PPARs IN THE REGULATION OF GENE EXPRESSION AND LIPID METABOLISM IN THE LIVER

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A doctoral thesis at a University in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These papers have already been published or are in manuscript at various stages (in press, submitted or in manuscript).
ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors, regulating genes involved in carbohydrate, lipid and lipoprotein metabolism. Synthetic PPAR\(\alpha\) agonists used in clinic are the fibrates, and their hypotriglyceridemic effect is believed to be the result of decreased VLDL triglyceride secretion and increased turnover of triglyceride-rich lipoprotein particles in the circulation. Synthetic PPAR\(\gamma\) agonists, used in clinic to treat type 2 diabetes, are the thiazolidinediones (TZDs), and PPAR\(\gamma\) in adipose tissue is believed to be the primary target for the TZDs.

TZD treatment of obese mice resulted in increased expression of proteins taking part in the peroxisomal fatty acid \(\beta\)-oxidation in the liver, whereas this effect was abolished in lean mice. This difference in effect might be explained by the finding that obese mice expressed higher levels of PPAR\(\gamma\)2 in the liver as compared to the lean mice. In primary mouse hepatocyte cultures, insulin and oleic acid were shown to increase the level of PPAR\(\gamma\)2, whereas PPAR\(\gamma\)1 expression was unaffected. The stimulatory effect of insulin on PPAR\(\gamma\)2 transcription was mediated via the phosphatidylinositol 3-kinase pathway. Both in vitro, in cultured hepatocytes, and in vivo, treatment with the PPAR\(\alpha\) agonist Wy14,643 (Wy) resulted in increased expression and activity of microsomal triglyceride transfer protein (MTP), which is rate limiting in the VLDL assembly and secretion. Increased MTP expression was paralleled by increased apoB100 secretion, suggesting that the stimulatory effect of Wy on apoB100 secretion could be mediated via increased MTP expression. Wy was also shown to increase the hepatic expression of adipose differentiation-related protein (ADRP) in vivo in mice and in vitro in cultured mouse hepatocytes. Wy increased the hepatic triglyceride content despite increased fatty acid oxidation and unchanged triglyceride synthesis. However, Wy was shown to inhibit the triglyceride secretion, which might contribute to the cellular triglyceride accumulation. Similar to Wy incubation, ADRP overexpression in mouse hepatocytes resulted in decreased triglyceride secretion and increased cellular triglyceride mass. In ADRP overexpressing cells, Wy further inhibited the triglyceride secretion although the ADRP protein expression was unaltered. Since the triglyceride content was unaffected by Wy in ADRP overexpressing cells, incubation with Wy did not result in lack of cellular triglycerides for VLDL secretion. Rather, PPAR\(\alpha\) activation prevents the availability of triglycerides for VLDL assembly, in part by increasing the expression of ADRP.

In summary, this study shows that the diabetes-associated increase in hepatic PPAR\(\gamma\)2 could be due to increased insulin signaling, and important for the effects of PPAR\(\gamma\) agonists in liver. Moreover, PPAR\(\alpha\) activation influences hepatic expression of MTP and ADRP that contributes to the increased secretion of triglyceride-poor apoB-containing lipoproteins following PPAR\(\alpha\) activation.
LIST OF PUBLICATIONS

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

I Rosiglitazone (BRL49653), a PPARγ-selective agonist, causes peroxisome proliferator-like liver effects in obese mice.
Ulrika Edvardsson, Monica Bergström, Maria Alexandersson, Krister Bamberg, Bengt Ljung and Björn Dahllöf.

II Insulin and oleic acid increase PPARγ2 expression in cultured mouse hepatocytes.
Ulrika Edvardsson, Anna Ljungberg and Jan Oscarsson.
Manuscript

III Activation of peroxisome proliferator-activated receptor α increases the expression and activity of microsomal triglyceride transfer protein in the liver.
Caroline Améen, Ulrika Edvardsson, Anna Ljungberg, Lennart Asp, Peter Åkerblad, Anna Tuneld, Sven-Olof Olofsson, Daniel Lindén and Jan Oscarsson.

IV PPARα activation increases triglyceride mass and adipose differentiation-related protein in hepatocytes.
Ulrika Edvardsson, Anna Ljungberg, Daniel Lindén, Lena William-Olsson, Helena Peilot-Sjögren, Andrea Ahnmark and Jan Oscarsson.
Submitted
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AADA</td>
<td>arylacetamide deacetylase</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACO</td>
<td>acyl-CoA oxidase</td>
</tr>
<tr>
<td>ADRP</td>
<td>adipose differentiation-related protein</td>
</tr>
<tr>
<td>Angptl</td>
<td>angiopoietin-like proteins</td>
</tr>
<tr>
<td>aP2</td>
<td>adipocyte fatty acid binding protein</td>
</tr>
<tr>
<td>aPKC</td>
<td>atypical protein kinase C</td>
</tr>
<tr>
<td>Apo</td>
<td>apolipoprotein</td>
</tr>
<tr>
<td>CE</td>
<td>cholesterol ester(s)</td>
</tr>
<tr>
<td>CETP</td>
<td>cholesterol ester transfer protein</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>COUP-TF</td>
<td>chicken ovalbumin upstream promoter transcription factor</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP responsive element binding protein</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DGAT</td>
<td>diacylglycerol acyltransferase</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FAS</td>
<td>fatty acid synthase</td>
</tr>
<tr>
<td>FATP</td>
<td>fatty acid transport protein</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’ balanced salt solution</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HL</td>
<td>hepatic lipase</td>
</tr>
<tr>
<td>HMG</td>
<td>3-hydroxy-3-methylglutaryl</td>
</tr>
<tr>
<td>HNF</td>
<td>hepatocyte nuclear factor</td>
</tr>
<tr>
<td>HSL</td>
<td>hormone sensitive lipase</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate-density lipoprotein</td>
</tr>
<tr>
<td>IRS</td>
<td>insulin receptor substrate</td>
</tr>
<tr>
<td>LCAT</td>
<td>lecitin cholesterol acyltransferase</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>L-FABP</td>
<td>liver fatty acid binding protein</td>
</tr>
<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
</tr>
<tr>
<td>LRP</td>
<td>low-density lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MTP</td>
<td>microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>NAFLD</td>
<td>non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NASH</td>
<td>non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>NEFA</td>
<td>non-esterified fatty acids</td>
</tr>
<tr>
<td>PAI</td>
<td>plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEMT</td>
<td>phosphatidylethanolamine N-methyltransferase</td>
</tr>
<tr>
<td>PEPCK</td>
<td>phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PI3-kinase</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>PLTP</td>
<td>phospholipid transfer protein</td>
</tr>
<tr>
<td>PMF</td>
<td>peptide mass fingerprint</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PPRE</td>
<td>peroxisome proliferator response element</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>SCD</td>
<td>stearoyl-CoA desaturase</td>
</tr>
<tr>
<td>SR-BI</td>
<td>scavenger-receptor B-I</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>TGH</td>
<td>triacylglycerol hydrolase</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumour necrosis factor α</td>
</tr>
<tr>
<td>TZD</td>
<td>thiazolidinedione</td>
</tr>
<tr>
<td>VLDL</td>
<td>very-low density lipoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
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INTRODUCTION

THE METABOLIC SYNDROME AND TYPE 2 DIABETES

The concept of the metabolic syndrome was first described in the 1920s by the Swedish physician Kylin, as the clustering of hypertension, hyperglycemia and gout (1). Later, in 1947, the upper body obesity (android or male-type obesity) was identified as the obesity phenotype that was related to metabolic abnormalities associated with type 2 diabetes and cardiovascular disease (CVD) (2). To achieve agreement of the definition of the metabolic syndrome and to provide a tool for clinicians and researchers, World Health Organization (WHO) defined the metabolic syndrome, in 1999, as the constellation of disturbances shown in Table 1. Several other manifestations have been identified that are related to the metabolic syndrome, and a new definition will most likely be applicable in the near future. Increased levels of pro-inflammatory cytokines and prothrombotic factors (fibrinogen and plasminogen activator inhibitor 1), in addition to non-alcoholic fatty liver disease (NAFLD) and/or non-alcoholic steatohepatitis (NASH) are all associated with the metabolic syndrome (3). Studies suggest that 20% to 30% of adults in the United States and other Western countries have excess fat accumulation in the liver. About 10% of these individuals are estimated to meet current diagnostic criteria for NASH (4).

The metabolic syndrome is also known as the insulin resistance syndrome, since the pathophysiology seems to be largely attributed to fatty-acid induced insulin resistance in the skeletal muscle (5). It has become increasingly evident that individuals with the metabolic syndrome, are at an increased risk of developing both CVD and type 2 diabetes, and the prevalence of the metabolic syndrome among type 2 diabetic patients is about 70% (6).

Table 1. WHO Clinical Criteria for Metabolic Syndrome.

<table>
<thead>
<tr>
<th>Insulin resistance, identified by 1 of the following:</th>
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<tr>
<td>• Type 2 diabetes</td>
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<tr>
<td>• Impaired fasting glucose</td>
</tr>
<tr>
<td>• Impaired glucose tolerance</td>
</tr>
<tr>
<td>• Impaired glucose uptake (hyperinsulinemic, euglycemic clamp)</td>
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</table>

<table>
<thead>
<tr>
<th>Plus 2 or more of the following:</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Hypertension, ≥140 mm Hg systolic or ≥90 mm Hg diastolic</td>
</tr>
<tr>
<td>• Plasma triglycerides ≥1.7 mmol/L</td>
</tr>
<tr>
<td>• HDL cholesterol &lt;0.9 mmol/L in men or &lt;1.0 mmol/L in women</td>
</tr>
<tr>
<td>• Obesity, BMI&gt;30 kg/m² or waist-to-hip ratio &gt;0.9 in men or &gt;0.85 in women</td>
</tr>
<tr>
<td>• Microalbuminuria, albumin excretion ≥20 µg/min</td>
</tr>
</tbody>
</table>
Atherogenic dyslipidemia in the metabolic syndrome and in type 2 diabetes
In the metabolic syndrome and in type 2 diabetes, atherogenic dyslipidemia is an important and common risk factor for coronary heart disease (CHD) that is the leading cause of morbidity and mortality worldwide (7,8). In addition to high plasma triglyceride levels and decreased high-density lipoprotein (HDL) levels (criteria for metabolic syndrome, Table 1), elevations of apolipoprotein (apo)B and small dense low-density lipoprotein (LDL) particles are further characteristics of atherogenic dyslipidemia. All these features are related to each other, and the underlying cause might be an increased production of triglyceride-rich very-low density lipoprotein (VLDL)1 particles in the liver. Due to the fact that VLDL1 is the precursor of LDL particles with prolonged turnover, an increased production of VLDL1 will give rise to elevated levels of apoB in plasma (see below, Lipoprotein metabolism). Decreased HDL levels are also related to increased VLDL1 production, by the fact that cholesterol ester transfer protein (CETP) mediates transfer of cholesterol esters (CE) from HDL in exchange for triglycerides from VLDL1, which results in triglyceride-rich HDL with faster turnover (9). Triglyceride enrichment of HDL has been shown to enhance apoAI clearance in healthy men (10), and it was demonstrated that hepatic lipase (HL) was required for the lipolysis, preceding apoAI clearance (11).

Treatment of atherogenic dyslipidemia
Diet, exercise and weight reduction are first line therapies in the treatment of atherogenic dyslipidemia, but there are also several alternative pharmacotherapies. The most commonly prescribed drugs for treatment of atherogenic dyslipidemia in type 2 diabetes are statins and fibrates.

Statins
Statins are cholesterol lowering drugs, and the number-one selling prescribed drug class on the market. Treatment with statins has been shown to reduce the risk for major cardiovascular events in high risk patients with metabolic syndrome by reducing all apoB-containing lipoproteins (12,13). Statins are 3-hydroxy-3-methylglutaryl (HMG) CoA reductase inhibitors that inhibit the synthesis of cholesterol, resulting in induced sterol regulatory element-binding protein (SREBP)-2 cleavage. Since SREBP-2 activates the transcription of the LDL receptor gene, treatment with statins gives rise to increased clearance of remnants and LDL particles from the circulation (14). At high LDL cholesterol levels, statins can be used in combination with cholesterol absorption inhibitors or bile acid sequestrants.
Fibrates
Fibrates have been used in clinic for treatment of dyslipidemia for almost 40 years. Treatment with fibrates results in decreased levels of plasma triglycerides and small dense LDL, and increased HDL cholesterol levels, and has been shown to be associated with a reduction in major cardiovascular events in the diabetic and insulin-resistant population (15,16). Fibrates are therefore a first line choice, as an alternative to statins, for treatment of dyslipidemia in patients with type 2 diabetes. In 1990, Isseman and Green identified peroxisome proliferator-activated receptor (PPAR)α as the therapeutic target of the fibrates (17). Thereafter, numerous studies have been performed to elucidate the mechanisms behind the therapeutic effects (see below).

Nicotinic acid
Nicotinic acid has been used for treatment of dyslipidemia for almost 50 years. Treatment with nicotinic acid increases HDL and decreases LDL cholesterol as well as triglycerides. This therapeutic profile makes nicotinic acid an ideal treatment of atherogenic dyslipidemia. However, due to several side effects this treatment is nowadays not commonly used. The mechanism of action for nicotinic acid is not completely understood. It is believed that inhibition of lipolysis in adipose tissue is the underlying cause for the therapeutic effect. The reduced lipolysis would decrease the flux of fatty acids to the liver and thereby reduce VLDL triglyceride secretion. Recently, the G protein-coupled receptor HM74A was identified as the receptor for nicotinic acid (18,19).

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS
The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors and members of the superfamily of nuclear hormone receptors. Three related PPAR subtypes have been identified, including PPARα, PPARβ/δ and PPARγ (17,20,21). The first cloned member of the PPAR family, PPARα, was a novel murine nuclear receptor that mediated transcriptional effects of peroxisome proliferators (17). The other closely related receptors, encoded by separate genes, were thereafter cloned and named PPARβ/δ and PPARγ (20). Despite their names, neither PPARβ/δ nor PPARγ responds to peroxisome proliferators. Similar to other nuclear receptors, PPARs possess a classic domain structure; a N-terminal domain that harbours a ligand-independent transcriptional activation function (AF-1); a DNA-binding domain; and a large C-terminal region that encompasses a ligand-binding domain, a dimerization interface and a ligand-dependent activation function (AF-2). The most conserved part is the DNA-binding domain, which contains two zinc-finger motifs that interact with specific recognition sites in the promoter of target genes, termed peroxisome proliferator response element (PPRE) (22).
PPRE is a direct repeat of six nucleotides that are separated by one nucleotide (consensus sequence AGGTCANAGGTCA), a so-called DR-1 element. To be transcriptionally active, PPAR has to heterodimerize with the retinoid X receptor (RXR) (23,24). The PPAR:RXR complex can be activated by the ligand of either receptor, however simultaneous exposure to both activators often results in a synergistic induction of gene expression (23). Natural ligands for the PPARs are unsaturated fatty acids and eicosanoids (25,26). Upon ligand binding, PPARs undergo a conformational change that dissociates corepressors and facilitates recruitment of coactivators to enable transcriptional activation. Other nuclear receptor complexes than PPAR:RXR have been shown to be able to bind to PPREs. RXR homodimers were demonstrated to bind to PPREs and activate transcription, and administration of the RXR ligand 9-cis retinoic acid to PPARα null mice could prevent hypothermia during fasting (27). Since a cross-talk exists between nuclear receptors recognizing DR-1 sequences, hepatocyte nuclear factor (HNF)-4 and chicken ovalbumin upstream promoter transcription factor (COUP-TF) have been shown to compete with PPAR signaling by displacement of the PPAR:RXR complex from its binding site (28-32). Therefore, one can conclude that binding to PPREs not always results in activation of target genes, but suppression may also occur.

**PPARα**

The PPARα receptor is highly expressed in tissues with high capacity of mitochondrial and peroxisomal β-oxidation, such as liver, heart, kidney and skeletal muscle (33-36). Synthetic ligands are a range of xenobiotic compounds, including e.g. hypolipidemic drugs (fibrates), plasticizers and herbicides (17). In rodents, treatment with PPARα activators results in peroxisome proliferation and tumour development, while the human liver is refractory to peroxisome proliferation and tumour development (37,38). Several factors may account for the fact that humans are less sensitive to peroxisome proliferation than rodents, such as lower levels of PPARα in the human liver (39), expression of a truncated dominant negative form of PPARα in humans that may interfere with PPARα function (40), differences in the mouse and human PPARα sequences, differences in the PPREs of target genes, and/or differences in expression pattern of corepressors/coactivators. The resistance of human hepatocytes to the effects of PPARα ligands, regarding peroxisomal β-oxidation and peroxisome proliferation, is based on studies performed on human primary hepatocytes and on human hepatoma cell lines (38,41-43). Studies on PPARα overexpressing HepG2 cells, have shown that these cells are resistant to peroxisome proliferation despite a PPARα expression level similar to the level in rodents. Overexpression of PPARα in these cells gave rise to increased levels of e.g. enzymes involved in the mitochondrial fatty acid oxidation and the ketogenic
enzyme mitochondrial HMG-CoA synthase, which were enhanced by incubation with the PPARα agonist Wy14,643. Conversely, the expression levels of genes associated with peroxisome proliferation in mouse liver were neither increased by PPARα overexpression nor up-regulated by Wy14,643 exposure (42,43). A PPRE of acyl-CoA oxidase (ACO), the rate-limiting enzyme in the peroxisomal fatty acid oxidation, have been identified in the human promoter (44), but the functionality of this PPRE has been questioned (45). However, overexpression of the human PPARα receptor in PPARα deficient mice, which are refractory to peroxisome proliferation, gave rise to development of peroxisome-proliferator induced responses (46), which establish that the human PPARα receptor is functionally competent in inducing peroxisome proliferation within the context of mouse liver environment.

**PPARα and lipid metabolism**
In the liver, PPARα activators are well-known for regulating genes involved in the uptake, transport and metabolism of fatty acids (reviewed in (47)). PPARα activation gives rise to up-regulation of fatty acid transport protein (FATP) (48,49) and acyl-CoA synthetase (50). Activation of fatty acids into acyl-CoA is required for further metabolism of fatty acids, including incorporation into triglycerides and fatty acid oxidation. In addition to the effects on peroxisomal fatty acid oxidation, PPARα activation gives rise to increased expression of genes involved in microsomal (51,52) and mitochondrial fatty acid oxidation (53-55), as well as ketogenesis (56). During fasting, glucocorticoids increase PPARα expression (57), which is paralleled by increased expression of genes involved in fatty acid oxidation and ketogenesis. In PPARα null mice, fasting leads to severe hypoglycemia, hypoketogenesis, hypothermia and elevated levels of free fatty acids, showing that PPARα plays a pivotal role in the regulation of these pathways (58). In addition, PPARα null mice display hypercholesterolemia and increased serum apoAI levels demonstrating that PPARα modulates lipoprotein metabolism (59). Furthermore, the phenotype of the aged PPARα null mice are characterized by hypertriglyceridemia, and female mice develop a more pronounced obesity than male mice, which instead show a marked liver steatosis (60). The increased obesity in the female PPARα null mice might be explained by increased hepatic apoB and triglyceride secretion that was not seen in the male mice (61).

The hypolipidemic effect of fibrates, is in part due to decreased VLDL triglyceride secretion, noticed in both humans and rats (62,63). Increased fatty acid oxidation is believed to result in reduced availability of triglycerides for VLDL assembly and thereby contribute to the hypotriglyceridemic effect of fibrates (64). However, there are other effects that also lead to a triglyceride lowering effect. Treatment with fibrates gives rise to increased lipoprotein lipase (LPL) expression (65) and
decreased apoCIII expression (66), which together results in increased hydrolysis of triglycerides and clearance of VLDL and chylomicrons in the circulation. ApoCIII suppression by PPARα activation is due to transcriptional inhibition of HNF-4 as well as displacement of HNF-4 from the apolipoprotein CIII promoter (66). Another explanation for decreased plasma triglyceride levels after treatment with fibrates could be an increased expression of the recently identified apoAV, which expression level has been shown to be negatively correlated to plasma triglycerides (67). A functional PPRE has been identified in the human apoAV promoter (68,69), and treatment of monkeys with a PPARα agonist resulted in increased apoAV levels in serum (70). In human, fibrates induce the expression of apoAI and apoAII, effects that are thought to contribute to the increased HDL level (71-73). However, treatment with fibrates in mice and rats resulted in decreased expression of apoAI (74,75), which in rat has been shown to be dependent on a non-functional PPRE and transcriptional repression by the nuclear receptor Rev-Erbα (75). Conversely to the effect seen in macrophages (76), fibrates have been shown to decrease the scavenger receptor B-I (SR-BI) expression in mouse liver, which correlated with enlarged HDL particles (77,78). This effect may counteract the reverse cholesterol transport, and is therefore a potential negative effect of PPARα agonists. However, there are contradictory results from studies on primary rat hepatocytes, showing an increased SR-BI protein expression upon activation by PPARα and PPARγ (79). However, the divergence could be due to the experimental models used, that might involve indirect effects on the liver in the in vivo situation.

**PPARγ**

PPARγ exists as two isoforms, PPARγ1 and PPARγ2, that arise from the same gene, but are generated through different promoter usage and splicing, resulting in 30 additional amino acids in the N-terminal of the PPARγ2 protein (80,81). PPARγ2 is expressed mainly in adipocytes, whereas PPARγ1 is more ubiquitously expressed, and beside adipose tissue also has been detected in lymphatic tissue, digestive tract and liver (33,82,83). A third PPARγ transcript, PPARγ3, encodes the same protein as PPARγ1 but is controlled by an alternate promoter (84). Recently, another isoform was identified, defined γORF4, which was translated into a protein lacking the ligand-binding domain encoded by exon 5 and 6. This isoform did not promote transcription, however it was shown to counteract the transcriptional activity of PPARγ. Since γORF4 was expressed in colorectal cancer cells, it was suggested to be related to tumorigenesis (85).

PPARγ is known as the master regulator of adipogenesis and fat cell function (86,87). The fact that PPARγ is an important regulator of glucose metabolism arose from the discovery that the insulin-sensitizing thiazolidinediones (TZD) are potent
INTRODUCTION

synthetic agonists for PPAR\(\gamma\) (88). Upon activation, PPAR\(\gamma\) promotes lipid storage in adipocytes and thereby reduces the levels of free fatty acids and triglycerides in the circulation. Since fatty acids have been shown to decrease the insulin-stimulated GLUT4 translocation in muscles, PPAR\(\gamma\) activation leads indirectly to increased glucose uptake in muscles (89). The increased lipid storage in adipocytes is a result of increased expression of genes promoting fatty acid uptake, storage and de novo lipogenesis, such as LPL (65), adipocyte fatty acid binding protein (aP2) (21), glycerol kinase (90,91), malic enzyme (92) and perilipin (93). During the last years it has become evident that the adipose tissue is not only a reservoir of energy but also an endocrine organ that secretes several hormones, so-called adipocytokines, such as leptin, adiponectin, tumour necrosis factor (TNF)\(\alpha\) and resistin (reviewed in (94)). Adiponectin is decreased in plasma in type 2 diabetic subjects as well as in diabetic and obese animals, and treatment with TZDs has been shown to increase the level of adiponectin (95-97). A functional PPRE has been identified in the human adiponectin promoter (97). The increase in adiponectin seems to play a role in the insulin-sensitizing effect of TZDs by enhancing fatty acid oxidation in muscle and liver (98-101) and by inhibiting the endogenous glucose production in liver (100,102,103).

It is reasonable to conclude that PPAR\(\gamma\) in adipose tissue is the primary target of the TZDs, and that the increased insulin sensitivity in muscle and liver derives from the effects in adipose tissue. However, in the A-ZIP/F-1 lipodystrophic mice, TZDs were shown to ameliorate hyperlipidemia, without affecting the high glucose or insulin levels (104). Additionally, in aP2/DTA lipodystrophic mice, treatment with TZD resulted in alleviated hyperglycemia, decreased insulin levels, and markedly decreased levels of serum cholesterol, triglycerides, and free fatty acids (105). Despite differences, these studies show that other tissues are of importance for the therapeutic effect of TZDs, at least in case of adipose tissue deficiency.

PPAR\(\beta/\delta\)

PPAR\(\beta/\delta\) is ubiquitously expressed, and the least characterized subtype of the PPARs (106). One of the best characterized function is related to the inflammatory response in keratinocytes. PPAR\(\beta/\delta\) expression is strongly induced and activated in mouse keratinocytes during wound healing, leading to keratinocyte proliferation and differentiation (107). In accordance, PPAR\(\beta/\delta\) \(+/-\) mice displayed impaired epidermal repair (107). Additionally, PPAR\(\beta/\delta\) has been shown to be important in lipid metabolism and energy homeostasis (for review see (108)). In studies using PPAR\(\beta/\delta\) null adipocytes, PPAR\(\beta/\delta\) was shown to be required for maximal lipid accumulation and differentiation during adipogenesis (109), which is in agreement with data showing that PPAR\(\beta/\delta\) induces PPAR\(\gamma\)2 in the early phase of adipocyte

15
differentiation (110). PPARβ/δ has also been shown to play a role in lipid and lipoprotein metabolism in liver. PPARβ/δ null mice on high fat diet displayed increased plasma triglyceride levels that was paralleled by decreased triglyceride content in the livers (111). Increased plasma triglycerides in these animals was explained by increased VLDL triglyceride production and decreased hydrolysis of VLDL by LPL, which partly might be a result of increased expression of the LPL-inhibiting angiopeitoin-like proteins (Angptl)3 and Angptl4 (112,113).

LIPOPROTEIN METABOLISM

Lipoproteins
Due to the extreme hydrophobicity, triglycerides and cholesterol esters have to be transported in the circulation in spherical lipoprotein particles. These particles consist of a hydrophobic core of triglycerides and cholesterol esters surrounded by an amphipatic monolayer of phospholipids, unesterified cholesterol and apolipoproteins. Lipoprotein particles are classified into chylomicrons, VLDL, LDL and HDL depending on the relative content of proteins and lipids that together determine the density of the particles (Table 2). In addition to the solubilization and stabilization effects of apolipoproteins many of them function as ligands for cell surface receptors and cofactors of enzymes.

Table 2. Density and composition of human lipoprotein particles.

<table>
<thead>
<tr>
<th></th>
<th>Chylomicrons</th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (g/ml)</td>
<td>&lt;0.95</td>
<td>0.95-1.006</td>
<td>1.006-1.019</td>
<td>1.019-1.063</td>
<td>1.063-1.21</td>
</tr>
<tr>
<td>Size (nm)</td>
<td>80-1200</td>
<td>30-80</td>
<td>25-35</td>
<td>18-25</td>
<td>5-12</td>
</tr>
<tr>
<td>Apolipoproteins</td>
<td>A, AII, AIV, B48 CI, CII, CIII, E</td>
<td>B100, CI, CII CIII, E</td>
<td>B100, E</td>
<td>B100</td>
<td>A, AII, CI, CII CIII, E</td>
</tr>
<tr>
<td>Composition (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>83</td>
<td>50</td>
<td>31</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Cholesterol + CE</td>
<td>8</td>
<td>22</td>
<td>29</td>
<td>45</td>
<td>30</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>7</td>
<td>18</td>
<td>22</td>
<td>21</td>
<td>29</td>
</tr>
<tr>
<td>Protein</td>
<td>1-2</td>
<td>10</td>
<td>18</td>
<td>25</td>
<td>33</td>
</tr>
</tbody>
</table>

Exogenous lipoprotein metabolism
Chylomicrons are assembled in the enterocytes of the gut for transport of dietary triglycerides to various tissues. ApoB is an essential component of chylomicrons, VLDL and LDL particles. However, for chylomicron assembly in the enterocytes, a truncated form of apoB is synthesized, called apoB48 referring to the N-terminal 48% of apoB100 (114,115). In circulation, triglycerides are hydrolyzed by LPL, which is bound to proteoglycans at the lumenal surface of the endothelial cells lining
the capillary bed. ApoCII in the chylomicron particle stimulates the LPL activity. During triglyceride hydrolysis, cholesterol ester rich chylomicron remnants are produced and catabolized in liver through uptake by the LDL receptor and the low-density lipoprotein receptor-related protein (LRP). The most important factor for clearance of the remnant particles is apoE, which interacts with heparan sulfate proteoglycans, and functions as the ligand for the LDL receptor and LRP (116). In apoE null animals, clearance of the remnant particles is negligible (117,118). An additional function of chylomicrons is to return bile cholesterol to the liver (119).

Endogenous lipoprotein metabolism
In the liver, a more dense triglyceride-rich apoB-containing lipoprotein, called VLDL, is produced. In the human liver, apoB100-containing VLDL particles are produced, while rodents produce both apoB48 and apoB100-containing VLDL particles. In conformity with chylomicrons, LPL hydrolyses VLDL triglycerides, but less efficiently than chylomicrons, resulting in slower plasma clearance of VLDL as compared to chylomicrons. In addition, studies using VLDL receptor deficient and transgenic mice have suggested a role for the VLDL receptor in peripheral clearance of VLDL (120). As a result of triglyceride hydrolysis, intermediate-density lipoprotein (IDL) and finally LDL particles are formed, which are taken up via the LDL receptor in liver, but also in extrahepatic tissues. Studies have shown that the liver accounts for 60-80% of the LDL removal from the plasma (121). The HDL particles, the so-called good cholesterol, has a central role in the transport of excess cholesterol from peripheral cells for elimination in the liver, referred to as reverse cholesterol transport (122). Precursors to HDL, lipid-poor apoAI is synthesized in liver and small intestine, and is converted to HDL particles in the circulation where excess of lipids and apolipoproteins from hydrolyzed VLDL and chylomicron particles are transferred by phospholipid transfer protein (PLTP). Additionally, lipid-poor apoAI interacts with the ABCA1 transporter, which mediates efflux of excess cholesterol and phospholipids to apoAI (123). The discoidal HDL particles are converted to spherical HDL particles by lecinin cholesterol acyltransferase (LCAT), which esterifies cholesterol in the HDL surface to form a neutral lipid core. Cholesterol-loaded HDL particles are transported to the liver for cholesterol uptake mainly via SR-BI.

VLDL assembly
The assembly of apoB-containing lipoproteins is a two-step process taking place in endoplasmic reticulum (ER) lumen and Golgi. The process is best characterized in liver cells, but the chylomicron assembly is probably similar in the enterocytes. In the first step, a partially lipidated apoB particle and a VLDL sized lipid droplet that lacks apoB are formed independently. In the second step, these two precursors are
fused to form a mature VLDL particle (124-127), which is transported through the Golgi apparatus and secreted from the cell. Pulse-chase studies in hepatocytes demonstrate that approximately 50% of the newly synthesized apoB is not secreted but is degraded intracellularly (128). Later studies have shown that the degree of lipidation of the apoB polypeptide chain is of great importance for the stability. If the lipidation is insufficient, apoB will be misfolded and, by retrograde translocation, directed to proteasomal degradation in the cytosol (129,130). However, there is also a non-proteasomal degradation of apoB that occurs post-ER. Insulin and fish oil are examples of activators of this degradation (131,132). Additionally, there are indications that insulin signaling, by reducing activation of phospholipase D activation, may inhibit the second step in the VLDL assembly process (133). Therefore, the overproduction of apoB in the insulin-resistant state may partly be a result of decreased degradation and increased maturation of VLDL particles.

**Microsomal triglyceride transfer protein**

Microsomal triglyceride transfer protein (MTP) is rate limiting in the production of apoB-containing lipoprotein particles in liver and intestine (134-137). Reduction of MTP activity in animals by MTP inhibitors (135) or liver-specific disruption of the MTP gene (138) lowers plasma levels of apoB-containing lipoproteins, whereas overexpression results in increased secretion of apoB-containing lipoproteins (139,140). The importance of MTP for secretion of apoB-containing lipoproteins in human was strengthened by the discovery that people with the rare genetic disease abetalipoproteinemia, who lack MTP, have plasma levels of apoB that are barely detectable (141). MTP interacts with the VLDL assembly process in several crucial steps. First, as a chaperone-like molecule it binds to the N-terminal of apoB, which may be required for the initiation of translocation (142-145). Second, MTP is necessary for co-translational lipid transfer to apoB, which directs apoB away from early proteasomal degradation and toward secretion (127,134,146,147). Third, MTP provides the bulk lipids for the formation of apoB-free lipid droplets that are fused with the pre-VLDL particle (138). However, the fusion of the pre-VLDL and the lipid droplet seems to be MTP-independent (148).

MTP is present in ER as a heterodimer together with protein disulfide isomerase (PDI). PDI is an ER luminal protein that catalyses formation of disulfide bonds during protein folding. However, the PDI subunit is inactive with respect to its isomerase activity in the MTP complex (149,150). Association of MTP with PDI is thought to retain MTP within the ER lumen, as MTP lacks the signal sequence that is required for retention within ER (151). Kinetic studies with model membranes show that MTP transfer lipids by a shuttle mechanism, whereby each MTP interacts transiently with a membrane, extracts lipids, dissociates and deliver lipids to the
second membrane, and becomes available for another cycle of lipid transfer (152). Although MTP is capable of transferring all of the lipid classes found in the apoB lipoproteins, *in vitro* analyses show that it strongly prefers triglycerides and cholesterol esters (153).

The MTP expression has been shown to be regulated by a number of factors. High-fat diet to hamsters and rats gave rise to increased MTP expression (154,155), whereas sterol depletion resulted in reduced MTP levels in HepG2 cells. This decrease was mediated by increased SREBP expression, which in turn repressed transcription of the MTP gene (156). In addition, HNF-4α activates the human MTP promoter (157), whereas the nuclear receptor COUP-TFII was demonstrated to suppress transcription of the MTP gene (157,158). COUP-TFII has previously been shown to repress transcription of several apolipoprotein genes (apoAI (159), apoCIII (160), and apoB (161,162)). In rats, the MTP expression was shown to differ between males and females as a consequence of the sex-differentiated secretory pattern of GH. When GH was administered as continuous infusion to male rats MTP expression was increased to the level of female rats (163). Moreover, insulin and glucose has been shown to decrease the expression of MTP in HepG2 cells (164), and the repressing effect of insulin can be explained by the identification of a negative response element in the human promoter (165). Conversely, hyperinsulinemic, diabetic animals have up-regulated MTP levels and increased apoB secretion (166,167), which might be explained by impaired insulin signaling and/or increased hepatic non-esterified fatty acid (NEFA) flux that may stimulate MTP gene expression.

**Access of triglycerides for VLDL assembly**

The assembly of apoB-containing lipoproteins requires the synthesis of apoB, triglycerides, cholesterol, cholesterol esters and phospholipids. However, the availability of triglycerides, has been shown to be an important determinant of the number and size of VLDL particles secreted into the plasma (128,168,169). There are three potential sources of fatty acids that enter the triglyceride storage pool in liver; *de novo* lipogenesis, plasma NEFA (originating mainly from adipose tissue) and remnant lipoproteins (170). Triglycerides for VLDL assembly has been shown to derive mainly from plasma NEFA (171). The process, by which intracellular triglycerides are mobilized and made available for VLDL assembly in ER is not completely understood. However, several studies have shown that the majority of VLDL triglycerides is derived from intracellular triglyceride stores that must undergo lipolysis before re-esterification and incorporation into VLDL particles (172-174). There are two distinct enzymes responsible for production of triglycerides from diacylglycerol and fatty acyl groups in hepatocytes. Overt
diacylglycerol acyltransferase (DGAT), associated to cytosolic facing ER, has been shown to esterify extracellular fatty acids and de novo synthesized endogenous fatty acids in the cytosolic pool, and these are not direct precursors to VLDL assembly (175). However, latent DGAT in the luminal facing ER is responsible for the re-esterification of fatty acids with diacylglycerol before channelling into the VLDL precursor particle (176). Recently, in mice overexpressing DGAT-1 and DGAT-2, DGAT-1 was shown to be associated to latent DGAT activity, while overt DGAT activity was increased in DGAT-2 overexpressing mice (177). The lipolytic mobilization of cytosolic triglycerides for VLDL assembly has been shown to be dependent upon the activity of triacylglycerol hydrolase (TGH) (178). Overexpression of TGH in the hepatoma cell line McArdle RH7777 resulted in increased VLDL triglyceride secretion as compared to control cells (179). In addition, inhibition of TGH activity in primary hepatocytes as well as in hepatoma cell lines was shown to decrease the secretion of apoB and triglycerides (180). Therefore, one can conclude that the intrahepatic triglyceride mobilization is an important determinant for hepatic VLDL output and a potential target for lipid-lowering therapy. In addition to TGH, arylacetamide deacetylase (AADA) has been proposed to be involved in triglyceride hydrolysis in liver (181). Quantitative studies have shown that there is a rapid turnover of the intracellular triglyceride pool, of which only a small proportion of the hydrolyzed fatty acid are re-esterified into VLDL particles, whereas the remainder is returned back to the cytosolic pool in a futile cycle (172,182). Insulin (172) and MTP inhibition (183) have been shown to increase the flux of re-esterified triglycerides back to the cytosolic pool, rather than into VLDL. For triglyceride mobilization and VLDL assembly see Figure 1.

**VLDL subclasses and formation of atherogenic small dense LDL particles**

Endogenous VLDL particles can be separated into two main subclasses; large triglyceride-rich buoyant VLDL1 particles and smaller and more dense VLDL2 particles. To date, it is not completely understood how the liver is able to regulate the amount of triglycerides that is incorporated into VLDL particles to produce either VLDL1 or VLDL2. However, key factors in the regulation of triglyceride incorporation into VLDL are; availability of lipids within the hepatocytes, MTP expression level and insulin signaling. At mild to moderate hypertriglyceridemia, defined as plasma triglyceride concentration >1.5 mmol/l, the total LDL cholesterol does not necessarily increase, but there is a sharp rise in small dense LDL (184), that are products of hydrolyzed VLDL1 particles (9,185). It might be that hydrolysis of VLDL1 gives rise to LDL particles with an altered apoB100 conformation and impaired binding to the LDL receptor, resulting in a prolonged residence time in the circulation and increased likelihood of remodelling (186). In humans, CETP facilitates inter-particle exchange of neutral lipids, and the preferred acceptor of
transferred cholesterol esters are large triglyceride-rich VLDL1 (187). Therefore, cholesterol esters from the LDL particles are transferred in exchange of triglycerides from VLDL1. Subsequently, HL removes triglycerides from the triglyceride-rich LDL particles, which results in generation of atherogenic small dense LDL. There are several factors that contribute to the atherogenic profile of small dense LDL. The small particle size favours the penetration of LDL particles into the arterial intima, and as a result of poor binding to the LDL receptor, the time for infiltration is prolonged (188). In addition, small dense LDL have higher binding affinity to intimal proteoglycans than large LDL particles, which promotes retention in the arterial wall (189,190). And finally, it might be an increased susceptibility of oxidation of small dense LDL particles (191), which in turn triggers the initiation of processes that finally leads to atherosclerosis.

**INSULIN EFFECTS IN LIVER**

Insulin is the most important physiological stimulus for energy storage, and affects a wide range of physiological processes. However, insulin is best known for its pivotal role in glucose homeostasis. The insulin receptor is expressed in many diverse cell types, but the number of receptors per cell is highest in hepatocytes and adipocytes. The insulin receptor belongs to the class of tyrosine kinase receptors, and consists of two extracellular α-units containing the insulin binding site, and two membrane
spanning β-subunits with intrinsic tyrosine kinase activity. Ligand-binding induces autophosphorylation of tyrosine residues of the β-subunits. This in turn leads to phosphorylation of the insulin receptor substrate (IRS) proteins (193), which function as interface between the insulin receptor and downstream effector molecules (194). In response to nutrient secretagogues, insulin is secreted from pancreas directly into the portal vein. The resulting high portal insulin concentration prime the liver for rapid alterations in hepatic carbohydrate and lipid metabolism. Many of these effects are mediated by fast changes in phosphorylation of enzymes and a more slow regulation of gene expression resulting in decreased gluconeogenesis and increased glycolysis and lipogenesis (195). Studies indicate that the enhanced expression of the lipogenic genes is mediated by the insulin-regulated transcription factor SREBP-1c (196). The importance of insulin action in liver has also been demonstrated in liver-specific insulin receptor knockout mice, which exhibit dramatic insulin resistance; severe glucose intolerance and a failure of insulin to suppress hepatic glucose production (197).

**Hepatic insulin resistance**

A hallmark of insulin resistance and type 2 diabetes is that the liver overproduces lipids as well as glucose, despite the fact that at normal physiological conditions, insulin increases lipid production and inhibits glucose production. An overproduction of both glucose and lipids establish a vicious cycle that aggravates hyperinsulinemia and insulin resistance. In insulin-resistant ob/ob mice and in lipodystrophic mice, chronic hyperinsulinemia was shown to result in down-regulation of IRS-2 (198). In contrast, these mice displayed an increased hepatic expression of SREBP-1c. Experiments in rat hepatocytes showed that insulin had a direct effect on IRS-2 expression, which varied inversely with insulin concentrations (198). It has also been shown that IRS-2 null mice have increased hepatic expression of SREBP-1 (199). Thus, decreased expression of IRS-2, as a result of hyperinsulinemia, is probably responsible for both enhanced gluconeogenesis and increased VLDL secretion seen in the hyperinsulinemic insulin-resistant state. Later studies have proposed a mechanism behind these contradictory effects in the insulin-resistant state. In insulin-resistant ob/ob mice, a discrepancy was demonstrated between activation of the downstream effectors of phosphatidylinositol 3-kinase (PI3-kinase); atypical protein kinase C (aPKC) and protein kinase B (PKB). Activation aPKC was maintained, whereas activation of PKB was impaired (200). This was in agreement with the fact that aPKC has been shown to mediate the insulin-induced expression of SREBP-1c (201). Moreover, PKB activation has been shown to mediate (i) insulin-stimulated glycogen synthesis (202) (ii) the suppressive effect of insulin on gluconeogenesis (203) (iii) the suppressive effect of insulin on glucose-6-phosphatase expression (204). Therefore, as increased activation of liver
aPKC in the hyperinsulinemic state possibly contributes to the development of hyperlipidemia, a conceivable therapeutic effort might be to inhibit aPKC activation in liver.

**ADRP – A LIPID DROPLET-ASSOCIATED PROTEIN**

Most cells are able to store neutral lipids in relatively small (< 1 µm diameter) storage lipid droplets for use as energy source or in membrane biogenesis. However, in the adipocyte that houses huge amounts of triglycerides, the droplets can exceed 50 µm in diameter (205). The core of neutral lipids are surrounded by a monolayer of phospholipids in which proteins are embedded. Studies have shown that numerous proteins are attached to the lipid droplets, suggesting that lipid droplets can be viewed as metabolically active cellular organelles (206,207). Major lipid-droplet associated proteins are perilipin and adipose differentiation-related protein (ADRP), belonging to the so-called PAT-family (Perilipin, ADRP (adipophilin) and TIP47), which exhibit strong sequence homology within their N-terminals (205,208). Perilipin is most abundantly expressed in adipose tissue (208), whereas ADRP appears to be ubiquitously expressed (209,210). Heid et al suggested ADRP to be a marker of lipid accumulation in a variety of cells (210), and increased ADRP protein levels were observed in livers of etomoxir treated rats as a result of inhibited fatty acid oxidation (211). It has been speculated whether increased ADRP expression promotes lipid deposition or if an increased ADRP level merely reflects the increased storage of lipid, i.e. lipid stabilization of ADRP. Both in fibroblasts (212) and in macrophages (213), overexpression of ADRP resulted in increased intracellular triglyceride content, demonstrating that ADRP promotes lipid storage, without induction of lipogenic genes (212). The mechanism behind this effect is not yet clarified. However, like perilipin (214), ADRP expression is suggested to be regulated by a post-translational mechanism that involves stabilization of the protein by association with lipid droplets since contradictory results have been observed comparing the ADRP mRNA and protein levels (205). Few studies have addressed the intracellular function of ADRP. In COS-7 cells, ADRP overexpression was shown to result in increased cellular uptake of long-chain fatty acids (215). Since, ADRP expression has been shown to be transcriptionally regulated by long-chain fatty acids (216), an increased uptake of fatty acids might result in increased ADRP level. Transcription of ADRP has also be shown to be activated by all members of the PPAR family in macrophages and in colorectal cancer cells (217-219), in addition to increased ADRP expression as a result of PPARα activation in kidney cells (220). PPREs were recently identified both in the human and murine ADRP promoters (221,222).
AIMS OF THE THESIS

The general aims of this thesis were to investigate effects of PPARα and PPARγ agonists on gene expression and lipid metabolism in mouse liver, and to study the regulation of PPARγ expression in mouse liver.

The specific aims of Paper I-IV were:

♦ To characterize the effect of rosiglitazone (PPARγ agonist) and Wy14,643 (PPARα agonist) on the expression of enzymes taking part in peroxisomal fatty acid β-oxidation in livers of lean and obese ob/ob mice.

♦ To identify factors that regulate PPARγ expression in primary mouse hepatocytes.

♦ To study whether PPARα activation affects MTP expression and activity in rat and mouse liver.

♦ To investigate if PPARα activation affects ADRP expression in mouse liver, and to explore the importance of changed ADRP expression for the effects of PPARα activation on triglyceride secretion and intracellular triglyceride content of mouse hepatocytes.
METHODOLOGICAL CONSIDERATIONS

Detailed descriptions of the methods are given in each paper. In this section specific issues regarding the major methods are discussed.

PROTEOMICS
- The combination of 2D-electrophoresis and biological mass spectrometry

During the last decade we have witnessed a revolution in molecular biology, and we now have the possibility to analyze the total transcript from a specific cell or tissue. However, since it is the proteins which actually “do the job” within the cell, it is valuable to be able to make a global analysis of the proteins of a specific cell or tissue at a given time under defined biological conditions (=proteomics). Due to the fact that eukaryotic cells express up to at least 10000 different proteins, it is technically challenging to make a proteome analysis. Two-dimensional electrophoresis with immobilized pH gradients (for review see (223)), combined with protein identification by mass spectrometry (MS) is one of the techniques used. The major steps of the work-flow include; sample preparation and protein solubilization, protein separation by 2D-electrophoresis, protein detection and quantification, computer assisted analysis of the 2D-patterns, and protein identification and characterization. However, there are limitations to be aware of.

2D-electrophoresis couples isoelectric focusing in the first dimension with SDS-polyacrylamide gel electrophoresis in the second dimension, and separate proteins according to their isoelectric point and molecular weight, respectively. Since proteins are separated according to the charge in the first dimension, non-ionic detergents are used for protein solubilization. However, this treatment is insufficient for solubilizing hydrophobic membrane proteins, which results in poor recovery of proteins belonging to this class. Moreover, current proteomic studies have revealed that the majority of identified proteins are high-abundant proteins, whereas low-abundant proteins such as receptor molecules are usually not detected. The detection of proteins that are present at low concentrations requires sample prefractionations coupled with narrow pH range gels in the first dimension. Other limitations regarding the 2D technique are; problems separating basic proteins (pI>10), and difficulties separating very large proteins, which do not enter the IPG strips readily in the first dimension because of their size. An advantage using proteomics, compared to global transcript analysis, is the ability to detect co- and post-translational modifications, truncations etc.

In Paper I, silver staining was used for detection and quantification of protein spots, which was the most commonly used sensitive (~1 ng) protein staining technique at that time. However, one drawback using silver staining is related to the
quantification of protein spots since the dynamic range is narrow. Therefore we chose the designation “semiquantitative data”. In a later study a global analysis was performed of the same livers as in Paper I using SyproRuby as staining dye (224). SyproRuby is as sensitive as silver staining but with the advantage of a quantitative linear dynamic range over three orders of magnitude, and compatible with in-gel tryptic digestion. The qualitative data and conclusions, regarding the expression of proteins taking part in the peroxisomal fatty acid oxidation, from that study was in agreement with the results in Paper I.

MS has become the technique of choice for identification of proteins from excised gel spots. Due to the high sensitivity (fmoles), a small amount of sample is required (225). Following in-gel tryptic digestion, identification can be made by peptide mass fingerprinting (PMF) (Fig. 2), which is based on the finding that a set of peptide masses, obtained by MS, matches peptide masses obtained from a theoretical tryptic digestion of an entry in the protein and nucleotide sequence databases queried, e.g. Swissprot and TrEMBL. As this technique is dependent on known sequences, PMF is effective when trying to identify proteins from species whose genomes are completely sequenced and well annotated, and vice versa for organisms whose genomes have not been completed. Another problem is to identify proteins with extensive post-translational modifications, since the peptides generated may not match with the unmodified protein in the database.

Figure 2. Principle of peptide mass fingerprinting. Proteins, separated by two-dimensional gel electrophoresis, are digested by in-gel tryptic digestion. Peptide masses, determined by mass spectrometry, are thereafter matched towards predicted peptide masses from hypothetical tryptic digestion of known protein sequences. Partly modified from PR Graves et al, Microbiol Mol Biol Rev 2002 (226).
DRUG TREATMENT IN VIVO

Doses of rosiglitazone and Wy14,643 in the *in vivo* experiments, were based on results from earlier dose-response studies (A. Kjellstedt, N. Oakes and B. Ljung, unpublished). In those studies, groups of *ob/ob* mice were treated with different doses of the drugs for one week, and plasma glucose, triglycerides and insulin were analyzed. The lowering effects of the compounds on plasma glucose, triglycerides and insulin were determined for each dose, and mean values of the lowering effect of the compounds on these three parameters were calculated. The mean values for the different doses were used to estimate the ED$_{25}$-value (the dose that lowered the mean of glucose, triglycerides and insulin by 25%), as shown in Figure 3. In Paper I, *ob/ob* mice on Umeå background were used. The doses of rosiglitazone (2.5 µmoles/kg/day) and Wy14,643 (180 µmoles/kg/day) were based on the results in Fig. 3A and 3B. In Paper I, the effects of the compounds were shown to be equally effective as in these initial experiments regarding lowering of plasma triglycerides and glucose. In Paper III and IV, C57BL/6 mice were treated with a dose of Wy14,643 (30 µmoles/kg/day) similar to 5xED$_{25}$ (~ED$_{50}$) in *ob/ob* mice on C57BL/6 background (Fig. 3C). In Paper III, it was shown that this dose of Wy14,643 resulted in a significant effect on serum triglycerides, in the same range as observed in the *ob/ob* mice on C57BL/6 background.

**Figure 3.** Determination of ED$_{25}$-values in *ob/ob* mice. Rosiglitazone on Umeå background (A), Wy14,643 on Umeå background (B), and Wy14,643 on C57BL/6 background (C). ED$_{25}$-values were determined after one week treatment with rosiglitazone or Wy14,643, based on mean values of the lowering effect of the compounds on plasma glucose, insulin and triglycerides at different doses (kindly obtained from Ann Kjellstedt).
HEPATOCELLULAR CULTURE

The use of cell cultures provides the opportunity to investigate direct effects on a specific cell type. Since transformed hepatic cell lines lack some differentiated liver functions (227), we have in our work used primary mouse and rat hepatocytes to study regulation of gene expression and lipid metabolism. As compared to transformed cell lines, primary cells maintain in vivo characteristics to a higher degree, even though they may lose some functions during culturing.

Primary hepatocytes were isolated by nonrecirculating collagenase perfusion through the vena porta of anaesthetized C57BL/6 mice, PPARα null mice (Sv129 background) and Sprague Dawley rats (61,228,229). During the first 6 minutes, the liver was perfused with Hanks’ balanced salt solution (HBSS) without calcium and magnesium, and supplemented with EGTA (37°C) at a flow rate of 20-30 ml/min in mice and 40-50 ml/min in rats. The cation-chelating agent EGTA removes calcium, leading to disruption of the Ca\(^{2+}\)-dependent cell-to-cell interactions. Thereafter, perfusion was continued for 7-9 minutes with Williams’ E medium with Glutamax supplemented with 400 mg/l collagenase IV (flow rate as above), which degrades collagen, resulting in a single-cell suspension within the liver capsule. After perfusion, the liver capsule was excised, and cells were filtered through a 250 µm pore size mesh nylon filter followed by a 100 µm pore size mesh nylon filter to remove undigested material. To remove collagenase and non-parenchymal cells, the cells were washed three times at 4°C in plating medium, by centrifugation at 50-60 x g. This procedure enrich the hepatocytes since they have a higher density than the other liver cells. The cells were thereafter counted and seeded at a density of ~105 000 cells/cm\(^2\) in 35 or 60 mm petri dishes coated with matrigel. The matrigel is rich in laminin, proteoglycans and collagen, which are components of the subendothelial space in the normal liver. Hepatocytes cultured on matrigel, have been shown to maintain the hepatic phenotype better compared to hepatocytes cultured on plastic or collagen (230-232). During plating for 16-19 hours, the medium contained a high concentration of insulin as this has been shown to improve plating efficiency and formation of monolayers (233). However, we have not investigated the importance of this procedure when using matrigel. After plating, the cells were cultured for up to 72 hours in medium as described in each paper. In experiments with oleic acid and palmitic acid, 0.75% (w/v) and 1% (w/v) essential fatty-acid free bovine serum albumin was included in the medium, respectively, which resulted in a molar ratio between albumin and oleic acid of 1:4, and between albumin and palmitic acid of 1:1. The effects in the hepatocyte cultures were related to the DNA content in each culture dish, and not to protein content as a consequence of culturing on a protein-rich matrix. However, when hepatocytes were collected for Western blot, the matrigel was removed by collecting the cells in PBS supplemented
with 5 mM EDTA. At normal conditions, cells were cultured in presence of 3 nM insulin. The insulin concentration in the portal blood of fasting rats is 0.34 nM and is several-fold higher at the fed state. The in vitro doses of insulin are therefore near or above the physiological range (234). The doses used of rosiglitazone and Wy14,643, in the in vitro studies, were based on data from reporter gene assays in which the ligand-binding domain of the murine PPARα and PPARγ receptor was the primary target (235). A concentration of 10 µM of each drug resulted in a selective and maximal activation of respective promoter. No agonistic effect of these drugs was observed in reporter gene experiments on PPARβ/δ.

QUANTITATIVE REAL TIME PCR

The advantages using quantitative real time PCR for measuring mRNA compared to other mRNA measurement techniques are that a very small amount of RNA is needed for each analysis and that the method has a high throughput.

SYBR Green was used for detection of the PCR products since the use of probe would have been more expensive. As SYBR Green detects the total amount of double-stranded DNA in the sample, the amplicon size was checked by electrophoresis. In addition, in most experiments dissociation curves were also used to check that a single amplicon was produced. All primers used were designed using the software Primer Express and guidelines from Applied Biosystems. The PCR efficiencies were tested for all primers used, and primer concentrations were used to achieve equal PCR efficiency for the housekeeping gene mouse acidic ribosomal phosphoprotein P0 (36B4) and the target gene. When the PCR efficiencies are similar for the target gene and the house-keeping gene, standard curves are not needed for quantification. Instead a relative quantification by calculating $2^{-\Delta Ct}$ for each sample was made, where $\Delta Ct$ is the difference in threshold cycle (Ct) values between the target gene and the housekeeping gene 36B4.

DETERMINATION OF APOB

The amount of apoB-containing lipoproteins secreted into the cell medium was estimated by $^{35}$S-labeling, followed by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Both apoB48 and apoB100 were precipitated and detected by this method. In a previous study, time course experiments was performed, which are the basis of the time points used in this study (61,236). Two hours $^{35}$S-labeling, results in an intracellular steady-state level of $^{35}$S-labeled apoB, which is followed by four hours chase that is sufficient for complete secretion of the $^{35}$S-labeled pool of apoB.
ADENO VIRAL-MEDIATED OVEREXPRESSION OF ADRP

Using adenovirus to overexpress genes in vitro and in vivo has been shown to be very effective. Since the hepatocytes express high levels of the coxsackie-adenovirus receptor, which mediates the adenoviral uptake, it is favourable to use this technique when overexpression is desired in these cells. The full-length sequence of ADRP or zsGreen was inserted into a adenovirus plasmid that lacks the E1 and E3 regions, which is necessary for replication and to counteract host defense mechanisms, respectively. The adenovirus expression vector was thereafter transfected into HEK cells which express E1, resulting in virus amplification. Following large-scale amplification, the virus were purified before infectious viral titers were determined.
SUMMARY OF RESULTS

PAPER I

Rosiglitazone (BRL49653), a PPARγ-selective agonist, causes peroxisome proliferator-like liver effects in obese mice.

Treatment with PPARα agonists in mice is known to result in increased expression of proteins involved in the oxidation of fatty acids. In a previous study, obese mice were treated with the PPARα agonist, Wy14,643, and liver proteins were analyzed by two-dimensional electrophoresis. Several protein spots were found to be up-regulated by treatment, and many of these were identified as proteins involved in the peroxisomal fatty acid β-oxidation (237). In this study we aimed to investigate if rosiglitazone affects the same set of proteins in liver as the PPARα agonist Wy14,643.

Obese diabetic ob/ob mice and lean littermates were treated with rosiglitazone and Wy14,643 for one week. The doses of the two compounds were equally effective regarding lowering of plasma glucose and triglycerides in obese mice. Liver proteins were separated by two-dimensional gel electrophoresis. The same set of protein spots (n=15), previously identified as proteins involved in the peroxisomal fatty acid β-oxidation and aP2 (237), were quantified and compared between the groups. In lean mice, Wy14,643 up-regulated all 15 spots, including ACO, peroxisomal bifunctional enzyme, 3-ketoacyl-CoA thiolase and aP2, whereas the expressions of these proteins were unaffected by rosiglitazone treatment. Interestingly, in obese mice Wy14,643 and rosiglitazone induced significant up-regulation of 14 and 9 spots, respectively. Since each enzyme was represented by several spots (discussed in Paper I) it is important to note that rosiglitazone enhanced the expression of at least one spot of each enzyme. Thus, rosiglitazone caused a similar, but less pronounced induction of peroxisomal enzymes as compared to Wy14,643. As PPARα and PPARγ has been shown to bind to a common consensus response element (PPRE) (238,239), it was conceivable that PPARγ, if expressed in liver, binds to same PPRE as PPARα and hence mimic PPARα-activated transcription. Therefore we measured PPARγ mRNA levels in liver of lean and obese mice, and found that PPARγ2 expression was markedly increased in livers of obese mice, while PPARγ1 expression was similar in obese and lean mice.

In conclusion, at equally effective doses, regarding hypoglycemic and hypolipidemic effects, rosiglitazone and Wy14,643 up-regulate proteins involved in peroxisomal fatty acid β-oxidation in livers of obese mice, whereas only Wy14,643 induces these changes in lean mice. Since livers of obese mice displayed higher expression of
**SUMMARY OF RESULTS**

*PPARγ2* mRNA levels, we suggest that *PPARγ2* activation contributes to the peroxisome proliferator-like effects of rosiglitazone in obese mice. These findings may suggest that, in obese diabetic mice, rosiglitazone has effects in liver that might contribute to the therapeutic effect.

**PAPER II**

**Insulin and oleic acid increase *PPARγ2* expression in cultured mouse hepatocytes.**

Hepatic expression of PPARγ has been shown to be elevated in several mouse models of obesity and diabetes (Paper I) (104,105,240-243). The physiological role of hepatic PPARγ expression has been studied in several models. Overexpression of PPARγ1 in PPARα null mice (244) and of PPARγ2 in AML 12 hepatocytes (245), gave rise to increased lipid accumulation. Conversely, in *ob/ob* mice (246) and in lipoatrophic A-ZIP/F-1 mice (242), liver specific PPARγ disruption was shown to alleviate liver steatosis, but resulted in impaired triglyceride clearance and insulin sensitivity. Together these results indicate a significant role for PPARγ in the development of fatty liver, but also that PPARγ in liver is important for normal triglyceride homeostasis. To get more insight into the mechanism, whereby PPARγ expression is induced in liver, we intended to identify factors that directly affect the expression of PPARγ in primary mouse hepatocytes.

Primary mouse hepatocytes were incubated in absence or presence of oleic acid (500 µM), insulin (30 nM) and high concentration of glucose (22 mM) for three days. We found that the expression of PPARγ2 mRNA, but not PPARγ1, was increased by insulin and to lesser extent by oleic acid. The effect of insulin on PPARγ2 mRNA was evident after 24 hours incubation with insulin, and stable for up to 72 hours. Actinomycin D blocked the effect of insulin, indicating a transcriptional effect. By incubating hepatocytes with different kinase inhibitors we showed that the stimulatory effect of insulin was mediated via the PI3-kinase pathway. To investigate if insulin influenced the effect of rosiglitazone we measured the expression of PPARγ1, PPARγ2, aP2 and ACO after three days incubation. The PPARα agonist Wy14,643 was also included in these studies, as it is known to increase the expression of aP2 and ACO in liver. The expression of all genes, except for PPARγ1, were up-regulated by insulin without addition of rosiglitazone or Wy14,643, which might be explained by the fact that PPARγ is activated by insulin in a ligand-independent manner (247). However, a direct effect of insulin on these genes can not be excluded. Neither insulin nor rosiglitazone affected the PPARγ1 expression, while PPARγ2 was induced by rosiglitazone insulin-dependently. The effect of oleic acid on PPARγ2 expression was independent of insulin, indicating
SUMMARY OF RESULTS

that different mechanisms are involved when comparing the stimulatory effects of rosiglitazone and oleic acid. The expression of aP2 was enhanced by rosiglitazone and Wy14,643, however the effect of rosiglitazone was shown to be potentiated in presence of insulin. A synergistic effect of TZD and insulin on aP2 expression has previously been reported in adipocytes (248). Both rosiglitazone and Wy14,643 increased the expression of ACO insulin-independently (2-fold and 20-fold, respectively). PPREs have been identified in the promoters of both aP2 (21) and ACO (249), but to the best of our knowledge a putative PPRE in the PPARγ2 promoter has not been identified.

In conclusion, this is the first study that shows a direct effect of insulin and oleic acid in the regulation of PPARγ expression in primary hepatocytes. The finding that only the PPARγ2 isoform was induced by insulin and oleic acid, help to understand the observations that the hepatic PPARγ2 expression is increased in several in vivo models of obesity and insulin resistance, as documented in Paper I. Future studies may reveal the mechanism behind the stimulatory effect of insulin on PPARγ2 expression, which might play an important role in the development of fatty liver.

PAPER III

**Activation of peroxisome proliferator-activated receptor α increases the expression and activity of microsomal triglyceride transfer protein in the liver.**

Previous studies, both in vivo and in vitro, have shown that PPARα agonists reduce the VLDL triglyceride secretion from liver and hepatocytes, respectively (63,229). However, the secretion of apoB100 from rat hepatocytes increased after incubation with the PPARα agonist Wy14,643, showing that an increased number of apoB-containing lipoprotein particles was secreted into the medium (229). Since MTP plays a crucial role in the assembly of apoB-containing lipoproteins, we aimed to investigate if PPARα activation resulted in increased MTP expression and activity.

Treatment of mice and rats with the PPARα agonist Wy14,643, resulted in increased MTP mRNA and protein levels, which was paralleled by increased MTP activity. The effect of Wy14,643 was shown to be PPARα dependent, since the effect was abolished in hepatocytes from PPARα null mice. However, the RXR ligand, 9-cis-retinoic acid, increased the MTP mRNA expression independently of PPARα, which might be a result of transcriptional activation mediated by RXR homodimers (27). The effect of Wy14,643 in rat hepatocytes was blocked by Actinomycin D, indicating a transcriptional effect. Additionally, McA-RH7777 cells were transfected with vectors containing either a conserved or mutated DR-1 element (158) of the MTP promoter that was coupled to the luciferase gene. In reporter gene assays,
SUMMARY OF RESULTS

Wy14,643 was shown to activate transcription of the wild type promoter in a dose-dependent manner, whereas the cells transfected with the mutated DR-1 element were unresponsive to Wy14,643. Finally, we measured apoB secretion from rat hepatocytes exposed to Wy14,643. In line with previous findings (229), Wy14,643 increased the apoB100 secretion whereas apoB48 secretion was unaffected. In time course studies, we showed that both MTP protein expression and apoB100 secretion was increased between 24 and 72 hours incubation with Wy14,643.

In conclusion, PPARα activation increases MTP expression in hepatocytes through transcriptional activation of the MTP gene. The PPARα-induced increase in apoB100 secretion was paralleled by increased MTP protein levels, indicating that the influence of PPARα agonists on apoB100 secretion could be mediated by increased expression of MTP.

PAPER IV

PPARα activation increases triglyceride mass and adipose differentiation-related protein in hepatocytes

In humans, treatment with PPARα agonists, i.e. fibrates, results in decreased plasma levels of triglycerides and increased plasma HDL cholesterol levels (15,250). The hypotriglyceridemic effect of fibrates is partly explained by down-regulation of hepatic apoC-III expression (66,251) and increased lipoprotein lipase expression (65), which results in increased turnover of VLDL particles. In addition, fibrates have been shown to decrease the VLDL triglyceride production both in vivo in human and rat (62,63), and in vitro in rat hepatocytes (229). Since PPARα agonists are known activators of fatty acid oxidation (55), the enhanced catabolism of fatty acids is believed to contribute to the reduced VLDL triglyceride secretion. ADRP is a lipid-droplet associated protein that has been shown to be regulated by PPARs in macrophages, colorectal cancer cells and in kidney (217-220), and PPREs have been identified both in the human and murine ADRP promoters (221,222). In this study we aimed to investigate the effects of the specific PPARα agonist Wy14,643, on the expression of ADRP in mouse liver in vivo and in primary mouse hepatocytes in vitro, and to determine the importance of changed ADRP expression for the effects of PPARα activation on triglyceride secretion and intracellular triglyceride accumulation.

Wy14,643 treatment was shown to increase the ADRP mRNA expression in mice fed chow or high-fat diet. However, ADRP protein levels were only clearly up-regulated in mice fed chow diet. High-fat diet per se was shown to induce the ADRP protein expression, and Wy14,643 had no further effect. Wy14,643 treatment
increased the hepatic triglyceride content by 33% in mice fed chow diet and to lesser
degree in mice fed high-fat diet (15%). The effect of Wy14,643 on ADRP
expression was shown to be mediated via PPARα activation since the effect was
abolished in the PPARα null mice. Also in primary mouse hepatocytes, three days
incubation with Wy14,643 resulted in increased ADRP mRNA and protein levels,
which was paralleled by increased hepatic triglyceride content. Since Wy14,643
stimulated the fatty acid oxidation in the hepatocytes, the increased triglyceride
content was surprising. Therefore, we studied the effect of Wy14,643 on triglyceride
synthesis, measured as 3H-palmitic acid incorporation. As Wy14,643 did not affect
the triglyceride biosynthesis, we further studied the triglyceride secretion. Wy14,643
incubation was shown to decrease the triglyceride secretion by 50%, indicating that
decreased secretion may contribute to the triglyceride accumulation in the cells.
Since ADRP overexpression has been shown to increase the intracellular triglyceride
level in other cell types (212,213), we hypothesized that the increased triglyceride
mass and decreased secretion might be a consequence of increased ADRP
expression. Indeed, overexpression of ADRP in hepatocytes resulted in decreased
triglyceride secretion. However, Wy14,643 incubation of ADRP overexpressing
cells resulted in a further decrease in triglyceride secretion, even though the ADRP
protein levels were unchanged, indicating that the increased ADRP expression was
not the sole mechanism for the decreased triglyceride secretion. Wy14,643 enhanced
the palmitic acid oxidation also in ADRP overexpressing cells, which might
contribute to decreased triglyceride secretion. However, the triglyceride content in
ADRP overexpressing cells was unaffected by Wy14,643, showing that increased
fatty acid oxidation did not result in lack of cellular triglycerides for VLDL
secretion in ADRP overexpressing cells.

In conclusion, PPARα activation increases ADRP protein expression and
triglyceride mass in hepatocytes. PPARα activation does not primarily decrease
triglyceride secretion via enhanced fatty acid oxidation. Rather, PPARα activation
prevents the availability of triglycerides for VLDL assembly, in part by increasing
the expression of ADRP.
DISCUSSION

PAPER I AND II

Effects of TZDs in liver
In Paper I, we showed for the first time that treatment with rosiglitazone caused a peroxisome proliferator-like liver effect in obese mice. Since an increased peroxisomal fatty acid oxidation may reduce the availability of triglycerides for VLDL secretion this effect might contribute to the therapeutic effect in obese mice. Thereafter, others have shown that both a TZD and a specific PPARβ/δ agonist were able to induce peroxisome proliferation in PPARα null mice (252). We have observed that rosiglitazone induces the expression of ACO in primary mouse hepatocytes, demonstrating a direct effect of a TZD on a typical PPARα target gene in the cells (Paper II). These results show that a functional overlap exists between the members of the PPAR family. Treatment with the PPARγ agonist GW1929 in Zucker diabetic fatty rats, gave rise to decreased hepatic mRNA levels of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase, whereas the expression of glucokinase increased, suggesting that PPARγ agonists decrease gluconeogenesis and increase glycolysis in the liver (253). These effects can of course be secondary to increased insulin sensitivity and maybe not a direct effect of hepatic PPARγ activation. However, in hepatocyte cultures, TZDs have been shown to increase the levels of GLUT2 and glucokinase, via activation of functional PPREs, suggesting that GLUT2 and glucokinase are direct targets of PPARγ in liver that upon activation may lead to increased glucose uptake and glycolysis, and also lipogenesis (254). Taken together, these studies propose that TZDs are able to directly affect both fatty acid and carbohydrate metabolism in liver cells.

In Paper I, we described effects of rosiglitazone in livers of obese animals, whereas the protein expression in livers of lean mice was unaffected. We suggested that this finding was a consequence of increased hepatic expression of PPARγ2 in the obese animals. In addition to our findings, several other studies have demonstrated elevated levels of PPARγ2, or total PPARγ, in livers of obese diabetic mice and in lipoatrophic A-ZIP/F-1 mice (104,240-243). In A-ZIP/F-1 mice, hepatic PPARγ disruption abolished the hypoglycemic and hypolipidemic effect of rosiglitazone, demonstrating that the liver is the primary site of TZD action in mice lacking adipose tissue (242). However, in wild type mice, made insulin-resistant by high-fat diet, the hypoglycemic and hypoinsulinemic effects of rosiglitazone were independent of hepatic PPARγ expression, showing that the major site of action in normal mice, is the adipose tissue (242). In accordance with our report, other studies have also shown that the effects of TZDs on hepatic gene expression were more
pronounced in \textit{ob/ob} and in A-ZIP/F-1, which is in line with a higher hepatic PPAR\(\gamma\) expression in these animals (242,243). Together, these findings show that the effects of TZDs in liver are more prominent when the PPAR\(\gamma\) expression is elevated. One can speculate that an increased PPAR\(\gamma\) expression gives rise to competitive replacement of DR-1 recognizing nuclear receptors that act as transcriptional suppressors, such as COUP-TFI (255).

**Regulation of PPAR\(\gamma\) expression in liver**

The endogenous signals that regulate hepatic PPAR\(\gamma\) expression have not previously been investigated in detail. However, high fat and high cholesterol diet have been shown to induce PPAR\(\gamma\) in livers of obese mice (82,256,257). In Paper II we demonstrated that insulin markedly increased the PPAR\(\gamma\)2 expression in primary mouse hepatocytes in a dose-dependent manner. Oleic acid was also shown to increase the PPAR\(\gamma\)2 expression, but to a lesser extent. Interestingly, the PPAR\(\gamma\)1 expression in cultured hepatocytes was unaffected by either of the treatments. We did not study the effects at different concentrations of oleic acid. However, the concentration of oleic acid was 500 \(\mu\)M, which is in the high range in the context of cell culturing. Therefore, the effect of oleic acid is most likely in the maximal range. Preliminary data from ADRP western blot analyses (not shown) indicate that the lipid content is markedly higher in cells exposed to oleic acid than to insulin, suggesting that PPAR\(\gamma\) expression is not directly correlated to the intracellular lipid content. These results are in agreement with studies in mice, showing that liver steatosis \textit{per se}, induced by choline deficient diet or fasting, failed to induce hepatic PPAR\(\gamma\) expression (244). In contrast to rosiglitazone, the stimulatory effect of oleic acid on PPAR\(\gamma\)2 expression was not dependent on insulin. This finding indicates that the effect of oleic acid was not mediated by PPAR\(\gamma\). However, one conceivable mediator of the effect of oleic acid could be PPAR\(\beta/\delta\), which has been shown to induce PPAR\(\gamma\)2 expression in 3T3 fibroblasts (110). The effect of insulin on PPAR\(\gamma\)2 expression was mediated via activation of the PI3-kinase pathway and was stable for up to 72 hours incubation in presence of 30 nM insulin. Since studies in rat hepatocytes showed that 100 nM of insulin for 24 hours resulted in an insulin-resistant hepatic phenotype (198), one may assume that the effect in our hepatocyte experiments occur despite a decreased insulin sensitivity. The insulin-induced SREBP-1c expression in liver has been shown to be mediated by aPKC, which acts downstream of PI3-kinase (201). Since aPKC signaling has been shown to be maintained in the livers of insulin-resistant \textit{ob/ob} mice (200), one may speculate that aPKC might play a role in the insulin-induced PPAR\(\gamma\)2 expression. Thus, the stimulatory effects of insulin and oleic acid on PPAR\(\gamma\)2 expression in cultured mouse hepatocytes might help to explain the elevated PPAR\(\gamma\)2 expression in insulin-resistant mouse models.
DISCUSSION

Several metabolic pathways are regulated in a reciprocal fashion by insulin and glucagon, and this might also be the fact for the regulation of PPARγ in liver. Herzig et al have shown that, in response to glucagon and cortisol, the transcription factor cAMP responsive element binding protein (CREB) activated the gluconeogenic and fatty acid oxidation programmes, whereas the PPARγ1 expression was suppressed in parallel with decreased triglyceride synthesis (258). Since glucagon decreased PPARγ1 expression one can speculate that insulin has the opposite effect on PPARγ1 in that model. Unfortunately, the PPARγ2 isoform was not studied in that paper. However, it might be that glucagon decreases only the PPARγ1 isoform whereas only the PPARγ2 isoform is induced by insulin.

Physiological role of PPARγ expression in liver
The significance of PPARγ expression in liver has been studied by hepatic overexpression of PPARγ1 in PPARα null mice. The resulting phenotype displayed increased expression of adipogenic and lipogenic genes accompanied with liver steatosis (244). Wild type mice, overexpressing PPARγ1 in liver, also exhibited hepatic lipid accumulation, but not as severe as seen in the PPARα null mice, indicating that the presence of PPARα reduced lipid accumulation. In addition, typical PPARα responsive genes, such as enzymes taking part in the peroxisomal fatty acid oxidation, were also up-regulated in the PPARγ1 overexpressing liver, again reflecting overlapping effects of the two receptors. Overexpression of PPARγ2 has been studied in vitro, in the murine hepatic cell line AML-12. As a consequence of increased PPARγ2 expression, these cells had elevated levels of lipogenic enzymes, such as SREBP-1, fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC), which was paralleled by increased triglyceride synthesis and lipid accumulation (245). The triglyceride synthesis was enhanced by troglitazone, which gave rise to an additional lipid accumulation in the PPARγ2 overexpressing cells.

Liver-specific disruption of PPARγ has been studied in ob/ob mice (246) and in lipoatrophic A-ZIP/F-1 mice (242), which both exhibit elevated hepatic PPARγ expression and liver steatosis in the control situation. In both strains, a loss of PPARγ resulted in reduced hepatic steatosis, but impaired muscle insulin resistance and triglyceride clearance. Interestingly, in parallel with the findings in the A-ZIP/F-1 and ob/ob mice, hepatic PPARγ disruption gave rise to impaired triglyceride clearance also in the wild type mice (242). With aging these mice also developed obesity, hyperlipidemia and insulin resistance (242), indicating that PPARγ expression in the liver is important for triglyceride and glucose homeostasis in the normal state. The target genes and molecular mechanisms by which PPARγ mediates lipid clearance remain largely unknown. However, there are several factors that may contribute to this effect. Expression of the LDL receptor was increased in
livers of PPARγ overexpressing mice (244), whereas the expression was decreased in A-ZIP/F-1 mice lacking PPARγ (242), which may indicate a role for PPARγ in the regulation of the LDL receptor. In addition, troglitazone has been shown to increase the expression and activity of the LDL receptor in HepG2 cells (259). The expression of CD36 in mouse liver has been shown to be up-regulated by TZDs (243,246), in agreement with a decreased CD36 expression in livers that lack PPARγ (242). The role of CD36 in the liver remains unclear, however, as a lipoprotein receptor/fatty acid transporter it might influence lipid uptake into the liver. In addition to an increased hepatic lipid uptake, an increased hydrolysis of triglyceride-rich lipoprotein particles would also give rise to enhanced triglyceride clearance. Postheparin LPL activity was not shown to be affected by liver PPARγ disruption in ob/ob mice (246). However, the level of apoCIII was not determined in that study, and a recently published paper demonstrated that the apoCIII production rate was reduced in type 2 diabetic subjects treated with pioglitazone (260), which most likely results in increased turnover of triglyceride-rich lipoprotein particles. To summarize, these data demonstrates that hepatic PPARγ plays a role in the triglyceride partitioning between liver and other tissues, contributing to regulation of body fat mass and glucose homeostasis in normal mice.

Is the PPARγ-mediated lipid accumulation in hepatocytes a species-specific phenomenon?
TZDs are thought to exert the therapeutic action partly by the “lipid steal mechanism”. Several studies, both in rats and humans, have demonstrated that TZDs prevent fatty-acid induced insulin resistance by repartitioning of fat from skeletal muscle and liver into adipocytes (261-263). Activation of PPARγ in adipose tissue results in decreased adipose tissue lipolysis (264) and increased adiponectin expression (101), which could counteract liver steatosis. However, in diabetic, obese mice and in lipoatrophic mice, treatment with TZDs gives rise to increased hepatic lipid accumulation (104,242,246,265,266). ob/ob mice, lacking hepatic PPARγ, did not develop liver steatosis by TZD treatment, indicating that the development of liver steatosis was mediated via hepatic PPARγ activation (246). The increased triglyceride accumulation in liver might be a result of increased fatty acid uptake and de novo lipogenesis, since genes, such as FAS, ACC and stearoyl-CoA desaturase (SCD)-1 were up-regulated in cells overexpressing PPARγ2 (245) and in mice treated with TZD (242,246). However, it must be pointed out that the effect of TZDs on lipid accumulation and gene expression only was noticed in mice with elevated PPARγ expression. This effect in mouse liver might be species-specific as the liver steatosis is alleviated by TZD in diabetic rats and humans (267-270). Moreover, treatment with TZDs has been shown to improve biochemical and histological features of NASH in clinical studies (271,272). Studies are required to elucidate the
mechanisms behind these species-differences. Since the effect of PPARγ overexpression has been studied in mice (244) and in a murine hepatic cell line only (245), it would be interesting to perform studies of PPARγ overexpression and TZD treatment also in human hepatic cells. In addition, these species-specific differences may also be a result of different effects in the adipose tissue that indirectly might affect the hepatic triglyceride level or that PPARγ is not up-regulated in the liver of diabetic humans.

PPARγ expression in human liver
In contrast to PPARα, PPARγ is most likely expressed at a higher level in human liver as compared to mouse and rat liver (83). However, data from other studies (one subject/study) indicate that the PPARγ expression in human liver can differ to a large extent between individuals (273,274). To the best of our knowledge, no studies have been performed to investigate hepatic PPARγ expression in type 2 diabetic subjects. Since population surveys have convincingly demonstrated an association between hyperinsulinemia and hepatic steatosis (275,276), one may speculate that one of the underlying mechanisms in the development of liver steatosis is an increased hepatic PPARγ expression in hyperinsulinemic individuals.

PAPER III AND IV

PPARα activation reduces the secretion of triglyceride-rich VLDL1 particles
An increased production of triglyceride-rich VLDL1 particles in the liver is believed to be the culprit underlying the development of atherogenic dyslipidemia. The mechanisms whereby the liver regulates the amount of triglycerides that is incorporated during the VLDL assembly is not completely understood, but key factors are: availability of lipids, MTP expression level and insulin signaling. Both in human and rat, treatment with fibrates has been shown to result in decreased VLDL triglyceride secretion from the liver (63,277). By lowering VLDL triglyceride content, fibrates inhibit the formation of slowly metabolized, potentially atherogenic small dense LDL particles (185). In rat hepatocytes, Wy14,643 was shown to increase apoB100 secretion, even though the triglyceride biosynthesis was decreased, resulting in secretion of increased number of apoB-containing lipoprotein particles with a higher density than VLDL (229). In rats, a reduced hepatic triglyceride secretion was observed despite unchanged palmitate incorporation (63), demonstrating that other mechanisms could be involved than decreased triglyceride synthesis.
DISCUSSION

Effects of PPARα activation on MTP expression and activity in the liver
Since MTP is rate-limiting in the assembly and secretion of apoB-containing lipoprotein particles, we aimed in Paper III to elucidate if changed MTP expression and activity could contribute to the increased apoB100 secretion that was previously noticed in rat hepatocytes (229). We found that treatment with Wy14,643 resulted in increased MTP expression and activity in mice and rats both in vitro and in vivo, which was accompanied with increased apoB100 secretion in rat hepatocytes. Moreover, we also identified a PPRE in the promoter of the MTP gene, that previously was identified to be responsive to RXRα (158). As PPARα is activated by fatty acids and fatty acid derivatives, one may speculate that the high-fat diet induced MTP expression (154,155) could in fact be mediated by PPARα. However, another conceivable mediator is HNFα, which is activated by fatty acids, and has been shown to stimulate transcription of the MTP gene (157). The increased MTP activity may help to explain the elevated LDL cholesterol levels that have been reported in some clinical studies with fibrates (278). In Paper III, we showed that the plasma level of apoB was decreased as a result of treatment with Wy14,643, even though an increased apoB100 secretion was noticed in cultured hepatocytes. However, the reduced apoB level is most likely a result of faster turnover of apoB-containing lipoproteins after Wy14,643 treatment, which in turn may be due to formation of triglyceride-poor lipoprotein particles with shorter half-life (185), in combination with decreased apoCIII level (66,251) and increased LPL activity (65). In contrast to the stimulatory effect of Wy14,643 on apoB100 secretion in cultured rat hepatocytes, apoB48 secretion was unaffected (Paper III), which is consistent with previous finding that only apoB100 secretion was induced (229). Studies on mice with liver-specific MTP disruption have shown that the secretion of apoB48 is less MTP dependent, since the plasma level of apoB48 was reduced only by 20% in these mice, as compared to 95% reduction of apoB100 (138). A minor effect on apoB48 secretion was also noticed in isolated hepatocytes lacking MTP, demonstrating that the results in vivo was not a confounding effect of apoB48 secretion from the intestine (138). Therefore, one can conclude that apoB48 can be secreted as lipid-poor particles from hepatocytes also in absence of MTP. The requirement of MTP for apoB100, in contrast to apoB48, may be explained by the finding that MTP inhibition did not block the secretion of apoB polypeptides of less than 65% of full length apoB100 in HepG2 cells, indicating that MTP is more important for secretion of longer apoB polypeptide chains (146). However, in mice overexpressing MTP, apoB48 and apoB100 contributed equally to the increased VLDL apoB secretion (139). Together, these studies indicate that, at different conditions, MTP contribute to a different degree to the production of apoB48. In contrast to our findings in rat hepatocytes (Paper III) (229), Fu et al showed that ciprofibrate inhibited apoB mRNA editing in mice, resulting in increased apoB100
secretion concomitantly with decreased apoB48 secretion (279). Since our experiments were performed in rat hepatocytes, one can not exclude that a species difference exist regarding the effect of PPARα activation on apoB mRNA editing. However, preliminary results from studies on mouse hepatocytes in our lab show that secretion of both apoB48 and apoB100 are increased as a result of PPARα activation, indicating that the increased apoB100 secretion is not a result of decreased apoB mRNA editing in mouse liver.

**Hepatic triglyceride accumulation and PPARα activation**

In Paper IV, we showed that Wy14,643 incubation resulted in decreased triglyceride secretion from mouse hepatocytes, which was in accordance with previous studies in rat hepatocytes (229). In contrast to the result in rat hepatocytes, the triglyceride biosynthesis was unaffected by Wy14,643 in cultured mouse hepatocytes. Even though Wy14,643 increased the fatty acid oxidation 4-fold in these cells, the cellular triglyceride mass was increased. Since the triglyceride biosynthesis was unaffected in the cells, we hypothesized that the increased triglyceride mass was a result of the reduced triglyceride secretion. Few studies have addressed the effect of PPARα activation on hepatic triglyceride content. In line with our results, fenofibrate treatment of rats resulted in elevated liver triglyceride levels (280). In addition, in rat hepatocytes bezafibrate was shown to induce the intracellular triglyceride content (281). Several studies have shown that fibrates induce the expression of lipogenic genes in liver (224,257,282), and Knight et al showed that Wy14,643 treatment in mice gave rise to increased fatty acid synthesis. However, the liver triglyceride or cholesterol ester content was not increased in that study (257), which might indicate a compensation by increased fatty acid oxidation or increased synthesis of phospholipids. Indeed, studies have been performed demonstrating that PPARα is required for maintenance of phospholipid homeostasis in the liver (283).

Our results that Wy14,643 increased the hepatic triglyceride content are contradictory to the effects of fibrates in animal models with liver steatosis, where alleviation of liver steatosis has been demonstrated (284-286). Interestingly, preliminary data from mouse hepatocytes experiments showed that ADRP expression was reduced by Wy14,643 in presence of oleic acid, indicating a decreased intracellular triglyceride content. Together, these data suggest that the effect of fibrates on hepatic triglycerides seems to be dependent on the metabolic situation and fatty acid load.

**Function of ADRP in liver cells**

In Paper IV, we showed that Wy14,643 increased the hepatic expression of ADRP in vivo and in cultured mouse hepatocytes. Since overexpression of ADRP has been
shown to induce triglyceride accumulation both in macrophages (213) and in fibroblasts (212) we hypothesized that the increased ADRP expression contributed to the increased triglyceride mass in hepatocytes. Indeed, in ADRP overexpressing hepatocytes (Paper IV), there was an increased triglyceride mass that, at least partly, might be due to a reduced triglyceride secretion. In addition, ADRP overexpression also resulted in decreased palmitic acid oxidation and increased triglyceride synthesis. An additive effect of ADRP on fatty acid uptake can not be excluded, since ADRP has been shown to promote uptake of long-chain fatty acids in COS-7 cells (215). The mechanism whereby ADRP inhibits triglyceride secretion is not elucidated. One study proposes that ADRP may decrease the hydrolysis of the cytosolic triglyceride pool (287), which is a prerequisite for VLDL production. Perilipin, another PPAR regulated member of the PAT family (93), which predominantly is expressed in adipose tissue, has been shown to reduce the access of cytosolic lipases to stored triglycerides in the fed state (288). In addition, studies have shown that perilipin is required to elicit hormone sensitive lipase (HSL) translocation to the surface of the lipid droplet, indicating that lipolysis in adipose tissue is a concerted action mediated by both HSL and perilipin (289). Further studies in ADRP overexpressing cells will elucidate if ADRP inhibits the triglyceride hydrolysis in hepatocytes. If that is the case, it is tempting to speculate that, in parallel with perilipin, an interaction may exist between ADRP and cytosolic facing lipases.

**Conceivable mechanisms behind the decreased triglyceride secretion by PPARα activation**

An increased fatty acid oxidation by fibrates (55) has, by many, been proposed to contribute to decreased VLDL secretion (64). However, in Paper IV we show for the first time that despite an increased oxidation of fatty acids, there is not a lack of triglycerides for the VLDL assembly, see Fig. 4. Therefore, one can conclude that other mechanisms are influenced by fibrates, resulting in decreased availability of triglycerides for VLDL assembly and secretion. Since ADRP overexpression resulted in decreased triglyceride secretion and increased intracellular triglyceride mass (Paper IV), we speculated that the increased ADRP expression by Wy14,643 incubation was the primary cause for the reduced triglyceride secretion. However, in ADRP overexpressing cells, Wy14,643 further reduced the triglyceride secretion despite unaffected ADRP protein level. These results indicated that the increased ADRP expression can not be the sole mechanism for decreased triglyceride secretion. Wy14,643 increased the fatty acid oxidation also in ADRP overexpressing cells, which might result in lack of triglycerides for VLDL production. However, the triglyceride mass was not decreased, suggesting that also other mechanisms are responsible for the prevention of the use of cytosolic triglycerides for VLDL
synthesis. There are several conceivable mechanisms that may contribute to this effect. Waterman et al have shown that fenofibrate raised overt DGAT activity but lowered that of latent DGAT in rat liver, which was paralleled by increased hepatic triglyceride content (280). These findings might be explained by a recently published paper, showing that overexpression of DGAT1 (latent) resulted in increased VLDL secretion, while overexpression of DGAT2 (overt) resulted in increased liver triglyceride content and unaffected VLDL secretion (177). These data suggest that DGAT1 plays an important role in VLDL synthesis, whereas increased DGAT2 activity has a role in steatosis. The triglyceride biosynthesis was not affected by Wy14,643 in cultured hepatocytes (Paper IV), however since we measured the total pool of newly synthesized triglycerides we can not exclude that there is a shift in the DGAT activities in the hepatocytes exposed to Wy14,643, resulting in an increased cytosolic pool of triglycerides.

Another possible mediator of the hypotriglyceridemic effect of fibrates is apoAV (68,69), and studies have been performed to elucidate the mechanism of its triglyceride lowering effect. In mice, adenovirus-mediated apoAV overexpression gave rise to increased LPL mediated hydrolysis as well as a decreased VLDL triglyceride secretion, despite unaffected apoB secretion (290). Studies in apoAV transfected COS-1 cells demonstrated that apoAV was poorly secreted and remained associated with the endoplasmic reticulum, suggesting that a main function of apoAV could be to modulate hepatic VLDL assembly and/or secretion (291). However, in human apoAV transgenic mice the hypotriglyceridemic effect was only attributed to increased LPL mediated hydrolysis of VLDL (292). Since different animal models were used, the discrepancy might be explained by an adaptive mechanism in mice continuously expressing high levels of apoAV in the liver, as well as functional dissimilarities between the mouse and human apoAV protein.

An additional conceivable mechanism of the fibrate-induced decreased VLDL secretion might be a reduced availability of other VLDL components than triglycerides. The availability of neutral lipids in the hepatocyte has a profound effect on VLDL synthesis and secretion (168,293,294). In addition, synthesis of phosphatidylcholine (PC), the major phospholipid component of VLDL, is also required for efficient VLDL production. PC is synthesized via the CDP-choline pathway, and also via the phosphatidylethanolamine (PE) methylation pathway in liver (295). Even though the CDP-pathway has been proposed to synthesize approximately 70% of the PC in liver (296), VLDL triglyceride and apoB100 secretion was decreased in mice lacking phosphatidylethanolamine N-methyltransferase (PEMT), which is responsible for the last step in the PE methylation pathway (297). Since the cellular PC level was unaffected by the lack of
PEMT, PEMT is believed to have an additional role in the VLDL production (297). The PEMT activity has been shown to be inhibited by fibrates in cultured rat hepatocytes, resulting in secretion of lipid-poor apoB-containing lipoprotein particles (298-300). Moreover, beside the effects on PEMT activity, Wy14,643 treatment in ob/ob mice resulted in decreased protein level of methionine adenosyltransferase (224), that is involved in the production of the methyl donor (S-adenosylmethionine) for methylation of PE into PC. Together these data indicate that PPARα activation inhibits the PE methylation pathway, which probably influence the load of lipids for VLDL assembly.

**Figure 4. Proposed mechanism of action of PPARα activation on intracellular fatty acid flux and VLDL synthesis in liver cells.** The cellular uptake of fatty acids (FA) is probably increased following PPARα activation via increased expression of FATP, L-FABP and acyl-CoA synthetase. Despite an increased fatty acid oxidation, PPARα activation results in intracellular triglyceride accumulation by preventing flux of substrate from cytosolic lipid droplets to the endoplasmic reticulum compartment, partly by increasing the expression of ADRP. Increased MTP expression and activity probably contributes to decreased co- or post-translational degradation of apoB 100 resulting in increased secretion of lipid-poor apoB-containing lipoproteins.
SUMMARY AND CONCLUSIONS

♦ Rosiglitazone and Wy14,643 increased the expression of proteins taking part in the peroxisomal fatty acid oxidation in obese mice, whereas only Wy14,643 affected the protein expressions in lean mice. Since obese mice displayed elevated levels of hepatic PPARγ2, we suggested that the effect of rosiglitazone on peroxisomal enzymes in obese mice was a result of increased hepatic PPARγ2 activation.

♦ Using primary mouse hepatocyte cultures, we showed that insulin, via the PI3-kinase signaling pathway, markedly increased the PPARγ2 expression, while PPARγ1 expression was unaffected. Additionally, oleic acid was shown to increase the PPARγ2 expression, but to a lesser extent. These results might help to understand the regulation of PPARγ expression in liver, which possibly plays a role in the development of liver steatosis.

♦ PPARα activation increased MTP expression and activity in mice and rats as well as in cultured hepatocytes by transcriptional activation of a DR-1 element in the MTP promoter. The effects of PPARα activation on MTP activity might explain the increased apoB100 secretion from rat hepatocytes, following Wy14,643 incubation.

♦ PPARα activation resulted in increased triglyceride mass and ADRP protein expression in mouse liver and in cultured mouse hepatocytes. Since the fatty acid oxidation was increased and triglyceride biosynthesis was unaffected, the increased triglyceride mass was suggested to be a result of decreased triglyceride secretion. ADRP overexpression resulted in decreased triglyceride secretion in parallel with increased cellular triglyceride mass. Wy14,643 incubation of ADRP overexpressing cells gave rise to a further decrease in triglyceride secretion, despite unaffected ADRP protein level, suggesting that the increased ADRP expression can not be the sole mechanism for the decreased triglyceride secretion following PPARα activation. In conclusion, PPARα activation prevents availability of cytosolic triglycerides for VLDL assembly and increases hepatic triglyceride content in part by increasing the expression of ADRP.
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