Exploring the activation and function of PPARα and PPARβ/δ using genomics

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Abstract

For many tissues fatty acids represent the major source of fuel. In the past few decades it has become evident that in addition to their role as energy substrates, fatty acids also have an important signaling function by modulating transcription of genes. An important group of transcription factors involved in mediating the effects of dietary fatty acids on gene transcription are the Peroxisome Proliferator-Activated Receptors (PPARs). PPARs are members of the superfamily of nuclear hormone receptors and regulate genes involved in numerous important biological processes, ranging from lipid metabolism to inflammation and wound healing. In the liver the dominant PPAR isoform has been show to be PPARα, although PPARβ/δ and PPARγ are expressed in liver as well.

The aim of this thesis was to further characterize the role of PPARα and PPARβ/δ in hepatic metabolism and study their activation by fatty acids. Even though PPARα as gene regulator in liver has been well described, a complete overview of its target genes has been lacking so far. By combining several nutrigenomics tools, we succeeded in creating a comprehensive list of PPARα-regulated genes involved in lipid metabolism in liver. Additionally, by using a unique design where mice were fed synthetic triglycerides consisting of one type of fatty acid, we could distinguish between different types of dietary unsaturated fatty acids in their ability to activate PPARα. Although it is well known that PPARα plays an important role in liver during fasting, no direct *in vivo* evidence exists that circulating free fatty acids are able to ligand activate hepatic PPARα. In our studies, we found that upregulation of gene expression by PPARβ/δ is sensitive to circulating plasma free fatty acids whereas this is not the case for PPARα. Not much is known about the function of PPARβ/δ in the liver. In order to better understand the role of this nuclear receptor, we compared the effects of PPARα and PPARβ/δ deletion on whole genome gene regulation and plasma and liver metabolites. Our results revealed that PPARβ/δ does not mediate an adaptive response to fasting, and pointed to a role for PPARβ/δ in hepatic glucose- and lipoprotein metabolism.

In conclusion, this thesis contributes to the important work of mapping the molecular mechanisms dictating lipid metabolism in the liver. By using several nutrigenomics tools, we are able to show that PPARα is a key mediator of the effect of dietary fatty acids on hepatic gene expression. In addition, we better define the roles of PPARα and PPARβ/δ in hepatic metabolism and provide a new concept for functional differentiation between PPARs in liver.

Contents

Chapter 1

General introduction

Nutrigenomics – a combination of molecular nutrition and genomics

Obesity is increasing world wide and has become one of the major health challenges globally. Several metabolic abnormalities are associated with obesity, including insulin resistance, high blood pressure, high blood cholesterol and high triglyceride levels. These conditions are collectively referred to as metabolic syndrome or syndrome X (1, 2). One of the important challenges for nutritional science today is to find effective solutions for this increasing problem of obesity and its related conditions.

Until recently, nutrition research focused mainly on physiological and epidemiological aspects of human health. The introduction of nutrigenomics has created large opportunities to increase our understanding of nutrition on a molecular level. Nutrigenomics, or nutritional genomics, studies the genome-wide influences of nutrition, and can be seen as a combination of molecular nutrition and genomics (3, 4). Within the field of nutrigenomics, nutrients are perceived as dietary signal molecules that bring about changes in gene-, protein- and metabolite expression. Presently, nutrigenomics research mainly targets disease prevention rather than curing already developed illnesses. A major focus is on chronic diseases, such as cardiovascular disease, metabolic syndrome and cancer, which are generally related to overconsumption of certain nutrients or food components. With the help of nutrigenomics, biomarker profiles can be created that allow separation of healthy and (early-) diseased states. The expectation is that in these early disease states nutritional intervention will suffice to restore health without the need for pharmacological therapy.

In order to reach the ambitious goals that nutrigenomics pursues, various genomics techniques are applied, including transcriptomics, proteomics, and metabolomics, as well as functional genomics tools such as transgenic and knockout mouse models. Genes that are particularly interesting to knockout in these models are those whose products function as nutrient sensors, including many members of the nuclear receptor superfamily. Transcriptomics has become a very important tool for the large scale analysis of biological processes. This technique measures the amounts of the various mRNAs that are being transcribed at any given moment in a cell or tissue. A transcriptome constitutes the basis for its corresponding proteome, and can vary a great deal in response to external stimuli such as exposure to certain nutrients or chemicals, or as a response to disease.

In the past decade, microarray technology has developed into a major tool for high-throughput transcriptomics. High-density oligonucleotide arrays, such as the Affymetrix GeneChip® Arrays, are able to measure the expression of the entire genome of an organism

in a single hybridization assay (5, 6). Affymetrix uses photolithography to attach millions of DNA probes onto a glass surface, creating an array with a massive amount of information on a small surface area (5). Each transcript in an RNA sample is represented by a set of 20 probe-pairs on the array, each probe built up of 25 nucleotides (7, 8). A probe-pair consists of a perfect match (PM) probe and a mismatch (MM) probe, which is identical to the PM probe with the exception that the middle nucleotide is substituted with its complement (7). The purpose of this design is to detect non-specific hybridization with the MM probe, whereas the PM probe will detect specific hybridization of transcripts from the gene of interest. Reliable results from microarray analyses are highly dependent on good quality control of the arrays and sound statistical tests of the outcome measurements (9).

The liver as metabolic regulator

The liver is of major importance in maintaining whole body metabolic homeostasis. In the fed state, a variety of substrates originating from the diet reach the liver, including glucose and fatty acids. The uptake of glucose from the hepatic portal vein into the hepatocyte occurs with the help of the GLUT2 transporter (10). When blood glucose levels rise after a meal, the pancreatic β-cells start producing and secreting insulin into the blood. The subsequent rise in plasma insulin stimulates several enzymes in the liver involved in the formation of glycogen, which functions as a storage form of glucose. This process will continue as long as there is enough glucose and insulin in the blood and until the glycogen storage in the liver has been saturated (11). An additional response to excess glucose is *de novo* synthesis of fatty acids by the liver. These fatty acids are excreted in the form of very low density lipoprotein (VLDL) particles into the blood stream to be used by other tissues such as adipose tissue (for storage in the form of triglycerides) and muscle, eventually returning to the liver as VLDL remnants.

The majority of fatty acids taken up by the liver are derived from endogenous triglyceride stores in the adipose tissue and circulate as free fatty acids. The remainder of fatty acids taken up are derived from the diet and arrive in the liver as free fatty acids via the portal vein in the case of short- or medium chain fatty acids, or as triglycerides incorporated into chylomicron remnants in the case of long chain fatty acids. Uptake of fatty acids into the hepatocyte occurs through the transporters fatty acid transport protein (FATP), fatty acid translocase (FAT/CD36), and possibly by diffusion, although the relative contribution of each of these processes remains to be determined (12).

The liver is a major site for fatty acid beta oxidation, which operates in both the mitochondria and peroxisomes of the hepatocyte and yields energy in the form of ATP. In certain obese individuals, excess hepatic uptake of fatty acids cannot be properly compensated by increased fatty acid breakdown or increased triglyceride secretion, giving rise to fatty liver or steatosis. This abnormal fat storage has been correlated with the metabolic syndrome and represents the early stage of non-alcoholic fatty liver disease (NAFLD). For reasons that are poorly understood, in some individuals the hepatic steatosis progresses towards the more severe non-alcoholic steatohepatitis (NASH), which may be followed by cirrhosis and ultimately liver failure.

In the fasted state, glucose and insulin levels in the blood decline and the liver will start using up the stored glycogen via a process called glycogenolysis (13). The high activity of glucose 6 phosphatase allows the liver to release glucose in the blood stream, which can be used by other tissues in the body as fuel. It is estimated that liver glycogen storage is sufficient to maintain glucose homeostasis in the blood for up to 10 hours (11). Once the glycogen reserves have been exhausted, the body relies upon the liver in particular to make glucose via the process of gluconeogenesis using lactate, gluconeogenic amino acids, and glycerol as substrate (11).

In response to low plasma insulin levels and high rates of hepatic fatty acid uptake, another process is initiated in the liver, which is ketogenesis. Ketogenesis describes the production of ketone bodies in the mitochondria from fatty acids released from white adipose tissue during fasting. The ketone bodies, which include β–hydroxybutyrate, acetoacetate and acetone, are transported from the liver to especially the brain, where they serve as important alternative substrates for energy production.

Nuclear receptors function as nutrient sensors

Nuclear receptors are transcription factors that mediate the effects of nutrients on gene expression. Upon binding of a ligand, which can be a natural or pharmacological compound, the receptor is activated, leading to binding of the receptor-ligand complex to DNA and increased transcription of specific target genes.

The superfamily of nuclear hormone receptors consists of 48 members and represents the most important group of nutrient sensors (3). Many members of this group bind nutrients and their metabolites, including retinoic acid (retinoic acid receptor RAR and retinoid X receptor RXR), vitamin D (vitamin D receptor VDR), bile salts (farnesoid X receptor

FXR), and fatty acids (peroxisome proliferator activated receptors PPARs). Chapter 2 describes in more detail the function of nuclear receptors in general and the PPAR family in particular.

Fatty acids – major biological regulators

Fat has traditionally been regarded exclusively as a way for the body to store energy. In the past decades it has become evident, however, that fatty acids function as signaling molecules with the ability to alter the regulation of genes involved in important biological processes.

Our diet consists mainly of saturated (SFA) and monounsaturated (MUFA) fatty acids, with a much smaller amount of polyunsaturated fatty acids (PUFA). A growing body of evidence indicates that an increased PUFA consumption can result in several health benefits, such as a decreased risk of cardiovascular disease, increased insulin sensitivity and protection of age-related neurological degeneration (14-16). Important dietary sources of PUFAs include fatty fish (eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA), and numerous vegetable oils.

However, it has been shown that dietary fatty acids have the ability to directly regulate genes involved in several inflammatory processes, lipid metabolism and energy utilization (17). PUFAs are able to increase fat oxidation via activation of peroxisome proliferator activated receptors, as well as suppress lipogenic genes via inhibition of sterol regulatory element-binding protein 1 (SREBP-1) (18, 19). Whether these mechanisms account for the beneficial effects of PUFAs on human health requires further investigation.

Outline of this thesis

The aim of the research described in this thesis is to determine the importance of the nuclear receptors PPARα and PPARβ/δ in fatty acid dependent gene regulation in liver, and to better characterize their functional roles in hepatic lipid metabolism. To this end, a nutrigenomics approach is followed using several common tools such as transgenic- and knockout mouse models and expression microarrays.

In **Chapter 2** a detailed overview is presented of the three members of the PPAR family and their important roles in metabolism and inflammation. **Chapter 3** is a descriptive profiling study designed for two purposes: 1) to generate a comprehensive overview of

PPARα-regulated genes relevant to lipid metabolism in liver and 2) to identify possible novel PPARα target genes and –pathways involved in lipid metabolism. **Chapter 4** addresses the overall role of PPARα in gene regulation by dietary fatty acids in liver using a unique experimental design based on synthetic triglycerides in combination with transcriptomics. In **Chapter 5**, we compare the activation of PPARα and PPARβ/δ in mouse liver during fasting and find that PPARβ/δ, but not PPARα, responds to increased plasma free fatty acid levels. In **Chapter 6** a comparative transcriptomics analysis is presented of the impact of PPARα and PPARβ/δ inactivation on liver gene expression during fasting. In **Chapter 7** the results of an *in silico* method for the screening of putative PPREs in the mouse genome are compared with results from gene expression analysis. Finally, in **Chapter 8** the general discussion and conclusions are presented.

Chapter 2

PPARs: important regulators in metabolism and inflammation

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Abstract

The ligand-activated family of peroxisome proliferator activated receptors (PPARs) consists of three members named PPARα, PPARδ and PPARγ. Each PPAR subtype is characterized by a specific tissue expression pattern, partially accounting for distinct biological functions. Analogous to many other nuclear receptors, PPARs form heterodimers with the retinoid X receptor and regulate DNA transcription by binding to specific response elements present in target genes.

PPARα (NR1C1) is highly expressed in liver, heart, intestine, skeletal muscle, and various immune cells. Agonists for PPARα include the lipid-lowering fibrate drugs as well as numerous fatty acids and eicosanoids. PPARα is known to play an important role in many different metabolic processes, especially under conditions of fasting, and has proven to be an extremely important regulator of inflammation via inhibition of gene expression.

PPARδ (NR1C2) is ubiquitously expressed but its function has mainly been studied in skin, heart, and skeletal muscle. In the past few years, it has become evident that PPARδ is involved in numerous biological processes including lipid metabolism, wound healing and inflammation.

The most studied PPAR subtype is PPARγ (NR1C3), which is expressed at high levels in adipose tissue, macrophages and vascular cells. PPARγ drives adipocyte differentiation, has important regulatory roles during fat storage and glucose metabolism, and is an important suppressor of inflammation. Importantly, it serves as the molecular target for the thiazolidionedione drugs.

In this chapter we provide an overview of the major functions of the three PPAR subtypes, and focus on their role in metabolic and inflammatory processes.

Introduction

The peroxisome proliferator activated receptors (PPARs) are ligand-activated transcription factors that belong to the superfamily of nuclear hormone receptors, and more specifically to the class II nuclear receptors. Three subtypes encoded by separate genes can be distinguished: PPARα, PPARβ/δ and PPARγ (20, 21). Until now, PPARs have been cloned from several species, including human, mouse, rat, chicken, fish, guinea pig, hamster and amphibian (21, 22).

PPARs, like many other nuclear receptors, bind to DNA and regulate transcription in the form of a heterodimer with the nuclear receptor retinoid X receptor (RXR) (Figure 1A). Recent evidence suggests that to a large extent PPARs and retinoid X receptors are associated even in the absence of ligand (23). Activation of target genes occurs through direct binding of the PPAR:RXR heterodimer to specific nucleotide sequences called peroxisome proliferator response elements (PPREs) (24). These response elements are of the direct repeat 1 (DR-1) type, which is defined by a direct repetition of the consensus sequence AGGTCA with a single nucleotide insertion between the two repeats (24). PPAR will bind to the 5' part of the response element, whereas RXR binds to the 3' half-site (24). A PPRE is commonly present as one or multiple copies located in or close to the promoter region of a target gene (24).

Members of the nuclear hormone receptor family are related to each other with respect to amino acid sequence and their molecular mode of function within cells. They share a highly conserved DNA binding domain (DBD) responsible for binding to the response element sequence in target genes. A reasonably well conserved ligand-binding domain (LBD) is present at the C-terminal end of the receptors, which binds ligands and interacts with coactivators. Crystallographic structures of PPARs reveal an exceptionally spacious ligand-binding pocket compared to other nuclear receptors, thereby explaining the promiscuity in ligand binding (25-27). Ligands of PPARs include numerous fatty acids and their derivates, as well as a large number of industrially synthesized compounds.

The ability of PPARs to regulate transcription is controlled by complex interactions involving coactivators and corepressors (Figure 1). PPARs are present in the nucleus of the cell, both in the presence and absence of ligand. In the latter case, compression of the chromatin is caused by corepressor proteins such as nuclear corepressors (NCoRs) and silencing mediator for retinoid and thyroid hormone receptor (SMRT), which connect PPARs with enzymes expressing histone deacetylase activity (28-30). Once ligand binding occurs, these corepressor protein complexes dissociate, and subsequent recruitment of

several coactivator proteins leads to a conformational change within the ligand-binding domain of the receptor (29, 30).

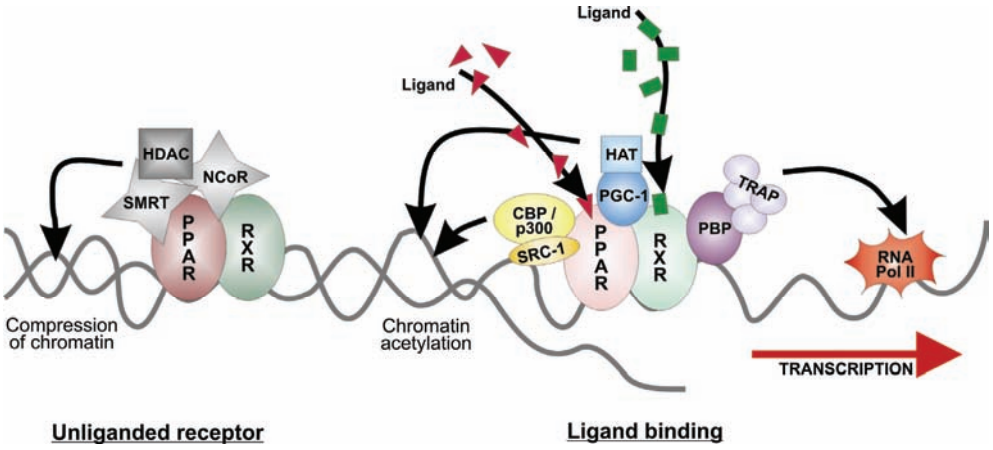

Figure 1. **Schematic depiction of the mechanism of gene regulation by PPARs.** Transcriptional regulation and the interplay between PPARs and cofactors. PPARs bind to DNA and regulate transcription as a heterodimer with the nuclear receptor retinoid X receptor (RXR). In the absence of ligand, corepressor proteins such as nuclear corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) connect the PPAR:RXR complex with enzymes expressing histone deacetylase activity (HDAC). This causes compression of the chromatin structure and a subsequent repression of gene transcription. Ligand binding is followed by a dissociation of the corepressor complexes and recruitment of coactivator proteins. CREB binding protein (CBP) and adenovirus E1A-associated protein (p300) are coactivators that possess histone acetyltransferase (HAT) activity, leading to remodeling of the chromatin structure and a facilitation of gene transcription. Steroid receptor coactivator 1 (SRC-1) forms a complex with CBP/p300 proteins, thereby recruiting HAT activity. PPAR gamma coactivator 1 (PGC-1) has the ability to recruit enzymes that posses HAT activity. PPAR binding protein (PBP) serves as an anchor within multi-subunit coactivator complexes, called TRAP complexes, which may function as docking platforms for RNA polymerase II during the transcriptional process.

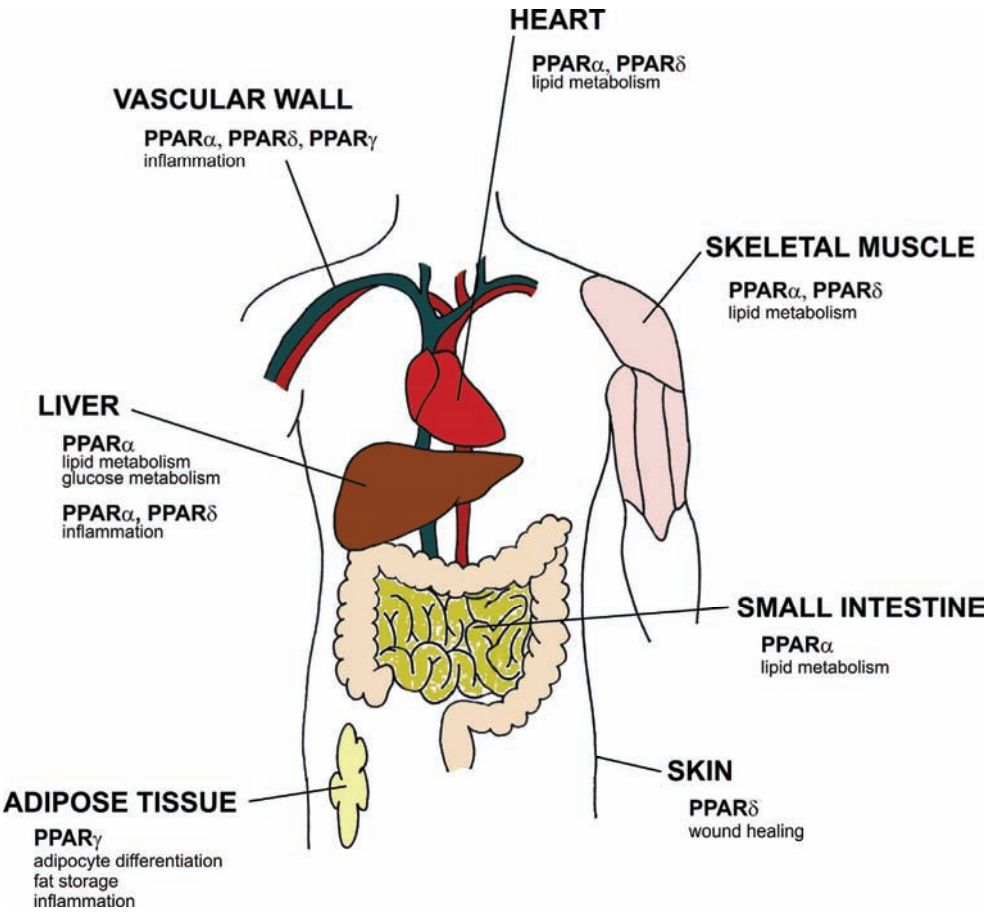

Figure 2. **The regulatory role of PPARs in different tissues.** Overview of tissue distribution and different processes where PPARs have proven to play an important regulatory role.

Several nuclear receptor coactivators have been identified in recent years. Coactivators contain a so-called LXXLL (L: leucine; X: any amino acid) motif, where direct binding with the ligand binding domain of the nuclear receptor takes place. Coactivators often have several different LXXLL motifs, suggesting that they are able to bind to several different nuclear receptors simultaneously.

Some coactivators possess histone acetyltransferase (HAT) activity (Figure 1). This group of coactivators is able to remodel chromatin structure and in this way facilitate gene transcription (30, 31). Examples of this type of coactivators are CBP (CREB binding protein) and p300 (adenovirus E1A-associated protein). SRC-1 (steroid receptor coactivator) is a coactivator that binds to the LBD on the receptor in a ligand-dependent manner and forms a complex with CBP/p300 proteins, thereby recruiting HAT activity.

A different group of coactivators consists of proteins that form multi-subunit coactivator complexes. These coactivators are recruited to the nuclear receptor in a ligand-dependent manner, and may function as docking platforms for RNA polymerase II during the transcriptional process. The most important protein in this context is the coactivator PBP (PPAR binding protein) which serves as an anchor inside the complex. Experiments involving liver specific knockouts of this gene showed a dysfunctional PPARα-mediated transcriptional response (32). Another important coactivator is PGC-1 (PPAR gamma coactivator 1). PGC-1 interacts with PPARγ to induce mitochondrial biogenesis and induction of a brown adipocyte-specific gene expression program (33). Additionally, it is able to coactivate PPARα-dependent gene regulation, especially towards fatty acid oxidation processes (34). This protein thus plays an important role during fasting in the liver by upregulating genes involved in gluconeogenesis, and fatty acid oxidation/ketogenesis (35, 36).

Each PPAR subtype is characterized by a specific tissue expression pattern (Figure 2), thereby accounting for their distinct biological functions. Within a tissue, PPARs exhibit differential activity towards target genes, which is partly due to differential availability of receptor-specific ligands and coactivators (24, 37, 38). Nevertheless, studies using PPAR agonists have shown considerable overlaps in gene regulation between PPARs, even within a certain tissue. To what extent this is an artifact of pharmacological activation of PPARs or reflects the limited receptor specificity of synthetic agonists remains to be determined. In this chapter we provide an overview of the major functions of PPARs, separated by PPAR subtype, and focus on their role in metabolic and inflammatory processes.

PPARα (NR1C1)

Of the three PPAR subtypes, PPARα was discovered first (20). Since then it has been cloned in several different species, including human (39, 40), rat (41), frog (42) and rabbit (43). Human PPARα is situated on chromosome 22, and its mouse namesake has been mapped to chromosome 15.

In rodent as well as in human, PPARα is expressed in many tissues that actively metabolize fatty acids (Figure 2). It is highly expressed in liver, with expression levels in mouse reportedly exceeding those in human (44, 45). In addition, PPARα is relatively well expressed in heart, kidney, intestine, skeletal muscle and brown adipose tissue (20, 38, 46-50). PPARα has also been found in different types of immune cells, such as macrophages and T and B cells (51-59). Finally, PPARα has been detected in vascular endothelial, vascular smooth muscle cells, and atherosclerotic lesions (51, 52, 60-62).

PPARα serves as a receptor for a structurally diverse set of compounds, both natural and synthetic. A major group of synthetic PPARα agonists are the fibrates, which comprises a cluster of lipid-lowering drugs used for treatment of dyslipidemia, including gemfibrozil, bezafibrate, clofibrate, fenofibrate and WY14643 (21, 24, 63-66). In addition, PPARα is activated by a variety of plasticizers, insecticides, and other rodent hepatic carcinogens. Endogenous ligands for PPARα include a variety of (long-chain polyunsaturated) fatty acids and eicosanoids (41, 67, 68). Recently, it was shown that the effects of dietary unsaturated fatty acids on hepatic gene expression are almost entirely mediated by PPARα and mimic those of synthetic PPARα agonists (69).

Metabolism

PPARα plays an important regulatory role in many different metabolic processes, especially under conditions of fasting (70-72) (Figure 2). In addition, it governs the metabolic response in liver to acute and chronic dietary fat feeding (69, 73). Here, we summarize the involvement of PPARα in nutrient metabolism, emphasizing pathways in liver.

Lipid metabolism

The first connection between PPARα and fatty acid catabolism was made in 1992 when it was shown that the peroxisomal acyl-coenzyme A (CoA) oxidase gene was a direct target gene of PPARα (42, 74). This enzyme carries out the first step of the oxidation of very long chain polyunsaturated fatty acids in peroxisomes. Since then, numerous genes involved in mitochondrial and peroxisomal fatty acid oxidation have been shown to be under control of PPARα, especially in the fasted state.

During fasting, free fatty acids (FFA) are released from adipose tissue storage and are transported to the liver. The liver, being a central player in maintaining metabolic homeostasis, responds by increasing the rate of fatty acid beta-oxidation and, after

prolonged fasting, ketogenesis. Fasting PPARα null mice exhibit a severe impairment in hepatic mitochondrial beta-oxidation leading to hypoketonemia, hepatic steatosis, myocardial lipid accumulation and hypoglycemia (70-72). It is now evident that PPARα not merely governs expression of a few key genes such as *Cpt1*, *Mcad*, and *Hmgcs2* (21, 75-77), but actually regulates entire pathways involved in different aspects of hepatic fatty acid metabolism, including fatty acid uptake across cell membranes (*Cd36*, *Fatp*) (78-81), intracellular fatty acid binding and transport (*Fabp*) (82-84), fatty acid activation (*Acsl*), microsomal fatty acid oxidation (*Cyp4a*), peroxisomal fatty acid oxidation (*Acaa*, *Acot*, *Ehhadh*, *Decr2*, *Acox1*), mitochondrial fatty acid oxidation (*Acad*, *Cpt*, *Hadh*, *Acot*), and ketogenesis (*Hmgcs2*, *Hmgcl*) (85). Furthermore, PPARα upregulates expression of several lipases (*Mgll*, *Lipg*, *Pnpla2*), as well as many genes involved in fatty acid synthesis/elongation/desaturation (*Fads*, *Agpat*, *Scd1*, *Dgat1*). Thus, PPARα can be considered a master regulator of hepatic lipid metabolism. Interestingly, although fasting increases delivery of free fatty acids to the liver, recent studies suggest that free fatty acids are unable to ligand-activate hepatic PPARα, in contrast to lipoprotein-derived fatty acids (86, 87).

PPARα has a similar, though less comprehensive role in regulation of lipid metabolism in cardiac and skeletal muscle (88-90). Moreover, recent studies also reveal a major role for PPARα in the small intestine (91) (Figure 2).

Glucose metabolism

A role of PPARα in glucose metabolism is supported by the severe hypoglycemia in fasted PPARα null mice (71, 72, 92-94). While it is tempting to relate the reduced plasma glucose to defective fatty acid oxidation, which in many fatty acid oxidation disorders gives rise to hypoglycemia, there is compelling evidence for direct regulation of glucose synthesis by PPARα. Several genes involved in synthesis of glucose from gluconeogenic precursors have been identified as direct PPARα targets, including *PCK1*, *Pcx*, and *Gpd*, although the former gene only in human (95). Specifically the conversion of glycerol into gluconeogenic intermediates is under direct control of PPARα (95). The effect of PPARα deletion on gluconeogenic fluxes reveals a more complex picture (92). It has been reported that PPARα null mice exhibit an increased gluconeogenic flux towards glycogen, thereby diminishing hepatic glucose output (96). Synthesis of glycogen is also affected in PPARα null mice, which is likely mediated via defective regulation of *Gys2* (97).

The hypoglycemia witnessed in PPARα null mice may also partially be due to an increased rate of glucose utilization (98). Decreased expression of *Pdk4*, which is a PPARα target

gene in liver, heart, kidney and skeletal muscle, may relieve the block on pyruvate oxidation and thus glucose utilization (99-103). Furthermore, regulation of glucose utilization may occur via PPARα in the brain (98).

Amino acid metabolism

In addition to lipid and glucose metabolism, PPARα also governs metabolism of amino acids (104, 105). The expression of numerous genes involved in the ammonia detoxification pathway and urea synthesis including *Cps1*, *Otc*, *Ass1*, and *Asl* are downregulated by PPARα. Consequently, plasma ammonia levels are increased after WY14643 treatment, while plasma urea levels are increased in PPARα null mice (105, 106). Currently, the molecular mechanisms behind this regulation remain elusive and require more detailed investigation (107).

Inflammation

A delayed inflammatory response to topical administration of leukotriene B4 and arachidonic acid provided the first evidence for a link between PPARα and inflammation (108). Follow-up studies have shown that PPARα is an extremely important regulator of inflammation, mainly by inhibiting inflammatory gene expression (Figure 2).

Hepatic inflammation

Numerous studies have shown a reduction of hepatic cytokine-stimulated inflammation and production of acute phase proteins upon PPARα activation. Several molecular mechanisms behind the anti-inflammatory effects of PPARα have been suggested. These include interference with several proinflammatory transcription factors including signal transducer and activator of transcription (STAT), activator protein-1 (AP-1), and NF-kB by PPARα (109). Further studies have revealed that PPARα diminishes the activity of the proinflammatory transcription factor CAATT/enhancer binding proteins (C/EBP) via sequestration of the coactivator glucocorticoid receptor-interacting protein-1/transcriptional intermediary factor-2 (GRIP1/TIF2) (110). Finally, PPARα can also inhibit cytokine signaling pathways via downregulation of the IL-6 receptor (111, 112) and upregulation of sIL-1 receptor antagonist (113), leading to diminished inflammatory responses. Its potent anti-inflammatory activity in liver may confer a protective role for PPARα against steatohepatitis. Indeed, several studies in mice have shown that activation of PPARα can slow down or even reverse progression of steatohepatitis (114-117). Part of the effect of PPARα may be linked to preventing upregulation of the *Cox2* gene, which has been directly linked to the progression of steatosis to steatohepatitis (118). Conversely, PPARα

ablation accelerates development of steatohepatitis in mice rendered obese by chronic high-fat feeding. PPARα may protect against steatohepatitis by a combination of reducing hepatic lipid storage and direct suppression of pro-inflammatory gene expression (119).

Inflammation in vascular wall

Inflammation in the arterial wall is an important contributor to atherogenesis (120). In addition to suppressing inflammatory responses in liver, PPARα also modulates inflammatory reactions in the arterial wall. As PPARα is expressed in various cell types present in atherosclerotic lesions, including smooth muscle cells, endothelial cells, and macrophages, the effect of PPARα on lesion development is rather complex. Immune-modulating effects of specific PPARα activation have been reported in these various cell types. However, some controversy still exists about the exact role of PPARα in the vascular wall as both pro- and antiatherogenic effects of PPARα have been demonstrated.
PPARα has been shown to suppress expression of several proinflammatory genes in the vascular wall of animals with extensive atherosclerosis, including monocyte chemotactic protein-1 (*Mcp-1*), tumor necrosis factor alpha (*Tnfα*), vascular cell adhesion molecule-I (*Vcam I*), intercellular adhesion molecule-I (*Icam I*), and interferon-γ (*Ifnγ*) (70). Other studies have shown that the anti-inflammatory role of PPARα in the vascular wall depends on the severity of inflammation or vascular lesion. In the absence of inflammation or in early lesions, the effects of PPARα are mainly proatherogenic (71, 72), whereas the development of severe lesions accompanied by inflammation is strongly reduced by PPARα activation.

Overall, it is clear that PPARα has a major impact on metabolic and inflammatory gene expression, especially in the liver and vascular wall. In general, these effects are positive in the context of specific metabolic diseases, including dyslipidemia and atherosclerosis.

PPARβ/δ (NR1C2)

The PPARβ/δ subtype was first identified in *Xenopus laevis* under the name PPARβ (42). Shortly thereafter the receptor was cloned in mouse (37, 121) and human as NUC1 or PPARδ (39). Throughout the remainder of this chapter we will refer to the receptor as PPARδ, which represents the official gene name. The human *PPARδ* gene has been mapped to chromosome 6, while its mouse counterpart is present on chromosome 17. Human and rodent PPARδ protein are highly homologous, sharing ~90% sequence identity in the LBD (65).

Similar to PPARα, PPARδ is ubiquitously expressed. High levels of mRNA have been found in the skin (122-125), heart (126, 127), skeletal muscle (128), adipose tissue (129, 130), small intestine (38, 47), and brain (38, 47, 131). A recent study indicates that PPARδ protein is especially abundant in mouse small intestine, followed by keratinocytes, liver, and at much lower levels in heart and skeletal muscle (132). In comparison to PPARα and PPARγ, the function of PPARδ is generally less well understood. However, in the past few years, using specific PPARδ agonists and/or PPARδ null mice, significant progress in the characterization of PPARδ has been made. It is now evident that PPARδ is involved in numerous biological processes including lipid metabolism (65, 131, 133-137), wound healing (138, 139), inflammation (140), placental development (65, 141-143), brain function and development (131, 144) and colon cancer (130, 145-148). Here the focus will be on the role of PPARδ in inflammatory and metabolic processes (Figure 2).

PPARδ can bind both endogenous and synthetic agonists. Endogenous PPARδ ligands include naturally occurring fatty acids (27, 149) as well as various eicosanoids such as prostaglandin A1 (PGA_1), prostaglandin D2 (PGD_2) and prostaglandin I2 (PGI_2) (68, 150, 151). Recently, evidence was provided that PPARδ also binds retinoic acid, which is selectively delivered to PPARδ via FABP5 (152).

Synthetic ligands of PPARδ are currently explored for their potential to improve plasma lipoprotein levels and include GW501516 and GW0742 (153). In a primate model for type 2 diabetes, GW501516 increased serum HDL cholesterol, improved insulin sensitivity and reduced adiposity in diet-induced obese mice (136). In addition, GW501516 was shown to reverse multiple abnormalities of the metabolic syndrome in mice and humans, causing significant reductions in plasma triglycerides, apolipoprotein B, LDL cholesterol, insulin, and glucose tolerance (137, 154-156). So far none of the PPARδ agonists have been launched onto the market, though GW510516 and MBX-8025 have entered phase 2 clinical trails.

Lipid metabolism

Analogous to PPARα and PPARγ, evidence is accumulating that PPARδ plays a role in the regulation of lipid metabolism (Figure 2). Consequently, PPARδ has become an interesting target for the treatment of metabolic syndrome. Effects of PPARδ have been demonstrated in several tissues, including skeletal muscle, heart, adipose tissue, and liver.

Lipid metabolism in skeletal muscle

Numerous *in vitro* studies have shown a stimulatory effect of PPARδ overexpression or activation on expression of genes involved in fatty acid catabolism, including mitochondrial- (*Lcad*, *Hadha*, *Decr*) and peroxisomal fatty acid oxidation (*Ech*), fatty acid transport (*Fatp*, *Lipe*, *Acsl*, *Cpt1*) and energy uncoupling (*Ucp1*, *-2*, *-3*) (93, 137, 157-159) (Figure 2). In line with these data, administration of GW510516 was found to induce fatty acid oxidation in skeletal muscle of C57BL/6J mice (137). Similarly, the PPARδ agonist GW610742X decreased muscle lipid content and shifted fuel use towards fatty acids, while inducing expression of specific genes (*Pdk4*, *Cpt1b*, *Ucp3*).

Besides governing fatty acid oxidation, PPARδ also determines muscle fiber type characteristics. Forced overexpression of PPARδ in skeletal muscle is associated with a selective increase in type 2a fast-oxidative fibers or, when expressed at supraphysiological levels, causes fiber type transformation towards type I fibers concurrent with an increase in endurance exercise performance (160, 161). Endurance exercise performance is also increased by GW501516, at least when combined with exercise training (162). Conversely, selective deletion of PPARδ in skeletal muscle myocytes is associated with a reduced muscle oxidative capacity and a switch in muscle fiber-type characteristics toward less oxidative fibers (128). Surprisingly, the role of PPARδ in skeletal muscle fatty acid oxidation is not supported by studies using whole body PPARδ null mice (163).

Lipid metabolism in heart

Similar to the situation in skeletal muscle, PPARδ appears to stimulate fatty acid oxidation in heart, although the picture is far from clear. Treatment of neonatal rat cardiomyocytes with PPARδ ligands L-165041 and GW501516 increases fatty acid oxidation rate and expression of selected genes involved in fatty acid catabolism (127). *In vivo*, the absence of PPARδ in heart leads to myocardial lipid accumulation and cardiomyopathy, as well as downregulation of several key fatty acid oxidation genes such as carnitine palmitoyltransferase 1 (*Cpt1*) and acyl-coenzyme A oxidase 1 (*Acox1*) (164). Conversely, treatment of rats with the PPARδ specific agonist GW610742X stimulated fatty acid oxidation rate. The metabolic changes were associated with increased expression of genes involved in lipid catabolism (*Cd36*, *Cpt1*, *Ucp3*) (165). Remarkably, transgenic overexpression of PPARδ in heart had little effect on fatty acid oxidation rate and expression of lipid catabolism genes, including *Fatp* and *Cd36*. Instead, PPARδ may stimulate myocardial glucose utilization, possibly via induction of *Slc2a4* (*Glut4*) and phosphofructokinase (126). Thus, while the importance of PPARδ in normal heart

functioning is evident, its specific impact on cardiac fatty acid catabolism remains somewhat ambiguous.

Lipid metabolism in adipose tissue

A gain of function study shows that PPARδ promotes fatty acid oxidation in adipocytes (155). On the other hand, PPARδ seems to have a facilitative, yet important role in lipo- and adipogenesis (166). Presently, the role of PPARδ in adipose tissue remains somewhat ambiguous.

Lipoprotein metabolism

Several studies support a role for PPARδ in lipoprotein metabolism. PPARδ agonists were shown to increase plasma HDL levels in mice (135, 167), rhesus monkeys (136), and human subjects (168). Although the complete mechanism behind this effect is unknown, the effect may be mediated by the cholesterol transporter *Abca1*, which is a target gene of PPARδ (136).

Apart from elevated levels of plasma HDL, primates also show a decrease in plasma triglycerides upon PPARδ activation (136, 168). Consistent with these data, plasma TG levels are increased in PPARδ null mice (169). Due to their beneficial effect on plasma lipoproteins, PPARδ agonists are currently explored for the treatment of dyslipidemia.

Wound healing

PPARδ is the dominant PPAR subtype in the skin and has been shown to be involved in different phases of the healing process of epidermal wounds (49, 139, 170) (Figure 2). PPARδ becomes induced in keratinocytes at the wound edge of damaged skin and, in contrast to PPARα, which is expressed during the early inflammatory phase of the healing, PPARδ remains active until the wound healing process has been completed (139). Induction of PPARδ expression is mediated by inflammatory cytokines, which via induction of stress-associated kinase pathway target a AP-1 site in the PPARδ promoter (171). The increase in PPARδ activity promotes keratinocyte differentiation and protects against apoptosis, thereby stimulating wound closure. Suppression of apoptosis is mediated by PPARδ-dependent upregulation of integrin-linked kinase and 3-phosphoinositide-dependent kinase-1 (*Pdpk1*), which phosphorylates protein kinase B-alpha (*Akt1*) (172). In addition, PPARδ stimulates wound healing by altering actin cytoskeleton plasticity and integrin function, resulting in increased cell migration (173). At later stages in wound

healing, normal PPARδ expression is restored by a TGFβ and SMAD3-mediated suppression of c-JUN binding to the PPARδ promoter (174).

Inflammation

The role of PPARδ in inflammation has primarily been studied in the context of atherosclerosis (Figure 2). Treatment with synthetic PPARδ agonist has been repeatedly shown to suppress inflammation in atherosclerotic lesions and lipid loaded macrophages, possibly by down-regulating expression of the chemoattractant *Ccl2* (*Mcp-1*) (175-178). Recent studies suggest that the inflammatory properties of PPARδ extend to other cell types present in the vascular wall, including endothelial cells and vascular smooth muscle cells (179, 180). Inhibition of inflammation likely accounts for the marked reduction in atherosclerotic lesion size upon PPARδ activation as observed in several but not all studies (169, 170, 178, 181). Remarkably, macrophage-specific deletion of PPARδ is also associated with a significant reduction in atherosclerotic lesion size (175). Together, these data suggest a complex role for PPARδ in atherosclerosis via its presence in macrophages and other cells that may involve both pro- and anti-inflammatory activities, as well as specific effects on plasma lipoproteins (175). Possibly, the effects of PPARδ on these pathways are compounded by its ability to promote lipid accumulation in macrophages via induction of genes involved in lipid uptake and storage (*CD36*, *MSR1*) (182).

The general anti-inflammatory properties of PPARδ are further substantiated by recent data showing that PPARδ promotes alternative activation of macrophages resident in liver and adipose tissue, which confers a protection against insulin resistance and hepatic steatosis (183, 184).

PPARγ (NR1C3)

PPARγ is clearly the most widely studied PPAR subtype, which is explained by it serving as the molecular target for the insulin-sensitizing thiazolidionedione drugs (TZDs). PPARγ also plays a key role in adipogenesis and consequently has been extensively studied for its involvement in obesity development.

Four distinct transcript variants of PPARγ are known, PPARγ1 through PPARγ4, that yield two protein variants differing at their N-terminus by the addition of 28 (human PPARγ2) or 30 (mouse PPARγ2) amino acids (42, 185-189). Whereas PPARγ2 is expressed selectively

in adipose tissue, (42, 185, 190-194), PPARγ1 has a broader expression pattern and is found in gut, brain, vascular cells and macrophages (185, 191, 192, 195).

For the human *PPARγ* gene, both common and rare sequence variants are known. The common Pro12Ala variant has been shown to be associated with a lower BMI, improved insulin sensitivity and reduced incidence of type 2 diabetes (196-200). Rare sequence variants of PPARγ lead to the formation of a dysfunctional protein that via dominant negative action interferes with transcriptional activation, possibly by sequestering coactivator proteins (201). Afflicted patients suffer from a form of lipodystrophy characterized by loss of fat from the gluteal region, dyslipidemia, hepatic steatosis, and severe insulin resistance (202).

Similar to other PPARs, PPARγ is able to bind both endogenous and synthetic ligands. The endogenous ligands for PPARγ remain poorly characterized. In contrast to PPARα and PPARδ, dietary (poly-unsaturated) fatty acids appear to be relatively weak ligands for PPARγ (65, 67, 203, 204). Ligand-activation of PPARγ in specific cell types may occur by fatty acid nitration products (205), as well as by oxidized fatty acids such as the linoleic acid metabolites 9-HODE and 13-HODE (206). It has been shown that prostaglandin 15d-PGJ2 efficiently binds and activates PPARγ, yet due to its low concentration its relevance as a physiological PPARγ agonist can be questioned. As mentioned above PPARγ is activated by synthetic ligands belonging to the antidiabetic thiazolidionedione drugs, which include troglitazone, rosiglitazone and pioglitazone (207-212). These drugs improve insulin sensitivity and are used in the treatment of type 2 diabetes (208-214). In addition, tyrosine derivative drugs like glitazars (215), as well as NSAIDs like ibuprofen and fenoprofen (216) have been identified as PPARγ ligands.

Metabolism

PPARγ is best known for its ability to stimulate adipocyte differentiation, fat storage, and glucose metabolism (217-222) (Figure 2). Moreover, PPARγ suppresses inflammation. Although it is becoming more evident that metabolism and inflammation are intertwined, for the sake of simplicity the impact of PPARγ on the two processes will be discussed separately.

Adipose tissue

Studies with PPARγ null mice as well as PPARγ null stem cells have shown that PPARγ is absolutely required for adipocyte differentiation (223-226). For detailed coverage of the

role of PPARγ in adipogenesis the reader is referred to several excellent reviews (217-222). Here the focus will be on the impact of PPARγ on the fully developed adipose tissue.

In the mature adipocyte, PPARγ stimulates the expression of numerous genes involved in fatty acid uptake (*Cd36*, *Slc27a1*, *Slc27a3*), fatty acid synthesis (*Elovls*, *Mogat*, *Acly*), lipolysis (*Lipe*, *Pnpla2*, *Mgll*, *Lpl*), lipid droplet proteins (*Cidea*, *Cidec*, *Adfp*, *Plin*, *S3-12*), glucose metabolism (*Pck1*, *Pdk4*, *Gys2*, *Slc2a4*), and glycerol metabolism (*Aqp7*, *Aqp3*, *Gpd1*, *Gyk*, *Pck1*). Additionally, a set of miscellaneous genes is regulated by PPARγ (*G0s2*, *Ucp1*, *Ucp2*, *Abca1*, *Rxrg*). The overall effect is enhanced extraction of fatty acids from circulating triglycerides, enhanced fatty acid uptake, enhanced fatty acid re-esterification, and enhanced storage as triglycerides. In addition, uptake of glucose as well as its conversion to fatty acid and glycerol phosphate is stimulated, contributing to increased energy storage. From a physiological perspective, PPARγ is thus particularly important in the fed state to drive storage of consumed nutrients in the adipose tissue.

From a clinical perspective, removal of fatty acids and triglycerides from the circulating pool towards storage in the adipose tissue improves dyslipidemia and minimizes ectopic fat storage. Indirectly, these changes may also promote insulin sensitivity. This is exemplified by the phenotype of hypomorphic PPARγ mice (227) and more distinctly adipose tissue specific PPARγ null mice, which show inborn and progressive lipodystrophy characterized by accumulation of TG in non-adipose tissue such as liver and skeletal muscle, leading to insulin resistance (228, 229). The effect of PPARγ on plasma FFA and ectopic fat storage may provide a mechanistic basis for the insulin sensitizing effect of TZDs.

In addition to its primary function as an energy storage organ, adipose tissue produces various hormones such as leptin, adiponectin, resistin, and TNFα that play an active role in the regulation of energy metabolism. The insulin-sensitizing effect of TZDs may be partially accounted for by altered production of these hormones. Indeed, adiponectin, production of which is elevated by PPARγ, promotes insulin responsiveness and glucose uptake. Other so-called adipokines that are under control of PPARγ and that may mediate effects of PPARγ activation include RBP4 and ANGPTL4 (230).

In addition to the mechanisms eluded to above, alternative explanations for the insulin sensitizing effect of PPARγ agonists include an increase in the number of small, insulin sensitive adipocytes (231), as well as a direct effect on macrophages, as will be further discussed below.

Non-adipose tissue

PPARγ has been shown to induce macrophage expression of scavenger receptor CD36, which is involved in uptake of oxidized LDL into the macrophage (206, 232). Overall, much overlap is observed between the effect of PPARγ on gene expression in macrophages and adipocytes. In addition, macrophage PPARγ governs cholesterol esterification and intracellular cholesterol distribution, and stimulates cholesterol removal from the macrophage via the target genes *ABCA1*, *ABCG1*, caveolin and *APOE1* (53, 233-237). Collectively, these effects of PPARγ beneficially impact macrophage foam cell formation (176).

Although PPARγ is only weakly expressed in skeletal muscle and liver, muscle or liver specific PPARγ null mice show a major and complex metabolic phenotype. Presently, the relative importance of skeletal muscle PPARγ in TZD-induced muscle glucose disposal remains undecided (238, 239). In liver, PPARγ expression increases during over- and high fat feeding, concurrent with development of hepatic steatosis, which is aggravated in PPARα null mice (73, 226, 240). Hepatic PPARγ is also upregulated in models of lipoatrophy and leptin deficiency, and studies employing PPARγ overexpression or deletion indicate that PPARγ is necessary and sufficient for inducing fatty liver (241-244).

Inflammation

Inflammation has become a prime area of interest as a candidate process linking obesity to many of its comorbidities. Numerous studies suggest that obesity is associated with a state of low grade inflammation, which likely originates from white adipose tissue and has been suggested to impact insulin sensitivity. In addition, inflammation importantly contributes to the process of atherosclerosis.

Atherosclerosis

Macrophages play an important role in both innate and adaptive immune responses, including phagocytosis of pathogens and defective or dying cells. In addition, by taking up oxidized LDL and converting into foam cells macrophages are key contributors to development of atherosclerosis.

Although PPARγ may stimulate uptake of oxidized LDL into macrophages, which is pro-atherogenic, the anti-atherogenic effects of PPARγ via suppression of inflammation seem to dominate. PPARγ activation reduces arteriosclerotic lesions at least partially by inhibiting inflammatory gene expression in macrophages, including *MCP-1*, *VCAM-1*,

ICAM-1, *IFNγ* and *TNFα* (176). Furthermore, reduced amounts of cytokines (245), nitric oxide and macrophage-scavenger receptor class A (SRA) (246) have been observed upon PPARγ activation.

Anti-inflammatory effects resulting from PPARγ activation has been shown both in human and mouse macrophages. Treatment with PPARγ agonist 15d-PGJ2 was shown to have an anti-inflammatory effect by decreasing production of inflammatory cytokines such as interleukin IL1h, IL6 and TNFα in human peripheral blood mononuclear cells (PBMCs) (245). Furthermore, treatment of activated peritoneal macrophages with 15d-PGJ2 reduced expression of inducible nitric oxide synthase, gelatinase B and scavenger receptor A genes, partially by inhibiting the transcription factors AP-1, STAT and NFKB (247). PPARγ activation was also shown to suppress expression of *COX2*, mainly via preventing activation and translocation of NFκB (248-250). However, because of its limited specificity for PPARγ, many experimental outcomes following 15d-PGJ2 treatment may be only partially dependent of PPARγ, which complicates interpretation of the data.

Nevertheless, studies with synthetic PPARγ agonists support a general anti-inflammatory effect of PPARγ (251-253), which plays a role in the anti-atherogenic effects of PPARγ, as assessed by measurement of carotid arterial intimal and medial complex thickness (254-256).

Several molecular mechanisms have been proposed underlying the anti-inflammatory effects of PPARγ. A major mechanism involves transrepression, which describes the DNA-binding independent protein-protein interaction between PPARγ and other (pro-inflammatory) transcription factors such as NFκB, STAT and AP-1, causing a change in transcriptional activity (247, 257). In addition, PPARγ may compete with pro-inflammatory transcription factors for limited amounts of coactivators such as SRC-1, TIF2, AIB-1, CBP, p300, TRAP220, and DRIP205 in the cell (258). Another possibility involves binding of PPARγ to nuclear receptor corepressor (NCoR)- histone deacetylase-3 (HDAC3) complexes, thereby preventing the removal of these corepressor complexes from promoter regions of inflammatory genes and causing a suppression of gene transcription (259). Binding of PPARγ to NCoR is initiated by ligand-dependent SUMOylation of the PPARγ ligand binding domain (260, 261).

Adipose tissue

Macrophages are abundant in adipose tissue and together with adipocytes contribute to the secretion of a variety of pro- and anti-inflammatory cytokines. It is now evident that in

obese individuals adipocyte hypertrophy leads to the recruitment of macrophages in adipose tissue, thereby altering its secretory profile (262-267). Recent evidence suggests that these macrophages are primarily classically activated and mainly secrete pro-inflammatory cytokines (268). Indeed, compared with lean individuals, obese persons have been observed to have a higher expression of tumor necrosis factor alpha, interleukin 6, monocyte chemotactic protein 1, inducible nitric oxide synthase and transforming growth factor ß1 (269-279). Presently, the trigger leading to the infiltration of macrophages is unclear but may involve local hypoxia as well as adipose cell death.

Recent studies support a major role for PPARγ in regulating not only the amount of macrophages present in adipose tissue but also their phenotype and secretory profile. Treatment of mice with PPARγ agonist stimulated infiltration of alternatively activated macrophages into adipose tissue, thereby reducing pro-inflammatory gene expression (280). Conversely, macrophage-specific deletion of PPARγ decreased expression of markers of alternatively activated macrophages in adipose tissue, and increased inflammatory gene expression (281, 282). The increased abundance of classically activated macrophages led to worsening of insulin resistance, especially after high fat feeding (281, 282). These data suggest that macrophage PPARγ plays a major role in determining macrophage polarization in adipose tissue, and may mediate the effect of TZDs on insulin sensitivity.

Overall, it is evident that PPARγ has a major influence on metabolic and inflammatory gene expression, especially in adipocytes and macrophages. In general, these effects are positive in the context of specific metabolic diseases, including insulin sensitivity and plausibly atherosclerosis.

Concluding remarks

It is now evident that the primary functions of PPARs are at the level of regulating metabolic processes and inflammation. Presently, PPARα and PPARγ serve as therapeutic targets for dyslipidemia and insulin resistance, respectively, and synthetic ligands of PPARδ are currently being explored for their potential to improve plasma lipoprotein levels. As our understanding of the link between inflammation and metabolism advances, better insight will be obtained into the mechanism underlying the therapeutic actions of PPAR agonists.

Chapter 3

Comprehensive analysis of PPARα-dependent regulation of hepatic lipid metabolism by expression profiling

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Abstract

PPARα is a ligand-activated transcription factor involved in the regulation of nutrient metabolism and inflammation. Although much is already known about the function of PPARα in hepatic lipid metabolism, many PPARα-dependent pathways and genes have yet to be discovered.

In order to obtain an overview of PPARα-regulated genes relevant to lipid metabolism, and to probe for novel candidate PPARα target genes, livers from several animal studies in which PPARα was activated and/or disabled were analyzed by Affymetrix GeneChips. Numerous novel PPARα-regulated genes relevant to lipid metabolism were identified. Out of this set of genes, eight genes were singled out for study of PPARα-dependent regulation in mouse liver and in mouse, rat, and human primary hepatocytes, including thioredoxin interacting protein (*Txnip*), electron-transferring-flavoprotein β polypeptide (*Etfb*), electron-transferring-flavoprotein dehydrogenase (*Etfdh*), phosphatidylcholine transfer protein (*Pctp*), endothelial lipase (*EL*, *Lipg*), adipose triglyceride lipase (*Pnpla2*), hormone-sensitive lipase (*HSL*, *Lipe*), and monoglyceride lipase (*Mgll*). Using an *in silico* screening approach, one or more PPAR response elements (PPREs) were identified in each of these genes. Regulation of *Pnpla2*, *Lipe*, and *Mgll*, which are involved in triglyceride hydrolysis, was studied under conditions of elevated hepatic lipids. In wildtype mice fed a high fat diet, the decrease in hepatic lipids following treatment with the PPARα agonist WY14643 was paralleled by significant upregulation of *Pnpla2*, *Lipe*, and *Mgll*, suggesting that induction of triglyceride hydrolysis may contribute to the anti-steatotic role of PPARα.

Our study illustrates the power of transcriptional profiling to uncover novel PPARα-regulated genes and pathways in liver.

Introduction

The peroxisome proliferator-activated receptors (PPARs) play a pivotal role in the regulation of nutrient metabolism. PPARs are ligand-activated transcription factors that belong to the superfamily of nuclear hormone receptors (24, 63, 283). They share a common mode of action that involves formation of heterodimers with the nuclear receptor RXR, followed by binding to specific DNA-response elements in the promoter of target genes. The genomic sequence recognized by PPARs, referred to as PPAR response element or PPRE, consists of a direct repeat of the consensus hexameric motif AGGTCA interspaced by a single nucleotide. Binding of ligands to PPARs leads to recruitment of coactivators and causes chromatin remodeling, resulting in initiation of DNA transcription and upregulation of specific PPAR target genes (284, 285). Ligands for PPARs include both endogenous compounds, such as fatty acids and their eicosanoid derivatives, and synthetic agonists. Three different PPAR subtypes have been identified: PPARα, PPARβ/δ, and PPARγ. The latter isotype, which is most highly expressed in adipose tissue, is known to play an important role in adipocyte differentiation and lipid storage (221, 225, 286). It is a target for an important class of antidiabetic drugs, the insulin-sensitizing thiazolidinediones. Expression of PPARβ/δ is ubiquitous and has been connected to wound healing, cholesterol metabolism, and fatty acid oxidation in adipose tissue and muscle (135, 155, 161, 172). Finally, PPARα is highly expressed in liver where it stimulates fatty acid uptake and activation, mitochondrial β-oxidation, peroxisomal fatty acid oxidation, ketogenesis, and fatty acid elongation and desaturation. In addition, it has a major role in glucose metabolism (95) and the hepatic acute phase response (111, 112). Importantly, PPARα is the molecular target for the hypolipidemic fibrate class of drugs that lower plasma triglycerides and elevate plasma HDL (high density lipoprotein) levels.

In recent years, microarray technology has emerged as a powerful technique to study global gene expression. In theory, microarray analysis is a terrific tool to map PPARα-dependent genes and further characterize PPARα function. In practice, microarray yields a huge amount of data, the analysis and interpretation of which can be very difficult. Numerous studies have examined the effect of synthetic PPARα agonists on global gene expression using microarrays. While these studies uncovered many possible PPARα target genes, the manner in which the data were presented often rendered interpretation difficult. Part of the complexity is due to the size of the PPARα-dependent transcriptome in liver, which easily exceeds one thousand genes.

The aim of the present study was twofold: (1) to generate a comprehensive overview of PPARα-regulated genes relevant to hepatic lipid metabolism and (2) to identify possible

novel target genes and target pathways of PPARα connected with lipid metabolism. To that end, we (1) combined microarray data from several independent animal experiments involving PPARα-/- mice (in these experiments, mice were either given WY14643 or fasted for 24 hours), (2) focused on upregulation of genes by PPARα in conformity with the general paradigm of transcriptional regulation by nuclear hormone receptors, and (3) reduced complexity by progressively moving from the complete PPARα-dependent transcriptome towards genes relevant to lipid metabolism, and finally to the identification of possible PPARα target genes involved in lipid metabolism.

Materials and Methods

Materials. WY14643 was obtained from ChemSyn Laboratories (Lenexa, KS). Recombinant human insulin (Actrapid) was from Novo Nordisk (Copenhagen, Denmark). SYBR Green was from Eurogentec (Seraing, Belgium). DMEM, fetal calf serum, calf serum, and penicillin/streptomycin/fungizone were from Lonza Bioscience (Verviers, Belgium). Otherwise, chemicals were from Sigma (Zwijndrecht, the Netherlands).

Animals. Male pure-bred Sv129 and PPARα-/- mice on a Sv129 background were used at 3-5 months of age (Jackson Laboratories, Bar Harbor, ME). Animals were fed normal laboratory chow (RMH-B diet, Arie Blok animal feed, Woerden, the Netherlands). Study 1: fed mice were killed at the end of the dark cycle. Fasting was started at the onset of the light cycle for 24 hours (n=5 per group). Studies 2 and 4: wildtype and PPARα-/- mice were fed with WY14643 for 5 days by mixing it in their food (0.1%, n=5 per group). Studies 2 and 4 were carried out independently and 2 years apart. Study 3: wildtype and PPARα-/- mice fasted for 4 hours received a single dose of WY14643 (400µl of 10mg/ml WY14643 dissolved in 0.5% carboxymethylcellulose) and were killed 6 hours later (n=5 per group). Study 5: wildtype and PPARα-/- mice at 2-3 months of age were given a high-fat diet (D12451, Research Diets, New Brunswick, NJ) for 20 weeks (composition available at http://www.researchdiets.com/pdf/Data%20Sheets/DIO%20Series.pdf). During the last week, half of the mice were given WY14643 for 7 days by mixing it in their food (0.1%, n=5 per group). Livers were dissected and immediately frozen in liquid nitrogen.

All animal experiments were approved by the animal experimentation committee of Wageningen University and were carried out in conformity with the public health service (PHS) policy on humane care and use of laboratory animals.

Primary hepatocytes. Rat (Wistar) and mouse (Sv129) hepatocytes were isolated by two-step collagenase perfusion as described previously (287). Cells were plated on collagen-coated six-well plates. Viability was determined by Trypan Blue exclusion, and was at least 75%. Hepatocytes were suspended in William's E medium (Lonza Bioscience, Verviers, Belgium) supplemented with 10% (v/v) foetal calf serum, 20m-units/ml insulin, 50nM dexamethasone, 100U/ml penicillin, 100μg/ml of streptomycin, 0.25μg/ml fungizone, and 50μg/ml gentamycin. The next day, cells were incubated in fresh medium in the presence or absence of WY14643 (10μM) dissolved in DMSO for 24 hours, followed by RNA isolation.

Human hepatocytes and Hepatocyte Culture Medium Bulletkit were purchased from Lonza Bioscience (Verviers, Belgium). Human hepatocytes were isolated from a single donor. Cells were plated on collagen-coated six-well plates. Upon arrival of the cells, the medium was discarded and was replaced by Hepatocyte Culture Medium. The next day, cells were incubated in fresh medium in the presence or absence of WY14643 (50μM) dissolved in DMSO for 12 hours, followed by RNA isolation.

Affymetrix microarray. Total RNA was prepared from mouse livers and primary hepatocytes using TRIzol reagent (Invitrogen, Breda, the Netherlands). RNA was either pooled per group or treatment (studies 1 and 2, primary hepatocytes), or used individually (studies 3 and 4), and further purified using RNeasy micro columns (Qiagen, Venlo, the Netherlands). RNA integrity was checked on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) using 6000 Nano Chips according to the manufacturer's instructions. RNA was judged as suitable for array hybridization only if samples exhibited intact bands corresponding to the 18S and 28S ribosomal RNA subunits, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number >8.0). Ten micrograms of RNA were used for one cycle cRNA synthesis (Affymetrix, Santa Clara, CA). Hybridization, washing, and scanning of Affymetrix Genechip MOE430 (studies 1 and 2) or mouse genome 430 2.0 arrays (studies 3 and 4) was according to standard Affymetrix protocols.

Scans of the Affymetrix arrays were processed using packages from the Bioconductor project (288). Expression levels of probesets were calculated using GCRMA (289), followed by identification of differentially expressed probesets using Limma (290). Comparison was between fasted wildtype and fasted PPARα-/- mice (study 1) or between WY14643-treated wildtype and WY14643-treated PPARα-/- mice (studies 2–4). P-values were corrected for multiple testing using a false discovery rate method (291). Probesets that

satisfied the criterion of FDR <1% (q-value <0.01) and fold change >1.5 were considered to be significantly regulated. Functional clustering of the array data was performed by a method based on overrepresentation of Gene Ontology (GO) terms (292).

For the primary hepatocytes, expression levels were calculated applying the multichip-modified gamma model for oligonucleotide signal (multi-mgMOS) (293) and a remapped chip description file (294).

All microarray datasets were deposited to gene expression omnibus (GEO). The GEO series accession numbers are as follows: study 1: GSE8290, study 2: GSE8291, study 3: GES8292, study 4: GSE8295, primary hepatocytes: GSE8302.

RNA isolation and qRT-PCR. Total RNA was extracted from tissues with TRIzol reagent (Invitrogen, Breda, the Netherlands). 1µg of total RNA was reverse-transcribed with iScript (Bio-Rad, Veenendaal, the Netherlands). cDNA was PCR-amplified with Platinum Taq DNA polymerase (Invitrogen) on a Bio-Rad iCycler orMyIQ PCR machine. Primers were designed to generate a PCR amplification product of 100-200 bp and were taken from PrimerBank (http://pga.mgh.harvard.edu/primerbank). Specificity of the amplification was verified by melt-curve analysis and evaluation of efficiency of PCR amplification. The sequence of primers used is available upon request. The mRNA expression of all genes reported was normalized to *36B4* or cyclophilin gene expression.

***In silico* screening of putative PPREs using a PPRE classifier.** Genomic sequences for mouse genes spanning 20KB centered at the transcriptional start site (TSS) were extracted from the Ensembl database (NCBI36) and screened for DR1-type REs with predicted binding strength of at least 1%. The binding strength prediction was based on a PPRE classifier that uses a database of *in vitro* binding data for PPARs to assign predicted binding strength according to a classification scheme (295). The conservation of the putative PPREs between mouse, human, dog, and rat were evaluated using the Vertebrate Multiz Alignment and Conservation track available from UCSC genome browser (NCBI releases for human and mouse genomes, hg18 and mm8, February 2006).

Histological examination of liver. 5µ sections were cut from frozen liver pieces. For oil red O staining, sections were air dried for 30 minutes, followed by fixation in formal calcium (4% formaldehyde, 1% $CaCl_2$). Oil red O stock solution was prepared by dissolving 0.5g oil red O in 500ml isopropanol. An oil red O working solution was prepared by mixing 30ml oil red O stock with 20ml dH_2O. Sections were immersed on

working solution for 10 minutes followed by extensive washes in H_2O. Haematoxylin and eosin staining of frozen liver sections was carried out as described (http://www.ihcworld.com/histology.htm).

Results

Global analysis of PPARα-dependent gene regulation

We analyzed the data from four independent microarray studies to obtain a comprehensive picture of PPARα-dependent upregulation of gene expression in mouse liver. In the first study, mRNA was compared between livers of 24 hour fasted wildtype and PPARα-/- mice. In the second study, mRNA was compared between liver of wildtype mice and PPARα-/- mice fed the PPARα agonist WY14643 for 5 days. In these two studies, RNA was pooled from 4-5 mice and hybridized to Affymetrix MOE430A GeneChip arrays. Since no biological replicates were analyzed, only a fold change threshold criteria could be applied. Using a cutoff of 1.5-fold, expression of a total of 1847 probesets was lower in 24 hour fasted PPARα-/- mice compared with 24 hour fasted wildtype mice (Figure 1A) (http://nutrigene.4t.com/microarray/ppar2007). Using the same cutoff, 2234 probesets were at least 1.5-fold lower in the livers of PPARα-/- mice fed WY14643 compared to wildtype mice fed WY14643 (http://nutrigene.4t.com/microarray/ppar2007). The number of probesets that overlapped between the two groups was 569. A large proportion of these genes, which are thus under control of PPARα under pharmacological and physiological conditions, may represent target genes of PPARα.

In the third study, mRNA was compared between livers of wildtype mice and PPARα-/- mice treated with WY14643 for 6 hours, while in the fourth study mRNA was compared between livers of wildtype mice and PPARα-/- mice fed WY14643 for 5 days. Study 4 was carried out independently of study 2 in a different set of mice. For these two studies, biological replicates (4-5 mice per group) were run using Affymetrix mouse genome 430 2.0 GeneChip arrays, enabling statistical analysis of the data which was not possible for studies 1 and 2. Applying a false discovery rate of 0.01 and a 1.5-fold cutoff, 1679 probesets were lower in the livers of PPARα-/- mice compared to wildtype mice 6 hours after treatment with WY14643, and 2207 probesets after 5 days of feeding WY14643 (Figure 1B) (http://nutrigene.4t.com/microarray/ppar2007). While the majority of genes regulated by PPARα after 6 hours of WY14643 treatment were also, and generally more significantly, regulated after 5 days of WY14643 treatment (overlap of 1001 probesets), many genes were specifically or more significantly regulated after 6 hours, including the

direct PPAR target *G0s2* and the *EL* gene, respectively. The complete set of data from studies 2 and 4, which includes up- and downregulated genes, has been submitted to the Peroxisome Proliferators compendium assembled by Dr. J.C. Corton (US EPA, Research Triangle Park, USA). They will be analyzed in conjunction with numerous other microarray experiments involving peroxisome proliferators to obtain the "peroxisome proliferator transcriptome." In addition, the datasets have been submitted to GEO.

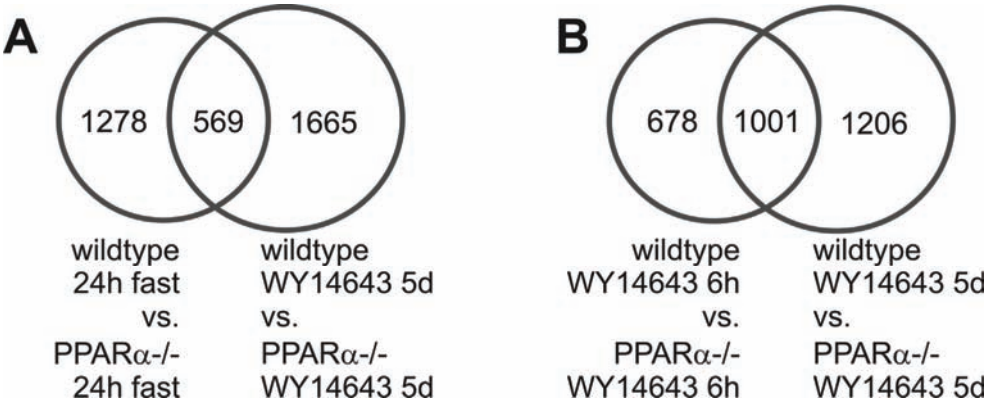


Figure 1. **Microarray analysis of PPARα-dependent gene regulation in mouse liver.** (A) Venn diagram showing the number of differentially expressed probesets between livers of 24-hour fasted wildtype and PPARα-/- mice, and between wildtype and PPARα-/- mice treated with the PPARα agonist WY14643 for 5 days. Pooled RNA was hybridized to Affymetrix MOE430A Arrays. A fold change of >1.5 was used as cutoff. (B) Venn diagram showing the number of differentially expressed probesets between livers of wildtype and PPARα-/- mice treated with the PPARα agonist WY14643 for 6 hours, and between wildtype and PPARα-/- mice treated with WY14643 for 5 days. RNA from individual mice was hybridized to Affymetrix Mouse 430 2.0 Arrays. Probesets that satisfied the criteria of fold change >1.5 and FDR <0.01 were considered to be significantly regulated.

Pathway analysis of PPARα-dependent gene regulation

Functional clustering analysis of the microarray data by Gene Ontology classification indicated that numerous Gene Ontology classes were overrepresented among the genes that were >1.5-fold upregulated in 24 hour fasted wildtype compared to 24 hour fasted PPARα-/- mice. The same was true for the comparison between wildtype and PPARα-/- mice treated with WY14643 for 5 days. Among the overrepresented Gene Ontology classes, we found many classes that are known to be governed by PPARα, including fatty acid β-oxidation, acyl-CoA metabolism, leukotriene metabolism, and peroxisome organization and biogenesis (http://nutrigene.4t.com/microarray/ ppar2007). Interestingly, we also noticed that numerous Gene Ontology classes were specifically upregulated by PPARα under fasting conditions or by WY14643 feeding. The data suggest, for example, that pyruvate metabolism and posttranslational protein targeting to membrane are specifically regulated in a PPARα-dependent manner by WY14643 but not by fasting. Indeed, it is clear that some genes (e.g., *Acot2* and *Cd36*) are PPARα-dependently regulated by WY14643 and much less so by fasting, whereas others (e.g., *Gpam*, *Hmgcs2*) are PPARα-dependently regulated by fasting and much less so by WY14643. However, it is important to emphasize that the ErmineJ Gene Ontology classification, as any functional clustering analysis, needs to be interpreted carefully.

The Gene Ontology classification analysis of the comparison wildtype versus PPARα-/- mice treated with WY14643 for 6 hours (study 3) was almost identical to the analysis for mice treated with WY14643 for 5 days (study 4), suggesting that most of the gene expression changes elicited by WY14643 treatment are fast transcriptional responses in correspondence with direct regulation of gene expression by PPARα. One notable exception was the class representing the acute phase response, which was regulated by 5 day but not 6 hour treatment with WY14643.

Comprehensive list of PPARα targets involved in lipid metabolism

Using these lists of genes that are upregulated by PPARα in mouse liver, we were able to create a comprehensive picture of PPARα-regulated genes connected with lipid metabolism. Genes in bold are PPARα-dependently regulated by WY14643 and during fasting, representing a conservative list of PPARα targets (Figure 2). Genes in normal font are PPARα-dependently regulated in any of the four studies included. From this picture, it is evident that rather than merely regulating the rate limiting enzyme in fatty acid oxidation, PPARα appears to regulate virtually every single step in the peroxisomal and mitochondrial fatty acid oxidation pathway. Furthermore, many genes involved in fatty acid binding and activation, lipid transport, and glycerol metabolism were controlled by

PPARα. What is remarkable is that PPARα also governs the expression of numerous genes involved in the synthesis of fats, which runs counter to the idea that PPARα mainly regulates fat catabolism. Several genes belonging to the lipogenic pathway have previously been recognized as PPARα targets, including *Mod1* and *Scd1*, yet the extent of regulation by PPARα is unexpected (21). Regulation of lipogenesis by PPARα was mainly observed after WY14643 treatment, and to a much lesser extent after fasting.

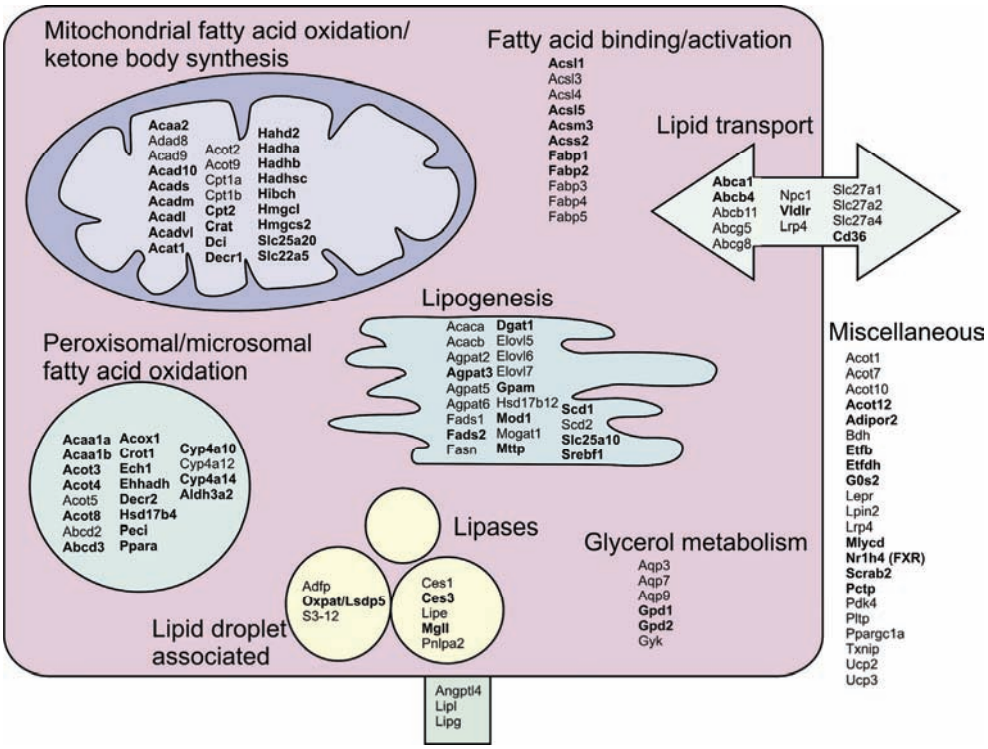


Figure 2. **Overview of PPARα-regulated genes involved in hepatic lipid metabolism.** Genes in bold are PPARα-dependently regulated during fasting and by WY14643, representing a conservative list of PPARα targets. Genes in normal font are PPARα-dependently regulated in any of the four studies included. Functional classification is based on a self-made functional annotation system of genes involved in lipid metabolism (http://nutrigene.4t.com/microarray/ppar2007).

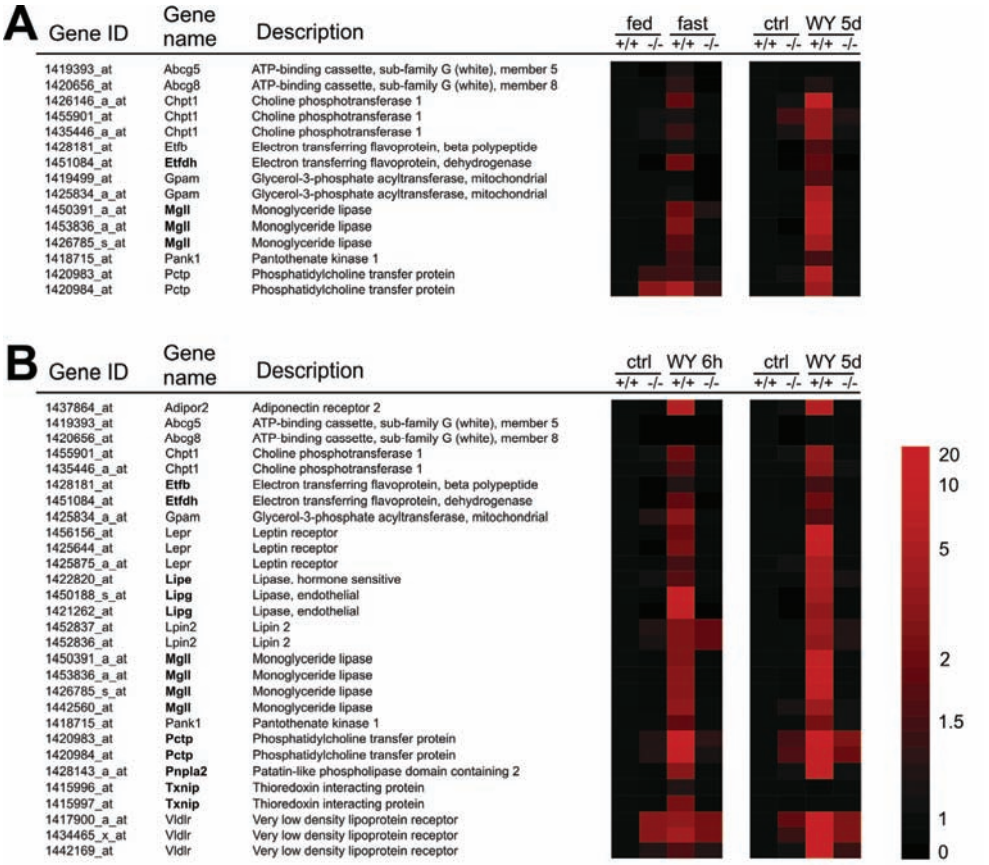


Figure 3. **PPARα-dependent regulation in mouse liver of selected genes involved in lipid metabolism as shown by heat map.** The (GCRMA normalized) expression data were derived from four separate microarray studies. Expression levels are shown as fold change according to color scale compared to wildtype mice without treatment (set at 1). (A) Expression data derived from studies 1 and 2. (B) Expression data derived from studies 3 and 4. Genes in bold were selected for expression analysis by qRT-PCR and *in silico* screening for putative PPREs.

Novel putative targets of PPARα involved in lipid metabolism

In addition to providing an overview of PPARα-dependent gene regulation, we were interested in identifying novel PPARα-regulated genes that are implicated in lipid metabolism. To that end, we went through the array data from studies 1 and 2 on the one hand, and studies 3 and 4 on the other hand, and selected a number of genes to generate a heat map showing their PPARα-dependent upregulation by fasting and/or WY14643 (Figure 3). To our knowledge, none of the genes shown, all of which are involved in hepatic lipid metabolism, has yet been reported to be regulated by PPARα. This includes phosphatidylcholine transfer protein (lipoprotein metabolism), glycerol-3-phosphate acyltransferase (triglyceride synthesis), very low-density lipoprotein receptor, choline phosphotransferase (phosphatidylcholine synthesis), and leptin receptor. Since all of these genes, except *Abcg5*, *Abcg8*, and *Lipe*, were upregulated 6 hours after WY14643 treatment, they possibly represent novel direct target genes of PPARα in liver, although PPREs have yet to be identified in their respective gene promoters.

Eight genes (shown in bold, Figure 3) were selected for more detailed investigation of PPARα-dependent gene regulation. Three of these genes are expected to be involved in the breakdown of hepatic triglycerides towards fatty acids: adipose triglyceride lipase (*Pnpla2*), hormone sensitive lipase (*Lipe*), and monoglyceride lipase (*Mgll*). Recent studies suggest that this threesome of genes is responsible for adipose tissue lipolysis (296-298). In addition, we selected endothelial lipase (*EL*, *Lipg*), a recently identified member of triglyceride lipase gene family that is a major determinant of plasma HDL cholesterol (299-301), and electron transferring flavoprotein dehydrogenase (*Etfdh*) and electron transferring flavoprotein β polypeptide (*Etfb*), which are components of the electron transport chain and accept electrons from at least nine mitochondrial matrix flavoprotein dehydrogenases (302, 303). Finally, we selected phosphatidylcholine transfer protein (*Pctp*), which is involved in lipoprotein metabolism, and thioredoxin interacting protein (*Txnip*), which was recently identified as a major regulator of the hepatic response to fasting, similar to PPARα. The selection of these genes was based entirely on perceived novelty and potential functional importance of the observed regulation. Using real-time quantitative PCR (qRT-PCR), we confirmed that the expression of all eight genes in liver was increased by WY14643 feeding in a PPARα-dependent manner (Figure 4A). In addition, we measured regulation of expression of this set of genes by PPARα during the course of fasting (Figure 4B). Expression of all eight genes went up during fasting which, except for *Pnpla2*, was PPARα-dependent. However, the pattern of expression was remarkably different between the various genes, suggesting for each gene a complex and unique interplay between several fasting-dependent transcription factors, including PPARα.

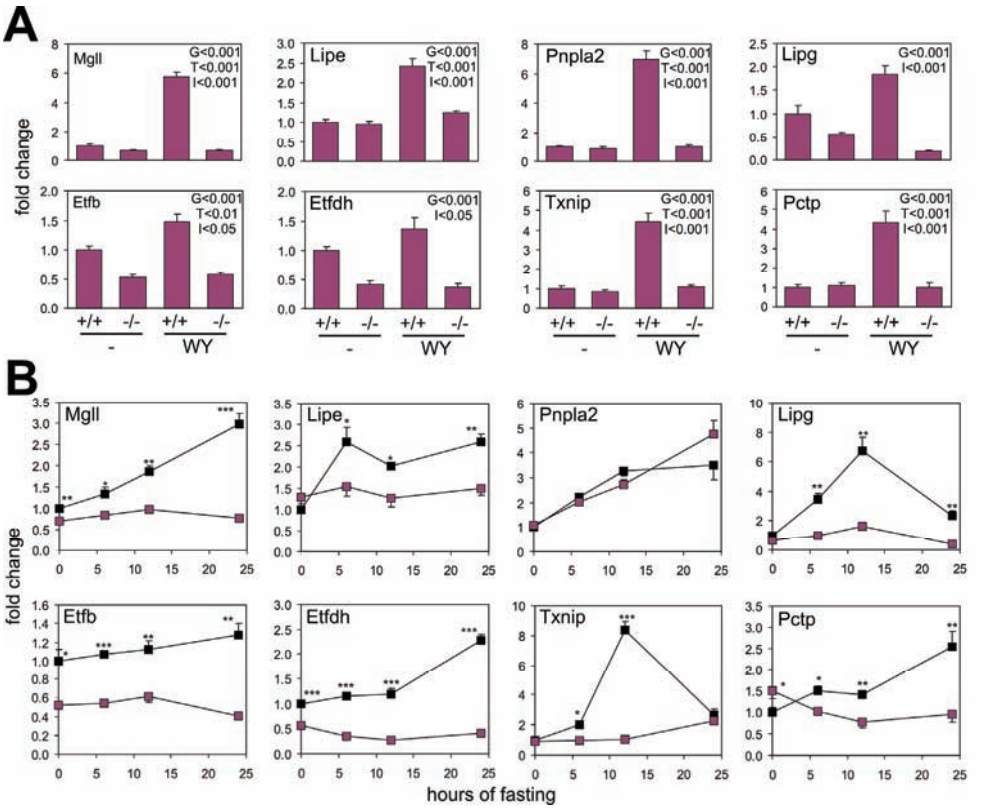


Figure 4. **PPARα governs expression of selected genes in mouse liver.** (A) Regulation of expression of selected genes by WY14643 feeding (5 days) in liver of wildtype (+/+) and PPARα-/- mice (-/-), as determined by qRT-PCR. Error bars represent SEM. Differences were evaluated statistically using two-way ANOVA. Significance (P-value) of effect of genotype (G), treatment (T) and interaction (I) between genotype and treatment is indicated in each figure. (B) Regulation of expression of selected genes by fasting in liver of wildtype (black boxes) and PPARα-/- mice (purple boxes), as determined by qRT-PCR. Error bars represent SEM. Differences in expression between wildtype and PPARα-/- mice at each time point were evaluated by student's t-test. *P < 0.05; **P < 0.01; ***P < 0.001.

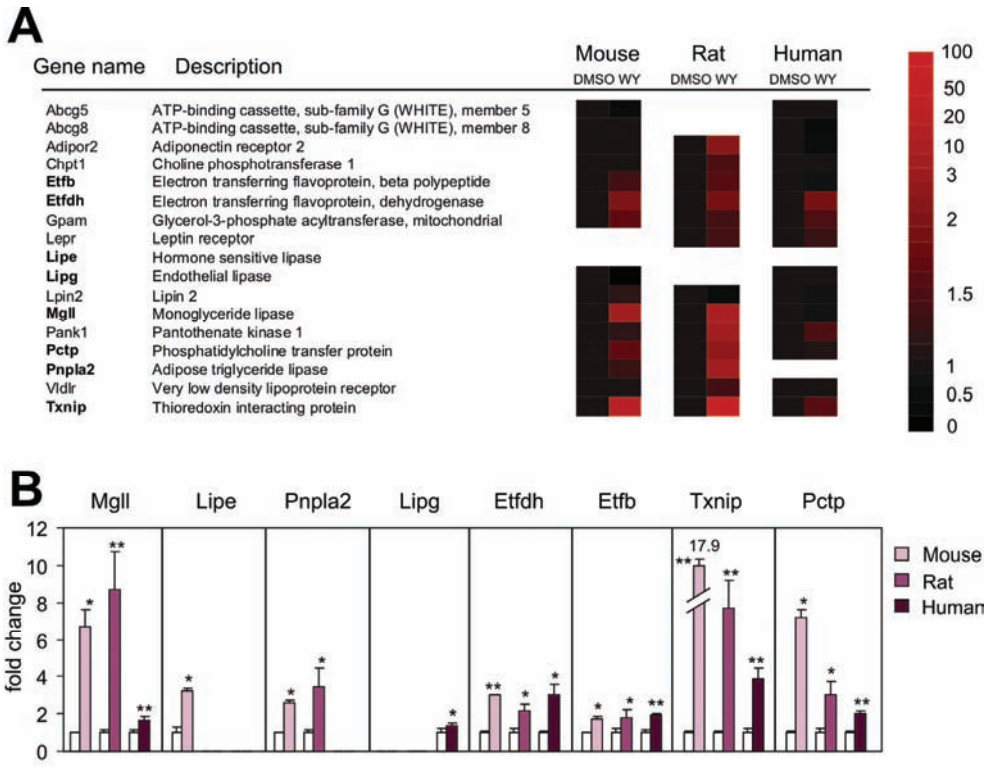
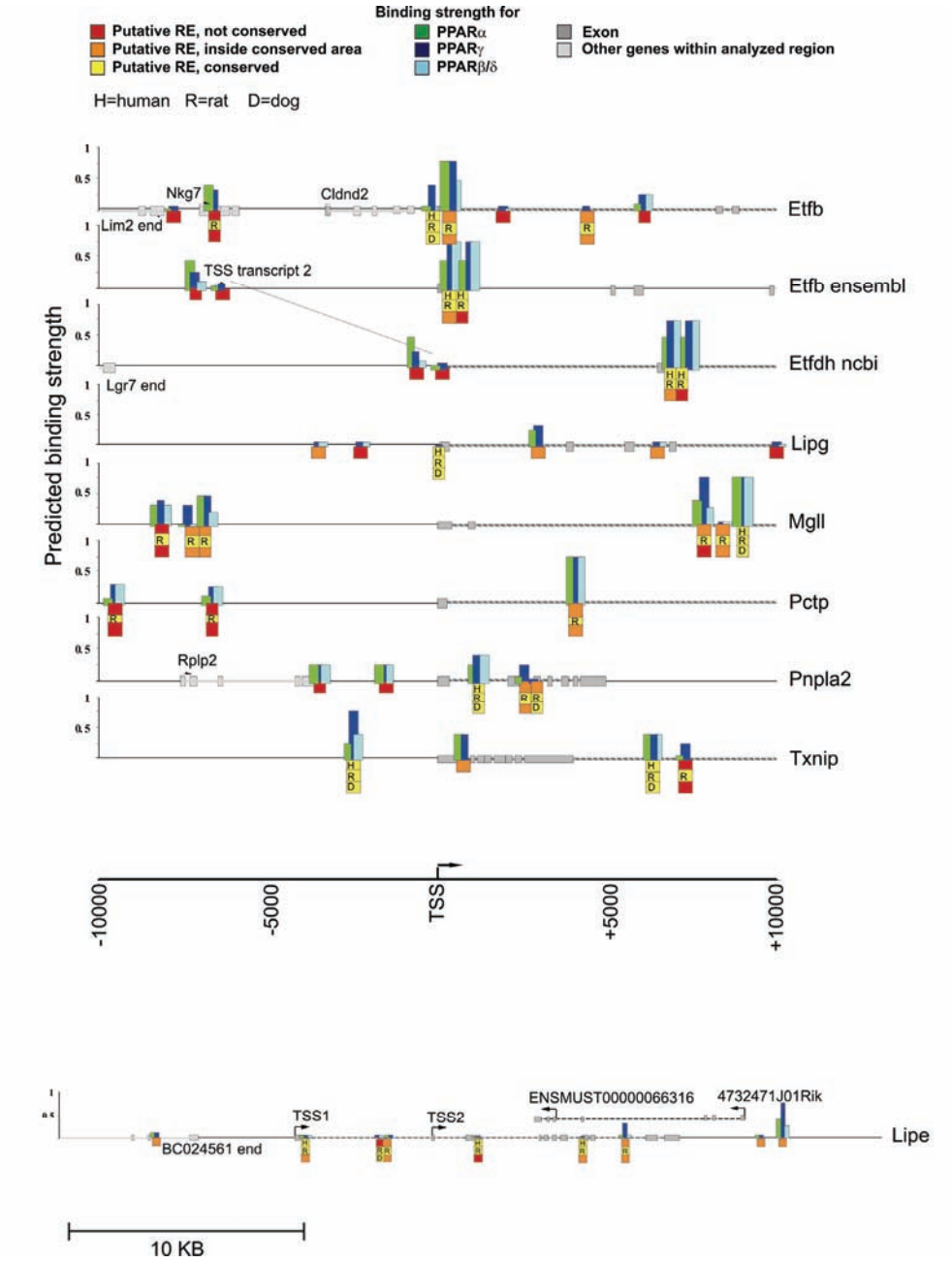


Figure 5. **Regulation of selected genes involved in lipid metabolism in primary hepatocytes by WY14643.** (A) Microarray-based heatmap showing relative expression levels of genes calculated using a multichip modified gamma model for oligonucleotide signal (multi-mgMOS) and a remapped chip description file. Expression levels in the absence of ligand were set at 1. (B) Relative induction of expression of selected genes in primary hepatocytes by WY14643, as determined by qRT-PCR. The primary hepatocytes used for qRT-PCR and microarray analysis were from independent experiments. Genes were not included when expression was extremely low (Ct > 30). Error bars represent SD. The effect of WY14643 on gene expression was evaluated by student's t-test. *P < 0.05; **P < 0.01.

Figure 6 (next page). In silico **screening for putative PPREs for the selected eight genes.** 10KB up- and downstream of the transcriptional start site were examined for the presence of putative PPREs. For each putative PPRE identified, the predicted PPAR subtype specific binding strength was determined, as reflected by the height of the bar. The sequence conservation of the PPRE among various species is indicated.


Binding strength for
Putative RE, not conserved
Putative RE, inside conserved area
Putative RE, conserved
PPARα
PPARγ
PPARβ/δ
Exon
Other genes within analyzed region
H=human R=rat D=dog
Predicted binding strength
Nkg7
Cldnd2
Lim2 end
Etfb
TSS transcript 2
Etfb ensembl
Etfdh ncbi
Lgr7 end
Lipg
Mgll
Pctp
Rplp2
Pnpla2
Txnip
-10000
-5000
TSS
+5000
+10000
TSS1
TSS2
ENSMUST00000066316
4732471J01Rik
BC024561 end
Lipe
10 KB

PPARα-dependent regulation in primary hepatocytes

To examine whether the PPARα-dependent regulation of the set of genes shown in Figure 3 was not an indirect consequence of metabolic perturbations elicited by the experimental challenge, we studied the effect of PPARα activation in primary mouse, rat, and human hepatocytes. Gene expression was first analyzed by microarray (Figure 5A), followed by targeted analysis of the selected eight genes by qRT-PCR (Figure 5B). Expression levels were calculated by applying a multichip modified gamma model for oligonucleotide signal (multi-mgMOS) (293) and a remapped chip description file (294) to allow for parallel analysis of the same gene within different species. Expression of almost every gene studied was highly upregulated by WY14643 in mouse and rat hepatocytes, compared to a more modest or no induction in human hepatocytes. For reasons that are not completely clear, in human hepatocytes, data from qRT-PCR and microarray did not always perfectly align. Overall, the data indicate that the PPARα-dependent regulation observed *in vivo* can be reproduced in primary hepatocytes. Furthermore, the data suggest that expression of six genes is governed by PPARα in human as well.

In silico screening of putative PPREs

To evaluate whether the selected eight genes represent possible direct PPAR target genes, the (mouse) genes were analyzed for the presence of putative PPREs using an *in silico* screening method (Figure 6). Ten KB up- and downstream of the TSS were examined. For each putative PPRE identified, the predicted PPAR subtype specific binding strength was determined. For each gene, at least one PPRE was identified that was conserved among rat, dog, and human. The *Etfdh* and *Txnip* genes were characterized by the presence of two very strong putative PPREs that were conserved in human. Up to six putative PPREs were identified in the *Mgll* gene, only one of which was conserved in human. A similar picture was found for *Pnpla2*. The putative PPREs located in the *EL* gene were weak and generally not conserved. Interestingly, a strong putative PPRE was identified in the *Pctp* gene, which however was not conserved in human. Conversely, the human *PCTP* gene contained several putative PPREs that were not conserved in mouse (data not shown).

PPARα activation prevents hepatic lipid storage after fasting

Our data extend the role of PPARα in hepatic lipid metabolism and suggest that PPARα may govern triglyceride hydrolysis. To find out whether activation of the triglyceride hydrolysis pathway by PPARα is associated with a decrease in hepatic triglyceride stores, we compared wildtype and PPARα-/- mice fed a high fat diet (HFD) for 20 weeks, followed by treatment for one week with WY14643. Numerous studies, including ours

(72), have shown that chronic HFD increases hepatic triglyceride stores. In wildtype mice fed the HFD, treatment with WY14643 markedly decreased hepatic lipids (Figures 7A and 7B), as shown by smaller lipid droplets, which was paralleled by significant induction of expression of *Pnpla2*, *Lipe*, and *Mgll* (Figure 7C). These data suggest that induction of the triglyceride hydrolysis pathway may contribute to the overall reduction in liver triglycerides elicited by PPARα activation.

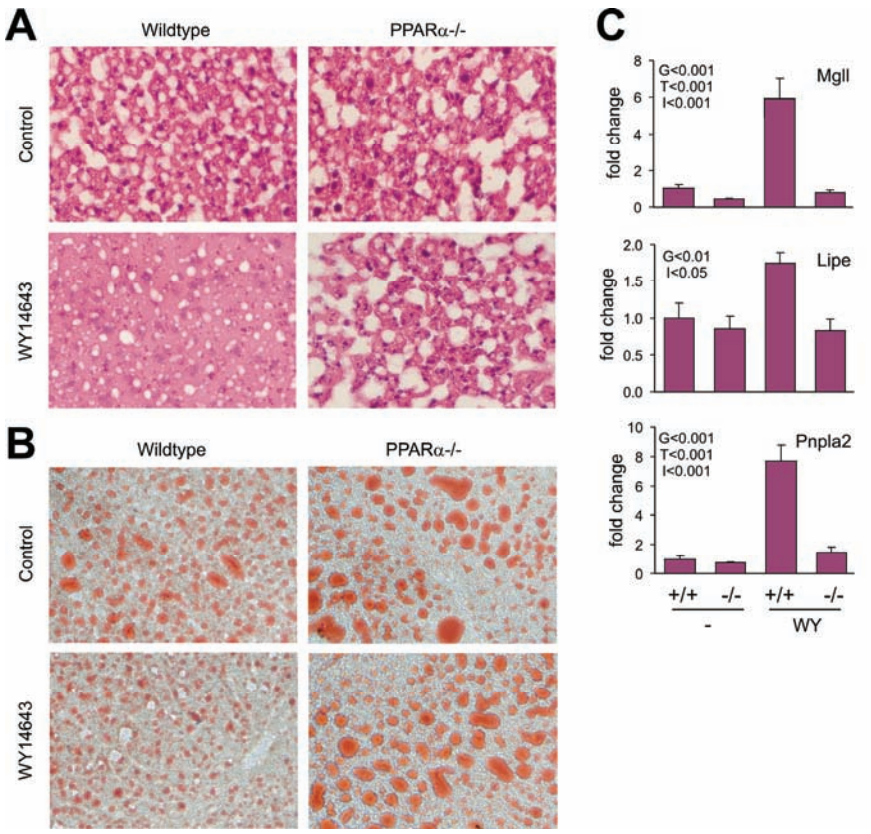


Figure 7. **Induction of the triglyceride hydrolysis pathway by WY14643 is paralleled by a decrease in hepatic lipid stores.** Hematoxilin and eosin staining (A) and oil red O staining (B) of representative liver sections of wildtype and PPARα-/- mice treated or not with WY14643 for 7 days (magnification 200X). All mice were given a high fat diet for 20 weeks prior to WY14643 treatment. (C) Hepatic expression of *Mgll*, *Lipe*, and *Pnpla2* in the four experimental groups as determined by qRT-PCR. Error bars represent SEM. Differences were evaluated statistically using two-way ANOVA. Significance (P-value) of effect of genotype (G), treatment (T), and interaction (I) between genotype and treatment is indicated in each figure.

Discussion

The aim of our study was twofold: (1) to generate a comprehensive overview of PPARα-regulated genes relevant to hepatic lipid metabolism, and (2) to identify possible novel target genes and target pathways of PPARα connected with lipid metabolism.

It can be argued that to identify possible novel PPARα targets, the proper comparison should have been between wildtype and wildtype treated with WY14643, as opposed to wildtype treated with WY14643 and PPARα-/- treated with WY14643, in order to avoid inclusion of genes that are differentially expressed between wildtype and PPARα-/- mice under basal conditions (and could represent genes indirectly regulated by PPARα). The rationale behind our decision was that we wanted to be open-minded about the PPARα-dependent transcriptome and not exclude genes that are solely regulated by PPARα under basal conditions. For example, opting for the comparison wildtype versus wildtype treated with WY14643 would have led to the exclusion of *Etfdh*, which according to our data represents a prime candidate PPARα target gene in mouse and human. Furthermore, to enable comparison between the effects of fasting and WY14643, it was essential to include the PPARα dependency, since the majority of genes regulated by fasting are regulated in a PPARα-independent manner.

Gene Ontology classification analysis showed that numerous pathways and biological processes beyond lipid metabolism were regulated by PPARα. We observed that the expression of almost 1700 probesets was significantly increased 6 hours after a single oral dose of WY14643. Although not all genes regulated may represent direct PPARα targets, and even though the functional consequences of the observed regulation still needs to be demonstrated, these data at least suggest a major role for PPARα in hepatic gene expression and overall liver homeostasis.

In agreement with the first aim, we created a comprehensive overview of hepatic PPARα-regulated genes connected to lipid metabolism (Figure 2). A functional PPRE has been found in the promoter of several of these genes, classifying them as direct PPARα target genes, and many more genes have been shown to be upregulated by PPARα without a functional PPRE having been identified (21). It can be presumed that the far majority of genes presented in Figure 2 (as well as the other genes that were shown to be regulated by PPARα) are actually direct target genes of PPARα, but it is beyond the scope and capacity of the present study to address this issue in more detail. Our hope is that by combination of expression arrays with global analysis of promoter occupancy by PPARα using chromatin

immunoprecipitation and tiling or promoter arrays (so-called ChIP-on-chip analysis), the complete picture of direct PPARα target genes will be available in the future.

The second aim of our study was to identify possible novel target genes of PPARα representing specific steps in lipid metabolism unknown to be governed by PPARα. As part of this effort, we identified several genes for which a link with PPARα has not yet been reported, including VLDL receptor, leptin receptor, and choline phosphotransferase. We focused our energy on eight genes for which regulation by PPARα was deemed most novel and functionally interesting. All eight genes, except for *Lipe*, were significantly upregulated 6 hours after treatment with WY14643.

Using an *in silico* method to screen for PPREs, for each gene several putative PPREs could be located within 10KB of the transcriptional start site. Within this region, at least one PPRE was identified that was conserved among rat, dog, and human. The presence of multiple strong putative PPREs within the mouse *Mgll* gene is in correspondence with the marked regulation of *Mgll* expression in mouse liver and isolated hepatocytes. To a lesser extent, this is also true for the *Pnpla2* and *Pctp* genes. Furthermore, the predicted presence of two strong, well-conserved putative PPREs in the *Etfdh* and *Txnip* genes is in agreement with the highest fold-induction of these genes by WY14643 in primary human hepatocytes. Although *in silico* screening may not be able to substitute for analysis of direct promoter binding by ChIP, the predictive power of the method explored has been shown to be remarkably robust (295). Our results also substantiate the developing notion that PPAR-dependent gene regulation is generally mediated by multiple PPREs, rather than a single PPRE.

One remarkable outcome of the global analysis of gene regulation by PPARα is that PPARα appears to play a major role in governing lipogenesis. While several genes involved in lipogenesis were already known as PPARα targets, including Δ5 and Δ5 desaturase (*Fads*), stearoyl-CoA desaturase (*Scd*), microsomal triglyceride transfer protein (*Mttp*), and malic enzyme (*Mod1*) (21), the extent of regulation of lipogenesis is somewhat surprising, especially since PPARα is generally considered to stimulate fat catabolism rather than fat synthesis. It can be speculated that upregulation of fatty acid desaturation and elongation enzymes by PPARα might serve to stimulate production of PPARα ligands, and is part of a feed forward action of PPARα that also includes auto regulation of gene expression.

Although the triglyceride hydrolysis pathway in liver still has to be fully elucidated, it may very well be similar to the pathway operating in adipose tissue (296). Adipose tissue

triglycerides are likely hydrolyzed in a three-step process catalyzed by adipose triglyceride lipase (*Pnpla2*), hormone sensitive lipase (*Lipe*), and monoglyceride lipase (*Mgll*) (296-298, 304). Remarkably, deletion of the *Pnpla2* gene in mice not only results in more adipose mass but also causes a marked increase in lipid storage in a variety of organs, including liver and heart, suggesting that the triglyceride hydrolysis pathway is conserved between various organs (296). Disabling the PPARα gene is known to increase hepatic triglyceride accumulation, especially under conditions of fasting (71, 72, 305). Conversely, treatment with PPARα agonists lowers hepatic triglyceride levels in various models of hepatic steatosis (306-309). The antisteatotic effect of PPARα has generally been ascribed to stimulation of fatty acid oxidation, which, by decreasing intracellular fatty acid levels, will act as a drain on intracellular triglyceride stores. However, our data suggest that PPARα may directly govern the triglyceride hydrolysis pathway in liver via upregulation of lipases *Pnpla2*, *Lipe*, *Mgll*, and possibly *Ces1* and *Ces3* (Figure 2). Although it is impossible to provide definite experimental proof that induction of the triglyceride hydrolysis pathway by PPARα, or induction of fatty acid oxidation for that matter, is necessary and sufficient for its hepatic triglyceride-lowering effect, it likely contributes to the overall reduction in liver triglycerides elicited by PPARα agonists.

Our data suggest that expression of *EL* is under control of PPARα. *EL*, synthesized in endothelial cells, plays an important role in governing plasma lipoprotein concentrations and is a major determinant of plasma HDL cholesterol and apoAI concentrations. Indeed, overexpression of *EL* in the liver results in a significant decrease in HDL cholesterol and apoAI (299-301). *EL* has been shown to have some triglyceride lipase but mainly phospolipase activity (310). Although *in silico* screening failed to detect a strong PPRE in this gene, in our study *EL* expression was highly increased by 6 hours WY14643 treatment and by fasting in a PPARα-dependent manner, suggesting that *EL* may be a direct PPARα target gene. As *EL* expression was minimal in primary hepatocytes, *EL* transcripts likely originated from liver epithelial cells rather than liver parenchymal cells. Although further work is necessary, we suspect that *EL* may be a direct PPARα target in endothelial cells. Considering that, in contrast to *EL*, PPARα agonists raise plasma HDL, the functional importance of regulation of *EL* by PPARα needs to be further validated.

Another novel PPARα-regulated gene of relevance to lipoprotein metabolism is *Pctp*. *Pctp* is a steroidogenic acute regulatory-related transfer domain protein that binds phosphatidylcholines with high specificity. Studies with Pctp-null mice suggest that it may modulate HDL particle size and rates of hepatic clearance (311). According to our data, expression of *Pctp* increases during fasting, which is abolished in PPARα-/- mice.

WY14643 markedly upregulated *Pctp* mRNA in mouse liver as well as in mouse, rat, and human hepatocytes, suggesting it may represent a novel PPARα target gene.

Etfdh and *Etfb* are essential components of the oxidative phosphorylation pathway. They are responsible for the electron transfer from at least nine mitochondrial flavin-containing dehydrogenases to the main respiratory chain (302, 303). According to our data, expression of *Etfdh* and *Etfb* is governed by PPARα, suggesting that besides the β-oxidation pathway, PPARα also regulates components of the respiratory chain involved in the transfer of electrons from fatty acids and other molecules.

The last gene that we studied in more detail was *Txnip*, which is also known as *Hyplip1*. A spontaneous mutation within the *Txnip* gene gives rise to a complex phenotype that resembles familial-combined hyperlipidemia, including hypercholesterolemia and hypertriglyceridemia (312). Recent studies suggest that *Txnip* plays an important metabolic role in the fasting-feeding transition by altering the redox status of the cell, which results in stimulation of the tricarboxylic acid cycle at the expense of ketone body or fatty acid synthesis (313). Indeed, Txnip-deficient mice show elevated plasma ketones, elevated free fatty acids, hypercholesterolemia, and hypertriglyceridemia, yet decreased glucose levels (312, 314). The phenotype is very similar to that of PPARα-/- mice with the exception of the elevated plasma ketones. Since hepatic expression of *Txnip* is decreased in PPARα-/- mice, it can be hypothesized that part of the effect of PPARα deletion on lipid and glucose metabolism is mediated by downregulation of *Txnip* in liver, which subsequently might affect redox status. It is unclear to what extent *Txnip* expression is affected by PPARα deletion in tissues other than liver.

In conclusion, our data indicate that the role of PPARα in hepatic lipid metabolism is much more extensive than previously envisioned. By generating a schematic overview of PPARα-dependent gene regulation in mouse liver, and, for a selected set of genes, by providing evidence for direct regulation by PPARα in rodents and human, we have extended the role of PPARα in the control of hepatic lipid metabolism.

Acknowledgements

We would like to thank Wei Zhu, Rene Bakker, and Oskari Uski for their valuable contributions.

Chapter 4

Effect of synthetic dietary triglycerides: a novel research paradigm for nutrigenomics

Linda M. Sanderson, Philip J. de Groot, Guido J.E.J. Hooiveld, Arjen Koppen, Eric Kalkhoven, Michael Müller and Sander Kersten

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Abstract

The effect of dietary fats on human health and disease are likely mediated by changes in gene expression. Several transcription factors have been shown to respond to fatty acids, including SREBP-1c, NF-KB, RXRs, LXRs, FXR, HNF4α, and PPARs. However, it is unclear to what extent these transcription factors play a role in gene regulation by dietary fatty acids *in vivo*.

Here, we take advantage of a unique experimental design using synthetic triglycerides composed of one single fatty acid in combination with gene expression profiling to examine the effects of various individual dietary fatty acids on hepatic gene expression in mice. We observed that the number of significantly changed genes and the fold-induction of genes increased with increasing fatty acid chain length and degree of unsaturation. Importantly, almost every single gene regulated by dietary unsaturated fatty acids remained unaltered in mice lacking PPARα. In addition, the majority of genes regulated by unsaturated fatty acids, especially docosahexaenoic acid, were also regulated by the specific PPARα agonist WY14643. Excellent agreement was found between the effects of unsaturated fatty acids on mouse liver versus cultured rat hepatoma cells. Interestingly, using Nuclear Receptor PamChip® Arrays, fatty acid- and WY14643-induced interactions between PPARα and coregulators were found to be highly similar, although several PPARα-coactivator interactions specific for WY14643 were identified.

We conclude that the effects of dietary unsaturated fatty acids on hepatic gene expression are almost entirely mediated by PPARα and mimic those of synthetic PPARα agonists in terms of regulation of target genes and molecular mechanism. Use of synthetic dietary triglycerides may provide a novel paradigm for nutrigenomics research.

Introduction

Dietary fatty acids have multiple functions in the human body. They are an important energy source, form an essential part of the phospholipid bilayer of membranes, and function as precursors to several signaling molecules, such as the eicosanoids. A huge body of literature collected in the past few decades provides compelling evidence that changes in the dietary fatty acid composition can profoundly influence health and disease. For example, it is well established that replacing dietary saturated fatty acids with n-6 mono- and polyunsaturated leads to a decrease in plasma concentration of low density lipoprotein, which is a well-known risk factor for atherosclerosis (315). Likewise, increased consumption of n-3 fatty acids, especially eicosapentaenoic acid and docosahexaenoic acid present in fish oil, is associated with decreased plasma triglyceride (TG) concentrations (316), may prevent against cardiac arrhythmias (317), and improves visual acuity in preterm infants (318). Numerous molecular mechanisms may underlie the effects of dietary fatty acids on parameters of health. While historically the main focus was on changes in plasma membrane fluidity as a result of changes in phospholipid composition, the discovery of nuclear receptors has progressively shifted the emphasis to regulation of gene expression.

The superfamily of nuclear receptors encompasses a related but diverse set of transcription factors that share a number of structural and functional features (319). They consist of a central DNA-binding domain that directs the receptor to specific DNA sequences within a gene promoter, and a ligand-binding domain, which can accommodate a variety of different compounds. Roughly, nuclear receptors can be divided into three main groups: the endocrine receptors that bind steroid hormones, the adopted orphan receptors that bind dietary lipids, and the orphan receptors, for which no ligand exists or still has to be identified (320). The adopted orphan receptors share a common mode of action that involves heterodimerization with the nuclear retinoid X receptor (RXR). Binding of ligands to the receptor leads to recruitment of coactivators and dissociation of corepressors, resulting in chromatin remodeling followed by initiation of DNA transcription. Adopted orphan receptors mainly function as lipid sensors by altering the rate of transcription of specific genes in response to changes in lipid concentration (320). These lipids include oxysterols, bile acids, and fat soluble vitamins. In addition, many adopted orphan receptors have been shown to bind fatty acids and alter transcription in response to changes in fatty acid concentration and/or composition, including RXR, peroxisome-proliferator activated receptors (PPARα, β/δ and γ), hepatic nuclear factor 4α (HNF-4α), liver X receptor (LXR) α and β, and farnesoid X receptor (321, 322). Other receptors that mediate the effects of dietary fatty acids on gene expression include the sterol regulatory element binding protein

1, and the nuclear factor kappaB (321). However, the relative contribution of all these receptors to fatty acid-dependent gene regulation *in vivo* remains completely unclear.

Here, we take advantage of a unique experimental design using synthetic triglycerides composed of one single fatty acid in combination with gene expression profiling to examine the effects of individual dietary fatty acids on hepatic gene expression in mice. By conducting these experiments in wildtype and PPARα-/- mice, we were able to explore the specific contribution of PPARα. We conclude that the effects of dietary unsaturated fatty acids on hepatic gene expression are almost exclusively mediated by PPARα and mimic those of synthetic PPARα agonists in terms of target genes regulation and molecular mechanism.

Materials and Methods

Materials. WY14643 was purchased from ChemSyn Laboratories (Lenexa, KS, USA). Triolein was from Fluka (Zwijndrecht, the Netherlands). Trilinolein, trilinolenin, tridocosahexaenoin and trieicosapentaenoin were from Nu-Chek-Prep, Inc. (Elysian, MN, USA). Fetal bovine serum and penicillin/streptomycin were from Cambrex Bioscience (Seraing, Belgium). SYBR Green was purchased from Eurogentec (Seraing, Belgium). All other chemicals were from Sigma (Zwijndrecht, the Netherlands).

Animals. Male pure-bred Sv129 and PPARα-/- mice (2-6 months of age) on a Sv129 background were used. Two weeks before start of the experiment, the animals were switched to a run-in diet consisting of a modified AIN76A diet (corn oil was replaced by olive oil) (Research Diet Services, Wijk bij Duurstede, the Netherlands). Starting at 5 a.m. the animals were fasted for 4 hours followed by an intragastric gavage of 400µl synthetic triglyceride (triolein, trilinolein, trilinolenin, trieicosapentaenoin or tridocosahexaenoin) (Figure 1). WY14643 and fenofibrate were given as 10mg/ml suspension in 0.5% carboxymethyl cellulose, which also served as control treatment (400µl). Four to five mice per group were used, adding up to 78 mice in total. 6 hours after gavage, mice were anaesthetized with a mixture of isofluorane (1.5%), nitrous oxide (70%) and oxygen (30%). Blood was collected by orbital puncture, after which the mice were sacrificed by cervical dislocation. Livers were removed, snap-frozen in liquid nitrogen and stored at -80°C until further analysis. For RNA analyses, tissue from the same part of the liver lobe was used. The animal studies were approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University.

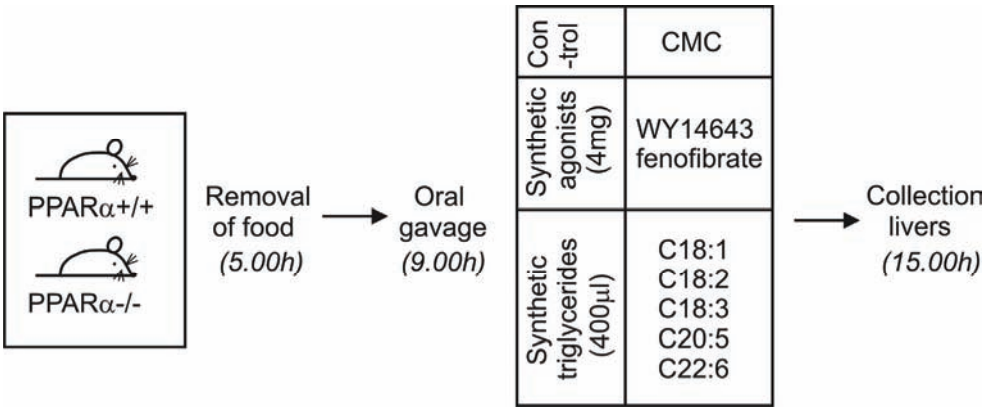

Figure 1. **Schematic representation of dietary intervention.** Wildtype and PPARα-/- mice fasted for four hours were given a single oral dose of different synthetic triglycerides composed of one single unsaturated fatty acid (400µl), or one of the PPARα agonists WY14643 or fenofibrate (4mg). After six hours, the livers were used for gene expression profiling using Affymetrix Mouse Genome 430 2.0 Microarrays (~45000 probesets) on biological replicates. CMC = carboxymethyl cellulose.

Lipid absorption and tissue distribution. Measurement of intestinal lipid absorption was carried out exactly as previously described (323). For the lipid loading test wildtype mice were fasted for 4 hours followed by administration of 400µl olive oil via intragastric gavage. Blood was collected by tail bleeding every 2 hours for plasma TG measurement. Tissue uptake of [^{3}H]-labeled TG packaged into VLDL-like emulsion particles was measured as previously described (324). The data shown reflect percentage of bolus radioactivity taken up after 30 minutes by a specific tissue expressed per gram tissue.

Triglycerides. Plasma and liver triglycerides were measured with a commercially available kit from Instruchemie (Delfzijl, the Netherlands). Livers were weighed and homogenized in a buffer (pH 7.5) containing 250mM sucrose, 1mM EDTA and 10mM Tris, with a final tissue concentration of 5%. 2µl of plasma or liver homogenate was used to determine TG.

RNA isolation and qRT-PCR. Total liver RNA was isolated with TRIzol reagent (Invitrogen, Breda, the Netherlands) according to manufacturer's instructions. A NanoDrop ND-1000 spectrophotometer (Isogen, Maarssen, the Netherlands) was used to determine RNA concentrations. 1µg of total RNA was reverse transcribed using iScript (Bio-Rad, Veenendaal, the Netherlands). cDNA was amplified on a Bio-Rad MyIQ or iCycler PCR machine using Platinum Taq DNA polymerase (Invitrogen, Breda, the Netherlands). PCR primer sequences were taken from the PrimerBank (325) and ordered from Eurogentec (Seraing, Belgium). Sequences of the primers used are available upon request.

Affymetrix microarray. Total RNA from mouse liver was extracted with TRIzol reagent, and purified and DNAse treated using the SV Total RNA Isolation System (Promega, Leiden, the Netherlands). RNA quality was assessed on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) with 6000 Nano Chips using the Eukaryote Total RNA Nano assay. RNA was judged as suitable for array hybridization only if samples showed intact bands corresponding to the 18S and 28S rRNA subunits, displayed no chromosomal peaks or RNA degradation products, and had a RIN (RNA integrity number) above 8.0. Five micrograms of RNA were used for one cycle cRNA synthesis (Affymetrix, Santa Clara, CA). Hybridization, washing and scanning of Affymetrix Mouse Genome 430 2.0 Arrays was carried out according to standard Affymetrix protocols.

Packages from the Bioconductor project were used for analyzing the scanned Affymetrix arrays (288). Arrays were normalized using quartile normalization, and expression estimates were compiled using GC-RMA applying the empirical Bayes approach (289). A non-specific filtering step was applied to remove probesets with low variation, as they provide no discriminating power (326). Only those probesets were included that had an inter-quartile range (IQR) across the samples of at least 0.25 on the log2-scale. Differentially expressed probesets were identified using linear models, applying moderated t-statistics that implement empirical Bayes regularization of standard errors (327).

Comparisons were made between wildtype treated and untreated (control) and also between PPARα-/- treated and untreated animals. Probesets that presented a P-value <0.01 were considered to be significantly changed by treatment. If a probeset was significantly changed in the wildtype but not the PPARα-/- mouse, it was considered to be PPARα-dependent (also probesets that were significantly changed in the PPARα-/- mouse, but had a fold change <1.5 of the fold change in the wildtype mouse were included in this category).

Functional analysis of the array data was performed by a method based on overrepresentation of Gene Ontology (GO) terms, where the functional class score (FCS) method was used (292, 328, 329).

Cell culture. Rat hepatoma FAO cells were grown in DMEM containing 10% (vol/vol) fetal bovine serum, 100U/ml penicillin and 100µg/ml streptomycin. Cells were incubated with albumin-bound fatty acids (100µM) dissolved in ethanol or synthetic PPARα ligands dissolved in DMSO (5µM WY14643, 50µM fenofibrate). Incubation continued for 24 hours and was followed by RNA isolation and qRT-PCR.

Cofactor recruitment assay. Nuclear Receptor PamChip® Arrays (PamGene, s'Hertogenbosch, the Netherlands) were used according to the manufacturer's instructions. Upon binding a ligand, PPARα undergoes a conformational change which promotes the formation of a cofactor binding pocket, subsequently allowing interaction with the so-called LxxLL motif within some coregulators. The PamChip® arrays consist of 48 peptides encompassing the LxxLL motifs of 19 different coregulator proteins ((330), Koppen et al. 2007. Micro Array assay for Real-time analysis of Coregulator-Nuclear receptor Interaction. Manuscript submitted.) Briefly, the arrays were incubated with glutathione-S-transferase (GST)-tagged PPARα-LBD (Invitrogen, Breda, the Netherlands) in the presence or absence of ligand. Quantification of interaction between PPARα and coregulators was made using Alexa488-conjugated anti-GST rabbit polyclonal antibody (Invitrogen). As ligands, either a negative control (EtOH), the synthetic PPARα agonist WY14643 or one of the fatty acids C18:1, C18:2, C18:3, C20:5 or C22:6 were used.

Results

Mice that were fasted for 4 hours were given a single oral dose (400µl) of synthetic triglycerides (TGs) consisting of one single fatty acid, followed by collection of tissues 6 hours thereafter (Figure 1). A parallel treatment in mice lacking PPARα was performed to enable estimation of the importance of PPARα in gene regulation by dietary fatty acids. The fatty acids studied were oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), eicosapentaenoic acid (C20:5), and docosahexaenoic acid (C22:6). No saturated fatty acids were included because triglycerides composed of common dietary saturated fatty acids are solid at room temperature and could not be administered orally. The 6 hour time point was chosen because in an independent oral fat load experiment, plasma triglyceride levels peaked 2 hours after the fat load and almost returned back to baseline

after 6 hours (Figure 2A), indicating that at that point most of the fat bolus has been cleared from the blood and taken up by the tissues. Indeed, we observed that 6 hours after oral dosing plasma TG levels had almost returned to baseline (Figure 2B), and were similar in wildtype and PPARα-/- mice, suggesting no major differences in plasma TG kinetics between the various fatty acids and between wildtype and PPARα-/-. Also, no major differences in the rate of intestinal TG absorption were observed between wildtype and PPARα-/- mice (Figure 2C). Finally, while as expected liver TG levels were higher in the PPARα-/- mice compared to wildtype mice, in the wildtype mice liver TG levels were similar between the various fatty acids (Figure 2D). These data argue against major differences in metabolic processing of dietary fat between wildtype and PPARα-/- mice and between different dietary fatty acids.

The focus of the present study is on liver since we observed that, when expressed per gram organ weight, the liver and heart take up most of the fatty acids present in TG-rich lipoproteins (Figure 2E). A future publication will address the effect of dietary fatty acids on gene expression and the involvement of PPARs in heart.

PPARα-dependent gene regulation by dietary unsaturated fatty acids

Expression profiling was carried out on individual mouse livers. Use of Affymetrix Mouse Genome 430 2.0 Arrays (whole mouse genome array), which contain more than 45000 probesets corresponding to over 34000 genes, allows for a genome-wide analysis of the number of significantly changed genes in the various treatment groups. After inter-quartile range (IQR) filtering, 11463 probesets (equivalent to 7231 genes) were left for analysis. A regularized t-test was performed to analyze changes in gene expression between the control and oral triglyceride group. The regularized t-test statistic has the same interpretation as an ordinary t-test statistic, except that the standard errors have been moderated across genes, i.e. shrunk to a common value, using a Bayesian model (327). A probeset was found to be significantly changed after treatment if $P<0.01$. All microarray results have been deposited into the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/) and can be accessed online under series number GSE8396. Quantitative real-time PCR was carried out on ~30 genes in order to confirm the results from the microarray, and the results were found to be in close agreement with the microarray data (Figure 3).

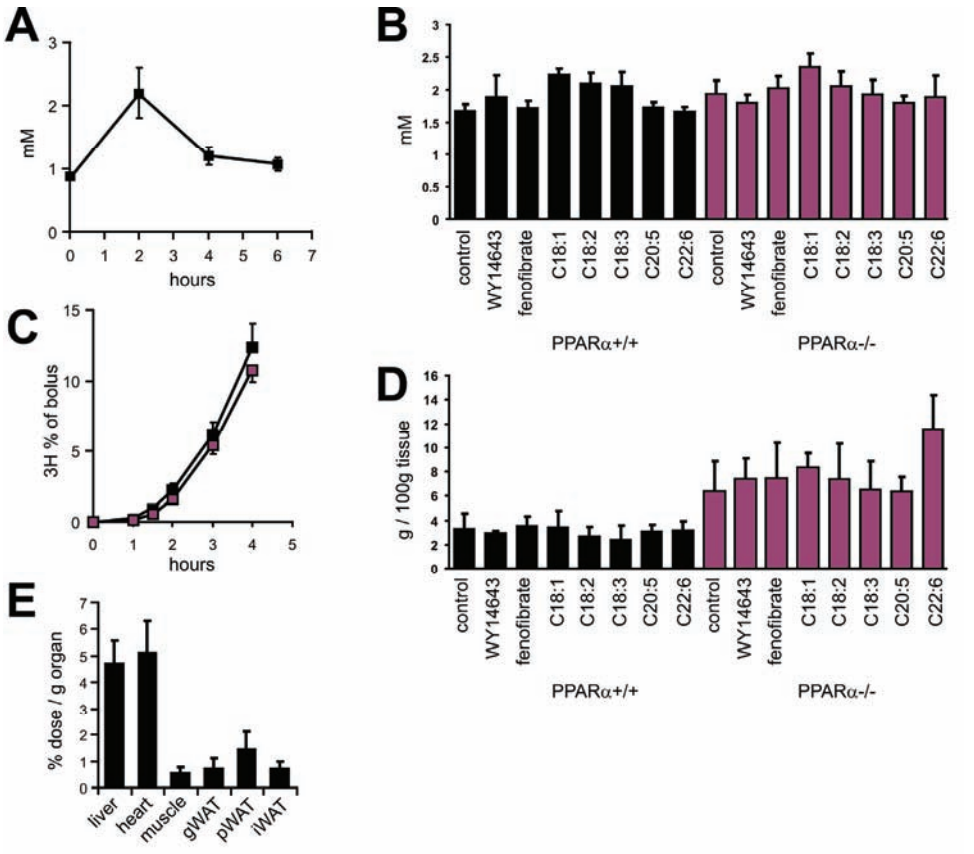

Figure 2. **Metabolic processing of dietary triglycerides.** (A) Wildtype mice were given an oral fat load of 400µl olive oil via intragastric gavage. TG levels were measured in plasma collected via the tail vein at the indicated time points. Errors bars represent SEM (n=11). (B) Plasma TG of wildtype and PPARα-/- mice sacrificed 6 hours after intragastric gavage with synthetic triglycerides, WY14643, or fenofibrate. Error bars represent SD (n=4-5 per group). (C) Intestinal triglyceride absorption rate was determined in 5h fasted wildtype (black boxes) and PPARα-/- (purple boxes) mice by measuring the appearance of [^{3}H] in plasma after intragastric gavage with 7uCi glycerol-tri[^{3}H]oleate mixed with olive oil (200µl). Immediately before the gavage, mice received an intraorbital injection of tyloxapol (Triton WR1339) dissolved in saline at 500mg/kg bodyweight. Blood was sampled via the tail vein at the indicated time points for measurement of ^{3}H-activity. Error bars represent SEM. (D) Liver TG of wildtype and PPARα-/- mice sacrificed 6 hours after intragastric gavage with synthetic triglycerides, WY14643, or fenofibrate. Error bars represent SD (n=4-5 per group). (D) Tissue uptake of radiolabeled VLDL-like emulsion particles. VLDL-like particles labeled with glycerol tri[^{3}H]oleate were injected into anesthetized mice. After 30 minutes, mice were euthanized and tissues collected for measurement of ^{3}H-activity. Error bars represent SEM (n=4).

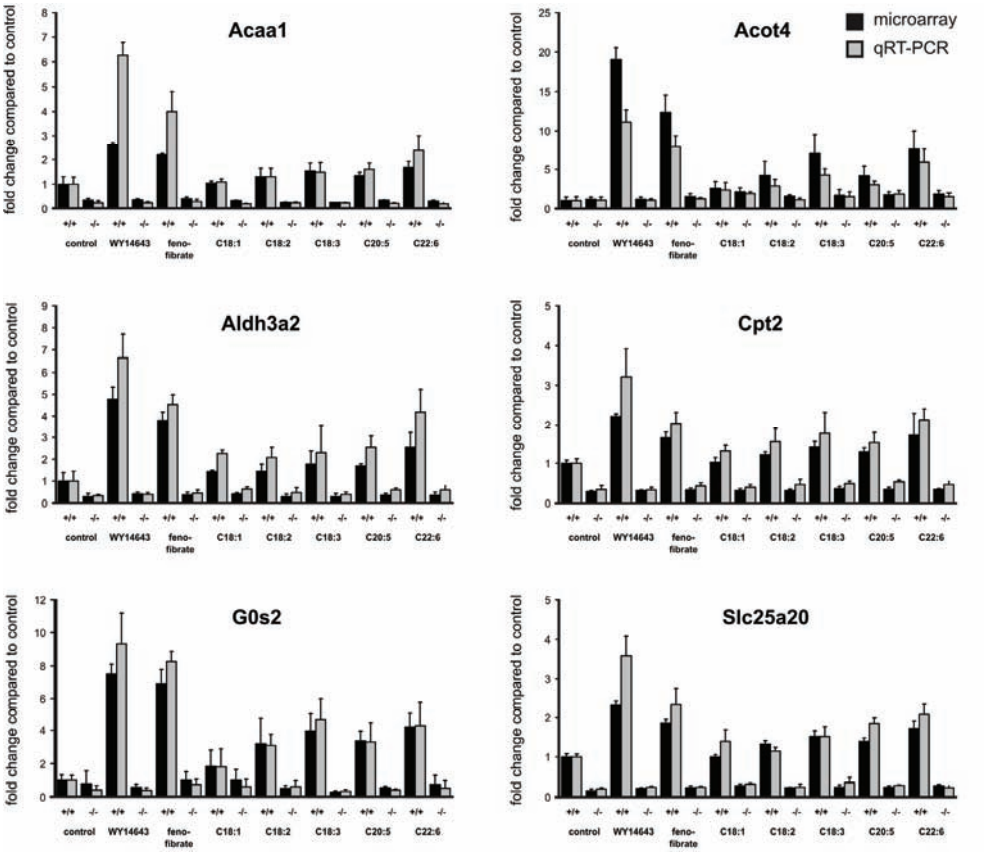

Figure 3. **Close agreement between microarray and qRT-PCR data.** mRNA expression of several genes was measured by quantitative real-time PCR to confirm the results from microarray. Results are shown as fold change compared to wildtype control. Error bars represent SD.

The highest number of statistically significantly changed genes was found after treatment with C22:6 (519, $P<0.01$), followed by, in turn, C18:3 (400), C18:2 (287), C20:5 (280) and C18:1 (114) (Figure 4 and Table 1). These numbers are relatively low in comparison with the synthetic PPARα agonists WY14643 (1674) and fenofibrate (1005). The data indicate that of all fatty acids studied, C22:6 is the most potent activator of gene expression.

Regulation of gene expression by dietary fatty acids or synthetic agonists was defined as PPARα-dependent when expression was statistically significantly up- or downregulated in wildtype but not PPARα-/- mice. As expected, gene regulation by WY14643 and fenofibrate in wildtype mice was almost completely abolished in PPARα-/- mice. Surprisingly, a similar though slightly less extreme picture was observed for dietary unsaturated fatty acids. Indeed, the far majority of genes regulated by dietary unsaturated fatty acids in wildtype mice did not show regulation in PPARα-/- mice, indicating PPARα-dependent regulation. This was highest for C20:5 (94.6%), followed by C22:6 (93.1%), C18:1 (88.6%), C18:2 (87.1%) and C18:3 (84.0%) (Figure 4 and Table 1). Similar numbers were obtained for up- and downregulation of gene expression. The few genes that were up- or downregulated by dietary unsaturated fatty acids independently of PPARα included *Lpin2* and *Srebp-1*, respectively. Together, these data suggest that the (short term) effects of dietary unsaturated fatty acids on hepatic gene expression are almost exclusively mediated by PPARα.

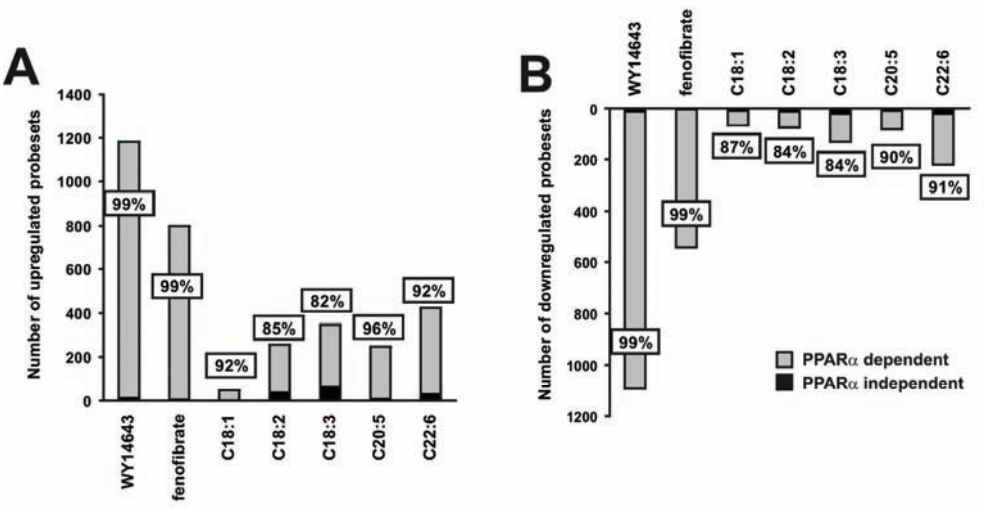

Figure 4. **PPARα-dependent regulation of gene expression by dietary unsaturated fatty acids.** Bars show number of up- (A) and downregulated (B) probesets in the different treatment groups. The number of probesets regulated by unsaturated fatty acids in a PPARα-dependent manner (purple bars, not changed in the PPARα-/- mouse), or PPARα-independent manner (black bars, changed in wildtype and PPARα-/- mice) are shown, with percentage PPARα dependence indicated. Probesets were considered statistically significantly regulated if $P<0.01$.

Table 1. Total number as well as PPARα-dependent up- and downregulated probesets and corresponding genes for each treatment group (P<0.01).

	Probesets			Genes			
	Total number	**PPARα-dep**	**% dep**	**Total number**	**PPARα-dep**	**% dep**	
WY14643	1180	1165	98.7%	797	789	99.0%	**Upregulated**
fenofibrate	800	794	99.3%	549	544	99.1%	
C18:1	51	47	92.2%	47	44	93.6%	
C18:2	260	221	85.0%	212	186	87.7%	
C18:3	349	285	81.7%	283	237	83.7%	
C20:5	249	238	95.6%	201	194	96.5%	
C22:6	425	393	92.5%	313	296	94.6%	
WY14643	1092	1081	99.0%	877	867	98.9%	**Downregulated**
fenofibrate	540	537	99.4%	456	454	99.6%	
C18:1	69	60	87.0%	67	57	85.1%	
C18:2	77	65	84.4%	75	64	85.3%	
C18:3	133	112	84.2%	117	99	84.6%	
C20:5	84	76	90.5%	79	71	89.9%	
C22:6	221	201	91.0%	206	187	90.8%	
WY14643	2272	2246	98.9%	1674	1656	98.9%	**Up- and downregulated**
fenofibrate	1340	1331	99.3%	1005	998	99.3%	
C18:1	120	107	89.2%	114	101	88.6%	
C18:2	337	286	84.9%	287	250	87.1%	
C18:3	482	397	82.4%	400	336	84.0%	
C20:5	333	314	94.3%	280	265	94.6%	
C22:6	646	594	92.0%	519	483	93.1%	

Overlap in gene regulation between dietary unsaturated fatty acids and WY14643

To further explore the role of PPARα in regulation of gene expression by dietary unsaturated fatty acids, the overlap in gene regulation between fatty acids and WY14643, which specifically targets PPARα, was studied. Remarkably, C22:6 showed a huge overlap in gene regulation with WY14643 (Figure 5A). Quantitatively, 84% of genes upregulated and 76% of genes downregulated by C22:6 (P<0.01) were also regulated by WY14643 (average 80.5%), suggesting that C22:6 impacts mainly PPARα target genes. Much less overlap was observed between C18:1 and WY14643 (average 32.4%), suggesting that gene

regulation by C18:1 may be less dependent on PPARα, or alternatively the existence of PPARα target genes specifically regulated by C18:1 (Figure 5A). An intermediate degree of overlap was observed between WY14643 and the other fatty acids studied (Table 2).

To further compare the effects of WY14643 and C22:6 on gene expression, for all probesets left after IQR-filtering the fold changes in expression in response to WY14643 and C22:6 were plotted against each other, with each probeset represented by a single dot (Figure 5B). The vast majority of probesets ended up in the lower left or upper right compartments, indicating that genes up- or downregulated by WY14643 were also up- or downregulated by C22:6, respectively, thus confirming the overlap in gene regulation between C22:6 and WY14643. Additionally, the positioning of the dots around a straight line with slope <1 shows that the relative magnitude of gene induction by C22:6 related to WY14643 was remarkably constant across all probesets. Thus, compared to WY14643, C22:6 behaves as an almost equally specific, yet less potent PPARα agonist. Nevertheless, several genes could be identified that were upregulated disproportionally strongly by WY14643 including *Cd36*, *Fabp4* (*aP2*), and *Cpt1b*, or by C22:6 including *Prlr* and *Txnip* (Figure 5B). A much more scattered picture was observed for the comparison between WY14643 and C18:1, indicating that these compounds have much less in common in terms of gene regulation. Again, the other fatty acids gave an intermediate picture (data not shown).

Table 2. Overlap in gene regulation between dietary unsaturated fatty acids and WY14643. Genes were considered statistically significantly regulated if P<0.01.

Treatment	Number of changed genes	Number of changed genes overlapping with WY14643	% changed genes overlapping with WY14643
WY14643	1674		
fenofibrate	1005	875	87.1%
C18:1	114	37	32.4%
C18:2	287	142	49.5%
C18:3	400	244	61.0%
C20:5	280	220	78.6%
C22:6	519	418	80.5%

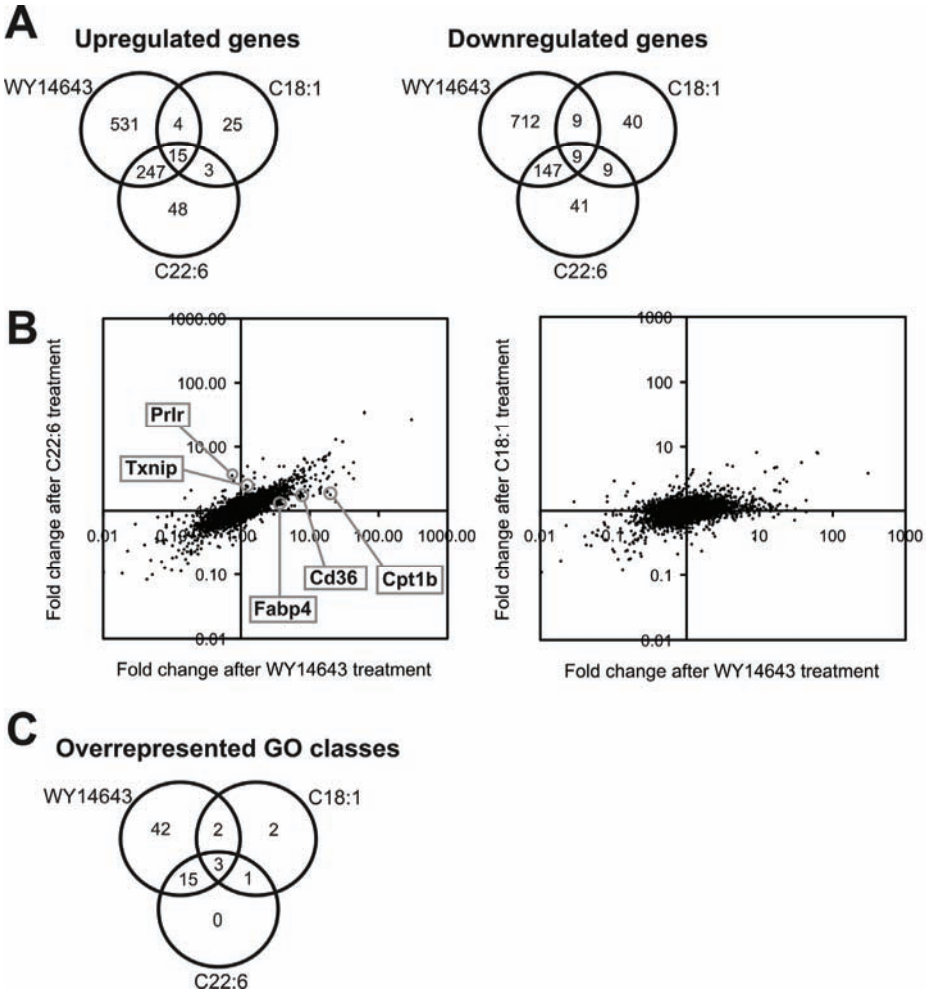

Figure 5. **Similarities between two dietary unsaturated fatty acids and the synthetic PPARα agonist WY14643.** (A) Venn diagrams showing the overlap in up- (left panel) and downregulated (right panel) genes after treatment with WY14643, C22:6 and C18:1. Genes were considered statistically significantly regulated if P<0.01. (B) Scatter plots demonstrating similarities in gene regulation between C22:6 and WY14643. Graphs show fold change in gene expression after treatment with WY14643 compared to C22:6 and C18:1. Genes that are upregulated disproportionally strongly by WY14643 (*Cd36*, *Fabp4* (*aP2*), and *Cpt1b*), or by C22:6 (*Prlr* and *Txnip*) are marked. In constructing the scatter plots, all probesets left after IQR-filtering were used. (C) Overlap in overrepresented Gene Ontology classes between C22:6, C18:1, and WY14643, based on a functional class score (FCS) method. The GO class unique to C22:6 and C18:1 is GO:0016070 (RNA metabolism), whereas the GO classes unique to C18:1 are GO:0007409 (axonogenesis) and GO:0016072 (rRNA metabolism).

Table 3. Overlap in overrepresented Gene Ontology classes between dietary unsaturated fatty acids and fenofibrate and WY14643 based on analysis with Functional Class Score method, FDR <0.0001.

Treatment	No. over-represented GO classes	No. GO classes overlapping with WY14643	% GO classes overlapping with WY14643	GO classes not in WY14643
WY14643	62			
fenofibrate	48	40	83.3%	GO:0006084 - acetyl-CoA metabolism; GO:0006519 - amino acid and derivative metabolism; GO:0006695 - cholesterol biosynthesis; GO:0006816 - calcium ion transport; GO:0009308 - amine metabolism; GO:0009725 - response to hormone stimulus; GO:0016126 - sterol biosynthesis; GO:0043283 - biopolymer metabolism
C18:1	8	5	62.5%	GO:0007409 - axonogenesis; GO:0016070 - RNA metabolism; GO:0016072 - rRNA metabolism
C18:2	11	9	81.8%	GO:0007167 - enzyme linked receptor protein signaling pathway; GO:0009725 - response to hormone stimulus
C18:3	13	11	84.6%	GO:0006445 - regulation of translation; GO:0006928 - cell motility
C20:5	13	13	100%	-
C22:6	19	18	94.7%	GO:0016070 - RNA metabolism

An alternative approach to study similarities in gene regulation is via determining the overlap in Gene Ontology (GO) classes overrepresented in the respective treatment groups (Table S1). P-values derived from t-test for all ~45000 probesets on the microarray were used for the GO-based functional clustering. The comparisons were made between the control group and each treatment group in wildtype mice. Out of a total of 19 GO classes overrepresented after C22:6 treatment, only one class (GO:0016070, RNA metabolism) was not shared between C22:6 and WY14643 (Figure 5C). Interestingly, this GO class was shared between C22:6 and C18:1 suggesting it may be specifically regulated by dietary unsaturated fatty acids and not WY14643. The remainder of fatty acids studied, except for perhaps C18:1, similarly showed a high degree of overlap with WY14643 (Table 3), thereby corroborating the very large resemblance in gene regulation between WY14643 and the dietary fatty acids studied. Overall, these data support the dominant role of PPARα in gene regulation by dietary unsaturated fat.

Hierarchy between dietary unsaturated fatty acids

Of all fatty acids studied, the number of significantly changed genes was highest for C22:6, followed by C18:3. The number was about equal for C20:5 and C18:2, while much fewer genes were changed after C18:1 treatment. Since the dietary fatty acids regulated gene expression principally via PPARα, the data are indicative of a hierarchy in *in vivo* PPARα-activating potency between dietary unsaturated fatty acids. Direct evidence for this notion came from comparison of fold changes in expression of PPARα target genes between the various fatty acid treatments. Genes involved in two major PPARα-regulated pathways were examined: mitochondrial fatty acid oxidation and peroxisomal fatty acid oxidation (Figure 6). These functional classes were created in house for various pathways within lipid metabolism and were specifically designed for Affymetrix GeneChip analysis (available at http://nutrigene.4t.com/microarray/ppar2007). By visualizing the changes in gene expression in the form of a heatmap, a clear hierarchy in PPARα-activating potency can be observed between the various treatments, which can be expressed as WY14643>fenofibrate>C22:6>C20:5=C18:3>C18:2>C18:1.

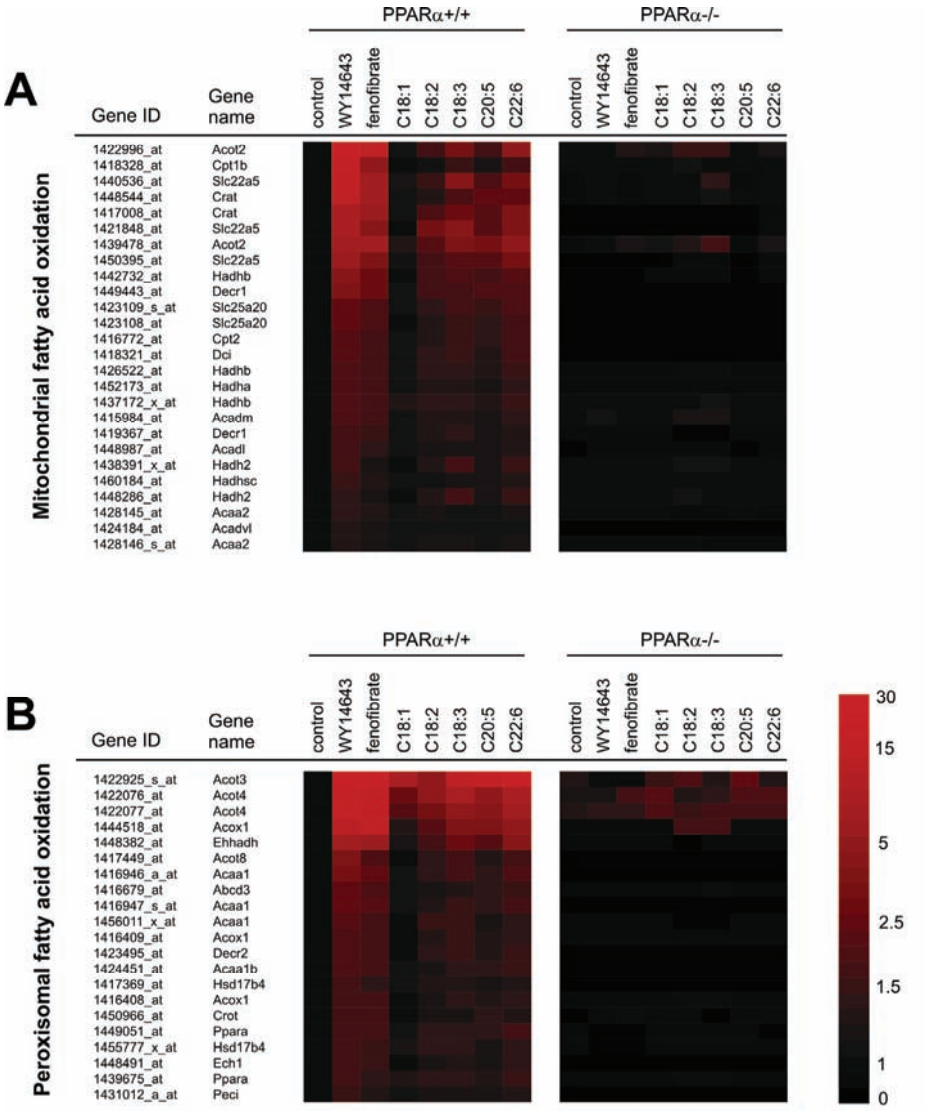

Figure 6. **Differential induction of genes involved in fatty acid oxidation between various dietary fatty acids.** (A) Genes involved in mitochondrial fatty acid oxidation. (B) Genes involved in peroxisomal fatty acid oxidation. The heatmaps were generated directly from the microarray data, using for each probeset the mean signal from 4-5 biological replicates. The scale represents fold-induction relative to wildtype control, which was set at 1. Only probesets showing significant ($P<0.01$) upregulation by WY14643 were included in the analysis. A list of probesets belonging to the functional class of mitochondrial fatty acid oxidation can be found at http://nutrigene.4t.com/microarray/ppar2007.

Since a direct comparison between synthetic agonists and dietary fatty acids is complicated by differences in dosage (4mg vs. 400µl), further comparisons were made between fatty acids only. For all probesets shown in the heatmaps as well as probesets belonging to the lipogenesis pathway we estimated the relative induction by each fatty acid expressed as a percentage of induction by C22:6. The median for all probesets within a functional class was calculated for each treatment group (Figure 7, bar). These data indicate that C22:6 is the most potent activator of PPARα-dependent gene regulation in mouse liver, while C18:1 is the least active.

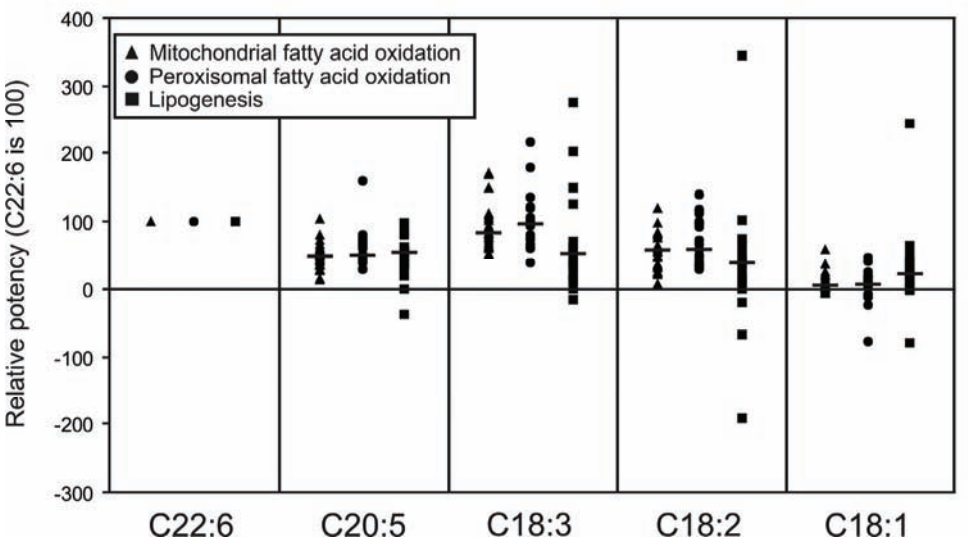

Figure 7. **Differential induction of genes involved in lipid metabolism between dietary unsaturated fatty acids.** For each probeset, the induction of expression by each fatty acid was expressed as a percentage relative to C22:6 (100%), using the mean signal from 4-5 biological replicates. Each dot represents one probeset. The horizontal bars represent the median percentage of induction relative to C22:6 calculated separately for each pathway and fatty acid. Only probesets showing significant ($P<0.01$) upregulation by WY14643 were included in the analysis. A list of probesets belonging to the three functional classes can be found at http://nutrigene.4t.com/microarray/ppar2007.

To examine whether the difference in *in vivo* PPARα-activating potency between the dietary fatty acids could be reproduced *in vitro*, cultured rat FAO hepatoma cells were treated with various unsaturated fatty acids. It was observed that the pattern of regulation of PPARα targets *Pdk4*, *Ehhadh* and *Cyp4A14* by unsaturated fatty acids was highly similar between the FAO cells and intact mouse liver (Figure 8). These data provide additional evidence that differences in metabolic processing of fatty acids are unlikely to explain differential fold-induction of genes between dietary fatty acids observed *in vivo*. Rather, they indicate an intrinsic difference in PPARα-activating potency between dietary unsaturated fatty acids, which is supported by published *in vitro* data.

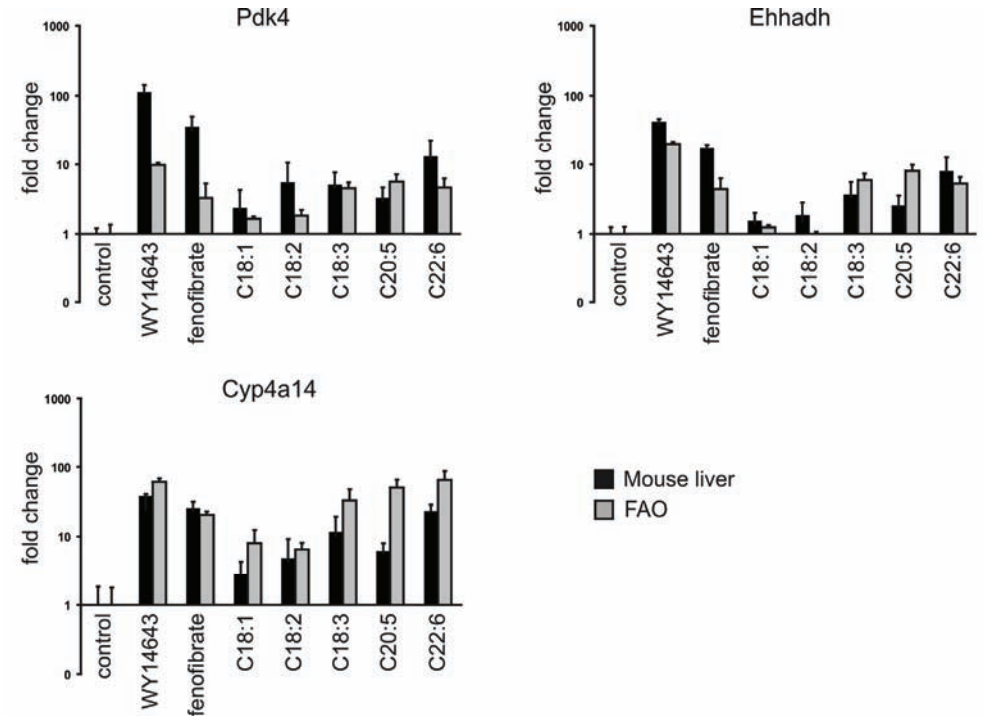

Figure 8. **Close agreement between fatty acid-dependent gene regulation *in vivo* and *in vitro*.** mRNA expression of three genes (*Pdk4*, *Ehhadh* and *Cyp4A14*) was determined in mouse liver and in rat hepatoma FAO cells using quantitative real-time PCR. Results are shown as fold change compared to control group. Error bars represent SD.

While in terms of target gene regulation dietary unsaturated fatty acids thus generally mimic the effect of the synthetic PPARα agonist WY14643 except for being less potent, it is unclear whether these different compounds activate PPARα and stimulate transcription of target genes via the exact same mechanism. To explore this issue we used Nuclear Receptor PamChip® Arrays to identify differences in coregulator recruitment between WY14643 and C22:6. In this system the interaction between nuclear receptors and immobilized peptides corresponding to specific coregulator-nuclear receptor binding regions is studied. Both C22:6 and WY14643 promoted the interaction between PPARα and numerous coregulator peptides. Interestingly, no PPARα-coregulator interactions unique to C22:6 could be identified. However, at least four interactions, representing the coregulator proteins TRIP3, TRIP8, RIP140, and the nuclear receptor SHP1, seemed to be elicited specifically by WY14643 (Figure 9). No differences in PPARα-coregulator interaction patterns could be observed between the various fatty acids studied (data not shown).

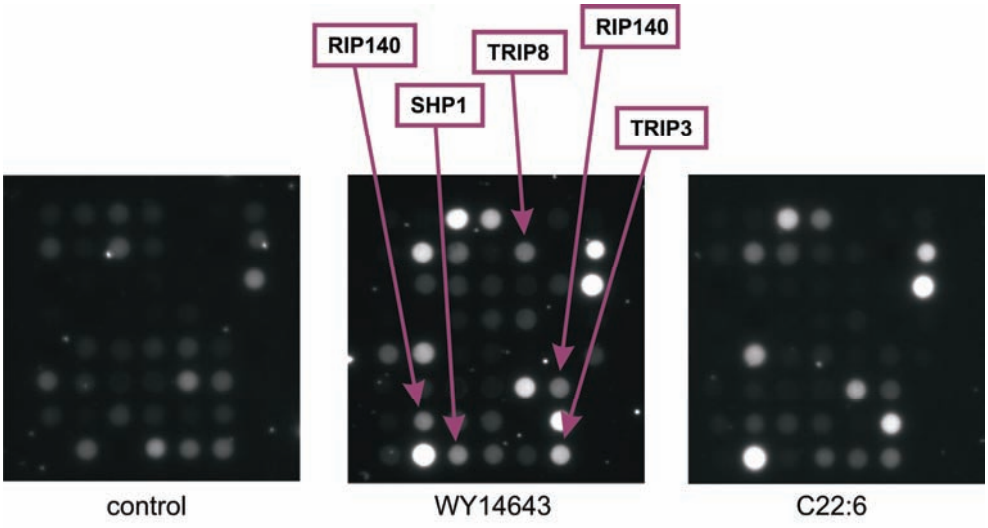

Figure 9. **Cofactor recruitment assay with WY14643 and C22:6.** The Nuclear Receptor PamChip® assay was used to measure the interaction between PPARα and immobilized peptides corresponding to specific coregulator-nuclear receptor binding regions. Measurements were performed in the presence of control (EtOH), WY14643 (5μM) or C22:6 (100μM). Arrows point to those coactivators selectively recruited by WY14643 but not C22:6. All images were taken after 100 msec exposure time.

Discussion

Dietary fats have numerous effects on human health. Current dietary guidelines strongly discourage consumption of saturated and trans fatty acids, whereas consumption of unsaturated fatty acids, especially n-3 fatty acids present in fish oil, is promoted (331-333). It is believed that dietary fatty acids mainly influence biological processes by altering DNA transcription. In the present paper, using a unique dietary intervention protocol consisting of a single dose of synthetic triglycerides composed of a single fatty acid, we show that in mouse liver PPARα dominates gene regulation by dietary unsaturated fat. Furthermore, we demonstrate that dietary polyunsaturated fatty acids (PUFAs), especially docosahexaenoic acid, are the most potent activators of PPARα *in vivo*. These latter data align well with *in vitro* experiments showing that in general PUFAs are more potent PPARα ligands compared to mono- and saturated fatty acids, although the results may depend somewhat on the method used (27, 41, 67, 68, 204, 334, 335).

It can be argued that our data and conclusions may be biased due to possible differential absorption and metabolic processing between the various fatty acids and between wildtype and PPARα-/- mice. Unfortunately, the unavailability of radioactive TG besides triolein makes it impossible to get complete and comparative information on the kinetic behavior of the various fatty acids used. However, several lines of evidence argue against major differences in kinetic behavior between the fatty acids: 1) it has been previously demonstrated that hepatic uptake of fatty acids from chylomicron remnants is unaffected by the fatty acid composition (336); 2) at the moment of sacrifice, plasma TG levels were highly similar for the various fatty acid groups; 3) fatty acid treatment *in vivo* and *in vitro* revealed a similar hierarchy in PPARα-activating potency between the fatty acids and in both analyses C22:6 emerged as the most potent PPARα agonist.

In addition, no major differences in the kinetics of dietary fat metabolism are expected between wildtype and PPARα-/- mice as: 1) wildtype and PPARα-/- mice show similar rates of intestinal TG absorption; 2) at the moment of sacrifice, plasma TG levels were highly similar between wildtype and PPARα-/- mice; 3) while synthetic PPARα agonists are known to stimulate plasma TG clearance (337), no evidence is available that points to differences in plasma TG clearance and tissue fatty acid uptake between wildtype and PPARα-/- mice; 4) genes that are upregulated by fatty acids in a PPARα-independent manner were induced to the same extent in wildtype and PPARα-/- mice (data not shown), suggesting that the dietary fatty acids were taken up at the same rate in liver of wildtype and PPARα-/- mice.

While PPARα activity is known to respond to changes in dietary fat content and composition (73, 338, 339), the large dominance of PPARα in fatty acid-dependent gene regulation in liver is surprising given that the activity of numerous transcription factors can be modulated by fatty acids, including SREBP-1, HNF4α, LXRs, FXR, RXRs, NF-KB, as well as PPARβ/δ and PPARγ (340-352). For several of these proteins, including RXRs and HNF4α, physical binding by fatty acids or fatty acyl-CoAs has been demonstrated (345-347, 353-357). RXR forms a permissive heterodimer with PPARα and accordingly it may be theorized that transcriptional activation of PPAR target genes by fatty acids may occur via their binding to either the PPARα and/or RXR moiety. The loss of fatty acid-dependent gene regulation in PPARα-/- mice, the very large overlap in gene regulation between unsaturated fatty acids and WY14643, and the less potent binding of fatty acids to RXR relative to PPARα strongly suggest a dominant role for PPARα in gene regulation by unsaturated fatty acids (343, 346, 358, 359). However, an additional role for RXR is hard to exclude as the effects of RXR activation seem to occur primarily via PPARα (360). It remains to be investigated to what extent the dominant role of PPARα in gene regulation by unsaturated fatty acids extends to tissues other than liver. Likely, the relative role of other transcription factors is related to their relative expression in a particular tissue.

Although it is clear that gene regulation by unsaturated fatty acids is highly dependent on PPARα, genes that are regulated in a PPARα-dependent manner do not necessarily represent direct PPARα targets. Some regulation is also expected to occur indirectly via activation of other transcription factors that are under direct control of PPARα. Analysis of the microarray data showed very little changes in the expression of other nuclear receptors in response to the intervention with the exception of CAR, which was upregulated, and RXRα and AhR, which were downregulated, although not necessarily in all treatments. The nuclear receptor CAR was recently identified as a PPARα target (361), suggesting that some genes may be regulated by PPARα and fatty acids via CAR. Secondary gene regulation was likely kept to a minimum by harvesting the livers only six hours after the oral gavage. It should also be noted that none of the putative fatty acid responsive transcription factors were significantly decreased in PPARα-/- mice, suggesting that their transcriptional regulatory function is not intrinsically suppressed in PPARα-/- mice.

Our study shows a clear hierarchy between unsaturated dietary fatty acids in terms of number of significantly changed genes and fold-induction of genes, with especially C22:6 behaving as a highly potent inducer of PPARα-dependent gene expression. The difference in *in vivo* PPARα-activating potency between the dietary fatty acids was reproduced *in vitro* and thus suggest an intrinsic difference in PPARα-activating potency between dietary unsaturated fatty acids, which is supported by *in vitro* receptor binding and transactivation

studies and thus likely reflects differences in binding affinity for PPARα (27, 340-344). Even though C18:2 was not the most potent inducer of gene expression, one could speculate that it likely represents the quantitatively most important dietary activator of PPARα, as the average intake of C18:2 is much higher than that of C18:3, C20:5 and C22:6.

In recent years, the concept of Selective PPAR Modulators (SPPARM) has emerged by analogy to Selective Estrogen Receptor Modulators (SERM). According to this concept, different PPAR agonists would induce differential gene expression based on selective receptor-coregulator interactions. While recent evidence supports the concept of selective PPARγ modulation (362-364), only limited data are available on PPARα (365). The design of our study allowed us to explore the concept of SPPARM in the comparison between unsaturated fatty acids and synthetic agonists. We hypothesized that fatty acids and synthetic PPARα agonists, while both activating PPARα, may induce differential gene expression patterns possibly via selective receptor-coregulator interactions.

In our analysis we found that almost every gene significantly up- or downregulated by C22:6 was also significantly up- or downregulated by WY14643, respectively. Clearly, the reverse was not true, illustrating that WY14643 is a more potent PPARα agonist than C22:6. Importantly, the scatter plot indicated that across all probesets the relative induction of gene expression by C22:6 when related to WY14643 was remarkably constant, suggesting that C22:6 behaves as a less potent, yet almost equally specific PPARα agonist. Nevertheless, several genes could be identified that were upregulated disproportionally strongly by WY14643 including *Cd36*, *Fabp4* (*aP2*), and *Cpt1b*, or by C22:6 including *Prlr* and *Txnip* (Figure 5B). Thus, differences in gene regulation between C22:6 and WY14643 could not entirely be accounted for by the lesser potency of C22:6. Interestingly, using the Nuclear Receptor PamChip® assay, at least four interactions, representing the coregulator proteins TRIP3, TRIP8, RIP140, and the nuclear receptor SHP1, seemed to be stimulated specifically by WY14643. However, no PPARα-coregulator interactions could be identified that were stimulated specifically by C22:6 and not WY14643.

Overall, similar observations were made for the other fatty acids studied, although compared to C22:6 they were less potent and/or less specific activators of PPARα, especially C18:1. The data indicate that in general dietary PUFAs mimic the effect of WY14643 on hepatic gene expression in terms of regulation of target genes and molecular mechanism, including coregulator interactions. In addition to being a more potent PPARα agonist in comparison with unsaturated fatty acids, WY14643 disproportionally induces expression of specific genes, which may be mediated via interactions with specific

coactivator proteins including RIP140. Thus, our data underscore the concept of selective PPARα modulation when comparing WY14643 with endogenous PPARα agonists, e.g. PUFAs.

Currently, one major drawback when performing microarray analyses on data derived from dietary intervention studies is the lack of proper statistical tools. The statistical methods developed to cope with the huge amount of data derived from microarray analyses work sufficiently well for stronger interventions, such as drug studies. When dealing with nutrition, however, changes in gene expression are often weak although no less important. Multiple testing methods normally used in microarray analyses to correct for false positives include FDR (false discovery rate) and Q-value (366-368). These methods are usually too restrictive for nutritional intervention, however, and will result in a loss of important results, as became apparent in the present study. Use of Q-value instead of P-value resulted in loss of a considerable amount of important information (data not shown). Numerous quantitative real-time PCR reactions have been carried out on the livers from this study supporting the use of the P-value.

In conclusion, dietary unsaturated fatty acids, especially docosahexaenoic acid and other PUFAs, acutely influence gene expression in mouse liver which, despite the presence of numerous other putative fatty acid-dependent transcription factors, is almost entirely mediated by PPARα. Consequently, dietary PUFAs largely mimic the effect of synthetic PPARα agonists on hepatic gene expression, both in terms of regulation of specific target genes and molecular mechanism including coregulator interactions, although compared to WY14643 and fenofibrate they are clearly less potent PPARα agonists. Our analysis underscores the power of a (nutri)genomics approach to investigate the potential molecular mechanisms underlying the effect of specific dietary components on (biomarkers of) health.

Acknowledgements

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Appendix Chapter 4

Table S1. Overrepresented GO classes in each treatment group based on analysis with Functional Class Score method, FDR <0.0001.

	GO ID	GO Class	Probes in class	Genes in class	Raw score	FDR
1	GO:0006637	acyl-CoA metabolism	24	14	4.56	3.16E-11
2	GO:0001676	long-chain fatty acid metabolism	11	9	3.60	8.87E-05
3	GO:0007031	peroxisome organization and biogenesis	31	16	3.08	2.89E-11
4	GO:0006732	coenzyme metabolism	63	35	2.87	2.41E-11
5	GO:0006635	fatty acid beta-oxidation	21	14	2.79	8.15E-05
6	GO:0019395	fatty acid oxidation	38	21	2.53	2.73E-11
7	GO:0006090	pyruvate metabolism	42	20	2.46	3.26E-11
8	GO:0019752	carboxylic acid metabolism	215	115	2.36	4.40E-11
9	GO:0019319	hexose biosynthesis	43	21	2.27	4.04E-11
10	GO:0008654	phospholipid biosynthesis	92	34	2.19	3.37E-11
11	GO:0006469	negative regulation of protein kinase activity	70	26	2.12	7.22E-11
12	GO:0009408	response to heat	51	25	1.94	2.53E-10
13	GO:0046467	membrane lipid biosynthesis	92	34	1.86	9.19E-11
14	GO:0006869	lipid transport	118	60	1.85	2.25E-11
15	GO:0009266	response to temperature stimulus	68	38	1.77	5.95E-11
16	GO:0006725	aromatic compound metabolism	60	34	1.71	2.35E-11
17	GO:0006644	phospholipid metabolism	116	45	1.68	4.21E-11
18	GO:0008203	cholesterol metabolism	122	55	1.64	5.62E-11
19	GO:0006986	response to unfolded protein	138	64	1.63	1.12E-10
20	GO:0006333	chromatin assembly or disassembly	110	37	1.59	8.72E-05
21	GO:0051789	response to protein stimulus	123	56	1.57	1.01E-09
22	GO:0045859	regulation of protein kinase activity	83	33	1.57	8.29E-05
23	GO:0009607	response to biotic stimulus	145	73	1.57	2.53E-11
24	GO:0007050	cell cycle arrest	122	49	1.55	2.11E-11
25	GO:0012502	induction of programmed cell death	182	81	1.53	2.66E-11
26	GO:0008202	steroid metabolism	210	104	1.53	2.06E-11
27	GO:0008610	lipid biosynthesis	230	108	1.52	1.44E-10
28	GO:0016125	sterol metabolism	114	52	1.51	1.94E-11
29	GO:0006694	steroid biosynthesis	119	56	1.45	5.32E-11
30	GO:0043065	positive regulation of apoptosis	283	117	1.44	2.47E-11
31	GO:0043067	regulation of programmed cell death	238	107	1.43	8.43E-11
32	GO:0045786	negative regulation of progression through cell cycle	273	108	1.41	2.15E-11
33	GO:0035023	regulation of Rho protein signal transduction	145	63	1.41	3.06E-11
34	GO:0045087	innate immune response	83	56	1.39	1.26E-10
35	GO:0006260	DNA replication	282	108	1.39	2.97E-11
36	GO:0006917	induction of apoptosis	241	106	1.38	6.32E-11
37	GO:0006006	glucose metabolism	86	47	1.38	8.43E-05
38	GO:0007507	heart development	255	102	1.36	5.06E-11
39	GO:0001525	angiogenesis	209	92	1.30	1.91E-11

	GO ID	GO Class	Probes in class	Genes in class	Raw score	FDR	
35	GO:0006260	DNA replication	282	108	1.39	2.97E-11	
36	GO:0006917	induction of apoptosis	241	106	1.38	6.32E-11	
37	GO:0006006	glucose metabolism	86	47	1.38	8.43E-05	
38	GO:0007507	heart development	255	102	1.36	5.06E-11	
39	GO:0001525	angiogenesis	209	92	1.30	1.91E-11	
40	GO:0007243	protein kinase cascade	278	109	1.30	3.49E-11	
41	GO:0008284	positive regulation of cell proliferation	209	94	1.29	3.61E-11	
42	GO:0007266	Rho protein signal transduction	207	87	1.29	1.84E-11	
43	GO:0006916	anti-apoptosis	189	75	1.28	5.06E-10	
44	GO:0016568	chromatin modification	284	107	1.27	1.01E-10	
45	GO:0043066	negative regulation of apoptosis	284	115	1.26	3.74E-11	
46	GO:0042060	wound healing	106	62	1.26	8.57E-05	
47	GO:0045934	negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	179	70	1.26	2.30E-11	
48	GO:0016481	negative regulation of transcription	274	109	1.25	1.87E-11	WY14643
49	GO:0006814	sodium ion transport	198	105	1.24	2.02E-11	
50	GO:0000165	MAPKKK cascade	214	85	1.23	1.98E-11	
51	GO:0007067	mitosis	268	114	1.21	6.74E-11	
52	GO:0048514	blood vessel morphogenesis	236	98	1.19	4.81E-11	
53	GO:0051242	positive regulation of cellular physiological process	201	91	1.18	4.60E-11	
54	GO:0040008	regulation of growth	215	89	1.18	2.81E-11	
55	GO:0019538	protein metabolism	215	101	1.17	1.81E-11	
56	GO:0030036	actin cytoskeleton organization and biogenesis	299	112	1.17	1.69E-10	
57	GO:0001558	regulation of cell growth	228	94	1.16	2.20E-11	
58	GO:0051056	regulation of small GTPase mediated signal transduction	195	86	1.16	2.02E-10	
59	GO:0006325	establishment and/or maintenance of chromatin architecture	299	112	1.15	7.78E-11	
60	GO:0000087	M phase of mitotic cell cycle	239	104	1.15	3.89E-11	
61	GO:0006092	main pathways of carbohydrate metabolism	206	99	1.15	3.37E-10	
62	GO:0045045	secretory pathway	288	115	1.10	2.59E-11	
1	GO:0006637	acyl-CoA metabolism	24	14	4.66	4.04E-11	
2	GO:0001676	long-chain fatty acid metabolism	11	9	2.87	1.26E-10	
3	GO:0006732	coenzyme metabolism	63	35	2.78	3.06E-11	
4	GO:0006084	acetyl-CoA metabolism	28	9	2.58	9.54E-05	
5	GO:0007031	peroxisome organization and biogenesis	31	16	2.46	3.61E-11	
6	GO:0006635	fatty acid beta-oxidation	21	14	2.31	2.30E-11	
7	GO:0009725	response to hormone stimulus	35	13	2.20	3.37E-10	
8	GO:0006090	pyruvate metabolism	42	20	2.12	4.21E-11	fenofibrate
9	GO:0019395	fatty acid oxidation	38	21	1.98	3.49E-11	
10	GO:0019752	carboxylic acid metabolism	215	115	1.90	6.74E-11	
11	GO:0019319	hexose biosynthesis	43	21	1.85	5.95E-11	
12	GO:0006519	amino acid and derivative metabolism	57	27	1.76	3.89E-11	
13	GO:0006695	cholesterol biosynthesis	50	22	1.72	5.06E-10	
14	GO:0008654	phospholipid biosynthesis	92	34	1.68	4.60E-11	
15	GO:0016126	sterol biosynthesis	59	25	1.66	1.12E-10	
16	GO:0009308	amine metabolism	65	30	1.65	2.81E-11	
17	GO:0008203	cholesterol metabolism	122	55	1.45	8.43E-11	
18	GO:0046467	membrane lipid biosynthesis	92	34	1.39	1.69E-10	
19	GO:0006725	aromatic compound metabolism	60	34	1.37	2.97E-11	

	GO ID	GO Class	Probes in class	Genes in class	Raw score	FDR	
20	GO:0006869	lipid transport	118	60	1.27	2.89E-11	
21	GO:0007050	cell cycle arrest	122	49	1.27	2.66E-11	
22	GO:0016125	sterol metabolism	114	52	1.26	2.47E-11	
23	GO:0043283	biopolymer metabolism	88	39	1.26	9.72E-05	
24	GO:0008610	lipid biosynthesis	230	108	1.26	2.53E-10	
25	GO:0006644	phospholipid metabolism	116	45	1.25	6.32E-11	
26	GO:0006694	steroid biosynthesis	119	56	1.19	7.78E-11	
27	GO:0006986	response to unfolded protein	138	64	1.17	2.02E-10	
28	GO:0007243	protein kinase cascade	278	109	1.15	4.81E-11	
29	GO:0043067	regulation of programmed cell death	238	107	1.15	1.44E-10	
30	GO:0045786	negative regulation of progression through cell cycle	273	108	1.14	2.73E-11	
31	GO:0008202	steroid metabolism	210	104	1.12	2.59E-11	
32	GO:0012502	induction of programmed cell death	182	81	1.06	3.37E-11	
33	GO:0007507	heart development	255	102	1.06	7.22E-11	**fenofibrate**
34	GO:0006092	main pathways of carbohydrate metabolism	206	99	1.06	1.01E-09	
35	GO:0006260	DNA replication	282	108	1.05	3.74E-11	
36	GO:0043065	positive regulation of apoptosis	283	117	1.04	3.16E-11	
37	GO:0007266	Rho protein signal transduction	207	87	1.03	2.35E-11	
38	GO:0043066	negative regulation of apoptosis	284	115	1.03	5.32E-11	
39	GO:0006816	calcium ion transport	208	86	1.02	4.40E-11	
40	GO:0007067	mitosis	268	114	1.02	1.01E-10	
41	GO:0006917	induction of apoptosis	241	106	1.01	9.19E-11	
42	GO:0045934	negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	179	70	1.00	9.91E-05	
43	GO:0008284	positive regulation of cell proliferation	209	94	1.00	5.06E-11	
44	GO:0016481	negative regulation of transcription	274	109	0.99	2.41E-11	
45	GO:0000087	M phase of mitotic cell cycle	239	104	0.98	5.62E-11	
46	GO:0000165	MAPKKK cascade	214	85	0.97	9.36E-05	
47	GO:0006814	sodium ion transport	198	105	0.97	2.53E-11	
48	GO:0045045	secretory pathway	288	115	0.96	3.26E-11	
1	GO:0016072	rRNA metabolism	149	75	0.83	5.06E-10	
2	GO:0016070	RNA metabolism	187	75	0.81	1.44E-10	
3	GO:0007266	Rho protein signal transduction	207	87	0.81	1.26E-10	
4	GO:0000087	M phase of mitotic cell cycle	239	104	0.79	2.53E-10	**C18:1**
5	GO:0007067	mitosis	268	114	0.78	3.37E-10	
6	GO:0007409	axonogenesis	275	112	0.77	1.01E-09	
7	GO:0043066	negative regulation of apoptosis	284	115	0.75	2.02E-10	
8	GO:0007243	protein kinase cascade	278	109	0.73	1.69E-10	
1	GO:0009725	response to hormone stimulus	35	13	1.81	5.06E-10	
2	GO:0006637	acyl-CoA metabolism	24	14	1.78	1.69E-10	
3	GO:0006333	chromatin assembly or disassembly	110	37	1.16	1.12E-10	
4	GO:0035023	regulation of Rho protein signal transduction	145	63	0.96	1.44E-10	
5	GO:0007266	Rho protein signal transduction	207	87	0.94	9.19E-11	**C18:2**
6	GO:0008284	positive regulation of cell proliferation	209	94	0.93	2.02E-10	
7	GO:0051056	regulation of small GTPase mediated signal transduction	195	86	0.91	1.01E-09	
8	GO:0019752	carboxylic acid metabolism	215	115	0.91	2.53E-10	
9	GO:0001558	regulation of cell growth	228	94	0.85	1.01E-10	

	GO ID	GO Class	Probes in class	Genes in class	Raw score	FDR	
10	GO:0043067	regulation of programmed cell death	238	107	0.82	3.37E-10	C18:2
11	GO:0007167	enzyme linked receptor protein signaling pathway	305	115	0.82	1.26E-10	
1	GO:0006637	acyl-CoA metabolism	24	14	2.48	1.26E-10	
2	GO:0006732	coenzyme metabolism	63	35	1.45	8.43E-11	
3	GO:0019752	carboxylic acid metabolism	215	115	1.11	2.02E-10	
4	GO:0008203	cholesterol metabolism	122	55	1.02	3.37E-10	
5	GO:0006445	regulation of translation	133	48	0.99	1.12E-10	
6	GO:0006986	response to unfolded protein	138	64	0.94	1.01E-09	
7	GO:0006260	DNA replication	282	108	0.89	1.01E-10	C18:3
8	GO:0006928	cell motility	225	84	0.88	7.78E-11	
9	GO:0051242	positive regulation of cellular physiological process	201	91	0.83	2.53E-10	
10	GO:0043066	negative regulation of apoptosis	284	115	0.82	1.69E-10	
11	GO:0043065	positive regulation of apoptosis	283	117	0.81	9.19E-11	
12	GO:0006917	induction of apoptosis	241	106	0.79	5.06E-10	
13	GO:0007243	protein kinase cascade	278	109	0.78	1.44E-10	
1	GO:0006637	acyl-CoA metabolism	24	14	2.20	1.01E-10	
2	GO:0006732	coenzyme metabolism	63	35	1.33	8.43E-11	
3	GO:0019752	carboxylic acid metabolism	215	115	0.90	1.44E-10	
4	GO:0006986	response to unfolded protein	138	64	0.86	5.06E-10	
5	GO:0048514	blood vessel morphogenesis	236	98	0.83	1.69E-10	
6	GO:0009607	response to biotic stimulus	145	73	0.82	9.19E-11	
7	GO:0007243	protein kinase cascade	278	109	0.82	1.12E-10	C20:5
8	GO:0007067	mitosis	268	114	0.81	2.02E-10	
9	GO:0001525	angiogenesis	209	92	0.81	7.78E-11	
10	GO:0016568	chromatin modification	284	107	0.80	3.37E-10	
11	GO:0000087	M phase of mitotic cell cycle	239	104	0.78	1.26E-10	
12	GO:0030036	actin cytoskeleton organization and biogenesis	299	112	0.77	1.01E-09	
13	GO:0006325	establishment and/or maintenance of chromatin architecture	299	112	0.76	2.53E-10	
1	GO:0006637	acyl-CoA metabolism	24	14	2.77	9.19E-11	
2	GO:0006732	coenzyme metabolism	63	35	1.74	5.32E-11	
3	GO:0009408	response to heat	51	25	1.50	5.06E-10	
4	GO:0019395	fatty acid oxidation	38	21	1.50	7.22E-11	
5	GO:0019752	carboxylic acid metabolism	215	115	1.25	1.26E-10	
6	GO:0006986	response to unfolded protein	138	64	1.23	2.02E-10	
7	GO:0051789	response to protein stimulus	123	56	1.21	1.01E-09	
8	GO:0009266	response to temperature stimulus	68	38	1.16	1.44E-10	
9	GO:0009607	response to biotic stimulus	145	73	1.15	5.95E-11	
10	GO:0016070	RNA metabolism	187	75	0.97	7.78E-11	C22:6
11	GO:0012502	induction of programmed cell death	182	81	0.95	6.74E-11	
12	GO:0045045	secretory pathway	288	115	0.94	6.32E-11	
13	GO:0043066	negative regulation of apoptosis	284	115	0.93	1.12E-10	
14	GO:0008610	lipid biosynthesis	230	108	0.93	2.53E-10	
15	GO:0030036	actin cytoskeleton organization and biogenesis	299	112	0.91	3.37E-10	
16	GO:0007243	protein kinase cascade	278	109	0.91	1.01E-10	
17	GO:0043065	positive regulation of apoptosis	283	117	0.90	5.62E-11	
18	GO:0007067	mitosis	268	114	0.88	1.69E-10	
19	GO:0006260	DNA replication	282	108	0.87	8.43E-11	

Chapter 5

PPARβ/δ but not PPARα serves as plasma free fatty acid sensor in liver

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Abstract

PPARα is an important transcription factor in liver that can be activated physiologically by fasting or pharmacologically using high-affinity synthetic agonists. Here we initially set out to elucidate the similarities in gene induction between WY14643 and fasting.

Numerous genes were commonly regulated in liver between the two treatments, including many classical PPARα target genes such as *Aldh3a2* and *Cpt2*. Remarkably, several genes induced by WY14643 were upregulated by fasting independently of PPARα, including *Lpin2* and *St3gal5*, suggesting involvement of another transcription factor. Using chromatin immunoprecipitation, *Lpin2* and *St3gal5* were shown to be direct targets of PPARβ/δ during fasting, whereas *Aldh3a2* and *Cpt2* were exclusive targets of PPARα. Binding of PPARβ/δ to the *Lpin2* and *St3gal5* genes followed the plasma FFA concentration, consistent with activation of PPARβ/δ by plasma FFAs. Subsequent experiments using transgenic and knockout mice for *Angptl4*, a potent stimulant of adipose tissue lipolysis, confirmed the stimulatory effect of plasma FFAs on *Lpin2* and *St3gal5* expression via PPARβ/δ. In contrast, the data did not support activation of PPARα by plasma FFA.

The results identify *Lpin2* and *St3gal5* as novel PPARβ/δ target genes and show that upregulation of gene expression by PPARβ/δ is sensitive to plasma FFA levels. In contrast, this is not the case for PPARα, revealing a novel mechanism for functional differentiation between PPARs.

Introduction

Hepatic lipid metabolism is governed by a complex interplay between hormones, transcription factors, and energy substrates, allowing for rapid adaptations to changes in metabolic needs (369). According to the traditional view, energy substrates such as fatty acids influence lipid metabolism by promoting flux through a particular pathway via mass action. However, it has become clear that energy substrates can also directly govern the transcription of enzymes involved in lipid metabolism via mechanisms analogous to that of many hormones. Indeed, it is now evident that glucose and fatty acids play a major regulatory role in hepatic lipid metabolism via direct activation or inhibition of specific transcription factors, including ChREBP (370, 371), SREBP1 (348-350, 372, 373), and PPARα (21).

Although numerous transcription factors have been shown to be activated by fatty acids *in vitro*, recent data suggest that PPARα is dominant in mediating the effects of dietary fatty acids on gene expression in liver (69). PPARα is a member of the superfamily of nuclear receptors and closely related to the other PPAR isoforms β/δ and γ (374) . Similar to several other nuclear receptors, PPARs function as heterodimers with the retinoid X receptor (RXR) and bind to specific sequences on the DNA referred to as PPAR-response elements (PPREs) (24, 63, 283). Numerous studies have shown that fatty acids can directly bind to PPARs and activate DNA transcription (39, 41, 67, 68, 204, 335). Binding of fatty acids changes the conformation of the PPAR protein (27, 149, 375, 376), and leads to recruitment of coactivator proteins (69, 204). Besides fatty acids and their derivatives, PPARs bind synthetic agonists including the thiazolidionediones, which serves as agonist for PPARα, and the fibrates, which are PPARα agonists (377).

Most of the information about the function of PPARα in liver and its impact on target genes is based on studies that have used high-affinity synthetic PPARα agonists. These pharmacological studies have shown that PPARα regulates a remarkably large number of genes, many of which are involved in hepatic lipid metabolism, thereby explaining the positive effect of synthetic PPARα agonists on plasma lipid parameters (21, 337). However, PPARα did not evolve as a receptor for fibrates but rather as a fatty acid sensor. Accordingly, the question arises to what extent results from pharmacological studies reflect the physiological function of PPARα.

Physiological experiments using PPARα-/- mice have shown that PPARα is especially important for the adaptive response to fasting. During fasting, the absence of PPARα elicits a complex phenotype characterized by fatty liver, hypoketonemia, hypoglycemia,

hypothermia, and elevated plasma FFA levels (70-72, 96). Furthermore, the hepatic induction of numerous metabolic genes during fasting is abolished in PPARα-/- mice. While both pharmacological and physiological studies thus support a major role for PPARα in hepatic lipid metabolism, evidence is suggesting that there is only partial overlap between genes upregulated by PPARα during fasting and genes upregulated by synthetic PPARα agonists (85). One possible explanation is that PPARα responds differently to pharmacological compared to physiological activation. Additionally, there may be a role for other PPAR subtypes. Besides PPARα, PPARβ/δ has been shown to be well expressed in hepatocytes (44, 47). However, the functional role of PPARβ/δ in hepatocytes and its physiological mechanisms of activation remain unknown.

Here we initially set out to elucidate the similarities and discrepancies in gene regulation in liver between pharmacological PPARα activation by WY14643 and physiological PPARα activation by fasting. While our data reveal major overlap between the effects of WY14643 and fasting, the data also indicate that a number of pharmacological PPARα target genes are induced by fasting independently of PPARα. Subsequent analysis uncovered a role for PPARβ/δ in hepatic gene regulation and revealed different mechanisms of activation of PPARα versus PPARβ/δ in mouse liver. Specifically, we find that upregulation of gene expression by PPARβ/δ is sensitive to plasma FFAs while this is not the case for PPARα.

Materials and Methods

Materials. Tridocosahexaenoin were from Nu-Chek-Prep, Inc. (Elysian, MN, USA). SYBR Green was purchased from Eurogentec (Seraing, Belgium). Protease inhibitor cocktail was purchased from Roche Diagnostics (Almere, the Netherlands), sonicated salmon sperm DNA was from Invitrogen (Breda, the Netherlands) and Proteinase K was from Fermentas (St.Leon–Rot, Germany). All other chemicals were from Sigma (Zwijndrecht, the Netherlands).

Animals. Pure-bred Sv129 PPARα-/- mice (129S4/SvJae) and corresponding wildtype mice (129S1/SvImJ) were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). The PPARβ/δ-/- mice were on a mixed background (Sv129/C57Bl/6) and have been previously described (378). The *Angptl4*-/-, +/-, and transgenic mice were on a C57Bl/6 background and have been previously described (324, 379, 380). Angptl4-transgenic mice overexpress Angptl4 in numerous tissues including adipose tissue (324, 379), while the *Angptl4*-/- lack Angptl4 expression in all tissues (380). Only male mice were used at 4-10

mice per group. For the fasting experiment, food was withdrawn for 24 hours starting at the onset of the light cycle.

PPARα ligand: Wildtype and PPARα-/- mice were fasted for 4h, and thereafter given an intragastric gavage of 400µl WY14643 (10 mg/ml in 0.5% carboxymethyl cellulose). Control treatment was 400µl 0.5% carboxymethyl cellulose. Livers were collected after 6h. Oral lipid load: Wildtype and PPARα-/- mice were given an intragastric gavage of 400µl synthetic triglyceride (tridocosahexaenoin) after a four hour fast. Control treatment was 0.5% carboxymethyl cellulose (400µl). Livers were collected 6h after gavage. PPARβ/δ ligand: Wildtype mice were given a single oral gavage of 150µg GW501516. Alternatively, PPARα-/- mice were fed 0.025% (wt/wt) L165041 mixed in food for 5 days.

Mice were anaesthetized with a mixture of isofluorane (1.5%), nitrous oxide (70%) and oxygen (30%). Blood was collected by orbital puncture, after which the mice were sacrificed by cervical dislocation. Livers were dissected and directly frozen in liquid nitrogen. For RNA analyses, tissue from the same part of the liver lobe was used.

The animal studies were approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University, the Netherlands and the University of Lausanne, Switzerland.

Affymetrix microarray. Total RNA from mouse liver was extracted with TRIzol reagent, and purified and DNAse treated using the SV Total RNA Isolation System (Promega, Leiden, the Netherlands). RNA quality measurements were performed on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) using 6000 Nano Chips in combination with the Eukaryote Total RNA Nano assay. RNA was judged as suitable for array hybridization only if samples showed intact bands corresponding to the 18S and 28S rRNA subunits, displayed no chromosomal peaks or RNA degradation products, and had a RIN (RNA integrity number) above 8.0. Five micrograms of RNA were used for one cycle cRNA synthesis (Affymetrix, Santa Clara, CA, USA). Hybridization, washing and scanning of Affymetrix Mouse Genome 430 2.0 arrays / Affymetrix NuGO mouse arrays was carried out according to standard Affymetrix protocols.

Packages from the Bioconductor project were used for analyzing the scanned arrays (381). Arrays were normalized using quantile normalization, and expression estimates were compiled using GC-RMA applying the empirical Bayes approach (382). A non-specific

filtering step was applied to remove genes with low variation, and included only those genes that had an inter-quartile range (IQR) across the samples of at least 0.25 on the log2-scale (326).

Gene set enrichment analysis (GSEA) was used to relate changes in gene expression to functional changes between mice treated with the PPARα agonist WY14643 for 6h and mice fasted for 24h. GSEA takes into account a broad context of physically interacting networks in which gene products function, including biochemical, metabolic and signal transduction routes (383). Gene sets with a FDR P-value <0.1 were considered significantly overrepresented.

Plasma metabolites. Plasma was obtained from blood by centrifugation for 10 minutes at 10000g. Plasma triglycerides and glycerol concentration in cell culture medium were determined using kits from Instruchemie (Delfzijl, the Netherlands). Plasma free fatty acids were determined using a kit from WAKO Chemicals (Instruchemie, Delfzijl, the Netherlands).

Fat explants. Epididymal adipose tissue was excised and cut into 0.2 – 0.3 cm^3 pieces. The explants were subsequently incubated for 15 min at 37ºC in DMEM containing 1% lipid-free BSA and 1 mg/ml collagenase type 1. Fat cells were liberated by gentle stirring followed by centrifugation of the cell suspension for 1 min at 400g. Fat cells were isolated from the surface and washed once in PBS. Subsequently, fat cells were incubated in DMEM containing 1% lipid-free BSA and 1µM isoproterenol at 37ºC with 5% CO_2. Medium was collected at various time points and frozen for measurement of free fatty acids and glycerol (kits from Instruchemie, Delfzijl, the Netherlands).

RNA isolation and qRT-PCR. Total liver RNA was isolated with TRIzol reagent (Invitrogen) according to manufacturer's instructions. A NanoDrop ND-1000 spectrophotometer (Isogen Life Science, IJsselstein, the Netherlands) was used to determine RNA concentrations. 1µg of total RNA was reverse transcribed using iScript (Bio-Rad, Veenendaal, the Netherlands). cDNA was amplified on a Bio-Rad MyIQ or iCycler PCR machine using Platinum Taq DNA polymerase (Invitrogen). PCR primer sequences were taken from the PrimerBank (384) and ordered from Eurogentec. Sequences of the primers used are available upon request.

Transactivation assay. Conserved PPREs were identified at 1291 or 23333 nucleotides downstream of the TSS of the mouse *Lpin2* or *St3gal5* gene, respectively, using a published algorithm (295). A 201-nucleotide and 183-nucleotide fragment surrounding the putative PPRE within the *Lpin2* and *St3gal5* genes, respectively, was PCR amplified from mouse genomic DNA (strain C57Bl/6) and subcloned into the KpnI and BglII sites of the pGL3 promoter vector (Promega, Leiden, the Netherlands). The reporter vector $(PPRE)_3$-TK-luciferase was included as a positive control. Reporter vectors were transfected into human hepatoma HepG2 cells together with an expression vector (pSG5) for mPPARβ/δ, in the presence or absence of GW501516 (1μM). A β-galactosidase reporter vector was co-transfected to normalize for differences in transfection efficiency. Transfections were carried out using Nanojuice (Novagen, Nottingham, UK). Luciferase activity was measured 24h post-transfection using the Promega luciferase assay kit (Promega) on a Fluoroskan Ascent FL apparatus (Thermo labsystems, Breda, the Netherlands). β-galactosidase activity was measured in the cell lysate by a standard assay using 2-nitrophenyl- β D-galactopyranoside as a substrate.

Chromatin Immunoprecipitation (ChIP) assay. It is becoming increasingly apparent that most nuclear receptor binding sites, including PPREs, are not found in proximity of the annotated transcriptional start site (TSS) of a gene but are often located quite distant (385-387). Nuclear receptors bound to such distal sites likely contact the basal transcription machinery via DNA looping. Binding of PPAR to distant PPREs can thus be demonstrated by showing cross-linking of PPAR to the TSS (388, 389).

Wildtype and PPARα-/- on a Sv129 background were fed or fasted for 24 hours (n = 3). Transgenic mice overexpressing *Angptl4* (*Angptl4*-Tg), wildtype (*Angptl4*+/+) and homozygous knockout (*Angptl4*-/-) mice (n=3) were fasted for 24h. At the end of the fasting period, mice were killed by cervical dislocation and livers were extracted. Livers were cut in smaller pieces and directly put in PBS containing 1% formaldehyde. Cross-linking was stopped after 15 minutes by adding glycine to a final concentration of 0.125M for 5 minutes at room temperature. After a short centrifugation to collect the liver pieces, two washing steps with ice-cold PBS were carried out. Livers were homogenized and thereafter centrifuged. After supernatant was removed, liver homogenate was resuspended in lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1, protease inhibitors) and the lysates were sonicated with a Bioruptor™ (Diagenode, Liège, Belgium) to achieve a DNA length of 300 – 1000 bp. After removal of cellular debris by centrifugation, supernatants were diluted 1:10 in ChIP dilution buffer (150mM NaCl, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 7.5, protease inhibitors). Chromatin was incubated

overnight at 4ºC with 2µg antibody, 25µl BSA (10mg/ml) and 2.4µl sonicated salmon sperm (10mg/ml). Antibodies used were anti-PPARα (sc-9000), anti-PPARGC1α (sc-13067) and anti-PPARβ/δ (sc-7197), all of which were obtained from Santa Cruz Biotechnologies (Heidelberg, Germany). Immunocomplexes were collected with 25µl MagaCell® Protein A Magnetic beads (Isogen Life Science) for 1 hour at room temperature, and subsequently washed sequentially with 700µl of the following buffers: ChIP wash buffer 1 (150mM NaCl, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH8, protease inhibitors) two times, ChIP wash buffer 2 (500mM NaCl, 1% Triton X-100, 2mM EDTA, 0.1% SDS, 20mM Tris-HCl pH8, protease inhibitors), ChIP wash buffer 3 (250mM LiCl, 1% NP40, 1% Deoxycholate, 1mM EDTA, 10mM Tris-HCl pH8), and two times TE buffer (1mM EDTA, 10mM Tris-HCl pH8). Elution of immunocomplexes were carried out in 250µl elution buffer (10mM EDTA, 0.5% SDS, 25mM Tris-HCl pH7.5) at 64ºC for 30 minutes. After collection of supernatant, elution was repeated with 250µl elution buffer at room temperature for 2 minutes. After combining the supernatants, cross-linking was reversed at 64°C overnight with 2.5µl Proteinase K (20mg/ml) for digestion of any remaining proteins. Genomic DNA fragments were recovered by phenol-chloroform extraction with phase lock gel (Eppendorf, Wesseling-Berzdorf, Germany), followed by salt-ethanol precipitation. Samples were diluted in sterile H_2O, and analyzed with quantitative PCR.

Primers were from Eurogentec and designed to cover the transcription start sites (TSS) of the following genes: *Aldh3a2* (F: 5'-CAGGTGAGGGAGCACAGTAC-3', R: 5'-CGCTTGGCTCTTTTCTGAAG-3'); *Cpt2* (F: 5'-GCCAGTCACGCAACAGCAG-3', R: 5'-TAGTTTAGAGACCGCTTCCG-3'); *Lpin2* (F: 5'-CCGTCTTGTGATTGGGCAGG-3', R: 5'-GAAGGAAACTCACCAGAATCC-3'); *St3gal5* (F: 5'-GCCTTCCACTATCTAATCACG-3', R: 5'- GTGTCCGCTCTGCCGACTG-3') and *Rplp0* (F: 5'- CGAGGACCGCCTGGTTCTC-3', R: 5'- GTCACTGGGGAGAGAGAGG-3').

Results

Overlap in gene regulation between pharmacological and physiological PPARα activation

PPARα in liver can be activated pharmacologically using synthetic agonists such as WY14643 or physiologically by fasting. To assess the similarities and discrepancies in gene regulation between these two stimuli, we compared microarray data from livers of mice treated with the synthetic PPARα agonist WY14643 for 6h and mice subjected to 24h fasting. Gene set enrichment analysis (GSEA) showed great similarity and overlap in top-regulated pathways between fasting and WY14643 treatment, almost all of which corresponded to pathways of lipid metabolism (Figure 1A). Much less overlap was observed at the individual gene level (Figure 1B). Nevertheless, a substantial number of genes upregulated by WY14643 were also induced by fasting. Many of these genes represent classical PPARα target genes involved in fatty acid catabolism such as *Acox1*, *Cpt2*, *Aldh3a2*, *Acot8*, *Ehhadh* and *Hmgcs2*. Consistent with an important role of PPARα, induction of classical PPARα target genes by fasting was abolished in PPARα-/- mice (Figure 1C, red dots; Figure 1D, upper panel). In contrast, a number of WY14643-responsive genes could be identified that were more significantly upregulated by fasting in PPARα-/- mice compared to wildtype mice, suggesting PPARα-independent regulation during fasting (Figure 1C, blue dots; Figure 1D, lower panel). Overall, these data indicate that targets of pharmacological PPARα activation exhibit diverse responses following physiological PPARα activation by fasting, being either up- or downregulated and showing a variable dependence on PPARα.

To explore the possible mechanism underlying the more significant induction by fasting of a number of pharmacological PPARα targets in PPARα-/- mice compared to wildtype mice, two representative genes were investigated in more detail: *Lpin2* and *St3gal5*. Remarkably, in contrast to classical PPARα targets *Cpt2* and *Aldh3a2* (Figure 2A and Figure 2C), induction of *Lpin2* and *St3gal5* by fasting and dietary fatty acids was largely maintained in PPARα-/- mice (Figure 2B and Figure 2D). These results imply that the effects of fasting and dietary fatty acids on hepatic expression of *Lpin2* and *St3gal5* may be partially mediated by a transcription factor other than PPARα. On the contrary, effects of fasting and dietary fatty acids on *Cpt2* and *Aldh3a2* are entirely mediated by PPARα. It should be mentioned that the expression profiles of *Cpt2* and *Aldh3a2* are representative of a large set of classical PPARα targets (see Figure 1D).

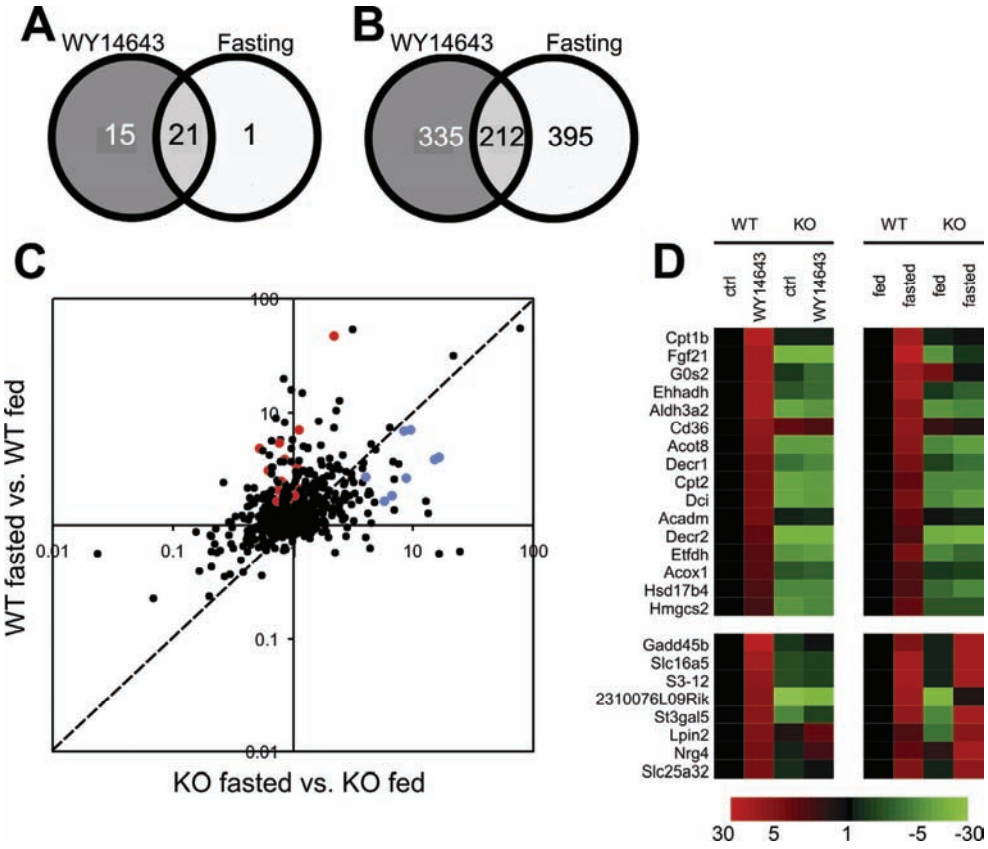


Figure 1. **Hepatic genes activated by WY14643 and fasting show a variable dependence on PPARα.** Livers from wildtype and PPARα-/- mice treated with the PPARα agonist WY14643 for 6h or fasted for 24h were used for gene expression profiling (n=4-5 mice per group). A) Overlap in top-regulated pathways between WY14643 treatment and fasting according to Gene Set Enrichment Analysis. Genesets with an FDR P-value <0.1 were considered significant. B) Overlap of upregulated genes between WY14643 treatment and fasting (criteria for inclusion: P < 0.01 and fold change > 1.5). C) Scatter plot showing the effect of fasting in genes significantly upregulated by WY14643. The Y-axis and X-axis show the effect of fasting in wildtype and PPARα-/- mice, respectively. Red dots represent classical PPARα target genes, while blue dots are WY14643-responsive genes that are more significantly upregulated by fasting in the PPARα-/- mouse compared to wildtype. D) Heatmap showing fold changes of genes compared to wildtype control / fed state. Upper panel: classical PPARα target genes, showing a PPARα-dependent increase in gene expression upon WY14643 treatment as well as fasting. The lower panel of genes exhibit a PPARα-dependent induction upon WY14643 treatment, but are induced independently of PPARα upon fasting.

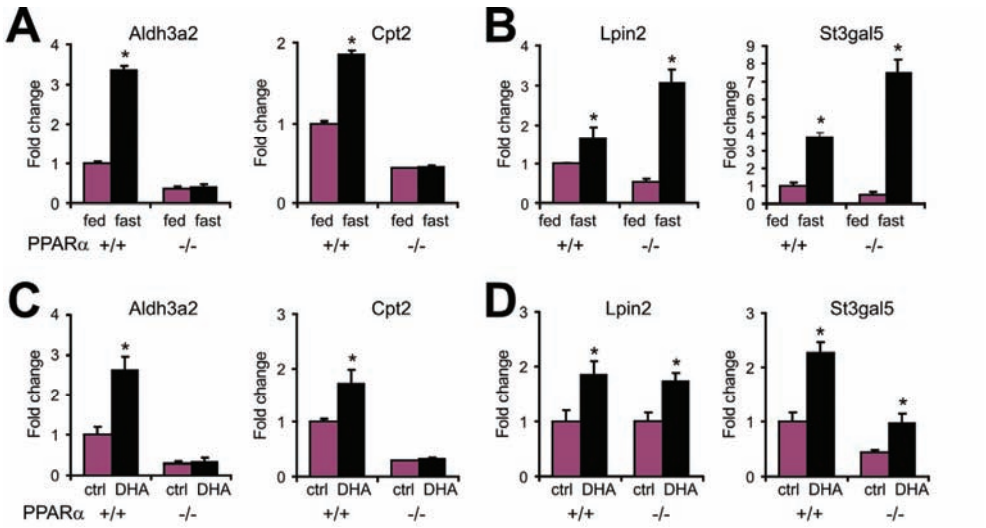


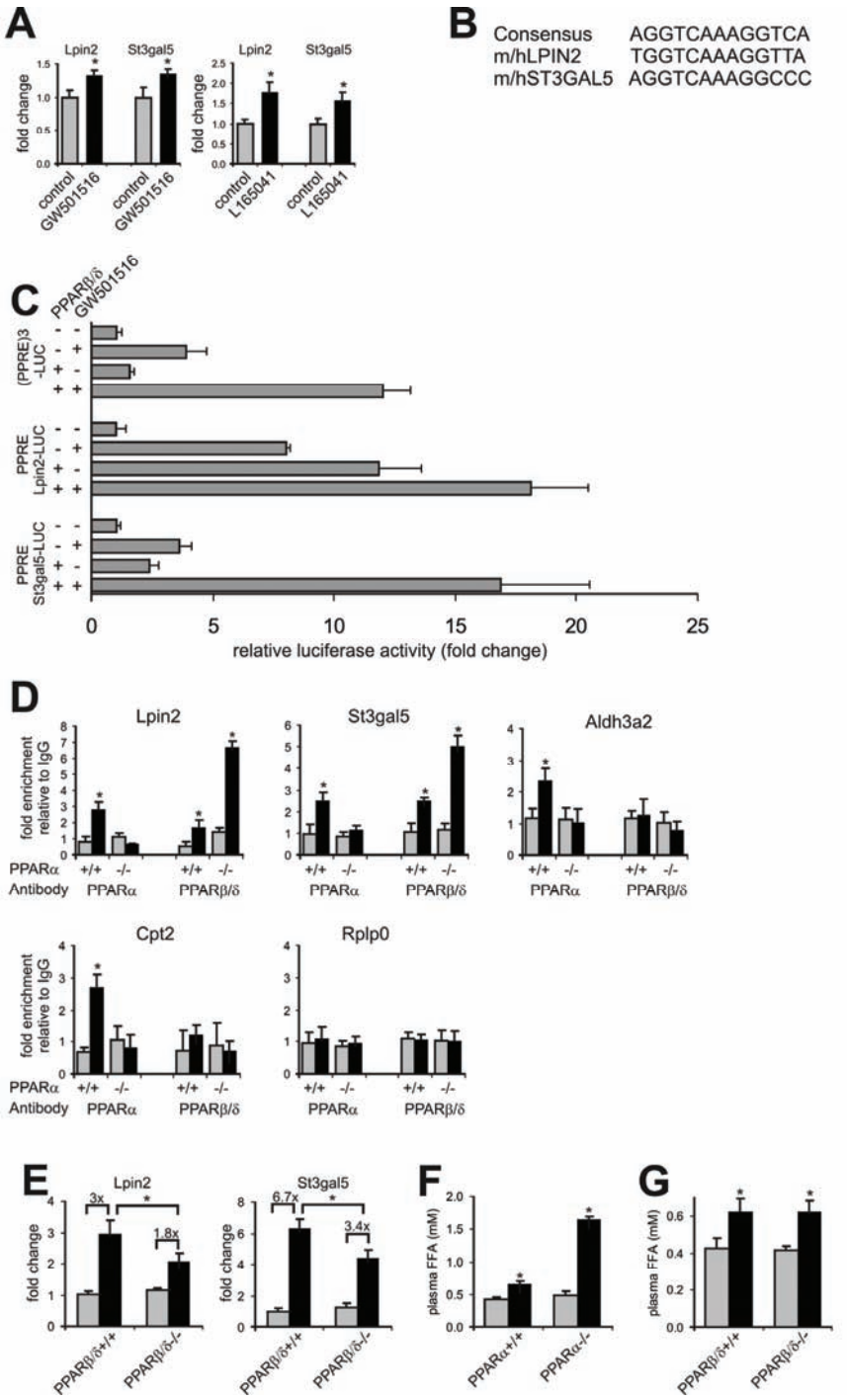
***Figure 2. Lpin2* and *St3gal5* are induced during fasting independently of PPARα.** Livers from wildtype and PPARα-/- mice fasted for 24h or treated with tridocosahexaenoin (DHA) for 6h were used for gene expression profiling (n=4-5 mice per group). Gene expression of classical PPARα targets *Aldh3a2* and *Cpt2* (A) and *Lpin2* and *St3gal5* (B) in fed and fasted wildtype and PPARα-/- mice. Gene expression of *Aldh3a2* and *Cpt2* (C) and *Lpin2* and *St3gal5* (D) after treatment with the dietary fatty acid DHA. Error bars represent SEM. * = significantly different according to student's t-test ($P<0.05$).

PPARβ/δ as an alternative transcription factor to PPARα in mouse liver during fasting

One obvious candidate alternative transcription factor is PPARβ/δ, which is well expressed in liver (47, 132). Supporting regulation of *Lpin2* and *St3gal5* by PPARβ/δ, the PPARβ/δ agonists GW501516 and L165041 significantly induced *Lpin2* and *St3gal5* mRNA (Figure 3A). To establish whether *Lpin2* and *St3gal5* are direct PPAR target genes, we identified a conserved PPRE within the *Lpin2* and *St3gal5* genes (Figure 3B) and cloned a genomic region encompassing the PPRE in front of a luciferase reporter to perform transactivation assays. GW501516 significantly increased reporter activity for the *Lpin2* and *St3gal5* genomic regions, which was further enhanced by co-transfection with PPARβ/δ (Figure

3C). In subsequent chromatin immunoprecipitation (ChIP) experiments, PPARβ/δ as well as PPARα could be cross-linked to the transcriptional start site (TSS) of the *Lpin2* and *St3gal5* genes, at least in the fasted state, which provides evidence for the presence of a distant functional PPRE (Figure 3D). These data suggest that *Lpin2* and *St3gal5* genes represent direct PPAR target genes. Interestingly, while fasting increased binding of both PPARα and PPARβ/δ to the *Lpin2* and *St3gal5* genes, fasting increased binding of only PPARα to the *Aldh3a2* and *Cpt2* genes (Figure 3D). No binding of PPARα and PPARβ/δ to the negative control gene *Rplp0* was observed. All together these data suggest that *Lpin2* and *St3gal5* are dual targets of PPARα and PPARβ/δ, whereas *Aldh3a2* and *Cpt2* are exclusive targets of PPARα. In agreement with this notion, induction of *Lpin2* and *St3gal5* by fasting was partially abolished in PPARβ/δ-/- mice (Figure 3E).

Figure 3 (next page). **PPARβ/δ as alternative transcription factor to PPARα in mouse liver.** A) *Lpin2* and *St3gal5* expression in liver of wildtype mice (n=5) treated with the PPARβ/δ agonist GW501516 for 6 hours or PPARα-/- mice (n=5) treated with the PPARβ/δ agonist L165041 for 5 days. Error bars represent SEM. B) PPREs conserved between mouse and human were identified 1291 bp and 23333 bp downstream of the TSS of the *Lpin2* and *St3gal5* genes. C) HepG2 cells were transfected with a PPARβ/δ expression vector and a SV40 reporter vector containing a 201-nucleotide and 183-nucleotide fragment containing the putative PPRE within the *Lpin2* and *St3gal5* genes, respectively. The reporter vector $(PPRE)_3$-TK-luciferase served as positive control. Luciferase and β-galactosidase activities were determined 24 hours after exposure of the cells to 1μM GW501516. Error bars represent SEM. D) Chromatin was extracted from livers of fed and 24 hour fasted wildtype and PPARα-/- mice (n=3 per group). ChIP was performed with antibodies against PPARα and PPARβ/δ on the TSS of *Lpin2*, *St3gal5*, *Aldh3a2*, *Cpt2* and *Rplp0*. Rabbit IgGs was used as a specificity control. Grey bars = fed state; black bars = 24h fasted state. Error bars represent SD. E) Expression of *Lpin2* and *St3gal5* in livers of fed and 24 hour fasted wildtype and PPARβ/δ-/- mice (n=4-5 per group). Relative induction by fasting is indicated. Error bars represent SEM. Plasma FFA levels in wildtype and PPARα-/- mice (F) or wildtype and PPARβ/δ-/- mice (G) sacrificed in fed or 24 hour fasted state. Error bars represent SEM. * = significantly different according to student's t-test ($P<0.05$).


A
Lpin2
St3gal5
fold change
control
GW501516
control
GW501516
Lpin2
St3gal5
fold change
control
L165041
control
L165041
B
Consensus AGGTCAAAGGTCA
m/hLPIN2 TGGTCAAAGGTTA
m/hST3GAL5 AGGTCAAAGGCCC
C
PPARβ/δ GW501516
(PPRE)3 -LUC
PPRE Lpin2-LUC
PPRE St3gal5-LUC
0 5 10 15 20 25
relative luciferase activity (fold change)
D
Lpin2
St3gal5
Aldh3a2
fold enrichment relative to IgG
PPARα +/+ -/- +/+ -/-
Antibody PPARα PPARβ/δ
Cpt2
Rplp0
E
Lpin2
St3gal5
fold change
3x
1.8x
6.7x
3.4x
PPARβ/δ+/+
PPARβ/δ-/-
F
plasma FFA (mM)
PPARα+/+
PPARα-/-
G
plasma FFA (mM)
PPARβ/δ+/+
PPARβ/δ-/-

Given the more pronounced induction of *Lpin2* and *St3gal5* by fasting in PPARα-/- vs. wildtype mice, we speculated that either expression of PPARβ/δ may be upregulated in PPARα-/- mice as a compensatory mechanism, or that ligand-activation of PPARβ/δ is enhanced in PPARα-/- mice. While we could not detect a change in PPARβ/δ mRNA in PPARα-/- mice (data not shown), consistent with the second scenario plasma FFA levels were markedly elevated in fasted PPARα-/- mice (Figure 3F), which was associated with marked induction of PPARβ/δ binding to the *Lpin2* and *St3gal5* promoter (Figure 3D). These data suggest that in the absence of PPARα plasma FFAs can induce *Lpin2* and *St3gal5* expression via PPARβ/δ. It should be noted that induction of plasma FFAs by fasting is unaltered in PPARβ/δ-/- mice (Figure 3G).

Circulating FFAs activate PPARβ/δ but not PPARα in mouse liver

Importantly, recent evidence suggests that PPARα in liver cannot be (ligand)-activated by plasma FFA, while it can be activated by fatty acids synthesized *de novo* (86). To study the activation of PPARα and PPARβ/δ by plasma FFA, we modulated fasting plasma FFA levels by taking advantage of a unique transgenic model system based on whole body overexpression or inactivation of the mouse *Angptl4* gene, which encodes a pro-lipolytic factor involved in lipid metabolism (379, 390). Previously, intravenous injection of Angptl4 was shown to cause an immediate increase in plasma FFA (391). In support, Angptl4 increased release of glycerol from 3T3-L1 adipocytes (Figure 4A).

Consistent with a pro-lipolytic effect of Angptl4, Angptl4 overexpression in mice was associated with a significant increase in release of fatty acids and glycerol from adipose tissue, whereas the opposite was observed in Angptl4-/- mice (Figure 4B). In agreement with these data, fasting plasma FFA levels were increased or decreased upon Angptl4 overexpression or inactivation, respectively (Figure 4C). In fact, the fasting-induced increase in plasma FFA was entirely blunted in Angptl4-/- mice. Plasma TG levels were increased or decreased upon Angptl4 overexpression or inactivation (Figure 4D), respectively, reflecting the well-documented inhibitory effect of Angptl4 on LPL activity (392). Finally, the defective lipolysis in Angptl4+/- and -/- mice was supported by the absence of changes in adipocyte cell size upon fasting, in contrast to Angptl4-Tg and wildtype mice (Figure 4E). These results corroborate the stimulatory effect of Angptl4 on adipose tissue lipolysis, which we exploited to study the effect of plasma FFAs on hepatic gene expression.

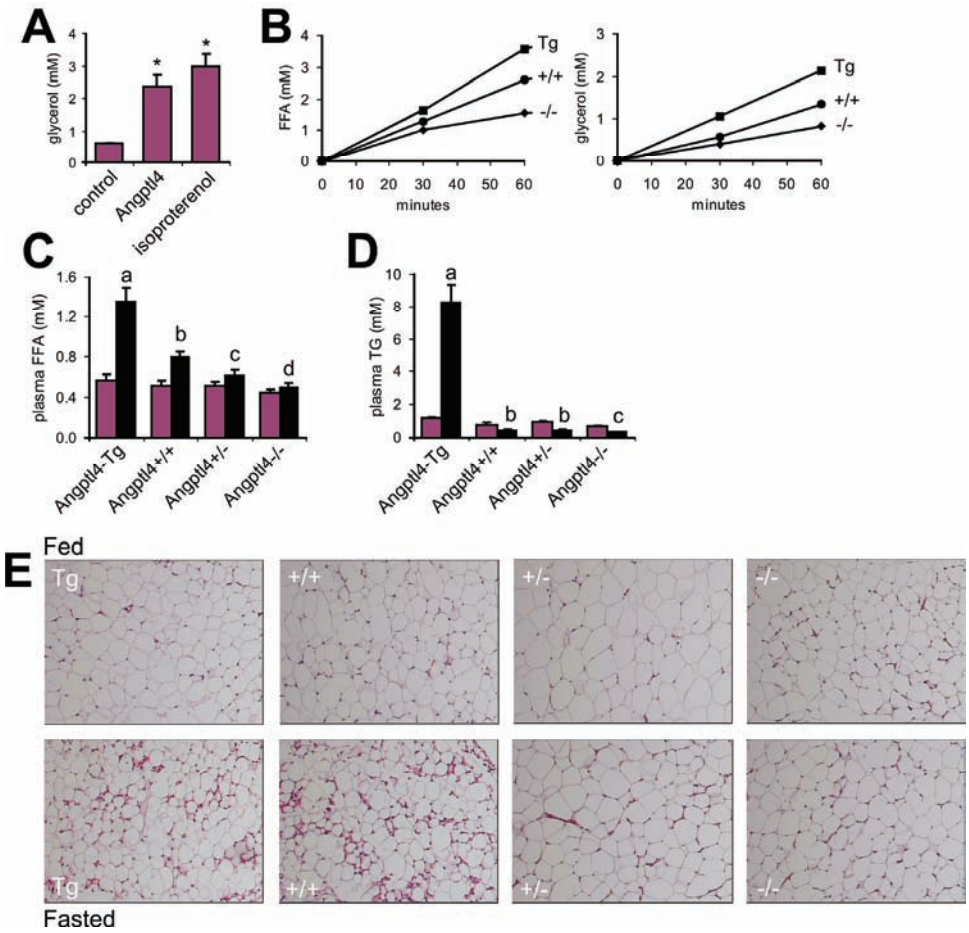


Figure 4. **Angptl4 stimulates adipose tissue lipolysis.** A) Glycerol concentration in medium of 3T3-L1 cells treated for 30 minutes with isoproterenol or with concentrated conditioned medium of HEK293 cells transfected with mAngptl4. Control cells were treated with condition medium of non-transfected HEK293 cells. Error bars represent SEM. * = significantly different according to student's t-test ($P<0.05$). B) Increase in fatty acid and glycerol concentration in medium of adipose tissue explants from transgenic mice overexpressing *Angptl4* (Tg), wildtype (+/+) and homozygous knockout (-/-) mice. Values are corrected for weight of explants. Plasma FFAs (C) and triglycerides (D) in transgenic mice overexpressing *Angptl4* (*Angptl4*-Tg), wildtype (*Angptl4*+/+), heterozygous (*Angptl4*+/-) and homozygous (*Angptl4*-/-) mice in fed or 24 hour fasted state (n=5). Grey bars = fed state; black bars = 24 hour fasted state. Error bars represent SEM. Different letters indicate statistically significant difference (student's t-test, $P<0.05$). E) Eosin and hematoxylin staining of epididymal adipose tissue.

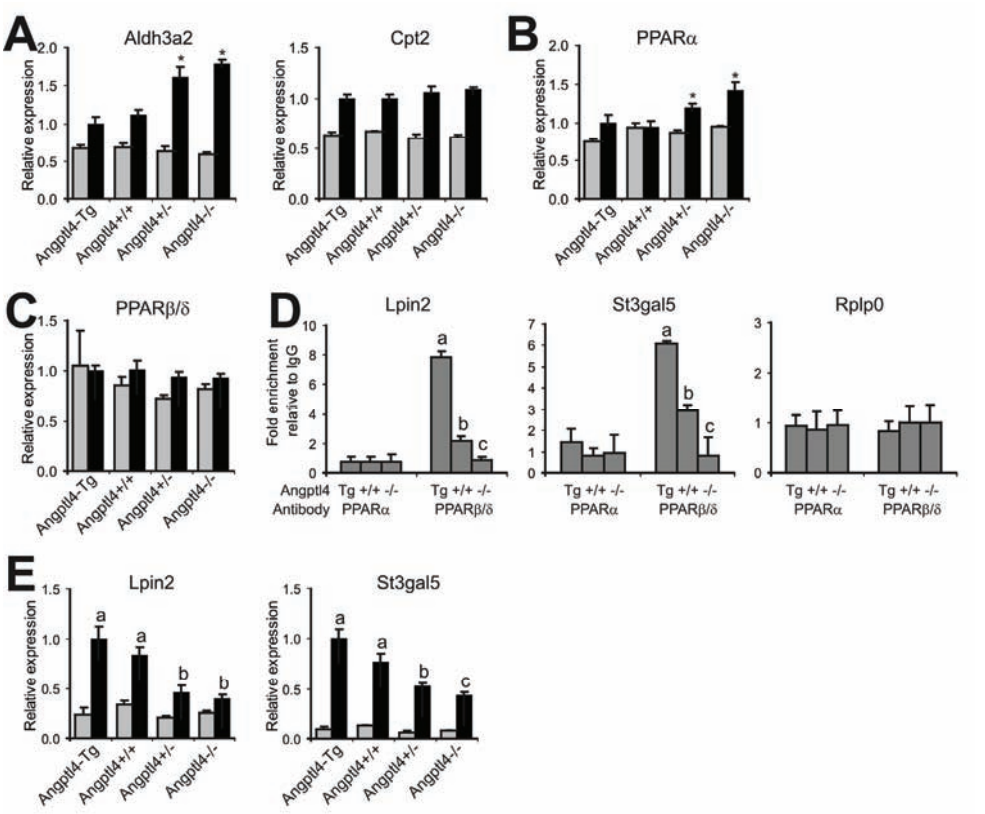


Figure 5. **Plasma FFAs do not activate hepatic PPARα.** Transgenic mice overexpressing *Angptl4* (*Angptl4*-Tg), wildtype (*Angptl4*+/+), heterozygous (*Angptl4*+/-) and homozygous knockout (*Angptl4*-/-) mice were sacrificed in fed state or after a 24 hour fast (n=5). Hepatic gene expression of classical PPARα targets *Aldh3a2* and *Cpt2* (A), *Pparα* (B) and *Pparβ/δ* (C). Grey bars = fed state; black bars = 24 hour fasted state. Error bars represent SEM. * = P<0.05. C). D) Chromatin was extracted from livers of 24 hour fasted transgenic mice overexpressing *Angptl4* (Tg), wildtype (+/+) and homozygous Angptl4 knockout (-/-) mice (n=3 per group). ChIP was performed with antibodies against PPARα and PPARβ/δ on the TSS of *Lpin2*, *St3gal5*, or *Rplp0*. Rabbit IgGs was used as a specificity control. Error bars represent SD. Different letters indicate statistically significant difference (student's t-test, P<0.05). E) Hepatic gene expression of *Lpin2* and *St3gal5*. Grey bars = fed state; black bars = 24 hour fasted state. Error bars represent SEM. Different letters indicate statistically significant difference (student's t-test, P<0.05).

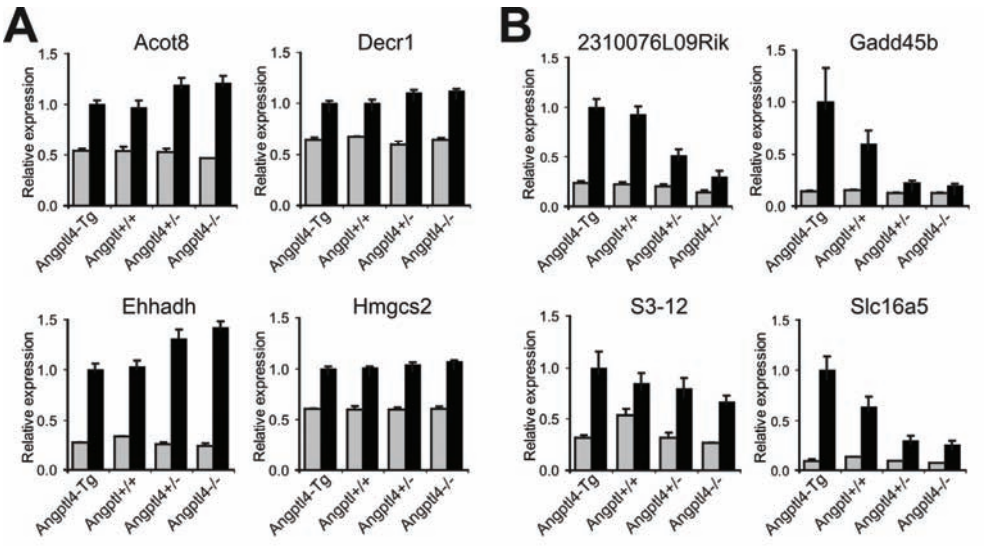


Figure 6. **Classical PPARα target genes do not follow plasma FFAs.** Transgenic mice overexpressing *Angptl4* (*Angptl4*-Tg), wildtype (*Angptl4*+/+), heterozygous (*Angptl4*+/-) and homozygous knockout (*Angptl4*-/-) mice were sacrificed in fed state or after a 24h fast (n=5). A) Gene expression of a representative set of classical PPARα target genes. Grey bars = fed state; black bars = 24h fasted state. Error bars represent SEM. B) Gene expression of a representative set of other WY14643-induced genes whose expression followed plasma FFA concentration independent of PPARα. Grey bars = fed state; black bars = 24h fasted state. Error bars represent SEM.

If hepatic PPARα is activated by plasma FFAs, expression of classical PPARα target genes during fasting would be expected to be proportional to the plasma FFA level throughout the various Angptl4 mouse models. Remarkably, rather than going down, gene expression of classical PPARα targets *Aldh3a2*, *Cpt2* and others was stable or went up as plasma FFAs decline (Figure 5A, Figure 6A). Expression of *Pparα* itself, which is auto-regulated, followed a very similar pattern (Figure 5B), suggesting that regulation of classical PPARα targets is determined by PPARα expression level. Supporting the use of the Angptl4 mouse models to study hepatic gene regulation by FFA, hepatic expression of *Srebp1*, which is known to be suppressed by fatty acids, negatively correlated with plasma FFA concentration (data not shown). In combination with previously published data (86), these data strongly suggest that PPARα is not activated by plasma FFA in mouse liver.

While plasma FFAs seemingly do not activate hepatic PPARα, data presented above suggested that FFAs induce *Lpin2* and *St3gal5* expression by activating PPARβ/δ, at least in PPARα-/- mice. To assess activation of PPARβ/δ by FFA in the presence of PPARα, we determined binding of PPARβ/δ to the *Lpin2* and *St3gal5* genes in the Angptl4 mouse models using ChIP. Importantly, independent of PPARβ/δ gene expression levels, which remained constant (Figure 5C), binding of PPARβ/δ to the *Lpin2* and *St3gal5* genes was proportional to the plasma FFA concentration and mimicked fasting *Lpin2* and *St3gal5* expression levels (Figure 5D and Figure 5E). In contrast, binding of PPARα to the *Lpin2* and *St3gal5* genes was minimal and did not follow the plasma FFA concentration (Figure 5D). Again, no binding of PPARα and PPARβ/δ to negative control gene *Rplp0* was observed. These data suggest that PPARβ/δ can be activated by plasma FFA. Other WY14643-induced genes whose expression followed plasma FFA concentration independent of PPARα included lipid droplet proteins *2310076L09Rik* (*MLDP*) and *S3-12*, as well as *Slc16a5* and *Gadd45b*, suggesting they might represent targets of PPARβ/δ as well (Figure 6B).

PPARα target genes may be upregulated during fasting via induction of PGC1α

If elevated plasma FFAs can not account for the induction of classical PPARα activation during fasting, the question arise what other mechanism may be responsible. One possibility is increased coactivator expression. The coactivator PGC1α plays a major role in the liver during fasting by upregulating genes involved in gluconeogenesis and fatty acid oxidation/ketogenesis, mediating activation by several transcription factors including PPARα (34-36). In agreement with previous data (35), expression of *Pgc1a* went up significantly during fasting (Figure 7A). Importantly, fasting markedly enhanced binding of PGC1α to the TSS of the PPARα target genes *Aldh3a2* and *Cpt2*, which was abolished in PPARα-/- mice (Figure 7B). No binding of PGC1α to *Rplp0* was observed. These data suggest that upregulation of *Pgc1α* mRNA may contribute to induction of classical PPARα target genes during fasting via increased PPARα-dependent binding of PGC1α to gene promoters.

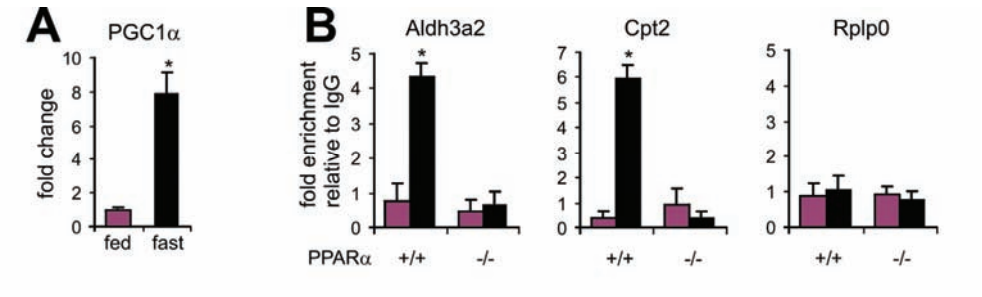


Figure 7. **PPARα activation during fasting may be mediated by PGC1α upregulation.** A) Expression of *Pgc1a* in livers of fed or fasted wildtype mice (n=5). Fasting statistically significantly induced gene expression of *Pgc1α* (P<0.05). Error bars represent SEM. B) Chromatin was extracted from livers of fed and 24 hour fasted wildtype and PPARα-/- mice (n=3 per group). ChIP was performed with antibodies against PGC1α on the TSS of *Aldh3a2* and *Cpt2*, and negative control gene *Rplp0*. Rabbit IgGs was used as a specificity control. Purple bars = fed state; black bars = 24 hour fasted state. Error bars represent SD. Fasting significantly induced binding of PGC1α in wildtype but not PPARα-/- mice (P<0.05).

Discussion

It has been clearly established that PPARα governs the fasting-induced upregulation of numerous genes involved in hepatic fatty acid oxidation, many of which are direct PPARα target genes (70-72). However, it has remained unclear whether elevated plasma FFAs themselves are responsible for the induction of hepatic fatty acid catabolism via enhanced ligand-activation of PPARα (70-72). Recently, using mice with liver-specific inactivation of the *Fasn* gene, Chakravarthy et al. showed that unlike dietary fatty acids and *de novo* synthesized fatty acids, circulating FFAs fail to activate hepatic PPARα (86). In the present study, using mice differentially expressing *Angptl4* we arrive at essentially the same conclusion.

Importantly, our data also suggest that in contrast to PPARα, hepatic PPARβ/δ can be activated by plasma FFA, which accounts for the plasma FFA- and fasting-dependent upregulation of several genes in wildtype and PPARα-/- mice, including *Lpin2* and *St3gal5*. Indeed, we demonstrate that *Lpin2* and *St3gal5* expression and binding of PPARβ/δ to the *Lpin2* and *St3gal5* promoter closely mirror plasma FFA levels. The role of PPARβ/δ in

gene regulation by plasma FFA during fasting was substantiated by the observation that induction of *Lpin2* and *St3gal5* by fasting is reduced in PPARβ/δ-/- mice.

In contrast to plasma FFAs, evidence abounds indicating that dietary fatty acids are able to activate PPARα (73, 338, 339). Recently, it was shown that the effects of dietary fatty acids on hepatic gene expression are quantitatively almost entirely mediated by PPARα (69). Additionally, the present data suggest that dietary fatty acids can also activate PPARβ/δ as induction of *Lpin2* and *St3gal5* by dietary fat was entirely or partially maintained in PPARα-/- mice.

It may be argued that the lack of effect of declining plasma FFAs on hepatic PPARα activation may be because PPARα, in contrast to PPARβ/δ, is already saturated with fatty acids at low plasma FFA levels, thus allowing no further activation. Previously, it has been shown that fatty acids bind to PPARβ/δ with an about 5 to10-fold lower affinity compared to PPARα (27). However, as treatment with synthetic agonists clearly results in more pronounced PPARα activation compared to fasting (73), the argument of PPARα saturation is only tenable if we assume fatty acids to act as partial agonists that do not elicit full PPARα activity compared to synthetic agonists. Saturation of PPARα is also not supported by Chakravarthy (86).

Alternatively, it is conceivable that fatty acids are present in hepatocytes in distinct pools, which have different activity towards PPARα and PPARβ/δ. In this context, it should be realized that dietary fatty acids present in chylomicron remnants are internalized differently compared to plasma FFAs. Whereas the former are liberated after endosomal and lysosomal degradation of cholesteryl-esters and triglycerides, plasma FFA are likely internalized via diffusion as well as via specific fatty acid transport proteins, including CD36 and FATPs. The third contributor to the hepatic fatty acid pool is *de novo* lipogenesis, a process which occurs in the cytosol. It is presently unclear to what extent these three sources of fatty acids undergo similar metabolic fates. Recent studies support the existence of distinct hepatic fatty acid pools that are differentially shuttled into various metabolic pathways, including oxidation and incorporation into VLDL-triglycerides (393). For example, there is evidence that fatty acids generated by *de novo* lipogenesis only marginally contribute to VLDL-triglycerides, in contrast to plasma FFAs (394). Our present and previous data suggest that in terms of gene regulation, a similar type of segregation occurs between the three sources of fatty acids (86). The mechanism underlying the differential activity of fatty acids from distinct pools towards PPARα and PPARβ/δ remains unknown. One could hypothesize a role for fatty acid binding proteins (FABPs). It can be speculated that FABP1, which has been shown to interact with PPARα

(82), picks up lipoprotein-derived fatty acids and shuttles them to PPARα, whereas another FABP expressed in liver such as FABP2 may selectively bind free fatty acids coming from plasma and shuttle them to PPARβ/δ.

An important lingering question is that if plasma FFAs do not activate hepatic PPARα during fasting, what mechanism accounts for activation of PPARα-dependent gene regulation during fasting? Previously, a role for PGC1α in fasting-dependent upregulation of hepatic mitochondrial fatty acid oxidation and ketogenesis was shown (34, 36). Our ChIP analysis indicates enhanced recruitment of PGC1α, which itself is upregulated by fasting, to classical PPARα target genes during fasting. Accordingly, activation of PPARα by fasting may be driven by the increase in PGC1α expression, although an important role of other coactivators cannot be excluded. Recently, it was shown that PGC1α cooperates with BAF60a (SMARCD1) to activate transcription of PPARα target genes involved in peroxisomal and mitochondrial fatty acid oxidation genes (395).

While our data suggest that PPARβ/δ mediates the effect of plasma FFAs on a small set of genes in liver, the overall importance of PPARβ/δ in hepatic gene regulation by FFAs remains unclear. The same is true for the actual functional role of PPARβ/δ in liver. Presently, combined transcriptomics and metabolomics analyses of livers of PPARα-/- and PPARβ/δ-/- mice are underway to gain more understanding about the role of PPARβ/δ in liver and to determine the extent to which PPARα and PPARβ/δ regulate distinct sets of genes and govern distinct metabolic pathways, especially under physiological circumstances.

In this study we have used variable expression of the *Angptl4* gene to create variations in fasting plasma FFA levels in mice. Angptl4 is a potent inhibitor of lipoprotein lipase and hepatic lipase and decreases uptake of lipoprotein remnants by the liver, thereby decreasing hepatic uptake of dietary fatty acids (324). In addition, it stimulates adipose tissue lipolysis, as shown by the acute increase in plasma FFA upon injection of recombinant Angptl4 (391), and by elevated plasma FFAs and glycerol levels in mice overexpressing Angptl4 (379). The pro-lipolytic effect of Angptl4 is supported by recent data in humans (390). In the present paper, Angptl4 markedly induced glycerol release from 3T3-L1 adipocytes. Activation of lipolysis by Angptl4 was further substantiated by the altered release of fatty acids and glycerol from adipose tissue explants from Angptl4-Tg and Angptl4-/- mice, as well as by the lack of an increase in FFA during fasting in Angptl4-/- mice. As a consequence, hepatic VLDL production is reduced in Angptl4-/- mice (396). By inhibiting LPL and stimulating adipose tissue lipolysis, Angptl4 promotes switching of hepatic fatty acid uptake from remnant-derived fatty acids towards plasma FFAs (379). Importantly, the

variations in plasma FFAs in the Angptl4 mouse models are specifically elicited by fasting, permitting study of the impact of differential plasma FFAs on hepatic gene expression during fasting.

While *in vivo* and *in vitro* studies using synthetic PPARα agonists are extremely relevant to assess the toxicological and pharmacological impact and significance of PPARα, it is unclear to what extent they report on the physiological role of PPARα in liver. Our results reveal that several genes upregulated following pharmacological PPARα activation are not induced by PPARα under physiological conditions such as fasting, or are induced by fasting independently of PPARα but dependently of PPARβ/δ. It is well known that in reporter assays PPARα and PPARβ/δ (and PPARγ) can activate the same genes, suggesting that all PPARs share an intrinsic ability to transactivate any given PPAR target gene. The present data on *Lpin2* and *St3gal5* are consistent with the notion that *in vivo* the dominant receptor in the regulation of a particular PPAR target is context dependent and importantly may differ between pharmacological and physiological stimuli. Genes other than *Lpin2* and *St3gal5* that have been shown to be activated by both PPARα and PPARβ/δ include *Adfp* (397), *G0s2* (398) and *Pdk4* (388). Overall, the data imply that studies using high-affinity synthetic PPAR agonists are not perfectly suited to assess the functions of PPARs during normal physiology.

Acknowledgements

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Chapter 6

PPARβ/δ regulates glucose utilization and lipoprotein metabolism in liver

Linda M. Sanderson, Mark Boekschoten, Béatrice Desvergne, Michael Müller and Sander Kersten

Manuscript submitted

Abstract

The nuclear hormone receptors PPARα and PPARγ are major drug targets for treatment of metabolic disorders. However, much less is known about PPARβ/δ. Here we set out to better characterize the role of PPARβ/δ in liver by comparing the effect of PPARα and PPARβ/δ deletion on liver function. In fed state, PPARα and PPARβ/δ were expressed in mouse liver at about equal levels, while in fasted state PPARα expression was markedly higher. Consistent with these data, the number of genes altered by PPARα and PPARβ/δ deletion was similar in fed state, whereas in fasted state the effect of PPARα deletion was much more pronounced. Minor overlap was found between PPARα- and PPARβ/δ-dependent gene regulation in liver. According to Gene Set Enrichment Analysis (GSEA), pathways upregulated by PPARβ/δ deletion were connected to innate immunity. Pathways specifically downregulated by PPARβ/δ deletion included lipoprotein metabolism and various pathways related to glucose utilization, which correlated with elevated plasma glucose and triglyceride levels and reduced plasma cholesterol levels in PPARβ/δ-/- mice. Downregulated genes that likely underlie these metabolic alterations include *Pklr*, *Fbp1*, *Apoa4*, *Vldlr*, *Lipg*, and *Pcsk9*, which may represent novel PPARβ/δ target genes. In contrast to PPARα-/- mice, no changes in plasma FFA, plasma β-hydroxybutyrate, liver triglycerides and liver glycogen were observed in PPARβ/δ-/- mice. Overall, the results reveal that unlike PPARα, PPARβ/δ does not mediate an adaptive response to fasting. Our data point to a role for PPARβ/δ in hepatic glucose utilization and lipoprotein metabolism, as well as innate immunity.

Introduction

Disturbances in lipid metabolism are at the basis of many chronic disorders, including obesity, diabetes, non-alcoholic fatty liver disease and atherosclerosis. Regulation of lipid metabolism is mainly coordinated by the liver, which therefore is a key target organ for the pharmacological treatment of the abovementioned diseases. Lipid metabolism is governed via a complex interplay between hormones, transcription factors, and energy substrates, allowing for rapid adaptations to changes in metabolic requirements (369). An important class of ligand-activated transcription factors involved in regulation of hepatic lipid metabolism are the nuclear hormone receptors, more specifically the farnesoid X receptor (FXR), the liver X receptors (LXR), and the peroxisome proliferator activated receptors (PPAR) (63, 399). Specific biological processes under control of FXR, LXRs and PPARs in liver include bile acid synthesis and metabolism, lipogenesis, lipoprotein metabolism, and fatty acid degradation. Additionally, FXR, LXRs and PPARs have been implicated in glucose metabolism, although the mechanisms and specific target genes involved remain poorly defined. FXR, LXRs and PPARs activate gene transcription by forming a complex with the retinoid X receptor, RXR, followed by binding of the heterodimeric complex to response elements in the DNA (24). Binding of ligand to the nuclear receptors results in the recruitment of coactivators and dissociation of corepressors, leading to chromatin remodeling and subsequent initiation of DNA transcription.

The PPAR group of nuclear receptors can be further separated into three subtypes: PPARα, PPARβ/δ and PPARγ (20, 21). All three PPARs are activated by fatty acids and by a variety of fatty acid-derived compounds including eicosanoids, oxidized fatty acids, and fatty acid amides. PPARα has been shown to be a key regulator of hepatic fatty acid metabolism, a role which is especially prominent during fasting. Indeed, lack of PPARα in fasted mice is associated with pronounced hepatic steatosis, a lack of increase in plasma ketone bodies, decreased plasma glucose, hypothermia, and elevated plasma free fatty acid (FFA) levels (42, 70-72, 74). These severe metabolic disturbances are the result of decreased expression of a large number of genes involved in hepatic lipid metabolism, many of which have been identified as direct PPARα target genes (78, 79, 81, 82, 85). Despite the relatively low expression level of PPARγ in liver, evidence suggests that PPARγ is critical for development of hepatic steatosis (73, 241, 243). Surprisingly, very limited information is available on the function of PPARβ/δ in liver, even though PPARβ/δ is well expressed in liver (47, 132). PPARβ/δ expression has been shown to be highest in the endothelial cells and hepatocytes, followed by liver resident macrophages (Kupffer cells) (44). Recent studies indicate that PPARβ/δ may influence the inflammatory properties of Kupffer cells (184). Other studies have linked PPARβ/δ to proliferation of

stellate cells and vitamin A metabolism (400, 401). Finally, activation of PPARβ/δ was shown to impact plasma lipoprotein levels (135, 136, 167). However, the overall role of PPARβ/δ in hepatic gene regulation remains poorly defined.

Here we set out to better elucidate the role of PPARβ/δ in hepatic function. The results reveal that unlike PPARα, PPARβ/δ does not mediate an adaptive response to fasting. Our data point to a role for PPARβ/δ in hepatic glucose utilization and lipoprotein metabolism.

Materials and Methods

Animals. A breeding colony of pure-bred Sv129 PPARα-/- mice (129S4/SvJae) and corresponding wildtype mice (129S1/SvImJ) was purchased from Jackson Laboratory (Bar Harbor, Maine, USA) and further expanded in our local animal facility. The PPARβ/δ-/- mice were on a mixed background (Sv129/C57Bl/6) and have been previously described (378).

Male mice (n=4-5 per group) were either fed or fasted for 24 hours. At the end of the experiment, mice were anaesthetized with a mixture of isofluorane (1.5%), nitrous oxide (70%) and oxygen (30%). Blood was collected by orbital puncture, after which the mice were sacrificed by cervical dislocation. Livers were dissected, snap frozen in liquid nitrogen and kept at -80ºC until further analysis. For RNA analyses, tissue from the same part of the liver lobe was used.

The animal studies were approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University, the Netherlands and the University of Lausanne, Switzerland.

RNA isolation and qRT-PCR. Total liver RNA was isolated with TRIzol reagent (Invitrogen, Breda, the Netherlands) according to manufacturer's instructions. RNA concentrations were measured on a NanoDrop ND-1000 spectrophotometer (Isogen Life Science, IJsselstein, the Netherlands). 1µg of total RNA was reverse transcribed using iScript (Bio-Rad, Veenendaal, the Netherlands). cDNA was amplified on a Bio-Rad MyIQ or iCycler PCR machine using Platinum Taq DNA polymerase (Invitrogen). PCR primer sequences were taken from the PrimerBank (325) and ordered from Eurogentec (Seraing, Belgium). Primer sequences are available upon request.

Affymetrix microarray. Total RNA from mouse liver was extracted with TRIzol reagent (Invitrogen), and subsequently purified and DNAse treated using the SV Total RNA Isolation System (Promega, Leiden, the Netherlands). RNA quality was measured on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) using 6000 Nano Chips according to manufacturer's instructions. RNA was judged as suitable for array hybridization only if samples showed intact bands corresponding to the 18S and 28S rRNA subunits, displayed no chromosomal peaks or RNA degradation products, and had a RIN (RNA integrity number) above 8.0. Five micrograms of RNA were used for one cycle cRNA synthesis (Affymetrix, Santa Clara, CA, USA). Hybridization, washing and scanning of Affymetrix NuGO Mouse Arrays (wildtype and PPARα-/- mice) and Affymetrix GeneChip® Mouse Exon 1.0 ST Arrays (wildtype and PPARβ/δ-/- mice) was carried out according to standard Affymetrix protocols.

Scans of the Affymetrix arrays were processed using packages from the Bioconductor project (288). Arrays were normalized with quantile normalization, and expression levels of probesets were calculated using the Robust Multichip Average (RMA) method (402, 403). Differentially expressed probesets were identified using Limma, and genes were considered to be significantly changed when raw $P<0.01$ (327). The dendrograms were created with the hclust command in R (stats library) utilizing the Ward clustering algorithm and the Pearson correlation measure (404). Functional analysis of the array data was performed using Gene Set Enrichment Analysis (383).

Microarray data were analyzed through the use of Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). Canonical pathway analysis identified the pathways from the Ingenuity Pathways Analysis library that were differentially expressed between knockout and wildtype mice. Genes from the data set that met the cut-off of $p < 0.01$ and were associated with a canonical pathway in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fisher's exact-test was used to calculate a P-value for each pathway. Pathways were considered to be differentially expressed between genotypes when the Fischer's exact P-value < 0.05.

Plasma metabolites. Plasma was obtained from blood by centrifugation for 10 minutes at 10000g. Plasma free fatty acids were measured with a commercially available kit from WAKO Chemicals (Sopachem, Wageningen, the Netherlands). Plasma glucose concentration was determined using a kit from Elitech (Sopachem, Wageningen, the Netherlands). Plasma and liver triglycerides, plasma β-hydroxybutyrate and plasma cholesterol were determined using kits from Instruchemie (Delfzijl, the Netherlands).

For measurement of liver glycogen, liver pieces were dissolved in 10 volumes of 1M NaOH and incubated at 55ºC. After 1-2 hours an equal volume of 1M HCl was added, followed by centrifugation for 5 min at 3000 rpm. Subsequently, 5µl of supernatant was added to 50µl of amyloglucosidase (1000U/ml in 0.2M sodium acetate buffer pH 4.8) and incubated for 2 hours with shaking (700 rpm) at 42ºC. After short centrifugation, glucose was measured using standard glucose assay (Glucose PAP SL, Elitech).

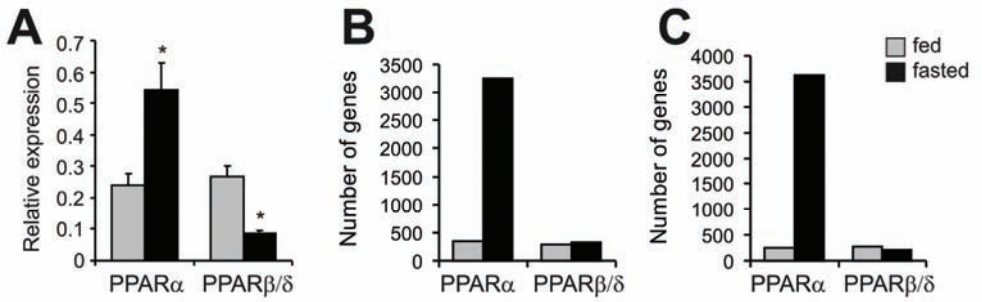

Figure 1. **Basal gene expression of PPARα and PPARβ/δ and whole genome expression profiling from wildtype, PPARα-/- and PPARβ/δ-/- mouse liver.** (A) Relative expression of PPARα and PPARβ/δ vs. the housekeeping gene *36b4* was measured with qRT-PCR. Error bars represent SEM. * = significantly different from fed mice according to student's t-test ($P<0.05$). (B) Number of upregulated genes between knockout and wildtype mice. (C) Number of downregulated genes between knockout and wildtype mice. Grey bars are fed mice (n=5), black bars are fasted mice (n=5).

Results

We first determined basal gene expression of PPARα and PPARβ/δ in mouse liver using qRT-PCR. Hepatic expression of PPARα and PPARβ/δ was similar in fed state. As previously shown (47, 72), PPARα expression increased during fasting whereas the opposite was true for PPARβ/δ (Figure 1A). As a consequence, PPARα mRNA clearly exceeded PPARβ/δ mRNA in fasted state.

In order to compare the role of PPARα and PPARβ/δ in gene regulation in liver, we performed whole genome expression profiling experiments on livers of fed and fasted wildtype and PPARα-/- or PPARβ/δ-/- mice. Since two different types of Affymetrix arrays were used, only genes present on both arrays (15004 genes in total) were included in the analysis. As expected, the number of genes differentially expressed between PPARα-/- mice vs. wildtype mice was much higher in the fasted state than in the fed state (Figure 1B and 1C). Interestingly, the number of genes differentially expressed between PPARβ/δ-/- mice vs. wildtype mice was about equal in fasted and fed state. Dendrogram of hierarchical clustering of the various groups showed that fasted PPARα-/- mice formed a highly distinct group, illustrating the dramatic impact of PPARα deletion specifically in fasted mice (Figure 2). In contrast to the situation for PPARα-/- mice, differences between wildtype and PPARβ/δ-/- were more pronounced in fed state compared to fasted state. Together, these data demonstrate that PPARα becomes much more important during fasting, whereas the opposite appears to be true for PPARβ/δ.

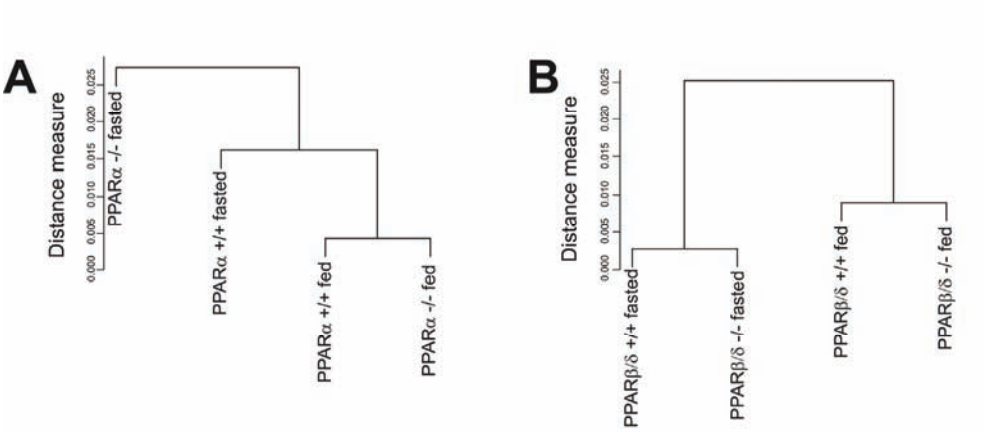

Figure 2. **Hierarchical clustering of fed and fasted wildtype, PPARα-/- and PPARβ/δ-/- mice.** Dendrograms showing PPARα (A) or PPARβ/δ (B). n = 5 per group.

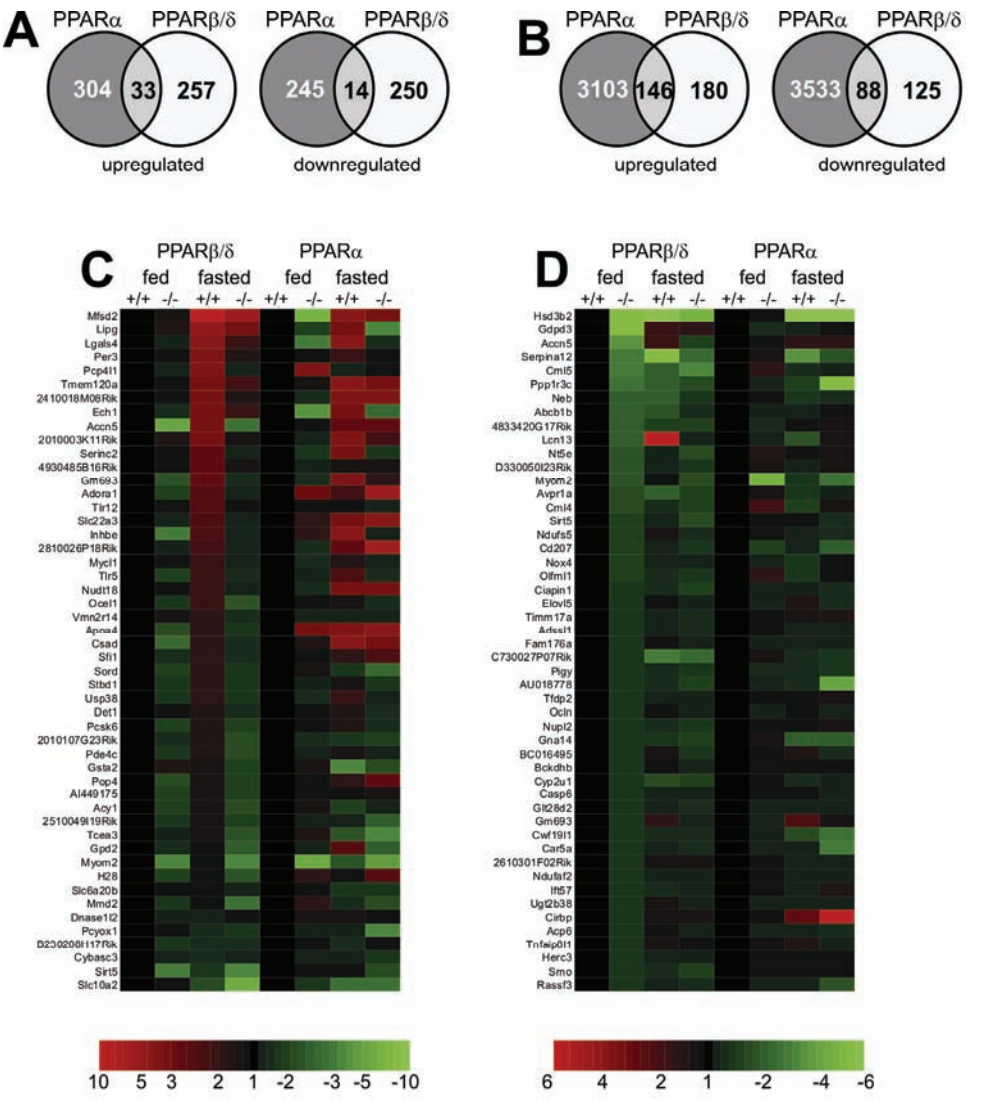

Figure 3. **Overlap in genes altered upon PPARα and PPARβ/δ deletion and top 50 of genes most significantly downregulated in PPARβ/δ mice.** Venn diagrams showing overlap in changed genes between PPARα and PPARβ/δ in the fed (A) and fasted (B) state. Heatmaps showing downregulated genes in fasted (C) and fed (D) state. Gene regulation is shown as fold change compared to wildtype fed mice according to color scale. Genes were included in analysis if $P < 0.01$ (C: PPARβ/δ-/- fasted vs. wildtype fasted; D: PPARβ/δ-/- fed vs. wildtype fed). n = 5 per group.

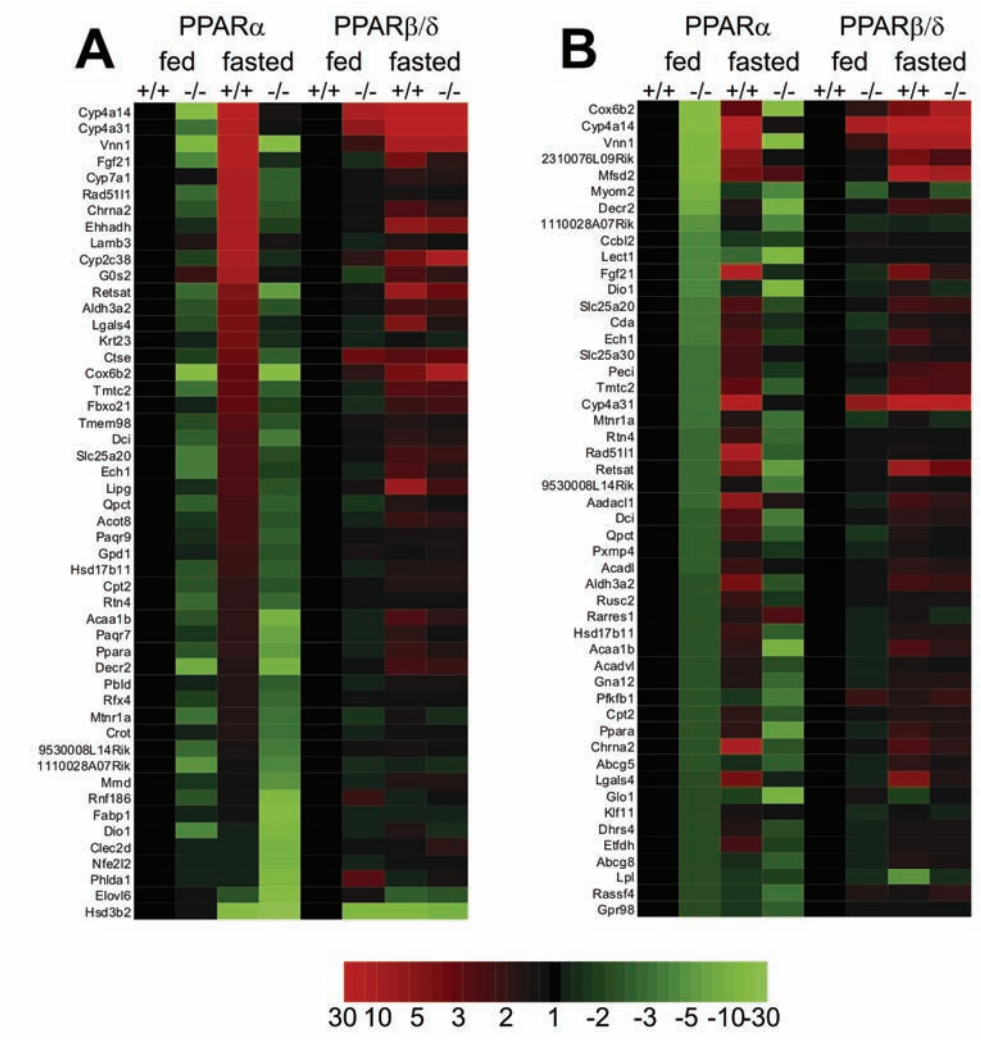

Figure 4. **Top 50 of genes most significantly downregulated in PPARα mice.** Heatmaps showing downregulated genes in fasted (A) and fed (B) state. Gene regulation is shown as fold change compared to wildtype fed mice according to color scale. Genes were included in analysis if $P < 0.01$ (A: PPARα -/- fasted vs. wildtype fasted; B: PPARα -/- fed vs. wildtype fed), n = 5 per group.

To examine to what extent PPARα and PPARβ/δ govern the same genes, we created Venn diagrams showing the overlap in genes altered upon PPARα and PPARβ/δ deletion. In the fed state, little overlap is observed between PPARα and PPARβ/δ deletion (Figure 3A). In the fasted state, however, a relatively large proportion of the genes altered upon PPARβ/δ deletion were also altered upon PPARα deletion, suggesting common regulation (Figure 3B). This finding is further illustrated by examining the top 50 of genes most significantly downregulated in PPARβ/δ-/- mice in fasted state. Indeed, several genes in the list were also reduced upon PPARα deletion, including *Lgals4*, *Serinc2*, and *Tlr5*. In contrast, few genes within the top 50 of downregulated genes in PPARα-/- mice in fasted state were also affected by PPARβ/δ deletion (Figure 3C, 3D and Figure 4). The expression of a number of individual genes representing specific profiles of regulation is illustrated in Figure 5. *Aldh3a2*, representing the group of classical PPARα target genes, was upregulated during fasting in a PPARα-dependent and PPARβ/δ-independent manner. An opposite pattern was observed for *Apoa4*. Interestingly, the fasting-induced expression of a number of genes, including *Lgals4* and *Lipg*, was dependent on both PPARα and PPARβ/δ. Overall, the data suggest limited functional overlap between the two PPARs in liver in fed state, whereas more significant overlap was observed in fasted state.

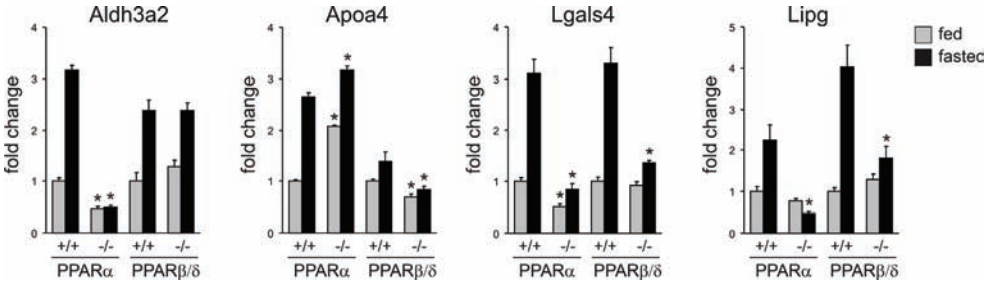

Figure 5. **Gene expression of four genes representing specific profiles of regulation.** Gene expression is shown as fold change vs. wildtype fed mice. Grey bars = fed state; black bars = fasted state. Error bars represent SEM. n = 5 per group. * = significantly different from corresponding wildtype mice according to student's t-test ($P<0.05$).

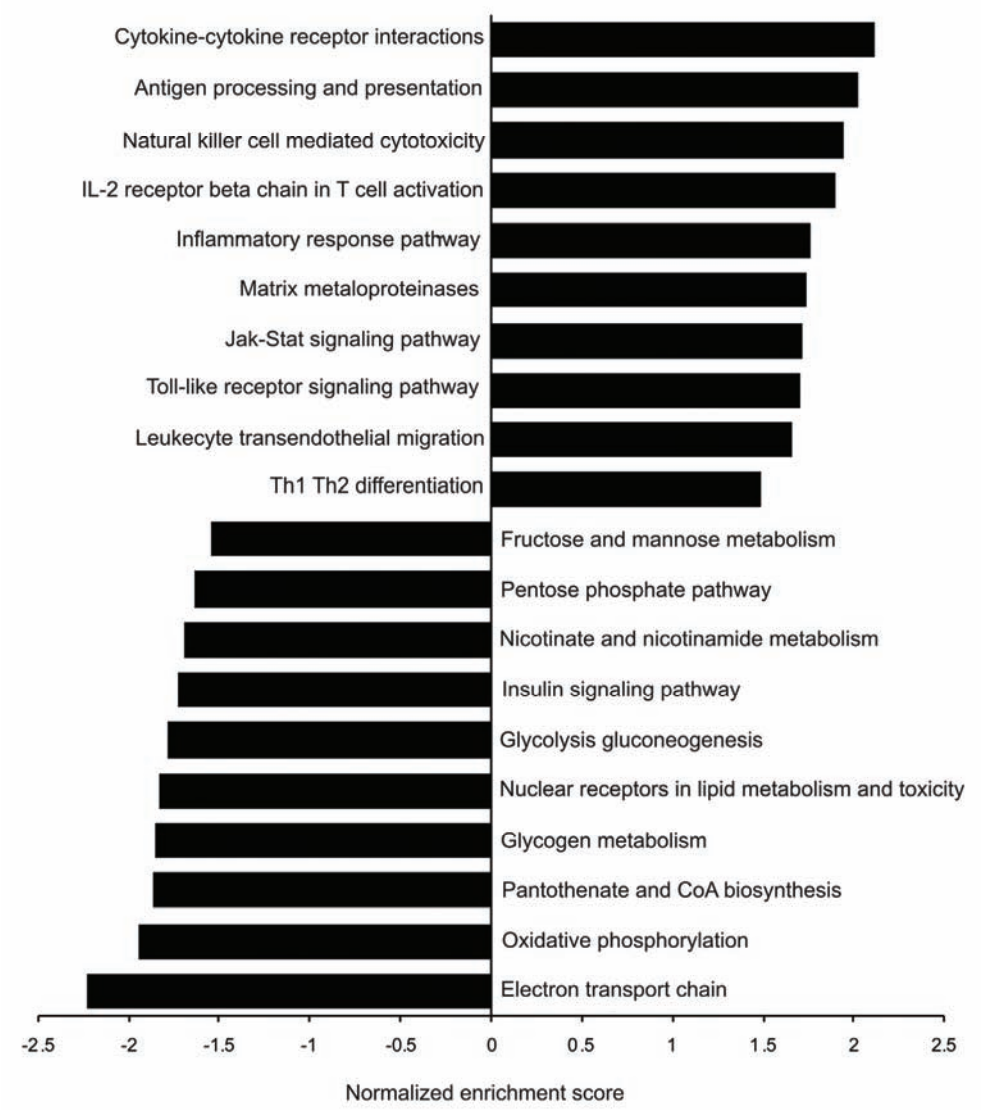

Figure 6. **Selected pathways identified by Gene Set Enrichment Analysis.** Only pathways are shown that had a false discovery rate Q-value of < 0.15. The normalized enrichment score reflects the degree to which a gene set is overrepresented at the top (upregulated) or bottom (downregulated) of the ranked gene list and is corrected for gene set size. Data are for the fed state.

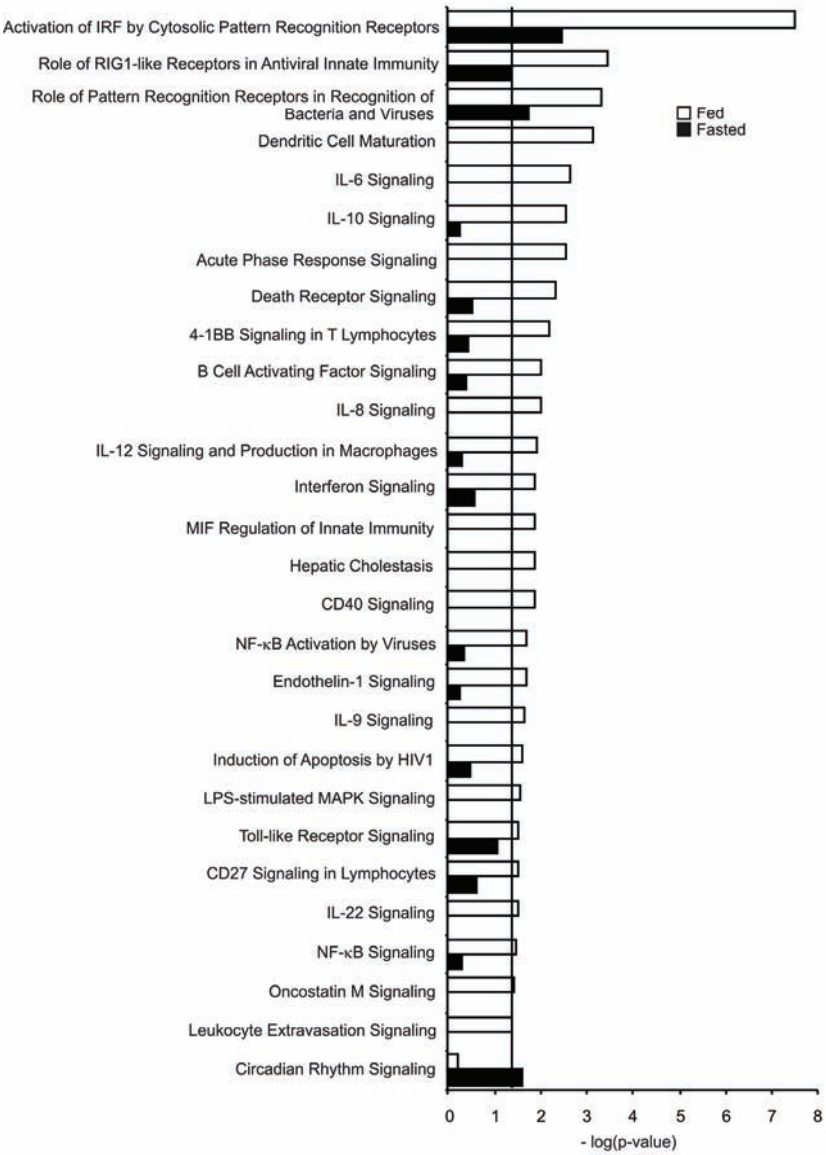

Figure 7. **Signaling pathways that are differentially expressed in PPARβ/δ-/- mice compared to wildtype mice in both fed (open bars) and fasted state (black bars).** Pathways were identified with the Canonical pathways feature of Ingenuity Pathway Analysis. Only significantly regulated signaling pathways are shown. The –log(P-value) shown on the X-axis was calculated by Fischer's exact t-test. The line indicates –log(P-value) = 1.3, which corresponds to a P-value of 0.05.

Functional analysis of microarray data

To investigate the functional role of PPARβ/δ in mouse liver, we used the pathway analysis tool GSEA, which determines whether an a priori defined set of genes shows statistically significant concordant differences between wildtype and PPARβ/δ-/- mice. The results from fed mice show that PPARβ/δ deletion is associated with induction of genes involved in various innate immunity and inflammation-related processes, including antigen processing and presentation, Toll-like receptor signaling pathway, and natural killer cell mediated cytotoxicity (Figure 6). These results were supported by Ingenuity pathway analysis, which pointed towards changes in innate immune system, and numerous other cytokine and inflammation related pathways (Figure 7). In agreement with the suggested role of PPARβ/δ in oxidative metabolism, the electron transport chain and oxidative phosphorylation pathways were decreased in PPARβ/δ-/- mice in fed state (Figure 6). Interestingly, in both fed and fasted state PPARβ/δ deletion was associated with a decrease in several pathways related to carbohydrate metabolism, including fructose and mannose metabolism, glycogen metabolism, glycolysis-gluconeogenesis and the pentose phosphate pathway, suggesting a role for PPARβ/δ in governing carbohydrate metabolism (Figure 6 and data not shown). Similarly, the lipoprotein metabolism pathway was downregulated in PPARβ/δ-/- mice in fed and fasted state (Figure 6 and data not shown). These changes were corroborated by decreased expression of specific genes within the abovementioned pathways, including liver pyruvate kinase (*Pklr*), fructose-1,6-bisphosphatase 1 (*Fbp1*), *Apoa4*, *Pcsk9*, *Pcsk6* (PACE4) and VLDL receptor (*Vldlr*) (Figure 8). Interestingly, the nicotinate and nicotinamide metabolism pathway was also downregulated in PPARβ/δ-/- mice in fed and fasted state. Within this pathway the gene most significantly downregulated was *Sirt5* (Figure 8).

Metabolic similarities and differences between PPARα and PPARβ/δ

To assess whether changes in expression of genes involved in carbohydrate and lipoprotein metabolism functionally impacted nutrient metabolism, we studied the metabolic response to fasting. To enable comparison with the role of PPARα, a parallel analysis was performed in PPARα-/- and PPARβ/δ-/- mice. As expected, fasting plasma FFAs were increased in PPARα-/- mice while fasting plasma levels of β-hydroxybutyrate were dramatically reduced (Figure 9A and 9B). No changes in plasma FFA or β-hydroxybutyrate were observed in PPARβ/δ-/- mice. In agreement with reduced expression of numerous genes involved in lipoprotein metabolism, plasma triglycerides were significantly elevated in PPARβ/δ-/- mice compared to wildtype mice in fed state, which resembled the situation in PPARα-/- mice (Figure 9C). In the fasted state, plasma cholesterol levels were reduced in PPARβ/δ-/- mice, whereas no change was observed in PPARα-/- mice (Figure 9D).

Consistent with reduced expression of genes involved in glucose utilization such as pyruvate kinase and fructose 1,6 bisphosphatase, fasting plasma glucose levels were significantly increased in PPARβ/δ-/-mice, whereas they were decreased in fasted PPARα-/- mice (Figure 9E). No effect of PPARβ/δ deletion on liver glycogen levels and triglyceride levels was observed (Figure 9F and 9G). In contrast, PPARα deletion gave rise to marked hepatic steatosis. Taken together, it is evident that PPARβ/δ deletion influences hepatic glucose and lipid metabolism, although the effects are less pronounced compared to PPARα deletion. The data support a functional role for PPARβ/δ in regulation of hepatic glucose and lipoprotein metabolism.

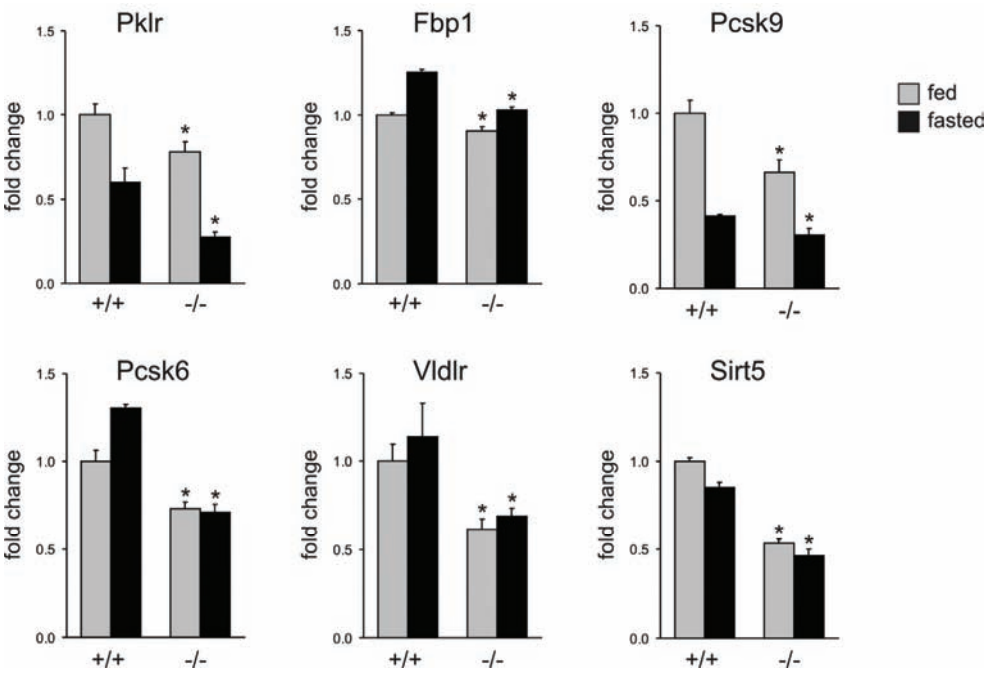

Figure 8. **Decreased expression of specific genes involved in several pathways related to carbohydrate- and lipoprotein metabolism.** Expression of selected genes is shown in wildtype (+/+) and PPARβ/δ-/- mice. Grey bars = fed state; black bars = fasted state. Error bars represent SEM. n = 5 per group. * = significantly different from corresponding wildtype mice according to student's t-test ($P<0.05$).

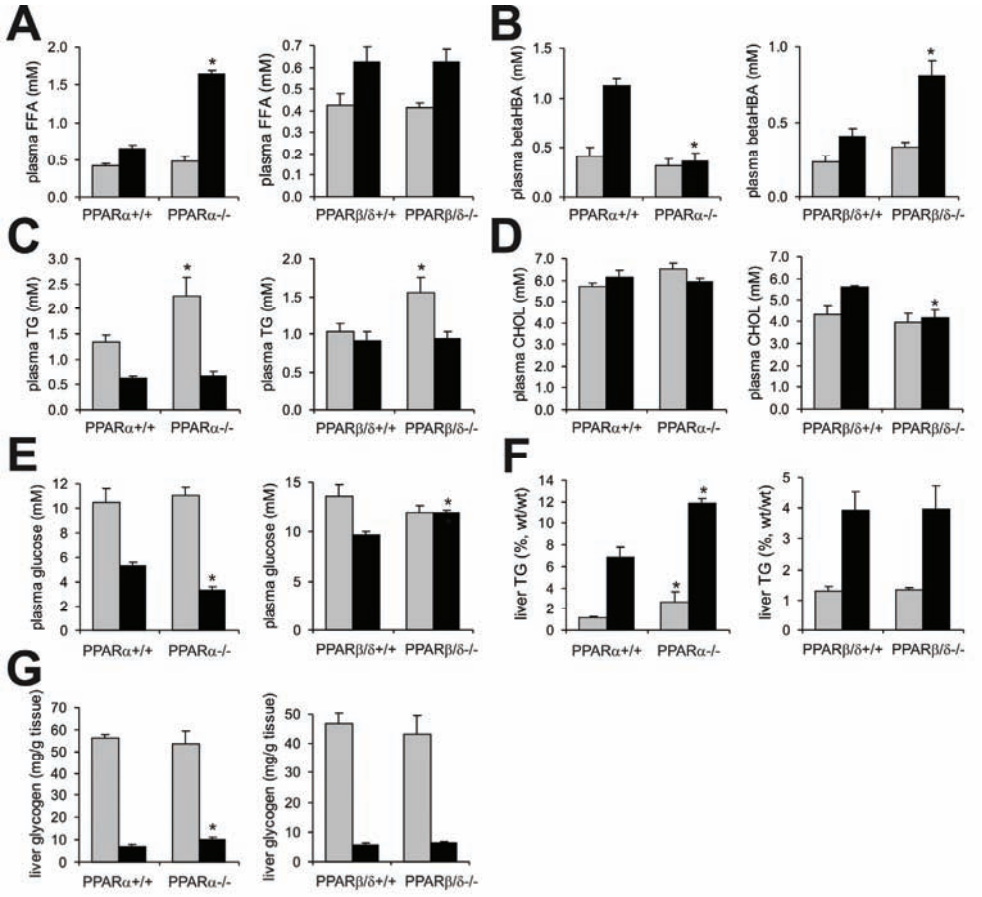

Figure 9. **Metabolic similarities and differences between PPARα and PPARβ/δ.** Plasma and liver metabolites were analyzed in fed and fasted wildtype, PPARα-/- and PPARβ/δ-/- mice. (A) plasma free fatty acids; (B) plasma β-hydroxybutyrate; (C) plasma triglycerides; (D) plasma cholesterol; (E) plasma glucose; (F) liver triglycerides; (G) liver glycogen. Grey bars = fed state; black bars = fasted state. n = 5 per group. * = significantly different from corresponding wildtype mice according to student's t-test ($P<0.05$).

Discussion

In this paper we have used PPARα and PPARβ/δ-/- mice in combination with Affymetrix microarray and analysis of plasma and liver metabolites to investigate the role of PPARβ/δ in liver. In fed liver, PPARα and PPARβ/δ expression levels are highly similar. Based on the number of genes differentially expressed between wildtype and PPARα or PPARβ/δ-/- mice, it can be argued that PPARα and PPARβ/δ are about equally important in the fed state. Upon fasting, however, the number of genes affected by PPARα deletion grows dramatically, whereas this is not the case for PPARβ/δ. These data suggest that in contrast to PPARα, PPARβ/δ does not mediate an adaptive response to fasting. Instead, PPARβ/δ is involved in basal regulation of a number of metabolic pathways. This notion is supported by analysis of several plasma metabolites.

Previously it was shown that PPARβ/δ deletion leads to embryonic lethality due to a placental defect, which was found in three independent PPARβ/δ-/- mouse lines (130, 134, 378). Despite embryonic lethality, breeding colonies could be created from surviving mice, enabling study of the role of PPARβ/δ in adult animals. It is unclear what type of mechanism allows the surviving mice to overcome the placental defects. However, we assume that this mechanism does not confound the data collected in adult PPARβ/δ-/- mice as presented here.

So far the most extensively documented roles of PPARβ/δ are as regulator of cell proliferation, cell differentiation and inflammation in the gastrointestinal tract (reviewed in (405)), and as a critical intermediate in skin wound healing (reviewed in (406)). Research on the metabolic role of PPARβ/δ has focused on regulation of fatty acid oxidation in skeletal muscle. Numerous *in vitro* studies have shown a stimulatory effect of PPARβ/δ overexpression or activation on genes involved in fatty acid catabolism (93, 137, 157-159). Forced overexpression of PPARβ/δ in skeletal muscle was shown to alter muscle fiber type characteristics towards more oxidative fibers (160, 161). Consistent with these data, administration of a synthetic PPARβ/δ agonist induced fatty acid oxidation, decreased muscle lipid content, and improved exercise performance (137, 162, 407). Interestingly, a recent study using PPARβ/δ-/- mice does not support a role for PPARβ/δ in fatty acid oxidation in skeletal muscle, at least in normal physiology (163). The reason for this discrepancy is not clear but may reflect differences between the pharmacological and physiological function of PPARβ/δ in muscle. Using gain of function models, PPARβ/δ has also been shown to induce fatty acid oxidation in adipose tissue (155). Our data clearly suggest that the key regulator of fatty acid oxidation in liver is PPARα and not PPARβ/δ. Hepatic PPARβ/δ is also not able to compensate for the lack of PPARα in PPARα-/- mice.

Instead, the primary metabolic influence of PPARβ/δ in liver is at the level of carbohydrate and lipoprotein metabolism. We found that PPARβ/δ deletion leads to downregulation of numerous pathways of carbohydrate metabolism, including pentose-phosphate pathway, mannose and fructose metabolism and especially glycolysis. Genes in the latter pathway that were clearly decreased in PPARβ/δ-/- mice included pyruvate kinase (*Pklr*) and fructose 1,6 bisphosphatase (*Fbp1*). Whether *Pklr* and *Fbp1* represent direct PPARβ/δ targets requires further investigation. Decreased flux of glucose through glycolysis might account for the elevated plasma glucose levels in fasted PPARβ/δ-/- mice as well as the reported impaired glucose tolerance (156). Overall, the data are consistent with previous data showing a stimulatory effect of the synthetic PPARβ/δ agonist GW510516 on glucose consumption and the pentose phosphate pathway (156).

In addition to glucose metabolism, PPARβ/δ deletion was associated with decreased expression of a number of genes connected with lipoprotein metabolism, including *Apoa4*, *Lipg*, and very low density lipoprotein receptor (*Vldlr*). A similar observation was made in hearts of PPARβ/δ-/- mice (our unpublished data). Confirming a previous report and in line with the plasma triglyceride-lowering effect of PPARβ/δ agonists in primates, plasma triglyceride levels were elevated in PPARβ/δ-/- mice, at least in the fed state (136, 169). In contrast, plasma total cholesterol was reduced in PPARβ/δ-/- mice in the fasted state. Elevated plasma triglyceride levels in PPARβ/δ-/- mice have been suggested to be related to a combination of increased VLDL production and decreased plasma triglyceride clearance, as evidenced by a decrease in postheparin LPL activity and increased hepatic expression of LPL inhibitors Angptl3 and Angptl4 (169). Based on the data presented here, it can be speculated that elevated plasma triglycerides may also be due to decreased expression of VLDL receptor and/or changes in production of various apolipoproteins, including Apoa5, Apoa4, and Apoc1. Interestingly, *Pcsk9* expression was decreased in PPARβ/δ-/- mice, which may contribute to the lowering of plasma cholesterol levels. Again, whether these genes represent direct PPARβ/δ target genes requires further investigation.

The changes in hepatic gene expression in PPARβ/δ-/- mice reported here are the combined effect of absence of PPARβ/δ in numerous cell types including endothelial cells, hepatocytes and Kupffer cells, all of which express ample levels of PPARβ/δ (44). While the alterations in glucose and lipoprotein metabolism are probably related to absence of PPARβ/δ in hepatocytes, changes in innate immunity and inflammation-related pathways are likely explained by the absence of PPARβ/δ in Kupffer cells. Recently, it was shown that PPARβ/δ is required for the acquisition of the metabolic and immune phenotypes of

alternatively activated macrophages in liver (184). Thus, PPARβ/δ appears to be a major modulator of Kupffer cell function.

Since PPARα mRNA was about 38% decreased in fasted PPARβ/δ-/- mice compared to fasted wildtype mice (data not shown, $p<0.05$), downregulation of several genes in fasted PPARβ/δ-/- mice vs. fasted wildtype might reflect indirect regulation via PPARα. However, since typical, highly sensitive PPARα targets such as *Cyp4a14* and *Aldh3a2* were completely unaffected by PPARβ/δ deletion during fasting, the overall impact is likely to be modest.

In conclusion, our data suggest that the roles of PPARα and PPARβ/δ in liver gene regulation only mildly overlap. While PPARα mediates the adaptive response to fasting, this is not the case for PPARβ/δ. Importantly, our study reveals that PPARβ/δ governs hepatic glucose utilization and lipoprotein metabolism.

Acknowledgements

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Chapter 7

Poor match between genome wide *in silico* screening of PPARα targets and actual gene regulation in the mouse

Linda M. Sanderson, Merja Heinäniemi, Guido J.E.J. Hooiveld, Carsten Carlberg, Michael Müller and Sander Kersten

Manuscript submitted

Abstract

PPARs are ligand-activated nuclear receptors that induce target gene expression by binding to specific response elements (PPREs) in the DNA. Based on chance alone, putative PPREs are expected to be present throughout the whole genome at reasonably high frequency. However, these PPREs can not all be functional and mediate PPAR-dependent gene regulation. Accordingly, it is unclear what sequence determinants or other genomic information are relevant for dictating whether a PPRE will be functional. Here we use an *in silico* screening method to identify putative PPARα binding sites in the mouse genome. In an attempt to investigate possible correlations between the putative PPREs identified and actual gene regulation, we compared the results from the *in silico* screen with results from microarray analysis of mouse livers and mouse primary hepatocytes treated with the PPARα agonist WY14643.

Our results show that the presence of a PPRE close to the transcription start site (TSS) of a gene based on *in silico* screening correlates very poorly with induction of gene expression by PPARα agonist as measured by microarray. This result did not change when considering conservation of PPRE between species, the number of PPREs situated close to a gene, the binding strength of PPARα to the PPRE, or the proximity of the PPRE to the TSS. Our data, therefore, question the utility of *in silico* screening to predict gene regulation by PPARs and to identify novel PPAR target genes. Screening for conserved PPREs within genes known to be induced by PPAR may be a valuable tool to identify functional PPREs.

Introduction

Peroxisome proliferator activated receptors (PPARs) are ligand-activated nuclear receptors that play a major role in the regulation of nutrient metabolism, including fatty acid-, glucose-, and amino acid metabolism (21, 217). Three different PPAR subtypes encoded by separate genes have been identified: PPARα, PPARγ and PPARβ/δ (20, 21). Each subtype has a distinct biological function related to a unique tissue expression pattern and a preferential activity towards specific target genes (24, 37, 38). PPARα is highly expressed in the liver where it governs metabolism of lipids including fatty acid uptake, mitochondrial and peroxisomal fatty acid oxidation, ketogenesis and fatty acid elongation (42, 72, 74, 408). In addition, PPARα is involved in the regulation of glucose metabolism and inhibits expression of genes involved in inflammation (92, 94, 108). PPARγ is the subtype most highly expressed in white adipose tissue and plays a key role in adipogenesis. It is best known for its ability to stimulate adipocyte differentiation, fat storage, and glucose metabolism (217, 219, 221, 223). Finally, PPARβ/δ is expressed in a large number of tissues and has been shown to be involved in several biological processes including cell proliferation and differentiation, wound healing and fatty acid oxidation (122, 409, 410).

PPARs, like many other nuclear receptors, bind to DNA as a heterodimer with the nuclear receptor RXR (retinoid X receptor). Gene activation by PPAR requires binding of the PPAR:RXR heterodimer to specific DNA sequences located in the promoter region of a target gene (24). These sequences, called peroxisome proliferator response elements (PPREs), are composed of a direct repeat of the consensus sequence AGGTCA with a single nucleotide spacer between the two repeats (24).

Subsequent to DNA binding, transcriptional regulation by PPARs involves complex interactions between coactivators and corepressors. In the absence of ligand, compression of the chromatin is caused by corepressor proteins such as nuclear corepressors (NCoRs) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) (28, 29). These corepressor proteins recruit enzymes expressing histone deacetylase activity, keeping the chromatin structure “closed” and inactive. When a ligand binds, the corepressor protein complexes dissociate and a recruitment of several coactivators occurs (29, 30), resulting in a more “open” and active chromatin structure which in turn leads to initiation of DNA transcription and upregulation of PPAR target genes.

According to the traditional view, PPREs are located in the promoter region of target genes. Indeed, several PPAR target genes possess functional PPREs in close proximity to the transcriptional start site (TSS). However, recent studies suggest that functional PPREs are

also found in introns (97, 230, 411, 412). Furthermore, recent ChIP-on-chip experiments support the presence of PPREs and other nuclear receptor binding sites distant from the TSS (385-387). It is believed that nuclear receptors bound to distant response elements may access the TSS via formation of loop-like structures within chromatin units (413, 414).

Based on chance alone, putative PPREs are predicted to be distributed throughout the whole genome at reasonably high frequency. However, these PPREs can not all be functional and mediate PPAR-dependent gene regulation. Accordingly, it is unclear what sequence or other genomic determinants are relevant for dictating whether a PPRE will be functional. Here we use an *in silico* screening method (295) to identify putative PPARα binding sites in the mouse genome. In order to investigate possible correlations between the putative PPREs and gene regulation, we compared the results from the *in silico* screen with results from microarray analysis of mouse livers and mouse primary hepatocytes treated with the PPARα ligand WY14643 for six hours.

In conclusion, together with previous data (415), the results of our study imply that screening for conserved PPREs within genes that are induced by PPAR may be a valuable tool to identify functional PPREs. However, screening of putative PPREs cannot predict gene regulation by PPARα nor do we find that PPARα-induced genes are enriched with PPREs, regardless of the selection criteria employed.

Materials and Methods

Materials. WY14643 was purchased from ChemSyn Laboratories (Lenexa, KS, USA). Recombinant human insulin (Actrapid) was from Novo Nordisk (Copenhagen, Denmark). William's E medium, foetal calf serum and penicillin/streptomycin/fungizone were from Lonza Bioscience (Verviers, Belgium). All other chemicals were from Sigma (Zwijndrecht, the Netherlands).

Animals. Pure-bred Sv129, BALB/C, C57BL6J, DBA, FVB and NMRI mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred at the animal facility of Wageningen University. All animal experiments were approved by the Local Committee for Care and Use of Laboratory Animals at Wageningen University.

Experimental design. Two independent studies were performed. Study A: Male Sv129 mice were given an intragastric gavage of 400µl WY14643 (10mg/ml in 0.5%

carboxymethyl cellulose) after a four hour fast. Six hours after gavage, mice were anaesthetized with a mixture of isofluorane (1.5%), nitrous oxide (70%) and oxygen (30%). Blood was collected by orbital puncture, after which the mice were sacrificed by cervical dislocation. Livers were removed, snap-frozen in liquid nitrogen and stored at -80°C until further analysis. For RNA analyses, tissue from the same part of the liver lobe was used. Study B: Primary hepatocytes were isolated from six different mouse strains (Sv129, BALB/C, C57BL6J, DBA, FVB and NMRI) by two-step collagenase perfusion as described previously (287). Cells were plated on collagen-coated six-well plates. Viability was determined by Trypan Blue exclusion, and was at least 75%. Hepatocytes were suspended in William's E medium supplemented with 10% (v/v) foetal calf serum, 20mU/ml insulin, 50nM dexamethasone, 100U/ml penicillin, 100µg/ml of streptomycin, 0.25µg/ml fungizone and 50µg/ml gentamycin. The next day, cells were incubated in fresh medium in the presence or absence of WY14643 (10µM) dissolved in DMSO for six hours, followed by RNA isolation.

RNA isolation and quality control. Total RNA was isolated from both studies with TRIzol reagent (Invitrogen, Breda, the Netherlands) according to manufacturer's instructions. RNA was further purified and DNAse treated using RNeasy micro columns (Qiagen, Venlo, the Netherlands). A NanoDrop ND-1000 spectrophotometer (Isogen, Maarssen, the Netherlands) was used to determine RNA concentrations. RNA quality was assessed on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) with 6000 Nano Chips using the Eukaryote Total RNA Nano assay. RNA was judged as suitable for array hybridization only if samples showed intact bands corresponding to the 18S and 28S rRNA subunits, displayed no chromosomal peaks or RNA degradation products, and had a RIN (RNA integrity number) above 8.0.

Affymetrix microarray. Five micrograms of RNA were used for one cycle cRNA synthesis (Affymetrix, Santa Clara, CA). Hybridization, washing and scanning of Affymetrix Mouse Genome 430 2.0 arrays was carried out according to standard Affymetrix protocols. Packages from the Bioconductor project (288), integrated in an in-house developed online management and analysis database for multiplatform microarray experiments (Gavai AK, de Groot PJ, Lin K, Boekschoten MV, Liu Y, Nijveen H, Neerincx PBT, Hooiveld GJEJ, Muller M and Leunissen JAM. MADMAX - Management and analysis database for multi-platform microarray experiments (2009). Submitted.), were used to analyze the scanned microarrays. Quality control based on various advanced quality metrics, diagnostic plots, pseudo-images and classification methods was performed to assure only excellent quality arrays were included in further statistical analyses (8). Arrays

were normalized using quantile normalization, and expression levels were calculated using GC-RMA applying the empirical Bayes approach (416) and a remapped chip description file (CDF) (294).

Comparisons were made between WY14643-treated and control animals (study A) and WY14643 and DMSO treated hepatocytes (study B). Differentially expressed genes from study A and B were combined to create one dataset. Genes with a P-value <0.01 and fold change >1.5 were considered significantly upregulated by treatment.

***In silico* screening of putative PPREs.** The *in silico* screening for putative PPREs was performed according to (295). Briefly, the consensus sequence of AGGTCA was used to create 39 different PPREs with single nucleotide variations. These variations were tested for the *in vitro* binding of PPAR-RXR heterodimers and subsequently used to calculate binding strength. The variations were divided into two categories based on medium (20-38%) or strong (>39%) binding. Genomic sequences for the entire mouse genome were extracted from the Ensembl data base (Ensembl release 44, April 2007). These were screened for the varying PPREs using in-house software named RESearch. A list of all medium and strong PPRE variants can be found in (295).

Conservation analysis of identified putative PPREs was carried out using cis-regulatory element annotation system (CEAS) (417). The CEAS model uses PhastCons (418) to assign conservation scores to each putative PPRE region. These scores are based on multiz alignment of human, chimp, mouse, rat, dog, chicken, fugu and zebrafish genomic DNA. PPREs with a conservation score >0.8 were considered to be conserved.

Distance to closest gene was calculated using PinkThing (http://pinkthing.cmbi.ru.nl; F. Nielsen, M. Kooyman, M. Huynen; paper in preparation). PinkThing compares the position of each putative PPRE against the location of genes.

Results

In silico screening of putative PPREs

Previously, we characterized the *in vitro* binding preference of PPARα on a panel of 39 systematic single nucleotide variations of the consensus DR1-type PPRE (AGGTCAAAGGTCA). PPREs were subsequently classified according to strength of binding by PPARα and grouped into strong or medium binding strength PPREs. Putative

PPREs were identified throughout the mouse genome via an *in silico* screening method (see methods).

For PPARα, we found a total of 113598 medium PPREs and 49373 strong PPREs. The number of PPREs identified within a chromosome was highly correlated with the size of the chromosome (Figure 1A). As it has been suggested that the functionality of nuclear receptor binding sites is determined by species conservation (415), we removed non-conserved PPREs, which drastically reduced the number of PPREs to approximately 4% of the original search. Interestingly, conserved PPREs appeared to be a bit more abundant on chromosome 11 relative to other chromosomes (Figure 1B).

We next examined the distance of the PPREs to the closest gene. The results indicated that most medium PPREs were situated 1-5KB up-or downstream from a TSS. Most strong PPREs were positioned more than 10KB from the TSS (Figure 2A). Interestingly, most conserved PPREs were situated more than 10KB from the TSS (Figure 2B), which was true for both medium and strong PPREs.

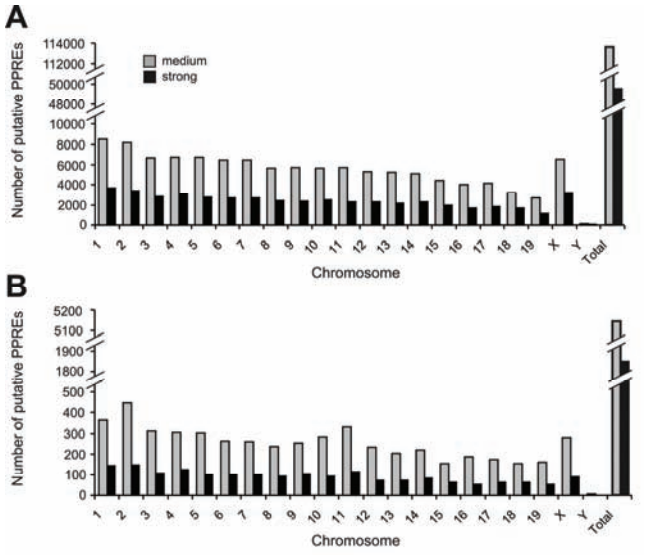

Figure 1. **Number of putative PPREs in the mouse genome.** The graphs show the number of PPREs for PPARα per chromosome and in total for all chromosomes. Grey bars are medium PPREs, black bars strong PPREs. (A) All PPREs resulting from the *in silico* screen. (B) Conserved PPREs with a conservation score of >0.8. Conservation scores were calculated using the CEAS method (417).

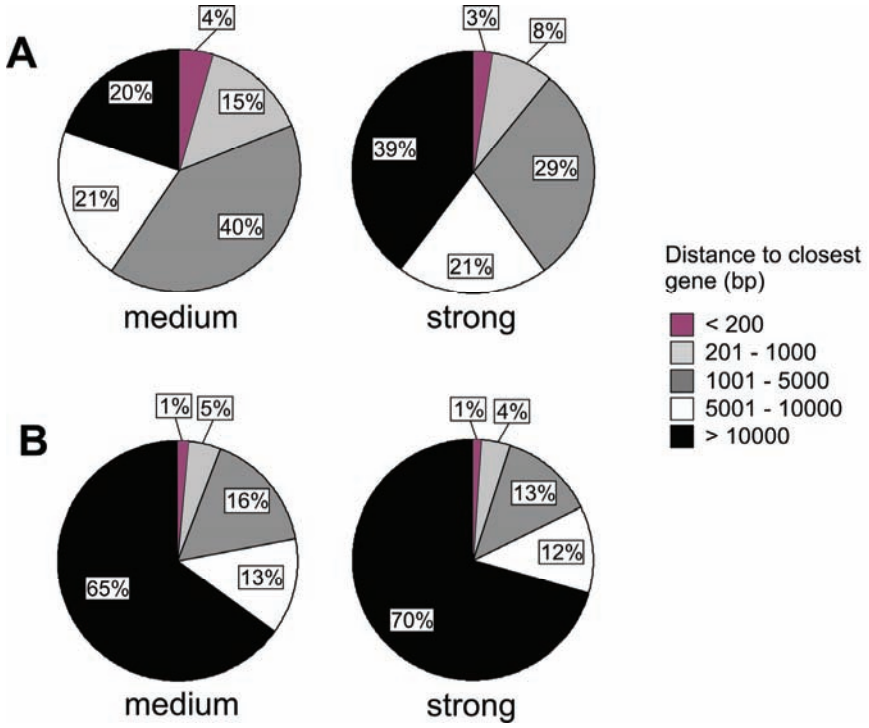

Figure 2. **Distance between PPREs and the closest gene.** Pie graphs showing the distance between PPREs for PPARα and the closest gene. (A) All PPREs resulting from the *in silico* screen are included. (B) Conserved PPREs with a conservation score of >0.8. Conservation scores were calculated using the CEAS method (417).

To better appreciate the number of PPREs present in and around an average single gene, for every gene we calculated how many putative PPREs were present within ±2KB, ±10KB or ±50KB from TSS (Figure 3). The majority (80%) of all genes have no PPRE, medium or strong, within ±2KB from the TSS. In contrast, close to 100% of genes contain at least one medium PPRE within ±50KB from the TSS, and almost 90% of genes contain at least one strong PPRE within ±50KB from the TSS. Conserved PPREs, however, are much more rare. Indeed, around 80% of all genes contain no conserved PPRE within ±50KB from the TSS.

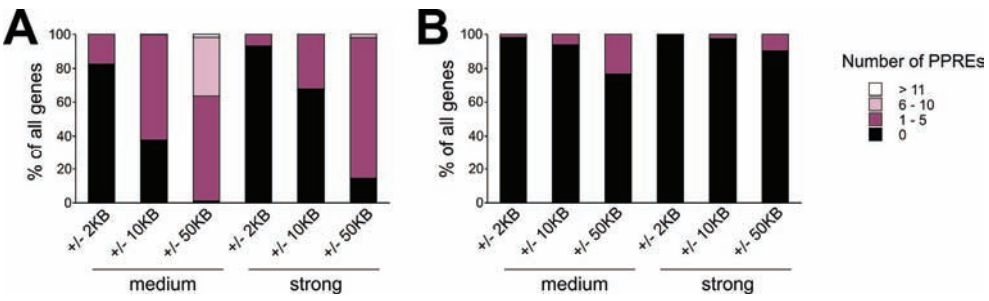

Figure 3. **Percentage of genes with a certain number of PPREs within different ranges from TSS.** Graph shows the percentage of all genes with a certain number of medium and strong PPREs for PPARα, according to scale, within ±2KB, ±10KB and ±50KB from TSS. (A) All PPREs resulting from the *in silico* screen are included. (B) Conserved PPREs with a conservation score of >0.8. Conservation scores were calculated using the CEAS method (417).

No correlation between in silico screening and PPARα regulation in vivo

By definition, functional PPREs mediate induction of gene expression by PPAR. To explore the connection between the presence of PPREs within a gene and gene regulation by PPARα, we first determined the set of genes upregulated by PPARα. To that end, Affymetrix microarray analysis was performed on liver samples from mice treated with the PPARα agonist WY14643 for 6 hours, as well as on primary hepatocytes treated with WY14643 for 6 hours. Out of 13935 genes included in the analysis, 598 genes were significantly upregulated ($P<0.01$, $FC>1.5$) upon WY14643 treatment in either mouse liver, primary hepatocytes, or both.

We next examined whether genes upregulated upon PPARα activation are enriched with PPREs. This analysis was performed separately for PPREs within ±2KB, ±10KB or ±50KB from TSS, and also for conserved PPREs. Importantly, we found that PPREs were not more abundant in genes significantly upregulated by PPARα activation compared to all genes on the array (Figure 4). This result was obtained independently of the type of PPRE (medium, strong) and whether the PPRE was conserved. The results indicate that genes that are upregulated by PPARα activation and thus represent putative PPARα target genes are not

enriched with PPREs, irrespective of whether PPREs are conserved, suggesting that presence of PPREs is disconnected from gene induction.

It can be hypothesized that not the presence of PPREs but rather their distance to the TSS is important for gene regulation. However, we found that upregulated genes were not specifically enriched with PPRE within ±2KB of TSS compared to nonregulated genes or all genes (Figure 4). In fact, there were hardly any regulated genes that contained a PPRE within ±2KB of TSS. Additionally, we did not find a relationship between the distance of gene to nearest PPRE and fold change in expression of the gene upon PPARα activation, again regardless of the type of PPRE and whether it was conserved (Figure 5).

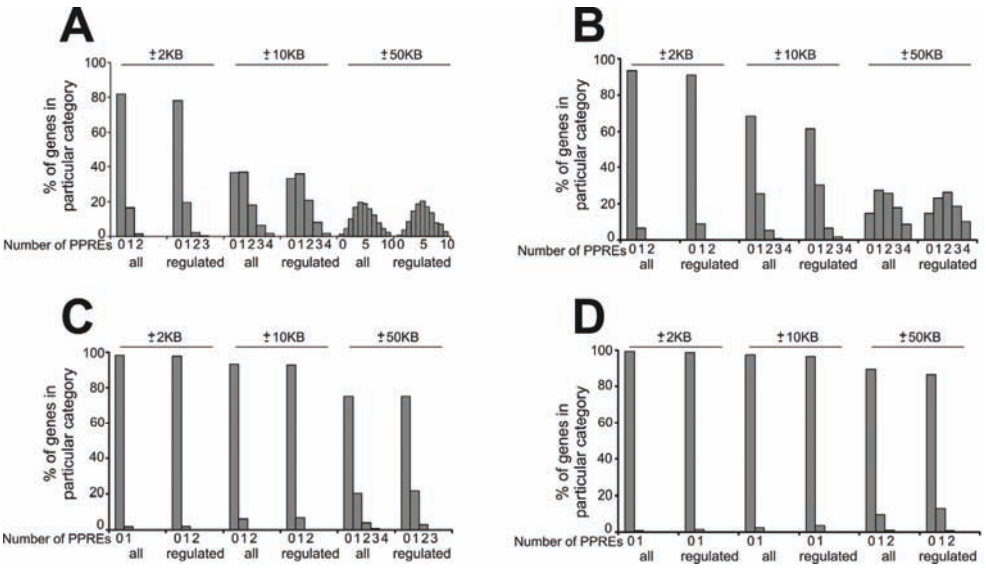

Figure 4. **PPARα-regulated genes are not enriched with PPREs.** Percent genes with certain number of PPREs (according to scale) within ±2KB, ±10KB and ±50KB from TSS. The X-axis compares all genes on the array with significantly upregulated genes upon PPARα activation (P<0.01, FC>1.5). (A) non-conserved medium PPREs; (B) non-conserved strong PPREs; (C) conserved medium PPREs; (D) conserved strong PPREs.

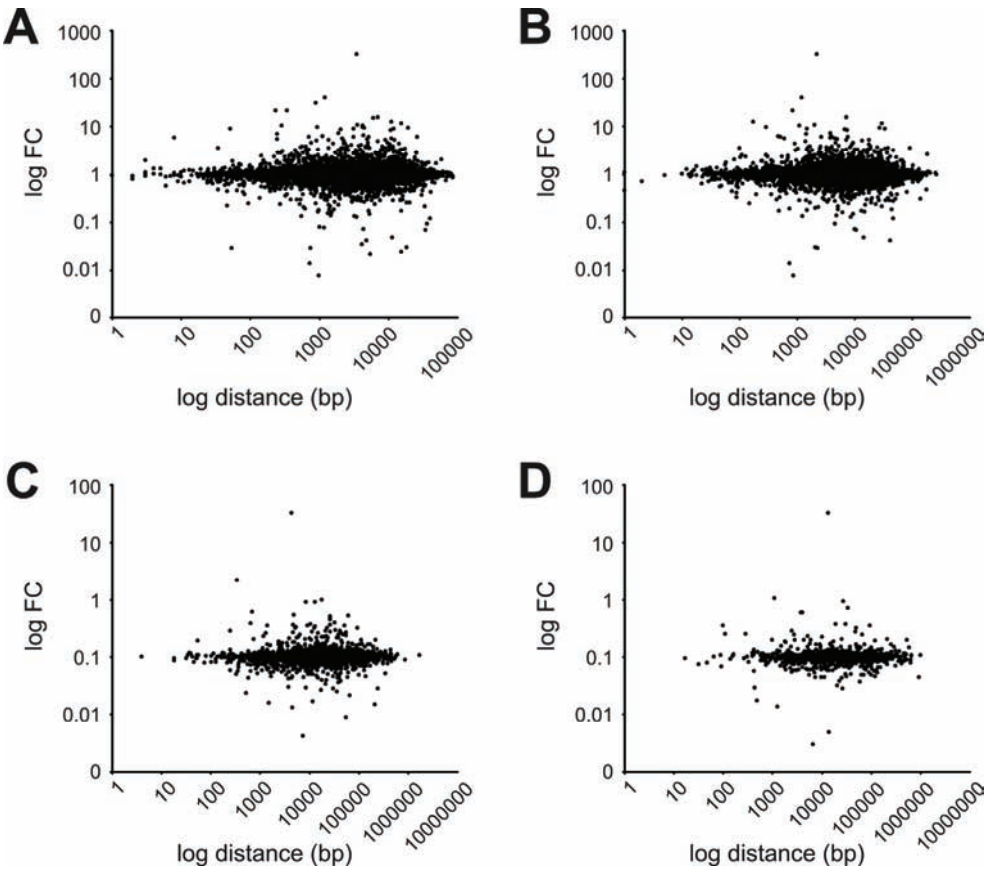

Figure 5. **No correlation between *in vivo* gene regulation and distance to closest PPRE.** Scatter plots showing the relationship between log fold change in gene expression (Y-axis) and log distance to closest PPRE for PPARα (X-axis). (A) medium PPREs, (B) strong PPREs, (C) medium conserved PPREs, (D) strong conserved PPREs. PPREs with a conservation score of >0.8 are considered to be conserved. Conservation scores were calculated using the CEAS method (417).

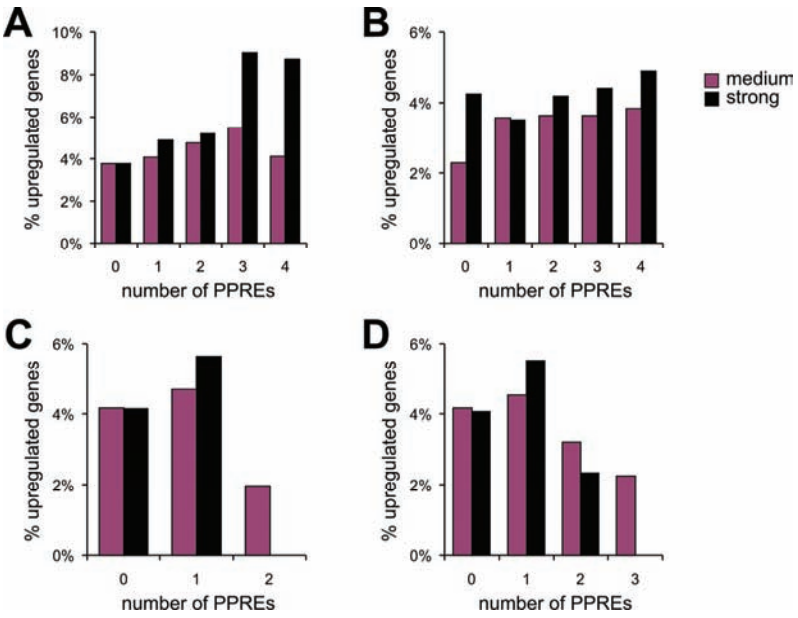

Figure 6. **Percentage of significantly upregulated genes with a certain number of PPREs.** Graph shows the percentage of genes, compared to all genes, (Y-axis) that have a certain number of PPREs for PPARα within ±10KB (A: non-conserved; C: conserved) or ±50KB (B: non-conserved; D: conserved) from TSS (X-axis). PPREs with a conservation score of >0.8 are considered to be conserved. Conservation scores were calculated using the CEAS method (417). Purple bars are medium PPREs, black bars are strong PPREs.

No correlation between number of PPREs for each gene and regulation by PPARs

Finally, we explored the possible correlation between the number of PPREs for each gene and gene regulation by PPARs. If gene regulation by PPARα requires several PPREs, one would expect that as the number of PPREs for a gene increases, the likelihood of regulation by PPARα increases accordingly. For non-conserved strong but not medium PPREs, there seems to be a minor trend that as the number of PPREs increases, the chance that a gene is regulated increases as well. No clear trend was observed for conserved PPREs. Our data argue against a clear relationship between the number of PPREs connected with a gene and its chance of being upregulated by PPARα activation (Figure 6).

Additionally, we explored the possible correlation between the number of PPREs for each gene and the fold change in expression of the gene upon PPARα activation. To quantify the number of PPREs and take into account the strength of predicted PPARα binding, we created a PPRE score for each gene. This PPRE score was calculated by giving each medium PPRE the value 0.5 and each strong PPRE the value 1. Then, by adding up the number of medium and strong PPREs for each gene within a certain distance from TSS (PPREscore = #strongPPREs * 1 + #mediumPPREs * 0.5), we obtained PPRE scores ranging from 0 to 16. We next plotted the PPRE score of a gene against the fold change in expression of the gene upon PPARα activation (Figure 7). The data show a lack of correlation between PPRE score and fold change in expression. In fact, the relationship between PPRE score and fold change resembled the overall frequency distribution of PPRE scores among all genes (compare left and right panels). This correlation was also not improved when only conserved PPREs were included. Taken together, our analysis does not support a correlation between the number of PPREs in proximity of a gene and its regulation by PPARα.

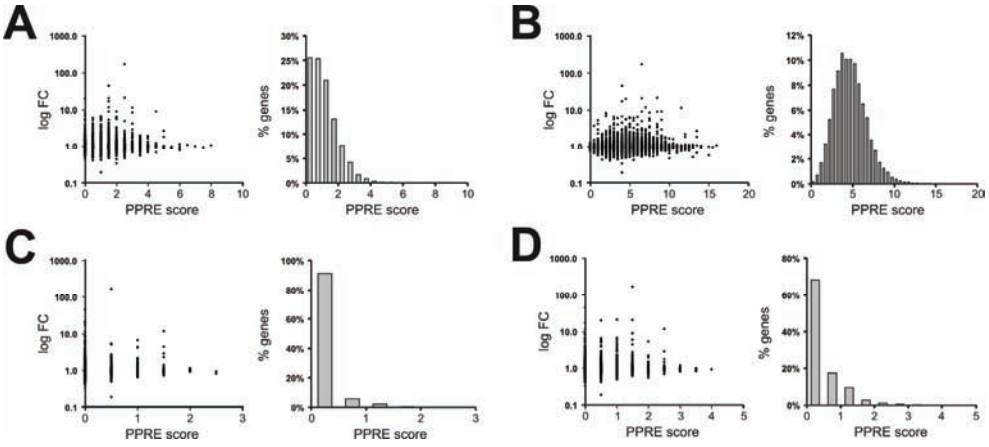

Figure 7. **No correlation between *in vivo* gene regulation and number of PPREs for each gene.** A PPRE score was calculated for each gene within a certain distance from TSS (PPREscore = #strongPPREs * 1 + #mediumPPREs * 0.5). This PPRE score (X-axis) was put in relation to log fold change (Y-axis) in scatter plots (left panels). Right panels show PPRE score (X-axis) in relation to percentage of genes (Y-axis). (A) PPREs within ±10KB from TSS, (B) PPREs within ±50KB from TSS, (C) conserved PPREs within ±10KB from TSS, (D) conserved PPREs within ±50KB from TSS. PPREs with a conservation score of >0.8 are considered to be conserved. Conservation scores were calculated using the CEAS method (417).

Discussion

The primary aim of this paper was to investigate the utility of whole genome screening for PPAR binding sites to help identify target genes of PPARs in mouse. A secondary aim was to find out the properties that determine the functionality of putative PPAR response elements. The major outcome of this study was that presence of PPREs in the vicinity of the TSS of a gene based on *in silico* screening correlates very poorly with actual gene regulation as determined by microarray analysis. This is true regardless of the number of PPREs present around a gene, their predicted strength of PPAR binding, their proximity to the TSS, and their conservation between species. Our data question the utility of *in silico* PPRE screening for the identification of novel PPAR target genes.

The applicability of an *in silico* screening method ultimately depends on setting appropriate criteria for screening in order to optimize sensitivity and selectivity. We screened the genomic region ±2KB, ±10KB and ±50KB from the TSS of a gene and performed separate analyses for medium and strong PPREs, the latter property being based on the predicted *in vitro* PPARα binding affinity of the PPRE. Indicating a lack of sensitivity, it was observed that 99% of all mouse genes carried at least one medium PPRE within ±50KB from the TSS. Setting more stringent criteria for PPREs in terms of location, predicted PPARα binding strength and number of PPREs, however, failed to improve matching with the data on gene regulation.

As an additional criterion to sort out the functional PPREs from our screen, we addressed sequence conservation across species. Recently, it was shown that sequence conservation of putative glucocorticoid receptor binding sites across species predicted actual *in vivo* occupancy at glucocorticoid-induced genes (415). These findings imply that conservation may be helpful in determining the functionality of PPAR binding sites within PPAR-regulated genes. However, whether the conservation of putative PPREs surrounding a gene may predict its upregulation remains unclear. In our study we assessed conservation using a method available online which is based on comparison between eight different species, including human, mouse and rat. While taking into account sequence conservation led to a dramatic decrease in the number of putative PPREs, it did not improve the correlation with the gene expression data.

To compose a list of genes upregulated by PPARα we combined microarray data of livers from mice treated with the PPARα agonist WY14643 for 6 hours and microarray data of primary hepatocytes treated with WY14643 for 6 hours. Previously we established that the effects of WY14643 on gene regulation in mouse liver are exclusively (>99%) mediated by

PPARα (69). The time point of 6 hours was chosen to largely avoid inducing indirect mechanisms of gene regulation involving another transcription factor. Although our list of PPARα-induced genes will certainly not be complete nor be devoid of false positives, the list should be highly enriched with PPARα target genes compared to the set of genes not induced by PPARα activation. Consistent with this notion, careful inspection of the list of PPARα-induced genes allowed us to identify virtually all established direct target genes of PPARα. Nevertheless, no differences in the number of PPREs present around a gene and their proximity to the TSS were found between genes upregulated and not upregulated by PPARα. These results were not altered when taking into account sequence conservation.

Our results raise the question to what extent upregulation of gene expression by PPARα is mediated by PPREs or may be achieved via an alternative mechanism. While several mechanisms have been put forward to explain downregulation of gene expression by PPARα (111), to our knowledge no evidence is available suggesting that PPAR can upregulate gene expression independently of PPRE binding.

It should be emphasized that the discrepancy between actual gene regulation and *in silico* prediction of putative PPAR targets based on presence of PPRE does not imply lack of PPARα binding to the PPREs identified. It is conceivable that PPARα is capable of binding to the putative PPREs, yet for reasons that remain to be defined binding may not translate into transcriptional regulation. To assess whether PPARα is bound to the PPREs identified, our analysis should be coupled to direct measurement of *in vivo* binding of PPAR to DNA using ChIP-on-chip or ChIP-Seq.

It is worth noting that conserved PPREs were absent in a number of well-known PPARα-induced genes. These include *Fgf21* and *Cidec*, both of which were recently shown to be transcriptional targets of PPAR (419-421). While promoter-reporter studies led to the identification of a functional PPRE in the mouse *Fgf21* and *Cidec* promoters (419, 422), it is unclear whether these PPREs are conserved in human.

One question that was not addressed in the present study is whether the presence of several PPREs in close proximity (so-called PPRE clusters) may be predictive of gene regulation by PPARα. An example of such a PPRE cluster consisting of four adjacent PPREs is present in intron 3 of the *Angptl4* gene, which represents a highly sensitive PPAR target. However, it is difficult to assign criteria for a PPRE cluster. Furthermore, PPRE clusters are expected to be extremely rare. Another aspect that we did not address is the proximity of PPREs to other transcription factor binding sites. It is expected that functional PPREs

are primarily found in promoter regions characterized by the presence of numerous transcription factor binding sites.

In the present paper we concentrated the analysis on PPARα. However, a parallel analysis was performed for PPARγ using microarray data from 3T3-L1 adipocytes and mouse adipose tissue treated with rosiglitazone which essentially yielded the same results. No enrichment of (conserved) PPREs was found among PPARγ-regulated genes.

In conclusion, together with previous data (415), our study suggests that screening for conserved PPREs may be a valuable tool to identify functional PPREs within genes known to be induced by PPAR. However, screening of putative PPREs cannot predict gene regulation by PPARα nor do we find that PPARα-induced genes are enriched with PPREs, regardless of the selection criteria employed.

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Chapter 8

General discussion

The objective of this thesis was to better characterize the roles and modes of activation of PPARα and PPARβ/δ in the liver. To achieve this aim, a nutrigenomics approach was pursued by combining microarray technology with several transgenic- and knockout mouse models in the context of a specific dietary intervention.

The concept of nutrigenomics rests on the knowledge that diet is the most important environmental factor impacting the transcriptome. As a consequence, in the past few years transcriptomics has taken the field of molecular nutrition by storm. Since transcriptomics first became available in the late twentieth century, a huge number of studies have been published reporting on the effects of nutrients at the whole genome level. However, while this technology allows for a very large number of genes to be analyzed simultaneously, thus giving a comprehensive picture of changes in gene expression, the gain of insight into the underlying molecular processes is generally limited. To fully understand and interpret whole genome gene expression data, it is highly recommended to combine transcriptomics analysis with specific functional genomics tools, thereby giving more specific information about the mechanism of gene regulation. Thus, rather than merely studying the effect of a dietary compound or drug, functional genomics tools should be implemented to alter the expression of potential molecular mediators, whether it is a membrane receptor, transcription factor, or signaling kinase. This concept, while commonplace in biomedical science, is just gaining ground within nutritional science. This thesis represents a successful example of such an approach, based on a combination of microarray analysis and knockout mouse models.

Since PPARα was first described in 1990 (20), its role in hepatic lipid metabolism has been extensively studied (42, 69, 73, 74). It is well established that PPARα is able to bind and be activated by dietary fatty acids. Indeed, several intervention studies in mice have demonstrated activation of PPARα in response to feeding with different fats. So far, however, only diets consisting of several types of fats have been used (such as a high fat diet, a Mediterranean diet, or a fish oil diet), which makes it impossible to distinguish the effects of the individual fatty acids on gene regulation by PPARα. In this thesis, we used a unique nutrigenomics design based on synthetic triglycerides composed of one single fatty acid. By feeding these synthetic triglycerides to wildtype and PPARα-/- mice, followed by determination of the gene expression profiles in the livers of these mice, we were able to investigate the effect of several types of dietary unsaturated fatty acids on hepatic gene expression and ascertain the specific role of PPARα in fatty acid-dependent gene regulation. This type of study can be considered a mixture between a nutritional and pharmacological intervention, since it concerns a dietary macronutrient yet is administered in relatively high dose in purified form. Whereas conventional nutrigenomics studies often

are based on a summary of effects by several components, in our study changes in gene expression can be traced back to one single fatty acid. In this thesis we successfully studied the effects of these synthetic triglycerides on gene expression in mouse liver. This approach can also be applied to other tissues such as heart and intestine, and may involve knockout models for transcription factors other than PPARα.

Using PPARα-/- mice, it has been clearly established that PPARα governs the fasting-induced upregulation of numerous genes involved in hepatic fatty acid oxidation, many of which have been identified as direct PPARα target genes (70-72). The PPARα-dependent induction of fatty acid oxidation during fasting, the ability of PPARα to be activated by fatty acids, and the increase in circulating FFAs during fasting have led to a generally accepted model in which the elevated plasma FFAs themselves are responsible for the induction of hepatic fatty acid catabolism via enhanced ligand-activation of PPARα (423). Although the evidence supporting direct activation of PPARα in liver by FFA is circumstantial, the model has been readily adopted since it makes sense physiologically and provides a mechanistic basis for the observation that the rate of fatty acid utilization is generally proportional to the plasma FFA concentration. We were able to show, however, that circulating FFAs are not able to ligand-activate PPARα in mouse liver. Since it is well established that dietary fatty acids are indeed able to bind and activate PPARα in liver (69, 73, 338, 339), the question remains how the two fatty acid signaling pathways are separated within the hepatocyte. The basis for the physical separation within hepatocytes lies in differences in the mechanisms of cellular uptake between the two fatty acid sources. While plasma FFAs enter the hepatocyte as fatty acids, dietary fatty acids are predominantly taken up in the form of triglycerides as remnant particles, which are internalized and degraded to liberate free fatty acids. We envision that a different mechanism of uptake is linked to distinct pools of fatty acids inside the hepatocyte, which may serve as substrates or ligands for distinct metabolic or regulatory pathways. The exact mechanisms responsible for intracellular transport of fatty acids largely remain elusive, although an important role of fatty acid binding proteins (FABPs) is commonly assumed. Several studies using co-immunoprecipitation and transactivation assays have indicated a direct interaction between liver FABP1 and PPARα (82, 424-426). Interestingly, a LXXLL domain characteristic to proteins that are capable of binding PPARs and other nuclear receptors has not been identified in FABP1 (82), suggesting that PPARα may interact with FABP1 through a domain yet to be identified. FABPs represent a large family of proteins that bind long chain fatty acids and LCFA-CoA with high affinity (427-429). Different members of this family are known to exist, each characterized by a specific pattern of expression among tissues. Although the various FABPs are still often referred to by the tissue in which they were originally identified (liver – FABP1, intestine – FABP2, heart – FABP3, adipose tissue –

FABP4, epidermis – FABP5 and brain – FABP7), several FABPs often co-exist in one single tissue. For example, different FABPs are known to be expressed in the liver. Recently, it was suggested that the nuclear receptor ligand retinoic acid is able to activate either retinoic acid receptor (RAR) or PPARβ/δ depending on whether it is bound to cellular retinoic acid binding protein (CRABP) or FABP5, respectively (83). In analogy it is conceivable that after uptake plasma FFAs bind to a different FABP compared to fatty acids derived from plasma triglycerides, which in turn is coupled to a different PPAR. According to this scenario, plasma free fatty acids might bind to FABP2 or FABP5 and interact with PPARβ/δ, while plasma triglyceride-derived fatty acids bind FABP1 and interact with PPARα. Future research will need to address whether this mechanism might explain the differential activation of the two PPAR subtypes by different fatty acids.

Experimental approaches to characterize these mechanisms are hampered by the lack of appropriate *in vitr*o hepatocyte model systems for studying gene regulation by fatty acids. For this thesis, several attempts were made to activate PPARα in primary hepatocytes by means of incubation with fatty acids. Although fatty acids efficiently upregulated gene expression in the FAO liver cell line, including upregulation of established PPARα target genes, we were not able to get a PPARα response in primary cells. Microarray analysis revealed that the fatty acids were indeed taken up by the hepatocytes, since the expression of many genes were changed upon treatment. None of these genes were known PPARα targets, however. Careful analysis of the genes regulated by fatty acids in primary hepatocytes did point to several target genes of HNF4a, which has previously been shown to bind LCFA-CoAs (340, 430-432). Although the reason for the lack of PPARα activation by fatty acids is not clear, there are a few possibilities that come to mind. First of all, it is possible that the unresponsiveness of PPARα is related to the fact that we incubated the cells with albumin bound FFAs, which based on our *in vivo* analysis were suggested to be unable to activate PPARα in liver. Alternatively, we found that expression of FABP1 and FABP2 in hepatocytes declined very quickly after culturing the cells. Finally, the lack of activation of PPARα targets by fatty acids might be connected to a high mitochondrial fatty acid oxidation activity, thereby limiting the amount of fatty acids available in the cell to activate PPAR. However, the latter speculation does not explain why our microarray analysis revealed hundreds of genes changed upon treatment with fatty acids.

Overall, this thesis illustrates the power of using microarrays. This is especially true when microarrays are combined with other experimental tools, such as transgenic- and knockout mouse models, siRNA, metabolic measurements and measurement of DNA binding. One important remaining problem, however, is the lack of proper statistical tools to deal with the low signals typically achieved with nutritional interventions. Direct application of

standardized techniques used for stronger interventions, such as pharmacological studies, often results in major loss of information. Rather than performing statistics at the level of individual genes, nutritional interventions call for a more comprehensive type of analysis that takes into account parallel regulation of genes within a single biological pathway.

This thesis illustrates that microarrays are not only useful for generating hypotheses but can be equally applied towards testing research hypotheses. Overall, the work described in this thesis has greatly expanded our understanding of the role of PPARα and PPARβ/δ in the liver. Furthermore, it has provided important new leads for future research. Finally, our nutrigenomics type of analysis has demonstrated the extremely important role of PPARs in gene regulation by fatty acids.

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Summary

Since obesity and its related metabolic diseases are rapidly increasing world-wide, studies aimed at uncovering the molecular mechanisms that control lipid metabolism are becoming more and more important. Within the field of nutrigenomics, researchers study how nutrition influences gene-, protein- and metabolite expression on a genome-wide scale. This type of research is mainly focused on preventing disease, rather than curing already developed illnesses and holds the expectation that nutritional intervention early on will prevent the need for pharmacological therapy. Common tools used within the field of nutrigenomics include gene expression microarrays, where the entire genome can be measured simultaneously on a small chip, and transgenic- and knockout mouse models, where a gene of interest can be either overexpressed or completely shut down.

As indicated above, within the field of nutrigenomics there is special interest in understanding the effect of dietary components on gene expression. Effects of dietary components on gene expression are partially mediated by members of the nuclear receptor superfamily, including the peroxisome proliferator activated receptors (PPARs). Three PPAR isoforms are known to exist: PPARα, PPARβ/δ and PPARγ. PPARα has been well described as a major regulator of hepatic lipid metabolism and is of great importance in the liver during fasting. Much less is known about PPARβ/δ in liver, although some studies point to an involvement of this transcription factor in certain metabolic processes.

The aim of the research carried out in this thesis was to determine the importance of PPARα in fatty acid dependent gene regulation in liver. Moreover, we set out to better characterize the functional roles of PPARα and PPARβ/δ in hepatic gene regulation and function.

With the help of various nutrigenomics tools, we were able to gather several important results. Firstly, we were able to create a comprehensive list of PPARα target genes in the liver, and demonstrated that the role of PPARα in hepatic lipid metabolism is much more extensive than previously described. Furthermore, using a unique nutrigenomics design that involves feeding mice synthetic triglycerides composed of one single fatty acid, we were able to determine the effect of individual fatty acids on gene regulation in liver. By performing a parallel analysis in wildtype and PPARα-/- mice, we were able to show that

the effects of dietary unsaturated fatty acids on hepatic gene expression are almost completely mediated by PPARα.

In the second part of this thesis we show that contrary to what has been generally assumed, circulating free fatty acids are not able to ligand-activate PPARα. We offer an alternative theory based on involvement of other transcription factors, especially PPARβ/δ. By using gene expression arrays in combination with analysis several metabolic parameters, we managed to appoint a role for PPARβ/δ in hepatic glucose utilization and lipoprotein metabolism.

Finally, we utilized an *in silico* method to screen for putative PPAR binding sites in the mouse genome. In an attempt to investigate a possible correlation between the identified putative PPREs and actual gene regulation, we compared the results from the *in silico* screen with results from microarray analysis of mouse livers and mouse primary hepatocytes treated with the PPARα agonist WY14643. Our data, however, question the utility of *in silico* screening to predict gene regulation by PPARs and to identify novel PPAR target genes. Screening for conserved PPREs within genes known to be induced by PPAR may be a valuable tool to identify functional PPREs.

In conclusion, this thesis demonstrates the power of microarray analysis in combination with specific knockout models to determine the impact and mechanisms of gene regulation by nutrients. This thesis extends our knowledge of PPARα as a dietary fatty acid sensor in liver and provides important new information on the role of PPARβ/δ in liver metabolism.

Samenvatting (Summary in Dutch)

Wereldwijd neemt de prevalentie van obesitas en aan obesitas gerelateerde ziekten nog steeds toe. Het is daarom belangrijk dat er onderzoek gedaan wordt naar de moleculaire mechanismen die betrokken zijn bij de regulering van het vetmetabolisme. Het vakgebied van Nutrigenomics is erop gericht met behulp van grootschalige technieken de effecten van voeding op het niveau van gen, eiwit en metaboliet te onderzoeken. Dit type onderzoek wil uiteindelijk bereiken dat door middel van voedingsinterventies ziekten kunnen worden voorkomen. Belangrijke technieken die binnen nutrigenomics worden toegepast zijn microarrays, waarmee de mRNA expressie van vrijwel het hele genoom bepaalt kan worden, alsmede transgeen en knock-out diermodelen waarbij een gen tot overexpressie wordt gebracht of volledig wordt uitgeschakeld.

Binnen nutrigenomics is er speciale aandacht voor de effecten van voedingsstoffen op de expressie van genen. Deze effecten komen voornamelijk tot stand via een groep van receptoren die bekend staan als nucleaire hormoon receptoren, en dan in het bijzonder de peroxisome proliferator activated receptors (PPARs). Er zijn 3 verschillende typen PPARs: PPARα, PPARβ/δ en PPARγ. PPARα reguleert het vetmetabolisme in de lever en is vooral van groot belang tijdens vasten. Over PPARβ/δ is veel minder bekend. Volgens een aantal studies is deze transcriptiefactor betrokken bij bepaalde metabole processen.

Het doel van dit promotieonderzoek was om uit te zoeken welk belang PPARα inneemt bij de regulering van gen expressie door vetzuren in de lever. Tevens was het onderzoek erop gericht de rol van PPARα en PPARβ/δ in de lever op te helderen.

Het onderzoek heeft een aantal belangrijke resultaten opgeleverd. Ten eerste zijn we erin geslaagd een uitgebreide lijst van PPARα target genen in de lever op te stellen die liet zien dat de rol van PPARα in de lever veel verder gaat dan tot dusver werd aangenomen. Daarnaast hebben we gebruik gemaakt van een uniek ontwerp waarbij muizen eenmalig werden blootgesteld aan synthetische triglyceriden bestaande uit één specifiek vetzuur. Door experimenten in parallel in wildtype and PPARα knock-out muizen uit te voeren konden we laten zien dat de effecten van onverzadigde vetzuren in de voeding op genexpressie in de lever vrijwel volledig tot stand kwamen via PPARα.

Het tweede deel van dit promotieonderzoek laat zien dat vrije vetzuren die in het bloed circuleren niet in staat zijn PPARα te activeren, in tegenstelling tot wat algemeen wordt aangenomen. Daarentegen kan PPARβ/δ in de lever wel door vrije vetzuren in het bloed worden geactiveerd. Door middel van microarray analyse in combinatie met meting van diverse metabolieten konden we laten zien dat PPARβ/δ in de lever betrokken is bij het metabolisme van lipoproteinen en glucose.

Tenslotte beschrijft dit proefschrift de resultaten van een *in silico* screen voor bindingsplaatsen van PPARα in het genoom. De resultaten van de screen zijn vergeleken met de resultaten van analyse van gen expressie in muizen levers en primaire hepatocyten behandeld met de PPARα agonist WY14643. Onze analyse plaatst vraagtekens bij de voorspellende waarde van *in silico* screening voor het vaststellen van PPARα target genen. Daarentegen kan het zoeken naar geconserveerde PPARs van nut zijn om functionele PPAR response elementen te identificeren binnen genen waarvan bekend is dat ze door PPARα worden gereguleerd.

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Utrecht, 17-08-2009

Curriculum vitae

Linda Marjatta Kjellberg was born on September 6th, 1976 in Boo, Sweden. After finishing high school in 1995, she began her studies in biology at Umeå University in the north of Sweden. As part of her Master's thesis, she spent six months at the University of Amsterdam, the Netherlands. Upon returning to Sweden, she decided to complement her biology studies with courses at a more molecular level and spent six months at Uppsala University studying chemistry and pharmacology. In 2003 she moved back to the Netherlands to get a Master's degree in Drug Innovation at Utrecht University. As part of this study, she worked on projects at Slotervaart Hospital in Amsterdam and the Department of Pharmaceutical Sciences at Utrecht University, dealing with pharmacogenomics and toxicity of drugs related to genomic make up. In 2005, she began her PhD studies at the Nutrition, Metabolism and Genomics (NMG) group at Wageningen University. Results of this research are described in this thesis. In 2005 she also married Eric and changed her last name to Sanderson. Their son Elliot was born in June 2009.

List of publications

Sanderson LM and Kersten S (2009) PPARs: important regulators in metabolism and inflammation. *In:* Bunce C and Campbell MJ, editors. *Nuclear receptors: current concepts and future challenges.* Springer.

Sanderson LM, de Groot PJ, Hooiveld GJ, Koppen A, Kalkhoven E, Müller M and Kersten S (2008) Effects of synthetic dietary triglycerides: a novel research paradigm for nutrigenomics. *PLoS ONE* **3**:e1681.
— Winner of TIFN Publication Prize 2008 (Nutrition and Health).

Rahkshandehroo M, Sanderson LM, Matilainen M, Stienstra R, Carlberg C, de Groot PJ, Müller M and Kersten S (2007) Comprehensive analysis of PPARα-dependent regulation of hepatic lipid metabolism by expression profiling. *PPAR Res* **2007**:26839.
— Rahkshandehroo and Sanderson are joint first authors.

Meijerman I, Sanderson LM, Smits P, Beijnen JH and Schellens JHM (2007) Pharmacogenetic screening of the gene deletion and duplications of CYP2D6. *Drug Metab Rev* **39**:45-60.

Bosch TM, Kjellberg LM, Bouwers A, Koeleman BP, Schellens JH, Beijnen JH, Smits PH and Meijerman I (2005) Detection of single nucleotide polymorphisms in the ABCG2 gene in a Dutch population. *Am J Pharmacogenomics* **5**:123-131.

Sanderson LM, Degenhardt T, Desvergne B, Müller M and Kersten S (2009) PPARβ/δ but not PPARα serves as plasma free fatty acid sensor in liver. Manuscript submitted.

Sanderson LM, Boekschoten M, Desvergne B, Müller M and Kersten S (2009) PPARβ/δ regulates glucose utilization and lipoprotein metabolism in liver. Manuscript submitted.

Sanderson LM, Heinäniemi M, Hooiveld GJEJ, Carlberg C, Müller M and Kersten S (2009) Poor match between genome wide in silico screening of PPARα targets and actual gene regulation in the mouse. Manuscript submitted.

Educational activities

Discipline specific activities

Wageningen Nutritional Sciences Forum 2009 (Arnhem, the Netherlands)
International Advanced Course on Epigenesis and Epigenetics 2008 (Wageningen, the Netherlands)
Benelux Nuclear Receptor Meeting 2008 (Utrecht, the Netherlands)
ICBL Conference on the Bioscience of Lipids 2008 (Maastricht, the Netherlands)
FEDERA Symposium on Obesity 2008 (Leiden, the Netherlands)
Centre for Integrative Genomics (CIG) Symposium 2008 (Lausanne, Switzerland)
Masterclass AMC 2007 and 2008 (Amsterdam, the Netherlands)
Keystone Symposium on Nuclear Receptors and Metabolic Syndrome 2007 (Steamboat Springs, CO, USA)
NuGO Advanced Course in Microarray Analysis 2006 (Maastricht, the Netherlands)
NWO Nutrition Meetings 2005, 2006 and 2007 (Papendal/Deurne, the Netherlands)
NuGO Week 2005, 2006 and 2007 (Tuscany, Italy; Oxford, UK and Oslo, Norway)
Masterclass Nutrigenomics 2005 (Wageningen, the Netherlands)

General Courses

Course on Laboratory Animal Science 2007 (Utrecht, the Netherlands)
Techniques for Writing and Presenting a Scientific Paper 2007 (Wageningen, the Netherlands)
VLAG PhD Week 2005 (Bilthoven, the Netherlands)
NuGO PhD Introduction Course 2005 (Marseille, France)

Optional Activities

Human Nutrition Journal Club (every four weeks)
NMG Journal Club (every two weeks)
NMG Scientific Meetings (every week)
Nutrigenomics Consortium (NGC) Scientific Meetings (every two months)
Human Nutrition PhD Tour 2005 (UK and Ireland)

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