2.3. Methods for studying adipose tissue metabolism in humans

Adipose tissue metabolism can be studied indirectly by measuring whole body lipid metabolism, or more directly by *in vivo* methods or by *in vitro* assays using isolated adipocytes. These techniques all have their inherent advantages and disadvantages. *In vitro* studies provide information of adipocytes that have been removed from their natural surroundings to study basic metabolic and mechanistic steps without ‘disturbance’ of other local factors, whereas *in vivo* assays are regarded as more suitable when studying adipose tissue as a part of the interacting organs in whole body physiology. These assays should not be regarded as mutually exclusive, but rather they provide complementary information.

2.3.1. Microdialysis technique *in vivo*

Principles. Microdialysis is widely used to study adipose tissue metabolism *in situ*. A microdialysis device is an artificial blood vessel system (Arner 1999), where the

microdialysis probe mimics the function of a capillary blood vessel with respect to its permeability to substances (Lafontan and Arner 1996) (Figure 2). The probe is made of semipermeable dialysis membrane, which is connected to inlet and outlet tubing. After skin disinfection, the probe/probes are implanted into subcutaneous adipose tissue with the aid of a cannula without local anaesthesia (Lafontan and Arner 1996). The probe is then continuously perfused with a neutral dialysis solvent, usually saline (0.9 g NaCl/l) or Ringer solution (Arner 1999), at low perfusion speed (0.1 – 10 μ l/min). The low perfusion speed allows solvent to equilibrate with the liquid surrounding the dialysis membrane by diffusion in both directions. Thus, outgoing dialysate mirrors the composition of extracellular fluid. Dialysate is collected in fractions and the concentrations of agents of interest are measured. It must be noted that the dialysate reflects the net sum of fluxes of compounds in the extracellular space, depending on adipocyte metabolite uptake/release, and delivery from/transport away by the microcirculation (Hagström-Toft 1991).

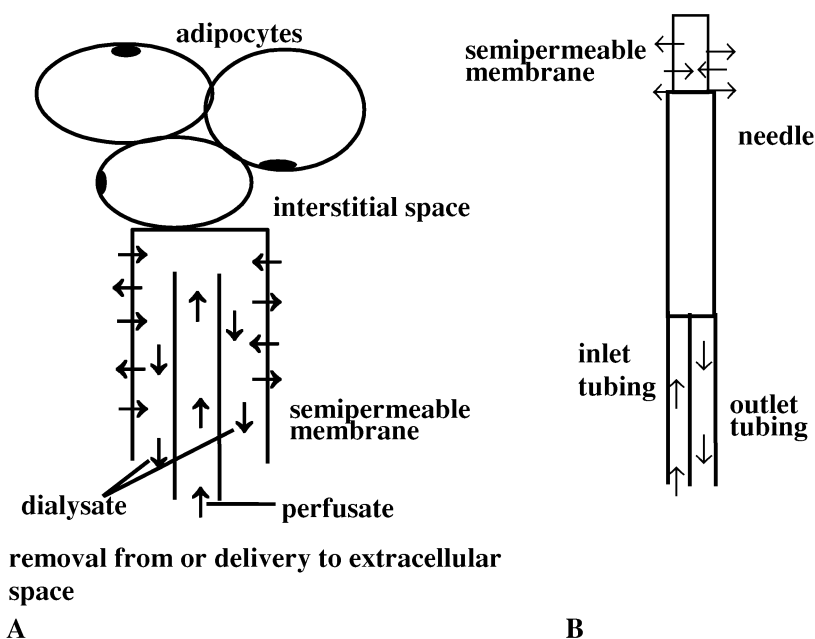


Figure 2. Schematic figure of the microdialysis system in the tissue (A) and the microdialysis probe (B). Modified from Lafontan and Arner 1996.

Performance. Under most circumstances, microdialysis allows assessment of only a fraction of a concentration of the substances in the extracellular space (Lafontan and Arner 1996). Because of the constant flow inside the microdialysis tube, complete equilibrium of the substances across the microdialysis membrane is usually not reached. The recovery can be improved by the use of long microdialysis membrane and a low perfusion rate (Arner 1999). Small and water-soluble molecules pass easily into the dialysate, whereas large, lipophilic molecules pass only in small amounts if at all (Arner 1995). In addition, small molecules that bind to carrier proteins pass the membrane more slowly. Usually, the membrane pore size is 1000 – 20 000 daltons (Da) (Lafontan and Arner 1996).

Local tissue factors are of great importance for dialysate recovery, i.e. the amount of substance of interest found in dialysate compared to concentration in the extracellular fluid. However, in kinetic studies, where the main aim is to follow changes in the concentration of tissue molecules over short period of time, it is often not necessary to consider recovery problems (Arner 1999). Instead, the change in relation to the baseline level is the major focus of the kinetic studies (Arner and Bülow 1993). As described in the next paragraph, it is possible to determine the true interstitial concentration of a particular substance by the microdialysis, although it is time-consuming (2 – 3 hrs) (Arner and Bülow 1993).

Determination of the extracellular concentration. When using ‘the mass transfer method’, the perfusion speed is gradually decreased and changes in the dialysate concentration are measured (Jacobson et al. 1985). With the aid of mathematical formulas, the concentration at zero perfusion speed corresponding the actual tissue concentration can be estimated (Jacobson et al. 1985). In equilibrium dialysis (Lönnroth et al. 1989), the tissue is perfused with different concentrations of the molecule of interest. The relationship of in- and outgoing concentrations is determined by regression analysis, where the equilibrium between in- and outgoing concentrations equals tissue concentration. Finally, true tissue concentration can be determined by using a low perfusion speed ($\leq 0.3 \mu\text{l}/\text{min}$) with a long dialysis membrane ($\geq 30 \text{ mm}$) (Bolinder et al. 1992). Under such conditions, recovery reaches approximately 100 % (Hagström-Toft 1991).

Blood flow. It is necessary to take into account the influence of local blood flow, when studying adipose tissue metabolism by microdialysis. This is because the tissue concentration of the agent of interest is always the net sum of local production, and

input / output by local blood flow (Hagström-Toft 1991). Thus, changes in blood flow greatly influence the concentrations of substances measured, such as glycerol and glucose. The most common way to estimate blood flow is the so called ethanol dilution technique, where ethanol is added to the perfusate (Hickner et al. 1991). The ratio of ethanol in dialysate vs. perfusate is determined to describe the relative changes in blood flow. The gold standard, using radiolabelled xenon to measure Xe-washout enables one to study actual blood flow rate. The ethanol dilution technique has been shown, however, to be as sensitive as Xe-washout method when measuring pharmacological stimuli (Hickner et al. 1991, Felländer et al. 1996), although Xe-washout may be more discriminative in its ability to monitor acute physiological changes (Karpe et al. 2002).

2.3.2. Catheterization technique *in vivo*

Adipose tissue metabolism can also be studied *in vivo* by measurements of arterio-venous differences (Frayn 1999). In humans this is most often done by catheterization of venous drainage of the subcutaneous adipose tissue depot of the anterior abdominal wall (Frayn et al. 1993). A catheter is introduced to a visible vein and advanced until its tip lies superior to the inguinal ligament. Another catheter is introduced percutaneously into the radial artery (Simonsen et al. 1994). This technique allows the determination of arterio-venous differences in the concentrations of lipophilic molecules, such as non-esterified fatty acids (NEFA) and TG.

In order to study adipose tissue metabolism with the catheterisation technique, blood flow determination is necessary (Frayn 1999, Macdonald 1999). By using the converse of Fick's principle, it is possible to estimate the rate of metabolism of a known substance by the tissue (Macdonald 1999). If the blood flow through the tissue and arterial and venous concentrations of the substance of interest are measured, the rate of uptake of the substance can be calculated:

$$\text{Net uptake} = \text{blood flow} \times (\text{arterial} - \text{venous concentration})$$

$$\text{Net output} = \text{blood flow} \times (\text{venous} - \text{arterial concentration})$$

However, Fick's principle assumes that blood flow should be stable, and the arterial concentration and tissue metabolism of the substrate should be constant (Macdonald 1999). Net flux, fractional extraction, and clearance of the substance of interest can be estimated (Macdonald 1999).

Some limitations of the arterio-venous difference technique must be noted. The studies are limited to abdominal subcutaneous adipose tissue and, further, the catheterization of the venous drainage is technically difficult (Frayn 1999). To overcome these limitations, a combination of tracer methods for measuring absolute

rates of NEFA uptake and release with selective catheterization has been developed (Frayn 1999).

Microdialysis and catheterisation give comparable information of local adipose tissue metabolism of glucose, glycerol, oxygen and carbon dioxide, whereas different results have been reported with regard to lactate (Simonsen et al. 1994). In a more recent study (Summers et al. 1998), these techniques were compared. The rate of glycerol release from adipose tissue in postprandial state evaluated with microdialysis method was systematically 40 % lower than with catheterisation technique (Summers et al. 1998). Bearing in mind the advantages and disadvantages of both methods (Table 2), the microdialysis and arterio-venous-difference technique produce, however, complementary information (Simonsen et al. 1994, Summers et al. 1998).

Table 2. Advantages and disadvantages of *in vivo* methods used to study adipose tissue metabolism. Modified from Frayn et al. 1993, Arner 1999, Frayn 1999.

Method	
Microdialysis:	
Advantages	Simple Minimal discomfort Long-term measurement Allows local pharmacological experiments Allows studies on several tissues and several regions of the body at the same tissue Many different molecules can be investigated at the same time
Disadvantages	Rather expensive Calibration problems Difficulties in studying large molecules Hydrophobic compounds cannot be studied Difficulties in studying molecules with strong protein-binding properties Not possible to study intra-abdominal adipose tissue
Catheterization:	
Advantages	Can be used to study hydrophobic molecules and lipoprotein particles Rather easy to quantify, but assessment of blood flow necessary
Disadvantages	Invasive Technically demanding Restricted to subcutaneous abdominal adipose tissue Not possible to study intra-abdominal adipose tissue

2.3.3. *In vitro* assays

The *in vitro* methods have some advantages compared to the *in vivo* methods. Sometimes these may also be considered as disadvantages depending on the aim of the study. First, the tissue environment can be controlled. This is especially important when studying hormonal regulation (Arner 1995). Furthermore, multiple metabolic investigations can be performed simultaneously and biochemical and molecular biology experiments can be done on the same tissue preparation in parallel with the metabolic investigations (Arner 1995). The disadvantages are that the effect of local tissue factors are difficult to estimate. Moreover, the removal of tissue from its natural surroundings may also alter the metabolism.

The choice of preparation. Isolated adipocytes have long been used in studies of adipose tissues metabolism, after the presentation of the technique by Rodbell (1964). The first crucial step is the digestion of connective tissue with collagenase, where high concentration of collagenase and shaking are usually needed (Arner 1995). Since the large adipocytes are more fragile, they may be lost during the isolation procedure (Ohisalo et al 1992).

In isolated adipocyte preparations, the metabolic response is rapid and pronounced (Arner 1995). Thus, it is ideal when studying the effect of pharmacological substances. Since lipolysis is clearly related to cell size (Reynisdottir et al. 1997, Large et al. 1998, Berger et al. 1999), the loss of large adipocytes may, however, cause some artifacts and problems in interpreting the data. Moreover, the artefactual leakage of adenosine may disturb the metabolism by inhibiting lipolysis (Arner 1995). Its leakage may sometimes be enough to cause total inhibition of lipolysis (Schwabe et al. 1973). One way to control this leakage is to add adenosine deaminase into the incubation medium to break down adenosine (Ohisalo et al. 1992). Another way is to use very dilute cell incubations in order to minimize the effect of adenosine (Arner 1995).

It is possible to use also adipose tissue fragments for *in vitro* studies. Adipose tissue is cut into small pieces and several pieces are incubated together (Arner 1995). The advantage is that tissue is intact and there is no loss of cells of a certain size. However, the responses to hormones or active agents tend to be sluggish and metabolic rates slow (Arner 1995). This makes it difficult to study pharmacological responses in tissue fragments.

Lipolysis. Lipolysis is examined by measuring end products, glycerol and FFA from the incubation medium. Glycerol determination is a more accurate index of lipolysis

than FFA release (Arner 1995), because glycerol is not re-utilized by adipocytes. The simultaneous measurement of glycerol and FFA is preferable. FFA re-esterification is one of the metabolic futile cycles, which can be determined by:

$$\text{re-esterification} = 3 \times \text{moles of glycerol release} - \text{moles of FFA released}$$

The assumptions are that there should be minimal or no incomplete hydrolysis of TG (usually < 10 % of total rate of lipolysis in humans) and oxidation of FFA and re-utilization of glycerol must be insignificant (Arner 1995).

The determination of the turnover of TG in adipocytes is difficult, since the total size of TG pool in lipid droplet of adipocytes cannot be determined with sufficient precision (Arner 1995). However, indirect radioactive methods can be used.

It can be considered that parallel use of *in vivo* and *in vitro* methods, where these assays are performed for the same subjects, will give complementary information. Thus, it is possible to study mechanistic biochemical cascades *in vitro* and to combine the information with *in vivo* data of the effect of local tissue factors and possible pathophysiological status of the subject. However, this can be reached only when the same subjects participate in both protocols.

2.3.4. Methods for studying gene mRNA expression

There are some methods that are commonly used for the quantification of transcription. Northern blotting is the only method providing information about mRNA size, alternative splicing and the integrity of RNA samples (Bustin 2000). RNase protection assay is useful for mapping transcript initiation and termination sites and intron/exon boundaries, whereas *in situ* hybridisation allows location of transcripts of specific cells within tissue (Bustin 2000). The reverse transcriptase polymerase chain reaction (RT-PCR) is an appropriate method when studying small tissue samples and information about *in vivo* regulation of mRNA expression of certain known genes is wanted (Auboeuf and Vidal 1997). RT-PCR is the most sensitive and most flexible of the quantification methods and can be used to compare the levels of mRNAs of different populations, to characterise patterns of mRNA expression, and to discriminate between closely related mRNAs (Bustin 2000, Moniotte et al. 2001). The possibility to analyse the mRNA expression of hundreds or even thousands of genes at the same time is provided by the rather new technique of DNA hybridization arrays (Moreno-Aliaga et al. 2001).

Reverse transcriptase competitive polymerase chain reaction (RT-cPCR). The idea of the RT-cPCR method is to add known amounts of competitor molecule, which differs

in sequence length from the target mRNA sequence, into the reaction medium (Auboeuf and Vidal 1997). The competitor molecule can be synthetic exogenous RNA added to the RT-reaction or DNA added to the amplification reaction. Competitors are coamplified in competition with the target in the same test tube, standardizing the amplification process (Auboeuf and Vidal 1997). This leads to the condition where the ratio between the target and the competitor remains constant, enabling the determination of the initial amount of target on the basis of the known amount of the competitor (Auboeuf et al. 1997). Thus, it is evident that the target and the competitor should amplify with the same efficiency and the same sense and antisense primers should be used (Auboeuf et al. 1997).

RT-cPCR allows an accurate quantification of RNA over a wide range of concentrations (Auboeuf and Vidal 1997). It has been suggested that the lower range of the RT-cPCR assays is 0.01 amol (Auboeuf and Vidal 1997). The method has also a relatively good reproducibility varying from 5 to 10 percent depending on the mRNA to be detected and the competitor (Auboeuf and Vidal 1997). However, the reproducibility of the RNA isolation and correct quantification of the concentration of RNA preparation is of major importance (Gilliland et al. 1990), since the reproducibility of the assay depends on these factors. Furthermore, for the reliability of the assay, the efficiency of the RT step must be optimised and different amplification reactions with changing amounts of the competitor flanking the assumed initial amount of target cDNA to determine the equivalence point accurately (Auboeuf and Vidal 1997, Freeman et al. 1999).

Real time PCR. The recent introduction of fluorescence based kinetic RT-PCR, real-time PCR, might be able to overcome the problems of conventional RT-PCR, such as problems in sensitivity, reproducibility and specificity (Bustin 2000). Nonetheless, the method depends on careful experimental design, application and validation to achieve accurate quantitative measurements of transcription (Bustin 2000).

In real-time PCR, fluorescence techniques are applied together with instrumentation where amplification, detection, and quantification have been combined. With this method, fluorogenic probes and/or interchelating dyes are used in the reaction so that the fluorescence emission of the generated PCR products is continuously monitored (Freeman et al. 1999, Bustin 2000). Real-time RT-PCR assays represents a significantly less variable technique than conventional RT-PCR, the coefficient of variation being 2 % compared to 10 % with the conventional technique (Bustin 2000).

DNA hybridisation array. By using DNA hybridisation array technology, potentially thousands of genes can be examined by one hybridisation (Landers 1999). Thus, it is possible to identify and compare patterns of gene expression for example in different disease states or in different patient groups (Yang et al. 1999). cDNA microarrays or oligonucleotide arrays are used (Nadler and Attie 2001). In cDNA arrays, 3'-end expressed sequence tags and/or known genes are spotted on the glass and are subsequently probed with fluorescently-labelled cDNAs from experimental samples (Nadler and Attie 2001). Oligonucleotide arrays are produced by short oligonucleotides complementary to the expressed genes (Nadler and Attie 2001). Total RNA from both the test and reference samples are isolated and labelled with fluorescent tags by a single round reverse transcription (Moreno-Aliaga et al. 2001). The matrix with either cDNA tags or oligonucleotides are then hybridised with two fluorescently-labelled cDNAs in a competitive manner and finally, the fluorescence is scanned (Moreno-Aliaga et al. 2001). The fluorescence at each spot correlates with the amount of mRNA in the original sample (Nadler and Attie 2001). However, the analyses and handling of array data is difficult due to the huge amount of data produced by a single hybridisation (Moreno-Aliaga et al. 2001). Moreover, this method is still poorly validated (King and Sinha 2001). Another difficulty is the high cost of the method (Moreno-Aliaga et al. 2001) and the need to verify analyses by using a reference method, such as real time PCR.

2.4. Adipose tissue metabolism

The main role of adipose tissue is to store energy when energy intake exceeds expenditure and release it when energy is needed by other tissues for oxidation (Frayn 2002). Numerous studies have shown that adipose tissue is also a site for multiple hormonally regulated metabolic reactions (Havel 2000, Kim and Moustaid-Moussa 2000, Frühbeck et al. 2001). Adipose tissue produces and secretes several active agents that take part in regulating energy homeostasis, enabling the body to adapt to metabolic challenges such as starvation, stress, infection, and energy excess, and, moreover, interacting with other tissues such as skeletal muscle, liver, kidneys, endothelium, immune system, and brain (Havel 2000, Kim and Moustaid-Moussa 2000, Frühbeck et al. 2001).

2.4.1. Adipocyte differentiation

Adipocyte differentiation (=adipogenesis) is dependent on the balance between factors that influence the differentiation either positively or negatively (Auwerx 1999).

Once preadipocytes enter the terminal phase of adipogenesis, coordinated regulation of adipose specific gene expression will result in mature fat cells that are highly specialized for energy storage and homeostasis (Gregoire et al. 1998). Peroxisome proliferator activated receptor (PPAR) and CCAAT/enhancer binding protein (C/EBP) families of transcription factors must function cooperatively to transactivate adipocyte genes and achieve adipocyte differentiation (Auwerx 1999). Regulation of adipogenesis is crucial to keep the body in energy balance. This is emphasized when one keeps in mind that both excessive amount of stored fat, i.e. obesity, as well as lipodystrophy are associated with severe, but paradoxically similar metabolic alterations (Frayn 2002).

The current knowledge of adipocyte differentiation is mainly based on *in vitro* models of white adipocyte studies, where either mouse cell lines or human or other primary cell cultures have been used (Koutnikova and Auwerx 2001).

Transcription factors. In the preadipocyte stage, preadipocyte factor-1 (Pref-1) is expressed (Gregoire et al. 1998). This protein inhibits adipocyte differentiation (Koutnikova & Auwerx 2001) and it has been suggested that Pref-1 may be a link between extracellular and intracellular signals to keep cells at the preadipocyte stage (Gregoire et al. 1998).

PPARs belong to the subfamily of nuclear hormone receptors (Spiegelman 1998). There are three related, but distinct PPAR proteins, PPAR α , PPAR δ (also called PPAR β), and PPAR γ (Tontonoz et al. 1994, Amri et al. 1995, Kersten 2002). Two different protein products PPAR γ 1 and PPAR γ 2 are produced from three separate mRNA-forms, γ 1, γ 2, γ 3 and γ 4 (Spiegelman 1998, Sundvold and Lien 2001). PPAR γ 2-protein contains 28 additional amino acids in its NH₂-terminal that are coded by the unique exon B. The protein is expressed almost exclusively in white adipose tissue in humans (Tontonoz et al. 1994, Auboeuf et al. 1997, Fajas et al. 1997, Vidal-Puig et al. 1997). PPAR γ 1 and PPAR γ 3 give rise to the same protein product (Fajas et al. 1998). PPAR γ interacts with two other transcription factors, C/EBP and adipose determination and differentiation factor 1/sterol regulatory element binding protein 1c (ADD1/SREBP1c) during adipocyte differentiation (Fajas et al. 1999, Rosen et al. 2000) (Figure 3).

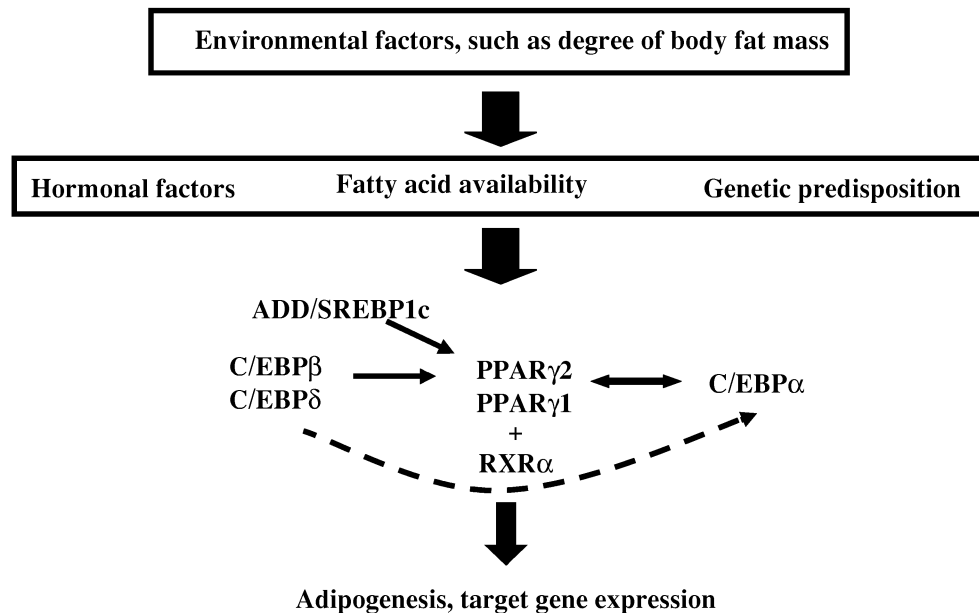


Figure 3. Modification of model for adipocyte differentiation. Environmental factors together with physiological and genetic factors modify adipogenesis. The transcriptional control of adipogenesis involves the activation of several families of transcriptional factors. Modified from Spiegelman 1998, Rosen et al. 2000.

There are also three C/EBP proteins, C/EBP- β , C/EBP- δ and C/EBP- α (Fajas et al. 1998). C/EBP- δ and C/EBP- β are expressed early in adipocyte differentiation, just before the actual differentiation takes place (MacDougald and Lane 1995). C/EBP- δ and C/EBP- β expression are repressed during the later phase of the differentiation (Hwang 1997). C/EBP- β has been shown to induce activity in the promoter of PPAR γ 2, whereas no induction was seen in PPAR γ 1 (Saladin et al. 1999), although its binding site has also been found in PPAR γ 1 (Spiegelman 1998). C/EBP α expression is induced during the terminal phase of adipogenesis, prior to the expression of adipose tissue specific genes (Hwang et al. 1997). Saladin and coworkers (1999) suggested that C/EBP- β cause the early induction of PPAR γ 2, when adipogenesis is initiated, and later, induction of C/EBP- α to maintain PPAR γ 2 expression. However, C/EBP- α may also induce adipogenesis independently, at least in the *in vitro* setting (Spiegelman 1998). It seems that the PPAR γ 1 level increases later and to a lesser extent than PPAR γ 2 levels during adipogenesis (Saladin et al. 1999). This finding is in line with another finding that

PPAR γ 2 is about 10-fold more active than PPAR γ 1 as a ligand independent transcriptional activator (Werman et al. 1997).

The expression of retinoid X receptor (RXR) is also induced a few hours after the initiation of the differentiation process (Chawla et al 1994). RXR α forms heterodimers with PPAR γ 2 to have full transcriptional activity (Spiegelman 1998). Retinoic acid (RA) forms a heterodimer with RXR α competing with PPAR γ , and thus preventing PPAR γ target gene expression (Koutnikova & Auwerx 2001). RA can also inhibit adipogenesis by interfering C/EBP β action (Koutnikova & Auwerx 2001). However, the RA level decreases along the differentiation process, and thus, RXR α is available for PPAR γ 2 (Saladin et al. 1999).

Another factor having an effect on the differentiation process is ADD1/SREBP1c, which is one of three SREBP isoforms, SREBP1a, SREBP1c and SREBP2 (Hua et al 1993, Yokoyama et al. 1993). SREBP1a and 1c are generated from a single gene through alternative splicing (Yokoyama et al. 1993). SREBPs belong to the basic helix-loop-helix leucine zipper family and they are synthesized in a membrane bound form (Brown and Goldstein 1993). SREBP1c expression is regulated by the sterol content within the cell. During sterol deprivation, SREBPs are cleaved to enter the nucleus and activate the transcription of genes involved in cholesterol and fatty acid synthesis, such as lipoprotein lipase (LPL) and fatty acid synthase (Tontonoz et al. 1993, Brown & Goldstein 1997, Boizard et al. 1998, Latasa et al. 2000, Koutnikova & Auwerx 2001). SREBP1c is the major form of mRNA expressed in white adipose tissue in human, mice and cultured adipocyte cell lines (Shimomura et al. 1997). SREBP1c can induce PPAR γ transcription through response elements in γ 1 and γ 3 promoters (Fajas et al 1999). It has also been suggested that via the action of SREBP1c, endogenous ligands for PPAR γ activation are produced to enhance the transactivation of PPAR γ 1 and γ 2 (Kim et al. 1998). PPAR γ appears also to stimulate SREBP-1 expression (Kersten 2002). Since SREBP-1 strongly stimulates the expression of lipogenic genes (Rosen et al. 2000), the interaction between PPAR γ and SREBP-1 is aimed at promoting lipogenesis in adipose tissue (Kersten 2002). Thus, it is suggested that PPAR γ has a role in adipogenesis as well as lipogenesis in mature adipocytes (Kersten 2002).

The interaction between C/EBP- α , PPAR γ and SREBP1c finally leads to the expression of several adipocyte specific target genes, including lipogenic enzymes, fatty acid binding proteins, and secreted factors to associate adipocytes for example with the regulation of whole body energy homeostasis and glucose and insulin metabolism (Auwerx 1999). Target genes of PPAR γ include, among others, LPL, adipocyte fatty

acid binding protein aP2, phosphoenolpyruvate carboxykinase, uncoupling protein, phosphoinositol 3-kinase, and glucose transporter protein GLUT4 (Koutnikova and Auwerx 2001). During this terminal phase of differentiation, adipocytes reach their typical phenotype with their characteristic lipid droplets and start acting as mature adipocytes.

Endocrine factors. Hormonal inducers of adipocyte differentiation include insulin, insulin like growth factor-1 (IGF-1), growth hormone (GH), glucocorticoids, triiodothyronine (T_3) and cAMP (Hwang et al. 1997, Gregoire et al. 1998). IGF-1 or pharmacological concentrations of insulin are needed for adipocyte differentiation (Gregoire et al. 1998). Insulin might exert its effects through IGF-1, which, in turn, appears to be necessary for adipocyte differentiation (Hwang et al. 1997, Gregoire et al. 1998), although IGF-1 has also inhibitory effects on the differentiation in the early stages of the cell cycle (Koutnikova and Auwerx 2001). It should be kept in mind, that insulin induces the expression of both PPAR γ and SREBP-1 (Kersten 2002). During the terminal phase of differentiation, maturing adipocytes become sensitive to insulin, when the insulin receptor number increases by 20-fold during differentiation (Reed and Lane 1980).

Glucocorticoids, cAMP and T_3 are also regarded as activators of adipogenesis. In 3T3-L1 mouse white preadipocytes, dexamethasone, a synthetic glucocorticoid, induces expression of C/EBP- δ , one of the early transcription factors of the differentiation process (Yeh et al. 1995). cAMP induces C/EBP- β expression (Yeh et al. 1995).

Studies investigating the role of GH have resulted in contradictory findings. In mouse cell lines, removal of GH from the culture medium blocked differentiation (Hwang et al. 1997). Within human primary preadipocytes, however, GH seems to lack the differentiating function (Gregoire et al. 1998). It has been speculated that human primary cells have already been exposed to the effect of GH before removal from the tissue environment (Hwang et al 1997).

A number of other polypeptide growth factors have been shown to inhibit adipocyte differentiation, including epidermal growth factor, transforming growth factor- α and - β , and basic fibroblastic growth factor (Hwang et al. 1997, Gregoire et al. 1998).

Paracrine factors. Interestingly, adipocytes, by themselves, produce agents that influence the differentiation/recruitment of new adipocytes. Acylation stimulating

protein (ASP) has been shown to stimulate the differentiation of preadipocytes into mature adipocytes and to promote TG synthesis in adipocytes (Cianflone 1994).

Angiotensinogen has an effect on both the differentiation of new adipocytes and on the function of mature adipocytes (Hwang et al. 1997). Angiotensinogen expression in adipose tissue appears to increase with increasing fat mass (Dusserre et al. 2000), to decrease in fasting, and to increase in refeeding (Frederich et al. 1992). It affects adipogenesis through stimulation of the formation of prostanoids (Hwang et al. 1997). For example, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 is one of the natural ligands for PPAR γ and, thus, is an inducer of adipocyte differentiation (Spiegelman 1998, Auwerx 1999).

Tumor necrosis factor α (TNF- α) is a strong inhibitor of adipogenesis (Koutnikowa and Auwerx 2001). Not only does it block the differentiation, but it also represses the phenotype of mature adipocyte (Hwang et al. 1997). In addition, it has been reported that TNF- α may down-regulate the expression of PPAR γ and C/EBP- α (Stephens and Pekala 1991, Xing et al. 1997, Kurebayashi et al. 2001).

2.4.2. Fat deposition into adipose tissue

Adipocytes synthesize TG from fatty acids delivered to them in chylomicrons and chylomicron remnants from dietary sources and in VLDL from liver (Frayn et al. 1994). Several mechanisms take part in TG synthesis in adipocytes. During the postprandial period, fatty acids from chylomicrons or VLDL particles are deposited and stored in adipocytes via TG synthesis (Frayn 2002). Lipogenesis refers to processes of fatty acid synthesis and subsequent TG synthesis that takes place both in liver and adipocytes (Kersten 2001). In humans, however, de novo lipogenesis does not contribute significantly to fat balance in persons consuming a typical Western diet (Guo et al. 2000, McDevitt et al. 2001). In adipose tissue, stored TG undergo continuous and simultaneous synthesis and breakdown (Edens et al. 1990). Both glycerol and fatty acids are released from adipocytes. Part of the released fatty acids is entrapped by adipocytes to be re-esterified (Coppack et al. 1994, Frayn et al. 1995). Glycerol is not, however, taken up by adipocytes (Low 1961). Glucose, instead, is used to form glycerol-3-phosphate for TG synthesis. Primary re-esterification is the total amount of FFA that is re-esterified during a given situation and reflects the TG synthesis capacity of the adipocytes (Edens et al. 1990). Fractional re-esterification is the proportion of FFA re-esterified in relation to the amount of FFA formed by lipolysis (Campbell et al. 1992).

Insulin. Insulin has long been known to be the main hormonal factor promoting fat deposition, especially postprandially (Coppack et al. 1989). Insulin acts by suppressing the action of hormone sensitive lipase (HSL), activating LPL and by stimulating glucose uptake in adipocytes (Coppack et al. 1989). Insulin also stimulates fractional re-esterification (Campbell et al. 1994, Evans et al. 1999).

Lipolysis in normal subjects is extremely sensitive to insulin action (Large and Arner 1998). The mechanism by which insulin inhibits the release of FFA from adipocytes involves phosphatidylinositol 3-kinase- (PI3K) and phosphodiesterase 3B (PDE3) pathways (Degerman et al. 1997, Van Harmelen et al. 1999). PDE3 may be stimulated by insulin receptor signaling which leads to the activation of PI3K (Zierath et al. 1998). PDE3 also causes lowering of the intracellular cAMP concentration (Degerman et al. 1997), which leads to decrease in HSL activity. Dephosphorylation of HSL may be further enhanced through insulin-mediated activation of protein phosphatases (Holm et al. 2000). Stimulation of re-esterification seems to be mediated by the PI3K pathway (Zierath et al. 1998).

Lipoprotein lipase. LPL is an enzyme, which hydrolyses and releases fatty acids from chylomicrons or VLDL particles (Frayn et al. 1994). LPL is synthesized in adipocytes as well as other cell types, from where it is secreted either in an active or inactive form (Eckel 1989). LPL is attached to heparan sulphate proteoglycans at the luminal surface of the vascular endothelium (Eckel 1989). In adipocytes, there is a large pool of the enzyme, much of which is not active (Pradines-Figueres et al. 1990). A large proportion of stored LPL is destined for degradation without export (Fielding & Frayn 1998). Increase in LPL mRNA expression levels, however, parallels the rate of synthesis and precedes the increases in LPL activity at protein level (Eckel 1989, Fried et al. 1993). During feeding, LPL levels increase (Eckel 1989). Since high carbohydrate diets are effective in promoting LPL activity on adipose tissue, insulin is thought to be a major regulator of feeding effect (Eckel 1989). In fact, it has been shown that insulin stimulates LPL activity in a dose- and time-dependent manner (Eckel 1989, Coppack et al. 1992, Frayn et al. 1994). Insulin up-regulates LPL expression and enhances its activity in adipose tissue. The activation of LPL by insulin is, however, a slow effect that is seen after several hours, contrary to the effect of insulin on fatty acid release (Frayn 2002).

Acylation stimulating protein. Along with the findings that subjects with complete LPL deficiency have normal bodily phenotype, and that their fat cells are filled

normally with lipid (Brun et al. 1989, Peeva et al. 1992), it became evident that other important factors acting on fat deposition must exist. ASP has a strong stimulative effect on TG synthesis, (Cianflone et al. 1989, Cianflone et al. 1994). ASP is generated *in vivo* by human adipocytes (Saleh et al. 1998). Adipocytes synthesize and secrete proteins of the so called alternate complement pathway: the third component of complement (C3), factor B, and factor D (=adipsin) (Cianflone et al. 1994). These proteins interact to produce a 77-amino acid derivative of C3, from which the terminal arginine is removed to produce ASP (Baldo et al. 1993). The extent to which cultured differentiating adipocytes produce ASP is proportional to the degree to which they have accumulated triglycerol mass during differentiation (Cianflone et al. 1994).

The effects of ASP are additive to and independent of those of insulin. Thus, ASP and insulin seem to be the two most potent *in vitro* stimulants of TG synthesis in adipocytes by controlling the rate at which the fatty acids can be taken up by adipocytes (Sniderman et al. 1998). The effect of ASP is also mediated via the PDE3 pathway (van Harmelen et al. 1999), and the expression of ASP is increased postprandially (Saleh et al. 1998) similarly to that of LPL. ASP acts by increasing translocation of glucose transporters to plasma membrane and by increasing the activity of diacylglycerol acyltransferase that catalyzes the final, rate-limiting step in TG synthesis (Sniderman et al. 1998).

Other factors. GH and leptin inhibit fat storage and lipogenesis (Kersten et al. 2001). GH lowers adipose tissue LPL activity, although its effect might also be related to the reduction of fat mass seen after GH treatment in GH subjects (Richelsen 1999, Richelsen et al. 2000).

Leptin expression in adipose tissue and plasma leptin concentrations increase with increasing fat mass (Maffei et al. 1995, Considine et al. 1996). Through its effects in the central nervous system, leptin decreases food intake and increases or maintains energy expenditure via the activation of the sympathetic nervous system, leading to activation of lipolysis (Campfield et al. 1995, Havel 2000). Thus, leptin has a reducing effect on fat mass. A leptin infusion has also been shown to lower adipose tissue LPL activity in leptin deficient obese mice to the level seen in lean mice (Picard et al. 1998). In human studies, the concept of stimulation of lipolysis by leptin has been challenged, since no effect was observed on basal or stimulated lipolysis by leptin in isolated human adipocytes (Aprath-Husmann et al. 2001).

2.4.3. Lipolysis and release of fatty acids

Lipolysis is a cascade of events that starts with a hormonal or some other stimulus that activates receptors in the plasma membrane of adipocytes and finally leads to release of fatty acids and glycerol from adipocytes (Figure 4). Lipolysis is affected by hormonal and physiological factors such as gender, stress, exercise, and aging (Arner 1996). The rate of lipolysis is also affected by tissue blood flow (Arner 1996). Most of lipolysis takes place in adipose tissue, but both cardiac and skeletal muscle and liver have lipolytic activity so that they can perform the local oxidation of FFA (Coppack et al 1994). In fact, it has been shown that during prolonged fasting as much as 15 – 20 % of glycerol released originates from nonadipose sources, such as intravascular and intramyocellular hydrolysis of TG (Jensen et al. 2001). It has been suggested that TG stores in liver and muscle serve as a buffer that compensates for differences in FFA supply and oxidative demand of lipids (Coppack et al. 1994).

Stimulation of lipolysis. Lipolysis in adipose tissue is mainly controlled by the activity of the sympathetic system and by plasma insulin (Holm et al. 2000). Catecholamines stimulate lipolysis in humans (Figure 4). Adrenaline reaches adipose tissue via the circulation whereas noradrenaline is produced by sympathetic nerve endings (Large and Arner 1998). Their effects are mediated via β -adrenergic receptors (AR), which can be divided into at least three different subtypes (Large and Arner 1998, Holm et al. 2000). In studies with human fat cells *in vitro* as well as in microdialysis studies on humans, it has been shown that stimulation is mainly mediated by β_2 -receptors, but β_1 -AR may also play a role (Barbe et al. 1996, Large and Arner 1998, Langin et al 2000). Further, microdialysis studies confirm that β_2 -ARs function is predominant (Barbe et al. 1996), but the relative importance of β_3 -AR in humans remains unclear (Enocksson et al. 1995, Barbe et al. 1996, Langin et al. 2000). There is evidence that the β_3 -AR is mainly expressed in visceral fat (Large and Arner 1998), where it seems to have a strong lipolytic function (Arner 2001). β_3 -AR seems to have minor importance in stimulating lipolysis in subcutaneous white adipose tissue (Tavernier et al. 1996). Stimulation of β -ARs leads also to an increase in local blood flow that further enhances the lipolytic effect (Barbe et al. 1996, Millet et al. 1998).

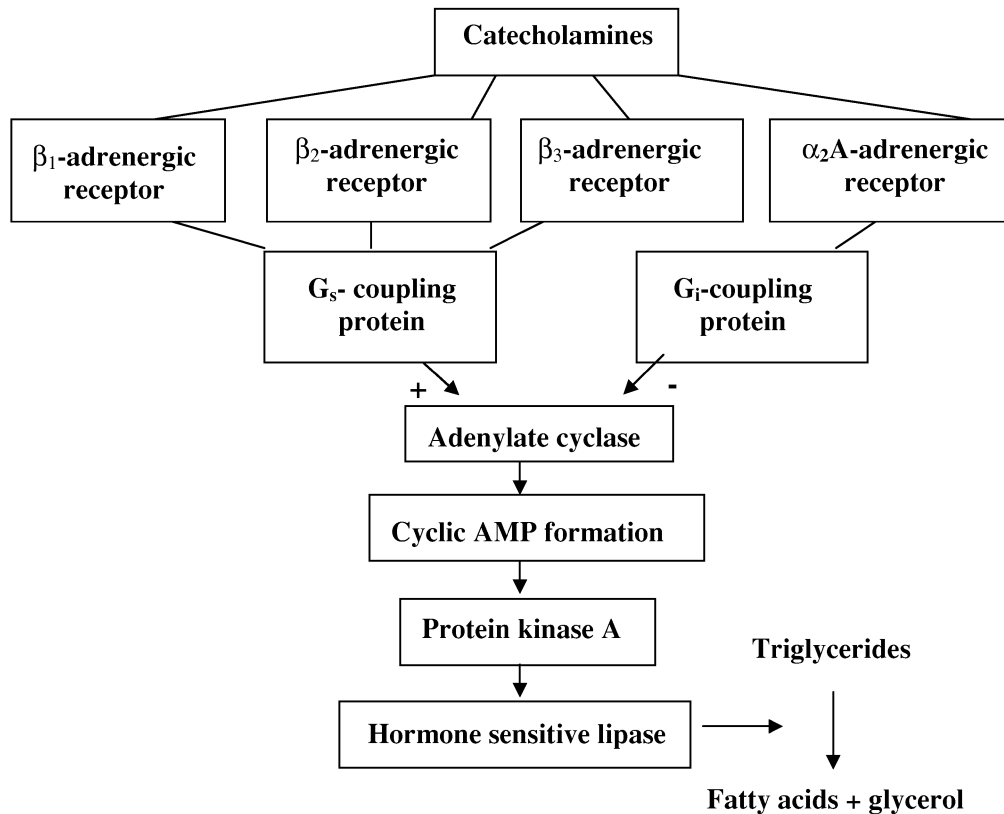


Figure 4. Catecholamine receptor signal transduction in human adipocytes. Modified from Arner 2001.

During prolonged exposure to catecholamines, β -ARs are desensitized which is seen as rapid decrease in the β -AR number and their responsiveness (Arner 1992). Moreover, individual variations in the sensitivity to β -AR action have been detected (Lönnqvist et al. 1992). In an *in vitro* study with subcutaneous adipocytes (Lönnqvist et al. 1992), lean subjects with low isoprenaline (non-subtype selective β -AR agonist) sensitivity, appeared to have a lower number and less mRNA expression of β_2 -AR compared to lean subjects whose adipocytes showed high isoprenaline sensitivity. No difference was observed in β_2 -AR affinity or β_1 -AR number or affinity, or mRNA expression between the groups. This finding further underlines the predominant role of β_2 -ARs in human adipose tissue.

β -ARs are positively coupled to adenylylase by the G_s -proteins (Large and Arner 1998) (Figure 4). Activation of adenylylase leads to cAMP accumulation in adipocytes. This leads, in turn, to the activation of protein kinase A (PKA) (Holm et al. 2000). PKA phosphorylates HSL which leads to the activation of the enzyme. Finally, HSL hydrolyses stored TG into di- and monoglycerides that are hydrolysed by monoacylglycerol lipase into FFA and glycerol to be released from adipocytes (Large and Arner 1998). In addition to their action on lipolysis, β -ARs also inhibit insulin-induced glucose transport, favouring the release of fatty acids because of lower re-esterification rate (Large and Arner 1998).

There are also other hormones that have a permissive effect on the catecholamine stimulative action, such as glucocorticoids, thyroid hormones, and GH (Large and Arner 1998). Some hormones may possibly mediate their effects also through G_s -protein-coupled receptors, such as thyroid-stimulating hormone, glucagon, cholecystokinin and parathyroid hormone, although their physiological roles are unclear (Large and Arner 1998). TNF α increases lipolysis in humans *in vivo* (Starnes et al. 1988) and in primary cell cultures (Hauner et al. 1995, Hotamisligil 1999,).

Recently, it was shown that atrial natriuretic peptide (ANP) can exert potent lipolytic effects in adipose tissue (Sengenès et al. 2000). The ANP receptor was found in human adipose tissue and further *in vitro* and microdialysis studies confirmed its lipolytic effect in human abdominal subcutaneous adipose tissue (Sengenès et al. 2000, Langin et al. 2000, Sengenès et al. 2002). ANP operates via cGMP (Sengenès et al. 2000), but it is unclear how this pathway participates in HSL activation.

Hormone sensitive lipase. HSL is the rate-limiting enzyme in the cascade of lipolysis (Figure 4), whose activation is mediated by phosphorylation. Upon stimulation by catecholamines, HSL is translocated from the cytoplasmic compartment to the surface of the lipid droplet (Holm et al. 2000, Kraemer and Shen 2002). In addition to HSL, PKA phosphorylates proteins called perilipins (Holm et al. 2000, Langin et al. 2000), which surround the surface of lipid droplets in adipocytes (Langin et al. 2000). When they are phosphorylated, physical alterations take place on the droplet surface that facilitate the action of active, phosphorylated HSL (Langin et al. 2000). HSL has also been suggested to interact with two other proteins, with adipocyte lipid-binding protein (ALBP or aP2) (Shen et al. 1999) and lipotransin (Suy et al. 1999). The interaction of ALBP might prevent the accumulation of fatty acids, decreasing the product inhibition of HSL, and facilitate FFA delivery out of the adipocytes (Holm et al.

2000, Kraemer and Shen 2002,). It has been suggested that ALBP and HSL constitute a lipolytic complex that increases the hydrolytic activity of HSL (Kraemer and Shen 2002). Lipotransin, instead, seems to dock HSL to the lipid droplet surface and might play a role in insulin-mediated inhibition of lipolysis (Holm et al. 2000, Langin et al. 2000, Kraemer and Shen 2002,). HSL is inactivated via dephosphorylation mediated primarily by the reducing effect of insulin on the cAMP level in adipocytes, as described before (Saltiel and Kahn 2001).

HSL expression is a major determinant of maximal lipolytic capacity in the human adipocyte and further, HSL expression is markedly affected by the adipocyte cell size (Reynisdottir et al. 1997, Large et al. 1998, Berger and Barnard 1999). Moreover, adipocyte cell size has a major influence on the rate of lipolysis (Arner 1996), larger cells being lipolytically more active. Thus, there is an association between cell size, HSL expression and lipolytic activity in human adipose tissue.

Inhibition of lipolysis. Catecholamines can also inhibit lipolysis via α_2 -ARs (Arner 1992), of which the α_2A -subtype seems to be predominant in adipose tissue (Arner 2001) (Figure 4). α_2 -ARs are negatively coupled to adenylate cyclase by G_1 -proteins that inactivate adenylate cyclase (Holm et al. 2000). The net effect of the catecholamines depends on the functional balance between stimulating β -ARs and inhibiting α_2 -AR (Large and Arner 1998). In human subcutaneous adipocytes, α_2 -AR numerically predominate over β -AR (Mauriège et al. 1987, Lafontan et al. 1997). Adrenaline seems to be the preferred amine for the α_2 -AR (Stich et al. 1999), and adrenaline-dependent inhibition of lipolysis has been described recently *in vitro* (Langin et al. 2000). The number of α_2 -ARs seems to be directly correlated with cell size, and a reduction in adipocyte size has been associated with a reduction in α_2 -AR (Lafontan and Berlan 1995). Indeed, adrenaline and noradrenaline have higher affinity at low concentrations for α_2 -ARs than for β -ARs (Lafontan and Berlan 1995, Stich et al. 1999). In spite of this, the β -response predominates, though in some situations α_2 -ARs become more active (Arner 2001). It has been suggested that α_2 -ARs could be involved in modulation of lipolysis at rest or during states of increased plasma adrenaline levels, such as mental stress or exercise (Lafontan et al. 1995, Large and Arner 1998). A recent *in vivo* microdialysis study showed that adrenaline has a specific role in the control of lipolysis in human subcutaneous adipose tissue via the antilipolytic action of the α_2 -AR during exercise-induced lipolysis (Stich et al. 1999). Further antilipolytic action is mediated by α_2 -ARs' decreasing effect on local blood flow

(Galitzky et al. 1993, Millet et al. 1998). There is a recent evidence that α_1 -ARs increase glucose uptake and lactate production in human adipose tissue (Bochman et al. 2002) as shown also in rat white adipose tissue (Faintrenie and G elo en 1996, Faintrenie and G elo en 1998).

Insulin is the most potent antilipolytic hormone inhibiting HSL. The suppression capacity of fatty acid release by insulin is highlighted by the rapidity of its action after a meal: fatty acid release can go from its highest rate, after an overnight fast to almost zero after breakfast within 90 minutes (Frayn 2002). Adenosine is another factor that also inhibits the action of HSL (L onnroth et al. 1989). Adenosine is released from nerve terminals and produced intracellularly by dephosphorylation of AMP and by hydrolysis of S-adenosylhomocysteine (Ohisalo 1987). Adenosine receptors (A receptor) are located in the plasma membrane (LaNoue and Martin 1994). The predominant A receptor in adipocytes is the A_1 -receptor (LaNoue and Martin 1994). The A_1 -receptor is coupled to adenylate cyclase via the G_i -protein (Carey 1998). Adenosine has insulin-like effects in adipocytes: it stimulates LPL, inhibits lipolysis (Ohisalo 1987), stimulates glucose uptake and increases insulin sensitivity (Schwabe et al. 1973). Thus, it is sometimes called local insulin. Moreover, insulin-like growth factor-1, prostaglandins E1 and E2, neuropeptide Y, peptide YY and ketone bodies inhibit lipolysis (Large and Arner 1998).

2.5. Effects of obesity and weight reduction

It has been shown that obesity leads to adaptive changes in adipose tissue metabolism (Kaartinen et al. 1991, Ohisalo et al. 1992, Coppack et al. 1992, Kaartinen et al. 1995, Frayn et al. 1996, Large and Arner 1998, Arner 1999, L ofgren et al. 2002) and gene expression (Vidal-Puig et al. 1997, Millet et al. 1997, Lefebvre et al. 1998, Kubota et al. 1999, Vidal 2001, L ofgren et al. 2002), possibly to overcome the potential health risks of increasing fat mass or to prevent further weight gain. It is not known whether these changes might be primary, causing obesity as such, or secondary changes, due to obesity itself. Currently, it seems that the alterations might be mainly secondary, since some of them have been reported to be reversible after weight loss.

2.5.1. Adipocyte differentiation

Since obesity is accompanied by an enlarged fat mass, it would be tempting to think that the action of transcription factors involved in adipogenesis might lead to obesity as such. Although an obese person may have increased fat cell number, increased fat cell number as such, without positive energy balance and enhanced storage of fat, is not,

however, identical to obesity (Spiegelman 1998). In fact, a failure in the capability to form new adipocytes might lead to metabolic disturbances leading to development of diabetes (Anand and Chada 2000).

There are, however, possible links between adipocyte differentiation and obesity or excess energy-linked signals. Obesity is accompanied by changes in hormone levels that ensure the storage of excess energy as fat (Spiegelman and Flier 1996). In addition to its action on LPL and HSL, insulin stimulates the action of fatty acid synthase and glycerophosphate dehydrogenase (Spiegelman and Flier 1996), and as mentioned before, expression of PPAR γ and SREBP-1 (Kersten 2002). It is also known that glucocorticoid levels increase in the obese state favouring fat deposition (Spiegelman and Flier 1996, Björntorp and Rosmond 2000). Moreover, one potential link is the presence of natural ligands for PPAR γ , which are derived from arachidonic or other polyunsaturated fatty acids (Spiegelman and Flier 1996).

Some studies have focused on the expression of transcription factors in obesity, mostly PPAR γ . No difference in the PPAR γ 2 expression in subcutaneous adipose tissue was found among obese, lean, and type 2 diabetic subjects, when subcutaneous adipose tissue was studied (Auboeuf et al. 1997, Rieusset et al. 1999). No difference in PPAR γ expression was found among obese, post obese and nonobese subjects either in intraperitoneal or in subcutaneous adipose tissue (Krempler et al. 2000). However, a large individual variation was observed in the level of expression, regardless of the amount of fat mass (Auboeuf et al. 1997, Rieusset et al. 1999). Another study (Vidal-Puig et al. 1997) showed, however, increased PPAR γ 2 expression in subcutaneous abdominal adipose tissue in obese subjects, but no difference was seen in PPAR γ 1 expression. Moreover, in comparison of different adipose tissue depots, omental and subcutaneous abdominal adipose tissue expressed similar levels of PPAR γ , when obese subjects were studied (Lefebvre et al. 1998). However, in subjects with BMI under 30, kg/m² omental adipose tissue expressed lower levels of PPAR γ (Lefebvre et al. 1998). The difference might be associated with enlargement of visceral fat depot in obesity (Lefebvre et al. 1998). In another study, on the other hand, this difference was not seen when overweight (mean BMI 28 \pm 7 kg/m²) and lean subjects were studied (Montague et al. 1998).

It is still unclear how PPAR γ may be linked to obesity. One of the mechanisms which might be involved is leptin (Kadowaki et al. 2002, Walczak and Tontonoz 2002). Leptin, as an adipocyte secreted hormone, controls energy balance through its effects on hypothalamus and paracrine effects on adipose tissue itself (Campfield et al. 1995,

Havel 2000). PPAR γ reduces leptin expression and thus, might increase food intake providing substrates to be stored in adipose tissue (Kallen et al. 1996, Hollenberg et al. 1997). Furthermore, PPAR γ agonists seem to down-regulate TNF- α expression (Hofman et al. 1994, Haman et al. 1995). Human isolated adipocytes show, however, decreased expression of PPAR γ , when treated with TNF- α (Sewter et al. 2002). By decreasing leptin and TNF- α concentrations, PPAR γ also could induce adipocyte sustaining endocrine, paracrine and autocrine responses (Auwerx 1999). In obesity, leptin expression and secretion is, however, increased. In rats, it was shown that leptin treatment stimulates PPAR γ expression (Qian et al. 1998) that might then be predisposing to obesity. It is unclear whether this relationship could also play a role in human obesity.

The expression of SREBP1c was down-regulated in subcutaneous adipose tissue of obese subjects as compared to lean subjects (Ducluzeau et al. 2001). This has also been shown in obese rodents (Nadler et al. 2000, Soukas et al. 2000). The change seemed to be paradoxical due to increased fat mass in obesity, but it has been suggested to be caused by down-regulation of lipogenesis to prevent further fat accumulation (Nadler et al. 2000, Sadler et al. 2000). The expression of C/EBP α , instead, is thought to be higher in intra- and extra-peritoneal adipose tissue in obese than in nonobese or postobese subjects (Krempler et al. 2000). One interesting finding was that TNF- α also reduced SREBP1c expression (Sewter et al. 2002).

Four interesting mutations of PPAR γ have been studied in connection to obesity (Yen et al. 1997, Vigoroux et al. 1998, Ristow et al. 1998, Barroso et al. 1999). A rare Pro115Gln mutation in NH₂-terminal ligand independent domain of PPAR γ leads to more active PPAR γ function and thus has been found in a few massively obese subjects (Ristow et al. 1998). Two other, extremely rare mutations (Pro467Leu and Val290Met) have been connected with insulin resistance, but with relatively normal body weight in three individuals (Barroso et al. 1999).

Another, more frequent mutation, the Pro12Ala change in exon B of PPAR γ 2, has yielded more controversial results (Yen et al. 1998, Beamer et al. 1998, Deeb et al. 1998, Valve et al. 1999, Lei et al. 2000, Clement et al. 2000, Altshuler et al. 2000, Lindi et al. 2001, Nicklas et al. 2001). Deeb and coworkers (1998) showed that the Alanine allele produced lower transcriptional activity *in vitro*. There are studies that associate Pro12Ala polymorphism with lower BMI (Deeb et al. 1998), or with higher BMI or increased weight gain (Beamer et al. 1998, Valve et al. 1999, Lindi et al. 2001). No association with body weight has been found in some studies (Clement et al. 2000,

Evans et al. 2000). Thus, it seems probable that environmental factors such as the degree of obesity or the quality of diet might influence the phenotypes associated with the Pro12Ala polymorphism (Ek et al. 1999, Luan et al. 2001).

There are only a few studies that have investigated transcription factor expression in connection to weight reduction. The expression of PPAR γ 2 (Vidal-Puig et al. 1997, Bastard et al. 1999, Ribot et al. 2001) and SREBP1c (Ribot et al. 2001) have been reported to decrease during weight loss. During weight maintenance, however, PPAR γ 2 expression increased to the levels found before weight loss (Vidal-Puig et al. 1997).

2.5.2. Fat deposition

Insulin. The effects of insulin are impaired in obesity (Frayn 2002) and this seems to associate with the increased amount of visceral fat (Hickner et al. 1999, Frayn et al. 2000). Impaired insulin sensitivity, i.e. insulin resistance, has been found in skeletal muscle, liver and adipose tissue (Hotamisligil 2000, Kahn and Flier 2000). In adipose tissue of obese individuals insulin resistance leads to impaired suppression of fat mobilization from adipose tissue (Eckel 1989, Coppack et al. 1992, Campbell et al. 1994, Hickner et al. 1999) and impaired activation of LPL (Eckel 1989, Ong and Kern 1989, Coppack et al. 1992). This condition usually leads to compensation by an increase in insulin secretion, which, in turn, leads to hyperinsulinemia (Kahn and Flier 2000).

In obese subjects, continuous FFA release has been shown after a meal despite existing hyperinsulinemia, in contrast to normal weight subjects, in whom FFA release was almost completely abolished (Coppack et al. 1992). This effect in obese subjects was due to a low fractional re-esterification, defective activation of LPL, and partly impaired suppression of HSL (Coppack et al. 1992) showing impaired action of insulin. In keeping with this, Frayn et al. (1996) have reported that plasma concentrations of insulin, glucose, and TG increased more in obese than in lean subjects after a meal. Thus, the insulin resistance caused obese subjects to fail to deposit TG in adipose tissue (Frayn et al. 1996), and hyperinsulinemia associated with obesity did not seem to be sufficient to produce normal adipose tissue metabolism in the postprandial period (Frayn 2002). However, hyperinsulinemia has also been reported to compensate for the effects of insulin resistance in obesity (Campbell et al. 1994).

After weight loss, the sensitivity of post-obese women to insulin was shown to be increased compared to women with normal weight and no history of obesity (Toubro et al. 1994). This might favour fat storage and lower lipid oxidation postprandially and thus, contribute to a propensity to obesity (Toubro et al. 1994).

Tumor necrosis factor α (TNF- α) has been suggested to hamper the effects of insulin on adipose tissue metabolism promoting insulin resistance in obesity (Hotamisligil et al. 1996). These effects of TNF- α have, however, been questioned recently (Saltiel and Kahn 2001). TNF- α also has a stimulating effect on lipolysis (Large and Arner 1998). In fact, mRNA levels of TNF- α in adipose tissue are higher in obese than in non-obese subjects (Hotamisligil et al. 1995), and weight reduction was associated with a decrease in mRNA levels (Hotamisligil et al. 1995).

ASP. Although obese subjects become resistant to the action of insulin, they remain responsive to ASP (Walsh et al. 1989). Fasting plasma ASP level has been reported to correlate positively with percent body fat (Weyer and Partley 1999), and, further, plasma ASP and adipose tissue C3 mRNA levels have been reported to be increased in obese subjects (Maslowska et al. 1999, Koistinen et al. 2001). Furthermore, during the postprandial period, ASP release from subcutaneous adipose tissue was significantly greater in obese than in normal weight subjects which resulted in enhanced fatty acid trapping in obese (Kalant et al. 2000). These findings indicate that ASP contributes to the metabolic changes and adaptations in obesity (Maslowska et al. 1999, Kalant et al. 2000). Plasma ASP concentrations has been reported to decrease during weight reduction (Sniderman et al. 1991, Sniderman and Cianflone 1994).

LPL. The levels of LPL in adipose tissue seem to affect the maintenance of adipocyte cell size, body weight, and obesity (Eckel 1989). However, data on the effects of obesity on LPL activity and expression are conflicting. Although LPL mRNA levels might be higher in obese compared to normal weight subjects (Eckel 1989), the enzyme activity does not necessarily parallel the level of LPL (Ong and Kern 1989, Imbeault et al. 1999). In some studies, higher LPL enzyme activity in obese subjects compared to normal weight subjects has been shown (Eckel 1989, Ong and Kern 1989, Berman et al. 1999), whereas other studies have shown blunted activity (Coppack et al. 1992, Reynisdottir et al. 1997), especially in subjects with insulin resistance (Reynisdottir et al. 1997). When the response of LPL activity to a meal was compared between obese and lean subjects (Ong and Kern 1989), the increase of activity was evident in lean subjects, but no change after the meal was observed in obese subjects. No changes or differences in LPL mRNA were observed (Ong and Kern 1989).

The situation is still more complex when activity and expression after weight loss have been studied. Some studies have found both increased LPL activity and mRNA

level after weight loss (Kern et al. 1990, Kern et al, 1995), but also a lack of effect of weight loss on LPL enzyme activity has been reported (Richelsen et al. 2000). Moreover, it has been reported that the higher the basal level of adipose tissue LPL activity, the greater the reduction in its activity after weight loss (Imbeault et al. 1999).

2.5.3. Lipolysis

It has been difficult to determine the rate of lipolysis in obesity compared to the state of normal weight since many other factors, such as regional and gender differences, phenotype of obesity, and changes in hormonal influences are also involved (Arner 1999). In general, the rate of the whole body lipolysis is decreased when calculated per fat mass in obesity (Lillioja et al. 1986). However, when calculated per fat free mass, lipolysis is increased in obese subjects (Coppack et al. 1994). In a microdialysis study, no modification of the rate of lipolysis was found in obese subjects after an overnight fast (Jansson et al. 1992). When studied *in vitro* in subcutaneous adipocytes, an increase in basal lipolysis related to cell number has been shown, but no change was found when interpreted by weight of fat cells (Large and Arner 1998). One factor influencing this discrepancy might be cell size, and in *in vivo* studies, also blood flow, which both have a strong effect on the rate of lipolysis (Barbe et al. 1996, Reynisdottir et al. 1997, Large et al. 1998, Millet et al. 1998).

Catecholamine induced lipolysis. In a number of *in vivo* and *in vitro* studies, the lipolytic action of catecholamines has been shown to be blunted in obesity (Connacher et al. 1991, Kaartinen et al. 1995, Horowitz and Klein 1996, Bougneres et al. 1997, Arner 1999). In subcutaneous adipocytes, the defect is caused by multiple changes in signal transduction. In obese women, almost 10-fold decrease in noradrenaline sensitivity in adipocytes due to reduction in cell surface density of β_2 -AR has been found when compared to lean women (Reynisdottir et al. 1994). Instead, mRNA levels of β_2 -AR, and the lipolytic sensitivity of β_1 - and α_2 -ARs were normal (Reynisdottir et al. 1994). Adipocytes from men with insulin resistance syndrome were resistant to the effect of catecholamines this being detected as a decrease in sensitivity to β_2 -AR selective agonist and a 50 % reduction in the number of receptors (Reynisdottir et al. 1994). Blunted stimulation of adenylate cyclase by β -ARs *in vitro* has also been found in some other studies (Kaartinen et al. 1995, Horowitz and Klein 1996). Moreover, the G_s -protein number was 63 times higher than the β -AR number in obese, suggesting that the defect was not at the level of receptor coupling to the G-protein (Kaartinen et al.

1995). In a recent *in vivo* study (Schiffelers et al. 2001), the blunted catecholamine action in obesity was confirmed to be due to impaired function of β_2 -AR. No difference was found in the β_1 -AR response (Schiffelers et al. 2001).

Enhanced α_2 -AR antilipolytic action in obese subjects has been reported, i.e. they had five to six times lower exercise induced glycerol release *in vivo* than in lean subjects (Stich et al. 2000). In another study (Karttinen et al. 1991), however, impaired α_2 -AR inhibition of lipolysis and an attenuated response to the effects of adenosine in obese subjects were reported.

Hormone sensitive lipase. HSL mRNA and protein expression are dependent on fat cell size regardless of adipose tissue depot or gender (Reynisdottir et al. 1997). HSL mRNA, protein activity and maximum lipolytic capacity were, however, decreased in the subcutaneous adipocytes of obese subjects compared to normal weight subjects independently of cell size (Large et al. 1999). The effect was less pronounced at the mRNA level (Large et al. 1999).

Controversial findings have been reported when the response of HSL expression and activity to weight reduction has been studied. During a very low calorie diet, HSL expression was increased (Stich et al. 1997), whereas in another study no change was seen (Hellström et al. 1996). After a similar diet and weight stabilization period, however, activity and expression were lower than before weight reduction (Reynisdottir et al. 1995, Klein et al. 1996). These changes were seen despite the decrease in fat cell size. This indicates that the regulation of HSL expression differs between the phase of active weight loss and the maintenance phase.

Genetic differences in lipolysis. In an extensive study (Mauriège et al. 1992), identical twins were overfed during a period of 100 days with ingestion of 1000 extra kcal per day. Mean body weight increased 8.4 kg and the gain of body fat reached 5.6 kg (Mauriège et al. 1992). Adipose tissue samples were taken before and after weight gain. No changes were detected in basal or catecholamine-induced lipolysis. Interestingly, intrapair similarities were observed in these variables, highlighting the important role of genotype in regulating the response of abdominal adipose tissue lipolytic activity to caloric excess. Moreover, first-degree non-obese relatives of obese subjects had a 50 % reduction in maximum activity of HSL compared to those without obesity in the family (Hellström et al. 1996). Thus, a strong genetic component could have an effect on adipose tissue metabolism and development of obesity. Sequence

variations in β -ARs, α_2 -AR and HSL and their effects on lipolytic activity have also been reported (Large et al. 1997, Arner 2001, Hamann et al. 2001, Rydén et al. 2001, Ukkola et al. 2001) as well as interaction between these variations (Ukkola et al. 2000, Ukkola et al. 2001).

The effect of weight reduction on lipolysis. There are only a few studies on lipolysis after weight reduction. Contrasting findings have been reported in women when basal and maximal lipolysis rates have been studied (Reynisdottir et al. 1995, Hellström et al. 1996, Stich et al. 1997). In the study of Mauriège et al. (1999), there was no change in basal lipolysis in either women or men, but enhanced lipolytic efficiency and β_1 - and β_2 -AR sensitivity in response to weight reduction in both genders were reported. Similarly, an increased response to isoprenaline and natriuretic peptides was seen after a 4-week low calorie diet (Sengenès et al. 2002).

α_2 -AR sensitivity has also been reported to decrease during consumption of a very low calorie diet promoting lipid mobilization (Hellström et al. 1997). When obese women were divided into groups, according to the rate of weight loss, those losing weight more rapidly showed 10-fold higher sensitivity to noradrenaline and had a 10-fold decrease in α_2 -AR sensitivity compared to those with a low rate of weight loss (Hellström et al. 1997). Inhibition or sensitivity to inhibition did not, however, respond to moderate weight loss in another study (Mauriège et al. 1999). Weight loss has also been found to improve the inhibitory effect of adenosine in obese subjects (Ohisalo et al. 1992).

2.6. Differences in regional adipose tissue metabolism

Many studies have shown that human adipose tissue from different sites of body varies in its metabolism and responsiveness to different stimuli. In normal weight subjects, visceral adipose tissue depot had the highest lipolytic activity induced by catecholamines, followed by the abdominal subcutaneous adipose tissue depot, which is the major body fat depot, whereas the lowest activity was found in femoral adipose tissue (Leibel et al. 1989) (Table 3). This difference across the depots has not, however, been so clear in all studies in non-obese subjects (Hoffstedt et al. 1997). Thus, regional differences in adipose tissue metabolism could be more pronounced in obesity or may be an obesity-related feature (Large and Arner 1998). This may also contribute to the frequently reported association between enlarged intra-abdominal fat stores and features of insulin resistance (Deprès et al. 1989, Marcus et al. 1999, Frayn 2002).

Table 3. Regional differences in induced lipolytic activity. Modified from Arner 1997 and Large and Arner 1998.

Hormone	Action on lipolysis	Regional differences
Catecholamines	Stimulating	visc>sc abd>sc glf
Insulin	Inhibiting	sc>visc
Prostaglandins E1, E2	Inhibiting	sc>visc
Adenosine	Inhibiting	sc>visc

visc=visceral, sc=subcutaneous, abd=abdominal, glf=gluteofemoral

2.6.1. Comparison of regional adipose tissue metabolism

The higher rate of catecholamine induced lipolysis in visceral adipose tissue compared to that in subcutaneous fat depots seems to be due mainly to increased expression, density and sensitivity of β_1 - and β_2 -AR as well as reduced α_2 -AR affinity and number in visceral adipose tissue (Arner et al. 1990, Lönnqvist et al. 1995, Mauriège et al. 1995, Vikman et al. 1996, Mauriège et al. 1999). Moreover, α_2 -AR sensitivity was decreased, β_3 -AR sensitivity increased, and cAMP activation of HSL was increased in isolated visceral adipocytes (Lönnqvist et al. 1997). Furthermore, β_3 -AR sensitivity has been found to be clearly higher in visceral than abdominal subcutaneous adipose tissue in obese subjects (Lönnqvist et al. 1995).

In visceral adipocytes isolated from obese subjects, the rates of FFA and glycerol release induced by catecholamines were about two fold higher compared to non-obese subjects, and no reutilization of fatty acids was observed (Lönnqvist et al. 1995). This is also in line with the reports, where the suppressive effect of insulin and adenosine on lipolysis was found to be less pronounced in visceral than in subcutaneous adipose tissue (Vikman et al. 1991, Shon et al. 1999). This might be due to reduced insulin receptor autophosphorylation and signal-transduction through the insulin receptor substrate-1 (IRS-1) associated PI3K pathway in visceral adipose tissue (Zierath et al. 1998). At the gene expression level, however, no regional differences in IRS-1 or PI3K mRNA levels have been observed (Lefebvre et al. 1998). Furthermore, the capability of insulin to increase LPL mRNA levels and activity was not observed in the visceral depot in obese men and women, whereas in the subcutaneous abdominal depot, the effect was seen (Fried et al. 1993). Indeed, in the basal state, the mRNA level of LPL in omental adipose tissue was lower (-20 %) than in subcutaneous abdominal adipose

tissue (Panarotto et al. 2000). LPL activity was reported to be lower in subcutaneous depot (Pedersen et al. 1994).

In spite of the higher lipolytic capacity in visceral adipose tissue, HSL activity, mRNA expression, and the level of maximum lipolytic capacity seem to be higher in subcutaneous abdominal depot compared to omental adipocytes in subjects with a wide range of BMI (22-50 kg/m²) (Reynisdottir et al. 1997). This could be due to the larger cell size in subcutaneous adipose tissue (Reynisdottir et al. 1997).

When subcutaneous adipose tissue depots have been studied in non-obese women, femoral adipose tissue has been found to be less responsive and less sensitive to β -AR stimulation than abdominal adipose tissue (Guo et al. 1997), but again, this has not been found in all studies (Millet et al. 1998). It was shown *in vitro* that abdominal adipocytes from non-obese subjects were much more sensitive to β -AR stimulation compared to gluteal cells (Wahrenberg et al. 1989). In obese women, higher β -AR affinity and lower α_2 -AR density in the abdominal depot compared to the gluteal depot was reported leading to higher lipolytic activity in abdominal adipose tissue (Berman et al. 1998). When obese men were studied, α_2 -AR responsiveness was much higher than in the non-obese group in the abdominal area, whereas no such difference was found in the gluteal depot (Mauriège et al. 1991).

In a study by Johnson et al. (2001), insulin resistance was evident in the abdominal subcutaneous fat depot, but surprisingly, to a lesser extent in the gluteal depot, when subcutaneous abdominal and femoral adipose tissue depots were compared in upper-body obese women. However, in another study, the resistance was not evident in either of the subcutaneous adipose tissue depots, despite the insulin resistant state (Hickner et al. 1999).

2.6.2. Gender differences in regional adipose tissue metabolism

Visceral fat accumulation is often associated with the metabolic disturbances known as the metabolic syndrome (Arner 1997, Desprès 2001), but criticisms have also been raised. Thus, it has been claimed that subcutaneous adipose tissue as a major source of FFA (Frayn 2000), or total body fat together with the effects of deep subcutaneous and visceral adipose tissue (Smith et al. 2001) are the major contributors to the metabolic disturbances occurring in obesity. Visceral adipose tissue is more lipolytically active and more resistant to insulin in men than in women (Large and Arner 1998). This leads to a higher fatty acid release from the upper body depots in men (Blaak 2001), resulting in a greater risk due to obesity in men (Lemieux et al. 1993). In fact, the lipolytic β_3 -AR

sensitivity was 12-times higher and the antilipolytic α_2 -AR sensitivity was 17-times lower in obese men than in obese women in omental adipose tissue (Lönnqvist et al. 1997). This resulted in higher fatty acid mobilization rate to the portal venous system in men (Lönnqvist et al. 1997). In another study, a gender difference was reported in response of LPL activity to weight loss in subcutaneous adipose tissue depots (Imbeault et al. 1999). LPL activity did not change in men, but decreased in women. No change or gender differences were, however, found at the level of mRNA expression (Imbeault et al. 1999).

The difference between abdominal and gluteal adipose tissue metabolism may be more pronounced in women than in men, since gluteal adipose tissue in women is more sensitive to α_2 -AR inhibition of lipolysis (Wahrenberg et al. 1989). Women have also a greater number of α_2 -ARs compared to abdominal area (Wahrenberg et al. 1989). After weight loss, however, α_2 -AR antilipolytic sensitivity was reduced in both regions in women, but only in the abdominal depot in men (Mauriège et al. 1999). β_1 -AR and β_2 -AR sensitivity and density were increased in parallel with weight loss in both genders, and these changes were more marked in abdominal than in gluteal adipose tissue (Mauriège et al. 1999). After 2 – 3 –years of weight loss, however, lipolytic capacity was reported to be decreased to the level of normal weight subjects in women (Löfgren et al. 2002). In men, no changes ascribed to obesity or weight loss were seen (Löfgren et al. 2002).

3. AIMS OF THE STUDY

Since adipose tissue may play a central role in the development of obesity, the present studies were carried out to examine adipose tissue metabolism both before and after weight reduction. The focus was on severely obese subjects who are considered most likely to reveal the metabolic alterations related to the pathophysiology of obesity. In addition to some genes of potential interest, these studies deal with the basic methodologies and changes in lipolysis and gene expression in adipose tissue. The specific aims of the studies were

1. To compare the microdialysis method *in vivo* and freshly isolated adipocytes *in vitro* when studying adipose tissue metabolism in severely obese subjects (I).
2. To study the effect of weight reduction on two key enzymes regulating lipolysis, hormone sensitive lipase (HSL) and lipoprotein lipase (LPL) by determining their mRNA expression in adipose tissue in severe obesity (II).
3. To study adipose tissue metabolism before and after one year of weight loss and its connection to HSL and LPL mRNA expression (II).
4. To study depot specific sterol regulatory element binding protein 1c (SREBP-1c) mRNA expression before and after one year of weight reduction (III).
5. To study the effect of weight reduction on depot specific mRNA expression of peroxisome proliferator activated receptor (PPAR) γ 1 and γ 2 (additional results).
6. To study the effect of Pro12Ala polymorphism in PPAR γ 2 gene on the PPAR γ total and PPAR γ 2 mRNA expression and its target gene expression (IV).

4. SUBJECTS AND METHODS

4.1. Subjects

Altogether 33 massively obese subjects (12 men (BMI range 38.0 – 74.0 kg/m²) and 21 women (BMI range 37.5 – 65.9 kg/m²)) were recruited to participate in the study. The subjects attended Kuopio University Hospital for the gastric banding operation (Figure 5) due to their massive obesity, and they underwent thorough medical examinations before the operation. Twenty-five subjects (8 men, 17 women) had normal or slightly increased fasting glucose concentrations, while 8 (4 men, 4 women) had been previously diagnosed as type 2 diabetic patients. Among the subjects, 25 (11 men (BMI 29.9 – 51.3 kg/m²) and 14 women (BMI 35.4 – 59.4 kg/m²)), of whom 7 men and 12 women were non-diabetic, participated in the follow-up study. Two of the subjects had a cardioselective β -blocking medication (atenolol). A 2-hour oral glucose tolerance test (OGTT) with a glucose load of 75 g was performed only in the non-diabetic obese subjects 7 - 56 days before and one year after gastric banding operation. The basic characteristics of the subjects are presented in Table 4. In the four separate studies some of the subjects were excluded.

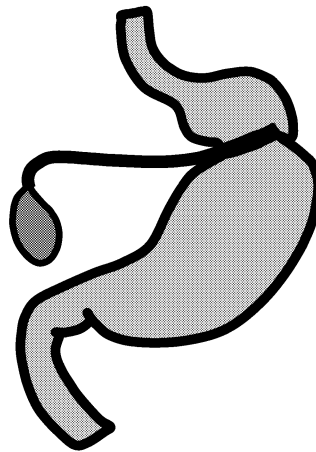


Figure 5. Gastric banding. On the left, the container enables the band to be tightened. Kindly provided by prof. M. Savolainen.

Six healthy normal weight subjects took part in Study **III** as a control group. Normal weight subjects were healthy volunteers, who went through the same laboratory examinations as the obese subjects, except the OGTT.

Study I: Altogether 22 massively obese subjects, 7 men and 15 women participated in the study. Based on OGTT, all the subjects were normoglycemic. The weight of the subjects was stable from the time of OGTT to the time of the gastric banding operation.

Study II: Seventeen massively obese non-diabetic subjects (8 men and 9 women), whose weight change was at least 10 % (mean change – 19 %), were included in the study.

Study III: Twenty-eight massively obese subjects (10 men and 18 women) participated in the study. Twenty subjects (6 men and 14 women) had normal fasting glucose concentrations while 8 had type 2 diabetes. Among the 28 obese subjects, 20 (8 men and 12 women), of whom 4 men and 10 women, were normoglycemic, participated in the one year follow-up study.

Study IV: Thirty massively obese subjects (10 men and 20 women) participated in the study. Twenty-three subjects (6 men and 16 women) had normal fasting plasma glucose concentrations while 8 (4 men and 4 women) had type 2 diabetes.

Table 4. Basic characteristics of the subjects before and after one year of weight reduction. Mean±SD.

	MEN		WOMEN	
	Before (n=12)	After (n=11)	Before (n=21)	After (n=14)
Age (yrs)	48±8		41±9	
Weight (kg)	158.5±37.0	123.9±22.0 ^c	139.2±23.4	118.2±19.1 ^c
BMI (kg/m ²)	50.0±9.6	39.9±6.2 ^c	50.9±7.8	43.5±6.0 ^c
Percent of fat	36.8±5.7	34.5±8.5 ^d	44.1±3.2 ^a	41.8±3.0 ^{b,d}
Fasting serum free fatty acids (mmol/l)	0.73±0.34	0.66±0.27	0.79±0.28	0.72±0.27
Fasting serum glycerol (mmol/l)	0.21±0.11	0.19±0.06	0.23±0.07	0.24±0.09
Fasting serum glucose (mmol/l) ¹	6.7±1.4	6.2±2.0	6.1±1.4	5.2±1.0
Fasting plasma insulin (mU/l) ¹	32±18	21±18 ^d	33±29	17±9 ^d
Fasting serum leptin (ng/ml)	34.8±19.9	16.2±9.6 ^c	58.3±18.1 ^a	39.9±11.3 ^{a,c}
Subcutaneous fat cell size (pl)	478±61	358±68 ^c	489±101	462±94 ^a
Omental fat cell size (pl)	496±73	ND	487±83	ND

^a p < 0.01 between genders, ^b p < 0.05 between genders, ^c p < 0.01 within the gender, ^d p < 0.05 within the gender

4.2. Ethical considerations

All the subjects received both oral and written information about the study. After having received the information, subjects signed a written consent to participate in the study. The ethics committee of the University of Kuopio and Kuopio University Hospital approved the study.

4.3. Study Design

The subjects visited the out patient clinic of the Kuopio University Hospital before the gastric banding operation (84 - 168 days before) and one-year after it. At both visits, body composition was determined by a bioelectrical impedance method (Bioelectrical Impedance, Body comp II, Version 1.5, RJL Systems Inc., Detroit, Michigan) in the fasting condition. Two microdialysis studies were carried out one week before and one-year after the operation at the Department of Clinical Nutrition, University of Kuopio. No drug treatment was allowed for at least 24 hours before the microdialysis study. Adipose tissue samples for the preparation of isolated adipocytes and for the determination of mRNA levels were taken during the gastric banding operation from subcutaneous abdominal and femoral and from omental adipose tissue region under general anesthesia. One year after surgery, subcutaneous abdominal and femoral adipose tissue biopsies were taken under local anaesthesia (Lidocain® 10 mg/ml) at the out-patient clinic. Local or general anaesthesia has been found not to affect adipose tissue metabolism in isolated adipocytes (Large et al. 1997). The samples for isolated adipocyte preparation were immediately placed in 0.9 % saline and the isolation procedure was started within the following 30 minutes, followed immediately by lipolysis studies. Adipose tissue samples for the mRNA expression studies were immediately frozen in liquid nitrogen and stored at -70° C for later analyses.

4.4. Methods

4.4.1. Anthropometric measurements

Height, weight and body composition were determined from each of the subjects. Body composition was not determined from one of the subjects due to technical difficulties. All measurements were done in the morning after a 12-h fast with standardized methods. Standing height and weight were measured in light clothing without shoes. Weight was measured to the nearest 0.1 kg with a digital scale (Seca delta, model 707, Dayton, Espoo, Finland). Height was measured to the nearest 0.5 cm. BMI was calculated [BMI = weight (kg) / height (m)²]. Body composition was determined by the bioelectrical impedance method (RJL systems, Detroit, MI, USA).

4.4.2. Microdialysis

Adipose tissue metabolism *in vivo* was studied after an overnight fast at rest using a microdialysis technique (Arner et al.1990, Arner 1995, Lafontan and Arner 1996). A dialysis membrane (cut-off point 20 000 mol wt, 13 mm x 0.5 mm) was glued to the end of a double Teflon tube (inlet tubing, cut off point 150 μm , outlet tubing, cut off point 200 μm) that passed through a steel needle. The perfusion solvent entered the probe through the inlet Teflon tube, and streamed upward in the space between the Teflon tube and the outer dialysis membrane. The dialysate then streamed into the probe and left it through the outlet Teflon tube via the sidearm from which it was collected.

Each microdialysis study lasted for three and a half hours. Three microdialysis probes were inserted without local anaesthesia percutaneously into abdominal subcutaneous adipose tissue left from the umbilicus with the help of a plastic canula (Venflon®2). The distance between the probes was 3 - 5 cm. The probes were sterilised before the microdialysis study by immersing them in 70 % ethanol solution. The inlet tubing of the probe was connected to a microdialysis pump (CMA 100, Carnegie Medicine, Sweden) and the sterile solutions of the agents diluted into Ringer's solution were continuously perfused into the tissue (1.5 $\mu\text{l}/\text{min}$). Ethanol (250 μl of 96 % ethanol) was added into 100 ml Ringer's solution for the estimation of local blood flow with the ethanol escape method (Hickner et al. 1991, Lafontan and Arner 1996,). Ethanol concentrations were analysed from the perfusate and dialysate. The changes in the concentration of the ethanol dialysate / perfusate –ratio describe the changes in the local blood flow (Arner 1995). The dialysate was collected as twenty-minute fractions.

During the first hour of the microdialysis Ringer's solution was perfused into the adipose tissue to monitor the basal state using the three probes. The basal state was determined from the dialysate samples collected during the last 40 minutes of the first hour in every probe separately. For studying the stimulation of lipolysis, 10 and 30 $\mu\text{mol}/\text{L}$ isoprenaline (Isoprenaline®) was infused. Inhibition of lipolysis was studied using 1, 5, and 10 $\mu\text{mol}/\text{l}$ of adenosine (Adenocor®), and 10 $\mu\text{mol}/\text{L}$ of α_2 -adrenergic agonist, clonidine (Catapresan®). Between each solution and different concentration of the agent, Ringer's solution was perfused for twenty minutes into tissue for wash out. Glycerol concentrations were analysed from the dialysate to estimate lipolysis. The level of stimulation was expressed as the ratio by dividing the isoprenaline simulated glycerol concentration in dialysate by basal glycerol concentration (measured during the last 40 minutes of the first hour of the study).

4.4.3. Lipolysis in isolated adipocytes

The adipose tissue samples from subcutaneous abdominal region were immediately placed in 0.9 % saline solution. Adipocytes were isolated by the modification of Ohisalo et al. (1992) of the method of Rodbell (1964) in the presence of collagenase (0.5 mg/ml) under constant shaking at 2 Hz at 37 ° C in a buffer containing 125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L CaCl₂, 2.5 mmol/L MgCl₂, 1 mmol/L KH₂PO₄, 4 mmol/L glucose, 2 % bovine serum albumin, and 25 mmol/L Tris at pH 7.4. After 60 minutes, cells were filtered through a nylon cloth and washed three times with the same buffer without collagenase. Cell diameter was estimated by direct microscopy of isolated cells. The study of lipolysis was performed with isolated, intact adipocytes.

For the studies of lipolysis, isolated adipocytes were diluted at 1:6 –ratio into the same buffer without collagenase. An aliquot (250 µl) of this cell suspension, containing 40 µl isolated adipocytes, was incubated for 50 minutes at 37° C under constant shaking (80 strokes per minute). Adenosine deaminase (1 µg/ml) was added into the incubation medium to remove endogenous adenosine. Basal and maximally stimulated (1 mmol/l forskolin) lipolysis was determined. Different concentrations of the effectors were added (Ohisalo et al. 1992): 10 nmol/l, 10² nmol/l, 10³ nmol/l and 10⁴ nmol/l concentrations of isoprenaline and adrenaline to study stimulation, and α₂-adrenergic agonist UK-14304 and N⁶-phenyl-isopropyladenosine to study inhibition of glycerol release from isolated adipocytes. Boiling the samples for two minutes terminated the incubations. Glycerol release values are given as pmol/µl of cells/min and for as µmol/10⁷ cells/50 min. The relative stimulation values with each concentration were also calculated by dividing stimulated glycerol release by basal glycerol release. In Study II, effective agonist concentrations causing 50 % of maximal effect (EC50) values (Östman et al. 1984, Mauriège et al. 1999) were calculated separately for the group of women and for the group of men. Individual values could not be calculated due to large individual variation in responses to different agents, and due to the low number of the concentrations used in the lipolysis study.

4.4.4. Preparation of total RNA and quantitation of target mRNAs

For total RNA preparation, adipose tissue samples were pulverized in liquid nitrogen. Total RNA from the frozen powder was prepared using RNeasy total RNA kit (Qiagen). The amount of total RNA was quantified spectrophotometrically at 260 nm. The ratio of absorption (260/280 nm) of all preparations was between 1.8 and 2.0. Total RNA was suspended into water and stored at -80° C.

The levels of mRNAs of interest (Auboeuf and Vidal 1997) were quantified by the reverse transcription reaction followed by competitive polymerase chain reaction (RT-cPCR). A detailed description of the method has been published previously (Auboeuf and Vidal 1997). The construction of the competitor molecule, the validation of the assay, and the sequences of the primers have been reported (Laville et al. 1996). To improve the analysis of the competitive PCR products, the sense primers were 5'-end labeled with the CY-5 fluorescent probe.

For each mRNA assay, a specific first-strand cDNA synthesis was synthesized from 0.1 µg of total RNA with 2.5 U of thermostable reverse transcriptase (*Tth* DNA polymerase; Promega Corp., Charbonnier, France) and with a specific antisense primer (Table 5) in a final volume of 20 µl. The medium was overlaid with mineral oil and incubated in the thermocycler (Minicycler PTC150, MJ Research, Waterton, MA) for 3 min at 60° C, followed by 15 min at 70° C and 5 min at 99° C as previously recommended (Auboeuf and Vidal 1997). After chilling in ice, 4 µl of water was added to the RT medium from which 20 µl were sampled for cDNA quantification by competitive PCR. The 20 µl of RT medium were added to a 100 µl PCR mix (final volume) containing 45 pmol of the corresponding sense primer and 30 pmol of the antisense primer. Four aliquots (20 µl) were then transferred to four microtubes containing 5 µl of a defined working solution of the competitor cDNA. Four concentrations of competitor ranged from lower to higher concentration than that of the estimated target concentration. The medium was overlaid with mineral oil and PCR amplification was processed for 40 cycles in conditions that have been described in detail previously (Auboeuf and Vidal 1997, Laville et al. 1996). The PCR products were analyzed in denaturing 4 % acrylamide-gel (Ready-mix, Pharmacia, Uppsala, Sweden). The electrophoresis was run with an ALF Express DNA sequencer (Pharmacia). The fluorescence of the target and competitor cDNA peaks were evaluated using the Fragment Manager software (Pharmacia). The logarithm of the competitor on the target amount ratio was plotted versus the logarithm of the initial amount of the competitor added in the PCR. The target mRNA concentration was calculated at the competition equivalence point (Auboeuf and Vidal 1997).

Table 5. Sequences of sense and antisense primers used in RT-cPCR.

Gene	Primer sequence	Size of the product	
		cDNA (bp)	Competitor (bp)
HSL:			
Sense	5'-TCTTCTGCACCAGCCACAAC-3'	254	308
Antisense	5'-AGATGGTCTGCAGGAATGGS-3'		
LPL:			
Sense	5'-ACACAGCTGAGGACACTTGC-3'	229	269
Antisense	5'-CACTGGGTAATGCTCCTGAG-3'		
SREBP-1c:			
Sense	5'-GCGGAGCCATGGATTGCAC-3'	311	331
Antisense	5'-CTCTTCCTTGATACCAGGCC-3'		
PPARγ:			
Sense	5'-TCTCTCCGTAATGGAAGACC-3'	474	400
Antisense	5'-GCATTATGAGACATCCCCAC-3'		
PPARγ2:			
Sense	5'-CTGATACTGTCTGCAACA-3'	580	506
Antisense	5'-GCATTATGAGACATCCCCAC-3'		
p85 αPI 3-kinase:			
Sense	5'-TGACGCTTTCAAACGCTATC-3'	247	222
Antisense	5'-CAGAGAGTACTCTTGCATTC-3'		
UCP2:			
Sense	5'-GACCTATGACCTCATCAAGG-3'	290	235
Antisense	5'-ATAGGTGACGAACATCACCACG-3'		

4.4.5. Screening of the Pro12Ala polymorphism in the exon B of the PPAR γ gene

DNA was prepared from the peripheral blood leukocytes by the salting out method (Miller et al. 1988). Exon B of the PPAR γ gene was amplified by PCR with the forward primer 5'-GACAAAATATCAGTGTGAATTACAGC-3' and the reverse primer 5'-CCCAATAGCCGTATCTGGAAGG-3' (product size, 167 bp). PCR was performed in a 6 μ l volume containing 50 ng genomic DNA, 3 pmol of each primer, 10 mmol/L Tris-HCl (pH 8.8), 50 mmol/L KCl, 1.5 mmol/L MgCl, 0.1 % Triton X-100, 100 μ mol/L deoxy (d)-NTP, 0.25 U DNA polymerase (Dynazyme DNA polymerase, Finnzymes, Espoo, Finland) and 0.55 μ Ci [32 P]dCTP. PCR conditions were denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s and annealing at 66°C for

1 min, with final extension at 72°C for 6 min. Variants were detected by single strand conformation polymorphism analysis. PCR products were first diluted 4- to 10-fold with 0.1 % SDS and 10 mmol/L ethylenediamine tetra-acetate and then mixed (1:1) with loading dye mix (95% formamide, 10 mmol/L ethylenediamine tetra-acetate, 0.05 % bromophenol blue and 0.05 % xylene cyanol). After denaturing at 98°C for 3 min, samples were immediately placed on ice. Two µl of each sample were loaded onto nondenaturing polyacrylamide gels (acrylamide/N,N'-methylene-bis-acrylamide ratio, 49:1; 6%) containing 10 % glycerol. Samples were run at temperatures that were shown to discriminate between the variants in the previously sequenced samples of exon B (37-38C) most accurately. The gel was dried and autoradiographed overnight at -70°C with intensifying screens.

4.4.6. Biochemical measurements

Fasting blood samples for the analyses of serum FFA, glycerol, glucose, leptin and plasma insulin concentrations were obtained before the microdialysis study. Serum glycerol and glucose were determined also after each microdialysis. Blood samples for measurement of fasting, 60 minutes and 120 minutes plasma glucose and insulin concentration were taken during the OGTT. Serum glucose was analyzed by a glucose dehydrogenase method (CV% 2.5) (Merck, cat no. 12193). The plasma glucose concentration was analyzed using the glucose oxidase method (Daiichi Co., Kyoto, Japan). Serum FFA were measured by a turbidometric analyzer (CV% 1.5) (Kone Ltd, Espoo, Finland). Radioimmunoassays were used for the analysis of plasma insulin (CV% 6) (Phasedeph insulin RIA 100; Pharmacia Diagnostics, Uppsala, Sweden) as well as for the analysis of serum leptin (CV% 13) (Linco Research Inc., St.Louis, MO, USA). Serum and microdialysis perfusate concentrations of glycerol were analyzed by a modification of the enzyme assay method of Kather & Wieland (1984) and the NADH generated was measured fluorometrically (CV% 6). Ethanol was analyzed by an enzymatic method (CV% 4) (Boehringer Mannheim, cat.no 176 290) and conventional fluorometry.

4.4.7. Statistical analysis

Calculations were performed using the SPSS/WIN program version 8.0 (I, II), 9.0 (III) and version 10.0 (IV) (SPSS Inc., Chicago, III, USA).

Study I: Pearson analyses of correlation were used for the assessment of associations between the variables of interest. Partial correlation analyses were used to correct the correlations for cell size or cell size and fat mass.

Study II: Wilcoxon nonparametric test was used for studying differences before and after weight reduction within gender group and Mann-Whitney test for studying difference between the genders. When analysing the data of adipose tissue metabolism *in vivo* or *in vitro*, a general linear model for repeated measures, corrected with cell size, was used for studying the differences between genders. Friedman nonparametric analysis of variance test was used for testing the difference in responses before and after weight reduction within the gender. Spearman nonparametric correlation was used to study the connection between the variables of interest. When appropriate, partial correlation was used for correcting analysis with cell size and/or fat mass.

Study III: Wilcoxon nonparametric paired t-test was used for studying differences among different adipose tissue depots at baseline as well as the differences within the adipose tissue depot before and after weight reduction. Mann-Whitney's nonparametric t-test was used for studying the difference in mRNA level between the genders. The difference in the change of mRNA levels along with weight loss between genders was studied using general linear model for repeated measures. Fat mass (kg) was used as covariate when necessary. Spearman's nonparametric correlation analysis was used for studying the associations. When corrected with fat mass (kg) or percent of body fat, the partial correlation analysis was used. The correction of Bonferroni was used because of multiple correlation analysis.

Study IV: Nonparametric Mann-Whitney test was used for studying the difference in mRNA level between groups. Analyses according to genotype groups were carried out by nonparametric Wilcoxon paired sample t-test. When analyzing solely gender differences, independent sample T-test was used. The correction of Bonferroni was used to take into account the effect of multiple comparisons by multiplying each test value by three according to the number of adipose tissue sites, i.e. omental, subcutaneous abdominal and femoral region.

The difference in the changes of mRNA levels accompanying weight loss between the groups of polymorphism within the gender was studied using a general linear model for repeated measures. The general linear model for univariate analysis was used when the analysis was corrected with fat mass. When PPAR γ 1 and PPAR γ 2 expression was compared among men, women and normal weight subjects Scheffe's post-hoc test, available in General Linear Model, was used for taking into account the multiple comparisons.

5. RESULTS

5.1. Complementary results from *in vivo* and *in vitro* studies (Study I)

The overall responses to stimulation and inhibition of lipolysis were modest in the subcutaneous abdominal adipocytes before weight reduction (Figure 6). The response to stimulation was clearer in the microdialysis study compared to the basal state (**Study I**: Figure 1, Figure 2). However, there was no response to inhibition in the microdialysis study before weight reduction. When studying the stimulation of lipolysis, the results from the *in vivo* and *in vitro* correlated positively with each other (**Study I**: Table 2).

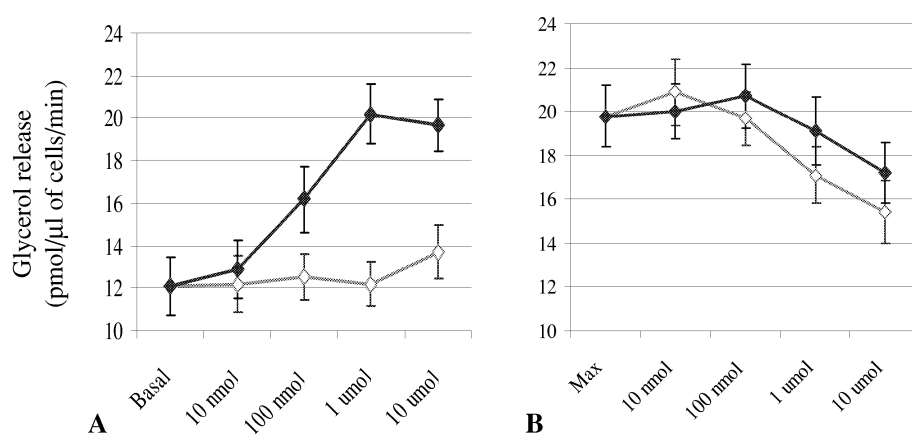


Figure 6. Glycerol release stimulated by adrenaline (grey line) or isoprenaline (black line) (A) and inhibited by UK-14304 (grey line) or N6-phenylisopropyladenosine (black line) (B) in subcutaneous abdominal adipocytes isolated from men and women before weight reduction. Mean ± SEM.

5.2. Changes in lipolysis after one year of weight reduction (Study II)

Mean weight reduction for men was $-20 \pm 3\%$ and for women $-18 \pm 2\%$ (**Study II**: Table 1). The reductions in fat mass in men and women were $-28 \pm 8\%$ and $-22 \pm 4\%$, respectively. The reduction of fat mass resulted also in a decrease in the cell size ($-21 \pm 5\%$ in men, $-15 \pm 5\%$ in women).

Women had a larger cell size than men both before and after weight reduction. Significant decreases in fasting serum glucose, insulin, and leptin concentrations were seen in both genders along with weight loss (**Study II**: Table 2).

With respect to glycerol release, the responses in subcutaneous adipocytes isolated from men and women were somewhat different. Surprisingly, there was no change in women in either basal or maximally stimulated glycerol release along with weight loss. This was also true for both stimulated and inhibited glycerol release in women.

In men, however, both basal and maximally stimulated glycerol release decreased significantly along with weight loss (**Study II**: Table 3). Although stimulated glycerol release remained similar both before and after weight reduction, the inhibited glycerol release in men showed clearly improved responses to α_2 -agonist and adenosine analog after weight reduction compared to those before weight reduction (**Study II**: Figure 2). A parallel change in response to inhibition in men was also seen *in vivo* in the microdialysis study.

The better response to inhibition of glycerol release by the α_2 -agonist was shown in adipocytes isolated from women than in those isolated from men before weight reduction (Figure 7). It is important to note that the difference was not seen after weight reduction. The adenosine analog induced inhibition in glycerol release did differ between genders after weight reduction, men showing a better response to inhibition. A difference was also seen in the response to inhibition by the α_2 -agonist in the microdialysis study between the genders, with men showing a stronger response (**Study II**: Figure 3).

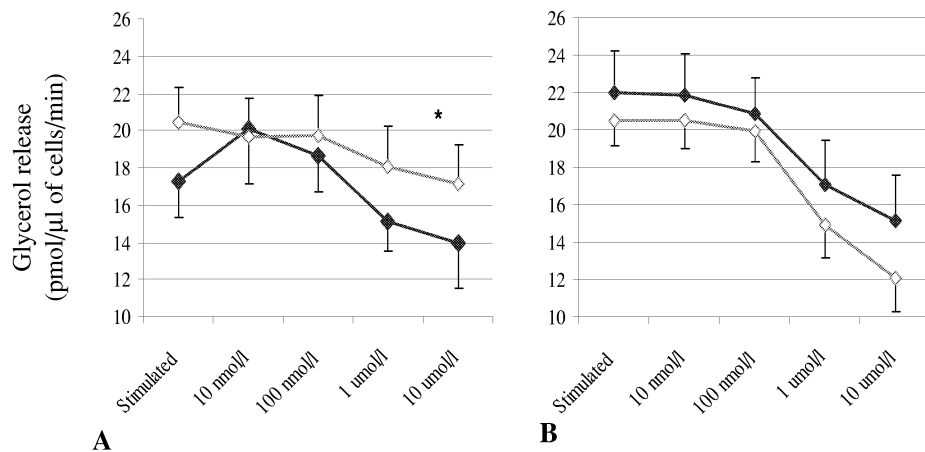


Figure 7. Glycerol release inhibited by the α_2 -agonist UK-14304 in subcutaneous abdominal adipocytes isolated from men (grey) or women (black) before (A) and after (B) weight reduction. Lipolysis was stimulated by 1 mmol/l forskolin. Mean \pm SEM.

5.3. Effect of weight reduction on rate-limiting enzymes for fat deposition and lipolysis in adipose tissue (Study II)

Hormone sensitive lipase (HSL) and lipoprotein lipase (LPL) mRNA expression responded quite differently in men and women in conjunction with the weight reduction (Study II: Figure 1). At baseline, women had a higher expression of HSL mRNA than men (Figure 8). HSL expression decreased, however, significantly in women along with weight loss, whereas in men no change was observed.

LPL mRNA expression was similar in men and women before weight reduction. The nonsignificant increase in LPL level in women and the slight decrease in men resulted in a gender difference in the expression after one year of weight loss (Figure 8). However, this difference was of borderline statistical significance ($p = 0.054$)

The different responses of expression to reduction of fat mass in genders resulted in a dramatic increase in the LPL/HSL ratio in women, whereas in men no change in the ratio was seen. In women, the HSL mRNA level was positively associated with basal and maximally stimulated glycerol release before weight reduction. In men, the HSL mRNA concentration was associated with body fat and cell size.

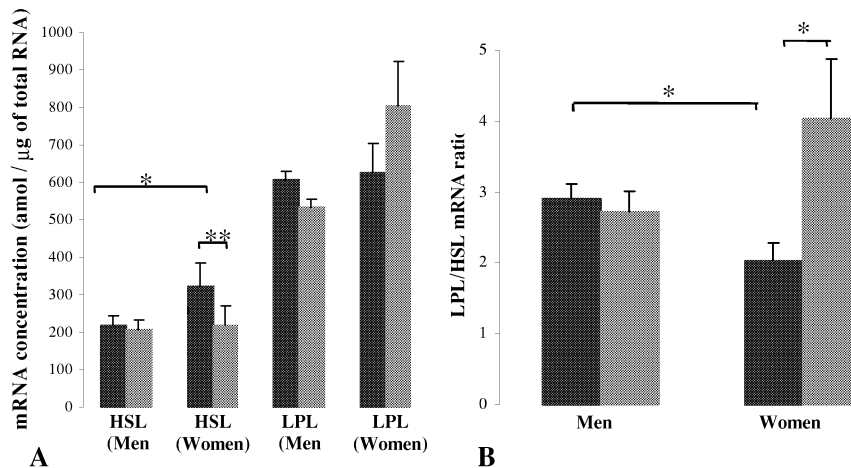


Figure 8. Mean HSL and LPL mRNA expression and LPL/HSL ratio in men and women before (black) and after (grey) weight reduction. ** $p < 0.01$, * $p < 0.05$.

5.4. Effect of massive obesity and weight reduction on Sterol Regulatory Binding Protein 1c (SREBP-1c) mRNA expression (Study III)

The obese nondiabetic and diabetic subjects did not differ from each other in anthropometric or biochemical data (**Study III**: Table 1). Diabetic subjects had higher fasting serum glucose concentrations both before and after weight reduction (**Study III**: Table 1). Normal weight control subjects, participating in this study, had normal values for anthropometry and biochemistry.

SREBP-1c mRNA expression was similar in adipose tissue samples isolated from nondiabetic and diabetic subjects. The depot-specific data combining nondiabetic and diabetic subjects are shown in Table 6. SREBP-1c mRNA expression was markedly lower in omental adipose tissue than in either subcutaneous abdominal or femoral depots (Table 6). Furthermore, men and women had similar mRNA expression of SREBP-1c. When the baseline mRNA concentrations were compared to those of the normal weight subjects, the values in subcutaneous depots from the obese subjects were significantly lower. After one year of weight loss, the SREBP-1c mRNA concentration was increased markedly in both nondiabetic and diabetic men and women (**Study II**: Figure 1). This increase was so marked that the difference to normal weight subjects was no longer significant. This was also true when diabetic and nondiabetic subjects were compared separately to normal weight subjects.

Table 6. SREBP-1c mRNA concentrations (amol/ μ g of total RNA) in obese subjects before and after weight reduction. Mean \pm SEM.

	MEN		WOMEN	
	Before	After	Before	After
N	10	8	18	12
Abdominal SC depot	3.2 \pm 0.3 ^a	5.1 \pm 0.5 ^c	3.7 \pm 0.5 ^b	5.4 \pm 0.8 ^d
Omental depot	1.8 \pm 0.4	ND	1.8 \pm 0.2	ND
Femoral SC depot	3.9 \pm 0.4 ^a	5.1 \pm 0.5 ^c	3.7 \pm 0.4 ^b	4.9 \pm 1.0

^a p < 0.05 difference from SREBP-1c mRNA concentration in omental adipose tissue

^b p < 0.01 difference from SREBP-1c mRNA concentration in omental adipose tissue

^c p < 0.05 difference from the level before weight reduction

^d p < 0.01 difference from the level before weight reduction

SC = subcutaneous

5.5. Effect of massive obesity and weight reduction on PPAR γ 1 and PPAR γ 2 mRNA expression

Reductions in weight, fat mass, fasting serum glucose, and plasma insulin and leptin were significant within the study groups along with weight loss (Table 4). There were no significant differences in the body weight changes between diabetic and nondiabetic subjects.

The expression levels of PPAR γ 1 and PPAR γ 2 mRNAs were lower in the omental adipose tissue compared to either of the subcutaneous depots before weight reduction ($p = 0.001$ across the depots and for γ 1 and γ 2, **Study IV**: Table 2). Both subcutaneous depots, however, showed a similar expression level. This depot related difference was due to obese women who were characterized by their higher expression levels of PPAR γ 1 and PPAR γ 2 in subcutaneous adipose tissue depots than in omental adipose tissue (Figure 9, **Study IV**: Table 3). In men, no regional difference was found and the values were similar to those found in the omental depot in women. This resulted in a gender difference in PPAR γ 2 mRNA concentration in femoral and abdominal subcutaneous adipose tissue (Table 7, Figure 9), women having higher expression.

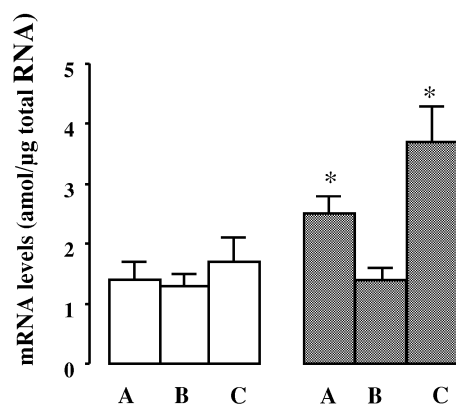


Figure 9. Mean PPAR γ 2 expression in men (white) and women (grey) before weight reduction in three different adipose tissue depots (A= abdominal subcutaneous, B=omental, C=femoral subcutaneous).

* $p < 0.05$ between men and women.

When the mRNA concentration in obese subjects was compared to normal weight subjects, PPAR γ 1 concentration was higher in abdominal subcutaneous depot in normal weight than in obese subjects (Table 7).

Table 7. PPAR γ 1 and PPAR γ 2 mRNA concentrations (amol/ug of total RNA) in obese men and women and normal weight subjects. Mean \pm SEM.

	Men	Women	Normal weight subjects
N	10	20	6
PPARγ1:			
Abdominal SC depot	13.2 \pm 1.3	14.7 \pm 0.9	20.8 \pm 2.3 ^b
Femoral SC depot	13.7 \pm 1.5	13.6 \pm 1.1	18.0 \pm 1.9
PPARγ2:			
Abdominal SC depot	1.45 \pm 0.31	2.54 \pm 0.33 ^a	1.92 \pm 0.14
Femoral SC depot	1.71 \pm 0.38	3.65 \pm 0.67 ^a	1.58 \pm 0.19

^a p < 0.05 difference from men

^b p < 0.01 difference from obese subjects, after correction for multiple comparisons by Scheffe p= 0.006 - 0.016

SC = subcutaneous

PPAR γ 1 expression level did not change in conjunction with weight reduction, whereas PPAR γ 2 mRNA expression decreased significantly in femoral adipose tissue (p=0.018).

5.6. Effect of the Pro12Ala polymorphism on the PPAR γ 2 gene mRNA expression before (Study IV) and after one year weight loss

We did not find any major effects of the Pro12Ala polymorphism on the γ 2 expression in the whole study group (Study IV: Table 2). Borderline difference (p = 0.083 for subcutaneous abdominal depot) was found in PPAR γ 2 mRNA expression between the groups of polymorphism in subcutaneous abdominal adipose tissue that suggested the subjects with Pro12Ala genotype have a higher expression. The effect of the polymorphism was also analyzed separately for men and women, because of the differences between men and women. These analyses revealed that the trend was due to the significantly higher mRNA level in men with the Ala-allele (p=0.011) (Study IV:

Table 3). Thus, the effect of polymorphism in exon B in PPAR γ 2 on the expression of the gene itself was seen only in men.

No marked changes were seen in the expression of PPAR γ 2 within either group in conjunction with the weight loss. The expression of PPAR γ 2 was similar between the groups of polymorphism after the weight loss.

5.7 Effect of the Pro12Ala polymorphism on the PPAR γ 2 target genes (Study IV)

The LPL, phosphoinositol 3-kinase (p85 α PI 3-kinase), uncoupling protein 2 (UCP2) and PPAR γ 1 mRNA expression levels were studied as target genes. p85 α PI 3-kinase mRNA expression was lower in omental adipose tissue in the Pro12Ala group compared to the Pro12Pro group (**Study IV**: Table 2). There were no other differences in the target gene expressions between the groups. There was no marked variation in the mRNA levels of PPAR γ 1, LPL, p85 α PI 3-kinase or UCP2 in parallel with the weight reduction. Moreover, no differences between the genotype groups were observed after the weight loss.

5.8 Connection between PPAR γ and SREBP-1c, two adipogenic and lipogenic transcription factors

Interestingly, SREBP-1c and PPAR γ correlated strongly in all adipose tissue depots before weight reduction. The linear correlation remained significant in femoral depot also after one year of weight reduction (Figure 10).

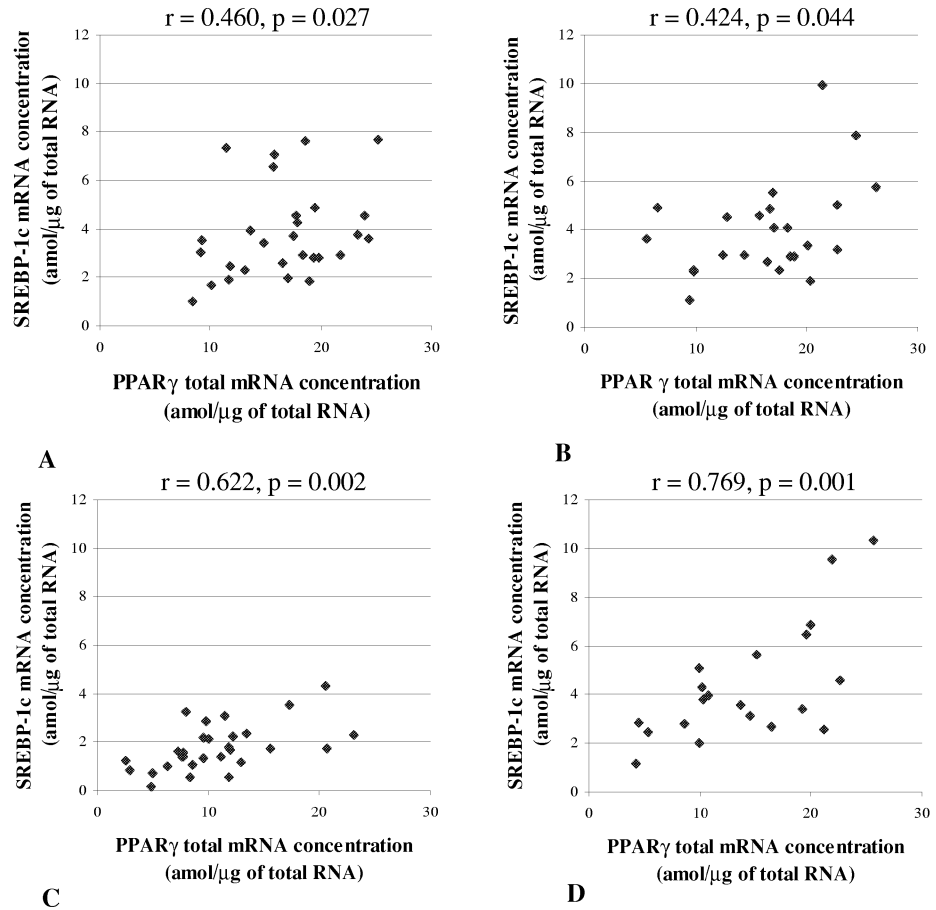


Figure 10. Association between PPAR γ total and SREBP-1c mRNA in subcutaneous abdominal (A), femoral (B), omental (C) before weight reduction and subcutaneous femoral (D) adipose tissue after weight reduction with fat mass corrected correlation coefficient and significance.

6. DISCUSSION

6.1. Methodological considerations

Subjects

Patients with severe obesity, who were selected to undertake the surgical operation for weight reduction, were examined in the present study. The subjects were well characterized for the purpose of the study, and were recruited in the order that they were on the waiting list for the operation.

Only two of the subjects were using a cardioselective β -blocking medication (atenolol). Type 2 diabetic subjects had also oral medication for diabetes. At this state of the obesity, it may be difficult to recruit large populations without medication. In the present study, 11 subjects did not have any regular medication.

The heavy study protocol and small number of weight reducing operations made the recruitment of a larger patient group difficult. The study population is, however, fairly large, compared with previous studies (Coppack et al. 1992, Fried et al. 1993, Kaartinen et al. 1995, Hellström et al. 1996, Imbeault et al. 1999, Large et al. 1999, Mauriège et al. 1999). Moreover, the subjects participated in a large number of other examinations that also could limit the number of those who were willing to participate in the present study. The drop out rate in the one year follow-up examinations was 24 %. These subjects did not differ from the other subjects in the examinations done before the operation. The reasons for the drop out were problems with the adjustable gastric banding causing complications and operations for correction or removal of the banding or unwillingness to participate in the follow-up studies.

Anthropometric measures

Anthropometric measures were done for the assessment of the state of obesity. For the purpose of our study, bioelectrical impedance method gives satisfactory estimates of fat mass (Rissanen 2002). This method is also the most convenient for its quickness and ease of performance for the patients in whom the supine position might cause difficulties in breathing. It was difficult to make a more specific characterization of the type of obesity (i.e. distribution of adipose tissue) at this level of the obesity with the methods available. It was not possible to use computer tomography technique due to size limitations.

Study design

Studies were planned according to the schedule of the operation. The microdialysis study was conducted within a week before the operation or the biopsy at the outpatient

clinic. This was considered important for attaining as parallel information as possible from *in vivo* and *in vitro* studies. The samples for the *in vitro* and mRNA expression analyses were also taken at the same time.

Adipose tissue samples in the follow-up examinations after one year weight loss were taken at the out-patient clinic under local anaesthesia. Differences in sample taking might distort the metabolic parameters studied. One previous study (Large et al. 1997) found no difference in the lipolysis between samples taken under general or local anaesthesia.

One year of weight reduction was considered as valid for the analyses of the changes in the adipose tissue metabolism. This was based on the previous experience of the amount of weight reduction after gastric banding. All the operations were done using the laparoscopic technique. Our subjects were, however, still moderately to severely obese after one year of weight reduction. Moreover, subjects might have been in different state of obesity after weight reduction or in the phase of slow weight reduction or even gaining weight that might have an effect on the individual variation that was seen in the measurements. In previous studies (Stich et al. 1997, Hellström et al. 1996, Vidal-Puig et al. 1997, Imbeault et al. 1999, Mauriège et al. 1999, Ribot et al. 2001), follow-up periods have been, however, often much shorter than in the present study. One recent study (Löfgren et al. 2002) had a 2 – 3 year follow-up, but a large variation was still seen, as in other previous studies.

Methods used to study adipose tissue metabolism

Microdialysis study. The microdialysis technique was chosen to study adipose tissue metabolism *in vivo* because it has proven to be a relatively easy and reliable method for studying subcutaneous adipose tissue (Arner et al. 1990, Arner 1995, Lafontan and Arner 1996). This technique, however, depends on the measurement of blood flow, which affects considerably the glycerol content of the tissue *in situ* (Hagström-Toft 1991, Arner and Bülow 1993). Blood flow estimation was based on the ethanol clearance method using the ratio of in- and outgoing ethanol concentration. The ethanol clearance method is reliable, when some pharmacological stimulus is measured, as in the present study (Hickner et al. 1991).

Due to the incomplete recovery, microdialysis allows assessment of only a fraction of a concentration of the substances in extracellular space (Lafontan and Arner 1996). In kinetic studies, like the present study, where the main aim was to follow changes in the concentration of tissue molecules over a short period of time, it is often not necessary to consider recovery problems (Arner 1999). Instead, the change in relation to baseline

level is the major focus of the kinetic studies (Arner and Bülow 1993). In the present study, the aim of the best possible recovery was taken into account by the use of a long length microdialysis membrane and a low perfusion rate, which are known to improve the recovery (Arner 1999). This caused, however, limited amount of the dialysate to be available for the glycerol and ethanol measurements, and measurements of glucose had to be excluded.

Studies on isolated adipocytes. For the *in vitro* studies, the classical isolation method (Rodbell 1964) and lipolysis studies in isolated adipocytes were used (Ohisalo et al. 1992). The advantage of *in vitro* studies is that the cell environment can be carefully controlled. This can, however, be also a disadvantage, since the effects of local tissue factors are not included. Thus, we decided to combine the analyses with isolated adipocytes with the microdialysis study *in situ*.

The isolation procedure as such may alter the metabolism, especially since it is necessary to use collagenase for removing the connective tissue (Rodbell 1964). This and mechanical shaking may break large adipocytes that are more fragile than the other cells (Arner 1995) and that, in turn, may cause selectivity among the cells.

Another important aspect to be considered is the artificial leaking of adenosine from isolated adipocytes (Ohisalo et al. 1992, Arner 1995). In the present study, adenosine deaminase was added into the incubation medium, to prevent the inhibitory effect of adenosine (Schwabe et al. 1973). Thus, the basal lipolysis was analysed in adipocytes that were not treated with any agent other than adenosine deaminase. Some studies have, however, been done without this addition (Reynisdottir et al. 1994, Mauriège et al. 1999). In this case, the removal of adenosine has sometimes been considered to be a stimulation of lipolysis. However, this measure may be variable depending on the amount of broken adipocytes and thus, the leaking adenosine in the incubation medium. Thus, the removal of adenosine was used to standardise the conditions.

mRNA expression studies. Expression was studied with RT-cPCR with a well validated method (Aubeouf and Vidal 1997). Since the synthesis of cDNA in the RT reaction is known to be sensitive to variation, synthetic DNA competitor molecules were added only into the PCR reaction. Thus, cDNA and competitor were amplified in similar conditions. All the assays for the genes analysed were validated by using known amounts of synthetic RNA in the RT reaction and then as a cDNA in the PCR reaction.

RT-cPCR is a widely used method for expression analyses. However, it may produce large individual variation. The reproducibility of the RT-cPCR is based on the

RNA isolation and estimation of the concentration of the RNA preparation (Gilliland et al 1990). The isolation procedure is difficult with the adipose tissue due to high amount of fat and low amount of RNA. Thus, the yield of good quality RNA is mostly dependent on the quality of the sample, but also on the standardized method of isolation. Since RNA is easily degraded, problems in isolation may produce poor quality of RNA and hamper the assay as such. Degradation of RNA may also affect the present results. Other factors with potential effects on the individual variation in expression levels, are the stability of weight of subjects at the time of sample taking, careful primer design and possibility for contamination (Bustin 2000).

6.2. General discussion

6.2.1. Lipolysis in severely obese subjects *in vivo* and *in vitro*

Concordance of the methods

Stimulation of the lipolysis using unselective β -AR agonists isoprenaline or adrenaline *in vitro* and isoprenaline *in vivo* gave concordant results in the present study. *In vitro* methods are widely used in studies of adipose tissue metabolism, but microdialysis and other *in vivo* methods are used more and more routinely nowadays. These methods have often given discrepant results and thus, caution should be taken when extrapolating *in vitro* results to the *in vivo* circumstances. However, only a few studies have examined the actual comparability of the methods when adipose tissue metabolism is investigated (Lillioja et al. 1986, Leibel and Edens 1990). These studies resulted in discrepant results between the methods. Comparison of FFA turnover and lipid oxidation rates *in vivo* with *in vitro* lipolytic rates of isolated abdominal fat cells showed increasing lipolysis *in vitro*, but decreasing whole body lipolytic activity *in vivo* with increasing degree of obesity (Lillioja et al. 1986). In another study (Leibel and Edens 1990), weight loss and changes in nutritional status had no effect on the re-esterification rate *in vitro*, although a clear increase in lipolytic activity was seen *in vivo*. In these studies, the whole body lipolytic activity and the effect of weight loss on the re-esterification rate were, however, measured by FFA turnover and lipid oxidation (Lillioja et al. 1986, Leibel and Edens 1990) and not by microdialysis. Thus, those results are not clearly comparable with our study, although in both earlier studies isolated adipocytes were used for *in vitro* results. In the present study, instead, methods focused on adipose tissue metabolism were applied and both measures were analysed in the same patients in highly comparable situations, and this might well have accounted for the concordant results we obtained. Other recent studies (Galitzky et al. 2001, Gesta

et al. 2001, Sengenès et al. 2002) also show concordant results with microdialysis and *in vitro* isolated adipocytes when done for the same subjects, although these studies are not specially designed for comparing the methods. Thus, it seems, that when the same subjects are studied and the same adipose tissue depot is assayed *in vivo* and *in vitro*, both methods give concordant results.

Effect of the weight reduction on lipolysis

Marked gender differences in the effect of the weight reduction on the lipolysis were found in the present study. Obesity may attenuate the responses in adipose tissue due to desensitization (Kaartinen et al. 1991, Arner 1999), or enhanced basal lipolysis. In the present study, the level of basal lipolysis decreased in men together with improved responsiveness and sensitivity to inhibition both *in vivo* and *in vitro* along with weight reduction. This indicates the reversibility of obesity-induced changes in adipose tissue metabolism in men. In women, however, no changes in basal or maximal lipolysis or in other measures of lipolysis were observed after a long-term weight loss, in spite of the decrease in cell size. It may be that a more pronounced weight loss would be needed to observe a similar change in women. Surprisingly, no changes were seen in β -AR responses in either gender.

Contrasting findings have been reported with respect to basal and maximal lipolysis in conjunction with weight loss (Reynisdóttir et al. 1995, Hellström et al. 1996, Stich et al. 1997, Löfgren et al. 2002). This also holds true for catecholamine induced lipolysis. Unchanged (Hellström et al. 1996, Mauriège et al. 1999), enhanced (Hellström et al. 1996, Sengenès et al. 2002) or reduced (Crampes et al. 1989) catecholamine induced lipolysis have been reported after weight loss. The effects of weight loss have been mostly studied after short-term weight loss (4 – 15 weeks), some of them shortly after very low calorie diet, and after moderate weight loss, up to 10 kilograms. The duration of these experiments may be limiting for conclusive results. Moreover, studying adipose tissue metabolism immediately after the very low calorie diet mirrors the highly active phase of fatty acid release from adipose tissue compared to state of established weight status. In the present study, the effects of long-term weight loss (one year) and marked change in weight (for men: -30 kg, and for women: -25 kg) were studied. The weight loss had, however, greatly slowed down already after the first six months in our subjects. So, it can be assumed that their weights were reasonably stable during follow-up examinations. However, the subjects were still severely obese after one year of

follow-up, and this might have prevented us from seeing any changes in β -AR sensitivity.

In a recent study (Löfgren et al. 2002), men had no marked changes in lipolytic measures, i.e. basal, maximal or catecholamine induced lipolysis after two years of bariatric surgery. However, women showed marked normalization of these measures when compared to lean controls. It should be noted that these women had more drastic changes in lipolysis, compared to men and especially to lean controls before weight reduction, that may partly interfere with the results (Löfgren et al. 2002). These kinds of differences between genders were not seen in our study before weight reduction. Unfortunately, we did not have lean controls in this particular study to clarify our results further. The study by Löfgren et al. (2002) and our study were the first to show marked gender differences in lipolysis due to obesity (Löfgren et al. 2002) and weight loss (Study II and Löfgren et al. 2002). The cause of the gender difference, however, remains to be studied further after long-term weight loss and in stabilized weight balance.

6.2.2. Gene expression in adipose tissue related to adipocyte differentiation, lipolysis and fat deposition

Hormone sensitive lipase and lipoprotein lipase

HSL expression is the major determinant of maximal lipolytic capacity in human adipocytes and is markedly affected by the cell size (Reynisdottir et al. 1997, Large et al. 1998, Berger and Barnard 1999). Controversial findings have, however, been reported on the effect of weight loss on HSL expression showing either increase (Stich et al. 1997), decrease (Reynisdottir et al. 1995) or no change (Hellström et al. 1996) in women. Löfgren et al. (2002) reported a slight decrease in HSL protein content (i.e. lipolytic capacity) in men, while the expression remained unchanged in women along with weight loss. These results are contradictory to our results. The different stages of HSL expression and activity were, however, measured i.e. mRNA level (Study II) and total HSL protein content (Löfgren et al. 2002). Both measurements are used and mirror the HSL enzyme activity, but only indirectly. It has to be kept in mind, that HSL activation in adipocyte depends on its phosphorylation state (Large et al. 1998) and translocation on the surface of the lipid droplet in the adipocyte (Holm et al. 2000, Kraemer and Shen 2002). Thus, we cannot conclude whether the differences would also be seen in HSL enzyme activity. Furthermore, when combining the results on HSL expression with lipolysis data, it is not possible to explain the results of lipolysis or its

gender differences by the changes in HSL expression either in our study or in the study by Löfgren et al. (2002).

In our study, along with the decrease in HSL expression, a slight increase in LPL expression was seen in women. Discordant findings have also been reported concerning LPL expression along with weight loss (Schwartz et al. 1981, Kern et al. 1990, Imbeault et al. 1999, Nicklas et al. 2000). It has been suggested that the increase in LPL activity and expression after weight loss might be explained by the improvement in insulin sensitivity which accompanied the weight reduction (Preiss-Landl et al. 2002). This may also explain the decrease in HSL mRNA expression in women in our study, since it is known that insulin can enhance the action of LPL whereas it inhibits HSL activity (Eckel 1989, Degerman et al. 1997).

Sterol regulatory element binding protein 1c

Low SREBP-1c expression in subcutaneous adipose tissue depots in massively obese subjects compared with normal weight controls in Study III has also been reported in obese rodents (Nadler et al. 2000, Soukas et al. 2000) as well as before in obese human subjects (Ducluzeau et al. 2001, Oberkofler et al. 2002), and recently also in omental adipose depot in obese compared to lean subjects (Oberkofler et al. 2002). It has been speculated (Nadler et al. 2000, Soukas et al. 2000) that this rather paradoxical reduction may reflect the down-regulation of pathways leading to lipogenesis in order to prevent further fat accumulation. SREBP-1c is, however, directly controlled by insulin (Kim et al. 1998, Flier and Hollenberg 1999, Azzouts-Marniche et al. 2000) also in humans (Ducluzeau et al. 2001). Thus, low expression in obesity might also be a consequence of insulin resistant state commonly seen in obese subjects (Kahn and Flier 2000), although no correlation between plasma insulin and SREBP-1c mRNA was found by us or others (Oberkofler et al. 2002).

After long-term substantial weight reduction, SREBP-1c expression increased in subcutaneous adipose tissue nearly to the levels of normal weight subjects in the present study. Oberkofler et al. (2002) reported a similar increase in subcutaneous, but also in omental adipose tissue following a nearly similar weight reduction. In another study (Ribot et al. 2001), weight loss was associated, however, with a decreased expression of SREBP-1c. The present study and the study by Oberkofler et al. (2002) support the hypothesis of the major role of insulin in controlling SRBEP-1c expression, since the expression increased along with the improvement of insulin sensitivity induced by weight loss. The discrepancy to the study by Ribot et al. (2001) could be explained by moderate weight loss and short-term weight reduction program used in that study. Other

factors may also contribute to the changes in SREBP-1c expression, such as changes in nutritional habits and calorie intake after bariatric surgery. In fact, it has been shown that SREBP-1c expression could be strongly affected by fasting and re-feeding in mouse white adipose tissue (Kim et al. 1998). Furthermore, as suggested by Oberkofler et al. (2002), leptin may suppress lipogenic pathways by reducing SREBP-1c expression in obesity, since leptin treatment of ob/ob and wild-type mice resulted in reduction of their SREBP-1c mRNA levels (Soukas et al. 2000). Thus, the reduction of leptin levels by weight loss may allow SREBP-1c expression to increase to its normal level.

Lower expression of SREBP-1c in omental than in subcutaneous adipose tissue was shown in Study III. This was, again, shown also by Oberkofler et al. (2002) in obese but not in lean subjects. Omental adipose tissue is lipolytically more active (Arner 1996), and insulin-mediated effects are weaker in omental than in other adipose tissue depots (Bolinder et al. 1983). Thus, it may be speculated that the weak response to insulin could contribute to low expression of SREBP-1c in omental adipose tissue in obesity.

Peroxisome proliferator activated receptor γ

According to previous studies, the effects of obesity and weight reduction on PPAR γ expression seem to be quite contradictory (Auboeuf et al. 1997, Vidal-Puig et al. 1997, Hotta et al. 1998, Lefebvre et al. 1998, Montague et al. 1998, Bastard et al. 1999, Rieusset et al. 1999, Ribot et al. 2001, Sewter et al. 2002). No difference in expression between obese and normal weight subjects (Auboeuf et al. 1997, Rieusset et al. 1999,) as well as higher (Vidal-Puig et al. 1997) and lower (Montague et al. 1998) expression levels in obese than in lean subjects have been reported. An association with obesity has been shown also in other primates (Hotta et al. 1998) and in some models of obesity in rodents (Vidal-Puig et al. 1996). The present study detected no difference in PPAR γ 2 expression between obese and lean subjects, whereas PPAR γ 1 expression was lower in obese subjects. In a recent study (Sewter et al. 2002), PPAR γ 1 mRNA expression was inversely correlated with BMI, such that the concentration was significantly lower in morbidly obese than in lean subjects. Instead, PPAR γ 2 mRNA expression was high in morbid obesity (Sewter et al. 2002). Thus, the finding of low PPAR γ 1 expression in the present study is in line with the results of Sewter et al. (2002). Although no statistical difference in PPAR γ 2 expression between lean and obese subjects was found, there was a tendency towards higher expression of PPAR γ 2 in obese women compared to lean controls in our study, showing results quite similar to those reported by Sewter et al. (2002). The reasons for the discrepancies between our results and some other studies are

unclear. Sewter et al. (2002) speculate that this could be attributable to the difference in the material used, i.e. isolated adipocytes vs. the whole adipose tissue. This does not seem, however, to explain the controversy in the case of our study, since we did use the whole adipose tissue similarly to most of the previous studies.

Furthermore, studies on depot specific differences in the expression of PPAR γ have yielded discordant findings (Lefebvre et al. 1998, Montague et al. 1998, Sewter et al. 2002) showing either no difference between omental and subcutaneous adipose tissue depots (Montague et al. 1998, Sewter et al. 2002,) or reduced expression of PPAR γ in omental depot in lean and slightly obese subjects (BMI <30 kg/m²) (Lefebvre et al. 1998). In obese subjects, no such difference was found (Lefebvre et al. 1998).

In Study IV, higher expression of PPAR γ 1 and PPAR γ 2 in the subcutaneous than in omental adipose tissue depot was found in obese women, but not in men, resulting in higher expression of PPAR γ in subcutaneous adipose tissue in women. This gender difference has been shown previously (Vidal-Puig et al. 1997), although not in all studies (Auboeuf et al. 1997, Rieusset et al. 1999, Sewter et al. 2002). The reason for the gender difference is not clear on the basis of the present study, but it is not due to difference in the fat mass between genders. This gender difference might be speculated to be one of the reasons why women tend to store fat mainly in subcutaneous adipose tissue in contrast to men. In other studies, the depot-related differences in the PPAR γ expression were not observed possibly because the genders have not been analysed separately (Lefebvre et al. 1998, Montague et al. 1998, Sewter et al. 2002). The observed gender difference is interesting, since a depot specific difference in the expression of other genes has been reported before, but mainly in women (Fried et al. 1987, Pedersen et al. 1994, Vidal 2001, Study II).

Weight reduction seems to reduce PPAR γ and PPAR γ 2 expression (Bastard et al. 1999, Ribot et al. 2001), although during the weight maintenance period, the expression might increase again to the former level (Vidal-Puig et al. 1997). Instead, we did not detect any effect of weight reduction on PPAR γ 1 mRNA expression, but PPAR γ 2 expression decreased in the femoral adipose tissue depot. The minor effect of weight reduction on the expression was probably because our subjects were still obese after one year of weight loss. Another factor that might explain why we failed to observe an effect due to weight loss, could be the large individual variation in the expression levels of PPAR γ , that has also been reported before (Auboeuf et al. 1997).

PPAR γ 1 and SREBP1c expression correlated positively before and in femoral adipose tissue also after weight loss in the present study. It has been shown that

SREBP1c induces PPAR γ transcription directly through the response elements in γ 1 promoter (Fajas et al. 1999) and indirectly by producing endogenous ligands for PPAR γ activation to enhance transactivation of PPAR γ 1 and γ 2 (Shimano et al. 1996). Moreover, PPAR γ seems also activate SREBP-1 expression (Kersten 2002). Thus, the interaction between these two transcription factors aims at adipogenesis, but also lipogenesis, as suggested before (Rosen et al. 2000, Kersten 2002). The positive correlation between SREBP1c and PPAR γ 1 in our study is in line with these studies, highlighting their involvement in adipose tissue metabolism and the connection between these two factors also in morbid obesity.

Effect of Pro12Ala polymorphism on PPAR γ 2 and its target gene expression

There were no major effects of Pro12Ala polymorphism at position 12 of the PPAR γ 2 gene on the expression of this gene in the present study. There was a tendency for higher expression of PPAR γ 2 mRNA in the subcutaneous adipose tissue depots in the subjects with Pro12Ala than with Pro12Pro genotype, this being especially apparent in the men with the Pro12Ala genotype. This finding is in contrast with the results *in vitro* (Deeb et al. 1998), which showed that the Ala12 allele is connected with the decreased PPAR γ 2 expression. Thus, it might be that the presence of both alleles, i.e. Pro12 and Ala12 in our subjects, could counteract the effect of the mutation. It is possible that those subjects homozygous for the mutation could have presented more pronounced differences in the expression compared to subjects with the Pro12Pro genotype. On the basis of the *in vitro* study (Deeb et al. 1998), they might not develop a state of massive obesity due to reduced function of PPAR γ 2. This hypothesis is not, however, supported by studies pointing out an association of the Pro12Ala polymorphism with obesity and obesity-associated metabolic alterations (Beamer et al. 1998, Koch et al. 1999, Valve et al. 1999, Hasstedt et al. 2001, Lindi et al. 2001, Nicklas et al. 2001, Stumvoll et al. 2001).

The investigations of target genes were focused on LPL, p85 α PI 3-kinase, UCP2 and PPAR γ 1 gene. The response element for PPAR γ 2 was found in LPL gene among the first target genes (Schoonjans et al. 1996). In p85 α PI 3-kinase the actual response element has not been found. However, its expression was recently shown to be increased by the thiazolidinedione, rosiglitazone (Rieusset et al. 1999) and by the activation of PPAR γ in human adipocytes (Rieusset et al. 2001). Moreover, UCP2 has been shown to be activated by the PPAR γ ligands in human adipocytes (Rieusset et al.

1999, Viguerie-Bascands et al. 1999). PPAR γ 2 has been reported to be able to activate PPAR γ 1 gene during the course of adipogenesis (Saladin et al. 1999).

PPAR γ 2 target gene expression did not generally differ between the groups with the polymorphism. The only significant difference between the genotypes was the lower expression of p85 α PI 3-kinase mRNA in the omental adipose tissue depot in the subjects with the Pro12Ala genotype. p85 α PI 3-kinase is an important part of the insulin signaling pathway (Saltiel and Kahn 2001), mediating the antilipolytic action of insulin (Rieusset et al. 2001). Although it is not possible to detect biological significance of this difference on the basis of this study, it might be speculated that the difference could be connected to the difference in insulin sensitivity in omental adipose tissue between the genotypes. This could mean better insulin sensitivity in this particular adipose tissue depot in Pro12Ala carriers as suggested previously (Deeb et al. 1998) or a decreased effect of insulin on the gene expression in lipolytically more active adipose tissue. Furthermore, reduced LPL activity has been shown to be associated with the Ala12 allele (Schneider et al. 2002). We did not see this effect at the level on mRNA expression. It has been suggested that the Pro12Ala variant might act as a recessive mutation, since a recent report (Hasstedt et al. 2001) showed that BMI, blood pressure and plasma TG in the subjects with Pro12Ala genotype were closer to the values in subjects with the Pro12Pro genotype. It may also be that this variant may act via an interaction with environmental factors such as physiological and nutritional conditions (Luan et al. 2001, Lindi et al. 2002,), and that this may account for the inconsistent findings regarding this polymorphism.

6.3. Concluding remarks

Adipose tissue has proven to be one of the central tissues affecting the development of obesity and other obesity-related diseases. It secretes several agents that affect directly or indirectly not only the whole body energy homeostasis but also adipose tissue energy metabolism. Thus, the purpose of this study was to examine metabolic alterations in obesity, concentrating on adipose tissue metabolism at the level of gene expression and the adipocyte itself. Massively obese subjects were chosen as our study group because they were assumed to present the most pronounced impact of extra fat mass and weight loss on adipose tissue metabolism.

Adipose tissue metabolism was investigated using the microdialysis method *in vivo* and isolated adipocyte assays *in vitro*. The results obtained in the present series of studies were similar with both methods which have not been shown before. These

methods provide different perspectives of adipose tissue metabolism and thus, they are not inter-changeable, rather they are complementary giving broader view of the phenomenon of interest.

Long-term weight reduction was associated with clear gender differences in adipose tissue lipolysis. Although we did not see any general reversal of changes along with weight loss, our and previous findings indicate that metabolic changes are mainly reversible after weight loss, and thus, they are secondary to obesity. Our subjects were still severely obese after weight loss, which may be the reason why we did not observe more clear reversal of changes.

LPL and HSL are two key enzymes of fat deposition and lipolysis, respectively, that are regulated in coordinated manner. There was a clear gender difference in the expression of these enzymes. Expression of HSL mRNA in women decreased along with the weight loss, whereas no changes were observed in men. Together with the decrease in HSL expression, LPL expression increased in women that might favor deposition of fatty acids into adipose tissue and hamper further weight loss or maintenance of reduced weight.

The regulation of SREBP-1c in adipose tissue is affected by the metabolic alterations evoked by weight change. Obesity decreases SREBP-1c expression, whereas weight loss normalizes the level of expression. These effects seem to reflect insulin resistance in obesity and the improvement of insulin sensitivity accompanying weight reduction as well as the weak effect of insulin on omental depot.

Obesity was associated with reduced PPAR γ 1 expression, whereas no clear difference was seen in PPAR γ 2 expression. In obese women, PPAR γ expression was higher in subcutaneous than in omental adipose tissue resulting in gender difference men having lower expression in subcutaneous depot than women. Although the reason for this gender difference remains to be resolved, it is not caused by the difference in the amount of fat mass. This could, however, indicate one reason for women having a tendency to increase more subcutaneous fat depots instead of fat in the visceral depot. Weight loss did not affect the PPAR γ expression.

The Pro12Ala polymorphism in position 12 in the PPAR γ 2 gene did not have any major effect on the expression of the gene itself or on its target genes. The minor effect on gene expression observed *in vivo* suggests that the presence of both alleles might counteract the impact of the mutation. It is suggested that the common Pro12Ala polymorphism in its heterozygous form has little influence on adipocyte function in morbidly obese patients.

7. SUMMARY

Study I: In severely obese subjects microdialysis method *in vivo* and lipolysis assay with freshly isolated adipocyte *in vitro* yielded concordant results in terms of lipolysis.

Study II: In women, HSL mRNA expression decreased, whereas LPL mRNA slightly increased along with weight reduction and decrease in cell size. In men, HSL and LPL mRNA levels remained unchanged after weight loss, but men had a decrease in their basal and maximal lipolysis and an increase in their sensitivity to inhibition of lipolysis after weight loss.

Study III: SREBP-1c mRNA expression in subcutaneous adipose tissue depots was lower in obese than in normal weight control subjects. Weight reduction resulted in an increase of SREBP-1c expression close to the level found in the normal weight subjects. Omental adipose tissue showed lower expression of SREBP-1c than subcutaneous depots in obese subjects.

Study IV and additional results: PPAR γ 1 mRNA expression was low in obesity compared with normal weight subjects, whereas no statistically significant difference was seen in PPAR γ 2 mRNA expression. In women, PPAR γ expression was higher in subcutaneous than in omental adipose tissue. Weight loss did not affect PPAR γ expression. The Pro12Ala polymorphism had no major effect on the expression of the gene itself or on its target genes. However, p85 α PI 3-kinase expression was lower in omental adipose tissue in the subjects with the Pro12Ala genotype.

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