Linköping University Medical Dissertations

No. 1123

On the importance of fat cell size, location and signaling in insulin resistance

Niclas Franck

Linköping University
FACULTY OF HEALTH SCIENCES

Linköping 2009

Printed in Sweden by LiU-Tryck, Linköping, 2009.

ISBN 978-91-7393-640-8
ISSN 0345-0082

To my beloved family

ABSTRACT

Obesity has reached epidemic proportions worldwide and is associated with insulin resistance, type 2 diabetes and cardiovascular disease. During the past decades, substantial evidence has demonstrated that not only the amount of adipose tissue constitutes a major determinant in the development of metabolic disorders, but also the distribution. The visceral adipose tissue has shown to be stronger correlated with insulin resistance, type 2 diabetes and cardiovascular disease than the subcutaneous depot. When we measured the activity of the nuclear receptor PPARγ in visceral and subcutaneous adipocytes, we found considerably lower activity in fat cells obtained from the visceral portion. This finding provides additional evidence to the unfavorable consequences of visceral obesity. The common PPARγ polymorphism Pro12Ala was studied in type 2 diabetic patients. We found that men with the Ala isoform exhibited higher sagittal abdominal diameter, waist circumference and body weight compared with homozygotes for the Pro isoform. However, no differences in either gender with regard to blood pressure or markers of cardiovascular disease and organ damage could be observed.

In addition to an excessive visceral adipose tissue mass, obese subjects with enlarged adipocytes display an increased risk for developing metabolic disorders compared with individuals exhibiting smaller fat cells but a similar degree of adiposity. The insulin responsiveness in small and large adipocytes obtained from the same subject was examined. Upon insulin stimulation, we found approximately a 2 fold increase of GLUT4 at the plasma membrane in small adipocytes, whereas the large fat cells were refractory to insulin induced GLUT4 translocation. This finding demonstrates a causal relationship between the accumulation of large fat cells in obese subjects and reduced insulin responsiveness.

Caloric restriction in humans ameliorates insulin responsiveness in liver and muscle prior to any substantial weight loss. By combining gene expression profiles of adipose tissue and adipocytes from human subjects undergoing either caloric restriction or overfeeding, we identified genes regulated by changes in caloric intake independent of weight loss *per se*. We found several genes under the control of mTOR and SREBP1 as well as genes involved in β-oxidation, liberation of fatty acids and glyceroneogenesis to be regulated during the interventions. These genes may indicate pathways and mechanisms mediating the effects of nutrient deprivation and obesity on morbidity and mortality

LIST OF PAPERS

This thesis is based on the following scientific papers, which will be referred to by their Roman numerals:

I. Sauma, L., Franck, N., Paulsson, J.F., Westermark, G.T., Kjølhede, P., Strålfors, P., Söderström, M., Nystrom, F.H. *Peroxisome proliferator activated receptor gamma activity is low in mature primary human visceral adipocytes*. Diabetologia, 2007. **50**(1): p. 195-201.

II. Franck, N., Stenkula, K.G., Öst, A., Lindström, T., Strålfors, P., Nystrom, F.H. *Insulin-induced GLUT4 translocation to the plasma membrane is blunted in large compared with small primary fat cells isolated from the same individual*. Diabetologia, 2007. **50**(8): p. 1716-22.

III. [1]Franck, N., [1]Gummesson, A., Jernås, M., Glad, C., Svensson, P.A., Guillot, G., Rudemo, M., Nystrom, F.H., Carlsson, L.M., Olsson, B. *Identification of adipose tissue genes regulated by caloric intake*. Submitted.

IV. Franck, N., Länne, T., Åstrand, O., Engvall, J., Lindström, T., Östgren, C.J., Nystrom, F.H. *The Ala isoform of the PPARγ Pro12Ala polymorphism is related to increased abdominal obesity in men but has little impact on cardiovascular risk markers in patients with type 2 diabetes*. Submitted.

[1] Contributed equally.

TABLE OF CONTENTS

ABSTRACT I

LIST OF PAPERS III

ABBREVIATIONS VII

BACKGROUND 1

The adipose tissue 1

Insulin action in adipocytes 2

Visceral and subcutaneous adipose tissue 6

Adipocyte size 9

PPARγ 13

PPARγ and adipocyte differentiation 14

PPARγ and insulin sensitivity 16

Genetic variants of PPARγ 19

AIMS 21

MATERIALS AND METHODS 23

RESULTS AND DISCUSSION 27

Paper I 27

Results 27

Discussion 27

Paper II 29

Results 29

Discussion 29

Paper III 31

Results 31

Discussion 32

Paper IV 33

Results 33

Discussion 34

CONCLUSIONS 35

ACKNOWLEDGEMENTS 37

REFERENCES 39

ABBREVIATIONS

11β-HSD1	11β-hydroxysteroid dehydrogenase 1
ACS	acetyl-CoA synthase
aP2	adipocyte fatty acid binding protein 2
AS160	Akt substrate of 160 kDa
C/EBP	CAAT/enhancer binding protein
CAP	Cbl associated protein
CPT1	carnitine palmitoyltransferase 1
CREB	cAMP response element binding protein
CrkII	CT10 related kinase II
DAG	diacylglycerol
DGK	diacylglycerol kinase
FATP-1	fatty acid transport protein 1
FOXO	forkhead protein
GLUT4	glucose transporter 4
Grb2	growth factor receptor-binding protein 2
HSL	hormone sensitive lipase
IFN-γ	interferon γ
IGF-1	insulin–like growth factor 1
IKKβ	inhibitor of nuclear factor-κB kinase
IL	interleukin
JNK	c-Jun N-terminal kinase
LC-CoA	long-chain acyl CoA
LPL	lipoprotein lipase
MAP	mitogen activated protein
MCD	malonyl CoA decarboxylase
MCP-1	monocyte chemoattractant protein 1
mTOR	mammalian target of rapamycin
N-CoR	nuclear receptor co-repressor
PAI-1	plasminogen activator inhibitor 1
PDE3B	phosphodiesterase 3B
PDK1	phosphoinositide-dependent kinase 1
PEPCK	phosphoenolpyruvate

PGC-1α	peroxisome proliferator activated receptor co-activator 1α
PGI_2	prostaglandin I_2
PI3K	phosphatidylinositol-3-kinase
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
PKA	protein kinase A
PKC	protein kinase C
PPAR	peroxisome proliferator activated receptor
PPRE	peroxisome proliferator response element
PTGIR	prostacyclin receptor
RBP4	retinol binding protein 4
RXR	retinoid X receptor
S6K1	ribosomal protein S6 kinase 1
SH2	Src homology 2
SIRT1	deacetylase sirtuin 1
SMRT	silencing mediator of retinoic acid and thyroid hormone receptor
SOS	son of sevenless
SREBP1	sterol response element binding protein 1
TCA	tricarboxylic acid
TAG	triacylglycerol
TNFα	tumor necrosis factor α
TZD	thiazolidinedione

BACKGROUND

Obesity has reached epidemic proportions worldwide and is associated with insulin resistance, type 2 diabetes (T2D) and cardiovascular disease, pathological conditions that account for a significant number of premature deaths every year. The most prominent feature of T2D is insulin resistance in liver, muscle and adipose tissue thus resulting in a diminished response to insulin and an impaired glucose uptake. The adipose tissue constitutes a central component in the development and progression of T2D that despite its limited part of total glucose uptake, now is accepted as a novel regulator of whole body insulin sensitivity and metabolism.

This thesis will focus on different mechanisms and responses in human primary adipocytes with regard to size, location and signaling in relation to insulin resistance and the impact of a common PPARγ polymorphism on obesity, blood pressure and markers of cardiovascular disease and organ damage in T2D subjects.

The adipose tissue

Throughout evolution, the adipose tissue has functioned as a fundamental survival system during periods of negative energy balance by releasing fatty acids to utilize as fuel, thus providing energy to seek more food. In accordance with the variations in energy availability, the human genome exhibits a large set of genes that favors storage of energy and few that protects against the effects of affluence. However, in modern society where caloric intake often exceeds energy expenditure, this evolutionary mechanism has instead become a burden by facilitating excessive lipid accumulation. As the adipose tissue expands, more fatty acids are released into the circulation and contribute to the development of insulin resistance by attenuating insulin stimulated glucose uptake in muscle and adipose tissue and glycogen synthesis in liver [1-3]. Although once considered as a passive energy storing depot, the adipose tissue is now also recognized as an endocrine organ that may communicate with the brain and peripheral tissues through adipokines such as leptin, adiponectin, tumor necrosis factor α (TNFα), resistin, interleukin 6 (IL-6) and retinol binding protein 4 (RBP4) that regulate appetite, metabolism and insulin sensitivity [4, 5]. During the past decades, substantial evidence has demonstrated that the release of fatty acids and adipokines to a large extent depend on the location of the adipose tissue (visceral or subcutaneous) as well as on the adipocyte size (small or large).

The correlation between obesity and insulin resistance is firmly established, particularly for the visceral fat depot or large adipocytes. Nevertheless, nutrient availability can modulate insulin sensitivity without the requirement of an obese phenotype. Overfeeding in rodents rapidly induces insulin resistance [6] whereas caloric restriction in humans ameliorates insulin responsiveness in liver and muscle [7], improvements that appear to occur prior to any substantial weight loss [8]. Thus, relative small fluctuations in energy balance may alter insulin sensitivity without any major effect on weight. Various integrated physiological feedback systems optimize energy uptake and storage during caloric deprivation whereas overfeeding conditions induce the cell to limit nutrient uptake, thus implying the existence of cell specific mechanisms that can detect changes in energy balance or nutritional availability. Indeed, several intrinsic pathways implicated in the modulation of insulin action respond to fluctuations in caloric load such as deacetylase sirtuin 1 (SIRT1) [9, 10], mammalian target of rapamycin (mTOR) [11] and malonyl CoA [12]. Increased activity of mTOR or elevated levels of malonyl CoA is associated with insulin resistance in both rodents and humans [11-13]. In contrast, SIRT1 appears to be a positive regulator of insulin sensitivity [14]. Studies in humans have demonstrated increased expression of SIRT1 during caloric restriction [10] whereas diet induced insulin resistance in mice decreases SIRT1 expression in conjunction with a decrease in insulin sensitivity [9].

Insulin action in adipocytes

In response to food intake, glucose levels increase and stimulate insulin secretion from the pancreatic β-cells into the circulation. Insulin signaling is characterized by a complex, highly integrated network that controls several processes such as glucose uptake, glycogenesis, lipogenesis and protein synthesis [15]. In adipocytes, the insulin receptor is located in small invaginations of the plasma membrane termed caveolae [16-18]. Binding of insulin to the extracellular ligand binding domain of the receptor induces autophosphorylation of the intracellular portion and activation of intrinsic tyrosine kinase activity [19] that further transmits the metabolic signal through the insulin receptor substrate (IRS) proteins, phosphatidylinositol-3-kinase (PI3K) and protein kinase B (PKB). The IRS family includes several members, although functional knockout studies have demonstrated that IRS1 constitutes the major mediator of insulin action in muscle and adipose tissue, whereas IRS2 mainly functions in liver [20]. The C-terminal of IRS1 contains a number of potential tyrosine phosphorylation residues that upon phosphorylation by the insulin receptor serves as docking

sites for proteins containing Src homology 2 (SH2) domains such as p85, the regulatory subunit of PI3K [21]. In addition to tyrosine phosphorylation, IRS proteins also undergo serine phosphorylation at multiple residues in response to insulin, cytokines and fatty acids [22]. Serine phosphorylation of IRS1 is increased in insulin resistant subjects and therefore considered as a negative regulator of insulin signaling [22]. Several IRS kinases including ribosomal protein S6 kinase 1 (S6K1) [23] and c-Jun N-terminal kinase (JNK) [24] are activated by insulin, thus indicating that serine phosphorylation of IRS1 may represent a negative feedback mechanism for the insulin signaling pathway. However, other studies have demonstrated an attenuated serine phosphorylation of IRS1 at specific residues in T2D subjects accompanied by reduced insulin induced tyrosine phosphorylation [25]. Upon association of p85 with IRS1, the catalytic subunit of PI3K p110 catalyzes the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) from phosphatidylinositol-4,5-bisphosphate (PIP_2) [26]. Notably, the regulatory subunits of PI3K are suggested to exist as monomers or heterodimers bound to p110 [27, 28]. In response to insulin stimulation, p85:p110, p55:p110 or p50:p110 binds to IRS1 and mediates normal insulin signaling, whereas the association of the monomeric forms with IRS1 appears to attenuate insulin action [27, 28]. Further supporting this, obese and T2D subjects display higher levels of p85, p55 and p50 in muscle compared with lean individuals [29], whereas caloric restriction in rodents reduces the abundance with a concomitant enhancement in glucose uptake [30]. Several proteins can bind to PIP_3 including members of the AGC family of serine/threonine kinases such as phosphoinositide-dependent kinase 1 (PDK1) that induces phosphorylation and activation of PKB at the plasma membrane [31, 32]. Similar to PDK1, PKB is a serine/threonine kinase and regulates glucose uptake by inhibiting the Rab-GTPase activating protein Akt substrate of 160 kDa (AS160) [33], thereby promoting cytoskeletal reorganization and translocation of the glucose transporter 4 (GLUT4) from intracellular vesicles to the plasma membrane where it facilitates glucose uptake into the cell (Figure 1) [34]. The expression of GLUT4 is almost exclusively restricted to muscle and adipose tissue, whereas the GLUT1 isoform is ubiquitously expressed although most abundant in erythrocytes and endothelial cells [35]. In the basal state, the majority of GLUT1 in muscle and adipose tissue localizes at the plasma membrane where it mediates basal glucose uptake during non insulin stimulated conditions [36]. In contrast to GLUT1, GLUT4 cycles between the plasma membrane and intracellular compartments [37]. Upon insulin stimulation, exocytosis of GLUT4 increases by 10-20 fold whereas endocytosis is only slightly reduced [37], thus resulting in a net increase of the glucose transporter at the plasma membrane.

Although the PI3K pathway is required for GLUT4 translocation to the plasma membrane, other studies indicate that the CAP/Cbl pathway may provide a second signal for insulin induced glucose uptake independent of PI3K activity [38]. Upon insulin stimulation, the insulin receptor promotes tyrosine phosphorylation of Cbl and its adaptor protein Cbl associated protein (CAP) [39], thus inducing the formed Cbl:CAP complex to dissociate from the receptor and migrate to lipid raft domains at the plasma membrane [40]. The signal is further transmitted via CT10 related kinase II (CrkII) and the Rho family member GTP-binding protein TC10 that through various effectors regulate insulin induced GLUT4 trafficking, docking and fusion at the plasma membrane (Figure 1) [41]. However, other studies have failed to confirm the importance of this pathway [42].

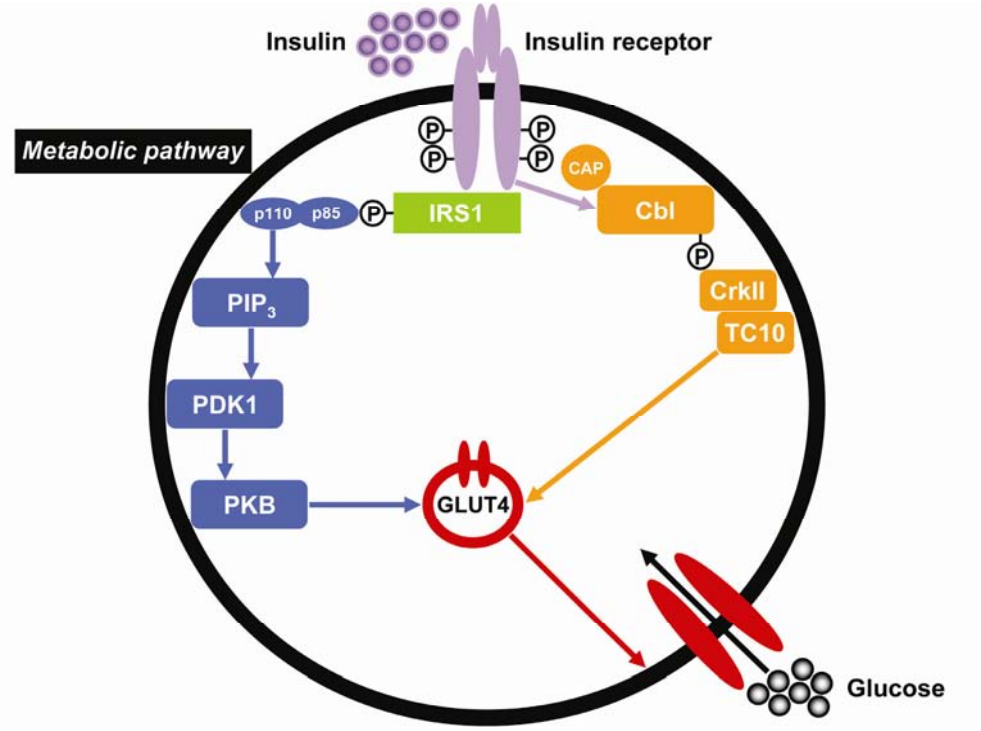

Figure 1. Metabolic effects of insulin. In response to increased glucose levels in the circulation, insulin binds to the insulin receptor that catalyzes the phosphorylation of IRS1 and Cbl. IRS1 and Cbl then interacts with other signaling molecules through their SH2 domains, thus resulting in a diverse series of signaling events such as activation of PI3K and the Cbl:CAP complex that act in a coordinate fashion to regulate glucose uptake via GLUT4.

Similar to other growth factors, insulin induces general gene expression, differentiation and cellular growth through the mitogen activated protein (MAP) kinase pathway. In adipocytes, the mitogenic effects are mainly mediated via the IRS proteins [43, 44]. Upon insulin induced tyrosine phosphorylation, IRS1 interacts with the SH2 adapter protein growth factor receptor-binding protein 2 (Grb2) [45] that in turn recruit Son of sevenless (SOS) to the plasma membrane. SOS functions as a guanine nucleotide exchange factor for the activation of Ras [46] that promotes the stepwise activation of Raf, MEK1/2 and ERK1/2 [47]. Activated ERK1/2 then migrate to the nucleus where they catalyze the phosphorylation of specific transcription factors that regulate differentiation and cellular growth (Figure 2) [48, 49].

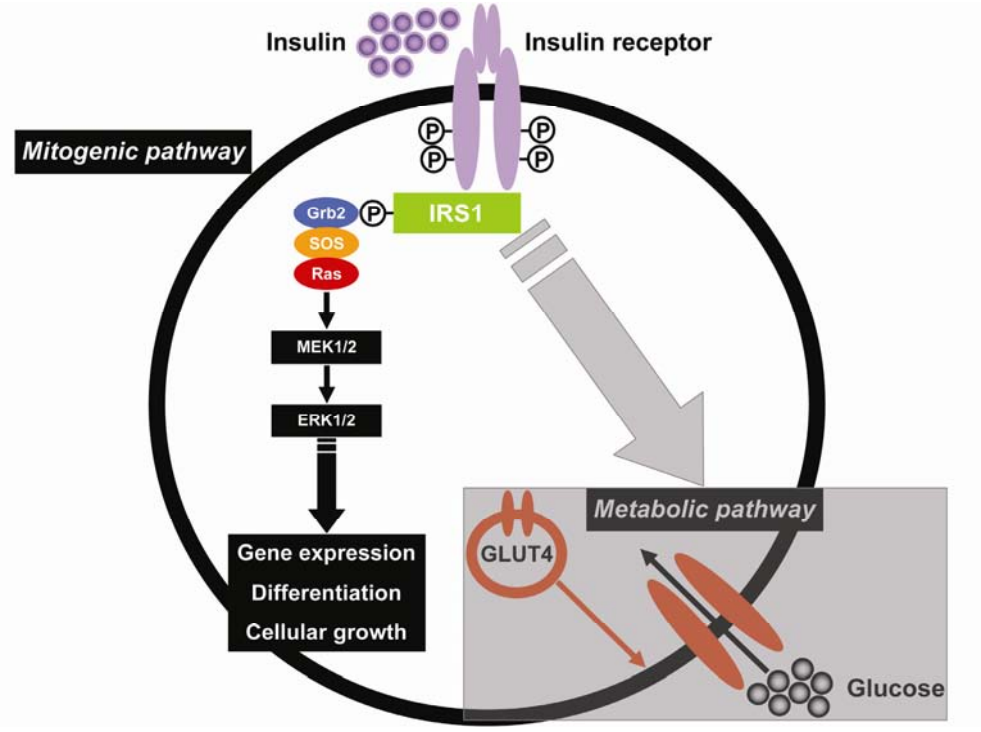

Figure 2. Mitogenic effects of insulin. In response to increased glucose levels in the circulation, insulin binds to the insulin receptor that catalyzes the phosphorylation of IRS1. IRS1 then interacts with the SH2 domain containing protein Grb2 that regulates gene expression, differentiation and cellular growth through the MAP kinase pathway.

Visceral and subcutaneous adipose tissue

Several epidemiological studies have demonstrated an increased prevalence of insulin resistance, T2D and cardiovascular disease among subjects with a high body mass index (BMI) [50, 51]. However, approximately 20 percent of all clinically obese individuals (BMI $\geq$30 kg/m^2) exhibit normal insulin sensitivity without any metabolic dysfunctions, a group collectively termed as metabolically healthy but obese (MHO) subjects [52]. In contrast, a considerable large part of the general population, approximately 18 percent, are normal with regard to BMI ($\leq$25 kg/m^2) but display several features of the metabolic syndrome [52]. Moreover, the majority of all subjects suffering from lipodystrophy, a syndrome characterized by partial or total loss of body fat, are insulin resistant and develops an abnormal metabolic profile similar to that observed in T2D [53]. During the past decades, substantial evidence has demonstrated that not only the amount of adipose tissue constitutes a major determinant in the development of insulin resistance and T2D, but also the distribution. The visceral adipose tissue, which mainly surrounds the internal organs, has been recognized as a more reliable predictor of metabolic and cardiovascular disorders than the subcutaneous depot [54]. Severely obese subjects display enhanced insulin sensitivity in response to surgical removal of the omentum, a part of the visceral adipose tissue that corresponds to less than 1 percent of the total body fat mass [55]. In contrast, surgical removal of 20 percent of the abdominal subcutaneous adipose tissue in obese glucose tolerant or T2D subjects appears to have limited impact on insulin sensitivity in liver, muscle and adipose tissue [56]. The underlying mechanisms responsible for this paradoxical outcome include anatomical factors as well as metabolic and hormonal differences between the two fat depots. In subcutaneous adipocytes, insulin exerts its anti-lipolytic effects through PKB dependent phosphorylation of phosphodiesterase 3B (PDE3B) that promotes hydrolysis of cyclic adenosine monophosphate (cAMP) and subsequently attenuated cathecolamine induced activity of protein kinase A (PKA) and hormone sensitive lipase (HSL) [57]. In contrast, visceral adipocytes are more responsive to cathecolamine induced activation of β-adrenergic receptors [58] and less sensitive to the anti-lipolytic effects of insulin [59, 60]. The impaired response to insulin may be attributed to a reduced binding capacity and autophosphorylation of the insulin receptor and a decreased phosphorylation of IRS1 and activation of PI3K [61]. However, opposite to the situation *in vitro*, activation of the insulin receptor, PKB and ERK1/2 in visceral adipocytes *in vivo* appears to be more rapid and intense [62] with a concomitant increase in insulin induced glucose uptake [62-64].

The visceral adipose tissue only accounts for a minor part of whole body production of circulating fatty acids, but an excessive abdominal fat accumulation, as clinically manifested by an increased waist circumference, is associated with hepatic insulin resistance in obese and T2D subjects [65, 66]. Due to its anatomical location, fatty acids originating from the visceral depot drain the liver through the portal vein and inhibit insulin induced suppression of glucose output [67, 68]. In addition, increased endogenous fatty acid synthesis and high fat diet may also contribute to hepatic insulin resistance [69]. Upon uptake in liver or muscle, fatty acids are subjected to mitochondrial β-oxidation or stored as triacylglycerol (TAG). It has been hypothesized that when the storage capacity of fatty acids as TAG or the ability to dispose them via oxidation reach past a critical limit during obesity or overfeeding conditions, metabolic intermediates such as long-chain acyl CoAs (LC-CoAs), diacylglycerol (DAG) and ceramide may accumulate in the cytosol and interfere with normal insulin action [70, 71]. In liver, the accumulation is suggested to be regulated by the glucose induced increase of malonyl CoA, the immediate precursor of *de novo* lipogenesis and main inhibitor of carnitine palmitoyltransferase 1 (CPT1), the rate limiting enzyme for import of LC-CoAs into the mitochondria [12]. As a result, LC-CoAs are redirected away from mitochondrial β-oxidation toward TAG synthesis, thereby promoting the formation of DAG and ceramide. Consistent with this model, overexpression of malonyl CoA decarboxylase (MCD) in liver, the enzyme that catalyzes the degradation of malonyl CoA, lowers circulating fatty acid levels and ameliorates diet induced insulin resistance in both liver and muscle in rodents [72]. Further contributing to the redirection toward TAG synthesis, insulin inhibits the expression of enzymes involved in β-oxidation by suppressing the activity of peroxisome proliferator activated receptor co-activator 1α (PGC-1α), a key regulator of mitochondrial biogenesis [73]. The lipid derived metabolites impair insulin sensitivity primarily by modulating the activity of components in the insulin signaling pathway. Both DAG and ceramide can activate several inhibitory serine kinases such as JNK, protein kinase C (PKC) and inhibitor of nuclear factor-κB kinase (IKKβ) that negatively regulates IRS1 and IRS2 through serine phosphorylation (Figure 3) [24, 74, 75], thereby attenuating downstream insulin signaling and subsequently suppression of glucose output.

Although an increased lipid intake is considered as the major determinant in diet induced insulin resistance, overfeeding is also often accompanied by high protein intake. Obese T2D subjects display elevated levels of amino acids in the circulation [76], and *in vivo* infusion of amino acids in animal models or humans attenuates glucose uptake in muscle, increases

hepatic gluconeogenesis and impairs insulin action [23, 77-79]. Recent studies indicate that the ability of amino acids to interfere with insulin responsiveness particularly involve branched chain amino acids that may activate mTOR and its downstream target S6K1, thus resulting in serine phosphorylation of IRS1 through similar mechanism as by JNK and PKC [23].

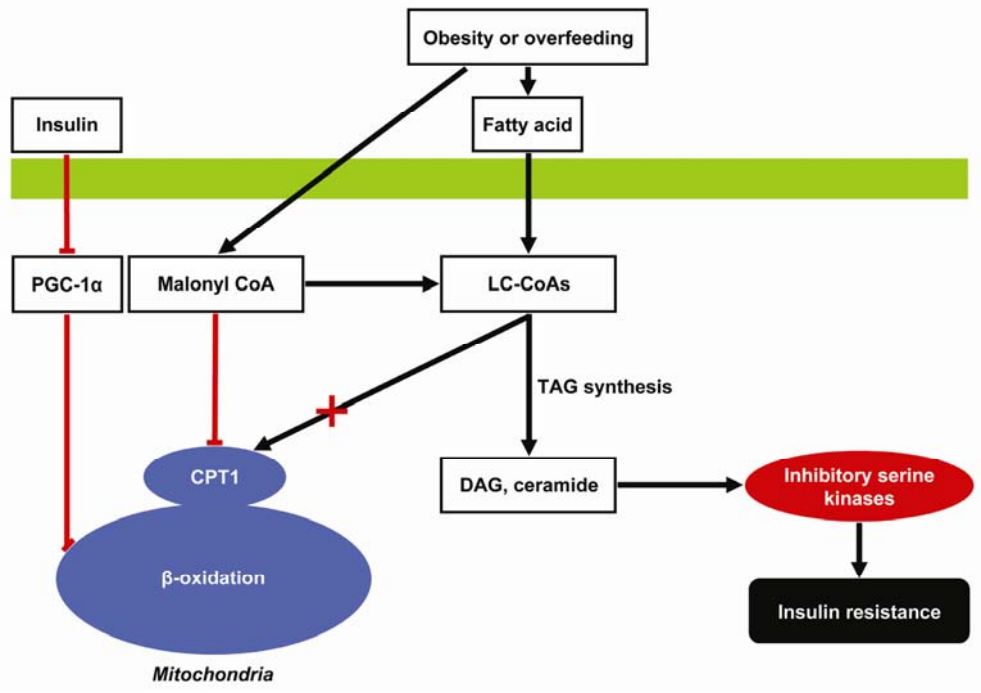

Figure 3. Fatty acid induced insulin resistance in liver. Excessive fatty acid uptake in liver leads to an attenuated β-oxidation and increased TAG synthesis, thus resulting in the accumulation of metabolic intermediates that interfere with insulin action. Insulin inhibits PGC-1α, thus further contributing to the reduced lipid oxidation and accumulation of metabolic intermediates.

Other depot specific differences implicated in the unfavorable consequences of an enlarged visceral adipose tissue mass involve the production and secretion of adipokines. Visceral adipocytes secrete more IL-6, IL-8, RBP4, plasminogen activator inhibitor 1 (PAI-1) and less adiponectin compared with subcutaneous fat cells [50, 80-85]. Furthermore, macrophage infiltration is more pronounced in the visceral adipose tissue and associated with hepatic inflammatory lesions [86].

Adipocyte size

The correlation between visceral obesity and insulin resistance is firmly established. However, the adipose tissue distribution alone cannot explain the differences in propensity for developing metabolic and cardiovascular disorders. Obese subjects with enlarged subcutaneous abdominal adipocytes display elevated levels of insulin and are more glucose intolerant compared with individuals exhibiting smaller fat cells but a similar degree of adiposity [87-90]. Thus, it appears as obese subjects with enlarged adipocytes (hypertrophic obesity) are more prone to develop insulin resistance compared with equally obese individuals but with an increased number of adipocyte (hyperplastic obesity) [87, 88]. Further supporting this, pharmacological activation of the peroxisome proliferator activated receptor γ (PPARγ) improves several components of the metabolic syndrome, effects partly mediated by a general decrease of the mean adipocyte size [91, 92]. Intriguingly, the number of adipocytes appears to be determined during childhood to adolescence [93], indicating that the differences in fat cell number between lean and obese subjects are established at a relative early age [94]. The mean adipocyte size varies according to sex, level of adiposity and anatomical location of the adipose tissue depot. Visceral and subcutaneous adipocytes increase in size with obesity in both men and women [95, 96]. However, normal weight to moderately obese women displays 20-30 percent smaller visceral adipocytes compared with their subcutaneous fat cells, whereas men appear to exhibit similar fat cell size in both depots independent of adiposity [95, 96].

The adipocyte displays a unique capacity to synthesize and store TAG in response to food intake as well as to hydrolyze TAG and release fatty acids between meals. During periods of nutrient deprivation, there is a dynamic equilibrium between the release of fatty acids into the circulation and oxidation in peripheral tissues such as liver or muscle where the majority of the utilization takes place. As caloric intake increases and exceeds energy expenditure, the adipocyte expands in size due to the increased TAG deposition. At early stages of obesity, the expanding adipocyte manages to actively store additional fatty acids by increasing the expression of enzymes involved in TAG synthesis, as well as to maintain nearly normal lipolytic rate during fasting [97]. The levels of circulating fatty acids may increase, but muscle still exhibits normal insulin sensitivity. However, as the enlargement further progresses, the biological activity of the adipocyte changes and affects whole body insulin sensitivity. Recent studies indicate that large adipocytes are more insulin resistant [88, 98, 99] and metabolically active with regard to lipolysis [100] compared with fat cells of smaller mean size. Indeed, enlarged adipocytes are more frequently found in obese and T2D subjects

compared with normal weight individuals [88, 101] and one of the main characteristics of obese subjects are high rates of fatty acid breakdown and uptake (fatty acid flux) in the adipose tissue [102].

Considering that the subcutaneous adipose tissue constitutes approximately 80 and 95 percent of the total fat mass in men and women, respectively [103], the detrimental effects of circulating fatty acids and adipokines on muscle is most likely mediated by those originating from the subcutaneous depot whereas the visceral portion mainly affects liver metabolism. *In vivo* infusion of fatty acids promotes accumulation of TAG in muscle and induces peripheral insulin resistance in insulin sensitive subjects [104, 105], whereas administration of lipid lowering drugs such as Acipimox improves glucose tolerance in both lean and obese subjects as well as in obese T2D individuals [106]. Indeed, levels of lipid metabolites such as LC-CoAs, DAG, ceramide and malonyl CoA in muscle are positively correlated with TAG content and negatively correlated with insulin sensitivity in obese animal models and humans [70, 107-109]. Moreover, T2D subjects display decreased expression of diacylglycerol kinase δ (DGKδ) and reduced activity of total diacylglycerol kinase (DGK) [110], enzymes that catalyze the phosphorylation of DAG to phosphatidic acid (PA), thus further contributing to the accumulation of DAG. Similar to the situation in liver, obese and insulin resistant subjects display reduced activity of CPT1 and impaired oxidation capacity in muscle [111, 112] as well as decreased expression of PGC-1α [113, 114]. Consistent with this, overexpression of CPT1 in animal models enhances fatty acid oxidation and ameliorates diet induced insulin resistance [115, 116]. Thus, the reduced ability to utilize fatty acids in muscle during conditions of high fatty acid flux such as obesity or overfeeding appears to induce accumulation of metabolic intermediates that promote insulin resistance by similar mechanisms as in liver. Conversely, enhanced muscle oxidation capacity would therefore reduce the levels of metabolic intermediates with a concomitant improvement in insulin responsiveness. However, although several studies have demonstrated a reduced oxidation of fatty acids and an impaired mitochondrial function [117], obese, insulin resistant and T2D subjects in other studies display normal oxidation capacity [118, 119] as well as normal or increased mitochondrial function compared with normal weight individuals [120, 121]. Furthermore, highly insulin sensitive athletes exhibit similar or higher levels of TAG in muscle compared with those observed in obese or T2D subjects [122], and exercise intervention in T2D individuals improves insulin responsiveness in muscle without a coordinated decrease in LC-CoA content [123]. Thus, it is suggested that during obesity or

overfeeding conditions, intermediates derived from an increased fatty acid oxidation rather than biosynthesis accumulate in the mitochondria and interfere with insulin signaling. Further supporting this, diet induced obesity in rodents cause increased expression of genes involved in β-oxidation but without a concomitant increase in downstream metabolic pathways such as the tricarboxylic acid (TCA) cycle and electron transport chain [124, 125]. As a result, due to the reduced ability of the TCA cycle to utilize acetyl-CoA derived from the overloaded β-oxidation, metabolic intermediates such as acylcarnitine accumulate in the mitochondria [124]. Notably, exercise intervention appears to reverse this accumulation by increasing TCA cycle activity [124].

Recently, another hypothesis has emerged suggesting an impaired ability to switch from fatty acid to glucose oxidation during the transition from negative to positive energy balance, known as metabolic inflexibility, to be involved in obesity induced insulin resistance [112]. This has been further demonstrated at both the whole body level and in isolated muscle mitochondria obtained from rodents subjected to high fat diet [126]. Moreover, specific ablation of MCD in human muscle cells increases malonyl CoA levels accompanied by a reduced lipid oxidation activity, thereby promoting the transition to glucose oxidation [127]. Intriguingly, the increased glucose utilization is not accompanied by enhanced insulin signaling, thus suggesting that MCD ablation in muscle promotes glucose uptake without the requirement of insulin induced activation and phosphorylation of IRS1, PI3K and PKB [127]. Thus, fatty acid induced insulin resistance in muscle provides conflicting results whereas a large body of evidence supports the hypothesis that an impaired oxidation with subsequent accumulation of cytosolic lipid intermediates constitute the major determinant in hepatic insulin resistance. In contrast, overloaded lipid supply to muscle may result in both an increased diversion of LC-CoAs into cytosolic lipid products and an enhanced mitochondrial accumulation of incomplete oxidation intermediates (Figure 4).

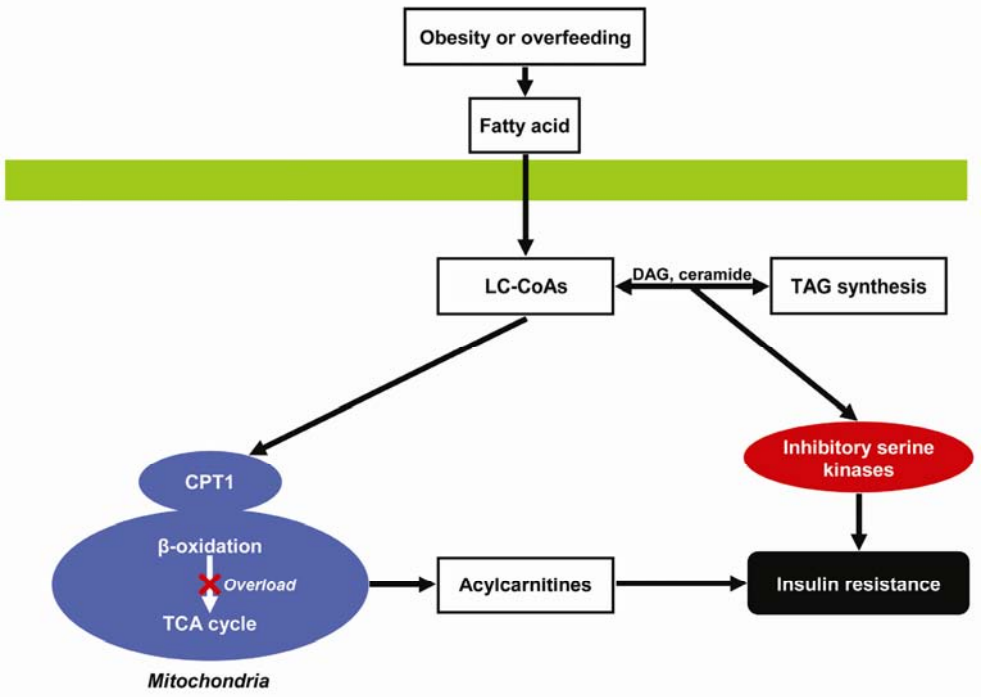

Figure 4. Fatty acid induced insulin resistance in muscle. Excessive fatty acid uptake in muscle leads to an overloaded β-oxidation and increased TAG synthesis, thus resulting in the accumulation of metabolic intermediates that interfere with insulin action.

Similar to visceral compared with subcutaneous adipocytes, large and small fat cells differ in their production and secretion of adipokines. Large adipocytes are suggested to secrete more leptin, TNFα, IL-1β, IL-6, IL-8 and less adiponectin compared with fat cells of smaller mean size [82, 128-132]. Further demonstrating the unfavorable consequences of enlarged adipocytes, conditioned media from human fat cells reduce insulin induced phosphorylation of PKB in a BMI and adipocyte size dependent manner [133]. Moreover, hypertrophic adipocytes secrete large amounts of monocyte chemoattractant protein 1 (MCP-1) [134]. Consistent with the increased MCP-1 secretion, macrophage content in adipose tissue from obese subjects may constitute up to 50 percent of the total cell number, compared with 5-10 percent in lean individuals [135]. In addition, recruited macrophages also secrete TNFα that further accelerates the development of inflammation in the adipose tissue [136] and release of fatty acids by inhibiting tyrosine phosphorylation of IRS1 [137].

PPARγ

The hallmarks of insulin resistance and T2D are a diminished response to insulin and decreased glucose induced insulin secretion. Synthetic ligands of the PPARγ receptor, a class of compounds collectively termed as thiazolidinediones (TZDs), display strong antidiabetic properties and are in clinical use for treatment of T2D. The peroxisome proliferator activated receptors (PPARs) form a subfamily of the nuclear receptor superfamily. The PPARs exists as three isoforms; PPARα, PPARδ and PPARγ, each encoded by separate genes [138]. All PPAR subtypes are ligand dependent transcription factors that regulate the expression of specific target genes upon binding to peroxisome proliferator response elements (PPREs) [138]. In contrast to steroid hormone receptors that function as homodimers, PPARs bind to PPREs as heterodimers with the retinoid X receptor (RXR) [139]. Similar to PPARs, RXRs exists as three isoforms; RXRα, RXRβ and RXRγ, all of which are activated by endogenous 9-cis retinoic acid [140]. The PPARγ receptor is highly expressed in adipose tissue and regulates several genes of importance for adipocyte differentiation, lipid metabolism and insulin sensitivity [141, 142]. The PPARγ1 splice variant is ubiquitously expressed although most abundant in adipose tissue [143, 144] whereas PPARγ2 is restricted almost exclusively to adipocytes [143, 144]. In the absence of ligand, the PPARγ:RXR heterodimer binds to co-repressor protein complexes such as nuclear receptor co-repressor (N-CoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) [145-147]. The co-repressors function to repress gene transcription through histone deacetylases that stabilize the chromatin structure and thus prevent transcription factors from associating with the DNA (Figure 5) [145]. Conversely, upon ligand binding to the ligand binding domain (LBD), the PPARγ receptor undergoes a conformational change that promotes degradation of bound co-repressor protein complexes and recruitment of transcriptional co-activator protein complexes that initiate gene expression by inducing acetylation of histones and chromatin remodeling (Figure 5) [145].

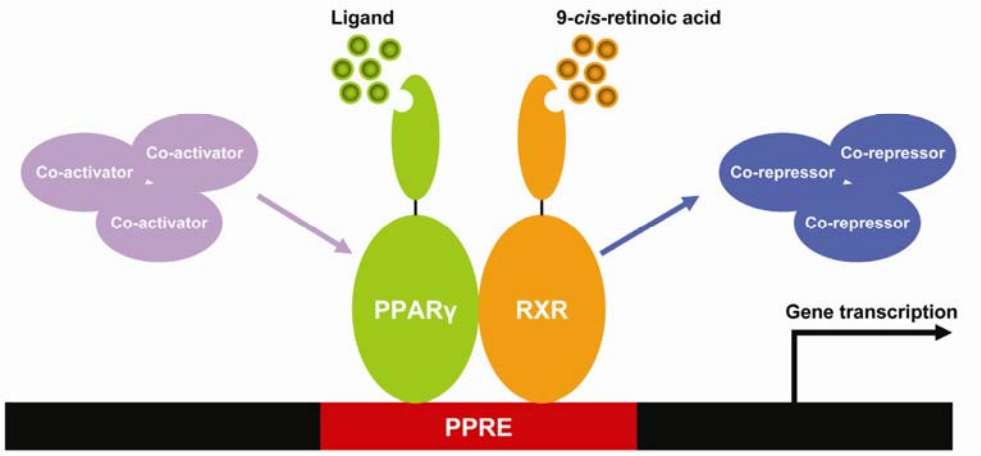

Figure 5. Activation of PPARγ target genes. In the absence of ligand, the PPARγ:RXR complex located on the PPARγ response element (PPRE) is associated with co-repressor protein complexes that prevent gene transcription. Binding of ligand to PPARγ and RXR induces degradation of the co-repressor protein complexes and recruitment of co-activator protein complexes that promote chromatin remodeling and binding of transcription factors for the initiation of gene transcription.

PPARγ and adipocyte differentiation

During differentiation, the adipocyte develops from a fibroblast like preadipocyte into a mature fat cell, a process characterized by lipid accumulation and an increased expression of fat cell specific markers such as adipocyte fatty acid binding protein 2 (aP2) and lipoprotein lipase (LPL), both being target genes of PPARγ [142]. TZD administration enhances insulin sensitivity, a mechanism partly mediated by stimulating the differentiation of preadipocytes, thereby increasing the number of small and more insulin responsive fat cells in the subcutaneous adipose tissue portion [148], although other studies suggest a similar impact on adipogenesis in both fat depots [149]. The expression and activation of PPARγ is sufficient to induce adipogenesis [150] and its importance has been further demonstrated in both functional and knockout studies [151-153]. Although PPARγ is considered as the master regulator, the receptor exerts its adipogenic effects in a cooperative fashion with other transcription factors such as sterol response element binding protein 1 (SREBP1) and members of the CAAT/enhancer binding protein (C/EBP) family [142]. The differentiation process is often divided into two separate phases. The first wave (determination phase) involves activation of C/EBPβ and C/EBPδ that promote the conversion of pluripotent stem

cells to preadipocytes [154]. At this stage, the preadipocyte lacks the potential to differentiate into other cell types, but still exhibits a similar morphological appearance as its precursor cell. In the second wave (terminal differentiation phase), the preadipocyte finalizes its maturation into a fully functional fat cell, a process mediated by C/EBPα and PPARγ [150, 155]. SREBP1 alone lacks significant impact on adipogenesis, but participates in the differentiation process by increasing the expression of PPARγ [156], PPARγ ligands [157] and fat cell specific genes [158-160].

Synthetic ligands of the PPARγ receptor such as TZDs display strong adipogenic properties, but the signals that stimulate differentiation *in vivo* remain to a large extent uncharacterized. A diet rich in saturated fatty acids induces both hypertrophy and hyperplasia, although the importance of these compounds in the *de novo* differentiation still remains unknown. Polyunsaturated fatty acids exhibit strong adipogenic properties *in vitro* by their ability to act as ligands for PPARγ, but are much less potent to initiate differentiation of preadipocytes *in vivo* [161, 162]. Opposite to the general effects of polyunsaturated fatty acids, prostaglandins appear to more specifically target adipogenesis. Prostaglandin I_2 (PGI_2) is a major metabolite of arachidonic acid in adipose tissue. Upon binding to the prostacyclin receptor (PTGIR) with subsequent activation of adenylyl cyclase, cAMP levels increase and induce C/EBPβ and C/EBPδ to initiate the determination phase of adipogenesis [163]. Adipocytes also secrete large amounts of the angiotensin precursor angiotensinogen, which promotes release of PGI_2 from fat cells and thereby further stimulating differentiation [164]. Glucocorticoids are potent inducers of preadipocyte differentiation *in vitro*. Stimulation with glucocorticoids increase the expression of C/EBPδ and PPARγ in 3T3-L1 cells [165, 166] and glucocorticoid receptors are expressed on human preadipocytes [167]. The enzyme 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) sensitizes tissues to glucocorticoids and is produced in human preadipocytes. During differentiation of 3T3-L1 cells, the synthesis of 11β-HSD1 is markedly increased [168], presumably via the transcriptional induction by C/EBPα [169]. Insulin is an anabolic hormone that is highly involved in preadipocyte differentiation. At early stages adipogenesis, the abundance of the insulin receptor is low and insulin functions mainly through insulin–like growth factor 1 (IGF-1) receptors, although this ratio shifts as the differentiation progresses [170]. Upon insulin stimulation, IGF-1 relays the signal via IRS1, PI3K and PKB [171-174] that through a complex network of different effectors such as cAMP response element binding protein (CREB) and forkhead protein 1 and 2 (FOXO1 and FOXO2) promote preadipocyte differentiation [175].

The majority of factors that regulate differentiation are of endocrine nature, but local signals that reflect TAG content in adjacent adipocytes may also contribute to the fate of immature fat cells. Conditioned media from enlarged adipocytes contain factors that induce proliferation and differentiation of preadipocytes [176]. Thus, it has been hypothesized that when adipocytes expand in size past a critical lipid content, they start to produce different protein and lipid metabolites [130, 177] that function to initiate differentiation of neighboring preadipocytes [87, 178].

The fate of preadipocytes is tightly regulated by a delicate balance between pro-adipogenic and anti-adipogenic signals. The most extensively studied factors that inhibit preadipocyte differentiation *in vitro* include several of inflammatory cytokines such as TNFα, interferon γ (IFN-γ), IL-1 and IL-6 [179-181], although the impact of these mediators on adipogenesis *in vivo* still remains uncharacterized.

PPARγ and insulin sensitivity

The PPARγ receptor is highly expressed in adipose tissue, whereas the expression in liver and muscle is low [143, 144]. Nevertheless, TZD administration improves glucose homeostasis in T2D subjects by enhancing insulin sensitivity in liver and muscle [182-184]. Furthermore, animal models lacking adipose tissue are refractory to the insulin sensitizing effects of TZDs [185] and muscle specific ablation of PPARγ expression in mice results in hepatic insulin resistance but is reversed by TZD administration [186], thus suggesting PPARγ in adipose tissue as the major target for the insulin sensitizing effects of TZDs. At the core of this, a "lipid steal" hypothesis has emerged [187]. Insulin resistance and T2D are associated with elevated levels of circulating fatty acids and ectopic lipid deposition that compromises systemic glucose disposal in insulin sensitive tissues [70, 71, 107, 108]. However, upon TZD mediated PPARγ activation, a large set of genes involved lipid uptake and storage such as aP2, LPL, CD36, phosphoenolpyruvate (PEPCK), acetyl-CoA synthase (ACS) and fatty acid transport protein 1 (FATP-1) act in a coordinate fashion to sequester fatty acids and accumulated TAG away from the circulation, liver and muscle into the adipose tissue (Table 1) [142, 188-193], thereby attenuating the overloaded TAG synthesis in liver and lipid oxidation or TAG synthesis in muscle. Consistent with this, TZDs lower systemic levels of fatty acids [184, 194] and induce a modest weight gain [195-197].

In addition to promote lipid uptake and storage in the adipose tissue, TZD mediated PPARγ activation also modulates the production and secretion of several adipokines implicated in the pathogenesis of insulin resistance and T2D. Adiponectin, a plasma protein exclusively produced in the adipose tissue is negatively correlated with adipose tissue mass and positively correlated with insulin sensitivity [198, 199]. The adiponectin gene is a direct target for regulation by PPARγ [200] and consequently, TZD administration increases plasma levels in humans [201, 202]. In animal models, adiponectin administration suppresses hepatic glucose output, promotes oxidation of fatty acids in muscle and improves whole body glucose homeostasis [203-206], presumably by reducing TAG content in liver and muscle. TZDs also reduce secretion of the adipose tissue specific hormone leptin [207, 208] that functions to suppress food intake and increase energy expenditure [209]. Administration of leptin to animal models promotes oxidation of fatty acids [210] and glucose disposal [211, 212], which is accompanied by a reduced accumulation of TAG in liver and muscle [213]. However, elevated levels are associated with obesity and insulin resistance in humans, thus suggesting leptin resistance in target tissues [214, 215]. In contrast to induction of insulin sensitizing adipokines, TZDs also suppress the secretion of diabetogenic factors such as TNFα [216, 217]. Levels of TNFα are elevated in obese subjects [218] and similar to fatty acids, exogenously administrated TNFα attenuates both insulin signaling and glucose transport in muscle, partly by promoting serine phosphorylation of IRS1 [219, 220]. However, *in vivo* neutralization of TNFα by anti-TNFα antibodies or TNFα antagonists seems to have limited impact on insulin sensitivity in humans [221-224].

Table 1. Genes regulated by PPARγ.

Upregulated genes	Function
Adipocyte fatty acid binding protein (aP2)	Intracellular fatty acid binding
Acyl-CoA synthetase (ACS)	Lipogenesis and/or catabolism
Phosphoenolpyruvate (PEPCK)	Glycerol synthesis for triacylglycerol
Lipoprotein lipase (LPL)	Hydrolysis of triacylglycerol
Thrombospondin receptor (CD36)	Cell surface fatty acid transporter
Fatty acid transport protein 1 (FATP-1)	Cell surface fatty acid transporter
Cbl-associated protein (CAP)	Insulin signaling toward glucose uptake
Glucose transporter 4 (GLUT4)	Glucose uptake
Pyruvate dehydrogenase kinase 4 (PDK4)	Inhibits glucose oxidation
Adiponectin	Improves insulin sensitivity
Downregulated genes	**Function**
Tumor necrosis factor α (TNFα)	Promotes insulin resistance
Resistin	Promotes insulin resistance
Leptin	Regulates appetite

Genetic variants of PPARγ

Hypertension is a complex disorder of the cardiovascular system and closely associated with insulin resistance. The prevalence of hypertension is approximately 2 fold higher among T2D subjects compared with the general population [225]. Furthermore, TZD administration lowers blood pressure in insulin resistant, obese and T2D subjects [226, 227]. Considering that lower blood pressure correlates with decreased insulin levels, the blood pressure lowering effect may partly be mediated by the improved insulin sensitivity [226]. A number of genetic variants in the PPARγ gene have been identified. The very rare gain of function polymorphism, where a proline is substituted to a glutamine (Pro115Gln), is associated with obesity but not insulin resistance (Figure 6) [228]. The Pro115Gln mutation inhibits serine phosphorylation at position 114 in the ligand-independent activation function 1 (AF-1) region of the PPARγ2 splice variant, thus resulting in a constitutively active protein and an increased differentiation of preadipocytes with a concomitant accumulation of cellular TAG content [228]. In contrast, carriers of the more highly prevalent PPARγ polymorphism Pro12Ala, where a proline is substituted to an alanine at position 12, display lower affinity for PPRE and thus a reduced transcriptional activity of PPARγ target genes (Figure 6) [229]. The polymorphism, also located in the AF-1 region, occurs with extremely variable frequencies in populations of different origins [230]. Carriers of the Ala variant display a modest reduction in BMI, slightly improved insulin sensitivity and reduced prevalence of T2D compared to wild type [229]. However, other studies have failed to confirm these results [231-234].

Figure 6. Genetic polymorphisms in PPARγ2. The Pro12Ala and Pro115Gln polymorphisms located in the AF-1 domain of PPARγ2 are associated with a reduced and gain of function respectively. AF-1, ligand-independent activation function 1; DBD, DNA binding domain; LBD, ligand binding domain.

AIMS

- To evaluate the PPARγ activity in visceral compared with subcutaneous human primary adipocytes obtained from the same subjects.

- To evaluate the insulin responsiveness in small compared with large human primary subcutaneous adipocytes obtained from the same subjects.

- To evaluate the gene expression response in human primary subcutaneous adipocytes during caloric restriction and overfeeding.

- To evaluate the impact of the PPARγ Pro12Ala polymorphism on obesity, blood pressure and markers of cardiovascular disease and organ damage in T2D subjects.

MATERIALS AND METHODS

Isolation of adipocytes (Paper I-III)

Visceral and/or subcutaneous abdominal human adipose tissue was removed during surgery (Paper I-II) or by needle aspiration (Paper III). The adipose tissue was cleared from vascular and fibrous structures, rinsed in 0.9% (w/v) NaCl and digested in an equal volume of Krebs Ringer solution (0.12 M NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4) containing 20 mM HEPES, pH 7.4, 3.5% (w/v) fatty acid-free bovine serum albumin (Roche, Mannheim, Germany), 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 a gauze web. The adipocytes were then washed with Krebs Ringer solution containing 20 mM HEPES, pH 7.4, 1% (w/v) fatty acid-free bovine serum albumin, 200 nM adenosine and 2 mM glucose and incubated at 37°C in a water bath with agitation until further analysis. Mouse adipocytes were isolated as described above (Paper I).

Separation of small and large adipocytes (Paper II)

Adipocytes were separated into one small and one large fraction using nylon filters (Millipore, Billerica, MA, USA). To collect the small adipocyte fraction, 1 ml of fat cells were suspended in 45 ml of Krebs Ringer solution and gently run through a nylon filter with a pore size of 60 μm. The fat cells that passed through the filter constituted the fraction of small adipocytes. The adipocytes that were trapped on the 60 μm filter were passed through a second nylon filter with a pore size of 120 μm. Those fat cells that were retained on this second nylon filter were collected and constituted the fraction of large adipocytes. After separation, each fraction was allowed to recover overnight at 37°C, 10% CO_2 in Krebs Ringer solution mixed with an equal volume of Dulbecco´s modified Eagle´s medium, pH 7.5, containing 25 mM glucose, 50 UI/ml penicillin, 50 μg/ml streptomycin, 200 nM phenylisopropyladenosine, 7% (w/v) fatty acid-free bovine serum albumin and 25 mM HEPES. Aliquots from each fraction were stained using Mayer's Haematoxylin to visualise the nuclei of adipocytes, thereby distinguishing from other particles such as lipid droplets. The cell diameters of the adipocytes were measured using Scion Image (Scion Corporation, Frederick, MD, USA). Representative pictures were taken at 10-20× magnification using an E800 Eclipse Microscope (Nikon, Tokyo, Japan).

Electroporation and Luciferase assay (Paper I)

Each 0.4-cm gap electroporation cuvette was filled with 200 µl of the adipocyte solution and an additional 200 µl of PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.5) containing (1) a plasmid encoding firefly luciferase cDNA under the control of a PPARγ response element from the acyl-CoA oxidase gene, termed pAOX-Luc (2 µg/cuvette) or (2) a plasmid containing the ligand binding domain of PPARγ fused to the yeast GAL4 DNA binding domain, termed GAL4-PPARγ (2 µg/cuvette) together with a plasmid encoding firefly luciferase cDNA under the control of a GAL4 response element, termed 5xGAL4-TK-LUC (2 µg/cuvette). In addition, the adipocytes for each condition were transfected with a plasmid that constitutively express renilla luciferase, termed pRluc (0.1 µg/cuvette). Electroporation of human and mice adipocytes were performed with one square wave pulse at 400 V 4 ms using a Bio-Rad GenePulser II (Bio-Rad Laboratories, Hercules, CA, USA). Cells for each condition were then pooled and incubated at 37°C in 10% CO_2. After 1 h of incubation, an equal volume of Dulbecco´s modified Eagle´s medium, pH 7.5, containing 25 mM glucose, 50 UI/ml penicillin, 50 µg/ml streptomycin, 200 nM phenylisopropyladenosine, 7% (w/v) fatty acid-free bovine serum albumin and 25 mM HEPES was added and adipocytes incubated until luciferase assay. In some experiments with adipocytes transfected with the pAOX-Luc plasmid, different concentrations of rosiglitazone were added 1 h after electroporation. After 18 h incubation, the adipocytes were lysed and assayed for firefly and renilla luciferase using the Dual-Luciferase Reporter Assay System (Promega, WI, USA). Cell lysates were prepared with 200 µl of supplied lysis buffer by passing through a 25-gauge needle. The measurement of firefly luciferase was initiated by adding 100 µl of Luciferase Assay Reagent II to each aliquot of 50 µl cell lysate. After quantification, the reaction was quenched and measurement of renilla luciferase initiated by adding 100 µl Stop & Glo Reagent. The activity of both firefly and renilla luciferase was measured using Victor 1420 multilabel counter (Wallac, Turku, Finland). The induced amount of firefly luciferase was normalized according to the constitutively expressed renilla luciferase, thus correcting for any differences in transfection efficiency.

Immunoblotting (Paper I-II)

Adipocytes 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 okadic acid, 1% (v/v) Triton X100 and 0.1% (w/v) SDS by passing through a 25-gauge needle and then centrifuged at 400 g for 10 min, 4°C (Paper I). The small and large fraction of adipocytes was treated with or

without 10 nM of insulin for 10 min at 37°C in a water bath with agitation. The incubation was terminated by separating the fat cells from medium by centrifugation at 5000 g through dinonylphthalate. To minimize post incubation changes in signaling and protein modifications, the adipocytes were immediately dissolved in SDS and β-mercaptoethanol with PMSF, 0.7 µg/ml pepstatin, 6.5 µg/ml aprotinin, 5 µg/ml leupeptin and 100 nM okadic acid, frozen within 10 s and thawed in boiling water (Paper II). Equal amounts of whole cell lysate (Paper I) or cells according to lipocrit (Paper II) were subjected to SDS-PAGE and transferred to polyvinylidine diflouride membranes (Paper I-II) or nitrocellulose membranes (Paper I). Membranes were incubated with antibodies against the protein of interest. Bound antibodies were detected by the secondary horseradish peroxidase (HRP) conjugated anti-IgG antibody using the ECL+ system (Amersham Biosciences, Amersham, UK) and quantified by chemiluminescence imaging (Las 1000, Image Gauge, Fuji, Tokyo, Japan).

Immunofluorescence confocal microscopy (Paper II)

Plasma membrane sheets were prepared as described [18] and fixed in phosphate buffer (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.5) containing 3% (v/v) paraformaldehyde for 30 min at room temperature. After blocking in bovine serum albumin, membranes were incubated with anti-GLUT4 or anti-caveolin-1 antibodies. The primary antibody was detected using a fluorescent secondary antibody (Molecular Probes, Carlsbad, CA, USA) and quantified with a DMIRE2 microscope (Leica). Labeling against GLUT4 was measured in 40 cells from each patient (10 in each adipocyte fraction, with or without insulin, respectively) with the same microscopic and software settings for all membranes. No labeling was observed in the absence of the primary antibody. Fluorescence intensity was measured with Scion Image (Scion Corporation, Frederick, MD, USA)

Total RNA isolation (Paper I, III)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then further purified with RNeasy mini columns (Qiagen, Hilden, Germany). The concentration was determined by spectrophotometer (Paper I, III) and high-quality RNA was confirmed using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) (Paper III).

Microarray (Paper III)

Labeled cRNA from reverse transcription of total RNA was fragmented and hybridized to the HG-U133 Plus 2.0 arrays according to manufacturers instructions (Affymetrix, Santa Clara, CA, USA). The microarrays were scanned with Affymetrix confocal laser scanner (GeneArray scanner GCS3000) and visualized using the Gene Chip Operating Software (GCOS, Affymetrix). Global background correction of probe intensities was performed using a method implemented in the robust multi-array average method (RMA) [235].

Real Time PCR (Paper III-IV)

Reagents for gene expression (Paper III) and genotyping (Paper IV) experiments were obtained from Applied Biosystems (Foster City, CA, USA) and used according to the manufacturers instructions. For gene expression analysis (Paper III), products were amplified and detected with the ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using default cycle parameters. Gene expression changes were evaluated according to the comparative C_T method. All standards and samples were analyzed in triplicate. For genotyping analysis (Paper IV), the PPARγ polymorphism Pro12Ala was detected with the ABI Prism 7500 Sequence Detection System (Applied Biosystems) using default cycle parameters and analyzed with SDS Software (Applied Biosystems).

RESULTS AND DISCUSSION

Paper I

In this study, we wanted to investigate the PPARγ activity in visceral compared with subcutaneous human primary adipocytes obtained from the same subjects.

Results

Basal PPRE and PPARγ activity in visceral and subcutaneous adipocytes

Basal PPRE activity displayed a median of 6.6 fold higher activity in subcutaneous compared with visceral adipocytes. To exclude the influence from other possible transcription factors present in the subcutaneous adipocytes, cells were transfected with a PPARγ fusion protein to measure the activity of the receptor. Similarly to PPRE, basal activity of the PPARγ fusion protein displayed a median of 6.2 fold higher activity in the subcutaneous compared with visceral adipocytes.

Quantification of endogenous PPARγ protein and co-activator/repressor mRNA levels

Levels of endogenous PPARγ receptor protein were 55 percent higher in the visceral adipocytes. The mRNA levels of different co-activators were similar in adipocytes from the two fat depots. However, the expression of the co-repressor N-CoR was approximately 2 fold higher in the visceral adipocytes. This difference was not however detected at the protein level.

Depot specific PPRE activity in response to rosiglitazone stimulation

Stimulation with different concentrations of the synthetic PPARγ ligand rosiglitazone demonstrated a dose dependent increase in PPRE activity in both visceral and subcutaneous adipocytes. However, PPRE activity in the visceral adipocytes was generally lower compared with the subcutaneous fat cells for all concentrations of rosiglitazone.

Discussion

We found a significantly lower basal PPRE activity in visceral compared with subcutaneous adipocytes obtained from the same subjects. There is however a possibility that other transcription factors present in the subcutaneous fat cells could bind to the transfected PPARγ response element and contribute to the higher activity. In order to exclude that, we used a

PPARγ fusion protein that allowed us to measure the activity of the transfected receptor. Similar to PPRE, the transfected PPARγ receptor displayed considerably lower basal activity in the visceral adipocytes. The lower visceral activity of PPARγ supports previous studies proposing that the effects of TZD administration are to a large extent restricted to the subcutaneous adipose tissue depot [148]. The lower PPARγ activity was not due to different amounts of endogenous PPARγ receptor protein since protein quantification demonstrated approximately 55 percent higher levels in visceral adipocytes compared with fat cells from the subcutaneous depot. Another possible explanation for the difference in activity could have been lower levels of endogenous ligands in the visceral adipocytes. However, stimulation with the highest rosiglitazone concentration could not induce PPRE activity in the visceral adipocytes to reach even basal levels in the subcutaneous fat cells. Visceral adipocytes have been shown to be more metabolic active with regard to lipolysis [61] and pharmacological activation of PPARγ promotes lipid uptake, thereby lowering the levels of circulating fatty acids [184]. Thus, low PPARγ activity may contribute to the increased secretion of fatty acids to the liver from the visceral depot, thus affecting liver metabolism [67, 68]. To explore possible mechanistic explanations, we measured mRNA levels of known co-activators and co-repressors of PPARγ. Similar mRNA expression was found in the two fat depots except for the co-repressor N-CoR that displayed approximately a 2 fold higher expression level in the visceral adipocytes. This difference was not however seen at the protein level.

Paper II

In this study, we wanted to investigate the insulin responsiveness in small compared with large human primary subcutaneous adipocytes obtained from the same subjects.

Results

Separation into small and large adipocytes

Separation into small and large adipocyte fractions was achieved using nylon filters of different pore sizes. After separation, the fraction of small cells was on average 81.3 μm and the large fraction on average 114 μm in diameter.

Insulin induced activation of the insulin receptor, IRS1 and PKB

The insulin induced activation of the insulin receptor, IRS1 and PKB in small and large adipocytes was analyzed by immunoblotting. Insulin stimulation significantly induced activation of the insulin receptor, IRS1 and PKB compared with non insulin stimulated cells. However, no differences between the small and large fractions could be observed.

Insulin induced GLUT4 translocation in small and large adipocytes

The insulin induced translocation of GLUT4 was analyzed by immunofluorescence confocal microscopy in small and large adipocytes. Insulin induced approximately a 2 fold increase of GLUT4 at the plasma membrane in small adipocytes, whereas no such increase was detected in the large fat cells.

Amount of the insulin receptor, IRS1, GLUT4 and caveolin-1

The protein levels of insulin receptor, IRS1, GLUT4 (immunoblotting) and caveolin-1 (immunofluorescence) in small and large adipocytes were examined. The amount of all proteins was similar in the two fat cell fractions.

Discussion

Enlarged adipocytes have been shown to be a strong predictor of T2D independent of obesity [98]. We found approximately a 2 fold insulin induced increase in the amount of GLUT4 at the plasma membrane in small adipocytes, whereas the large fat cells did not reveal any increase following insulin stimulation. This novel finding is in line with the effects of insulin sensitizing agents such as rosiglitazone, which improves several components of the metabolic syndrome in parallel with a general decrease in adipocyte size [91, 92]. Indeed, large

adipocytes have previously been associated with an impaired response to insulin [88, 98, 99], although these studies could not exclude potential differences in environmental or genetic factors. Similar to visceral compared with subcutaneous adipocytes, large fat cells have been suggested to be less responsive to the PKB mediated anti-lipolytic effects of insulin compared with small adipocytes [100]. We found no difference between small and large adipocytes with regard to phosphorylation of the insulin receptor, IRS1 or PKB, thus suggesting that the anti-lipolytic effects of insulin are still intact in large fat cells. However, we cannot exclude that a dose response curve with multiple concentrations of insulin concentrations could reveal a size difference with regard to phosphorylation of PKB between the two fractions.

Paper III

In this study, we wanted to investigate the gene expression response in human primary subcutaneous adipocytes during caloric restriction and overfeeding.

Results

Changes in energy intake and body weight

In the very low caloric diet (VLCD) study, the mean energy intake at baseline was 2307±568 kcal. After 8 weeks of VLCD (450 kcal), subjects decreased in weight on average 18.6 kg. During refeeding (week 16-18) when ordinary food was re-introduced, subjects increased their caloric intake considerably from 450 to 1407±557 kcal per day, accompanied by only a modest increase in weight of on average 0.8 kg.

In the Fast Food Study (FFS), the mean energy intake at baseline was 2323±550 kcal. During 4 weeks of fast food based hyperalimentation, subjects increased their caloric intake from 2723±550 to 5226±1109 kcal per day, accompanied by an increase in weight of on average 7.3 kg.

Identification of genes transcriptionally regulated in response to changes in energy balance

Genes that displayed decreased expression between baseline and week 8 in the VLCD study, increased expression between weeks 16 and 18 in the VLCD study and increased expression between baseline and week 4 in FFS, were defined as being positively regulated by caloric intake. Genes regulated in opposite directions were defined as being negatively regulated by caloric intake. Using these selection criteria, 50 genes were defined as positively regulated and 52 genes defined as negatively regulated by caloric intake. Overrepresented biological processes among the genes either positively or negatively regulated by caloric intake were evaluated using The Protein ANalysis THrough Evolutionary Relationship (PANTHER). We found that lipid and fatty acid metabolism was the major biological process positively regulated by caloric intake, whereas genes negatively regulated by caloric intake mainly were involved in protein synthesis and metabolism. SCD1, GPX3, C6 (real-time PCR) and PEDF (ELISA) were selected for verification of the microarray analysis. Results were in accordance with the effects observed in the microarray data.

Discussion

Caloric restriction enhances insulin sensitivity in humans [7] prior to any major weight loss and within a relative short time frame [8]. However, it can be difficult to discriminate genes regulated by diet from those that follows weight change during such interventions. In the present study, we identified genes regulated by changes in caloric intake by combining gene expression data from subjects undergoing either caloric restriction or overfeeding. We found several genes under the control of mTOR and SREBP1 that influence lipogenesis, glycolysis, and protein synthesis as well as cell size, to be regulated by changes in caloric intake. Furthermore, important members of the intrinsic defense against oxidative stress, β-oxidation and glyceroneogenesis displayed an altered expression during the interventions. This study provides important information of how adipose tissue is regulated at the transcriptional level by changes in caloric intake and may indicate pathways and mechanisms mediating the effects of nutrient deprivation and obesity on morbidity and mortality.

Paper IV

In this study, we wanted to investigate the impact of the PPARγ Pro12Ala polymorphism on obesity, blood pressure and markers of cardiovascular disease and organ damage in T2D subjects.

Results

Pro12Ala frequency

The total cohort constituted 408 participants in CArdiovascular Risk factors in Patients with DIabetes – a Prospective study in the Primary health care setting (CARDIPP) in which the Pro12Ala isoform of PPARγ was successfully analyzed. Among women one (0.7%) subject was Ala/Ala, whereas 33 (22.3%) were heterozygotes for the Ala variant. Corresponding figures for men were eight (3.1%) Ala/Ala, and 43 (16.8%) heterzygotes for the Ala variant. Of the Ala variant (homo- or heterozygotes) 26.5% were on diet only, 38.6% were treated with oral antidiabetic medication (OAD) and 34.9% received insulin treatment. Corresponding figures for the Pro isoform were 29.1%, 38.0% and 32.9%. In men 27.8% received diet only, 38.4% were treated with OAD and 33.7% with insulin. Corresponding figures for women were: diet only 29.9%, OAD in 37.5% and insulin in the remaining 32.6%.

Pro12Ala in relation to major anthropometric data and findings related to diabetes

Men with Ala isoform exhibited higher sagittal abdominal diameter (SAD), waist circumference and body weight compared with homozygotes for the Pro isoform. There were no differences with respect to glycemic control measured as HbA1c in either gender.

Pro12Ala in relation to blood pressure levels and noninvasive measures of cardiac size, intima-media thickness in the carotid arteries and carotid-femoral pulse wave velocity

No differences in blood pressure, cardiac size, intima-media thickness (IMT) in the carotid arteries or carotid-femoral pulse wave velocity (PWV) were found with respect to the Pro12Ala isoforms. There were also no differences in systolic or diastolic ambulatory blood pressure levels in men or women when analyzed separately in subjects with a BMI of < or > 30 kg/m^2.

Discussion

We found higher weight and SAD in men with the Ala isoform compared with homozygotes of the Pro isoform. In contrast, BMI only exhibited a tendency to be higher in the same subgroup. While blood pressure and echocardiographic determination of left ventricular mass index (LVMI) are manifestations of cardiovascular disease that can be reversed and treated with medication, PWV and IMT are markers of vascular disease of a more irreversible nature. However, we found no differences related to the presence of the Ala isoform, which would have been in line with more obesity, with respect to PWV or IMT in either males or females.

The relationship between the Ala isoform and risk for cardiovascular disease, in excess of that to increased abdominal obesity in men, could potentially have been offset by treatment. However, there were no differences in the use of antihypertensive drugs in either gender, or in the frequency of prescribed cholesterol lowering statins related to Pro12Ala isoforms. The proportions of patients treated with diet only, OAD or insulin treatment were also similar when analyzed with respect to the PPARγ Pro12Ala polymorphism.

The Pro12Ala polymorphism has previously been associated with lower office diastolic blood pressure [236]. In contrast, we found a non significant tendency in the opposite direction in subjects with the Ala variant. Indeed, ambulatory blood pressure levels are generally considered to be considerably more reproducible than office blood pressure, and also display a more robust relationship with organ damage, morbidity and mortality [237]. Thus, the lack of differences in the markers of hypertensive organ damage further supports the findings that there was no apparent linkage between the PPARγ Pro12Ala isoforms and blood pressure load.

CONCLUSIONS

- The activity of PPARγ is significantly lower in human primary visceral compared with subcutaneous adipocytes. This finding further supports the strong association between visceral obesity and insulin resistance.

- The insulin induced GLUT4 translocation is blunted in large compared with small human primary subcutaneous adipocytes. This finding provides further evidence for the unfavorable consequence of hypertrophic obesity.

- Several genes involved in the regulation of cell size, β-oxidation, liberation of fatty acids and glyceroneogenesis respond to changes in caloric intake in human primary subcutaneous adipocytes and may indicate pathways and mechanisms mediating the effects of nutrient deprivation and obesity on morbidity and mortality.

- The PPARγ Pro12Ala polymorphism is associated with sagittal abdominal diameter, waist circumference and body weight in men with T2D but not with blood pressure or markers of cardiovascular disease and organ damage in either gender. This finding suggests that determination of Pro12Ala in clinic practice do not provide any major information on cardiovascular risk or circulatory organ damage in T2D subjects.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the support from my colleagues. Especially I would like to thank:

My supervisor **Fredrik Nyström** and co-supervisor **Torbjörn Lindström,** for your endless support and encouragement during the years. Your scientific knowledge has been a major source of inspiration.

Peter Strålfors, for your scientific knowledge, enthusiasm and support during the years.

Mats Söderström, my very own molecular biology guru. Thank you for answering the same questions over and over again, your patience has been invaluable to me.

Siri Fagerholm, for being so kind and for your never ending smile. I hope you one day will understand the greatness of Thåström.

Cecilia Johansson, for our conversations about everything but research, I will truly miss them. Good luck with your married life and I know you will be a supercool mom!

Åsa "B-Bob" Svensson, for being such a cool chick and for giving Sing Star dancing a new meaning. To be the only music nerd on the floor would have been a very heavy burden to carry.

Sophie Huertel, for making such delicious vegetarian food.

Lilian Sauma, my former companion in the "Bunker" and one of the kindest persons I know. Thank you for our endless conversations about everything and nothing and many laughs together. I wish you the very best in life.

Anna Danielsson, for many laughs together in the lab. I will call you when I find a suitable place for our "Lanthandel".

Anita "Lilla Gumman" Öst, for our conversations about life, research and everything in between. It has been a privilege to get to know you. See you in Stockholm when you are awarded with the Nobel price.

Kristoffer Svensson, for increasing the testosterone level in our group and for taking care of "Lilla Gumman".

Tobias Strid, for making such tasty pasta carbonara at 3.30 in the morning, respect!

Gunnar Cedersund, for giving curiosity and enthusiasm a new meaning.

Cecilia Karlsson, for understanding the beauty of Norrköping.

Sebastian "Ulla" Schultz, Jana Sponarova, Sofia Nyström and Marie Oskarsson, for singing so beautiful.

Sven Hammarström and **Gunilla Westermark,** for contributing to a dynamic research atmosphere.

My former colleagues **Jesper Svartz, Unn Örtegren Kugelberg, Nabila Aboulaich, Hans Thorn, Veronika Patcha Brodin, Johan Paulsson, Karin Stenkula** and **Elisabeth Hallin,** for being such nice co-workers.

Jag vill speciellt tacka mina närmaste vänner och familj, skulle aldrig ha klarat detta utan er…

Salvador Rivas, så lika men ändå så olika. Peace brother!

David Ahlqvist, min vän sedan 20 år tillbaka. Tack för din vänskap och lojalitet, du vet hur mycket den betyder för mig. Om jag tröttnar på forskningen ska vi starta ett rockband!

Maria Kingbäck, tack för att du finns baby…

Mina syskon **Toni** och **Therese,** och mina föräldrar **Göran** och **Birgitta**. Tack för all er kärlek och stöd. Denna avhandling är tillägnad till Er.

REFERENCES

1. Boden, G., *Effects of free fatty acids (FFA) on glucose metabolism: significance for insulin resistance and type 2 diabetes.* Exp Clin Endocrinol Diabetes, 2003. **111**(3): p. 121-4.
2. Griffin, M.E., et al., *Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade.* Diabetes, 1999. **48**(6): p. 1270-4.
3. Boden, G., *Role of fatty acids in the pathogenesis of insulin resistance and NIDDM.* Diabetes, 1997. **46**(1): p. 3-10.
4. Kershaw, E.E. and J.S. Flier, *Adipose tissue as an endocrine organ.* J Clin Endocrinol Metab, 2004. **89**(6): p. 2548-56.
5. Rosen, E.D. and B.M. Spiegelman, *Adipocytes as regulators of energy balance and glucose homeostasis.* Nature, 2006. **444**(7121): p. 847-53.
6. Wang, J., et al., *Overfeeding rapidly induces leptin and insulin resistance.* Diabetes, 2001. **50**(12): p. 2786-91.
7. Kelley, D.E., et al., *Relative effects of calorie restriction and weight loss in noninsulin-dependent diabetes mellitus.* J Clin Endocrinol Metab, 1993. **77**(5): p. 1287-93.
8. Assali, A.R., et al., *Insulin resistance in obesity: body-weight or energy balance?* J Endocrinol, 2001. **171**(2): p. 293-8.
9. Sun, C., et al., *SIRT1 improves insulin sensitivity under insulin-resistant conditions by repressing PTP1B.* Cell Metab, 2007. **6**(4): p. 307-19.
10. Civitarese, A.E., et al., *Calorie restriction increases muscle mitochondrial biogenesis in healthy humans.* PLoS Med, 2007. **4**(3): p. e76.
11. Krebs, M., et al., *The Mammalian target of rapamycin pathway regulates nutrient-sensitive glucose uptake in man.* Diabetes, 2007. **56**(6): p. 1600-7.
12. Rasmussen, B.B., et al., *Malonyl coenzyme A and the regulation of functional carnitine palmitoyltransferase-1 activity and fat oxidation in human skeletal muscle.* J Clin Invest, 2002. **110**(11): p. 1687-93.
13. Um, S.H., et al., *Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity.* Nature, 2004. **431**(7005): p. 200-5.
14. Milne, J.C., et al., *Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes.* Nature, 2007. **450**(7170): p. 712-6.
15. Saltiel, A.R. and C.R. Kahn, *Insulin signalling and the regulation of glucose and lipid metabolism.* Nature, 2001. **414**(6865): p. 799-806.
16. Thorn, H., et al., *Cell surface orifices of caveolae and localization of caveolin to the necks of caveolae in adipocytes.* Mol Biol Cell, 2003. **14**(10): p. 3967-76.
17. Karlsson, M., et al., *Colocalization of insulin receptor and insulin receptor substrate-1 to caveolae in primary human adipocytes. Cholesterol depletion blocks insulin signalling for metabolic and mitogenic control.* Eur J Biochem, 2004. **271**(12): p. 2471-9.
18. Gustavsson, J., et al., *Localization of the insulin receptor in caveolae of adipocyte plasma membrane.* FASEB J, 1999. **13**(14): p. 1961-71.
19. Cheatham, B. and C.R. Kahn, *Insulin action and the insulin signaling network.* Endocr Rev, 1995. **16**(2): p. 117-42.
20. Kido, Y., et al., *Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2.* J Clin Invest, 2000. **105**(2): p. 199-205.

21. Myers, M.G., Jr., et al., *IRS-1 activates phosphatidylinositol 3'-kinase by associating with src homology 2 domains of p85.* Proc Natl Acad Sci U S A, 1992. **89**(21): p. 10350-4.
22. Zick, Y., *Ser/Thr phosphorylation of IRS proteins: a molecular basis for insulin resistance.* Sci STKE, 2005. **2005**(268): p. pe4.
23. Um, S.H., D. D'Alessio, and G. Thomas, *Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1.* Cell Metab, 2006. **3**(6): p. 393-402.
24. Hirosumi, J., et al., *A central role for JNK in obesity and insulin resistance.* Nature, 2002. **420**(6913): p. 333-6.
25. Danielsson, A., et al., *Attenuation of insulin-stimulated insulin receptor substrate-1 serine 307 phosphorylation in insulin resistance of type 2 diabetes.* J Biol Chem, 2005. **280**(41): p. 34389-92.
26. Fruman, D.A., R.E. Meyers, and L.C. Cantley, *Phosphoinositide kinases.* Annu Rev Biochem, 1998. **67**: p. 481-507.
27. Mauvais-Jarvis, F., et al., *Reduced expression of the murine p85alpha subunit of phosphoinositide 3-kinase improves insulin signaling and ameliorates diabetes.* J Clin Invest, 2002. **109**(1): p. 141-9.
28. Chen, D., et al., *p50alpha/p55alpha phosphoinositide 3-kinase knockout mice exhibit enhanced insulin sensitivity.* Mol Cell Biol, 2004. **24**(1): p. 320-9.
29. Bandyopadhyay, G.K., et al., *Increased p85/55/50 expression and decreased phosphotidylinositol 3-kinase activity in insulin-resistant human skeletal muscle.* Diabetes, 2005. **54**(8): p. 2351-9.
30. McCurdy, C.E., R.T. Davidson, and G.D. Cartee, *Calorie restriction increases the ratio of phosphatidylinositol 3-kinase catalytic to regulatory subunits in rat skeletal muscle.* Am J Physiol Endocrinol Metab, 2005. **288**(5): p. E996-E1001.
31. Corvera, S. and M.P. Czech, *Direct targets of phosphoinositide 3-kinase products in membrane traffic and signal transduction.* Trends Cell Biol, 1998. **8**(11): p. 442-6.
32. Rameh, L.E. and L.C. Cantley, *The role of phosphoinositide 3-kinase lipid products in cell function.* J Biol Chem, 1999. **274**(13): p. 8347-50.
33. Sano, H., et al., *Insulin-stimulated phosphorylation of a Rab GTPase-activating protein regulates GLUT4 translocation.* J Biol Chem, 2003. **278**(17): p. 14599-602.
34. Tanti, J.F., et al., *Potential role of protein kinase B in glucose transporter 4 translocation in adipocytes.* Endocrinology, 1997. **138**(5): p. 2005-10.
35. Khan, A.H. and J.E. Pessin, *Insulin regulation of glucose uptake: a complex interplay of intracellular signalling pathways.* Diabetologia, 2002. **45**(11): p. 1475-83.
36. James, D.E., R.C. Piper, and J.W. Slot, *Targeting of mammalian glucose transporters.* J Cell Sci, 1993. **104 (Pt 3)**: p. 607-12.
37. Pessin, J.E., et al., *Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. Location! Location! Location!* J Biol Chem, 1999. **274**(5): p. 2593-6.
38. Pessin, J.E. and A.R. Saltiel, *Signaling pathways in insulin action: molecular targets of insulin resistance.* J Clin Invest, 2000. **106**(2): p. 165-9.
39. Ribon, V. and A.R. Saltiel, *Insulin stimulates tyrosine phosphorylation of the proto-oncogene product of c-Cbl in 3T3-L1 adipocytes.* Biochem J, 1997. **324 (Pt 3)**: p. 839-45.
40. Baumann, C.A., et al., *CAP defines a second signalling pathway required for insulin-stimulated glucose transport.* Nature, 2000. **407**(6801): p. 202-7.
41. Chiang, S.H., et al., *Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10.* Nature, 2001. **410**(6831): p. 944-8.

42. Mitra, P., X. Zheng, and M.P. Czech, *RNAi-based analysis of CAP, Cbl, and CrkII function in the regulation of GLUT4 by insulin.* J Biol Chem, 2004. **279**(36): p. 37431-5.
43. Parpal, S., et al., *Cholesterol depletion disrupts caveolae and insulin receptor signaling for metabolic control via insulin receptor substrate-1, but not for mitogen-activated protein kinase control.* J Biol Chem, 2001. **276**(13): p. 9670-8.
44. Stenkula, K.G., et al., *Expression of a mutant IRS inhibits metabolic and mitogenic signalling of insulin in human adipocytes.* Mol Cell Endocrinol, 2004. **221**(1-2): p. 1-8.
45. Pelicci, G., et al., *A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction.* Cell, 1992. **70**(1): p. 93-104.
46. Skolnik, E.Y., et al., *The function of GRB2 in linking the insulin receptor to Ras signaling pathways.* Science, 1993. **260**(5116): p. 1953-5.
47. Avruch, J., et al., *Ras activation of the Raf kinase: tyrosine kinase recruitment of the MAP kinase cascade.* Recent Prog Horm Res, 2001. **56**: p. 127-55.
48. Boulton, T.G., et al., *ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF.* Cell, 1991. **65**(4): p. 663-75.
49. Prusty, D., et al., *Activation of MEK/ERK signaling promotes adipogenesis by enhancing peroxisome proliferator-activated receptor gamma (PPARgamma) and C/EBPalpha gene expression during the differentiation of 3T3-L1 preadipocytes.* J Biol Chem, 2002. **277**(48): p. 46226-32.
50. Wajchenberg, B.L., *Subcutaneous and visceral adipose tissue: their relation to the metabolic syndrome.* Endocr Rev, 2000. **21**(6): p. 697-738.
51. Kissebah, A.H. and G.R. Krakower, *Regional adiposity and morbidity.* Physiol Rev, 1994. **74**(4): p. 761-811.
52. Karelis, A.D., et al., *Metabolic and body composition factors in subgroups of obesity: what do we know?* J Clin Endocrinol Metab, 2004. **89**(6): p. 2569-75.
53. Reitman, M.L., et al., *Lipoatrophy revisited.* Trends Endocrinol Metab, 2000. **11**(10): p. 410-6.
54. Giorgino, F., L. Laviola, and J.W. Eriksson, *Regional differences of insulin action in adipose tissue: insights from in vivo and in vitro studies.* Acta Physiol Scand, 2005. **183**(1): p. 13-30.
55. Thorne, A., et al., *A pilot study of long-term effects of a novel obesity treatment: omentectomy in connection with adjustable gastric banding.* Int J Obes Relat Metab Disord, 2002. **26**(2): p. 193-9.
56. Klein, S., et al., *Absence of an effect of liposuction on insulin action and risk factors for coronary heart disease.* N Engl J Med, 2004. **350**(25): p. 2549-57.
57. Kitamura, T., et al., *Insulin-induced phosphorylation and activation of cyclic nucleotide phosphodiesterase 3B by the serine-threonine kinase Akt.* Mol Cell Biol, 1999. **19**(9): p. 6286-96.
58. Arner, P., et al., *Beta-adrenoceptor expression in human fat cells from different regions.* J Clin Invest, 1990. **86**(5): p. 1595-600.
59. Bolinder, J., et al., *Differences at the receptor and postreceptor levels between human omental and subcutaneous adipose tissue in the action of insulin on lipolysis.* Diabetes, 1983. **32**(2): p. 117-23.
60. Marette, A., et al., *Regional variation in adipose tissue insulin action and GLUT4 glucose transporter expression in severely obese premenopausal women.* Diabetologia, 1997. **40**(5): p. 590-8.

61. Zierath, J.R., et al., *Regional difference in insulin inhibition of non-esterified fatty acid release from human adipocytes: relation to insulin receptor phosphorylation and intracellular signalling through the insulin receptor substrate-1 pathway.* Diabetologia, 1998. **41**(11): p. 1343-54.
62. Laviola, L., et al., *Insulin signaling in human visceral and subcutaneous adipose tissue in vivo.* Diabetes, 2006. **55**(4): p. 952-61.
63. Lundgren, M., et al., *Glucocorticoids down-regulate glucose uptake capacity and insulin-signaling proteins in omental but not subcutaneous human adipocytes.* J Clin Endocrinol Metab, 2004. **89**(6): p. 2989-97.
64. Virtanen, K.A., et al., *Glucose uptake and perfusion in subcutaneous and visceral adipose tissue during insulin stimulation in nonobese and obese humans.* J Clin Endocrinol Metab, 2002. **87**(8): p. 3902-10.
65. Gastaldelli, A., et al., *Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects.* Gastroenterology, 2007. **133**(2): p. 496-506.
66. Nielsen, S., et al., *Splanchnic lipolysis in human obesity.* J Clin Invest, 2004. **113**(11): p. 1582-8.
67. Lafontan, M. and J. Girard, *Impact of visceral adipose tissue on liver metabolism. Part I: heterogeneity of adipose tissue and functional properties of visceral adipose tissue.* Diabetes Metab, 2008. **34**(4 Pt 1): p. 317-27.
68. Wahren, J. and K. Ekberg, *Splanchnic regulation of glucose production.* Annu Rev Nutr, 2007. **27**: p. 329-45.
69. Carpentier, A., et al., *Sensitivity to acute insulin-mediated suppression of plasma free fatty acids is not a determinant of fasting VLDL triglyceride secretion in healthy humans.* Diabetes, 2002. **51**(6): p. 1867-75.
70. Holland, W.L., et al., *Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance.* Cell Metab, 2007. **5**(3): p. 167-79.
71. Yu, C., et al., *Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle.* J Biol Chem, 2002. **277**(52): p. 50230-6.
72. An, J., et al., *Hepatic expression of malonyl-CoA decarboxylase reverses muscle, liver and whole-animal insulin resistance.* Nat Med, 2004. **10**(3): p. 268-74.
73. Li, X., et al., *Akt/PKB regulates hepatic metabolism by directly inhibiting PGC-1alpha transcription coactivator.* Nature, 2007. **447**(7147): p. 1012-6.
74. Gao, Z., et al., *Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex.* J Biol Chem, 2002. **277**(50): p. 48115-21.
75. Samuel, V.T., et al., *Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease.* J Biol Chem, 2004. **279**(31): p. 32345-53.
76. Felig, P., et al., *Splanchnic glucose and amino acid metabolism in obesity.* J Clin Invest, 1974. **53**(2): p. 582-90.
77. Krebs, M., et al., *Mechanism of amino acid-induced skeletal muscle insulin resistance in humans.* Diabetes, 2002. **51**(3): p. 599-605.
78. Krebs, M., et al., *Direct and indirect effects of amino acids on hepatic glucose metabolism in humans.* Diabetologia, 2003. **46**(7): p. 917-25.
79. Tremblay, F., et al., *Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability.* Diabetes, 2005. **54**(9): p. 2674-84.

80. Fain, J.N., et al., *Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans.* Endocrinology, 2004. **145**(5): p. 2273-82.
81. Bastelica, D., et al., *Stromal cells are the main plasminogen activator inhibitor-1-producing cells in human fat: evidence of differences between visceral and subcutaneous deposits.* Arterioscler Thromb Vasc Biol, 2002. **22**(1): p. 173-8.
82. Fried, S.K., D.A. Bunkin, and A.S. Greenberg, *Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid.* J Clin Endocrinol Metab, 1998. **83**(3): p. 847-50.
83. Bruun, J.M., et al., *Higher production of IL-8 in visceral vs. subcutaneous adipose tissue. Implication of nonadipose cells in adipose tissue.* Am J Physiol Endocrinol Metab, 2004. **286**(1): p. E8-13.
84. Kloting, N., et al., *Serum retinol-binding protein is more highly expressed in visceral than in subcutaneous adipose tissue and is a marker of intra-abdominal fat mass.* Cell Metab, 2007. **6**(1): p. 79-87.
85. Bacha, F., et al., *Adiponectin in youth: relationship to visceral adiposity, insulin sensitivity, and beta-cell function.* Diabetes Care, 2004. **27**(2): p. 547-52.
86. Cancello, R., et al., *Increased infiltration of macrophages in omental adipose tissue is associated with marked hepatic lesions in morbid human obesity.* Diabetes, 2006. **55**(6): p. 1554-61.
87. Krotkiewski, M., et al., *Impact of obesity on metabolism in men and women. Importance of regional adipose tissue distribution.* J Clin Invest, 1983. **72**(3): p. 1150-62.
88. Salans, L.B., J.L. Knittle, and J. Hirsch, *The role of adipose cell size and adipose tissue insulin sensitivity in the carbohydrate intolerance of human obesity.* J Clin Invest, 1968. **47**(1): p. 153-165.
89. Stern, J.S., et al., *Adipose-cell size and immunoreactive insulin levels in obese and normal-weight adults.* Lancet, 1972. **2**(7784): p. 948-51.
90. Salans, L.B., S.W. Cushman, and R.E. Weismann, *Studies of human adipose tissue. Adipose cell size and number in nonobese and obese patients.* J Clin Invest, 1973. **52**(4): p. 929-41.
91. Okuno, A., et al., *Troglitazone increases the number of small adipocytes without the change of white adipose tissue mass in obese Zucker rats.* J Clin Invest, 1998. **101**(6): p. 1354-61.
92. de Souza, C.J., et al., *Effects of pioglitazone on adipose tissue remodeling within the setting of obesity and insulin resistance.* Diabetes, 2001. **50**(8): p. 1863-71.
93. Spalding, K.L., et al., *Dynamics of fat cell turnover in humans.* Nature, 2008. **453**(7196): p. 783-7.
94. Knittle, J.L., et al., *The growth of adipose tissue in children and adolescents. Cross-sectional and longitudinal studies of adipose cell number and size.* J Clin Invest, 1979. **63**(2): p. 239-46.
95. Boivin, A., et al., *Regional differences in adipose tissue metabolism in obese men.* Metabolism, 2007. **56**(4): p. 533-40.
96. Tchernof, A., et al., *Regional differences in adipose tissue metabolism in women: minor effect of obesity and body fat distribution.* Diabetes, 2006. **55**(5): p. 1353-60.
97. Frayn, K.N., et al., *Regulation of fatty acid movement in human adipose tissue in the postabsorptive-to-postprandial transition.* Am J Physiol, 1994. **266**(3 Pt 1): p. E308-17.

98. Weyer, C., et al., *Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance.* Diabetologia, 2000. **43**(12): p. 1498-506.
99. Salans, L.B. and J.W. Dougherty, *The effect of insulin upon glucose metabolism by adipose cells of different size. Influence of cell lipid and protein content, age, and nutritional state.* J Clin Invest, 1971. **50**(7): p. 1399-410.
100. Engfeldt, P. and P. Arner, *Lipolysis in human adipocytes, effects of cell size, age and of regional differences.* Horm Metab Res Suppl, 1988. **19**: p. 26-9.
101. Miyazaki, Y., et al., *Effect of pioglitazone on abdominal fat distribution and insulin sensitivity in type 2 diabetic patients.* J Clin Endocrinol Metab, 2002. **87**(6): p. 2784-91.
102. Horowitz, J.F. and S. Klein, *Whole body and abdominal lipolytic sensitivity to epinephrine is suppressed in upper body obese women.* Am J Physiol Endocrinol Metab, 2000. **278**(6): p. E1144-52.
103. Wajchenberg, B.L., et al., *Depot-specific hormonal characteristics of subcutaneous and visceral adipose tissue and their relation to the metabolic syndrome.* Horm Metab Res, 2002. **34**(11-12): p. 616-21.
104. Bachmann, O.P., et al., *Effects of intravenous and dietary lipid challenge on intramyocellular lipid content and the relation with insulin sensitivity in humans.* Diabetes, 2001. **50**(11): p. 2579-84.
105. Itani, S.I., et al., *Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha.* Diabetes, 2002. **51**(7): p. 2005-11.
106. Santomauro, A.T., et al., *Overnight lowering of free fatty acids with Acipimox improves insulin resistance and glucose tolerance in obese diabetic and nondiabetic subjects.* Diabetes, 1999. **48**(9): p. 1836-41.
107. Hulver, M.W., et al., *Skeletal muscle lipid metabolism with obesity.* Am J Physiol Endocrinol Metab, 2003. **284**(4): p. E741-7.
108. Borkman, M., et al., *The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids.* N Engl J Med, 1993. **328**(4): p. 238-44.
109. Shulman, G.I., *Cellular mechanisms of insulin resistance.* J Clin Invest, 2000. **106**(2): p. 171-6.
110. Chibalin, A.V., et al., *Downregulation of diacylglycerol kinase delta contributes to hyperglycemia-induced insulin resistance.* Cell, 2008. **132**(3): p. 375-86.
111. Kim, J.Y., et al., *Lipid oxidation is reduced in obese human skeletal muscle.* Am J Physiol Endocrinol Metab, 2000. **279**(5): p. E1039-44.
112. Kelley, D.E., et al., *Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss.* Am J Physiol, 1999. **277**(6 Pt 1): p. E1130-41.
113. Mootha, V.K., et al., *PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes.* Nat Genet, 2003. **34**(3): p. 267-73.
114. Patti, M.E., et al., *Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8466-71.
115. Bruce, C.R., et al., *Overexpression of carnitine palmitoyltransferase-1 in skeletal muscle is sufficient to enhance fatty acid oxidation and improve high fat diet-induced insulin resistance.* Diabetes, 2008.
116. Bruce, C.R., et al., *Overexpression of carnitine palmitoyltransferase I in skeletal muscle in vivo increases fatty acid oxidation and reduces triacylglycerol esterification.* Am J Physiol Endocrinol Metab, 2007. **292**(4): p. E1231-7.

117. Morino, K., K.F. Petersen, and G.I. Shulman, *Molecular mechanisms of insulin resistance in humans and their potential links with mitochondrial dysfunction.* Diabetes, 2006. **55 Suppl 2**: p. S9-S15.
118. Bonen, A., et al., *Triacylglycerol accumulation in human obesity and type 2 diabetes is associated with increased rates of skeletal muscle fatty acid transport and increased sarcolemmal FAT/CD36.* FASEB J, 2004. **18**(10): p. 1144-6.
119. Steinberg, G.R., et al., *AMP-activated protein kinase is not down-regulated in human skeletal muscle of obese females.* J Clin Endocrinol Metab, 2004. **89**(9): p. 4575-80.
120. Bandyopadhyay, G.K., et al., *Increased malonyl-CoA levels in muscle from obese and type 2 diabetic subjects lead to decreased fatty acid oxidation and increased lipogenesis; thiazolidinedione treatment reverses these defects.* Diabetes, 2006. **55**(8): p. 2277-85.
121. Boushel, R., et al., *Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle.* Diabetologia, 2007. **50**(4): p. 790-6.
122. Goodpaster, B.H. and D.E. Kelley, *Skeletal muscle triglyceride: marker or mediator of obesity-induced insulin resistance in type 2 diabetes mellitus?* Curr Diab Rep, 2002. **2**(3): p. 216-22.
123. Bruce, C.R., et al., *Disassociation of muscle triglyceride content and insulin sensitivity after exercise training in patients with Type 2 diabetes.* Diabetologia, 2004. **47**(1): p. 23-30.
124. Koves, T.R., et al., *Peroxisome proliferator-activated receptor-gamma co-activator 1alpha-mediated metabolic remodeling of skeletal myocytes mimics exercise training and reverses lipid-induced mitochondrial inefficiency.* J Biol Chem, 2005. **280**(39): p. 33588-98.
125. Muoio, D.M. and C.B. Newgard, *Obesity-related derangements in metabolic regulation.* Annu Rev Biochem, 2006. **75**: p. 367-401.
126. Koves, T.R., et al., *Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance.* Cell Metab, 2008. **7**(1): p. 45-56.
127. Bouzakri, K., et al., *Malonyl CoenzymeA decarboxylase regulates lipid and glucose metabolism in human skeletal muscle.* Diabetes, 2008. **57**(6): p. 1508-16.
128. Van Harmelen, V., et al., *Leptin secretion from subcutaneous and visceral adipose tissue in women.* Diabetes, 1998. **47**(6): p. 913-7.
129. Winkler, G., et al., *Expression of tumor necrosis factor (TNF)-alpha protein in the subcutaneous and visceral adipose tissue in correlation with adipocyte cell volume, serum TNF-alpha, soluble serum TNF-receptor-2 concentrations and C-peptide level.* Eur J Endocrinol, 2003. **149**(2): p. 129-35.
130. Sopasakis, V.R., et al., *High local concentrations and effects on differentiation implicate interleukin-6 as a paracrine regulator.* Obes Res, 2004. **12**(3): p. 454-60.
131. Skurk, T., et al., *Relationship between adipocyte size and adipokine expression and secretion.* J Clin Endocrinol Metab, 2007. **92**(3): p. 1023-33.
132. Lagathu, C., et al., *Long-term treatment with interleukin-1beta induces insulin resistance in murine and human adipocytes.* Diabetologia, 2006. **49**(9): p. 2162-73.
133. Skurk, T., C. Alberti-Huber, and H. Hauner, *Effect of conditioned media from mature human adipocytes on insulin-stimulated Akt/PKB phosphorylation in human skeletal muscle cells: role of BMI and fat cell size.* Horm Metab Res, 2009. **41**(3): p. 190-6.
134. Curat, C.A., et al., *From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes.* Diabetes, 2004. **53**(5): p. 1285-92.
135. Weisberg, S.P., et al., *Obesity is associated with macrophage accumulation in adipose tissue.* J Clin Invest, 2003. **112**(12): p. 1796-808.

136. Cartier, A., et al., *Visceral obesity and plasma glucose-insulin homeostasis: contributions of interleukin-6 and tumor necrosis factor-alpha in men.* J Clin Endocrinol Metab, 2008. **93**(5): p. 1931-8.
137. Engelman, J.A., et al., *Tumor necrosis factor alpha-mediated insulin resistance, but not dedifferentiation, is abrogated by MEK1/2 inhibitors in 3T3-L1 adipocytes.* Mol Endocrinol, 2000. **14**(10): p. 1557-69.
138. Berger, J. and D.E. Moller, *The mechanisms of action of PPARs.* Annu Rev Med, 2002. **53**: p. 409-35.
139. Miyata, K.S., et al., *The peroxisome proliferator-activated receptor interacts with the retinoid X receptor in vivo.* Gene, 1994. **148**(2): p. 327-30.
140. Mangelsdorf, D.J., et al., *Characterization of three RXR genes that mediate the action of 9-cis retinoic acid.* Genes Dev, 1992. **6**(3): p. 329-44.
141. Ferre, P., *The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity.* Diabetes, 2004. **53 Suppl 1**: p. S43-50.
142. Rosen, E.D., et al., *Transcriptional regulation of adipogenesis.* Genes Dev, 2000. **14**(11): p. 1293-307.
143. Vidal-Puig, A., et al., *Regulation of PPAR gamma gene expression by nutrition and obesity in rodents.* J Clin Invest, 1996. **97**(11): p. 2553-61.
144. Fajas, L., et al., *The organization, promoter analysis, and expression of the human PPARgamma gene.* J Biol Chem, 1997. **272**(30): p. 18779-89.
145. Glass, C.K. and M.G. Rosenfeld, *The coregulator exchange in transcriptional functions of nuclear receptors.* Genes Dev, 2000. **14**(2): p. 121-41.
146. Heinzel, T., et al., *A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression.* Nature, 1997. **387**(6628): p. 43-8.
147. Alland, L., et al., *Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression.* Nature, 1997. **387**(6628): p. 49-55.
148. Adams, M., et al., *Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation.* J Clin Invest, 1997. **100**(12): p. 3149-53.
149. van Harmelen, V., et al., *Increased lipolysis and decreased leptin production by human omental as compared with subcutaneous preadipocytes.* Diabetes, 2002. **51**(7): p. 2029-36.
150. Tontonoz, P., E. Hu, and B.M. Spiegelman, *Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor.* Cell, 1994. **79**(7): p. 1147-56.
151. Barak, Y., et al., *PPAR gamma is required for placental, cardiac, and adipose tissue development.* Mol Cell, 1999. **4**(4): p. 585-95.
152. Zhang, J., et al., *Selective disruption of PPARgamma 2 impairs the development of adipose tissue and insulin sensitivity.* Proc Natl Acad Sci U S A, 2004. **101**(29): p. 10703-8.
153. Rosen, E.D., et al., *PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro.* Mol Cell, 1999. **4**(4): p. 611-7.
154. Tang, Q.Q. and M.D. Lane, *Activation and centromeric localization of CCAAT/enhancer-binding proteins during the mitotic clonal expansion of adipocyte differentiation.* Genes Dev, 1999. **13**(17): p. 2231-41.
155. Lin, F.T. and M.D. Lane, *CCAAT/enhancer binding protein alpha is sufficient to initiate the 3T3-L1 adipocyte differentiation program.* Proc Natl Acad Sci U S A, 1994. **91**(19): p. 8757-61.
156. Fajas, L., et al., *Regulation of peroxisome proliferator-activated receptor gamma expression by adipocyte differentiation and determination factor 1/sterol regulatory*

element binding protein 1: implications for adipocyte differentiation and metabolism. Mol Cell Biol, 1999. **19**(8): p. 5495-503.
157. Kim, J.B., et al., *ADD1/SREBP1 activates PPARgamma through the production of endogenous ligand.* Proc Natl Acad Sci U S A, 1998. **95**(8): p. 4333-7.
158. Ericsson, J., et al., *Identification of glycerol-3-phosphate acyltransferase as an adipocyte determination and differentiation factor 1- and sterol regulatory element-binding protein-responsive gene.* J Biol Chem, 1997. **272**(11): p. 7298-305.
159. Tabor, D.E., et al., *Identification of conserved cis-elements and transcription factors required for sterol-regulated transcription of stearoyl-CoA desaturase 1 and 2.* J Biol Chem, 1999. **274**(29): p. 20603-10.
160. Kim, J.B., et al., *Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1.* J Clin Invest, 1998. **101**(1): p. 1-9.
161. Gaillard, D., et al., *Requirement and role of arachidonic acid in the differentiation of pre-adipose cells.* Biochem J, 1989. **257**(2): p. 389-97.
162. Forman, B.M., J. Chen, and R.M. Evans, *Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta.* Proc Natl Acad Sci U S A, 1997. **94**(9): p. 4312-7.
163. Aubert, J., et al., *Prostacyclin IP receptor up-regulates the early expression of C/EBPbeta and C/EBPdelta in preadipose cells.* Mol Cell Endocrinol, 2000. **160**(1-2): p. 149-56.
164. Ailhaud, G., *Cross talk between adipocytes and their precursors: relationships with adipose tissue development and blood pressure.* Ann N Y Acad Sci, 1999. **892**: p. 127-33.
165. Cao, Z., R.M. Umek, and S.L. McKnight, *Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells.* Genes Dev, 1991. **5**(9): p. 1538-52.
166. Wu, Z., N.L. Bucher, and S.R. Farmer, *Induction of peroxisome proliferator-activated receptor gamma during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPbeta, C/EBPdelta, and glucocorticoids.* Mol Cell Biol, 1996. **16**(8): p. 4128-36.
167. Joyner, J.M., L.J. Hutley, and D.P. Cameron, *Glucocorticoid receptors in human preadipocytes: regional and gender differences.* J Endocrinol, 2000. **166**(1): p. 145-52.
168. Napolitano, A., et al., *11Beta-hydroxysteroid dehydrogenase 1 in adipocytes: expression is differentiation-dependent and hormonally regulated.* J Steroid Biochem Mol Biol, 1998. **64**(5-6): p. 251-60.
169. Williams, L.J., et al., *C/EBP regulates hepatic transcription of 11beta -hydroxysteroid dehydrogenase type 1. A novel mechanism for cross-talk between the C/EBP and glucocorticoid signaling pathways.* J Biol Chem, 2000. **275**(39): p. 30232-9.
170. Smith, P.J., et al., *Insulin-like growth factor-I is an essential regulator of the differentiation of 3T3-L1 adipocytes.* J Biol Chem, 1988. **263**(19): p. 9402-8.
171. Sakaue, H., et al., *Posttranscriptional control of adipocyte differentiation through activation of phosphoinositide 3-kinase.* J Biol Chem, 1998. **273**(44): p. 28945-52.
172. Garofalo, R.S., et al., *Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta.* J Clin Invest, 2003. **112**(2): p. 197-208.
173. Tseng, Y.H., et al., *Differential roles of insulin receptor substrates in brown adipocyte differentiation.* Mol Cell Biol, 2004. **24**(5): p. 1918-29.
174. Miki, H., et al., *Essential role of insulin receptor substrate 1 (IRS-1) and IRS-2 in adipocyte differentiation.* Mol Cell Biol, 2001. **21**(7): p. 2521-32.

175. Rosen, E.D. and O.A. MacDougald, *Adipocyte differentiation from the inside out.* Nat Rev Mol Cell Biol, 2006. **7**(12): p. 885-96.
176. Marques, B.G., D.B. Hausman, and R.J. Martin, *Association of fat cell size and paracrine growth factors in development of hyperplastic obesity.* Am J Physiol, 1998. **275**(6 Pt 2): p. R1898-908.
177. Le Lay, S., et al., *Cholesterol, a cell size-dependent signal that regulates glucose metabolism and gene expression in adipocytes.* J Biol Chem, 2001. **276**(20): p. 16904-10.
178. Faust, I.M., et al., *Diet-induced adipocyte number increase in adult rats: a new model of obesity.* Am J Physiol, 1978. **235**(3): p. E279-86.
179. Gregoire, F., et al., *Interferon-gamma and interleukin-1 beta inhibit adipoconversion in cultured rodent preadipocytes.* J Cell Physiol, 1992. **151**(2): p. 300-9.
180. Ohsumi, J., et al., *Troglitazone prevents the inhibitory effects of inflammatory cytokines on insulin-induced adipocyte differentiation in 3T3-L1 cells.* Endocrinology, 1994. **135**(5): p. 2279-82.
181. Xu, H., J.K. Sethi, and G.S. Hotamisligil, *Transmembrane tumor necrosis factor (TNF)-alpha inhibits adipocyte differentiation by selectively activating TNF receptor 1.* J Biol Chem, 1999. **274**(37): p. 26287-95.
182. Kersten, S., *Peroxisome proliferator activated receptors and obesity.* Eur J Pharmacol, 2002. **440**(2-3): p. 223-34.
183. Inzucchi, S.E., et al., *Efficacy and metabolic effects of metformin and troglitazone in type II diabetes mellitus.* N Engl J Med, 1998. **338**(13): p. 867-72.
184. Boden, G., et al., *Thiazolidinediones upregulate fatty acid uptake and oxidation in adipose tissue of diabetic patients.* Diabetes, 2005. **54**(3): p. 880-5.
185. Chao, L., et al., *Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones.* J Clin Invest, 2000. **106**(10): p. 1221-8.
186. Norris, A.W., et al., *Muscle-specific PPARgamma-deficient mice develop increased adiposity and insulin resistance but respond to thiazolidinediones.* J Clin Invest, 2003. **112**(4): p. 608-18.
187. Ye, J.M., et al., *Direct demonstration of lipid sequestration as a mechanism by which rosiglitazone prevents fatty-acid-induced insulin resistance in the rat: comparison with metformin.* Diabetologia, 2004. **47**(7): p. 1306-13.
188. Sfeir, Z., et al., *Regulation of FAT/CD36 gene expression: further evidence in support of a role of the protein in fatty acid binding/transport.* Prostaglandins Leukot Essent Fatty Acids, 1997. **57**(1): p. 17-21.
189. Tontonoz, P., et al., *PPAR gamma 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene.* Mol Cell Biol, 1995. **15**(1): p. 351-7.
190. Schoonjans, K., et al., *PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene.* EMBO J, 1996. **15**(19): p. 5336-48.
191. Schoonjans, K., et al., *Induction of the acyl-coenzyme A synthetase gene by fibrates and fatty acids is mediated by a peroxisome proliferator response element in the C promoter.* J Biol Chem, 1995. **270**(33): p. 19269-76.
192. Martin, G., et al., *Coordinate regulation of the expression of the fatty acid transport protein and acyl-CoA synthetase genes by PPARalpha and PPARgamma activators.* J Biol Chem, 1997. **272**(45): p. 28210-7.
193. Tontonoz, P., et al., *mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer.* Genes Dev, 1994. **8**(10): p. 1224-34.

194. Maggs, D.G., et al., *Metabolic effects of troglitazone monotherapy in type 2 diabetes mellitus. A randomized, double-blind, placebo-controlled trial.* Ann Intern Med, 1998. **128**(3): p. 176-85.
195. Chaput, E., et al., *Fenofibrate and rosiglitazone lower serum triglycerides with opposing effects on body weight.* Biochem Biophys Res Commun, 2000. **271**(2): p. 445-50.
196. Akazawa, S., et al., *Efficacy of troglitazone on body fat distribution in type 2 diabetes.* Diabetes Care, 2000. **23**(8): p. 1067-71.
197. Carey, D.G., et al., *Effect of rosiglitazone on insulin sensitivity and body composition in type 2 diabetic patients [corrected]*. Obes Res, 2002. **10**(10): p. 1008-15.
198. Hu, E., P. Liang, and B.M. Spiegelman, *AdipoQ is a novel adipose-specific gene dysregulated in obesity.* J Biol Chem, 1996. **271**(18): p. 10697-703.
199. Weyer, C., et al., *Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia.* J Clin Endocrinol Metab, 2001. **86**(5): p. 1930-5.
200. Berg, A.H., T.P. Combs, and P.E. Scherer, *ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism.* Trends Endocrinol Metab, 2002. **13**(2): p. 84-9.
201. Maeda, N., et al., *PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein.* Diabetes, 2001. **50**(9): p. 2094-9.
202. Yu, J.G., et al., *The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects.* Diabetes, 2002. **51**(10): p. 2968-74.
203. Yamauchi, T., et al., *Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase.* Nat Med, 2002. **8**(11): p. 1288-95.
204. Yamauchi, T., et al., *The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity.* Nat Med, 2001. **7**(8): p. 941-6.
205. Tomas, E., et al., *Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation.* Proc Natl Acad Sci U S A, 2002. **99**(25): p. 16309-13.
206. Berg, A.H., et al., *The adipocyte-secreted protein Acrp30 enhances hepatic insulin action.* Nat Med, 2001. **7**(8): p. 947-53.
207. Kallen, C.B. and M.A. Lazar, *Antidiabetic thiazolidinediones inhibit leptin (ob) gene expression in 3T3-L1 adipocytes.* Proc Natl Acad Sci U S A, 1996. **93**(12): p. 5793-6.
208. De Vos, P., et al., *Thiazolidinediones repress ob gene expression in rodents via activation of peroxisome proliferator-activated receptor gamma.* J Clin Invest, 1996. **98**(4): p. 1004-9.
209. Ahima, R.S. and J.S. Flier, *Leptin.* Annu Rev Physiol, 2000. **62**: p. 413-37.
210. Muoio, D.M., et al., *Leptin directly alters lipid partitioning in skeletal muscle.* Diabetes, 1997. **46**(8): p. 1360-3.
211. Kamohara, S., et al., *Acute stimulation of glucose metabolism in mice by leptin treatment.* Nature, 1997. **389**(6649): p. 374-7.
212. Minokoshi, Y., M.S. Haque, and T. Shimazu, *Microinjection of leptin into the ventromedial hypothalamus increases glucose uptake in peripheral tissues in rats.* Diabetes, 1999. **48**(2): p. 287-91.
213. Unger, R.H., Y.T. Zhou, and L. Orci, *Regulation of fatty acid homeostasis in cells: novel role of leptin.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2327-32.
214. Considine, R.V., et al., *Serum immunoreactive-leptin concentrations in normal-weight and obese humans.* N Engl J Med, 1996. **334**(5): p. 292-5.

215. Ren, J., *Leptin and hyperleptinemia - from friend to foe for cardiovascular function.* J Endocrinol, 2004. **181**(1): p. 1-10.
216. Peraldi, P., M. Xu, and B.M. Spiegelman, *Thiazolidinediones block tumor necrosis factor-alpha-induced inhibition of insulin signaling.* J Clin Invest, 1997. **100**(7): p. 1863-9.
217. Steppan, C.M., et al., *The hormone resistin links obesity to diabetes.* Nature, 2001. **409**(6818): p. 307-12.
218. Hotamisligil, G.S., et al., *Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance.* J Clin Invest, 1995. **95**(5): p. 2409-15.
219. Bouzakri, K. and J.R. Zierath, *MAP4K4 gene silencing in human skeletal muscle prevents tumor necrosis factor-alpha-induced insulin resistance.* J Biol Chem, 2007. **282**(11): p. 7783-9.
220. Plomgaard, P., et al., *Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation.* Diabetes, 2005. **54**(10): p. 2939-45.
221. Paquot, N., et al., *No increased insulin sensitivity after a single intravenous administration of a recombinant human tumor necrosis factor receptor: Fc fusion protein in obese insulin-resistant patients.* J Clin Endocrinol Metab, 2000. **85**(3): p. 1316-9.
222. Di Rocco, P., et al., *Lowered tumor necrosis factor receptors, but not increased insulin sensitivity, with infliximab.* Obes Res, 2004. **12**(4): p. 734-9.
223. Ofei, F., et al., *Effects of an engineered human anti-TNF-alpha antibody (CDP571) on insulin sensitivity and glycemic control in patients with NIDDM.* Diabetes, 1996. **45**(7): p. 881-5.
224. Bernstein, L.E., et al., *Effects of etanercept in patients with the metabolic syndrome.* Arch Intern Med, 2006. **166**(8): p. 902-8.
225. Simonson, D.C., *Etiology and prevalence of hypertension in diabetic patients.* Diabetes Care, 1988. **11**(10): p. 821-7.
226. Ogihara, T., et al., *Enhancement of insulin sensitivity by troglitazone lowers blood pressure in diabetic hypertensives.* Am J Hypertens, 1995. **8**(3): p. 316-20.
227. Nolan, J.J., et al., *Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone.* N Engl J Med, 1994. **331**(18): p. 1188-93.
228. Ristow, M., et al., *Obesity associated with a mutation in a genetic regulator of adipocyte differentiation.* N Engl J Med, 1998. **339**(14): p. 953-9.
229. Deeb, S.S., et al., *A Pro12Ala substitution in PPARgamma2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity.* Nat Genet, 1998. **20**(3): p. 284-7.
230. Yen, C.J., et al., *Molecular scanning of the human peroxisome proliferator activated receptor gamma (hPPAR gamma) gene in diabetic Caucasians: identification of a Pro12Ala PPAR gamma 2 missense mutation.* Biochem Biophys Res Commun, 1997. **241**(2): p. 270-4.
231. Mori, Y., et al., *Effect of the Pro12Ala variant of the human peroxisome proliferator-activated receptor gamma 2 gene on adiposity, fat distribution, and insulin sensitivity in Japanese men.* Biochem Biophys Res Commun, 1998. **251**(1): p. 195-8.
232. Ringel, J., et al., *Pro12Ala missense mutation of the peroxisome proliferator activated receptor gamma and diabetes mellitus.* Biochem Biophys Res Commun, 1999. **254**(2): p. 450-3.

233. Mancini, F.P., et al., *Pro12Ala substitution in the peroxisome proliferator-activated receptor-gamma2 is not associated with type 2 diabetes.* Diabetes, 1999. **48**(7): p. 1466-8.
234. Beamer, B.A., et al., *Association of the Pro12Ala variant in the peroxisome proliferator-activated receptor-gamma2 gene with obesity in two Caucasian populations.* Diabetes, 1998. **47**(11): p. 1806-8.
235. Irizarry, R.A., et al., *Exploration, normalization, and summaries of high density oligonucleotide array probe level data.* Biostatistics, 2003. **4**(2): p. 249-64.
236. Ostgren, C.J., et al., *Peroxisome proliferator-activated receptor-gammaPro12Ala polymorphism and the association with blood pressure in type 2 diabetes: skaraborg hypertension and diabetes project.* J Hypertens, 2003. **21**(9): p. 1657-62.
237. Boggia, J., et al., *Prognostic accuracy of day versus night ambulatory blood pressure: a cohort study.* Lancet, 2007. **370**(9594): p. 1219-29.