The role of PPARs in inflammation and obesity

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Dit onderzoek is uitgevoerd binnen de onderzoeksschool VLAG
(Voeding, Levensmiddelentechnologie, Agrobiotechnologie en Gezondheid).

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Proefschrift

Ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. M.J. Kropff,
in het openbaar te verdedigen
op maandag 18 juni 2007
des namiddags te vier uur in de Aula.

Rinke Stienstra (2007)

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Thesis Wageningen University, Wageningen, The Netherlands
With abstract – references – with summary in Dutch

ISBN: 978-90-8504-677-6

Abstract

As the number of obese individuals worldwide keeps rising steadily, a global epidemic of obesity-related complications is on the horizon. Obesity greatly increases the risk for cardiovascular disease and Type II diabetes, especially when excess bodyfat is present in and around the abdomen. To prevent the obesity epidemic from getting completely out of control, effective strategies to reduce the incidence of obesity are urgently needed.

Seemingly, obesity can be easily treated or prevented by reducing food intake and increasing physical activity levels. However, the low success rate of these strategies together with the prospect of potential huge financial rewards has shifted the emphasis to pharmalogical approaches to reverse obesity and obesity-related complications. One important group of molecular targets for the treatment of obesity and related disorders are the Peroxisome Proliferator Activated Receptors (PPARs), a group of ligand-activated transcription factors that consists of three members: PPARα, PPARβ/δ and PPARγ. Each PPAR governs the expression of specific sets of target genes involved in various cellular processes ranging from inflammation to glucose and lipid metabolism. Currently, synthetic agonists for PPARs are prescribed for the treatment of dyslipidemia and insulin resistance.

Apart from metabolic abnormalities, obesity is also accompanied by a chronic low grade inflammation, which is generally believed to originate from expanding adipose tissue. Elevated secretion of pro-inflammatory factors from adipose tissue has been linked to the development of atherosclerosis and insulin resistance. Since PPARs have important anti-inflammatory properties in a wide variety of cell types, they might protect against obesity-induced inflammation and its complications.

Although the PPARα isotype is known to suppress inflammation, relatively little is known about the specific inflammatory pathways that are governed by PPARα in liver. By using microarray analysis, we compared the expression profiles of inflamed and PPARα-activated liver. Whereas inflammation up-regulated numerous pro-inflammatory genes, activation of PPARα in liver resulted in an opposite expression profile. We were able to identify interleukin receptor 1 antagonist, which suppresses the effect of IL-1, as a direct target gene of PPARα. To investigate whether PPARα is involved in controlling obesity-induced inflammation, both Wildtype and PPARα -/- mice were fed either a low fat diet or a high fat diet. After the diet intervention, liver gene expression profiles were compared by microarray analysis. In Wildtype mice, HFD significantly increased the hepatic and adipose

expression of numerous genes involved in inflammation. Importantly, this effect was amplified in PPARα-/- mice, suggesting an anti-inflammatory role of PPARα in liver and adipose tissue. Further studies were carried out to investigate the mechanism behind the anti-inflammatory role of PPARα. These studies led to the conclusion that PPARα protects against obesity-induced chronic inflammation in liver by reducing hepatic steatosis, by direct down-regulation of inflammatory genes, and by attenuating inflammation in adipose tissue.

PPARγ is highly expressed in adipose tissue and has therefore been extensively studied in the context of obesity. However, less is known about the impact of PPARγ on obesity-induced inflammation. Accordingly, we tested whether activation of PPARγ by rosiglitazone could reverse the inflammatory status of adipose tissue observed in obese mice. PPARγ activation resulted in suppression of pro-inflammatory gene expression and led to remodeling of adipose tissue. Surprisingly, the number of macrophages in adipose tissue was increased after rosiglitazone treatment. However, gene expression changes obtained via qPCR analysis suggested that these macrophages were alternatively activated and might contribute to cell proliferation and remodeling of the adipose tissue.

Finally, the last part of this thesis describes the identification of Glycogen Synthase 2, the rate-limiting enzyme in the conversion of glucose to glycogen, as a novel PPAR target gene in liver and adipose tissue. The complexity of regulation by the hepatocyte nuclear factor 4α and PPARs via different response elements is shown.

Contents

Chapter 1

General introduction

Outline of this thesis

The first part of this thesis focuses on the role of PPARs in the control of inflammatory reactions. PPARα is known to have important immune-suppressive effect, which first became apparent by a study in 1996 showing a prolonged inflammatory responses in animals lacking PPARα after exposure to LTB4 (1). In chapter 3, the role of PPARα in the regulation of pro-inflammatory genes in liver is investigated, which is followed by detailed investigation of the putative anti-inflammatory role of PPARα in the context of obesity-induced inflammation (chapter 4). Chapter 5 describes the results of ongoing analysis of the role of PPARγ in governing obesity-induced inflammation in adipose tissue. Finally, the last part of this thesis describes the identification of Glycogen Synthase 2 as a novel PPAR target gene in liver and adipose tissue.

The current chapter serves as an introduction to obesity and its connection with low grade chronic inflammation. It aims at defining the molecular mechanisms of obesity-induced inflammation and provides a brief overview of our current knowledge on the connection between PPARs and inflammatory control. In chapter 2, a more detailed overview of the role of PPARs in the regulation of obesity-induced inflammation is presented.

Obesity and the Metabolic Syndrome

The prevalence of obesity has increased dramatically in the past decades (2), which is mostly due to changes in lifestyle and easy accessibility of food (3) (4). In the United States, 60% of the population is affected by overweight and obesity combined (BMI ≥ 25), whereas 27% of US adults are obese (BMI ≥ 30). Although the prevalence of obesity varies widely within Europe, the incidence of overweight and obesity is rapidly increasing in most European countries as well (3).

If energy intake exceeds energy expenditure, the surplus of energy will be primarily stored in adipose tissue in the form of triglycerides. Concurrent with the development of obesity, other metabolic abnormalities arise, including hypertriglyceridemia, elevated blood pressure and insulin resistance, which are clustered into the metabolic syndrome or Syndrome X. In 2001, the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) introduced clinical criteria for defining and diagnosing metabolic syndrome (5). These clinical criteria include elevated waist circumference (≥ 102 cm in men, ≥ 88 cm in women), elevated triglycerides (≥ 150 mg/dL (1.7 mmol/L)), reduced HDL-C (≤ 40 mg/dL (1.03 mmol/L) in men, ≤ 50 mg/dL (1.3 mmol/L) in women), elevated blood pressure (≥ 130 mm Hg systolic blood pressure or ≥ 85 mm Hg diastolic blood pressure) and elevated fasting glucose (≥ 100 mg/dL). When 3 out of 5 factors are present, individuals are diagnosed with metabolic syndrome and are highly predisposed to the development of type II diabetes and cardiovascular disease. Insofar as type II diabetes and cardiovascular diseases are major causes of death in Western societies (6) (7), effective strategies to reduce the prevalence of metabolic syndrome are urgently needed.

It is generally believed that the predominant risk factor predisposing to the development of metabolic syndrome is obesity and the associated expansion of fat depots (8). While growth of several fat depots appears to be relatively harmless, excess visceral fat depots is considered a major risk factor for the development of obesity-related metabolic abnormalities (9). Part of the metabolic complications of obesity may be due to storage of triglycerides in tissues other than adipose tissue, such as heart, pancreas and liver. Indeed, elevated fat accumulation in liver is commonly observed in obese individuals leading to fatty liver disease (10).

Chronic low grade inflammation and obesity

Changes in inflammatory status are nowadays believed to play an important role in the development of metabolic syndrome. Indeed, obesity is currently viewed as a state of low grade chronic inflammation elicited by changes in the secretion profile of adipose tissue (11). The first evidence for a link between obesity and chronic inflammation was provided by Hotamisligil and colleagues, who showed that expression of Tumor Necrosis Factor α (TNFα) is significantly higher in obese compared to lean subjects (12). Subsequently, it was shown that plasma levels of pro-inflammatory proteins are increased during obesity, whereas levels of anti-inflammatory proteins such as adiponectin are decreased (13) (14) (15) (16). Many of the inflammatory parameters contributing to the pro-inflammatory state in obesity are believed to originate from adipose tissue. Consequently, our view of adipose tissue as merely an energy storage compartment has changed drastically (8). Growth of adipose tissue leads to changes in its morphology including adipocyte hypertrophy and macrophage infiltration (17, 18), both of which contribute to altered secretion of a battery of pro-inflammatory adipocytokines including TNFα, IL-6 and resistin. Since adipose tissue is well vascularized (19), secreted adipocytokines can readily reach the circulation and act in an endocrine fashion (See figure 1).

Recent studies have provided insight into the mechanisms underlying the link between inflammation and insulin resistance. In mouse models and in vitro studies, obesity-induced inflammation strongly inhibited insulin signalling by blocking Insulin Receptor Substrate-1 phosphorylation (20) (21) (22) (23). The obesity-induced pro-inflammatory cytokine TNFα can oppose insulin action via different mechanisms including disruption of protein kinase C interaction with Insulin Receptor Substrate-1. The discovery of a molecular link between inflammatory mediators and insulin signalling has led to the notion that inflammation is a primary cause for the development of insulin resistance. Similarly, atherosclerosis is currently considered as an ongoing inflammatory process (24) (25) in which adipose-derived inflammatory factors might play an important role.

Although the link between obesity and inflammation is unequivocal, the underlying mechanism(s) remain poorly defined. It is also not fully clear yet whether inflammation is a physiological response to overeating, or a patho-physiological response that has the potential to do great harm, although recent evidence points towards the latter. Since inflammation stimulates adipose tissue lipolysis (27), inflammation may be elicited during obesity as a feedback mechanism to keep bodyweight within normal boundaries. Another theory argues

that the stresses of obesity are similar as the stresses of an infection thus causing a similar reaction of our immune system (28). Emerging data show that obesity causes endoplasmic reticulum stress which may subsequently lead to activation of inflammatory pathways (29).

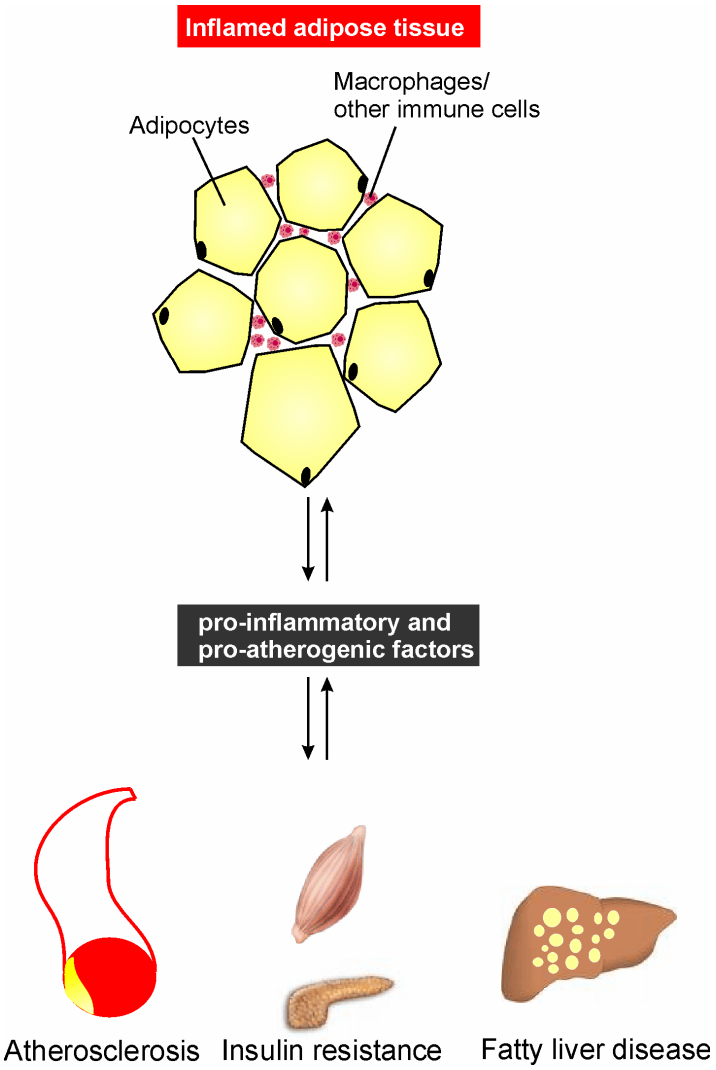


Figure 1 **Obesity and subsequent enlargement of adipose tissue results in a chronic low state of inflammation.** Obesity-induced changes in adipose tissue leading to a pro-inflammatory secretion profile can influence the progression of insulin resistance, atherosclerosis and fatty liver disease.

Alternatively, the adipocyte hypertrophy that accompanies obesity may result in reduced nutrient supply leading to adipocyte death and the subsequent recruitment of macrophages. Finally, the discovery that fatty acids can efficiently bind to Toll Like Receptors and activate our immune system (30) revealed that our body is capable of recognizing the damaging potential of overeating (31) and reacts by inducing an inflammatory response.

PPARs

Peroxisome Proliferator-Activated Receptor (PPARs) are ligand activated transcription factors that belong to the superfamily of nuclear hormone receptors, which also includes receptors for vitamin D, vitamin A, thyroid hormone, bile acids, and steroid hormones (33). Three different PPARs can be distinguished: PPARα, PPARβ/δ, and PPARγ, each of which has a different expression pattern partially accounting for their specific functions. Since the initial discovery of the PPARα-isotype in 1990 (32), an impressive amount of literature on these receptors has accumulated. This has led to a broad understanding of the role of PPARs in numerous biological processes, ranging from cell differentiation and proliferation to inflammation and energy homeostasis.

PPARs mainly operate by governing the expression of specific sets of genes. Analogous to many other nuclear receptors, PPARs bind to DNA and regulate transcription as a heterodimer with the nuclear receptor Retinoid X Receptor (RXR). PPARs recognize DNA sequences called PPAR Response Elements (PPREs), which are mostly located in promoters of target genes. Functional PPREs have been identified in genes involved in a variety of functions including lipid metabolism, glucose metabolism, detoxification, and inflammatory control (36).

Activation of transcription by PPARs is achieved by binding of specific ligands to the Ligand Binding Domain (LBD), followed by recruitment of co-activator proteins and dissociation of co-repressors (34). Numerous co-activators involved in PPAR-dependent gene transcription have been identified, including Peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1), CREB Binding Protein (CBP) and the Glucocorticoid Receptor interacting protein/transcriptional intermediary factor (GRIP/TIF). Co-activator recruitment generally leads to an increase in enzymatic activity of histone acetyltransferase, histone methyltransferase and subsequent nucleosome remodeling (NRM), activities which are essential to initiate transcription of PPAR target genes (39).

X-ray crystallographic analysis of the PPAR LBDs has provided important information about the structure-function relationship of PPARs. The exceptionally spacious ligand-binding pocket within the LBD allows for binding of a wide variety of both natural and synthetic ligands. Among the natural ligands, polyunsaturated fatty acids and eicosanoids are potent inducers of PPAR activity. Synthetic ligands for PPARs are commonly used clinically for the treatment of diabetes-related metabolic abnormalities. Fibrates, which are potent inducers of PPARα, correct (diabetic) dyslipidemia by lowering blood TGs and increasing plasma High Density Lipoprotein (HDL) concentrations (35), whereas the thiazolidinediones, which bind PPARγ, ameliorate insulin sensitivity (35).

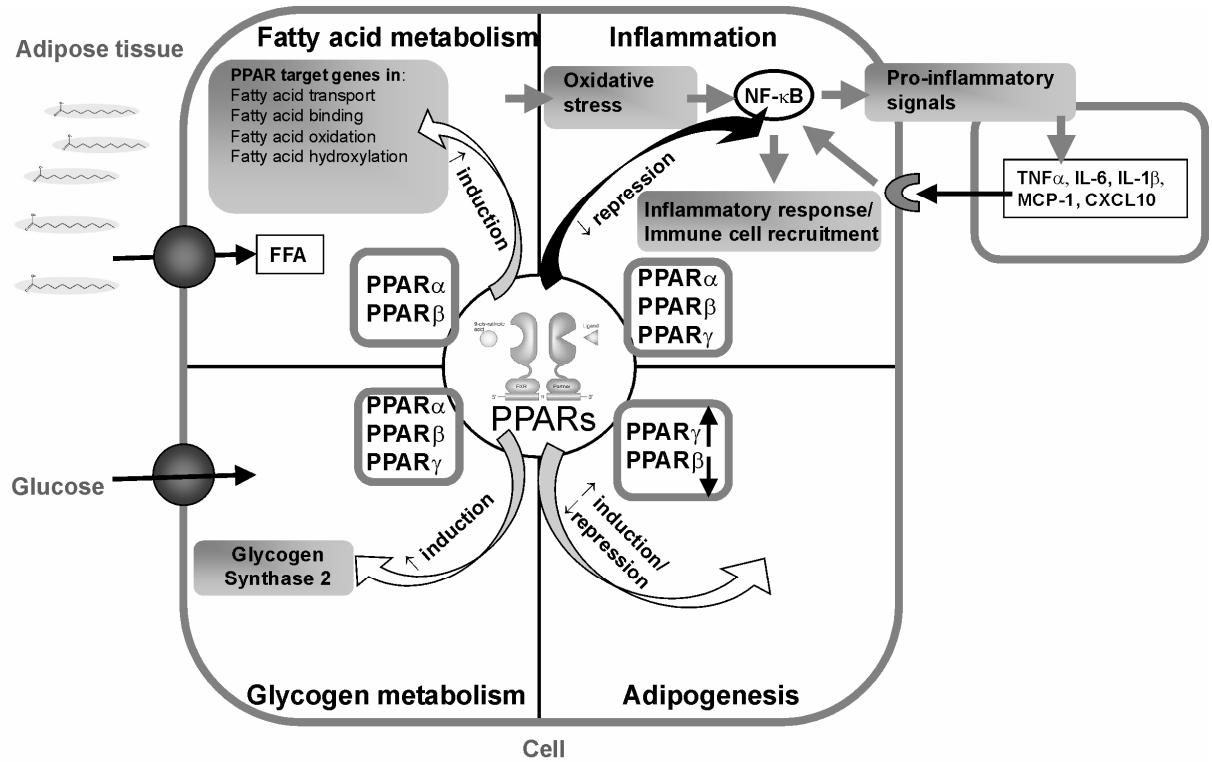
All three PPARs show unique tissue expression profiles that provide important clues about their biological functions. PPARα is highly expressed in liver, intestine and heart, and enhances fatty acid transport, binding, and oxidation (41). The important role of PPARα in these processes is illustrated by the inability of mice lacking PPARα to properly respond to food deprivation. Consequently, fasted PPARα -/- mice show elevated free fatty acid levels, hypoglycemia and increased liver lipid content (42). PPARγ is predominantly expressed in adipose tissue in which it tightly controls adipogenic differentiation (43). Target genes of PPARγ include fatty acid binding protein 2, lipoprotein lipase, phosphoenolpyruvate carboxykinase and glycerol kinase (44).
In contrast to the restricted expression profile of PPARα and PPARγ, PPARβ/δ is ubiquitously expressed. Gain and loss of function studies reveal a role of PPARβ/δ in fatty acid catabolism and energy uncoupling in adipose tissue and muscle. PPARβ/δ target genes include UCP-1 and 3, CPT-1 and PDK4 (45).

In addition to serving an important role in the regulation of energy metabolism (36), PPARs also govern inflammatory responses (46). However, whereas the target genes involved in lipid and glucose metabolism are positively regulated by PPARs, inflammatory processes are down-regulated by PPARs via mechanisms of transrepression (47), which is true for PPARα, PPARβ/δ and PPARγ (48) (49). The anti-inflammatory activities of PPARs are partially effectuated by inhibition of Nuclear factor κB (NF-κB), which represents one of the master transcription factors responsible for initiating inflammatory responses in various tissues (50). Inactive NF-κB subunits are localized in the cytosol tightly bound to IκB, thereby preventing nuclear translocation of the complex. Upon activation by pro-inflammatory cytokines such as TNFα and IL-6, the complex enters the nucleus and activates the expression of inflammatory genes (51). PPARs are able to attenuate NF-κB function either by interfering

with the transcription activating capacity of the NF-κB complex or by regulating genes that suppress the activation of NF-κB. By physical interaction with components of the NF-κB complex, PPARα impairs binding of NF-κB to the DNA and subsequent activation of inflammatory genes (52). Secondly, PPARα activation results in increased expression of IκB, the inhibitory protein that prevents the transfer of NF-κB to the nucleus (53). Aside from interference with NF-κB, additional molecular pathways by which PPARβ/δ and PPARγ inhibit inflammation have been identified. PPARβ/δ activation indirectly regulates inflammatory reactions by releasing the inflammatory suppressor protein B cell lymphoma-6 protein (BCL-6) (48), while PPARγ inhibits the transcription of pro-inflammatory genes by preventing the release of corepressor complexes from promoters of pro-inflammatory genes thereby inhibiting their transcription (54).

Inasmuch as obesity is characterized by increased fat storage and chronic low grade inflammation, which are targeted by PPARs, PPARs represent important targets to prevent or decrease obesity-related metabolic abnormalities. In chapter 2, the protective role of PPARs in reducing obesity-induced inflammation is discussed in more detail.

Figure 2 **PPARs are involved in controlling diverse cellular pathways.**



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Chapter 2

Review

PPARs, obesity, and inflammation

Rinke Stienstra, Caroline Duval, Michael Müller, Sander Kersten

This chapter has been published in **PPAR Research**.

Abstract

The worldwide prevalence of obesity and related metabolic disorders is rising rapidly, increasing the burden on our health care system. Obesity is often accompanied by excess fat storage in tissues other than adipose tissue, including liver and skeletal muscle, which may lead to local insulin resistance and may stimulate inflammation, as in steatohepatitis. In addition, obesity changes the morphology and composition of adipose tissue, leading to changes in protein production and secretion. Some of these secreted proteins, including several pro-inflammatory mediators, may be produced by macrophages resident in the adipose tissue. The changes in inflammatory status of adipose tissue and liver with obesity feed a growing recognition that obesity represents a state of chronic low-level inflammation. Various molecular mechanisms have been implicated in obesity-induced inflammation, some of which are modulated by the Peroxisome Proliferator Activated Receptors (PPARs). PPARs are ligand activated transcription factors involved in the regulation of numerous biological processes, including lipid and glucose metabolism, and overall energy homeostasis. They are activated by a variety of different endogenous ligands such as fatty acids and eicosanoids, and serve as the molecular targets of the insulin-sensitizing thiazolidinedione and hypolipidemic fibrate drugs. Importantly, PPARs also modulate the inflammatory response, which makes them an interesting therapeutic target to mitigate obesity-induced inflammation and its consequences. This review will address the role of PPARs in obesity-induced inflammation specifically in adipose tissue, liver and the vascular wall.

Abbreviations used: ADAM8: A Disintegrin And Metallopeptidase Domain 8; ADRP: Adipose Differentiation Related Protein; AP-1: Activator Protein–1; BCL-6: B-Cell Lymphoma-6; BMI: Body Mass Index; C/EBP: CCAAT/Enhancer Binding Protein; CRP: C-Reactive Protein; CVD: Cardiovascular Disease; GRIP1/TIF2: Glucocorticoid Receptor-Interacting Protein 1/Transcriptional Intermediary Factor 2; HDAC3: Histone Deacetylase 3; HFD: High Fat Diet; ICAM I: Intercellular Adhesion Molecule I; IFNγ: Interferon γ; IL-1β: Interleukin 1β; IL-6: Interleukin 6; IP-10/CXCL10: Chemokine (C-X-C motif) ligand 10; IRS-1: Insulin Receptor Substrate 1; LDLR: Low Density Lipoprotein Receptor; MAC-1: Macrophage antigen-1; MCP-1: Monocyte Chemoattractant Protein-1; MIP-1α: Macrophage Inflammatory Protein 1α; NAFLD: Non-Alcoholic Fatty Liver Disease; NASH: Non-Alcoholic Steatohepatitis; N-CoR: Nuclear Receptor Co-Repressor; NF-κB: Nuclear Factor-κB; PPAR: Peroxisome Proliferator Activated Receptor; SAA: Serum Amyloid A; SREBP: Sterol Regulatory Element Binding Protein; STAT: Signal Transducer and Activator of Transcription; TGF-β1: Transforming Growth Factor-β1; TNFα: Tumor Necrosis Factor α; VCAM I: Vascular Cell Adhesion Molecule I; WAT: White Adipose Tissue.

Introduction

The prevalence of obesity worldwide has progressively increased over the past decades. In 2000 it was estimated that more than half of US adults were overweight, while the frequency of obesity, which is defined by a BMI (Body mass index) ≥30 kg/m^2, was 20%, reflecting an increase of 61% within 10 years (1). Not only have more and more adults become obese, obesity is also striking at a much younger age leading to a high number of obese children and adolescents (2). Unless drastic action is taken, many countries will face a decline in life expectancy in the 21st century due to the obesity epidemic.

Obesity is the direct result of an imbalance between energy intake and energy expenditure. The excess energy is primarily stored in adipose tissue in the form of triglycerides. Although adipocytes are specifically designed to store energy and easily fill up with fat, the morphological changes associated with adipose tissue growth are not without consequences for the organism as a whole (3). Evidence has accumulated suggesting that in response to adipocyte hypertrophy during development of obesity, adipose tissue function is compromised.

Obesity also provokes structural and metabolic alterations in other organs, including skeletal muscle and liver. Indeed, obesity is closely linked to fat storage in liver and is nowadays considered as a major risk factor for the development of fatty liver diseases. The incidence of non-alcoholic fatty liver disorders (NAFLD) and obesity are therefore intimately linked. It has been estimated that about 75% of obese subjects have NAFLD while 20% develop Non Alcoholic Steatohepatitis (NASH), which is defined as fatty liver disease with inflammation (4). The amount of fat stored in liver is determined by the balance between fatty acid uptake, endogenous fatty acid synthesis, triglyceride synthesis, fatty acid oxidation, and triglyceride export. Changes in any of these parameters can affect the amount of fat stored in liver.

The excessive fat accumulation in adipose tissue, liver and other organs strongly predisposes to the development of metabolic changes that increase overall morbidity risk. The metabolic abnormalities that often accompany obesity include hypertension, impaired glucose tolerance, insulin resistance leading to hyperinsulinemia, and dyslipidemia. Collectively, these abnormalities have been clustered into the metabolic syndrome or Syndrome X (5). Individuals that are diagnosed with metabolic syndrome have a significantly increased risk of developing cardiovascular disease (CVD) and type II diabetes. Inasmuch as CVD is the major cause of death in industrialized countries, effective strategies to curtail the number of

individuals with metabolic syndrome are badly needed. Visceral obesity, which is characterized by excess fat storage in and around the abdomen, is the prime cause of the metabolic abnormalities and therefore represents an important target in the treatment of metabolic syndrome (6).

In recent years, it has become clear that obesity also gives rise to a heightened state of inflammation. The link between obesity and inflammation was first established by Hotamisligil and colleagues who showed a positive correlation between adipose mass and expression of the pro-inflammatory gene Tumor Necrosis Factor α (TNFα) (7). The link between obesity and inflammation has been further illustrated by the increased plasma levels of several pro-inflammatory markers including cytokines and acute phase proteins like C-Reactive Protein (CRP) in obese individuals (8) (9). Nowadays, CRP is considered as an independent biomarker for the development of CVD (10) which emphasizes the connection between inflammation, obesity and CVD. Many of the inflammatory markers found in plasma of obese individuals appear to originate from adipose tissue (8). These observations have led to the view that obesity is a state of chronic low grade inflammation that is initiated by morphological changes in the adipose tissue.

One consequence of the elevated inflammatory status is insulin resistance. Pro-inflammatory cytokines originating from fat have been shown to directly interfere with insulin signalling pathways (11). For example, TNFα causes insulin resistance by inhibiting tyrosine phosphorylation of Insulin Receptor Substrate-1 (IRS-1) (12). Other mechanisms of inhibition of IRS-1 phosphorylation by inflammatory mediators include chronic activation of JNK, PKC and IKK (13-15).

Besides TNFα, adipose tissue produces a host of other adipokines with well described effects on metabolism and inflammation. Resistin, adiponectin, leptin and Monocyte Chemoattractant Protein-1 (MCP-1) are among a group of secreted proteins from adipose tissue with immune-modulating functions (16). The production and secretion of these adipokines is altered during obesity, resulting in a more pro-inflammatory or atherogenic secretion profile. Indeed, whereas secretion of MCP-1, resistin and other pro-inflammatory cytokines is increased by obesity, the adipose secretion of the anti-inflammatory protein adiponectin is decreased (17).

Although increased visceral fat depots (6) and adipocyte hypertrophy (3) had been linked to a higher degree of adipose inflammation, until recently the exact pathways leading to a pro-inflammatory state of adipose tissue in obese individuals remained unidentified. However, recently much attention has been diverted to the role of macrophages. In 2003, two

papers published back to back showed that diet-induced obesity is associated with infiltration of macrophages into white adipose tissue (18) (19). Infiltrated macrophages, which are part of the stromal vascular fraction of adipose tissue, are subsequently responsible for the production of a wide variety of pro-inflammatory proteins including MCP-1, TNFα and Interleukin-6 (IL-6). The development of insulin resistance in adipocytes was closely linked to the infiltration of macrophages. However, if and how entry of macrophages into white adipose tissue (WAT) leads to systemic insulin resistance remains unclear, although it is increasingly believed that altered secretion of adipokines by WAT during obesity may represent an important piece of the puzzle.

One of the other tissues that is affected by the enlargement and pro-inflammatory secretion profile of adipose tissue is the liver. Chronic activation of the master regulator of inflammation Nuclear Factor-κB (NF-κB) by cytokines has been directly linked to the development of insulin resistance in liver (20, 21). It has also been shown that adipose-specific over-expression of MCP-1 increases hepatic triyglyceride content (22). Although steatosis is a common occurrence in obese individuals, the role of inflamed adipose tissue in development of steatosis needs further exploration.

Initially characterized by excess fat storage, steatosis can progress to steatohepatitis and finally lead to cirrhosis and structural alterations of the liver (23). The molecular mechanisms underlying the development of steatosis and progression to steatohepatitis remain poorly understood. Whereas some patients only develop steatosis, others develop steatohepatitis and fibrosis. Lipid peroxidation, cytokines and other pro-inflammatory compounds are believed to play a vital role in the transition (4). In addition, the role of the expanding adipose tissue might also prove relevant to the development of steatohepatitis.

Recently, the elevated inflammatory status of adipose tissue and concurrent increased production of adipose tissue-derived cytokines has also been connected with atherosclerosis. Initially defined as a pathological lipid deposition, the atherosclerotic process is nowadays considered as an ongoing inflammatory process in which numerous cytokines, chemokines, and inflammatory cells participate (24). Independent of its connection to the metabolic syndrome, obesity itself is a known risk factor for the development of atherosclerosis and CVD (25).

In summary, obesity represents a major health threat and effective therapies to minimize obesity-related co-morbidities are sorely needed. By targeting the inflammatory component, the progression of obesity towards insulin resistance and CVD might be slowed down.

The ligand-activated transcription factors belonging to the Peroxisome Proliferator Activated Receptor (PPAR)-family are involved in the regulation of inflammation and energy homestasis and represent important targets for obesity, obesity-induced inflammation and metabolic syndrome in general. These receptors share a common mode of action that involves heterodimerization with the nuclear receptor RXR and subsequent binding to specific DNA-response elements in the promoter of target genes. Binding of ligands to PPARs leads to recruitment of co-activators and chromatin remodeling, resulting in initiation of DNA transcription (26) (27). Currently, synthetic PPAR-agonists are widely used for the treatment of insulin resistance and dyslipidemia. This review will explore the role of PPARs in governing chronic inflammation with special emphasis on the connection with metabolic syndrome. The link with obesity and inflammation will be discussed separately for the three PPAR isoforms: PPARα, PPARβ/δ and PPARγ.

PPARα

PPARα is well expressed in metabolically active tissues including liver, brown adipose tissue, muscle and heart. In addition, PPARα is expressed in cells involved in immune responses including monocytes, macrophages and lymphocytes (28). Activation of PPARα occurs through a variety of natural agonists, including unsaturated fatty acids and eicosanoids, whereas fibrate drugs act as synthetic agonists. In liver, PPARα plays a pivotal role in fatty acid catabolism by up-regulating the expression of numerous genes involved in mitochondrial fatty acid oxidation, peroxisomal fatty acid oxidation, and numerous other aspects of fatty acid metabolism in the cell (28). As a consequence, activation of PPARα can prevent and decrease hepatic fat storage (29) (30) (31, 32). Other metabolic pathways under control of PPARα include gluconeogenesis (33), biotransformation (34) and cholesterol metabolism (35). While the function of PPARα in mouse liver is relatively well defined, much less is known about its role in human liver. Experiments with "humanized" PPARα mice have revealed that there are intrinsic differences in the properties of the human and mouse PPARα protein (36). In general, research on the role of PPARα in human liver is hampered by the low expression levels of PPARα in human hepatoma cell lines (37).

Besides governing metabolic processes, PPARα also regulates inflammatory processes, mainly by inhibiting inflammatory gene expression. Hepatic PPARα activation has been repeatedly shown to reduce hepatic inflammation elicited by acute exposure to cytokines and other compounds. In recent years, several molecular mechanisms responsible for the immunosuppressive effects of PPARα have been uncovered (38). These include interference with several pro-inflammatory transcription factors including Signal transducer and activator of transcription (STAT), Activator protein–1 (AP-1) and NF-κB by PPARα (39). The latter mechanism involves stimulation of expression of the inhibitory protein IκBα, which retains NF-κB in a non-active state, leading to suppression of NF-κB DNA-binding activity (40). Detailed molecular studies have further revealed that PPARα diminishes the activity of the pro-inflammatory transcription factor CAATT/enhancer binding proteins (C/EBP) via sequestration of the coactivator glucocorticoid receptor-interacting protein 1/transcriptional intermediary factor 2 (GRIP1/TIF2) (41). Finally, PPARα can also inhibit cytokine signalling pathways via down-regulation of the IL-6 receptor (42) and up-regulation of sIL-1 receptor antagonist (Stienstra *et al*. in press), leading to diminished inflammatory responses. Interestingly, in humans, specific PPARα activation using fenofibrate has been shown to

decrease plasma levels of several acute phase proteins that are normally increased during inflammatory conditions (42).

PPARα and steatosis

In mice fed a high fat diet, proper functioning of PPARα is essential to prevent the liver from storing large amounts of fat (43). By inducing mitochondrial, peroxisomal and microsomal fatty acid oxidation, PPARα reduces hepatic fat accumulation in the liver during the development of fatty liver disease and thus prevents steatosis (44) (31) (45). It can be hypothesized that since PPARα has a potent anti-inflammatory activity in liver, the progression of steatosis towards steatohepatitis might be counteracted by PPARα. Indeed, several studies in mice have shown that PPARα activation is able to reduce or even reverse steatohepatitis induced by feeding a methionine- and choline-deficient (MCD) diet (31, 45, 46).

In a mouse model of steatohepatitis the presence and activation of PPARα prevented the induction of COX-2 expression (47). Since up-regulation of COX-2 is seen in alcoholic steatohepatitis and non-alcoholic steatohepatitis and has been directly linked to the progression of steatosis to steatohepatitis, the inhibitory effect of PPARα on COX-2 may reduce steatohepatitis. An anti-inflammatory role of PPARα in the development of steatohepatitis is further supported by a study in which wild-type and PPARα-/- mice were fed a high fat diet to induce obesity. Although both genotypes developed a fatty liver after chronic high fat feeding, animals lacking PPARα developed steatohepatitis accompanied by an increased number of infiltrated lymphocytes and macrophages. By suppressing the expression of specific chemokines involved in attracting macrophages and other immune-related cell types, PPARα might moderate steatohepatitis (Stienstra et al. submitted). These results are in line with a study performed in APOE2 knock-in mice fed a western-type high fat diet (48). When the animals were co-treated with fenofibrate, macrophage infiltration of the liver was prevented.

PPARα and atherosclerosis

Inflammation in the arterial wall is known to promote the process of atherosclerosis (49). In addition to suppressing the inflammatory response in liver, PPARα may also influence

inflammatory reactions in the arterial wall. As PPARα is expressed in various cell types present in atherosclerotic lesions, the effect of PPARα on lesion development is rather complex. Immune-modulating effects of specific PPARα activation have been reported in various cell types. However, some controversy still exists about the exact role of PPARα in the vascular wall as both pro- and anti-atherogenic effects of PPARα have been demonstrated. An anti-atherogenic effect of PPARα via suppression of several pro-inflammatory genes like MCP-1, TNFα, Vascular cell adhesion molecule I (VCAM I), Intercellular adhesion molecule I (ICAM I) and Interferon γ (IFNγ) has been reported in the vascular wall of animals with extensive atherosclerosis (50). Other studies have shown that the anti-inflammatory role of PPARα in the vascular wall seems to be dependent on the severity of inflammation or vascular lesion. In the absence of inflammation or in early lesions, the effects of PPARα are mainly pro-atherogenic (51) (52), whereas the development of severe lesions accompanied by inflammation is strongly reduced by PPARα activation.

Several acute phase proteins have been linked to the development of atherosclerosis (53). This includes CRP, which is currently used as a marker for systemic inflammation and linked to CVD, and Serum Amyloid A (SAA), which has been shown to be involved in the development of atherosclerosis (54). As PPARα activation down-regulates plasma concentrations of acute phase proteins including CRP and SAA in humans (42), it might indirectly prevent or slow down the progression of atherosclerosis.

PPARα and adiposity

Although expression of PPARα in WAT is much lower compared to PPARγ, evidence abounds that PPARα may also influence adipose tissue function. It has been shown that PPARα -/- mice gain more adipose mass compared to wild-type animals (55), which may be via local or systemic effects of PPARα. An anti-obesity role for PPARα is supported by several studies in which obese rodents were administered synthetic PPARα agonists (56) (57) (58). While it is true that PPARα agonists have a clear anorexic effect resulting in decreased food intake, evidence is accumulating that PPARα may also directly influence adipose tissue function, including its inflammatory status.

A recent study revealed that treatment of obese diabetic KKAy mice with Wy-14643 decreased adipocyte hypertrophy as well as macrophage infiltration (59). In PPARα -/- mice chronically fed a High fat diet (HFD), expression of inflammatory genes in adipose tissue was more pronounced compared to wild-type mice. In addition, fractionation of adipose tissue in

adipocytes and stromal vascular cells revealed higher gene expression levels of the specific macrophage marker F4/80+ in the stromal vascular fraction of PPARα -/- mice (Stienstra et al. submitted).

PPARα may govern adipose tissue inflammation in three different ways: 1) by decreasing adipocyte hypertrophy, which is known to be connected with a higher inflammatory status of the tissue (11) (59) (3), 2) by direct regulation of inflammatory gene expression via locally expressed PPARα, or 3) by systemic events likely originating from liver. Full clarification of the role of locally expressed PPARα in adipose tissue will have to await the availability of adipose tissue-specific PPARα -/- mice.

Thus, while evidence is mounting that PPARα activation reduces adipose inflammation as observed during obesity, it is unclear whether the anti-inflammatory effects of PPARα in WAT are caused by direct or indirect mechanisms.

PPARβ/δ

Compared to PPARα and PPARγ, much less is known about PPARβ/δ and its natural ligands. Due to its ubiquitous expression profile, lack of specific ligands and, until recently, lack of availability of knock-out models, the role of PPARβ/δ in many tissues has been poorly explored. Fortunately, the recent generation of PPARβ/δ -/- mice has provided a strong impetus for the characterization of the function of PPARβ/δ (60). Several abnormalities have been observed in mice lacking PPARβ/δ which include impaired wound healing, a decrease in adipose mass, and disturbed inflammatory reactions in skin (61).

PPARβ/δ has been directly linked to the development of obesity. Indeed, several groups have reported a decrease in adiposity after PPARβ/δ activation. By stimulating fatty acid oxidation, PPARβ/δ activation leads to loss of adipose mass in different mouse models of obesity (62). Similar effects on fatty acid oxidation have been observed in heart, resulting in improved muscle contraction (63). In addition to increasing fatty acid oxidation, activation of PPARβ/δ in muscle also increases the number of type I muscle fibers, which leads to enhanced endurance performance (64).

The number of studies that have addressed the role of PPARβ/δ during inflammation is limited. So far, an anti-inflammatory effect has been observed in macrophages suggesting a possible role for PPARβ/δ in the process of atherogenic inflammation. It appears that PPARβ/δ acts as an inflammatory switch in which inactivated PPARβ/δ is pro-inflammatory and activated PPARβ/δ promotes an anti-inflammatory gene expression profile. The proposed switch of PPARβ/δ is linked to the B cell lymphoma-6 (BCL-6) protein which functions as inflammatory suppressor protein (65). In the unliganded state, BCL-6 is part of the PPARβ/δ -RXRα transcriptional complex. Upon ligand activation, co- repressors including BCL-6 are dissociated and PPARβ/δ-dependent gene transcription ensues. The released BCL-6 subsequently acts as a repressor of pro-inflammatory gene expression in macrophages.

PPARβ/δ and steatosis

It can be hypothesized that the stimulatory effect of PPARβ/δ on fatty acid oxidation in muscle and adipose tissue might also extend to liver, which would render PPARβ/δ an anti-steatotic role in liver. Within the liver PPARβ/δ expression is found in different cell types although the highest levels are found in hepatic endothelial cells (66).

According to a recent report by Nagasawa *et al*., activation of PPARβ/δ may diminish fatty liver disease. In this study mice were fed a MCD diet to induce steatohepatitis. Administration of the PPARβ/δ agonist GW501516 not only decreased hepatic lipid content yet also reduced inflammatory gene expression. PPARβ/δ decreased fat storage in liver mainly by activation of genes involved in fatty acid oxidation. Furthermore, the elevated mRNA levels of Transforming growth factor-β1 (TGF-β1), TNFα, MCP-1 and Interleukin 1β (IL-1β) that accompany the development of steatohepatitis were counteracted by PPARβ/δ activation (67). Which liver cell types and molecular mechanisms contribute to the observed regulation is unknown.

PPARβ/δ and atherosclerosis

Due to the anti-inflammatory properties of PPARβ/δ in macrophages, it is plausible that atherosclerosis is affected by PPARβ/δ -activation. By feeding low density lipoprotein receptor (LDLR) -/- mice a hypercholesterolemic diet supplemented with a specific PPARβ/δ ligand, it was shown that PPARβ/δ is able to interfere with the inflammatory process underlying the development of atherosclerosis. Whereas lesion development itself was not prevented by PPARβ/δ activation, inflammatory gene expression was blunted compared to untreated mice (50). The anti-inflammatory action of PPARβ/δ was mainly achieved by a strong inhibition of VCAM-1, MCP-1, and IFNγ expression, genes that are associated with the development of atherosclerosis. A recent study in which LDLR -/- mice were treated with the PPARβ/δ agonist GW0742X revealed an anti-atherosclerotic effect of PPARβ/δ, in addition to an anti-inflammatory effect. Lesion development was strongly inhibited and inflammatory gene expression in macrophages was decreased (68).

While in mice there is compelling evidence for an anti-inflammatory role of PPARβ/δ in the atherosclerosis, the role of PPARβ/δ in humans is relatively unknown. Remarkably, PPARβ/δ was shown to strongly promote lipid accumulation in human macrophages thereby supporting the development of atherosclerosis (69). Whether PPARβ/δ influences inflammatory gene expression in human cells needs further study.

PPARβ/δ and adiposity

Recently, it was shown that activation of PPARβ/δ in adipose tissue causes a marked decrease in fat mass which is mainly achieved by activation of fatty acid oxidative pathways (62). Moreover, high fat diet-induced adiposity was strongly inhibited by activation of PPARβ/δ in adipose tissue. Whether PPARβ/δ is able to control inflammatory gene expression in WAT during diet-induced obesity is still unclear. Inasmuch as inflammatory gene expression is linked to adiposity, it could be hypothesized that inflammatory gene expression will be suppressed by PPARβ/δ activation. Also, since expression of IL-1β, MCP-1 and TNFα are controlled by PPARβ/δ in liver (67), it is tempting to speculate that inflammatory gene expression is under control of PPARβ/δ in adipose tissue as well.

PPARγ

PPARγ is considered the master regulator of adipogenesis and accordingly has been extensively studied in the context of obesity. In humans PPARγ is most highly expressed in adipose tissue, yet reasonable levels of PPARγ mRNA can also be found in other organs including skeletal muscle, colon and especially lung (70). The latter is probably due to the abundance of macrophages in lung. At least two different isoforms of PPARγ are known: PPARγ1, which is the form expressed in non-adipose tissues, and PPARγ2, which is adipose-tissue specific. Unsaturated fatty acids and several eicosanoids serve as endogenous agonists of PPARγ, while anti-diabetic drugs belonging to the thiazolidinediones act as synthetic agonists of PPARγ. Target genes of PPARγ are involved in adipocyte differentiation, lipid storage, and glucose metabolism, and include lipoprotein lipase, CD36, phosphoenolpyruvate carboxykinase, aquaporin 7, and adiponectin (71).

Gain and loss of function studies have shed more light on the specific functions of PPARγ in different tissues. While homozygous PPARγ-deficient animals are embryonically lethal, specific ablation in adipose tissue revealed the indispensable role of PPARγ in adipocyte differentiation and function (72). In liver, PPARγ is involved in triglyceride homeostasis and contributes to steatosis. At the same time, hepatic PPARγ protects other tissues from triglyceride accumulation and insulin resistance (73).

Similar to PPARα, PPARγ is involved in governing the inflammatory response, especially in macrophages. Currently, two different molecular mechanisms have been proposed by which anti-inflammatory actions of PPARγ are effectuated: 1) via interference with pro-inflammatory transcription factors including STAT, NF-κB and AP-1 (74), and 2) by preventing removal of co-repressor complexes from gene promoter regions resulting in suppression of inflammatory gene transcription (75). This mechanism involves ligand-dependent SUMOylation of PPARγ followed by binding of PPARγ to nuclear receptor co-repressor (NCoR)-histone deacetylase-3 (HDAC3) complexes localized on inflammatory gene promoters. The binding of PPARγ prevents the removal of co-repressor complexes thus retaining inflammatory genes in a suppressed state.

PPARγ and adiposity

PPARγ is indispensable for adipocyte differentiation both in vivo and in vitro (76) (77) (78). In spite of its vital role in adipogenesis and lipogenesis, PPARγ expression itself is not

strongly influenced during obesity. As discussed above, diet-induced obesity is associated with increased inflammatory gene expression in adipose tissue via adipocyte hypertrophy and macrophage infiltration. It has been shown that PPARγ is able to reverse macrophage infiltration and subsequently reduce inflammatory gene expression (18). Adipose expression of inflammatory markers A disintegrin and metallopeptidase domain 8 (ADAM8), Macrophage inflammatory protein 1α (MIP-1α), Macrophage antigen-1 (MAC-1), F4/80+ and CD68 was down-regulated by specific PPARγ activation. Inflammatory adipokines mainly originate from macrophages which are part of the stromal vascular fraction of adipose tissue (18) (19), and accordingly, the down-regulation of inflammatory adipokines in WAT by PPARγ probably occurs via effects on macrophages. By interfering with NF-κB signalling pathways, PPARγ is known to decrease inflammation in activated macrophages (74). PPARγ may also influence inflammatory gene expression via effects on adipocyte morphology. Indeed, smaller adipocytes are known to secrete less inflammatory markers compared to larger adipocytes (3). Treatment of obese rats with the synthetic PPARγ agonist troglitazone dramatically reduced the size of adipocytes without changing the total weight of WAT. In parallel, the expression levels of the inflammatory marker TNFα were normalized compared to those of untreated rats (79). Furthermore, by directly inducing the expression of adiponectin in adipocytes (80), PPARγ may directly contribute to suppression of chronic inflammation accompanying obesity.

Summarizing, the anti-inflammatory effects of PPARγ activation in adipose tissue are presumably achieved by effects on both adipocytes and adipose tissue-resident macrophages. Interestingly, PPARγ is induced both during macrophage and adipocyte differentiation (71). Since pre-adipocytes that are present in adipose tissue have the ability to differentiate towards macrophage type cells and towards adipocytes depending on the local environment (81), the role of PPARγ in determining the fate of pre-adipocytes is of interest. It can be hypothesized that activation of PPARγ might favor adipocyte differentiation resulting in a decreased inflammatory status of adipose tissue during obesity.

PPARγ and atheroslerosis

PPARγ is expressed in white blood cells and differentiated macrophages and has been implicated in the process of atherosclerosis. Initially, PPARγ activation was proposed to be pro-atherogenic by stimulating uptake and storage of oxidized lipids in macrophages via up-regulation of the scavenger receptor/fatty acid transporter CD36. This process leads to foam

cell development and is a key event in the development of atherosclerosis (82). In contrast, treatment with thiazolidinediones has been shown to reduce the development of atherosclerosis in mouse models (71) (50), suggesting that PPARγ is anti-atherogenic. The inhibitory effect on atherosclerosis may be mediated by up-regulating expression of the ABCA1 transporter in macrophages, thereby promoting cholesterol efflux. Furthermore, PPARγ activation strongly reduces inflammatory gene expression in macrophages, including MCP-1, VCAM-1, ICAM-1, IFNγ and TNFα (50). Several human studies also point to anti-atherogenic effects of PPARγ in type II diabetic patients. Daily administration of 400 mg troglitazone or 30 mg pioglitazone for 6 months resulted in a reduction of common carotid arterial intimal and medial complex thickness which is used as a non-invasive method to monitor early atherosclerotic lesions (83, 84). In a randomized controlled trial using 5238 patients with type II diabetes, treatment with 15 mg to 45 mg pioglitazone improved cardiovascular outcome (85). Whether these protective effects in humans are achieved by inhibiting inflammation remains to be determined.

PPARγ and steatosis

It has been well established that in mouse models of steatosis the development of fatty liver is associated with increased hepatic expression of PPARγ. In a non-fatty liver, the role of PPARγ appears to be limited and is probably restricted to stellate cell function during liver injury-induced fibrogenesis (86). During the development of steatosis, hepatocytes become lipid-loaden and gain phenotypical characteristics of adipocytes which include the formation of large lipid droplets. In parallel, expression of adipogenic and lipogenic genes such as Sterol regulatory element binding protein (SREBP), Adipose differentiation related protein (ADRP) and PPARγ is strongly up-regulated in steatotic livers (87) (88). Likely, the up-regulation of PPARγ contributes to the phenotype, since adenoviral-mediated hepatic over-expression of PPARγ1 on a PPARα -/- background dramatically increases hepatic lipid accumulation and adipogenic gene expression in mice (89). Also, marked up-regulation of PPARγ in livers of PPARα -/- mice fed a high fat diet leads to increased expression of adipocyte markers and might contribute to the fatty liver phenotype (43). In contrast, mice that specifically lack PPARγ in liver are protected from hepatic steatosis and show decreased expression levels of lipogenic genes compared to wild-type mice (90) (73). Thus, PPARγ induction appears to be necessary and sufficient for hepatic steatosis.

The development of steatosis and progression into steatohepatitis is closely linked to an increased inflammatory state of the liver (4). Recent data suggest that activation of PPARγ in fatty liver may protect against inflammation. Microarray analysis revealed that several inflammatory genes that are up-regulated in fatty livers of mice fed a high fat diet were strongly down-regulated by PPARγ over-expression in liver (89). These genes include SAA, Chemokine (C-X-C motif) ligand 10 (CXL10)/IP10 and Interferon γ inducible protein, 47 kDa. Data from our own group showed that hepatic PPARγ activation by rosiglitazone under steatotic conditions results in down-regulation of multiple pro-inflammatory genes. Thus, although activation of PPARγ in liver contributes to the development of steatosis, inflammatory gene expression is suppressed.

Several small clinical human studies have been performed to evaluate the effects of thiazolidinediones in patients diagnosed with NASH. After treatment, the degree of steatosis and inflammation improved in a number of patients indicating that PPARγ may be an interesting pharmacological target (91). Apart from weight gain, no side-effects were reported in these studies. However, more studies are needed to assess the potentially beneficial effects of PPARγ activation on liver function.

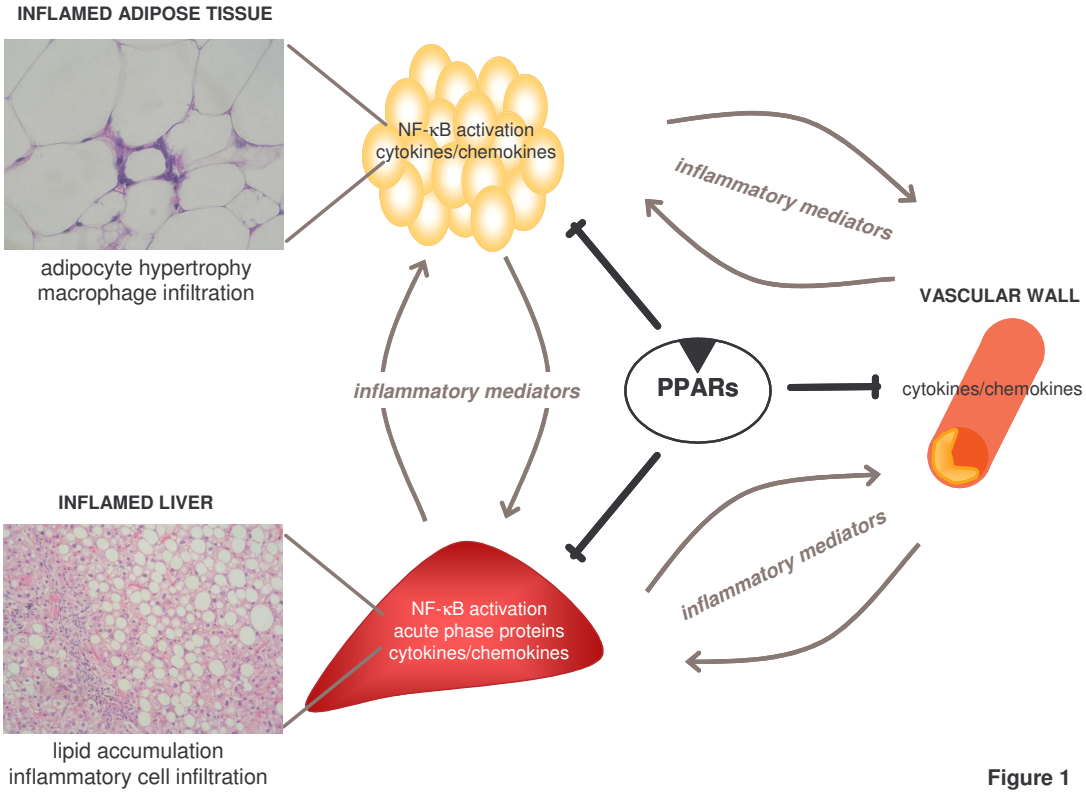

Figure 1

Figure 1 **Central role of PPARs in obesity-induced inflammation.** (Visceral) obesity and associated fatty liver stimulate inflammation in adipose tissue and liver via increased recruitment and infiltration of macrophages, resulting in increased production of pro-inflammatory cytokines. By down-regulating pro-inflammatory genes in liver, adipose tissue and the vascular wall, PPARs have a major influence on the progression of obesity-related inflammation and its complications.

Conclusion

An elevated inflammatory status is increasingly believed to be an important mediator that links excess (visceral) fat mass with numerous metabolic abnormalities, including insulin resistance. PPARs may influence the inflammatory response either by direct transcriptional down-regulation of pro-inflammatory genes via mechanisms involving transrepression, or indirectly via their transcriptional effects on lipid metabolism. Numerous animal studies have demonstrated a role for PPARs in counteracting obesity-induced inflammation in liver, adipose tissue and the vascular wall. The ability to reduce inflammatory cell infiltration further underlines the central role of PPARs in obesity-induced inflammation (Figure 1).

A growing number of studies strongly support anti-inflammatory properties of PPARs in human obesity as well. Several clinical trials in type II diabetic or hyperlipidemic patients have clearly shown that PPARα agonists including fenofibrate, ciprofibrate and gemfibrozil can effectively reduce circulating levels of TNFα, IL-6, Fibrinogen and CRP (92). Rosiglitazone, a selective PPARγ agonist, exerts anti-inflammatory effects in both obese and type II diabetic individuals by decreasing plasma concentrations of C-reactive protein, serum amyloid A, and matrix metalloproteinase (93) (94).

Since synthetic PPARα and PPARγ agonists independently ameliorate obesity induced inflammation, agonists that activate both PPARα and PPARγ (so called dual PPARα/PPARγ agonists) might be even more effective. Unfortunately, the development and clinical trials of these compounds have been hampered by serious concerns regarding their safety. Many dual PPARα/PPARγ agonists once in clinical development have since been abandoned, often for reasons of toxicity, including most recently the dual agonist tesaglitazar.

In conclusion, although more work is needed to evaluate their full potential in humans, especially in terms of safety, PPAR agonists nevertheless represent a promising strategy to mitigate obesity-associated inflammation.

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Chapter 3

Research article

The Interleukin 1 receptor antagonist is a direct target gene of PPARα in liver

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This chapter has been published in the **Journal of Hepatology**.

Abstract

The Peroxisome Proliferator-Activated Receptor (PPAR) α belongs to the superfamily of Nuclear Receptors and plays an important role in numerous cellular processes, including lipid metabolism. It is known that PPARα also has an anti-inflammatory effect, which is mainly achieved by down-regulating pro-inflammatory genes. The objective of this study was to further characterize the role of PPARα in inflammatory gene regulation in liver.
According to Affymetrix micro-array analysis, the expression of various inflammatory genes in liver was decreased by treatment of mice with the synthetic PPARα agonist Wy14643 in a PPARα-dependent manner. In contrast, expression of Interleukin-1 receptor antagonist (IL-1ra), which was acutely stimulated by LPS treatment, was induced by PPARα. Up regulation of IL-1ra by LPS was lower in PPARα -/- mice compared to Wt mice. Transactivation and chromatin immunoprecipitation studies identified IL-1ra as a direct positive target gene of PPARα with a functional PPRE present in the promoter. Up-regulation of IL-1ra by PPARα was conserved in human HepG2 hepatoma cells and the human monocyte/macrophage THP-1 cell line. In conclusion, besides down-regulation of pro-inflammatory gene expression, PPARα suppresses the inflammatory response by direct up-regulation of genes with anti-inflammatory properties.

Abbreviations used: IFN, Interferon; IL-1ra, Interleukin-1 receptor antagonist; IL-6R, Interleukin 6 Receptor; IL-1RacP, Interleukin-1 Receptor accessory Protein; LIFR, Leukaemia Inhibitory Factor Receptor; LPS, Lipopolysaccharide; PMA, Phorbol Myristic Acid; PPAR, Peroxisome Proliferator-Activated Receptor; PPRE, PPAR Response Elements; RXR, Retinoid X Receptor; SAA, Serum Amyloid A; STAT3, Signal Transducer and Activator of Transcription 3; Wt, Wildtype.

Introduction

Inflammation describes the comprehensive reaction of the host to various types of injury, which is generally protective and aimed at promoting tissue repair. The inflammatory response is mediated by a diverse group of cytokines and other signaling molecules that are able to profoundly influence cellular function. At the cellular level, numerous signaling pathways and transcription factors conspire in a complex network to produce the appropriate response. In recent years it has become clear that the Peroxisome Proliferator Activated Receptors (PPARs) modulate this response in a variety of organs. PPARs are members of the superfamily of Nuclear Receptors that play a pivotal role in mediating the effect of small lipophilic ligands on gene transcription (1). The three isotypes of the PPAR-family, PPARα, PPARβ/δ and PPARγ, have been implicated in numerous processes, including lipid and glucose metabolism, and inflammation. Activation of the receptor occurs by binding of various ligands, ranging from natural compounds such as fatty acids to highly specific synthetic agonists. Upon ligand-activation, binding to so called PPAR Response Elements (PPRE) located in the promoter of target genes results in increased gene transcription. To accomplish activation of gene transcription it is essential that PPAR forms a heterodimer with the Retinoid X Receptor (RXR) (2).

Besides their ability to enhance gene transcription (3), PPARs are also able to suppress gene expression. For example, it has been shown that activated PPARα lowers the expression of several enzymes connected with amino acid metabolism (4), although the mechanism behind the observed down-regulation remains unknown. In addition, the effects of PPARα on inflammation are mainly achieved by suppressing gene expression. Since the initial observation that PPARα-/- mice have a prolonged inflammatory response (5), an important role for PPARs in regulating inflammatory responses has clearly emerged. Although numerous studies have demonstrated the protective and anti-inflammatory effects of PPARα-activation in liver (6) (7), information about the precise molecular mechanisms involved is somewhat limited. One of the mechanisms by which this nuclear receptor exerts its anti-inflammatory action is through modulation of the NF-κB pathway. Physical interaction of PPARα with NF-κB prevents its activation and downstream pro-inflammatory effects (8). Moreover, PPARα has been shown to up-regulate the expression of IκB, the natural NF-κB inhibitor that prevents the nuclear translocation and activation of the pro-inflammatory transcription factor (9).

Anti-inflammatory properties have also been assigned to the other two PPAR isotypes. Activation of PPARγ controls the inflammatory status of the intestinal tract (10) and is responsible for the down-regulation of a specific subset of pro-inflammatory genes in macrophages (11). The recent generation of macrophages lacking PPARβ/δ has also revealed a specific role for PPARβ/δ in regulating inflammatory processes (12).

To better understand the regulatory role of PPARα in liver and to identify possible new target genes under the control of PPARα, we studied PPARα-dependent gene expression levels in mouse liver by means of Affymetrix microarray analysis. Activation of PPARα was achieved by treating Wildtype (Wt) and PPARα -/- mice with the synthetic PPARα agonist Wy-14643. While numerous inflammatory genes were found to be down regulated by PPARα activation, the IL-1 receptor antagonist, however, was highly up-regulated by PPARα. Additional experiments indicated that the IL-1 receptor antagonist is a direct target gene of PPARα. Our data suggest that PPARα may modulate inflammation by direct up-regulation of target genes.

Material and Methods

Chemicals

Wy14643 was obtained from ChemSyn Labarotories (Lenexa, Kanasa). Cell culture medium, fetal calf serum and penicillin/streptomycin/fungizone were from Cambrex Bioscience (Seraing, Belgium). SYBR green was from Eurogentec (Seraing, Belgium).
The human and mouse antibody against IL-1ra and the recombinant hIL-1β were from R&D systems (R&D Systems Europe Ltd, Abingdon, UK). Otherwise, chemicals were from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Animal experiments

Sv129 PPARα-/- mice and corresponding Wt mice were purchased at the Jackson Laboratory (Bar Harbor, Maine, USA). For the fasting experiment, male mice were fasted for 24 hours starting at the onset of the light cycle. For the feeding experiments with Wy14643 (0.1%), L165041 (0.025%) and Rosiglitazone (0.01%), ligands were mixed in the food and given to female mice for 5 days. Liver was dissected and directly frozen into liquid nitrogen. Lipopolysaccharide (LPS) (*E. coli*; Sigma-Aldrich, St-Louis, MO) was administered at a dose of 1 mg/kg IP. After 3 hours or 16 hours of treatment, liver was dissected and frozen into liquid nitrogen. The animal experiments were approved by the animal experimentation committee of the Wageningen University, the Netherlands, the district government of Lower Saxony, Germany and the Institutional Animal Care and Use Committee of Emory University, United States.

Oligonucleotide microarray

Total RNA was isolated from mouse liver using Trizol reagent (Invitrogen, Breda, The Netherlands) following the supplier's protocol. For the microarray experiment, 10 μg of total liver RNA pooled from 5 or 3 mice was used for cRNA synthesis. To confirm integrity of the RNA, bioanalyzer (Agilent, Amsterdam)) analysis was done before the hybridization process was started. The Affymetrix Mouse Expression array 430A was used and results were analyzed using Microarray Suite and Data Mining Tool (DMT) software following

instructions of the manufacturer. Heat Map analysis was done using Spotfire DecisionSite software (Spotfire Inc, Sommerville, MA).

RNA Isolation and RT-PCR

RNA from animal tissue or cells was extracted with Trizol reagent using the supplier's instructions. After treatment with Dnase I amplification grade (Invitrogen), 4 or 5 μg of RNA was used for reverse transcription with Superscript II RT Rnase H (Invitrogen) using oligo (dT) primers following manufacturer's instructions.

Real Time Quantitative PCR

PCR was performed with platinum Taq polymerase (Invitrogen) and SYBR green using an iCycler PCR machine (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). The primers were designed using Primer3 software (http://cbr-rbc.nrc-cnrc.gc.ca/cgi-bin/primer3_www.cgi) and are listed in table I. Only primer pairs yielding unique amplification products were used for real-time PCR analysis. Generated PCR-product sizes were between 90-260 bp. As an internal control, the expression of the housekeeping gene β-actin was measured which remained constant during all of the experimental conditions studied.

Plasmids and DNA constructs

Mouse genomic DNA (mouse strain C57/B6) was used to PCR-amplify 1900 bp of the soluble IL-1ra promoter. The forward primer sIL-1ra-Fprom 5'CCGCTCGAGCGGTGAGCAAATAGAATAGTC 3' and the reverse primer sIL-1ra-Rprom 5' CCCAAGCTTGGGACAGAAGGATGAGAAGGA 3' including restrictions sites for both XhoI and HinDIII were used to PCR-amplify 1900 bp of the sIL-ra promoter. The generated fragment was subcloned into the XhoI and HinDIII sites of the pGL-3 basic vector (Promega Corp., Leiden, The Netherlands). Mutations of the PPRE were obtained using two separate partially overlapping PCR fragments generated using the wildtype sIL-1ra promoter as a template. The forward primer sIL-1ra-mutF 5' TTTCTCTAGGGCTGAGGACAGCAAACTTCT 3' combined with primer sIL-1ra-Rprom and the reverse primer sIL-1ra-mutR 5' AGAAGTTTGCTGTCCTCAGCCCTAGAGAAA 3'

combined with primer sIL-1ra-Fprom were used to generate the two partially overlapping PCR fragments. In a final PCR, the two fragments with overlapping ends were used to amplify the mutated sIL-1ra promoter with the forward primer sIL-1ra-Fprom and the reverse primer sIL-1ra-Rprom.The final product was cloned into the XhoI and HindIII sites of the pGL-3 basic vector. cDNA corresponding to mPPARα was cloned into pSG5 (Stratagene). After cloning, fragments were sequenced to confirm the integrity of the constructs. The RXRα expression plasmid was a generous gift of Dr. S.A. Kliewer.

Primary mouse hepatocyte isolation

Primary mouse and rat hepatocytes were isolated as described previously (13). Briefly, after cannulation of the portal vein, the liver was perfused with calcium free HBSS which was pre-gassed with 95% O_2 /5% CO_2. Next, the liver was perfused with a collagenase solution until swelling and degradation of the internal liver structure was observed. The hepatocytes were released, filtered and washed several times using Krebs buffer. The viability was assessed by tryptan blue staining and was at least 80%. Cells were cultured in William's Medium E supplemented with 10% FCS, penicillin/streptomycin/fungizone, insulin and dexamethasone. Cells were plated in collagen (Serva Feinbiochemica, Heidelberg, Germany) coated wells with a density of 0.5 x 10^6 cells/ml. After 4 hours of incubation, the medium was removed and replaced with fresh medium. The next day, hepatocytes were used for experiments and treated with IL-1β (5ng/ml) for 24h.

Cell Culture and transfection

Human hepatoma HepG2 cells were obtained from the ATCC (Manassas, VA, USA) and grown in DMEM containing 10% FCS and PSF. THP-1 cells were from ATCC and grown in RPMI-1640 containing 10% FCS and PSF. HepG2 cells were transfected using calcium phosphate precipitation. A β-galactosidase reporter was co-transfected to normalize for differences in transfection efficiency. After transfection, cells were treated with the PPARα ligand Wy14643 at 50 μM or vehicle (DMSO) for 24 hours prior to lysis. Promega luciferase assay (Promega) and standard β-galactosidase assay with 2-nitrophenyl-BD galactopyranoside were used to measure the relative activity of the promoter. For expression experiments in HepG2 cells, FCS was removed from the medium when ligand or cytokines

were added. THP-1 cells were differentiated towards macrophages using phorbol myristic acid (Sigma-Aldrich) at a concentration of 100 μM.

Chromatin Immunoprecipitation (ChIP)

Wt or PPARα -/- mice were used and fed by gavage with either Wy14643 (50 mg/kg/day) or vehicle (0.5 % carboxymethyl cellulose) for 5 days or fasted for 24h. After treatment, mice were sacrificed by cervical dislocation and the liver was perfused with prewarmed (37 °C) phosphate-buffered saline for 5 minutes followed 0.2% collagenase for 10 min. The liver was diced and forced through a stainless steel sieve and the hepatocytes were collected into DMEM containing 1% formaldehyde. After incubation at 37 °C for 15 min, the hepatocytes were pelleted and ChIP was performed using a mouse PPARα-specific antibody as previously described (14). The sequences of the primers used for PCR were 5'-CAGATGCAGAATTGGGAAAAGATG-3' for the forward primer and 5'-GCAAGCAATAGGGCCTGGTGAAC-3' for the reverse primer. Control primers used were 5'-CTCCCTTTCCCCTTCTGTCCCTCTCATT-3' for the forward primer and 5'-TTCCCAAACTCCCCACCCCATCC-3' for the reverse primer.

Western Blot

Western blotting was carried out using an ECL system (Amersham Biosciences) according to the manufacturer's instructions. Acetone precipitated protein from equal amounts of medium from HepG2 cells or equal amounts of mouse total liver cell lysates as determined by Bio-Rad Protein Assay reagent (Bio-Rad Laboratories BV) were used and resolved by SDS/PAGE on a 12% polyacrylamide gel. A protein marker (Invitrogen) was used to determine the sizes of the separated proteins. Separated proteins were transferred to Immobilon-P transfer membranes (Millipore).The primary antibody was used at a dilution 1:1000 and the membranes were incubated overnight at 4 °C. The secondary antibody was used at a dilution of 1:5000. All incubations were performed in 1X Tris-buffered saline, pH 7.5, with 0.1 % Tween 20 and 5% dry milk. In the final washings, dry milk was removed from the solution.

Statistical analysis

The Student's T-test was used to calculate statistically significant differences.

Table 1 Primer sequences used for Real Time PCR

Gene	*Forward primer*	*Reverse primer*	*Product size*
Mouse sIL-1ra	AAATCTGCTGGGGACCCTAC	TGAGCTGGTTGTTTCTCAGG	164 bp
Mouse icIL-1ra	CAGTTCCACCCTGGGAAGGT	AGCCATGGGTGAGCTAAACAGGACA	128 bp
Mouse STAT3	GACCCGCCAACAAATTAAGA	TCGTGGTAAACTGGACACCA	214 bp
Mouse IL-18	ACAACTTTGGCCGACTTCAC	GGGTTCACTGGCACTTTGAT	127 bp
Mouse IL-1RAcP	TTGCCACCCCAGATCTATTC	CCAGACCTCATTGTGGGAGT	122 bp
Mouse SAA	GCGAGCCTACACTGACATGA	TTTTCTCAGCAGCCCAGACT	121 bp
Mouse LIF-receptor	AGGAATGCCACAATCAGAGG	AACCCGGAAAGTGTATGCAG	90 bp
Mouse IL-6 receptor	CAGCGACACTGGGGACTATT	AGTCACTCTTCCCGTTGGTG	220 bp
Mouse β-actin	GATCTGGCACCACACCTTCT	GGGGTGTTGAAGGTCTCAAA	139 bp
Human sIL-1ra	GCCTCCGCAGTCACCTAAT	TCCCAGATTCTGAAGGCTTG	108 bp
Human β-actin	AACACCCCAGCCATGTACG	ATGTCACGCACGATTTCCC	254 bp

Results

PPARα, but not PPARβ/δ and PPARγ, is involved in the negative regulation of inflammatory related-genes in liver-To screen for potential novel target genes of PPARα involved in inflammation in liver we performed micro-array analysis. A comparison was made between liver RNA from Wt and PPARα-/- mice treated or not with the synthetic PPARα ligand Wy14643. The expression of a large number of genes involved in inflammation was decreased by Wy14643 in Wt but not PPARα-/- mice (Fig. 1A). As shown in the Fig. 1B, the expression of all of these genes was up-regulated during Lipopolysaccharide (LPS)-induced acute inflammation. These included serum amyloid A, orosumucoid, metallothionein, Signal Transducer and Activator of Transcription 3, IL-18, and IL-1RAcP. An exception to this pattern of regulation was IL-1Ra, which in contrast to the numerous other genes induced by LPS, was up-regulated by PPARα activation. Real-time quantitative PCR (qPCR) confirmed the marked suppression of the selected genes involved in inflammation by Wy14643 in Wt but not PPARα-/- mice (Fig. 2).

To evaluate whether the down-regulation of inflammatory genes in liver is specific to PPARα activation, we studied the effect of synthetic PPARβ/δ and PPARγ agonists. Whereas Wy14643 markedly reduced expression of the selected set of genes, no changes or an opposite response was observed after administration of L165041 and rosiglitazone (Fig. 3).

PPARα activation enhances gene expression of the anti-inflammatory IL-1 receptor antagonist-While many genes involved in inflammation were down-regulated by Wy14643, hepatic expression of Interleukin 1 receptor antagonist (IL-1ra) was highly increased by PPARα activation (Fig. 1). IL-1ra is believed to have mainly anti-inflammatory properties (15). The IL-1ra gene is expressed in multiple forms using two different promoters, resulting in three intracellular forms transcribed via the 5' promoter and one soluble form transcribed via a different promoter (16). To more closely examine the regulation of IL-1ra by PPARα in liver, specific primers were developed to distinguish between the intracellular (icIL-1ra) and soluble (sIL-1ra) form of IL-1ra. QPCR showed that the soluble form but not the intracellular form is regulated by Wy14643 and fasting in mouse liver in a PPARα-dependent manner (Fig. 4A,B).

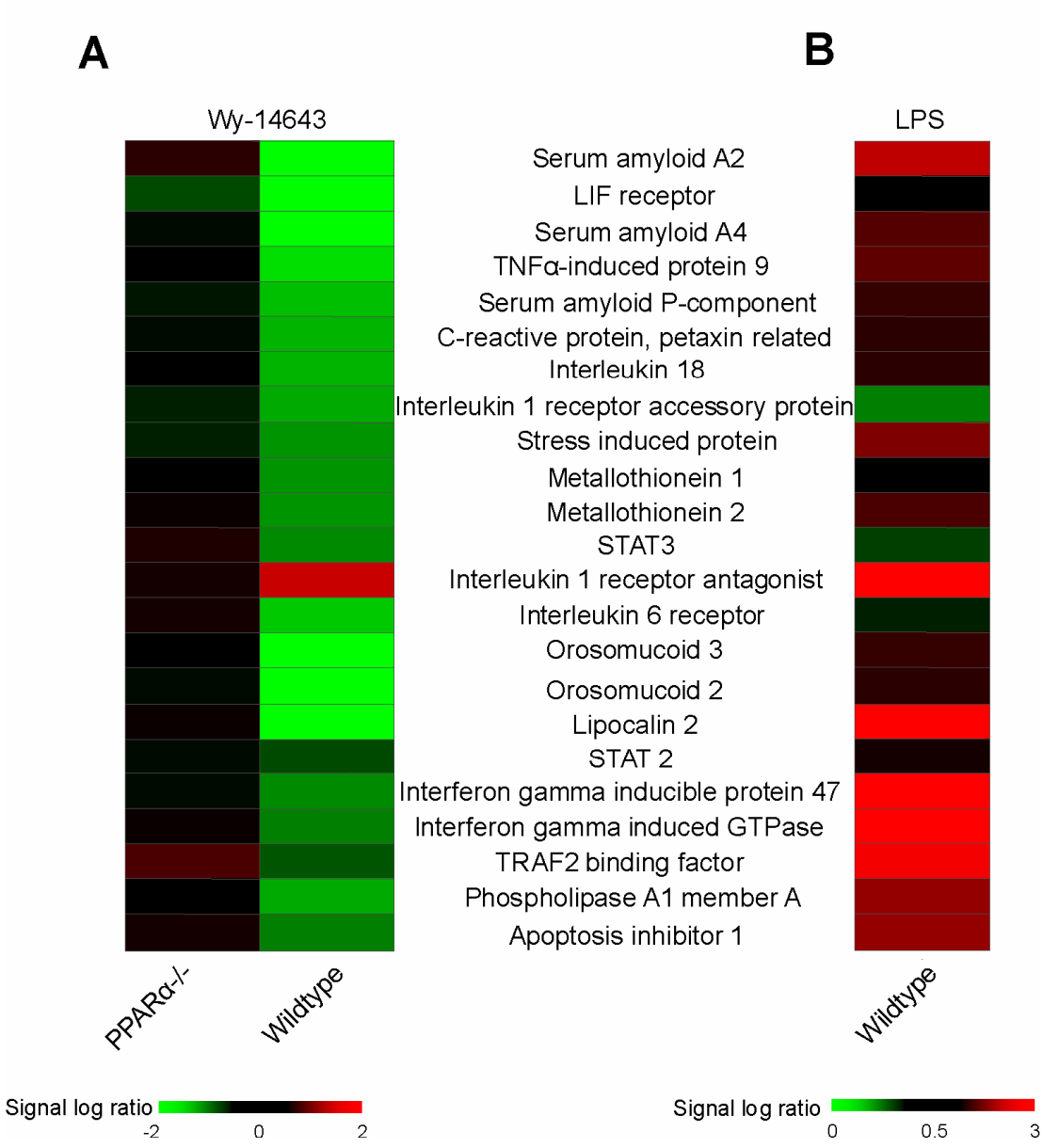


Figure 1 **Microarray analysis of mouse liver after PPARα activation and LPS treatment.**

Wildtype mice and PPARα-/- mice were exposed to Wy14643 0.1% for 5 days after which RNA was isolated. Pooled liver RNA (n=5) was used for microarray analysis with the Affymetrix Mouse Expression Array 430A (A). Wt mice were exposed to LPS for 3h and pooled liver RNA (n=3) was analyzed using microarray (B). SLR= Signal log ratio, the actual fold change can be calculated by using fold change = $2^{(SLR)}$ and represents the fold change between mice receiving either Wy14643 or LPS and untreated mice. Fold-changes for all genes shown were statistically significant.

It should be mentioned that icIL-1ra mRNA was difficult to detect by qPCR compared to sIL-1ra and to our knowledge no function has been assigned to icIL-1ra in liver. Further analysis showed that only the PPARα isotype is able to upregulate sIL-1ra expression in liver (Fig. 4C). Protein levels of IL-1ra analyzed in liver cell lysates of PPARα -/- and Wt mice treated or not with Wy14643 matched the gene expression data (Fig. 5).

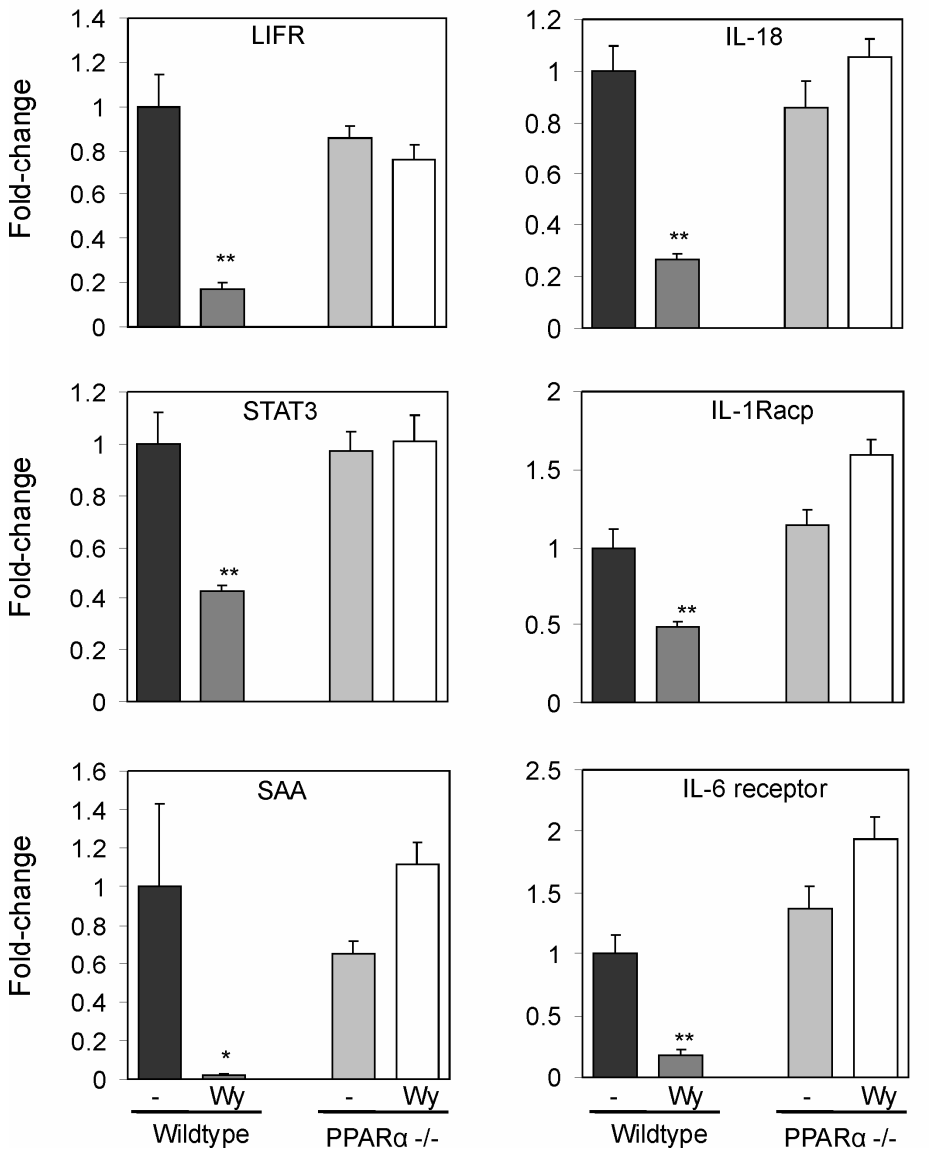


Figure 2 **Confirmation of gene expression results from the microarray experiment using qPCR.** Gene expression results of LIFR, STAT3, SAA, IL-18, IL-1RacP, and the IL-6 receptor were confirmed by qPCR on liver of Wt and PPARα-/- animals fed the PPARα agonist Wy14643. Expression of the Wt control animals was set at 1. Differences between Wt mice treated with Wy14643 and Wt control mice were evaluated by Student's T-test (** =P<0.005, * =P<0.05). Error bars represent SEM (n=4).

PPARα is essential to induce expression of sIL-1ra during inflammatory conditions in liver- To investigate whether PPARα is involved in the regulation of sIL-1ra during inflammatory conditions, Wt and PPARα -/- mice were exposed to LPS. As expected, LPS treatment increased sIL-1ra gene expression, however the increase was significantly higher in Wt mice compared to PPARα -/- mice (Figure 6A). Induction of IL-1β expression is similar between Wildtype and PPARα -/- mice after exposure to LPS (data not shown). Subsequently, the ratio of IL-1ra to IL-1β, which is often used to determine the activation of IL-1 signaling pathways, was drastically improved in Wt mice compared to PPARα -/- mice after exposure to LPS, suggesting a shift towards a more anti-inflammatory phenotype (Figure 6A). Treatment of Wt and PPARα -/- primary mouse hepatocytes with IL-1β to induce sIL-1ra gene expression gave comparable results, as shown by a more pronounced increase in sIL-1ra gene expression in hepatocytes expressing PPARα compared to PPARα -/- hepatocytes (Figure 6B).

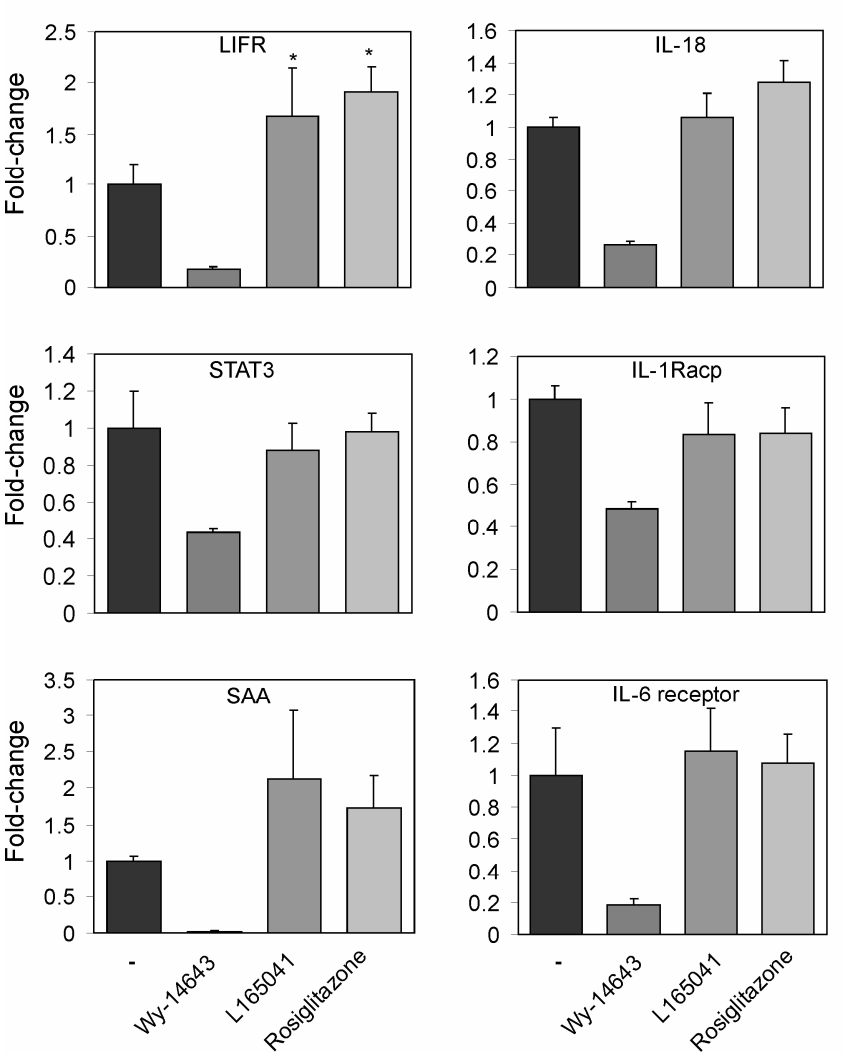


Figure 3 **PPARα is mainly responsible for the gene expression changes in liver.** Wildtype mice were fed the PPARα agonist Wy14643, the PPARβ/δ agonist L165041 or the PPARγ agonist Rosiglitazone. Liver RNA was isolated and qPCR was performed for LIFR, STAT3, SAA, IL-18, IL-1RAcP and the IL-6 receptor. Gene expression levels from the animals receiving vehicle only were set at 1. * Significantly different between control and treated animals (Student's T-test, P<0.05). Error bars represent SEM (n=5).

sIL-1ra is a true target gene of PPARα in mouse liver-To determine whether the sIL-1ra promoter is directly regulated by PPARα, 1.9 kb of promoter region of the soluble isoform was cloned into a luciferase reporter vector and used in transactivation assays. Co-transfection

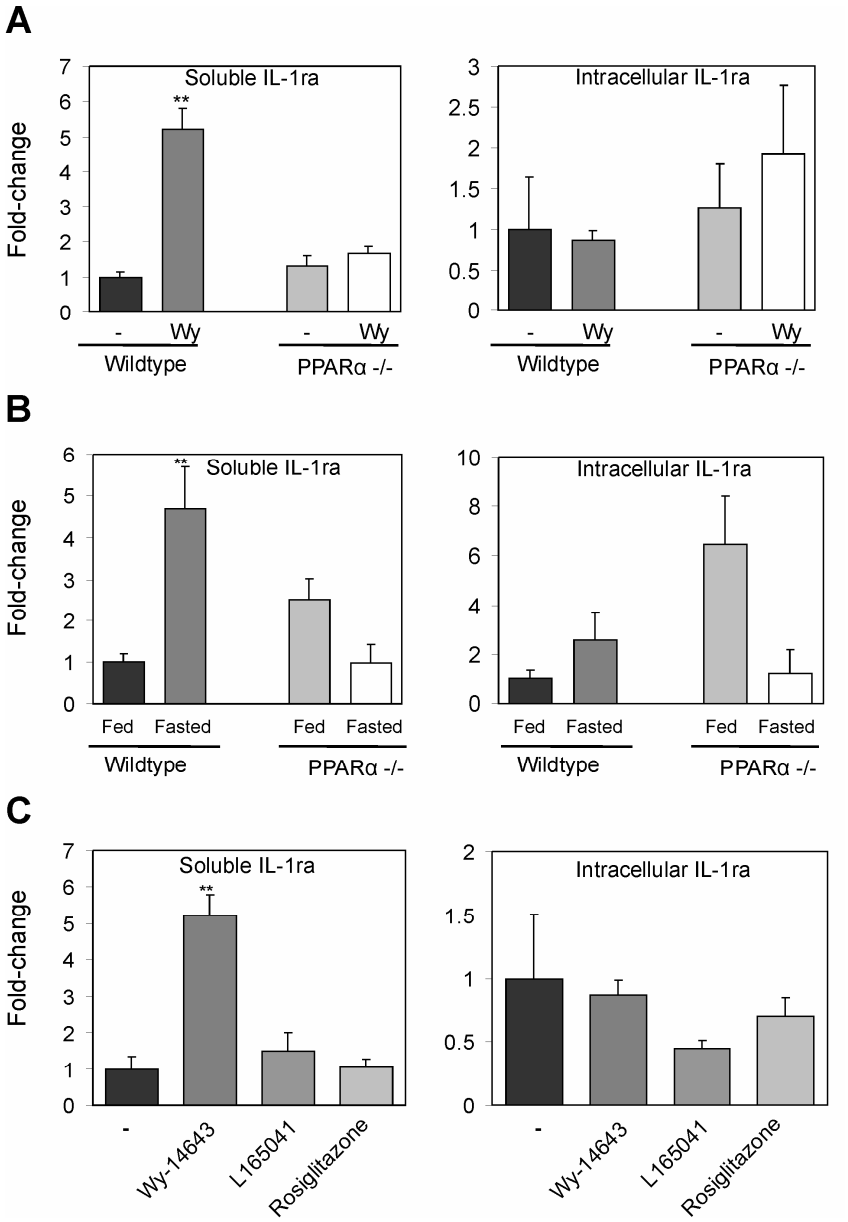


Figure 4 **PPARα regulates gene expression of sIL-1ra in mouse liver.** Expression of sIL-1ra and icIL-1ra was determined by qPCR in Wt and PPARα null mice after feeding with Wy14643 (A) and in fed and 24-hour fasted state (B). (C) Mice were given Wy14643, L165401 and Rosiglitazone and sIL-1ra and icIL-1ra expression was determined in mouse liver. ** Significantly different between control and fasted or Wy14643 fed animals (Student's T-test, P<0.005). Error bars represent SEM (n=4).

with PPARα and RXRα expression plasmids revealed that while RXRα alone did not have any effect on the promoter activity (data not shown), transfection of both PPARα and RXRα expression plasmids increased the activity of the sIL1-ra promoter (Fig. 7A). This effect was further enhanced by treatment of cells with Wy14643. In silico analysis of the sIL-1ra promoter using Nubiscan V2.0 software identified a possible PPRE located at around 700 bp upstream of the transcription start site. To investigate whether this PPRE may be responsible

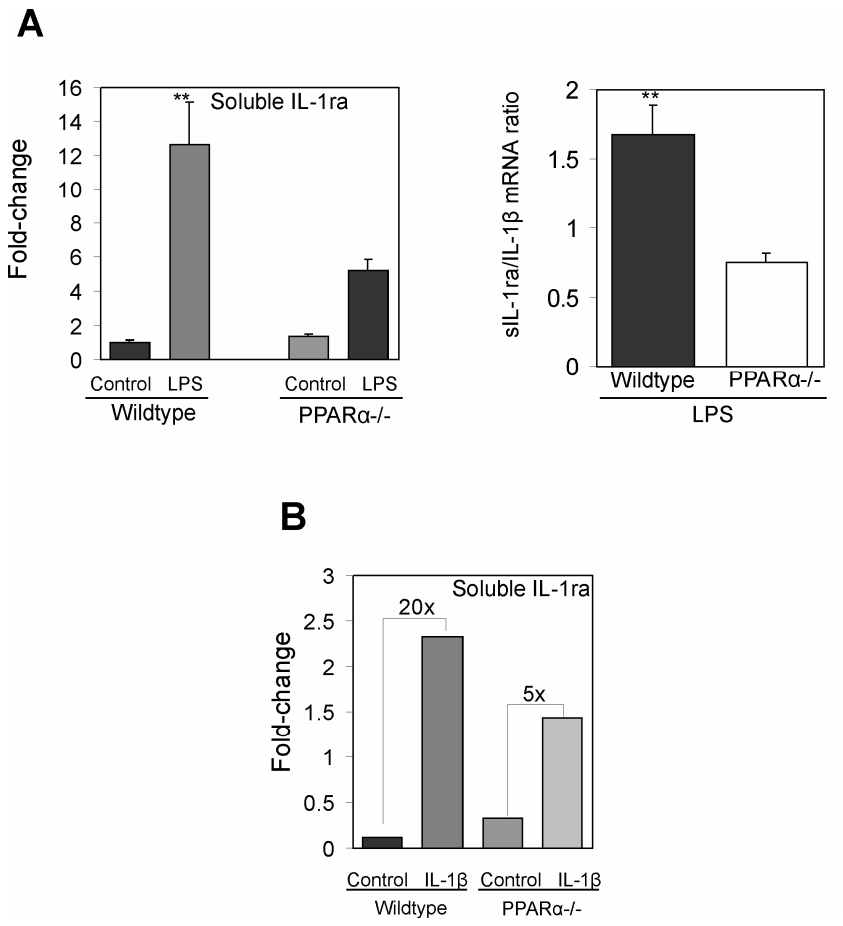
Figure 5 **Protein levels of IL-1ra are PPARα-dependently increased in mouse liver.** Equal amounts of total liver cell lysates from Wt and PPARα -/- animals fed the PPARα agonist Wy14643 were analysed by Western Blot using a mouse IL-1ra antibody. Migration of molecular-weight markers is shown.

for PPARα-mediated transactivation of the sIL-1ra gene, the response element was disabled by site-directed mutagenesis (Fig. 7B). After mutating the PPRE, the response to PPARα and Wy14643 was completely abolished, suggesting that the effects of PPARα are mediated by the PPRE identified (Fig. 7C).

Finally, to investigate if PPARα is bound to the PPRE identified in mouse liver, *in vivo* chromatin immunoprecipitation (ChIP) was performed using an anti-PPARα antibody. In mice, treatment with Wy14643 and fasting increased the binding of PPARα to this PPRE in Wt but not PPARα -/- mice. No binding was observed to a control sequence located 1800 base pairs upstream from the PPRE (Fig. 7D). Taken together, these data indicate that the PPRE identified mediates the effects of PPARα on sIL-1ra gene expression.

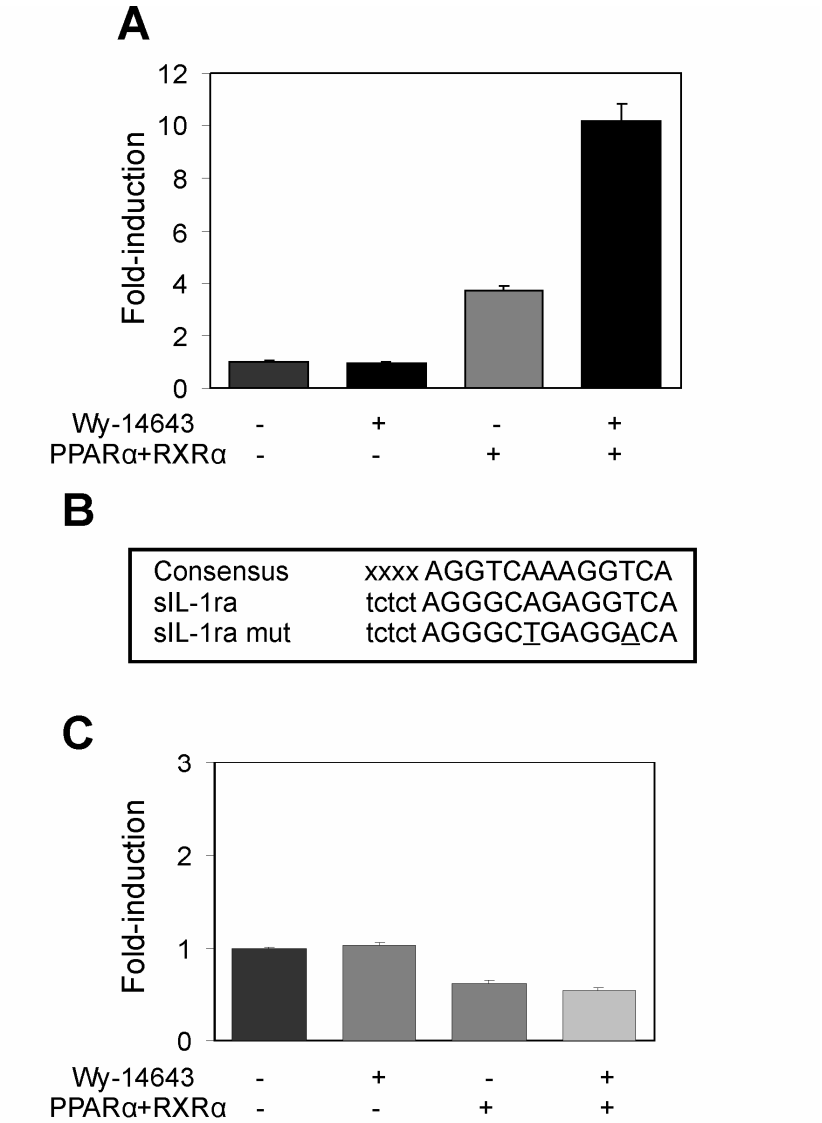
sIL-1ra is also regulated by PPARα in human cells-To evaluate whether the effects seen in mouse liver can also be reproduced in human liver cells, HepG2 cells were used. HepG2 cells only express the soluble form of the IL-1ra gene (17) and treatment of the cells with IL-1β, an inducer of sIL-1ra expression, caused the expected increase in gene expression levels (Fig. 8A). Incubation with RXRα and PPARα agonists alone did not result in major changes in gene expression. However, combined treatment with both IL-1β and PPARα/RXRα agonists synergistically increased sIL-1ra expression. Since soluble IL-1ra is secreted, medium from

Figure 6 **PPARα is essential for the induction of sIL-1ra in liver during LPS-induced inflammation.** (A) Wt and PPARα -/- animals were exposed to LPS or saline for 16h. Liver RNA was isolated and expression of sIL-1ra was measured by qPCR. Significant differences for sIL-1ra expression between Wt and PPARα -/- mice treated with LPS were observed (Student's T-test, P<0.01). The sIL-1ra to IL-1β ratio after exposure to LPS was calculated in Wildtype and PPARα -/- mice after LPS treatment. IL-1β expression was determined via qPCR. sIL-1ra to IL-1β ratios of Wildtype and PPARα -/- mice were significantly different (Student's T-test, P<0.002). Error bars represent SEM (n=4). (B) Primary mouse hepatocytes from Wt and PPARα -/- mice were treated with IL-1β (5ng/ml) or vehicle. After 24h, expression levels of sIL-1ra were determined via qPCR.



treated HepG2 cells was analyzed for IL-1ra protein concentration. IL-1ra protein levels nicely followed the sIL-1ra mRNA induction in these cells (Fig 8B) with the upper band representing the glycosylated form of the sIL-1ra protein and the lower band the unglycosylated protein (18). Next, the expression of sIL-1ra was examined in the human derived monocyte/macrophage THP-1 cell line (Fig 8C). Since it is known that PPARα localization shifts from hepatocytes towards Kupffer cells during hepatic inflammation (19), it was of interest to test the ability of PPARα to regulate the expression of sIL-1ra in this cell type. The expression of sIL-1ra was increased by differentiation of the cells towards macrophages using phorbol myristic acid (PMA). In contrast to the expression levels in monocytes, the expression level of sIL-1ra in macrophages was increased by Wy14643. These data indicate that sIL-1ra is a target of PPARα in human hepatocytes and macrophages.

Figure 7 **Up regulation of sIL-1ra by PPARα is regulated by a PPRE present in the sIL-1ra promoter.** (A) HepG2 cells were transfected with the Wt sIL-1ra promoter and expression plasmids for PPARα and RXRα. Cells were treated with Wy14643 (50 μM) for 24h after which luciferase and β-galactosidase activities were determined in the cell lysates. The relative luciferase activity of the DMSO treated cells was set to 1. Error bars represent SEM (n=3) (B) Alignment of the consensus PPRE with the sequence found in the mouse soluble promoter and the mutated PPRE used in the transfection experiment. (C) HepG2 cells were transfected with the mutant sIL-1ra promoter and expression plasmids for PPARα and RXRα and treated with Wy14643 (50 μM) for 24h. Thereafter, relative luciferase activity of the cell lysates were determined. (D) In vivo Chromatin Immunoprecipitation (CHIP) of the PPRE in the sIL-1ra mouse promoter was performed using an antibody against PPARα. The gene sequence spanning the putative PPRE and a random control sequence were analyzed by PCR in the immunoprecipitated chromatin of mouse liver. Livers of fasted and Wy14643 fed animals were used.


A
Fold-induction
12
10
8
6
4
2
0
Wy-14643 - + - +
PPARα+RXRα - - + +
B
Consensus xxxx AGGTCAAAGGTCA
sIL-1ra tctct AGGGCAGAGGTCA
sIL-1ra mut tctct AGGGCTGAGGACA
C
Fold-induction
3
2
1
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Wy-14643 - + - +
PPARα+RXRα - - + +
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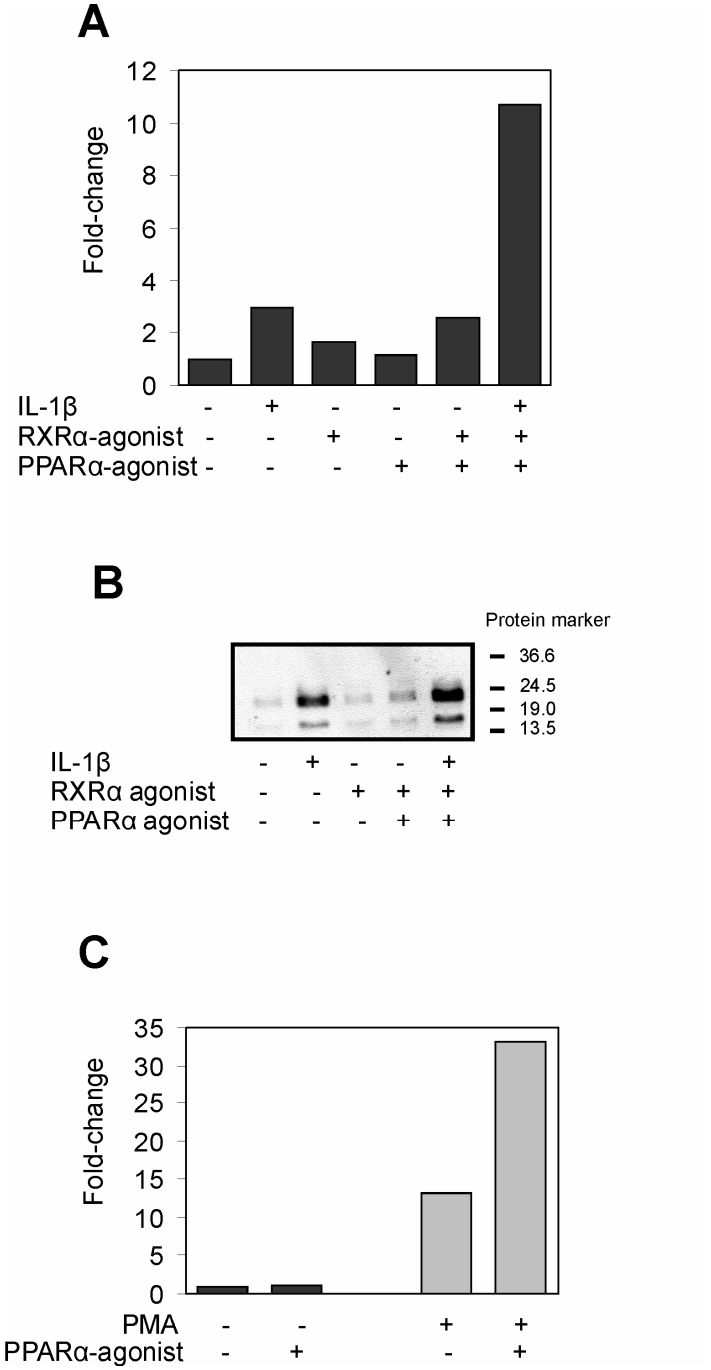

Figure 8 **Human sIL-1ra gene expression is affected by PPARα.** (A) HepG2 cells were treated with IL-1β (10 ng/ml), RXR-agonist (5 μM) and PPARα- agonist (100 μM Wy14643) for 24h. sIL-ra gene expression was determined using qPCR. The relative expression from the DMSO treated cells was set on 1. (B) The culture medium was analyzed for IL-1ra protein levels by Western blotting using a human IL-1ra antibody. Migration of molecular-weight markers is shown. (C) Undifferentiated THP-1 cells and THP-1 cells differentiated to macrophages using PMA (100 μM) were treated with Wy14643 (50 μM) for 36h. sIL-ra gene expressed was measured by qPCR. The relative expression of undifferentiated THP-1 cells treated with DMSO was set at 1.

Discussion

Since the initial discovery indicating that PPARα is involved in inflammation (5), considerable progress has been made towards resolving the regulatory role of PPARα during inflammation (8, 9, 20). It is now clear that PPARα mainly governs inflammation by down-regulating expression of genes, including several acute phase genes.

Here, we show that soluble Interleukin 1 Receptor antagonist is a direct target gene of PPARα with a functional PPRE in its promoter, suggesting that PPARα may also govern inflammation by direct up-regulation of target genes. IL-1ra is an inhibitor of cytokine signaling and is produced by many cells including hepatocytes. During the acute phase response, the expression of IL-1ra is strongly induced and IL-1ra is therefore referred to as a positive acute phase protein. In our hands, hepatic sIL-1ra expression was highly up-regulated after LPS treatment. By binding to the IL-1 receptor with almost equal affinity as IL-1, it prevents activation of the pro-inflammatory IL-1 pathway and downstream NF-κB activation (21). Under non-inflammatory conditions, PPARα activation by Wy14643 or fasting markedly increased sIL-1ra gene expression in liver, which was not observed in PPARα -/- mice. Thus, PPARα seems to be able to increase the expression of sIL-1ra in liver independently of any inflammatory stimulus. Our observation that induction of sIL-1ra during LPS-induced inflammation is lower in animals lacking PPARα strongly suggests that PPARα is also necessary for the induction of sIL-1ra in liver during acute inflammatory conditions. Importantly, after LPS treatment the ratio of sIL-1ra to IL-1β, which can be considered as a marker for activation of IL-1 signaling pathways, was significantly improved in Wt compared to PPARα -/- mice. These data suggests that PPARα promotes a shift towards a more anti-inflammatory phenotype. Transactivation and chromatin immunoprecipiation experiments indicated that PPARα regulates sIL-1ra expression via a PPRE located around 700 bp upstream of the transcription start site, thus clearly establishing IL-1ra as a direct PPARα target gene.

In human HepG2 cells PPARα activation increased sIL-1ra gene expression when cells were co-treated with IL-1β, which is known to induce expression of sIL-ra via binding of NF-κB and C/EBP to the sIL-1ra promoter (22). In THP-1 cells differentiated towards macrophages, PPARα also induced sIL-1ra expression. Since macrophage/monocyte type cells including Kupffer cells have an important role in controlling hepatic inflammation (23), the PPARα-dependent regulation of the IL-1ra gene in these cells might contribute to the immune-modulating functions of PPARα in liver. Together, these data indicate that sIL-1ra is a direct

target gene in mouse and human hepatocytes, as well as in human macrophages. By inhibiting binding of IL-1β to its receptor via increased expression of its natural antagonist, PPARα might prevent or counteract the activation of the IL-1β-signalling cascade including the pro-inflammatory NF-κB pathway.

The availability of microarray techniques offers the opportunity to screen for new PPARα controlled genes and pathways with the aim of elucidating the effects of PPARα on inflammation. Using this approach, our initial analysis focused on inflammation-related genes that are regulated by PPARα activation in liver under normal conditions. Many of the genes that were decreased in liver of mice treated with Wy14643 were increased during acute inflammation mimicked by LPS administration. Several acute phase response genes, cytokine receptor components, and intracellular signaling transducers were affected. The results for several of these genes corresponds well with earlier studies (24, 25), whereas for other genes the linkage to PPARα is entirely novel.

IL-18, a pro-inflammatory cytokine with an established role in liver injury (26), is one of the genes that was decreased after PPARα activation. Furthermore, the LIF-receptor, which belongs to the IL-6 receptor family, was strongly decreased after PPARα-agonist treatment in mouse liver. Downstream of this pathway, PPARα is also ably to suppress the expression of STAT3, a transcription factor implicated in the regulation of inflammatory signaling in liver (27). Finally, PPARα seems to have a role in regulating IFNγ-activated genes.

Our microarray analysis provides experimental support for a previous study showing that the expression of specific components of the inflammatory IL-6 signaling cascade was suppressed by PPARα agonists. Subsequent exposure to IL-6 led to a diminished acute phase response in liver (24). The microarray analysis also reveals several additional candidate genes by which PPARα might exert its potent anti-inflammatory effects in liver.

In conclusion, by negatively regulating multiple components of inflammatory signalling pathways ranging from cytokines to receptor complexes and downstream target genes, PPARα activation may render the liver less susceptible to the effects of inflammation. Our data show that up-regulation of soluble IL-1ra by PPARα during hepatic inflammation may contribute to its anti-inflammatory effects.

***Acknowledgements*-**We would like to thank Jolanda van der Meijde for performing the microarray experiment and René Bakker for assisting with the animal experiments. We thank Dr. Mark Leibowitz (Ligand Pharmaceuticals) for the kind gift of Lg100268. This study was supported by the Centre for Human Nutrigenomics, with additional support by the Netherlands Organization for Scientific Research (NWO), the Wageningen Center for Food Sciences, the Swiss National Science Foundation and Etat de Vaud and the National Institutes of Health grant GM46897.

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Chapter 4

Research article

PPARα protects against obesity-induced hepatic inflammation

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This chapter has been published in **Endocrinology**.

Abstract

Recently it has become evident that obesity is associated with low grade chronic inflammation. The transcription factor PPARα has been shown to have a strong anti-inflammatory action in liver. However, the role of PPARα in obesity-induced inflammation is much less clear. Therefore, the aim of our study was to determine whether PPARα plays a role in obesity-induced hepatic inflammation.

To induce obesity, Wildtype sv129 and PPARα-/- mice were exposed to a chronic high fat diet (HFD), using a low fat diet (LFD) as control. In Wildtype mice, HFD significantly increased the hepatic and adipose expression of numerous genes involved in inflammation. Importantly, this effect was amplified in PPARα-/- mice, suggesting an anti-inflammatory role of PPARα in liver and adipose tissue. Further analysis identified specific chemokines and macrophage markers, including MCP-1 and F4/80+, that were elevated in liver and adipose tissue of PPARα-/- mice, indicating increased inflammatory cell recruitment in the knock-out animals. When all groups of mice were analyzed together, a significant correlation between hepatic TG and expression of inflammatory markers was observed. Many inflammatory genes that were up-regulated in PPARα-/- livers by HFD were down-regulated by treatment with the PPARα ligand Wy-14643 under normal non-steatotic conditions, either in vivo or in vitro, suggesting an anti-inflammatory effect of PPARα that is independent of reduction in liver TG.

In conclusion, our results suggest that PPARα protects against obesity-induced chronic inflammation in liver by reducing hepatic steatosis, by direct down-regulation of inflammatory genes, and by attenuating inflammation in adipose tissue.

Abbreviations: ALT, Alanine aminotransferase; HFD, High Fat Diet; IFI-47, Interferon gamma inducible protein 47; IL-1β, Interleukin-1β; LFD, Low Fat Diet; MCP-1, Monocyte Chemotactic Protein 1; MIP-1α, Macrophage Inflammatory Protein 1α; PPAR, Peroxisome Proliferator Activated Receptor; PPRE, PPAR Response Element; RXR, Retinoid X Receptor; SAA, Serum Amyloid A; TNFα, Tumor Necrosis Factor α; VCAM-1, Vascular Cell Adhesion Molecule-1; WAT, White Adipose Tissue; Wt, Wildtype.

Introduction

The Peroxisome Proliferator Activated Receptors (PPARs) comprise a subgroup of nuclear receptors that govern a variety of cellular processes, including lipid metabolism and inflammation. Three isotypes have been identified, PPARα, -β/δ and –γ, which share a common molecular structure and mechanism of action (1). PPARs are ligand-activated transcription factors that regulate gene transcription by binding to specific DNA sequences, known as PPAR Response Elements (PPRE), generally present in the promoter of genes. Binding to DNA and thus transcriptional activation is dependent upon formation of a heterodimer between PPAR and its indispensable partner Retinoid X Receptor (RXR), another member of the Nuclear Receptor Superfamily.

The PPARγ-isotype is mainly expressed in white adipose tissue (WAT) and regulates the expression of numerous genes involved in adipocyte differentiation and energy storage (2). PPARβ/δ is more widely expressed and has been connected with diverse functions ranging from regulation of fatty acid oxidation and inflammation to wound healing in skin (3). PPARα is highly expressed in metabolically active tissues including liver, muscle and brown adipose tissue. In hepatocytes, the liver cell type with the highest expression level of PPARα, PPARα governs lipid metabolism, gluconeogenesis and amino acid metabolism (4).

In addition to governing metabolism, in recent years it has also become evident that PPARα has an important role in regulating inflammatory responses in liver. By suppressing expression of pro-inflammatory genes PPARα controls and inhibits inflammation (5). One of the molecular mechanisms responsible for the immunosuppressive effects of PPARα is direct physical interaction with NF-κB (6), resulting in deactivation of this pro-inflammatory signaling pathway. Genes that are negatively regulated by PPARα in this fashion include acute phase genes and inflammatory signaling components like the IL-6 receptor (7).

Growing evidence has pointed to the involvement of a variety of inflammatory processes in the development of obesity and obesity-associated pathology (8). The recent demonstration that obesity is accompanied by a marked increase in macrophage infiltration of WAT has been instrumental in advancing our thoughts about the origin of inflammatory changes during obesity (9) (10). Indeed, it is now clear that obesity is strongly associated with an increase in circulating levels of acute phase proteins and cytokines, which mainly originate from WAT (11) (12). The influential role of inflammation in liver has been supported by studies showing that NF-κB activation is a crucial step in the development of obesity-induced insulin resistance (13) (14).

Inasmuch as PPARα is able to suppress many of these inflammatory pathways, it might be an attractive target to reduce the inflammatory burden caused by obesity. However, so far the role of PPARα and its ability to interfere with these inflammatory processes in liver has not been studied in the context of obesity-induced inflammation. To explore this function of PPARα in liver, Wildtype (Wt) and PPARα-/- mice were chronically fed a low fat diet (LFD) or high fat diet (HFD). The HFD was given to induce moderate adiposity, resembling obesity. Our results indicate that the presence of PPARα in liver protects against chronic inflammation induced by chronically feeding a HFD.

Material and Methods

Animals and diet

Sv129 PPARα-/- mice and corresponding Wt mice were purchased at the Jackson Laboratory (Bar Harbor, Maine, USA). Male mice received a high fat diet or low fat diet for 6 months. The diets provided either 10 or 45% energy percent in the form of lard fat (D12450B or D12451, Research Diets, New Brunswick, USA). Table I shows the composition of the diets. In another experiment, male Wt and PPARα-/- mice received Wy-14643 (Chemsyn Laboratories, Lenexa, KS) mixed in the food (0.1%) for 5 days. After both feeding experiments, liver and white adipose tissue were dissected, weighed, and directly frozen into liquid nitrogen. The animal experiments were approved by the animal experimentation committee of Wageningen University.

Table I Composition of diets

	Low fat diet	**High fat diet**
Protein (g/100g)	19.2	24
Carbohydrate (g/100g)	67.3	41
Starch	29.9	8.5
Sucrose	33.2	20.1
Fat (g/100g)	4.3	24
Soybean oil	2.4	3
Lard	1.9	21

RNA isolation and quality control

Total RNA was isolated from mouse liver using TRIzol reagent (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. RNA was treated with DNAse and purified using RNeasy columns (Qiagen, Hilden, Germany). Concentrations and purity of RNA samples were determined on a NanoDrop ND-1000 spectrophotometer (Isogen, Maarssen, the Netherlands). RNA integrity was checked on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) with 6000 Nano Chips 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.

Affymetrix GeneChip oligoarray analysis

Pooled RNA samples from 5 mice per experimental group were used for microarray analysis. Samples were hybridized on Affymetrix GeneChip Mouse Genome 430A arrays. Expression levels were calculated applying the multi-chip modified gamma model for oligonucleotide signal (multi-mgMOS) (15) and a remapped Gene Chip Description (CDF) File (16). Heat maps were made in Spotfire DecisionSite software (Spotfire Inc, Sommerville, MA). Detailed descriptions of the applied methods are available on request.

Real-Time PCR

RNA from animal tissue or cells was extracted with Trizol reagent (Invitrogen) using the supplier's instructions. 1 μg of RNA was used for reverse transcription with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). Real-Time PCR was done with platinum Taq polymerase (Invitrogen) and SYBR green using an iCycler PCR machine (Bio-Rad Laboratories BV). Melt curve analysis was included to assure a single PCR product was formed. The primers used are listed in table II.

Table II Primer sequences used for qPCR

Gene	*Forward primer*	*Reverse primer*
36B4	AGCGCGTCCTGGCATTGTGTGG	GGGCAGCAGTGGTGGCAGCAGC
SAA	GCGAGCCTACACTGACATGA	TTTTCTCAGCAGCCCAGACT
Interleukin-1β	TGGTGTGTGACGTTCCCATT	CAGCACGAGGCTTTTTTGTTG
TNFα	CAACCTCCTCTCTGCCGTCAA	TGACTCCAAAGTAGACCTGCCC
Interleukin-6	CTTCCATCCAGTTGCCTTCTTG	AATTAAGCCTCCGACTTGTGAAG
STAT1	GTACAGCCGCTTTTCTCTGG	TCCTGGAGATTACGCTTGCT
STAT3	GACCCGCCAACAAATTAAGA	TCGTGGTAAACTGGACACCA
Metallothionein 2	GCCTGCAAATGCAAACAATGC	AGCTGCACTTGTCGGAAGC
CXCL10/IP-10	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA
Lipocalin 2	TGGAAGAACCAAGGAGCTGT	GGTGGGGACAGAGAAGATGA
MCP-1	CCCAATGAGTAGGCTGGAGA	TCTGGACCCATTCCTTCTTG
MIP1α	CCTCTGTCACCTGCTCAACA	GTAGACTCACATGGCGCTGA
CD68	CCAATTCAGGGTGGAAGAAA	CTCGGGCTCTGATGTAGGTC
F4/80+	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
ADAM8	CACCACTCCCAGTTCCTGTT	AGCTGGTCACCTCTTCTGGA
ICAM-I	TGTGCTTTGAGAACTGTGGCA	TGGCGGCTCAGTATCTCCTC
VCAM-I	AGTTGGGGATTCGGTTGTTCT	CCCCTCATTCCTTACCACCC
Leptin	AGAAGATCCCAGGGAGGAAA	TGATGAGGGTTTTGGTGTCA

Plasma analysis

Serum SAA levels were determined by ELISA (Biosource International, Breda, the Netherlands) following manufacturer's instructions. Serum ALT (Alanine Transferase = Glutamate Pyruvate Transaminase) activity was measured using a commercially available kit from Human (Human GmbH, Wiesbaden, Germany). Plasma concentrations of multiple chemokines were measured with Luminex xMAP techniques (Luminex, Texas, USA).

Liver Triglycerides

Liver triglycerides were determined in 10% liver homogenates prepared in buffer containing Sucrose 250 mM, EDTA 1 mM, Tris-HCl 10 mM pH 7.5 using a commercially available kit from Instruchemie (Delfzijl, The Netherlands).

Immunohistochemistry

Cryosections of 5 μm from frozen liver were made. The coupes were dried overnight at room temperature followed by fixation in acetone for 5 minutes and acetone with 0.15% H_2O_2 for 5

minutes. For detection of macrophages/monocytes, an F4/80+ antibody (Serotec, Oxford, UK) was used. After pre-incubation with 20% normal goat serum, sections were incubated overnight at 4 °C with the primary antibody diluted 1:50 in PBS/ 1% Bovine Serum Albumin (BSA). After incubation with the primary antibody, a goat anti rat IgG conjugated to horseradish peroxidase (Serotec) was used as secondary antibody. Visualization of the complex was done using 3,3'-diaminobenzidene for 5 minutes. Negative controls were used by omitting the primary antibody. Oil-red O and Haematoxylin and Eosin staining of liver sections were done using standard protocols.

Primary hepatocyte isolation

Primary mouse hepatocytes from Wt and PPARα-/- mice were isolated as described previously (17). Briefly, after cannulation of the portal vein, the liver was perfused with calcium free HBSS which was pregassed with 95% O2/5% CO2. Next, the liver was perfused with a collagenase solution (Sigma-Aldrich, Zwijndrecht, the Netherlands) until swelling and degradation of the internal liver structure was observed. The hepatocytes were released, filtered and washed several times using Krebs buffer. The viability was assessed by using tryptan blue (Sigma-Aldrich) and was around 80%. Cells were brought into culture using Williams E Medium supplemented with 10 % FCS (Cambrex, Verviers, Belgium), penicillin/streptomycin/fungizone, insulin and dexamethasone. Cells were plated on collagen (Serva Feinbiochemica, Heidelberg, Germany) coated wells with a density of 0.5 x 10^6 cells/ml. After 4 hours of incubation, the medium was removed and replaced with fresh medium. The next day, hepatocytes were used for experiments. IL-1β and TNFα were from R&D systems (R&D Systems Europe Ltd, Abingdon, UK).

Isolation of adipocytes and stromal vascular cells

Freshly isolated epididymal adipose tissue was used for the isolation of adipocytes and stromal vascular cells. Minced adipose tissue was digested using collagenase (Sigma-Aldrich) at a concentration of 5 mg/ml dissolved in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % FCS. Tissues were incubated for 45 minutes at 37 ºC and were subsequently filtered through a 250 µM nylon mesh filter. After centrifugation, the floating cells were collected as adipocytes and the pelleted cells as stromal vascular cells. Both cell fractions were washed with PBS and RNA was isolated using Trizol reagent (Invitrogen).

Immunoblot analysis

Immunoblotting was carried out using an ECL system (Amersham Biosciences, Diegem, Belgium) according to the manufacturer's instructions. Equal amounts of liver cell lysates as determined by Bio-Rad Protein Assay reagent (Bio-Rad Laboratories BV) were resolved by SDS/PAGE on a 12% polyacrylamide gel. The F4/80+ antibody (Serotec) and the actin antibody (Sigma-Aldrich) were used at a dilution 1:1000 and the membranes were incubated overnight at 4 °C. The secondary antibodies (goat anti-Rat or rat anti-Rabbit IgG, Peroxidase, Sigma-Aldrich) were used at a dilution of 1:5000. All incubations were performed in 1X Tris-buffered saline, pH 7.5, with 0.1 % Tween 20 and 5% dry milk. In the final washings, dry milk was removed from the solution.

Statistical analysis

Statistical significant differences were calculated using two-way ANOVA or Student's T-test. Correlations between gene expression signals and liver TG content were assessed by Pearson's correlation coefficient. The cut-off for statistical significance was set at a P-value of 0.05 or below.

Results

Microarray and qPCR analysis reveals markedly increased inflammatory gene expression in PPARα-/- vs. Wt mice fed a HFD- First, we assessed the overall change in bodyweight at the end of the diet intervention. HFD feeding caused significantly more bodyweight gain compared to LFD feeding (Figure 1A). Changes in liver and adipose tissue weight in response to both diets were also evaluated. In comparison with LFD, HFD significantly increased adipose tissue to body weight ratio in Wt and PPARα-/- mice (Figure 1B). In contrast, HFD increased liver to body weight ratio only in PPARα-/- mice (Figure 1C). Overall, liver weights were higher in PPARα-/- mice.

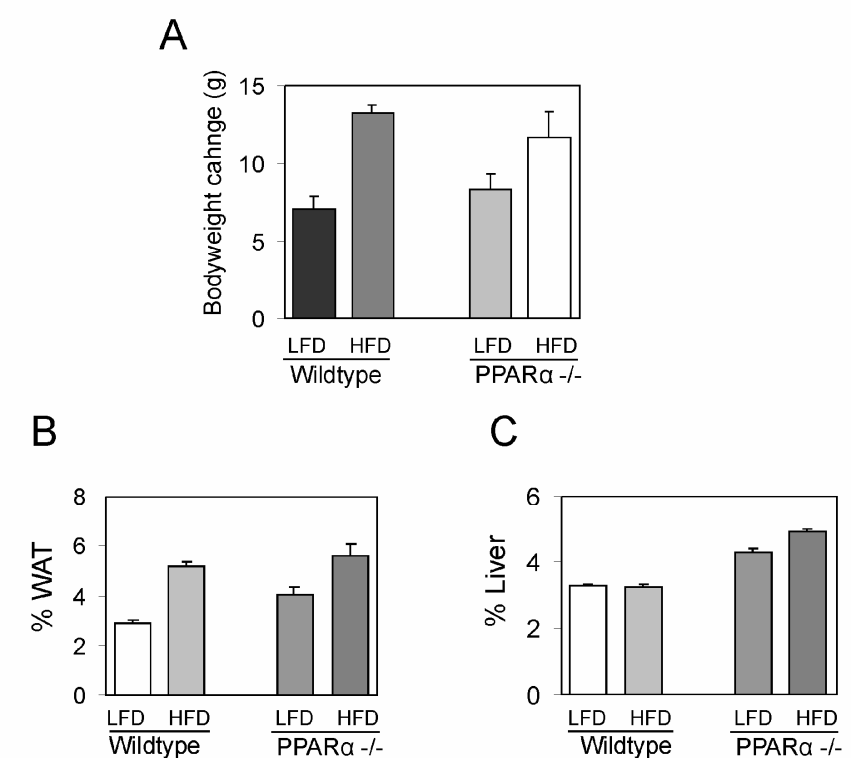

Figure 1 **Adipose and liver weights are altered by HFD and PPARα deletion.** (A) Bodyweight changes of mice are determined by comparing bodyweight values at the beginning of the diet intervention and after LFD or HFD intervention. Significant effects were observed using two-way ANOVA for diet ($p=0.0001$) but not for genotype or interaction between both parameters. (B) Weight of epididymal adipose tissue after the diet intervention expressed as a percentage of total body weight. Significant effects were observed for diet ($p<0.0001$) and genotype ($p=0.02$). (C) Liver weight after the diet intervention expressed as a percentage of total body weight. Significant effects were observed for diet ($p=0.0009$), genotype ($p<0.0001$) and the interaction between both parameters ($p=0.0005$). Error bars represent SEM.

To evaluate the potential role of PPARα in obesity-induced hepatic inflammation, changes in gene expression in Wt and PPARα-/- mice after HFD were studied by Affymetrix GeneChip analysis. Wt and PPARα-/- mice showed increased expression of inflammatory genes in liver after HFD feeding compared to LFD feeding (Figure 2). However, the effect of HFD feeding on inflammatory gene expression was more pronounced in the PPARα-/- animals compared to the Wt animals (Figure 2). Genes that were highly increased in PPARα-/- mice fed the HFD included hepatic acute phase genes such as SAA and Orosomucoid, chemokines, and macrophage-related genes. These results suggest a higher degree of hepatic inflammation and the recruitment of pro-inflammatory cells including macrophages to the liver and indicate an anti-inflammatory effect of PPARα in livers of mice fed the HFD.

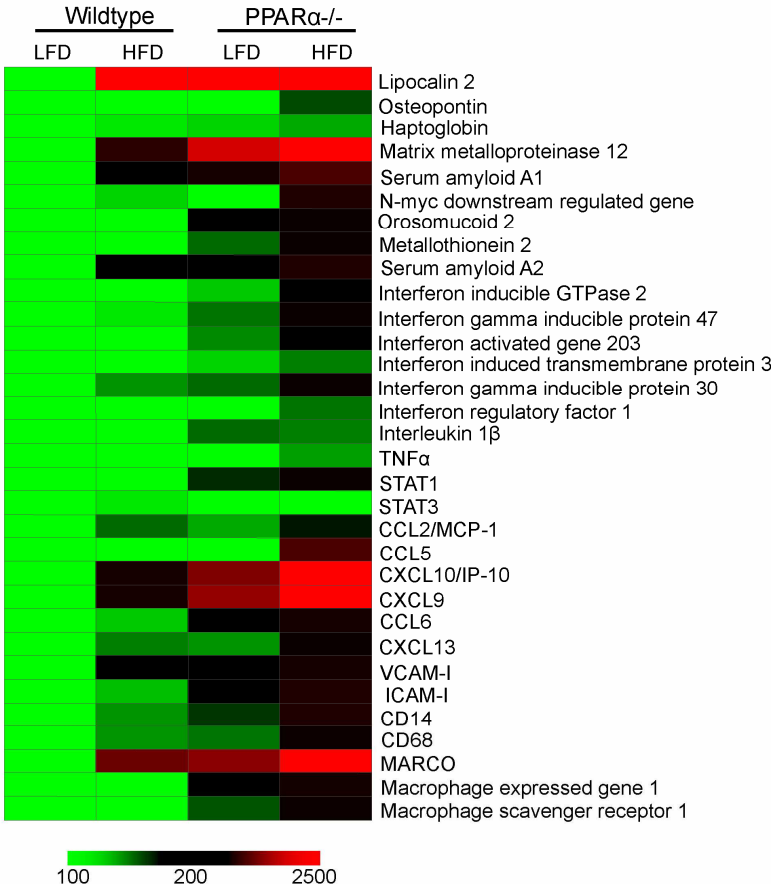

Figure 2 **Microarray analysis reveals higher inflammatory gene expression signals in liver of PPARα-/- mice fed a HFD.** Microarray analysis was performed on liver mRNA comparing the gene expression signals nduced by the different diets (LFD and HFD) in both genotypes (Wt and PPARα-/-). The expression signals from the Wt animals receiving the LFD were arbitrarily set at 100.

To validate the gene expression changes obtained from the microarray analysis (Figure 2) and to study the expression of transcription factors and cytokines possibly involved in the regulation of these genes, quantitative real-time PCR (qPCR) analysis was performed. The majority of inflammatory genes that were analyzed showed an increased expression in the HFD group that was amplified in the PPARα-/- mice (Figure 3).

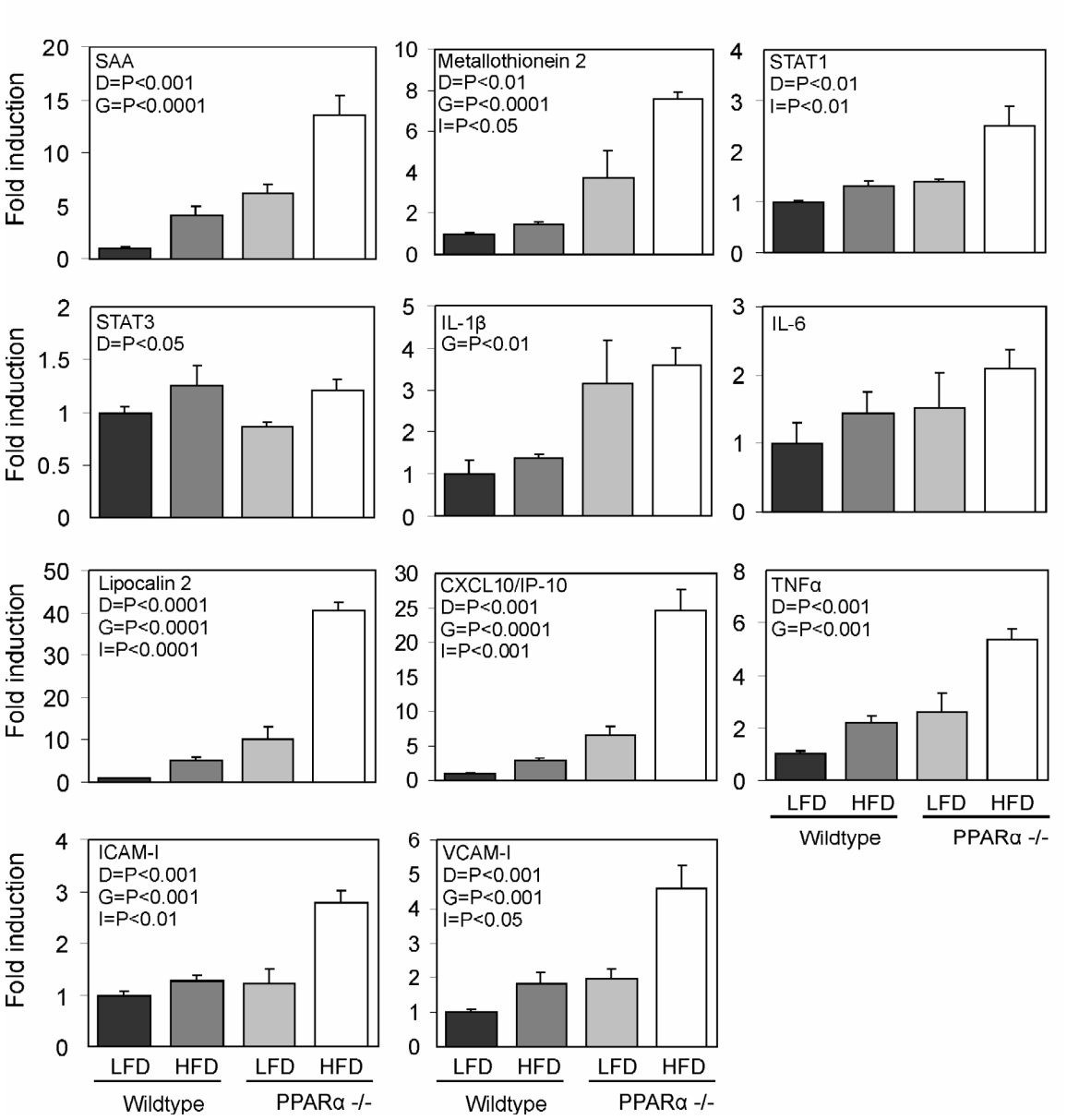

Figure 3 **QPCR reveals elevated expression of inflammatory genes in liver of PPARα-/- vs. Wt mice fed a HFD.** mRNA expression in liver was determined by qPCR (n=4 per group). Statistically significant differences were observed using two-way ANOVA for diet (D), genotype (G) or the interaction between both parameters (I) and are indicated at the top of each figure. Error bars represent SEM.

Expression of Serum Amyloid A (SAA), Lipocalin and CXCL10/IP-10 was 15-, 40-, and 25-fold higher, respectively, in PPARα-/- on HFD compared to Wt mice on LFD. Similar but somewhat mitigated changes were observed for Metallothionein 2, Vascular Cell Adhesion Molecule-1 (VCAM-1) and several other genes. To assess which molecular pathways are implicated in the regulation of these genes, we analyzed the expression of several transcription factors and cytokines including STAT1 and 3, IL-6, IL-1β and Tumor Necrosis Factor α (TNFα). Significant effects of both diet and genotype were only found for TNFα. Together, these data point towards a protective effect of PPARα on obesity-induced hepatic inflammation.

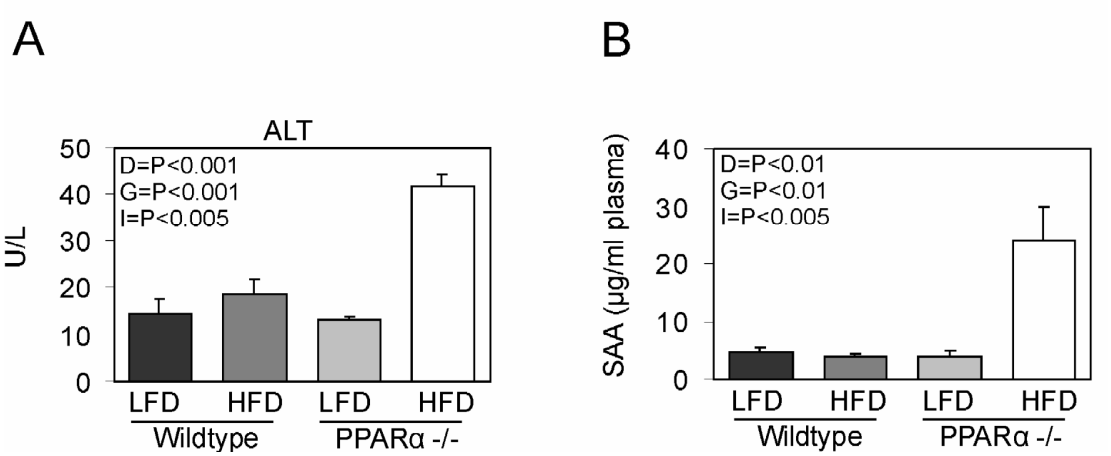

Figure 4 **Plasma markers of liver injury and inflammation are increased in PPARα-/- vs. Wt mice fed a HFD.** Plasma levels of ALT (A) and Serum Amyloid A (B) were determined (n=5 per group) (B). Statistically significant differences were observed using two-way ANOVA for diet (D), genotype (G) or the interaction between both parameters (I) and are indicated at the top of each figure. Error bars represent SEM.

Plasma levels of ALT and Serum Amyloid A (SAA) are increased in PPARα-/- mice fed the HFD-To examine if the changes in inflammatory gene expression in liver were translated into an increased state of inflammation in the circulation, the plasma levels of ALT and the acute phase protein SAA were measured. After HFD, serum ALT activity levels were significantly higher in the PPARα-/- compared to the Wt mice, indicating more liver injury (Figure 4A). In parallel with the gene expression results, in the HFD group plasma SAA levels where markedly higher in the PPARα-/- compared to the Wt mice (Figure 4B). The markedly

elevated plasma levels of inflammatory marker SAA may point towards a chronic state of inflammation in the PPARα-/- animals after feeding the HFD (18).

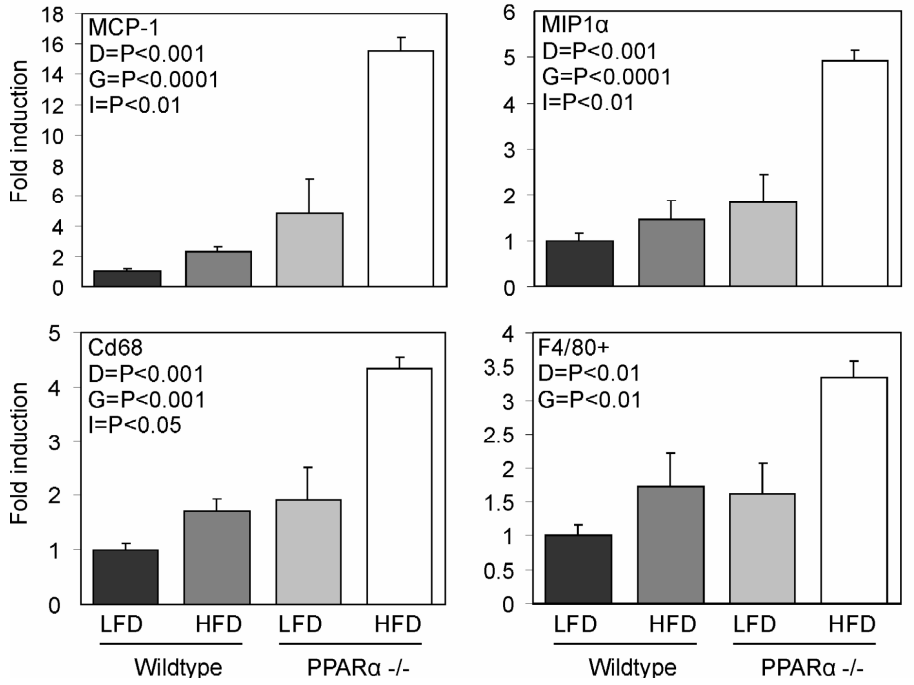

Figure 5 **Significantly higher expression levels of macrophage/monocyte markers in liver of PPARα-/- vs. Wt mice fed a HFD.** mRNA expression in liver was determined by qPCR (n=4 per group). Statistical significant differences were observed using two-way ANOVA for the effect of diet (D), genotype (G) or the interaction (I) between both parameters.

Increased presence of macrophage/monocyte markers in PPARα-/- vs. Wt mice fed the HFD-Close investigation of the microarray data also revealed that the expression of numerous chemokines and macrophage/monocyte markers were noticeably increased in the liver of PPARα-/- mice fed the HFD, suggesting an increase in inflammatory cell recruitment. QPCR analysis corroborated these data by showing markedly increased expression in PPARα-/- mice fed the HFD of two key genes implicated in macrophage/monocyte type cells recruitment: MCP-1 and MIP1α (Figure 5). A similar expression pattern was observed for CD68 and F4/80+, two genes specifically expressed by macrophages. To determine changes in plasma concentrations of several chemokines, a multiplexing analysis was performed (Table III): Except MIP1α, all chemokines showed the highest plasma concentration in the PPARα-/- mice fed the HFD. In particular, plasma concentrations of MCP-1 and CXCL10/IP-10 were highly induced in HFD fed PPARα-/- mice. MCP-1 belongs to the subclass of CC

chemokines whereas CXCL10/IP-10 is part of the CXC subclass. Both subclasses are known to be involved in the recruitment of different inflammatory cell types (19) (20).

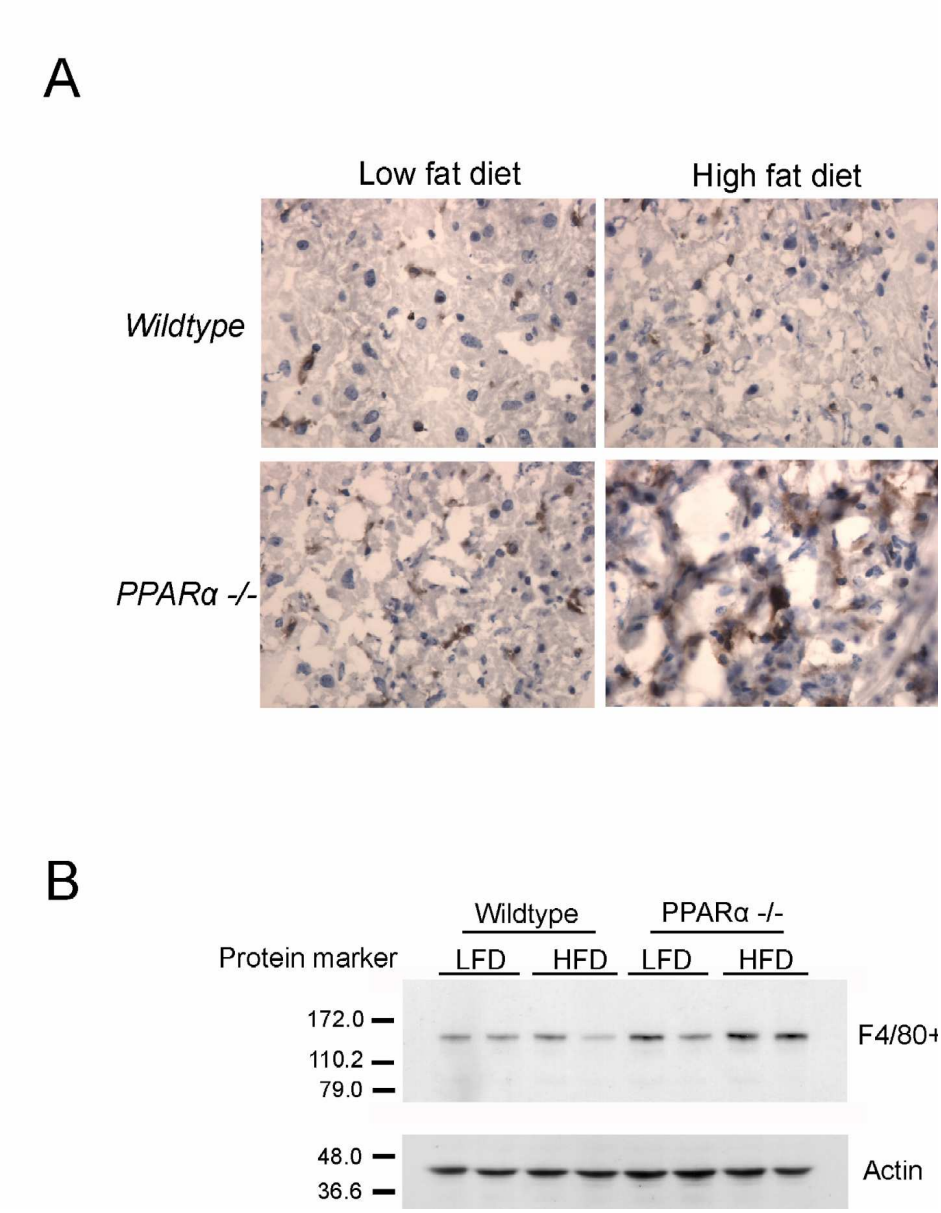

Figure 6 **Immunohistochemistry reveals increased abundance of macrophages in liver of PPARα-/- vs. Wt mice fed a HFD.** (A) Immunohistochemical staining of liver tissue was carried out using an antibody against the macrophage/monocyte specific marker F4/80+. Original magnification: 640x. (B) Equal amounts of total liver cell lysates were analysed for F4/80+ or actin protein by immunoblot. Molecular mass sizes are given in kDa.

Table III Pooled mouse plasma (n=5) was used for determining the concentration of multiple chemokines. Concentrations are in pg/ml plasma, except for Mip-1γ (ng/ml plasma).

	Wildtype		*PPARα-/-*	
	Low fat diet	High fat diet	Low fat diet	High fat diet
MCP-1 (CCL2)	18	29	37	75
Mip1α (CCL3)	260	260	200	160
Mip-1β (CCL4)	28	12	43	69
MCP-3 (CCL7)	52	54	52	70
Eotaxin (CCL11)	793	780	753	879
MCP-5(CCL12)	23	22	25	36
Mip-1γ (CCL15)	8.6	9.8	14	14
Mip-3β (CCL19)	140	110	110	160
MDC (CCL22)	82	84	92	99
Mip-2 (CXCL2)	12	15	14	18
IP-10 (CXCL10)	28	31	46	109

Although the analysis of plasma concentrations of chemokines was performed on pooled plasma samples which precluded statistical analysis of the data, plasma concentrations of MCP-1 and CXL10/IP-10 perfectly fit with the hepatic gene expression data obtained by qPCR of individual mice. To investigate whether the increased expression of genes involved in macrophage recruitment as well as macrophage marker genes in the PPARα-/- mice fed the HFD was associated with macrophage infiltration, macrophages in liver were visualized using an antibody against F4/80+, a marker for mature macrophages (21). The number of activated macrophages present in the liver was found to be increased in the PPARα-/- mice compared to the Wt mice fed the HFD (Figure 6A). No staining was observed in the absence of primary antibody (data not shown). Analysis of total liver cell lysates for F4/80+ protein content by immunoblot gave similar results (Figure 6B). Together, these data indicate that the lack of PPARα promotes obesity-induced macrophage infiltration in liver.

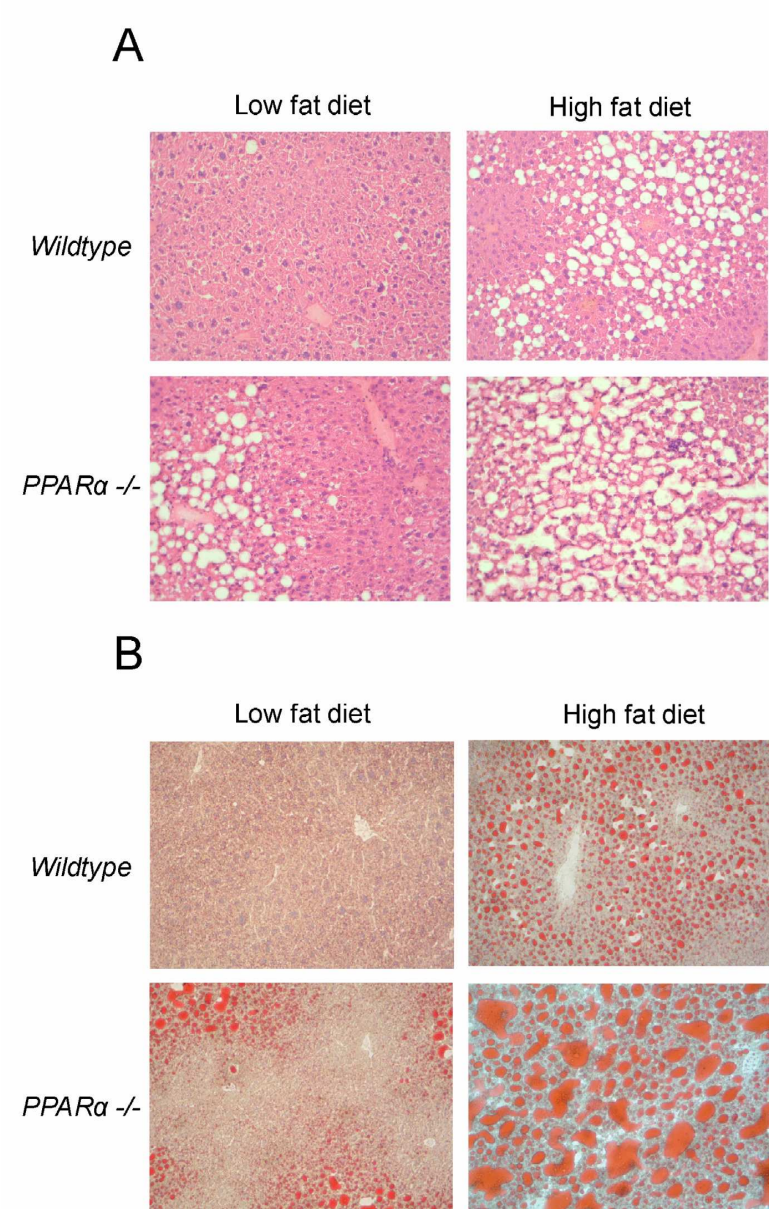

Figure 7 **Staining of the liver shows markedly increased fat storage in PPARα-/- vs. Wt mice fed a HFD.** Hematoxylin and Eosin (A) and Oil red O (B) staining of representative mouse liver sections was performed. Original magnification: 200x

Changes in expression of inflammatory genes are positively correlated with liver TGs-As expected, HFD feeding increased lipid accumulation in liver in Wt mice. Similarly, hepatic lipid levels were elevated in PPARα-/- mice on the LFD, which is consistent with the important function of PPARα in fatty acid catabolism (22). Remarkably, the combination of PPARα deletion and HFD feeding caused massive hepatic lipid accumulation, as shown by the appearance of large lipid droplets in histological sections of liver (Figure 7A), which were

visualized by Oil Red O staining (Figure 7B). While Wt mice fed the HFD and PPARα-/- mice on LFD developed hepatic steatosis, PPARα-/- mice fed the HFD displayed clear signs of steatohepatitis with inflammatory cells including lymphocytes infiltrating the liver.

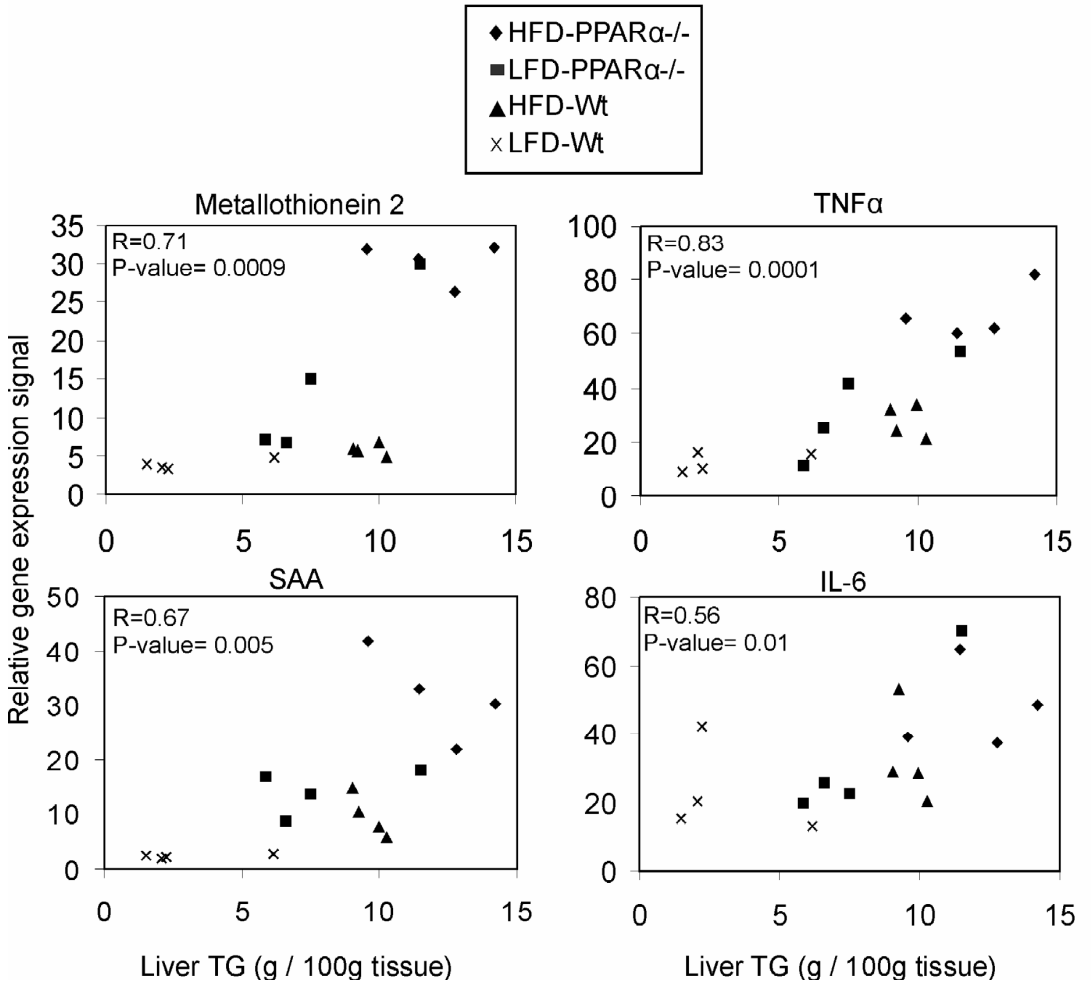

Figure 8 **A positive correlation was observed between liver triglycerides and hepatic gene expression of inflammatory markers.** Gene expression of TNFα, SAA, IL-6 and Metallothionein 2 were assessed by qPCR (n=4 per group). Correlation was assessed using the Pearson's correlation coefficient and results are shown in each graph.

To ascertain whether the up regulation of inflammatory genes in PPARα-/- mice on HFD may be connected to hepatic lipid accumulation, we studied the correlation between inflammatory gene expression and hepatic lipid content. A highly significant positive correlation ($P<0.009$-0.01, $R=0.83$-0.56) was observed between the expression of numerous inflammatory genes and liver triglyceride content (Figure 8) suggesting that the increase in triglyceride storage in the liver may be responsible for the elevated inflammatory status of the liver.

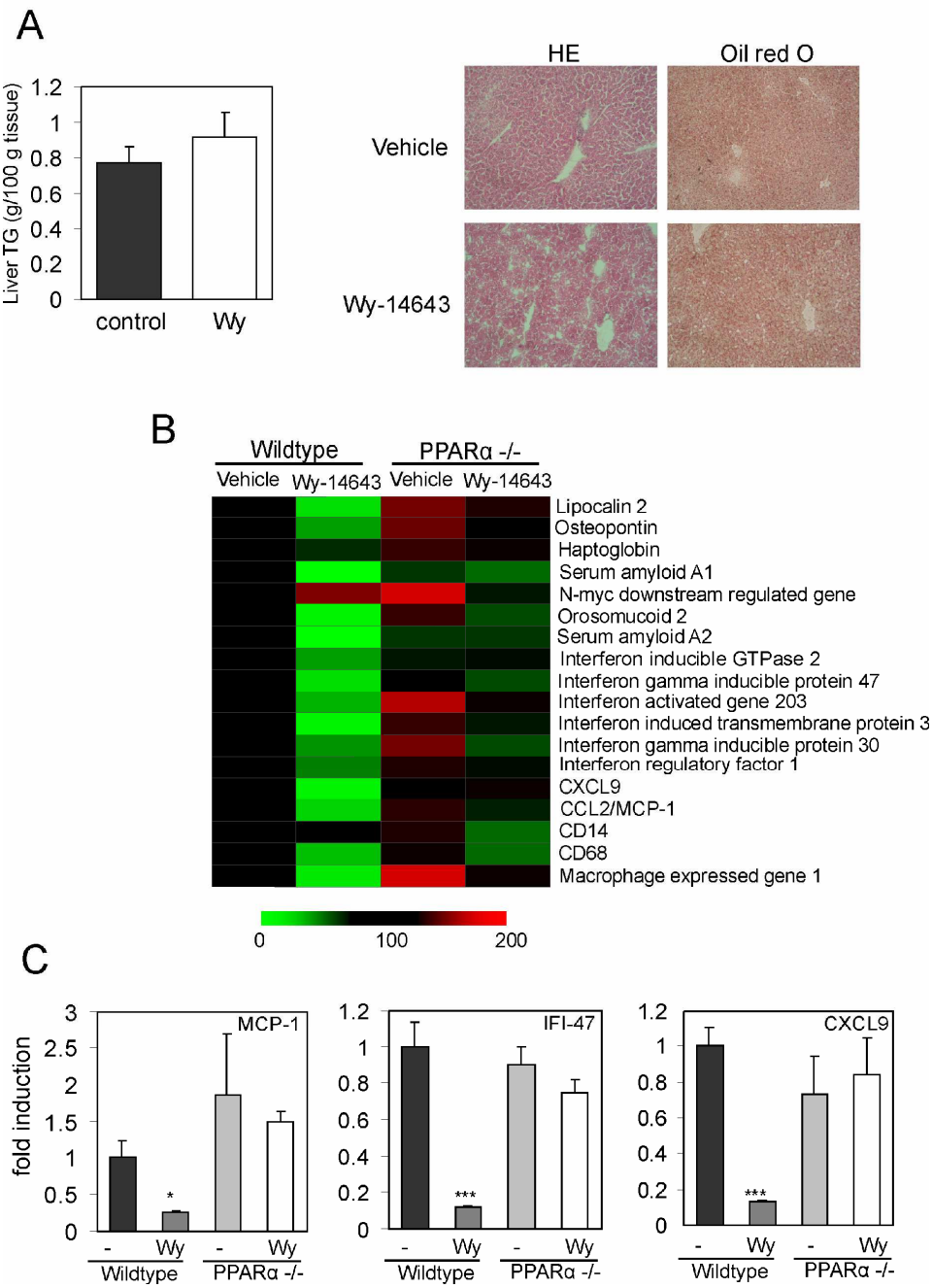

Figure 9 **PPARα is able to down-regulate expression of inflammatory genes independent of its effects on hepatic lipid content.** (A) Triglyceride content and HE and Oil red O staining of liver from Wt mice after 5 days of Wy-14643 treatment. No significant effect of Wy-14643 treatment on the triglyceride content of liver was observed. (B) Microarray gene expression signals of Wt and PPARα-/- mice treated or not with the synthetic ligand Wy-14643 for 5 days. The expression signals from the Wt mice that did not receive Wy-14643 were arbitrarily set at 100. (C) Q-PCR analysis of CCL2/MCP-1, Interferon gamma inducible protein 47 (IFI-47) and CXCL-9 confirms changes obtained from the microarray analysis. Changes in expression signal between Wt control and Wt mice treated with Wy-14643 were evaluated using Student's T-test. Error bars represent SEM. * p=0.01, *** p<0.001

PPARα is able to down-regulate expression of inflammatory genes in liver independent of its effect on hepatic lipid storage-Data presented in Figure 8 suggest that PPARα indirectly inhibits inflammation by preventing fat accumulation in liver. To examine whether PPARα might also directly suppress inflammatory gene expression independent of its influence on hepatic lipid storage, the effect of the PPARα agonist Wy-14643 on inflammatory gene expression in liver under non-steatotic conditions was studied by Affymetrix GeneChip analysis. Mice were fed normal chow for 5 days with or without Wy-14643 mixed in their food. Whereas treatment of the mice with Wy-14643 did not result in significant changes in hepatic triglyceride content (Figure 9A), numerous inflammatory genes were down regulated by Wy-14643 in a PPARα-dependent manner (Figure 9B). The results from microarray were confirmed for several genes by qPCR (Figure 9C). Since not all genes analyzed after the HFD intervention are expressed under normal conditions, not all genes shown in Figure 2 are presented here.

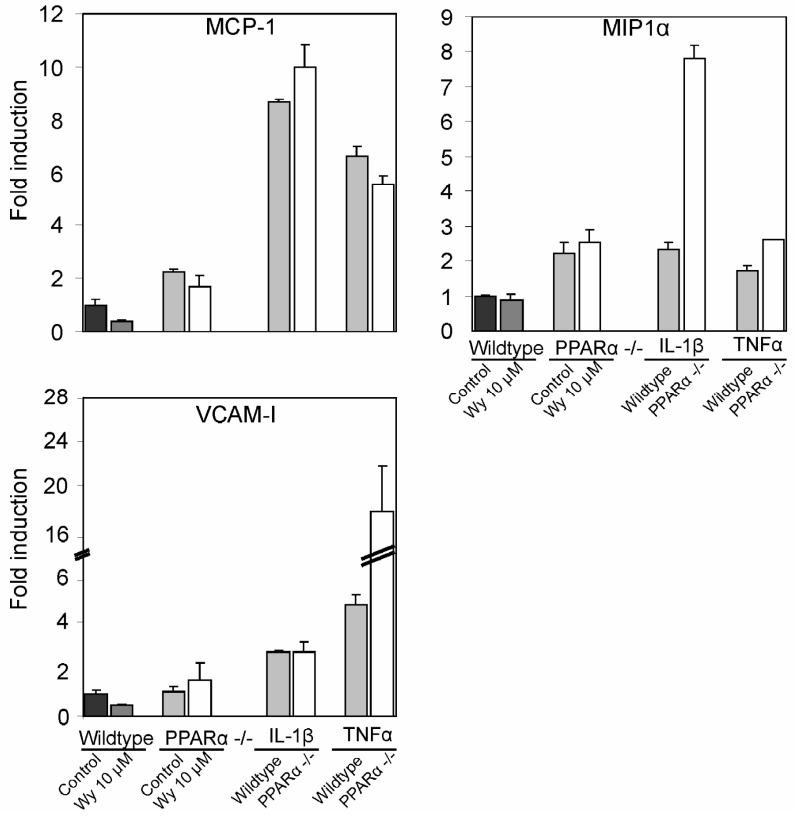

Figure 10 **PPARα suppresses inflammatory gene expression in mouse primary hepatocytes.** Expression of MCP-1, MIP1α, and VCAM-1 was determined by qPCR in Wt and PPARα-/- mouse hepatocytes treated for 24h with Wy-14643 (10 μM) for PPARα activation or 24h with IL-1β (10 ng/ml) and TNFα (10 ng/ml) for activation of inflammatory pathways.

To further establish an inhibitory effect of PPARα on inflammatory gene expression in liver independent of its influence on hepatic lipid storage, the ability of PPARα to regulate genes involved in inflammatory cell recruitment was studied in primary mouse hepatocytes. Basal expression of MCP-1 and MIP-1α was much higher in hepatocytes lacking PPARα, strengthening the possible involvement of PPARα in controlling the expression of these genes (Figure 10). Furthermore, pharmacological PPARα activation using Wy-14643 resulted in decreased expression of MCP-1 and VCAM-1, changes which were not observed in hepatocytes cultured from PPARα-/-. To evaluate the role of PPARα in the regulation of these genes during inflammatory conditions, hepatocytes from both genotypes were treated with IL-1β and TNFα (Figure 10). Expression of both cytokines was increased in liver of PPARα-/- mice after HFD feeding (see Figure 3). Expression of MIP1α and VCAM-1 were much higher in PPARα-/- hepatocytes incubated in the presence of IL-1β or TNFα, respectively. In summary, these data suggest that PPARα is able to down regulate the expression of numerous inflammatory genes in liver independent of its effect on hepatic lipid accumulation.

PPARα governs inflammatory gene expression in adipose tissue- To establish if HFD feeding induced chronic inflammation in adipose tissue, the expression of several inflammatory genes was measured by qPCR. HFD significantly increased adipose expression of several cytokines as well as several genes involved in inflammatory cell recruitment and macrophage markers. Similar to what was observed in liver, the effects of HFD were amplified in PPARα-/- mice (Figure 11A). The elevated expression of the macrophage marker gene F4/80+ in PPARα-/- mice on HFD was specific to the stromal vascular fraction of WAT (Figure 11B), suggesting increased macrophage infiltration in the adipose tissue of the PPARα-/- mice after HFD feeding. The successful separation of stromal vascular cells from adipocytes is shown by the dominant expression of leptin in the adipocyte fraction. These data suggest that PPARα influences expression of inflammatory genes in adipose tissue, including cytokines, chemokines, and macrophage markers.

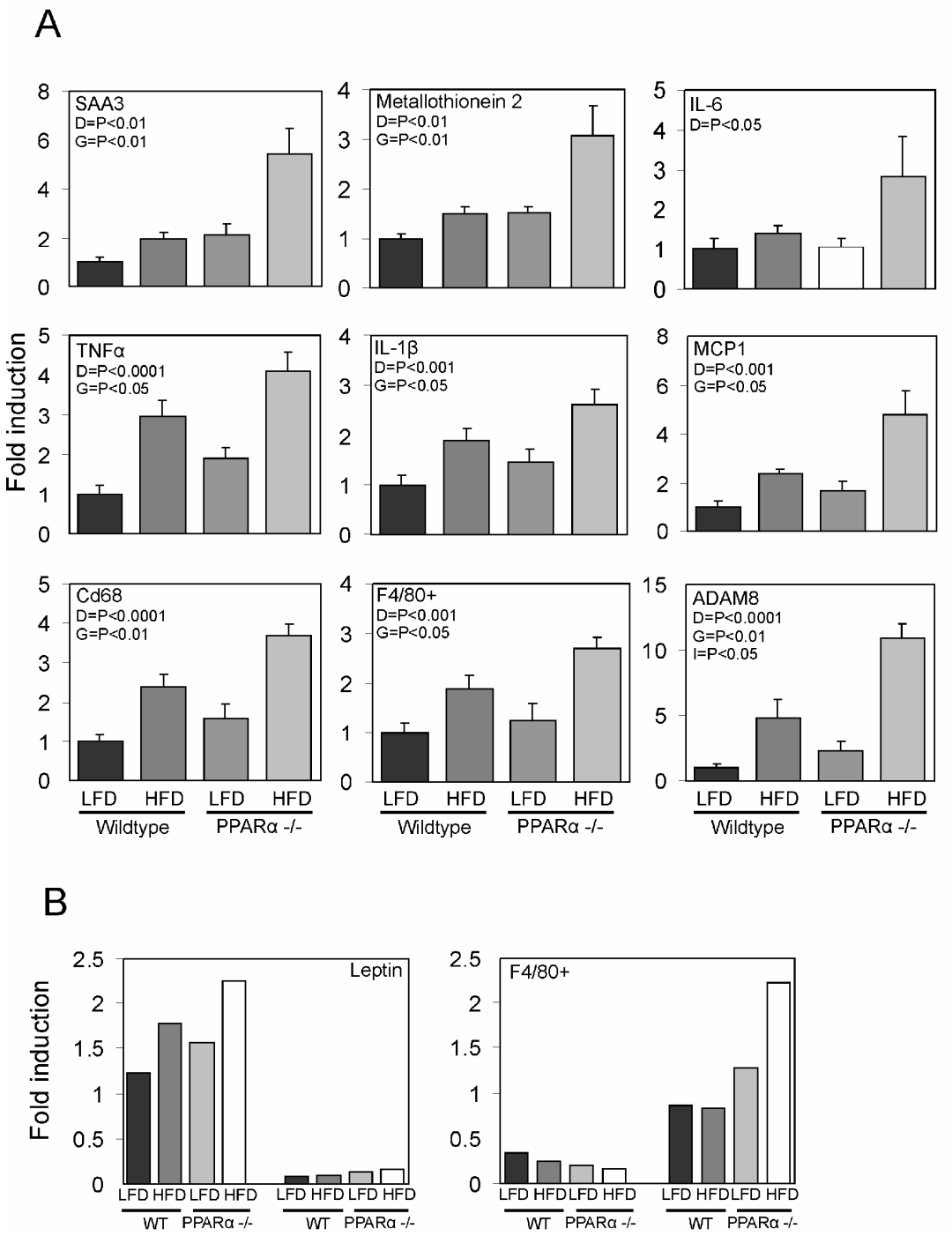

Figure 11 **QPCR analysis reveals elevated expression of inflammatory genes in adipose tissue of PPARα-/- vs. Wt mice fed a HFD.** (A) mRNA expression in white adipose tissue was determined by qPCR (n=4 per group). Statistically significant differences were observed using two-way ANOVA for diet (D), genotype (G) or the interaction between both parameters (I) and are indicated at the top of each figure. Error bars represent SEM. (B) mRNA expression in isolated adipocytes and stromal vascular cells was analyzed by qPCR (n=2 per group).

Discussion

Recent studies suggest that obesity is associated with low grade chronic inflammation in which numerous tissues, including adipose tissue and liver, are implicated (9) (10) (23). In adipose tissue, increased lipid storage may alter the production of various adipocytokines. Furthermore, in response to tissue growth macrophages may infiltrate adipose tissue and cause increased production of inflammatory mediators. In liver, inflammatory processes are mostly activated in parallel with increased local fat accumulation, as observed in non-alcoholic steatohepatitis (24). In steatohepatitis, the activity of pro-inflammatory transcription factors such as NF-κB is increased, which may lead to the development of insulin resistance (13).

The nuclear receptor PPARα has a major role in liver by altering the transcription of numerous target genes, many of which are involved in fatty acid oxidation. In line with its importance in fatty acid catabolism, positive effects of PPARα activation in the prevention and reversal of steatosis have already been demonstrated (25) (26). In addition to these diverse functions in hepatic metabolism, in the past decade a role for PPARα in controlling inflammation has clearly emerged (27) (28) (29). Since then, several molecular mechanisms by which PPARα exert its anti-inflammatory effects in liver and vascular wall have been uncovered (30) (31). Thus, while the connection between PPARα and inflammation is strong, little to no information is available yet on whether PPARα may modulate obesity-induced inflammation in liver. The aim of the present study was to determine whether PPARα may play a role in obesity-induced hepatic inflammation. Obesity was induced by chronically feeding Wt and PPARα-/- mice a HFD. Several lines of evidence suggest that PPARα protects against hepatic inflammation under conditions of obesity: 1) micro-array and qPCR analysis indicated that expression of numerous genes involved in inflammation was markedly up-regulated in PPARα-/- mice fed a HFD compared to Wt mice fed a HFD. This included several acute phase genes and other inflammatory markers 2) in plasma of PPARα-/- mice vs. Wt mice fed a HFD, markedly higher levels of serum amyloid A protein and ALT were measured, suggesting increased liver inflammation and injury 3) livers of PPARα-/- mice vs. Wt mice fed a HFD showed significantly increased infiltration of macrophages, as indicated by elevated presence of macrophage markers at the gene expression and protein level. These observations indicate that the presence of PPARα in liver protects against inflammation elicited by chronically feeding mice a HFD.

It can be theorized that PPARα may protect against obesity-induced hepatic inflammation by decreasing lipid storage in liver, which contributes to hepatic inflammation as observed in steatohepatitis. Alternatively, it is conceivable that PPARα suppresses the inflammatory response in liver by directly down-regulating the expression of target genes involved in inflammation. Finally, PPARα may act indirectly by suppressing inflammation in adipose tissue, thereby decreasing the secretion of adipokines that may promote hepatic inflammation. With respect to the former hypothesis, we observed markedly higher hepatic lipid storage in PPARα-/- vs. Wt mice fed a HFD, indicating the protective effect of PPARα against steatosis. When all mice were grouped together, highly significant correlations were observed between hepatic triglyceride concentration and gene expression levels of several acute phase proteins and cytokines, suggesting that the two are causally linked. These data would argue that PPARα influences obesity-induced hepatic inflammation mainly by decreasing lipid storage. However, we also observed that under non-steatotic conditions, either in vivo or in vitro, activation of PPARα by a synthetic agonist consistently down-regulated the expression of numerous inflammatory genes in a PPARα-dependent manner. This is in line with previous data showing a suppressive effect of PPARα on the expression of numerous genes implicated in hepatic inflammation in the absence of steatosis (32). Hence, PPARα suppresses inflammation regardless of changes in lipid storage in liver. Inasmuch as obesity is invariably connected with elevated hepatic lipid storage, and PPARα automatically decreases lipid storage by stimulating fatty acid catabolism, it is difficult to separate the relative contribution of these mechanisms to the overall effect of PPARα on obesity-induced hepatic inflammation.

Another possible mechanism directly implicates adipose tissue as the primary initiator of elevated hepatic inflammation. In mice fed the HFD, deletion of PPARα was associated with markedly elevated expression of numerous inflammatory genes in adipose tissue. This included cytokines, chemokines, and macrophage markers. Thus, it is possible that the elevated hepatic inflammation is secondary to events originating in the adipose tissue. These events are directly or indirectly governed by PPARα but are also independent of adiposity, as inflammatory gene expression was higher in HFD-fed PPARα-/- vs. Wt mice despite comparable fat mass. It should be emphasized that although the expression of PPARα in adipose tissue is low compared to PPARγ, this does not necessarily mean it is non-functional. Accordingly, it can be envisioned that similar to its role in liver and the vascular wall PPARα directly regulates expression of inflammatory genes in adipose tissue.

The development of chronic inflammation associated with obesity has partly been attributed to the infiltration of macrophages in WAT. Indeed, it was shown that after 16 weeks of HFD feeding, infiltration of macrophages occurred resulting in elevated production of several pro-inflammatory mediators by WAT (9) (10). Whether similarly obesity may also lead to increased macrophage infiltration in liver is not very clear. Our data suggest that the presence of PPARα prevents macrophage infiltration in liver in a mouse model of obesity. While Kupffer cells represent the natural macrophage population in liver and play an important role in the immune defense, the higher expression levels of various chemokines and macrophage marker genes, including MCP-1, MIP1α, CXCL10/IP-10, VCAM and F4/80+, together with enhanced immunohistological staining for macrophage markers in the PPARα-/- mice fed the HFD, strongly suggests an increase in macrophage recruitment in liver (33) (34). As discussed above, PPARα may reduce macrophage infiltration by direct regulation of target genes involved in this pathway or indirectly by decreasing hepatic lipid storage, and by attenuating inflammation in adipose tissue.

We have previously shown that HFD feeding increases hepatic PPARα expression and activation, leading to the induction of classical PPARα target genes involved in fatty acid oxidation (35). The present data add a novel twist by showing that while activation of PPARα down-regulates inflammatory gene expression, HFD increased inflammatory gene expression. The importance of PPARα is demonstrated by our finding that the increase in inflammatory gene expression caused by HFD becomes more dramatic in mice lacking PPARα. The observation that in terms of regulation of inflammatory gene expression the HFD-induced adiposity overrules the potential effect of HFD on PPARα activation attests to the notion that the effect of chronic HFD on inflammation is mediated by increased obesity.

In conclusion, PPARα exerts a marked anti-inflammatory effect in liver in a mouse model of obesity, which appears to be at least partially achieved by decreasing activation and infiltration of inflammatory cells in liver. PPARα may reduce obesity-induced hepatic inflammation by diminishing fatty liver, which is tightly linked to elevated inflammatory status, by directly regulating inflammatory gene expression, or by suppressing inflammation in adipose tissue. Since PPARα automatically decreases steatosis via its effect on fatty acid catabolism, it is difficult to separate the relative contribution of these mechanisms to the overall effect of PPARα on obesity-induced hepatic inflammation.

***Acknowledgements*-**The authors would like to thank Jolanda van der Meijde for excellent technical assistance, Prof J.H.J.M. van Krieken for assisting in the immunohistochemical analysis, Wilma Blauw and Rene Bakker for helping out with the animal experiments and Guido Hooiveld for the analysis of the microarray data. This study was supported by the Centre for Human Nutrigenomics, with additional support by the Netherlands Organization for Scientific Research (NWO), the Dutch Diabetes Foundation and the Wageningen Centre for Food Sciences.

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Chapter 5

Research article

PPARγ activation uncouples inflammatory gene expression from macrophage infiltration in adipose tissue

Rinke Stienstra, Caroline Duval, Sander Kersten, Michael Müller

This chapter is **in preparation**.

Abstract

It is increasingly clear that obesity is associated with chronic low-grade inflammation. The primary factors are likely adipocyte hypertrophy and infiltration of macrophages leading to increased secretion of pro-inflammatory mediators. Recently, it was shown that during diet-induced obesity the phenotype of adipose-resident macrophages changes from alternatively activated macrophages towards a more classical and pro-inflammatory phenotype. Here, we explore the effect of PPARγ-activation on obesity-induced inflammation in mice by short term oral administration of rosiglitazone after 20 weeks of dietary intervention with high fat diet. As expected, high fat feeding led to an increase in bodyweight gain, elevated adipose mass and hepatic steatosis. Treatment with rosiglitazone further increased adipose mass and changed adipose morphology towards smaller adipocytes. Surprisingly, despite down-regulation of pro-inflammatory gene expression, rosiglitazone increased the number of macrophages in adipose tissue, as shown by immunohistochemical analysis and quantification of macrophage marker genes. Gene expression analysis of the stromal vascular fraction of adipose tissue revealed higher levels of markers from alternatively activated macrophages upon rosiglitazone treatment. In conclusion, our results suggest that short term rosiglitazone treatment increases macrophage infiltration in adipose tissue, while suppressing inflammation. The alternatively activated macrophages may play a role in PPARγ-dependent expansion and remodeling of adipose tissue.

Introduction

The number of obese people worldwide is steadily growing (1). Unless major action is taken, obesity will increasingly strain our health care resources over the next few decades, as obesity is connected with several metabolic disturbances that greatly increase morbidity risk. This is especially true for central obesity, which is characterized by increased adipose mass in the abdomen. These metabolic disturbances are collected in the metabolic syndrome and include hypertension, dyslipidemia, and insulin resistance (2). Each serves as an independent risk factor for atherosclerosis and associated coronary heart disease.

Although the overall negative impact of central obesity and metabolic syndrome on morbidity is evident, it has been very difficult to get a handle on why some individuals are obese seemingly without any damaging consequences for health whereas others are afflicted by a range of metabolic abnormalities. Clearly, our understanding of the chain of events that leads to metabolic syndrome, although growing, is still remarkably scarce. Recent studies suggest an important role for inflammatory processes. Indeed, it has been found that obesity is associated with a state of chronic low grade inflammation, which is likely caused by adipocyte hypertrophy together with infiltration of macrophages into adipose tissue (3). As a result, the secretion of pro-inflammatory mediators such as TNFα and IL-6 from adipose tissue is increased, leading to disruption of normal homeostatic control of metabolism either locally or systemically (4) (5) (6). Why macrophages infiltrate adipose tissue during obesity is currently unclear although it has been suggested that macrophage localization and infiltration is strongly linked to adipose cell death (7). More recently, it has been proposed that adipose tissue resident macrophages itself undergo phenotypic changes during obesity. In adipose tissue of mice rendered obese by high fat feeding, macrophages appear to be mainly activated according to "classical activation" whereas macrophages present in adipose tissue of lean mice are "alternatively activated" (8). Classically activated macrophages express high levels of pro-inflammatory mediators including TNFα which may contribute to insulin resistance. In contrast, alternatively activated macrophages are considered anti-inflammatory by expressing genes such as IL-10, IL-1 receptor antagonist, and arginase I (9).

The Peroxisome Proliferator Activated Receptor γ is ligand-activated transcription factor that is part of the superfamily of nuclear receptors. It regulates gene transcription by forming a heterodimer with the retinoid X receptor followed by binding to specific DNA sequences in and around the promoter of target genes. PPARγ serves as the molecular target for an important class of anti-diabetic drugs, the thiazolidinediones. In addition, PPARγ binds

and is activated by poly-unsaturated fatty acids and fatty acid-derived molecules. Expression of PPARγ is highest in adipose tissues where it plays a pivotal role in the process of cell differentiation and acquisition of the adipocyte phenotype (10) (11). Indeed, target genes of PPARγ identified so far are mainly involved in adipogenesis and lipogenesis, including fatty acid binding protein 4, GLUT4, glycerol 3-phosphate dehydrogenase, lipoprotein lipase, glycerol kinase, and aquaporin 7 (12).

As mentioned above, PPARγ is the molecular target for the insulin-sensitizing thiazolidinediones, which effectively lower plasma glucose levels by promoting responsiveness of tissues to insulin and thus stimulating glucose uptake. Thiazolidinediones also reduce plasma free fatty acid concentrations, although this effect is mainly evident in rodents (13) (14).

In addition to its role in adipocyte differentiation and lipid metabolism, PPARγ also has potent anti-inflammatory activity. Treatment of mice with rosiglitazone causes a significant decrease in expression of numerous pro-inflammatory mediators, including TNFα and IL-6 (15). In adipose tissue, induction of adipocyte differentiation by PPARγ is paralleled by the appearance of smaller adipocytes (16), which may partially account for the inhibitory effect of PPARγ on inflammatory gene expression (17). Furthermore, PPARγ may suppress inflammation associated with diet-induced obesity by lowering the number of macrophages present in adipose tissue (18) (5).

Here we studied the effect of rosiglitazone in mice chronically fed a high fat diet. Remarkably, it was observed that rosiglitazone stimulated adipose infiltration of macrophages, while diminishing pro-inflammatory genes. Our data indicate that PPARγ activation uncouples inflammatory gene expression from macrophage infiltration in adipose tissue.

Material and Methods

Animal study

Sv129 mice were purchased at the Jackson Laboratory (Bar Harbor, Maine, USA). Male mice received a high fat diet or low fat diet for 21 weeks. The diets provided either 10 or 45% energy percent in the form of palm oil (D12450B or D12451, Research Diets, New Brunswick, USA). In the last week of diet intervention, half of the group of mice receiving the HFD, was switched to the HFD supplemented with Rosiglitazone. At the end of the feeding experiment, liver and white adipose tissue were dissected, weighed, and directly frozen into liquid nitrogen. The animal experiments were approved by the animal experimentation committee of Wageningen University.

RNA isolation and quality control

Total RNA was isolated from adipose tissue using TRIzol reagent (Invitrogen, Breda, the Netherlands) according to the manufacturer's instructions. Concentrations and purity of RNA samples were determined on a NanoDrop ND-1000 spectrophotometer (Isogen, Maarssen, the Netherlands). RNA integrity was checked on an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, the Netherlands) with 6000 Nano Chips.

Real-Time PCR

1 μg of RNA was used for reverse transcription with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). Real-Time PCR was done with platinum Taq polymerase (Invitrogen) and SYBR green using an iCycler PCR machine (Bio-Rad Laboratories BV). Melt curve analysis was included to assure a single PCR product was formed. The primers used are listed in table I.

Table I Primers sequences used for qPCR

Gene	*Forward primer*	*Reverse primer*
36B4	AGCGCGTCCTGGCATTGTGTGG	GGGCAGCAGTGGTGGCAGCAGC
UCP1	CCTGCCTCTCTCGGAAACAA	TGTAGGCTGCCCAATGAACA
CPT-1b	TCTCCTACCACGGGTGGATGTT	CCATGACCGGCTTGATCTCTTC
Gyk	ATCCGCTGGCTAAGAGACAACC	TGCACTGGGCTCCCAATAAGG
Cox7a1	ACAATGACCTCCCAGTACACT	GCCCAAGCAGTATAAGCAGTAGG
CD68	CCAATTCAGGGTGGAAGAAA	CTCGGGCTCTGATGTAGGTC
F4/80+	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
CyclinA2	CTTTACCCGCAGCAAGAAAAC	ACGTTCACTGGCTTGTCTTCTA
Col1a1	TGTGTGCGATGACGTGCAAT	GGGTCCCTCGACTCCTACA
Arginase 1	TGGCTTGCGAGACGTAGAC	GCTCAGGTGAATCGGCCTTTT
IL-1ra	AAATCTGCTGGGGACCCTAC	TGAGCTGGTTGTTTCTCAGG
IL-18	GACTCTTGCGTCAACTTCAAGG	CAGGCTGTCTTTTGTCAACGA
MCP-1	CCCAATGAGTAGGCTGGAGA	TCTGGACCCATTCCTTCTTG
SAA3	GTTCACGGGACATGGAGCAGAGGA	GCAGGCCAGCAGGTGGGAAGTG
Fibr-β	AGTGTTGTGTCCTACGGGATG	CTGAGGAGGTATCGGAAACAGA
IL-6	CTTCCATCCAGTTGCCTTCTTG	AATTAAGCCTCCGACTTGTGAAG
Mt-2	GGAGAACGAGTCAGGGTTGT	AAAGAGGCTTCCGACAAGTG
Leptin	AGAAGATCCCAGGGAGGAAA	TGATGAGGGTTTTGGTGTCA

Histology/Immunohistochemistry

For detection of macrophages/monocytes, an F4/80+ antibody (Serotec, Oxford, UK) was used. Sections were pre-incubation with 20% normal goat serum followed by overnight incubation at 4 °C with the primary antibody diluted 1:50 in PBS/ 1% Bovine Serum Albumin (BSA). After incubation with the primary antibody, a goat anti rat IgG conjugated to horseradish peroxidase (Serotec) was used as secondary antibody. Visualization of the complex was done using 3,3'-diaminobenzidene for 5 minutes. Negative controls were used by omitting the primary antibody. Oil-red O and Haematoxylin and Eosin staining of sections were done using standard protocols.

Dapi staining

To visualize DNA content of adipose tissue, sections were incubated with 4',6-diamidino-2-phenylindole (Sigma-Aldrich) for 30 minutes at room temperature. Sections were analysed using a fluorescence microscope.

Liver triglycerides

Liver triglycerides were determined in 10% liver homogenates prepared in buffer containing 250 mM sucrose, 1mM EDTA and 10 mM Tris-HCl at pH 7.5 using a commercially available kit from Instruchemie (Delfzijl, The Netherlands).

Plasma insulin

Plasma levels of insulin were measured using a commercially available kit from Linco (Linco Research, St Louis, MO, USA).

Fractioning of adipose tissue

Freshly isolated epididymal adipose tissue was used for the isolation of adipocytes and stromal vascular cells. Minced adipose tissue was digested using collagenase (Sigma-Aldrich) at a concentration of 5 mg/ml dissolved in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % FCS (Cambrex, Verviers, Belgium). Tissues were incubated for 45 minutes at 37 ºC and were subsequently filtered through a 250 µM nylon mesh filter. After centrifugation, the floating cells were collected as adipocytes and the pelleted cells as stromal vascular cells. Both cell fractions were washed with PBS and RNA was isolated using Trizol reagent (Invitrogen).

Statistical analysis

Statistical significant differences were calculated using a Student's T-test. The cut-off for statistical significance was set at a P-value of 0.05 or below.

Results

Rosiglitazone increased adipose mass together with the up-regulation of PPARγ target genes- Mice were fed a low fat diet or high fat diet for 20 weeks. Although energy intake was similar (Figure 1C), mice on high fat diet (HFD) showed a significantly higher bodyweight gain compared to mice on low fat diet (LFD) during the diet intervention (Figure 1A). Adipose tissue weight at the end of the study was also significantly higher in animals fed the HFD (Figure 1B). Furthermore, HFD significantly increased plasma insulin (Figure 1D) suggesting development of insulin resistance, as well as liver triglycerides (Figure 1E). Treatment with rosiglitazone during the last week of the HFD intervention further increased adipose tissue weight (Figure 2A), and caused a decrease in plasma insulin (Figure 2B) and hepatic triglycerides (Figure 2C). The expression levels of several PPARγ target genes, including UCP-1, CPT-1b, Gyk and Cox7a1 was markedly increased in adipose tissue after rosiglitazone treatment (Figure 2B).

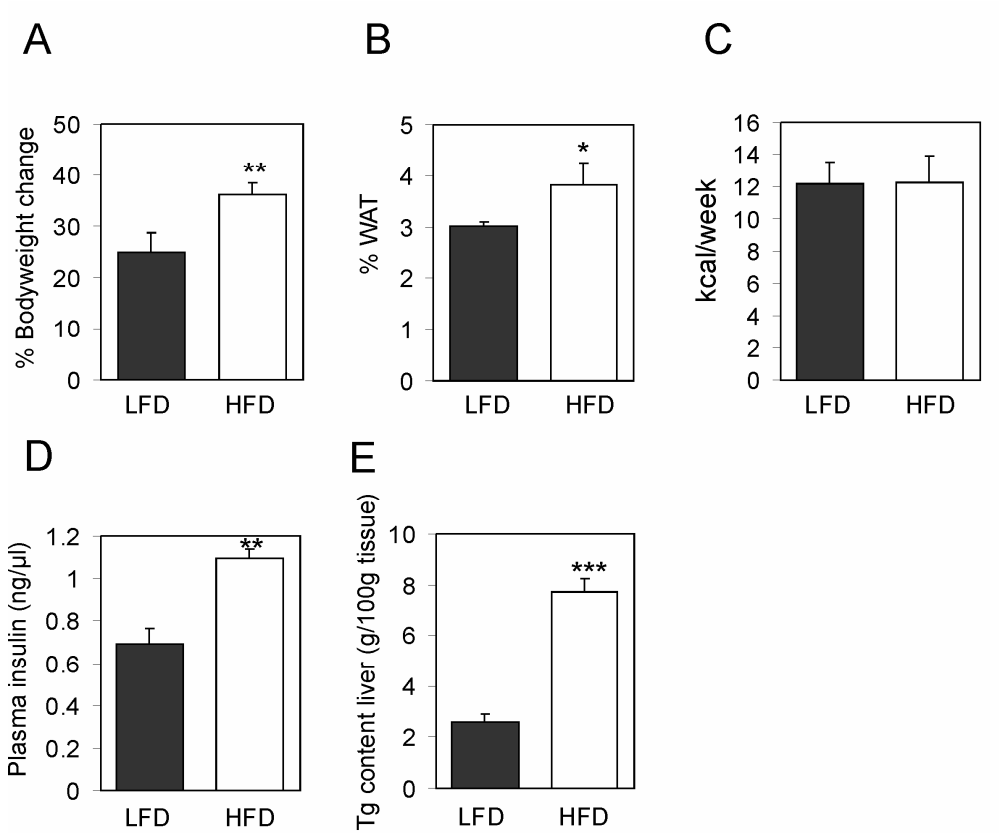

Figure 1 **Mice fed a high fat diet develop obesity-** Mice were fed a low fat or high fat diet for 20 weeks. (A) % of total bodyweight gain (P = 0.009) and (B) % of white adipose tissue mass (P = 0.03) were significantly increased in mice fed a high fat diet treatment compared to animals receiving a low fat diet. (C) Caloric intake in low fat diet and high fat diet fed mice was similar. Error bars represent SEM. (D) Plasma insulin levels are increased after high fat diet intervention (P = 0.001) (E) Triglyceride content of liver is measured after diet intervention. A significant increase in hepatic fat accumulation in the liver is observed after in high fat diet fed animals (P-value = 0.0001). Error bars represent SEM.

Rosiglitazone down-regulates expression of pro-inflammatory genes in adipose tissue- In line with the anti-inflammatory properties of PPARγ, adipose expression of the pro-inflammatory genes SAA3, fibrinogen β, and Metallothionein 2 was significantly down-regulated by rosiglitazone, as shown by Q-PCR. There was a trend towards decreased expression of IL-6 as well (p = 0.06) (Figure 3).

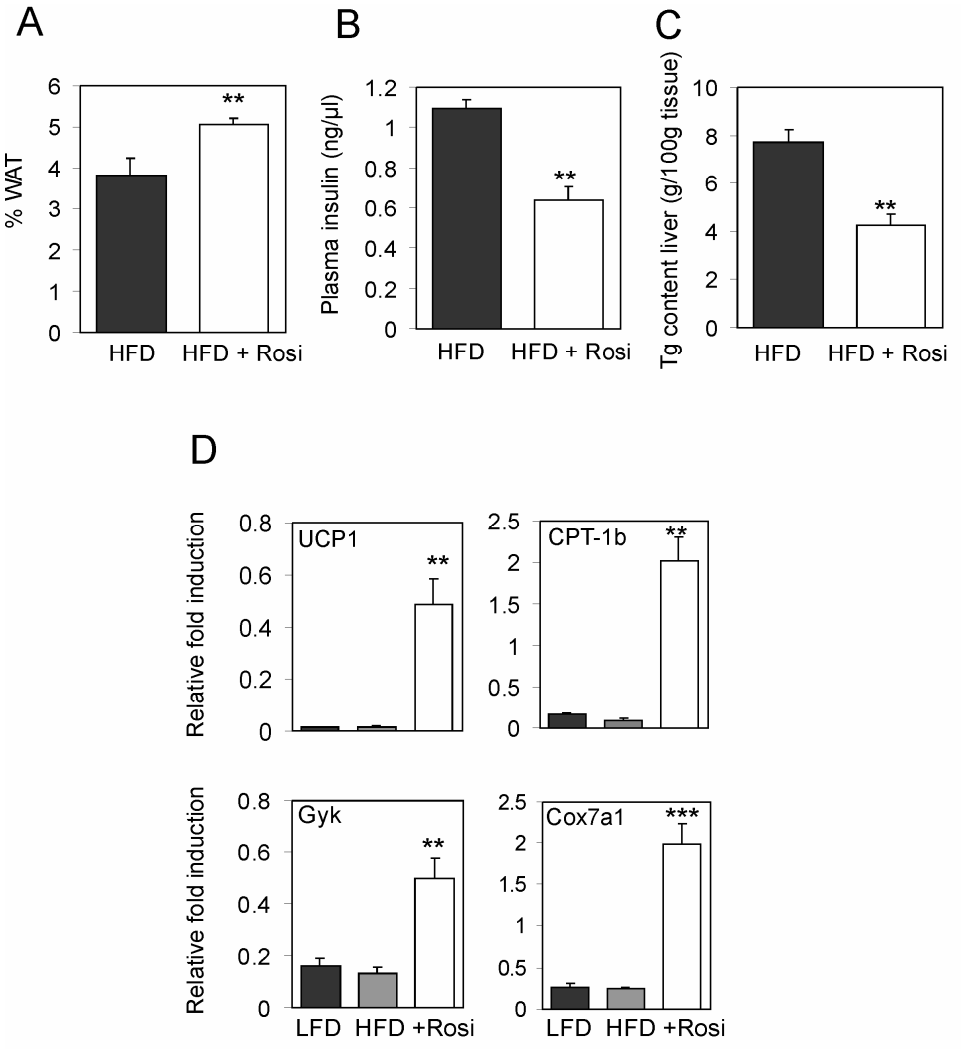

Figure 2 **High fat diet + rosiglitazone leads to a further increase in adipose mass and up regulation of PPARγ target genes-** (A) Addition of rosiglitazone to the high fat diet led to a significant increase in white adipose tissue mass (P-value = 0.009) compared to animals only receiving high fat diet. (B) Plasma insulin levels were downregulated in animals receiving HFD + Rosiglitazone compared to mice receiving HFD only (P-value = 0.0015). (C) Liver TG content is decreased after HFD + Rosiglitazone treatment (P-value = 0.0001) (D) rosiglitazone resulted in a significant up regulation of PPARγ target genes in white adipose tissue including UCP-1 (P-value = 0.002), CPT-1b (P-value = 0.002), Glycerol kinase (P-value = 0.004) and Cox7A1 (P-value = 0.0001) compared to mice receiving high fat diet. Error bars represent SEM.

Rosiglitazone causes remodeling of adipose tissue- Despite an increase in adipose mass following rosiglitazone treatment, an overall change in morphology of adipose tissue towards smaller adipocytes was observed (Figure 4A). In line with previously published results (16), rosiglitazone caused the formation of clusters of smaller adipocytes (Figure 4A). Surprisingly, DAPI nuclear staining revealed clusters of cells surrounding adipocytes in adipose tissue of mice treated with rosiglitazone, suggesting the presence of cell aggregates (Figure 4B).

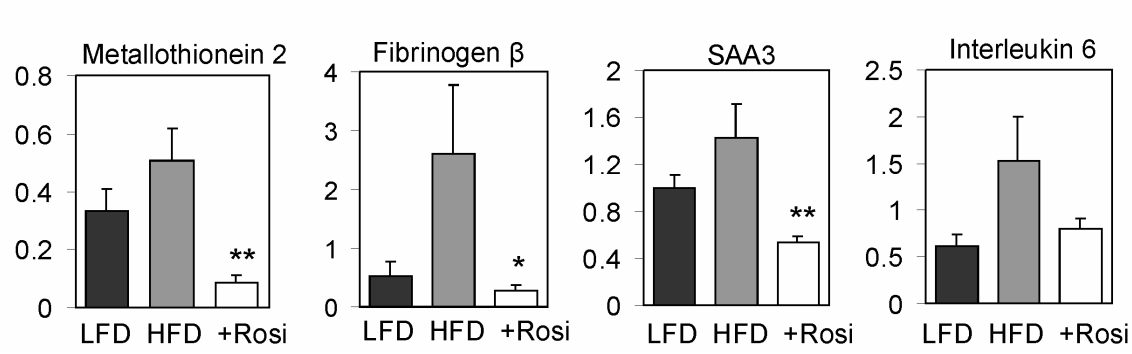

Figure 3 **Inflammatory genes in white adipose tissue are down-regulated after High fat diet + rosiglitazone treatment**- Several pro-inflammatory genes were down regulated in adipose tissue of HFD + rosiglitazone treated mice compared to HFD fed animals. Expression levels of SAA3 (P-value = 0.005), Fibrinogen β (P-value = 0.03) and Metallothionein (P-value = 0.002) were significantly down regulated compared to HFD fed mice. Error bars represent SEM.

Rosiglitazone stimulates macrophages infiltration in adipose tissue- The clusters of cells visualized using DAPI nuclear staining strongly resembled so-called Crown-like structures that are formed by aggregated macrophages in adipose tissue during obesity (7). To analyze if more macrophages were present in adipose tissue after rosiglitazone treatment, immunohistochemical staining using the specific macrophage marker F4/80+ was performed.

As shown in Figure 5A, rosiglitazone treatment increased staining for F4/80+, suggesting the presence of more macrophages. These results were corroborated by quantitative analysis of adipose expression of F4/80+ and CD68, another macrophage specific gene (Figure 5B). These data suggest that rosiglitazone stimulates macrophages infiltration in adipose tissue

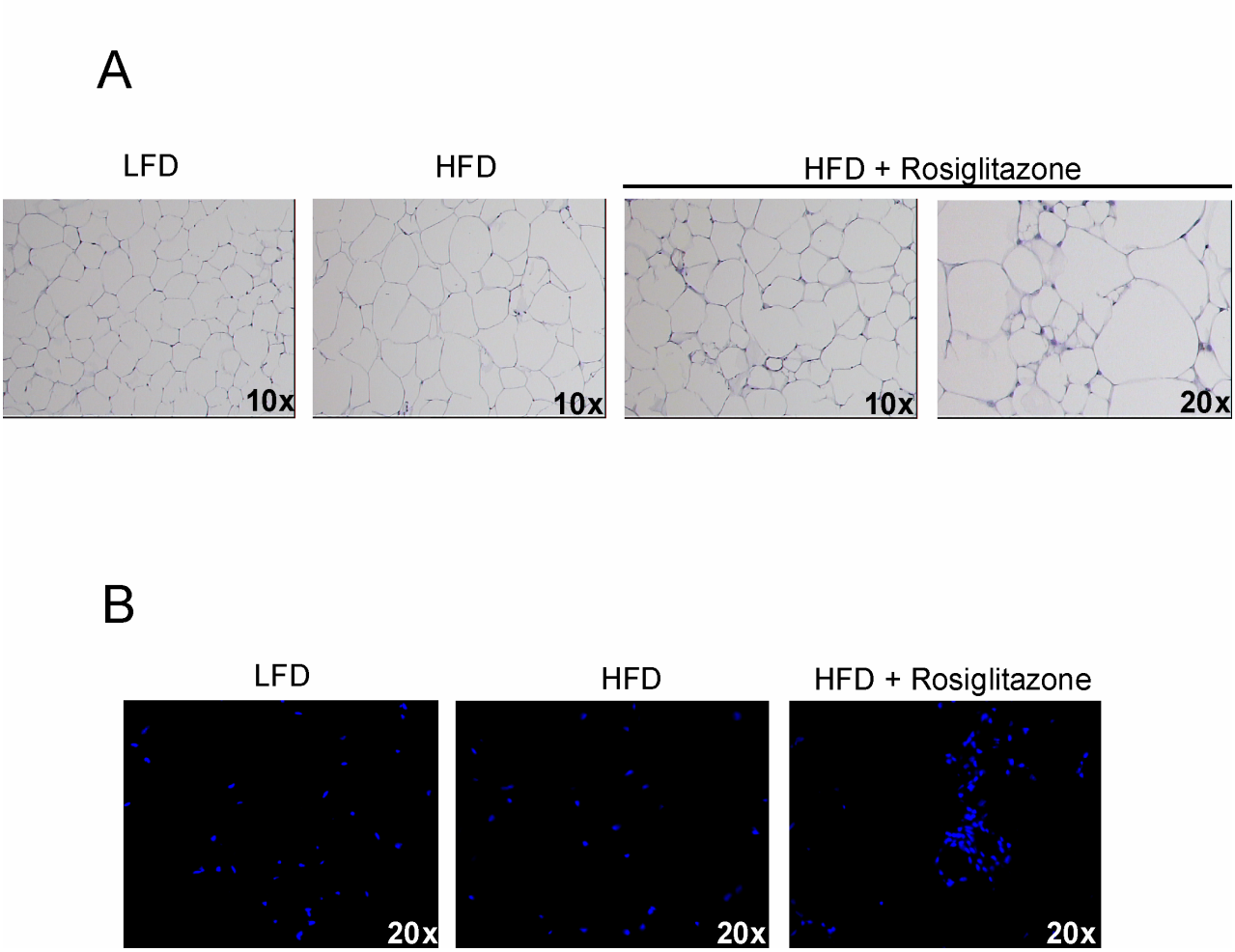

Figure 4 **Remodeling of adipose tissue after 1 week of rosiglitazone treatment**- (A) Representative HE-staining shows morphological changes in adipose tissue in HFD + rosiglitazone treated mice. Clusters of small adipocytes are present. (B) Dapi-staining of adipose tissue revealed the presence of cell clusters in adipose tissue of HFD + rosiglitazone treated animals compared to low fat or high fat diet fed mice.

Rosiglitazone increases adipose abundance of alternatively activated macrophages- Macrophages can either be activated by T helper 1 (Th1) type responses via IFNγ, and by alternative activation via Th2-type cytokines. The latter response, which is stimulated by IL-4 and IL-13, elicits a macrophage phenotype connected with tissue repair and blockage of inflammation (9).

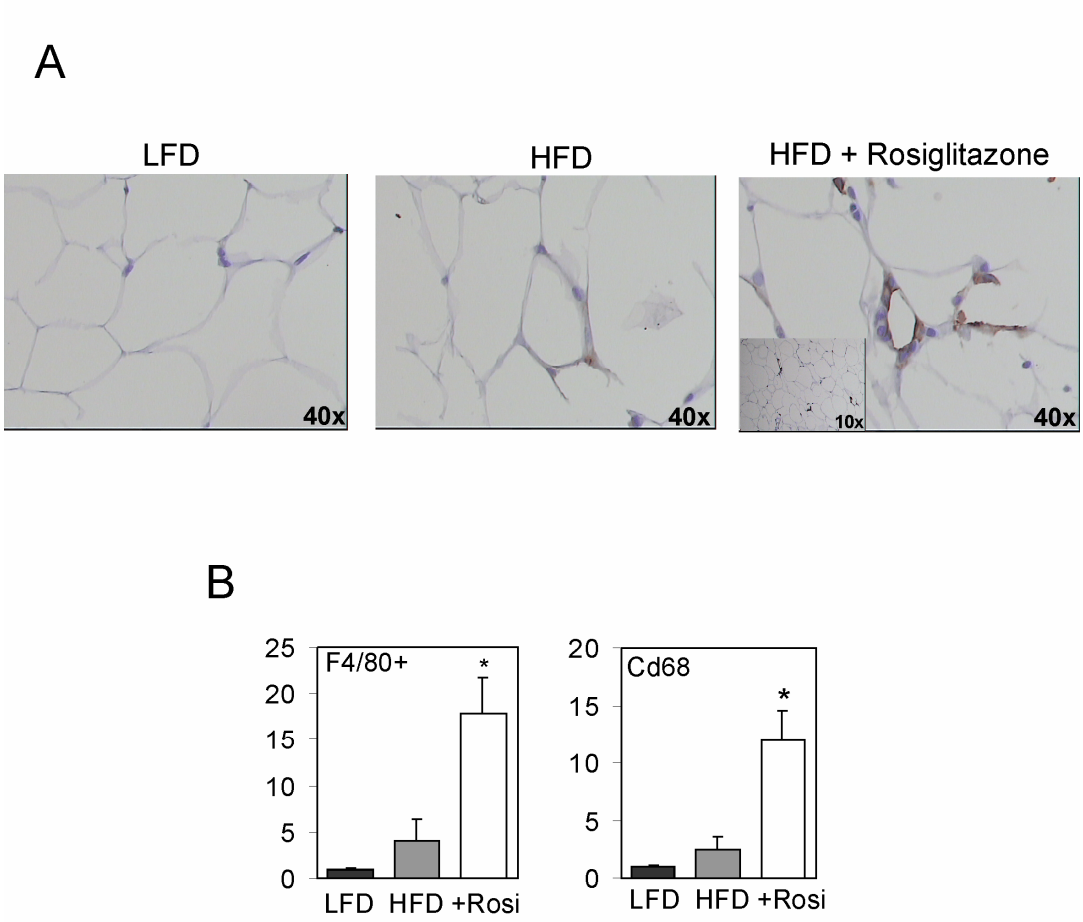

Figure 5 **More macrophages are present in adipose tissue after in HFD + rosiglitazone treated mice compared to HFD fed animals**- *(*A) Representative immunohistochemical staining of adipose tissue using the specific macrophage marker F4/80+. Magnification is indicated at the right bottom corner. (B) Expression levels of CD68 (P-value = 0.01) and F4/80+ (P-value = 0.01) were analyzed in adipose tissue by qPCR. Significantly higher expression levels for both markers are found in mice fed HFD + rosiglitazone compared to animals fed HFD only. Error bars represent SEM.

To investigate the effects of rosiglitazone on adipose tissue macrophages (ATMs), adipose tissue was fractionated into adipocytes and stromal vascular cells containing ATMs. Leptin was predominantly expressed in the adipocyte fraction confirming successful fractionation (Figure 6A). Remarkably, in the stromal vascular fraction, expression of Arginase I, which serves as a marker of alternatively activated macrophages, was significantly up-regulated by rosiglitazone. The same was true for the typical M2 marker gene sIL-1ra (9) and MCP-1, which act as a chemoattractant. In contrast, expression of IL-18, a cytokine linked to the classical activation of macrophages via Th1 type responses (19), was down-regulated by rosiglitazone. Together, these data suggest that rosiglitazone increases the presence of alternatively activated macrophages in adipose tissue.

It has been reported that alternatively activated macrophages stimulate cell proliferation and collagen production (9). Therefore, expression levels of Cyclin A2 and procollagen 1a1 were analysed in total WAT. Interestingly, expression of both genes was significantly increased by rosiglitazone, suggesting increased cell proliferation and collagen production.

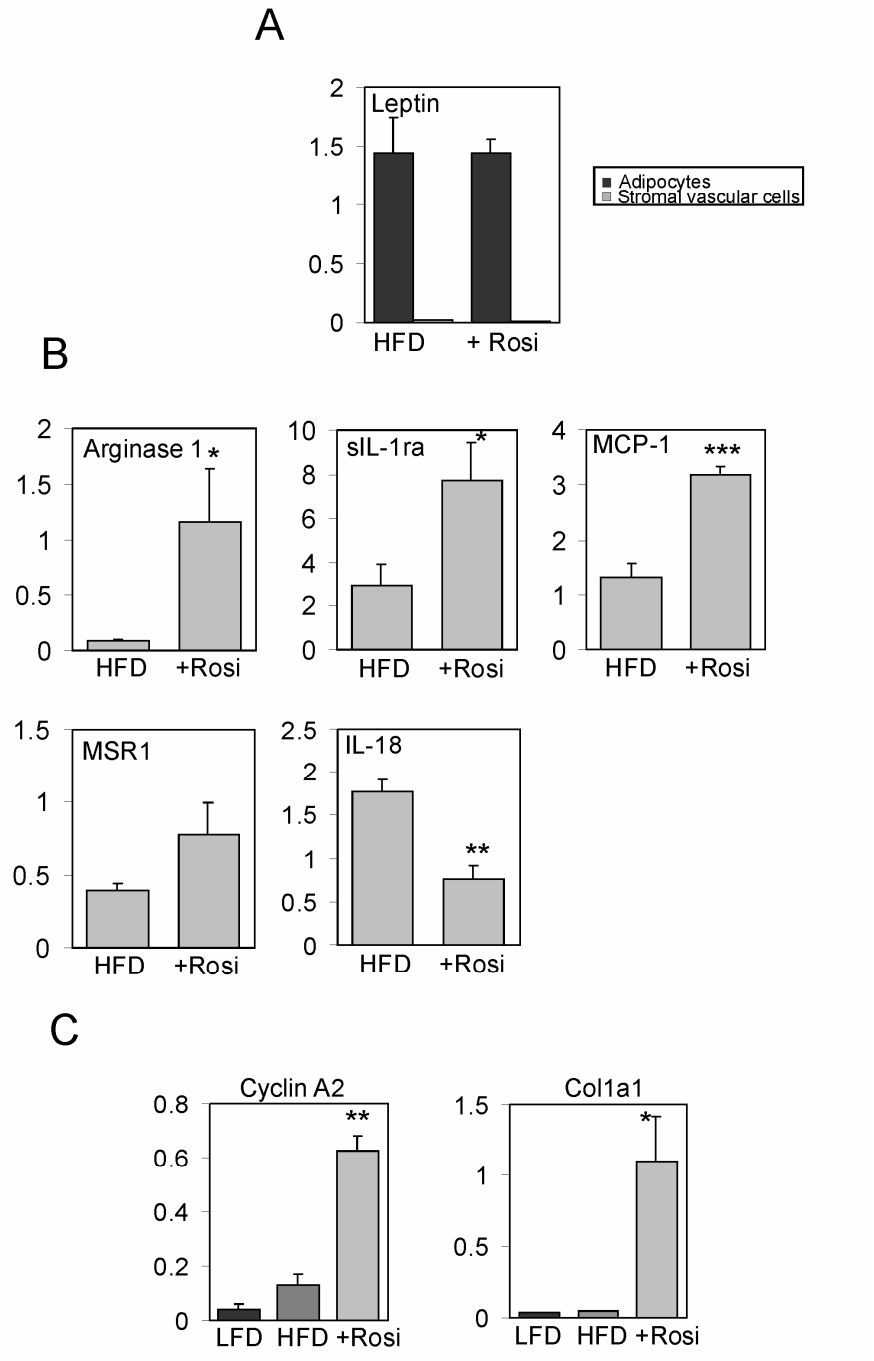

Figure 6 **Stromal vascular fraction of HFD + rosiglitazone fed mice shows higher expression levels of markers from alternatively activated macrophages compared to HFD fed animals**- (A) Expression of leptin is determined in adipocytes and stromal vascular fraction of adipose tissue from HFD and HFD + rosiglitazone fed mice. Expression of leptin is mainly localized in adipocytes. (B) Several markers indicative of the activation state of macrophages are measured by qPCR in stromal vascular fraction of adipose tissue of HFD and HFD + rosiglitazone fed animals. Significant changes in gene expression after rosiglitazone treatment were observed for Arginase I (P-value = 0.045), IL-1ra (P-value = 0.025), MCP-1 (P-value = 0.00016) and IL-18 (P-value = 0.001). (C) Expression of Cyclin A2 and Collagen 1A1 was measured in total WAT by qPCR analysis. Both genes (Cyclin A2, P-value = 0.0017; Collagen 1A1, P-value = 0.012) were significantly increased after rosiglitazone treatment. Error bars represent SEM

Discussion

In our study, one week of rosiglitazone treatment in mice increased adiposity, while simultaneously reducing hepatic triglyceride accumulation. Furthermore, rosiglitazone significantly decreased plasma insulin levels, suggesting improved insulin sensitivity. Surprisingly, treatment with rosiglitazone was associated with a marked increase in the number of macrophages present in adipose tissue, despite a clear down-regulation of pro-inflammatory gene expression. In the stromal vascular fraction of adipose tissue, we observed elevated expression of markers characteristic of alternatively activated macrophages. These data suggest that PPARγ activation disconnects inflammatory gene expression from macrophage infiltration in adipose tissue.

Obesity has been shown to be associated with the infiltration of macrophages into adipose tissue that may contribute to an elevated inflammatory status by secreting a variety of pro-inflammatory mediators. MCP-1 has been identified as an important chemo-attractant responsible for the infiltration of immune cells into adipose tissue (6) (20). Previous studies suggests that PPARγ can counteract obesity-induced inflammation in adipose tissue via several mechanisms including down-regulation of chemo-attractant and pro-inflammatory genes (5), apoptosis of adipose-resident macrophages (18), and changing the morphology of adipose tissue towards smaller adipocytes (21). In addition, PPARγ activation during obesity increases the oxidative capacity of adipocytes via mitochondrial remodeling (22). While our study supports an anti-inflammatory effect of PPARγ in adipose tissue, in contrast to previous studies we find that expression of MCP-1 is increased by rosiglitazone treatment, concurrent with an increase in the number macrophages present in adipose tissue.

Recently, it was reported that obesity leads to polarization of adipose-resident macrophages towards a M1 phenotype. M1 polarized macrophages secrete higher levels of pro-inflammatory genes and likely contribute to obesity-induced inflammation (8). The higher expression levels of Arginase I and sIL-1ra in rosiglitazone-treated animals is indicative of repolarization of adipose macrophages towards a M2 phenotype. Inasmuch as M2 or alternatively activated macrophages possess mainly anti-inflammatory properties, the switch in macrophage phenotype may be partially responsible for the suppression of inflammatory gene expression by rosiglitazone.

Furthermore, adipose macrophages might have a role in PPARγ-dependent remodeling of adipose tissue. The higher level of arginase I expression in adipose tissue of rosiglitazone-treated mice is indicative of a shift in macrophage metabolism towards a higher production of

ornithine and away from the generation of nitric oxide. Alternatively activated macrophages have been linked to tissue repair and cell proliferation. Indeed, co-culture experiments have shown that M2 activated macrophages can positively influence proliferation and collagen production of neighboring cells (23). In the mice treated with rosiglitazone, we observed a marked increase in the expression of genes related to cell cycle progression and collagen production. Insofar as PPARγ promotes energy storage, recruitment of M2 activated macrophages may contribute to PPARγ-induced remodeling and expansion of adipose tissue, serving the needs of the growing fat mass.

It is not fully clear why our data are at odds with two recent studies, both of which showing reduced adipose macrophages after PPARγ activation. Xu *et al.* reported that 2 weeks of rosiglitazone treatment decreased the expression of the typical macrophage marker F4/80+ in adipose tissue of ob/ob mice (5). Similarly, Bodles *et al.* showed that treatment of obese individuals with pioglitazone for 10 weeks (18) resulted in apoptosis of adipose-resident macrophages and a decrease in total macrophage numbers. Possibly, the response to PPARγ agonists may be dependent upon the animal model used.

Alternatively, it is possible that the response is related to the duration of treatment. Short term treatment with PPARγ agonist might cause a transient increase in adipose infiltration of macrophages, which will assist in the remodeling of adipose tissue. After remodeling, prolonged stimulation of PPARγ might remove surplus macrophages via apoptosis. Importantly, the increased presence of macrophages in adipose tissue does not lead to elevated inflammation. Experimental support for such a scenario will require systematic analysis of the time-dependent effect of PPARγ activation on adipose tissue morphology an function.

In conclusion, we show that short term PPARγ activation during HFD-induced obesity promotes macrophage infiltration into adipose tissue, while simultaneously reducing pro-inflammatory gene expression. The majority of the newly recruited macrophages appears to be alternatively activated and may play an important role in PPARγ-dependent adipose tissue remodeling and expansion.

***Acknowledgements*-**The authors would like to thank Rene Bakker for helping out with the animal experiments. This study was supported by the Centre for Human Nutrigenomics and Top Institute (TI) Food and Nutrition.

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Chapter 6

Research article

Glycogen Synthase 2 is a novel target gene of Peroxisome Proliferator-Activated Receptors

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This chapter has been published in **Cellular and Molecular Life Sciences**.

Abstract

Glycogen synthase 2 (Gys-2) is the rate-limiting enzyme in the storage of glycogen in liver and adipose tissue, yet little is known about regulation of Gys-2 transcription. The Peroxisome Proliferator-Activated Receptors (PPARs) are transcription factors involved in the regulation of lipid and glucose metabolism and might be hypothesized to govern glycogen synthesis as well. Here, we show that Gys-2 is a direct target gene of PPARα, PPARβ/δ and PPARγ. Expression of Gys-2 is significantly reduced in adipose tissue of PPARα -/-, PPARβ/δ -/- and PPARγ +/- mice. Furthermore, synthetic PPARα, β/δ, and γ agonists markedly up-regulate Gys-2 expression in mouse 3T3-L1 adipocytes. In liver, PPARα deletion leads to decreased glycogen levels in the refed state, which is paralleled by decreased expression of Gys-2 in fasted and refed state. Two putative PPAR response elements (PPREs) were identified in the mouse Gys-2 gene: one in the upstream promoter (DR-1prom) and one in intron 1 (DR-1int). It is shown that DR-1int is the response element for PPARs, while DR-1prom is the response element for Hepatic Nuclear Factor 4 alpha (HNF4α). In adipose tissue, which does not express HNF4α, DR-1prom is occupied by PPARβ/δ and PPARγ, yet binding does not translate into transcriptional activation of Gys-2. Overall, we conclude that mouse Gys-2 is a novel PPAR target gene and that transactivation by PPARs and HNF4α is mediated by two distinct response elements.

Introduction

Metabolic syndrome describes a combination of metabolic abnormalities that include central obesity, dyslipidemia, hypertension, insulin resistance, and a pro-inflammatory and pro-thrombotic state. An important group of pharmacological targets for the treatment of metabolic syndrome are the Peroxisome Proliferator Activated Receptors (PPARs). PPARs are ligand-activated transcription factors belonging to the superfamily of nuclear receptors, which include numerous cellular receptors for nutrients and steroids. So far, three PPAR isotypes (α, β/δ, γ) have been identified in a wide range of species, each displaying a different tissue distribution and ligand specificity (1). PPARs share a similar structure and a common molecular mechanism of action by forming an obligate heterodimer with the 9-cis retinoic acid receptor RXR. PPAR-RXR heterodimers selectively bind genomic sequences consisting of a direct repeat of the hexameric nucleotide sequence AGGTCA separated by 1 nucleotide (Direct Repeat-1). These so called peroxisome proliferator response elements (PPRE) are located in the promoter of PPAR target genes or in intronic regions (2) (3) (4) (5) .

The PPARα isotype (NR1C1) is highly expressed in liver and governs the adaptive response to fasting (6) (7) (8). PPARα is an extremely important regulator of hepatic nutrient metabolism including fatty acid oxidation (peroxisomal and mitochondrial), fatty acid uptake, amino-acid metabolism, glycerol metabolism, and lipoprotein assembly and transport (9) (10) (11). In addition, PPARα potently suppresses the hepatic inflammatory response (12) (13), an effect which is also observed in extra-hepatic tissues such as the vascular wall (14). Much less is known about the role of PPARα in other tissues, although evidence is accumulating that PPARα induces cardiac and skeletal muscle fatty acid oxidation (15) (16). Importantly, PPARα mediates the effects of hypolipidemic fibrate drugs, which decrease plasma triglycerides and increase plasma HDL concentrations. In contrast to PPARα, PPARγ (NR1C3) is highly expressed in white adipose tissue where it promotes lipid storage. PPARγ is a key transcription factor in the adipogenesis program and is essential for adipocyte survival (17) (18). It also serves as the molecular target for the thiazolidinedione class of insulin-sensitizing drugs that are widely used in the treatment of type 2 diabetes. PPARγ promotes whole body glucose utilization, however it has been difficult to identify the molecular mechanisms behind this effect. Much of the attention has been focused on possible cross-talk between adipose tissue and skeletal muscle, as muscle is responsible for the major share of whole body glucose utilization. However, adipose tissue is a large organ, especially in the obese, and accordingly it

can also be envisioned that the insulin sensitizing effect of TZDs on glucose uptake is partially exerted at the adipose tissue level.

While PPARα and PPARγ have been extensively studied over many years, much less is known about the function of the PPARβ/δ isotype (NR1C2). Studies with genetically modified PPARβ/δ mice have illustrated the importance of this nuclear receptor in white adipose tissue and skeletal muscle, two organs that have a key role in glucose homeostasis (19) (20) (21) (22). It was shown that activation of PPARβ/δ in adipose tissue protects against adiposity and hyperlipidemia by inducing fatty acid catabolism (21). Moreover, pharmacological activation as well as specific constitutive over-expression of PPARβ/δ leads to a shift in muscle fiber composition towards type I muscle fibers, resulting in increased muscle oxidative capacity (20, 22). PPARβ/δ has also been shown to stimulate hepatic VLDL production, influence wound healing, and affect colon carcinogenesis (23) (24) (25). However, whether PPARβ/δ has a functional role in glucose homeostasis, in analogy with other PPAR isotypes, remains to be firmly established.

Here we show that Glycogen synthase 2 (Gys-2), the rate limiting enzyme for glycogen synthesis in liver and adipose tissue, is a target gene of PPARα, PPARβ/δ and PPARγ. Transcriptional regulation is achieved via a PPAR response element present in the first intron. We shown that an additional direct repeat response element identified in the Gys-2 promoter mediates transactivation by Hepatic Nuclear Factor 4α (HNF4α).

Material and methods

Chemicals

Wy14643 was obtained from Eagle Picher Technologies laboratories (Lenexa, Kansas, USA). Rosiglitazone was from Alexis (Breda, the Netherlands). SYBR Green was from Eurogentec (Seraing, Belgium). Dulbecco's Modified Eagles Medium (DMEM), fetal calf serum (FCS), calf serum and penicillin/streptomycin/fungizone were from Cambrex Bioscience (Seraing, Belgium). Otherwise, chemicals were from Sigma (Zwijndrecht, the Netherlands).

Animal experiments

PPARβ/δ mutant null mice (PPARβ/δ -/-) and PPARγ heterozygous mice (PPARγ +/-) were on a mixed background (Sv129/C57BL/6) and have been described previously (26) (27). Wild-type littermates served as control animals. PPARα -/- mice and corresponding wild-type mice on Sv129 background were purchased at Jackson Laboratories (Bar Harbor, Maine, USA). Liver-specific HNF4α-null mice were generated as described previously. Livers were collected from 45-day old HNF4α$^{flox/flox}$ X albumin-Cre+ (KO) and HNF4α$^{flox/flox}$ X albumin-Cre- (FLOX) mice (28). Mice were maintained at 20°C with a 12h light-dark cycle. All mice were between 3-6 months of age. For the fasting experiment, 3-month old male mice were fasted for different periods of time starting at the onset of the light cycle. For the refeeding experiment, mice were fasted for 24 hours after which they were put back on chow for 7 hours before sacrifice. After sacrificing the animals, tissues were immediately frozen in liquid nitrogen. The animal experiments were approved by the animal experimentation committee of Wageningen University or the Etat de Vaud (Switzerland).

Oligonucleotide micro-array

Total RNA was prepared from epididymal WAT of wild-type and PPARβ/δ-/- mice (five animals of each genotype) using Trizol reagent (Invitrogen, Breda, the Netherlands) and subsequently pooled per group. Pooled RNA was further purified using Qiagen RNeasy columns and the quality was verified using Bioanalyzer 2100 (Agilent, Amsterdam). 10 μg of RNA was used for one cycle cRNA synthesis (Affymetrix, Santa Clara, USA). Hybridization,

washing and scanning of Affymetrix Genechip mouse genome 430 2.0 arrays was according to standard Affymetrix protocols. Fluorimetric data were processed by Affymetrix GeneChip Operating software and the gene chips were globally scaled to all the probe sets with an identical target intensity value. Further analysis was performed by Data Mining Tool (Affymetrix).

3T3-L1 adipogenesis assay

3T3-L1 fibroblasts were grown in DMEM plus 10% calf serum and plated for final differentiation in DMEM plus 10% FCS. Two days after reaching confluence, the medium was changed and the following compounds were added: Isobutyl methylxanthine (0.5 mM), dexamethasone (1 µM), and insulin (5 µg/mL). After 3 days, the medium was changed to DMEM plus 10% FCS and insulin (5 µg/mL). After 6 days the medium was changed to DMEM plus 10% FCS, which was changed every 3 days.

Primary mouse or rat hepatocyte isolation

Primary mouse and rat hepatocytes were isolated as described previously (29). Briefly, after cannulation of the portal vein, the liver was perfused with calcium free HBSS which was pre-gassed with 95% O_2 /5% CO_2. Next, the liver was perfused with a collagenase solution until swelling and degradation of the internal liver structure was observed. The hepatocytes were released, filtered and washed several times using Krebs buffer. The viability was assessed by tryptan blue staining and was at least 80%. Cells were cultured in William's Medium E supplemented with 10% FCS, penicillin/streptomycin/fungizone, insulin and dexamethasone. Cells were plated in collagen (Serva Feinbiochemica, Heidelberg, Germany) coated wells with a density of 0.5 x 10^6 cells/ml. After 4 hours of incubation, the medium was removed and replaced with fresh medium. The next day, hepatocytes were used for experiments.

RNA isolation, Reverse-Transcription, and Real-time quantitative PCR

Total RNA was extracted from tissues with TRIzol reagent (Invitrogen, Breda, the Netherlands). 1 µg 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 or MyIQ 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. Expression was related to the control gene 36B4, which did not change under any of the experimental conditions studied. The following primer pairs were used: mGys-2 (forward): CCAGCTTGACAAGTTCGACA, mGys-2 (reverse): ATCAGGCTTCCTCTTCAGCA, m36B4 (forward): AGCGCGTCCTGGCATTGTGTGG, m36B4 (reverse): GGGCAGCAGTGGTGGCAGCAGC, mPPARα (forward): TATTCGGCTGAAGCTGGTGTAC, mPPARα (reverse): CTGGCATTTGTTCCGGTTCT, mPPARβ (forward): TTGAGCCCAAGTTCGAGTTTG, mPPARβ (reverse): CGGTCTCCACACAGAATGATG, mPPARγ (forward): CACAATGCCATCAGGTTTGG, mPPARγ (reverse): GCTGGTCGATATCACTGGAGATC.

Transactivation assay

The proximal part of the mouse Gys-2 promoter was PCR amplified from mouse genomic DNA (strain C57/B6) using the forward primer: 5' CTTGCTGCCTTTCAGGAGAGGGCAG 3' and reverse primer: 5' TTCTCTTTAGCCATTAAGATAG 3'. The resulting 553 base pair fragment was used for a second PCR amplification step introducing HindIII and KpnI sites which were used for subcloning into the pGL-3 Basic vector (Invitrogen).

A 156 bp nucleotide fragment surrounding the putative PPRE within the mGys-2 promoter was PCR amplified from mouse genomic DNA (strain C57/B6) and subcloned into KpnI/BglII sites of the pGL3 SV40 promoter vector (pGL3-tk-LUC, Promega, Leiden, the Netherlands) using the forward primer: 5'AAATCGCAGCTGAAACCT 3', and reverse primer: 5' CTCCTGCTTGTGCTTCTGC 3'. A 314 nucleotide fragment surrounding the putative PPRE within intron 1 of the mouse Gys-2 gene was PCR amplified from mouse genomic DNA (strain C57/B6) and subcloned into the KpnI and BglII sites of the pGL-Tk-Luc reporter gene. Reporter vectors were transfected into human hepatoma HepG2 cells, together with an expression vector (pSG5) for mPPARα, mPPARβ, or mPPARγ1, in the presence or absence of Wy14643 (50 μM), L-165041 (5 μM), or rosiglitazone (5 μM), respectively. Transfections were carried out using the calcium-phosphate precipitation method. A β-galactosidase reporter vector was co-transfected to normalize for differences in transfection efficiency. Luciferase activity was measured 24 hours 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-βD-galactopyranoside as a substrate. To disable the mouse Gys-2 PPRE within the mGys-2 promoter, two separate (A and B) partially overlapping PCR fragments were generated using the wild-type mGys-2 promoter as a template. Primers sets used to generate part A of the mutated mGys-2 promoter fragment were: 5'-TTTGGTCTAAAGGCCTTTGGCCAAAGG-3' and 5'-CTTGCTGCCTTTCAGGAGAGGGCAG-3'. Primers sets used to generate part B of the mutated mGys-2 promoter fragment were: 5'-CCTTTGGCCAAAGGCCTTTAGACCAAA-3' and 5' TTCTCTTTAGCCATTAAGATAGGGATTG-3'. PCR was carried-out using the two upper DNA fragments and the following primers: 5'-cccaagcttCTTGCTGCCTTTCAGGAG-3' and 5'-ggggtaccTTCTCTTTAGCCATTAAGATAG-3'. The PCR fragment was subsequently cloned into the pGL3 basic reporter vector (HindIII/KpnI cloning site) and verified by automated sequencing.The hHNF4α expression plasmid was constructed by amplifying human hepatoma HepG2 cDNA using the following primers, forward primer 5'-GAATGCGACTCTCCAAAACC-3' and reverse primer 5'ATCCTTCCCATTCCTGCTCT-3', followed by subcloning of the resulting PCR product into an pGEM-Teasy vector (Promega). The insert was excised by NotI digestion and further subcloned into pcDNA3.1/V5-HisA (Invitrogen). The nucleotide sequence was verified by automated sequencing.

Gel shift

hRXRα and mPPARγ proteins were generated from pSG5 expression vectors, using the TNT coupled in vitro transcription/translation system (Promega). The following oligos were annealed to generate the double stranded DNA probe; for DR-1int: 5'-CAGGACTTTGGTGACCTCTGGCCTATAT -3' and 5'-ACACATATAGGCCAGAGGTCACCAAAGTC -3'. For non-specific competition, the following primers were used: etsF 5'-TGGAATGTACCGGAAATAACACCA-3', etsR 5'-TGGTGTTATTTCCGGTACATTCCA-3'. Oligonucleotides were annealed and labeled by Klenow filling (New England Biolabs, Leusden, the Netherlands) using Redivue [α-^{32}P] dCTP (3000 Ci/mmol) (Amersham, Roosendaal, the Netherlands). *In vitro* translated proteins

(0.5-0.8 µl per reaction) were pre-incubated for 15 min on ice in 1x binding buffer (80 mM KCl, 1 mM DTT, 10 mM Tris-Cl pH 7.4, 10% glycerol, plus protease inhibitors) in presence of 2 µg of poly[dI.dC], 5 µg of sonicated Salmon sperm DNA and competitor oligonucleotides in a final volume of 20 µl. Then 1 ng (1ng/µl) of radiolabeled oligo was added and incubation continued for another 10 min at room temperature. Complexes were separated on a 4% polyacrylamide gel (acrylamide/bisacrylamide 37.5:1) equilibrated in 0.5x TBE at 25 mA.

Chromatin Immunoprecipitation (Chip)

Chromatin immunoprecipitation on 3T3-L1 cells and mouse liver was carried out as described previously (11). Sequences of primers used for PCR were 5'-TCTGGCAGGCATAAGGACCCGAGTT-3' and 5'-GGAAGCCAGGACAGAGTGCAAATACAAT -3' for DR-1int (Intron). For DR-1prom, the following primers were used: 5'-AAAACTGCTTGTGTCTGAGGGAAAC-3' and 5'-AGAGGACAGACTGAGCATGACAAGAG-3'. Control primers used were 5'-GCTGCGAGATCCATCACCCACTAAAC-3' and 5'-AGCCATCTCACCAGCCCCAACTT-3'. Antibodies against PPARs were from Santa Cruz Biotechnology (Santa Cruz, California, U.S.A.). Chromatin immunoprecipitation on rat hepatocytes was done using a commercially available kit (Active Motif, Rixensart, Belgium). The HNF4α antibody was from Tebu-Bio (Heerhugowaard, The Netherlands). The primers used to amplify the sequence surrounding the DR-1prom were 5'-GAATGCCGCTGTGCCTGAGGGAAAC-3' and 5'-AGAGGACAGAAGAAGAGTGACAAGAG-3'. For DR-1int: 5'-TCTGTCAGGCATAAGGACCTGGGTT-3' and 5'-ATTGTATTTGAACTCTGTCCTGGTCTCT-3'.

Histology

Liver tissue from Wildtype and PPARα-/- mice was embedded in Tissue-Tek O.C.T. Compound from Sakura Finetek (Zoeterwoude, the Netherlands) and frozen. Cryosections of 5 µm from frozen liver were made and analyzed for glycogen accumulation using the periodic acid–Schiff (PAS) reaction. Hematoxylin and eosin (H&E) staining of liver cryosections was done using standard protocols.

Western-blot

A mouse anti-glycogen synthase monoclonal antibody was used (clone GS-7H5 MAB3106) (Chemicon International, Hampshire, UK). Western blotting was carried out as previously described (4). The primary antibody was used at a dilution of 1:1000 and the secondary antibody (anti-mouse IgG, Dako, Glostrup, Denmark) was used at a dilution of 1:8000.

Table 1 Genes involved in glucose and lipid metabolism that were differentially expressed between white adipose tissue of wild-type and PPARβ/δ -/- mice.

Gene	Product	Fold-decrease	
		micro-array	Q-PCR
Glucose metabolism			
Gys2	glycogen synthase 2	13.27	9.28
Pik3r1	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	2.99	
Pik3r1	phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)	2.64	
Gys3	glycogen synthase 3, brain	2.25	
Ppp1r3c	protein phosphatase 1, regulatory subunit 3C, protein targeting to glycogen	2.19	
Slc2a4	solute carrier family 2 (facilitated glucosetransporter), member 4	2.16	1.56
Slc2a4	solute carrier family 2 (facilitated glucosetransporter), member 4	2.04	1.56
PFK-2	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase	2.01	
Gdc1	glycerol phosphate dehydrogenase 1, cytoplasmicadult	1.93	
Ppp1r3c	protein phosphatase 1, regulatory subunit 3C, protein targeting to glycogen	1.91	
Gdc1	glycerolphosphate dehydrogenase 1, cytoplasmicadult	1.84	
Pygl	liver glycogen phosphorylase	1.71	
Lipid Metabolism			
Pparg	peroxisome proliferator activator receptorgamma	2.99	3.63
C5D	sterol-C5-desaturase	2.85	
Lrp1	low density lipoprotein receptor-related protein1	2.22	
Cd36	CD36 antigen	2.20	3.09
Fads3	fatty acid desaturase 3	2.06	
Lipe	lipase, hormone sensitive	1.96	1.66
Lrp2	low density lipoprotein receptor-related protein 2	1.91	
Fabp5	fatty acid binding protein 5, epidermal	1.87	
Dgat1	diacylglycerol acyltransferase	1.87	
Decr1	2,4-dienoyl CoA reductase 1, mitochondrial	1.85	
Slc27a1	solute carrier family 27 (fatty acidtransporter), member 1	1.85	
Phyh	phytanoyl-CoA hydroxylase	1.77	
Dgat2l1	diacylglycerol O-acyltransferase 2-like 1	1.75	
Ppargc1	peroxisome proliferative activated receptor, gamma, coactivator 1	1.74	1.97

Results

Expression of glycogen synthase-2 in white adipose tissue is regulated by PPARs- Our initial aim was to identify novel putative target genes of PPARβ/δ in white adipose tissue (WAT). Accordingly, we compared gene expression in WAT of wild-type versus PPARβ/δ -/- mice using Affymetrix micro-array analysis. The expression of several genes involved in glucose and lipid metabolism was down-regulated in PPARβ/δ -/- mice, including PPARγ, PGC-1α, and GLUT4, which was confirmed for several genes by real-time quantitative PCR (qPCR) (table 1). Expression of glycogen synthase 2 (Gys-2) was most significantly down-regulated in PPARβ/δ -/- mice and therefore Gys-2 was selected for more detailed investigation. Q-PCR confirmed the marked down-regulation of Gys-2 mRNA in WAT of PPARβ/δ -/- mice (Fig. 1A). Furthermore, expression of Gys-2 also appeared to be down-regulated in WAT of PPARγ +/- and PPARα -/- mice, although the former result did not achieve statistical significance. In PPARβ/δ -/- mice, the decrease in Gys-2 mRNA was paralleled by a significant down-regulation of PPARα and PPARγ expression, while in PPARα -/- mice expression of PPARβ/δ was significantly down-regulated (Fig. 1B). These data shown that PPARs are crucial for maintaining Gys-2 expression in fat, although it is difficult to ascertain which PPAR isotype is the main regulator of Gys-2 expression in WAT.

To investigate whether expression of Gys-2 in adipocytes may be under direct control of PPARs, the effect of PPAR ligands on Gys-2 mRNA was studied in differentiated mouse 3T3-L1 adipocytes. It was observed that the PPARβ/δ agonist L165041, and the PPARγ agonists ciglitazone and rosiglitazone significantly induced Gys-2 mRNA levels (Fig. 1C). This effect was confirmed at the protein level (Fig. 1D). Thus, Gys-2 may represent a direct target gene of PPARγ and PPARβ/δ in adipocytes.

PPARα governs Gys-2 hepatic expression- A link between PPARs and glycogen has been previously made. It was observed that liver glycogen levels were reduced in refed PPARα -/- mice compared to refed wild-type mice (30) (31), which we confirmed using histochemical staining (Fig. 2). Furthermore, hepatic expression of Gys-2 was decreased in PPARα -/- mice (32). Expression of Gys-2 is highest in liver, followed by WAT (our unpublished data).

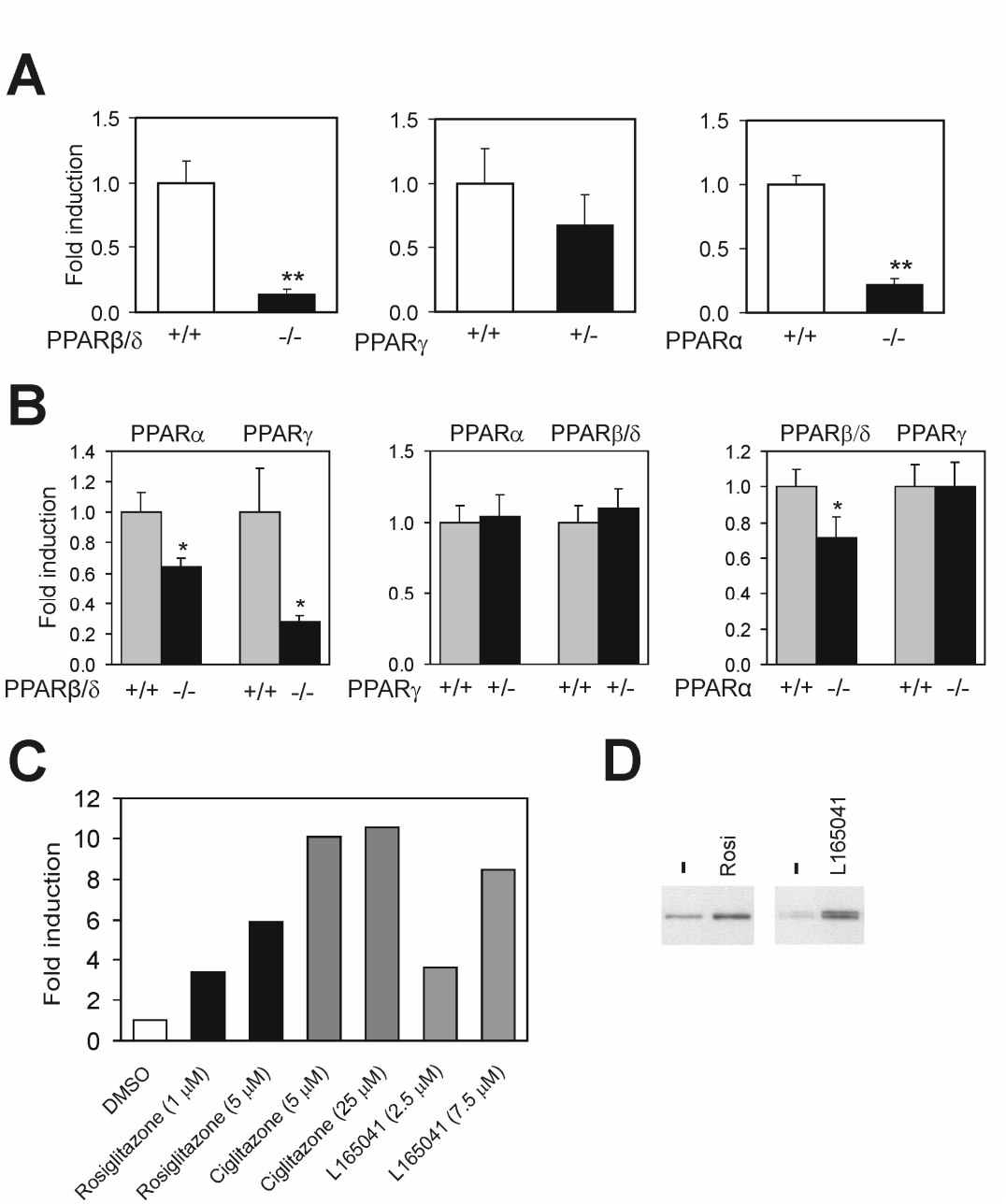


Figure 1 **Expression of Gys-2 in WAT is regulated by PPARs**. Expression of Gys-2 (A) and PPARs (B) in WAT of PPARβ/δ -/- mice, PPARγ +/- mice, and PPARα -/- mice, as determined by qPCR. The effects of PPAR deletion were evaluated by students t-test (* = $p<0.05$; ** = $p<0.01$). Error bars represent SEM (n=5). (C) Differentiated 3T3-L1 adipocytes were treated with the synthetic PPARγ agonists rosiglitazone or ciglitazone, or the PPARβ/δ agonist L165041 for 24h. Expression of Gys-2 was determined by qPCR. Expression of cells treated with vehicle (DMSO) was set at 1. Data shown are representative results from three independent experiments. (D) Gys protein expression was analysed in lysates from 3T3-L1 adipocytes treated with either PPARβ/δ (L165041, 2,5 μM) or PPARγ (rosiglitazone, 1 μM) agonist.

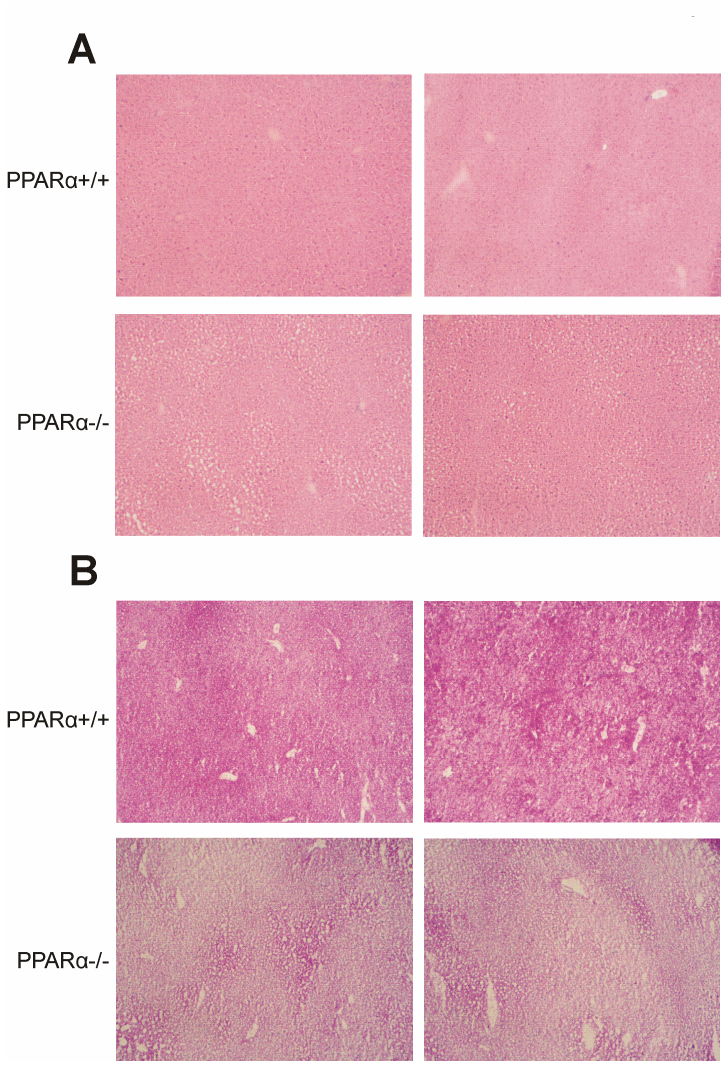


Figure 2 **Staining for hepatic glycogen is higher in refed Wildtype mice compared to refed PPARα -/- mice.** (A) Representative hematoxylin and eosin staining of liver from two Wildtype and two PPARα -/- mice. (B) Representative PAS staining of liver from two Wildtype and two PPARα -/- mice. Mice were fasted for 24 hours followed by refeeding for 7 hours before sacrifice.

We confirm that hepatic Gys-2 mRNA is markedly reduced in PPARα -/- mice, however only in the 24h fasted and refed state (Fig. 3A). To further examine the role of PPARα in Gys-2 expression, primary hepatocytes from wild-type and PPARα -/- mice were treated with the synthetic PPARα agonist Wy14643, allowing for a direct evaluation of the effect of PPARα activation on Gys-2 expression. Basal expression of Gys-2 was about 4-fold reduced in PPARα -/- hepatocytes, indicating a requirement for PPARα (Fig. 3B). Furthermore,

Wy14643 stimulated Gys-2 expression in wild-type but not in PPARα -/- hepatocytes (Fig. 3B). Induction of Gys-2 expression by synthetic PPARα agonists was also observed in rat primary hepatocytes (Fig. 3C). Together, these data suggest a direct role of PPARα in governing hepatic Gys-2 expression.

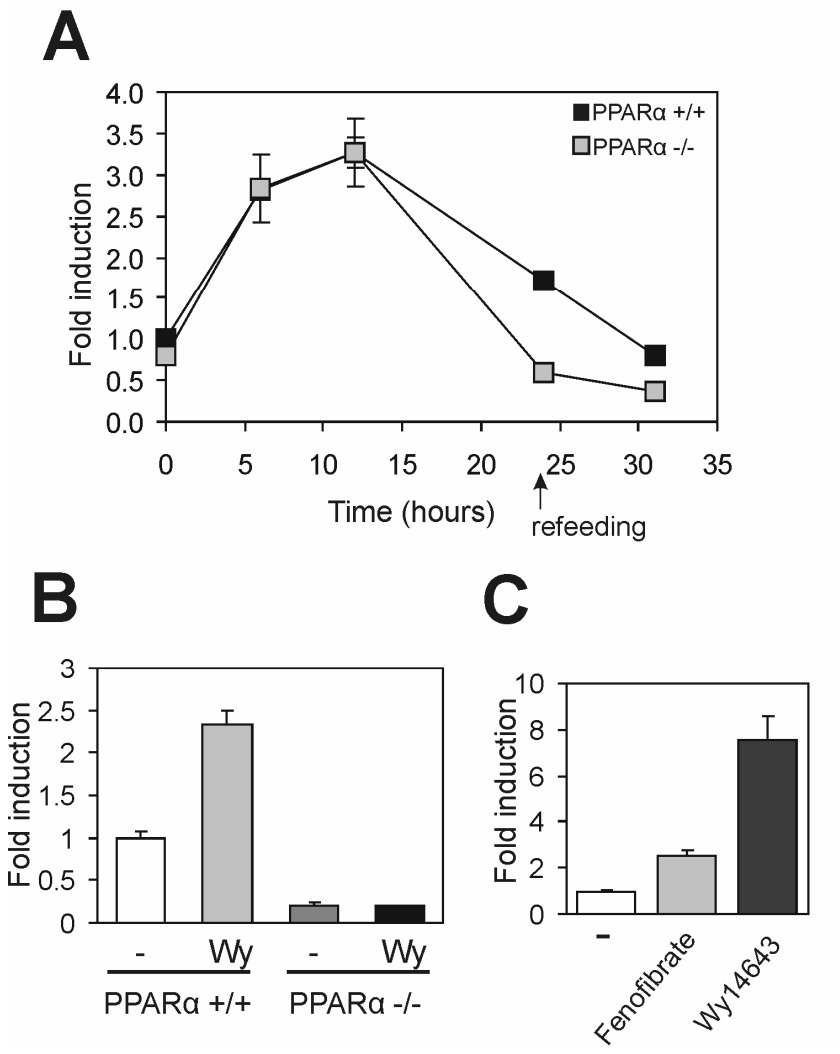


Figure 3 **PPARα governs hepatic expression of Gys-2.** (A) Relative expression of Gys-2 in fed, fasted and refed wild-type and PPARα -/- mice, as determined by qPCR. Mice were fasted for 0h, 6h, 12h, 24h, or 24h followed by refeeding for 7h. Significant differences between Wildtype and PPARα -/- mice were observed in the 24h fasted and the refed state (B) Relative expression of Gys-2 in freshly isolated wild-type and PPARα -/- hepatocytes treated for 24h with vehicle (DMSO) or Wy14643 (10 μM), as determined by qPCR. Significant effects were observed by two-way ANOVA for genotype ($p<0.001$), and for the interaction between the genotype and Wy14643 ($p<0.05$). (C) Relative expression of Gys-2 in freshly isolated rat hepatocytes which were treated for 24h with either vehicle (DMSO), Wy14643 (50 μM) or fenofibrate (50 μM). The effects of Wy14643 and fenofibrate was statistically significant (Students t-test: $p<0.01$). Error bars represent SEM.

Identification of a putative PPRE in the proximal promoter of the mouse Gys-2 gene- To determine what genomic region could be responsible for the PPAR-induced up-regulation of Gys-2 mRNA, the mouse Gys-2 gene was scanned for potential PPREs (NUBIScan algorithm and Hidden Markov Model framework) (33) (34). A Direct Repeat-1 motif (DR-1prom) was localized to the proximal Gys-2 gene promoter, about 169 base pairs upstream from the transcription start site. With the exception of two nucleotides, DR-1prom is identical to the consensus sequence, suggesting that this sequence could serve as a functional PPRE (Fig. 4A). DR-1prom was conserved between mouse and rat.

To examine whether the promoter region containing the putative PPRE is responsible for PPAR-dependent up-regulation of Gys-2 expression, a 553 nucleotide-fragment of the mouse Gys-2 promoter gene was cloned in front of a luciferase reporter gene and transactivation studies carried out in HepG2 cells. Surprisingly, co-transfection of PPARα or PPARγ1 expression vectors in combination with PPAR agonists slightly decreased luciferase activity, while PPARβ/δ activation had little effect (Fig. 4B). Transactivation assays performed with a small genomic fragment surrounding DR-1prom cloned in front of SV40-luciferase led to a similar overall PPAR mediated-repression for PPARα and PPARγ1, while PPARβ/δ had little effect (Fig. 4C). Co-transfection of RXR or of different co-activators such as CBP and PGC1α did not change this pattern (data not shown). Thus, the PPRE identified in the Gys-2 promoter probably does not mediate the effect of PPARs on Gys-2 expression. Nevertheless, chromatin immunoprecipitation (ChIP) experiments carried out in 3T3-L1 cells indicated that 1) PPARγ was bound to DR-1prom in mature adipocytes, but not in pre-adipocytes (Fig. 4D), and 2) PPARβ/δ was bound to DR-1prom in pre-adipocytes and, more strongly, in mature adipocytes (Fig. 4E). Thus, despite DR-1prom behaving poorly as a PPAR response element in classical transactivation assay, it binds both PPARγ and PPARβ/δ in adipocytes. This suggests that in vivo binding of PPARγ and PPARβ/δ to DR-1prom does not translate into transcriptional activation of the Gys-2 gene and accordingly that activation of Gys-2 expression by PPARs may be mediated by another genomic region. It should be mentioned that ChIP did not reveal any binding of PPARα to DR-1prom in hepatocytes (data not shown).

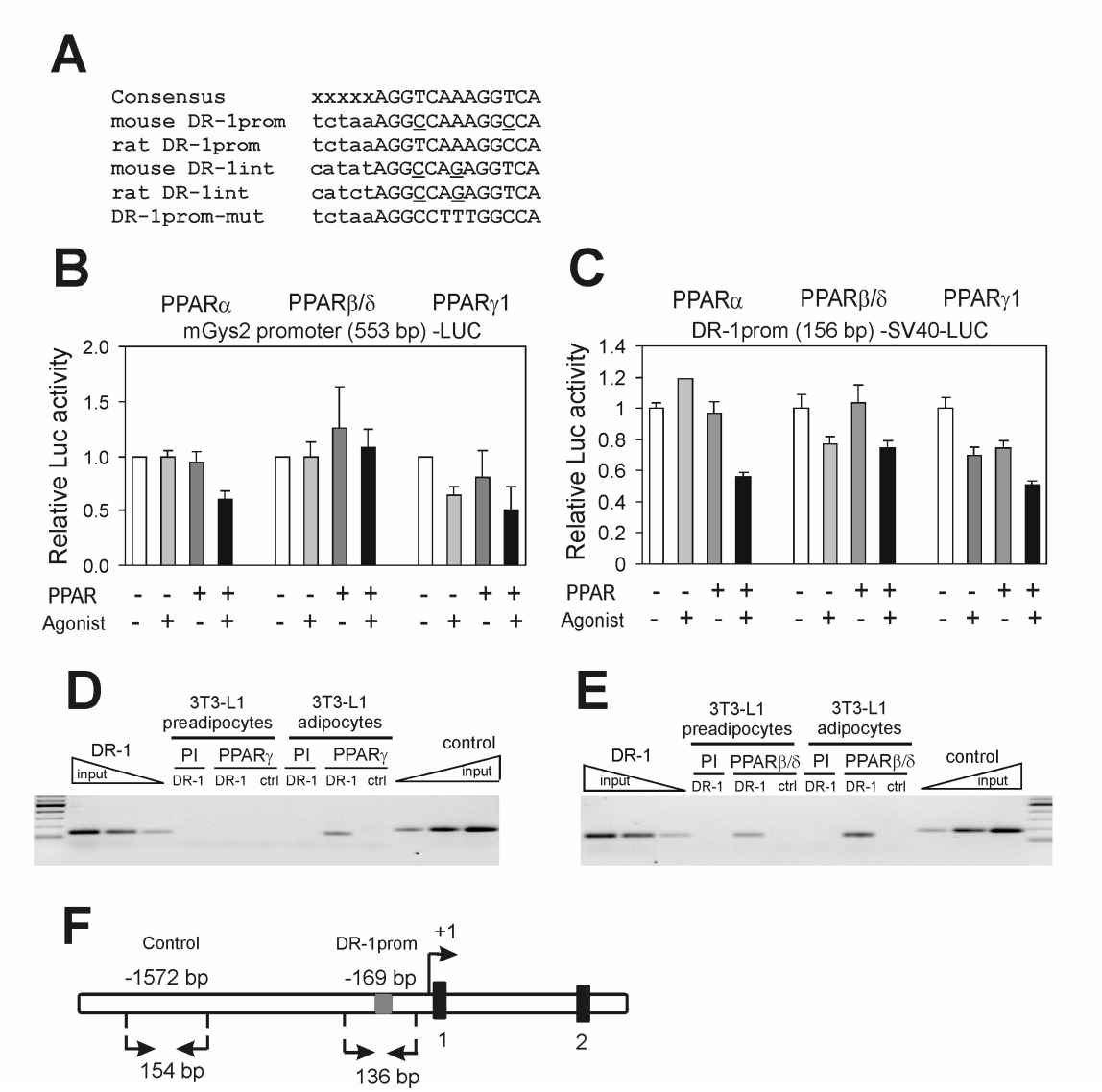


Figure 4 **The DR-1 present in the Gys2 promoter does not mediate PPAR-dependent transactivation.** (A) Alignment of the consensus PPRE sequence with the sequence of rat and mouse Gys-2 DR-1prom and Gys-2 DR-1int. (B) HepG2 cells were transfected with a reporter vector containing a 553-nucleotide fragment of the proximal mouse Gys-2 promoter gene and PPAR expression vectors. (C) HepG2 cells were transfected with a SV40 reporter vector containing an isolated 156-nucleotide fragment surrounding DR-1prom of the proximal mouse Gys-2 promoter gene and PPAR expression vectors. Luciferase and β-galactosidase activities were determined 24h after exposure of the cells to different PPAR agonists: 50 μM of Wy14643, 5 μM of L-165041 and 10 μM of rosiglitazone. Error bars represent SEM. Chromatin immunoprecipitation of DR-1prom using antibodies against mPPARγ (D) or mPPARβ/δ (E). The gene sequence spanning DR-1prom and a random control sequence were analyzed by PCR in the immunoprecipitated chromatin of 3T3-L1 preadipocytes and mature adipocytes. Preimmune serum was used as a control. *PI*, preimmune serum, *Cntl*, random control sequence. (F) Schematic overview of localization of primers used for amplification of immunoprecipitated DNA.

Interestingly, using the same strategy as described above, a putative PPRE that is homologous to the consensus DR-1 sequence was identified in intron 1 of the mouse Gys-2 gene (Fig. 4A). To assess whether DR-1int was able to mediate PPAR-dependent transactivation, a 314 nucleotide genomic fragment surrounding DR-1int was cloned in front of the SV40 promoter followed by a luciferase reporter gene. In HepG2 cells treatment with the synthetic PPARα agonist Wy14643 induced reporter activity and this activation was further enhanced upon co-transfection of mPPARα (Fig. 5A). Similar inductions of reporter activity were observed for PPARβ/δ and PPARγ and their respective agonists (Fig. 5A). Thus, DR-1int is able to mediate PPAR-dependent transactivation, irrespective of the PPAR isotype, suggesting that it may at least be partially responsible for PPAR-dependent regulation of Gys-2 expression.

In agreement with the transactivation data, PPARα, PPARβ/δ and PPARγ proteins were able to specifically bind DR-1int in gel shift experiments. A retarded heterodimeric complex was observed only in the presence of both PPAR and obligate binding partner RXRα (Fig. 5B). The complex disappeared in the presence of an excess of cold specific oligonucleotide, but not non-specific oligonucleotide.

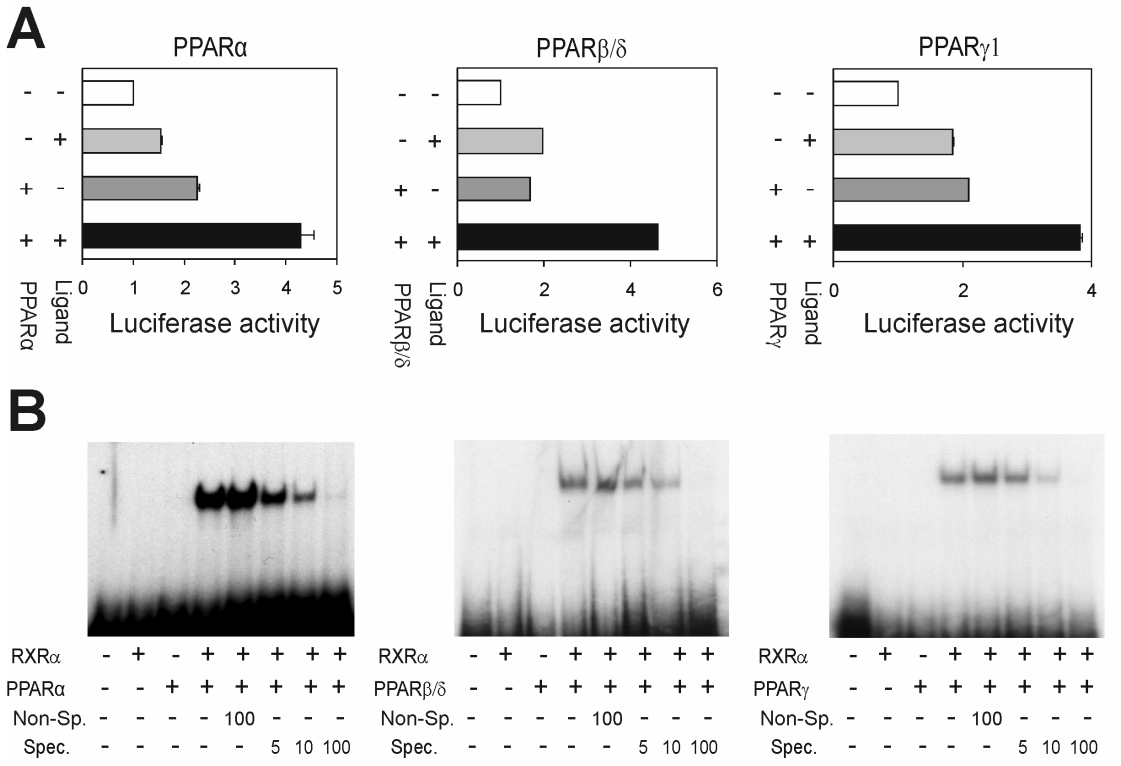


Figure 5 **Gys-2 upregulation by PPARs is mediated by a PPRE present in intron 1 of the Gys-2 gene (DR-1int).** (A) HepG2 cells were transfected with a 314-nucleotide fragment of intron 1 of the mouse Gys-2 gene and PPAR expression vectors. Luciferase and β-galactosidase activities were determined 24h after exposure of the cells to different PPAR agonists: 50 μM of Wy14643, 5 μM of L-165041 and 10 μM of rosiglitazone. Error bars represent SEM. (B) Binding of the PPAR/RXR heterodimers to DR-1int as determined by gel shift assays. A double-stranded response element containing Gys-2 DR-1int was incubated with *in vitro* transcribed/translated mPPARα protein (left panel), mPPARβ/δ protein (middle panel) and mPPARγ1 protein (right panel) together with *in vitro* transcribed/translated hRXRα protein. Fold excess of specific (malic enzyme PPRE) or nonspecific (ETS oligonucleotide) cold probe is indicated.

Examination of in vivo PPAR binding to DR-1int by ChIP yielded very similar results as for DR-1prom: PPARγ was bound to DR-1int in mature 3T3-L1 adipocytes, but not in pre-adipocytes (Fig. 6A), whereas PPARβ/δ was bound to DR-1int in both pre- and mature adipocytes (Fig. 6B). In liver, ChIP analysis demonstrated binding of PPARα to DR-1int in wild-type but not PPARα -/- mice, and binding was enhanced by fasting and Wy14643 (Fig. 6C). Together, these data indicate that mouse Gys-2 is a direct PPAR target gene and that regulation by PPARs is at least partially mediated by a PPRE present in intron 1.

Gys-2 is a novel direct target of the liver enriched factor HNF4α. As explained above, PPARα caused a reduction in Gys-2 promoter activity via DR-1prom (Fig. 4B and 4C). A similar decrease of promoter activity in response to PPARα despite the presence of a putative PPRE has been reported for other genes. Indeed, it was found that PPARα decreases expression of the apoCIII and transferrin genes via competition with the hepatocyte nuclear factor 4α (HNF4α). Since HNF4α is known to recognize DR-1 sequences as well, we examined whether HNF4α might control the expression of Gys-2 in liver, possibly via DR-1prom. Gys-2 mRNA levels were markedly decreased in liver specific HNF4α null mice, thus supporting a role for HNF4α in regulating Gys-2 expression (Fig. 7A). In transactivation assays using the Gys-2 promoter, HNF4α markedly activated reporter activity, suggesting the presence of a HNF4α response element within the 0.55 kb promoter fragment (Fig. 7B). Mutating DR-1prom resulted in an approximately 50% reduction in HNF4α–dependent activation of the Gys-2 promoter (Fig. 7C), which suggests that a) HNF4α responsiveness is partially mediated by DR-1prom, or b) the mutations with DR-1prom only partially disabled HNF4α responsiveness.

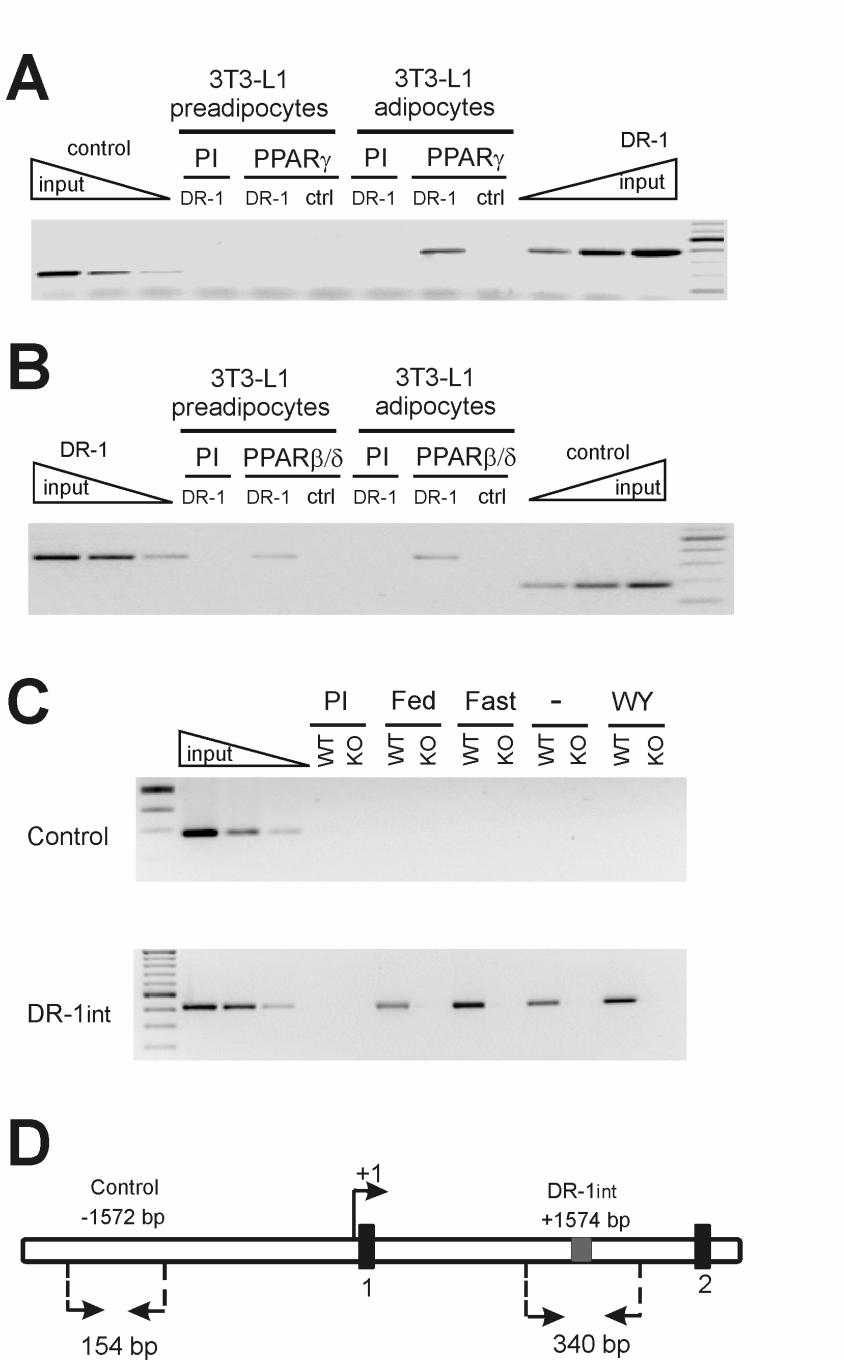
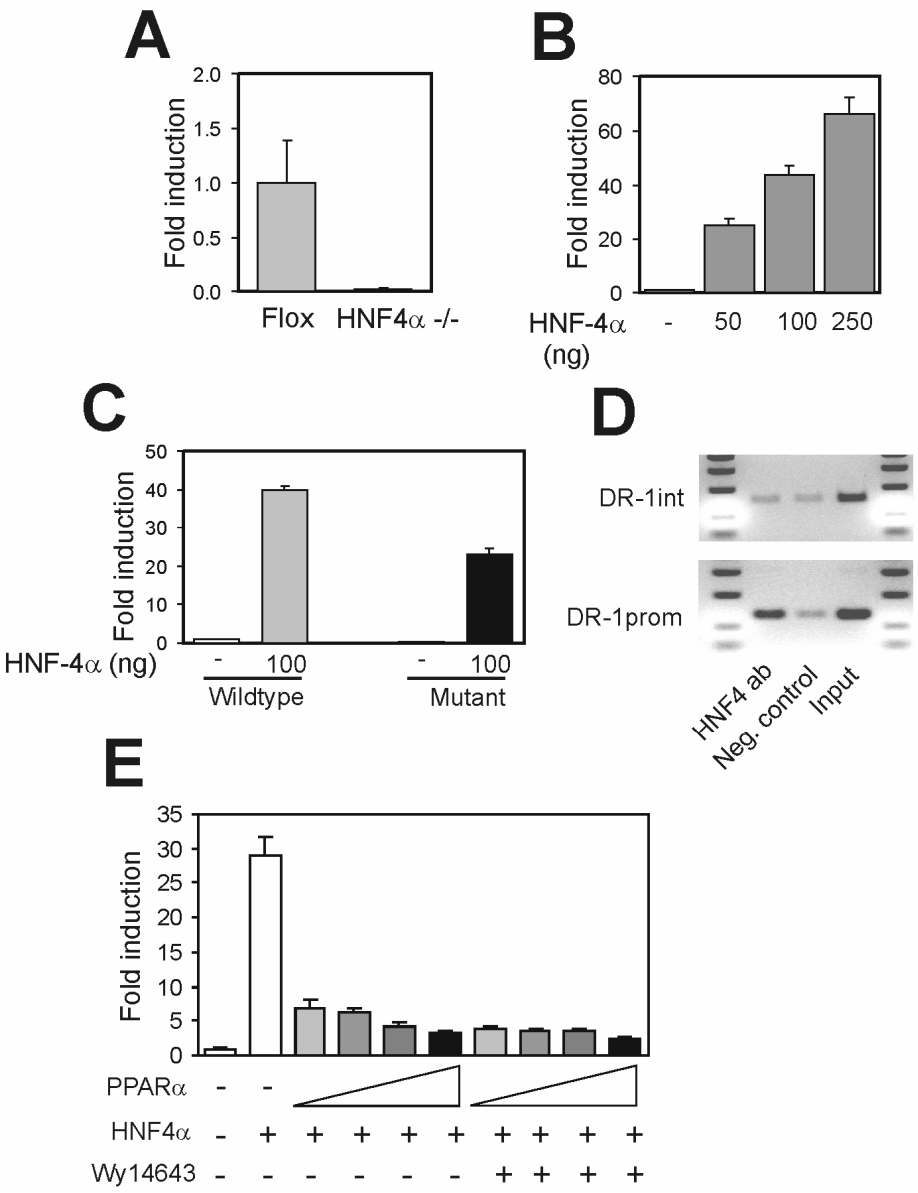


Figure 6 **PPARβ/δ, PPARγ, and PPARα bind to the Gys-2 DR-1int in vivo.** Chromatin immunoprecipitation of Gys-2 DR-1int using antibodies against mPPARγ (A), mPPARβ/δ (B) or mPPARα (C). The gene sequence spanning DR-1int and a random control sequence were analyzed by PCR in the immunoprecipitated chromatin of 3T3-L1 preadipocytes and mature adipocytes (A,B) or mouse liver (C). Preimmune serum was used as a control. *PI*, preimmune serum, *Cntl*, random control sequence. (D) Schematic overview of localization of primers used for amplification of immunoprecipitated DNA.

Regardless of these explanations, these data suggest that HNF4α directly regulates the hepatic expression of Gys-2 at least partially via DR-1prom. Finally, ChIP clearly showed HNF4α binding to DR-1prom but not DR-1int in rat primary hepatocytes (Fig. 7D).

Cross-talk between PPARα and HNF4α in the transcriptional control of Gys-2- Whereas HNF4α activates the Gys-2 promoter via DR-1prom, PPARα does the opposite, suggesting that PPARα may interfere with Gys-2 promoter activation by HNF4α. To examine whether this is the case, the effect of PPARα on HNF4α-mediated transactivation of the 0.55 kb Gys-2 promoter was studied. PPARα activation significantly reduced HNF4α-dependent transactivation, indicating competition between HNF4α and PPARα in the regulation of the Gys-2 promoter (Fig 7E). As already mentioned above, we failed to find any evidence for binding of PPARα to DR-1prom in hepatocytes. Thus, the inhibitory effect of PPARα on transcriptional activation of Gys-2 by HNF4α likely does not occur via competition with HNF4α for actual binding to DR-1prom.

Figure 7 **Gys-2 DR-1prom is a binding site for the nuclear receptor HNF4α.** (A) Gys-2 mRNA levels in liver of liver-specific HNF4α-null (HNF4α -/-) and wild-type (FLOX) mice were analyzed by qPCR (n=3 per group). (B) HepG2 cells were transfected with a reporter vector containing 553 bp of the mouse Gys-2 proximal promoter and increasing amounts of hHNF4α expression vector. (C) HepG2 cells were transfected with a reporter vector containing 553 bp of the wild-type and mutated mGys-2 proximal promoter and hHNF4α expression vector. (D) Chromatin immunoprecipitation of Gys-2 DR-1prom using antibodies against HNF4α. The gene sequence spanning DR-1prom and a control sequence were analyzed by PCR in the immunoprecipitated chromatin of rat primary hepatocytes. (E) HepG2 cells were transfected with a reporter vector containing 553 bp of the mouse Gys-2 proximal promoter, an expression vector for hHNF4α, and increasing amounts of mPPARα expression vector. Normalized luciferase activity of the mGys-2 reporter vector in the absence of mPPARα, hHNF4α and Wy14643 was set at 1. Error bars represent SEM.


A
Fold induction
2.0
1.5
1.0
0.5
0.0
Flox
HNF4α -/-
B
Fold induction
80
60
40
20
0
HNF-4α (ng)
-
50
100
250
C
Fold induction
50
40
30
20
10
0
HNF-4α (ng)
-
100
-
100
Wildtype
Mutant
D
DR-1int
DR-1prom
HNF4 ab
Neg. control
Input
E
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35
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PPARα - -
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Discussion

In the present paper we have identified the mouse Gys-2 gene as a direct PPAR and HNF4α target gene. It is shown that the effects of PPARs and HNF4α on Gys-2 expression occur via two distinct response elements. Indeed, while transcriptional activation of the Gys-2 gene by PPARs was found to be mediated by a PPRE present in intron 1 of the mGys-2 gene (DR-1int), the stimulatory effect of HNF4α was mediated by a response element in the immediate upstream promoter (DR-1prom).

Our data are suggestive of the following scenario: In liver, which expresses high amounts of HNF4α, DR-1prom is occupied by HNF4α but not PPARα, while DR-1int is bound by PPARα but not HNF4α. Hence, HNF4α and PPARα activate Gys-2 expression via different response elements. Nevertheless, important negative cross-talk between the two nuclear receptors was observed. In the absence of any mutual binding to the response elements, it can be hypothesized that competition may take place at the level of binding to common co-activator proteins in a mechanism that is often referred to as squelching. In adipose tissue, which does not express HNF4α, DR-1prom is occupied by PPARβ/δ and PPARγ but this does not result in transcriptional activation. Rather, transactivation occurs via binding of PPARβ/δ and PPARγ to DR-1int.

Our data indicate that HNF4α is an extremely powerful activator of mouse Gys2 transcription, explaining the marked reduction in hepatic Gys-2 expression in liver-specific HNF4α null mice (35). As mentioned above, regulation of mGys-2 expression by HNF4α at least partially occurs via DR-1prom. A recent study that combined ChIP with promoter micro-arrays showed that the Gys-2 promoter is bound by HNF4α in human liver (36), thus establishing Gys-2 as a direct target of HNF4α in human as well. It is not very clear why disabling the DR-1prom reduced HNF4α-dependent transactivation by only 50%. It is possible that the 0.55 kb Gys-2 promoter fragment contains an additional HNF4α response element, although in silico analysis failed to reveal such an element. Alternatively, it is possible that mutating the wild-type DR-1prom (AGGCCAAAGGCCA) into a mutated DR-1prom (AGGCCTTTGGCCA) only partially disabled the response element.

While functional PPREs are commonly located within regulatory sequences, i.e. proximal promoters, PPREs have also been identified in intronic sequences. Examples are PPREs within intron 3 of human/mouse Angptl4 and rat peroxisomal thiolase B genes, and within intron 1 of the rat acyl-CoA binding protein gene and human carnitine

palmitoyltransferase 1A (2) (3) (4) (5). Our data demonstrate that regulation of Gys-2 expression by PPARs is also mediated by an intronic PPRE.

In the past few years, our understanding of the function of PPARβ/δ in vivo has improved greatly thanks to studies using various transgenic mouse models (19) (37). At the level of metabolism, PPARβ/δ over-expression promotes skeletal muscle fatty acid oxidation and type I fiber content in mice, resulting in improved endurance exercise performance (22). Conversely, deletion of PPARβ/δ in cardiomyocytes is associated with impaired fatty acid oxidation and expression of fatty acid oxidative genes, whereas glucose uptake is increased (38). In WAT, PPARβ/δ stimulates fatty acid oxidation and uncoupling, thereby diminishing adiposity (21). It is thus clear that PPARβ/δ plays a pivotal role in governing fatty acid oxidation in a variety of tissues. In contrast, data linking PPARβ/δ to regulation of glucose homeostasis remain scarce (39) (20) (40). Our data reveal that PPARβ/δ is a critical regulator of the adipose expression of the glycogen synthase 2 gene. Furthermore, micro-array and qPCR analysis indicated that expression of numerous other genes involved in lipid and glucose metabolism was markedly down-regulated in PPARβ/δ null mice, including GLUT4, p85, and CD36. Since PPARγ and PGC-1α were significantly down-regulated as well, it is possible that many of the observed changes are not linked to the absence of PPARβ/δ per se but rather reflect indirect effects mediated via decreased PPARγ and PGC-1α mRNA. Although such an effect may contribute to some extent to the down-regulation of Gys-2 in PPARβ/δ null mice, the in vitro studies leave no doubt that Gys-2 is a direct target gene of PPARβ/δ, as well as of PPARγ.

Glycogen is stored in many tissues, yet it is particularly abundant in liver, muscle, and adipose tissue. In liver glycogen serves to maintain blood glucose levels between meals, while skeletal muscle glycogen is used to fuel muscle contractions. In contrast, adipose tissue glycogen serves as a source of glycerol 3-phosphate which is required for (re)-esterification of fatty acids into triglycerides (41). Several alternative pathways exist to produce glycerol 3-phosphate, including synthesis from glucose, and conversion of gluconeogenic precursors (glyceroneogenesis). Since expression and activity of glycerol kinase are very low in adipose tissue (42), direct phosphorylation of glycerol is not considered a major pathway to generate glycerol 3-phosphate. However, recent studies suggest that this may change after treatment with synthetic PPARγ agonists, which markedly up-regulate glycerol kinase expression in human and mouse adipocytes (42) (11). In fact, it has been hypothesized that stimulation of

glycerol kinase expression by thiazolidinediones, resulting in increased fatty acid re-esterification, may at least partially account for the suppressive effect of thiazolidinediones on plasma free fatty acid levels. Stimulation of fatty acid esterification is part of a general lipogenic and adipogenic effect of PPARγ in the adipocyte. Since adipose glycogen stores yield glycerol 3-phosphate as a precursor for fatty acid (re-)esterification, up-regulation of Gys-2 expression by PPARγ can be placed in the context of the lipogenic role of PPARγ in the adipocyte, which is aimed at promoting energy storage.

Besides contributing to lipogenesis, synthesis of glycogen permits continued uptake of glucose uptake into cells. Accordingly, it can be speculated that up-regulation of adipose glycogen synthase 2 by PPARγ might partially account for the stimulation of glucose uptake into adipocytes by PPARγ agonists.

It is currently still ambiguous whether PPARβ/δ serves a general anabolic or catabolic function in the adipocyte. On the one hand, it has been reported that PPARβ/δ promotes fatty acid oxidation in adipocytes (20) (21). On the other hand, PPARβ/δ also seems to have a facilitative, yet important role in lipo- and adipogenesis (43). As discussed above for PPARγ, up-regulation of Gys-2 expression by PPARβ/δ may indicate a role for PPARβ/δ in fatty acid (re-) esterification, thus contributing to a lipogenic role for PPARβ/δ.

The highest levels of glycogen are found in liver and fluctuate with nutritional status. The hepatic synthesis of glycogen from glucose is catalyzed by Gys-2 (44). Remarkably, expression of Gys-2 in liver increases during fasting, at the same time when glycogen stores are actively broken down (32). The reason behind this seemingly counterintuitive regulation is not very clear but it may serve to prime the glucose synthesizing system for when dietary glucose becomes available again. In the absence of PPARα, we observed that the expression of Gys-2 drops markedly during prolonged fasting and refeeding. The reduced Gys-2 expression is likely responsible for the diminished rate of glycogen formation upon refeeding, as observed by us and previously by others. Indeed, the effect of PPARα deletion on liver glycogen is minor except under conditions of refeeding (32) (30) (31). It has been reported that after a short term fast the gluconeogenic flux in PPARα null mice is directed more towards glycogen, leading to a decrease in hepatic glucose output. However, it is unclear what happens to the gluconeogenic flux toward glycogen in the fasted-refed state, although our and other data clearly indicate that total glycogen synthesis is decreased in PPARα -/- mice.

Mutation of the GYS2 gene in humans leads to lower hepatic glycogen levels and fasting hypoglycemia (45), biochemical features which are also observed in PPARα -/-

animals (31). However, opposite to what is observed in patients with a dysfunctional GYS2 gene, PPARα -/- mice show low plasma ketones, which is explained by the stimulatory effect of PPARα on fatty acid oxidation and ketogenesis.

Overall, our data suggest that the decreased hepatic glycogen levels in PPARα -/- and liver-specific HNF4α null mice (28) (35) may be due to decreased activation of Gys-2 expression via DR-1int and DR-1prom, respectively. Although PPARα and HNF4α stimulate Gys-2 expression via different response elements, important interplay exist between signalling of the two nuclear receptors.

In conclusion, we show that Glycogen synthase 2 is a direct target gene of PPARs. Transcriptional regulation is achieved via a PPAR response element present in the first intron. An additional direct repeat response element identified in the Gys-2 promoter mediates transactivation by Hepatic Nuclear Factor 4α (HNF4α).

Acknowledgements- We would like to thank Marco Alves for the synthesis of L165041 compound, Mechteld Grootte-Bromhaar and Jolanda van der Meijde for performing the micro-array experiment. This study was supported by the Netherlands Organization for Scientific Research (NWO), with additional support from the Dutch Diabetes Foundation, the Royal Netherlands Academy of Art and Sciences (KNAW), Wageningen Center for Food Sciences, the Centre for Human Nutrigenomics, the Swiss National Science Foundation and the Human Frontier Science Programme.

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Chapter 7

General discussion

The first aim of the research presented in this thesis was to further characterize the role of Peroxisome Proliferator Activated Receptors (PPARs) in the regulation of gene expression during obesity, acute inflammation and obesity-induced inflammation. The second aim was to identify novel PPAR target genes involved in lipid and glucose metabolism by using PPARα, β/δ and γ knockout models combined with genomics techniques. Results of these studies are discussed in this chapter and some recommendations for future experiments are provided.

Since the initial discovery in 1990 as a receptor activated by peroxisome proliferators (1), innumerable studies have been published that collectively have greatly expanded our knowledge of PPARs. Most importantly from a nutritional point of view, PPARs have been identified as nutrient sensors that can be potently activated by dietary polyunsaturated fatty acids (2). In addition to these natural ligands, synthetic ligands of PPARs exist and are widely used to treat metabolic abnormalities including dyslipidemia and insulin resistance (3) (4).

Although initially PPARs were thought to function mainly in the regulation of energy metabolism, the appearance of a study by Devchand *et al.* (5) in 1996 revealed an important function of the PPARα isotype in governing inflammation. Besides PPARα, the two other isotypes PPARβ/δ and PPARγ have also been shown to be involved in immune-modulation (6) (7).

Albeit the physiological role of PPARs has been extensively documented and numerous target genes have been identified (8), there is still much to learn about the specific processes that are under control of PPARs. The availability of microarray techniques combined with the development of different genetically engineered mouse models offers the opportunity to easily screen for potential new PPAR target genes. By applying this approach we were able to show PPARα-dependent regulation of numerous genes involved in the hepatic inflammatory response (Chapter 3). Subsequently, an important role of PPARα (Chapter 4) and PPARγ (Chapter 5) in the regulation of obesity-induced inflammation was uncovered. Finally, microarray analysis helped us to identify Glycogen Synthase 2, the rate limiting enzyme in the conversion of glucose into glycogen, as a novel PPAR target gene in liver and adipose tissue (Chapter 6).

PPARα, inflammatory control, and obesity

Many Western countries are currently faced with an obesity epidemic, as rates of obese individuals have rapidly escalated. Obesity strongly predisposes to the development of Metabolic syndrome, which is defined by a cluster of metabolic abnormalities, including insulin resistance, dyslipidemia, and hypertension (9). In addition, it has become evident that obesity is also associated with a state of low grade chronic inflammation (10). The enlargement of adipose mass leads to increased macrophage infiltration and elevated production of adipocytokines (11). Apart from an increase in adipose mass, obesity is also characterized by fat accumulation in liver which can eventually lead to the production of pro-inflammatory cytokines (12). It has been shown that obesity-induced pro-inflammatory cytokines like TNFα cause metabolic abnormalities including insulin resistance that are characteristic of the metabolic syndrome (13) (14).

Since PPARs control the expression of genes involved in energy metabolism and inflammation and can be relatively easily activated, they are viewed as important targets in the treatment of metabolic syndrome and related disorders. An overview of the current knowledge of PPARs in the control of obesity and inflammation is provided in Chapter 2.

All three PPAR-isotypes have been linked to inflammatory control in different cell types and via different mechanisms. However, the molecular pathways underlying the regulation of inflammatory responses by PPARs remain relatively poorly characterised. In addition, it is currently unclear if and possibly how PPARs might control obesity-induced inflammation.

To increase our understanding of how PPARα controls hepatic inflammation, we compared hepatic gene expression profiles generated by Affymetrix microarray analysis from PPARα-activated versus LPS-induced acute inflamed mouse liver (Chapter 3). Whereas inflammation up regulated numerous pro-inflammatory genes, PPARα activation in liver resulted in an opposite expression profile. Genes that were up regulated by inflammation and down regulated by PPARα activation included Serum Amyloid A, Interleukin-6 receptor and Interleukin 18.

In contrast, expression of Interleukin-1 receptor antagonist (IL-1ra), which was acutely stimulated by LPS treatment, was induced by PPARα. Several studies have previously shown that IL-1ra has an important role in regulating the duration and severity of inflammatory responses (15) (16). Detailed molecular analysis revealed that PPARα is involved in the positive regulation of IL-1ra both during normal and inflammatory conditions in liver. Since

IL-1ra binds to the IL-1 receptor without any downstream effect, up regulation via PPARα can block the pro-inflammatory IL-1 signalling pathway. Together with the down regulation of pro-inflammatory genes, PPARα activation in liver leads to an anti-inflammatory expression profile. The positive regulation of IL-1ra implies that PPARα does not only control inflammation by down-regulating inflammatory gene expression but also by up regulation of anti-inflammatory genes. The identification of genes that are under control of PPARα will assist in elucidating the molecular mechanism underlying the regulation of inflammatory responses by PPARα. Our results are in line with previous studies showing that PPARα is involved in regulation of inflammatory responses (5) (17) (18).

Using a genomics approach to compare the inflammatory response between Wildtype and PPARα -/- mice may help to further clarify the role of PPARα in regulating inflammation. Moreover, generation of organ specific gene expression signatures will allow for a comparison between tissue-specific functions of PPARα during inflammatory conditions.

In liver it would be interesting to study the importance of different cell types during an inflammatory response. Kupffer cells, the liver resident macrophages, are indispensable to drive hepatic inflammatory responses (19). Although PPARα is predominantly expressed in hepatocytes, (20) it has been shown that expression of PPARα shifts towards Kupffer cells during inflammation in liver (21). Thus, it is reasonable to consider the possibility that PPARα may govern Kupffer cell gene expression during inflammatory conditions in liver. However, previous studies have reported that expression of PPARα and important cofactors is down-regulated during LPS-induced inflammation in vivo and in vitro (22) (23). These results suggest a minor role of PPARα due to its lower expression levels during hepatic inflammatory responses. Nevertheless, re-localization of PPARα expression into Kupffer cells during hepatic inflammation strongly suggests involvement of PPARα in regulating inflammatory reactions.

To investigate whether PPARα is involved in controlling obesity-induced inflammation, both Wildtype and PPARα -/- mice were chronically fed a low fat diet consisting of 10 energy% from fat or a high fat diet consisting of 45 energy% from fat (Chapter 4). Although both genotypes became obese due to the high fat diet, the degree of hepatic steatosis was significantly higher in animals lacking PPARα. This was accompanied by an increase in hepatic inflammatory gene expression and immune cell recruitment in PPARα -/- mice. Similar to what was observed in liver, adipose tissue of PPARα -/- mice fed the HFD displayed a higher degree of inflammatory gene expression compared to HFD fed Wildtype

mice. Our results suggest that PPARα controls obesity-induced inflammation either by 1: preventing the development of severe steatosis via induction of fatty acid oxidation, 2: direct inhibition of pro-inflammatory gene expression or 3: by controlling the inflammatory status of adipose tissue.

Several studies have previously shown that hepatic PPARα activation via Wy14643 results in a strong reduction of hepatic TG accumulation , liver injury and inflammation in animals fed a MCD diet to induce steatohepatitis (24) (25). Our results show that the presence of hepatic PPARα in liver prevents the development of severe steatosis and steatohepatitis. PPARα -/- animals fed the HFD clearly developed sings of steatohepatitis and displayed a higher number of activated macrophages in liver. Interestingly, differences in adipose tissue inflammatory gene expression between Wildtype and PPARα -/- animals were observed. The function of PPARα in adipose tissue homeostasis is somewhat controversial. It has been reported that PPARα-/- mice develop increased adipose tissue mass compared to Wildtype counterparts (26) which might be due to local or systemic effects. More recently, it was shown that Wy14643 treatment of diabetic KKAy mice potently reduced the inflammatory status of adipose tissue (27). Finally, it has been shown that PPARα agonists reduce adiposity in obese animals (28) (29). In summary, evidence suggesting a regulatory role of PPARα in adipose tissue function is rapidly accumulating. However, more work is clearly needed to establish if and via which cell types PPARα controls adipose tissue gene expression.

In conclusion, results from our diet-intervention study suggest that PPARα is needed to inhibit obesity-induced inflammation. Although the importance of hepatic PPARα in controlling diet-induced inflammation has been clearly established, it is currently unknown which liver cell types are of relevance. It might be theorized that Kupffer cells, which secrete pro-inflammatory mediators, have a dominant role in inducing liver abnormalities during the development of obesity. The possible interaction between Kupffer cells and hepatocytes in liver and the role of PPARα in this process are interesting topics that will be addressed in future studies.

In contrast to the role of PPARα in mouse liver, the function of PPARα in human liver in the development of steatosis and progression towards steatohepatitis is virtually unknown. Although some studies have evaluated the effects of PPARα activation in patients with NASH (12) (30), little is known about PPARα target genes in liver or about the regulation of PPARα expression itself. Theoretically, impaired expression of PPARα in liver of obese individuals might play a role in the pathogenic process underlying the development of steatosis and the

progression towards hepatic inflammation and steatohepatitis as obese mice lacking PPARα suffer from steatosis and inflammation. When expression is reduced during the development of fatty liver, PPARα is unable to maintain its biological functions leading to fatty acid build-up and extensive inflammation, which will eventually promote the development of fatty liver disease. Studying the regulation of PPARα itself together with the identification of human hepatic PPARα target genes will help us to establish the importance of this nuclear receptor in the prevention and treatment of obesity-related abnormalities in liver.

PPARγ, obesity, and adipose tissue

PPARγ is an adipogenic master regulator and is indispensable for adipocyte differentiation (31)(32). As a result of its high expression level and important function in adipose tissue, the role of PPARγ during the development of obesity has been extensively studied. Synthetic PPARγ ligands are widely used in the treatment of metabolic abnormalities mainly due to their insulin-sensitising effect (33).

Similar to PPARα, PPARγ has important anti-inflammatory properties, which is especially evident in macrophages (34)(7). Consequently, the development of atherosclerosis, in which inflammatory macrophages play a predominant role, is inhibited by PPARγ activation via reduction of pro-inflammatory gene expression (35). The anti-inflammatory role of PPARγ has also been studied in the context of obesity-induced inflammation. Activation of PPARγ via Rosiglitazone in ob/ob mouse models has been shown to down-regulate inflammation and up-regulate the oxidative capacity of adipose tissue (11)(36). Down-regulation of adipose inflammation was accompanied by a decrease in the number of macrophages in adipose tissue. In addition, PPARγ activation has been shown to result in remodeling of adipose tissue with the appearance of smaller adipocytes (37). Smaller adipocytes are considered to be less harmful compared to larger adipocytes due to a decrease in secretion of pro-inflammatory proteins (38).

To learn more about the role of PPARγ and to identify novel pathways that may mediate the effect of PPARγ on adipose tissue homeostasis during obesity, sv129 mice were fed a high fat diet to induce obesity. In the last week of the dietary intervention, the PPARγ activator Rosiglitazone was administered (Chapter 5). Animals fed the HFD plus Rosiglitazone displayed a reduction in liver lipid accumulation and an increase in adipose mass. This is in line with previous studies showing that PPARγ activation leads to a decrease in liver fat both in humans and mice (39)(40). However, the liver lipid lowering effects of PPARγ activation strongly depend on low hepatic expression levels of PPARγ. Over expression of PPARγ in liver has opposite effects leading to the development of severe steatosis (41)(42).

Rosiglitazone treatment of obese mice led to an increase in adipose mass suggesting a redistribution of liver fat towards adipose tissue. Surprisingly, histological analysis of adipose tissue revealed that Rosiglitazone treatment resulted in an increase in the number of macrophages. However, microarray analysis showed that this was not accompanied by a higher level of inflammatory gene expression. Detailed analysis of the microarray data

revealed that several genes that are currently used as markers of alternatively activated macrophages were increased by Rosiglitazone treatment. Macrophages that are alternatively activated are involved in tissue remodeling and cell proliferation (43). Subsequently, these macrophages might play a role in PPARγ-dependent remodeling of adipose tissue resulting in smaller adipocytes which have a lower pro-inflammatory gene expression profile. Recently, a study by Lumeng *et al.* (44) revealed that obesity leads to a phenotypical switch of adipose macrophages towards classical activated macrophages that express high levels of TNFα. It might be theorized that PPARγ activation during conditions of obesity leads to the attraction of alternative activated macrophages which are involved in remodeling of adipose tissue and suppression of inflammation. By removing apoptotic larger adipocytes and stimulating the development of smaller adipocytes, alternative activated macrophages might have an important role in PPARγ-mediated adipose tissue remodeling and inhibition of inflammation. However, more studies are clearly needed to establish the effects of Rosiglitazone on macrophage biology in adipose tissue. Future experiments including gene expression profiling of adipose-specific macrophages treated with Rosiglitazone will further expand or knowledge of PPARγ functioning in adipose tissue during obesity.

PPARs and glycogen metabolism

Besides anti-inflammatory roles, PPARs are also involved in regulating lipid and glucose metabolism. As mentioned earlier, PPARα and γ-activators are effectively used in the treatment of obesity-related metabolic abnormalities including plasma lipid disorders and early stages of Type II diabetes (45)(46) although it has remained difficult to identify the molecular mechanisms behind these effects. The role of PPARβ/δ, the third PPAR-isotype, in controlling genes involved in energy metabolism is relatively unexplored. However, the recent generation of knockout models has effectively helped us to understand PPARβ/δ biology (47)(48). Studies with specific agonists have suggested that activation of PPARβ/δ might be an effective pharmacological strategy to correct metabolic abnormalities.

By starting with comparing adipose expression profiles from Wildtype and PPARβ/δ -/- animals using Affymetrix micro-array analysis, we identified several genes implicated in energy metabolism that were differentially regulated (Chapter 5). Expression of Glycogen Synthase 2 (Gys 2), the rate-limiting enzyme in the conversion of glucose to glycogen, was most significantly down-regulated in adipose tissue of PPARβ/δ -/- mice. Further molecular analysis identified Gys 2 as a positive PPARα and γ target gene in adipose tissue as well. In liver, cross talk between HNF4α and PPARα was shown to determine the expression levels of Gys 2.

Despite the fact that PPARs have been previously linked to glycogen metabolism (49), our results have identified Gys 2 as a novel target gene with two PPREs present in both the promoter and an intronic sequence via which PPARs might influence glycogen metabolism. Importantly, cross talk between PPARα and HNF4α exists and is determinative for the activation of Gys 2 expression in liver.

Besides the identification of Gys 2 as a novel PPAR target gene, microarray analysis revealed that many other genes involved in energy metabolism were differentially regulated in adipose tissue of Wildtype and PPARβ/δ -/- mice. CD36, PPARγ and PGC1γ were among a list of potentially interesting genes to be studied in future experiments.

Conclusion

The work described in this thesis further characterizes the role of PPARα in regulating inflammatory responses. Besides controlling acute inflammatory conditions, PPARα also prevents the development of obesity-induced chronic inflammation, which was observed in liver and adipose tissue. Future experiments including detailed analysis of cell-type specific expression of PPARα during conditions of inflammation will advance our understanding of PPARα biology. The generation of adipose-specific PPARα -/- mice will further elucidate the role of PPARα in regulating adipose tissue gene expression.

In contrast to PPARα, the role PPARγ in adipose tissue homeostasis has been clearly established. Our preliminary data show that Rosiglitazone treatment of obese mice results in a redistribution of liver fat towards adipose tissue. However, the morphology of adipose tissue was changed resulting in the appearance of smaller adipocytes. In addition, an up regulation of genes involved in oxidative capacity was observed. Most surprisingly, more macrophages were present in adipose tissue of Rosiglitazone-treated obese animals. However, gene expression analysis of adipose tissue suggested that macrophages were polarized towards an alternative phenotype. These alternative activated macrophages might have a role in PPARγ-activated remodeling of adipose tissue which may eventually lead to a more healthier total body status during obesity.

Finally, Glycogen Synthase 2, the rate-limiting enzyme in the conversing of glucose to glycogen, was identified as a novel PPAR-target gene.

Overall, results presented in this thesis have clearly shown that PPARs are versatile regulators of cellular functioning with important functions in both energy metabolism and immunity. Moreover, as integrators of metabolism and inflammatory signalling, PPARs might prove to be important therapeutic targets in the treatment of metabolic syndrome and related pathologies.

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Samenvatting

Overgewicht en de ontwikkeling van chronische ontsteking

In dit proefschrift worden verschillende studies beschreven, die voornamelijk gericht zijn op de negatieve effecten in lever en vetweefsel veroorzaakt door overgewicht (obesitas). Het aantal mensen met overgewicht in de westerse wereld is de laatste jaren spectaculair gestegen. Omdat de ontwikkeling van overgewicht vaak gepaard gaat met het ontstaan van allerlei gezondheidsproblemen is het bestuderen van de onderliggende mechanismen belangrijk. In de eerste plaats hebben mensen met overgewicht vaak last van allerlei metabole afwijkingen zoals hoge glucose- en triglyceridenwaarden in het bloed. Daarnaast zijn ook de cholesterolwaarden in het bloed vaak verstoord. In combinatie met overgewicht ontstaat hierdoor een sterk verhoogd risico op het ontwikkelen van diabetes type II en hart- en vaatziekten.

Naast deze metabole verstoringen is uit onderzoek ook duidelijk geworden dat mensen met overgewicht een chronische ontsteking ontwikkelen. Deze chronische ontsteking blijkt voornamelijk te ontstaan in het vetweefsel dat zich bevindt in de buikholte rond organen. Doordat vetcellen groter worden en inflammatoire cellen zoals macrofagen in het vetweefsel infiltreren, raakt het vetweefsel ontstoken. De grotere vetcellen en macrofagen zijn verantwoordelijk voor de afgifte van een groot aantal pro-inflammatoire stoffen die via de circulatie ook andere organen kunnen bereiken.

Net als het vetweefsel is de lever ook zeer gevoelig voor de effecten van overgewicht. Opslag van vet in de lever, ook wel steatosis genoemd, komt vaak voor bij obese individuen. Alhoewel vetopslag alleen relatief ongevaarlijk is, kan steatosis zich verder ontwikkelen tot een leverontsteking en uiteindelijk zelfs leiden tot een sterk verlies in leverfunctioneren met alle nadelige gevolgen van dien.

Een van de meest eenvoudige manieren om gewicht te verliezen is het verminderen van de energie-inname en het verhogen van het energieverbruik d.m.v. een verhoogd activiteitsniveau. Deze manier blijkt echter bij veel mensen moeilijk vol te houden, waardoor de ontwikkeling van andere methoden om overgewicht en de bijbehorende gezondheidsprobleem te verminderen erg belangrijk is.

PPARs

PPARs zijn transcriptie factoren die zich bevinden in de kern van cellen. Omdat onverzadigde vetzuren uit onze voeding kunnen binden aan de PPARs en ze daarbij activeren worden PPARs ook wel 'vetsensoren' genoemd. Geactiveerd PPAR bindt aan specifieke stukken van het DNA wat uiteindelijk leidt tot het afschrijven van genen, vertaling in eiwitten en verandering in functie. Veel van de genen die worden gereguleerd door PPAR zijn betrokken bij de energiehuishouding en bij het verwerken van vetten en koolhydraten uit onze voeding. In een deel van dit proefschrift wordt beschreven hoe de synthese van glycogeen in lever en vetweefsel door PPARs gereguleerd wordt. Uit onze resultaten blijkt namelijk dat PPARs de expressie van het enzym Glycogen Synthase 2 (Gys2) te stimuleren, wat snelheidheisbepalend is voor de glycogeen vorming.

Naast een rol in de energiehuishouding heeft PPAR ook een belangrijke ontstekingsremmende functie. Dit gebeurt voornamelijk door de expressie van genen die betrokken zijn bij het ontstaan van een ontsteking te onderdrukken. De ontstekingsremmende werking van PPAR is inmiddels aangetoond in verschillende weefsels waaronder de lever en het vetweefsel.

Omdat een verstoorde energiehuishouding en een verhoogde ontstekingsgraad vaak voorkomen bij mensen met overgewicht, kan activatie van PPAR mogelijk een gunstig effect teweeg brengen.

PPARs, ontsteking en overgewicht

Alhoewel al langer duidelijk is dat PPAR ontstekingsremmend werkt in de lever, is het lastig gebleken een duidelijk inzicht te krijgen in de onderliggende mechanismen. Daarnaast is het nog onduidelijk via welke celtypen PPARs de ontstekingsreactie kunnen remmen.
Door een vergelijking te maken tussen genen die worden gereguleerd tijdens ontsteking en genen die worden gereguleerd door PPAR in muizen, is gepoogd een beter idee te krijgen van de ontstekingsremmende werking van PPAR. Genen die geactiveerd werden tijdens ontsteking bleken sterk te worden geremd in de lever door activatie van PPAR, wat zou kunnen leiden tot een onderdrukking van ontstekingsreacties in de lever door PPAR. Eén gen in de lever week af van dit patroon en werd zowel geactiveerd tijdens ontsteking en door PPAR in lever. Verder onderzoek beschreven in dit proefschrift toont aan dat dit gen, Soluble

Interleukin 1 receptor antagonist genaamd, een ontstekingsremmende werking heeft en mede wordt gereguleerd door PPAR in de lever.

Om meer te leren over de rol van PPAR tijdens overgewicht-geïnduceerde ontsteking hebben we muizen een hoog vet dieet gegeven. Als gevolg van dit hoog vet dieet ontwikkelden de muizen overgewicht. Door naast muizen met PPAR ook muizen zonder PPAR (zogenaamde PPAR knock-outs) te gebruiken was het mogelijk om te bepalen wat de precieze functie is van PPAR tijdens de ontwikkeling van overgewicht en het ontstaan van een chronische ontsteking. Het bleek dat beide typen muizen eenzelfde mate van overgewicht ontwikkelden door het hoog vet dieet. In lever en vetweefsel waren daarentegen grote verschillen te zien tussen muizen met en zonder PPAR. In de lever leidde de afwezigheid van PPAR tot meer steatosis. Ook was er een verhoogde expressie van allerlei ontstekingsgenen meetbaar en waren het aantal geactiveerde macrofagen in de lever van muizen zonder PPAR sterk verhoogd. In het vetweefsel waren vergelijkbaren effecten meetbaar.

In het laatste deel van het onderzoek hebben we de rol onderzocht van PPAR in macrofagen. Aangezien deze cellen een belangrijke rol spelen in het induceren van ontsteking in mensen met overgewicht is het belangrijk meer te weten te komen over het precieze functioneren van macrofagen. Door het activeren van PPAR bleken deze cellen minder ontstekingsgenen tot expressie te brengen en daarnaast een remmend effect te hebben op ontsteking in het vetweefsel.

Uit deze resultaten blijkt dat de afwezigheid of niet goed functioneren van PPAR leidt tot een sterk verhoogde ontsteking in obese dieren, terwijl activatie van PPAR ontsteking vermindert. Dat laatste wekt de suggestie dat activatie van PPAR de schadelijke effecten van overgewicht mogelijk kan beperken.

Samenvattend, uit het onderzoek beschreven in dit proefschrift blijkt dat PPARs een zeer diverse rol spelen in de cel. Niet alleen zijn PPARs betrokken bij het reguleren van energiemetabolisme en belangrijk voor de regulatie van Gys2, tevens spelen deze nucleaire receptoren een rol bij het remmen van ontsteking in lever en vetweefsel. Hierdoor heeft activatie van PPAR mogelijk een gunstig effect in mensen met overgewicht door het verminderen van ontsteking en bijbehorende nadelige gezondheidseffecten. Toekomstig onderzoek zal zich moeten richten op de specifieke rol van PPARs in verschillende celtypen aanwezig in de lever en het vetweefsel om zo meer te leren over de precieze mechanismen achter de beschermende werking van PPARs tegen overgewicht-geïnduceerde ontsteking.

Dankwoord

Zonder de hulp van vele mensen had dit proefschrift er nooit geweest.

Allereerst wil ik Michael bedanken. Niet alleen heb je me de mogelijkheid gegeven om als AIO te beginnen, ook de vrijheid die ik heb gekregen tijdens het doen van onderzoek heb ik altijd erg gewaardeerd. De vele werkbesprekingen waren altijd nuttig en leerzaam.
Sander, zonder jouw hulp had ik niet zover gekomen. Je hebt me geleerd hoe je goed onderzoek moet doen. Bedankt voor het corrigeren van al mijn werk ('Wat is dit nu weer voor onzin?!') en het plannen en uitvoeren van vele proeven. De 'burrito en comedy-channel' reis, onze begroeting elke morgen en de vele fietstochtjes zal ik niet snel vergeten.
Ben, bedankt dat ik 'je AIO' mocht zijn.
Stef, thanks for all the fun we had in the lab (écoute….). Besides teaching me very useful French words, you have learned me a lot about PPARs and molecular biology. I am very happy your are going to be my 'parrain'. Die schande!
Laeticia, thank you for being my 'paranimf '. It is a lot of fun being your roommate. Mijn andere (oud)-kamergenoten, Jolanda, Elke, Bart, Mark, Maryam, Marleen, bedankt voor alle gezelligheid, gesprekken en de leuke tijd.
Elgin, ook al was je maar redelijk kort betrokken bij het project, je hebt me heel goed op weg geholpen. Bedankt daarvoor. Ik wil ook graag Fokko, Dave (thanks for all the cycling-trips), en alle ander aio's van NMG (Heleen, Meike, Saskia, Linda, David, Anastasia, Susan) bedanken. Daarnaast wil ik natuurlijk ook alle andere mensen van het 'NMG-lab' bedanken voor hun hulp. De gezellige werksfeer heb ik altijd erg gewaardeerd. Een paar mensen wil ik in het bijzonder noemen; Guido, zonder jouw hulp waren veel dingen nooit gelukt (array analyse, hepatocyten, oplossen van computerproblemen), bedankt daarvoor. Shohreh, thanks for your help in isolating RNA, making sections and setting up new techniques. Caroline, thank you for your help and all the discussions about our projects. Rene, Chris en alle andere studenten die ik mede heb mogen begeleiden, bedankt voor jullie bijdrage.
De AIOs van het CHN, bedankt voor alle nuttige bijeenkomsten en het delen van onze ervaringen.
De AIO-reis die ik heb mogen maken naar Australië was ook een bijzondere ervaring en daarvoor wil ik alle mede AIO-reisgenoten en begeleiders bedanken.

Ik wil de mensen van het secretariaat op de derde bedanken voor alle hulp bij het versturen van pakketen, maken van afspraken en allerlei andere praktische zaken.
Rene, Bert, Judith, Wilma en alle andere mensen van het dierenlab, bedankt voor jullie hulp en inzet. Cathy, zonder jouw kennis waren de vele kleuringen nooit gelukt.

Naast collega's wil ik ook mijn vrienden (Wageningse voedingsgroep), familie en schoonfamilie niet vergeten die bewust of onbewust ook hebben bijgedragen aan dit proefschrift. Bedankt voor alle interesse, ondersteuning en gezellige tijd.
Pa en ma, zonder jullie steun had ik dit nooit kunnen doen. Jullie staan altijd voor me klaar.
Als laatste wil ik Marloes bedanken. Jij bent het allerbelangrijkste in mijn leven en zonder jouw humor, relativeringsvermogen en liefde had ik dit proefschrift nooit kunnen maken.

Rinke

About the author

Rinke Stienstra was born on the 26th of July 1979 in Dordrecht, the Netherlands. After finishing secondary school in Dordrecht at the 'Titus Brandsma College' in 1997, he studied Medicine at the University of Leuven, Belgium for 1 year. In 1998, he started his university training in Human Nutrition at Wageningen University. Specializing in Nutrigenomics, he performed 2 study projects. The first project was carried out at the laboratory of the Nutrition, Metabolism and Genomics group of the Wageningen University were he studied the role of PPARs in the regulation of gene expression in several cell lines. During his second training period, he joined the laboratory of Metabolic Disorders led by Prof. R. Wanders at the Academic Medical Center in Amsterdam. After receiving his MSc degree in Human Nutrition in September 2002, he started his PhD project in the same year at the Nutrition, Metabolism and Genomics group of the Wageningen University. Results of this research are described in this thesis. His project was funded by the Center for Human Nutrigenomics (CHN), the Nutrigenomics Consortium and TI Food & Nutrition (TIFN). During his PhD period he joined the educational program of the graduate school VLAG offering advanced courses in Food Technology, Agrobiotechnology, Nutrition and Health Sciences.

List of publications

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Patsouris D, Stienstra R, Müller M, Kersten S, Cross-talks between LXRα, RXRα and PPARγ in the adipose tissue, *in preparation*.

Abstracts

Keystone symposium 2007

Stienstra R, Duval C, Kersten S, Müller M.

The role of PPARα and PPARγ in high fat diet induced inflammation

Digestive Disease Week 2006

Stienstra R, Mandard S, Patsouris D, Maass C, Kersten S, Müller M.

PPAR alpha protects against high fat diet induced hepatic inflammation

Gastroenterology 130 (4): A827-A827 Suppl. 2, 2006

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Stienstra R, Mandard S, Lichtenauer-Kaligis E, Kersten S, Müller M.

Identification of new PPAR alpha target genes support its anti-inflammatory function in liver.

European Journal of Gastroenterology & Hepatology 17 (1): A40-A40, 2005

Education statement of the Graduate school VLAG

Discipline specific activities

Courses

Microarray course Service XS, Leiden, 2002

Symposium Nutrim, Maastricht University, 2002

Masterclass Nutrigenomics, VLAG, 2003

Meetings

Conference European Association for the Study of Liver Disease, Tours, 2003

Ernst Klink symposium, Cologne, 2003

NWO Nutrition, Papendal/Deurne, The Netherlands 2002-2006

NVGE (Dutch Association for Gastroenterology), 2004-2005

PPAR conference, Monaco (Monte Carlo), 2005

Digestive Disease Week, Los Angeles, USA, 2006

Keystone Symposium, Nuclear Receptor Pathways to Metabolic regulation, USA, 2007

General courses

VLAG PhD week, 2002

Laboratory animal science, Utrecht University, 2003

Safe handling of radio-active materials and sources, Wageningen University, 2004

Optionals

Preparation PhD research proposal

PhD-tour Australia, Wageningen University, 2003

Journal Club, Division of Human Nutrition, Wageningen University, 2002-2006

Journal Club and work in progress-meetings, NMG group, Wageningen University, 2002-2006

The studies described in this thesis were supported by the Centre for Human Nutrigenomics, VLAG, Innovative Cluster Nutrigenomics and Top Institute Food & Nutrition.