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Karolinska Institutet, Stockholm, Sweden

NOVEL ASPECTS OF METABOLIC REGULATION AND INFLAMMATION IN HUMAN ADIPOCYTES

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Stockholm 2015

Published by Karolinska Institutet.
Printed by E-Print AB 2015

ISBN 978-91-7549-848-5

Novel aspects of metabolic regulation and inflammation in human adipocytes

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Till alla sanningssökare
"Tänka fritt är stort men tänka rätt är större"

ABSTRACT

The significance of adipose tissue and obesity has been recognized in numerous pathologies. However, the mechanisms behind this connection are not yet completely understood. The aim of this thesis was to investigate the roles of Liver X Receptor (LXR), V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB) and Salt-inducible kinases (SIKs) in primary human adipocytes with focus on metabolic regulation and inflammation. Our overall hypothesis was that these factors may influence metabolism and inflammation in human white adipose tissue (WAT).

In **paper I**, we studied the effects of LXR activation on human basal lipolysis. We show that stimulation of LXR using the LXR agonist GW3965 enhances spontaneous lipolysis, measured as glycerol released into culture medium. We demonstrate that hormone-sensitive lipase (HSL) and perilipin 1 (PLIN1), two proteins important for lipolysis, are downregulated on both mRNA and protein level by GW3965 treatment, which changes the morphology of the lipid droplet coating. Using chromatin immunoprecipitation we show that LXR binds to the proximal promoters of both *LIPE* (encoding HSL) and *PLIN1*. Furthermore, we demonstrate that LXRα is the isoform mediating increased lipolysis. PLIN1 knockdown in combination with GW3965 treatment indicate that PLIN1 downregulation was the main mechanism by which LXR activation increase glycerol release in human adipocytes.

In **paper II** we examined the role of LXR in human adipocyte glucose uptake. We identified three proteins important for insulin signalling – AKT2, c-CBL associated protein (CBL) and caveolin 1 (CAV1) – to be downregulated by LXR activation. LXR activation reduced AKT2 phosphorylation and GLUT4 translocation to the plasma membrane, which resulted in reduced insulin-stimulated glucose uptake. In addition, *AKT2* and *SORBS1* (encoding CAP) expression in human WAT was inversely correlating with BMI and severity of insulin resistance. We concluded that LXR is an inhibitor of insulin-stimulated glucose uptake in human adipocytes, likely via transcriptional suppression of *AKT2*, *SORBS1* and *CAV1*.

In **paper III**, we investigated the role of MAFB in human WAT. *MAFB* mRNA expression was upregulated in obesity and downregulated after weight loss in adipose tissue from four different human cohorts. We found that the expression of *MAFB* is increased during adipocyte differentiation and that it mediates effects of TNFα-stimulation on lipid accumulation and basal lipolysis. In turn, knockdown of MAFB in adipocytes resulted in reduced expression of pro-inflammatory factors. *MAFB* was differentially regulated in response to TNFα treatment in adipocytes (downregulated) vs. monocytes (upregulated). *MAFB* was mainly expressed in adipose tissue macrophages and its expression correlated positively with macrophage- and pro-inflammatory markers in WAT, and negatively with insulin-stimulated lipogenesis and stimulated lipolysis in human mature adipocytes, suggesting that it might be involved in the development of unhealthy glucose- and lipid metabolism.

In **paper IV**, we aimed to identify the role of SIKs in human obesity and adipose tissue. We found that expression of *SIK2* and *SIK3* mRNA in human WAT was downregulated in obesity, upregulated after weight loss and correlated negatively with insulin resistance. SIK2 was the most highly expressed SIK isoform in human adipocytes. Both basal and insulin-stimulated glucose uptake were inhibited by SIK inhibitor. SIK inhibition also lead to reduced phosphorylation of histone deacetylase 4 (HDAC4) and CREB regulated transcription coactivator 1 (CRTC2), which may potentially mediate the attenuation of glucose uptake. Taken together, SIK2 may have a protective role in obesity-induced loss of insulin sensitivity.

In summary, we have identified the roles of three factors in human adipocytes, all of which were previously uncharacterised in human WAT. We show that LXR enhances spontaneous lipolysis while inhibiting insulin-stimulated glucose uptake in human adipocytes, two features associated with an unfavourable metabolic profile. We also found that MAFB and SIK2 expression is regulated by obesity and inflammation. Finally, we demonstrate that MAFB is itself a regulator of inflammation in human adipocytes and that SIK2 positively regulates glucose uptake in human adipocytes. Our findings contribute to the understanding of the metabolic syndrome and its development in obesity.

LIST OF SCIENTIFIC PAPERS

I. Britta M. Stenson, Mikael Rydén, Nicolas Venteclef, Ingrid Dahlman, **Annie M. L. Pettersson**, Aline Mairal, Gaby Åström, Lennart Blomqvist, Victoria Wang, Johan W. E. Jocken, Karine Clément, Dominique Langin, Peter Arner, and Jurga Laurencikiene.
Liver X Receptor (LXR) Regulates Human Adipocyte Lipolysis
Journal of Biological Chemistry, 2011; vol 286; page 370-379

II. **Annie M. L. Pettersson**, Britta M. Stenson, Silvia Lorente-Cebrián, Daniel P. Andersson, Niklas Mejhert, Johan Krätzel, Gaby Åström, Ingrid. Dahlman, Alexander V. Chibalin, Peter Arner and Jurga Laurencikiene.
LXR is a negative regulator of glucose uptake
Diabetologia, 2013; vol 56; page 2044-2055

III. **Annie M.L. Pettersson***, Juan R. Acosta*, Johan Krätzel, Britta Stenson, Lennart Blomqvist, Nathalie Viguerie, Dominique Langin, Peter Arner and Jurga Laurencikiene.
MAFB as a novel regulator of human adipose tissue inflammation
Manuscript
* Shared first authorship

IV. Johanna Säll, **Annie M.L. Pettersson**, Emma Henriksson, Fredrik Linder, Yuedan Zhou, Ola Hansson, Daniel P. Andersson, Mikael Ekelund, Eva Degerman, Jurga Laurencikiene and Olga Göransson.
Salt-inducible kinase 2 (SIK2) is downregulated in obesity and is required for glucose uptake in human adipocytes
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

22-R-HC	22-R-Hydroxycholesterol
9cRA	9-cis retinoid acid
ACC	Acetyl CoA carboxylase
AMPK	AMP-activated protein kinase
AS160	Akt substrate of 160 kDa
ATGL	Adipose triglyercide lipase
BMI	Body mass index
CAGE	Cap Analysis of Gene Expression
CAP	c-Cbl associated protein
CAV1	Caveolin 1
CEBP	CCAAT/enhancer-binding proteins
ChREBP	Carbohydrate-response-element-binding protein
CIDEC	Cell death-inducing DNA fragmentation factor C
CRTC	CREB-regulated transcription coactivator (CRTC)
DNL	*De novo* lipogenesis
ELISA	Enzyme-linked immunosorbent assay
FA	Fatty acid
FAS	Fatty acid synthase
GLUT4	Glucose transporter
GSV	GLUT4 storage vesicle
HDAC	Histone deacetylase
hMSC	Human mesenchymal stem cell
HOMA-IR	Homeostatic model assessment of insulin resistance
HRP	Horseradish peroxidase
HSL	Hormone-sensitive lipase
IL	Interleukin
IRS	Insulin receptor substrate
LPL	Lipoprotein lipase
LRP10	Low density lipoprotein receptor-related protein 10
LXR	Liver X receptor
LXRE	Liver X Receptor response element
MAFB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
MCP-1	Monocyte chemoattractant protein 1
mTORC2	Mammalian target of rapamycin complex 2
NCoR	Nuclear Receptor Corepressor
NF-κB	Nuclear factor kappa B
NR	Nuclear receptor
PDE3B	Phosphodiesterase 3B
PI3K	Phosphatidylinositol-3 kinase
PIP2	Phosphatidylinositol-4,5 diphosphate
PIP3	Phosphatidylinositol-3,4,5 trisphosphate
PKA	Protein kinase A
PLIN1	Perilipin 1
PM	Plasma membrane
PPARγ	Peroxisome proliferator-activated receptor gamma

qRT-PCR	Quantitative real-time polymerase chain reaction
RXR	Retinoic X receptor
SCD	Stearoyl-CoA desaturase
SIK	Salt-inducible kinase
SMRT	Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor
SREBP-1c	Sterol regulatory element-binding protein-1c
SUMO	Small ubiquitin-like modifier
SVF	Stromal vascular fraction
TG	Triglyceride
TNF	Tumor necrosis factor
WAT	White adipose tissue

1 BACKGROUND

1.1 OBESITY

As obesity is growing into an epidemic worldwide [1], an endless number of diseases follow in its path. Obesity does not only cause serious metabolic disturbances such as type 2 diabetes and fatty liver but is also associated with higher rates of co-morbidities such as chronic low-grade inflammation, atherosclerosis, hypertension, asthma, cancer, depression, Alzheimer's disease, infertility, birth complications, sleep apnoea, premature aging and impaired physical ability. The grounds for this epidemic are numerous and include a sedentary lifestyle and overnutrition, but also less obvious causes such as sleep deprivation, fat-promoting viruses, an obesogenic gut flora springing from excessive hygiene and/or unhealthy foods, effective indoor temperature regulation, around-the-clock feeding, toxins (e.g. bisphenol), stress, anxiety, and the availability of highly palatable foods which disrupt the satiety system [2-7]. Three well-known methods of weight reduction are diet, exercise and bariatric surgery. However, they all have their limitations. Classical calorie-reducing dieting and exercise seldom produce long-lasting results and demand immense willpower from affected individuals. Bariatric surgery is mainly available to individuals with morbid obesity, and comes with the risk of side-effects and complications such as nutritional malabsorption, dumping syndrome, and reflux [8]. Obesity is characterised by an excess of adipose tissue and the level of obesity is defined by body mass index (BMI) which is calculated by dividing an individual's weight in kilograms by their squared height in centimetres. The BMI scale is divided into categories listed in table 1.

Table 1. Definition of BMI classes

BMI class	BMI (kg/m^2)
Underweight	<18.5
Normal weight	18.5-24.9
Overweight	25.0-29.9
Obese	>30
Morbidly obese	>40

Some of the medical treatments widely used today in the management of obesity-related health issues target insulin resistance and glucose intolerance. These treatments – such as insulin injections, metformin and, more rarely, thiazolidinediones – come with a range of side-effects, including weight gain, osteoporosis or intestinal distress [9]. Although lifestyle change can have great impact on the weight of some individuals, there will always be a category where diet and exercise will not be an attractive or possible alternative. This category includes persons with severe depression and those with physical disabilities or additional diseases that complicate exercise ability. Therefore, there is a need for further characterisation of adipose tissue and for the development of safe and effective drugs to treat both obesity itself and its secondary ailments.

1.2 WHITE ADIPOSE TISSUE

White adipose tissue (WAT) is what most of us have in mind when we think about body fat. Brown adipose tissue, which is located in different areas, largely possesses distinct properties from WAT and will not be addressed in this thesis. WAT develops at numerous locations throughout the body and have many different functions such as energy storage

and maintaining energy homeostasis, insulation and hormonal signalling. WAT is not only located in visual depots such as legs, belly, arms and breasts but also in the foot soles, bone marrow, eye, and surrounding the heart and other inner organs. There are two major divisions of WAT; subcutaneous WAT which is located peripherally beneath the skin, and visceral WAT which is embedded among the internal organs. Visceral WAT is more metabolically active than subcutaneous WAT in that it exhibits a higher flux of fatty acids (FAs) which is released directly into the portal circulation and to the liver. Visceral WAT is also considered more prone to inflammation. [10] WAT is a complex organ of great heterogeneity and consists of a number of cell types described below. When the term adipose tissue is used in this thesis, it refers to subcutaneous WAT containing all cell types.

1.2.1 Adipocytes

Adipocytes are one of the body's largest cell types and make up 20-40% of the total cell number in adipose tissue, but over 90% of the tissue volume. The renewal rate for adipocytes in humans is about 10% per year [11]. Their main function is to store energy in the form of lipids and they largely consist of a large lipid droplet which constitutes about 90% of the cell mass. A narrow cytoplasm surrounds the lipid droplet and the nucleus is located near the plasma membrane [12]. The functions of the adipocyte are described in detail in chapter 1.3.

1.2.2 Adipocyte precursor cells

Adipocyte progenitor cells are found in the stromal vascular fraction (SVF) and include mesenchymal stem cells and pre-adipocytes. Mesenchymal stem cells in adipose tissue have the ability to differentiate into chondrocytes, osteocytes, myocytes and adipocytes. Pre-adipocytes are small adipocyte-precursor cells destined to develop into adipocytes *in vivo*. Pre-adipocytes display distinct gene expression profiles depending on their depot of origin, which they retain after isolation and *in vitro* differentiation [13]. A complex transcriptional network is activated in the adipogenic process. This network includes the master regulator of adipogenesis peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer-binding proteins (CEBPs) and sterol regulatory element-binding protein-1c (SREBP-1c) [14].

1.2.3 Macrophages

Macrophages develop from monocytes and their main function is to eliminate cells affected by infection through either phagocytosis or by cytokines [15]). Macrophages are a natural and necessary component of adipose tissue. They proliferate in adipose tissue during periods of increased lipolysis, such as weight loss and fasting, in order to take care of an excess of released FAs [16-18]. They also take up FAs released from apoptotic adipocytes, protecting the surroundings from lipotoxic effects (further described in section 1.3.3) [19]. When adipose tissue expands, adipocytes and macrophages which are already present start to secrete proteins promoting macrophage proliferation [18] (see section 1.4.2). The macrophages also go through an identity switch from M2 (anti-inflammatory) to M1 (pro-inflammatory). M1 macrophages initiate secretion of elevated amounts of pro-inflammatory cytokines which leads to impaired insulin sensitivity both locally and systemically [20]. However, the M1/M2 division is increasingly considered as an oversimplification.

1.2.4 Additional adipose tissue cell types

Adipose tissue also contains endothelial cells, lymphoid cells, mast cells, fibroblasts and leukocytes. These cell types will not be addressed in this thesis.

1.3 FUNCTIONS OF THE ADIPOCYTE

The main functions of the adipocyte is energy storage after feeding and in times of abundance, and energy release between meals and in times of food shortage. Storage and release are operated by lipogenesis and lipolysis, respectively. Insulin plays a crucial part in the regulation of these processes.

1.3.1 Adipocytes as targets of insulin signalling

Insulin is a peptide released from pancreatic beta cells in the islets of Langerhans in response to elevated post-prandial blood glucose levels. Many cell types respond to insulin signalling, including adipocytes. In adipocyte biology, insulin is one of the most important hormones, controlling differentiation, growth and energy homeostasis. Insulin is strongly pro-adipogenic and energy homeostasis is controlled by insulin through regulation of lipogenesis, FA uptake and lipolysis.

The control of glucose homeostasis by insulin is a tightly regulated process, and when dysregulated or completely lacking (such as in type 1 diabetes) it poses immediate and long-term danger. Malfunctioning insulin signalling leads to insulin resistance, which is described in section 1.5.

The immediate effect of an activated insulin signalling cascade is glucose uptake, where the ultimate target is translocation of the glucose transporter protein GLUT4 from intracellular stores to the plasma membrane. GLUT4 translocation and recycling is a complex process which involves as many as 70 proteins and a number of phosphorylation events. Two insulin signalling pathways have been described; the phosphatidylinositol-3 kinase (PI3K)-dependent pathway and the non-canonical CBL-TC10 pathway. The pathways are depicted in Fig. 1. Both cascades are initiated when insulin binds extracellularly to the insulin receptor with subsequent tyrosine auto-phosphorylation of the intracellular parts of the receptor.

In the PI3K pathway, this attracts insulin receptor substrate (IRS) proteins which bind the tyrosine phoshorylated residues and are themselves tyrosine phosphorylated. IRS proteins serve as docking sites for PI3K which catalyses the conversion of phosphatidylinositol-4,5-diphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). Phosphoinositide Dependent Protein Kinase 1 (PDPK1) and AKT then dock at PIP3 in the plasma membrane. AKT is phosphorylated by PDK1 at Thr308 and by mammalian target of rapamycin complex 2 (mTORC2) at Ser473 [21, 22]. AKT is thereby fully activated and catalyses an inhibitory phosphorylation of the RAB GAP Akt substrate of 160 kDa (AS160). By inactivating AS160, GLUT4-containing vesicles are released from their intracellular sequesterisation and can translocate to the plasma membrane.

The CBL-TC10 pathway initiates with the binding of APS, CBL and C-cbl associated protein (CAP) to the insulin receptor. CBL is phosphorylated by the receptor which stimulates the binding of CRK and the guanine nucleotide exchange factor C3G to the plasma membrane. The small GTPase TC10 is activated by C3G and facilitates the translocation of GLUT4 to the PM. [23-25]. The importance of the CBL-TC10 pathway has, however, been questioned [26]. An array of proteins are involved in the final stages of translocation and recycling of GLUT4 but will not be further described [22]. Basal glucose uptake is, independently of insulin signalling, mediated by the ubiquitously expressed glucose transporter GLUT1.

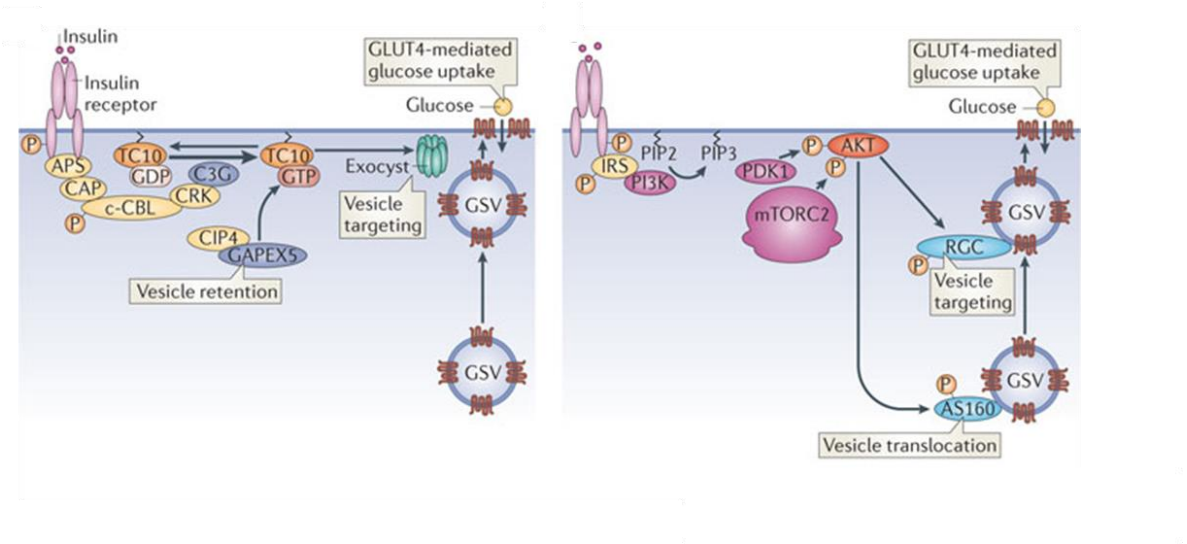


Fig.1. CBL-TC10- and PI3K –dependent insulin signalling pathways. Source: Leto & Saltiel, Nature Reviews. Molecular Cell Biology [22]. Printed with permission from the publisher.

AKT has been described as a central hub, with a key role in insulin signalling. There are three isoforms of AKT; AKT1, -2 and 3. AKT2 is the major isoform important for glucose uptake. AKT1 has primarily been implicated in cellular growth and anti-apoptosis but may have a compensatory role in glucose uptake in case of loss of AKT2. AKT3 is important for neuronal development. [21]. AKT2 has been repeatedly shown to be crucial for glucose uptake. In contrast, one study suggests that AKT2 is *de facto* expressed in abundance and that minor alterations in AKT2 expression does not affect glucose uptake [27]. Of the IRS isoforms IRS1 and -2, IRS1 has been shown to be the major isoform mediating glucose uptake in adipocytes [28].

In addition to the signalling cascades caveolin 1 (CAV1) has been found to be important for insulin effects in adipose tissue [29]. CAV1 is a building block of caveolae located in the PM where it regulates insulin-signalling [30] and acts in a stabilising manner on the insulin receptor and GLUT4 by preventing their degradation [31].

It is important to mention that only about 10% of insulin-stimulated glucose uptake occurs in adipose tissue, while the major target tissue is skeletal muscle [32]. However, insulin sensitivity in adipose tissue is vital for systemic metabolic homeostasis [33-35].

1.3.2 Lipogenesis

Energy is stored in adipocytes in the form of triglycerides (TGs) in the lipid droplet. Two pathways contribute to TG synthesis, a process also known as lipogenesis. The major pathway in adipocytes involves the uptake of TGs in the form of FAs from blood. Besides promoting glucose uptake, insulin also powerfully stimulates FA uptake. Thus, although FAs from the diet are the greatest contributor to lipogenesis and fat tissue growth, this process is heavily dependent on insulin, which is released mainly in response to carbohydrate intake. TGs in blood are transported in chylomicrons or very low density lipoprotein (VLDL). Lipoprotein lipase (LPL) hydrolyses the TGs into FAs which adipocytes are able to take up. The expression and activity of LPL is also positively regulated by insulin. The FAs enter the adipocytes through the FA transporter FAT/CD36 or passive diffusion. FAs are then subject to re-esterification, forming TGs intracellularly, which in turn are joined to the lipid droplet. In this pathway, glucose is used for synthesis of glycerol – the TG backbone. [36]

The second pathway is denoted *de novo* lipogenesis (DNL) and involves synthesis of TGs from glucose via acetyl-CoA. This process is not very pronounced in adipocytes and does not largely contribute to energy storage, and is generally considered to be more important in liver, especially in the development of non-alcoholic fatty liver disease. However, DNL in adipose tissue may serve as means of producing lipid signalling molecules [37, 38]. Unexpectedly, adipocyte DNL takes place in situations of caloric restriction rather than after excess energy intake. Adipose tissue DNL was also reported to exert positive effects on glucose homeostasis. [39] Fatty acid synthase, (FAS), acetyl CoA carboxylase (ACC) and stearoyl-CoA desaturase (SCD) are important enzymes in the DNL pathway. These enzymes are transcriptionally regulated by the lipogenic transcription factors SREBP-1c and carbohydrate-response-element-binding protein (ChREBP), of which the latter is more important in adipocytes. [38]

1.3.3 Lipolysis

Energy is mobilised from adipocytes and released in the form of FAs to be utilised mainly by muscle. This process is denoted lipolysis, a name derived from the hydrolysis of TGs from the lipid droplet. TGs are hydrolysed from the lipid droplet by three enzymes situated on its surface but the process is also regulated by a number of non-enzymatic factors. TGs are converted into diacylglycerol (DG) via adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL). DGs are hydrolysed into monoacylglycerol (MG) by HSL and the final FA is liberated by monoacylglycerol lipase (MGL). The FAs then either travel bound to albumin to its target tissues or can be re-esterified. In addition, one glycerol molecule is released per original TG and is transported out of the adipocyte as adipocytes do not express the enzyme necessary for phosphorylation of glycerol which is needed for formation of the glycerol backbone in the making of triglycerides. [40, 41]

Basal lipolysis occurs continuously at low rates in adipocytes. Lipolysis can also be triggered by hormones. In detail, stimulated lipolysis is initiated with the binding of pro-lipolytic factors to their respective receptors. The most physiologically relevant ones are the catecholamines adrenaline and noradrenaline which bind to the adrenergic receptors. The adrenergic lipolytic pathway is illustrated in Fig. 2. Other factors are also known to stimulate lipolysis but will not be addressed here. *β* adrenergic receptors have a stimulatory effect on lipolysis while the *α2* adrenergic receptor has an inhibitory effect. The net effect is dependent on the relative expression of *β* versus *α2* adrenergic receptors, although *β* adrenergic signalling is normally dominant in human adipocytes [42]. Upon binding by catecholamines to the *β* adrenergic receptors, adenylate cyclase is activated and converts ATP into cAMP. cAMP in turn binds and activates protein kinase A (PKA) which phosphorylates and activates HSL.

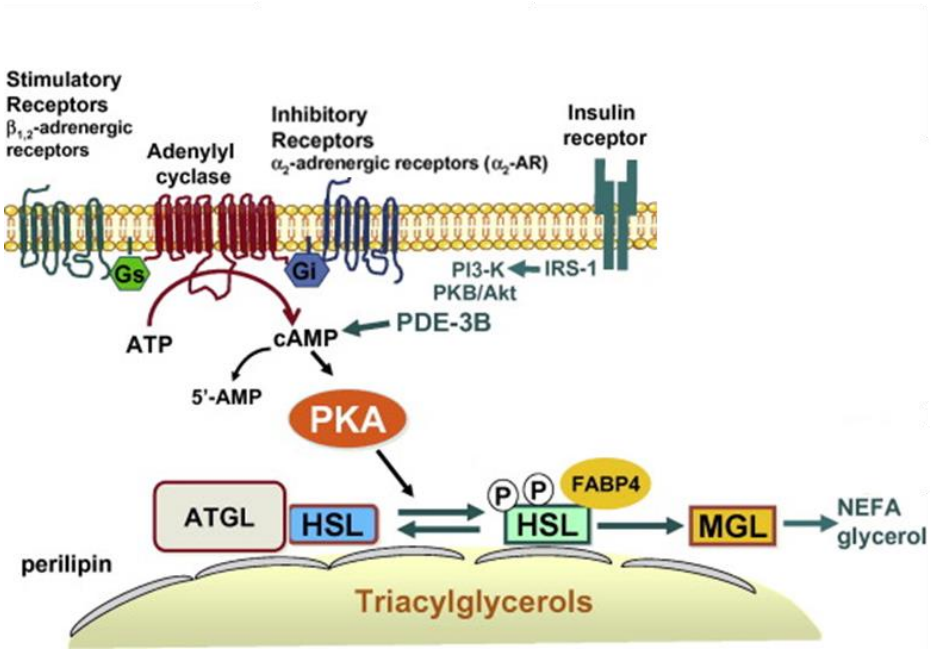


Fig. 2. The adrenergic lipolytic pathway in human adipocytes. Source: Lafontan & Langin, Progress in Lipid Research [40]. Printed with minor changes and with permission from the publisher.

Besides HSL, ATGL is also regulated hormonally through its cofactor CGI-58. Perilipin 1 (PLIN1) is a lipid-droplet associated protein which in the unstimulated state physically obstructs the lipid droplet from the lipolytic enzymes, thereby blocking lipolysis. In the basal state PLIN1 binds CGI-58. When lipolysis is triggered by catecholamines, PLIN1 is phosphorylated by PKA upon which CGI-58 is released to activate ATGL and the hydrolysis of TGs [43]. PLIN1 also goes through a conformational change itself, which recruits HSL to the surface of the lipid droplet [44].

In obesity, the lipolytic response is often dysregulated; basal lipolysis is increased whereas stimulated lipolysis is blunted. Elevated basal lipolysis, or an inability of the adipocyte to store lipids, lead to lipid spillover and lipotoxicity. FAs can aggregate ectopically on muscle, heart, liver and pancreas, as well as cause internal hepatic fat accumulation. This contributes to an impaired ability to clear the blood from glucose, a state termed insulin resistance [42] (further described in section 1.5).

1.3.4 The endocrine function of adipocytes

Adipocytes cross-talk with their close environment and with distant tissues through autocrine, paracrine and endocrine processes. This cross-talk is mediated by adipokines; peptides secreted from adipocytes. Many adipokines are pro-inflammatory and are increasingly expressed as weight gain proceeds (see section 1.4.). These negatively affect metabolism, fat distribution, blood pressure and endothelial health. However, not all adipokines are of malicious character. For example, leptin is an adipokine almost solely expressed by adipocytes. It is of key importance in appetite and energy regulation. Leptin travels from adipose tissue to the hypothalamus where it promotes satiety [45]. Along with leptin, adiponectin is another highly expressed adipokine with beneficial properties. Its expression is decreased in obesity and it has insulin sensitising effects. Adiponectin also improves dyslipidemia and inflammation [46].

1.4 INFLAMMATION

In addition to leptin and adiponectin described above, adipose tissue and adipocytes secrete a large number of peptides [47-53]. Three of the most important pro-inflammatory adipokines which are also studied in this thesis will be described below. All three are regulated by nuclear factor kappa B (NF-κB) as well as expressed and secreted by both adipocytes and macrophages [54-56].

1.4.1 Tumor necrosis factor alpha

Tumor necrosis factor alpha (TNFα) is an extensively studied cytokine and has been connected to a number of pathologies. TNFα works in an auto- and paracrine manner and promotes the expression and secretion of other adipokines. TNFα secretion is elevated in obesity, correlates negatively with insulin sensitivity [57] and reduces glucose uptake in human adipocytes [58]. Its mechanism of action is an inhibition of IR autophosohorylation and of IRS1 signalling [57]. TNFα antagonizes PPARγ and thereby inhibits lipid storage and glucose uptake by transcriptional regulation. IR, IRS-1 and GLUT4 are examples of genes with reduced expression levels as a result of elevated levels of TNFα [59, 60]. Moreover, TNFα knockout mice are protected from obesity-induced insulin resistance [61]. A second mechanism promoted by TNFα with detrimental effects on health is increased basal lipolysis, which leads to augmented FA-levels, in turn also affecting insulin resistance, as discussed above.

1.4.2 Monocyte chemoattractant protein 1

Monocyte chemoattractant protein 1 (MCP-1) is encoded by the CCL2 gene. Similarly to TNFα, MCP-1 increases in obesity [62] and is of diabetogenic nature [63]. By paracrine and autocrine effects, MCP-1 stimulates macrophage proliferation within adipose tissue. Activated macrophages secrete more MCP-1, consolidating a chronic inflammation. [18].

1.4.3 Interleukin-6

Interleukin (IL)-6 secreted from adipose tissue has been established in several pathologies of obesity, including insulin resistance. Mice receiving acute infusions of IL-6 develop insulin resistance without being obese [64]. IL-6 also impairs insulin sensitivity in humans [65] and is positively associated with BMI, insulin and occurrence of type 2 diabetes in human subjects [66].

1.5 METABOLIC SYNDROME

The metabolic syndrome comprises multiple interconnected subconditions such as insulin resistance, visceral adipocity, hypertension, dyslipidemia, and high blood pressure. Insulin resistance is a major element of the diagnosis. Insulin resistance is characterised by the body's inability to respond properly to insulin and subsequently take up glucose from the blood, and precedes type 2 diabetes. High glucose levels are detrimental due to the inherent toxicity of glucose. Elevated blood glucose leads to increased reactive oxygen species which damage cells and tissues, it glycosylates proteins indiscriminately, thereby changing their properties (for example low density lipoprotein), and cause blood vessels stiffness. [67] Insulin resistance and insulin sensitivity can be measured by several different methods. One method appearing in this thesis is the homeostasis model assessment of insulin resistance (HOMA-IR) [68]. The model is based on measurements of fasting plasma insulin and glucose in mmol/L and the HOMA-IR index is calculated by the following formula:

$$\text{HOMA-IR} = \frac{\text{plasma glucose} \cdot \text{plasma insulin}}{22.5}$$

In this model, a higher value indicates a higher level of insulin resistance.

Multiple organs are involved in the development of insulin resistance. The condition has several possible roots, of which one is a high sugar diet. When sugar intake is high, pancreatic beta cells produce high levels of insulin to promote glucose disposal. When insulin levels remain high, muscle cells and adipocytes begin to lose their sensitivity to insulin. This is a molecular process occurring on several levels. As a result, in insulin resistant muscle and adipose tissue, the insulin signalling pathway is impaired and glucose uptake is dysfunctionally low. Subsequently, pancreatic beta cells produce even higher levels of insulin to compensate for the low blood glucose clearance rate. Simultaneously, hepatic insulin resistance develops, causing impaired suppression of hepatic gluconeogenesis, which results in high glucose output from the liver – despite concurrent high levels of circulating insulin and glucose. [69] Eventually, beta cell insulin production gradually fails – due to defect pancreatic insulin signalling, glucose toxicity and beta cell inflammation – and the condition known as type 2 diabetes has been reached [70]. Below is listed a number of molecular events contributing to insulin resistance. They often occur simultaneously and many are promoted by the pro-inflammatory environment emerging in obesity, in particular by TNFα.

- A negative feedback loop triggered by prolonged insulin receptor stimulation results in serine phosphorylation of IRS proteins by AKT and other kinases, which inhibits their ability to bind PI3K and terminates insulin signal transduction [71, 72].
- Degradation or inhibitory phosphorylation of IRS on serine residues by cytokines, impairing the insulin signalling pathway [57, 72, 73].
- Transcriptional suppression of the insulin receptor, IRS and GLUT4 by e.g. TNFα [59, 60], possibly by inhibiting transcription and activity of PPARγ [74].
- Inhibition of insulin receptor-mediated tyrosine phosphorylation of IRS via the TNFα/NF-κB/SOCS pathway [75, 76].
- TNFα antagonises suppression of lipolysis by insulin via downregulation of phosphodiesterase 3B (PDE3B) [77, 78].
- TNFα also directly affects lipolysis through suppression of HSL expression [79] and downregulaton of perilipins via NF-KB [80].
- When insulin resistance has been established, subsequent failure by insulin to suppress basal lipolysis results in higher FA flux and subsequent lipotoxicity which further consolidates the insulin resistant state.

To be noted, researchers have identified numerous origins of insulin resistance, besides a high sugar diet. Obesity itself can be the tipping point, causing dysregulated FA metabolism, oxidative stress or inflammation which in turn impair the function of organs involved in glucose disposal [81]. Inflammation with other origins may also contribute to insulin resistance, including failing to consume a diet sufficient in anti-inflammatory components such as polyunsaturated fatty acids or antioxidants. In line with this, inhibiting inflammation with salicylates or aspirin has positive effects on obesity-induced insulin resistance [82]. In addition, lifestyle factors such as night-time feeding also contribute to insulin resistance. In many cases, multiple pre-diabetic conditions co-occur and exacerbate each other until a vicious circle has developed.

1.6 REGULATION OF CELLULAR RESPONSE

A cell can respond in multiple ways to internal signals or environmental changes. Metabolism and inflammation can be regulated on different levels and in different time-frames. Regulation on of e.g. lipolysis or glucose uptake can take place via immediate stimulation of hormones which trigger signalling cascades, leading to a metabolic event, often within minutes. In other cases, the organism needs to adapt to a more permanent situation. This adaptation is often mediated by changes in gene expression via transcription factors, working in a time-frame of hours to days. One important subclass of transcription factors are nuclear receptors, which can be described as sensors of changes in hormonal or nutritional status and gene expression. In contrast, protein kinases are important regulators of both short-term effects and long-term regulation of the cellular response. In this thesis, we make a closer examination of the role of the nuclear receptor LXR, the transcription factor MAFB and the SIK kinases in human adipose tissue.

1.7 NUCLEAR RECEPTORS AND LIVER X RECEPTOR

Nuclear receptors (NRs) are transcription factors that can be activated by compounds – ligands – from the diet or products of internal metabolism such as bile acids, retinoids, FAs and cholesterol, but also by hormones such as estrogen and steroids such as vitamin D. Each ligand activates a distinct nuclear receptor. When activated, NRs regulate, by stimulating or inhibiting transcription, a large number of processes in the body, such as metabolism, circadian rhythm, inflammation, development, growth and reproduction. [83]. Humans express 48 different NRs divided into six families (NR1-6) [84]. Several NRs have been implicated in metabolic disease.

NRs have a distinct mechanism of action. Once bound and activated by their ligands, they bind the DNA at their respective response elements and promote or inhibit transcription. Most NRs are permanently present in the nucleus and presumed to be constitutively bound to the DNA, whereas some (mostly steroid receptors) translocate to the nucleus upon extranuclear ligand binding in the cytoplasm. Co-factors are an important part of the process and are proteins that can act as co-repressors and co-activators. Co-repressors bind as protein complexes to the NR when there is no ligand bound. When a ligand binds, the co-repressor complex dissociates and is replaced by co-activators. Subsequently, this promotes arrival and binding by the transcriptional machinery, and transcription is initiated. [85] The capacity of nuclear receptors to turn gene expression on or off, and to be bound by ligands make them appealing candidates for the drug industry and the treatment of diseases such as type 2 diabetes and atherosclerosis.

The NR1 family of nuclear receptors comprises e.g. PPARγ – a master regulator of adipogenesis – and the Liver X Receptors (LXRs). The Liver X Receptors (LXR) belong to the largest superfamily, NR1 [86]. Its two isoforms (LXRα and LXRβ) are encoded by the *NR1H3* and *NR1H2* genes, respectively, and share 78% identity in amino acid sequence in their DNA- and ligand-binding domains [87]. LXRβ is ubiquitously expressed while LXRα is highly expressed in tissues with metabolic function such as liver, muscle and adipose tissue [88]. When the term LXR is used in this thesis without specification of isoform, both isoforms are intended.

LXR can be endogenously activated by oxysterols such as 22-R-hydroxy-cholesterol (22-R-HC). Oxysterols form when cholesterol is oxidized which takes place both enzymatically and by auto-oxidation [89]. LXR can also be activated by synthetic ligands, of which GW3965 and

T0901317 are commonly used. LXR dimerises with retinoic X receptor (RXR), then bind as a heterodimer to LXR response elements (LXREs) in the DNA and promote or transrepress transcription of its target genes. LXREs consist of direct repeats of the sequence 5'-AGGTCA-3' which is separated by four nucleotides. LXR can heterodimerise with RXR both in the absence or presence of RXR ligands and it belongs to the Class II NRs, which are constitutively located in the nucleus. LXR has a number of co-factors. Among the best characterised are the co-repressors Nuclear Receptor Corepressor (NCoR) and Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor (SMRT). NCoR/SMRT is a co-repressor complex which binds to LXR in the absence of ligands. The complex is released and dissociates after ligand binding, allowing transcription occur. In the event of transrepression, LXR is targeted by small ubiquitin-like modifier (SUMO)-ylation in the combination of ligand binding, upon which co-repressors bind and transcription of the target gene is inhibited [90].

LXR has been shown to be an important regulator of cholesterol metabolism, especially reverse cholesterol transport, a process where cholesterol is transported from peripheral tissues to the liver by efflux through specific transporters such as ATP-binding cassette proteins ABCA1 and ABCG1. LXR strongly promotes transcription of both transporters. In addition, LXR has also been shown to repress certain inflammatory genes [91] and have protective properties in neurodegenerative disease [92]. Moreover, LXR has been implicated in lipid and glucose metabolism. In the liver, LXR stimulates the expression of lipogenic genes such as *SREBP1c*, *SCD1* and *FASN*. In human adipocytes, LXR influences both lipid and glucose oxidation [93]. In a study using 3T3-L1 adipocytes and mice, LXR has also been shown to induce lipolysis and lead to elevated serum glycerol [94]. In murine models, LXR has also been reported to be a regulator of glucose metabolism. Most studies observe a positive effect on glucose uptake and insulin action in adipocytes, but data from different laboratories are largely inconsistent. Both human and mice possess an LXRE in the GLUT4 promoter [95] and its expression is promoted by LXR in 3T3-L1 adipocytes [96]. In line with those data, LXR was found to increase basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes [94, 96]. Although, it has also been suggested that the positive effect on glucose uptake by LXR might be short-lived since upregulation of GLUT4 expression was transient in mice fed LXR-agonist [95]. In one study, only basal glucose uptake was increased, whereas insulin-stimulated uptake was unaltered [94]. Another study reports an increase in GLUT4 expression after LXR stimulation but no effect on glucose uptake in subcutaneous adipose tissue from rats [97]. The few studies focusing on LXR in human adipocytes also observe conflicting outcomes. In the human adipocyte-like cell line SGBS, LXR stimulation promoted expression of GLUT4 [95], and in LiSa cells (a visceral adipocyte model) LXR restored insulin sensitivity when cells were simultaneously stimulated with TNFα [98]. Conversely, a different study on the role of LXR in SGBS cells observed a *downregulation* of GLUT4 upon LXR activation [99]. In addition, in a report from our group, LXRα was shown to be more highly expressed in obese adipose tissue compared to lean [100], whereas GLUT4 is generally known to be suppressed in obesity. In conclusion, data on the regulation of glucose uptake by LXR are inconsistent. Despite the controversy, LXR has been suggested a potential drug target in the treatment of type 2 diabetes.

1.8 V-MAF MUSCULOAPONEUROTIC FIBROSARCOMA ONCOGENE HOMOLOG B

V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB) is a transcription factor belonging to the Maf family of basic leucine zipper transcription factors – a family of oncogenes – and is expressed in many tissues in both mice and humans [101, 102]. MAFB has emerged as a factor important for brain segmentation [103]. In addition, MAFB has

been shown to be a regulator of cell fates, as it governs differentiation paths for a number of cell types, including macrophages, blood cells, beta cells and osteoclasts [104-108]. Moreover, MAFB may have a role in inflammation. In the human monocyte/macrophage cell line THP-1, TNFα stimulation has been reported to induce MAFB expression. In the same study, MAFB in turn promotes expression of MCP-1 and thus drives inflammation [109]. MAFB has been previously implicated in metabolic processes such as insulin and glucagon transcription [110, 111], which poses an interesting link to diabetes. Moreover, CCAAT/enhancer binding protein δ (CEBPδ) – a transcription factor implicated in early adipocyte differentiation – controls transcription of MAFB [112]. However, little is known about MAFB as a regulator of other metabolic processes, and MAFB was completely uncharacterised in adipose tissue.

1.9 SALT-INDUCIBLE KINASAES

The Salt-inducible kinases (SIKs) comprise a family of three members (SIK1, -2 and -3) which are related to AMP-activated protein kinase (AMKP). The name originates from the discovery of SIK1 in the adrenal glands of high salt fed rats. SIK2 and -3 were added to the family based on sequence homology [113, 114]. AMPK is a master regulator of cellular energy homeostasis and metabolism. Similarly to AMPK, the SIKs have been found to be regulators of metabolism and to be phosphorylated by Liver Kinase B1 [115]. An increased intracellular AMP/ATP ratio activates SIK2 in 3T3-L1 adipocytes [116], a situation which mimics a cellular energy deficit and also activates AMPK. SIKS have been implicated in the regulation of a large number of cellular processes in different tissues. SIK2 has been shown to regulate insulin secretion [117], and suppress gluconeogenesis in the liver [118] as well as inhibit lipogenesis in 3T3-L1 adipocytes[116]. Together, this indicates a possible role of SIKs in metabolic disease.

SIKS exert their mechanism of action through phosphorylation of transcriptional regulators and have a number of different targets. CREB-regulated transcription coactivator (CRTC) -1, -2 and -3 and histone deacetylase (HDAC) -4 and -5 (Class II HDACs) are among the established substrates for the SIK proteins. CRTC phosphorylation by SIKs sequesters them in the extranuclear space. When SIKs are phosphorylated and inactivated by PKA, CRTCs translocate to the nucleus where they regulate CREB targeted transcription. In a similar fashion, HDACs are also prevented from entering the nucleus when phosphorylated by SIKs. HDACs suppress transcription by deacetylation of histones, which results in local condensation of chromatin and reduced gene expression. Consequently, this suppression is inhibited by SIKs. [119-123]

SIK1 is most highly expressed in adrenal glands whereas SIK3 is widely expressed in a number of tissues. SIK2 is most abundant in adipose tissue, although its role in adipocytes has only recently been studied more extensively. SIK2 expression is increased during adipogenesis and is higher in adipose tissue from the obese and diabetic db/db mice than in wild-type mice [114]. Recently, CRTC2, CRTC3 and HDAC4 have been confirmed as SIK2 substrates in murine and human adipocytes. In the same study, basal glucose uptake was augmented in primary rat adipocytes expressing wild-type SIK2 compared with adipocytes expressing mutated loss-of-function SIK2. However, insulin-stimulated glucose uptake in these cells was not affected. [124] In another recent investigation, SIK2 knockout mice displayed higher glucose levels in the blood than wild-type mice, and had suppressed glucose tolerance. GLUT4 was downregulated in the SIK2 knockout mice and glucose uptake in adipocytes was impaired [125]. Taken together, SIK2 emerges as an interesting candidate

in obesity and diabetes research. However, the role of SIK2 in obesity and adipocyte glucose metabolism is incompletely understood and has not been previously investigated in humans.

2 AIMS AND HYPOTHESES

2.1 GENERAL AIMS

The general aim of the work comprising this thesis is to extend the knowledge about adipose tissue, an organ of immense importance for the development of modern diseases, yet incompletely characterised in humans. More specifically, finding factors that may serve as possible drug targets or markers for metabolic disease originating in adipose tissue could be of importance for future diagnosis and treatment strategies. We have studied three factors with potential roles in obesity-associated metabolic complications; 1) LXR, which has previously been implicated in adipocyte metabolism in rodent models, 2) MAFB and 3) SIK2 which we found to have differential expression in adipose tissue from obese humans compared to lean. This differential expression might indicate involvement in the development of obesity or its secondary complications. In this thesis, we wished to establish the role of these factors in adipose tissue function.

2.2 STUDY-SPECIFIC AIMS

I. The aim of the first study was to investigate the role of LXR in the regulation of lipolysis.
II. The aim of study two was to study how LXR affects human adipocyte glucose uptake.
III. The aim of the third study was to characterise the function of MAFB in human adipocytes and monocytes.
IV. The aim of study four was to examine the regulation of SIKs by obesity and the influence on glucose uptake by SIK2.

3 METHODOLOGY

The methods and materials used for each study have been described in their corresponding papers. In this chapter, interesting aspects of a few selected methods not covered therein will be addressed.

3.1 CELL CULTURE MODELS

3.1.1 Primary adipocytes

For the majority of the experiments in this thesis, primary cells isolated from human adipose tissue were used. Adipose tissue was obtained from liposuctions or biopsies. In total, three different primary cell culture systems listed below were employed.

3.1.1.1 Primary in vitro *differentiated pre-adipocytes*

Total SVF of adipose tissue was cultured and differentiated *in vitro* into adipocytes, without proliferation. After 3-4 days in adipogenic media, immune cells die off and are washed away during media changes. Only cells with adipogenic capacity remain in the culture. The cells with adipogenic capacity within SVF are stimulated to develop into adipocytes by activating the adipogenic program. Rosiglitazone is a synthetic ligand of PPARγ, the main factor driving adipogenesis, and a crucial component of adipogenic culture medium. In addition, insulin is also pro-adipogenic and added to the medium. The major advantage of SVF-derived adipocytes is that some characteristics of the donor tissue are preserved even after *in vitro* differentiation [126, 127]. Thus, experiments conducted on this cell system retain a certain degree of biological variation, in this aspect resembling freshly isolated mature adipocytes. The major disadvantages of this adipocyte system are the time consuming isolation procedure and limited supply of donor tissue. In this thesis, the term SVF-derived adipocytes will be used to describe these cells.

3.1.1.2 Human mesenchymal stem cells

Human mesenchymal stem cells (hMSCs) were isolated from the stromal vascular fraction (SVF) of adipose tissue, expanded *in vitro* and differentiated into adipocytes. hMSCs can be differentiated into myocytes, osteocytes, chondrocytes and adipocytes. FGF2 is added to the culture medium to stimulate proliferation and adipogenesis is induced using rosiglitazone and insulin as for SVF-dervied adipocytes. Adipocytes differentiated from hMSCs display an adipocyte phenotype in their lipolytic response, secretion of adipokines and response to insulin [127-130]. hMSC cultures are expandable and advantageous to use when large amounts of cells are needed and when a minor degree of biological variation is desired.

3.1.1.3 Freshly isolated mature adipocytes

Mature adipocytes were isolated from adipose tissue by collagenase. Mature adipocytes do not attach to cell culture dishes due to their high lipid content and have to be cultured floating. Methods of assessing e.g. glucose uptake in these cells have been evaluated [131]. The advantage of studying mature adipocytes from human donors is that they more than other adipocyte cell systems resemble adipocytes *in vivo*. The drawback is their fragility in culture medium and that they cannot be cultured longer than 72 h, as determined empirically in our lab. Also, there are limitations to the type of treatments and experimental procedures that can be applied for mature adipocytes.

Both hMSC-derived adipocytes and SVF-derived adipocytes in culture differ from primary mature *ex vivo* adipocytes. For example, they have multiple lipid droplets whereas mature adipocytes have one large integral TG reservoir. This may affect their function. Further, a study comparing gene expression profiles assessed during *in vitro* and *in vivo* adipogenesis reveal a number of genes that are differentially regulated [132]. In addition, the differentiation cocktail and culture conditions of all cell cultures lack the diversity of molecules that surrounds cells *in vivo* and this may have an indiscernible impact on their function.

3.1.2 Cell lines

In paper I and IV we have used immortalised cell lines.

3.1.2.1 3T3-L1 adipocytes

The murine 3T3-L1 adipocyte cell line was employed for one comparative experiment in paper I. In the field of adipocyte research, it is a commonly employed culture model. 3T3-L1 is an immortalised cell line of pre-adipocytes originally derived from mouse embryos [126]. Like human SVF-derived preadipocytes differentiated *in vitro*, 3T3-L1 adipocytes also exhibit multiple lipid droplets. In its character of a cell line, it has an altered cell cycle mechanism and a high degree of spontaneous mutations. The main advantage of this model is its cost-effectiveness and low degree of biological variation. A drawback of using 3T3-L1 cells is their murine origin. Rodents and humans biology differ substantially in some aspects, and there are known differences between murine and human adipose tissue function. Interspecies differences will be discussed in section 3.2. Unlike SVF-derived and hMSC adipocytes, 3T3-L1 are committed and pre-programmed for adipogenic differentiation.

3.1.2.2 THP-1 cells

The human monocyte cell line THP-1 was used for paper III. As 3T3-L1, THP-1 is an immortalised cell line. THP-1 monocytes can be differentiated into macrophages in culture. In adipose tissue *in vivo*, fully differentiated macrophages exhibit different polarizations – M1 for classically activated (pro-inflammatory) macrophages, and M2 for alternatively activated (anti-inflammatory) macrophages. However, after inducing differentiation according to the standard protocol, THP-1 cells display a non-polarized, non-activated (M0) phenotype [133]. This concern is however not applicable to this thesis since THP-1 cells were studied as undifferentiated monocytes. As mentioned in section 1.2.3, the macrophage population *in vivo* is most likely more complex than the M1/M2 division implies.

3.2 INTERSPECIES DIFFERENCES

When selecting cell system or animal model for a research study, awareness of the advantages and disadvantages of different options is important. Using *in vivo* animal (often mouse) models provides the obvious advantage of being able to study a factor in the physiological context, and is a necessary step prior to human drug trials. However, there are a number of known differences between human and mouse biology. A few examples of discrepancies between human and mice specific for adipose tissue function are listed below.

- Epididymal fat is an often studied adipose tissue depot in mice, partly because it is easy to isolate. However, there is no corresponding fat depot in humans and as different fat depots have been shown to differ in certain aspects this raises concerns

about the relevance of epididymal fat for studies where results are extrapolated to humans [10].

- Omental fat – a rather large and important visceral fat depot in humans –, on the other hand, is barely present in mice [10].
- In humans, lipolysis can be negatively regulated by *α2* adrenergic receptor signalling. However, this pathway does not exist in rodents [42].
- In human adipose tissue, lipolysis can be stimulated by natriuretic peptides, whereas this function has not been found in rodents [134].
- Genome-wide analyses of PPARg binding sites reveal that a high number of binding sites are different in the mouse and human genome [135, 136].
- Interestingly, mice have a higher adipocyte turnover – 0.6% per day in mice [137] vs. 8% per year in humans [11]. Although, this might simply reflect their shorter life-span.
- The immune system of mouse models often employed in obesity-studies differs from that of humans in a number of respects [138]. Since immune cells populate adipose tissue, and there is extensive cross-talk between adipocytes and immune cells, this may be of importance to adipocyte research.

An additional concern specific to obesity research is the often studied obese mouse models which lack leptin production (ob/ob mice) or a functional leptin receptor (db/db mice). They are highly convenient to work with but differ from common human obesity. Leptin deficiency is a rare condition in humans and does not contribute to the epidemic of obesity. As mentioned in section 1.3.4, leptin stimulates satiety. But it also affects energy expenditure, atherogenesis, fertility, insulin sensitivity, and neurodegenerative disease [46]. This may influence study results in ways that are hard to predict.

Conclusively, when a factor has solely been studied in animal models, it is often considered a limitation. However, choosing to work exclusively with human material is time-consuming and costly. Yet there are major important advantages to use human cells when possible.

3.3 STUDYING GENE EXPRESSION

The phenotype of a cell is formed by its gene expression profile. Gene expression controls everything from physical appearance to its function. The study of gene expression is an especially important part of thesis as three of the studies concerns transcription factors. We have used two different methods for mRNA quantification and two methods for quantification of protein. Advantages and disadvantages of these methods will be briefly discussed below.

3.3.1 Microarray profiling

Genome-wide mRNA expression profiling by microarray provides the possibility to compare mRNA or miRNA expression of a large number of transcripts (>20 000) between different conditions. In paper I and II, microarrays are used to examine global mRNA and miRNA expression. Although other alternative methods to determine global levels of transcripts were available at the time, such as RNA sequencing (RNA-seq) or Cap Analysis of Gene Expression (CAGE), they were not as established and evaluated as microarrays. Microarrays also has additional advantages; they are cheaper, require less biological material and expression data are easily comparable between different data sets. Microarray data is easily processed using computer software and information on signalling pathways and biological processes can easily be extracted. Like with other methods determining RNA expression,

comparison of the absolute quantification between genes is not possible due to distinct binding affinities of the different mRNA/miRNA transcripts to their respective probes. Following microarray profiling, quantitative real-time polymerase chain reaction (qRT-PCR) is often used as a validation method for specific genes of interest.

3.3.2 Quantitative real-time PCR

Quantitative real-time PCR **(**qRT-PCR) is used to quantify certain transcripts of interest using specific primers and nucleotide-binding dyes. In short, RNA is purified from cells or tissue and reverse transcribed into cDNA. The transcript is amplified during multiple cycles by Taq polymerase and primers that bind to a sequence which is specific to the transcript of interest. In the papers of this thesis, we have used two different methods for detection; Taqman and SYBR green assays. In a Taqman assay, probes specific to a certain sequence are labelled with a fluorescent dye and a quencher, which absorbs the fluorescence. During elongation, the probe is bound to the cDNA and as the polymerase reaches the probe, it is degraded and the quencher is simultaneously released from the fluorophore, allowing it to emit fluorescence. Taqman assays require a larger amount of RNA and is expensive, compared to the SYBR green method. In a SYBR green assay, the dye binds non-specifically to double-stranded DNA. Due to the unspecific nature of the dye, it also binds indiscriminately to any primer-dimers and unspecific PCR products that might form. Therefore, SYBR green primers should be verified in a dissociation curve to detect any unspecific products. Fluorescence is recorded by a PCR instrument and compared by the researcher to that of a housekeeping gene in the same samples.

3.3.3 Western blot

Western blot is a method widely used to measure protein expression based on protein size compared to a known standard. Proteins are separated by size using electrophoresis and transferred to a membrane which is incubated with primary antibodies binding the protein of interest. Incubation with a secondary antibody conjugated to enzymes or fluorophores then binds to the primary antibody and detection is performed using chemiluminescence or fluorescence. A densiometric comparison of protein band size and density can be made on the resultant blot. The band density can also be measured by computer software and compared to that of a housekeeping protein. However, it is not only semi-quantitative due antibody-target properties. It also has other drawbacks; it is dependent on specific antibodies that bind the denatured protein with high affinity. Post-translational modifications of a protein may alter the perceived protein size compared to the known standard. In addition, it is time-consuming and the success of the procedure can be unpredictable.

3.3.4 Enzyme-linked immunosorbent assay

In contrast to Western blot, enzyme-linked immunosorbent assay (ELISA) is highly quantitative and quick to perform. In ELISA, protein lysates are loaded onto a microplate pre-coated with antibodies binding the target protein. The protein is subsequently probed with a detection antibody and a secondary antibody linked to an enzyme, e.g. horseradish peroxidase (HRP). HRP will catalyse a colour change of a substrate, upon which this colour can be measured spectrometrically. Colour density indirectly corresponds to protein expression and is often normalised to the amount of loaded protein. ELISA requires less material than western blot, and conditioned medium can be used for detection of secreted proteins. ELISA is dependent on a very high specificity of the antibody. Should unspecific

binding to other proteins occur, this is not by any means visible and the quantification will be faulty. Nevertheless, ELISA serves a good compliment to Western blots.

3.3.5 Correlations of mRNA levels and corresponding protein expression

mRNA levels do not always reflect the protein levels. Regulation of translation or translation rate, protein half-life and secretion are some events that all lead to a non-linear relationship between mRNAs and protein, although this varies greatly between individual proteins [139]. In addition, the amount of protein does not translate to protein function and protein activity should ideally be investigated. Therefore, one should whenever possible quantify both mRNA and protein amounts as well as protein activity when studying the biological function associated with the protein in question. However, mRNA levels alone do provide interesting clues despite not always being accompanied by the matching variations in protein expression, since it offers information about the transcriptional regulation of a certain gene, or of the activity of a certain transcription factor.

3.4 METABOLIC METHODS

3.4.1 Glucose uptake

Three of the four thesis papers include measurements of glucose uptake. There are several methods to study glucose uptake into adipocytes. In paper II and IV, we used a classical glucose uptake protocol designed for adipocytes in culture. In this protocol, 2-Deoxy-D-[1-^{3}H]-glucose is added to the cells in the absence or presence of insulin, upon which basal and insulin-stimulated glucose uptake can be quantified. 2-Deoxy-D-[1-^{3}H]-glucose is a radioactive, non-metabolisible form of glucose. Thus, it cannot be incorporated into lipids and the only factor being measured is glucose uptake itself. However, for paper III we developed a second protocol, measuring total glucose uptake based on a lipogensis protocol [140]. For this procedure, we use D-[3-^{3}H]glucose which is metabolised by the cell and incorporated into lipids. Also, insulin stimulation is prolonged compared to the glucose uptake protocol described above – 2 hours vs. 15 minutes. This provides a higher and more robust insulin-response. In human adipocytes, glucose uptake is rate-limiting for lipogenesis at the concentrations of glucose used in this protocol [141] and thus, measuring total glucose uptake after stimulation of lipogenesis reflects the cell's ability to transport glucose across the plasma membrane. Consequently, when stimulating with insulin, we are indirectly measuring insulin signalling and GLUT4 translocation.

Insulin is added to the adipocyte culture medium as an inducer of adipogenesis. To reset insulin sensitivity in our cells prior to glucose and lipogenesis assessments, we removed insulin for a period of time prior to insulin stimulation. In SVF-derived adipocytes, 3 h incubation in insulin-free medium was enough to produce an insulin response. However, hMSC-derived adipocytes have a higher insulin concentration in culture medium and require a longer time in insulin-free medium to reverse their insulin resistance. Thus, we optimised incubation times in medium without insulin and found 48-72 h to be optimal [127].

3.4.2 Lipolysis

In paper II we studied the effects of LXR stimulation on adipocyte lipolysis. Lipolysis was assessed during 3 h incubation of cultured adipocytes in medium with or without lipolytic agents. Isoprenaline is a *β* adrenergic agonist with structural resemblance to adrenaline and was used in our study to stimulate lipolysis. When assessing lipolysis in adipocytes,

glycerol release into the conditioned medium is quantified. The reason for this is briefly discussed earlier – adipocytes do not express the enzyme required for re-esterification of glycerol to FAs into TGs [41]. Thus, released glycerol represents an accurate account for the rate of lipolysis. Glycerol is also easily measurable enzymatically or using a bioluminescence method. Of note is that albumin has to be supplied into the conditioned medium as a carrier for the non-water soluble liberated FAs, or the cells will be subject to lipotoxic effects.

4 RESULTS

4.1 PAPER I

As mentioned in the introduction, lipolysis is one of the main functions of the adipocyte, resulting in the release of FAs to be utilised by other tissues. In obesity, this process is dysregulated and while stimulated lipolysis is blunted, basal lipolysis is enhanced. LXR has previously been implicated in the regulation of lipid turnover in mice and murine cell models. When activating LXR in 3T3-L1 adipocytes, basal lipolysis is augmented [94]. However, the role of LXR in lipolysis in human adipocytes had not been studied. We hypothesized that LXR also regulates lipolysis in human adipocytes. We therefore set out to determine the effects of LXR activation on lipolysis in human fat cells.

Initially, we stimulated human SVF-derived adipocytes using the LXR specific agonist GW3965 and observed a concentration-dependent increase in lipolysis as measured by glycerol released into conditioned medium. Using mRNA microarray profiling, qRT-PCR and western blot, we identified two genes with major importance for lipolysis which were downregulated by GW3965 treatment; hormone-sensitive lipase HSL and PLIN1.

Next we investigated the effects of LXR activation on the cellular localisation of HSL and PLIN1 by immunostaining. Interestingly, lipid droplet-associated PLIN1 proteins decreased in number. Also, when quantifying the ratio of HSL to PLIN present in the cells, it turned out to increase dramatically upon LXR stimulation. This indicated that HSL activation was enhanced, despite the observed downregulation of HSL expression. It also implies that the general lipid droplet morphology was altered.

We continued to investigate HSL sites that are targets of phosphorylation and activation of its lipolytic function. When quantifying the total downregulation of HSL by LXR and comparing it to the levels of phosphorylated HSL, the ratios were not altered upon LXR-stimulation. We further stimulated adipocytes with GW3965 with or without a combination of the HSL inhibitor BAY. Intriguingly, inhibiting HSL activity completely blocked the effects of LXR on glycerol release. Hence, we could conclude that enhanced lipolysis by LXR in adipocytes still relied on HSL activity, despite the downregulation of HSL expression.

We continued to explore the role of PLIN1 in LXR-mediated lipolysis. PLIN1 knockdown lead to increased glycerol release. When combining PLIN1 knockdown with GW3965 treatment, the effects of GW3965 were gone. From this we drew the conclusion that PLIN1 was a main player in the regulation of lipolysis by LXR. Using chromatin immunoprecipitation we also found that LXR is recruited to the proximal promoter of PLIN1, and binds to intron 1 of the *LIPE* gene (encoding HSL). We were also able to demonstrate that LXRα is the major isoform mediating the effects or LXR on lipolysis.

In summary, we are able to demonstrate that LXR augments basal lipolysis in primary human adipocytes. The findings of paper I are depicted in Fig. 3.

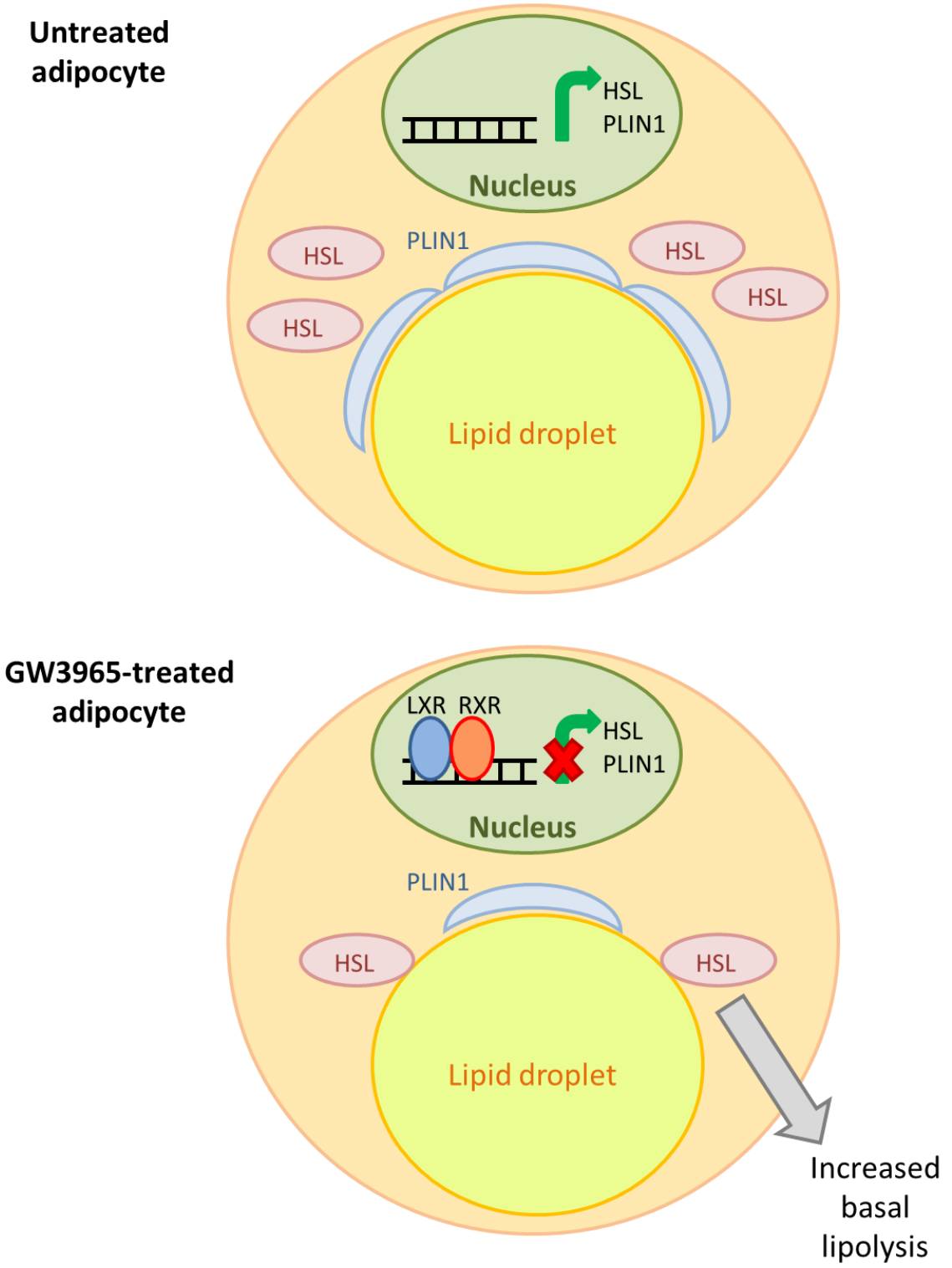


Fig. 3. Proposed mechanism of LXR-mediated effects on lipolysis.

4.2 PAPER II

As discussed in section 1.5, the diagnosis of type 2 diabetes means in part that muscle and adipose tissue have lost their ability to properly take up glucose from the blood. LXR has been extensively studied in the context of glucose metabolism in murine adipose models such as 3T3-L1 mouse-derived adipocytes and LXR knockout mice. Several studies have found that LXR promotes glucose uptake in murine adipocytes (summarised in section 1.7). This has opened up interest for LXR as a drug target in the treatment of diabetes. However,

the role of LXR on glucose uptake has only briefly been studied in human adipocytes. Thus, we decided to investigate the effects of LXR stimulation on glucose uptake and insulin signalling in primary human adipocytes. For this study we used three different human adipocyte cell systems; 1) SVF-derived adipocytes, 2) hMSCs-derived adipocytes and 3) primary mature *ex vivo* adipocytes.

Initially, we performed a genome-wide analysis of the effects on mRNA and miRNA expression after LXR stimulation, to search for transcriptional alterations of any genes or miRNAs known to be involved in glucose uptake. This was performed by microarray profiling of human adipocytes treated with the LXR agonist GW3965. Thus, we focused on mRNA expression. The overall results of the mRNA microarray analysis placed metabolic pathways in the top of listed regulated biological processes.

A closer examination of the insulin signalling pathway revealed that LXR stimulation negatively altered the expression levels of several genes of the insulin signalling cascade. More specifically*, AKT2, SORBS1* (encoding CAP) and *CAV1* – all important for insulin signalling – were downregulated compared to control treatment. GLUT1 (encoded by *SLC2A1*) and GLUT4 (encoded by *SLC2A4*) are glucose transporters responsible for basal and insulin-stimulated glucose uptake in adipocytes, respectively. *SLC2A1* was upregulated by LXR activation, whereas *SLC2A4* (encoding GLUT4) was unaffected. Thus, we were able to confirm the upregulation of *SLC2A1* observed in previous studies. Interestingly, the lipogenic genes *ACC1, SCD1* and *FAS* were unaffected by LXR activation in this microarray, contrary to previous reports in hepatocytes and murine cell lines. AKT2, CAP and CAV1 were also downregulated on protein level, as determined using western blot.

Next, we studied the effects of LXR activation on glucose uptake in human adipocytes. We stimulated SVF-derived adipocytes with the LXR agonist GW3965 and measured basal and insulin-stimulated glucose uptake. Basal uptake remained unaffected whereas insulin-stimulated glucose uptake was suppressed upon LXR activation. We were curious to investigate if the weight status of the human donor subjects would have an effect on the outcome. We discovered that LXR significantly suppressed glucose uptake in adipocytes from overweight donors only, in both SVF-derived adipocytes and mature *ex vivo* adipocytes.

AKT2 is universally considered a protein of very high importance for transduction of the insulin signalling pathway. Therefore, we turned our focus to this protein. We studied effects of LXR activation on phosphorylation of AKT2 at Ser474. As expected, AKT2-P-ser474 increased dramatically upon insulin stimulation. In combination with GW3965 stimulation, phosphorylation at this site was reduced to same extent as the downregulation of total AKT2 protein, suggesting that LXR mainly affects protein expression and not activation of AKT2. The fact that we observed the same level of LXR-mediated inhibition of insulin-stimulated glucose uptake as of phosphorylation of AKT2 also suggests that AKT2 could be the major factor through which LXR impairs insulin-stimulated glucose uptake. We also demonstrate that LXRα turned out to be the main isoform mediating the suppression on AKT2 expression.

Reduced insulin-stimulated glucose transport should in theory be associated with reduced translocation of GLUT4 to the plasma membrane. Therefore, we stimulated adipocytes with LXR agonist and isolated the plasma membrane fraction. A western blot using plasma

membrane proteins showed that LXR stimulation indeed caused a diminished translocation of GLUT4.

In summary, we demonstrate that LXR-stimulation inhibits insulin-induced glucose uptake in adipocytes from humans on the obese spectrum. This inhibition may be mediated by suppression of AKT2, CAP and CAV1 expression.

The findings of paper II are illustrated in Fig. 4.

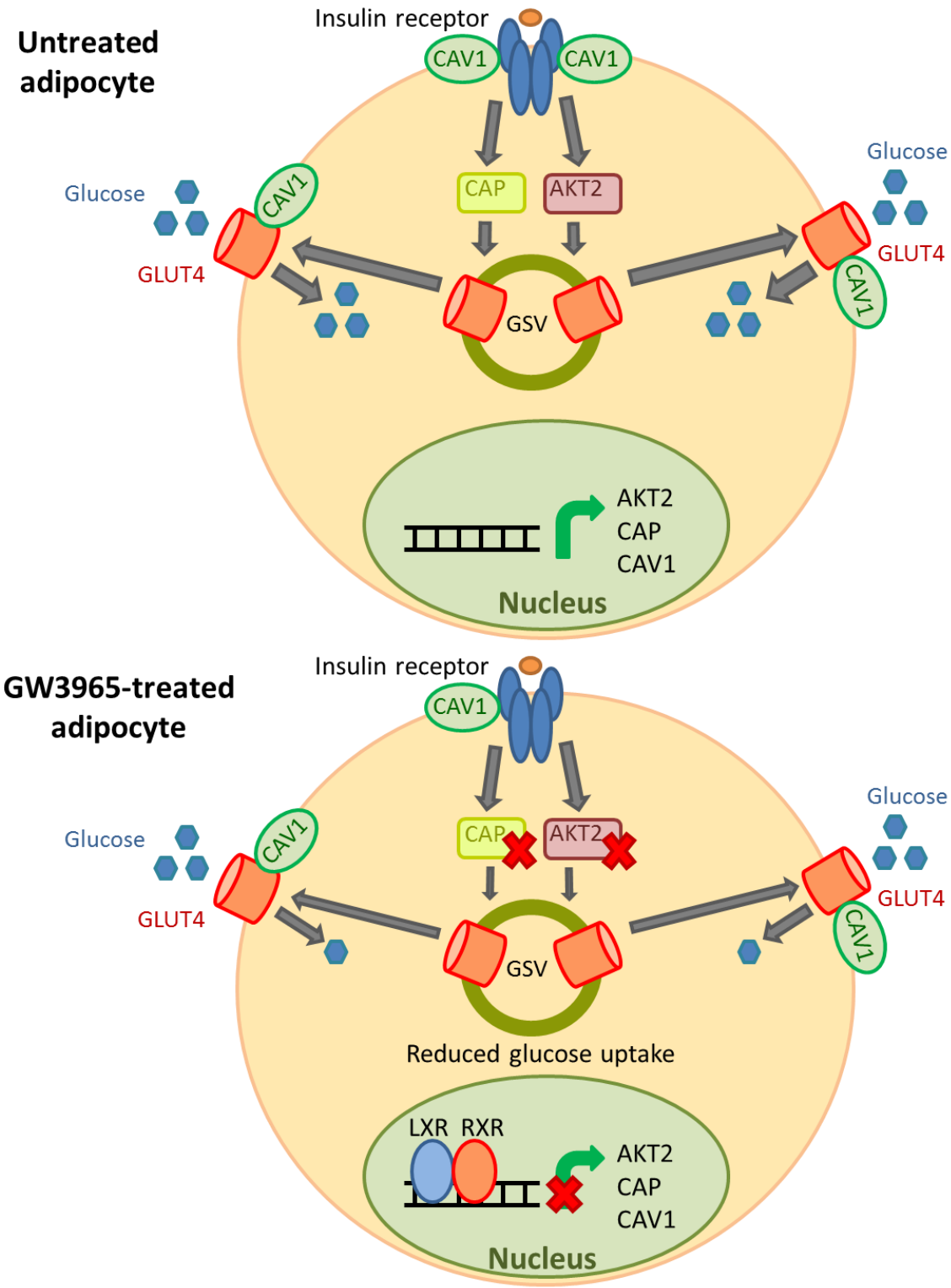
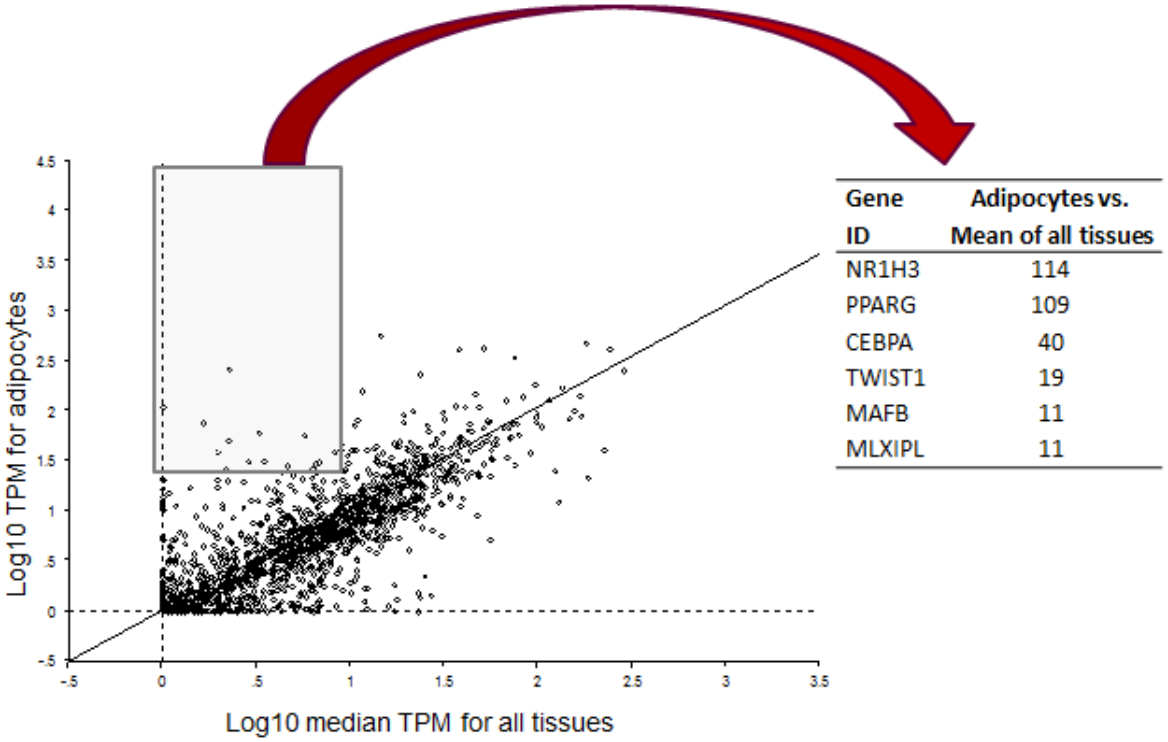


Fig. 4. Proposed mechanism of LXR-mediated effects on glucose uptake. GSV; GLUT4 storage vesicle.

4.3 PAPER III (MANUSCRIPT)

After publishing paper II, interesting data emerged from the functional annotation of the mammalian genome 5 project, where CAGE analysis was used to quantify genome-wide transcriptional profiles of 150 human tissues and cell types [142]. Extracting the expression of known human transcription factors from this data set reveals LXRα as the factor with the highest enrichment in mRNA expression in adipocytes (114x higher as compared to the median expression in all tissues). MAFB turned up in the top five with the highest expression

in adipocytes. (Fig. 5.) This lead to our hypothesis that MAFB may be important in adipocyte biology. In a previously published microarray dataset comparing gene expression in adipose tissue between obese and non-obese individuals, we also found *MAFB* to be regulated by obesity. MAFB had not been implicated in obesity or studied in adipose tissue, and together, these initial findings intrigued us to characterise its role in human adipose tissue.



Gene ID	Adipocytes vs. Mean of all tissues
NR1H3	114
PPARG	109
CEBPA	40
TWIST1	19
MAFB	11
MLXIPL	11

Fig. 5. Enrichment of *MAFB* mRNA expression in adipocytes relation to the median expression in 150 human tissues. TPM; tags per million. Image prepared by Niklas Mejhert.

Initially, we confirmed higher expression of *MAFB* in obese subjects compared to lean in a second cohort. This cohort also comprised obese individuals diagnosed with the metabolic syndrome, and in these subjects, *MAFB* was even more highly expressed than in metabolically healthy obese subjects. The same regulation pattern occurred in a third and a fourth cohort where *MAFB* was downregulated after weight loss. These findings possibly imply that MAFB might be involved in the dysregulation of metabolism and/or inflammation.

Next, we investigated if *MAFB* was associated with any metabolic features of human subjects in a cohort of 56 subjects. *MAFB* correlated negatively with insulin sensitivity measured as insulin-stimulated lipogenesis in adipocytes, as well as with isoprenaline-induced lipolysis which indicates that MAFB might be associated with an unfavourable metabolic profile.

The findings described above intrigued us to make an extensive investigation of the role of MAFB in adipocyte function and metabolism. We found that *MAFB* was strongly induced during adipocyte differentiation. To study the importance of MAFB for metabolism, we performed siRNA-mediated knockdown of *MAFB*. *MAFB* knockdown did not affect adipogenesis, lipid accumulation, basal lipolysis, nor basal or insulin-stimulated lipogenesis.

However, *MAFB* knockdown did reverse the unfavourable effects caused by TNFα treatment on lipid accumulation and lipolysis. This implies that MAFB is required to fully mediate the effects of TNFα in these conditions and this cell system. Thus, MAFB is a mediator of inflammation in human adipocytes.

We further studied the role of MAFB in the regulation of inflammatory gene expression in adipocytes and found that MAFB mediates TNFα-induced IL6 expression. In line with the reversal of TNFα-mediated effects on lipolysis and lipid accumulation, MAFB is seemingly necessary for TNFα signalling to fully induce IL-6 expression.

MAFB itself has also been reported to be regulated by TNFα in macrophages [109]. Since TNFα release is increased in adipose tissue in obesity, we wished to study the effects of TNFα on MAFB expression in adipocytes. Thus, we stimulated both monocytes – the precursors of macrophages – and adipocytes with TNFα and measured *MAFB* expression. Interestingly, in contrast to monocytes, *MAFB* was downregulated by TNFα in adipocytes. The effects were obvious already after 6 hours of incubation, from which we drew the conclusions that the effects were a result of direct TNFα signalling.

Finally, we observed that *MAFB* is 13 times more highly expressed in monocytes/macrophages than in adipocytes from primary adipose tissue, and correlates strongly and positively with the macrophage markers *CD14* and *CD68* as well as *TNFα* mRNA expression in adipose tissue explants. All in all, these data suggest that MAFB is a marker of inflamed adipose tissue as well as the level of macrophages infiltration.

The findings of paper III are depicted in Fig. 6.

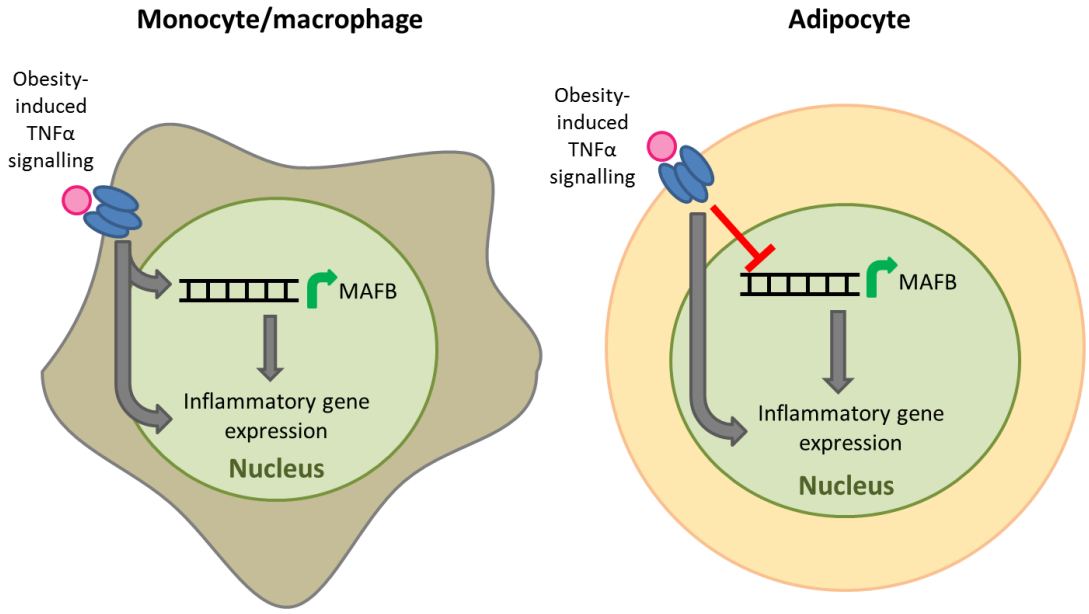


Fig. 6. Proposed regulation and mechanism of action of MAFB in monocytes/macrophages and adipocytes.

4.4 PAPER IV (MANUSCRIPT)

The Salt-inducible kinases (SIKs) SIK1, 2 and 3 are related to AMPK and influence gene expression by phosphorylating proteins that in turn regulate transcription such as HDACs

and CRTCs. SIKs (mainly SIK2 and SIK3) have previously been shown to be highly expressed and to regulate various metabolic functions in murine adipose tissue. However, SIK data from human adipose tissue is lacking. We hypothesized that SIKs are of importance in human adipose tissue and aimed to investigate its regulation by obesity and its role in glucose uptake.

Initially, we wished to investigate the relative expression levels of the three SIK proteins in adipocytes from human subjects. The by far most predominant isoform expressed in adipocytes was *SIK2*. Next, we quantified the levels of *SIK2* and *3* in intact subcutaneous adipose tissue from non-obese and obese subjects. Interestingly, *SIK2* and *SIK3* expression was lower in obese. In addition, *SIK2* and *SIK3* mRNA was increased after weight loss by bariatric surgery. *SIK2* mRNA expression also correlated negatively with HOMA-IR. These data suggest that SIK2 might be a factor associated with a favourable metabolic profile in human adipose tissue.

From this point in the investigation, focus turned to SIK2 as it was most highly expressed in adipocytes, and also was affected by obesity and weight loss more dramatically than SIK3. We thus set out to further characterise the role of SIK2 in obesity.

We hypothesised that the reason for suppressed SIK2 expression in obesity could be related to the pro-inflammatory environment. Therefore, we mimicked the influence of inflamed tissue on adipocytes by treating them with TNFα and studied the effects on SIK2 mRNA and protein expression. In line with our hypothesis, SIK2 expression was downregulated by TNFα on both mRNA and protein level.

Since SIKs have been implicated in the regulation of glucose metabolism in rodents, we wished to determine the role of SIK2 on glucose uptake in human adipocytes. We treated adipocytes in culture with a pan-SIK inhibitor and measured glucose uptake. Since the expression level of SIK2 greatly exceeded that of SIK1 and -3, we made the assumption that any effects observed following SIK-inhibition treatment in all probability could be attributed to inhibition of SIK2. Interestingly, both basal and insulin-stimulated glucose uptake was attenuated upon SIK inhibition. These data support the significance of the correlations with HOMA-IR and decreased expression of SIK2 in obese subjects. In addition, phosphorylation of the SIK substrates HDAC4 and CRTC2 was similarly reduced upon inhibitor treatment.

The findings of paper IV are illustrated in Fig. 7.

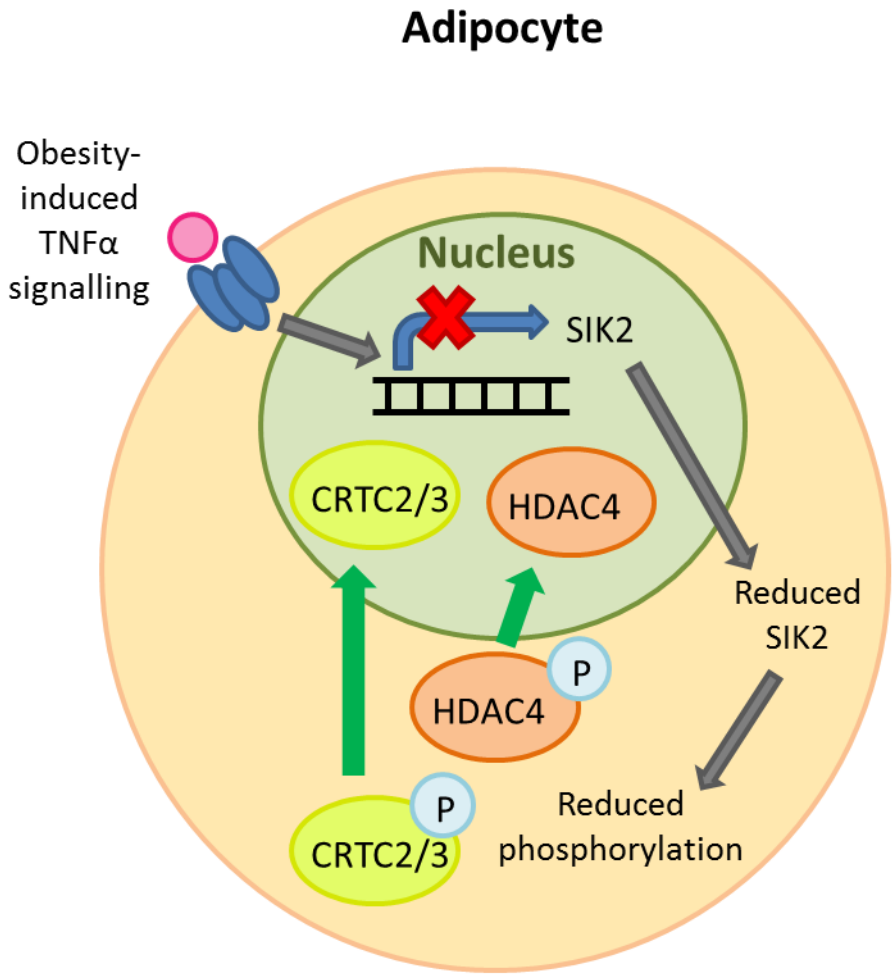


Fig. 7. TNFα-regulation of SIK2 and SIK2-mediated effects on gene expression in adipocytes.

In addition to the data presented in paper IV, we have investigated correlations of *SIK2* mRNA with other variables available in our clinical cohorts which could give clues to the function of SIK2 in adipose tissue. In unpublished data, *SIK2* mRNA expression correlated positively with adipocyte cell volume from humans with varying BMI. We also found a negative correlation between *SIK2* and *SLC2A4* mRNA (Fig. 8.)

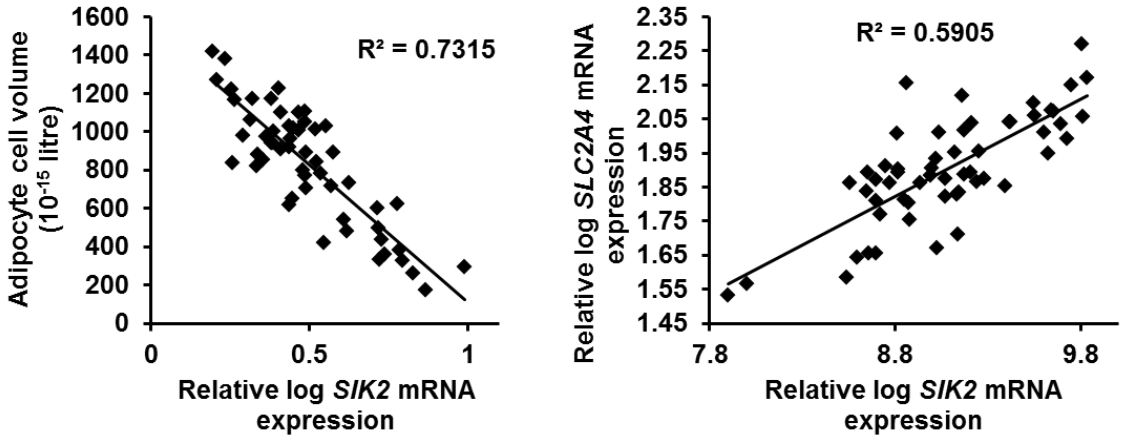


Fig. 8. Correlations of *SIK2* mRNA with *SLC2A4* mRNA expression and cell volume.

5 DISCUSSION

In **paper I and II**, we find that LXR is an inducer of basal lipolysis and a suppressor of insulin-stimulated glucose uptake in human adipocytes.

In **paper I**, we found that PLIN1 and HSL were downregulated by LXR in our cell system. However, the level of HSL phosphorylation was not affected. Our observation of increased spontaneous lipolysis with a simultaneous drop in PLIN1 levels has been reported previously [143-145]. With regards to HSL, knockdown of this protein has been shown to either suppress [146] or not affect spontaneous lipolysis [79, 147]. Since HSL phosphorylation was not affected by LXR, and HSL knockdown might not alter basal lipolysis, it is possible that suppression of PLIN1 alone (leading to an increase in HSL to PLIN1 ratio) is mediating the observed effects of LXR stimulation on lipolysis. Interestingly, TNFα has similar effects on adipocyte lipolysis – downregulating both HSL and PLIN1 [79, 80] – with a net effect of augmented spontaneous lipolysis. It is important to mention that HSL is known to be phosphorylated on multiple sites and other residues not investigated in this paper may be affected by LXR treatment.

ATGL mRNA expression was not altered by LXR on the microarray, and although its co-factor *CGI-58* was downregulated on mRNA level, we were not able to confirm it on protein level. It has however been reported that reduced expression of PLIN1 could lead to release of CGI-58 from the lipid droplet, where it resides in the unstimulated state, and subsequent activation of ATGL. Thus, it is possible that ATGL or CGI-58 is involved in LXR-mediated lipolysis.

We further found that LXR binds to the human PLIN1 and HSL proximal promoters. However, we did not identify any LXREs in the PLIN1 promoter and LXR did not bind to the weak LXREs found in the HSL promoter. Consequently, LXR can evidently bind to additional sequences or possibly interact with DNA through another factor. LXR has been shown to LXREs have predominantly been associated with positive regulation of gene expression [148], and as LXR binds to other sequences in the proximal promoters of *LIPE* and *PLIN1*, these sequences could in theory specifically promote negative regulation of LXR target genes.

An mRNA microarray analysis was used both in **paper I and II** to identify LXR-regulated genes of potential interest in human adipocytes. Using computer software to highlight biological processes that were regulated by LXR in our cells revealed that metabolic pathways were among the top of regulated processes. This confirms two previous studies conducting genome-wide mRNA profiling of LXR effects in human cells, in hepatocytes [149] and macrophages [150], both found that one of the main processes regulated by LXR is lipid- and cholesterol metabolism. In this genome-wide analysis, a mere 364 genes were upregulated, whereas 1.161 genes were downregulated upon LXR activation. Since LXREs reportedly primarily mediate a positive gene regulation (as discussed above), this could hypothetically indicate that the human genome contains a large number of alternative binding sites for LXR.

The role of LXR in the expression of insulin signalling proteins is previously unexplored. Therefore, when analysing the specific metabolic pathways regulated by LXR on the microarray, it was interesting to find that the expression of three proteins of presumed high importance for insulin signalling (AKT2, CAP, CAV1) was altered by LXR stimulation. In a previous report, the authors performed a microarray analysis on adipose tissue from LXR-

agonist fed mice [151]. However, the insulin signalling pathway was not affected by LXR activation in that study. This implies the presence of species-specific differences in the response to LXR stimulation. Interestingly, Kotokorpi and co-authors [149] also found that the effects of LXR stimulation differed in human and murine hepatocytes, confirming our observation of interspecies differences.

In the microarray, *SLC2A1* was upregulated by LXR activation, which is a confirmation of findings in previous studies. However, translation into its protein GLUT1 remained unaltered by LXR. This is in line with unaltered basal glucose uptake after LXR activation. Possible reasons for the discrepancy between mRNA transcription and protein expression from the same gene are discussed in section 3.3.5. In addition, the total expression level of GLUT1 is low in adipocytes. Also, some GLUT4 is believed to be present on the plasma membrane even in the non-stimulated state and could contribute to basal glucose uptake.

In both **paper I and II**, LXRα was the isoform mediating the effects of LXR activation on lipolysis and glucose uptake. Since LXRβ is expressed in most tissues and LXRα expression is restricted to a few tissues which are all lipid-metabolising, it is not entirely unexpected that LXRα is the major isoform involved in lipolysis and glucose uptake. LXRα has also previously been denoted the most important isoform in adipocyte metabolism [94, 95, 152].

In **paper II** we shortly address an interesting aspect of cell biology. We show that primary pre-adipocytes from human subjects react in a BMI-specific manner to LXR stimulation, an observation that was repeated with the same outcome in mature *ex vivo* adipocytes. This implies that pre-adipocytes retain a metabolic memory even after two weeks of *in vitro* culturing. It also demonstrates the long-lasting effects of epigenetic imprinting and the importance of epigenetics in the future of individualised medicine, a topic not addressed in this thesis. Furthermore, we also made an interesting observation in the regulation of GLUT4 mRNA expression by LXR in different individuals. Since GLUT4 has been reported to be upregulated in human SGBS adipocyte-like cell line, it was surprising to observe that it remained unaltered in our primary cells. When performing a closer examination of the data, it emerged that GLUT4 was slightly downregulated in SVF-derived adipocytes from three of the subjects and slightly upregulated in the remaining three subjects – on average unchanged. This might imply that LXR-regulation of *SLC2A4* varies between individuals and once again points to epigenetic mechanisms in the regulation of LXR-mediated transcription.

To be mentioned, LXRα is more highly expressed than LXRβ in our human adipocyte cell systems (unpublished observation). In addition, LXRα is subject to positive autoregulation whereas LXRβ is not. Thus, when stimulating cells with an LXR agonist, LXRα expression is further increased. This difference in expression levels is important to have in mind since LXRα-specific effects may dominate when stimulating LXR activity.

The main finding of **paper I**, that LXR upregulates lipolysis in adipocytes, is in line with studies in murine models. Increased basal lipolysis is a complication of obesity and the metabolic syndrome and could result in ectopic FA disposition on other organs, impairing their function. This implies that any drug trials investigating LXR as a pharmaceutical target must proceed with awareness of this potential side-effect. In addition, obese subjects have a much greater likelihood than lean to develop insulin resistance, and thus, the patients who would be considered for a possible treatment for type 2 diabetes with LXR-stimulating agents would be obese. Therefore, the finding that LXR downregulates glucose uptake only in adipocytes from obese subjects could be of clinical significance, and is the opposite of the desired outcome of such a treatment.

In **paper III**, we show that MAFB expression is increased in obese adipose tissue, and is enriched in macrophages, and that MAFB mediates inflammation in adipocytes.

MAFB has been previously shown to regulate differentiation of a number of cell types. However, MAFB does not influence adipogenesis in human adipocytes in our study. On the other hand, its expression is strongly induced by adipocyte differentiation, indicating an important function in the mature adipocyte. As CEBPδ has been previously demonstrated to regulate MAFB in keratinocytes, it is possible that CEBPδ is mediating the upregulation of MAFB in adipocytes as well, which may be determined in future studies.

Since *MAFB* expression is highly enriched in macrophages compared to adipocytes isolated from primary adipose tissue, and *MAFB* mRNA correlates strongly with macrophage markers within adipose tissue, we conclude that MAFB is a marker of macrophage abundance. Thus, elevated *MAFB* expression observed in adipose tissue from obese individuals likely come from increased macrophage content. Despite MAFB being downregulated in adipocytes, this downregulation does not likely affect total expression in adipose tissue to any large extent due to the massive MAFB expression originating from macrophages.

The major role of MAFB in adipocytes appears to be as a mediator of inflammation. Its downregulation by TNFα in adipocytes could speculatively be interpreted as a negative feedback process, to stabilise the inflammatory response. Inflammation has a tendency to promote itself, and in order to prevent a vicious cycle of an ever amplified inflammatory response, such a feedback system might be necessary for the organism.

In **paper IV** we show that SIK2 is differentially regulated by obesity in subcutaneous adipocytes and is a positive regulator of glucose uptake. To be noted, *SIK2* expression was not altered by obesity in omental adipocytes from human subjects. This implies that SIK2 is either differentially regulated by weight gain in subcutaneous vs. omental fat, or that SIK2 has different functions in these two depots. This would have to be determined further but a different regulation or role of SIK2 in omental fat is not unlikely considering recent research increasingly identifying differences between different fat depots.

We also show that SIK2 is downregulated by TNFα. TNFα has been shown to suppress glucose uptake by a number of mechanisms discussed above, and our data indicate that inhibition of SIK2 expression might be added to the list. The specific mechanism of this suppression remains to be determined. A reduction in SIK2 protein expression is visible on western blot already after 3 h of TNFα treatment. This implies that SIK2 either has a short half-life and its expression is inhibited by TNFα, or that TNFα is stimulating SIK2 protein degradation.

In our study, phosphorylation of HDAC4 and CRTC2 were diminished upon SIK inhibitor treatment. Although we can hypothesise that diminished phosphorylation of HDAC4 and CRTC2 might be involved in the suppression of glucose uptake, such a connection would have to be further investigated. However, SIK2 and HDAC4 have been previously implicated in the regulation of GLUT4 expression [125, 153] and a similar mechanism may be present in the cell system used in this study. When calculating the ratio of insulin-stimulated expression to basal in vehicle- and SIK inhibitor treated samples, it is unaltered. This implies that the insulin signalling cascade may not be involved in the SIK2-mediated regulation of glucose uptake. However, it does not automatically exclude a role of GLUT4, since a certain

amount of GLUT4 is present in the plasma membrane even in the unstimulated state, and may mediate basal glucose uptake alongside GLUT1.

In unpublished data, *SIK2* mRNA expression correlated positively with *SLC2A4* mRNA. This implies that SIK2 either is associated with a favourable metabolic profile in general, or possibly is a positive regulator of GLUT4 expression. Moreover, *SIK2* mRNA strongly and negatively correlated with adipocyte cell volume from humans with varying BMI. This is in line with recent findings where SIK2 knockout mice displayed increased adipocyte size [125]. A large adipocyte size is much more prominent in obesity and has been connected to dysregulated metabolism. Thus, as concluded based on data in **paper IV**, SIK2 seems to be associated with a healthy adipocyte phenotype.

6 EXTENDED DISCUSSION AND FUTURE PERSPECTIVES

We use clinical cohorts to investigate correlations with clinical parameters and differential expression in obese/diabetic. This can provide clues on factors of importance in adipocyte biology, the development of obesity or its complications, and can often be the initiation point of a new investigation. All three factors studied in this thesis were found to be differentially expressed in obese adipose tissue compared to lean. In addition, having access to data from large clinical cohorts enriches studies as you can investigate the clinical relevance of your *in vitro* findings, place them in a context and strengthen your hypothesis. On the other hand, one must interpret statistical correlations with some degree of scepticism as correlation does not prove causation. In addition, one must be careful to statistically adjust for variables that might influence the association between two factors. In our cohorts, BMI and/or age, depending on the context, might serve as suitable variables to add into a regression analysis.

NRs are compelling drug targets because of their function as binders of ligands, and the diversity of their effects. However, this diversity also poses a risk. Administering an exogenous molecule to an organism may indiscriminately and uncontrollably activate a receptor in all tissues simultaneously. A drug cannot be metabolised and controlled in the same way as the availability and abundance of endogenous ligands, which can be produced locally in certain tissues or be controlled in what way they are allowed to enter specific cell types. Schupp and Lazar [83] conclude that “some of the most effective therapeutic agents available today are derived from endogenous ligands of NRs, with the great example of anti-inflammatory corticosteroids”. However, whereas corticosteroids are indeed highly effective in their suppression of inflammation, they also come with numerous side-effects, and tend to lose their power over time.

LXR is an established regulator of cholesterol metabolism and a transrepressor of inflammatory genes. Therefore, it has been suggested as a pharmaceutical target. The role of LXR in other metabolic processes is somewhat controversial, and the findings of **paper I** and II add to the complexity. There seems to be important inter-species differences in LXR effects regarding glucose uptake, as concluded in **paper II**, where insulin signalling and glucose uptake is inhibited by LXR in human adipocytes. In addition, we show in **paper I** that stimulation of LXR in human adipocytes augments spontaneous lipolysis. Despite its beneficial effects on atherosclerosis and neurodegenerative diseases, activating LXR systemically may thus produce serious side-effects. In fact, the LXR agonist LXR-623 has been evaluated in a clinical phase one drug trial using healthy human subjects. The most common side-effects concerned the central nervous system, which was not the intended target tissue. The study did not report on glucose metabolism or lipolysis, but a brief mention on glucose levels stated that glucose levels did not exhibit abnormalities.

An alternative approach to overcome negative effects of systemic NR activation is to investigate and take advantage of differential expression of NR subtypes, which opens up for tissue-specific drug development [154]. As the negative impacts on basal lipolysis and glucose transport has mainly been attributed to LXRα in **paper I** and **II**, isoform-specific targeting of LXRβ using a selective agonist might be a promising strategy to circumvent adverse metabolic side-effects in the treatment of e.g. neurodegenerative illness. Others suggest that targeting the co-factors of NRs might be an efficient approach [155].

Indeed, an important aspect of LXR biology is the importance of co-factors. The discrepancies in LXR effects emerging when comparing data from different laboratories might depend on the relative expression levels of co-factors in the respective cell systems. In turn, these levels might depend on their origin, minor differences in differentiation protocols or other unknown factors. They might also fluctuate depending on experimental timing in mice, as many genes are known to be under the control of the circadian rhythm.

In an attempt to summarise the physiological effects of LXR activation in the human context –considering that LXR stimulated spontaneous lipolysis and suppresses glucose uptake in adipocytes – one might speculate that stimulation of LXR mimics a situation where glucose and FAs are in demand for oxidation elsewhere, such as muscle. In line with this, it has been reported that LXR stimulates the uptake of both glucose and FA in human muscle cells [156, 157]. In addition, the lipogenic genes *FASN*, *SCD-1* and *ACC1* were unaltered in our microarray profiling analysis, suggesting that lipogenesis was not stimulated by LXR agonist treatment. Our findings are further supported by the observation that LXR activation leads to smaller adipocytes [97] which goes in line with elevated FA release and decreased glucose uptake.

In section 3.2, differences between animal models and human biology are discussed and a number of discrepancies between human and murine adipose tissue function are listed. The effect of LXR in glucose uptake adds to the list. In addition, the decreased SIK2 expression observed after weight loss in human adipocytes in **paper IV**, points to differential regulation between species in obesity as SIK2 has been shown to be *upregulated* in obese mice compared to wild-type [114]. This supports the use of human material in medical research in the initial investigations of a new factor. In the worst case scenario, a factor if importance for human health could in theory be dismissed as irrelevant based on initial findings in animal models. In other cases, huge amounts of research funding could be lost on studies in mice on a factor of little importance in human pathology.

As mentioned earlier, macrophages are a necessary component of adipose tissue. Ablation of FA uptake by macrophages results in an unfavourable phenotype in mice [158]. This is also true for inflammation, which is likely needed for optimal function of the organism. In support of this, mice deficient in the immune-response mediating CD40 receptor are insulin resistant [159]. However, excessive levels of inflammatory factors contribute to a chronic inflammatory state and metabolic disease. In this aspect, MAFB might very well contribute to the unfavourable phenotype of obesity. In support of this, MAFB correlates negatively with insulin sensitivity. However, the role of MAFB in metabolic dysregulation is still relatively unexplored, and needs adipocyte and/or in vivo models where MAFB expression is manipulated. Therefore, it is too soon to draw any conclusions about MAFB as a potential drug target or marker for metabolic disease. Future strategies in MAFB research could include identification of the distinct molecular mechanisms regulating MAFB in adipocytes and macrophages.

SIKs share many characteristics and functions with the metabolic master switch AMPK. As AMPK, SIKs are phosphorylated and regulated by LKB1 and SIK2 is activated by increased AMP levels in 3T3-L1 adipocytes. However, this was not confirmed by other studies [160-162]. Further, SIK substrates overlap with those of AMPK, and SIK2 phosphorylates the same site as AMPK on e.g. IRS [114, 163]. As AMPK, SIK2 appears important for metabolism. Although there is some controversy about the role of AMPK in adipocyte glucose uptake [164], AMPK was shown in one study to stimulate basal glucose uptake [165]. The same conclusion is drawn for SIK2 in **paper IV**. AMPK is activated by metformin, the most widely

used drug in type 2 diabetes treatment. Future research might determine if SIKs are of the same importance to human metabolism as AMPK and a potential drug target. Also, SIK2 regulation by inflammation is intriguing and future studies might elucidate which pathway is mediating SIK2 downregulation by TNFα. Finally, SIK2 is relatively unexplored in adipose tissue and it would be of interest to further determine the mechanism by which SIK2 inhibition suppresses glucose uptake.

To conclude the findings of this thesis, we have shown that LXR, MAFB and SIK2 are regulators of human adipocyte metabolism and inflammation. We have found a deleterious role of LXR in the regulation of adipocyte lipolysis and glucose uptake. We further have identified the importance of MAFB and SIK in inflammation and metabolism, respectively – two proteins previously uninvestigated in human adipocytes. We show that MAFB and SIK2 expression is altered in obesity and by TNFα treatment. In addition, MAFB it is required for mediating some of TNFα's detrimental effects on adipocyte function. At last, we show that SIK2 is a positive regulator of glucose transport in human adipocytes. Together, these findings have contributed to the understanding of adipocyte function and could lead to the generation of interesting hypotheses and new projects.

7 ACKNOWLEDGEMENTS

Thank you Jurga, for being a great supervisor. For generously sharing your time and effort to teach me science, for always keeping your door open, and for encouraging my own contributions and ideas. Not everyone are as lucky with their supervisors!

Thank you Agné and Peter for always being kind and supporting co-supervisors, and for always answering my questions. I have a lot of respect for your skills.

Thank you Ulf, my external mentor, for teaching me scientific skills and ethics, for invaluable support in personal matters, and for calling me “russinet i kakan”.

Thank you Gaby, Eva, Elisabeth and Kerstin for being a great support in the lab and for your kindness. How could the lab ever work so smoothly without you?

Thank you Christel and Daniel for friendship and support beyond colleagueship, and Daniel for all the fun in Visby.

Thank you Britta, Yasmina, Juan, Niklas, Paul and Johan K – my fellow PhD/master students, for many laughs and fun discussions. I will miss sharing a room with you!

Thank you Anna for answering all my questions and for sharing my interest in (sometimes odd) conversation topics.

Thank you Ingrid, Mikael, Hui, Johan Henriksson, Johan Hoffstedt, Patrik, Lena, Clara, Silvia, Jesper, Katharina, Britt-Marie and Yvonne for good times at lab getaways and dinners, and for guidance and help when I needed it.

Thanks everyone in the lab for friendship and good times! I have learned something from everyone and I’m grateful for the helpful and sharing atmosphere that characterises the Lipidlab.

Thank you to collaborators from other labs for your ideas and contributions to the papers of this thesis.

Thank you mr Fox for moral PhD support, scientific inspiration all the way from abroad and all the fun we’ve had and will have. No matter how much I worked, I always knew you worked more.

Thank you fellow PhD students in the Doctoral Students’ Association and the members of the Board of Doctoral Education for letting me take part in your work and listening to my ideas, not to forget all the fun at the getaways.

Thank you to my childhood friends for all the good times in the past and for those to come, and for keeping your feet on the ground.

Thank you my Uplands and Uppsala friends for endless fun and letting me into the crowd where I feel I belong.

Thank you to my entire family who’ve always been ridiculously supportive (I’m not actually Einstein, you know), and simply being the best in all possible ways.

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