REGULATION OF IL-6-SIGNALING AND INFLAMMATORY RESPONSE - ROLE OF INSULIN, FOXO1 AND PKCδ

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The aim of this thesis was to investigate if and how insulin antagonizes interleukin-6 (IL-6)-signaling and action using both 3T3-L1 mouse adipocytes (*Paper I*) and HepG2 human hepatocytes (*Paper III*). We also investigated the importance of PKCδ in 3T3-L1 adipocytes and mouse embryonic fibroblasts (MEFs) lacking protein kinase C-δ (PKCδ-/-) (*Paper II*).

Obesity is associated with a low grade chronic inflammation in the adipose tissue as enlarged adipocytes and macrophage infiltration increase the secretion of inflammatory molecules, such as IL-6, which further enhance the inflammatory response in the adipose tissue and liver.

Insulin was found to exert an anti-inflammatory effect on IL-6-signaling in 3T3-L1 adipocytes by reducing the tyrosine phosphorylation of the transcription factor STAT3, increasing the serine phosphorylation of STAT3 and, furthermore, reducing the nuclear translocation and the transcriptional activity of STAT3. In addition, we found that insulin both induced activation of the phosphatase SHP2, which dephosphorylates STAT3, and synergistically increased gene expression of the suppressor of cytokine signaling (*Socs3)* and thus, impairing IL-6-signaling. These effects also reduced IL-6-induced gene expression of inflammatory genes such as serum amyloid A 3 *(Saa3)* and haptoglobin *(Hp)*. The effect of insulin was mediated through a MEK-mitogen-activated protein kinase (MAPK) pathway since PD98059 (MEK-inhibitor) reduced the inhibitory effects of insulin (*Paper I*).

The anti-inflammatory effect of insulin was also observed in HepG2 hepatocytes. Insulin reduced the IL-6-induced transcription of *SAA1*, *SAA2*, *HP*, plasmin activator inhibitor 1 (*PAI-1)* and *orosomucoid 1 (ORM1)*. However, the signaling mechanism for how insulin exerts its anti-inflammatory effect in HepG2 hepatocytes differed from that of 3T3-L1 adipocytes as insulin also stimulates nuclear exit of forkhead box O1 (FOXO1); a co-activator of STAT3 (*Paper III*).

Furthermore, the tyrosine and serine phosphorylation of STAT3 was found to be dependent on the serine and threonine kinase PKCδ, as the specific PKCδ inhibitor rottlerin reduced these phosphorylations in 3T3-L1 adipocytes. Consequently, the nuclear translocation of STAT3, the IL-6-induced gene transcription of *Socs3*, *Il-6*, *Saa3* and *Hp* as well as the protein secretion of SAA3, were all reduced. Furthermore, PKCδ was found to

translocate to the nucleus following IL-6 and this was reduced by rottlerin. In agreement with the effect of rottlerin, PKCδ-/- MEFs also displayed a markedly reduced ability of IL-6 to activate the gene transcription of *Saa3*, *Hp*, *Socs3* and *Il-6* genes when compared to wild type (wt) MEFs. These results associated with a reduced nuclear translocation and phosphorylations of STAT3 (*Paper II*).

In conclusion, we have found that insulin exerts anti-inflammatory effects by antagonizing IL-6-signaling and action in both 3T3-L1 adipocytes and HepG2 heptocytes. PKCδ was also found to play an important role in STAT3 activation and for IL-6-induced inflammation in 3T3-L1 adipocytes while FOXO1 seems of importance as a co-activator in HepG2 cells. Future studies should be focused on the interplay between PKCδ and FOXO1, which can increase our knowledge of cytokine-induced inflammation and development of new anti-inflammatory treatments.

Keywords: Type 2-Diabetes, inflammation, obesity, IL-6, STAT3, PKCδ, FOXO1

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LIST OF PUBLICATIONS

The thesis is based on the following papers that will be referred to their roman numerals:

I. Andersson CX, Sopasakis VR, **Wallerstedt E**, Smith U. *Insulin antagonizes interleukin-6 signaling and is anti-inflammatory in 3T3-L1 adipocytes*. J Biol Chem. 2007 Mar 30;282(13):9430-5.

II. **Wallerstedt E**, Smith U, Andersson CX. *Protein kinase C-δ is involved in the inflammatory effect of IL-6 in mouse adipose cells*. Diabetologia. 2010 Feb 12. [Epub ahead of print].

III. **Wallerstedt E**, Sandqvist M, Smith U, Andersson CX. *Anti-inflammatory effect of insulin in the human hepatoma cell line HepG2*. Submitted 2010.

LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette transporter 1
AMPK	5´-AMP-activated protein kinase
aP2	Adipocyte protein 2
BAT	Brown adipose tissue
BCA	Bicinchonic acid
BMI	Body mass index
BP	Blood pressure
C/EBP-β	CCAAT/enhancer binding protein-β
C3	Complement factor 3
CNS	Central nervous system
CRP	C-reactive protein
CVD	Cardiovascular disease
DAG	Diacylglycerol
DDT	Dichlorodiphenyltrichloroethane
EGR-1	Early growth response factor-1
eNOS	Endothelial nitric oxide synthases
ES	Embryonic stem cells
FFA	Free fatty acids
FOXO1	Forkhead box O1
FPG	Fasting plasma glucose
G6Pase	Glucose-6-phosphatase
GLUT2, 4	Glucose transporter 2, 4
gp130	Glycoprotein 130
HDL	High density lipoprotein
HP	Haptoglobin
HRP	Horseradish peroxidase
ICV	intracerebroventricular
IGF-1	Insulin-like growth factor-1
IKK	IκB kinase
IL-6	Interleukin-6
IL-6R	IL-6 receptor
IP	Intraperitoneal
IR	Insulin receptor
IRS-1, 2	Insulin receptor substrate-1, 2
IκB	Inhibitor of κB
JAK	Janus kinase
JNK	c-JUN NH_2-terminal kinase
LPL	Lipoprotein lipase
MAPK	Mitogen-activated protein kinase

MCP-1	Monocyte chemotactic protein-1
MEF	Mouse embryonic fibroblast
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF-κB	Nuclear factor κB
NO	Nitric oxide
ORM1	Orosomucoid 1
PAI-1	Plasmin activator inhibitor 1
PDK1, 2	Pyruvate dehydrogenase kinase isozyme 1, 2
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1
PHA	Phytohaemagglutinin
PI3-kinase	Phosphoinositide 3-kinases
PIAS	Protein inhibitor of STAT3
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PIP_3	Phosphatidylinositol 3, 4, 5-trisphosphate
PKB	Protein Kinase B
PKCδ	Protein kinase C-δ
PKCδKN	Kinase-negative PKCδ
PPARα, γ	Peroxisome proliferator-activated receptors α, γ
PTB1B	Protein tyrosine phosphatase 1B
ROS	Reactive oxygen species
SAA	Serum amyloid
SDS-PAGE	Sodium dodecyl sulphate polyacrylmide gel electrophoresis
SH-2	Scr homology-2
SHP2	SH-2-domain containing tyrosine phosphatase
sICAM-1	Soluble intercellular adhesion molecule-1
SOCS3	Suppressor of cytokine signaling
SREBP-1c	Sterol regulatory element binding protein-1c
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
T2D	Type 2 diabetes
TG	Triglyceride
TLR2, 4	Toll like receptor 2, 4
TNFα	Tumor necrosis alpha
TTP	Tristetraprolin
UCP-1	Uncoupling protein-1
VLDL-TG	Very low-density lipoprotein- triglyceride
vSMC	Vascular smooth muscle cells
WAT	White adipose tissue
WT	Wild type

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INTRODUCTION

Type 2 diabetes (T2D) and insulin resistance

The prevalence for diabetes is increasing throughout the world and it is expected to rise from 171 million cases in 2000 to 366 million in 2030. The greatest increase will be seen in developing countries. The main reasons are the increased incidence of obesity and a sedentary lifestyle with physical inactivity (1).

Insulin resistance is a common underlying hallmark associated with obesity and T2D and plays an important pathophysiological role in both conditions (2). Insulin resistance is characterized by a decreased ability of insulin to act on peripheral tissues such as adipose tissue, skeletal muscle and liver leading to elevated glucose levels and hyperinsulinemia due to the reduced glucose uptake by insulin-sensitive cells and increased hepatic glucose production (2). T2D is a complex disorder caused by both environmental and genetic factors and where the development of the disease is a multistep process that is initiated by the impaired insulin sensitivity in the peripheral tissues. As a consequence, the β-cells in the pancreas produce more insulin to overcome the tendency for elevated glucose levels in the blood, leading to β-cell dysfunction with impaired insulin secretion and increased hepatic glucose production (3).

Insulin resistance in the adipose tissue is important for T2D development as the larger and expanded tissue mass results in increased release of free fatty acids (FFA) from the stored triglycerides in the adipocytes (lipolysis) (4, 5). Under normal conditions insulin suppresses adipose tissue lipolysis and stimulates blood TG uptake thorough lipoprotein lipase (LPL). This is impaired by the insulin resistance leading to increased FFA in the circulation that further promote the systemic insulin resistance and, as a consequence, higher levels of FFA are observed in the circulation (6). Furthermore, increased FFA levels in blood have also been shown to impair hepatic insulin action leading to insulin resistance in the liver. This is due to increased hepatic glucose production together with increased triglyceride synthesis and storage concomitant with secretion of excess triglycerides from the liver by very low-density lipoprotein-triglyceride (VLDL-TG) particles (7). In muscle, increased FFA in the circulation leads to increased FFA influx with TG deposition that negatively affects expression of genes that, for example, are involved in the mitochondrial function (8). Increased FFA levels contribute to insulin resistance by inhibiting insulin-stimulated glucose uptake and reducing glycogen synthesis (9).

The Metabolic Syndrome (MetS) includes several risk factors associated with cardiovascular disease (CVD) and T2D such as obesity and increased plasma levels of lipids and glucose. In 2006, the International

Diabetes Federation defined the Metabolic Syndrome as central obesity together with two of the following factors: raised TG levels, reduced high density lipoprotein (HDL)-cholesterol, raised blood pressure or raised fasting plasma glucose (10) (Table 1).

Table 1. International Diabetes Federation´s (IDF) definition of the Metabolic Syndrome (2006)

Central obesity (defined by waist circumference ≥ 94 cm for Europid men and ≥ 80 cm for Europid women, with ethnicity specific values for other groups) Plus any two of the following factors below:
1. Raised triglyceride (TG) level: ≥ 1.7 mmol/l (150 mg/dl), or specific treatment for this lipid abnormality
2. Reduced HDL-cholesterol < 1.03 mmol/l (40 mg/dl) in males and < 1.29 mmol/l (50 mg/dl) in females, or specific treatment for this lipid abnormality
3. Raised blood pressure (BP): systolic: ≥ 130 mmHg or diastolic: ≥ 85 mmHg, or treatment of previously diagnosed hypertension
4. Raised fasting plasma glucose (FPG) ≥ 5.6 mmol/l (100 mg/dl), or previously diagnosed Type 2 diabetes

Function of insulin in adipocytes

Insulin is a pleiotrophic hormone that exerts several critical control steps on anabolic processes in fat by stimulating glucose and FFA uptake, inhibiting lipolysis and stimulating *de novo* fatty acid synthesis in adipocytes. Insulin is also important for regulation of adipose tissue growth and differentiation by inducing gene expression of different adipokines and fat-specific transcription factors such as the transcription factor sterol regulatory element binding protein-1c (SREBP-1c) and peroxisome proliferator-activated receptors γ (PPARγ) (11, 12).

Insulin signaling is initiated by binding of insulin to the receptor α-subunits of the insulin receptor (IR). IR is a transmembrane receptor with intrinsic tyrosine kinase activity (13). Interaction between insulin and IR leads to autophosphorylation of IR within the receptor β-subunits and recruitment of intracellular docking proteins (14), including insulin receptor substrate-1 and insulin receptor substrate-2 (IRS-1 and IRS-2) (15). Furthermore, activated IR phosphorylates IRS molecules on multiple tyrosine residues (16). Tyrosine phosphorylated IRS molecules associate with Src homology-2 (SH-2) containing proteins like the p85 regulatory subunit of phosphoinositide 3-

kinases (PI3-kinase). This, in turn, catalyses the phosphorylation of phosphatidylinositol 4, 5-bisphosphate (PIP_2) to phosphatidylinositol 3, 4, 5-trisphosphate (PIP_3). Protein Kinase B (PKB), also known as Akt, interacts with PIP_3 and this interaction enables pyruvate dehydrogenase kinase isozyme 1 (PDK1) to associate and activate PKB by phosphorylating Thr-308. PKB is also phosphorylated on Ser-473 by a putative pyruvate dehydrogenase kinase isozyme 2 (PDK2). This kinase is still unidentified but more than 10 kinases have been proposed to function as potential PDK2s (17, 18). Moreover, AS160, a protein that is phosphorylated at several sites by insulin-activated PKB has been shown to regulate the GLUT4 translocation in adipocytes (19).

Activation of insulin signaling by the mechanisms mentioned above also leads to activation of glucose transport. In the adipose tissue and skeletal muscle, the insulin-sensitive glucose transporters 4 (GLUT4) are synthesized and, following insulin stimulation, they are translocated from intra-cellular vesicles to the plasma membrane where they mediate glucose uptake (20, 21) (Fig.1).

The glucose transporter protein responsible for the increased glucose disposal by the liver is glucose transporter protein 2 (GLUT2) (21, 22). The muscle tissue is important for glucose disposal as it, when measured during the euglycemic hyperinsulinemic clamp, accounts for 60-70% of the glucose uptake whereas the liver accounts for approximately 30% and the adipose tissue for approximately 10 % (23).

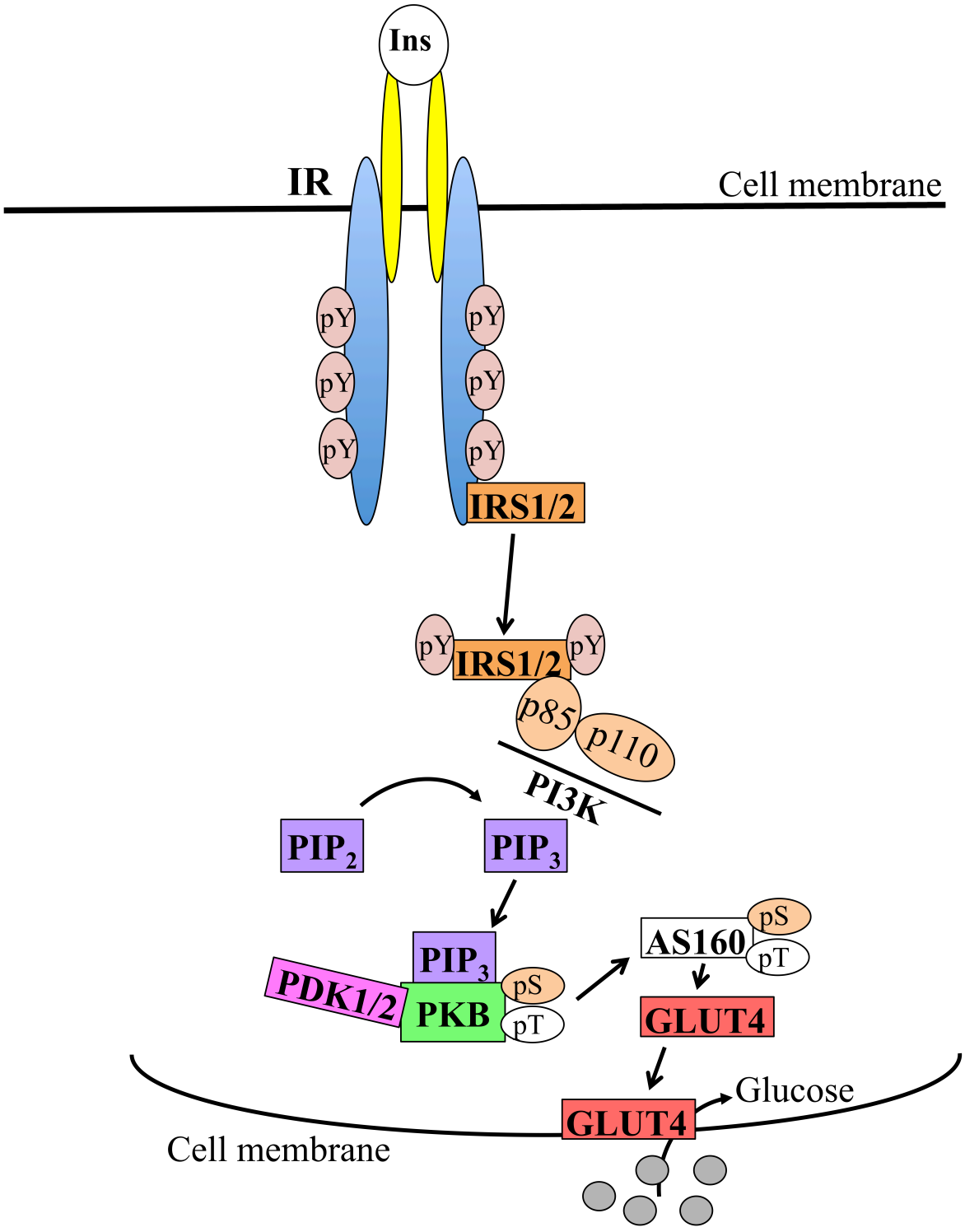

Fig.1. An schematic overview of the insulin signaling pathway. Binding of insulin leads to activation of down-stream molecules with increased glucose uptake into the cell.

Adipose tissue as an endocrine organ

In mammals, the adipose tissue consists mainly of white adipose tissue (WAT) and the adipocytes in the WAT depot store surplus energy (24). Increased storage of excess energy as fat results in progressive development of obesity that is characterized by an increased adipocyte cell size and/or an expanded number of adipocytes. Obesity is accompanied by corresponding risk factors such as impaired insulin sensitivity and hyperglycemia that further can progress to the development of diabetes as well as cardiovascular disease (25).

The adipose tissue is not only a reservoir for fat storage as energy, it is also a highly active metabolic and endocrine organ consisting of preadipocytes, adipocytes, immune cells, endothelial cells, nerve tissue and connective tissue matrix (26). It is also a source of multipotent stem cells that are able under the right conditions to undergo differentiation into mature adipocytes (27).

The role of the adipose tissue as an endocrine organ was first started in 1994 with the discovery of leptin (28). Today it is well documented that the adipose tissue produces multiple bioactive molecules, called adipokines, that not only have paracrine and autocrine functions, but also serve other metabolic effects by secreting these adipokines into the circulation (29). Several adipokines have been identified as secreted peptides from the adipose tissue organ e.g. leptin, adiponectin, resistin, visfatin and proinflammatory cytokines such as monocyte chemotactic protein-1 (MCP-1), tumor necrosis alpha (TNFα) and IL-6 (30). This introduction will focus on the adipokines important for this thesis.

TNFα, a pro-inflammatory cytokine, is secreted by non-fat cells in the adipose tissue and infiltrated macrophages seem to be responsible for the increased TNFα expression in the adipose tissue of obese subjects (31, 32). TNFα has been shown to have several negative effects in the adipose tissue. It is known to inhibit lipogenesis through stimulation of lipolysis and decreased FFA uptake, impair insulin signaling through induction of serine phosphorylation of IRS-1, inhibit preadipocyte differentiation by suppressing expression of adipocyte-specific genes and stimulate an inflammatory response (31). TNFα has been shown to act via the nuclear factor κB (NF-κB) signaling pathway and, for example, stimulate adipocyte IL-6 production in adipocytes (33, 34).

IL-6 secretion from the adipose tissue is increased in human obesity and insulin resistance and the high levels of IL-6 in the circulation is a predictor of T2D (35, 36). One of its main functions is to regulate the hepatic production of inflammatory molecules such as C-reactive protein (CRP) and a positive relationship has been reported between IL-6 levels in the adipose tissue and circulating CRP levels (37).

IL-6-induced hepatic *SAA* expression is modest but it is synergized by IL-1 and TNFα (38). IL-6-induced secretion of PAI-1 and HP has also been observed in HepG2 cells (39, 40). In contrast, IL-6 negatively affects secretion of adiponectin in adipocytes, a hormone with insulin sensitizing effects (41) and also inhibits LPL activity. LPL plays an important role for the regulation of hydrolysis of triglycerides in the circulation and, thereby, stimulates the accumulation of fatty acids in the adipocytes (42).

The adipose tissue has been shown to contribute approximately 30% of the IL-6 levels in the circulation where the secretion from omental depots is 3-fold higher compared to subcutaneous adipose tissue. In the same study, IL-6 secretion from the adipocytes in the omental adipose tissue was higher compared to adipocytes in the subcutaneous depots (43, 44). As obesity is associated with a low chronic inflammation in the adipose tissue (45) and only 10% of the total IL-6 levels in the adipose tissue are derived from adipocytes (44), other cells in the adipose tissue seem to be important for the high secretion of IL-6 levels into the circulation. The stromal vascular cells probably play a key role for this, as obesity is associated with increased macrophage infiltration (46), recruited by MCP-1 secretion from the adipocytes (47).

PAI-1 is another adipokine that is prominently expressed in the liver, endothelial cells and in the adipose tissue. The main function of PAI-1 is to inhibit the activity of tissue plasminogen activator, a molecule that is involved in fibrinolysis; the physiological breakdown of blood clots. TNFα is chronically elevated in the adipose tissue of obese subjects and it has been shown that TNFα increased the gene expression of *Pai-1* in 3T3-L1 mouse adipocytes (48) and also that the plasma levels of PAI-1 are strongly correlated with body mass index (BMI) and visceral adipose tissue mass (49). Another study showed that the cells in the stromal vascular fraction are responsible for the increased plasma levels of PAI-1 to greater extent than the adipocytes in the adipose tissue (50). Increased plasma levels of PAI-1 are also an early inflammatory marker (51) and probably contributes to the development of cardiovascular disease due to the reduced activity of the fibrinolytic system (52).

SAA is an acute-phase protein that is mainly secreted from the liver (53) but also from adipocytes (54). *SAA1* and *SAA2* are expressed in humans whereas *SAA3* is a pseudogene in man (55). SAA is also involved in the transport of cholesterol by linking HDL-cholesterol to macrophages and, thereby, reduces the cardiovascular protective effect of HDL (56). Obesity has been shown to be associated with increased serum levels of both SAA1 and SAA2 (54). *Saa3* is highly up-regulated in differentiated 3T3-L1 mouse adipocytes when stimulated with pro-inflammatory molecules, such as IL-6 (57). In an obese mouse model of hyperglycemia and hyperinsulinemia, *Saa3* is drastically increased at the transcriptional level in the adipocytes compared to

wt mice. *Saa3* expression was relatively low in the liver in these mice as well as in wt mice compared to the adipose tissue (58). It has also been demonstrated that both macrophages and macrophage-derived "foam cells" express *SAA* (59, 60).

HP is an acute-phase protein mainly produced by the liver. It is a marker of inflammation when the plasma levels are increased (61). It binds free hemoglobin that is released during haemolysis in the plasma and, thereby, inhibits its oxidative activity (61). *HP* is also expressed in the adipose tissue and increased serum levels of HP are associated with obesity in humans (62, 63). Obesity-induced *Hp* expression in adipose tissue seems to be regulated by TNFα as obese mice deficient for TNFα or TNFα receptors did not display an increased *Hp* expression (64). TNFα has also been shown to be a more potent inducer of *Hp* expression in 3T3-L1 mouse adipocytes compared to IL-6 (62).

Adipose tissue inflammation

The link between obesity, insulin resistance and low chronic inflammation was proposed in 1993 (45). A deeper understanding of the increased secretion of pro-inflammatory molecules from the adipose tissue seen in obesity was the detection of macrophage infiltration in the adipose tissue of obese mice (46). The numbers of cells such as macrophages and monocytes can be as high as 40% of total cell number in the adipose tissue (65). There is also a strong relationship between macrophage infiltration and adiposity that may account for the increased secretion of pro-inflammatory molecules observed in obesity (46). A positive correlation in humans between BMI and adipose tissue macrophages in visceral adipose tissue has also been shown (66). Recently, Nishimura et al. showed that obese adipose tissue activates $CD8^+$ T cells, which, in turn, secrete factors that promote the recruitment and activation of macrophages in this tissue (67).

Obesity is associated with enlarged fat cells that further promote the formation of necrosis-like adipocytes cell death that probably depends on adipocyte hypertrophy. Ninety % of all macrophages in the WAT from obese mice and humans were localized to dead adipocytes (68). The mechanism for the initiation of the inflammatory response in obesity has recently been suggested (Fig. 2). Obesity renders enlargement of the adipocytes, which enhances secretion of FFA, IL-6 and MCP-1. The secretion of MCP-1 recruits macrophages into the adipose tissue (47). Furthermore, the released FFA by enlarged adipocytes promotes activation of infiltrated macrophages through binding of FFA to toll like receptor 4 (TLR4) on macrophages leading to activation of the NFκB pathway with further increased inflammatory response and, consequently impaired insulin signaling and insulin resistance (69). This process also promotes increased IL-6 levels in the circulation in obese subjects

(36). Moreover, plasma levels of TNFα are positively correlated with BMI, insulin resistance and T2D (70, 71). In addition, complement factor 3 (C3) is highly expressed in adipocytes and has been shown to be an activator of macrophages and thus, promote the inflammatory response in the adipose tissue (46, 72).

Preadipocytes display phagocytic activity and following appropriate stimuli (such as TNFα), they can transdifferentiate into macrophage-like cells, secrete pro-inflammatory molecules typical for macrophages but not for adipocytes and, thereby, contribute to the increased inflammatory response (73, 74). Furthermore, FFA can induce serine phosphorylation of IRS-1 through PKCθ activation in adipocytes with concomitantly reduced insulin signaling cascade (75). TNFα, due to its ability to induce serine phosphorylation of IRS-1 (76) and reduced *Irs-1* expression in response to IL-6 are, thus, potent mediators for impairing the further downstream insulin signaling (33).

In addition, the pro-inflammatory milieu in the adipose tissue impairs the recruitment of preadipocytes to differentiate into mature adipocytes, forcing further uptake of fat in to the existing adipocytes, causing the enlargement observed in obesity (77).

Obesity is associated with increased lipolysis of the enlarged adipocytes in the adipose tissue (4, 5). The enhancement of adipocyte hypertrophy due to impaired adipocyte differentiation also promotes necrosis-like adipocyte cell-death with release of cell debris and FFA that further recruit and activate macrophages into the adipose tissue and, consequently, stimulation of the inflammatory response (68). In addition, the presence of TNFα and IL-6 in the adipose tissue leads to maintained Wnt-signal in the preadipocytes. It is crucial to switch off the Wnt-signal for initiating the differentiation preadipocytes into mature adipocytes with lipid accumulation (78, 79). These findings further support that the increased levels of TNFα and IL-6 in obesity contribute to impaired differentiation of preadipocytes and an inflammatory response.

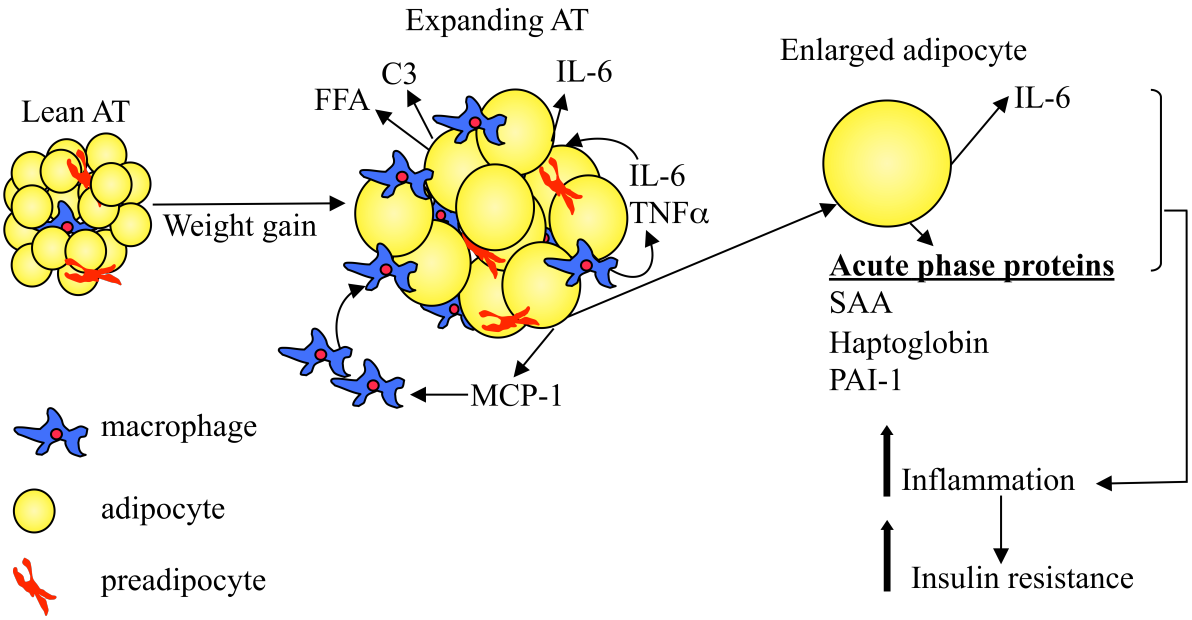

Fig.2. A mechanism for initiating the inflammatory response in the adipose tissue associated with weight gain and recruitment of macrophages into the adipose tissue.

Liver steatosis

The increased secretion of inflammatory mediators and increased FFA release from the adipose tissue in obesity affect the function of the liver by impairing the hepatic insulin action and leading to excessive lipid storage (80). It has been proposed that 95% of obese individuals and up to 70% of T2D patients have non-alcoholic fatty liver disease (NAFLD). Development of NAFLD is a progressive process, caused by the increased circulating levels of FFA and insulin resistance in the peripheral tissue (81).

Plasma levels of the insulin-sensitizing hormone adiponectin are reduced in individuals with NAFLD (82) as well as peroxisome proliferator-activated receptor α (PPARα) (83). The development of the more serious form non-alcoholic steatohepatitis (NASH) is a two-step-process that first involves lipid accumulation in the liver (steatosis) and the second process involves hepatocellular injury and inflammation (84, 85), which can lead to severe cirrhosis (86). The molecular mechanisms leading from steatosis to NASH are unclear but it has been demonstrated that liver resident cells (Kupffer cells) and recruited macrophages can initiate the inflammatory process in the liver (87).

IL-6-signaling

The cytokine IL-6 is a glycosylated polypeptide consisting of 184 amino acids with a molecular weight of approximately 21-28 kDa (88). It is produced by a variety of cells in different tissues, including adipose tissue, liver and skeletal muscle (89-91). IL-6 is a circulating, multifunctional cytokine that is involved in different cellular processes and due to its action in different cell types, it has both pro-inflammatory (in adipose tissue and liver) (56, 92) and anti-inflammatory properties (in the skeletal muscle) (93). IL-6-signaling is initiated by binding of IL-6 to its membrane-bound receptor, the IL-6 receptor (IL-6R) (Fig. 3). Binding of IL-6 to the IL-6R, consisting of one transmembrane domain, leads to homodimerization of the signal transducing receptor subunits glycoprotein 130 (gp130) following recruitment to the IL-6R-complex. Furthermore, interaction of the IL-6R-complex with gp130 leads to activation of the gp130-associated tyrosine kinase, from the Janus Kinas (JAK) family allowing phosphorylation of gp130 on several tyrosine residues in the cytoplasmic region. The phosphorylated tyrosine residues on the gp130 tail serves as docking sites for recruited STAT molecules, mainly STAT3 and STAT1 through their SH2-domains (88). In this thesis, STAT3 is mainly studied and described. Gp130-STAT3 interaction leads to activation of STAT3 through induction of Tyr-705 phosphorylation that is necessary for the homo- or heterodimerization to another tyrosine phosphorylated STAT3 or STAT1 molecule (92, 94). The homo- or hetero dimerization of STAT3 enables nuclear translocation where it directly or indirectly regulates the gene induction of specific genes (92, 94) such as *SAA*, *SOCS3* and *Hp*. STAT3 is also serine phosphorylated at serine residue 727 and a variety of serine/threonine kinases, such as PKCδ, ERK, JNK, p38 MAPK and mammalian target of rapamycin (mTOR) (95-102) have been reported to be involved in this phosphorylation. Depending on different cell types and stimuli, contradictory results have been reported regarding the importance of this phosphorylation. It has been shown to either increase (95, 98, 101, 102), decrease or have no effect on the transcriptional activation (96, 97, 99).

The IL-6-signaling pathway can be regulated in several ways. Tyrosine phosphorylation at position 759 on gp130 serves as docking site for the SH-2-domain containing tyrosine phosphatase (SHP2) (103, 104). Binding of SHP2 at position 759 on gp130 attenuates further down-stream signaling of IL-6 (104) probably through dephosphorylation of JAK (105). As a phosphatase, SHP2 also negatively regulates the JAK/STAT pathway by dephosphorylating STAT and gp130 (92). Further on, members of suppressors of cytokine signaling proteins (SOCS1-SOCS7) are negative feedback inhibitors of the IL-6-mediated signal. Primarily SOCS3 and SOCS1 are the most common in this family to affect IL-6-signaling. They affect the IL-6-mediated signaling negatively by interacting with their SH2 domain on tyrosine residue

759 (the same site as for SHP2) in the cytoplasmic tail of gp130 (104). SOCS3 has also been shown to interact with JAKs (106).

Another molecule affecting the signal transduction of IL-6 in a negative manner is protein inhibitor of STAT3 (PIAS) (107). It belongs to a family of proteins that exert small ubiquitin-like modifier (SUMO)-ligase activity (108). It has been shown that PIAS3 through a short amino acid sequence (82-132), is able to bind STAT3 and negatively regulate IL-6-mediated signaling by blocking the DNA-binding capacity of STAT3 and, thereby, inhibits gene transcription activity (107, 109).

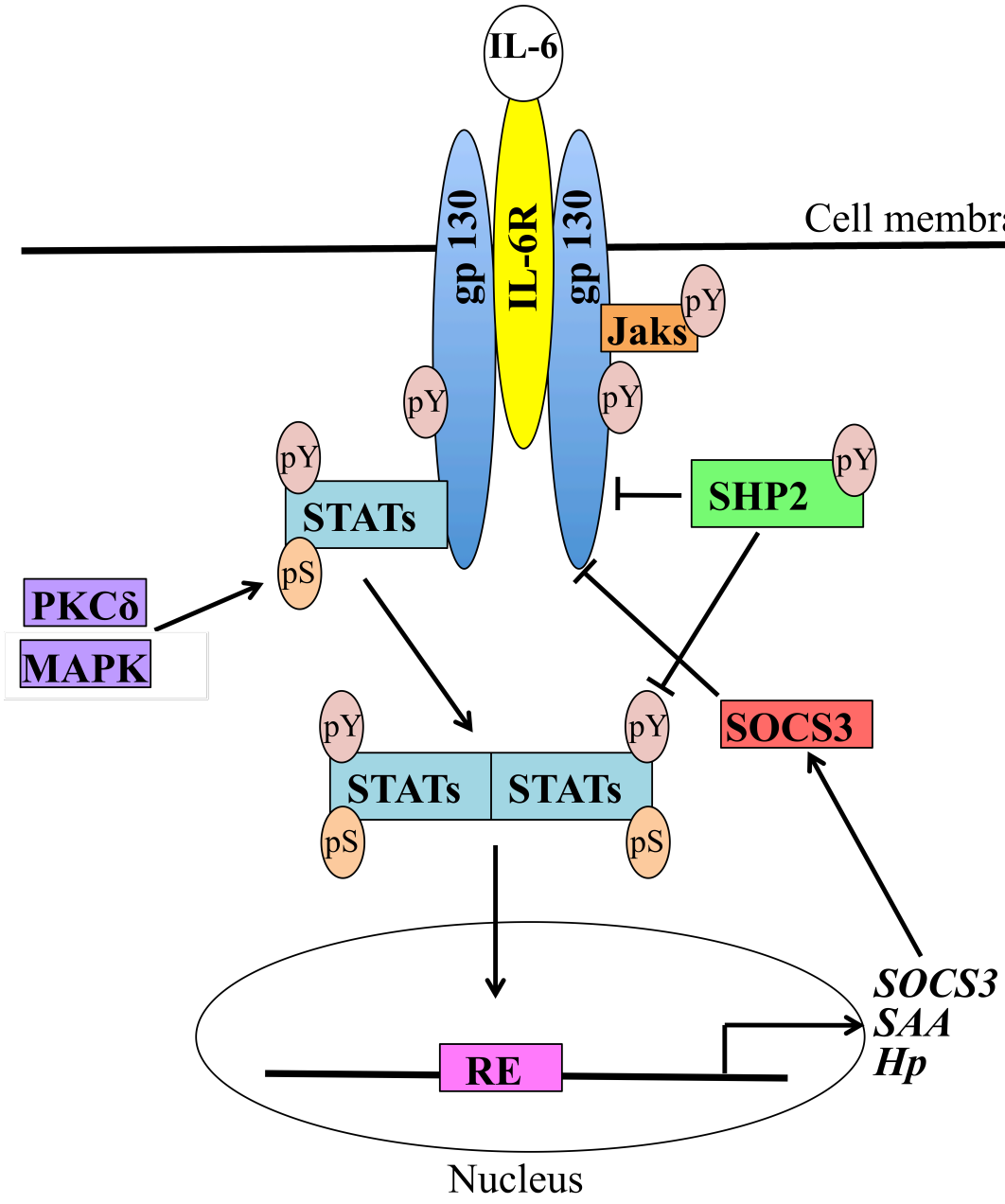

Fig.3. An schematic overview of the IL-6 signaling pathway.

Cross-talk between IL-6-signaling and insulin signaling

Activation of IL-6-signaling negatively affects the insulin signaling and action by reducing *Irs-1* and *Glut4* expression and also inhibiting tyrosine phosphorylation of IRS-1 protein (33). Induction of JAK/STAT-signaling leads to increased *Socs3* expression and SOCS1 and SOCS3 can bind to IR and further inhibit tyrosine phosphorylation of IRS and, thereby, impair the downstream insulin signaling (110, 111).

Moreover, Senn et al. have demonstrated that IL-6 negatively affects insulin signaling in hepatocytes through reduced tyrosine phosphorylation of IRS-1, decreased association of PI3K with IRS-1 and also inhibition of the PKB activity. The inhibitory effect on the insulin signaling pathway in response to IL-6 is suggested to depend on up-regulation of SOCS3 expression (112).

In addition, over-expression of IL-6 in mice skeletal muscle leads to hyperinsulinemia, impaired insulin-stimulated glucose uptake by the skeletal muscles, increased levels of serum SAA and infiltration of inflammatory cells in the liver (113).

In mononuclear cells from obese subjects, Dandona and coworkers have shown that insulin has anti-inflammatory properties by reducing intranuclear NFκB content and reactive oxygen species (ROS) generation. Consequently, this was associated with reduced plasma levels of inflammatory molecules and suppressed levels of the transcription factor early growth response factor 1 (EGR-1), which regulates the expression of *PAI-1* and, thus, suggesting that insulin also has en anti-atherotrombotic effect (114, 115).

Moreover, increased local adipose tissue inflammation is associated with endothelial dysfunction in obese subjects (116). IL-6 has also been shown to negatively affect the insulin signaling pathway in endothelial cells and thus, impair the vasodilating action of insulin. This was associated with increased serine phosphorylation of IRS-1 and consequently further impaired down-stream insulin signaling with decreased endothelial nitric oxide synthases (eNOS) activity and nitric oxide (NO)-production (117).

IL-6 levels in insulin resistant subjects

Obesity, insulin resistance and T2D are all associated with elevated levels of IL-6 in the circulation (118-120). There is also a correlation between circulating levels of CRP, SAA and the pro-inflammatory cytokines IL-6 and TNFα in overweight subjects (54, 119). The increased levels of IL-6 in the circulation observed in patients with the metabolic syndrome seem to originate to a large extent from omental adipose and visceral fat (121). It has been suggested that about 30% of the total circulating IL-6 originates from the adipose tissue (44). Elevated circulating IL-6 levels have been described to predict the

development of T2D in women (122). Moreover, reduction of body weight is correlated with reduction of pro-inflammatory cytokines such as IL-6 and TNFα (118).

The C174G polymorphism of the IL-6 gene is reported to be associated with insulin resistance and T2D. However, there are conflicting reports claiming different effects of the polymorphism. Several studies have shown that the IL-6-174C variant is associated with body fat mass (123) whereas other reports have demonstrated that this variant is associated with reduced levels of IL-6 in the circulation (124) and increased insulin sensitivity (125) in healthy Caucasian subjects. In addition, the IL-6-174G variant has been shown to be associated with insulin resistance in Caucasians (126). The contradictory results regarding the two distinct IL-6 polymorphisms are probably dependent on different genetic and/or ethnic predispositions to develop insulin resistance and T2D (127). Moreover, there is also evidence that the IL-6-174C variant is more prevalent in NAFLD patients compared to healthy subjects and, thus, associated with increased fasting insulin and homeostasis model of insulin resistance (HOMA-IR). IL-6-174C variant is an independent predictor of both NAFLD and NASH (127).

PKCδ

PKCδ was cloned from a rat cDNA library in 1987 (128) and belongs to the novel PKCs, characterized by diacylglycerol (DAG) activation, within the protein kinas family of serine-threonine kinases. All PKC isoforms consist of an N-terminal regulatory domain and a C-terminal catalytic domain. There are a variety of studies reported regarding the different functions of PKCδ, which seems to be dependent of cell type and specific stimuli. For example, phorbol esters have been shown to have both pro-and anti-apoptotic effects (129). Mice that lack PKCδ develop autoimmune diseases and display increased proliferation of B cells, which supports the concept that PKCδ negatively regulates the B-cell proliferation (130).

Moreover, PKCδ has been shown to play an important role in obesity and insulin-resistant states. A study in PKCδ deficient mice, fed a high fat diet, showed reduced levels of triacylglycerol in the liver due to reduced production of proteins involved in the lipid synthesis. They also displayed improved glucose tolerance as a consequence of increased insulin sensitivity compared to wt mice (131). In another study two different mouse strains were compared. One of them was more prone to develop obesity, becoming insulin resistant and glucose intolerant when fed a high fat diet. The *Pkcδ* expression in these mice was up-regulated in skeletal muscle, liver and fat (132).

Increased PKCδ activity in response to unsaturated fatty acids is associated with ATP-binding cassette transporter 1 (ABCA1) degradation. ABCA1 is a key protein involved in the HDL formation by mediating

transport of cholesterol and phospholipids to lipid-deficient HDL apolipoproteins (133), suggesting that PKCδ inhibits the removal of cholesterol and promotes development of atherosclerosis.

AIM

The overall aim with this thesis is to study the cellular and molecular mechanisms in differentiated 3T3-L1 mouse adipocytes and in HepG2 hepatocytes, with the main focus on the regulation of IL-6-signaling and IL-6-induced expression of inflammatory genes in response to insulin and the role of PKCδ.

The specific aims are:
Paper I: Examine the effect of insulin on IL-6-signaling in differentiated mouse 3T3-L1 adipocytes

Paper II: Investigate the role of PKCδ on STAT3 phosphorylation and activation of proinflammatory genes in response to IL-6 in differentiated 3T3-L1 adipocytes

Paper III: To examine the effect of insulin on IL-6-signaling in the human hepatoma cell line, HepG2

EXPERIMENTAL PROCEDURES

Cell culture

3T3-L1

In the mid of 1970s, Green and Kehinde established different sublines from the mouse fibroblast line 3T3 that were able to differentiate *in vitro* into mature adipocytes with lipid accumulation as droplets in the cytoplasm (134, 135). 3T3-L1 cells are today used as a standard model for *in vitro* studies of adipocyte differentiation and obesity (136). 3T3-L1 preadipocytes were grown until 90% confluent before addition of the differentiation cocktail and differentiated into mature adipocytes as described in *Paper I*.

Human hepatoma cell line, HepG2

The cell line was derived from liver tumour biopsies from a 15-year-old Causcasian American male from Argentina in 1975 (137). The HepG2 resembles liver parenchymal cells with the same morphological characteristics and epithelial cell shape and secretes many plasma proteins e.g. albumin, α-fetoproteins (AFP) and acute phase proteins (137, 138). Due to their specific properties they are commonly used for *in vitro* studies. They were grown and cultured as described in *Paper III*.

SHP2-/- MEFs and wt MEFs

Saxton et al. generated SHP2 deficient mice by insertion of a mutated SHP2 gene. Exon 3, encoding amino acids 46-110 was deleted in embryonic stem cells (ES) and replaced by a neomycin resistant cassette (139). The SHP-/- MEFs and SHP2+/+ MEFs used in this thesis were a kind gift from Dr. Reiner Lammers lab, Tubingen, Germany. MEFs were derived from embryonic mice and cultured for several passages until they reached immortalized condition. SHP2-/- MEFs, SHP2+/+ MEFs and wt MEFs were grown and cultured as described in *Paper I*.

PKCδ-/- MEFs and wt MEFs

Miyamoto et al. generated PKCδ-/- deficient mice by disrupting the PKCδ gene in ES by inserting a neomycin resistant cassette on the position for the first and second exon in the N-terminal SHP2 domain (130). The PKCδ-/- MEFs used in this thesis was a kind gift from M. E. Reyland, University of Colorado Health Sciences Center, Aurora, CO, USA (140). MEFs were derived from embryonic mice and cultured for several passages until they reached

immortalized condition. PKCδ-/- MEFs and wt MEFs were grown and cultured as described in *Paper II*.

Protein extraction

Whole cell extract

Cells were washed in phosphate-buffered saline and lysates were prepared as described in *Paper I*. The lysate was centrifuged and kept on ice followed by determination of the protein concentration that was quantified by the bicinchonic acid kit (BCA) (Pierce, Rockford, IL).

Subcellular fractionation

The nuclear and cytoplasmic fractions were prepared as suggested by the manufacturer of the STAT3 transcription factor assay kit (Active Motif, Carlsbad, CA) or (Chemicon International, Temecula, CA) (*Paper I, II, III*). The protein concentration was quantified by the BCA (Pierce, Rockford, IL) or Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Immunoblotting

Protein analyses were performed by the Western Blot technique as described in *Paper I*. The proteins were separated on SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylmide Gel Electrophoresis) (Lonza, Rockland, IL). Same amount of protein was boiled in sample buffer containing the detergents dichlorodiphenyltrichloroethane (DDT) and SDS to denaturate proteins molecules before it was applied on the polyacrylamide gel.

Following gel electrophoresis wet-blotting was performed at 80V for approximately 2 hr. The proteins in the polyacrylamide gel were transferred to nitrocellulose membrane, blocked in 5% non-fat dry milk and probed with primary antibodies to the proteins of interest followed by the corresponding secondary antibody linked to horseradish peroxidase (HRP) (Cell Signaling Technologies, Beverly, MA). Detection was made by Immun-STAR™ HRP chemiluminescence kit using ChemiDoc XRS detection system (Bio-Rad Laboratories, Hercules CA) or by enhancing chemiluminescence kit (Amersham Biosciences) using light sensitive films.

STAT3 Transcription assay

Nuclear extracts were prepared and analyzed on a 96-well plate coated with oligonucleotides corresponding to the STAT3 consensus binding site (*Paper I, II*). STAT3 primary antibody was added to the plate followed by HRP-

conjugated secondary antibody. The absorbance was read on a spectrophotometer. Notably, this kit measures nuclear STAT3 binding to its consensus binding site reflecting potential transcription activity of STAT3. The protocol was essentially as suggested by the manufacturer of the STAT3 transcription factor assay (Active Motif, Carlsbad, CA) or (Chemicon International, Temecula, CA).

RNA extraction and Reverse Transcriptase PCR

Cells were washed in RNAse-free PBS and mRNA was extracted using the RNeasy Mini Protocol (Qiagen, Valencia, CA). cDNA was synthesized from 0.4 μg total RNA using the High Capacity cDNA Reverse Transcriptase kit according to the supplier's protocol (Applied Biosystems, Foster City, CA) (*Paper I, II, III*).

Real-time PCR

Real-time PCR was performed by using the ABI Prism 7900 HT Sequencing Detection System (Applied Biosystems, Foster City, CA). The PCR reaction uses the 5´ nuclease activity of the Taq DNA polymerase to cleave the probe bound to its amplicon target. The probe contains a fluorescent reporter dye at the 5´ end and a quencher dye at the 3´ end and in the intact state the fluorescent signal from the reporter is eliminated by the quencher. After the reverse transcription, the temperature is increased leading to denaturation of the double-stranded cDNA followed by annealing of primers and probe at a lower temperature. Polymerisation occurs and when the polymerase reaches the labeled probe, it is cleaved and the reporter dye is separated from the quencher dye, which makes it possible for detection of the fluorescent signal. The fluorescent signal is directly proportional to the number of molecules present at the end of the previous or the beginning of the current cycle. Amplification of the PCR-products is exponential and the step first recorded statistically significant, the fluorescent signal above background is called C_t and it occurs always during the exponential phase of amplification (141). The reporter signal from the gene of interest is normalized to an endogenous internal control gene, 18S (the small subunit of ribosomal RNA (rRNA)), present in each sample (*Paper I, II, III*).

Statistics

Statistical analyses were performed in SPSS or Microsoft Excel with paired Wilcoxon´s test, unpaired Mann Whitney´s test or paired and unpaired Student´s *t* test as appropriate. A value of $p<0.05$ was considered statistically significant. Results shown in the figures are means ± SEM.

SUMMARY OF RESULTS

Transcriptional effect of IL-6 on proinflammatory genes

We investigated whether IL-6 induces transcription of proinflammatory genes and the negative feed-back inhibitor of IL-6, *Socs3*, in differentiated 3T3-L1 mouse adipocytes and in HepG2 hepatocytes. We found that IL-6 is a potent inducer of the proinflammatory genes *Saa3* and *Hp* in differentiated 3T3-L1 adipocytes *(Paper I)* and *(Paper II)*. Same results were detected in HepG2 cells where *SAA1*, *SAA2*, *PAI-1*, *ORM1* and *HP* were induced by IL-6 *(Paper III)*. In both cell types, IL-6 induced *Socs3* gene expression (*Paper I, II, III*).

Anti-inflammatory effect of insulin

Insulin has an anti-inflammatory effect on IL-6-signaling in differentiated 3T3-L1 adipocytes by reducing the Tyr-705 phosphorylation of the transcription factor STAT3, increasing the Ser-727 phosphorylation of STAT3 and, furthermore, reducing the nuclear translocation and the transcriptional activity of STAT3. Consequently, insulin stimulation also reduces the IL-6-induced gene expression of the inflammatory markers *Saa3* and *Hp*. The effect of insulin was found to be mediated through the MEK-MAPK pathway since PD98059 (MEK-inhibitor) reduced the anti-inflammatory effect of insulin. In addition, we also found that insulin induces the Tyr-542 phosphorylation of the phosphatase SHP2 that is able to dephosphorylate STAT3 and, thus, inhibit IL-6-signaling. Finally, insulin was also found to synergistically increase the gene expression of the negative feedback inhibitor of IL-6-signaling, *Socs3 (Paper I)*.

The anti-inflammatory effect of insulin was also seen in the human hepatoma cell line HepG2. Insulin stimulation reduced the IL-6-induced gene transcription of *SAA1*, *SAA2*, *HP*, *PAI-1* and *ORM1*. However, the signaling mechanism for how insulin exerts its anti-inflammatory effect in HepG2 cells differs from that seen in 3T3-L1 adipocytes. Insulin was neither a potent activator of Tyr-542 phosphorylation of the phosphatase SHP2 nor induced Ser-727 phosphorylation of STAT3. Furthermore, the inhibitory effect of insulin on Tyr-705 phosphorylation of STAT3 was not observed in HepG2 cells. A modest synergistic SOCS3 induction was seen in samples treated with both IL-6 and insulin. However, this result did not reach statistical significance.

FOXO1 has been found to act as a co-activator for IL-6-induced STAT3 transcription (142). It has been shown that insulin, through PKB activation, induces Thr-24 phosphorylation of FOXO1. Phosphorylation at this residue mediates nuclear exit of FOXO1 (143). We found that insulin induced Thr-24 phosphorylation of FOXO1 and translocated it from nucleus compared

to samples treated with IL-6 alone. This may contribute to the anti-inflammatory effect of insulin (*Paper III*). However, as no effect is seen on IL-6-induced Tyr-705 phosphorylation of STAT3 in the presence of insulin, the small induction of *SOCS3* is probably playing a minor role for the anti-inflammatory effect of insulin.

Involvement of PKCδ for the proinflammatory effect of IL-6

Both Tyr-705 and Ser-727 phosphorylation of STAT3 were found to be dependent on the serine/threonine kinase PKCδ as the specific PKCδ inhibitor rottlerin reduced these phosphorylations in differentiated 3T3-L1 adipocytes. Consequently, the nuclear translocation of STAT3, the IL-6-induced gene transcription of *Socs3, Il-6, Saa3 and Hp*, as well as the protein secretion of SAA3 were reduced by rottlerin. Furthermore, PKCδ was found to translocate to the nucleus following IL-6 stimulation, which could be reduced by rottlerin. In agreement with the effect of rottlerin, PKCδ-/- MEFs also displayed a markedly reduced ability of IL-6 to activate the transcription of *Saa3*, *Hp*, *Socs3* and *Il-6* genes when compared to wt MEFs. These results also were also associated with a reduced nuclear translocation and phosphorylation of STAT3 (*Paper II*).

Surprisingly, PKCδ was not important for activation of STAT3 signaling and transcription of inflammatory molecules in HepG2 cells since PKCδ knockdown in these cells neither reduced the IL-6-induced Tyr-705 and Ser-727 phosphorylation of STAT3 nor the gene transcription of *SAA1*, *SAA2*, *HP*, *PA1-1* and *ORM1* in response to IL-6 (*Paper III*).

DISCUSSION

Effects of IL-6 in different tissues

Obesity is associated with enlarged fat cells and an impaired preadipocyte differentiation into mature adipocytes (68, 77). Our lab has previously reported that large adipocytes secrete more IL-6 compared to small adipocytes and also that the interstitial concentration of IL-6 is ≈ 100-fold higher than in plasma which further supports the importance of IL-6 as a paracrine regulator of adipose tissue that negatively affect the gene expression of *aP2*, *ADIPONECTIN*, and *PPARγ2* in human adipose tissue (144) and *Glut4*, *Pparγ* and *Irs-1* in differentiated 3T3-L1 adipocytes (33). It has been suggested that approximately 30 % of the IL-6 in the circulation originates from the adipose tissue and also that adipocytes in the omental depot secrete more IL-6 than to subcutaneous adipose tissue (43, 44).

The role of IL-6 has been extensively debated and contradictory effects have been reported in different tissues e.g. adipose tissue, liver and skeletal muscle. In *Paper I, Paper II* and *Paper III* we show that long-term exposure of IL-6 is a potent inducer of inflammation in 3T3-L1 adipocytes and HepG2 hepatocytes. This supports that the elevated IL-6 levels observed in the adipose tissue of obese subjects can promote the development a low chronic inflammation. Furthermore, our results in *Paper III* support a previous report showing that the main function of IL-6 is to induce the hepatic production of inflammatory molecules (56) leading to an increased inflammatory response. Senn et al. showed that acute IL-6 stimulation of hepatocytes impaired insulin signaling through decreased tyrosine phosphorylation of IRS-1 and, thereby, decreased the association of the p85 subunit of PI3-kinase with IRS-1 which further resulted in a reduced activation of PKB and glycogen synthesis (112). The impaired IR signal transduction was found to be dependent on IL-6-induced expression of *Socs3* and the association of this molecule with IR (145).

Previously, our lab reported that short-term IL-6 infusion in rats did not lead to impaired insulin signaling in liver, skeletal muscle or adipose tissue (146) which further supports that the chronically elevated IL-6 levels observed in obesity (118) and T2D subjects (120) are important for the insulin resistance in peripheral tissues. In addition, chronically elevated IL-6 levels, through over-expression in skeletal muscle in mice, results in hyperinsulinemia, reduced body weight due to increased energy expenditure, impaired insulin-induced glucose uptake by the skeletal muscle and prominent inflammation in the liver (113). This finding was further supported by Nieto-Vazquez et al. who showed that insulin resistance in myocytes during long-term treatment with IL-6 was dependent on impairment at the level of IRS-1 by: 1) inducing a JNK-mediated serine phosphorylation of IRS-1, 2) impairing its tyrosine phosphorylation by SOCS3 and, 3) tyrosine

dephosphorylation of IRS-1 by activating the protein tyrosine phosphatase 1B (PTB1B) (147). However, short-term exposure of IL-6 to rat myocytes and L6 myocytes has been shown to mimic the putative positive effect of IL-6 on insulin sensitivity when produced and released during exercise. This effect of IL-6 *in vitro* seemed to be mediated through IL-6-induced AMPK (5´-AMP-activated protein kinase) activation (147, 148). Moreover, acute IL-6 infusion in humans has been shown to enhance the insulin-stimulated whole-body glucose disposal *in vivo* (148) but the IL-6 concentration in the circulation was much higher than the observed concentration of ~120 ρg/ml IL-6 after a marathon run (149) which may suggest that the effect of IL-6 in myocytes *in vitro* is a pharmacological effect of IL-6 rather than a physiological effect.

The effect of IL-6 in muscle is different compared to the observed effects in adipose tissue and liver since IL-6 is locally produced and secreted by skeletal muscle during exercise (150). The release of IL-6 during muscle contraction is not part of the inflammatory response and/or muscle damage, rather a signal from the muscle to increase glucose uptake when the glycogen availability is reduced (151, 152). It has been demonstrated that carbohydrate ingestion during exercise reduced the IL-6 release into plasma (153) and also that the plasma level of IL-6 peak at the end of exercise and then slowly declined (150). IL-6 has also been shown to inhibit glycogen synthesis in hepatocytes due to inhibition of glycogen synthase activity and accelerated glycogen phosphorylase activity and, thereby, stimulation of glycogen degradation (154). In accordance, recombinant IL-6 injection in humans has been shown to increase the hepatic glucose production (155) which further supports the possible explanation that increased IL-6 release during muscle contraction regulates the availability of glucose for the muscle in exercise.

The effect of IL-6 seems to be opposite in the central nervous system (CNS) compared to chronic exposure in adipose tissue, liver and skeletal muscle. IL-6-/- knockout mice developed obesity, displayed increased triglyceride levels in blood and were glucose intolerant. Fat mass was partly reversed with a single intracerebroventricular (ICV) administration of IL-6, probably due to increased energy expenditure whereas intraperitoneal (IP) administration had no effect (156). Wallenius et al. have also shown that chronic ICV administration of IL-6 to rats fed a high fat diet reduced the body weight (157), which further supports that the CNS is mainly the target for the anti-obesity effect of IL-6. Jansson et al. (158) hypothesize a possibility for the observed anti-obesity effect of IL-6 in the CNS, by suggesting that IL-6 in rodents stimulate the sympathetic nervous system followed by increased expression of uncoupling protein-1 (*Ucp-1*) in brown adipose tissue (BAT) leading to increased heat production. The IL-6 causing this effect in CNS is suggested to originate from the skeletal muscle that, during exercise, secrete IL-6 that penetrates into the CNS and exert anti-obesity effect. Other possibilities are that low secreted levels of IL-6 from WAT may reach the CNS

or that locally produced IL-6 in the CNS account for the effect of IL-6 (158). However, there are contradictory results as Di Gregorio et al. were not able to repeat the effects of IL-6 in seen knockout mice (159).

Anti-inflammatory effect of insulin

Our findings in *Paper I* and *Paper III* suggest that insulin has different effects on IL-6-signaling in the cell lines, 3T3-L1 and HepG2. We showed that insulin inhibits the IL-6-induced expression of inflammatory genes in both cell types but the mechanisms for the anti-inflammatory effect were different. In 3T3-L1 adipocytes, it was dependent on STAT3 signaling transduction whereas in the HepG2 hepatocytes the anti-inflammatory effect seemed to be mediated at the transcriptional level.

The finding that insulin has an important anti-inflammatory role was first described by Dandona and co-workers. They showed that infusion of a low dose of insulin in obese non-diabetic subjects reduced the ROS production and NFκB activation in mononuclear cells. This was also combined with reduced plasma levels of inflammatory molecules such as soluble intercellular adhesion molecule-1 (sICAM-1), MCP-1 and PAI-1 (114). The impaired PAI-1 levels were associated with suppressed nuclear EGR-1 levels, a transcription factor involved in the regulation of *PAI-1* expression and, thereby, inhibiting fibrinolysis. The effects of insulin on the fibrinolytic system further suggest that insulin also has a positive anti-atherotrombotic effect (115). Moreover, FFA release from enlarged adipocytes due to nutrition overload and further stimulation of lipolysis have been shown to bind TRLs on the cell surface of macrophages and, thereby, further stimulate the inflammatory response in the adipose tissue (69). In accordance, toll-like receptor 2 (TLR2) and TLR4 expression is increased in the adipose tissue of obese non-diabetic subjects compared to lean controls (160). Interestingly, Dandona and co-workers have also shown that insulin infusion in T2D subjects reduced the expression of TLR4 by 20-30% in mononuclear cells through reduced DNA-binding of the transcription factor PU.1 to a specific sequence in the TLR4 promotor (161), which further supports the anti-inflammatory role of insulin.

In *Paper I,* we present a novel mechanism for how insulin can regulate IL-6-signaling and thus, exert the anti-inflammatory effect on IL-6-induced expression of inflammatory genes in 3T3-L1 mouse adipocytes. Insulin was not a potent inhibitor of IL-6-induced *Socs3* gene expression. Instead, insulin synergistically increased the IL-6-induced *Socs3* expression, whereas insulin alone had no or a small effect, *Paper I*. This was not consistent with Emanuelli et al. (162) who found that insulin enhanced *Socs3* expression through activation of STAT5B (162). In order to elucidate the synergistic effect of insulin on IL-6-induced *Socs3* expression we examined the effect of insulin

on IL-6-induced activation of STAT5. Interestingly, we found that insulin increased the IL-6-induced tyrosine phosphorylation of STAT5, suggesting that STAT5 is involved in the synergistic up-regulation of *Socs3*, *Paper I*. These findings propose an additional mechanism for insulin to reduce the IL-6-signaling pathway, and also the insulin-signaling cascade since SOCS3 has been shown to act as a negative feedback inhibitor to the insulin signaling (111, 162). The finding that insulin has anti-inflammatory effect may be of physiological importance since both obesity and T2D are associated with increased inflammation. Hence, an anti-inflammatory role of exogenous insulin could therefore antagonize this inflammation and lead to improved systemic insulin sensitivity in the peripheral tissues. Insulin infusion in patients with acute myocardial infarction has reduced plasma levels of SAA, CRP and PAI-1 further supporting the anti-inflammatory effect of insulin (163).

In *Paper III* we present results suggesting that the signaling mechanism of how insulin impairs the IL-6-induced inflammation in HepG2 hepatocytes is mediated through a different mechanism compared to 3T3-L1 mouse adipocytes. Our results are partly consistent with what has been observed in rat hepatocytes. Jeschke et al. found that IP injection of insulin reduced the hepatic gene expression of *Il-6* and the proinflammatory signal transcription factors *Stat3*, *Stat5* and *C/EBP-β* (CCAAT/enhancer binding protein-β) in endotoxemic rats receiving LPS (164). Concordantly that insulin increased IL-6-induced *SOCS3* expression, *Paper III*, insulin has been shown to increase the LPS-induced *Socs3* expression, and reduced the gene expression of *Stats* in endotoxemic rats (164). However, we were not able to observe any inhibitory effect of insulin on STAT3 protein expression, which further suggests that the effect of insulin in the HepG2 hepatocytes is mediated on transcriptional level. Another possible explanation may be that the HepG2 cells are a human liver carcinoma cell line that differs from rat liver. It has been reported that HepG2 also differs from human liver cells with regard to some liver-specific functions (165) and also that STAT3 has been shown to be constitutively active in HepG2 cells (166). However, as no effect is seen on IL-6-induced Tyr-705 phosphorylation of STAT3 in the presence of insulin, the small induction of *SOCS3* is probably playing a minor role for the reduction of the IL-6-induced inflammation compared to the effect of FOXO1.

One potential protein involved in STAT3-mediated transcription in an insulin-dependent way is FOXO1. FOXO1 is not only a transcription factor involved in the regulation of gluconeogenic genes such as phosphoenolpyruvate carboxykinase (*PEPCK*), glucose-6-phosphatase (*G6Pase*) and peroxisome proliferator-activated receptor-γ coactivator-1α (*PGC-1α*) (143, 167). It is also found to act as a STAT3 co-activator in HepG2 hepatocytes and thereby, increase the expression of the acute phase plasma protein *α_2-macroglobulin* in response to IL-6 (142). In the same study, it was

also shown that the co-localization of STAT3 and FOXO1 in the nucleus was reduced by insulin, suggesting that activation of the insulin-signaling pathway impairs the IL-6-induced activation of inflammatory genes at the transcriptional level (142). In accordance with Kortylewski et al. (142), we also observed that FOXO1 is translocated out from the nucleus in insulin-treated HepG2 hepatocytes, *Paper III*. Upon insulin stimulation, FOXO1 is threonine phosphorylated at residue 24 by PKB kinase leading to nuclear export and, thus, inhibiting transcription (143, 167).

Interestingly, Schmitz-Pfeiffer and Biden recently showed that binding of insulin to insulin/insulin-like growth factor-1 (IGF-1) receptor also induced phosphorylation of FOXO1 and nuclear exit (168). Thus, we can not exclude that the observed anti-inflammatory effect of insulin may be exerted through the IGF-1 receptor leading to the reduced expression of inflammatory STAT3-regulated genes.

A hallmark of T2D is increased hepatic glucose production due to the hepatic insulin resistance (3) suggesting that nuclear FOXO1, by acting as a co-activator to STAT3 transcription and promoting inflammation, can be a mediator for the elevated blood glucose level. This hypothesis is also supported by Matsumoto et al. (169) as they have shown that mice lacking FOXO1 in the liver display reduced glucose levels suggesting that FOXO1 is required for insulin regulation of hepatic glucose output (169). Thus, the ability of insulin to induce the nuclear exit of FOXO1 may be an important mechanism for how insulin regulates hepatic glucose production in addition to the effect of IL-6-induced inflammation.

PKCδ, FOXO1 and inflammation

Our findings *In Paper II* suggest that PKCδ is important for the induction of inflammatory genes in response to IL-6 since other STAT3-regulated genes were not affected during rottlerin treatment in 3T3-L1 adipocytes. In contrast, PKCδ did not seem to be important for the IL-6-induced inflammatory response in HepG2 hepatocytes, (*Paper III*).

In accordance with our results many studies show that PKCδ is important in inflammation. Lymphocytes play an important role in allergic inflammation and rottlerin has been shown to inhibit phytohaemagglutinin (PHA)-induced peripheral blood mononuclear cell proliferation, suggesting that inhibition of PKCδ in the context of allergic inflammation may be important as a possible therapeutic target (170). It is also reported that neutrophil storage of elastase and recruitment of these cells are involved in inflammation-related lung disorders such as cystic fibrosis. This involves increased mucin secretion in response to neutrophil elastase (HNE), which is associated with PKCδ activation in normal human bronchial epithelial (NHBE) cells. Inhibition of PKCδ with rottlerin reduced HNE-stimulated mucin

secretion suggesting that PKCδ is involved in this process (171). Moreover, PKCδ has also been shown to be involved in the regulation of NF-κB-dependent expression of proinflammatory genes such as *IL-8* in a human airway epithelial cell line, probably through a mechanism that induces the nuclear translocation of NF-κB. This was assumed by the finding that rottlerin or a dominant negative PKCδ mutant abrogated TNFα-induced gene expression of *IL-8* (172). Another study also supports the involvement and importance of PKCδ in inflammatory processes. It was reported that PKCδ expression was increased in vessel wall samples from patients with abdominal aortic aneurysms. In the same study, they also show that TNF-α-induced production of MCP-1 was abrogated with rottlerin in rat normal vascular smooth muscle cells (vSMC) whereas over-expression of PKCδ in these cells resulted in an increased gene expression of *MCP-1* upon TNFα stimulation. These results may suggest that increased PKCδ expression in the aneurismia tissue may mediate *MCP-1* gene expression and recruitment of inflammatory cells such as macrophages into this abnormal tissue (173).

PKCδ has also been shown to elicit different responses in different cells (129, 174). A negative role of PKCδ on cell survival has been reported by Voss et al. (175). They showed that PKCδ activation is involved in the caspase-3 phosphorylation and promotes monocyte apoptosis and, thereby, inhibits the pro-longed life span that is observed in inflammation, differentiation and oncogenic transformation (175). Moreover, tristetraprolin (TTP) is known to bind to target genes and, thereby, inhibit the stabilization of the mRNA, leading to reduced protein production (176). This was confirmed in TTP knock-out animals that displayed increased stability of TNFα (177). Leppänen et al. reported that down-regulation of PKCδ inhibits gene expression of *TTP* in macrophages, thereby, suggesting that PKCδ is important for *TTP*-induction and consequently down-regulation of inflammatory reactions (174). Furthermore, mice that lack PKCδ were prone to develop autoimmune diseases and display increased proliferation of B cells, which supports the concept that PKCδ regulates the B-cell proliferation (130). The opposite functions of PKCδ seem to be dependent on particular cell type and specific stimuli.

In *Paper II,* we support the concept that PKCδ is important for the inflammatory responses, and in our case, the important role of PKCδ was associated with STAT3 activation and induction of inflammatory genes in response to IL-6 in 3T3-L1 adipocytes. As PKCδ surprisingly was not important for IL-6-induced inflammatory response in HepG2 hepatocytes, (*Paper III*), it would be interesting to further elucidate if other PKC isoforms are involved, since these cells also express PKCα, β, ε, ζ and θ (178, 179).

As obesity is associated with an increased inflammatory response in adipose tissue, our results suggest that PKCδ could be increased in obese subjects. There are a few reports showing that other isoforms of PKC are

increased in liver and skeletal muscle of obese insulin-resistant subjects compared to lean controls such as α, ε, ζ and β (180, 181). Moreover, an *in vivo* study in PKCδ knockout mice showed that these mice displayed reduced levels of triacylgycerol in the liver due to reduced production of proteins involved in the lipid synthesis. They were also less glucose intolerant as a consequence of increased insulin sensitivity compared to wt mice (131). We have also confirmed that lack of PKCδ is associated with impaired lipid accumulation, and found that PKCδ-/- MEFs displayed reduced lipid droplets after adipocyte differentiation compared to wt MEFs (data not shown). These results suggest that PKCδ may be involved in lipid accumulation and Kayali et al. have shown that PKCδ is highly expressed in 3T3-L1 adipocytes, 3T3-L1 preadipocytes as well as in mouse fat (182).

It has been widely debated whether rottlerin is a specific PKCδ inhibitor or not, due to its reported effects in mitochondria and on AMPK (183, 184). Our suggestion that rottlerin is an appropriate PKCδ inhibitor in 3T3-L1 adipocytes was further supported by siRNA PKCδ transfection in these cells, showing that IL-6-induced inflammation was reduced compared to non-transfected cells, *Paper II*. However, PKCδ has also been shown to play an important role *in vivo* for mediating histamine release in mast cells. In these cells, the process was due to ROS mediating Ca^{2+}-induced PKCδ activation. IP injection of rottlerin in a mouse model of allergic asthma reduced the release of histamine, suggesting that PKCδ inhibition would be valuable for attenuating the allergic inflammation associated with mast cell degranulation (185) and further supporting that PKCδ is involved in inflammatory responses.

We also show in *Paper II* that PKCδ is translocated into the nucleus in response to IL-6 in 3T3-L1 adipocytes. In contrast, Schuringa et al. showed that PKCδ is present in the nucleus independent of IL-6 stimulation (98) while Jain et al. found that PKCδ was localized in the cytoplasm (99). The reason for these discrepancies is currently unclear.

Lu et al. have recently shown that TNFα stimulates IL-6 secretion in human embryonic kidney 293T cells and this production was abrogated with rottlerin (186). Similar results were observed in siRNA PKCδ transfected human osteosarcoma U2OS cells, since they displayed reduced secretion of IL-6 in response to TNFα compared to non-transfected cells. These results suggest that PKCδ induces transcriptional activity of *NF-κB* to regulate IL-6 production in response to TNFα, supporting its presence in the nucleus. They propose a model for the activation of PKCδ and the NF-κB signaling pathway where by the subunits RelA/p65 of NF-κB and PKCδ are translocated from the cytoplasm into the nucleus upon TNFα stimulation. PKCδ and RelA/p65 then form a complex on the κB elements in the promoter and, thereby, induce the gene expression of *NF-κB* (186). In addition, our laboratory has previously demonstrated that TNFα is a potent inducer of *Il-6* gene expression in 3T3-L1

adipocytes (33) and, in *Paper II*, we showed that IL-6-induced expression of IL-6 itself is reduced with rottlerin. Together, these two results (98, 186) support our finding that nuclear translocation of PKCδ is important for induction of inflammation. It also illustrates a possibility for the increased TNFα secretion from adipose tissue macrophages observed in obesity to involve nuclear translocation of PKCδ followed by induction of the expression of *Il-6* and other cytokines and, consequently, an increased inflammatory response.

Moreover, a functional PKCδ is important for FOXO1-mediated nuclear events as recently published by Hennige et al. (187). Interestingly, they show that fatty acid-mediated apoptosis in β-cells is dependent on a functional PKCδ with concomitant nuclear translocation of FOXO1. In the same study they also showed that over-expression of a kinase-negative PKCδ (PKCδKN) enables Ser-256 phosphorylation of FOXO1 with concomitant nuclear exit. These mice were also protected against β-cell apoptosis suggesting that high fat diet-induced failure of the β-cells in wild-type mice is dependent on a functional PKCδ (187). These findings also support our finding that a functional PKCδ is important for IL-6-induced inflammation, *Paper II*.

Kortylewski et al. have shown that FOXO1 can act as a co-activator for STAT3 in response to IL-6 and, thereby, increase the gene expression of the acute phase protein, α_2*-macroglobulin* (142). In addition, we confirmed in *Paper III* that insulin regulates FOXO1 content in the nucleus. It is possible that PKCδ may play a key role for the inflammatory response and, together with FOXO1 accumulation in nucleus, act as complex to regulate the induction of STAT3-activated genes. It is crucial to further elucidate if FOXO1 may be a potential molecule for interaction with nuclear PKCδ and, thereby, stimulate the inflammatory response in 3T3-L1 adipocytes as well as in other proinflammatory cells.

CONCLUSION

This thesis shows a novel mechanism for how insulin can regulate IL-6-signaling in 3T3-L1 adipocytes and, thereby, exert an anti-inflammatory effect by antagonizing IL-6-induced inflammation. This finding is important since obesity is associated with a low chronic inflammation in the adipose tissue and may, therefore, provide an insight into the regulation of the inflammatory response in adipocytes. Furthermore, we show in these cells that PKCδ plays a key role for IL-6-induced STAT3 activation and induction of inflammatory genes. Consequently, the inflammatory response in the presence of IL-6 was impaired by PKCδ inhibition, which suggests that PKCδ may be a target for drug development; impairing IL-6-induced inflammation. Moreover, the results in HepG2 hepatocytes suggest a role for FOXO1 in the regulation of inflammation in these cells. Future studies should be focused on understanding the potential link between PKCδ and FOXO1 which may open up new avenues into our understanding of cytokine-induced inflammation and also provide opportunities for the development of new anti-inflammatory agents.

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