Interplay between hormones, nutrients and adipose depots in the regulation of insulin sensitivity

- an experimental study in rat and human adipocytes

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ABSTRACT

Obesity and specifically central obesity is related to insulin resistance, type 2 diabetes and other components of the so-called metabolic syndrome. The aim of this study was to elucidate the interplay between hormones, nutrients and adipose depots in normal and insulin-resistant fat cell metabolism.

High levels of free fatty acids (FFAs) induce insulin resistance in muscle and liver in vivo. In the present study, rat adipocytes were treated with high physiological levels of oleic or palmitic acid in vitro for 4-24 h. This treatment had no effect on basal or insulin-stimulated glucose uptake capacity in these cells, neither did it affect the levels of the insulin signalling proteins; insulin receptor substrate (IRS)-1 or –2, phosphatidylinositol 3-kinase (PI3-K), protein kinase B (PKB) or glucose transporter (GLUT) 4, or the regulation of lipolysis rate.

Visceral adiposity is considered to be more harmful than peripheral adiposity with respect to metabolic and cardiovascular complications. In adipose biopsies from subjects undergoing abdominal surgery, we found that glucose uptake capacity was elevated in omental as compared to subcutaneous adipocytes. The sensitivity (EC50) or maximum relative response to insulin, measured as % of basal, did however not differ between the depots. In women, subcutaneous adipocytes displayed a higher lipolysis rate following cAMP-stimulation than omental adipocytes, whereas there was a tendency towards the opposite in adipocytes from men. No differences were found between depots or sexes in the ability of insulin to inhibit lipolysis or in the levels of the lipolysis regulating proteins, i.e. protein kinase A (PKA), hormone sensitive lipase (HSL) and perilipin.

Glucocorticoids, e.g. cortisol, exert pronounced insulin-antagonistic effects and are associated with redistribution of fat from peripheral to central fat depots in humans. Treatment of human subcutaneous and omental adipocytes in vitro, with the cortisol analogue dexamethasone, resulted in a dose dependent down-regulation of basal and insulin-stimulated glucose uptake capacity in omental, but not in subcutaneous cells. Concomitantly, the levels of IRS-1 and PKB were decreased only in omental adipocytes after dexamethasone treatment. The relative effect of insulin to stimulate glucose uptake was however not altered by dexamethasone treatment. The cAMP-stimulated lipolysis rate was elevated by dexamethasone treatment in cells from the subcutaneous depot in women and tended to be elevated in omental cells from men. No alterations however, were seen in the levels of the assessed lipolysis regulating proteins.

Subcutaneous as well as omental fat cell size correlated negatively to insulin action in subcutaneous fat cells in vitro after adjusting for age, sex and body fat parameters in non-diabetic, but not in type 2 diabetic, subjects. Large subcutaneous fat cell size was strongly related to plasma leptin levels in non-diabetic and in type 2 diabetic subjects.

We conclude that 1) adipocytes seem to be less vulnerable to elevated levels of fatty acids than muscle and liver cells, 2) the interactions between glucocorticoids and insulin in the regulation of glucose uptake differ between adipose depots, 3) depot specific hormonal lipolysis regulation differs between sexes and 4) fat cell size is related to insulin action in subcutaneous fat cells and to circulating levels of leptin.

Key words: adipose tissue, cAMP, glucocorticoids, glucose, insulin, insulin resistance, lipolysis, subcutaneous fat, type 2 diabetes, visceral fat
LIST OF PAPERS

I) Lundgren M, Eriksson JW 2004 No in vitro effects of fatty acids on glucose uptake, lipolysis or insulin signaling in rat adipocytes. Horm Metab Res 36: 203-209


ABBREVIATIONS

AC  adenylyl cyclase
ADA  adenosine deaminase
AMPK  AMP activated kinase
ANOVA  analysis of variance
AR  adrenoceptor
ATP  adenosine triphosphate
BCA  bicinchoninic acid
BMI  body mass index
BSA  bovine serum albumin
cAMP  cyclic adenosine monophosphate
CNS  central nervous system
DMEM  Dulbecco’s modified Eagle’s medium
Dx  dexamethasone
FCS  foetal calf serum
FFA  free fatty acid
GLUT  glucose transporter
HbA1c  glycosylated haemoglobin A
HOMA-IR  homeostasis model assessment insulin resistance index
HPA  hypothalamic-pituitary-adrenal
HSL  hormone sensitive lipase
IL  interleukin
IR  insulin receptor
IRS  insulin receptor substrate
Om  omental
PBS  phosphate buffered saline
PDE  phosphodiesterase
PIA  N^6-(R-phenylisopropyl) adenosine
PI3-K  phosphatidylinositol 3-kinase
PKA  protein kinase A
PKB  protein kinase B
SBP  systolic blood pressure
Sc  subcutaneous
SDS-PAGE  sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM  standard error of the mean
TG  triglyceride
TNF-α  tumour necrosis factor alpha
Visc  visceral
VLDL  very low density lipoprotein
WHR  waist to hip ratio
INTRODUCTION

Type 2 diabetes

The prevalence of type 2 diabetes is rapidly increasing worldwide, especially in developing countries. This is thought to be mainly due to a more sedentary lifestyle including physical inactivity and overweight or obesity but also to a more stressful environment in modern society. Moreover, genetic factors are also involved, and unfavourable interactions between environmental and genetic components are of great importance in the development and progression of type 2 diabetes (1). The disease is characterized by chronic elevation of circulating glucose (hyperglycaemia), followed by development of macro vascular (coronary heart disease, cerebrovascular and peripheral artery disease) and micro vascular (retinopathy, nephropathy and neuropathy) complications. The chronic hyperglycaemia is due to insufficient production and release of insulin together with defects in insulin action, i.e. insulin resistance in target tissues, mainly muscle, liver and adipose tissue.

Insulin action and insulin resistance

Insulin resistance means that insulin's ability to produce metabolic effects in target tissues is impaired and this is thought to be a central defect in the pathogenesis of type 2 diabetes. In healthy subjects, as the glucose levels rise after a meal, insulin is released from pancreatic β-cells and transported to target tissues via the blood. In muscle, this promotes glucose uptake and glycogen storage. In the liver, insulin inhibits glucose production and release as well as promotes glycogen storage. In adipose tissue, glucose uptake is stimulated and release of free fatty acids (FFA) is inhibited. Insulin resistance in combination with insufficient production and release of insulin, therefore, leads to perturbations in glucose as well as lipid metabolism and hence, hyperglycaemia and dyslipidemia (Fig 1).
Figure 1. Tissues involved in insulin resistance. Insulin resistance in muscle, liver and adipose tissue leads to hyperglycaemia and hyperlipidemia which can induce or further aggravate insulin resistance. VLDL, very low density lipoprotein.

Adipose tissue and insulin action

The body’s largest store of energy is commonly situated in the different adipose tissue depots. The storage and utilization of the energy in these depots are extensively regulated depending on the individual’s metabolic status and demand. In the fasting state, energy is released in the form of FFA and glycerol and this is mediated by different hormones, e.g. catecholamines (adrenaline, noradrenalin). These hormones bind to inhibiting $\alpha$-, and upon further elevation also to stimulating $\beta$-adrenergic, receptors (AR) at the fat cell surface. Binding to $\beta$-adrenergic receptors starts a cascade of reactions inside the fat cell leading to elevation of the cAMP-levels. The elevated levels of cAMP activates protein kinase A (PKA) and in turn, hormone sensitive lipase (HSL) and perilipin. Perilipin is a protein associated with the lipid droplet and is thought to protect the lipids from being hydrolysed (2-4). Once activated, perilipin is translocated from, and instead HSL gains access to the lipid droplet where it promotes hydrolysis of triglycerides to glycerol and FFAs. Glycerol and FFAs are then released into the blood stream and transported to different tissues in need of energy (5-7) (Fig 2). FFAs are also transported to the pancreas and stimulate insulin release as a feedback mechanism to avoid uncontrolled lipolysis (8, 9).
Postprandially, elevated levels of insulin, by binding to the insulin receptor, inhibit this process by a mechanism involving phosphodiesterase (PDE) 3B. Activation of PDE3B, leads to hydrolysis of cAMP and in turn, decreased activation of PKA as well as perilipin and HSL and hence less release of FFAs and glycerol (5-7) (Fig 2). Simultaneously, insulin promotes glucose uptake and glycogen synthesis in the fat cell. This effect is exerted by activation of insulin receptor substrate (IRS)-1 and -2, along with phosphatidylinositol 3-kinase (PI3-K), protein kinase- B (PKB) and -C (PKC) and finally glucose transporter 4 (GLUT4). GLUT4 translocates to the cell surface upon activation, where it facilitates the insulin-dependent glucose transport into the cell (10) (Fig 2).

**Figure 2.** Lipolytic pathway and the major insulin signalling pathways in glucose transport and antilipolysis. α-AR, α-adrenergic receptors; AMP, adenosine monophosphate; β-AR, β-adrenergic receptors; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; FFA, free fatty acid; G_i, inhibitory GTP-binding protein; GLUT4, glucose transporter 4; G_s, stimulatory GTP-binding protein; HSL, hormone sensitive lipase; IR, insulin receptor; IRS1/2, insulin receptor substrate 1 and 2; PDE3B, phosphodiesterase 3B; PI3-K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol-3,4-bisphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; PKA, protein kinase A; PKB/Akt, protein kinase B; TG, triglyceride.

An insulin signalling pathway distinct from the above schematized, also mediated by the insulin receptor but via Cbl and Cbl associated protein (CAP), has recently been discovered in adipocytes in vitro (11). However, to what extent this pathway is of importance for glucose transport in humans is still largely unknown (12, 13).
Adipose tissue depots, free fatty acids and the “portal theory”

The different stores of adipose tissue and their lipids serve important functions in the body. For example, lipids and fatty acids form the skeleton of steroid hormones and intracellular signalling molecules and constitute lipid bi-layers in cellular membranes. In addition, adipose tissue depots protect the body against mechanical trauma, produce heat and act as energy reservoirs.

Large amounts of adipose tissue as seen in overweight and obese states is however closely related to insulin resistance, type 2 diabetes as well as cardiovascular disease and hypertension. Central or visceral obesity is considered especially harmful in this regard (14-18) and is therefore considered to be a powerful risk marker for the development of type 2 diabetes as well as cardiovascular diseases in both women and men (16, 19-21). Removal of visceral fat, either by surgery or by diet in combination with exercise leads to improved metabolic profile in animals and humans (22-24).

The relationship between visceral adiposity and e.g. cardiovascular disease and type 2 diabetes is not fully understood. At least three possible explanations are at hand; 1) Visceral fat could due to its location and drainage of e.g. FFA via the portal vein exert insulin-antagonizing effects directly on the liver. This is the so-called “portal theory”. 2) Visceral fat may have distinct biochemical properties making it especially harmful as adiposity evolves and this depot grows larger. This could be related to e.g. adipose tissue as an endocrine organ. 3) Finally, visceral adiposity could be secondary to some other pathological factor leading to both visceral fat accumulation and insulin resistance.

Fat cells from the visceral adipose depot have been shown to display higher lipolytic activity and lower sensitivity to the antilipolytic effects of insulin than subcutaneous cells (25-28). This is possibly due to a larger amount of β-adrenergic receptors on the cell surface (27, 29). Hypothetically visceral adipose tissue therefore releases proportionally more FFAs into the circulation than subcutaneous adipose tissue (18, 30). A large visceral fat depot would therefore lead to a greater FFA delivery selectively to the liver followed by impairment of the hepatic insulin clearance and action. As a result thereof, increased glucose and very low density lipoprotein (VLDL) output from this tissue would occur (31-33). This concept, the so-called “portal theory”, has however been challenged since it has been stated that the
quantitative contribution of visceral fat to whole body and hepatic FFA exposure probably is of minor importance (34). In addition, the release of different adipokines (described in detail below) has been shown to differ between adipose depots. This may be an additional mechanism whereby adipose tissue deposition plays a role in development of insulin resistance.

In this respect it should be mentioned that the amount and distribution of body fat is partly regulated by genes (18, 35-37) and by sex as well as ethnic background. Generally, men have a larger proportion of visceral fat than women (18, 38) and white women tend to have more visceral fat than black women, even after adjustments for waist hip ratio (WHR) (39). Profound differences in the lipolytic regulation of different subcutaneous depots have been seen depending on ethnicity and also on sex (40). Women, in general, also seem to be somewhat protected from the adverse effects of obesity, e.g. dyslipidemia, insulin resistance and coronary heart disease, as compared to men (16).

Regardless of their site of origin, prolonged elevation of plasma FFAs, as often seen in the obese, diabetic and insulin-resistant states (41, 42), elicit patophysiological effects. These effects include impaired whole-body glucose uptake (43) and impaired glycogen synthesis and glucose transport in muscle (44-46) in combination with elevated glucose production and reduced clearance of insulin in the liver (31-33). Chronically elevated levels of FFAs could also lead to impairment of the regulation of insulin secretion from pancreatic β-cells (47-50). Elevated levels of FFAs, by their effects on mainly muscle and liver, therefore promote hyperglycaemia as well as insulin resistance.

**Adipose tissue as an endocrine organ**

Adipose tissue has previously been considered to be merely a site for storage of excess energy. Recently however, adipose tissue has gained interest for its function as an endocrine organ, releasing substances with paracrine as well as endocrine effects of which some probably hitherto are unknown. What is known is that the different adipose tissue depots release a number of factors involved in regulation of e.g. food intake, energy expenditure and insulin action as well as in glucose and lipid metabolism. Adipocyte-secreted factors may be involved in the development of muscle insulin resistance as suggested by co-culture
experiments of myocytes and adipocytes in vitro (51). At high levels, these factors, commonly termed adipokines, can be divided into factors considered to be mainly beneficial (e.g. adiponectin, leptin) or unfavorable (e.g. IL-6, resistin, TNF-α) in terms of e.g. insulin sensitivity (52).

**Adiponectin**

Adiponectin was discovered in the nineteen nineties and is expressed in and released from adipose tissue. The circulating levels of this protein are generally high, especially in women (53) and are negatively correlated to weight gain, and above all, visceral adiposity (54, 55). Concomitantly, adiponectin levels decrease with development of insulin resistance and are low in subjects with type 2 diabetes as compared to healthy individuals (53). Low levels of adiponectin have even been proposed to predict development of type 2 diabetes (56). Furthermore, adiponectin levels are elevated after weight reduction in healthy as well as type 2 diabetic subjects (53). According to in vitro studies on myocytes and endothelial cells and in vivo studies on mice, adiponectin exerts beneficial effects on insulin action (57, 58) and have anti-atherogenic properties (53, 59). The insulin sensitizing effects of adiponectin are thought to be exerted, at least in part, by increasing the oxidation of fatty acids in muscle via activation of AMP activated kinase (AMPK) and by reduction of glucose output from the liver (60).

**Leptin**

Leptin is secreted from adipose tissue and was discovered in 1994 as the gene responsible for the obesity in ob/ob mice (61). It was then discovered that administration of leptin to leptin deficient animals (62) and humans (63, 64) leads to reduced food intake followed by weight reduction. Therefore, leptin was first proposed to be an anti-obesity hormone where high levels would signal to the central nervous system (CNS) to reduce food intake. However, overweight and obese subjects generally have elevated levels of leptin and only modest weight reductions are seen when leptin is administrated to these subjects (65). They are thus proposed to be leptin resistant (66). The levels of leptin in circulation are related to the degree of insulin resistance (67) and adiposity and are reduced upon weight loss (68), however, there
are marked inter individual differences with a high degree of variation not depending on adipose tissue mass (69). Furthermore pre- as well as post-menopausal women have higher leptin levels than men with comparable BMI (70-72) and possibly testosterone could be involved in this regulation, since leptin levels correlated negatively to this sex hormone in men of different ages (73).

A new, contrary to the first mentioned, mechanism of leptin action has been proposed based on this knowledge. When the energy stores are filled, the levels of leptin are high, but when energy stores are depleted, leptin levels fall and this signals to the CNS that it is time to eat (74). Leptin therefore, rather than to limit food intake, is proposed to act as a warning when energy stores are at risk of being depleted (69, 74, 75). This effect would then be mediated at low levels of leptin.

In addition, leptin, possibly by acting together with adiponectin, has insulin sensitizing properties in muscle. This is mediated by activation of AMPK, promoting fatty acid oxidation in mitochondria of skeletal muscle in mice in vivo (76) and in vitro (77).

**Visfatin**

Just recently, a novel adipokine was discovered that initially was suggested to be predominantly expressed and released by visceral adipose tissue: visfatin (78). This has however been challenged by one other study that could not find any differences in visfatin expression between subcutaneous and visceral adipocytes (79). Visfatin in circulation is positively correlated to the degree of visceral adiposity in humans and is upregulated during adipogenesis and by glucocorticoids in 3T3-L1 adipocytes in vitro (78, 80). In contrast, growth hormone and isoprenaline but not insulin down-regulates this protein (80). Visfatin is of interest with respect to insulin resistance since it lowers plasma glucose levels in mice in vivo and promotes insulin action on glucose uptake in adipocytes and myocytes in vitro (78). This effect is proposed to be mediated via the insulin receptor (IR) however by a mechanism separated from that by insulin (78).
Interleukins and TNF-α

Interleukins and TNF-α are inflammation factors released at least in part by adipose tissue. These factors are also of interest because in addition, they could be adiposity signals (81). For example, the circulating levels of IL-6 are correlated to BMI and are hence often elevated in obese and also in insulin resistant subjects (82, 83). Besides its role in the immune response, IL-6 has been found to elevate cortisol release from the hypothalamic-pituitary-adrenal (HPA) axis (84, 85) and in addition, IL-6 signals to the brain to reduce food intake and increase energy expenditure in rodents (81). The latter function, may however be mediated by locally produced IL-6 rather than IL-6 secreted from adipose tissue (86, 87). In humans, the levels of IL-6 in brain are, in contrast to circulating levels, negatively correlated to adiposity (88). IL-6 has been shown to enhance energy expenditure and lipolysis (89-92) as well as glucose levels (93) in humans in vivo and in vitro, and to induce insulin resistance in hepatocytes in vitro (94). IL-6 has also been shown to down-regulate the gene expression of adiponectin in vitro (95). Taken together, these results suggest a role for IL-6 in the regulation of whole-body insulin sensitivity and energy homeostasis.

The circulating levels of TNF-α and the expression of TNF-α in adipose tissue is elevated in obesity and type 2 diabetes (83, 96) and is reduced with weight loss and improved insulin sensitivity (52, 96). There is, however, no evidence that adipose tissue in humans would be a net exporter of TNF-α, as reflected in circulating levels (52, 82) suggesting that TNF-α has primarily paracrine/autocrine effects in adipose tissue. Nevertheless, TNF-α has been shown to elevate lipolysis (97) and to reduce glucose uptake capacity by interfering with the insulin signal and glucose transporters (98-100) in adipocytes in vitro. In rodents, TNF-α has also been shown to affect the expression of other adipokines (101), for example, to reduce the levels of adiponectin and elevate the levels of leptin released from adipose tissue (102-104).

Resistin

Resistin is a newly discovered adipokine that has gained a lot of interest, as it was proposed to cause insulin resistance and be “the” link between obesity and type 2 diabetes (105). This proposition was based on resistin causing insulin resistance in mice in vivo and that antibodies directed against this protein improved insulin sensitivity (105). Since then, further
studies have shown this protein to be of relevance for the development of insulin resistance in rodents, but what role if any it has in the development of insulin resistance in humans is still largely unknown (106-108).

Other adipose derived factors

In addition to the above-mentioned proteins and hormones, adipose tissue releases other known, and probably still unknown, factors related to fat mass and possibly to insulin resistance, hypertension and atherosclerosis in obesity (52, 109). Furthermore, adipose tissue possesses cortisol (cortisone to cortisol via 11β-HSD) and aromatase (testosterone to estrogen) converting properties with potential autocrine, paracrine and endocrine effects (110, 111).

A summary of the release pattern of the mentioned adipokines is presented in table 1.

**Table 1.** Adipokine release pattern, effect on insulin sensitivity and impact of obesity and glucocorticoids. From (78, 80, 107, 112-118).

<table>
<thead>
<tr>
<th>Adipokine</th>
<th>Main depot of origin</th>
<th>Main cell type of origin</th>
<th>Effect on insulin sensitivity</th>
<th>Impact of obesity</th>
<th>Impact of glucocorticoids</th>
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<tr>
<td>Adiponectin</td>
<td>Sc/Visc</td>
<td>Adipocytes (A) vs. Stromal (S)</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
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<tr>
<td>Leptin</td>
<td>Sc</td>
<td>Adipocytes (A)</td>
<td>↓↓</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Visfatin</td>
<td>Visc</td>
<td>Stromal vascular cells (S)</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>IL-6</td>
<td>Visc</td>
<td>Stromal vascular cells (S)</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Sc/Visc</td>
<td>Stromal vascular cells (S)</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Resistin</td>
<td>Sc/Visc</td>
<td>Stromal vascular cells (S)</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
</tr>
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</table>

Site of origin refers to predominant site of origin from the subcutaneous or visceral adipose tissue depot, stromal vascular cells or adipocytes. A, Adipocytes; S, Stromal vascular cells; Sc, Subcutaneous depot; Visc, Visceral depot; ↑↓↓, Different impacts in different tissues (leptin) or different results in different reports (resistin).
Ectopic fat and fat cell size

Ectopic fat

Closely related to the view of adipose tissue as an endocrine organ, is the theory of ectopic fat, i.e. fat stored in other tissues than adipose tissues. According to this theory, excess energy is stored in adipocytes that expand to a certain (individual) limit. When this limit is passed, FFAs spill over from the adipose tissues and are transported to other organs of the body, e.g. muscle and liver, where they are accumulated as ectopic fat (119). These tissues are not as well equipped as adipose tissue to take care of excess energy and this would, according to this theory, cause development of insulin resistance and type 2 diabetes. The FFA spillover is thought to occur due to inability of the adipose tissue to differentiate new fat cells. Large fat cell size would accordingly be a predictor for insulin resistance and type 2 diabetes as well as other characteristics of the metabolic syndrome (119).

Ectopic fat accumulation in muscle, liver and pancreas is a common phenomenon in subjects with insulin resistance and type 2 diabetes (120) but also in insulin resistant lipodystrophic subjects (121). A lot of evidence exists supporting a role for ectopic fat accumulation in the development of type 2 diabetes. For example, intra-myocellular lipid content seems to be closely related to insulin resistance in otherwise healthy subjects of different ages (122-124), in obese and type 2 diabetic subjects (125), in type 1 diabetic subjects with poor metabolic control (126) as well as in women with gestational diabetes (127) and in offspring to type 2 diabetic subjects (128, 129). Reduced intra-myocellular lipid content by administration of PPARγ-agonists or by weight reduction through biliopancreatic diversion (in morbidly obese patients), has been shown to be associated with improved insulin sensitivity, further supporting this association (130, 131).

However, despite this seemingly close relationship between intra-myocellular lipid content and insulin resistance, there are some contradictions. For example, the effect of intra-myocellular lipid content on insulin sensitivity is dependent on muscle fiber type (125) and aerobic fitness, i.e. athletes have large depots of intra-myocellular lipid and are very insulin sensitive (132). Furthermore, dietary interventions or increased physical activity can improve insulin sensitivity, independent of any effect on intra-myocellular lipid content (133-135). Sex and ethnicity may also be of importance in this respect (132, 136). Intra-myocellular lipid
content, therefore, may not cause, but rather be a consequence of insulin resistance, and for example, mitochondrial function may be of importance in this regard (132).

In the liver, lipid accumulation has been shown to be associated with insulin resistance in different animal models (137) and in healthy as well as type 2 diabetic human subjects (138, 139). Administration of leptin and adiponectin in the ob/ob mouse decreased intrahepatic lipid accumulation and reversed insulin resistance in these animals (140). The same results were seen with PPARγ-agonist administration in type 2 diabetic human subjects (141). In addition, lipid accumulation in pancreatic β-cells in vitro induces apoptosis (142, 143).

*Fat cell size*

Micro array studies reveal that expression of several genes involved in fat cell differentiation and proliferation are disturbed in insulin resistant relatives to type 2 diabetic subjects (144) and enlarged fat cells are also seen in these subjects (145) suggesting a deficient cell differentiation capacity. Enlarged subcutaneous fat cell size is related to insulin resistance, in vivo and in vitro, and has been shown to be predictive of type 2 diabetes (146-148). Furthermore, this fat cell enlargement is evident long before the disease (147). In addition, the antidiabetic drugs tiazolidinediones (TZDs) enhance insulin sensitivity in parallel with recruitment of new, small fat cells, especially in the subcutaneous depot (149-151). Implants of fat into lipodystrophic mice enhances insulin sensitivity in these animals (152) suggesting an important role for the adipose tissue in glucose as well as lipid metabolism.

Enlarged fat cells could also be of importance in development of whole-body insulin resistance by other mechanisms. For example, large fat cells are more insulin resistant than small ones (119) and have thus less glucose uptake capacity and potentially release more FFAs into circulation. This in turn, can result in whole-body, muscle and liver insulin resistance (31-33, 43-46). Furthermore, the pattern and levels of adipokines released by the fat cell could also be altered with enlargement of the cell (147) and for example IL-6 and leptin release from subcutaneous adipocytes is related to fat cell size (95, 153).
Enlarged fat cell size could of course also be a result of some other pathogenic factor that independently leads to both insulin resistance and large fat cells, e.g. insulin or glucocorticoids (147).

**Insulin-antagonistic hormones**

**Glucocorticoids**

Glucocorticoids, in humans mainly cortisol, are of great importance in e.g. regulating blood pressure and metabolism. They are also active players of food intake regulation through actions in hypothalamus (111). In healthy individuals, cortisol is released in a diurnal pattern with high levels in the early morning and low levels in the afternoon and at night. Short-term regulation of cortisol levels occurs in response to various physiological and psychological stimuli superimposed on this diurnal pattern. Cortisol rhythmicity is regulated by the HPA axis that in response to different stimuli signals via the pituitary to the adrenal cortex where cortisol is produced and released (154). There is evidence that this regulation is disturbed in central obesity and after long periods of stress (155, 156). In addition, not only circulating levels are of interest since there is a tissue-specific regulation (i.e. pre-receptor metabolism) of active glucocorticoids in target tissues, e.g. liver and adipose tissue (110, 111, 157).

Glucocorticoids are of interest with respect to development of insulin resistance and type 2 diabetes due to their ability to counteract insulin's effects. For example, glucocorticoids have been shown to induce insulin resistance in muscle in vivo (158) and in adipocytes in vitro (159) by perturbations in the glucose transport machinery. In addition, glycogen synthesis is disturbed (158), glucose output from the liver is elevated (160) and insulin output from pancreas is inhibited (161, 162) by cortisol.

Glucocorticoids stimulate lipoprotein lipase (LPL) activity (163), predominantly in the visceral depot (164), leading to central obesity. This is most obviously manifested in Cushing’s disease, a condition with chronically elevated levels of cortisol that leads to hypertension, hyperglycaemia and dyslipidemia (165). Cortisol’s permissive effects on lipolysis upon catecholamine-stimulation (166) could possibly induce the dyslipidemia. Glucocorticoids may also, in a depot and sex-specific manner, affect the release of various
adipokines from adipose tissue. For example, IL-6 release has been shown to be downregulated and leptin release up-regulated especially from visceral adipocytes, in vitro (112, 167) and in vivo (168, 169) by glucocorticoid treatment.

Catecholamines

Catecholamines, mainly adrenaline and noradrenaline, are released upon stimulation of the sympathetic nervous system, e.g. due to physical or psychological stress. This results in elevated circulating levels of adrenalin, noradrenalin and glucagon with mainly catabolic effects on glucose and lipid metabolism in muscle, liver and adipose tissue.

In muscle and fat, the insulin-independent glucose uptake capacity is elevated by low levels of noradrenaline-stimulation and inhibited by adrenaline-stimulation or higher levels of noradrenaline. The insulin-stimulated glucose uptake capacity is acutely reduced upon catecholamine-stimulation (170) and this in combination with elevated liver glucose output results in elevated blood glucose levels (171). Furthermore, following catecholamine-stimulation LPL activity is elevated in muscle and inhibited in adipose tissue (171). As outlined previously, lipolysis is increased in adipose tissue by elevation of cAMP and subsequent activation of HSL after catecholamine-stimulation (5-7).

According to one hypothesis, long-term stress leads to resistance to glucocorticoid effects, followed by a depletion or inflexibility in the glucocorticoid response to physical or psychological stimuli (154). This phenomenon is often seen in abdominal obesity and sympathetic nerve stimulation mediated by adrenaline and noradrenalin has been suggested as a compensatory mechanism in this situation (154).

Growth hormone

Growth hormone (GH) is released from the pituitary and affects carbohydrate metabolism. Prolonged elevation of GH levels for a few hours leads to insulin resistance and hyperglycaemia by reduced glucose uptake capacity in muscle and impaired suppression of glucose production in the liver, as well as elevated lipolysis (172-174). Concomitantly, GH
deficiency leads to improved insulin sensitivity and reduced levels of circulating glucose at least in children (174, 175). The exact mechanisms behind this are not fully understood but seems to be tissue-specific (176) involving alterations in insulin receptor levels and action as well as alterations in the insulin signalling cascade. GH may also exert its insulin-antagonistic effects by regulation of adipokines released from adipose tissue, for example adiponectin (177).

Glucagon

Glucagon is secreted from the pancreas, when glucose levels are low, and stimulates glucose production and release from the liver (178). In healthy subjects, glucagon release is suppressed after a meal as insulin and glucose levels rise (179, 180). In subjects with impaired glucose tolerance and in type 2 diabetic subjects, however, glucagon levels stays elevated after a meal, thereby contributing to postprandial hyperglycaemia (178-181).
AIMS

The aim of this study was to explore the role of hormonal and metabolic factors in different adipose depots for the development of cellular insulin resistance.

Specific aims:

1. To explore a possible impact of free fatty acid levels on insulin-mediated regulation of glucose and lipid metabolism in epididymal rat adipocytes (paper I).

2. To compare glucose metabolism and the effects of glucocorticoids between human subcutaneous and omental fat depots (paper II).

3. To compare lipolysis regulation, the effects of glucocorticoids and possible gender differences between human subcutaneous and omental fat depots (paper III).

4. To study whether fat cell size *per se* is associated with insulin resistance and circulating adipokine levels (paper IV).
METHODS

Animals (paper I)

Male Sprague-Dawley rats were housed at the animal facility at Umeå University Hospital. They were freely fed with standard laboratory rat chow and water and housed under a 12/12 h light/dark cycle. At the weight of 150-200g, the rats were sacrificed by decapitation and the epididymal fat pads were excised and transported to the laboratory. The Umeå Ethics Committee for animal research approved the study protocol.

Patients and healthy volunteers (paper II-IV)

The subjects in paper II and III were recruited among patients undergoing elective abdominal surgery, but otherwise healthy, at the Umeå University Hospital. Exclusion criteria were malignant or endocrine (except for treated primary hypothyroidism) disorders or acute disease conditions. The patients were fasted overnight and following anaesthesia at the beginning of surgery, biopsies were taken from the abdominal subcutaneous and omental fat depots, the latter representing a visceral fat depot. One to two months after surgery, the subjects attended the Metabolic Unit at the Umeå University Hospital for blood sampling and body composition assessments.

The subjects in paper IV were recruited as above, or among patients at the Diabetes Unit of Umeå University Hospital and by advertisement in a local newspaper. In either case the subjects attended the Metabolic Unit at the Umeå University Hospital at 08.00, following an overnight fast and blood samples were obtained. The majority of fat biopsies were taken with a needle biopsy of subcutaneous fat from the lower part of the abdomen after local dermal anaesthesia. In a subgroup of subjects however, biopsies was taken during abdominal surgery as described above. The Umeå University Ethics Committee approved the study protocols and the participants gave their informed consent.

The study groups in paper II and III consisted of non-diabetic subjects only. In paper IV, there were 83 non-diabetic subjects and 49 type 2 diabetic patients classified in accordance with the 1998 WHO criteria (182). Of the 49 subjects with type 2 diabetes, 10 were treated with non-
pharmacological measures, 33 were treated with oral antidiabetic drugs and the remaining six subjects were treated with insulin, alone or in combination with oral antidiabetic drugs. The subjects in paper IV were pooled from other studies performed by our group (148, 183, 184 and Lundgren et al., Renstrom et al., and Ruge et al., unpublished data).

**Adipocyte preparation and assessment of fat cell size**

Isolated fat cells were obtained by mincing and thereafter shaking (at ~150 rpm) the tissue samples in polypropylene containers at 37°C for approximately 1 h in medium 199, containing 5.6 mM glucose, 40mg/ml BSA and 0.6 mg/ml collagenase. Following collagenase digestion, cells and medium were filtered through a nylon mesh (pore-size; 0.3 mm in diameter) and washed four times with fresh medium.

Cell size was assessed according to the method of Smith et al. (185). In brief, ~0.5 ml of cell suspension was placed in a monolayer on a glass slide and the diameter of 100 consecutive cells was determined with a light-microscope equipped with an inbuilt ruler. Cell size is presented as the average cell diameter (μm) of 100 cells from the subcutaneous and omental adipose tissue, respectively.

**Adipocyte culture**

Isolated adipocytes were placed in flasks containing DMEM with 10% FCS, penicillin (100U/ml), streptomycin (100µg/ml) and with the cortisol analogue dexamethasone, D-glucose and FFAs as indicated. Cells were then incubated under a gas phase of 95% O₂ and 5% CO₂ for 24 h or as indicated.

**Preparation of fatty acid solutions**

Fatty acids are hydrophobic and are therefore under physiological conditions transported in the blood bound to albumin. Therefore, fatty acids to be used in the cell culture were conjugated with fatty acid free albumin. Sodium salts of the fatty acids to be examined were
first dissolved in heated (40-50°C) distilled water. To conjugate with BSA (FFA:BSA ratio 2.5:1 or 6:1) the FFA-solution was thereafter mixed with warm (~40°C) BSA dissolved in DMEM to produce a clear stock solution. The stock solutions were then frozen (-20°C) below N₂-gas to avoid oxidation of the fatty acids, until utilization.

**Glucose uptake assay**

The glucose uptake assay was performed essentially as previously reported (186). Following collagenase treatment or culture, cells were washed and thereafter incubated (lipocrit 3-5 %) at 37°C in vials containing ADA, BSA (4%), medium 199, and PIA, with or without insulin. After 15 min, D-[U-¹⁴C]glucose was added (0.7-1.0 µM) and the incubation continued for another 45 (paper II-IV) or 60 min (Paper I and IV). The cells were then transferred to pre-chilled tubes and separated from the glucose-containing medium by centrifugation through silicone oil, thereby terminating the assay. The fat pellet was collected and cell-associated radioactivity was determined by scintillation counting. Under these experimental conditions, using low concentrations of glucose, the glucose uptake is mainly determined by the rate of trans-membrane transport (187) and is calculated according to the following formula:

\[
\text{Cellular clearance of medium glucose} = \frac{\text{Cell-associated radioactivity} \times \text{Volume}}{\text{Radioactivity in medium} \times \text{Cell number} \times \text{Time}}
\]

**Lipolysis assay**

Following collagenase treatment or culture, cells were washed and thereafter incubated (lipocrit 1-3 %) for 60 min at 37°C in vials containing medium 199, ADA, BSA (4%), glucose (5.6 mM) and PIA with or without insulin. 8-bromo cAMP or isoprenaline was added as indicated. After 60 min, transferring cells and medium to pre-chilled tubes and immediate centrifugation through silicone oil terminated the assay. The medium was collected for determination of glycerol content according to Bradley and Kaslow (188). Glycerol was phosphorylated in the presence of glycerokinase and [γ-³²P]ATP for 30 min at 37°C. Residual
[γ-32P]ATP was then hydrolysed in perchloric acid at 95°C for 60 min and free 32P-phosphate was precipitated on ice in the presence of ammonium molybdate and triethylamine. After centrifugation, radioactivity of the supernatant was measured reflecting phosphorylated glycerol.

**Western blot analyses of proteins from total cellular lysates**

After culture for 24 h, cells were washed four times with fresh medium and incubated 10 min at 37°C in vials containing glucose, medium 199, BSA (4%), ADA and PIA with or without a maximal insulin concentration (1000 µU/ml). This procedure was used in order to study the insulin stimulated phosphorylation, and hence activation of PKB. Cells were then centrifuged through silicone oil (paper I-II) or transferred to cold PBS (paper III), collected and thereafter lysed with 0.1-0.2 ml lysis buffer. Insoluble substances were sedimented by centrifugation and supernatants were collected. Protein content was measured with the BCA protein assay kit (Pierce Chemical Co, Rockford, IL, USA) and bovine serum albumin as standard.

Separation of proteins was performed by SDS-PAGE. The total amount of protein added per lane was equal within each set of experiments. After separation, proteins were transferred to an Immobilon-P membrane, which was blocked overnight at 4°C in 5% dry milk dissolved in PBS. Detection of the various proteins (IRS-1, IRS-2, PI3-K, PKB, GLUT4, PKA, perilipin A and HSL) was performed using polyclonal antibodies and ECL Western blotting kit was used to visualize immunoreactive bands. β-actin was used as a housekeeping protein in some experiments to assure equal loading. The different culture conditions did not affect the levels of β-actin.

**Blood chemistry and insulin sensitivity in vivo**

Commercial enzymatic kits were used to analyse adiponectin and leptin (Linco Research Inc., St Charles, MO), FFA (Wako Chemical USA Inc., Richmond, VA), interleukin-6 (IL-6) and tumour necrosis factor (TNF)-α (R&D Systems Inc., Minneapolis, MN). Serum insulin concentrations were measured by micro particle enzyme immunoassay (Immulite 2000, DPC, Los Angeles, CA, or Abbot Imx, Abbott Laboratories, Abbott Park, IL). All other
measurements were done according to routine methods at the Department of Clinical Chemistry, Umeå University Hospital. Insulin resistance was estimated by using the Homeostasis Model Assessment Insulin Resistance index (HOMA-IR) calculated from fasting serum glucose and insulin concentrations \[\text{glucose (mM)} \times \text{Insulin (pM)} / 22.5\] (189). The M-value was calculated as the glucose uptake rate at steady state between 60-120 min of a 2 h euglycemic, hyperinsulinemic (56 mU/m² body surface area/min) clamp. This procedure was performed as previously described in detail (190).

**Statistical analyses**

The SPSS package (SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. Results are given as means ± S.E.M. ANOVA, t-test, linear simple and multiple regressions and Wilcoxon signed ranks were used as appropriate. Variables are logarithmically transformed where appropriate due to skewed distributions. The significance level was set at p-values less than 0.05 unless otherwise indicated.
SUMMARY OF RESULTS

Paper I

The aim of this study was to explore whether FFAs alone or in combination with high levels of glucose and insulin leads to insulin resistance in mature rat adipocytes cultured in vitro.

Treatment of isolated adipocytes with FFAs for 4 or 24 h had no effect on either the basal or the insulin-stimulated glucose uptake capacity and this was independent of fatty acid used (oleate, unsaturated or palmitate, saturated), concentration thereof (0.6 or 1.5 mM) or FFA to BSA ratio (2.5:1 or 6:1). Furthermore, FFAs produced no additional effects to the insulin-antagonistic actions exerted by high levels of glucose and insulin in the incubation medium.

The rate of basal and cAMP-stimulated lipolysis in these cells was not affected by incubation in the presence of FFAs and neither was the ability of insulin to inhibit lipolysis. Contents of insulin signalling proteins and GLUT4 also remained unaffected as well as insulin's ability to stimulate PKB phosphorylation.

We conclude that it is not likely that FFAs have direct insulin-antagonistic effects on adipocytes from rats and that this is also true for a model of cellular insulin resistance (high levels of glucose and insulin).

Paper II

The aim of this study was to compare isolated adipocytes from the human subcutaneous and omental fat depots with respect to glucose metabolism and regulation, with particular focus on the influence of glucocorticoids.

Fresh human omental adipocytes displayed a ~2 fold higher basal and insulin-stimulated glucose uptake capacity as compared to subcutaneous adipocytes. The relative response to insulin, measured as % of basal glucose uptake, was however similar and the EC$_{50}$ value did not differ between the two depots. The same pattern was seen after the cells had been cultured for 24 h, albeit the relative insulin response was somewhat lower.
Addition of the cortisol analogue dexamethasone to the incubation medium led to a significant decrease in both the basal and the insulin-stimulated glucose uptake capacity only in omental adipocytes. This effect was concentration dependent with a maximal effect at 0.3 μM dexamethasone. The relative effect of insulin, however, remained unaffected by dexamethasone treatment. Dexamethasone, independent of concentration used, had no consistent effects on glucose uptake capacity in subcutaneous adipocytes.

The cellular content of IRS-1 and PKB protein tended to be higher in fresh omental compared to subcutaneous adipocytes, but this difference was not statistically significant. The amount of GLUT4 was significantly higher in fresh omental compared to subcutaneous cells.

In omental adipocytes, incubation of the adipocytes for 24 h in the presence of dexamethasone led to a significant decrease in the cellular content of IRS-1 (~40%) and PKB (~20%). Insulin's ability to stimulate PKB phosphorylation however remained unaffected, as did the cellular content of GLUT4. In subcutaneous cells, dexamethasone had no consistent effects on the cellular content of the measured insulin signalling proteins or GLUT4.

Simple linear and multivariate stepwise regression analyses between maximal insulin-stimulated glucose uptake capacity vs. adipocyte cell size, clinical and biochemical characteristics revealed that omental adipocyte cell size alone or together with systolic blood pressure (SBP) was significantly correlated to insulin action in subcutaneous and omental adipocytes, respectively.

We conclude that the basal and insulin-stimulated glucose uptake capacity is higher in human omental compared to subcutaneous adipocytes. Treatment with dexamethasone for 24 h significantly decreased the cellular amounts of IRS-1 and PKB proteins in omental cells. This could be part of the explanation to the concomitant decrease in glucose uptake capacity observed in these cells.
Table 2. Summary of the results in paper II. Effects of dexamethasone on basal and insulin–stimulated glucose uptake capacity and on the levels of insulin signalling proteins.

<table>
<thead>
<tr>
<th>Depot</th>
<th>Glucose uptake</th>
<th>IRS-1</th>
<th>PKB</th>
<th>pSer\textsuperscript{473}-PKB</th>
<th>GLUT4</th>
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<tr>
<td>Sc</td>
<td>←</td>
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Om, Omental; pSer\textsuperscript{473}-PKB, insulin-stimulated pSer\textsuperscript{473}-PKB; Sc, Subcutaneous

Paper III

The aim of this study was to compare isolated adipocytes from the human subcutaneous and omental fat depots with respect to lipolysis regulation as well as susceptibility to the influence of glucocorticoids.

In fresh adipocytes, the basal rate of lipolysis was equal regardless of depot and sex. In women the rate of 8-bromo cAMP-stimulated lipolysis was significantly higher in subcutaneous compared to omental adipocytes, whereas no significant differences were found between the two depots in men. The same patterns were seen when isoprenaline, instead of 8-bromo cAMP, was used to stimulate lipolysis. The antilipolytic effect of insulin was similar between depots and sexes even though omental adipocytes tended to be slightly less insulin sensitive than subcutaneous adipocytes (n.s.).

In women, in vitro treatment of cells with dexamethasone for 24 h enhanced the ability of 8-bromo cAMP to stimulate lipolysis (~40%) in the subcutaneous but not in the omental depot. In men, dexamethasone had no consistent effects in either depot but tended to slightly elevate (by ~10%, n.s.) 8-bromo cAMP-stimulated lipolysis in omental cells. The same pattern was seen when lipolysis was stimulated with isoprenaline. Insulin's ability to inhibit lipolysis was not altered by dexamethasone and hence was equal between depots and sexes independent of previous treatment (with or without dexamethasone).

The cellular content of PKA, Perilipin A and HSL did not differ between adipose depots or between sexes and remained unaltered by dexamethasone treatment.
Simple linear and multivariate regressions between basal or 8-bromo cAMP-stimulated lipolysis and adipocyte cell size as well as clinical and biochemical characteristics, revealed that adiponectin was negatively and leptin positively related to the 8-bromo cAMP stimulated lipolysis rate in subcutaneous cells after adjusting for age and sex. Adiponectin was also negatively related to the 8-bromo cAMP-stimulated lipolysis in omental cells, but this relationship was lost after adjusting for age and sex.

We conclude that, in women, the catecholamine-stimulated rate of lipolysis is higher in the subcutaneous compared to the omental depot, and is even further elevated by the presence of glucocorticoids. This was not seen in adipocytes from men and hence, the hormonal regulation of lipolysis differs between adipocytes from different adipose tissue depots and also between sexes. PKA, Perilipin A and HSL protein levels are equal in adipocytes regardless of depot and sex and are not altered after treatment with dexamethasone. The differences in lipolytic rate could therefore not be attributed to the levels of these proteins.

Table 3. Summary of the results in paper III. Lipolysis activation by 8-bromo cAMP in fresh subcutaneous and omental adipocytes from women and men and after a 24 h culture with dexamethasone.

<table>
<thead>
<tr>
<th></th>
<th>Women</th>
<th></th>
<th>Men</th>
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<tr>
<td></td>
<td>Sc</td>
<td>Om</td>
<td>Sc</td>
<td>Om</td>
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<tr>
<td>Fresh cells</td>
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<td>⇓</td>
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<tr>
<td>Dx culture</td>
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Dx, dexamethasone; Om, Omental; Sc, Subcutaneous

Paper IV

The aim of this study was to study relationships between subcutaneous and omental fat cell size vs. anthropometric and biochemical characteristics with emphasis on circulating adipokine levels and insulin sensitivity in vivo and in vitro. This was done in non-diabetic subjects and in subjects with type 2 diabetes.

In non-diabetic subjects, after adjusting for age, sex and body composition, the subcutaneous as well as the omental fat cell size was independently and negatively correlated to insulin
action on glucose uptake in vitro in subcutaneous fat cells. There was, however, no relationship between fat cell size in either depot and insulin action on glucose uptake in omental fat cells in vitro, or insulin sensitivity assessed in vivo (M-value and HOMA-IR index). Furthermore, subcutaneous fat cell size was independently and positively correlated to the plasma levels of leptin, but not to any of the other assessed adipokines (adiponectin, IL-6 and TNF-α), in these subjects. Omental fat cell size was not associated to any of the assessed adipokines. Plasma leptin levels, but not any of the other assessed adipokines, was independently and negatively associated with insulin action on glucose uptake in vitro in subcutaneous fat cells.

In type 2 diabetic subjects, no independent relationships were found between fat cell size in any adipose tissue depot and any of the insulin sensitivity parameters assessed. Subcutaneous fat cell size however, was independently correlated to plasma levels of leptin after adjusting for age, sex and body composition.

We conclude that large fat cells may predict cellular insulin resistance and possibly, development of type 2 diabetes. However, after the development of type 2 diabetes, factors other than fat cell size probably become more important to promote insulin resistance.
DISCUSSION

It is generally considered that insulin resistance precedes type 2 diabetes and most patients with type 2 diabetes are insulin resistant. However, less is known about the early mechanisms behind insulin resistance. Adipocyte derived factors such as adipokines and FFAs could be involved, as well as other hormones in the circulation with potential insulin antagonistic properties.

Effects of free fatty acids on glucose and lipid metabolism

It is well established that the levels of FFAs in the circulation often are elevated in obese subjects and accompanied by insulin resistance and type 2 diabetes (41, 42). The effects of FFAs on different aspects of insulin action have therefore been extensively studied and those results showed impaired whole body and muscle glucose uptake and utilization (43-46) as well as enhanced glucose production and reduced insulin clearance in the liver (31-33). Perturbations in insulin secretion from pancreatic β-cells (47-50) are also a result of prolonged elevation of FFA levels.

The results of previous work where the effects of FFAs on adipocytes and adipose tissue metabolism have been studied are, however, not as convincing as studies on e.g. muscle and liver. FFA exposure of adipocytes for different time periods in vitro, has led to either enhanced (191-193), unchanged (193) or inhibited glucose uptake and utilisation (194-196) somewhat depending on fat cell type, length of the incubation period and fatty acid used (saturated or unsaturated) (193, 194). The contradictory results led us to perform an additional study on rat adipocytes cultured in vitro in the presence of either oleate or palmitate for 4 or 24 h.

We found no significant differences between control cells and cells treated with FFA, with respect to basal or insulin-stimulated glucose uptake capacity or in the levels of the insulin signalling proteins; IRS-1, PI3-K, PKB or GLUT4. Basal and 8-bromo cAMP-stimulated lipolysis as well as the ability of insulin to inhibit lipolysis remained unaffected by FFA treatment. These results were true regardless of the length of the incubation period, the fatty acid used or concentration thereof (corresponding to high physiological levels in humans and
Furthermore, no additional aggravation of the insulin resistance was seen by FFA exposure to adipocytes already rendered insulin resistant by high levels of glucose and insulin.

Some of the discrepancies between our and previously reported results could be accounted for by differences in study design, mainly in use of cell type (primary adipocytes or 3T3-L1 cells) and method for glucose transport assessment (\(^{3}\text{H}-2\text{-deoxyglucose}\) or \(^{14}\text{C}-\text{glucose}\) uptake). However, the effects of FFAs have been small and taken together, irreproducible.

Nevertheless, it cannot be excluded that high FFA levels despite our results in vitro exert insulin-antagonistic effects in adipose tissue in vivo. Furthermore, adipocytes from humans as well as cells from different adipose tissue depots could respond differently to FFA exposure than the cells used in this study. Our results suggest however, that elevated levels of FFAs, as often seen in obese and type 2 diabetic subjects, is a consequence rather than a cause of insulin resistance in fat cells. Hence, high levels of FFAs do not, from our point of view, seem to further aggravate a cellular defect in insulin action in fat cells. The mechanisms behind the primary adipose tissue insulin resistance are however not fully understood and could include neuroendocrine pathways, e.g. alterations in the autonomic nerve activity, the HPA-axis and cortisol release regulation (145, 159, 190, 197).

**Adipose tissue depot differences and the influence of sex**

Visceral adiposity is considered to be more harmful than peripheral adiposity with respect to metabolic and cardiovascular alterations. In adipose biopsies from subjects undergoing abdominal surgery we found, in accordance with previous studies in vivo and in vitro (28, 198, 199), omental adipocytes to have ~2-fold higher capacity of basal as well as insulin-stimulated glucose uptake as compared to subcutaneous adipocytes. In parallel, omental adipocytes displayed higher levels of the insulin signalling protein PKB and GLUT4 as compared to subcutaneous adipocytes. Possibly, the elevated level of GLUT4 in omental adipocytes could account for the higher glucose uptake capacity in this depot. This could, indirectly by substrate competition in situations with high calorie intake, promote triglyceride storage preferentially in the omental depot (200).
In adipocytes from women, the ability of cAMP or isoprenaline to stimulate lipolysis was higher in subcutaneous compared to omental cells, whereas in men there were no consistent differences between the depots. This is in accordance with some (201-203) but not all (25, 27) previous studies on human adipose tissue. However, sex is not always taken into account in the previous studies and this may be one explanation for the discrepant results. In addition, sex hormones such as estrogens and testosterone have permissive effects on catecholamine stimulated lipolysis (155) and may therefore be involved in sex-specific lipolysis regulation. The levels of these hormones are unfortunately not assessed in this study but a hint may be given by the fact that women, independent of age or treatment with gonadal hormone replacement therapy, displayed the same pattern of lipolysis regulation. Hence, estrogens may therefore be of minor importance in that sense.

Adiponectin and leptin, two adipose tissue-derived factors, correlated to lipolytic rate and they are displayed differently between the sexes and could therefore possibly also be involved. Adiponectin's potential effects on lipolysis are to our knowledge not known, but leptin has been shown to counteract insulin action on glucose uptake capacity and antilipolysis in adipocytes in vitro (204). Leptin levels may thus explain some of the differences in lipolytic activation between sexes since women in general have higher levels of leptin in circulation than men (70-72). The previously proposed high lipolytic activity in omental fat could also require local interaction with stromal-vascular cells since some of the older studies were mainly performed on adipose tissue rather than on isolated adipocytes (25, 26).

We had approximately the same results whether we used isoprenaline or a cAMP analogue acting on different steps (β-AR stimulation elevating intracellular cAMP-levels and by mimicking cAMP, respectively) in the lipolysis regulation, and would therefore suggest that the sex specific regulation is located beyond cAMP in the lipolysis-activating cascade. However, no differences in the levels of the lipolysis regulating proteins PKA, HSL and perilipin were found between sexes or depots and the literature does not provide convincing evidence for a regulation of lipolysis by the amount of these proteins (202, 205-210). It might therefore be speculated that the specific activity in, rather than the amount of, any of these proteins is regulated. Some other additional lipolysis activating mechanism could possibly also be involved as suggested by Lucas et al (211), since mice devoid of HSL still display
lipolysis (206, 212, 213) and since over-expression of HSL does not necessarily mean elevated levels of lipolysis (211).

Previous studies suggest an enhanced effect of insulin in subcutaneous compared to omental adipocytes (26, 28). In contrast, we found no consistent differences between the depots in the relative ability of insulin either to inhibit stimulated lipolysis or to stimulate glucose uptake. Omental cells however, tended to be slightly less sensitive to insulin's antilipolytic effect than the subcutaneous cells, but this difference was not statistically significant possibly due to the small cohort.

**Effects of glucocorticoids on glucose and lipid metabolism**

Glucocorticoids are known for their insulin-antagonistic effects as seen both in vivo and in vitro (158-160) and they are associated with redistribution of fat from peripheral to central fat depots in humans (165) possibly mediated by induction of elevated LPL-activity (163) predominantly in the visceral adipose depot (164). This redistribution of fat is a common phenomenon in Cushing's disease – a condition of chronic hypercortisolism. We used the cortisol analogue dexamethasone to study the effects of glucocorticoids on glucose and lipid metabolism in human subcutaneous and omental adipocytes, in vitro.

Consistent with our studies on rat adipocytes from epididymal fat pads (159), dexamethasone treatment resulted in a dose dependent inhibition of the glucose uptake capacity in both sexes, but only in omental cells. In parallel with the decreased glucose uptake capacity in omental cells after dexamethasone treatment, there were significantly decreased levels of the insulin signalling proteins IRS-1 and PKB.

There is evidence from the literature of perturbations in GLUT4 translocation after inhibition of PKB action and also after dexamethasone treatment (214-216). Possibly this could account for some of the perturbations in glucose uptake ability seen in these cells. However, a suppression of the overall glucose uptake capacity via inhibition of GLUT1-mediated basal glucose uptake is another possible explanation (217) since we cannot see perturbations in the insulin effect *per se*. Hence, the maximal insulin stimulated glucose uptake capacity, measured as % of basal was unaffected by dexamethasone treatment. Other insulin signalling
pathways, such as the quite recently discovered Cbl pathway (11) might also be of importance in this respect.

Furthermore, glucocorticoids have been shown to enhance lipolysis in subcutaneous adipose tissue in vivo (218, 219). Treatment of adipocytes in vitro with dexamethasone had, however, different effects on lipolysis in women and men. Whereas no effect on the basal lipolytic rate was seen in cells from either depot regardless of sex, cAMP- as well as isoprenaline-stimulated lipolysis was elevated in cells from the subcutaneous depot in women but not in men. No consistent effects of dexamethasone were seen in the omental depot from either sex, but the cAMP-stimulated lipolysis tended to be elevated in adipocytes from men. The relative effect of insulin to inhibit lipolysis was not altered by dexamethasone treatment. Hence, dexamethasone counteracted insulin's antilipolytic effect by elevating the degree of lipolytic stimulation exerted by catecholamines, and not by altering the insulin effect per se.

The mechanisms behind the glucocorticoid mediated effect on lipolysis are not known but our results do not suggest that it is regulated by the cellular amount of the lipolysis regulating proteins PKA, HSL or perilipin since the levels of these proteins are unaffected by dexamethasone treatment. However, glucocorticoids might enhance the lipolytic stimulation by altering the specific activities rather than the levels of these proteins. Modulation of PKA activity has for example been shown to be an effect of glucocorticoid treatment in ovarian granulosa cells and in brain from rats (220, 221).

Previous studies suggest that visceral adipose tissue is more sensitive to glucocorticoid exposure than subcutaneous adipose tissue when it comes to regulation of e.g. leptin production and LPL activity (164, 167, 222). This is also the case with glucose uptake capacity according to our study. However, our results on glucocorticoid effects on lipolysis regulation point to the importance of considering sex in the analyses, since the pattern of lipolysis displayed in the two depots are completely different in men and women, as are the effects of glucocorticoids. Furthermore, ethnicity, although our studies consist of predominantly Caucasian subjects, is a factor that should be taken into account in studies with human specimens.

The effects of glucocorticoids on adipocyte metabolism in vivo are probably similar to those demonstrated in vitro. Glucocorticoid excess can lead to elevated circulating levels of both


Effects of fat cell size on glucose and lipid metabolism

Enlarged subcutaneous fat cell size has been shown to be associated with insulin resistance both in vitro and in vivo in type 2 diabetic subjects and in subjects with type 2 diabetes heredity (145, 148). Large subcutaneous fat cells are also suggested to predict the risk for developing type 2 diabetes in non-diabetic individuals (147). The mechanisms behind this relationship are not established but could be a primary cellular insulin resistance in the adipose tissue followed by enhanced liver glucose output and in parallel muscle and whole-body insulin resistance (33, 44, 45, 119). It could also be due to an inadequate ability of fat cell differentiation leading to spill-over of FFAs from the adipose tissue and hence ectopic fat accumulation in muscle and liver, a common phenomenon in obese subjects with type 2 diabetes (9, 120), in parallel with development of insulin resistance in these tissues (122, 123, 129, 137, 138, 223). There is evidence for perturbations in adipogenesis in insulin resistance (144) and the anti-diabetic drugs glitazones, are thought to mediate their effects by an enhanced differentiation of new, small fat cells (150, 224) thereby reducing insulin resistance (151, 224), supporting this theory. Enlarged fat cells could also be a consequence of some other factor leading to both insulin resistance and fat cell growth.

Our results might suggest an active role for fat cell size in the development of cellular insulin resistance, since both omental and to a slightly lesser extent, subcutaneous fat cell size was inversely related to insulin action on glucose uptake in subcutaneous fat cells in vitro. After adjusting for body composition (e.g. BMI, WHR or % body fat) this was seen only in non-diabetic subjects. However, insulin action on glucose uptake in omental fat cells does not seem to be regulated by fat cell size in either depot. Possibly, cellular insulin resistance in glucose and FFAs (158, 166). Impairment of cellular glucose uptake and altered lipolysis regulation, respectively, are probably important underlying mechanisms. The exact impact of the apparently specific interaction with the glucose transport system in visceral adipocytes is not clear, but it certainly could be of importance in the link between visceral obesity and insulin resistance. In addition, glucocorticoids enhance LPL expression (163) mainly in the visceral adipose tissue (164), and that should promote fatty acid delivery and uptake in that depot. Thus there are several pathways whereby glucocorticoids could link chronic stress and the metabolic syndrome.
enlarged fat cells precedes whole-body insulin resistance and fat cell size could thereby be a marker that predicts and possibly also promotes the development of insulin resistance and type 2 diabetes. However, once type 2 diabetes has been established, factors other than fat cell size such as elevated glucose and lipid levels (225), may be of greater importance promoting insulin resistance. Indeed, small changes in insulin mediated glucose uptake capacity in adipose tissue may have major metabolic consequences. This is revealed by studies on transgenic mice where elevated adipose tissue glucose uptake capacity, mediated by over expression of GLUT4, is followed by increased whole–body glucose turnover (226, 227).

Even though enlarged fat cells were more insulin resistant with respect to glucose uptake, there is surprisingly no evidence in our material that enlarged adipocytes would release elevated levels of FFAs. Hence, we did not find any clear relationship between fat cell size and basal lipolytic rate in either fat depot. Neither did we find any relationship between fat cell size and FFA release upon stimulation. Furthermore, the antilipolytic effect of insulin and the circulating levels of FFAs were not related to fat cell size in either adipose tissue.

From the results in paper II, we hypothesised that subcutaneous and omental fat cell size could be markers for general insulin resistance. We also suggested that adipocyte-derived molecules such as IL-6, TNF-α, adiponectin and leptin might be signals from the enlarged fat cell to other tissues as well as to other adipose depots, in this respect. Therefore, in a larger cohort (paper IV), we studied the relationship between the plasma levels of adipokines and assessments of whole-body insulin sensitivity (M-value, HOMA-IR index) and in fat cells in vitro (insulin action on glucose uptake in subcutaneous and omental fat cells). Of the assessed adipokines (adiponectin, IL-6, leptin and TNF-α), only plasma levels of leptin were significantly related to subcutaneous fat cell size after adjusting for age, sex and body composition and this is in accord with previous work (153). This would suggest that if there are perturbation in adipokine release from enlarged fat cells this is, with leptin being an exception, not reflected in plasma adipokine levels. None of the assessed adipokines was associated to omental fat cell size, suggesting that the strong relationship between omental fat cell size and glucose uptake capacity in subcutaneous fat cells, is probably not due to elevated release of these adipokines from enlarged omental fat cells. However, it should be mentioned that only circulating adipokine levels are assessed and it can therefore not be excluded that interstitial levels of the various adipokines are associated to fat cell size or that autocrine or
paracrine mechanisms might be involved in regulation of the glucose uptake capacity in fat cells.

Leptin is mainly secreted from subcutaneous adipocytes (114, 116) and this could thus possibly explain the strong relationship between subcutaneous fat cell size and leptin levels. Most other adipokines are, however, either released predominantly from the non-adipocyte (stromal vascular) fraction or equally from adipocytes and non-adipocytes in adipose tissue (107, 113, 114, 116, 118).

Leptin has been shown to counteract insulin's ability to stimulate glucose uptake as well as inhibit lipolysis in fat cells in vitro (204). Leptin secretion is elevated with obesity (65) and according to our results, related to fat cell size. We found strong relationships between leptin levels vs. subcutaneous fat cell size and vs. insulin action on glucose uptake in subcutaneous fat cells in non-diabetic subjects. Leptin, hence, might possibly be a marker of cellular insulin resistance in adipose tissue.
SUMMARY

1. FFAs do not seem to have insulin-antagonizing effects in rat fat cells in vitro, either in normal cells or in a model of insulin resistance. High levels of FFAs are hence probably a consequence rather than a cause of insulin resistance in adipose tissue.

2. Human omental adipocytes display higher basal and insulin-stimulated glucose uptake capacity in parallel with higher GLUT4 protein level compared with subcutaneous adipocytes. Upon treatment with glucocorticoids, both the basal and insulin-stimulated glucose uptake capacity is inhibited in adipocytes from the omental but not from the subcutaneous depot. This is possibly explained by a concomitant down-regulation of the insulin signalling proteins IRS-1 and PKB.

3. In women, subcutaneous adipocytes display a higher cAMP-stimulated lipolysis than omental adipocytes and this appears to be amplified by glucocorticoid exposure. In men there were no differences between the depots but dexamethasone tended to elevate the stimulated lipolysis in omental, but not in subcutaneous, adipocytes. Glucocorticoids had no effects on the levels of the lipolysis regulating proteins PKA, HSL or perilipin.

4. Subcutaneous and omental fat cell size is independently and negatively associated with insulin's ability to stimulate glucose uptake in subcutaneous cells in vitro, but not to the in vivo assessments of insulin sensitivity; HOMA-IR index and M-value. This relationship was only seen in non-diabetic subjects and not in patients with type 2 diabetes. Therefore we suggest that fat cell size could be a marker that predicts and possibly also promotes the development of insulin resistance and type 2 diabetes. Other factors are of greater importance promoting insulin resistance when type 2 diabetes becomes established.
CONCLUDING REMARKS

Obesity is thought to be involved in the development of insulin resistance and central obesity seems to be especially harmful in this respect. In this work we show that there are differences between the subcutaneous and omental adipose tissue depots regarding glucose and lipid metabolism and that at least the latter is partly regulated by sex-specific mechanisms. We also show that glucocorticoids have insulin-antagonistic effects on glucose as well as lipid metabolism in fat cells, at least in vitro, and again the way these effects are manifested is dependent on adipose tissue depot and sex. A hypothetical overview on the interplay between genes, sex, hormones and adipose tissue depots in the regulation of insulin sensitivity is depicted in Fig 3.

Figure 3. Hypothetical scheme of the interplay between genes, sex, hormones and adipose tissue distribution in the regulation of insulin sensitivity. Genes as well as sex and stress influence adipose tissue mass, distribution and possibly fat cell size. These factors in turn, affect circulating adipokine and FFA levels as well as degree of ectopic fat accumulation, important for the development of insulin resistance. In addition, stress through the actions of cortisol may have direct effects on insulin sensitivity.
SAMMANFATTNING PÅ SVENSKA

Förekomsten av typ 2-diabetes (tidigare kallad åldersdiabetes) ökar kraftigt i hela världen. Denna utveckling antas främst bero på livsstilsförändringar, där minskad fysisk aktivitet, ökad övervikt och stress antas ha stor betydelse. Typ 2-diabetes kännetecknas av kroniskt förhöjda blodsockernivåer. Detta sker p.g.a. otillräcklig insulinproduktion och frisättning från bukspottkörteln i kombination med minskad effekt av insulin, s.k. insulinresistens, i insulinets målvävnader, t.ex. muskeln, lever och fettväv. De förhöjda blodsockernivåerna orsakar på sikt komplikationer med kärlskador som gemensam nämnare. Dessa kärlskador ger oftast upphov till synskador och problem med nerv- och njurfunktionen. Individer med typ 2-diabetes löper också kraftigt förhöjd risk att få andra kärlsjukdomar såsom hjärtinfarkt och stroke.

Insulin frisätts från bukspottkörteln och transportereras via blodet till kroppens olika organ då blodsockernivåerna stiger efter måltid. Insulinets funktioner är många, men förenklat kan sägas att i muskel stimuleras sockerupptag, i lever förhindras sockerproduktion och sockerfrisättning, samt i fettväv stimuleras sockerupptag och förhindras frisättningen av lagrad energi i form av fettsyror. Insulinresistens, som uppkommer tidigt i utvecklingen av typ 2-diabetes, leder därför till störningar i både socker- och fettmetabolismen med förhöjda nivåer av socker och fetter i blodet. Detta kan i sig också leda till försämrad insulineffekt i t.ex. muskel- och levervävnad vilket skapar en ond cirkel som på sikt antas orsaka typ 2-diabetes.

Övervikt är intimit förknippat med insulinresistens och typ 2-diabetes men även med hjärt/kärlsjukdom och högt blodtryck. Särskilt farligt anses det vara när fetten är placerat i buken. En teori om varför denna fettdepå skulle vara särskilt farlig baseras på att fettsyrorna från denna depå transporteras direkt till levern där de bl.a. ombildas till socker som frisätts till blodbanan. En stor mängd fett i buken skulle alltså kunna ligga bakom de förhöjda blodsocker- och blodfett niveauer som är så vanliga bland individer med insulinresistens och typ 2-diabetes.

Tidigare har visats att förhöjda nivåer av fettsyror i blodet kan leda till att insulin inte fungerar som det ska i t.ex. lever och muskel. Huruvida fettsyror är skadliga också i fettväven är okänt. I delarbete I använde vi råttfettceller som modell för att studera om förhöjda nivåer av fettsyror påverkar insulininkänsligheten även i fettvävnaden. Vi odlade cellerna i närvaro av
höga fysiologiska nivåer av antingen oljesyra eller palmitinsyra, två vanligt förekommande fettsyror i vår kost, under 24 h. Därefter mättes fettcellernas förmåga att ta upp socker och insulins förmåga att hämma frisättningen av fettsyror från fettcellerna. Mängden av ett antal viktiga proteiner som förmedlar insulins signaler i cellerna mättes också. Vi fann att fettsyror inte hade några effekter på fettcellernas ämnesomsättning eller proteinnivåer. Detta talar för att fettväven är bättre på att hantera höga nivåer av fettsyror än andra vävnader och att fettsyror därmed inte ytterligare förvärrar insulinresistensen i denna vävnad.

I delarbete II och III, ville vi studera om det finns skillnader i insulinkänslighet mellan två av kroppens olika fettdepåer. Vi studerade underhudsfett och s.k. visceralt fett (som sitter runt tarmarna inne i buken), och hur stresshormonet kortisol påverkar ämnesomsättningen i celler från dessa depåer. Vi fann i delarbete II att färska viscerala celler hade en avsevärt högre kapacitet att ta upp socker än celler från underhudsfettet. När vi odlade cellerna i närvaro av dexametason, ett kortisolliknande ämne, försämrades förmågan att ta upp socker i de viscerala cellerna, men inte i celler från underhudsfettet. Den försämrade förmågan att ta upp socker i de viscerala cellerna följdes av en minskad mängd av ett antal av de proteiner som förmedlar insulins signal inne i cellerna, vilket delvis skulle kunna förklara det försämrade sockerupptaget.

I delarbete III fann vi att den icke-stimulerade frisättningen av fettsyror från cellerna var ungefär densamma oavsett vilken depå cellerna kom ifrån. Hos män sågs heller inga skillnader mellan depåerna när frisättningen av fettsyror stimulerades för att likna ett ökat energibehov. Hos kvinnor var dock den stimulerade frisättningen av fettsyror betydligt högre från underhudsfettceller jämfört med viscerala celler. Efter odling i närvaro av dexametason ökade den stimulerade frisättningen av fettsyror från underhudsfettceller ytterligare hos kvinnor medan de viscerala fettcellerna från kvinnor och från båda fettdepåerna hos män var opåverkade av denna behandling. Mängden av de proteiner som reglerar frisättningen av fettsyror var lika oberoende av kön, fettdépå och dexametasonbehandling, varför förklaringen till den ökade frisättningen av fettsyror från underhudsfett hos kvinnor efter dexametasonbehandling måste sökas på annat håll. Insulins förmåga att förhindra frisättningen av fettsyror var densamma oavsett depå, kön eller dexametasonbehandling.

Sammanfattningsvis tyder resultaten i studie II och III på att viscerala celler är känsligare för dexametasonets effekter än underhudsfettceller, men bara när det gäller att ta upp socker.
Istället är det underhudsfettceller som påverkas mest av dexametason när det gäller effekter på frisättningen av fettsyror. Men detta kunde å andra sidan bara ses hos celler från kvinnor och inte från män. Alltså finns både depå- och könsspecifik reglering av ämnesomsättningen i fettceller och det är därför vanskligt att tala om att just den viscerala depån generellt skulle vara särskilt ogynnsam. De viscerala fettcellerna verkar inte, enligt våra studier, heller vara mer insulinresistenta än fettcellerna från underhudsfettet.


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This message will be repeated in English (see below).

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English version of the above; Thanks all!

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