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# Transcriptional activity of PPAR $\gamma$ in primary human adipocytes

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Cover picture/illustration: Basic mechanism of PPAR $\gamma$  as ligand-activated transcription factor in the adipocyte. Upon ligand binding, PPAR $\gamma$  forms a heterodimeric complex with RXR $\alpha$ , which binds to the PPRE (PPAR response element) and drives the transcription of target genes.

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*To my beloved family*



## ABSTRACT

The prevalence of obesity is increasing in most parts of the world and is a strong risk factor for the development of insulin resistance, type 2 diabetes and hypertension. Adipose tissue is mainly composed of adipocytes which store energy in the form of triglycerides and release it as free fatty acids. Adipose tissue is one of the major regulators of energy homeostasis in the body. Adipose tissue in different regions of the body has different characteristics and adipocytes in intra-abdominal fat depots are more associated with insulin resistance than adipocytes from subcutaneous fat depots.

Research performed during the past several years has led to an explosion in the understanding of adipose tissue and the active role that it plays in aspects of physiology and pathophysiology. One important discovery has been identification of the nuclear hormone receptor called peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ). Peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) is a transcription factor, which is highly expressed in adipocytes. PPAR $\gamma$  has been shown to affect several genes of importance for lipid metabolism, differentiation of fat cells and insulin sensitivity. The PPAR $\gamma$  receptor can be activated by thiazolidinediones (TZD), a class of insulin-sensitising drugs, which promote fatty acid storage in fat depots and decrease glucose levels in plasma, thus, demonstrating the importance of PPAR $\gamma$  activity in insulin resistance and metabolic syndrome.

This thesis has investigated the transcriptional activity of PPAR $\gamma$  in a clinically relevant cell type for insulin resistance and type 2 diabetes; the primary human adipocyte. For this purpose, a method for transfection of primary human adipocytes by electroporation and for measurement of the activity of PPAR $\gamma$  has been developed and optimised. This method has been used to study the effect of saturated and unsaturated fatty acids on the transcriptional activity of PPAR $\gamma$ . Interestingly, it was found that saturated fatty acids can activate PPAR $\gamma$ , thus promoting a protection against diabetes. The strongest activator was the monounsaturated palmitoleic acid. The transcriptional activity of PPAR $\gamma$  in primary human adipocytes from intra-abdominal and subcutaneous adipose tissues was also examined. It was found that PPAR $\gamma$  activity is considerably lower in adipocytes from visceral compared with subcutaneous fat from the same subject. Another reason for using human tissue to reach clinical relevance shown here was that the same difference in PPAR $\gamma$  activity could not be found between intra-abdominal and subcutaneous fat tissues in mice. This finding may serve as the basis of why excess intra-abdominal fat tissue is associated with high risk for development of type 2 diabetes and cardiovascular diseases.

The blood pressure regulating renin-angiotensin system (RAS) in human adipose tissue and in isolated adipocytes was examined and related to PPAR $\gamma$ . It was found that the production of angiotensin II, which is an important hormone for increasing the blood pressure, can be produced by isolated adipocytes and that the production is higher in adipocytes coming from omental than subcutaneous fat tissue. Further, it was shown that angiotensin II inhibits PPAR $\gamma$  activity in omental adipocytes, thus reducing the insulin sensitivity. Therefore, this study connects two of the major risk factors in obesity; diabetes and hypertension, and may also explain how drugs, which inhibit the RAS, can also be protective against diabetes. In conclusion, the findings in this thesis give new knowledge about regulating mechanisms of fat cells and its importance in diabetes and cardiovascular disease.

## SVENSK SAMMANFATTNING

Prevalensen av fetma ökar drastiskt i stora delar av världen och utgör en stor riskfaktor för att utveckla insulinresistens, typ 2 diabetes och hypertoni. Fett kan lagras i olika fettdepåer i kroppen. Fettet som inlagras inuti kroppen, intra-abdominellt fett, skiljer sig från fettväven som lagras direkt under huden (subkutant fett). Nyare rön visar att en stor mängd intra-abdominell fettvävnad är en särskilt stark riskfaktor för att utveckla insulinresistens och typ 2 diabetes, samt att avlägsnande av subkutant fett knappast alls påverkar riskfaktorer för kardiovaskulär sjukdom.

Under de senaste åren har forskningen lett till en djupare förståelse av fettvävnaden och dess aktiva roll i fysiologin och patofysiologin av insulinresistens. En viktig upptäckt har varit identifieringen av en nukleär receptor som kallas för PPAR $\gamma$  (peroxisome proliferator-activated receptor gamma). PPAR $\gamma$  receptorn uttrycks huvudsakligen i fettceller och är viktig för fettcelldifferentieringen och fettcellsfunktionen. Receptorn aktiveras av vissa läkemedel för behandling av insulinresistens och hyperglykemi, de så kallade tiazolidindionerna (avandia och actos finns på den svenska marknaden), som sänker blodsockret och även påverkar blodtrycket samt blodfetterna i gynnsam riktning. Detta utgör ett tydligt bevis för betydelsen av PPAR $\gamma$  aktiviteten vid insulinresistens och det metabola syndromet.

Den här avhandlingen studerar transkriptionsaktiviteten av PPAR $\gamma$  i en klinisk relevant celltyp för insulinresistens och typ 2 diabetes, den mänskliga fettcellen. För detta ändamål har en metod för transfektion av primära humana fettceller utvecklats. Metoden användes för att studera insulinsignaleringen i detalj och också för att mäta aktiviteten hos transkriptionsfaktorer. Aktiviteten av PPAR $\gamma$  i primära humana fettceller påverkades olika av olika mättade och omättade fettsyror, som alltså kan verka som hormoner. Intressant nog visades att mättat fett, som av många anses vara särskilt "onyttigt", i form av stearinsyra kan aktivera PPAR $\gamma$  och därmed tänkas medföra ett skydd mot diabetes. Den starkaste aktivatorn var enkelomättad palmoljesyra. Aktiviteten hos PPAR $\gamma$  i fettceller från

de två olika fettdepåerna, intra-abdominella och subkutana fettvävnaden, studerades. Aktiviteten av PPAR $\gamma$  i isolerade fettceller från intra-abdominellt fett befanns vara betydligt lägre än i subkutant fett från samma person. Som en ytterligare anledning att använda mänsklig vävnad för att nå klinisk relevans visades också av att möss inte har samma skillnad i PPAR $\gamma$  aktivitet mellan subkutant och intra-abdominellt. Fynden ger underlag till varför stor mängd intra-abdominellt fett är förknippat med hög risk för diabetes och därmed kopplad ökad kardiovaskulär risk.

Det blodtrycksreglerande renin-angiotensin systemet (RAS) i human fettvävnad och i isolerade fettceller och relationen till PPAR $\gamma$  studerades. Produktionen av angiotensin II, som är ett viktigt blodtryckshöjande hormon, producerades av isolerade human fettceller och produktionen var högre från fettceller som kommer från mänskligt omentfett än från subkutant fett. Vidare visades att tillsatt angiotensin II hämmade PPAR $\gamma$  aktiviteten i fettceller från omentfettet. Detta fynd kopplar alltså samman två av de stora riskfaktorerna vid fetma; diabetes och högt blodtryck. Det ger också nya intressanta infallsvinklar i hur blodtrycksläkemedel som hämmar reninsystemet kan tänkas skydda mot diabetesuppkomst.

Sammanfattningsvis visar denna avhandling att man kan transfektera primära humana fettceller och studera PPAR $\gamma$  aktivitet i denna celltyp, och att PPAR $\gamma$  aktiviteten kan styras av fettsyror, vilket alltså innebär att matkomponenter (fettsyror) har direkt hormonella effekter i kroppen. Omentfett visades ha särskilt låg PPAR $\gamma$  aktivitet. Slutligen befanns att fettväven och isolerade fettceller kan producera olika komponenter i RAS. Det är tydligt att dessa fynd tillsammans har givit upphov till viktig ny kunskap om fettcellens reglermekanismer och dess betydelse för diabetes och kardiovaskulär sjukdom.

## LIST OF PAPERS

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

**I Expression of a mutant IRS inhibits metabolic and mitogenic signalling of insulin in human adipocytes**

Karin G. Stenkula, Lilian Said, Margareta Karlsson, Hans Thorn, Preben Kjolhede, Johanna Gustavsson, Mats Söderström, Peter Strålfors, Fredrik H. Nystrom

*Molecular and Cellular Endocrinology; 2004, 221: 1-8.*

**II PPAR $\gamma$  response element activity in intact primary human adipocytes: effects of fatty acids**

Lilian Sauma, Karin G. Stenkula, Preben Kjolhede, Peter Strålfors, Mats Söderström, Fredrik H. Nystrom

*Nutrition; 2006, 22: 60-68.*

**III Peroxisome proliferator activated receptor gamma activity is low in mature primary human visceral adipocytes**

Lilian Sauma, Niclas Franck, Johan F. Paulsson, Gunilla T. Westermark, Preben Kjolhede, Peter Strålfors, Mats Söderström, Fredrik H. Nystrom

*Diabetologia; 2007, 50: 195-201.*

**IV Isolated primary human visceral fat cells release more angiotensin II than subcutaneous adipocytes**

Lilian Sauma, Niclas Franck, Preben Kjolhede, Per Sandström, Torbjörn Lindström, Fredrik H. Nystrom

*Submitted.*

## ABBREVIATIONS

AOX	acyl-coenzyme A oxidase
C/EBP $\alpha$	CCAT/enhancer binding protein $\alpha$
FFA	free fatty acids
GLUT4	glucose transporter 4
11 $\beta$ -HSD1	11 $\beta$ -hydroxysteroid dehydrogenase 1
HSL	hormone sensitive lipase
IL	interleukin
IRS	insulin receptor substrate
LBD	ligand-binding domain
MAP kinase	mitogen-activated protein kinase
N-CoR	nuclear receptor corepressor
PI3K	phosphatidylinositol 3 kinase
PKB	protein kinase B
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR response element
RAS	renin-angiotensin system
RBP4	retinol binding protein 4
RXR	retinoid X receptor
TNF $\alpha$	tumor necrosis factor $\alpha$
TZD	thiazolidinediones



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# INTRODUCTION

Obesity has reached epidemic proportions world wide and is one of the most visible of public health issues. The health consequences of obesity range from increased risk of premature death to development of various diseases, including insulin resistance, type 2 diabetes, hyperglycemia, abnormal blood lipid profiles and hypertension, all of which contribute to cardiovascular disease leading to stroke and myocardial infarction [1]. Indeed, cardiovascular disease is the major cause of death in the industrialized world, and much of this can be attributed to the excess accumulation of adipose tissue. Conversely, lack of adipose tissue, as in lipodystrophy, is also associated with insulin resistance, which reflects that the adipose tissue in fact, as a site for fatty acid storage, is critical for whole body insulin sensitivity [2].

Research performed during the past several years has led to an explosion in the understanding of adipose tissue and the active role that it plays in aspects of physiology and pathophysiology. One exciting discovery has been identification of the nuclear hormone receptor called peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), a transcription factor activated by various fatty acids and fatty acid metabolites, as a critical controller of fat cell differentiation and function [3]. Besides of being essential for fat cell development and the formation of adipose tissue, PPAR $\gamma$  is also a target of insulin-sensitising drugs for treatment of insulin resistance and hyperglycemia associated with type 2 diabetes [4].

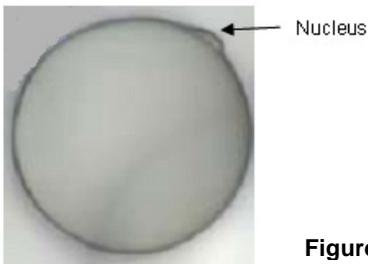
This thesis will focus on the presentation of a method to transfect primary human adipocytes for a molecular biological approach; for studying insulin signaling in adipocytes and measuring the activity of transcriptions factors. The transcriptional activity of PPAR $\gamma$  will be examined regarding the effects of different saturated and unsaturated fatty acids, and with respect to

intra-abdominal and subcutaneous adipose tissues. Because of the association between obesity and hypertension, the blood pressure regulating renin-angiotensin system (RAS) in adipose tissue and in isolated adipocytes will be examined and related to PPAR $\gamma$ .

# BACKGROUND

## The adipose tissue

The adipose tissue is an important regulator of energy balance and metabolism. It consists of fat cells, nerves, connective tissue, blood vessels, macrophages and immune cells. Adipocytes or fat cells can be very large cells often displaying a diameter over 100  $\mu\text{m}$ . Almost the entire intracellular space of these cells is filled with a big central lipid droplet, mainly consisting of triacylglycerols (commonly called triglycerides), occupying more than 95% of the cell volume and turning the cytosol into a thin film, which is only about 0.5  $\mu\text{m}$  thick. The nucleus of the fat cell protrudes since it is pushed to the side by the lipid droplet (Figure 1).



**Figure 1.** Isolated primary human adipocyte.

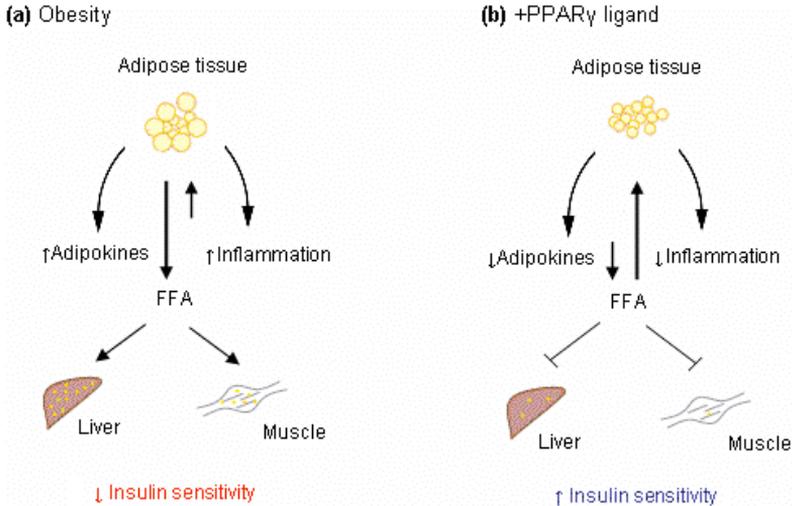
In addition to being an energy container and provider, the adipose tissue also functions as an endocrine organ, secreting different adipokines (hormones released from the adipocytes) that regulate metabolism, such as leptin, which affects energy balance by reducing food intake and increasing energy expenditure, and adiponectin, an adipocyte-derived plasma protein with insulin sensitising and anti-inflammatory properties [5]. Adipose tissue also synthesises and secretes other adipokines that cause insulin resistance for example resistin, tumor necrosis factor  $\alpha$  ( $\text{TNF}\alpha$ ), retinol binding protein 4 (RBP4) and interleukin (IL) 6 and 8 [6-8]. It has been reported that the plasma levels of these proteins are increased in human obesity

(Figure 2a), which in turn interferes with the action of insulin and its tissue sensitivity [7, 9]. In obesity, adipose tissue seems to be chronically inflamed and infiltrated by macrophages and together these cells produce inflammatory cytokines (reviewed in [10], Figure 2a).

Abnormalities in the storage and release of fatty acids in adipose tissue in obesity lead to elevated plasma fatty acid concentrations [11]. Obese individuals and type 2 diabetic patients have high levels of fatty acid in plasma, indicating a role of lipid metabolism in the pathogenesis of insulin resistance and type 2 diabetes. The inability of the adipose tissue to trap fatty acids leads to increased accumulation of triacylglycerols in nonadipose tissue such as liver and muscle (reviewed in [12], Figure 2a). The insulin producing  $\beta$ -cells in the pancreas initially increase the insulin secretion to compensate for the insulin resistance, which eventually can lead to  $\beta$ -cell failure and the development of type 2 diabetes (reviewed in [13]). This situation is improved by PPAR $\gamma$  ligands, called thiazolidinediones (TZD), a class of insulin-sensitising drugs, which promote fatty acid storage in fat depots and regulate the expression of adipokines that effect glucose homeostasis (reviewed in [14], Figure 2b).

In addition, the size of the adipocyte also plays an important role in insulin sensitisation. Obese subjects and individuals with type 2 diabetes have fat cells that are enlarged with less responsiveness to insulin [15-18]. Recently, it was shown that large adipocytes in non-diabetic subjects are associated with insulin resistance [19] and that the amount of glucose transporter 4 (GLUT4) in the plasma membrane of large fat cells did not increase following insulin stimulation, as it did in small fat cells obtained from the same individual [20]. In rodents, small adipocytes are shown to be more sensitive to insulin action and metabolically more active [21, 22], and treatment with TZD increases the number of small adipocytes and decreases the number of large adipocytes in adipose tissue in rats [23]. Thus, the

differentiation of adipocytes and thereby altering the number of adipocytes, which is dependent on the activation of PPAR $\gamma$ , appear to be an important mechanism by which increased levels of insulin resistance factors e.g. resistin and TNF $\alpha$ , and higher levels of free fatty acids (FFA) are normalised, leading to alleviation of insulin resistance (Figure 2b).



**Figure 2. Development of insulin resistance.** (a) Insulin resistance associated with obesity can be induced by adipokines, such as tumor necrosis factor  $\alpha$ , increased levels of free fatty acids (FFA), which inhibit insulin signalling in muscle and liver, and chronic inflammation in adipose tissue. (b) Regulation of gene expression by PPAR $\gamma$  ligands results in insulin sensitisation via: (i) retention of fatty acids in adipose tissue; (ii) regulation of adipocyte hormone gene expression; and (iii) improvement of glucose metabolism in adipocytes (not shown).

The human adipose tissue also expresses mRNA for the different components of RAS, such as angiotensinogen (AGT), angiotensin-converting enzyme (ACE) and angiotensin II type 1 (AT1) receptors [24-27]. Of the RAS components, the presence of proteins corresponding to AGT, ACE, AT1 receptors and angiotensin II have been demonstrated in human adipose tissue and isolated primary human adipocytes [27, 28]. Experimental studies suggest that the adipose RAS in mice is regulated by

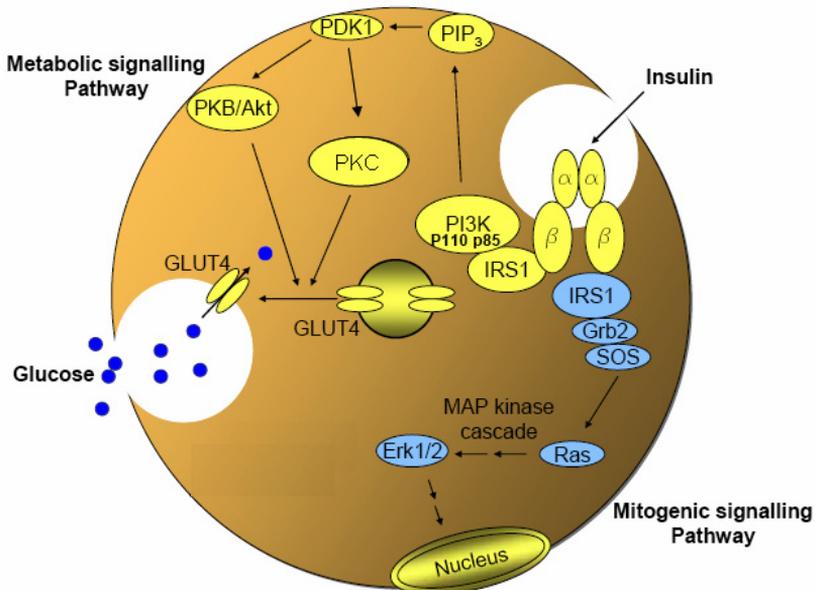
nutrition as well as hormonally and correlates with the degree of obesity [29] and that angiotensin II may alter adipose tissue blood flow, growth and metabolism (reviewed in [30]). Thus, an upregulated adipose RAS may contribute to insulin resistance and hypertension also in obese individuals.

### **Insulin signalling and metabolic regulation in adipocytes**

In normal physiology, adipose tissue stores energy in the form of triglycerides that can be hydrolysed (lipolysis) to release FFA. Different hormones, in particular insulin and catecholamines, control the storage and utilisation of energy in the triglyceride depots. Rise in circulating insulin levels promotes the uptake of glucose through an intracellular signal transduction pathway initiated by the activated insulin receptor. In adipocytes, the insulin receptor is localised in caveolae invaginations of the plasma membrane (Figure 3) [31-33]. In response to insulin, the intracellular signalling involves autophosphorylation of the receptor, which later phosphorylates insulin receptor substrate 1 (IRS1). Thus, phosphorylated IRS1 associates with the two Src homology (SH2) domains of the regulatory subunit (p85) of phosphatidylinositol 3 kinase (PI3K). The catalytic subunit (p110) of PI3K is thereby activated. PI3K converts PI(4,5)P<sub>2</sub> (an inositol phospholipid in the plasma membrane) to PI(3,4,5)P<sub>3</sub>, which in turn activates phosphoinositide-dependent protein kinase-1 (PDK1), and via protein kinase B/Akt (PKB/Akt) and protein kinase C (PKC) causes GLUT4-containing vesicles to translocate to the plasma membrane to increase the rate of glucose entering the cell (Figure 3, reviewed in [34, 35]).

In addition of being a controller of glucose transport, lipid, glycogen and protein metabolism through the metabolic signalling pathways, insulin also can control gene transcription and regulate adipose tissue growth and differentiation through the mitogenic signalling pathway [36, 37]. Binding of an adaptor protein, Grb2 to phosphorylated IRS stimulates the mitogenic

signalling pathway of insulin through a Ras/mitogen activated protein (MAP) kinase cascade [38], in which the MAP kinases Erk1/2 are being phosphorylated and activated (Figure 3). In turn, activation of the Erk1/2 results in the positive regulation of the transcription factor Elk-1. Insulin also regulates the expression and transcriptional activity of other fat-specific transcription factors, including sterol regulatory element-binding protein (SREBP) -1c, which regulates the expression of lipid synthesising enzymes [39], and of PPAR $\gamma$ , which plays a critical role in adipocyte function [39, 40]. PPAR $\gamma$  protein levels in differentiated 3T3-L1 preadipocytes are shown to be increased in response to insulin stimulation through the activation of the MEK/Erk signalling pathway [41].



**Figure 3. Insulin signalling pathways in an adipocyte.** Insulin binds to its transmembrane receptor in caveolae, leading to activation of two major signalling cascades, the Akt and the Erk pathways, which mediate most metabolic and transcriptional effects of insulin in adipocytes.

Insulin inhibits lipolysis by lowering the levels of intracellular cyclic-AMP (cAMP), which leads to decreased phosphorylation and activity of hormone sensitive lipase (HSL) and thus to less hydrolysis of triacylglycerol [42, 43]. Catecholamines, particularly noradrenalin, on the other hand, stimulate lipolysis by binding to  $\beta$ -adrenergic receptors on the cell surface. Activation of these receptors increases the concentration of intracellular cAMP, and thereby increased phosphorylation of HSL and perilipin, and an increased triacylglycerol hydrolysis and release of fatty acids [44, 45].

### **Visceral and subcutaneous adipose tissue**

In recent years it has become more obvious that not only the amount of adipose tissue is important for the development of insulin resistance, but also the distribution of body fat (reviewed in [46]). In humans, abdominal adiposity constitutes of visceral and subcutaneous adipose tissue. Visceral adipose tissue, surrounding the internal organs including the omentum, represents typically around 20% of total body fat in men, and 6% in women (reviewed in [47]). Subcutaneous adipose tissue on the other hand is the depot below the skin. Visceral adipocytes have been suggested to be smaller than subcutaneous cells [48, 49], although in some studies fat cells from these two fat cell populations were found to be of similar size [50, 51].

Excess visceral adipose tissue has appeared to be strongly linked to insulin resistance, type 2 diabetes, hypertension and dyslipidaemia, compared to excess subcutaneous adipose tissue [52-54]. Potential explanations proposed so far are multiple and include a higher metabolic rate of visceral adipose tissue. Due to its anatomical location, visceral fat drains directly to the liver via the portal vein, therefore exposing the liver to high concentrations of FFA from this depot. As mentioned above, high concentration of circulating fatty acids has been shown to induce insulin resistance in liver and muscle [55-57] and to reduce insulin secretion from pancreatic  $\beta$ -cells [58].

Several studies have compared insulin signalling and action in subcutaneous versus visceral fat (reviewed in [37]). Visceral adipocytes appear to be more responsive to catecholamine-stimulated lipolysis due to higher amount of  $\beta$ -adrenergic receptors [59] and less response to insulin's anti-lipolytic effect [60-62], due to reduced insulin receptor binding capacity and autophosphorylation and reduced signal transduction through an IRS1 associated PI3K pathway in human visceral adipocytes compared to subcutaneous adipocytes [50]. Therefore, individuals with greater visceral fat mass lose more visceral fat during diet or exercise, since visceral adipocytes have a higher lipolytic rate [63]. In contrast, *in vivo* studies in humans show that visceral fat cells have both higher protein expression as well as greater and earlier activation in insulin signalling via the insulin receptor, PKB/Akt and ERK1/2 signal mediators [51]. However, the level of IRS1 was not significantly different and the activation of the protein was not examined [51]. On the other hand, insulin-stimulated glucose uptake has been found to be approximately 2-fold higher in isolated omental compared with subcutaneous human adipocytes [48, 49], and this is associated with higher expression of GLUT4 in omental adipocytes.

Another possible explanation for the metabolically unfavourable consequences of excess visceral adipose tissue invokes some of the adipokines secreted by the adipose tissue, which via the blood stream can affect whole body energy homeostasis. It has been reported that visceral and subcutaneous fat secrete different amounts of adipokines (reviewed in [47]) and recently it was reported that visceral adipocytes secrete more RBP4 than subcutaneous adipocytes [64]. Visceral fat also seems to be more infiltrated by macrophages than subcutaneous fat [65]. In the case of adiponectin, which improves insulin sensitivity, plasma levels appear to be negatively correlated with visceral fat storage, suggesting that changes in plasma adiponectin might mediate some of the association between excess visceral fat and insulin resistance [66-68].

The importance of the intra-abdominal adipose tissue for the insulin sensitivity was demonstrated in a study where the omentum, which corresponds to less than 1% of the total body fat mass, was surgically removed [69]. This resulted in increased insulin sensitivity, while surgical removal of a large portion (18% of total body fat mass) of the subcutaneous fat tissue was without any effect on the insulin sensitivity [70]. Indeed, visceral adiposity, manifested as a high waist circumference, is now accepted as a major component of the metabolic syndrome [71].

## **Peroxisome proliferator-activated receptor $\gamma$ (PPAR $\gamma$ )**

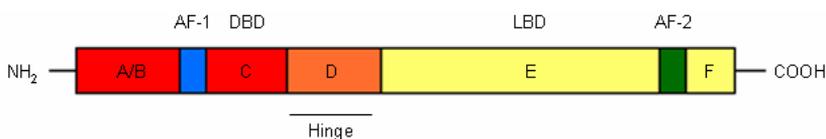
PPARs are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily, as are retinoic acid receptors, thyroid hormone receptors, and steroid receptors. In 1990, Issemann and Green [72] cloned a member of the steroid hormone receptor superfamily from mouse liver that could be activated by peroxisome proliferators. This nuclear receptor was named peroxisome proliferator-activated receptor, PPAR (later named PPAR $\alpha$ ). Shortly thereafter, three structurally and functionally similar receptors, encoded by separate genes, were cloned in *Xenopus*. These receptors were named PPAR $\alpha$  (NR1C1), PPAR $\beta/\delta$  (also called NUC1, NR1C2), and PPAR $\gamma$  (NR1C3) [73-75], although the latter two did not share the peroxisome proliferating characteristics. These receptors have since been shown to fulfil critical roles in lipid metabolism, glucose homeostasis, cell proliferation and differentiation, and control of inflammatory response (reviewed in [76]).

### **Structure and transcriptional activity**

The PPAR $\gamma$  gene maps to chromosome 3. It has 11 exons (coding regions) that span more than 140,000 bases [77]. There are two protein splice isoforms of PPAR $\gamma$ : PPAR $\gamma$ 1 and PPAR $\gamma$ 2 [78]. PPAR $\gamma$ 2 has 30 additional

N-terminal amino acids compared with PPAR $\gamma$ 1 in humans (28 in mice). Two additional mRNA splice variants PPAR $\gamma$ 3 [79] and PPAR $\gamma$ 4 [80] have also been reported that give rise to proteins identical to PPAR $\gamma$ 1. The biological relevance of these mRNA variants remains unclear.

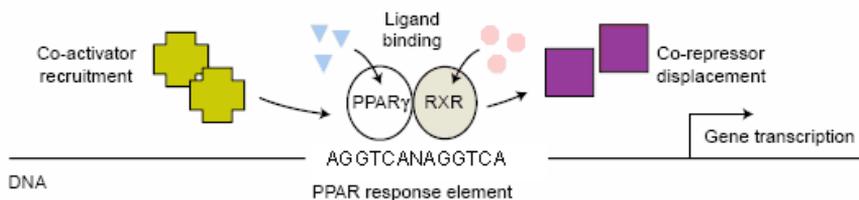
Like other nuclear receptors, PPAR $\gamma$  has a modular structure composed of functional domains [81]; a DNA binding domain (DBD) of ~70 amino acids, that specifically binds DNA in the regulatory region of PPAR-responsive genes, and a C-terminal ligand binding domain (LBD) of ~250 amino acids (Figure 4). The LBD appears to be quite large in comparison with those of other nuclear receptors [82, 83]. This difference may allow the PPAR $\gamma$  to interact with a broad range of structurally different naturally accruing and synthetic ligands. The N-terminal or A/B region of PPAR $\gamma$  contains a ligand-independent activation function 1 (AF-1) that is regulated by phosphorylation. Located in the C terminus of the LBD or E/F domain is the ligand-dependent activation function 2 (AF-2) domain (Figure 4). This region is involved in the generation of the receptors' coactivator binding site [82].



**Figure 4. PPAR $\gamma$  structure.** PPAR $\gamma$  displays the typical nuclear hormone receptor domain structures, including a central DNA-binding domain (DBD), located in the C-domain, a C-terminal ligand-binding domain (LBD), located in the E/F domain, and two activation domains (AF-1, ligand-independent transcriptional activation domain; AF-2, ligand-dependent activation domain). The D-domain (hinge) is believed to allow for conformational change following ligand binding.

PPARs are ligand-activated transcription factors that bind to specific DNA sequences known as peroxisome proliferator response elements (PPREs) located in the promoters of target genes. PPRE is composed of two direct

repeats of the consensus sequence AGGTCA separated by a single nucleotide, and is also known as a DR-1 (direct repeat) element (Figure 5). However, PPARs can not bind to PPREs in monomeric form; they strictly depend on retinoid X receptor (RXR) as a DNA-binding partner. Like PPARs, RXR exists as three distinct isoforms: RXR $\alpha$ ,  $\beta$ , and  $\gamma$ , all of which are activated by the endogenous agonist 9-cis retinoic acid [76]. In the absence of ligand, the transcriptionally inactive PPAR $\gamma$ /RXR $\alpha$  heterodimer is associated with a corepressor complex that contains histone deacetylase activity. Deacetylated histones keep the nucleosome in a state in which transcription is inhibited. Acetylation of histones is important in chromatin remodelling, which facilitates gene transcription. Upon binding of ligand, PPAR $\gamma$  undergoes a conformational change stabilising its interaction with RXR $\alpha$ . This allows for the dissociation of the corepressor complex and recruitment of a coactivator complex with histone acetylase activity [84]. These conformational changes stimulate the transcription of target genes (Figure 5).



**Figure 5. The gene action of PPAR $\gamma$ .** PPAR $\gamma$  binds to PPAR response element (PPRE) as a heterodimer with the retinoid X receptor (RXR). Ligand binding to either PPAR or RXR induces displacement of corepressors, recruitment of coactivators and transcriptional activation of target genes.

In addition to their ligand-induced regulation of activity, PPAR $\gamma$  activity can also be regulated by phosphorylation (reviewed in [85]). AF-1 domain contains a MAP kinase phosphorylation site [85-87] and phosphorylation at this site reduces the transcriptional activity of PPAR $\gamma$  by reducing its ability

to bind ligands [88]. Genetic prevention of PPAR $\gamma$  phosphorylation in mice has been shown to improve insulin sensitivity [89].

### **Cofactors**

As mentioned above, PPAR $\gamma$  transcriptional activity is regulated by proteins called cofactors. Such cofactors interact with nuclear receptors and they can either repress (corepressors) or enhance (coactivators) their transcriptional activities (Figure 5). Coactivators that have been shown to interact directly with PPAR $\gamma$  to initiate its transactivation include members of the p160 family of coactivators, which include steroid receptor coactivator 1 (SRC1), transcriptional intermediary factor 2 (TIF2) and p300/ CBP interacting protein (pCIP) [90], as well as other coactivators such as PPAR $\gamma$  coactivator 1 $\alpha$  (PGC1 $\alpha$ ), have been reported to interact with PPAR $\gamma$  and increase its activity [91, 92]. In contrast, corepressors such as the silencing mediator for retinoid and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (N-CoR) repress PPAR $\gamma$  mediated transcription [93].

### **Tissue distribution of PPAR $\gamma$**

PPAR $\gamma$  is highly expressed in both white and brown adipose tissues. Besides adipose tissue, also large intestine, placenta and haematopoietic cells express high levels of PPAR $\gamma$  mRNA and protein. Kidney, liver and small intestine show intermediate expression levels, whereas PPAR $\gamma$  is barely detectable in muscle or pancreas [77, 94]. Related to the subtype distribution, PPAR $\gamma$ 2 is much less abundant in all tissues analysed relative to PPAR $\gamma$ 1, the predominant PPAR $\gamma$  form. The only tissue expressing pronounced amounts of PPAR $\gamma$ 2 is the adipose tissue. The expression of PPAR $\gamma$ 3 mRNA is restricted to macrophages and the large intestine [93]. It is of interest to compare the tissue distribution of PPAR $\gamma$  with that of PPAR $\alpha$  and of PPAR $\delta$  for insight into their respective biological roles. PPAR $\alpha$  is most highly expressed in muscle and liver, where it regulates

fatty acid oxidation and the metabolic response to fasting [95]. PPAR $\delta$  is abundantly expressed in almost all tissues. Its precise biological role is less well understood than those of PPAR $\alpha$  and PPAR $\gamma$ , but recent work suggests that it is a potent inducer of fatty acid oxidation and that PPAR $\delta$  agonists improve plasma lipid profiles [96].

### **Ligands to PPAR $\gamma$**

As pointed out earlier, PPAR $\gamma$  has a larger ligand-binding site compared with other nuclear receptors; thereby allowing the transcription factor to have high promiscuity for a variety of ligands [82, 97]. Owing to the critical role that PPARs play in lipid metabolism, the search for natural ligands began with fatty acids and eicosanoids. According to *in vitro* studies, several fatty acids were indeed found to be able to bind and activate PPAR $\gamma$  at micromolar concentrations [83, 98]. However, little is known about such potential effects in primary human fat cells.

The strongest specific PPAR $\gamma$  activators that have been characterised so far are synthetic. These include the insulin sensitizers TZD or glitazones, which were identified as part of a drug-screening process for antidiabetic compounds in the early 1980s [99, 100] and only later found to act via PPAR $\gamma$  [101, 102]. These compounds have insulin-sensitising and antidiabetic activity in humans with type 2 diabetes or impaired glucose tolerance [76], and they also lower circulating levels of triglycerides and FFA. Two glitazones, rosiglitazone (Avandia) and pioglitazone (Actos), are currently approved for the treatment of human type 2 diabetes. A third glitazone, troglitazone (Rezulin) has been removed from the market due to hepatotoxicity.

Other drugs that can function as targets of PPAR $\gamma$  and have broad useful profiles with limited adverse effects are some angiotensin II type 1 receptor blockers (ARB) like telmisartan and irbesartan [103]. ARB have been

shown to induce adipogenesis and PPAR $\gamma$ -target gene expression in human adipocytes [104]. For instance, telmisartan is both an angiotensin type 1 receptor antagonist and PPAR $\gamma$  ligand [105]. Therefore, besides its antihypertensive effect, it could also improve carbohydrate and lipid metabolisms. Most importantly, telmisartan does not cause the side effects, such as fluid retention and even heart failure, that are associated with drugs of the TZD class [106].

### **Human genetic variants of PPAR $\gamma$**

Several mutations have been identified in the PPAR $\gamma$  gene. The most common variant is specific to PPAR $\gamma$ 2 and results in a proline to alanine substitution at position 12 (Pro12Ala) [107]. Carriers of the Ala variant, which has a somewhat lower DNA-binding capacity and decreased transcriptional activity than the Pro form, seem to have lower BMI, enhance insulin sensitivity, improve the lipid profile and to protect against development of diabetes, although this has not consistently been shown (reviewed in [108]).

A much rarer, “gain of function” mutation in PPAR $\gamma$  is Pro115Gln. Carriers of this mutation are extremely obese but insulin sensitive. This mutation is located in the N terminus making PPAR $\gamma$  constitutively active through the prevention of the phosphorylation status of PPAR $\gamma$  by MAP kinase at serine 114 [109]. In contrast, several dominant negative mutations, located in the LBD, i.e. Val290Met, Phe360Leu, Arg397Cys and Pro467Leu, have been associated with severe insulin resistance, diabetes and hypertension (reviewed by [110]), which clearly displays the importance of functional PPAR $\gamma$  for proper insulin action in humans.

## **Biological functions of PPAR $\gamma$**

### **PPAR $\gamma$ and adipogenesis**

PPAR $\gamma$  was initially recognised as a transcription factor involved in the activation of the adipocyte fatty acid binding protein (aP2), during the differentiation of adipocytes from undifferentiated precursor cells [78], and it was rapidly recognised as playing a central role in the process of adipogenesis [111]. It is now known that the presence of PPAR $\gamma$  is absolutely required for the formation of adipose tissue [112]. Zhang *et al.* [113] showed that specific PPAR $\gamma$ 2 knock-out mice, although having normal development of other tissues, display a total reduction of the white adipose tissue, less lipid accumulation and reduced expression of adipogenic genes. They also showed that cultured fibroblasts from the same mouse model had decreased adipogenic potential compared to fibroblasts from wild type mice. PPAR $\gamma$ 1 has also been shown to induce adipogenesis when overexpressed in fibroblasts. PPAR $\gamma$ 2, however, has better ability to induce adipocyte differentiation under conditions of limited availability of ligand in cell lines [114].

### **Lipid metabolism**

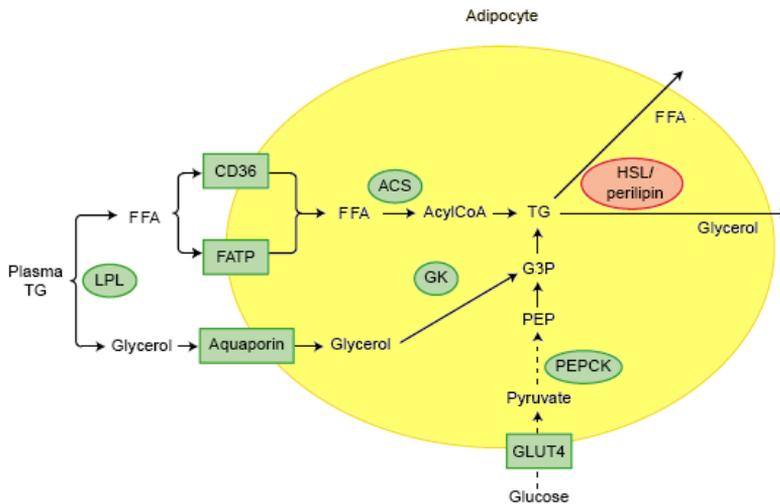
In adipocytes, PPAR $\gamma$  regulates the expression of several genes (Table 1). The products of these genes can be grouped functionally into: 1) fatty acid binding and uptake, lipogenesis and triglyceride synthesis, proteins involved in hydrolysis of plasma triglycerides; 2) proteins regulating lipolysis; 3) proteins directly implicated in insulin signalling and glucose uptake; and 4) regulation of adipokines.

**Table 1. Genes that are regulated by PPAR $\gamma$ .** Arrows indicate upregulation ( $\uparrow$ ) or downregulation ( $\downarrow$ ) of genes by PPAR $\gamma$  agonists. See text for details and references.

Gene	Function
Adipocyte fatty acid binding protein (aP2) $\uparrow$	Intracellular fatty acid binding
Acyl-CoA synthase (ACS) $\uparrow$	Lipogenesis and/or catabolism
CD36, Fatty acid transport protein (FATP) $\uparrow$	Cell surface fatty acid transporters
Lipoprotein lipase (LPL) $\uparrow$	Triglyceride hydrolysis
Phosphoenolpyruvate carboxykinase (PEPCK) $\uparrow$	Glycerol synthesis
Glycerol kinase (GK) $\uparrow$	Glycerol synthesis
Perilipin $\uparrow$	Lipolysis regulation
c-Cbl-associated protein (CAP) $\uparrow$	Insulin signalling toward glucose transport
Insulin receptor substrate 2 (IRS-2) $\uparrow$	Insulin receptor-mediated signalling
Glucose transporter 4 (GLUT4) $\uparrow$	Glucose uptake
Tumor necrosis factor $\alpha$ (TNF $\alpha$ ) $\downarrow$	Pro-inflammatory cytokine; mediator of insulin resistance
Adiponectin $\uparrow$	Adipokine that improves insulin sensitivity
Resistin $\downarrow$	Adipokine; mediator of insulin resistance
Leptin $\downarrow$	Fat-derived hormone that inhibits food intake
11 $\beta$ -hydroxysteroid dehydrogenase 1 (11 $\beta$ -HSD1) $\downarrow$	Controls intracellular conversion to active cortisol

Genes involved in lipid metabolism include aP2, phosphoenolpyruvate carboxykinase (PEPCK), acyl-CoA synthase (ACS), and lipoprotein lipase (LPL). PPAR $\gamma$  has also been shown to control expression of CD36 and fatty acid transport protein (FATP) (reviewed in [76]). These genes have all been shown to be upregulated by PPAR $\gamma$ . Plasma triglycerides are hydrolysed by LPL to FFA and glycerol (Figure 6). FFA uptake by adipocytes is

facilitated by the transporters CD36 and FATP; the aquaporin channel facilitates glycerol transport. In adipocytes, FFA are esterified via the action of ACS for storage as triglycerides, while glycerol is converted to glycerol 3-phosphate (G3P) by the action of glycerol kinase (GK). G3P can also be synthesised via the action of PEPCK (Figure 6). Thus, PPAR $\gamma$  may promote triglyceride synthesis by inducing transcription of genes involved in regulating plasma lipid uptake (LPL, CD36, FATP, aquaporin) and metabolism within the adipocyte (ACS, GK, PEPCK) [115-117]. Activation of PPAR $\gamma$  receptor can also influence lipolysis by inducing perilipin expression [118] (Figure 6). Perilipin covers the surface of mature lipid droplets.



**Figure 6. Effects of PPAR $\gamma$  ligands on lipid metabolism in the adipocyte.**

PPAR $\gamma$ -regulated gene products promoting lipid storage are highlighted in green, whereas those promoting lipolysis are highlighted in red. PPAR $\gamma$  ligands increase the expression of both lipoprotein lipase (LPL), which releases free fatty acids (FFA), and the levels of fatty acids transporters, fatty acid transport protein (FATP) and CD36, which transport FFA into the adipocyte. These fatty acids are esterified via the action of acyl-CoA synthase (ACS) into triglyceride (TG), while glycerol is converted to glycerol 3-phosphate (G3P) by the action of glycerol kinase (GK). In addition, G3P can be synthesised via the action of phosphoenolpyruvate carboxykinase (PEPCK). PPAR $\gamma$  can also stimulate lipolysis by inducing perilipin expression (Perilipin is an important determinant of hormone-sensitive lipase (HSL) activity).

## **PPAR $\gamma$ and insulin sensitivity**

Activation of PPAR $\gamma$  regulates the expression of several other genes that improve glucose metabolism in the adipocyte, including the insulin-responsive GLUT4 [119, 120] and c-Cbl-associated protein (CAP), which is crucial for GLUT4 translocation to the cell surface [121]. Expression of IRS2 is also increased in cultured adipocytes and human adipose tissue incubated with PPAR $\gamma$  agonists [122].

PPAR $\gamma$  activity has been associated with several genes that affect insulin action. Increased levels of TNF $\alpha$ , a pro-inflammatory cytokine that is expressed by adipocytes, has been associated with insulin resistance and diminished insulin signal transduction. mRNA and protein levels of TNF $\alpha$  are highly expressed in adipose tissue from obese and diabetic rodents [123, 124]. High expression of TNF $\alpha$  suppresses the transcription factor CCAT/enhancer binding protein (C/EBP)  $\alpha$  which, in turn, activates the GLUT4 gene [125]. C/EBP $\alpha$  has an essential role in hepatocyte and adipocyte differentiation. TNF $\alpha$  also suppresses the expression of PPAR $\gamma$  [126]. In addition, TNF $\alpha$  activates the JNK (c-Jun N-terminal kinase) pathway, mediating an increased serine phosphorylation of the IRS1 and thus, inhibiting its interaction with the insulin receptor and reducing insulin-stimulated tyrosine phosphorylation of the IRS1 [127]. Treatment with PPAR $\gamma$  agonists (TZD) has been shown to significantly decrease the plasma levels of TNF $\alpha$  in obese patients with type 2 diabetes [128]. However, blockade of TNF $\alpha$  in humans seems to be of little relevance for insulin resistance [129].

Activation of PPAR $\gamma$  also increases the expression of adiponectin [130]. Reduced levels of adiponectin are strongly associated with obesity and insulin resistance [131]. Conversely, adiponectin levels increase in obese patients after weight loss [132]. On the other hand, activation of PPAR $\gamma$  reduces leptin and resistin mRNA levels in adipocytes. Leptin is produced

and secreted by adipocytes and crosses the blood-brain barrier and interacts with neurons resulting in a decreased food intake. Mutation of the leptin gene (*ob*) results in the genetic form of obesity seen in *ob/ob* mice [133]. The leptin gene is regulated by C/EBP $\alpha$  and PPAR $\gamma$  activation by TZDs antagonizes the C/EBP $\alpha$ -mediated transactivation of the leptin promoter [134]. It has also been shown that circulating resistin levels, which are increased in diet-induced and genetic forms of obesity in mice, are decreased by rosiglitazone [135].

PPAR $\gamma$  agonists also reduce 11 $\beta$ -hydroxysteroid dehydrogenase 1 (11 $\beta$ -HSD1) expression in adipocytes and adipose tissue of type 2 diabetes mouse models [136]. 11 $\beta$ -HSD1 converts inactive cortisone to bioactive cortisol within tissues, and overexpression of this gene in mouse adipose tissue gives rise to central obesity, insulin resistance and hypertension [137]. Thus, some of the insulin-sensitising actions observed after activation of PPAR $\gamma$  may result from a decrease in adipose 11 $\beta$ -HSD1 levels.

In conclusion, insulin signalling in the adipose tissue is impaired in insulin resistance and the signal can be at least partially restored upon activation of PPAR $\gamma$  by TZD. Initiation of obesity-related insulin resistance can occur by several mechanisms (a) disruption of lipid homeostasis leading to an increase of plasma fatty acids concentrations, (b) activation of factors such as cytokines, that also cause insulin resistance, (c) decreased glucose uptake into adipocytes leading to decreased whole-body glucose removal, and (d) increase of large adipocyte; actions which can all be reduced by PPAR $\gamma$  activation. As a result, PPAR $\gamma$  activity plays a central role in maintaining an insulin-sensitive and functional adipose tissue.

## **SPECIFIC AIMS OF THE THESIS**

- To develop a method that allows the study of transcription factors such as Elk-1 and PPAR $\gamma$  in primary human adipocytes.
- To study the effect of saturated and unsaturated fatty acids on the transcriptional activity of PPAR $\gamma$  in primary human adipocytes.
- To examine and compare the transcriptional activity of PPAR $\gamma$  in primary human adipocytes from intra-abdominal and subcutaneous adipose tissues.
- To investigate the renin-angiotensin system in adipose tissue and in human adipocytes from intra-abdominal and subcutaneous adipose tissues, and to study the effect of angiotensin II on PPAR $\gamma$  activity.

## MATERIAL AND METHODS

Key methods and techniques used in this thesis are briefly described.

### Isolation of adipocytes

Subcutaneous and omental abdominal human adipose tissue were removed during surgery. The patients were either women (Paper I-III) operated for various gynaecological diseases, usually requiring hysterectomy or of both genders (Paper IV). Patients were not investigated with regard to insulin sensitivity, but subjects with the diagnosis of diabetes mellitus were excluded or studied separately (Paper II). The adipose tissue was cleared from vascular and fibrous structures and rinsed in 0.9 % (w/v) NaCl. The fat tissue (5-15 g) was cut with scissors into millimetre-sized pieces and digested in equal volume (1g/ml) of Krebs-Ringer solution (0.12 M NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>) containing 20 mM HEPES, pH 7.4, 3.5 % (w/v) fatty acid free bovine serum albumin, 200 nM adenosine, 2 mM glucose and 260 U/ml collagenase (Worthington Lakewood, NJ, USA) for 1.5 h at 37 °C in a water bath with agitation. After collagenase digestion the adipocytes were separated from connective tissue debris by filtering through gauze. The adipocytes were then washed in the Krebs-Ringer solution containing 20 mM HEPES, pH 7.4, 1% (w/v) fatty acid-free bovine serum albumin (Roche, Mannheim, Germany), 200 nM adenosine and 2 mM glucose, allowed to float by gravity during rinsing, and then kept in a water bath with agitation at 37 °C.

Mice adipocytes from the subcutaneous and visceral adipose tissue of standard lean male NMRI mice (B&K Universal, Södertälje, Sweden) were isolated by collagenase digestion, and incubated in Krebs-Ringer solution at 37°C on a shaking water bath as described above. Mice adipocytes from the

subcutaneous, epididymal and intra-abdominal fat tissue from appendix epipliocae of obese male mice derived from our own laboratory [138] were also isolated and rinsed in the same way as human adipocytes (Paper III).

## **Electroporation**

Each 0.4-cm gap electroporation cuvette was filled with 200  $\mu$ l of the adipocyte solution and an additional 200  $\mu$ l of PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.5) containing plasmid DNA of interest. For human adipocytes, each cuvette was electroporated with six exponentially decaying pulses at 600 voltages (or between 200-800 V for optimasition) at 25  $\mu$ F (Paper I), or with one square-wave pulse at 400 V 4 msec (Paper II-IV) using a Bio-Rad GenePulser II (Bio-Rad Laboratories Inc., Hercules, CA, USA). For mice adipocytes each cuvette was electroporated with one square-wave pulse at 400 V 4 msec (Paper III). Cells were pooled and transferred to petri dishes and kept at 37 °C in 10 % CO<sub>2</sub>. One hour after electroporation an equal volume of Dulbecco's modified Eagle's medium pH 7.5, containing 25 mM glucose, 50 UI/ml penicillin, 50  $\mu$ g/ml streptomycin, 200 nM phenylisopropyladenosine, 7% (w/v) fatty acid-free bovine serum albumin, and 25 mM HEPES was added. After 18 h of incubation the cells were collected and analyzed.

## **Luciferase assay**

For measurement of the activity of transcriptions factors such as Elk-1 and PPAR $\gamma$ , a luciferase based assay was used. In this assay, phosphorylation of a transfected GAL4 DNA-binding domain/Elk-1 activation domain fusion protein (plasmid: pFA-Elk, 0.5  $\mu$ g/cuvette) (Paper I) or the activity of a transfected human PPAR $\gamma$  ligand-binding domain fused to the yeast GAL4 DNA-binding domain (plasmid: GAL4-PPAR $\gamma$ , 2  $\mu$ g/cuvette) (Paper II and III), results in activation of a co-transfected GAL4 binding

sequence/luciferase reporter plasmid (plasmids: pFR-Luc, 1.0 µg/cuvette for pFA-Elk; 5xGAL4-TK-LUC, 2 µg/cuvette for GAL4-PPAR $\gamma$ ) resulting in increased luciferase expression. PPAR $\gamma$  activity was also measured by using a plasmid that encodes firefly luciferase cDNA under the control of a PPRE from the acyl-coenzyme A oxidase gene, called pAOX-luc (2 µg/cuvette) (Paper II-IV). The cells were also transfected with 0.1 µg/cuvette of a plasmid coding for Renilla luciferase; pRLuc (BioSignal Packard, CT, USA). Twenty hours after electroporation cells were incubated with 5 nM insulin, for 3 h (Paper I) or one hour after electroporation cells were incubated with different fatty acids (Paper II). The cells were lysed and assayed for firefly and Renilla luciferase using the Dual-Luciferase® Reporter Assay Systems (Promega, WI, USA), that allows separate analysis of the resulting light formed using a luminometer. Cell lysates were prepared with 200 µl of supplied buffer, by passing through a 25-gauge needle two times. Firefly luciferase assay was initiated by adding 100 µl of Luciferase Assay Reagent II to each 50 µl aliquot of cell lysate. After quantifying the firefly luminescence, the reaction was quenched and the Renilla luciferase reaction was activated by adding 100 µl Stop & Glo® Reagent. The firefly and Renilla luciferase activities were measured using a Victor 1420 multilabel counter (Wallac, Turku, Finland). The induced amount of firefly luciferase was normalised according to the constitutively expressed Renilla luciferase, thus correcting for differences in transfection efficiency.

## **Immuno-detection**

### **Immunoblotting**

Cells were lysed in 20 mM Tris, 1 mM EDTA, 9% (v/w) sucrose, 8.7 µg/ml PMSF, 0.7 µg/ml pepstatin, 6.5 µg/ml aprotinin, 5 µg/ml leupeptin, 100 nM okadaic acid, 1% (v/v) Triton-X100, and 0.1 % (w/v) SDS, by passing through a 25-gauge needle three times. The lysate was centrifugated at 400

× g for 10 min at 4°C, to pellet cell debris. The protein concentration of the whole cell lysates was determined using BCA Protein Assay (Pierce, Rockford, IL, USA). Proteins were then separated by electrophoresis using a SDS-polyacrylamide gel and transferred electrophoretically to polyvinylidene difluoride membranes (Papers I-IV) or nitrocellulose membranes for N-CoR blots (Paper III). The membranes were incubated with antibodies of interest. Bound antibodies were detected with the secondary horseradish peroxidase (HRP)-conjugated anti-IgG antibody according to ECL+ protocol (Amersham Biosciences, Amersham, UK), and quantified by chemiluminescence imaging (Las 1000, Image-Gauge, Fuji, Tokyo, Japan).

### **Immunohistochemistry**

Abdominal subcutaneous and omental human adipose tissues were fixed in 10% neutral buffered formalin and embedded in paraffin and sections were placed on glass slides (plus slides; Histolab, Gothenburg, Sweden) deparaffinised and rehydrated (Paper IV). Visualisation of immunoreactivity was performed by incubation of tissue sections in HRP/diaminobenzide (DAB) reaction. Slides were counterstained with Mayer's hematoxylin, dehydrated and mounted. The presence of ACE and angiotensin II was established using a Nikon Eclipse E800 (Nikon, Tokyo, Japan).

### **RNA extraction and amplification**

Total RNA was extracted from isolated subcutaneous and omental adipocytes by homogenisation in Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions (Paper III). First-strand cDNA synthesis was made from 1 µg of total RNA using a kit (Enhanced Avian RT First Strand Synthesis Kit; Sigma, Saint Louis, MO, USA). PCR amplification of the cDNA was done using ThermoWhite Taq DNA

polymerase (Saveen Werner, Malmö, Sweden). The amplified products were quantified by fluorescence imaging (LAS 1000; Fuji, Tokyo, Japan) after separation in 1.5% agarose gel, and normalised to mRNA levels of  $\beta$ -actin.

## RESULTS AND DISCUSSION

### **Development of a method for studying transcription factors (Paper I)**

In this paper, a method for transfection of primary human adipocytes that allowed the study of insulin signalling and activity of transcription factors was developed. The technique was based on an existing method for transfection of rat adipocytes using electroporation [139]. The amount of plasmid DNA, and the strength of the electric pulses were tested and optimised in order to achieve as high transfection efficiency as possible with a relatively low cell death. It was found that the highest protein expression in primary human adipocytes was reached at around 600 V. When performing electroporation with the optimised protocol the transfection efficiency was 10-15 % with a cell viability of approximately 90 %. The optimised technique was used to study the importance of IRS for mediating the insulin-induced signalling downstream of the insulin receptor, and the effects of expression of a mutant of IRS3 (IRS3-F4) on insulin signalling for metabolic and mitogenic control in primary human adipocytes. IRS3-F4 has disrupted binding sites for the regulatory subunit of PI3K (p85) with tyrosine substituted to phenylalanine in four YXXM motifs [140].

The IRS3-F4 mutant was co-transfected with PKB/Akt, GLUT4 or Elk-1. It was shown by immunoblotting that insulin could induce activation of PKB/Akt and increase the amount of GLUT4 at the plasma membrane under regular circumstances, while the co-expression of IRS3-F4 inhibited the insulin-induced activation of PKB/Akt and translocation of GLUT4. The insulin-induced activation of Elk-1, which is a downstream of the MAP-kinase ERK and responsible for regulation of gene transcription control, was analysed by a luciferase system, where activation of co-transfected Elk-1 binding domain results in luciferase activity of another

co-transfected binding domain/luciferase containing plasmid (see method). Insulin stimulation of Elk-1 in cells co-transfected with IRS3-F4 was totally inhibited.

Thus, the development of the method to transfect primary human adipocytes allowed us to study the importance of IRS for both metabolic and mitogenic signalling in human adipocytes, and to measure the activity of a transcription factors.

### **Activation of PPAR $\gamma$ by fatty acids (Paper II)**

In order to investigate whether several fatty acids, which are normally present in different food sources, could induce transcriptional activity of PPAR $\gamma$  in intact primary human adipocytes, cells were electroporated with square wave pulses using a further refined technique [141] and transfected with pAOX-luc (see method). The cells were co-transfected with a plasmid encoding renilla luciferase controlled by a constitutively active promoter (pRLuc). PPRE-inducible firefly luciferase activity was normalised according to renilla luciferase, thus correcting for differences in the amount of transfected cells. Transfected adipocytes were treated with different saturated and unsaturated fatty acids at concentrations of 1 and 10  $\mu$ M because *in vitro* assessment of ligand binding of mono- and polyunsaturated fatty acids have demonstrated a half maximal effective concentration lower than 10  $\mu$ M [83, 98] and that such levels, or higher, are encountered in human plasma [142].

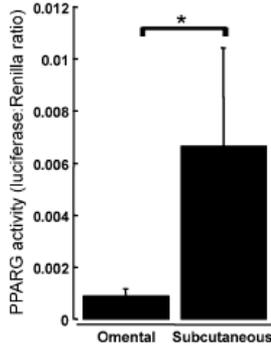
Treatment of primary human adipocytes with rosiglitazone induced a maximal fivefold increase in PPAR $\gamma$  transcriptional activity, and this maximal response to rosiglitazone was positively correlated to BMI. There was also a weak trend toward a linear negative relation between basal PPAR $\gamma$  activity and BMI. This implies that obese subjects are particularly

sensitive to the effects of rosiglitazone on PPAR $\gamma$  transcriptional activity due to a low endogenous activity of PPAR $\gamma$ .

The transcriptional activity of PPAR $\gamma$  was clearly increased when cells were incubated with the saturated fatty acids palmitic and stearic acids. In contrast, the polyunsaturated fatty acids linoleic acid,  $\gamma$  linolenic acid, and docosahexaenoic acid did not induce any statistically changes in PPAR $\gamma$  activity. The monounsaturated palmitoleic acid increased basal PPAR $\gamma$  activity by 35 % at 1  $\mu$ M, thus being more effective than the fish oil eicosapentaenoic acid in this regard. At 1  $\mu$ M, palmitoleic acid induces a transcriptional activity level of PPAR $\gamma$  in human adipocytes which corresponds to 54 % of the effect of therapeutic plasma levels of rosiglitazone [143]. Therefore, the activation of PPAR $\gamma$  induced by dietary fatty acids can potentially have great effect on insulin sensitivity and hence, on cardiovascular morbidity and mortality.

### **PPAR $\gamma$ activity in human subcutaneous and visceral adipocytes (Paper III)**

PPAR $\gamma$  activity in subcutaneous and visceral fat cells was compared in this study. Basal PPAR $\gamma$  activity differed largely in subcutaneous and omental adipocytes from the same subjects, displaying a median of 6.2-fold higher activity in subcutaneous than in intra-abdominal adipocytes. The difference between the two fat depots was not caused by a lower amount of the PPAR $\gamma$  receptor in the intra-abdominal fat cells, but rather by different activity levels of the PPAR $\gamma$  receptor as shown by transfecting the cells with a GAL4-PPAR $\gamma$  fusion protein and a second plasmid encoding luciferase under control of five copies of a GAL4 response element (5 $\times$ GAL4-TK-LUC) as reporters of PPAR $\gamma$  receptor activity, which confirmed the results (Figure 7).



**Figure 7. PPARE activity in omental and subcutaneous adipocytes.** Primary human adipocytes were transfected with plasmids encoding a GAL4-PPAR $\gamma$  fusion protein and firefly luciferase under control of the GAL4 response element and also the constitutively expressed RLuc. After 18 h of incubation luciferase activities were measured by luminometry. Light emission from firefly luciferase was normalised to that of RLuc. \* $p < 0.05$  in paired  $t$  test,  $n = 5$

To further study the potential mechanisms behind the difference in PPARE activity, the levels of mRNA of known coactivators and corepressors in subcutaneous and omental adipocytes were examined. The levels of mRNA for coactivators of PPAR $\gamma$  were similar in adipocytes from the two fat depots. However, there were twofold higher levels of N-CoR mRNA in the intra-abdominal than in the subcutaneous fat cells. This did not translate into a parallel difference in N-CoR protein levels as assayed by immunoblotting. However, in an individual cell, cofactors can be recruited by different transcription factors and since they are generally not abundant, a measurement of the absolute amount does not exclude the possibility that varying amounts are available to the particular transcription factor PPAR $\gamma$  [144].

Since many studies related to insulin signalling in primary fat cells are performed on epididymal cells obtained from male rodents, it was of interest to test PPAR $\gamma$  activity in fat cells from different abdominal depots in mice. In contrast to our findings in human adipocytes, PPAR $\gamma$  activity

was slightly higher in the visceral (appendices epiploicae) than in subcutaneous fat cells in these animals, while epididymal fat cell PPAR $\gamma$  activity was similar to that in subcutaneous adipocytes. These findings highlight important species differences between mice and humans with regard to PPAR $\gamma$  activity in fat cells and point to the importance of using intra-abdominal adipocytes from human when studying mechanisms behind the insulin resistance that is linked to visceral obesity in man.

### **RAS in human adipose tissue and adipocytes; relation to PPAR $\gamma$ (Paper IV)**

Since type 2 diabetes and hypertension are strongly linked to visceral obesity, and that antihypertensive drugs have been shown to reduce insulin resistance [145], components of RAS, specially angiotensin II, were studied in isolated primary human adipocytes derived from both subcutaneous and omental human adipose tissue.

Both human adipose tissue and isolated adipocytes were shown to produce angiotensin II, and the production was significantly higher in the isolated omental adipocytes. Treatment with rosiglitazone reduced angiotensin II levels in both subcutaneous and visceral adipocytes, which provides a link to the blood pressure lowering effects of this TZD. Furthermore, angiotensin II was found to reduce PPAR $\gamma$  activity in isolated visceral, but not in the subcutaneous adipocytes. The high levels of angiotensin II in medium from visceral adipocytes, and the effect of angiotensin II to induce lower PPAR $\gamma$  activity in these cells, confirm our earlier findings of much lower basal PPAR $\gamma$  activity in omental as compared with subcutaneous adipocytes [146]. Indeed, it is possible that the reverse is also true, that the reduced PPAR $\gamma$  activity in visceral fat cells could explain the higher angiotensin II production, since a high activity of PPAR $\gamma$ , by addition of rosiglitazone, reduced angiotensin II production.

## CONCLUSIONS

This thesis showed that measurement of the transcriptional activity of PPAR $\gamma$  indeed is feasible in primary human fat cell.

The main findings of the studies can be summarised as follows:

- ❖ A protocol for transfection of primary human adipocytes by electroporation and for measurement of the activity of several transcription factors was developed and optimised. The usefulness of the protocol for molecular biological studies was demonstrated.
- ❖ Saturated and monounsaturated fatty acid stimulated the transcriptional activity of PPAR $\gamma$ , which suggests that several components in the food can have direct hormonal effects in humans.
- ❖ The transcriptional activity of PPAR $\gamma$  was found to be lower in primary human adipocytes from visceral compared with subcutaneous fat, which relates to the fact that visceral adiposity is strongly linked to insulin resistance and type 2 diabetes.
- ❖ Adipose tissue and isolated adipocytes were found to produce components of RAS. Visceral adipocytes produced more angiotensin II than subcutaneous fat cells. Furthermore, angiotensin II was shown to inhibit PPAR $\gamma$  activity in visceral adipocytes, thus suggesting a new link between hypertension and insulin resistance.

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