Transcriptional regulation of nutrient metabolism by PPARα, γ and LXRα

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Abstract

Transcriptional regulation of nutrient metabolism by PPARα, γ and LXRα

PhD thesis by David Patsouris, Division of Human Nutrition, Wageningen University, Wageningen, The Netherlands

Peroxisome Proliferators Activated Receptors (PPARs) and Liver X Receptors (LXRs) are Nuclear Hormones Receptors that mediate the effect of nutrients on gene expression by acting as sensors for fatty acids and cholesterol-derived metabolites, respectively. In as much as metabolic diseases evolve by unfavorable genetics in combination with excess exposure to nutrients, investigation into the mode of action of PPARs and LXRs may provide important new leads for the pharmacological treatment of these diseases.

The work presented in this PhD thesis demonstrates that PPARα directly stimulates the hepatic conversion of glycerol into glucose, which was the first report showing that PPARα directly governs hepatic gluconeogenesis. Regulation of gluconeogenesis by PPARα likely explains why PPARα null mice develop severe hypoglycemia when fasted. This function of PPARα appears to be conserved and functional in human since activation of PPARα with synthetic PPARα ligand decreased the plasma glycerol levels.

In the second part of this PhD thesis work it is demonstrated that PPARα becomes activated in liver upon high fat diet and associated insulin resistance. Although activation of PPARα by high fat feeding was weak relative to treatment with synthetic PPARα agonist or fasting, consistent upregulation of PPARα target genes was observed that became evident after comprehensive expression profiling by micro-array. The results obtained illustrate the power of a focused nutrigenomics approach to promote our understanding of regulation of gene expression by nutrients and their specific role in governing nutrient metabolism.

In the last part of the manuscript we describe novel cross-talk between PPARγ, LXRα and their heterodimeric partner RXR in adipose tissue. We show that LXRα and its ligand T0901317 specifically repress the expression of cGPDH *in vivo* and in differentiated mature adipocytes. Further investigation of the molecular mechanism demonstrated that liganded LXRα prevents the binding of PPARγ to the PPREs of the cGPDH promoter by competing with PPARγ for their reciprocal partner RXR. Our data reveal novel cross-talk between PPARγ, LXRα and RXR in the control of gene expression in adipocytes.

Abbreviations

Nuclear Hormones Receptors and related proteins

HAT	Histone Acetyl Transferase
HDAC	Histone Deacetylase
HNF-4	Hepatic Nuclear Factor -4
LXR	Liver X Receptor
NHR	Nuclear Hormone Receptor
PGC1	PPAR Gamma Coactivator 1
PPAR	Peroxisome Proliferator Activated Receptors
RXR	Retinoid X Receptor
SREBP	Sterol Regulatory Element Binding Protein

Enzymes

AQP	Aquaporin
CAP	cCbl Associated Protein
cGPDH	cytosolic Glycerol Phosphate Dehydrogenase
mGPDH	mitochondrial Glycerol Phosphate Dehydrogenase
Gyk	Glycerol Kinase
PEPCK	Phosphoenolpyruvate Carboxykinase

Various

ChIP	Chromatin Immuno Precipitation
DHA	Docohexaenoic Acid
EPA	Eicosapentaenoic Acid
FFAs	Free Fatty Acids
IR	Insulin Resistance
PUFAs	Polyunsaturated Fatty Acids
Q- PCR	Quantitative PCR
TG	Triglyceride
TF	Transcription Factor
TZDs	Thiazolidinediones
WAT	White Adipose Tissue
WHO	World Health Organization

Contents

After the British defeat of the German Afrika Korps in Egypt in November 1942, during World War II, Winston Churchill stated, "Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."

Chapter 1

General introduction about insulin resistance and Nuclear Hormone Receptors

Type II diabetes, definitions and figures

Since especially the brain is critically dependent upon an adequate supply of glucose, it is clear that the plasma glucose levels needs to be tightly controlled (1). Maintenance of plasma glucose levels is achieved by the balanced action of several important metabolic hormones, the most notable of which is insulin. Insulin is the main anabolic hormone in the body and is in charge of storing lipids and glucose (2) (3) (4). The importance of insulin in governing plasma glucose levels is demonstrated by the severe metabolic phenotype observed when insulin is not produced, as in type I diabetes, or when the tissue fail to respond to insulin, as in type II diabetes. Both conditions cause elevation of plasma glucose levels that if not treated properly can lead to grave clinical consequences. Diabetes is defined by elevated fasting plasma glucose (FPG), which in 1999 was lowered from 7.8 to 7.0 mmol/l (World Health Organization (WHO) http://www.who.int/diabetes/). An additional indicator of diabetes is an impaired Oral Glucose Tolerance Test (OGTT) characterized by 2h plasma glucose levels exceeding 11.1 mmol/l. Elevated fasting glucose and impaired glucose tolerance might reflect different underlying physiological defects (5).

According to the WHO, an estimated 30 million people world-wide suffered from diabetes in 1985. By 1995, this number had gone up to 135 million. The latest WHO estimate for the year 2000 is 177 million, which corresponds to a prevalence of 2.8%. This number is expected to increase to at least 300 million people by 2025, corresponding to a prevalence of 4.4% (6). The number of yearly deaths attributed to diabetes was previously estimated at just over 800000. However, it has long been known that the number of deaths related to diabetes is considerably underestimated. A more plausible figure is likely to be around 4 million deaths per year that are related to the disorder, which translates into about 9% of the global total. Most of these diabetes-related deaths are from cardiovascular complications. Although the prevalence is more rapidly increasing in developing countries (prevalence of 3.3%, 3.5% in 1995, 2000 and estimated to 4.9% in 2025), industrialized countries do not escape the epidemic partly because of the aging population (6.2%, 6.9% in 1995, 2000 and 7.6% for 2025) ((7), WHO detailed figures: http://www.who.int/diabetes/actionnow/en/diabprev.pdf, WHO representative worldwide map: http://www.who.int/diabetes/actionnow/en/mapdiabprev.pdf). The Netherlands and France with an estimated prevalence for 2025 of 2.7% and 2.6%, respectively, are within the low ranges for the developed countries.

Type II diabetes, a complex disease

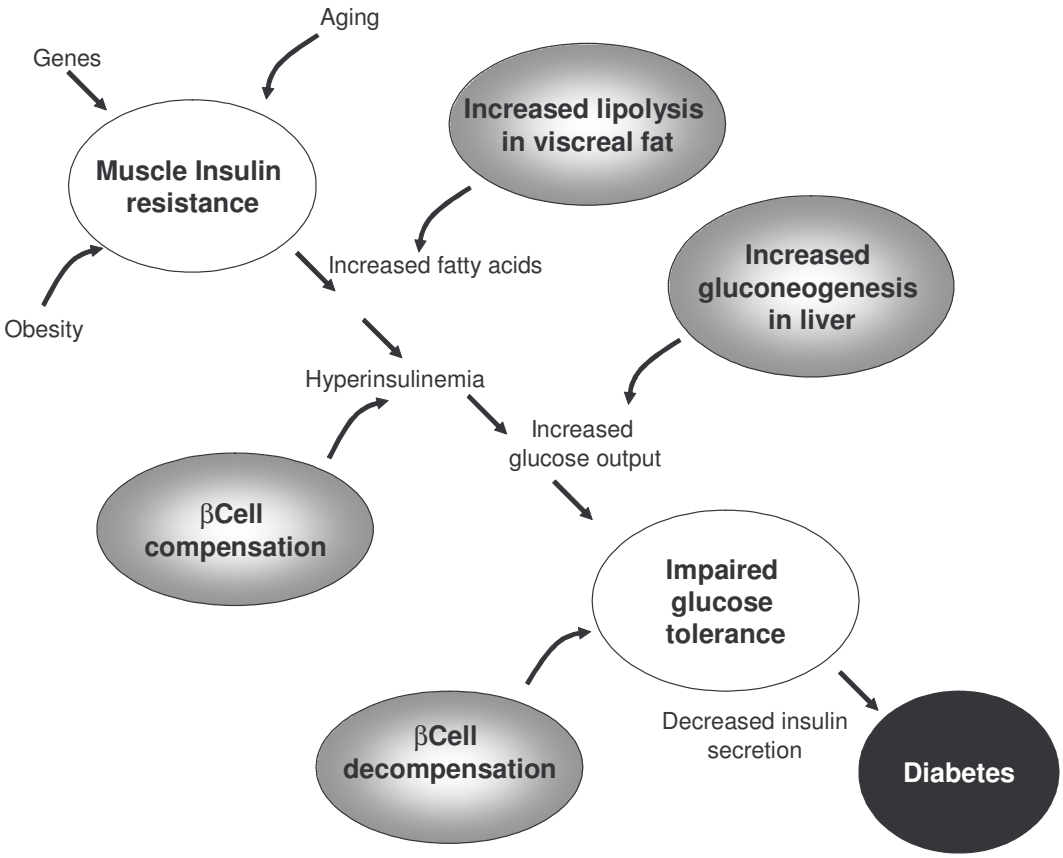
Overt type II diabetes is most often preceded by a state of insulin resistance, which is part of a collection of metabolic abnormalities united in the metabolic syndrome. According to the most recent criteria, for an individual to be defined as having the metabolic syndrome they need to suffer from abdominal obesity, plus two of the following four additional factors: elevated plasma triglycerides, reduced plasma HDL cholesterol, elevated blood pressure, and elevated fasting plasma glucose level (i.e. diabetes) (8). As such, the common central feature of the metabolic syndrome and all its components is obesity. This is surprising since adipose tissue does not secrete insulin nor is its main target. Instead, diabetes seems to evolve from the lack of responsiveness of its main tissue target, which is skeletal muscle, followed by a defect of the pancreatic β- cells that produce insulin. The disease progressively worsens as more and more organs become involved (see **table 1** and **figure 1**). The sequence of events leading to type II diabetes is generally considered as follows: At the initial stages of the disease, excess visceral adipose tissue somehow causes skeletal muscle to slowly fail in its response to insulin which, since muscle is the main organ responsible for insulin-dependent glucose uptake, would result in a rise in plasma glucose level (9). To overcome the resistance of skeletal muscle to insulin, the β- cell of the pancreas must release more insulin, allowing for normalization of plasma glucose levels. As the liver progressively becomes insulin resistant as well, additional stress is placed on the pancreas, as glucose output from the liver will increase. It is believed that eventually the β-cells from the pancreas "decompensate" due to chronic lipo- and glucotoxicity, resulting in elevated plasma glucose levels and whole-body glucose intolerance (10) (11) (12) (13).

Table1: Physiological defects during progression from Prediabetes to overt type 2 diabetes[A].

	Insulin Resistance (Muscle)	Insulin Deficiency (βCell)[C]	Glucose production (Liver)
Prediabetes (FPG[B] < 6.1mM)	+/-	-	normal
Impaired fasting glucose (FPG[B]: 6.1-6.9 mM)	+	-	normal
Diabetes Mild (FPG[B]: 7.0-7.8 mM)	+	+	normal ↑
Moderate/ severe (FPG[B] > 7.8mM)	+	+	

A: See Bajaj (2003 (13)) for a more complete discussion of this controversial subject.
B: FPG, fasting plasma glucose. Definitions are based upon the most recent recommendations of the Expert Committee of the American Diabetes Association.
C: Definition of insulin deficiency: Insulin levels are lower than in normal individuals, either in the fasting state or in response to ingestion of oral glucose. However, this definition does not exclude the possibility that defects in β-cell function are present prior to the development of insulin deficiency. Table adapted from Taylor SI (1999, (14)).

Figure 1: Metabolic Staging of Type 2 Diabetes.



Type 2 diabetes is characterized by a progressive decrease in insulin action, followed by an inability of the β cell to compensate for insulin resistance. Insulin resistance is the first lesion, due to interactions among genes, aging, and metabolic changes produced by obesity. Insulin resistance in visceral fat leads to increased fatty acid production, which exacerbates insulin resistance in liver and muscle. The β cell compensates for insulin resistance by secreting more insulin. Ultimately, the β cell can no longer compensate, leading to impaired glucose tolerance, and diabetes. Adapted from Saltiel, 2001 (15).

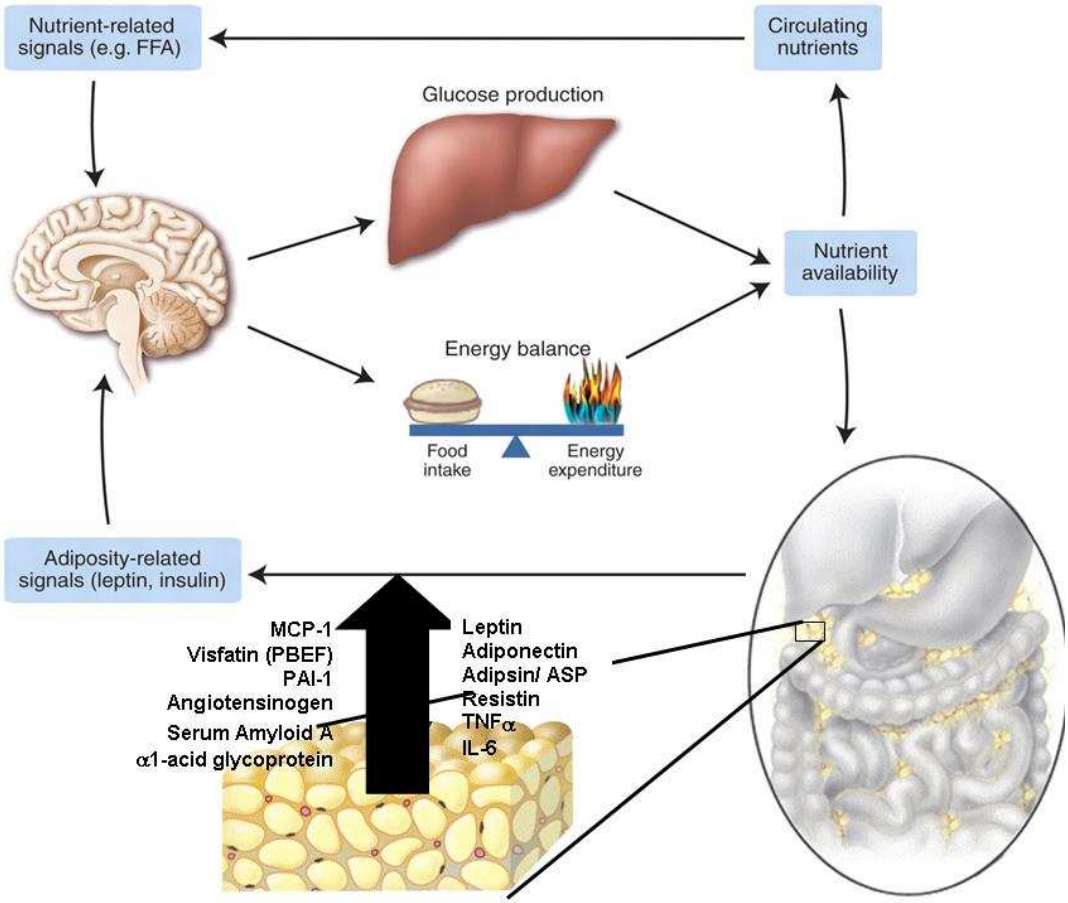
The White Adipose Tissue/ Liver axis in diabetes

For long, white adipose tissue was regarded as merely being able to store and release energy according to the fluctuations in energy availability. However, the association of numerous metabolic diseases with obesity has drawn increasing interests towards this tissue and has emphasized its role as a complex tissue involved in numerous metabolic functions. Indeed, it is currently acknowledged that excess adipose tissue likely predisposes to these diseases via altered release of factors, which has its origin in a disequilibrium between energy intake and energy expenditure (16). Intense efforts have been put together in order to identify the molecular causes for the imbalance. Current knowledge on the regulation of the energy intake highlights the importance of hunger/satiety hormones such as leptin and α-melanocyte stimulating hormone, impairment of which result in severe obesity in humans (17-19). Similarly, it is also clear that complex regulation takes place at the level of energy expenditure. It this context, it is important to realize that adipose tissue does not behave as a uniform tissue. In humans, two types of white adipose tissue, i.e. subcutaneous and visceral adipose tissue are distinguished. Excess visceral adipose tissue has appeared to be a much more important predictor of the onset of metabolic syndrome and diabetes compared to excess subcutaneous adipose tissue (20) (21) (22). Potential explanations proposed so far are multiple and include a higher metabolic rate of visceral adipose tissue which is partly explained by a weak sensitivity to insulin. This may result in an accelerated hydrolysis of the stored triglycerides (TGs), increasing the delivery of free fatty acid (FFAs) to the liver via the portal vein (23). Exposure of the liver to high concentrations of fatty acid will lead to lipid accumulation in liver and subsequent insulin resistance (lipotoxicity) (15) (24). However, the results of recent studies have down-scaled the importance of hepatic lipid accumulation as a direct cause for hepatic insulin resistance (25) (26) (27) (28).

Another potential explanation for the metabolically unfavorable consequences of excess visceral adipose tissue invokes some of the proteins secreted by adipose tissue, called adipokines or adipocytokines, which via the blood stream can affect whole body energy homeostasis. So far, the best-studied adipokines are adiponectin, resistin, TNFα, leptin and visfatin, the latter of which was reported to be specifically secreted by visceral fat (For review, see (29) and (30)). In the case of adiponectin, which promotes insulin sensitivity, plasma levels appear to be negatively correlated with visceral fat storage, suggesting that changes in plasma adiponectin might mediate the association between excess visceral fat and

insulin resistance (31) (32, 33). In general, functions that are influenced by these adipokines include glucose disposal by the fat and skeletal muscle, hepatic glucose production, fatty acid oxidation, fat storage, and satiety (**figure 2**) (34) (35) (36) (37).

Figure 2: Model depicting the control of energy homeostasis and hepatic glucose metabolism by adiposity- and nutrient-related signals.

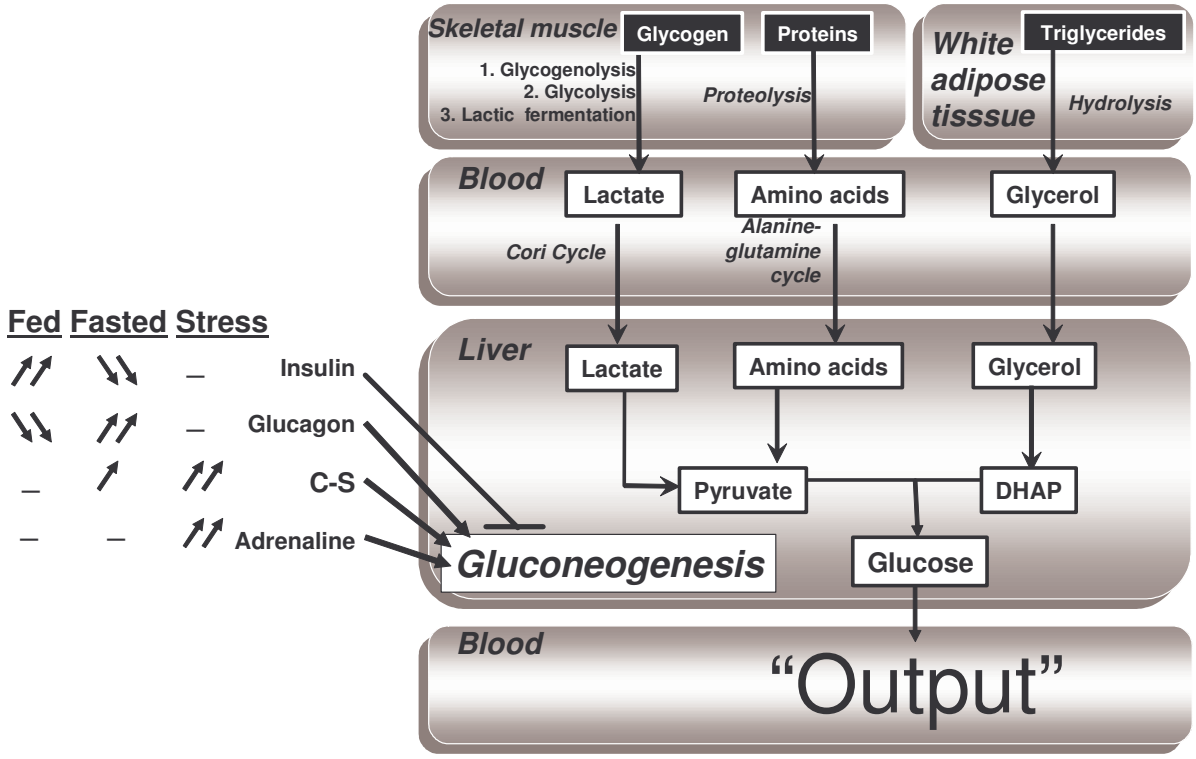


Neuronal systems sense and respond to input from hormones such as insulin, leptin and other adipokines that are secreted in proportion to body energy stores and from the metabolism of circulating nutrients (such as glucose and FFAs). In response to this input, adaptive changes occur in energy intake, energy expenditure, and hepatic glucose production. IL-6, Interleukin-6; MCP1, Monocyte Chemoattractant Protein-1; PAI-1, Plasminogen Activator Inhibitor- 1; PBEF, pre-B cell colony-enhancing factor; TNFα, Tumor Necrosis Factor α. Adapted from Schwatz, 2005 (38).

Unlike white adipose tissue and skeletal muscle, the liver and to some extent kidney and small intestine can synthesize glucose via gluconeogenesis and hence importantly contribute to maintenances of plasma glucose levels (39). Maintenance of plasma glucose levels assures a constant supply of glucose to the brain which is dependent on glucose for functioning. During fasting, gluconeogenesis is stimulated by several energy mobilizing hormones (e.g. glucocorticoids, glucagons), whereas during feeding this pathway in inhibited by insulin (40, 41). In liver, three different substrates serve as the major precursors for glucose synthesis. Gluconeogenic amino acids, released by skeletal muscle protein degradation, are regarded as the main precursor to generate glucose, especially under conditions of fasting (42-44) (45). Lactate, derived from the skeletal muscle breakdown of glycogen under anaerobic conditions is another important precursor for glucose synthesis (46) (47). Finally, glycerol provided by the breakdown of triglycerides from white adipose tissue can also be used to generate glucose (**Figure3**) (48) (49) (50). There are different reports claiming differences in the respective contribution of these precursors to the synthesis of glucose. In rodents, glycerol, whose conversion into glucose is strictly controlled by PPARα (see **chapter 3**), seems to account for most of gluconeogenesis during prolonged fasting (51).

The pathway of gluconeogenesis is under negative control by insulin. Accordingly, hepatic insulin resistance will result in increased hepatic liver output (**figure 1**, **2** and **3**). Recent studies have highlighted the importance of PPARα, PGC-1α, FOXO1 and HNF4α in mediating the suppression of hepatic gluconeogenesis by insulin (41) (52) (53, 54) (55).

Figure 3: Controlled regulation of hepatic gluconeogenesis



Gluconeogenesis, literally *de novo* synthesis of glucose, is mainly performed in liver. The metabolic pathway is fuelled by 3 different precursors which are lactate, gluconeogenic amino acids and glycerol. Hormones such as insulin but also the catabolic hormones glucagons and glucocorticoids (C-S) are involved in the regulation of this pathway (see (56)).

How important is heredity in determining the risk of type 2 diabetes?

There are no known immediate survival advantages of morbid obesity, and increased body fat is associated with increased mortality (57). Hence, natural selection is unlikely to have favored obesity per se. On the other hand, during periods of prolonged famine that plagued early human hunter-gatherers, a survival advantage would have been conferred by genes that favor the economical use and storage of energy: so-called "thrifty" genes (58, 59). The existence of thrifty genes was initially proposed by Neel, who focused on the efficient use of glucose as a biological fuel; he suggested that evolutionary pressure to preserve glucose for use by the brain during starvation led to a genetic propensity toward insulin resistance in peripheral tissues (60). Biological systems store energy most efficiently as fat and, hence, another function of thrifty genes is to promote an increase in adipose tissue. In the modern setting of sedentary lifestyles and unrestricted access to high-caloric foods, thrifty genes have been suggested to underlie the twin epidemics of obesity and diabetes (58). While it is undoubtedly true that human obesity and type 2 diabetes have a strong genetic component, the majority of cases are not due to a single genetic defect. Thus, rather than being monogenic diseases, obesity and type 2 diabetes are heterogeneous diseases that result from the interplay between environment and predisposing genetic variation (61, 62). Thus, there are likely to be multiple thrifty genes, and the inheritance of several polymorphisms (single-nucleotide polymorphisms (SNPs)) leading to small differences in protein function can render populations more or less susceptible to obesity and diabetes (62-64) (65).

The largest subgroup of these monogenic diseases is caused by defects in the pancreatic ß cell, resulting in a stable or progressive disorder of insulin secretion. Monogenic disorders that primarily impair insulin action either involve molecules in the insulin signal transduction cascade or result in abnormalities of fat tissue development (lipodystrophy) with secondary metabolic derangements leading to insulin resistance, see **Table 2** and (61, 62).

Table 2: Monogenic diseases leading to impaired glucose homeostasis in human

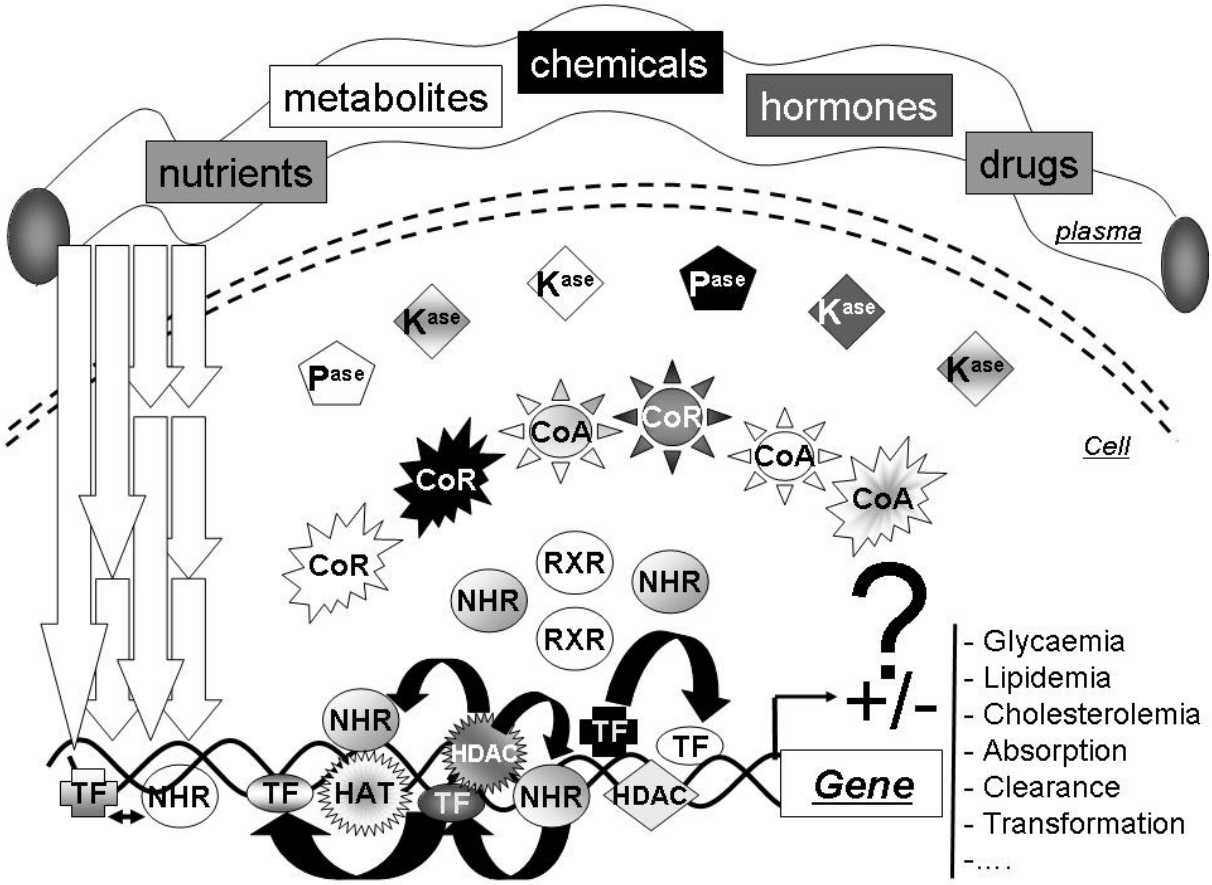
Gene	Monogenic disease	Polygenic type 2 diabetes
GCK	MODY2	
HNF1A	MODY3	Gly319Ser, OR = 1.97 in Oji-Cree
HNF1B	MODY5	
HNF4A	MODY1	Thr103Ile late-onset diabetes in Japanese (OR = 4.3), 5' SNPs increased risk in Finnish (OR = 1.33) and Ashkenazim (OR = 1.4), protective haplotype in UK Caucasian (OR = 0.83)
INS	Diabetes-type hyperglycemia with hyperinsulinemia	Excess paternal transmission of class III VNTR (69% versus expected 50%), 3p+9 in UK Caucasian (OR = 2.02 recessive model only)
INSR	Leprechaunism (Donahue syndrome)	Val985Met in the Netherlands (OR = 1.87), IVS6+43 (OR = 1.32) and haplotype in UK Caucasians (OR = 1.34)
	Rabson-Mendenhall syndrome	
	"Type A" insulin resistance	
IPF1	MODY4	
KCNJ11	Permanent neonatal 606176 diabetes mellitus (PNDM)	Glu23Lys OR = 1.18
Mitochondrial genome	Diabetes and deafness maternally inherited (MIDD)	Mitochondrial DNA 16189, OR = 1.6
PPARG	Familial partial lipodystrophy (FPLD3)	Pro12Ala, OR = 1.25

Human genes in which rare major missense and/or nonsense mutations result in a disorder of glucose homeostasis with a clear Mendelian (or mitochondrial) pattern of inheritance and for which large and/or replicated case-control studies have shown an association between diabetes risk and more common SNPs in or close to the gene. GCK, glucokinase HNF, Hepatic Nuclear Factor; INS, Insulin, INSR, Insulin Receptor; IPF1, Insulin Promoter Factor-1α; ; KCNJ 11, KATP channel subunits Kir6.2; OR, odds ratio of disease in carriers of the susceptibility allele versus noncarriers; PPARG, PPAR gamma; VNTR, variable number of tandem repeats. Table reproduced from O'Rahilly, 2005 (62) and (66).

Transcriptional control of important metabolic genes

In order to accurately respond to signals from the outside, complex biological networks involving numerous proteins have evolved. This complexity is particularly true for the control of genes with critical functions in the coordination of energy homeostasis. Key players involved in these networks include transcription factors (TFs and NHRs), several chromatin condensation modifying proteins, co-activators/repressors and kinases/phosphatases (a virtual representation is shown in **figure 4**). Investigation of the mode of action of these networks is important to better understand how signals from the outside can influence gene expression. This is especially relevant since these signals, which include nutrients, chemicals, and hormones, are known to importantly contribute to disease etiology and progression (see **paragraphs** on **heredity** and **nutritional "omics"**).

Figure 4. From the outside, to the inside: multiple layers model.



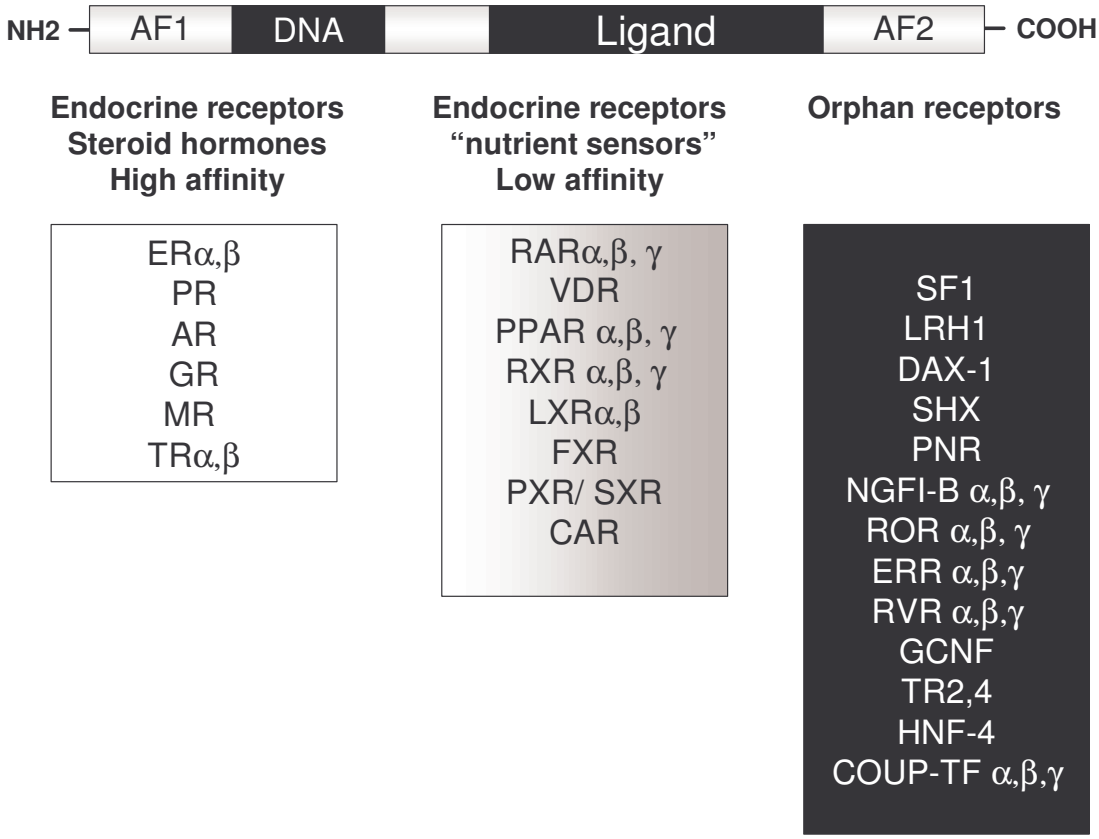
Schematic representation of pathways controlling the expression of a gene involved in key metabolic functions. The transcription of genes is usually ensured through the binding of Transcription Factors (TFs) and Nuclear Homones Receptors (NHRs) to the promoter region of the gene via specific recognition sequences. Additional factors directly influencing the expression of the genes are modifiers of the chromatin structure, which enable the accessibility of the promoter to the TFs and NHRs. These chromatin modifying enzymes include Histone Acetyl Transferases (HATs) but also Histone Deacetylases (HDACs). Some NHRs can act alone as monomer but also as homodimers or heterodimers with the Retinoid X Receptor (RXR). At another organization layer, these TFs and NHRs are usually associated with co-repressors proteins (CoRs), which prevent inappropriate transcription in absence of a signal from the outside. When the cell is triggered with the right signal, the CoR are dismissed from their interaction with the TFs/NHRs and co-activators (CoAs) will replace them enabling transcription to occur. Moreover, some kinases (K^{ases}) and phosphatases (P^{ases}) are also important factors that are able to modify the activity of all the various components within the networks. Finally, signals arising from the bloodstream, e.g. drugs, nutrients, chemicals, metabolites or hormones will elicit a response by governing the expression of genes, which contributes to maintenance of homeostasis.

NHRs in the metabolism

Nuclear hormone receptors function as ligand-activated transcription factors, and thus provide a direct link between small molecules that control these processes and transcriptional responses. In human, the NHR superfamily includes 48 members (67, 68), all of which conform to a common structural organization. The N-terminal region (A/B domain) is highly variable, and contains at least one constitutionally active transactivation region (AF-1) and several autonomous transactivation domains. The A/B domain is variable in length, ranging from less than 50 to more than 500 amino acids. The most conserved region is the DNA-binding domain (DBD, C domain), which contains the P-box, a short motif responsible for DNA-binding specificity, and is involved in dimerization of nuclear receptors. The largest part of the protein is taken up by the ligand-binding domain, which in addition to binding of ligand also mediates receptor dimerization. This dimerization includes formation of homodimers as well as heterodimers. The extreme C-terminus within the ligand binding domain is responsible for interactions with co-activator proteins.

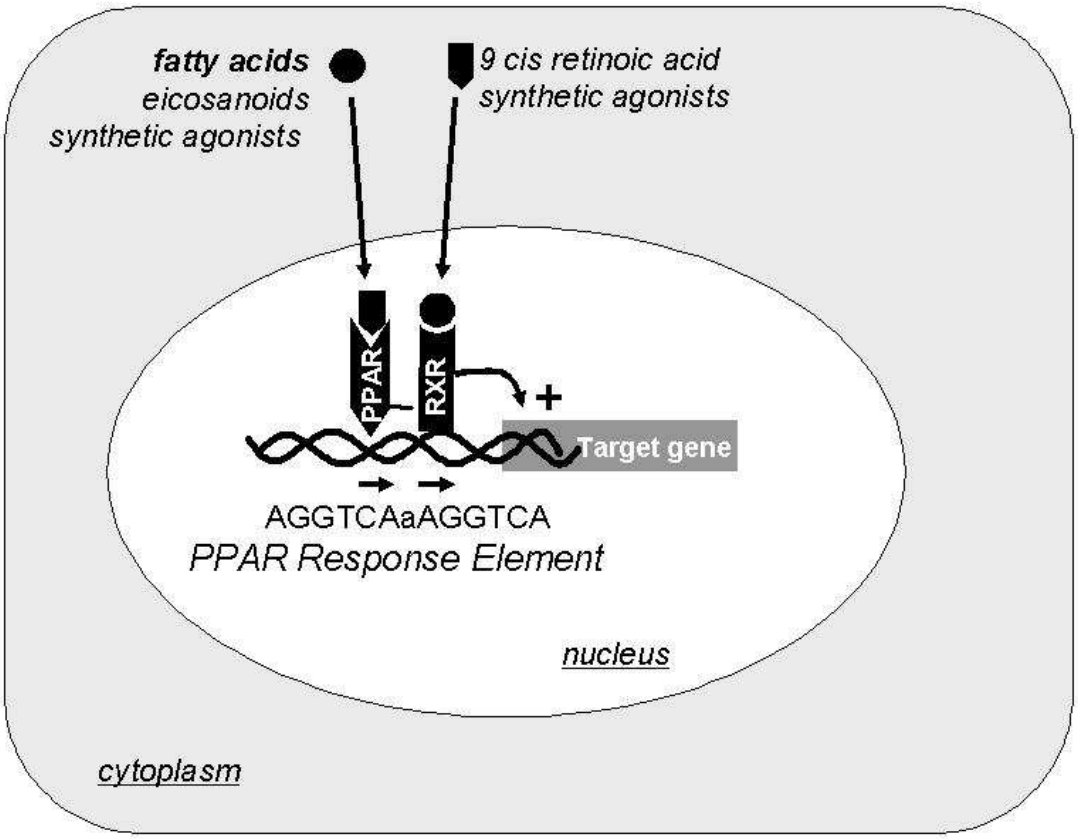
Within the superfamily, NHRs can be classified according to the class of ligands that they bind. NHRs within the first group bind classical hormones and include the receptors for estrogens, progesterone, and androgens. Receptors within the second group bind nutrients, usually with lower affinity, and include the Retinoid X Receptor, the Liver X Receptor, the Farnesoid X Receptor or PPARs (see **chapter 2** for more details). Finally, a large number of nuclear receptors have been identified through sequence similarity to known receptors, but have no identified natural ligand, and are referred to as “nuclear orphan receptors”. **Figure 5** illustrates the commune structures of the NHR superfamily and the classification of its members according to the type of ligand they bind. The signaling events leading to the activation of a typical NHR, PPAR are shown in **Figure 6**.

Figure 5. Classification of the NHRs superfamilly.



The upper part of the figure show a classical organization of domains conserved between NHR members. The two main domains characteristic of NHRs are a DNA Binding Domain (DBD) and a Ligand Binding Domain (LBD). Two other domains, the Activation Function 1 and 2 (AF1 and AF2) are involved in ligand-independent and -dependent activation of the NHRs, respectively. NHRs can be classified according to their ligand-binding properties. A distinction is made between NHRs that bind steroid hormones, nutrients and NHRs with no ligands identified (so far or not). ER, Estrogen Receptor; PR, Progesterone Receptor; AR, Androgen Receptor; GR, Glucocorticoïd Receptor; MR, Mineralocorticoïd Receptor; TR, Thyroid Hormone Receptor; RAR, Retinoic Acid Receptor; PPAR, Peroxisome Proliferator Activated Receptor; RXR, Retinoid X Receptor; LXR, Liver X Receptor; FXR, Farnesoid X Receptor; PXR/SXR, Pregnane and Xenobiotic X Receptor; CAR, Constitutive Androstane Receptor. Other abbreviations for "orphans receptors" can be found in Robinson-Rechavi, 2003 (68).

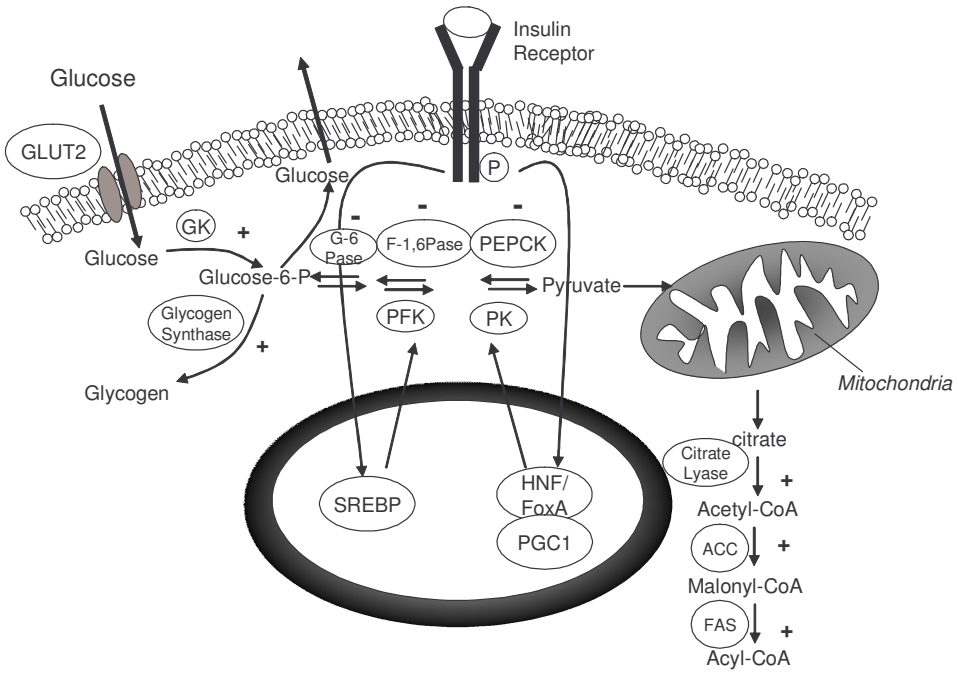
Figure 6. Signaling events leading to the activation of PPARs.



Physiological (fatty acids, eicosanoids, retinoic acid) or synthetic ligands (TZDs, Fibrates, LG100268) enter the cell and the nucleus and bind PPAR or its heterodimeric partner RXR. These associations occurs when PPAR/RXR is bound to specific Response Elements (PPREs) located within the promoter region, resulting in increased transcription of their hence-called target genes.

Since nuclear receptors bind small molecules that can easily be mimicked by drug design, and govern functions associated with major diseases (e.g. reproductive function, cancer, osteoporosis and diabetes), they are highly successful pharmacological targets. Additionally, they bind nutrients, which are known to influence the progression of metabolic diseases and via NHRs can modify the expression of genes implicated in these diseases (including some thrifty genes). These reasons together explain why the NHRs family has received a lot of attention and has been the subject of intense research since their discovery (69). Current knowledge on the functions of NHRs has largely been obtained from genetic studies in mice. These genetic approaches involve the deletion or otherwise inactivation of the NHR gene from the genome; this deletion can either be complete (classical Knocking- out) or spatially and temporally controlled (conditional Knocking- out). Additionally, it is also possible to over-express the NHRs in a given tissue (Transgenesis). These studies have clearly demonstrated that many of the effects of insulin, especially in governing gene expression, are mediated via the coordinated regulation of the activity and expression of some NHRs. Thus, in as much as type II diabetes is caused by defects in insulin signaling, with concomitant altered expression of its target genes, the NHRs represent some interesting candidates to bypass and overcome the insulin signaling defect. In **figure 7**, an example of coordinated regulation by insulin of the NHRs LXRα, HNF- 4 and their shared coactivator PGC1α, in the control of the expression of the thrifty genes acetyl-CoA carboxylase (ACC), fatty-acid synthase (FAS), phosphoenolpyruvate carboxykinase (PEPCK), Glucose- 6- Phosphatase (G-6-Pase), Fructose-1,6-Bisphosphatase (F-1,6-Pase) is shown ((54), (55), (70), (71), (72), (73), (74)).

Figure 7: Insulin control of hepatic gene expression via coordinate regulation of NHRs.

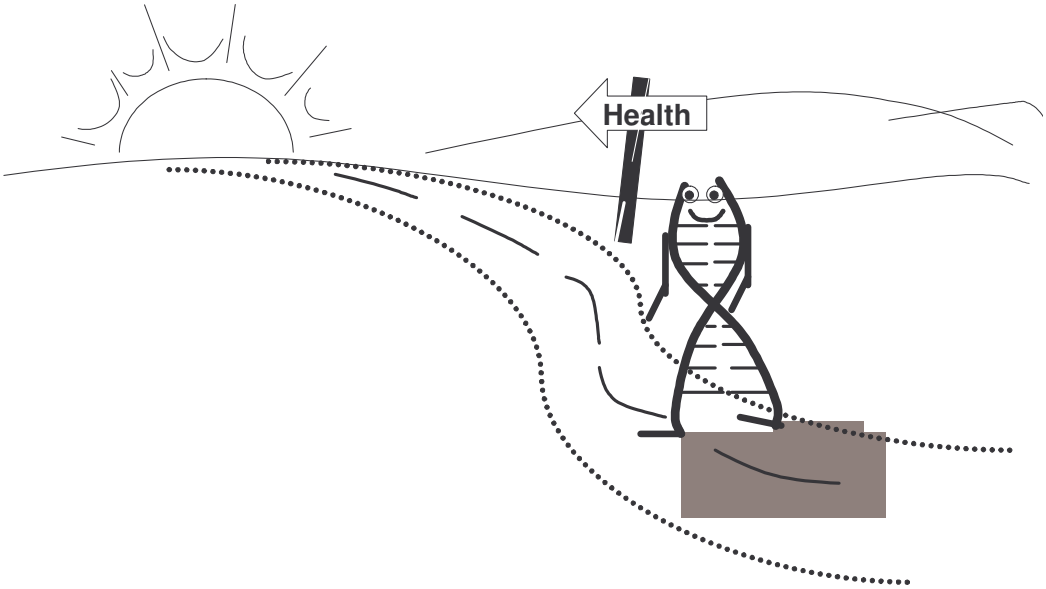


In the hepatocyte, insulin stimulates the utilization and storage of glucose as lipid and glycogen, while repressing glucose synthesis and release. This is accomplished through a coordinated regulation of some NHRs. Insulin stimulates (+) the expression of genes encoding glycolytic and fatty-acid synthetic enzymes, while inhibiting (-) the expression of those encoding gluconeogenic enzymes. These effects are mediated by a series of NHRs and co-factors, Liver X Receptor (LXRα), hepatic nuclear factor (HNF)- 4α and PPARγ co-activator 1 (PGC1). LXRα, which induces expression of some lipolytic enzymes and represses gluconeogenic genes, is activated by insulin. Conversely, HNF-4α, which stimulates gluconeogenic genes expression, is repressed. Insulin also regulates the activities of enzymes such as glycogen synthase and citrate lyase through changes in phosphorylation state. ACC, acetyl-CoA carboxylase; F-1,6-Pase, fructose-1,6-bisphosphatase; FAS, fatty-acid synthase; G-6-Pase, glucose-6-phosphatase; GK, glucokinase; Glucose-6-P, glucose-6-phosphate; PEPCK, Phosphoenolpyruvate Carboxykinase; PFK, phosphofructokinase; PK, pyruvate kinase. Adapted from Saltiel and Kahn, 2001 (75) and Finck and Kelly, 2006 (76).

Nutrional “omics”

Ecological studies, comparisons of migrant populations, cohort studies, and intervention trials clearly demonstrate that diet and physical activity have a major impact on the development of diabetes (77). More recent attention has focused on the possible effects of prenatal and early postnatal environment on diabetes risk (78). In fact, like for cancer (79) there is increasing evidences suggesting that diabetes is a multi-factorial disease, which involves the interaction of the environment (such as diet) with an individual’s genetics. Nutrigenetics is the study of individual differences at the genetic level that influence the response to diet. These individual differences are most likely at the level of single nucleotide polymorphisms. Nutrigenomics, which describes the study of how genes and dietary components interact to alter phenotype, will likely facilitate greater understanding of how nutrition affects metabolic pathways and how this process goes awry in diet-related diseases. It is envisaged that nutrigenetics may lead to individualised dietary advice, which is often referred to as “personalized nutrition” (**figure 8**) (80). Although this concept is still mostly hypothetical and studies so far have only revealed small changes in diabetes risk (40)(41),(81, 82) some private companies are already exploiting the field. An example is Sciona (www.sciona.com) who already sells kits to self-test the risk to develop diabetes. The kit analyzes for the presence of 13 common SNPs already associated with metabolic diseases including ApoC3, LPL, TNFα, eNOS or MTHFR (83-88) (89, 90) (91).

Figure 8: Cartoon illustrating the promise of nutritional "omics".



The dream of improving individual health through tailored nutritional recommendations has been well described in the *New York Times* in May 2003: 'A trip to the diet doc, circa 2013. You prick your finger, draw a little blood and send it, along with a $100 fee, to a consumer genomics lab in California. There, it's passed through a mass spectrometer, where its proteins are analyzed. It is cross-referenced with your DNA profile. A few days later, you get an email message with your recommended diet for the next four weeks. It doesn't look too bad: lots of salmon, spinach, selenium supplements, bread with olive oil. Unsure of just how lucky you ought to feel, you call up a few friends to see what their diets look like. There are plenty of quirks. A Greek co-worker is getting clams, crab, liver and tofu – a bounty of B vitamins to raise her co-enzyme levels. A friend in Chicago, a second-generation Zambian, has been prescribed popcorn, kale, peaches in their own juice and club soda. (This looks a lot like the hypertension-reducing 'Dash' diet, which doesn't work for everyone but apparently works for him.) He is allowed some chicken, prepared in a saltless marinade, hold the open flame – and he gets extra vitamin D because there's not enough sunshine for him at his latitude. (His brother's diet, interesting enough, is a fair bit different.) Your boss, who seems to have won some sort of genetic lottery, gets to eat plenty of peanut butter, red meat and boutique cheeses…Nobody is eating exactly what you are. Your diet is uniquely tailored. It is determined by the specific demands of your genetic signature, and it perfectly balances your micronutrient and macronutrient needs. Sick days become a foggy memory. (Foggy memory itself is now treated with extracts of ginkgo biloba and a cocktail of omega-3 fatty acids.)… Your cholesterol does not react much to diet so you can eat bacon sandwiches and don't need to spend money on vitamin supplements that aren't doing anything for you… You willingly take only the vitamins you need in precisely the right doses, which will postpone the onset of disease to which you are naturally susceptible' (Grierson, 2003). Picture Adapted from "it's not just your genes" by DeBusk R and Joffe Y, BKDR, Inc. Publishing; 1st edition (2006).

Objective and Outline of this thesis

The main objective of the research presented here was to probe the role of PPARα in the onset of hyperglycaemia in type II Diabetes. The project was originally based on accumulation of different clues that let us to believe that altered signaling via PPARα may contribute to the hepatic phenotype in type II diabetes: 1) Obesity greatly increases the risk for developing type II diabetes and it is well recognized that elevated plasma free fatty acid levels associated with obesity are a critical intermediate in the pathophysiology of type II diabetes. 2) Free fatty acids promote diabetes partly by stimulating hepatic gluconeogenesis (glucose output). 3) Fatty acids are able to bind to PPARα and activate the expression of its so-called target genes (i.e. fatty acids are ligands for PPARα). 4) Past data from our group had indicated that PPARα has a major impact on fasting plasma glucose levels. Consequently, we were interested to find out whether PPARα mediated the stimulatory effect of elevated circulating free fatty acids on hepatic glucose output by up-regulating the expression of genes involved in gluconeogenesis. By combining microarray with the use of PPARα null mice we were able to identify the stimulatory function of PPARα in the hepatic conversion of glycerol into glucose. Defects in this metabolic pathway likely contribute to the severe hypoglycemia in PPARα null mice when fasted. Moreover, this pathway was conserved and relevant in human. Detailed molecular investigations demonstrated that the action of PPARα on this pathway was direct. cGPDH was identified as a direct PPARα/ γ dual target gene in liver and adipose tissue, respectively (**Chapter 3**).

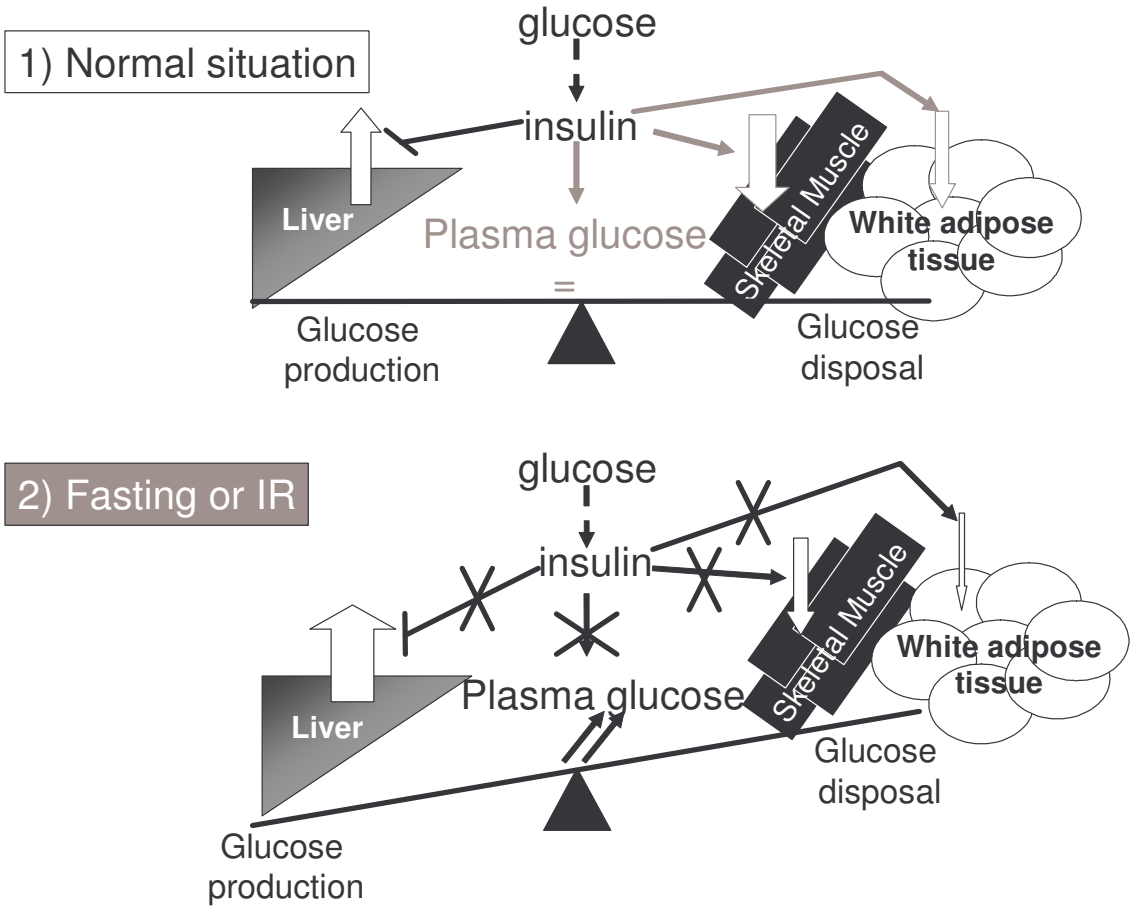
Secondly, we wanted to test whether the implication of PPARα in hepatic glucose formation was of relevance in the context of type II diabetes (see **figure 9** for analogy between fasting and diabetes). We used a nutritional intervention consisting of a high fat diet in order to induce insulin resistance. Analysis of the expression of PPARα target genes demonstrated that PPARα was activated upon high fat feeding. Furthermore, quantification in the activation of PPARα target genes showed that high fat diet weakly activates PPARα. However, acting in a chronic manner, the physiological changes can significantly alter glucose homeostasis (**chapter 4**).

Finally, we have been interested in the interplay between LXRα and PPARγ in the context of adipose tissue. The results obtained demonstrated that ligand-activated LXRα is able to specifically attenuate the expression of cGPDH and some other PPARγ target genes. At the

molecular level we were able to show that LXRα competes with PPARγ for their reciprocal partner RXRα, resulting in down-regulation of PPARγ target genes (**Chapter 5**).

Finally, the results of the studies above are discussed in **Chapter 6**.

Figure 9: Fasting as a model to investigate IR and hepatic glucose production.



In normal situation 1) insulin is able to tightly control plasma glucose levels. Overall, this action is achieved by increasing the uptake of glucose in the skeletal muscles and white adipose tissue. In addition, insulin suppresses hepatic glucose production. In case of fasting or insulin Resistance (IR) (2)), insulin signaling is impaired. The consequences of this defect are that glucose disposal is lowered while the hepatic glucose production is increased, which translate into a rise of plasma glucose. The width of the white arrows pointing at the different tissues represent the importance of the glucose fluxes and how they are modified between the physiological states.

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Chapter 2

PPAR ligands for the treatment of insulin resistance

David Patsouris, Michael Müller, and Sander Kersten

Current opinion in investigational drugs 2004; 5(10):1045-50

ABSTRACT

Peroxisome Proliferators Activated Receptors (PPARs) are a group of ligand-activated transcription factors that play critical roles in the regulation of energy metabolism. Synthetic agonists for these receptors are used for treatment of a variety of metabolic abnormalities, including type 2 diabetes mellitus and dyslipidemia. This article will critically review current data linking PPAR and/or PPAR ligands with the treatment of insulin resistance, focusing on important advances over the past year.

INTRODUCTION

According to the World Health Organization, diabetes mellitus is becoming an increasing cause of mortality and morbidity worldwide. Diabetes mellitus is defined by elevated plasma glucose levels, which in the long-term causes major damage to nerves and blood vessels. Diabetes mellitus is directly connected to an inability of the body to produce (diabetes type 1) or to respond to (diabetes type 2) the hormone insulin. [1]

Overt type 2 diabetes is most often preceded by a state of insulin resistance, which describes an impaired response to insulin, either in liver (hepatic insulin resistance), or skeletal muscle and adipose tissues (peripheral insulin resistance). Insulin resistance is almost invariably linked to obesity, and is often part of a combination of metabolic abnormalities united in the term metabolic syndrome. Although insulin resistance is most often at the basis of impaired glucose metabolism in metabolic syndrome, an effective pharmacological strategy to lower plasma glucose might bypass the insulin resistance and directly target the consequences of it, which are decreased glucose utilization and/or enhanced hepatic glucose production.

A group of molecular targets for insulin resistance that has received a lot of attention are the Peroxisome Proliferator Activated Receptors. PPARs are members of the super-family of Nuclear Hormone Receptors and consists of three members: PPARα, β/δ, and γ [2,3,4]. They serve as ligand-activated transcription factors, and translate the effect of small lipophilic compounds, including a variety of fatty acids and fatty acid-like molecules, on gene transcription. Each of the three PPARs activates a distinct set of (target) genes and therefore has a distinct biological function. In addition to serving as nuclear receptor for fatty acids, PPARs also are the molecular target for two important classes of synthetic agonists. While PPARα binds and is activated by the fibrate class of drugs, which are used in the treatment of low plasma HDL/high triglycerides, PPARγ binds and is activated by thiazolidinediones,

which have gained popularity in the treatment of type 2 diabetes and associated insulin resistance [3,4,5]. Although recent reports suggest a possible role for PPARβ/δ in the treatment of low plasma HDL, the pharmacological application of PPARβ/δ agonists needs to be explored further [3].

This article will critically review current data linking PPAR and/or PPAR ligands with the treatment of insulin resistance, focusing on important advances over the past year.

PPARγ AGONISTS

PPAR γ is mainly expressed in adipose tissue, and to a lesser extent in colon and macrophages [6,7]. Elaborate experiments over the past decade have indicated that expression of PPARγ is both necessary and sufficient for adipocyte differentiation [8]. For this reason PPAR γ is nowadays considered as the master regulator of fat differentiation. In addition, PPARγ functions in the mature adipocyte to promote lipid storage and regulate the synthesis of proteins secreted from fat tissue, the so-called adipocytokines [9,10,11].

PPARγ entered into the pharmaceutical spotlight in 1995 after it was discovered that a group of compounds called thiazolidinediones (TZDs) serve as agonists for PPARγ [4]. Numerous studies in a variety of animal models for diabetes and/or obesity have indicated that TZDs not only promote glucose uptake in peripheral tissues but also lower plasma triglycerides effectively [12]. Clinical studies with diabetic patients have confirmed these effects in humans, although in general they are a bit less pronounced than in rodents. The two TZDs that are approved for clinical use are pioglitazone (Actos) and rosiglitazone (Avandia), and a few others are presently in clinical development.

Although the effectiveness of TZDs toward stimulating glucose utilization is well established, many question marks still surround the mechanism by which this effect is achieved (Figure 1). In fat tissue TZDs increase the expression of genes involved in glucose transport (GLUT4) and insulin signaling (CAP, p85alphaPI-3K), yet adipose tissue is responsible for only a minor portion of whole body glucose disposal. In contrast, skeletal muscle accounts for the major portion of TZD-induced glucose disposal, yet expresses very little PPARγ. Numerous non-exclusive mechanisms have been put forward to explain this apparent paradox. It has been suggested that because of their ability to induce apoptosis as well as adipocyte differentiation, TZDs are able to cause enrichment of adipose tissue with smaller adipocytes at the expense of large adipocytes. Inasmuch as smaller adipocytes are more responsive to insulin, this remodeling of adipose tissue would be associated with

increased sensitivity to insulin. Alternatively, it is possible that the effects of TZDs on glucose disposal are directly linked to their ability to lower plasma free fatty acid levels. Plasma free fatty acids have received a lot of bad press for their purported role in linking obesity to insulin resistance. Indeed, evidence abounds indicating that elevated free fatty acids interfere with insulin signaling in skeletal muscle, and stimulate glucose production in liver [13,14,15]. Recent studies have indicated that TZDs may lower plasma free fatty acids by stimulating their re-esterification in adipose tissue via enhanced synthesis of glycerol 3-phosphate, either by inducing glycerol phosphorylation via glycerol kinase, by inducing glyceroneogenesis via phosphoenol-pyruvate carboxykinase, or by inducing synthesis of glycerol 3-phosphate from glucose via glycerol 3-phosphate dehydrogenase (Figure 2) [16,17]. Besides lowering free fatty acid release, TZDs also influence the secretion of several adipose-derived hormones (adipocytokines) that are implicated in glucose metabolism. Upregulation of factors that promote insulin sensitivity and suppress hepatic glucose production (adiponectin), in combination with down-regulation of factors that impair insulin action and glucose tolerance (TNFα, resistin) [9,10] may indeed be the dominant mechanism by which TZDs exert their effect (Figure 1 and 2).

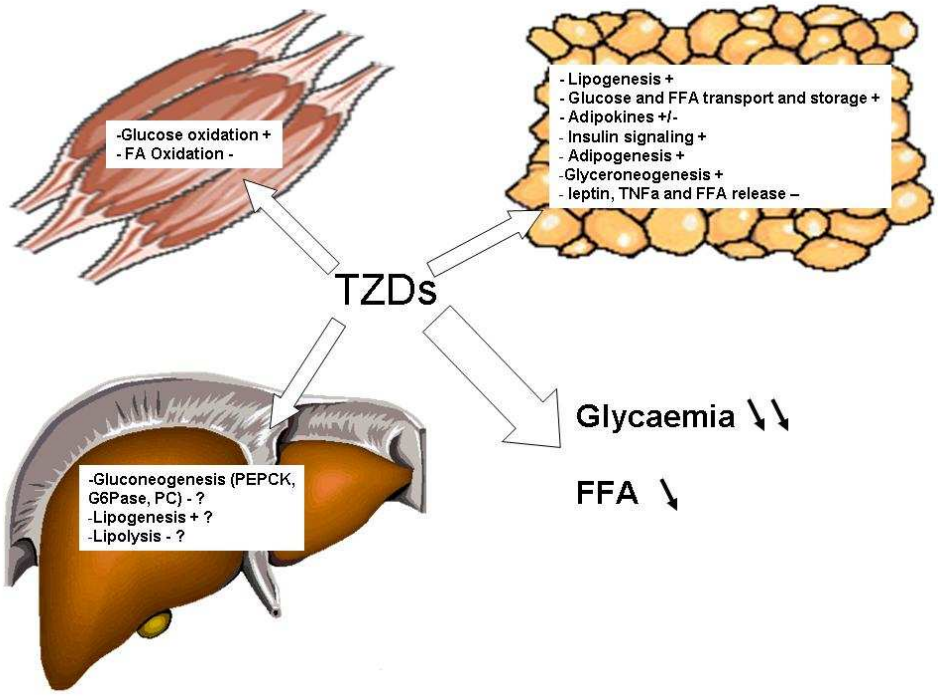

Figure 1: Effects of TZDs in various organs. Processes that are stimulated by TZDs are shown in green, processes that are inhibited by TZDs are shown in red. Synthesis of adipocytokines is both stimulated and inhibited by TZDs, depending on the adipocytokine. The overall effect is a decrease in plasma glucose, free fatty acids, and triglycerides

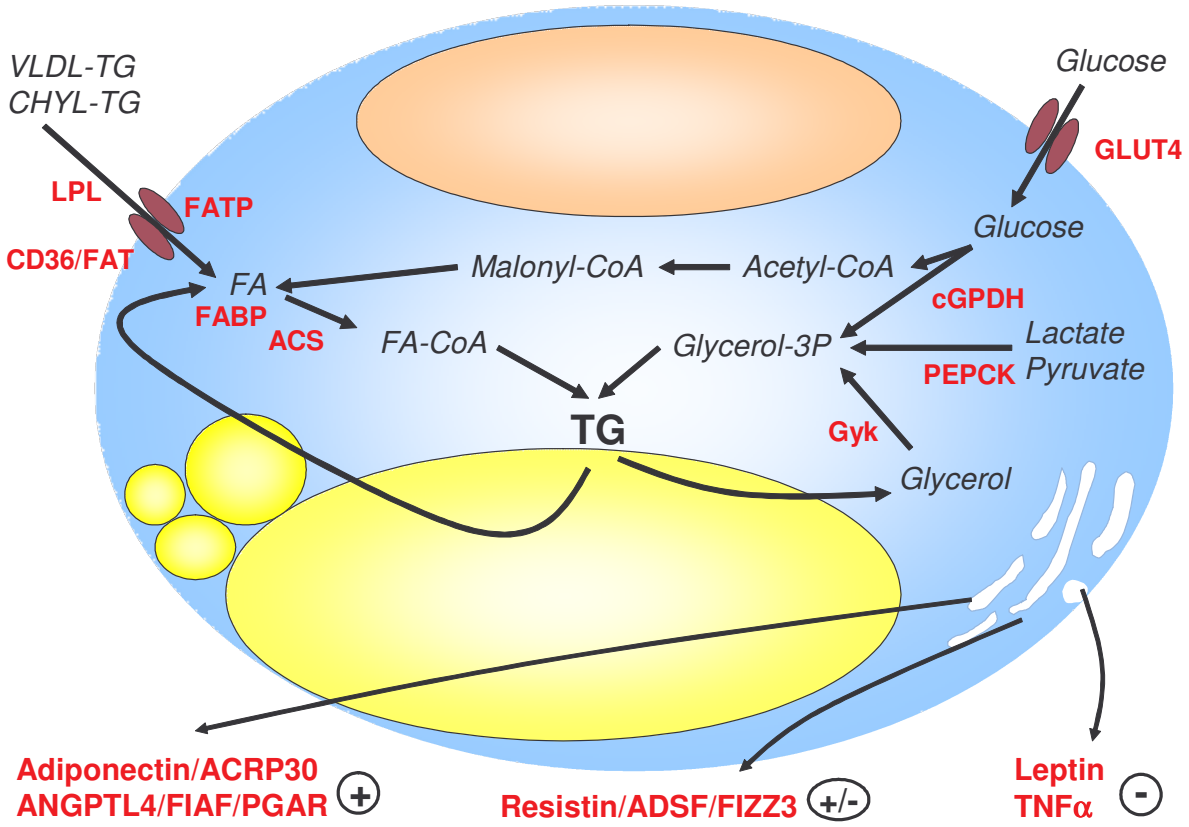

Figure 2: Regulation of adipocyte metabolism by TZDs. Specific target genes of PPARγ and thus TZDs are indicated in red. LPL = lipoprotein lipase, FATP = fatty acid transport protein, FABP = fatty acid binding protein, ACS = acyl-CoA synthetase, Gyk = glycerol kinase, PEPCK = phosphoenol-pyruvate carboxykinase, cGPDH = cytosolic glycerol 3-phosphate dehydrogenase, GLUL4 = Glucose transporter 4

A few years ago it was believed that mice that carry a tissue-specific deletion of PPARγ would be a fantastic asset to help sort out the mechanism by which TZDs promote whole body glucose disposal. However, rather than narrowing down the options, analysis of tissue-specific PPARγ null mice has further increased the complexity, not in the least because result from different studies were sometimes inconsistent and led to opposite conclusions.

To ascertain whether the presence of PPARγ in skeletal muscle is essential for the effect of TZDs on glucose disposal, two groups independently generated mice with a muscle–specific obliteration of PPARγ [18,19]. Both sets of mice suffer from increased adiposity and insulin resistance, the latter of which could be secondary to the increased fat mass. However, while the muscle-specific PPARγ null mice described by Norris et al. showed hepatic insulin resistance but no effect on insulin-stimulated whole body or muscle glucose disposal, the mice described by Hevener et al. displayed markedly diminished whole body glucose disposal. Furthermore, whereas Norris et al. observed that TZDs were able to revert insulin resistance induced by a high fat diet, leading to the conclusion that PPARγ in muscle is dispensable for the effects of TZDs, Hevener et al. observed no beneficial effect of TZDs, which led the authors to a completely opposite conclusion [18,19]. Accordingly, the role of muscle in TZD-induced glucose disposal remains ambiguous.

The analysis of mice with a specific deletion of PPARγ in adipose tissue or liver has been informative with regards to the role of PPARγ in these respective organs, yet has complicated the picture of the mechanism by which TZDs act. Confirming PPARγ's role as a master regulator of adipogenesis, specific disruption or deletion of the PPARγ gene in white adipose tissue induces adipose cell death and severely diminished fat mass (lipodystrophy) [20,21,22,23]. However, the latter observation means that it is impossible to separate the effects of PPARγ deletion per se from the effects of reduced fat mass, which by itself has major metabolic consequences. With regards to the mechanism of action of TZDs, deletion of PPARγ in adipose tissue does not seem to influence the stimulatory effect of TZDs on glucose disposal, suggesting that PPARγ in adipose tissue is dispensable for TZD-induced glucose disposal, although the picture is far from complete. Previously, it has been shown that in mice that are virtually devoid of adipose tissue troglitazone can still lower plasma glucose, improve glucose tolerance, and diminish hyperinsulinemia [24]. However, in a different animal model of lipodystrophy, TZDs failed to improve hyperglycemia and hyperinsulinemia [25]. Obviously, the issue is far from fully resolved.

Expression of PPARγ in liver is elevated in several mouse models of diabetes and fatty liver. Inasmuch as PPARγ upregulates the expression of a whole set of genes involved in fat synthesis, and excess fat storage in liver has been connected with hepatic insulin resistance, activation of PPARγ could promote fatty liver and thereby worsen hepatic insulin resistance [26]. However, it appears that liver-specific PPARγ null mice represent a mouse model in which hepatic insulin resistance and fatty liver are completely disconnected [27,28]. Indeed, while PPARγ deletion improves fatty liver yet aggrevates insulin resistance in liver, adipose tissue and muscle, TZD treatment promotes fatty liver but improves hyperglycemia. From these studies it can also be concluded that PPARγ in liver appears not to play a role in mediating the effects of TZDs on glucose metabolism.

Overall, tissue-specific PPARγ null mice have clearly established that PPARγ is required for adipogenesis, adipose cell survival, and partitioning of lipids between metabolic tissues. However, studies with these mice have failed to indicate whether the beneficial effect of PPARγ agonists on liver and muscle insulin sensitivity occurs via direct action on these tissues or via some systemic effects.

In human patients a major drawback of treatment with TZDs is that it promotes weight gain, which is probably caused by increased food intake in combination with increased fat synthesis. Ideally, one would like to develop PPARγ agonists that stimulates glucose utilization but have little effect on weight gain, although it is not completely clear whether these two effects can be separated in vivo as one may partially depend on the other. Analysis of genes induced by TZDs in a variety of organs by microarray demonstrates that while some genes are regulated by all TZDs, others are regulated specifically by a particular TZD. Accordingly, one would like novel PPARγ agonist to be neutral toward those genes involved in weight gain, yet activate those genes involved in inducing glucose disposal. Two compounds with such properties have been reported so far, one of which (FMOC-L-Leucine) was remarkably effective in ameliorating glucose tolerance in two diabetic mouse models [29,30].

PPARα AGONISTS

Whereas the effects of PPARγ agonists on glucose metabolism and the mechanisms involved are well studied, much less in known about PPARα agonists and glucose metabolism. The PPARα agonists that have been approved for use are gemfibrozil, clofibrate, bezafibrate, ciprofibrate and fenofibrate. PPARα is mainly expressed in liver, where it plays a

role in lipid metabolism and inflammation (Figure 3) [10]. PPARα was first linked with glucose metabolism by the observation that mice lacking PPARα suffer from severe hypoglycemia when fasted [31]. The hypoglycemia is most likely caused by defective hepatic glucose synthesis, or may be impaired secondary to defective fatty acid oxidation, which "fuels" glucose synthesis. Recent data have indicated that PPARα directly stimulates hepatic utilization of glycerol, which serves as a precursor for glucose synthesis (Figure 3) [16]. In addition to having a lower basal rate of glucose synthesis, PPARα null mice also display a higher peripheral and hepatic sensitivity to insulin [16,32,33,34]. Consequently, PPARα null mice are partially protected from insulin resistance brought about by a high fat diet [32,34]. Recent studies have drawn attention to TRB-3, which is a negative regulator of intracellular insulin signaling, as a potential mediator between PPARα deletion and improved insulin sensitivity [35].

Based on the above discussion, inhibiting PPARα using synthetic antagonists might be a fruitful strategy to lower hepatic glucose production and promote insulin sensitivity. However, numerous studies have shown that at least in rodent models of diabetes/obesity PPARα agonists significantly restore insulin sensitivity [36,37,38,39,40]. This beneficial effect is translated in a decrease of plasma glucose and insulin levels. This is probably achieved by stimulating glucose utilization in muscle and adipose tissue [36,37,39,41,42,43,44,45].

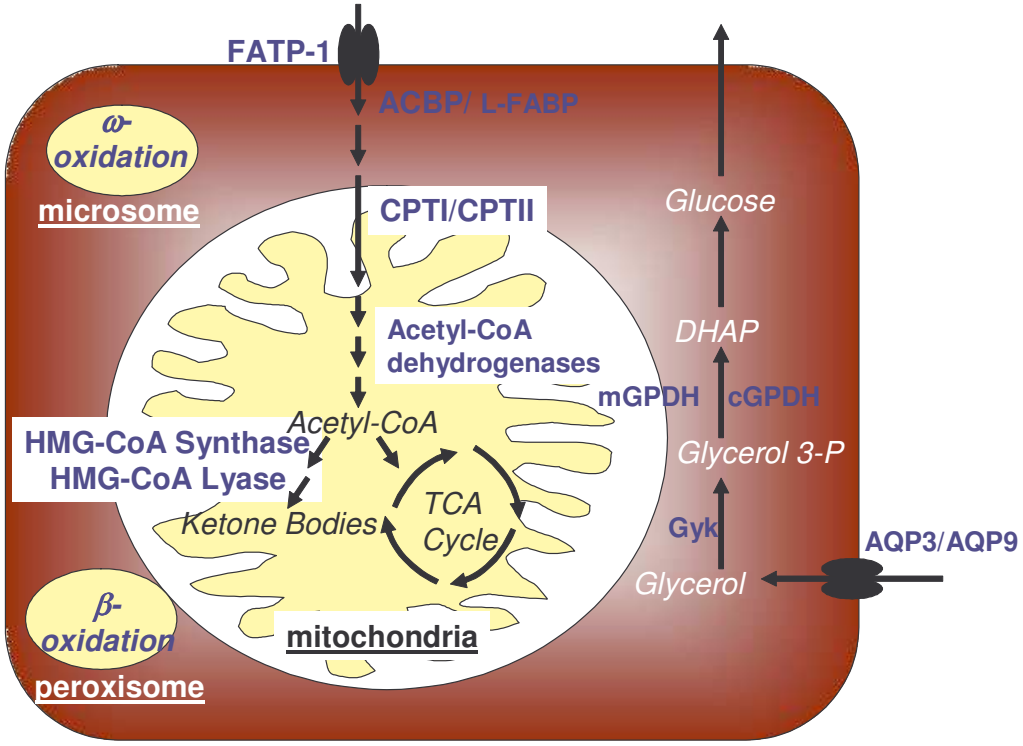

Figure 3: Regulation of hepatocyte metabolism by fibrates. Specific targets of PPARα and thus fibrates are indicated in blue. FATP = fatty acid transport protein, ACBP = Acyl-CoA binding protein. FABP = fatty acid binding protein, CPT = carnitine palmitoyl transferase, Gyk = glycerol kinase, GDPH = glycerol 3-phosphate dehydrogenase, AQP = aquaporin. The figure is not exhaustive but shows representative target genes.

An important question is how exactly fibrates improve insulin sensitivity and lower plasma glucose, considering that PPARα deletion causes a similar phenotype. It is possible that (part of) the effects of fibrates are secondary to their ability to diminish hepatic and muscle triglyceride levels, which are negatively associated with insulin sensitivity and which are typically elevated in mouse models of diabetes/obesity [43,44,45]. Ideally, one would like to suppress the stimulatory effect of PPARα on gluconeogenic gene expression in liver, while promoting its stimulatory effect on genes involved in fatty acid oxidation in muscle and liver, using selective PPAR modulators (SPPARM). Although it is clear that synthetic PPARα agonists have different and only partially overlapping effects on gene expression [46], it is uncertain whether such as compound can be developed.

With respect to the effectiveness of PPARα agonists toward insulin resistance in humans, a limited number of studies have so far reported positive results, thereby confirming

the animal data. However, more studies with long-term follow-up are needed. It should be noted that the data for total mortality data for some of the fibrates are a bit worrying. As it was introduced relatively recently, these types of data are still lacking for micronized fenofibrate (Tricor), but some concern is warranted.

DUAL PPARα/γ AGONISTS

Inasmuch as both PPARγ and PPARα agonists appear to promote insulin sensitivity and glucose disposal, agonists that activate both PPARγ and PPARα may prove to be even more effective in ameliorating insulin resistance. Combined with the fact that the metabolic abnormalities corrected by PPARα and PPARγ agonists partially overlap and often occur in a single patient, this has led to the development of numerous so called dual PPARα/γ agonists. Numerous animal studies with an array of different dual agonists have yielded very encouraging data, resulting in improved plasma lipid, cholesterol and glucose parameters, without major weight gain. Consequently, many compounds have entered the phase of human clinical trials. Although initial expectations were high, the discontinuation of clinical development of three dual agonists has been a major setback. Phase III clinical trials for the dual agonist ragaglitazar were suspended after long term animal studies showed development of bladder tumors, while trials for farglitazar were abandoned because of fluid retention problems. Clinical trials of another dual agonist (MK-767 or RP-297) were stopped because the compound was linked with rare malignant tumors in mice, although it is not known of what type. Other dual agonists such as tesaglitazar and muraglitazar are still in clinical development. If no untoward effects for these compounds can be demonstrated, approval for marketing may be expected in 2005-2006.

PPARβ/δ AGONISTS

Recent studies have indicated that PPARβ/δ stimulates fatty acid oxidation in skeletal muscle and adipose tissue [47,49,3]. Accordingly, agonists for PPARβ/δmight have potential for treatment of insulin resistance as well. However, at the present time it is unclear whether PPARβ/δ agonists have any effect on control of plasma glucose, either in humans or in animals. The results from such studies are eagerly awaited.

CONCLUSIONS

Although the exact mechanisms behind its effects are still not fully resolved, PPARγ agonists have proven effective in stimulating insulin-dependent glucose disposal in peripheral organs. Currently, there is a definite need for PPARγ agonists that cause less weight gain than the ones approved but it is unclear whether the effects on glucose disposal and weight gain can be separated. With regards to PPARα, there is increasing evidence for a direct link between this receptor and glucose metabolism. However, given the questionable track record of some fibrates on mortality, the stimulatory role of PPARα in gluconeogenesis, and the limited efficacy of PPARα agonists toward insulin resistance compared to PPARγ agonists, it is unlikely that any of the pure PPARα agonist will be approved for correcting insulin resistance. Instead, the focus will remain on dual PPARα/γ agonists. Although the clinical efficacy of dual agonists is very encouraging, there are increasing concerns about their toxicity. Finally, agonists for PPARβ/δ might have potential for treatment of insulin resistance but the validity of the concept first needs to be demonstrated in animal studies.

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Chapter 3

PPARα governs glycerol metabolism

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ABSTRACT

Glycerol, a product of adipose tissue lipolysis, is an important substrate for hepatic glucose synthesis. However, little is known about the regulation of hepatic glycerol metabolism. Here we show that several genes involved in the hepatic metabolism of glycerol, i.e., cytosolic and mitochondrial glycerol 3-phosphate dehydrogenase (GPDH), glycerol kinase, and glycerol transporters aquaporin 3 and 9, are upregulated by fasting in wild-type mice but not in mice lacking *PPAR*α. Furthermore, expression of these genes was induced by the PPARα agonist Wy14643 in wild-type but not *PPAR*α–null mice. In adipocytes, which express high levels of PPARγ, expression of cytosolic GPDH was enhanced by PPARγ and ß/δ agonists, while expression was decreased in *PPAR*$\gamma^{+/-}$ and *PPAR*$ß/\delta^{-/-}$ mice. Transactivation, gel shift, and chromatin immunoprecipitation experiments demonstrated that cytosolic GPDH is a direct PPAR target gene. In line with a stimulating role of PPARα in hepatic glycerol utilization, administration of synthetic PPARα agonists in mice and humans decreased plasma glycerol. Finally, hepatic glucose production was decreased in *PPAR*α-null mice simultaneously fasted and exposed to Wy14643, suggesting that the stimulatory effect of PPARα on gluconeogenic gene expression was translated at the functional level. Overall, these data indicate that PPARα directly governs glycerol metabolism in liver, whereas PPARγ regulates glycerol metabolism in adipose tissue.

INTRODUCTION

In most parts of the world, the prevalence of obesity is increasing rapidly. One of the most important secondary ailments of obesity is type 2 diabetes, which affects millions of people worldwide. It is well recognized that elevated plasma free fatty acid levels associated with obesity are a critical intermediate in the pathophysiology of type 2 diabetes (1). Free fatty acids promote diabetes partly by stimulating hepatic gluconeogenesis and glucose output (2-6). However, the mechanism(s) by which free fatty acids achieve this effect remains obscure.

Fatty acids are able to activate the expression of genes via PPARs (7). PPARs are ligand-activated transcription factors that belong to the superfamily of nuclear hormone receptors. Three PPAR isotypes are known: PPARα, PPARß/δ, and PPARγ. The latter isotype is mainly expressed in adipose tissue and plays an important role in adipocyte differentiation and lipid storage (8). It serves as a target for an important class of antidiabetic

drugs, the insulin-sensitizing thiazolidinediones. PPARß/ δ is expressed ubiquitously and thus far has been connected with wound healing, cholesterol metabolism, and fatty acid oxidation (9-11). Finally, PPARα stimulates hepatic fatty acid oxidation and ketogenesis, and regulates production of apolipoproteins. It serves as target for the hypolipidemic fibrate class of drugs, which include fenofibrate and gemfibrozil. Experiments with *PPAR*α-null mice have been invaluable in elucidating the physiologic role of PPARα and have indicated that hepatic PPARα is particularly important during fasting (12-14). Fasted *PPAR*α-null mice suffer from a variety of metabolic defects including hypoketonemia, hypothermia, elevated plasma free fatty acid levels, and hypoglycemia. The mechanism behind the fasting-induced hypoglycemia has so far remained elusive, but it is conceivable that PPARα directly regulates the expression of genes involved in gluconeogenesis. Since fatty acids are ligands for PPARα, the latter mechanism would be able to explain the stimulatory effect of elevated plasma free fatty acids on hepatic gluconeogenesis and glucose output.

In order to ascertain what metabolic steps or pathways are affected by *PPAR*α deletion, we performed microarray analysis with RNA from liver of fasted wild-type and *PPAR*α-null mice. Interestingly, it was found that the expression of several genes involved in gluconeogenesis was decreased in *PPAR*α-null mice compared with wild-type mice. Follow-up analysis indicated that PPARα stimulates the expression of a set of genes involved in the conversion of glycerol to glucose and that at least one of these genes, the cytosolic glycerol 3-phosphate dehydrogenase gene (GPDH) is a direct target of PPARα with a functional PPAR response element in its promoter. Our data demonstrate that PPARα directly regulates glycerol metabolism in liver.

RESULTS

*Regulation of gluconeogenic gene expression by PPAR*α. In agreement with previous data, hepatic PPARα expression was strongly induced by fasting (Figure 1A). Accordingly, it can be expected that the effects of PPARα on gene expression are especially evident during fasting. To pinpoint novel pathways regulated by *PPAR*α, we compared mRNA of livers of fed and fasted PPARα-null and wild-type mice by oligonucleotide microarray. As expected, the fasting-induced increase in expression of fatty acid oxidative and ketogenic genes was PPARα dependent (Figure 1B). Interestingly, a similar type of regulation was observed for

cytosolic GPDH (cGPDH) and mitochondrial GPDH (mGPDH), which are involved in the conversion of glycerol to glucose (Figure 1C). In contrast, phosphoenolpyruvate carboxykinase (PEPCK), which is considered to be the rate-limiting enzyme in gluconeogenesis from lactate/pyruvate, was upregulated during prolonged fasting in a PPARα-independent manner (Figure 1C). Real-time quantitative PCR (Q-PCR) confirmed that cGPDH and mGPDH were upregulated by fasting only in wild-type mice (Figure 2A). Interestingly, a similar type of regulation was observed for glycerol kinase, as well as aquaporin 3 (AQP3) and aquaporin 9 (AQP9). The latter two transporters are involved in the cellular uptake of glycerol (15), which, via the action of glycerol kinase, is phosphorylated to glycerol 3-phosphate, which in turn is converted to the gluconeogenic intermediate dihydroxyacetonphosphate via cGPDH and mGPDH. To establish that the decreased expression in fasted *PPAR*α-null mice is not an indirect consequence of metabolic perturbations in these mice, wild-type and *PPAR*α-null mice were fed for 5 days with the synthetic PPARα ligand Wy14643. Wy14643 consistently upregulated the expression of cGPDH, mGPDH, glycerol kinase, and AQP3 in wild type but not *PPAR*α-null mice (Figure 2B). No induction was observed for AQP9. Taken together, these results indicate that PPARα induces hepatic expression of genes involved in the conversion of glycerol to glucose.

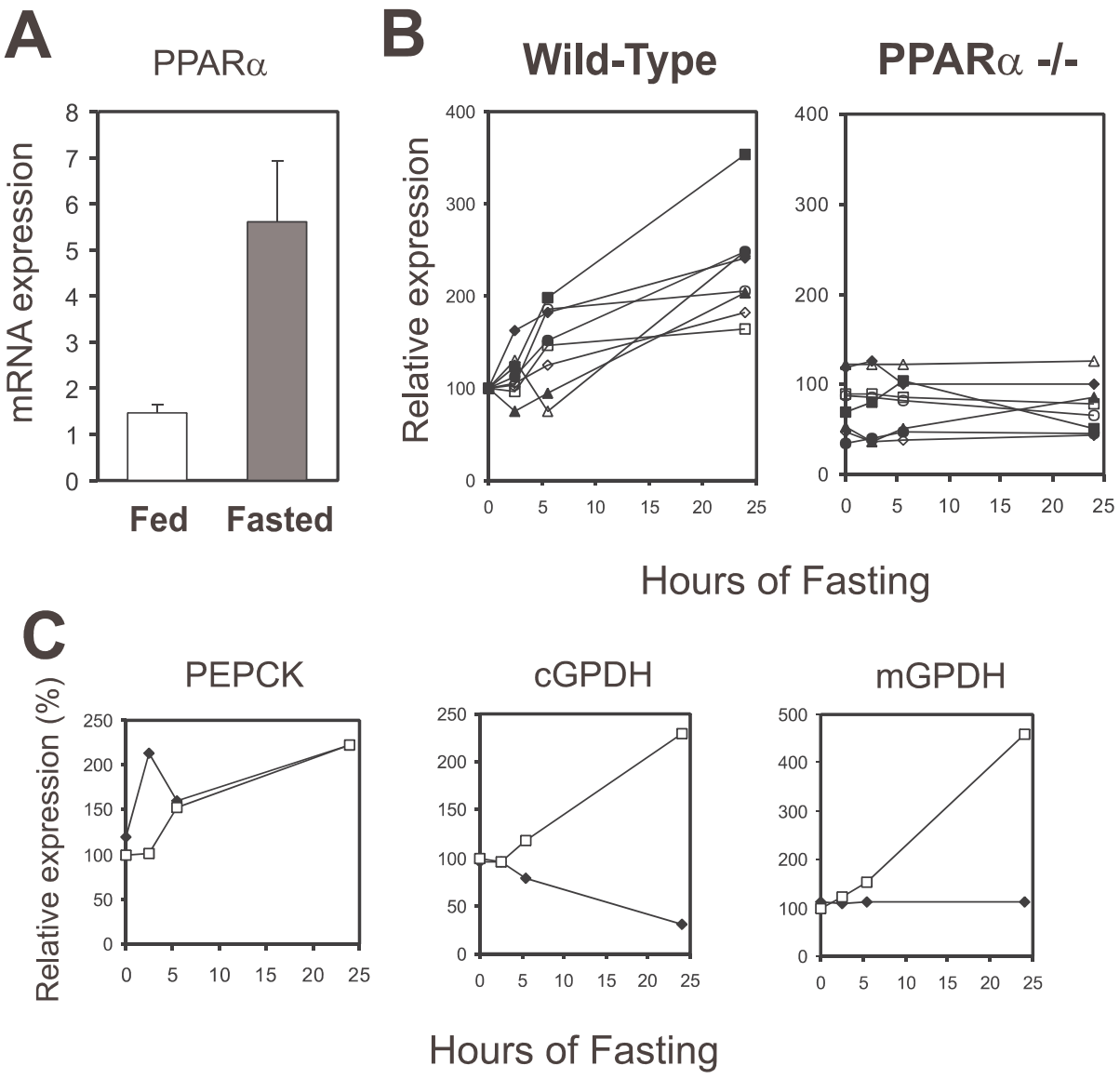

Figure 1 Oligonucleotide microarray analysis identifies novel putative PPARα target genes. (**A**) Relative expression of PPARα in liver was determined by Q-PCR in fed and 24-hour-fasted mice (n = 4). The difference was evaluated by Student's t test ($P < 0.01$). Error bars represent SEM. (**B**) Expression of genes involved in fatty acid oxidation and ketogenesis in livers of wild-type and *PPAR*α-null mice, as determined by oligonucleotide microarray (Affymetrix). The average difference (expression) of wild-type at 0 hours was arbitrarily set at 100. Filled diamonds: long-chain fatty acyl-CoA synthetase; open diamonds: carnitine palmitoyltransferase II; filled triangles: long-chain acyl-CoA dehydrogenase; open circles: short-chain acyl-CoA dehydrogenase; open triangles: medium-chain acyl-CoA dehydrogenase; filled circles: dodecenoyl-CoA / δ -isomerase; filled squares: HMG-CoA synthase; open squares: HMG-CoA lyase. (**C**) Hepatic expression of PEPCK (left), cGPDH (middle) and mGPDH (right) after 0, 2.5, 5.5 and 24 hours fasting in wild-type and *PPAR*α-null mice according to oligonucleotide microarray. The average difference (expression) of wild-type at 0 hours was arbitrarily set at 100.

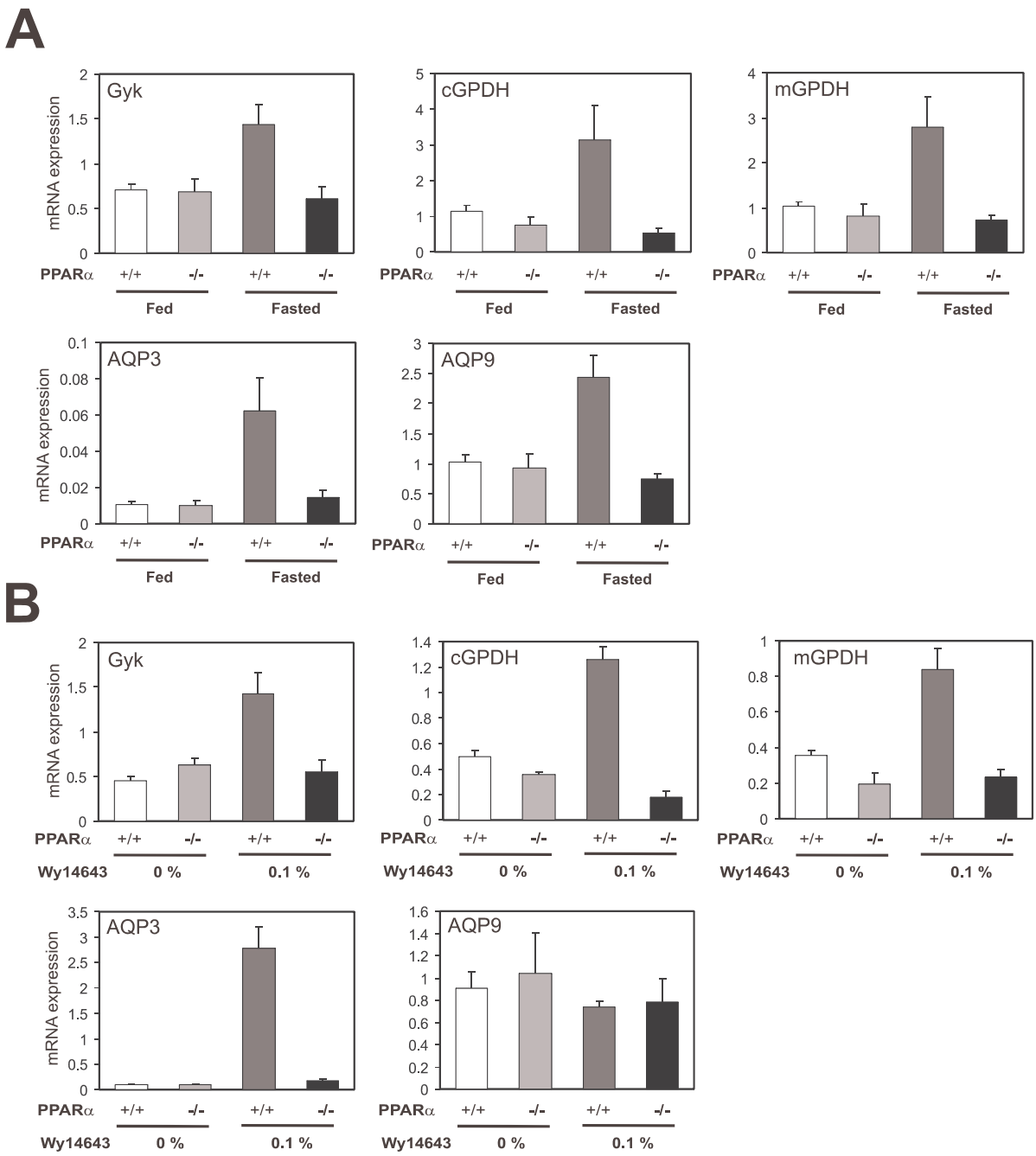

Figure 2 PPARα upregulates the expression of numerous genes involved in the conversion of glycerol to glucose. (**A**) Relative expression of glycerol kinase (Gyk), cGPDH, mGPDH, AQP3, and AQP9 were determined by Q-PCR in fed and 24-hour-fasted wild-type and *PPAR*α-null mice. Statistically significant effects were observed by two-way ANOVA for all genes for genotype ($P < 0.01$), and for the interaction between genotype and feeding status ($P < 0.05$). (**B**) Relative expression of Gyk, cGPDH, mGPDH, AQP3, and AQP9 were determined by Q-PCR in wild- type and *PPAR*α-null mice after feeding with Wy14643. Statistically significant effects were observed by two-way ANOVA for all genes for genotype and for Wy14643 treatment, and for the interaction between the two parameters ($P < 0.01$), except for AQP9. Error bars represent SEM.

PPARγ and PPARß/δ ligands induce cGPDH expression in adipocytes. The liver takes up glycerol to convert it into glucose, whereas adipose tissue takes up glucose and converts it into glycerol 3-phosphate, which becomes incorporated into triglycerides. In adipose tissue, the expression of PPARα is low, whereas PPARß/δ and PPARγ are well expressed. It is well established that the uptake of glucose into adipocytes and its conversion to triglycerides is stimulated by PPARγ (16, 17). To investigate regulation of glycerol metabolism in adipocytes by PPARγ and PPARß/δ, mature mouse (3T3-L1) and human (SGBS) adipocytes were incubated with PPARγ agonists ciglitazone or rosiglitazone or PPARß/δ agonist L165041. All ligands, in mouse and human adipocytes, significantly increased expression of cGPDH, glycerol kinase, and AQP7 (Figure 3, A and B). The known PPARγ target c-cbl–associated protein (CAP) was included as a positive control gene. cGPDH is highly expressed in adipocytes, where it functions in the synthesis of glycerol 3-phosphate from glucose (fed state) or gluconeogenic precursors (fasted state) (see Figure 7). It is often used as an adipogenesis marker. Glycerol kinase in adipocytes may catalyze recycling of glycerol, whereas AQP7 encodes a transporter that facilitates export of glycerol from the adipocytes. Supporting a role of PPARγ and PPARß/δ in regulating cGPDH expression in vivo, cGPDH mRNA was decreased in white adipose tissue (WAT) of *PPARγ*$^{+/-}$ and *PPARß/δ* $^{-/-}$ mice compared with wild-type mice (Figure 3C). Also, rosiglitazone, but not L165041, increased cGPDH mRNA in WAT of wild-type mice (Figure 3D). Taken together, these data suggest that, whereas PPARα induces glycerol utilization in liver, PPARγ and possibly PPARß/δ seem to be involved in the regulation of intracellular glycerol metabolism in adipose tissue.

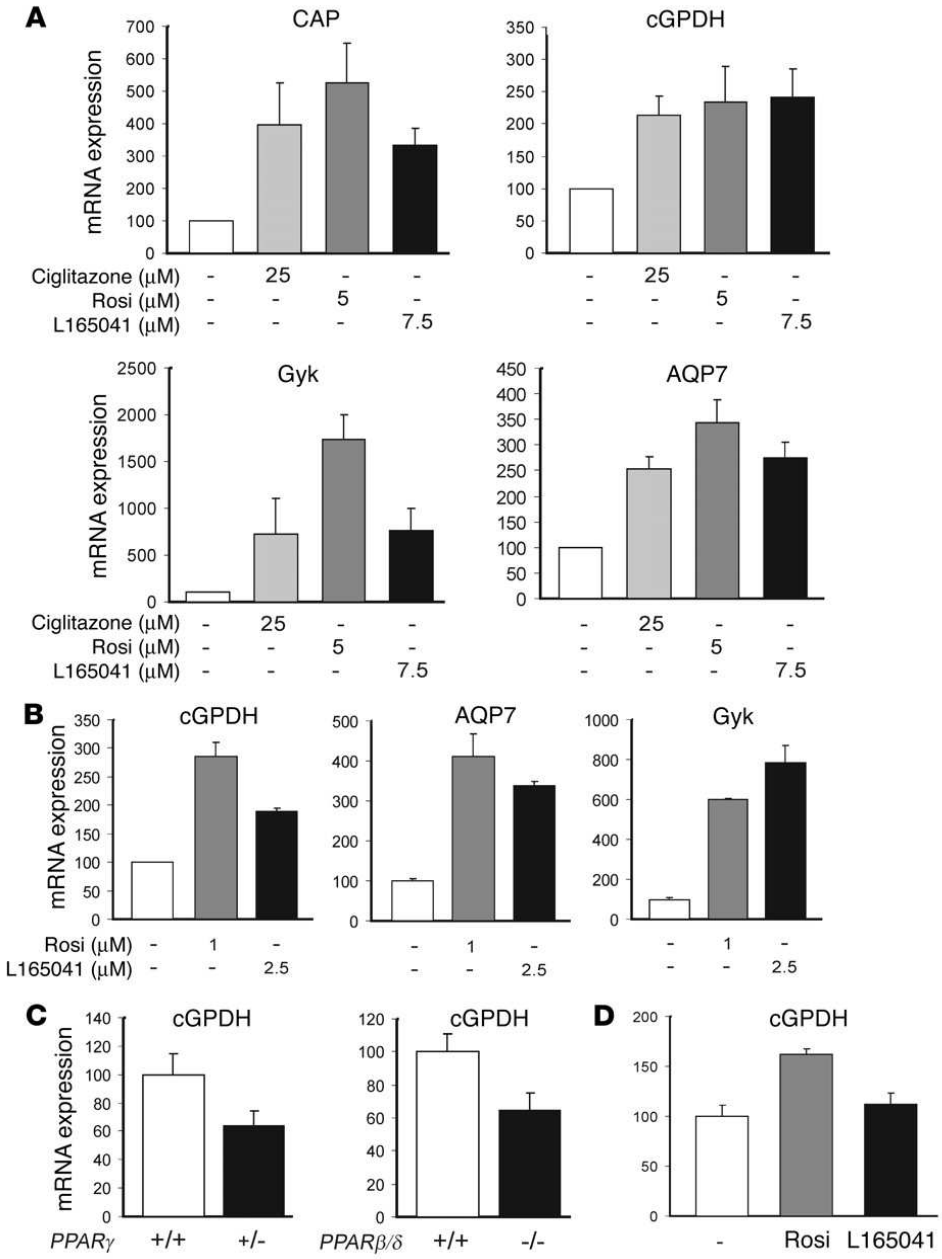

Figure 3 PPARγ and PPARß/ δ agonists induce cGPDH gene expression in adipocytes. (**A**) 3T3-L1 adipocytes at day 10 of differentiation were treated with the PPARγ agonists ciglitazone (25 μM) or rosiglitazone (Rosi) (5 μM), or the PPARß agonist L165041 (7.5 μM), and mRNA expression of the indicated genes was determined by Q-PCR. Results are expressed as percentage of control (DMSO). One-way ANOVA indicated that differences in expression were statistically significant for all four genes ($P < 0.05$). (**B**) Human SGBS adipocytes at day 13 of differentiation were treated with PPARγ agonist rosiglitazone (1 μM) or PPARß agonists L165041 (2.5 μM). Expression of the indicated genes was determined by Q-PCR. One-way ANOVA indicated that differences in expression were statistically significant for all three genes ($P < 0.05$). (**C**) Expression of cGPDH in WAT of *PPARγ*+/– and *PPARß*// δ –/– mice, as determined by Q-PCR. Differences were statistically significant (Student's *t* test, $P < 0.05$). (**D**) Expression of cGPDH in WAT of wild-type mice fed 0.01% rosiglitazone or 0.025% L165041, as determined by Q-PCR. The effect of rosiglitazone was statistically significant (Student's *t* test, $P < 0.01$). Error bars represent SEM.

cGPDH is a direct PPAR target gene. Our data so far suggest that cGPDH is a PPARα target gene in liver and a PPARγ (and possibly PPARß/δ) target gene in adipose tissue. To determine what genomic region is responsible for PPAR-induced upregulation of cGPDH expression, 2.2 kb of cGPDH promoter sequence immediately upstream of the transcription site was cloned in front of a luciferase reporter, and transactivation studies were carried out in NIH-3T3 cells. It was observed that cotransfections with a PPARα or PPARγ expression vector markedly increased luciferase activity, which was further enhanced by the addition of ligand (Figure 4, A and B). This response to PPARs and ligands was completely abolished in deletion constructs containing 0.5 or 0.25 kb of promoter sequence, suggesting that the PPAR responsive element was located in the region –2.2 to –0.5 kb. Screening of this genomic region yielded two putative PPAR response element (PPREs) about 1 kb upstream of the transcription start site, which differed little from the consensus PPRE (see Supplemental Figure 1).

To determine whether these PPREs are able to bind PPAR in vitro, we performed electrophoretic mobility shift assay. In the presence of only PPARα or retinoid X receptor α (RXRα), a single complex was observed, which originated from the reticulocyte lysate (Figure 4C, lanes 2 and 3). An additional, slower moving complex was observed only in the presence of both receptors (Figure 4C, lane 4), indicating that it represented a PPAR/RXR heterodimer. PPRE1 bound the heterodimer PPAR/RXR more efficiently than PPRE2 (Figure 4C, lane 4 vs. lane 12). Specificity of binding was demonstrated by competition with the nonradiolabeled PPRE of the malic enzyme promoter. In contrast, a response element for liver X receptor (LXR) was ineffective in competing for binding with the radiolabeled PPREs. These data demonstrate that the PPREs identified bind the PPAR/RXR heterodimer in vitro, further indicating that cGPDH is a direct PPAR target gene.

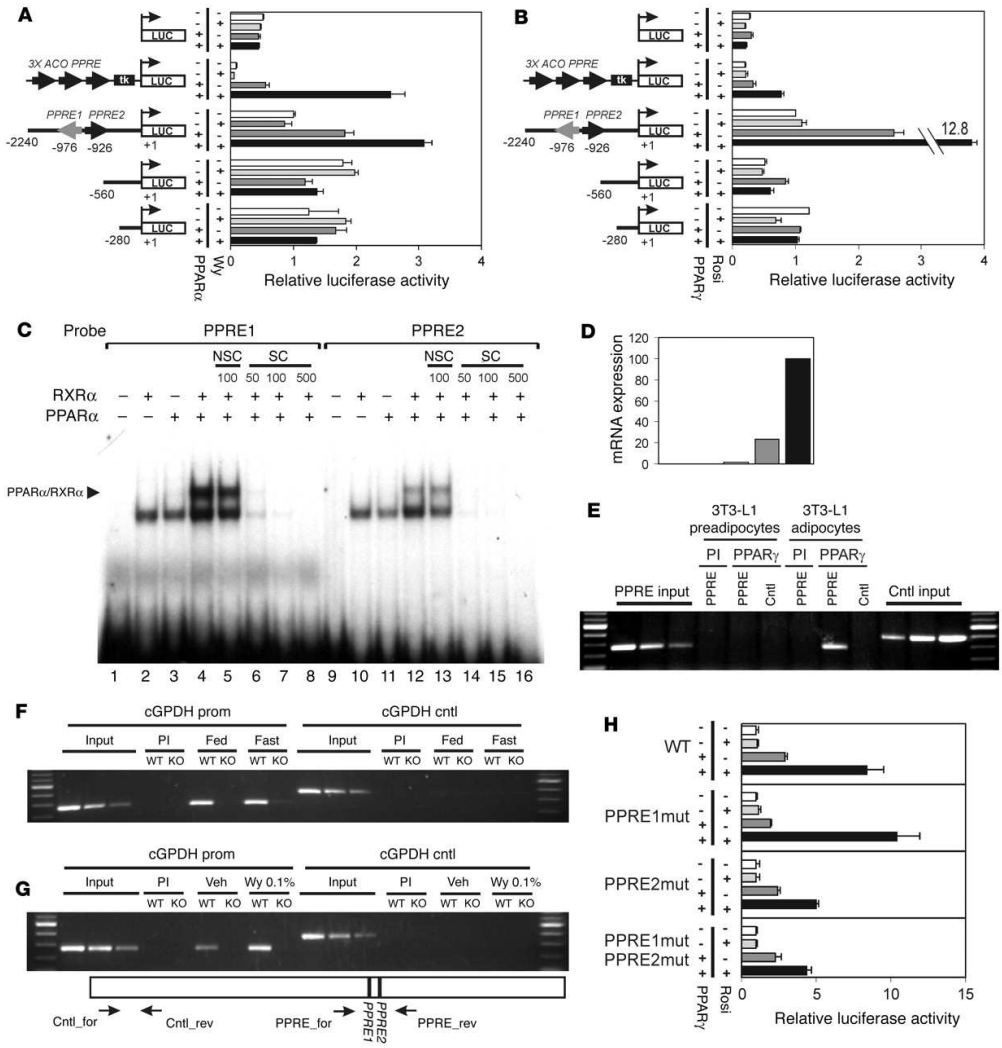

Figure 4 cGPDH is a direct PPARα/γ target gene. Mouse cGPDH reporter constructs containing 2240, 560, or 280 bp of immediate upstream promoter region were transfected into NIH-3T3 cells together with a PPARα (**A**) or PPARγ (**B**) expression vector. Normalized activity of the full-length cGPDH reporter in the absence of PPAR and ligands was set at 1. (**C**) Binding of the PPAR/RXR heterodimer to putative response elements, as determined by electrophoretic mobility shift assay. The double-stranded response elements cGPDH-PPRE1 (lanes 1–8). Fold-excess of specific (SC) or nonspecific (NSC) cold probe is indicated. (**D**) Expression of cGPDH during 3T3-L1 adipogenesis as determined by Q-PCR. Expression at day 8 was set at 100%. ChIP of PPRE within mouse cGPDH promoter using anti-mPPARγ or anti-mPPARα antibodies. Gene sequences spanning the putative PPREs (+1020 to +782) and a random control sequence (+2519 to +2124) were analyzed by PCR in the immunoprecipitated chromatin of 3T3-L1 preadipocytes and adipocytes (**E**), fed and fasted wild-type and *PPAR*α-null mice (**F**), and wild-type and *PPAR*α-null mice treated or not with Wy14643 (**G**). Preimmune serum was used as a control. (**H**) Transcriptional activity of site-directed mutants (mut) of the cGPDH promoter. Mouse cGPDH reporter constructs containing double nucleotide changes in PPRE1, PPRE2, or both, were transfected into HepG2 cells together with a PPARγ expression vector. Normalized activity of the reporter in the absence of PPAR and ligand was set at 1. Error bars in **A**, **B**, and **H** represent SEM. Cntl, random control sequence; PI, preimmune serum; prom, promoter; Veh, vehicle; Wy, Wy14643; for, forward primer; rev, reverse primer.

To find out whether PPARα and PPARγ are bound to these sequences in vivo, chromatin immunoprecipitation (ChIP) was performed using an anti-mPPARα or anti-mPPARγ antibody. Expression of cGPDH (and PPARγ) is highly upregulated during 3T3-L1 adipogenesis (Figure 4D). Using ChIP, we observed binding of PPARγ to a 238-bp sequence spanning the putative PPREs in differentiated 3T3-L1 adipocytes but not in preadipocytes (Figure 4E). There was no immunoprecipitation of the PPREs with preimmune serum, and no binding of PPARγ to a random control sequence was observed. In the fed and fasted mouse liver, PPARα was specifically bound to the PPRE sequence in wild-type but not *PPAR*α-null mice (Figure 4F). Treatment with Wy14643 enhanced binding of PPARα to the sequence, which was not observed in the *PPAR*α-null mice (Figure 4G). Because of the close proximity between the two PPREs, it was not possible to carry out ChIP for each putative PPRE separately. These data suggest that PPARα binds in vivo to the sequence containing the two PPREs.

Transactivation studies with cGPDH promoter constructs carrying mutations with the PPREs indicated that the most downstream PPRE (PPRE2) was particularly important for PPARγ-mediated promoter activation (Figure 4H). In contrast, mutating PPRE1 did not diminish promoter activation. These data suggest that PPRE2 but not PPRE1 is involved in mediating the effect of PPARγ on cGPDH promoter activity, although it cannot be ruled out that the double nucleotide changes introduced into PPRE1 failed to yield a dysfunctional PPRE. When the region encompassing PPRE1 and/or PPRE2 was placed in front of a heterologous promoter, significant PPARγ-dependent transactivation was observed for PPRE2, and for PPRE1 and PPRE2 together, but not PPRE1 alone (see Supplemental Figure 2). Together, these data suggest that PPRE2 at least partially mediates the PPARγ-dependent increase in cGPDH transcription.

PPARα activation decreases plasma glycerol levels in mice and humans. To examine whether induction of genes involved in the conversion of glycerol to glucose by PPARα has any functional consequences, we measured glycerol levels in plasma and urine. Inasmuch as fasting increases glycerol release from adipose tissue and at the same time stimulates hepatic glycerol utilization, it is difficult to interpret the effect of *PPAR*α deletion, which may affect both processes, on glycerol levels in fasted animals. We therefore focused on the effect of PPARα activation by Wy14643. First, it was established that the induction of GPDH, glycerol

kinase, and AQP3 gene expression by PPARα was translated at the enzyme activity or protein level (Figure 5, A–C). In line with the mRNA data indicating upregulation of glycerol utilization by PPARα, Wy14643 significantly decreased plasma glycerol concentration in wild-type but not *PPAR*α-null mice (Figure 5D). A similar pattern was observed in urine (Figure 5E). Furthermore, in human atherosclerotic patients, 4-week treatment with fenofibrate caused a mean decrease in plasma glycerol levels of 18% ($P < 0.01$; Figure 5F). Interestingly, a significant correlation was observed between the fenofibrate-induced decrease in plasma free fatty acids (likely mediated by a PPARα-induced increase in hepatic fatty acid utilization), and the decrease in plasma glycerol, suggesting a common mechanism (Figure 5G). These data provide compelling in vivo evidence that PPARα stimulates hepatic glycerol utilization.

*Hepatic glucose production is diminished in PPAR*α*-null mice.* Glycerol is one of the main precursors for hepatic glucose production, particularly during fasting. To find out whether the stimulatory effect of PPARα on hepatic glycerol utilization may translate into decreased hepatic glucose production in *PPAR*α-null mice, hyperinsulinemic clamp experiments were carried out. Both wild-type and *PPAR*α-null mice were fed Wy14643 for 12 days and fasted for 24 hours in order to maximize differences in gluconeogenic gene expression, and thus phenotype, between the two sets of mice. In the basal state (24-hour fast), plasma glucose was almost threefold lower and plasma free fatty acids almost threefold higher in *PPAR*α-null mice (Table 1). Supporting a stimulatory role for PPARα in gluconeogenesis during fasting, hepatic glucose production, which is equal to whole-body glucose utilization in the basal state, was markedly decreased in *PPAR*α-null mice compared with wild-type mice in both the basal and the hyperinsulinemic state (Figure 6A). These data suggest that the fasting-induced hypoglycemia in *PPAR*α-null mice is probably due to impaired gluconeogenesis. Alternatively, the hypoglycemia may be caused by increased whole-body glucose utilization in *PPAR*α-null mice. However, no evidence for this was found as glucose utilization was decreased in *PPAR*α-null mice compared with wild-type mice in the basal state and unchanged in the hyperinsulinemic state (Figure 6C). Overall, *PPAR*α-null mice appeared to be more sensitive to insulin as both the percentage stimulation of whole-body glucose utilization and the percentage inhibition of hepatic glucose output by insulin were augmented compared with wild-type mice (Figure 6, B and D).

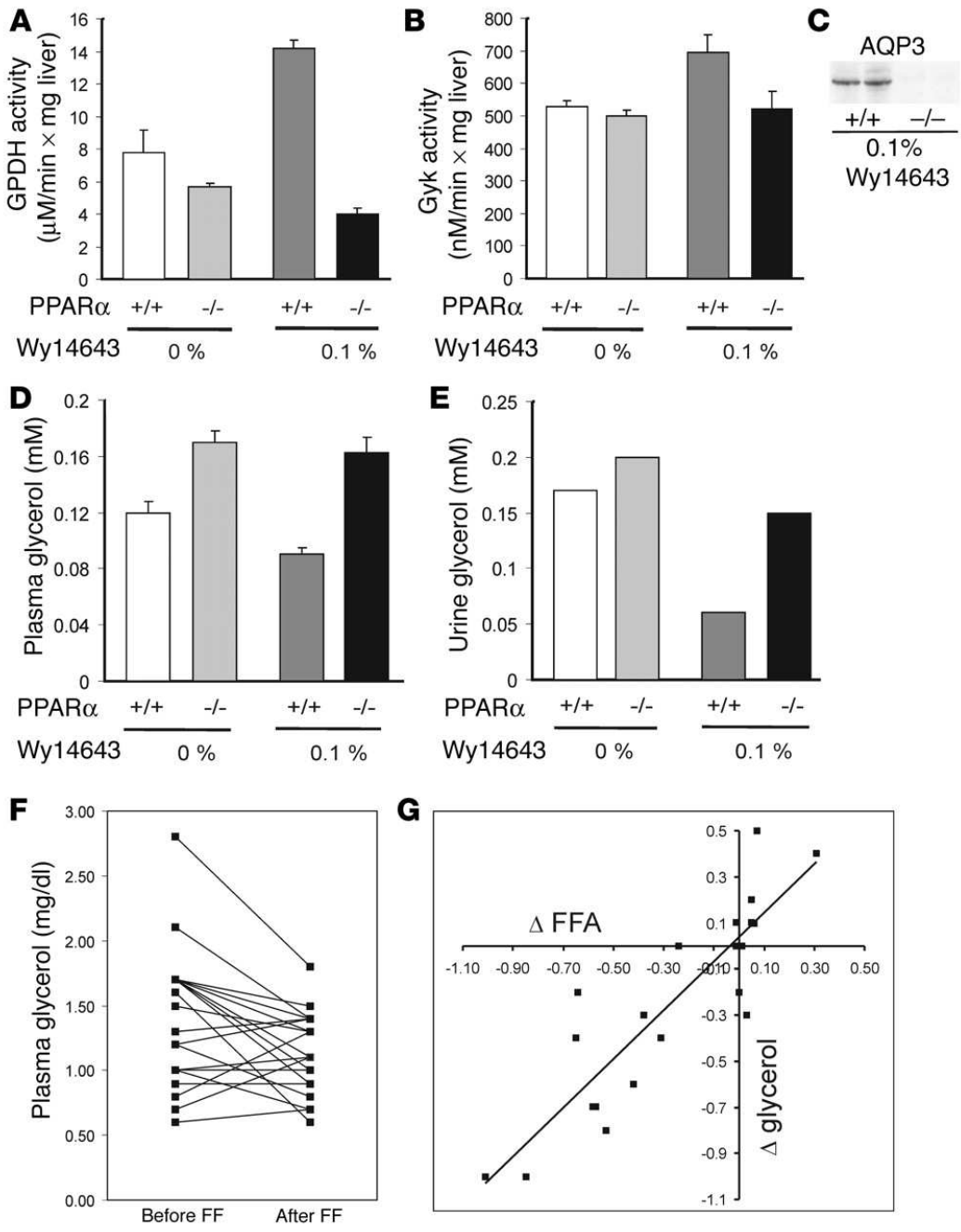

Figure 5 PPARα activation decreases plasma and urine glycerol levels. Enzyme activity of GPDH (**A**) or glycerol kinase (**B**) was determined in liver homogenates of wild-type and *PPAR*α-null mice after feeding with Wy14643 ($n = 4$ per group). Error bars represent SEM. (**C**) AQP3 protein was determined by Western blot in the membrane fraction of liver homogenates of wild-type and *PPAR*α-null mice treated with Wy14643. Equal amounts of protein were loaded. Glycerol was determined in plasma (**D**) ($n = 4$) and urine (**E**) (samples in each group were pooled and determined in duplicate) in wild-type and *PPAR*α-null mice after feeding with Wy14643. Significant effects were observed by two-way ANOVA for genotype and for Wy14643 treatment ($P < 0.05$). (**F**) Plasma glycerol levels decreased in atherosclerotic patients after 4-week treatment with micronized fenofibrate (FF) (250 mg/day). ($P < 0.01$, paired Student's *t* test) (**G**) Correlation between changes in plasma free fatty acids (FFA) and glycerol in atherosclerotic patients treated with fenofibrate.

Table 1

Plasma glucose and free fatty acid (FFA) concentration during hyperinsulinemic clamp (basal = 24-hour fast)

Genotype	Plasma glucose (mM)		Plasma FFA (mM)	
	Basal	Clamp	Basal	Clamp
PPARα$^{+/+}$	6.11 ± 0.43	7.75 ± 0.53	0.53 ± 0.05	0.34 ± 0.12
PPARα$^{-/-}$	2.13 ± 0.20[A]	8.38 ± 0.49	1.55 ± 0.10[A]	0.53 ± 0.12

Values are mean ± SEM. [A]Statistically significantly different from wild-type mice ($P < 0.0001$).

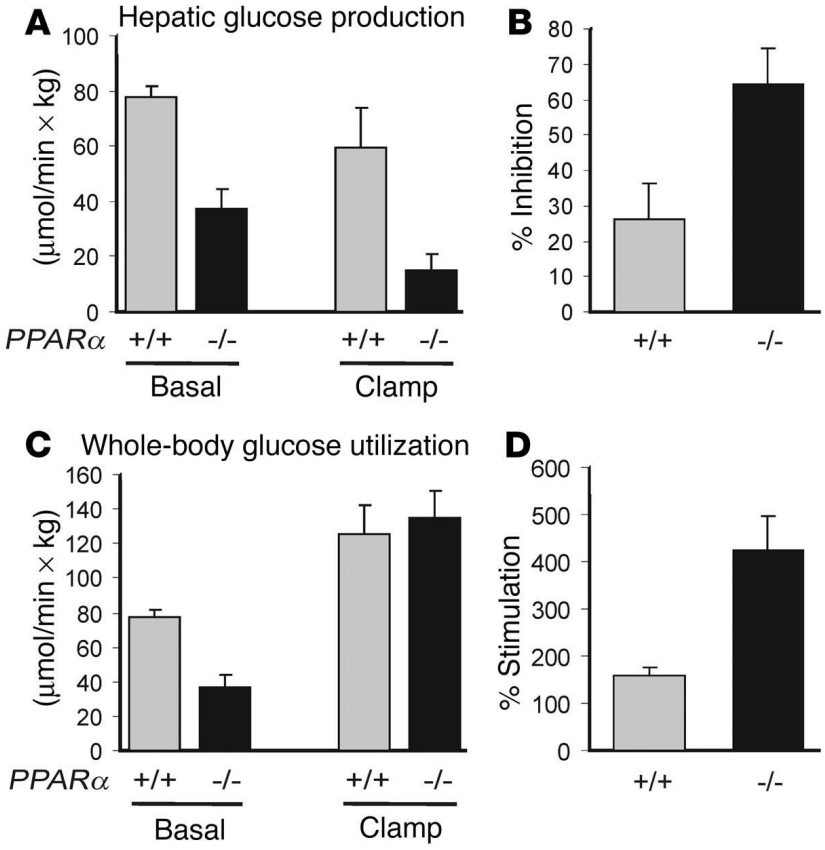

Figure 6 Decreased hepatic glucose production and increased insulin sensitivity in *PPARα*-null mice. Wild-type and *PPARα*-null mice administered Wy14643 and fasted were analyzed by hyperinsulinemic clamp technique. (**A**) Hepatic glucose production under basal and hyperinsulinemic conditions. (**B**) Percentage of inhibition of hepatic glucose production by insulin. (**C**) Whole-body glucose utilization under basal and hyperinsulinemic conditions. (**D**) Percentage of stimulation of whole-body glucose utilization by insulin. Differences between genotypes were statistically significant for all variables except glucose utilization under hyperinsulinemic conditions. $P < 0.05$, Mann-Whitney *U* test. Error bars represent SEM.

DISCUSSION

Although PPARα has mostly been connected with fatty acid catabolism, numerous lines of evidence indicate that it influences glucose homeostasis as well. First of all, fasting *PPAR*α-null mice display marked hypoglycemia (12-14). Furthermore, induction of insulin resistance in mice by high-fat feeding is mitigated in the absence of *PPAR*α (18, 19). Paradoxically, in a variety of diabetic animal models, activation of PPARα by synthetic agonists also improves glucose homeostasis (20), possibly by reducing endogenous glucose production (21, 22) and/or increasing glucose disposal (22-24). Recently, it was also observed that induction of the gluconeogenic genes PEPCK and glucose 6-phosphatase by dexamethasone is PPARα dependent (25). However, since PEPCK and glucose 6-phosphatase are not direct target genes of PPARα, the mechanism behind this regulation remains elusive. All together, it can be concluded that, although PPARα has an important influence on glucose metabolism, the mechanisms behind this regulation remain ill defined. Here, it is shown that PPARα decreases plasma glycerol levels in mice and humans by directly upregulating the expression of genes involved in hepatic gluconeogenesis from glycerol, including cGPDH, mGPDH, glycerol kinase, AQP3, and AQP9. The gluconeogenic gene cGPDH is identified as a direct target gene of PPARα with a functional PPAR response element in its promoter. The stimulatory effect of PPARα on gluconeogenic gene expression is associated with elevated hepatic glucose production during fasting. Our data support, extend, and provide a molecular explanation for the largely ignored observation that, in several rodent diabetic models, plasma glycerol levels are decreased by treatment with PPARα agonists (23, 24, 26, 27).

During prolonged fasting, when hepatic glycogen stores are depleted, plasma glucose levels are maintained exclusively by de novo glucose synthesis in liver (gluconeogenesis). The main precursors for hepatic gluconeogenesis are lactate, amino acids, and glycerol, which are converted into glucose via a series of reactions in the cytosol and mitochondria. The contribution of glycerol to hepatic glucose production greatly depends on the nutritional state and may vary from 5% postprandially in humans (28) to being the main gluconeogenic precursor in rodents after prolonged fasting (29). The significance of glycerol as a gluconeogenic precursor is supported by the episodic hypoglycemia observed in patients with isolated glycerol kinase deficiency (30) and by the phenotype of mice lacking both cGPDH and mGPDH (31), which suffer from elevated plasma glycerol concentrations and hypoglycemia before dying within the first week of life. Inasmuch as glycerol is an important

gluconeogenic precursor during fasting, and its conversion to glucose in liver is impaired in the absence of PPARα, defective synthesis of glucose from glycerol may explain the fasting-induced hypoglycemia in *PPAR*α-null mice (12-14). Indeed, it was observed that hepatic glucose production was impaired in fasted *PPAR*α-null mice, although the relative importance of defective conversion of glycerol to glucose is hard to estimate.

During feeding, in adipose tissue, PPARγ induces the expression of genes promoting the conversion of glucose to fatty acids, as well as the conversion of glucose to glycerol 3-phosphate (Figure 7). Glycerol 3-phosphate serves as the direct precursor for triglyceride synthesis. Moreover, PPARγ stimulates glycerol transport, glyceroneogenesis, and glycerol phosphorylation (16, 32). During fasting, lipolysis in adipose tissue releases glycerol and fatty acids into the blood, which are carried to the liver for further metabolism. PPARα plays a pivotal role in regulating the metabolism of fatty acids by stimulating hepatic fatty acid oxidation and ketogenesis (7). The present data show that the metabolic fate of glycerol is also under the control of PPARα, which stimulates its conversion to glucose in liver (Figure 7). Together with the previous finding that PPARα suppresses amino acid catabolism and ureagenesis (33), these combined data indicate that PPARα coordinates hepatic nutrient metabolism during fasting. Furthermore, by activating PPARα, fatty acids released from adipose tissue determine not only their own metabolic fate, but also that of other nutrients. Thus, PPARα serves as nutrient sensor that senses changes in feeding status and translates them into metabolic adjustments aimed at maintaining homeostasis. Under conditions of elevated plasma fatty acid concentrations, such as type 2 diabetes and obesity, it can be hypothesized that PPARα becomes permanently activated, resulting in enhanced conversion of glycerol into glucose. Although several aspects of this proposed mechanism remain to be demonstrated in humans, it provides an attractive molecular explanation for the observed link between elevated plasma free fatty acid levels and hepatic glucose production (4).

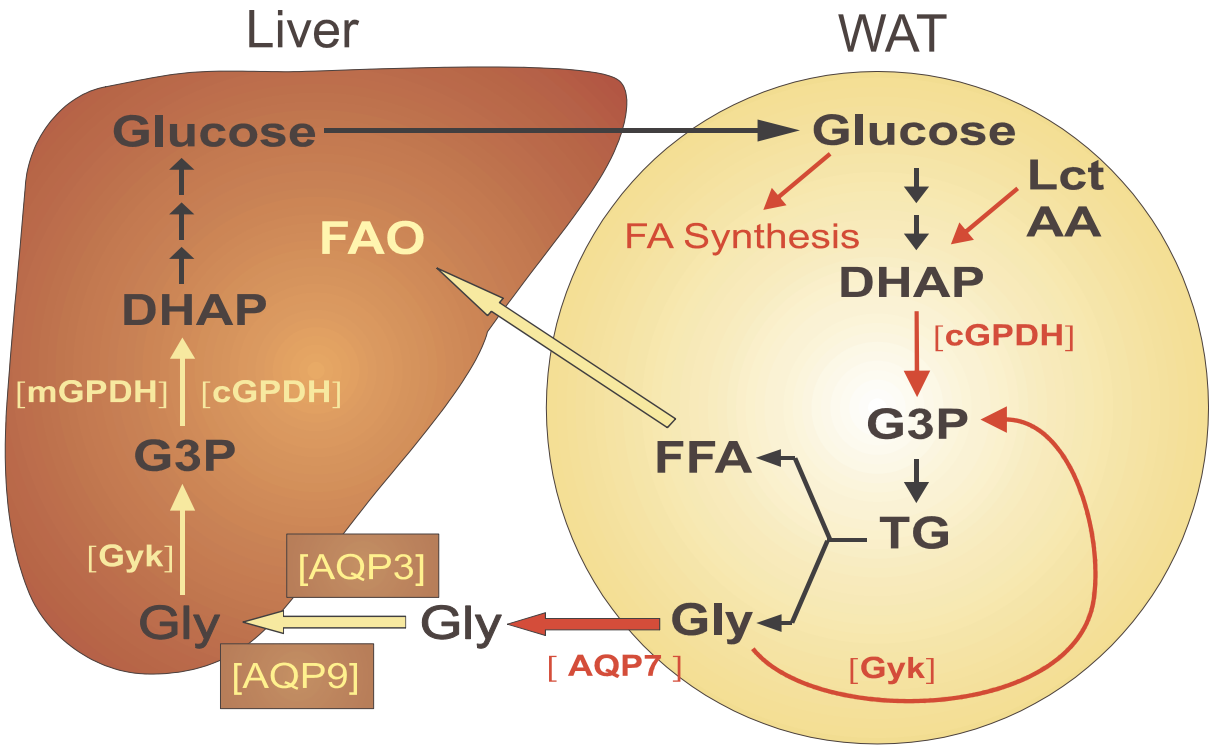

Figure 7 Proposed model integrating the roles of PPARα and PPARγ in glycerol (Gly) metabolism. Adipose tissue releases FFAs and glycerol. FFAs released by adipose tissue ligand-activate PPARα, whose hepatic expression is increased during fasting. Activation of PPARα induces expression of AQP3 and AQP9, which enable glycerol to enter the hepatocytes. Activation of PPARα also induces the expression of glycerol kinase, cGPDH, and mGPDH, which participate in the conversion of glycerol to glucose. In adipose tissue, PPARγ induces the expression of genes promoting the conversion of glucose to FFAs, as well as the conversion of glucose to glycerol 3-phosphate (G3P) from glucose. Glycerol 3-phosphate serves as the direct precursor for triglyceride (TG) synthesis. Moreover, PPARγ stimulates glycerol transport, glyceroneogenesis, and glycerol phosphorylation. Pathways regulated by PPARα are indicated in yellow, whereas those regulated by PPARγ are indicated in red. DHAP, dihydroxyacetonephosphate; Lct, lactate; FAO, fatty acid oxidation. Brackets indicate enzymes.

Our data support previous publications showing that insulin sensitivity is higher in *PPAR*α-null mice (18, 19), at least when the measurement is done under fasting conditions (34). While it is clear from the present study that, after fasting, the expression of gluconeogenic genes is lower in *PPAR*α-null mice, the molecular mechanisms explaining the heightened response to insulin by these mice remain elusive. At the same time, administration of PPARα agonists has also been shown to improve insulin sensitivity in various rodent models of obesity/diabetes (20-24). This situation is comparable to PPARγ, where partial deletion and ligand activation both reduce insulin resistance (35).

It is much harder to reconcile our data with those of Xu et al. (36), which show enhanced hepatic glucose production, as well as enhanced hepatic glucose production from glycerol, in fasted *PPAR*α-null mice compared with fasted wild-type mice. As the fasted *PPAR*α-null mice suffer from severe hypoglycemia, hepatic glucose production can only be enhanced if, at the same time, whole-body glucose utilization is hugely increased. However, the decreased whole-body glucose utilization after a 24-hour fast observed in the present study, combined with a lack of evidence that fatty acid oxidation is impaired in skeletal muscle (37), which would cause higher glucose utilization, indicates that this is unlikely to be the case. In contrast to Xu et al. (36), using a different method, we observed markedly decreased hepatic glucose production in fasted *PPAR*α-null mice. Possible explanations for these seemingly discrepant findings are differences in the background strain of the *PPAR*α-null mice (sv129 vs. C57/B6) and perhaps bias in the method of calculating glucose production by mass isotopomer distribution analysis (MIDA). According to a recent study that employed MIDA, it is possible that decreased hepatic glucose production in *PPAR*α-null mice is also partially due to preferential partitioning of glucose 6-phosphate toward glycogen rather than toward glucose (38).

Previous data have established that AQP7 and probably glycerol kinase are a direct PPARγ target genes in adipocytes (16, 39). Here we confirm upregulation of these genes by PPARγ (and probably PPARß/δ) and further demonstrate that the cGPDH gene is a direct target of PPARγ in adipocytes. Thus, whereas PPARα controls the hepatic utilization of glycerol, glycerol metabolism in adipocytes is under the control of PPARγ. Remarkably, cGPDH is upregulated by both PPARα and PPARγ, but since the role of cGPDH differs between liver and adipocytes, the effects of this regulation are very different.

PEPCK is often considered to catalyze the rate-limiting step in gluconeogenesis from pyruvate. Numerous transcription factors, including the glucocorticoid receptor, hepatic nuclear factor 3, and the retinoic acid receptor, regulate transcription of the PEPCK gene (40). Recent studies have shown that the PPARγ coactivator 1 (PGC1) stimulates the expression of PEPCK and that this effect is mediated by hepatocyte nuclear factor 4 (41). Since PGC1 is also a coactivator of PPARα, it is no surprise that it can enhance PPARα-mediated transactivation of the cGPDH promoter (unpublished data). In contrast, and in line with

previous observations (13), our analysis clearly indicates that the induction of expression of PEPCK during fasting is independent of PPARα.

Since the linkage between PPARα and glycerol metabolism was uncovered by microarray analysis, this study demonstrates the potential of genomics tools to elucidate novel pathways regulated by nuclear hormone receptors. However, to demonstrate a direct involvement of a nuclear hormone receptor in a particular pathway, the analysis should extend beyond merely descriptive data.

In conclusion, although an important role of PPARα in glucose metabolism has been demonstrated by numerous studies, the underlying mechanisms have remained elusive. Based on our study, it can be concluded that PPARα directly stimulates hepatic glycerol metabolism and, via this and other mechanisms, importantly influences hepatic glucose production during fasting. This effect of PPARα may account for the pronounced hypoglycemia in fasted *PPARα*-null mice.

METHODS

Oligonucleotide microarray. Total RNA was prepared from mouse livers using Trizol reagent (Invitrogen, Breda, The Netherlands). For the oligonucleotide microarray hybridization experiment, 10 µg of total liver RNA pooled from four mice was used for cRNA synthesis. Hybridization, washing and scanning of Affymetrix Genechip Mu6500 probe assays was according to standard Affymetrix protocols (Affymetrix, Santa Clara, California, USA). Fluorimetric data were processed by Affymetrix GeneChip3.1 software, and the gene chips were globally scaled to all the probe sets with an identical target intensity value.

Plasmid and DNA constructs. Based on sequences available in GenBank, a 2.3 kb fragment of the mouse cGPDH promoter was amplified by PCR from 3T3-L1 genomic DNA. Different-size fragments of the cGPDH promoter were cloned into the KpnI and BglII sites of pGL3 basic vector (Promega Corp., Leiden, The Netherlands). Site-directed mutations were introduced into the PPREs using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). The sequences of the primers used are provided in Supplemental Table 1. cDNA encoding for mPPARα, mPPARß, and rPPARγ2 were cloned into pSG5 (Stratagene). Nucleotide fragment surrounding the PPREs within the cGPDH promoter were amplified by PCR and subcloned into the KpnI and BglII sites of pTAL-SEAP

(BD Biosciences Clontech, Alphen aan den Rijn, The Netherlands). PPREtkLUC containing three copies of acyl-CoA oxidase PPRE was a generous gift from Ronald Evans (Salk Institute, La Jolla, California, USA).

Animal experiments. SV129 *PPAR*α-null mice and corresponding wild-type mice were purchased at the Jackson Laboratory (Bar Harbor, Maine, USA). For the fasting experiments, 5-month-old male mice were fasted for 0, 2.5, 5.5 or 24 hours starting at the onset of the light cycle. For the feeding experiments with Wy14643 (Chemsyn, Lenexa, Kansas, USA), 5-month-old female mice were fed 0.1% Wy14643 for 5 days by mixing it in their food. Blood was collected via orbital puncture. Livers were dissected and directly frozen in liquid nitrogen. For the clamp study, male 3-month-old wild-type (n = 4) and *PPAR*α-null mice (n = 5) were fed 0.1% Wy14643 for 12 days. Mice were fasted for 24 hours prior to the clamp studies. The hyperinsulinemic clamp and assays for blood glucose and plasma free fatty acids were carried out as previously described (42). The animal experiments were approved by the animal experimentation committee of the Etat de Vaud (Switzerland) or Wageningen University.

Cell culture and transfections. Mouse fibroblast NIH-3T3 cells or human hepatoma HepG2 cells were grown in DMEM containing 10% FCS. Cells were transfected with PPAR expression and luciferase reporter constructs using PolyFect (QIAGEN Inc., Leusden, The Netherlands) or calcium phosphate precipitation. After transfection, cells were incubated in the presence or absence of PPARs ligands (rosiglitazone 5 µM, Wy14643 10 µM) for 24–48 hours prior to lysis. Promega luciferase assay (Promega Corp.) and standard ß-galactosidase assay with 2-nitrophenyl-ß-D galactopyranoside were used to measure the relative activity of the promoter. 3T3-L1 fibroblasts were amplified in DMEM plus 10% calf serum and plated for final differentiation in DMEM plus 10% FCS. On day 0, which was 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). On day 3, the medium was changed to DMEM plus 10% FCS and insulin (5 µg/ml). On day 6, the medium was changed to DMEM plus 10% FCS, which was changed every 3 days. SGBS cell culture and induction of adipogenesis were performed exactly as previously published (43). 3T3-L1 adipocytes and SGBS adipocytes were incubated with synthetic PPAR agonists for 36–48 hours prior to RNA extraction.

Isolation of total RNA and Q-PCR. Total RNA was extracted from cells or tissue with Trizol reagent following the supplier's protocol. Total RNA 3–5 µg was treated with DNAse I

amplification grade and then reverse-transcribed with oligo-dT using Superscript II RT RNase H–. cDNA was PCR amplified with Platinum Taq DNA polymerase. (All these reagents were from Invitrogen.) Primer sequences used in the PCR reactions were chosen based on the sequences available in GenBank. Primers were designed to generate a PCR amplification product of 100–200 bp. Only primer pairs yielding unique amplification products without primer dimer formation were subsequently used for real-time PCR assays. PCR was carried out using Platinum Taq polymerase and SYBR green on an iCycler PCR machine (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). The sequence of primers used is available in Supplemental Table 1. The mRNA expression of all genes reported is normalized to ß-actin expression.

cGPDH enzymatic assay. cGPDH activity was assayed according to the spectrophotometric method of Wise and Green with some modifications (44). Livers were weighted, resuspended, and sheared in 20% homogenization buffer (w/v) (25 mM Tris-HC pH 7.5, 1 mM EDTA and 1 mM 2-mercaptoethanol). After brief sonication, cells were centrifuged at 4°C for 10 minutes at 16,000 *g*. The supernatant of cell lysate was used for determining the protein concentration by Bio-Rad Protein Assay reagent (Bio-Rad Laboratories BV) and for the enzymatic assay. The same amount of protein was incubated in standard reaction mixture (100 mM triethanolamine, 0.25 mM EDTA, 50 mM 2-mercaptoethanol, and 0.2 mM NADH). The reaction was initiated by the addition of dihydroxyacetone phosphate, and NADH disappearance was followed at 340 nm.

Glycerol kinase assay. Glycerol kinase activity was assayed according to the spectrophotometric method described by Leclercq et al. with some modifications (45). Whole livers were homogenized in 20% homogenization buffer (w/v) (10 mM Tris pH 7.5, 1 mM EDTA, 0.25 M sucrose plus Complete proteases inhibitor cocktail). Homogenates were then microcentrifuged at 14,000 rpm for 10 minutes at 4°C. Glycerol kinase activity in the supernatant was determined spectrophotometrically at 25°C.

Membrane fractionation and immunoblotting. Twenty per cent liver homogenates were centrifuged at 4,000 *g* for 15 min at 4°C, followed by centrifugation of the supernatant at 200,000 *g* for 1 hour. The pellet was resuspended in homogenization buffer and used for determining the protein concentration by Bio-Rad Protein Assay reagent (Bio-Rad Laboratories BV) or SDS/PAGE. Membrane fractions were resolved by SDS/PAGE on a 12% polyacrylamide gel. Western blotting was carried out as described by Kersten et al. (46). The

blot was incubated with rabbit anti-(rat)aquaporin 3 primary antibody (1:400; Chemicon Europe Ltd., Hofheim, Germany) for 16 hours at 4°C.

ChIP. Pure-bred wild-type or *PPAR*α-null mice on a Sv129 background were used. Mice were fed by gavage with either Wy14643 (50 mg/kg/day) or vehicle (0.5% carboxymethylcellulose) for 5 days. Alternatively, mice were fasted or not fasted for 24 hours. After the indicated treatment, mice were sacrificed by cervical dislocation. The liver was rapidly perfused with pre-warm (37°C) PBS for 5 minutes followed by 0.2% collagenase for 10 minutes. The liver was diced, forced through a stainless steel sieve, and the hepatocytes were collected directly into DMEM containing 1% formaldehyde. After incubation at 37°C for 15 minutes, the hepatocytes were pelleted, and ChIP was carried out using PPARα-specific antibodies as previously described (9). 3T3-L1 cells were differentiated as described above. After cell lysis and sonication, the supernatant was diluted 20-fold in re-ChIP dilution buffer (1 mM EDTA, 20 mM Tris-HCl, pH 8.1, 50 mM NaCl, and 1% Triton-X) prior to incubation with mouse PPARγ antibody. The remainder of the assay was carried out as described previously (9).

Electrophoretic mobility shift assay. Mouse PPARα and human RXRα proteins were generated from pSG5 expression vectors using a coupled in vitro transcription/translation system (Promega Corp.). The following oligonucleotides were used: GPDHPPRE1for (5'-AGGGAAGGAAGGTCAAAGGCCACTGGTGACAC-3'), GPDHPPRE1rev (5'-GTGTCACCAGTGGCCTTTGACCTTCCTTC-3'), GPDHPPRE2for (5'-GAGATTATCTGAG-GTGAAGGGGCAACCTGTGG-3') and GPDHPPRE2rev (5'-CCACAGGTTGCCCCTTCACCTCAGATAAT-3'). Oligonucleotides were annealed and labeled by Klenow filling (New England Biolabs (UK) Ltd., Leusden, The Netherlands) using Redivue [α-32P]dCTP (3000 Ci/mmol) (Amersham Biosciences Europe GmBH, Roosendaal, The Netherlands). Binding and electrophoresis was exactly performed as previously described (47), with the exception of unprogrammed lysate, where only 1/6 of the volume was used for binding.

Plasma and urine glycerol. Levels of glycerol in urine and plasma of mice were determined using the triglyceride assay from Beckman Coulter Nederland B.V. (Mijdrecht, The Netherlands) by omitting the first step (digestion with lipase). Measurements were carried out on a Synchron LX20 analyzer (Beckman Coulter Nederland B.V.). For measurement of

glycerol in human plasma, blood was taken after an overnight fast from 21 male subjects before and after a 4-week treatment with 250 mg of micronized fenofibrate daily. All subjects had significant coronary artery disease as documented by angiography.

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FOOTNOTES

Nonstandard abbreviations used: aquaporin (AQP); chromatin immuno-precipitation (ChIP); cytosolic GPDH (cGPDH); glycerol 3-phoshate dehydrogenase (GPDH); mass isotopomer distribution analysis (MIDA); mitochondrial GPDH (mGPDH); phosphoenolpyruvate carboxykinase (PEPCK); PPARγcoactivator 1 (PGC1); PPAR response element (PPRE); real-time quantitative PCR (Q-PCR); retinoid X receptor α (RXRα); white adipose tissue (WAT).

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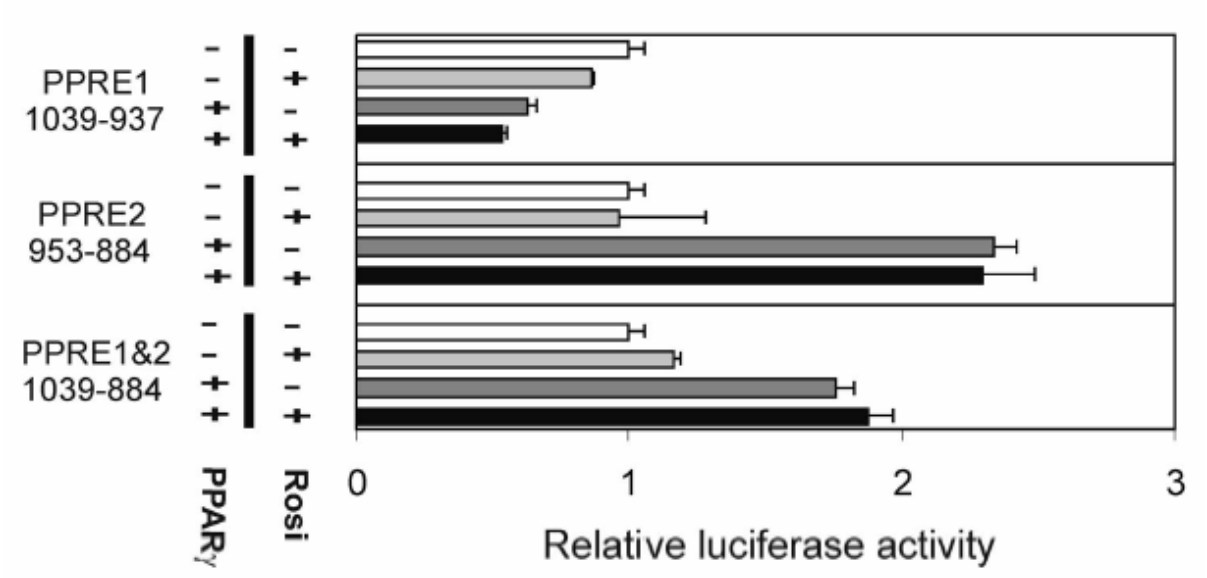
SUPPLEMENTAL DATA

	DR+1
Consensus	AGGTCAAAGGTCA
L-FABP	AGGCCATAGGTCA
Acyl-CoA oxidase	AGGACAAAGGTCA
HMG-CoA symthase	GGGCCAAAGGTCT
mouse cGPDH PPRE1 (-976/ 5'UTR)	aGgTCAAAGGCCA
mouse cGPDH PPRE2 (-926/ 5'UTR)	AGgTGaAGGGGCA
human Gyk PPRE (-969/ ATG)	AGGTCAAGGGAGA
mouse Gyk PPRE (-945/ ATG)	AGGTCAAATGAGA
human mGPDH PPRE (-973/ 5'UTR exon1A)	AGTTCTCAGGTGA
mouse mGPDH PPRE (-562/ 5'UTR exon1A)	AGTTCTCAGGTGA
human AQP9 PPRE (-1552/ ATG)	TGGTCATAGGTAA
mouse AQP9 PPRE (-1475/ ATG)	TGGTCATAGGAAA
human AQP3 PPRE1 (-672/ ATG)	AGGGAGAAGGGCC
human AQP3 PPRE2 (-1637/ ATG)	AGCATAGAGGGCA
mouse AQP3 PPRE (-673/ ATG)	AGGTCACAGGACA

Supplementary figure 1 In silico identification of potential PPREs within promoter of genes regulated by PPARα and PPARγ. Sequences corresponding to the human and mouse promoters were accessed via the Ensembl Genome Browser (http://www.ensembl.org/). Identification of the putatives PPREs was performed using NUBISCAN algorythm (http://www.nubiscan.unibas.ch/). With the exception of cytosolic GPDH for which the trancription initiation start has been extensively described and in order to prevent any ambiguity, the PPREs are indicated in relation to the translation start site (ATG). The mouse and human mitochondrial GPDH gene contain a conserved PPRE in the promoter A. Given that this promoter is located 29 Kb upstream of the ATG, the position of the PPREs are indicated in relation to exon1A. Nucleotides within PPRE1 and PPRE2 of cGPDH promoter that were mutated in Figure 4h are in lower case.\



Supplementary figure 2 The region encompassing PPRE2 but not PPRE1 of the cGPDH promoter is able to activate a heterologous promoter. Nucleotide fragments surrounding the PPREs within the cGPDH promoter were subcloned into pTAL-SEAP and transfected into HepG2 cells together with a PPARγ expression vector. Normalized activity of the reporter in the absence of PPAR and ligand was set at 1. Error bars are SEM.

SUPPLEMENTARY TABLE 1

Primers Q-PCR

Mouse/ Rat β–Actin :
Forward : 5'- CTGACTGACTACCTCATGAAGATCCT - 3'
Reverse : 5'- CTTAATGTCACGCACGATTTCC - 3'
Human β–Actin :
Forward : 5'- CTTCCTGGGCATGGAGTC - 3'
Reverse : 5'- GCCAGGGTACATGGTGGT - 3'
Mouse AQP3 :
Forward : 5'- GTGGCTCAGGTGGTGCTCAG - 3'
Reverse : 5'- CACATTGCGAAGGTCACAGCG - 3'
Mouse AQP7 :
Forward : 5'- GCATCCTTGTTACCGTCCTTGG - 3'
Reverse : 5'- GCACCCACCACCAGTTGTTTC - 3'
Human AQP7 :
Forward : 5'- ACA TTG TGG CGG GGC TTC C -3'
Reverse : 5'- TTC CTG GCA GTG CTG GGT TG -3'
Mouse AQP9 :
Forward : 5'- GAAGGACCGAGCCAAGAAGAAC - 3'
Reverse : 5'- AGCAATAGAGCCACATCCAAGG -3'
Mouse c-Cbl Associated Protein :
Forward : 5'- CAAGTCGCAGTGCCACTGTG - 3'
Reverse : 5'- AGTTCCAACTCATCATCGTTCTGT - 3'

Mouse cGPDH :
Forward : 5'- GCCTTCGCCAAGCTCTTCTG - 3'
Reverse : 5'- TAGCAGGTCGTGATGAGGTCTG - 3'
Human cGPDH :
Forward : 5'- ACC AAG GCG GCA GTG ATC C- 3'
Reverse : 5'- ACA CCA CAG CTC TCC AAG AAG G- 3'
Mouse mGPDH :
Forward : 5'- CTCGCCATCGCCCTCACTG - 3'
Reverse : 5'- ACCGCTCACTCGCTCTTTGC - 3'
Mouse Gyk :
Forward : 5'- ATCCGCTGGCTAAGAGACAACC - 3'
Reverse : 5'- TGCACTGGGCTCCCAATAAGG - 3'
Human Gyk :
Forward : 5'- AGCCCTCAATGCCCGAAACC- 3'
Reverse : 5'- GTTCAAACCGCTCCATCGTGAC- 3'
Mouse PPARα :
Forward 5'- ATTCGGCTGAAGCTGGTGTAC - 3'
Reverse 5'- CTGGCATTTGTTCCGGTTCT -3'

Primers ChIP

Control primers :
Forward 5' – GCA ATG CCT GCA GTT CTA CC -3'
Reverse 5' – ACA GAT GCC AGC TCA GTC AC -3'
PPRE primers :
Forward 5' – TTC CTG AAG CCT GGA AGG AG –3'
Reverse 5' – GCC AGC CTT GGT CTA CAG AG – 3'

Primers mutagenesis cGPDH promoter

PPRE1BclI :
Forward 5' - GTC ACC AGT GGC CTT TGA TCA TCC TTC CCT TTA GAG ACC -3'
Reverse 5' - GGT CTC TAA AGG GAA GGA TGA TCA AAG GCC ACT GGT GAC -3'
PPRE2PstI :
Forward 5' - GGA GAT TAT CTG AGC TGC AGG GGC AAC CTG TG -3'
Reverse 5' - CAC AGG TTG CCC CTG CAG CTC AGA TAA TCT CC -3'

Chapter 4

PPARα mediates the effects of high fat diet on hepatic gene expression

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ABSTRACT

Peroxisome proliferators activated receptors are transcription factors involved in the regulation of numerous metabolic processes. The PPARα isotype is abundant in liver and activated by fasting. However, it is not very clear what other nutritional conditions activate PPARα. To examine whether PPARα mediates the effects of chronic high fat feeding, wild-type and PPARα null mice were fed a low fat diet (LFD) or high fat diet (HFD) for 26 weeks. HFD and PPARα deletion independently increased liver triglycerides. Furthermore, in wild-type mice HFD was associated with a significant increase in hepatic PPARα mRNA and plasma free fatty acids, leading to a PPARα-dependent increase in expression of PPARα marker genes CYP4A10 and CYP4A14. Micro-array analysis revealed that HFD increased hepatic expression of characteristic PPARα target genes involved in fatty acid oxidation in a PPARα-dependent manner, although to a lesser extent than fasting or Wy14643. According to micro-array, there may be functional compensation for PPARα in PPARα null mice. Remarkably, in PPARα null mice on HFD, PPARγ mRNA was 20-fold elevated compared to wild-type mice fed a LFD, reaching expression levels of PPARα in normal mice. Adenoviral over-expression of PPARγ in liver indicated that PPARγ can up-regulate genes involved in lipo/adipogenesis but also characteristic PPARα targets involved in fatty acid oxidation. It is concluded that 1) PPARα and PPARα-signalling are activated in liver by chronic high fat feeding 2) PPARγ may compensate for PPARα in PPARα null mice on HFD.

INTRODUCTION

Diabetes mellitus type 2 has become a major health concern worldwide. Overt type 2 diabetes is most often preceded by a state of insulin resistance, which describes an impaired response to insulin, either in liver or peripheral tissues. Insulin resistance is almost invariably linked to obesity, and is often part of a combination of metabolic abnormalities united in the term metabolic syndrome, which also include dyslipidemia, hypertension, and a pro-inflammatory and pro-thrombotic state.

An important group of molecular targets for the treatment of insulin resistance are the Peroxisome Proliferator Activated Receptors. PPARs are ligand-activated transcription factors that activate the transcription of genes involved in many different processes, including lipid and glucose metabolism, inflammation, and wound healing. Three different PPAR isotypes are known to date: α, β/δ, and γ. In analogy with many other nuclear hormone receptors,

PPARs form heterodimers with the retinoid X receptor RXR and stimulate gene expression by binding to specific elements located in the promoter of target genes. All three PPARs bind and are activated by fatty acids, especially poly-unsaturated fatty acids, as well as by various eicosanoids (1) (2).

Most of the research on PPARs has concentrated on PPARγ since it binds and is activated by an important class of insulin-sensitizing drugs called thiazolidinediones, which include rosiglitazone and pioglitazone. Activation of PPARγ results in stimulation of peripheral glucose disposal and improves insulin sensitivity, possibly by lowering plasma free fatty acid levels and affecting plasma concentrations of adipocytokines (3, 4). PPARγ is mainly present in adipose tissue where it stimulates adipo- and lipogenesis by upregulating target genes such as FAT/CD36, aP2/FABP4, and lipoprotein lipase. Gain and loss of function experiments have demonstrated that PPARγ is absolutely required for adipocyte differentiation (5-7). In liver PPARγ is only very weakly expressed and does not appear to be influenced by feeding/fasting (8). Instead, hepatic PPARγ is up-regulated in animal models of leptin deficiency and lipoatrophy, concurrent with development of hepatic steatosis (9-11).

Whereas PPARγ promotes the storage of lipids, the PPARα isotype stimulates lipid catabolism. It is highly expressed in liver where it up-regulates numerous genes involved in fatty acid uptake and activation, mitochondrial β-oxidation, peroxisomal fatty acid oxidation (rodents only), ketone body synthesis, fatty acid elongation and desaturation, and apolipoprotein synthesis. In addition, it plays an important role in the hepatic acute phase response. PPARα is the molecular target for the hypolipidemic fibrate drugs, which are used for the treatment of (diabetic) dyslipidemia (12). Apart from lipid catabolism, there is increasing experimental support for an important connection between PPARα and glucose homeostasis. Indeed, mice lacking PPARα display pronounced fasting hypoglycemia, which can be attributed to increased insulin-mediated stimulation of whole body glucose utilization and inhibition of hepatic glucose output (13-15). Lowered hepatic glucose output is probably caused by a combination of impaired energization of gluconeogenesis due to defective fatty acid oxidation, impaired conversion of glycerol to glucose, and decreased glycogen stores (16). It has been reported that the effect of PPARα on hepatic insulin resistance may implicate the mammalian tribbles homolog TRB-3, which is a negative regulator of intracellular insulin signaling (17).

Under physiological conditions, the function of PPARα is mainly evoked during fasting, which is associated with increased hepatic PPARα mRNA expression and increased

plasma free fatty acid levels. Indeed, whereas in the fed state deletion of PPARα has few consequences, in the fasted state it induces a severe phenotype characterized by hypoglycaemia, hypoketonemia, hypothermia and a fatty liver (16) (18). Another physiological stimulus that may trigger PPARα function is obesity/insulin resistance, which can be modelled in mice by chronically feeding a high fat diet. High fat feeding augments fat mass, is associated with attenuated insulin signalling, and results in increased plasma free fatty acid levels and possibly increased hepatic PPARα expression levels. To determine if PPARα indeed mediates the effects of chronic high fat feeding, wild-type and PPARα null mice fed a high fat diet for several months were studied. The data show that 1) PPARα and PPARα-signalling are activated in liver by chronic high fat feeding 2) PPARγ may compensate for PPARα in PPARα null mice on HFD.

MATERIALS AND METHODS

Chemicals

Wy14643 was obtained from ChemSyn Laboratories. Recombinant human insulin (Actrapid) was from Novo Nordisk. SYBR Green was from Eurogentec. Dulbecco's modified Eagle's medium, fetal calf serum, calf serum, and penicillin/streptomycin/fungizone were from BioWhittaker Europe (Cambrex Bioscience). Otherwise, chemicals were from Sigma.

Animal experiments

SV129 PPARα null mice and corresponding wild-type mice were purchased at the Jackson Laboratory (Bar Harbor, Maine, USA). For the fasting experiments, 5-month-old male mice were fasted for 0 or 24 hours starting at the onset of the light cycle. For the feeding experiments with Wy14643 (Chemsyn, Lenexa, Kansas, USA), 5-month-old male mice were fed 0.1% Wy14643 for 5 days by mixing it in their food. For the diet intervention, 2-month-old male mice were fed with a low or high fat diet for 26 weeks. The respective diets provided either 10 or 45% energy percent in the form of lard fat (D12450B or D12451, Research Diets, New Brunswick, USA). Body weight and food intake were measured at regular intervals throughout the feeding intervention. An additional dietary intervention was performed with C57/B6 mice (Harlan, Zeist, the Netherlands), which were fed a low or high fat diet providing either 10 or 45 percent of fat from palm oil. At week 2, 4 and 16 of the intervention for the C57B6 mice or at the end of the dietary intervention for the SV129 mice, tissues were dissected, weighted and directly frozen in liquid nitrogen. Blood was collected via orbital

puncture. The animal experiments were approved by the animal experimentation committee of Wageningen University.

Plasma and tissue metabolites

Plasma was obtained from blood by centrifugation for 10 minutes at 10000 g. Plasma free fatty acids were determined using a kit from WAKO Chemicals (Sopachem, Wageningen, the Netherlands). Tissue triglycerides level was determined using a kit from Instruchemie (Delfzijl, the Netherlands).

Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was performed after 24 weeks on the experimental diets. After a 6 hour fast mice were injected intraperitoneally with glucose (2 g/kg bodyweight). Blood was collected by tail bleeding after 0, 20, 40, 60, 90 and 150 minutes and glucose measured using Accucheck compact (Roche Diagnostics, Almere, the Netherlands). The areas under the curves (AUCs) were determined with GraphPad Prism 4 software.

Cell culture

Rat hepatoma FAO cells were grown in Dulbecco's modified Eagle's medium containing 10 % (v/v) Fetal Calf Serum. Serum was depleted to 0.5% 12 hours prior to incubation with insulin. Cells were incubated with insulin at 0, 10 or 100 nM for 24 hours followed by RNA isolation.

Rat hepatocytes were isolated by two-step collagenase perfusion as described previously (19). Hepatocytes were suspended in William's E medium (Cambrex, Seraing, Belgium) supplemented with 10% fetal calf serum, 20 mU/mL insulin, 50 nM dexamethasone, penicillin-streptomycin, and 50 μg/mL gentamycin. After 4 hours medium was replaced by the same medium without insulin. The next day, cells were incubated in the presence or absence of insulin for 10 hours.

Adenoviral gene transfer

PPARα null mice were intravenously injected (tail vein) with virus particles of Ad/LacZ or Ad/mPPARγ1 and killed 6 days later as described (9) (20).

Isolation of total RNA and Q-PCR

Total RNA was extracted from cells or tissue with Trizol reagent following the supplier's protocol. Total RNA 3–5 μg was treated with DNAse I amplification grade and then reverse-transcribed with oligo-dT using Superscript II RT RNase H^-. cDNA was PCR amplified with Platinum Taq DNA polymerase (all from Invitrogen, Breda, the Netherlands) Primer sequences used in the PCR reactions were chosen based on the sequences available in GenBank. Primers were designed to generate a PCR amplification product of 100–200 bp (13) (21). Only primer pairs yielding unique amplification products without primer dimer formation were subsequently used for Q-PCR assays. PCR was carried out using Platinum Taq polymerase (Invitrogen) and SYBR green on an iCycler PCR machine (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). The mRNA expression of all genes reported is normalized to the ribosomal 36B4 gene expression.

Micro-array

RNA was prepared from liver of 4 mice per group using Trizol and subsequently pooled per group. Pooled RNA was further purified using Qiagen RNeasy columns and the quality verified by lab on a chip analysis (Bioanalyzer 2100, Agilent). 10 μg of RNA was used for one cycle cRNA synthesis (Affymetrix, Santa Clara, USA). Hybridization, washing and scanning of Affymetrix Genechip mouse genome 430A 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).

RESULTS

Male wild-type and PPARα null mice at 2-3 months of age were fed a low fat diet (10% fat, LFD) or high fat diet (45% fat, HFD) for 26 weeks. Energy intake throughout this period was identical in the four groups (Fig. 1A). Feeding a HDF caused significant weight gain in the wild-type mice, whereas the effect was much less evident in the PPARα null mice (Fig. 1B). Gonadal fat weight was increased by HFD in both wild-type and PPARα null mice, although somewhat less pronounced in the latter group, who already had higher levels on the LFD (Fig. 1C). Liver weight was higher in the PPARα null mice, which was further increased by HFD (Fig. 1D). This was partially due to elevated hepatic triglyceride levels, which were

increased by both PPARα deletion and HFD (Fig. 1E). In PPARα null fed a HFD, almost 15% of liver weight consisted of triglycerides, indicating a severe fatty liver.

During fasting both hepatic PPARα expression and plasma free fatty acid levels are increased, leading to activation of PPARα signalling. A similar situation may exist during HFD. Indeed, plasma free fatty acids, which serve as ligands for PPARα, were significantly increased (26%, $p<0.05$) in mice fed the HFD (Fig. 2A). Furthermore, hepatic PPARα mRNA levels were modestly but significantly increased by HFD, as determined by micro-array and quantitative PCR (Q-PCR) (Fig. 2B). A similar stimulatory effect of HFD on hepatic PPARα expression was observed in mice on a C57/B6 background (Fig. 2C). To examine whether PPARα up-regulation by HFD may be connected with insulin we measured the effect of insulin on PPARα expression in isolated rat hepatocytes and FAO rat hepatoma cells. In both cell-types PPARα expression was markedly decreased by insulin treatment (Fig. 2D,E). Thus, upregulation of hepatic PPARα by HFD may be attributed to a diminished response to insulin, although other mechanisms cannot be excluded.

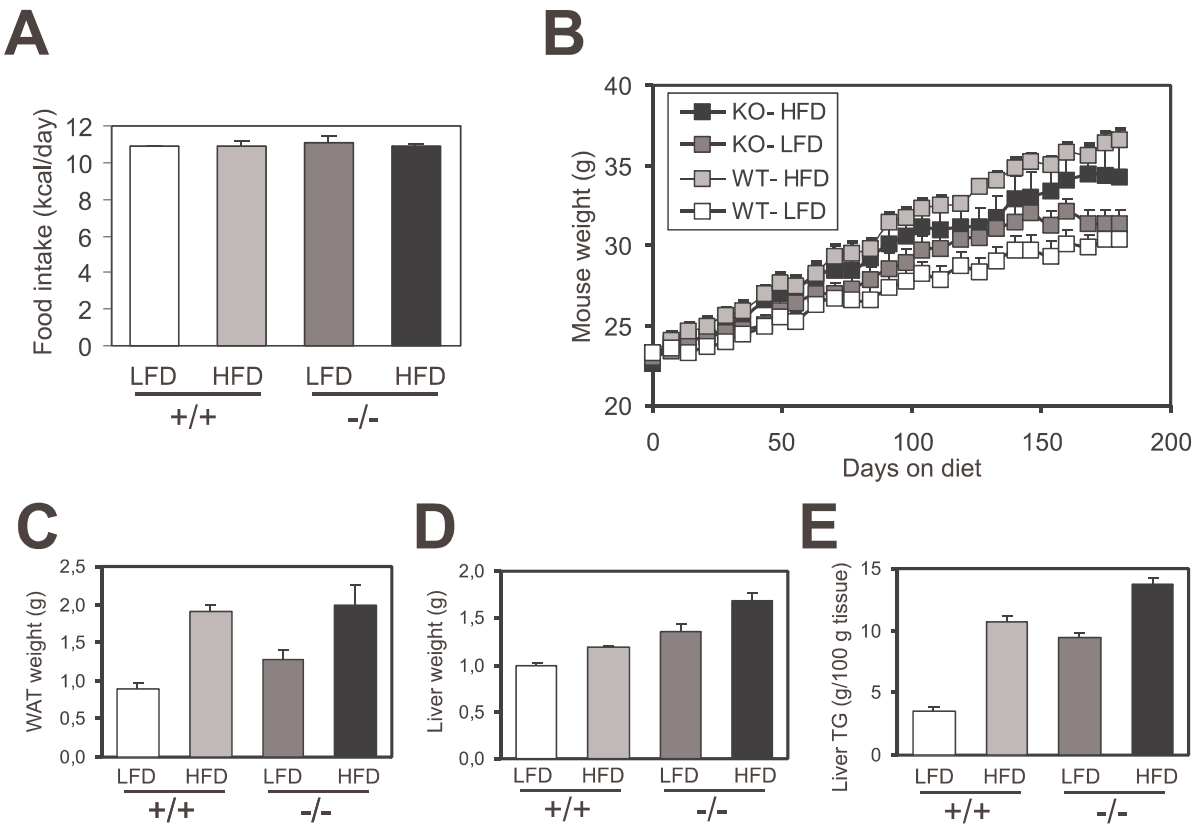

Figure 1 **High fat diet feeding of wild-type and PPARα null mice** A) Food intake (expressed as energy/day) of the four experimental groups. No significant differences were observed. B) Evolution of bodyweight during the experimental feeding. C) Weight of epididymal adipose tissue after 26 weeks on the diet. Significant effects were observed by two-way ANOVA for diet ($p<0.0001$) but not for genotype. D) Liver weight after 26 weeks on the diet. Significant effects were observed by two-way ANOVA for diet ($p<0.0001$) and for genotype ($p<0.0001$). E) Liver triglycerides after 26 weeks on the diet. Significant effects were observed by two-way ANOVA for diet ($p<0.0001$), for genotype ($p<0.0001$), and for the interaction between the two parameters ($p<0.005$). Error bars represent SEM.

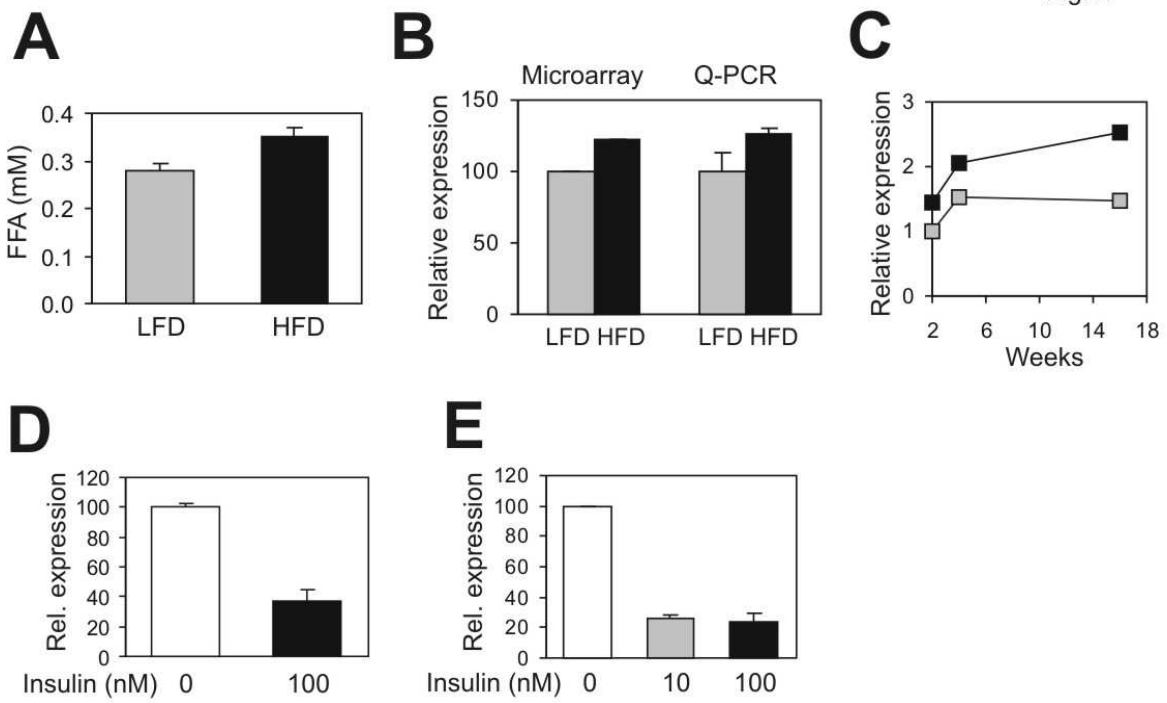

Figure 2 HFD increases plasma free fatty acids and hepatic PPARα mRNA A) Plasma free fatty acid concentration of wild-type Sv129 mice fed a LFD or HFD. Plasma FFAs were significantly increased by HFD (Student's t-test, $p<0.01$). B) PPARα mRNA levels in livers of wild-type Sv129 mice fed a LFD or HFD, as determined by microarray (pooled liver samples) or Q-PCR (individual mice). The effect of HFD was statistically significant (Student's t-test, $p<0.05$). C) PPARα mRNA levels in livers of wild-type C57/B6 mice fed a LFD (grey squares) or HFD (black squares), as determined by Q-PCR on pooled liver samples. Insulin down-regulates expression of PPARα in rat primary hepatocytes (D) or rat FAO hepatoma cells (E), as determined by Q-PCR (Student's t-test, $p<0.01$). Error bars represent SEM.

To examine whether HFD is associated with increased PPARα activity in liver, we measured mRNA expression of Cyp4A10 and Cyp4A14. Both are target genes that are extremely sensitive to the presence and activation of PPARα in mouse liver and can thus serve as markers of PPARα activity. Expression of Cyp4A10 and Cyp4A14 was highly induced by Wy14643 and by fasting in wild-type mice, whereas expression was very low throughout and not inducible in PPARα null mice (Fig. 3A, B). Feeding the HFD resulted in increased expression of both genes in wild-type but not PPARα null mice, indicating enhanced PPARα activity, although the effects were modest compared to Wy14643 and fasting (Fig. 3C).

Fig. 3

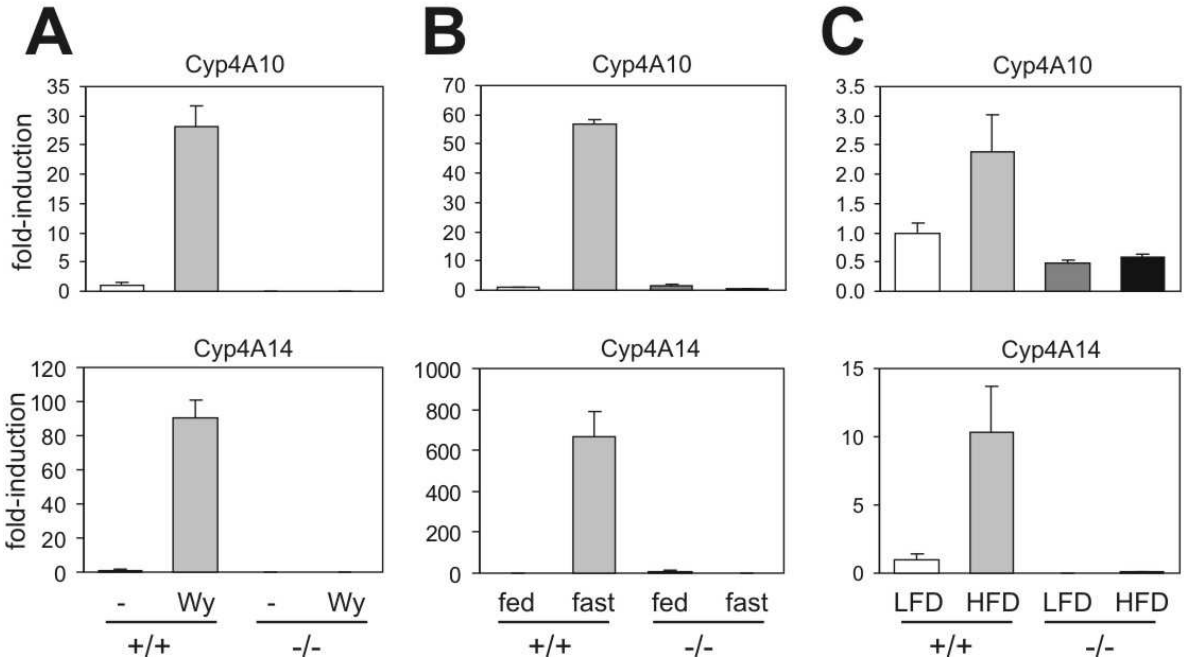

Figure 3 Upregulation of Cyp4A10 and Cyp4A14 by Wy14643, fasting and HFD is PPARα dependent A) Cyp4A10 (upper panel) and Cyp4A14 (lower panel) mRNA expression in livers of wild-type and PPARα null mice treated with Wy14643. B) Cyp4A10 and Cyp4A14 mRNA expression in livers of fed and fasted wild-type and PPARα null mice. C) Cyp4A10 and Cyp4A14 mRNA expression in livers of wild-type and PPARα null mice fed a LFD or HFD. Expression was determined by Q-PCR. Error bars represent SEM.

To examine whether this pattern of expression was similar for other classical target genes of PPARα, a comparative micro-array experiment was performed on liver of wild-type and PPARα null mice either fed Wy14643, fasted, or fed a HFD. Analysis of expression of characteristic PPARα target genes involved in peroxisomal and mitochondrial fatty acid oxidation revealed a clear PPARα-dependent regulation by Wy14643 and fasting (Fig. 4A,B and Table 1). Similar to Cyp4A10 and Cyp4A14, expression of this set of genes was increased by HFD in a PPARα-dependent manner, although again the effects were less pronounced compared to Wy14643 and fasting (Fig. 4C and Table 1).

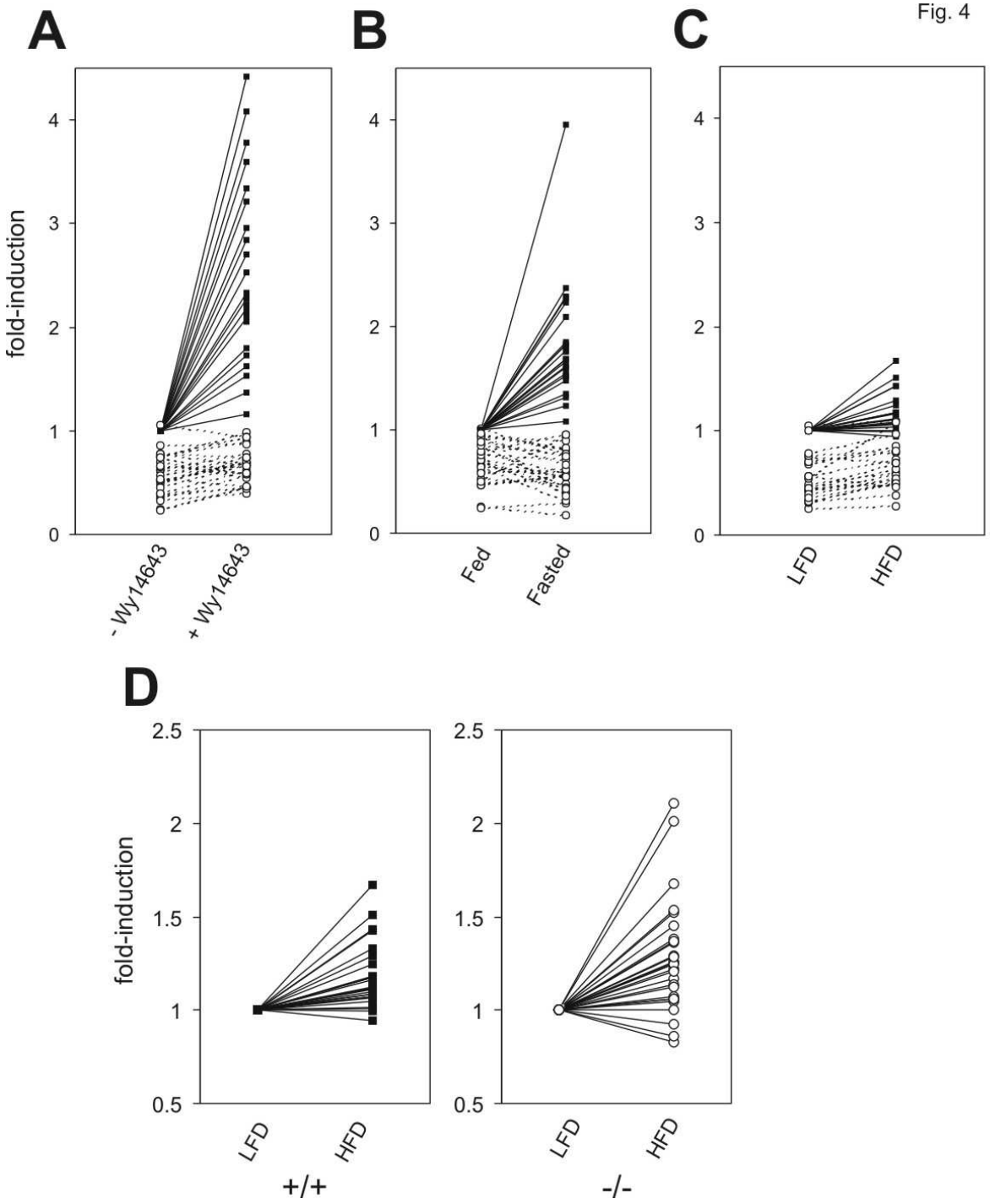

Figure 4 HFD upregulates expression of PPARα target genes involved in fatty acid catabolism A) Affymetrix microarray analysis of liver RNA of wild-type and PPARα null mice treated or not with Wy14643. B) Affymetrix microarray analysis of liver RNA of fed and fasted wild-type or PPARα null mice. C) Affymetrix microarray analysis of liver RNA of wild-type and PPARα null mice fed a LFD or HFD. Wild-type mice = filled squares and straight lines; PPARα null mice = open circles and dotted lines. For all genes, expression (Average difference) of untreated wild-type mice was set at 1 and expression in the other conditions was related to this value. All genes in the clusters "peroxisomal fatty acid oxidation" and "mitochondrial fatty acid oxidation" are included in the graph.

Table 1 Changes in expression of selective genes involved in metabolism according to micro-array analysis. Criteria for inclusion were a significant PPARα-dependent up-regulation of gene expression by Wy14643 and/or fasting. Expression in wild-type control mice (no Wy14643, fed, and on LFD) was set at 1. Changes in expression are expressed as fold-change in comparison with wild-type control mice. A= absent.

Probe Set ID	*Gene Symbol*	*WT-Wy*	*KO-veh*	*KO-Wy*	*WT-Fasted*	*KO-Fed*	*KO-Fasted*	*WT-HF*	*KO-LF*	*KO-HF*
peroxisomal fatty acid oxidation										
1416947_s_at	Acaa1	2.22	0.51	0.68	1.53	0.73	0.55	0.99	0.48	0.60
1416946_a_at	Acaa1	3.22	0.35	0.57	2.37	0.69	0.39	1.11	0.42	0.57
1456011_x_at	Acaa1	2.31	0.74	0.98	2.29	1.01	0.77	1.29	1.05	1.09
1424451_at	Acaa1	2.05	0.40	0.62	1.62	0.71	0.43	1.01	0.36	0.50
1450966_at	Crot	1.37	0.78	0.66	1.23	0.97	0.36	1.33	0.53	1.11
1423495_at	Decr2	2.14	0.25	0.45	1.52	0.26	0.17	1.51	0.25	0.27
1448491_at	Ech1	4.08	0.37	0.45	2.09	0.48	0.56	1.44	0.31	0.52
1448382_at	Ehhadh	4.42	0.76	0.94	3.95	0.93	0.67	1.01	0.70	0.70
1417449_at	Pte1	5.56	0.49	0.73	1.85	0.47	0.55	1.12	0.75	0.70
mitochondrial fatty acid oxidation										
1424184_at	Acadvl	2.18	0.45	0.67	1.67	0.58	0.62	1.18	0.45	0.48
1448987_at	Acadl	2.53	0.52	0.70	1.76	0.50	0.95	1.06	0.39	0.54
1460216_at	Acads	1.80	0.75	0.89	1.35	0.94	0.82	1.12	0.71	0.79
1422526_at	Acsl1	2.70	0.54	0.46	1.69	0.76	0.71	1.07	0.56	0.47
1450643_s_at	Acsl1	2.27	0.66	0.59	1.69	0.84	0.77	1.08	0.74	0.64
1416772_at	Cpt2	2.96	0.69	0.54	1.52	0.65	0.55	1.43	0.68	0.85
1418321_at	Dci	3.78	0.39	0.44	1.81	0.52	0.48	1.25	0.43	0.53
1419367_at	Decr1	2.84	0.61	0.59	1.48	0.65	0.55	0.94	0.56	0.73
1449443_at	Decr1	3.34	0.62	0.78	1.54	0.86	0.44	1.17	0.70	0.80
1452173_at	Hadha	2.34	0.78	0.74	1.60	0.93	0.90	1.07	0.76	0.81
1426522_at	Hadhb	2.33	0.75	0.58	1.82	0.79	0.96	1.12	0.73	0.88
1460184_at	Hadhsc	1.63	1.06	0.94	1.08	0.89	0.74	1.10	0.92	1.18
1431833_a_at	Hmgcs2	1.16	0.57	0.74	1.82	0.64	0.32	1.12	0.44	0.63
1423858_a_at	Hmgcs2	1.53	0.65	0.64	1.60	0.77	0.58	1.04	0.45	0.69
1424639_a_at	Hmgcl	2.09	0.86	0.88	1.31	0.95	0.82	1.09	0.78	0.97
1423108_at	Slc25a20	2.21	0.23	0.45	1.75	0.25	0.29	1.17	0.33	0.38
1423109_s_at	Slc25a20	1.72	0.33	0.40	2.28	0.46	0.56	1.16	0.33	0.50
microsomal fatty acid oxidation										
1415776_at	Aldh3a2	3.60	0.52	0.69	2.23	0.60	0.60	1.67	0.54	1.08
1424853_s_at	Cyp4a10	2.09	0.04	0.16	19.94	2.84	1.34	2.00	0.04	0.11
1424943_at	Cyp4a10	22.93	0.02	0.19	39.02	1.22	1.22	A	A	A
1423257_at	Cyp4a14	3.60	0.04	0.15	94.65	9.79	0.77	4.08	0.06	0.13
fatty acid binding proteins										
1417556_at	Fabp1	1.35	0.86	0.86	0.95	0.88	0.43	0.95	0.78	0.76
1418438_at	Fabp2	2.85	1.00	1.11	2.40	1.26	1.42	1.11	0.66	1.27
1416023_at	Fabp3	37.70	0.55	1.10	0.63	0.79	1.34	1.68	1.21	0.90
1417023_a_at	Fabp4	29.25	1.64	1.35	0.81	1.51	2.33	1.06	2.23	2.63
lipogenesis										
1420722_at	Elovl3	4.14	0.49	0.55	0.37	1.02	0.30	0.84	0.28	0.38
1419031_at	Fads2	1.79	0.68	0.80	1.45	0.78	0.87	0.98	0.58	0.66
1449325_at	Fads2	2.57	0.67	0.59	1.58	0.73	0.89	1.02	0.56	0.66
1416632_at	Mod1	3.63	0.71	0.36	0.69	0.88	0.60	0.23	0.15	0.03
1419399_at	Mttp	2.45	1.01	0.90	1.39	1.02	0.86	0.79	0.87	0.87
1415965_at	Scd1	1.61	0.55	0.20	0.24	0.65	0.07	0.24	0.24	0.05
glycerol metabolism										
1416204_at	Gpd1	2.78	0.71	0.52	1.83	0.89	0.52	1.11	0.66	0.73
1448249_at	Gpd1	2.23	0.79	0.69	1.51	0.94	0.46	1.02	0.62	0.75
1439396_x_at	Gpd1	1.90	0.66	0.91	1.44	1.18	1.09	1.36	0.74	0.82
1417434_at	Gdm2	3.32	0.93	0.90	1.37	0.96	0.52	0.79	0.85	0.75
1422704_at	Gyk	2.30	1.26	1.27	1.06	1.08	0.71	1.22	0.95	1.54
miscellaneous lipid metabolism										
1417130_s_at	Angptl4	2.14	0.33	0.30	3.93	0.39	2.80	0.67	0.46	0.50
1450883_a_at	Cd36	11.48	1.02	0.72	1.50	1.63	1.34	1.51	1.95	3.62
1450884_at	Cd36	5.11	0.78	0.56	0.92	1.36	0.89	1.18	1.38	1.64
1449065_at	Cte1	8.79	1.11	1.00	12.39	3.90	9.34	2.02	1.94	4.49
1422997_at	Cte1	13.04	0.79	0.92	16.24	4.13	10.22	1.12	2.00	3.76
1415904_at	Lpl	11.62	1.12	1.03	0.71	0.87	0.51	1.20	1.54	4.15
1431056_a_at	Lpl	23.36	0.71	0.89	0.52	0.90	0.33	1.09	1.75	3.51
1450391_a_at	Mgll	5.92	0.76	0.99	1.57	0.96	1.11	1.06	0.92	0.72
1426785_s_at	Mgll	4.37	0.70	0.68	1.62	0.70	0.69	0.80	0.91	0.59
1417273_at	Pdk4	56.49	1.62	1.83	1.52	3.29	3.58	1.24	4.96	3.29
1448188_at	Ucp2	3.91	1.15	1.20	1.09	0.97	2.11	1.35	1.75	1.75

Interestingly, when the changes in expression induced by HFD were expressed in relative terms for wild-type and PPARα null mice separately, thus correcting for differences in basal expression, the effect of HFD was similar for wild-type and PPARα null mice (data not shown and Table 1, compare last two columns). This suggests that there might be compensation for PPARα in the PPARα null mice, perhaps by other PPAR isotypes. Indeed, whereas PPARβ/δ expression was hardly affected by either HFD or PPARα deletion (Fig. 5), PPARγ showed a most remarkable pattern. Expression was elevated by both HFD and PPARα deletion, resulting in a 20-fold increase in PPARγ mRNA in PPARα null mice on HFD compared to wild-type mice on LFD (Fig. 5A). In PPARα null mice on HFD, levels of PPARγ mRNA reach almost 80% of that of PPARα in wild-type mice (Fig. 5B), suggesting that it may be functionally important. In support of a functional role of PPARγ in PPARα null mouse liver, expression of PPARγ targets CD36/FAT, LPL, aP2, and UCP2 mirrored that of PPARγ, suggesting that these genes are up-regulated by PPARγ in liver (Fig. 5A). Interestingly, induction of cGPDH and glycerol kinase by HFD was at least as strong in PPARα null mice compared to wild-type mice, even though basal expression was lower in the PPARα null mice. Overall, these data suggest that in mice lacking PPARα the effect of HFD on PPARα target genes may be mediated by PPARγ, which is highly up-regulated in PPARα null mice on a HFD.

To examine whether PPARγ is able to up-regulate characteristic PPARα target genes involved in fatty acid oxidation and catabolism, expression of these genes was determined in liver of PPARα null mice infected with PPARγ1-expressing adenovirus. Under these conditions, expression of PPARγ was highly up-regulated (Fig. 6A), whereas PPARβ/δ was not affected (Fig. 6B). It has previously been shown, and which is confirmed here, that PPARγ1 over-expression causes up-regulation of several PPARγ target genes, as well as other genes involved in lipo/adipogenesis (Fig. 6C) (20) (9).

However, PPARγ over-expression also led to the pronounced induction of numerous characteristic PPARα target genes involved in fatty acid oxidation and ketogenesis, including HMG-CoA synthase, bifunctional enzyme, very long chain acyl-CoA dehydrogenase and carnitine palmitoyl-transferase 2 (Fig. 6D), plus many others (9). These data demonstrate that, when expressed at a certain level, PPARγ is able to up-regulate classical PPARα target genes in liver.

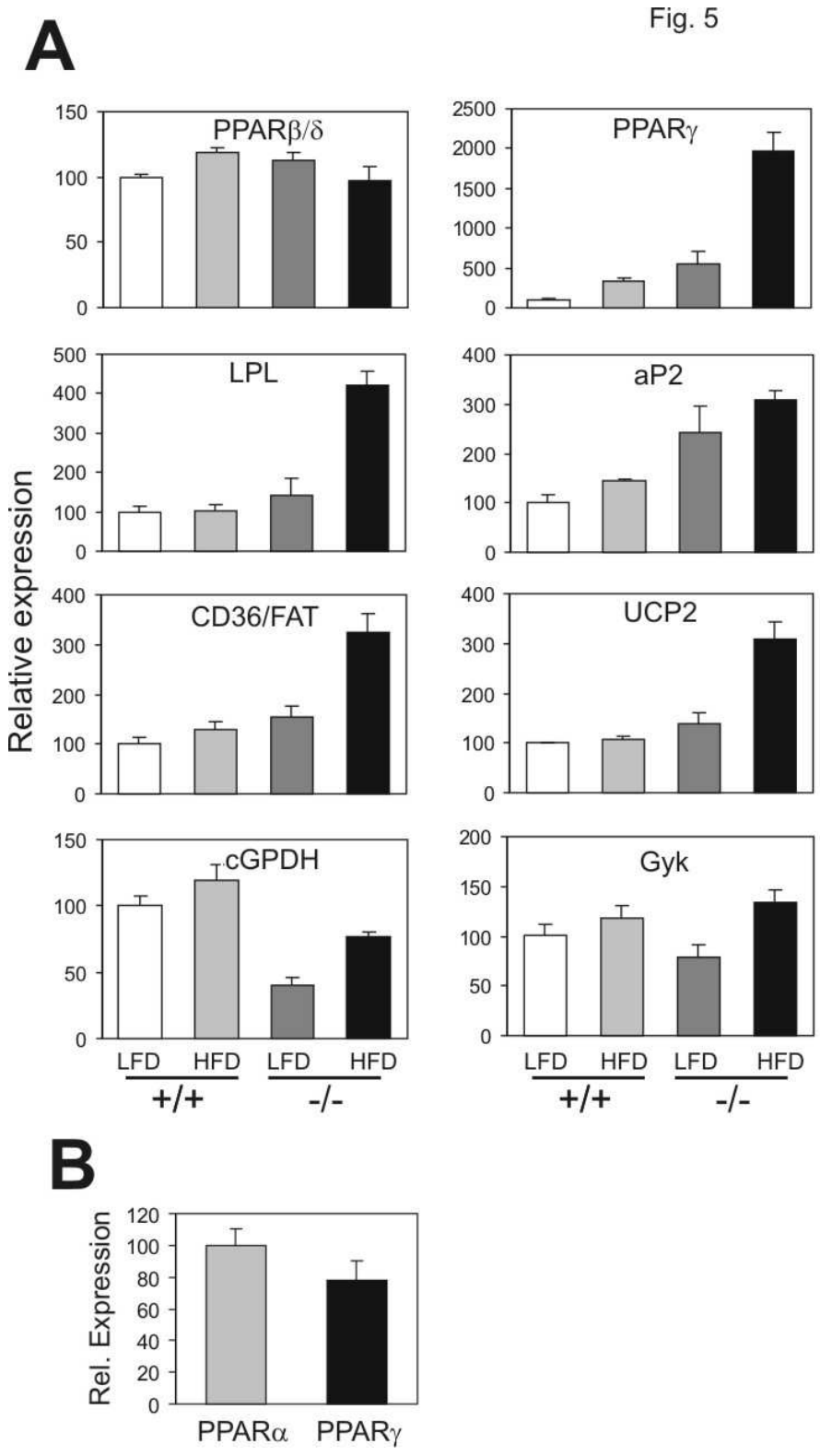

Figure 5 PPARγ and its targets are upregulated in livers of PPARα null mice fed a HFD A) Expression of PPARβ/δ, PPARγ, and several target genes of PPARα/PPARγ were determined by Q-PCR in liver of wild-type and PPARα null mice fed a LFD or HFD. B) Expression of PPARα in wild-type normal liver was related to expression of PPARγ in liver of PPARα null mice on HFD. Relative expression was calculated based on difference in Ct values (amplification efficiency was identical, 94%).

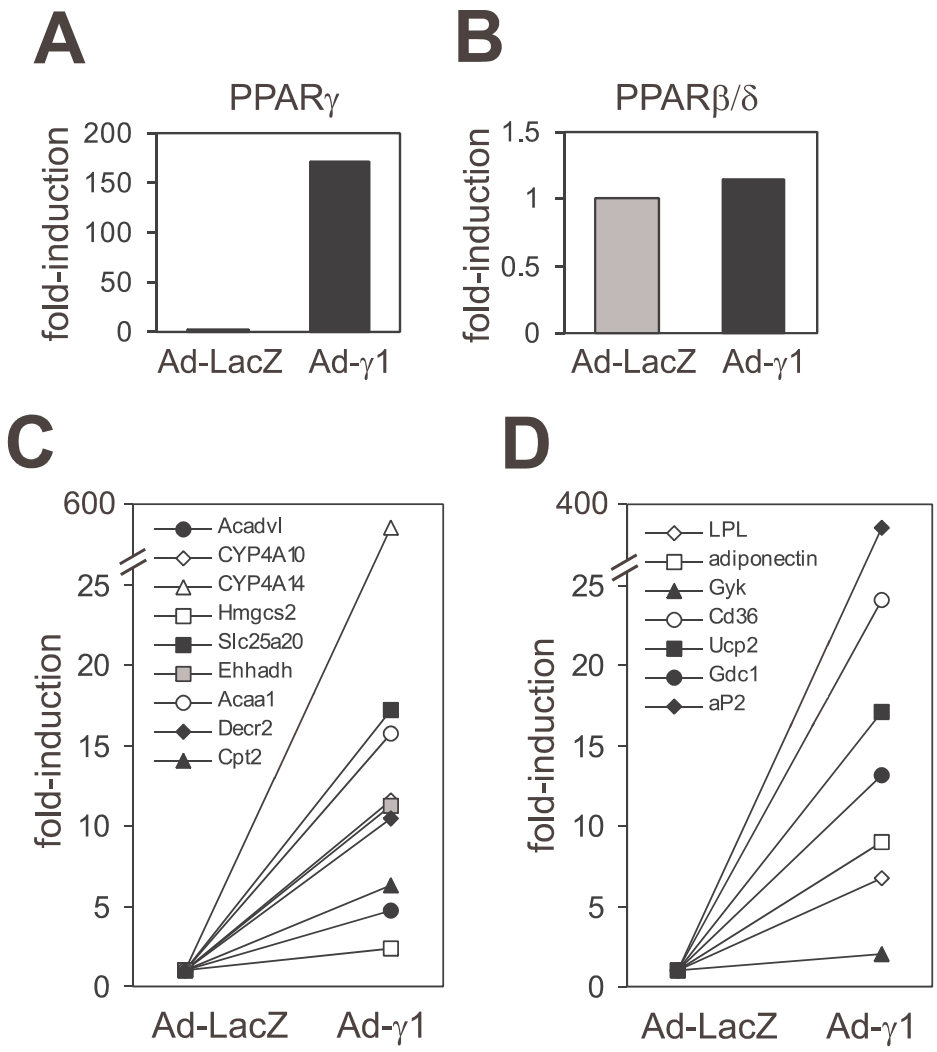

Figure 6 Adenoviral over-expression of PPARγ1 in liver leads to induction of PPARα target genes Expression of PPARγ (A) and PPARβ/δ (B) in liver of a PPARα null mouse infected or not with an adenovirus expressing PPARγ1. C) Expression of several classical PPARα targets (C) and PPARγ/α targets (D) in liver of a PPARα null mouse infected or not with an adenovirus expressing PPARγ1. Genes shown in panel C: very long chain acyl-Coenzyme A dehydrogenase; cytochrome p450 4A10; cytochrome p450 4A14; carnitine palmitoyltransferase 2; 2-4-dienoyl-Coenzyme A reductase 2; 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2; acetyl-Coenzyme A acyltransferase 1; mitochondrial carnitine/acylcarnitine translocase, member 20; enoyl-Coenzyme A hydratase/3-hydroxyacyl Coenzyme A dehydrogenase (bifunctional enzyme). Genes shown in panel D: lipoprotein lipase, cytosolic glycerol 3-phosphate dehydrogenase, UCP2, adiponectin, glycerol kinase, adipocyte fatty acid binding protein aP2, and CD36/fatty acid translocase.

Previous studies had shown that whereas HFD impairs glucose tolerance in wild-type mice, this is not the case in PPARα null mice, which thus appear to be protected from the effects of HFD (22). However, if indeed PPARγ is able to compensate for PPARα in the null mice as in our experiment, one would expect that HFD would cause deterioration of glucose tolerance in PPARα null mice as well. Indeed, although glucose tolerance was improved by PPARα deletion, HFD exacerbated glucose tolerance to a similar extent in wild-type and PPARα null mice (Fig. 7A,B). Interestingly, the area under the glucose tolerance curve (Fig. 7B) showed remarkable similarity with the expression pattern of PPARα target genes (Fig. 4C), including cGPDH (Fig. 5A).

DISCUSSION

Numerous in vivo and in vitro studies have shown that PPARα is activated by fatty acids (23),(24),(25),(26). It has also been clearly established that in liver PPARα stimulates the expression of a large set of genes involved in fatty acid catabolism (12). Accordingly, hepatic PPARα is considered to function as a fatty acid sensor that adjusts catabolism of fatty acids to the prevailing plasma fatty acid concentration. This is especially relevant during fasting, when plasma free fatty acids and the flux of fatty acids through the liver increases dramatically (16, 18).

While fasting has been an important evolutionary force shaping human energy metabolism, it is rarely encountered in modern industrialized societies. Instead, we are dealing with a crisis of over-nutrition, giving rise to obesity and associated ailments. Obesity and fasting appear to represent two ends of the metabolic spectrum, yet they are both associated with elevated hepatic fatty acid flux and diminished insulin signalling. Inasmuch as hepatic PPARα mediates an adaptive response to fasting, the aim of this study was to determine if PPARα may mediate some effects of chronic HFD, which is used as a model system for obesity/insulin resistance. Using expression profiling it is observed that HFD results in activation of PPARα target genes, probably via a combination of increased PPARα mRNA and elevated plasma FFA levels. Since the effects of HFD on gene expression are small, which is common in nutritional interventions, a pattern only emerges by analyzing all genes together, illustrating the power of micro-array analysis. While the effects are modest compared to what is observed after treatment with WY14643 or after fasting, HFD is a much more chronic exposure, suggesting that some of the long-term effects of HFD on lipid metabolism may be mediated by PPARα. Our data support and extend previous data by

Kroetz et al., which showed that induction of hepatic CYP4A during streptozotocin-induced diabetes requires PPARα (27).

The up-regulation of PPARα mRNA by HFD is expected to serve a physiological purpose similar to what happens during fasting. During HFD, increased amounts of fatty acids arrive at the liver and concomitantly there is an increased requirement for fatty acid oxidation. In spite of up-regulation of PPARα and numerous PPARα target genes involved in fatty acid oxidation, HFD causes fatty liver, suggesting that the up-regulation is not sufficient to efficiently catabolize the extra load of fatty acids. This is again analogous to what is observed during fasting, where there is spillover of fatty acids into the triglyceride synthesis pathway despite stimulation of fatty acid oxidation, causing a fatty liver (16, 18, 28). Importantly, deletion of PPARα resulted in more pronounced hepatic accumulation of TG during both fasting and HFD, suggesting that PPARα protects from lipid overload in these situations. This observation underscores the notion that PPARα in liver becomes especially important when the flux of fatty acids through the liver is increased.

Our data clearly demonstrate that insulin represses the expression of PPARα in hepatocytes. Accordingly, it can be hypothesized that the up-regulation of hepatic PPARα by fasting and HFD may be related to attenuation of insulin signalling. Supporting our data, de Fourmestraux et al. showed that feeding a HFD to C57Bl/6 mice resulted in up-regulation of hepatic PPARα mRNA together with some of its target genes involved in β-oxidation. Interestingly, the increase in PPARα only occurred in mice developing obesity-related diabetes but not those remaining lean and healthy, suggesting that PPARα up-regulation is connected with defective insulin action (29).

Recently, Lin et al. proposed that the transcription factor SREBP and co-activator PGC-1β may be involved in mediating the effects of high fat diet on lipogenesis (30). In contrast to Lin et al. we found PGC-1β expression to be decreased after high fat feeding. It is well established that high fat feeding is associated with suppression of endogenous fatty acid synthesis. In our experiment, we observed marked suppression of lipogenic genes by HFD, including fatty acid synthase, ATP-citrate lyase, Acetyl-CoA carboxylase and others. Interestingly, PGC-1β mRNA was decreased as well, suggesting that it may mediate suppression of lipogenesis by HFD.

Our data clearly show that hepatic PPARγ is highly up-regulated in PPARα null mice on a high fat diet, reaching an expression level that approximates PPARα. At that level of expression, PPARγ may compensate for PPARα by mediating the HFD-induced up-regulation

of characteristic PPARα target genes involved in fatty acid oxidation in PPARα null mice. Indeed, PPARγ does not appear to possess some intrinsic property that prevents it from activating classical PPARα targets, as indicated by the marked induction of PPARα target genes in liver by adenoviral PPARγ over-expression. Similarly, PPARα can act on behalf of PPARγ, since PPARα activation by Wy14643 causes marked hepatic up-regulation of classical PPARγ targets LPL, CD36, and aP2, as shown here and in previous studies (31)(32). Remarkably, in our study regulation of LPL, CD36, and aP2 by PPARα was not observed under conditions of physiological activation of PPARα by fasting.

Finally, by comparing the role of PPARα in mediating the effects of Wy14643, fasting and high fat feeding on gene transcription (Table 1) it becomes clear that the function of PPARα in hepatic gene regulation can not simply be extrapolated from pharmacological activation of PPARα using synthetic agonists. This is an extremely important conclusion that can clarify some of these discrepancies in the literature with respect to role of PPARα in hepatic gene regulation.

Compensation for PPARα by PPARγ is not necessarily limited to gene expression but may translate into functional consequences, such as fasting blood glucose levels and glucose intolerance, which are reduced in PPARα null mice. Previous studies by Guerre Millo et al. had shown that HFD impairs glucose tolerance in wild-type mice, but not in PPARα null mice, which thus appear to be protected from the effects of HFD (22). However, in our hands PPARα null mice were not protected from HFD-induced deterioration of glucose homeostasis, possibly thanks to up-regulation of PPARγ expression. The reason for the discrepancy is not very clear but may be related to the type of high fat diet. Importantly, compensation by PPARγ in PPARα null might not be limited to HFD. Indeed, Hashimoto et al. reported that after 72 hours of fasting, hepatic PPARγ mRNA was increased in PPARα null mice vs. wild-type mice (28). One can speculate that this may explain why PPARα null mice seems to experience a “second wind” after 24 hours of fasting rather than die from the severe metabolic disturbances.

Up-regulation of PPARγ mRNA in liver by HFD was associated with increased hepatic triglyceride levels. However, it is not exactly clear in what order they occurred: 1) increased triglyceride levels, either because of impaired fatty acid oxidation (PPARα null mice) or increased fat delivery (HFD) causes PPARγ expression to go up; or 2) increased PPARγ mRNA, either as a compensatory mechanism (PPARα null mice) or elicited by HFD,

stimulates lipogenesis and triglyceride storage. Probably, both mechanisms are working in concert to induce a vicious cycle of enhanced hepatic triglyceride storage. Previous studies have demonstrated that PPARγ over-expression is both necessary and sufficient to induce fatty liver (33) (11) (9). Hepatic PPARγ expression is up-regulated in animal models of severe obesity and lipoatrophy, concurrent with development of steatosis. Under those circumstances treatment with TZD further aggravate hepatic steatosis, whereas deletion of PPARγ decreases hepatic fat storage. This positive link between PPARγ and liver fat storage is supported by studies by Yu et al., which showed that PPARγ1 over-expression in liver causes hepatic steatosis and induction of adipocyte specific gene expression (9).

In muscle, elevated tissue triglyceride levels are associated with impaired insulin sensitivity, possibly via a mechanism that involves fatty acyl-CoA. Since in various animal models of obesity/diabetes impaired hepatic insulin sensitivity is associated with a fatty liver, it has been suggested that a similar mechanism may operate in liver. However, PPARα null display improved glucose tolerance and insulin sensitivity (13), in spite of markedly elevated hepatic triglyceride levels. This indicates that in PPARα null mice hepatic triglycerides and insulin resistance are disconnected. This also appears to be true in liver-specific PPARγ null mice, casting doubt on the impact of hepatic triglycerides on hepatic insulin resistance (33).

Overall, we conclude that 1) PPARα and PPARγ are activated in liver by high fat feeding, the latter mainly in the absence of PPARα 2) In PPARα null mice on a high fat diet PPARγ is able to compensate for PPARα, which might translate into functional consequences.

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Chapter 5

Cross- talks between LXRα, RXRα and PPARγ in the adipose tissue

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In preparation

ABSTRACT

PPARγ and LXRα are Nuclear Hormones Receptors that are highly expressed in adipose tissue. While the function of PPARγ as a master regulator of adipocyte differentiation and maturation is well described, the function of LXRα in adipose tissue remains controversial. We previously showed that the cGPDH gene, which is involved in triglyceride synthesis, is a direct PPARγ target gene in adipose tissue. Here we show that LXRα and its ligand T0901317 specifically repress the expression of cGPDH *in vivo* and in differentiated mature adipocytes. Further investigation of the molecular mechanism by transactivation and chromatin immunoprecipiation experiments demonstrated that liganded LXRα prevents the binding of PPARγ to the PPREs of the cGPDH promoter by competing with PPARγ for their reciprocal partner RXR. A similar mechanism likely applies to the down-regulation of AQP7 expression by LXR. Our data reveal novel cross-talk between PPARγ, LXRα and RXR in the control of gene expression in adipocytes.

INTRODUCTION

Adipocytes actively participate in whole-body energy homeostasis at several levels. Not only are adipocytes essential for the storage and release of fatty acids, they also secrete a variety of factors called adipocytokines, many of which are critically involved in energy homeostasis (1) (2). The energy storage function of adipose tissues as well as its endocrine function are carefully regulated, and directly respond to changes in nutritional status (3). Nevertheless, dysregulation is known to occur as in obesity, which is characterized by excess fat storage and altered production of numerous adipocytokines, or lipodystrophy, in which impaired adipose lipid storage leads to severe metabolic disturbances elsewhere in the body. Although much insight has been gained in the past few years about how the energy storage and protein secretion function of adipose tissue are regulated, significant gaps in our knowledge remain.

The 3T3-L1 and SGBS cell lines are well established *in vitro* models of mouse and human adipoblasts, respectively, which can be differentiated into mature adipocytes by certain hormonal stimuli. Combination of these models with studies in genetically engineered mice has led to a clear picture of the sequence of molecular events driving the adipogenesis process. It has been shown that the transcription factors belonging to the CCAAT/Enhancer Binding Proteins, as well as the Peroxisome Proliferator Activated Receptor gamma 2 play a key role in adipocyte differentiation (4) (5) (6) (7) (8) (9) (10). In fact, PPARγ2 is nowadays considered the master regulator of adipogenesis which orchestrates the diverse cellular events

required for growth arrest, clonal expansion, and subsequent lipid accumulation. PPARγ2 is a member of the nuclear hormone receptor family which also includes its close relatives PPARα and PPARβ/δ. PPARs regulate transcription by forming a heterodimeric complex with the partner Retinoid X Receptor and recognizing specific genomic DNA sequences named Peroxisome Proliferator Response Elements (PPRE), which are present in the upstream promoter or introns of target genes. The consensus PPRE consists of a direct repeat of the sequence AGGTCA spaced by a single nucleotide and is referred to as Direct Repeat 1 (DR-1) (11). Transcription is activated by binding of ligand, which includes unsaturated fatty acids and various eicosanoids, in addition to synthetic ligands belonging to the class of fibrates (PPARα) and thiazolidinediones (PPARγ). The latter compounds are approved for the clinical treatment of type 2 diabetes and promote peripheral glucose utilization (11-13) (14).
Important information about the role of PPARγ2 in the differentiating and mature adipocyte can be extrapolated from the various target genes that are under transcriptional control of PPARγ. This include genes encoding proteins involved in energy uptake, intracellular transport and storage (e.g. FABP4, LPL, PEPCK, GYK, PCx, CAP) (15) (16) (17) (18) (19), lipid droplets formation (ADRP, perilipin (20, 21), several adipocytokines (adiponectin, Angptl4) (22, 23), and others (AQP7, UCP1, 2 and 3, G0S2) (24-27) (16). Recently, we identified the cytosolic GPDH gene as a direct target gene of PPARγ. cGPDH is a key enzyme involved in the formation of triacylglycerols and has been extensively described as a marker of adipogenesis. In fact, the enzymatic activity of cGPDH has been assayed for decades to monitor the extent of adipocyte differentiation (28) (29) (30) (31). Two functional PPREs located within the proximal promoter of the mouse cGPDH gene were identified (16).

Like PPARγ, LXRα is a nuclear hormone receptor that forms a heterodimeric complex with RXR and is also highly expressed in adipose cells. Additionally, both PPARγ/RXR and LXRα/RXR are permissive heterodimers whose activity is affected not only by the respective ligands for PPARγ or LXR, but by RXR ligands as well. Natural ligands for LXR are oxysterols such as 22(R)-hydroxycholesterol, which can be synthesized from cholesterol (32) (33). However, while PPARγ/RXR binds Direct Repeat sequences spaced by one nucleotide (DR-1), LXRα/RXR as a rule recognizes DR-4 response elements (34) (35).
Unlike in liver, intestine or macrophages, where the function of LXRα has been well characterized, its role in adipose tissue remains somewhat ambiguous. Expression of LXRα is increased during adipogenesis yet appears late in comparison with PPARγ2 (5) (36). The

other isoform of LXR, LXRβ, is more ubiquitously expressed and remains stable during the adipogenesis process. Some functional redundancies between the two isoforms in adipose tissue may exist since only the double LXRα/β knock-out mice display an adipose phenotype, as shown by a reduction of their adipose mass (5) (36) (37). In contrast, feeding mice a synthetic agonist of LXR does not appear to influence adipose mass despite marked upregulation of genes involved in de novo lipogenesis in liver and adipose tissue (36) (38) (39) (40). Upregulation of lipogenesis in liver is associated with induction of a severe fatty liver phenotype. Target genes of LXRα in adipose tissue include FAS, SREBP1c, Spot14, and GLUT4 (5) (35) (38) (39) (41) (42) (43), all of which are involved in energy storage. Thus, it is clear that at least at the level of gene expression LXRα promotes lipogenesis in mature adipocytes. However, to what extent LXRα affects other aspects of adipocyte function, including differentiation, lipolysis, nutrient transport, and secretion of adipocytokines remains unclear (5) (36) (38) (39) (44).

Interestingly, recent observations have demonstrated the existence of cross-talks and competition between PPARα and LXRα in the liver. On the one hand, ligands for PPARα have been reported to repress LXR activity and target genes (45), while on the other hand LXR may influence PPARα-dependent gene regulation (46), (47). Furthermore, LXRα and PPARα have been reported to form stable heterodimers in solution (48). Whether similar cross-talk exists between PPARγ and LXRα in adipose cells has not yet been addressed. Here we demonstrate that LXRα and its ligand specifically repress basal and PPARγ-induced expression and activity of cGPDH in adipose cells. Furthermore, we provide evidence that this repression is direct and mediated via decreased binding of PPARγ and RXRα to the cGPDH promoter. A similar regulation was observed for AQP7. However, repression of these PPARγ target genes by LXR did not affect 3T3-L1 adipogenesis or lipid storage.

MATERIAL AND METHODS

Chemicals. Rosigliazone and T0901317 were from Alexis. Recombinant human insulin (Actrapid) was from Novo Nordisk. SYBR Green was from Eurogentec. Dulbecco's modified Eagle's medium, fetal calf serum, calf serum, and penicillin/streptomycin/fungizone were from Cambrex Bioscience. Tissue/plasma triglyceride kit was from Instruchemie. Anti-RXRα and -PPARγ antibodies were from Santa- Cruz (SC-553 and SC-7273). All other chemicals were from Sigma.

Animals. Adipose tissue from LXRα null mice and wild type counterparts, as well as adipose tissue from mice fed the LXR agonist GW3965 were obtained from Prof. Folkert Kuipers (University Medical Center Groningen, the Netherlands)

Cell culture and transfections. 3T3-L1 and NIH-3T3 cells were grown and amplified in DMEM supplemented with 10% Calf Serum. HepG2 cells were grown in DMEM supplemented with 10% Fetal Calf Serum. Amplification and differentiation of SGBS cells was performed as previously described (49). Cells were transfected with PPARγ, LXRα expression and luciferase reporter constructs using PolyFect (QIAGEN Inc., Leusden, The Netherlands) for NIH-3T3 cells or calcium-phosphate precipitation for HepG2 cells. After transfections, cells were incubated in the presence or absence of ligand(s) for 24–48 hours prior to lysis. Promega luciferase assay (Promega Corp.) and standard ß-galactosidase assay with 2-nitrophenyl-ß-D galactopyranoside were used to measure the relative activity of the promoter. Adipogenesis of 3T3-L1 was induced 2 days post confluence by incubating the cells for 2 days with isobutyl methylxanthine (0.5 mM), dexamethasone (1 µM), and insulin (5 µg/ml) in DMEM supplemented with 10% FCS. On day 2, the medium was changed to DMEM plus 10% FCS and insulin (5µg/ml). From day 4 the cells were grown in DMEM/ 10% FCS and the medium was renewed every two days. SGBS cells were grown, differentiated and treated as described in former publications (16) (27).

Isolation of RNA and Q-PCR. Total RNA was extracted from tissues with Trizol reagent (Invitrogen, Breda, The Netherlands). 1 µg of total RNA was reverse-transcribed with iScript (Biorad, Veenendaal, the Netherlands). cDNA was PCR amplified with Platinum Taq DNA polymerase (Invitrogen) on a Biorad iCycler apparatus. Primers were designed to generate a PCR amplification product of 100 to 150 bp. Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification. Sequences of primers used are available on request. The mRNA expression of all genes reported is normalized to beta-actin, cyclophilin or 18S gene expression, depending on which control gene was expressed at stable levels.

Plasmid and DNA constructs. The mouse cGPDH promoter constructs and PPAR expression vectors have been described elsewhere (16). The expression vector for mPGC-1α was created by amplifying mPGC-1α cDNA from mouse liver cDNA and subsequent cloning

into the SmaI and BamH1 sites of the pCMX vector. Human LXRα and mouse RXRα cloned into the pCMX vector were a generous gift from Hilde Nebb (Institute for Nutrition Reseach, Oslo, Norway). The PPRE-tk-LUC reporter vector containing three copies of the acyl-CoA oxidase PPRE was a generous gift from Ronald Evans (Salk Institute, La Jolla, California, USA).

cGPDH enzymatic assay. cGPDH activity was assayed according to the spectrophotometric method of Wise and Green with some modifications (31). The same amount of protein was incubated in standard reaction mixture (100 mM triethanolamine, 0.25 mM EDTA, 50 mM 2-mercaptoethanol, and 0.2 mM NADH). The reaction was initiated by the addition of dihydroxyacetone phosphate, and NADH disappearance was followed at 340 nm (16).

Chromatin Immunoprecipitation. Mature adipocytes (day 10) were treated with the indicated ligands for 24 hours. Five 3 cm plates of cells were pooled for each condition. The assay was performed with the ChIP-IT enzymatic shearing kit from Active Motif (Rixensart, Belgium). Immunoprecipitated chromatin was diluted 10 times prior to quantification by Q-PCR. Values corresponding to the immunoprecipitated chromatin were divided by the corresponding input values in order to represent the "relative DNA binding" of PPARγ and RXRα (4). An IgG binding control was included to test for the specificity of the antibodies. Primers used for the amplification of the region encompassing the PPREs within the mouse cGPDH promoter or a control sequence have previously been described (16). AQP7 primers encompassing the previously described PPREs from the proximal promoter were developed according to the sequences available from the EMBL genome browser. Sequences were AQP7 PPRE forward: 5'-GCGGTCTCCGAGTCCTCACT -3', AQP7 PPRE reverse: 5'-TCACAAGGGCTGCCTCAGAA -3' (24).

RESULTS

The cGPDH gene has previously been identified as a direct PPARγ target gene (16). Indeed, a dose-dependent increase in cGPDH expression was observed upon treatment of mouse 3T3-L1 adipocytes with the PPARγ agonist rosiglitazone. In contrast, treatment of adipocytes with a synthetic agonist for RXR, which together with PPARγ forms the heterodimeric complex that binds to the cGPDH promoter, caused a marked repression of cGPDH mRNA and furthermore prevented induction of cGPDH mRNA by rosiglitazone (Fig.1A). This initial observation led us to think that the RXR agonist might pull RXR away from the PPARγ-RXR

complex by strengthening the interaction of RXR with another permissive nuclear hormone receptor. One possible candidate was LXR, which is well expressed in adipocytes and forms a heterodimeric complex with RXR. Interestingly, treatment of mouse (3T3L1) and human (SGBS) mature adipocytes with the synthetic LXR agonist T0901317 led to a dramatic repression of cGPDH expression. This repression was dose dependent and reinforced by treatment with RXR agonist (Fig.1B and 1C). Changes in cGPDH mRNA were paralleled by changes in cGPDH enzyme activity (Fig.1D and 1E). Suppression of cGPDH expression by LXR agonist was already observed after 2 hours of treatment and was maintained in the presence of cycloheximide, a protein synthesis inhibitor (Fig. 1F and 1G). These data indicate that cGPDH is a negative target gene of LXR. In vivo evidence for this notion was provided by the demonstration that in mice, cGPDH expression is elevated in white adipose tissue of LXRα null mice (Fig. 2A). Furthermore, treatment of mice with the LXR agonist GW3965 significantly reduced adipose cGPDH expression (Fig. 2B).

To investigate how LXR activation may lead to suppression of cGPDH expression, we performed transactivation studies using the mouse cGPDH promoter placed in front of a luciferase reporter. As expected, combined PPARγ2 co-transfection and addition of rosiglitazone significantly stimulated reporter activity, yet this induction was dose-dependently repressed by T0901317 (Fig 3A). Since the NIH-3T3 cells used for transactivation studies do not express LXR, repression of reporter activity by T0901317 required co-transfection with LXRα (Fig. 3B). The PPARγ co-activator protein PGC1α potentiated the transactivation induced with PPARγ2 and its ligand. This potentiation was not sufficient to prevent the dose-dependent repression of cGPDH promoter activity by LXRα and its ligand.

According to our hypothesis, LXR agonists decrease cGDPH expression by trapping RXR in a complex with LXR rather than PPARγ, rendering RXR limiting for PPARγ-mediated gene activation. To investigate whether RXR is indeed limiting for cGPDH promoter activity, the effect of RXR co-transfection was studied. A marked increase in reporter activity by RXR was observed, suggesting that RXR may indeed be limiting for induction of the cGPDH promoter (Fig. 4A and 4B). In addition, over-expression of RXR attenuated the LXR agonist-mediated inhibition of cGPDH promoter activity, which supports our hypothesis that PPARγ and LXR compete for binding to a single partner: RXR.

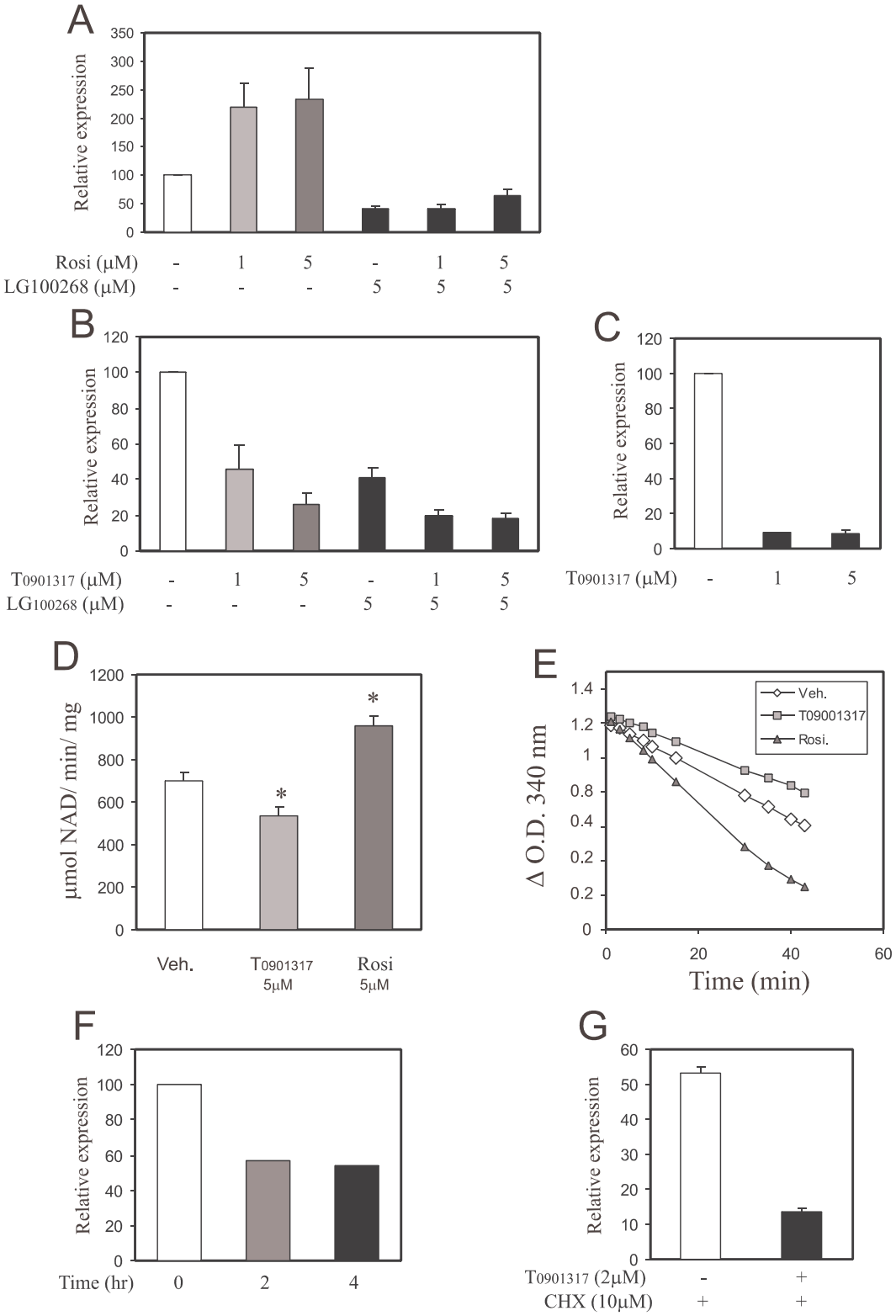

Figure 1: LXR ligands represses cGPDH expression in the adipose context.
A-C: Mouse and human mature adipocytes (day 10) were treated for 24 hours at the doses and time indicated. The decrease of cGPDH expression translates at the level of GPDH enzyme activity (D) and (E). The repression of cGPDH expression by LXR ligand is observed within several hours (F) and does not require de novo protein synthesis (G). 3 sets of independent experiments were performed for each graph. Q-PCR was used to measure the expression of cGPDH. β- Actin (A, B, C, F) and 18S (G) were used as control genes.

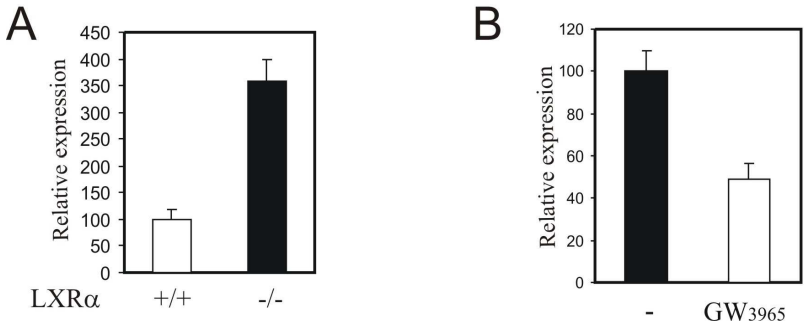

Figure 2: LXRα and ligands represses cGPDH expression *in vivo*.
A: Perirenal white adipose tissues from LXRα wild- type or null mice were extracted for subsequent quantitative measure of cGPDH expression. B: Wild- type mice were treated with GW3965 (at 0.03% and for 10 days) prior to RNA isolation from adipose tissue. cGPDH expression is normalized to β- actin.

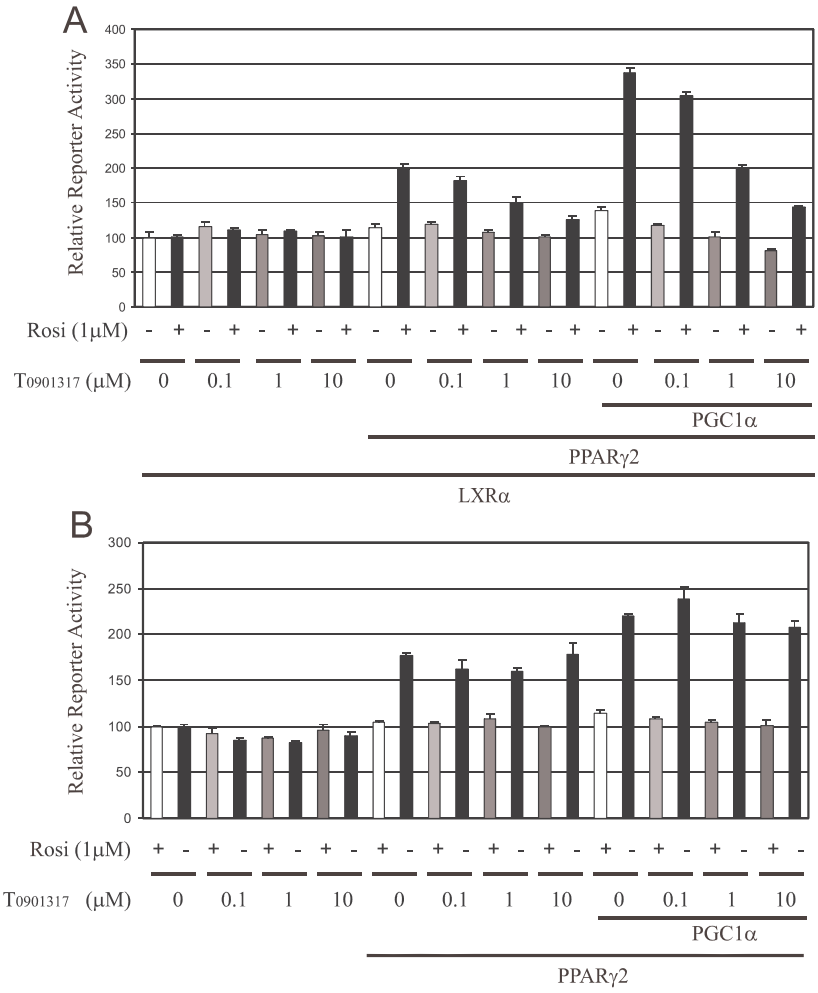

Figure 3: LXR activation suppresses cGPDH promoter activity.
A and B: NIH-3T3 cells were transfected with the mouse cGPDH promoter placed in front of a luciferase reporter, plus expression vectors for PPARγ2 and LXRα. PPARγ-mediated transactivation is dose dependently attenuated by T0901317 only when LXRα is co-transfected. Over-expression of the co-activator PGC1α does not attenuate the LXR-dependent repression of cGPDH promoter activity.

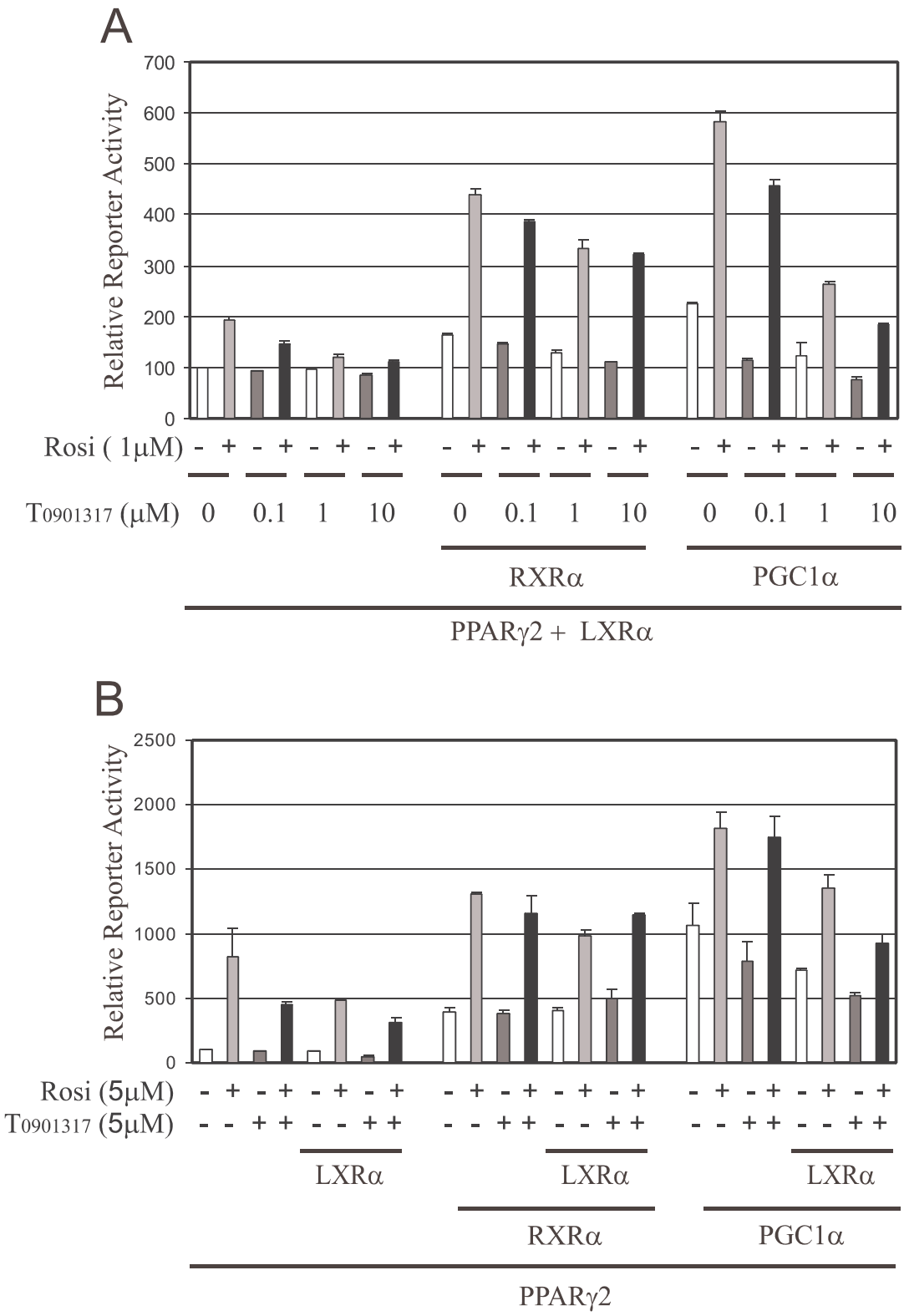

Figure 4: RXRα but not PGC1α attenuate LXRα/ ligands mediated repression of cGPDH promoter.

NIH-3T3 cells were transfected with the mouse cGPDH promoter placed in front of a luciferase reporter, plus expression vectors for PPARγ2, LXRα, RXRα and PGC1α. Transfections were carried out in NIH-3T3 cells (A) or HepG2 cells (B). RXRα but not PGC1α over-expression attenuated LXR-dependent repression of the cGPDH promoter. Experiments were performed at least three times and showed similar effects.

The above scenario also suggests that the suppression of cGPDH promoter activity by LXR co-localizes to the same promoter region as the induction of cGPDH promoter activity by PPARγ. Promoter deletion studies as well as promoter mutation analysis support this notion (Fig. 5A). It was observed that deletion of the cGPDH promoter region that contains the two functional PPREs not only abolished the response to PPARγ agonist but also to LXR agonist. Furthermore, disabling PPRE1 attenuated, while disabling PPRE1 and PPRE2 together completely abolished LXR agonist-mediated suppression of cGPDH promoter activity (Fig. 5B).

The above data indicate that suppression of cGPDH promoter activity by LXR is mediated by the same region within the cGPDH promoter that mediates PPARγ-dependent upregulation. To investigate whether LXR activation impairs binding of PPARγ and RXR to this genomic region, chromatin immunoprecipiation was performed. As shown in Fig. 6A and 6B, incubation of 3T3-L1 adipocytes with the LXR agonist T0901317 significantly reduced binding of PPARγ and RXR to the genomic region containing the two PPAR response elements. Control primers did not generate any products with the exception of samples corresponding to the input prior the immunoprecipitations steps. Because of the close proximity between the two PPREs, it is not possible to separate the effect on PPRE1 and PPRE2 using chromatin immunoprecipitation.

Taken together, these data indicate that LXR activation reduces cGPDH expression by inhibiting binding of the PPARγ-RXR heterodimer to the PPRE(s) within the cGPDH promoter.

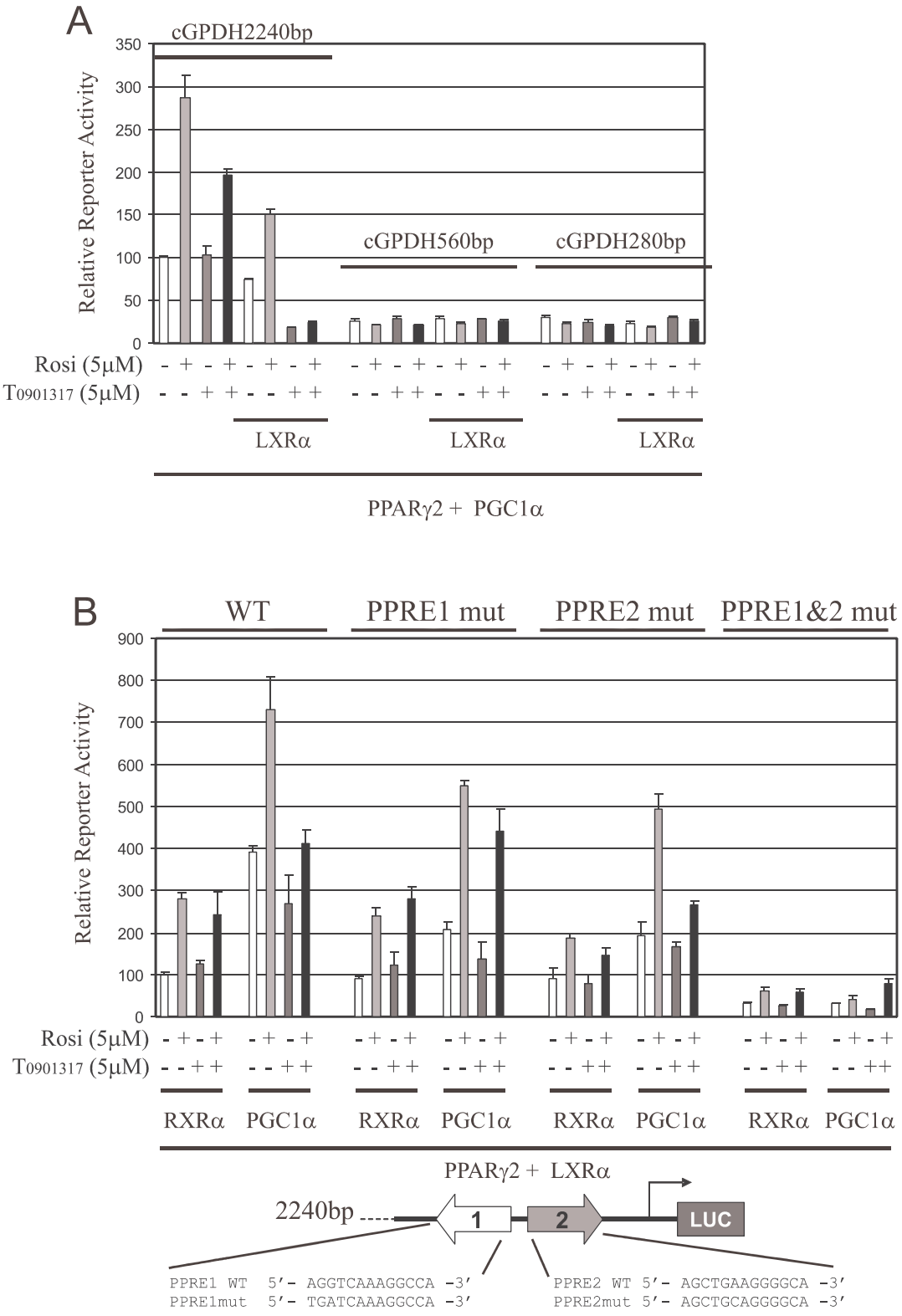

Figure 5: The cGPDH PPREs are needed for LXRα and T0901317 to repress cGPDH promoter.

NIH-3T3 cells were transfected with deletion constructs (A) or mutated constructs (B) of the mouse cGPDH promoter placed in front of a luciferase reporter, plus expression vectors for PPARγ2, LXRα, RXRα and PGC1α. The repressive effects of LXRα and its ligand is abrogated upon removal or mutation of the PPREs. NIH-3T3 cells were used in A and HepG2 cells in B. Experiments were performed at least three times and showed the similar effects.

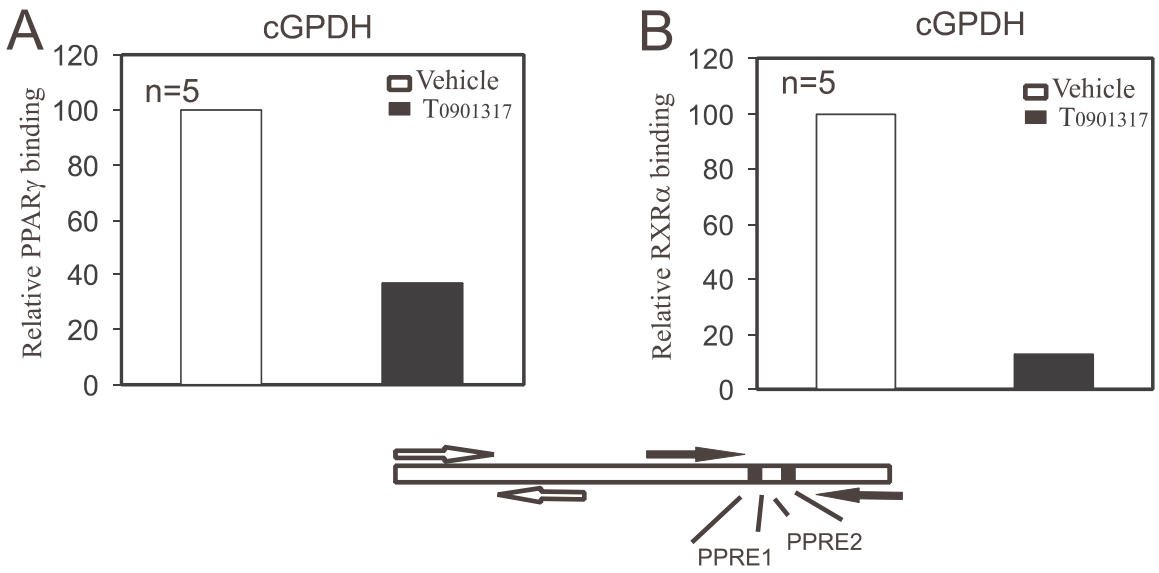

Figure 6: LXRα and T0901317 repress PPARγ and RXRα binding on the cGPDH promoter

Chromatin Immmunoprecipitations with PPARγ (A) and RXRα (B) antibodies were performed on mature 3T3-L1 adipocytes treated for 24 hours with rosiglitazone (1μM) or T0901317 (1μM) (n=5 pooled). Selected primers for amplifying the region containing the cGPDH PPREs (indicated in black) were used for quantification by Q-PCR. The relative binding of PPARγ and RXRα was obtained upon normalization with the input DNA (4). Primers designed for amplifying a control region not containing the cGPDH PPREs (indicated in white) did not give rise to quantifiable products in immunoprecipitated samples.

To find out whether the inhibitory effect of LXR activation extended to other PPARγ target genes, we studied the effect of LG100268 and T0901317 on the expression of several PPARγ targets in 3T3-L1 adipocyte. Interestingly, the expression of the glycerol transporter protein AQP7 was significantly decreased by LXR and RXR agonist (Fig. 7A and 7B), whereas other known PPARγ target genes including CAP, FIAF, GOS2 were either up-regulated or not changed (data not shown). Down-regulation of AQP7 by T0901317 was also observed in human SGBS adipocytes (Fig. 7C). Similar to the situation for cGPDH, chromatin immunoprecipitations revealed significantly reduced binding of PPARγ and RXR to the AQP7 promoter in 3T3-L1 adipocytes treated with T0901317 (Fig. 7D and E).

Since within adipocytes cGPDH and AQP7 are important for determining the concentration of glycerol 3-phosphate, which is an intermediate in the synthesis of triglycerides, we were interested to examine whether down-regulation of cGPDH and AQP7 translated into any changes in triglyceride storage in differentiating 3T3-L1 adipocytes.

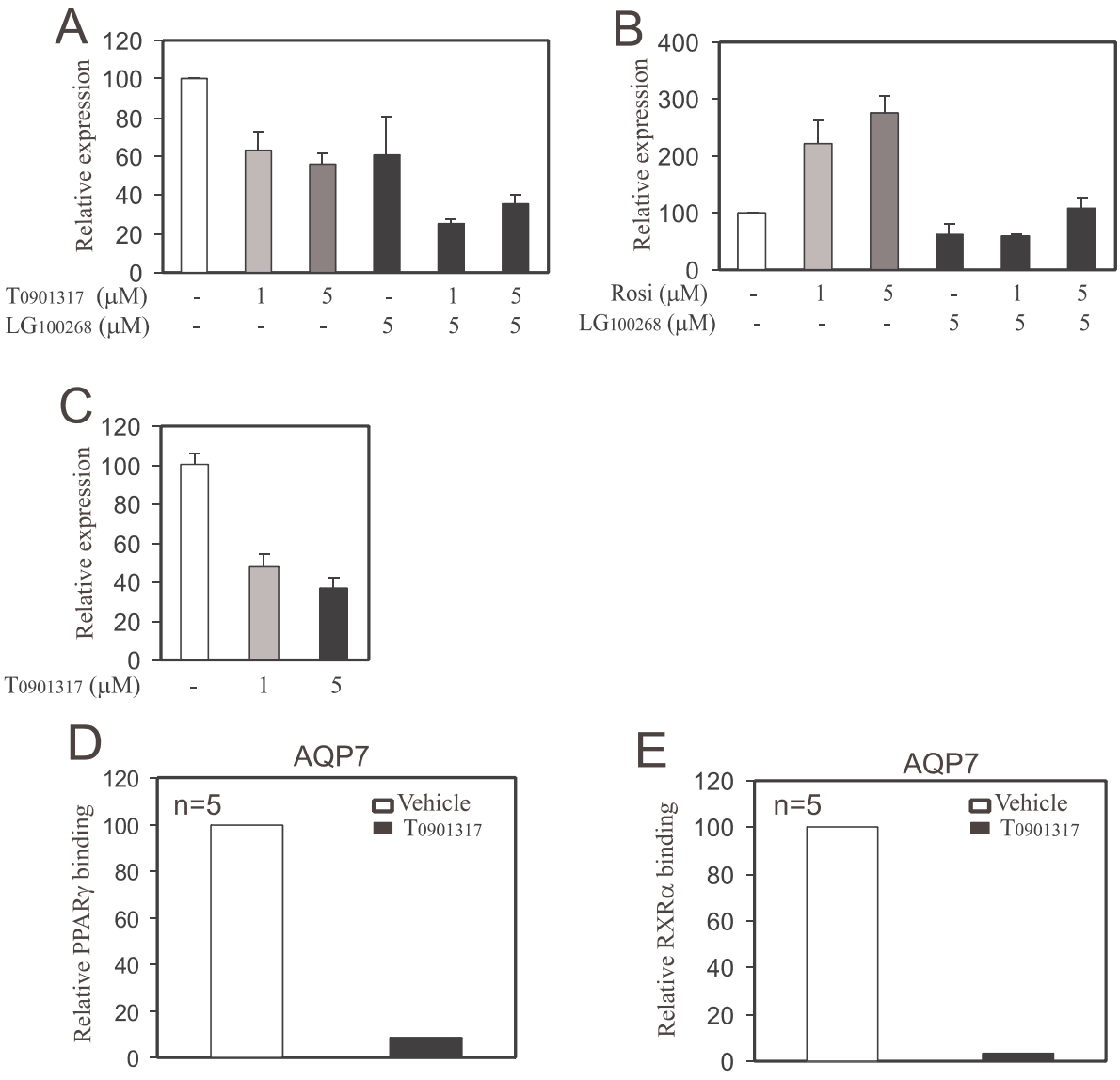

Figure 7: AQP7, a PPARγ target gene, is down- regulated by LXRα/ ligands in a similar manner as cGPDH.

Mouse 3T3-L1 (A and B) and human SGBS (C) mature adipocytes were treated with ligands for 24 hours at the doses and time indicated. T0901317 and RXR ligands down-regulated AQP7 expression (A and C). AQP7 expression was stimulated by rosiglitazone, which was attenuated by T0901317 (B). Chromatin Immmunoprecipitations performed with samples described in Figure 6 were used for quantification of PPARγ (D) and RXRα (E) binding to the AQP7 PPRE.

The experiments reported in Fig.1, showing reduced cGPDH expression after treatment with RXR and LXR ligands, were performed on mature adipocytes. However, numerous PPARγ target genes increase in expression during the adipogenesis process per se i.e. in early days of the differentiation. In as much as previous experiments in which adipocytes were treated with LXR ligands or LXRα expression levels were modified gave conflicting data ((36) (44) (5) (39) (38) (41) (42)), our observations prompted us to perform a co-treatment experiment with Rosiglitazone and the LXR ligand in a chronic manner throughout the adipogenesis process. The cells were pre- treated with ligands one day before induction of adipogenesis and for the full duration of the differentiation protocol. At day 5, the adipose triglyceride levels were quantified with Oil Red O staining (Fig. 8A) and an enzymatic assay (Fig. 8B). In agreement with previous observations (39) (44), there was no evidence of an effect of T0901317 alone on cellular lipid stores. In addition, LXR ligand also did not interfere with the induction of lipid storage promoted by Rosiglitazone. Interestingly, Q-PCR on a large number of PPARγ genes demonstrated that they were down regulated by T0901317 (Table 1). Furthermore, the genes that were down regulated by T0901317 also had a lower expression when cells were treated with rosiglitazone and T0901317 compared to rosiglitazone alone. The genes tested that shared this pattern were AQP7, cGPDH, Adiponectin, UCP2, and Gyk. In contrast, several other PPARγ target genes including PCx, PEPCK, CD36, Angptl4, GOS2 and CAP were not affected or even increased upon treatment with LXR ligands. LXR signaling was functional since expression of the direct LXRα target genes FAS and SREBP1c was up regulated by T0901317. Surprisingly, the down regulation of several PPARγ target genes by T0901317 did not translate into changes in the amount of triglycerides stored. However, this might be explained by a compensating induction of LXRα target genes involved in lipogenesis. Finally, in contrast with rosiglitazone which induced the expression of PPARγ2 and LXRα, T0901317 did not significantly modify the expression of these two genes.

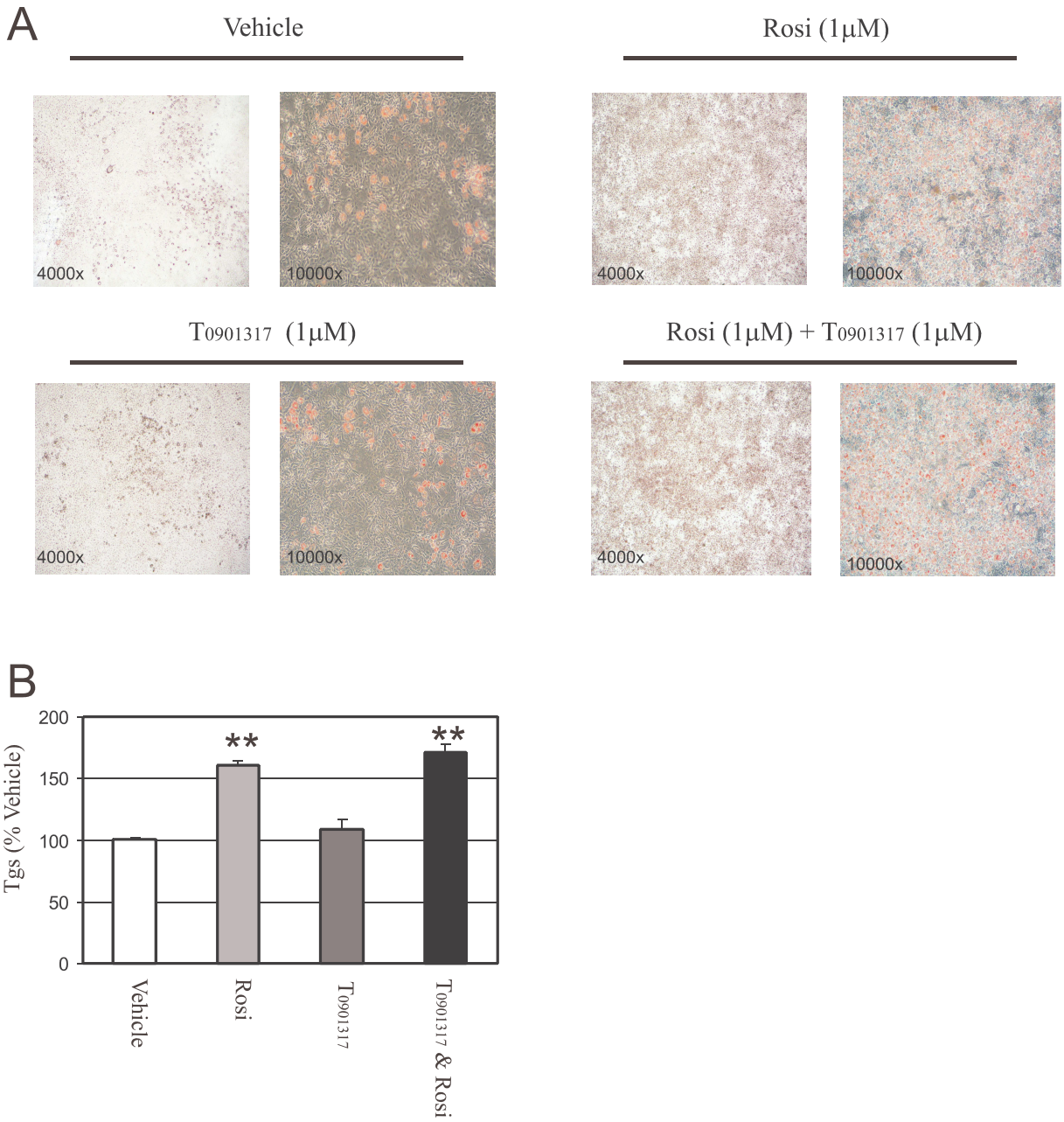

Figure 8: Chronic exposure of 3T3-L1 cells to T0901317 does not alter lipid stores. Confluents 3T3-L1 cells were pre- treated with rosiglitazone (1μM), T0901317 (1μM), or both, one day prior to the exposure with the adipogenic cocktail (IBMX, Dexamethasone, Insulin). The exposure with PPARγ or LXR ligand was performed for 5 days post-induction of differentiation. Oil Red O lipid staining was performed followed by examination with optical microscope (A) (n=3). Triglycerides (Tgs) stores were quantified with an enzymatic kit (B) (n=5).

Table I: Chronic exposure of 3T3-L1 cells with T0901317represses some PPARγ target genes.

	Vehicle		Rosiglitazone		T090137		Rosi & T0901307	
	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM
NHRs involved:								
PPARγ2	1	0	1,52	0,08	1,36	0,35	2,54	0,58
LXRα[$]	1	0,16	2,88	0,3	0,9	0,01	2,14	0,12
RXRα	1	0,36	1,6	0,19	1,7	0,08	1,71	0,01
PPARγ direct target genes down regulated by T090137:								
Gyk	1	0,31	48,14	3,29	0,29	0,08	37,04	5,72
cGPDH	1	0,09	6,35	0,39	0,64	0,02	3,5	0,26
Adiponectin	1	0,09	3,83	0,38	0,72	0,08	2,43	0,28
AQP7	1	0,06	6,67	0,67	0,87	0	6,14	0,13
UCP2	1	0,1	4,37	0,81	0,88	0,02	3,67	0,25
LXR direct target genes:								
SREBP1c	1	0,01	2,94	0,47	2,09	0,07	2,09	0,01
FAS	1	0,11	4,73	0,49	1,68	0,17	2,82	0,41
PPARγ target genes not affected or up regulated by T090137:								
aP2/ FABP4	1	0,14	5,96	0,22	0,93	0,04	5,17	0,26
GOS2	1	0,22	9,5	0,72	1,01	0,01	7,06	0,22
LPL	1	0,14	2,93	0,29	1,08	0,02	2,83	0,09
CAP	1	0,42	5,68	0,05	1,1	0	6,08	0,09
PEPCK	1	0,06	4,28	0,2	1,22	0,05	7,09	0,43
PCx	1	0,08	5,57	0,46	1,24	0,08	4,29	0,26
Perilipin	1	0,37	5,48	0,13	1,27	0,01	4,89	0,26
ADRP	1	0,34	6,12	0,61	1,37	0,01	8,02	0,14
FIAF/ Angptl4	1	0,43	7,91	0,13	1,39	0,21	9,21	0,13
CD36	1	0,12	1,97	0,15	1,44	0,09	6,15	0,09

Confluents adipoblats 3T3L1 cells were pre- treated with Rosiglitazone (1μM), T0901317 (1μM), or both ligands (1μM/ 1μM), one day prior to the exposure with the adipogenesis cocktail (IBMX, Dexamethasone, Insulin). The exposure with PPARγ, LXR ligands was performed for 5 days post the induction of differentiation. RNA was extracted and genes expression was measured with Q- PCR. Genes expression were normalized to the cyclophilin expression and set to 1 for the vehicles in order to represent the relative fold changes for each ligand treatment. ADRP, Adipocyte Differentiation Related Protein; AQP, Aquaporin; aP2/FABP4, aP2/Fatty Acid Binding Protein 4; CAP, C-cbl Associated Protein; CD36, Cluster of Differentiation 36; FAS, Fatty Acid Synthase; FIAF/ Angptl4, Fasting Induced Adipose Factor/ Angiopoietin like protein 4; GOS2, G0/G1 switch gene 2; cGPDH, cytosolic Glycerol Phosphate Dehydrogenase; Gyk, Glycerol kinase; LPL, Lipoprotein Lipase; LXR, Liver X Receptor; PCx, Pyruvate Carboxylase; PEPCK, Phosphoenolpyruvate Carboxykinase; PPAR, Peroxisome Proliferator Activated Receptor; SREBP, Sterol Regulatory Element-Binding Protein; UCP2, Uncoupling Protein 2. [$] LXRα is a PPARγ target gene (38).

DISCUSSION

Previous studies have demonstrated cross-talk between PPARα and LXRs in liver. Indeed, it has been shown that PPARα ligands such as PUFAs and fenofibrate bind and repress LXRα and β activity (47) (50) (51) (52). Additionally, T0901317, via LXRα and β, is able to down-regulate the activity and expression of PPARα target genes. This antagonism between LXRs and PPARα is reciprocal: the PPARα ligand Wy14643 was shown to repress LXR activity and target genes (45), (46). Furthermore, PPARα and LXRα are able to physically interact and form stable heterodimers (53).

To our knowledge, whether similar cross-talk exists between PPARγ and LXR has never been investigated. However, it was recently demonstrated that LXR can form a stable high affinity complex with the different PPAR isotypes in solution (48). The binding of LXRs to PPARs involved the ligand-binding domains of the receptors and in the case of PPARγ and LXR was highly enhanced in the presence of LXR ligands T0901317 and 22RHC. The interaction of PPARγ with liganded LXRα was very tight and more important than for LXRβ. Moreover, the affinity of the LXRα-LBD treated with T0901317 was higher for the PPARγ-LBD than for the PPARα– and PPARβ/δ–LBDs.

Our observations clearly demonstrate that liganded LXRα represses several PPARγ target genes (table 1). In the case of cGPDH and AQP7, this repression was fast, direct and involved decreased binding of PPARγ and RXRα to the PPREs present within the promoter. Our data are consistent with the explanation that LXR agonist promotes the formation of the LXR/RXR heterodimeric complex, thereby rendering RXR less available for interaction with PPARγ. Alternatively, it is possible that LXR ligand induces the formation of LXRα/PPARγ heterodimers, resulting in decreased binding of the PPARγ/RXRα heterodimer to the promoter. Theoretically, it is also possible that T0901317 might act as a PPARγ antagonist. However, this is unlikely since down-regulation by T0901317 was not observed for all target PPARγ targets. In addition, in transactivation assay the effect of T0901317 was dependent on co-transfection with LXRα (fig. 2C).

Treatement of adipocytes with the RXR ligand LG100268 minimized the induction of cGPDH and AQP7 by rosiglitazone. Furthermore, RXR ligands synergized with LXRα ligands in down-regulating the expression of these two PPARγ target genes. These results indicate that RXRα actively participates in the cross-talk between LXRα and PPARγ. In

transactivatin assay, over-expression of RXRα attenuated the LXRα–mediated repression of cGPDH promoter activity, further stressing the involvement of RXR (fig. 3A and 3B). We also tested the effect of the PPARγ co- activator PGC1α but failed to see any effect on inhibition by LXR. Consistent with our mechanism, Ide et al. reported similar observations on PPARα-induced transactivation of a PPREtkLUC reporter (46). Their observations demonstrated that RXRα but not co-activators like CBP and p300 could alleviate the LXRs/ T0901317 induced repression of PPARα activity.

The reported roles of LXR in adipose tissue homeostasis and metabolism are diverse and conflicting. Effects ranges from induction of lypolysis and inhibition of adipogenesis, stimulation of adipogenesis and lipogenesis, to stimulation of lipogenesis only. Discrepancies between our observations, which show a negligible effect of LXR agonist on 3T3-L1 adipocyte differentiation, and some other reports may rely on subtle differences in the growing conditions (serum) of 3T3-L1 cells or the differentiation protocol. However, the latter explanation is unlikely since we failed to find an effect of T0901317 in three different adipogenesis protocols. *In vivo* data demonstrate that only dual LXRα/β knock- out mice above the age of one year show a reduced adipose mass. This might indicate that LXRα/β may functionally compensate each other but are unlikely required for adipogenesis per se. Rather, LXRs appear to be involved in regulation of lipogenesis, similar to the situation in liver. However, whether this pathway is functionally relevant remains unclear since no changes in fat mass have been described so far. This is different from liver where LXR ligands strongly induce hepatic steatosis, which is accompanied by an increase of the liver mass and increased production of VLDL particles. Whether the differences in the net effect of LXRα ligands between liver and adipose tissue are because of the relatively low expression of PPARγ in the liver is not clear.

Our observations that LXRα reduces the expression of several PPARγ target genes without affecting adipose triglycerides stores might suggest that the induction of lipogenic LXR target genes might compensate for the suppression of PPARγ target genes, leading to no overall effect on triglyceride stores.

Our chromatin immunoprecipitation and transactivation experiments indicated that LXRα/ T0901317 wields its repressive activity via decreased binding of PPARγ/RXR to the PPREs. However, treatment with LXR ligands did not decrease the expression of all markers of

PPARγ activity in adipose tissue , including CD36, FIAF/Angptl4, CAP, or LPL (table1). A lack of effect on FIAF/Angptl4 and LPL is consistent with previous observations made by others (39). A recent study demonstrated that PPARγ/RXRα is constitutively bound to the PPRE(s) of their target genes (54) (55). This binding was unaltered upon treatment with rosiglitazone and transcriptional activation of target genes was strictly dependent on the net balance between co-activators/-repressors associated. Importantly, major differences in co-activator binding between the Gyk and aP2 promoter, which served as model target genes, could be identified. It is conceivable that the versatile interaction of PPARγ and RXRα on the cGPDH and AQP7 promoters might define a limited subset of PPARγ target genes. The specific association of co-activators/-repressors with PPARγ/RXRα or the configuration of the chromatin might strengthen or weaken the stability of PPARγ/RXRα to the PPREs, rendering them more or less susceptible to competition by LXRα/ T0901317.

The importance of the glycerol transporter AQP7 in adipose tissue function is increasingly recognized. Deletion of AQP7 is associated with increased triglyceride synthesis in adipose tissue, resulting in elevated adipose mass (56) (57) (58). This is most likely due to impaired glycerol efflux, which via glycerol kinase may lead to elevated intracellular concentrations of glycerol 3-phosphate, a precursor for triglyceride synthesis. According to our data, expression of AQP7 is suppressed by treatment with LXR ligand, which is in agreement with a lipogenic role of LXR in adipose tissue. Ross et al. observed that adenoviral LXRα over-expression in adipocytes concomitant with T0901317 treatment induces adipose lipolysis, releasing additional FFA but not glycerol into the medium (5). The lack of release of glycerol into the medium may be explained by repression of AQP7 expression by T0901317, which would cause entrapment of glycerol in the cells.

In conclusion, we have shown that LXR activation reduces the expression of selected PPARγ target genes in adipocytes, indicating important cross-talk between PPARγ and LXR. The suppressive effect of LXR, at least on the cGPDH promoter, is likely mediated by reducing the availability of RXR for heterodimeric interaction with PPARγ.

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Chapter 6

General Discussion

The objective of my PhD work was to investigate the functions of the NHRs PPARα, γ and LXRα in relation to glucose homeostasis. The work presented in this thesis adds important new information to this area of investigation. A general discussion of the composite data in the context of the contemporary scientific literature is presented here.

Novel function for PPARα

Chapter 3 describes detailed investigations into the function of PPARα in glucose homeostasis in fasted liver. We have used microarray in an attempt to elucidate the mechanisms behind the severe hypoglycemia in PPARα null mice when fasted. The results demonstrate that 3 key genes, i.e. cytosolic and mitochondrial GPDH, and glycerol kinase, all of which are involved in the gluconeogenic conversion of glycerol, fail to show a fasting-induced increase in expression in PPARα null mice. Furthermore, expression of the aquaglyceroporin 3 and 9 was also diminished in PPARα null mice. Therefore, the pathway representing the conversion of glycerol into glucose appeared to be tightly controlled and stimulated by PPARα. Basal and hyperinsulinemic clamp experiments demonstrated that fasted PPARα null mice had a reduced hepatic glucose production. Additionally, treatment with the PPARα agonist, fenofibrate lowered the plasma glycerol levels of patients. In combination with the previously observed hypoglycemia in fasted PPARα null mice, these data indicate that regulation of the glycerol pathway at the level of gene expression was translated at the functional level. Finally, these data also suggested that measurement of the plasma levels of glycerol might serve as a plasma marker for PPARα activity.

PPARα and the glycemia

It is difficult to estimate to what extent the hypoglycemia of fasted PPAR null mice can be attributed to impaired gluconeogenesis from glycerol. The relative involvement of the various gluconeogenic precursors in total glucose synthesis differs a lot between studies (1-6). The dogmatic view is that most of glucose is synthesized from lactate and amino acids. However, other reports claim that in case of prolonged fasting, glycerol becomes the major precursor (7).

In one of the current models on the progression of type II diabetes, visceral fat contributes to the rise in hepatic glucose production via excess delivery of FFA to the liver. These FFAs are generated from the breakdown of adipose TGs and are released together with glycerol.

Accordingly, it is tempting to speculate that glycerol may become more important as a gluconeogenic precursor when visceral fat stores are high.

Importantly, we have obtained evidence indicating that PPARα is involved in the fasting-induced expression of other gluconeogenic enzymes such Pyruvate Carboxylase (PCx), Lactate Dehydrogenase (LDHa) and Fructose 1,6 Bisphosphatase (FBP1) (unpublished data). Therefore, PPARα most likely not only participates in the conversion of glycerol to glucose but perhaps also in the conversion of other gluconeogenic precursors. Thus, rather than reflecting a requirement of gluconeogenesis for fatty acid oxidation to provide the energy and reducing equivalents for the pathway, our work indicates that the hypoglycemia in fasted PPARα null mice is due to a direct effect of PPARα on the expression of gluconeogenic enzymes ((8) and **chapter 2**).

Hyperinsulinemic clamp experiments (described in **figure 6** of **chapter 3**) provided important insight into the sensitivity of the PPARα null mice to insulin with respect to glucose disposal. It was shown that in response to hyperinsulinemia the increase in glucose disposal is more pronounced in PPARα null mice, suggesting elevated peripheral insulin sensitivity. Therefore, PPARα is likely also involved in the regulation of energy metabolism in peripheral tissues such as skeletal muscles and adipose tissue. However, PPARα mRNA is below detection in white adipose tissue (our observation), making any direct effect on this tissue unlikely. Rather, PPARα deletion may influence adipose metabolism indirectly by altering fat stores. While PPARα deletion thus appears to improve hepatic and peripheral insulin sensitivity and is associated with hypoglycemia, treatment with synthetic PPARα agonists has been reported to increase fatty acid oxidation in skeletal muscle of diabetic models which correlated with lower plasma glucose and insulin levels ((9), (10), (11), (12), (13), (14), (15)). These pleiotropic and seemingly discrepant effects of PPARα raise important conceptual questions about the comparison between PPARα deletion and treatment with synthetic agonists. An important difference is that treatment with ligand is a relatively transient stimulus where PPARα is very potently activated, whereas deletion of the PPARα gene leads to more subtle chronic effects. Also, both experimental stimuli may give rise to indirect side effects. For example, feeding with Wy14643 markedly induces the expression of PPARγ target genes in the liver of Wild- type mice (CD36, LPL, aP2, see **chapter 4**), whereas their expression is not changed in fasted PPARα null mice. Furthermore, feeding with PPARα agonists usually alters the feeding behavior and lowers food intake, leading to loss of adipose

tissue in rodents (12) (16) (17) (18) (19). Finally, during prolonged fasting (post 24 hours) and high fat diet, the expression of PPARγ increases the liver of PPARα null mice (20).

Another illustration of the complexity of regulation of glucose homeostasis by PPARα is provided by experiments made in PPARα$^{+/+}$LDLR$^{-/-}$ *or* PPARα$^{+/+}$LDLR$^{-/-}$ *double knock- out* mice that were treated with dexamethasone, which is a known inducer of insulin resistance. According to the published results, dexamethasone specifically induced the expression of the two gluconeogenic enzymes glucose-6-phosphatase and PEPCK in PPARα$^{+/+}$ LDL $^{-/-}$ mice but not in double knock-out mice. However, according to our and other data neither glucose-6-phosphatase nor PEPCK are under direct transcriptional control of PPARα ((21) and **chapter 3**).

The generation of tissue-specific and/or conditional knock-out mice, which for PPARα has not yet been realized in liver, skeletal muscle, or any other tissue for that matter, may provide important new insights into the physiological function of PPARα specifically in those tissues ((21) (22, 23) (24) (25)).

Physiological activation of PPARα

In addition to helping to identify novel functions for PPARα, microarrays have been fruitful to reveal under what physiological challenges PPARα becomes activated. Indeed, we have demonstrated, taking advantage of microarrays, that PPARα becomes activated not only by synthetic agonists or by fasting but also under conditions of High Fat Diet-induced insulin resistance. While the relative induction of gene expression by High Fat Diet was small compared to activation by Wy14643 and 24 hour fasting, significant upregulation of PPARα target genes was observed. PPARα activation likely occurs via two mechanisms: 1) increased hepatic expression of PPARα, which, since insulin is able to suppress the expression of PPARα, is likely connected to hepatic insulin resistance. 2) increased plasma levels of free fatty acids, which results in increased ligand-activation of PPARα in liver. Although it is well acknowledged that fatty acids are endogenous ligands for PPARα, recent data have called into question the importance of fatty acids released from adipose tissue as physiological activators of PPARα in liver. In this context, it is important to note that fatty acids can enter the liver from three main sources: 1) generated *de novo* in liver, 2) released from WAT and present in blood as albumin-bound free fatty acids, or 3) originating from the diet and delivered to the liver via chylomicron remnants (**figure 1** and (20), (25), (26), (20, 27, 28), (29)). A recent

study by Chakravarthy et al has called into question the idea of a single pool of intracellular fatty acids to which the various fatty acid sources can contribute (20) (25). However, this concept is poorly supported by the published evidence available. From a physiological point of view, activation of PPARα by ligand seems to be especially relevant under conditions of fasting, when hepatic fatty acid oxidation is elevated. Also, in case of excess load with dietary fatty acids, activation of PPARα and hepatic β-oxidation is required. Finally, de novo lipogenesis can only give rise to mono-unsaturated fatty acids, which are relatively poor ligands for PPARα compared to poly-unsaturated fatty acids.

It has been reported that dietary treatment with fatty acids results in the upregulation of a specific subset of PPARα target genes involved in fatty acid β-oxidation but not all of them (26), (30) (31)). Currently, this concept is currently further explored within the Nutrition, Metabolism and Genomics group. Preliminary data indicate that dietary fatty acids, especially poly-unsaturated fatty acids, are very powerful activators of PPARα in liver and that little to no differentiation between PPARα-target genes is observed.

Recently, Pawar (27) used primary hepatocytes and established that most of the polyunsaturated fatty acids (C18:1,n9; C18:2,n6; C18,n3; C20:4,n6; C20:5,n3; C22:5,n3; C22:6,n3) are able to activate PPARα within some physiological ranges, i.e. affinities IC_{50} ranging from 0.3 to 1.2μM. However, in agreement with some others *in vitro* assays, only the 22:5*n*-3 and 22:6*n*-3 (Eicosapentaenoic, EPA and Docohexaenoic, DHA) behaved as agonists and efficiently induced PPARα activation (32). Furthermore, the better these nutrients activate PPARα, the more their functional properties correspond with those of synthetic PPARα ligands such as fenofibrate. The functions which are shared between synthetic PPARα agonists and DHA/EPA include anti- inflammatory properties, lowering of plasma triglycerides and partial insulin sensitization in diabetes (26) (33), (34), (35), (36), (37). Accordingly, it would be interesting to test the effect of feeding a diet enriched in fatty acids on insulin resistance, hepatic glucose production, and inflammation in wild-type and PPARα null mice. Previously, it has been shown using PPARα null mice that PPARα does not mediate the plasma triglyceride-lowering effects of fish oil. Indeed, it should be realized that fatty acids regulate gene expression both PPARα dependently and independently. Other transcription factors that may be involved in mediating the effect of fatty acids on hepatic gene expression include LXRs, FXR (38), SREBP1c, RXRα and HNF4α (for review see see (26), (39) and (31)).

Besides fatty acids, PPARs may also be activated by other components of our diet or present in our environment. The latter property is shared with several other NHRs and the synthetic compounds that serve as NHR ligands are often referred to as endocrine disruptors. Although they were not originally made for this, these molecules may accumulate in the environment and are able to alter NHRs signaling. Endocrine Disruptors can act as full or partial agonists or even antagonists. Numerous endocrine disruptors modify the estrogen receptors (ERs) activities, yet all NHRs are potential targets. In the case of PPARα, the most important group of activators are plasticizers (phthalates) which are used to soften plastics. Although phthalates induce cancer in rodents, their carcinogenicity in human remains to be proven (40). The sources of endocrine disruptors are various and can either be "natural" like some phytoestrogens or derived from industrial activities like several herbicides or plasticizers ((41), (42), Endocrine Disruptor Knowledge Database: http://edkb.fda.gov/, CASCADE network: http://www.cascadenet.org/default.asp). From a historical point of view, the first PPARα ligands described are herbicides inducing hepatic peroxisomal proliferation (43) (44). Among the lists of side effects reported, more attention is being paid towards the onset of obesity and other metabolic diseases. For instance, birth weight is an established factor affecting the onset of obesity and its complications (45-47). The major environmental influence on birth weight has been considered to be *in utero* nutrition. Therefore, maternal nutrition and exposure to chemical toxins has been the focus of research into the fetal basis of diseases including obesity (48) (49). These chemicals include heavy metals, solvents, polychlorinated biphenols, organophosphates, phthalates, and bisphenol A. Additionally, these pollutants are often lipophilic and are therefore stored in the adipose tissue where they can alter the activity of NHRs such as PPARγ and ERs (50) (51).

Finally, numerous old remedies, especially prepared from herbs which are good sources of ligands for NHRs (52). Interestingly, their beneficial effects on health were known for ages far before the NHRs were described. These effects are again an illustration of the attractive link that NHRs hormones provide between health/ disease and the environment of the body. **Tables 1** and **2** non- exhaustively summarize known ligands for PPARα according to their sources (for reviews see (32) and (53)).

Table1: Reported natural ligands that target PPARα.

Subtype	Coumpound	Abbreviation	Reference
Fatty acids like			
	cis-9, trans-11 Conjugated Linoleic Acid	c9,t11 CLA	(54) (55) (56)
	trans-10, cis-12 Conjugated Linoleic Acid	t10,c12 CLA	(54) (55) (56)
	Oleylethanolamide	OEA	(18)
	Phytol		(57) (58)
	Phytanic acid		(57)
Other			
	Pseudolaric acid B		(52)
	Genistein		(59) (60)
	Epigallocatechin gallate		(61)
	Isoprenoids		(52)
	Formononetin		(60)
	Biochanin A		(60)
Unsaturated Fatty Acids			
	Eicosapentaenoic Acid	EPA	(54) (55) (56)
	Docohexaenoic Acid	DHA	(54) (55) (56)
	α- Linoleic Acid		(32)
	γ-Linoleic Acid		(32)
	Linoleic Acid		(32)
	Dihomo-γ- linoleic Acid		(32)
	Arachidonic Acid		(32)
	Palmitoleic Acid		(32)
	Oleic Acid		(32)
	Petroselinic Acid		(32)
Saturated Fatty Acids			
	Myristic Acid		(32)
	Palmitic Acid		(32)
	Stearic Acid		(32)
Eicosanoids			
	8-Hydroxyeicosapentaenoic Acids	8-HEPE	(32)
	Leukotriene B4		(32)

Table2: Reported synthetic ligands that target PPARα.

Subtype	Coumpound	Abbreviation	Reference
Prostaglandin 12 analogs			
	Carbaprostacyclin	cPGI	(32)
	Iloprost		(32)
LeukotrieneB4 analogs			
	Trifluoromethyl leukotriene B4		(32)
	ZK151657		(32)
	ZK 158252		(32)
Hypolipidemic agents			
	Clofibric Acid		(23) (24) (32) (62)
	Cipofibric Agents		(23) (24) (32) (62)
	Bezafibric Acid		(23) (24) (32) (62)
	Pirinixic Acid	Wy14643	(23) (24) (32) (62)
	Fenofibrate		(23) (24) (62)
	Gemfibrozil		(23) (24) (62)
	Eicosatetraynoic Acid	ETYA	(32)
Carnitine Palmitoyl transferase I Inhibitors			
	LY-171883		(32)
	2- Bromopalmitate	2Br-C16	(32)
	Tetradecylglycidic acid	TDGA	(32)
Fatty acyl-CoA dehydrogenase inhibitors			
	Ortylthiopropionic acid	OTP	(32)
	Tetradecylthiopropionic Acid	TTP	(32)
	Nonylthiopropionic Acid	NTA	(32)
	Tetradecylthioacetic Acid	TTA	(32) (54) (63)
Peroxisome Proliferators			
	nafenopin		(44) (53)
	trichloroacetic acid		(44) (53)
	methylclofenapate		(44) (53)
	mono(2-ethylhexyl) phthalate	MEHP	(44) (53)
	di-(2-ethylhexyl) phthalate	DEHP	(40) (53)
	di-(2-ethylhexyl) adipate	DEHA	(40) (53)
	Dehydroepiandrosterone	DHEA	(53) (64) (65)

More paradoxes in PPARα null mice

Hepatic triglycerides stores and inflammatory are known to be increased in association with type II diabetes. These has resulted in the so- called "two hits model", describing that a pro-inflammatory situation, in combination with an excess stores of triglycerides may lead to cirrhosis, liver failure and diabetes (66). In our high fat diet experiments (chapter 4), we observed a rise of hepatic triglycerides and an increased expression of acute- phase proteins such as Serum Amyloid and C- reactive proteins in the PPARα null mice (23) (24) (66) (67). According to the two hits model, one would expect the PPARα null mice to suffer from liver dysfunctions, including hepatic insulin resistance. Instead the IPGT demonstrates that these mice have a lower fasting glycemia with an adequate response to an oral glucose load. While the link between inflammation and diabetes/obesity is gaining ground (68), the purported association between hepatic triglycerides stores and insulin resistance is suffering. For example, liver specific PPARγ knock- out mice fed a high fat diet show some protection towards the induction of a fatty liver, yet their insulin sensitivity is worsened in liver (69) (70). Also, PPARα null mice store huge amounts of lipids in liver when fasted yet show improved hepatic insulin resistance under the same conditions. In skeletal muscle, the hypothesis has been put forward that rather than triglycerides per se, the culprits that impair intracellular insulin signaling are acyl-CoAs or diacylglycerol. This is because elevated triglyceride levels as observed in endurance athletes are associated with improved insulin signaling (71) (72) (73) (74) (75) (76) (77) (78).

Interplays and Tissue specific regulations of NHRs target genes:

In the **chapter 5**, we describe that LXRα and its ligand repress the expression of the two PPARγ target genes AQP7 and cGPDH. We demonstrate that the mechanism involves decreased binding of PPARγ and RXRα to the PPREs of these genes. In cultured adipocytes and in white adipose tissue of mice, LXRα and ligand did not repress the expression of many other PPARγ target genes, which suggests that for most PPARγ target genes the relative expression of RXRα may not be limiting in the system. Recently, Guan identified different subsets of PPARγ target genes in adipose cells (79). These findings where obtained using the technique of Chromatin Immunoprecipitation, and opened up some novel perspectives on the fundamental mode of action of NHRs. In the paper it was demonstrated that when PPARγ is expressed, the binding of PPARγ to the PPARγ-responsive promoters is constitutive (**chapter**

5). Transcription of the PPARγ target was only triggered by differential recruitment/release of specific co-activators/co-repressors. Hence, a possible explanation for our observations could be that AQP7 and cGPDH represent a particular subset of PPARγ target genes where PPARγ and RXR are weakly associated with the PPREs. In those conditions, any decrease in RXR availability may affect gene transcription. Additionally, their transcription might involve a common co-activators with LXRα, which would not be the case for the other PPARγ target genes.

There are numerous reports about dual PPARα/γ liver/adipose tissue target genes, including cGPDH, the Adipose differentiation related protein (ADRP), the Fasting Adipose Factor (FIAF), Gyk, Lipoprotein Lipase, CD36, and G0/G1 switch gene 2 (GOS2) (80) (81) (82) (83) (84). Nevertheless, several PPARγ-specific targets are also known, such as PEPCK and adiponectin (85). Since adiponectin is expressed exclusively in adipose tissue, it is no surprise that its expression in liver is not under control of PPARα (86). The regulation of PEPCK is highly interesting: it is highly expressed in liver, increases during fasting, yet is not under control of PPARα. In this context it is important to realize that PEPCK supports different function in liver (gluconeogenesis) and adipose tissue (glyceroneogenesis). However, the same is true for cGPDH (**chapter 3**).

In **chapter 4** we demonstrate that when expressed above a certain level, PPARγ is able to replace PPARα in the regulation of classical PPARα target genes involved in fatty acid catabolism. Together with the observation that PPARα and PPARγ are rarely co-expressed in the same tissue, this suggests that the concept of separate PPARα and PPARγ targets needs revision. According to our unpublished data, pyruvate carboxylase (PCx) is a direct PPARα target gene in the liver, while Jitrapakdee demonstrated that PCx is a PPARγ target gene in the adipose tissue (87). Interestingly, similarly to lipoprotein lipase (88), chromatin immunoprecipitation in mouse hepatocytes demonstrated that PPARα bind a different PPRE than PPARγ on the same proximal region of the PCx promoter 1B (our unpublished observations). Hence a possible explanation for the differential regional regulation of PPARα/ γ target genes might be the existence of different classes of PPREs, some of which are only accessible to PPARγ in the adipose tissue, while others can only bind PPARα in liver.

Co activators/ Co repressors: the next step beyond NHRs?

Amongst the co-activators/co-repressors, several appear to be ubiquitously expressed such as the co-repressors NcoR, SMRT, HDAC3 (79) or the co-activators CBP and p300 (79). On the other hand, several others seem to be expressed in a tissue-specific manner and direct the genetic program induced by NHRs. Important insight has been gained from experiments that demonstrate the involvement of PPARγ in governing the fate of undifferentiated mesenchymal stem cells. Whereas the association of PPARγ with TRAP220 and TIF-2 induces the cells to differentiate into adipose cells (89) (90) (91), the association with SRC- 1 or PGC1α induces mitochondrial proliferation and other oxidative metabolism, resulting in the formation of brown fat cells (92) (93). Moreover, TAZ specifically inhibits PPARγ, which stimulates osteogenesis (94), while Hic5 positively interact with PPARγ to promote the differentiation of epithelial cells from the gut (95).

Chromatin Immunoprecipitations is an elegant technique to study the mode of activation of NHR target genes. Importantly, they allow determination of which co-activators and co-repressors are involved in the transcriptional regulation of a certain gene. Several new perspectives on NHR function have been emerged from their use. Taking advantage of ChIP, the role of particular co-activators in governing expression of two different subsets of PPARγ target genes was recently reported (79). In liver, we made several observations suggesting the existence of different subsets of PPARα targets, each of which is supposedly under differential control by co-activators. For instance, the aquaporin 3 gene is highly upregulated by Wy164643 treatment but not by fasting (**chapter 3**). Conversely, the Aquaporin 9, lactate dehydrogenase A (LDHa) and fructose 1,6 bisphosphatase (FBP1) genes, both of which are involved in gluconeogenesis, are induced by fasting in a PPARα dependent manner yet are not upregulated in response to treatment with Wy14643. These observations suggest that fasting may be a necessary situation for PPARα to induce specific subsets of target genes. For this subset of genes, as with any other direct PPARα target genes, transcription may be strictly dependent on the net balance between the associations of co-activators/co-repressors to PPARα. For those PPARα target upregulated by fasting but not by Wy14643, the balance may be in favor of increased transcription only under the condition of fasting. It should be mentioned that the far majority of PPARα targets is PPARα-dependently upregulated by both fasting and Wy14643 (**chapter 3** and **4**). However, even for those genes, which includes the classical PPARα target genes involved in fatty acid oxidation, complex regulation occurs. For example, ChIP performed in liver (**chapter 3**) demonstrated that PPARα is constitutively

occupying the PPREs of the cGDPH promoter. Remarkably, treatment with Wy14643 but not fasting further stimulated binding of PPARα to the cGPDH promoter, even though expression of cGPDH responds similarly to fasting and Wy14643. Accordingly, it seems that fasting and treatment with Wy14643 generate two different modes of activation of PPARα target genes.

The PPAR binding protein (PBP) (96), PPAR alpha-interacting cofactor 285 (PRIC285) (97), peroxisome proliferator-activated receptor (PPAR gamma)-interacting protein (PRIP), CBP, p300 (98) and PPARγ Coactivator 1 (PGC1α and PGC1β) (93) (99) represent co-activators hat have been reported to interact and mediate PPARα dependent gene expression.

Most of the attention has been directed toward PGC1α (93) (99) (100) (101) (102). It was demonstrated that PPARα stimulates hepatic fatty β- oxidation genes via PGC1α (100). The mechanisms of action of PGC1α not only involves recruitment and interaction with the transcription machinery but also impacts on transcriptional elongation and processing of the target genes (79) (92) (103). The name PPARγ co-activator belies an association with numerous other NHRs and transcription factors, including ERRα, NRF-1, PPARγ, HNF4, GR, LXRα, FXRα and SIRT1, CREB and FOXO1 (93) (104) (105) (106) (107) (108) (109). PGC1β, the other PGC1 isoform, also interacts with PPARα *in vitro*. However, it has not yet been reported whether it can mediate PPARα-dependent gene expression *in vivo*. Instead, it may possibly mediate the effect of saturated FAs in liver via LXRα and SREBP1c (110).

It would be interesting to know which co-activator is involved in mediating the effects of PPARα on expression of gluconeogenic target genes. While we have evidence that PGC1α co-activates PPARα– and PPARγ-dependent activation of the cGPDH promoter, it is unclear whether this also occurs in physiological conditions, i.e. with endogenous levels of PGC1α and PPARα. In that regard, ChIP to assess binding of PGC1α to the cGPDH promoter would be very valuable and informative experiments to perform. However, to date there are no suitable antibodies available for PGC1α. An alternative would be to check for the expression levels of cGPDH and other PPARα target genes involved in gluconeogenesis in fasted PGC1α null mice.

Furthermore, studies revealing the functions of co-activators/repressors can be used to find out novel functions for PPARα. For instance, it has recently been demonstrated that PGC1α is involved in hepatic heme biosynthesis, via the induction of 5-aminolevulinate synthase (ALAS1) in fasting conditions (111). Interestingly, our microarray data demonstrate that PPARα also stimulate the expression of several key enzymes involved in heme biosynthesis

(our unpublished data). For example, the expression of ALAS-1, coproporphyrinogen oxidase, and porphobilinogen synthase is clearly impaired in fasted PPARα null mice. Therefore, it is likely that the interaction of PGC1α with PPARα is needed for the induction of genes involved in hepatic heme biosynthesis.

Conclusion, perspectives and recommendations for future work:

This thesis contributes important new information to our understanding of how the NHRs PPARα, PPARγ and LXRα are regulated and alter the expression of some key metabolic genes and processes. The work presented in this thesis also nicely illustrates the successful application of micro-array technology to answer important questions in nutrition and metabolism. By maintaining a well-defined focus and strategy throughout, rather than succumbing to the trend of pursuing a holistic approach, novel insights were gained that are superior to large amounts of descriptive information.

Importantly, the research presented in this thesis has drawn attention to the complexity of regulation by PPARα and has clearly revealed the existence of specific subsets of PPARα targets, displaying a specific pattern of regulation by PPARα under various pharmacological and nutritional conditions. This concept will be elaborated on in future studies, which will focus on the differential response between various endogenous ligands (e.g. EPA, DHA) and synthetic ligands (Wy14643, fenofibrate). These studies will reveal to what extent the effects of dietary fatty acids on hepatic gene expression are mediated via PPARα and may allow for generation of transcriptional finger prints for various fatty acids.

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Summary

Transcriptional regulation of nutrient metabolism by PPARα, γ and LXRα

The objective of this thesis was to investigate the mechanisms of transcriptional regulation by the Nuclear Hormones Receptors PPARα, γ and LXRα, with special emphasis on glucose homeostasis. An additional focus was on the role of these Nuclear Hormones Receptors in the adipose Tissue/ Liver axis, both of which are actively involved in the onset of type 2 diabetes.

The first part of my PhD thesis work concentrates on the role of PPARα in the adaptive response of the liver to fasting. The results obtained demonstrated that PPARα is a key player in hepatic glucose metabolism by governing the hepatic conversion of glycerol into glucose. Additionally, we have shown that a defect in this metabolic pathway likely contributes to the profound hypoglycemia in fasted PPARα null mice. We showed that this function of PPARα was conserved in human.

In the second part we have been interested in deciphering the role of PPARα in high fat diet induced type II diabetes. The results obtained show that PPARα is activated in liver upon high fat feeding. Moreover, this activation was functional and translated into specific up-regulation of PPARα target genes. Using micro-array analysis it was demonstrated that PPARα can be activated in liver in three different ways: 1) treatment with specific pharmacological ligands, 2) fasting, and 3) a nutritional intervention with a high fat diet. As anticipated, gene expression profiling and comparison of PPARα markers of activity demonstrated that PPARα was most potently activated by the synthetic PPARα agonist Wy14643. Fasting also resulted in marked activation of PPARα but to a lesser extent than Wy14643. Finally, high fat feeding weakly but consistently induced expression of PPARα target genes, which was only revealed by analyzing gene expression comprehensively using micro-array. The results indicate that a nutritional challenge is sufficient to cause reproducible and chronic, yet weak changes in gene expression.

Finally, we have investigated the role and interplay between LXRα and PPARγ in the context of adipose tissue. The results obtained demonstrated that ligand-activated LXRα is able to specifically attenuate the expression of cGPDH and other PPARγ target genes. We have carefully examined the molecular aspects of this down-regulation and demonstrate that LXRα competes with PPARγ for their common binding partner RXRα. However, so far we have not

been able to demonstrate any changes in adipocyte biology that could be linked to this regulation.

The results obtained illustrate the power of a focused nutrigenomics approach to promote our understanding of regulation of gene expression by nutrients and their specific role in governing nutrient metabolism. The studies described here should pave the way for future studies into the molecular regulation of energy metabolism by nutrients and the dysregulation of these pathways in metabolic syndrome.

Résumé

Régulation transcriptionelle du métabolisme des nutrients par PPARα, γ and LXRα

L'objectif de cette thèse était d'investir les mécanimes de régulation transcriptionnelle par les Récepteurs Nucléaires Hormonaux PPARα, γ et LXRα, avec une attention particuliére sur l'homésostasie glucidique. Un autre intérêt a été porté sur le rôle de ces Récepteurs Nucléaires Hormonaux dans l'axe tissus adipeux/ foie, lesquels sont impliqués dans le dévelopement du diabéte de type 2. La premiére partie de ma thèse se concentre sur le rôle de PPARα dans la réponse adaptative du foie au jeûn. Les résultats obtenus demontrent que PPARα, en régissant la conversion du glycérole en glucose, est un acteur clé du métabolisme hépatique du glucose. En addition, nous avons démontré qu'une défection de cette voie métabolique entraine une sévére hypoglycémie chez les souris PPARα knock- out. Cette fonction de PPARα est égallement conservée chez les humains.

Dans la deuxième partie, nous nous sommes interessé au rôle de PPARα dans l'insulino-resistance induite par un régime riche en graisses. Les résultats obtenus montrent que dans cette situation, PPARα est activé dans le foie. De plus, cette activation est fonctionnelle et se traduit par l'augmentation spécifique des génes cibles de PPARα. L'analyse des résultats de microarrays, démontre que PPARα peut être activé de trois différentes façons : 1) le traitement par des ligands pharmacologiques specifiques, 2) le jeûn, et finallement 3) une intervention nutritionelle consistant en un régime riche en graisses. Comme on pouvait le penser, la mesure de l'expression des génes cibles, marqueurs de l'activité de PPARα, montrent que PPARα est le plus fortement activé par le Wy14643, un de ses ligand pharmacologique. Également, le jeûn induit une activation prononcée de PPARα mais moindre comparativement au Wy14643. Finallement, un régime riche en graisses active modérément mais de façon consistante PPARα. Cette derniére découverte fût uniquement rendue possible par l'analyse fine et détaillée des résultats des microarrays. En résumé, les résultats démontrent qu'une intervention nutritionelle est suffisante pour entrainer des modifications modestes mais chroniques et reproductibles de l'expression de génes.

Finallement, nous avons étudié les rôles et interactions entre LXRα et PPARγ dans le contexte du tissue adipeux. Les résultats obtenus démontrent que LXRα, quand activé par un

ligand, peut diminuer l'expression de cGPDH et d'autres génes cibles de PPARγ. Nous avons examiné avec attention les aspects moléculaires de ces repressions et pu démontrer qu'ils résultent d'une compétition entre LXRα et PPARγ pour leur partenaire commun, RXRα. Toutefois, à ce jour, nous n'avons pu démontrer d'implication fonctionnelle de ces interactions sur les fonctions des adipocytes.

En résumé, les resultats obtenus illustrent la puissance d'une approche orientée dans le domaine de la nutrigénomique afin d'appréhender les régulations de l'expression génique par les nutrients et leurs rôles spécifiques dans le contrôle du métabolisme. Les études décrites ici devraient servir et permettre á d'autres, d'investir les régulations moléculaires du métabolisme énergétique par les nutrients et les dérègulations de ces mêmes voies dans le cadre du syndrôme mètabolique.

Aknowledegments

I would like to write the first few words of this section in french since I would like to address them to my family and dedicate this PhD Thesis manuscript to my two grand fathers. Chers papis, je n'ai pas la prétention d'avoir contribué a de découvertes majeures concernant d'éventuelles applications médicales mais, cela dit, ce travail me permet d'accéder au titre de docteur ce qui je l'éspére vous aurez rendu fier. Par la même occasion, j'aimerais trés sincèrement remercier toute ma famille et mes amis pour le fait d'avoir été et de toujours être de vrais « moteurs » et soutiens pour moi.

A PhD Thesis is of course involving a lot of people and with no exeption, the all members of the NMG group must gratefully be acknowledged for having provided a stimulating and yet friendly environment all over these 4 last years.

In fact, when I think about it, I can say that I have gladly seen our group expending. Since I started some people are already gone (Elke, Fokko, Marleen, Stephane) and some others will very soon be leaving; even before the publication of this manuscript (Anita, Jolanda). To these people, I would like to whish a friendly "good luck in your new life". Some of them (Anand, Caroline, Elgin, Guido, Hanneke, Heleen, Jan, Jenny, Karin, Laetitia, Linda, Lydia, Mark, Meike, Mechteld, Nicole, Philip, Rinke, Shoreh and Wilma) may either be staying or may move sometime, that's the way these science things are made; however, our scientific world is rather small and I am convinced that our roads will cross again.

In particular, I am very grateful to Michael for letting me joining his laboratory and providing some excellent facilities but also necessary freedom in thinking and directing my project. I have appreciated our meetings, always friendly and insightful; I have learned a lot, thanks again.

My next thought goes to Sander who was a fantastic supervisor, forever a mentor and a trustful friend as well. Especially, I am completely grateful to him for providing me some of his knowledge, allowing myself to find some confidence and at the same time, smoothly directing and organising my ideas in order to generate some hopefully interesting findings.

Additionnally, I would like to address some more acknowledgements to René Bakker and the other people from the animal facilities. Thank you for the friendly technical assistance with mice.

Finally, I would like to add a few words to all the PhD students and supervisors who got involved in the two PhD Tours I have joined. Our Tours in Australia and United Kingdom, both in different ways, were great and lovely experiences. In particular, I am directing these thoughts to the organizers of the PhD Tour in U.K. (Gerda, Marieke, Mark, Martinet, Pascalle and Renate).

« C'est la vie »

David

Curriculum Vitae

David Patsouris was born on the 18th of December 1976 in Pierrelatte, Drôme, France. In 1995, he joined the university Joseph Fourier, Grenoble, France, in order to study physiology, molecular and cellular biology. In 1999, he worked for a year in the Laboratory of Cytoskeletal Proteins (LPC), CNRS, Grenoble, on the centromere – kinetocohores complexes. In 2000, after getting his Diplôme d'Études Approfondies (DEA/ Master of Science), he moved to the Institut of Animal Biology (IBA), Lausanne, Switzerland, in order to work on the molecular mechanisms associated with the differentiation of adipocytes. Finally, in 2002, after gathering some strong interests for the fields of Nuclear Hormones Receptors, and metabolism, he joined the Nutrition, Metabolism and Genomics Group (NMG), Wageningen, The Netherlands, and started a PhD project under the direction of Dr Sander Kersten and Prof Michael Müller.

Publications

- Mandard S, Zandbergen F, Tan NS, Escher P, Patsouris D, Koenig W, Kleemann R, Bakker A, Veenman F, Wahli W, Muller M, Kersten S. The direct peroxisome proliferator-activated receptor target fasting-induced adipose factor (FIAF/PGAR/ANGPTL4) is present in blood plasma as a truncated protein that is increased by fenofibrate treatment. *J Biol Chem.* (2004) 279:34411-20.

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- Zandbergen F, Mandard S, Escher P, Tan NS, Patsouris D, Jatkoe T, Rojas-Caro S, Madore S, Wahli W, Tafuri S, Muller M, Kersten S. The G0/G1 switch gene 2 is a novel PPAR target gene. *Biochem J.* (2005) 392(Pt 2):313-24.

- Patsouris D, Reddy JK, Müller M, Kersten S. PPARα mediates the effects of high fat diet on hepatic gene expression. *Endocrinology* (2006) 147(3):1508-16.

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

Training and Supervision Plan

Courses

Masterclass Nutrigenomics, Wageningen, the Netherlands, 2003

Symposium Nutrim, Universiteit Maastricht, the Netherlands, 2003

Training period at Center for Integrative Genomics, Lausanne, Switzerland

Advance Course on Bioinformation Technology, Wageningen, the Netherlands, 2003

CASCADE Summer School on Nuclear Receptors, Lyon, France, 2004 (poster)

Meetings

Ernst Klenk symposium, Univerisity of Cologne, Germany 2003

Voedings dagen, Papendal, the Netherlands, 2003

Voedings dagen, Papendal, the Netherlands, 2004 (poster)

Ernst Klenk symposium, Univerisity of Cologne, Germany 2005

Voedings dagen, Papendal, the Netherlands, 2004 (oral)

NUGO Week, Wageningen, the Netherlands, 2004 (poster)

PPARS Meeting, Monte- Carlo, 2005 (poster)

4th Endo-Neuro-Psycho meeting, Doorwerth, The Netherlands, 2005 (poster)

Voedings dagen, Papendal, the Netherlands, 2005

NUGO Week, Tuscany, Italy, 2005

The Ernst Klenk Lecture, University of Cologne, Germany, 2005

General courses

Journal Club and Research presentations NMG, the Netherlands, 2002-2006

Journal Club Human Nutrition Division, Wageningen, the Netherlands, 2002-2006

Optionals

Preparation PhD research proposal

PhD study Tour Asutralia, 2003 (oral)

PhD study Tour United Kingdom, 2005 (oral and poster)

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